Study of anticancer properties of novel synthetic compound(s) on breast cancer cell lines

THESIS SUBMITTED TO JAWAHARLAL NEHRU UNIVERSITY

FOR THE AWARD OF THE DEGREE OF

DOCTOR OF PHILOSOPHY

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July, 2019

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CERTIFICATE

The research work embodied in the thesis entitled ***"Study of anticancer properties of novel synthetic compound(s) on breast cancer cell lines"*** has been carried out in School of Life Sciences, Jawaharlal Nehru University, New Delhi.

This work is original and has not been submitted so far, in part or in full, for award of any degree or diploma of any university.

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15/7/15

Dedicated

to my

Parents……..!

ACKNOWLEDGEMENTS

" ॐ नमः शिवाय "

First and foremost, I am thankful to Lord Shiva for being most merciful to me and watching over me through all the hardships faced by me in the life so far. I hope he will continue to bless me in this way.

I am grateful to almighty for giving me opportunity and strength to accomplish my parent's dreams. This research work is the result of many years of hard work, during which I got the opportunity to work with many people who were more than supportive. It's a great pleasure to convey my sincere gratitude to them in my acknowledgement.

I wish to express the words of gratitude and the endless respect for my supervisor, Dr. Neelima Mondal, under whose supervision and constant encouragement, I was able to complete this Ph.D. work. She was always there with as a source of inspiration, encouragement and guidance through my work. Her high levels of professional excellence, eye for detail and unending enthusiasm will always inspire even in future as a role model. I am very thankful to Prof. S. K. Dhar, SCMM, JNU for his invaluable suggestions and support from time to time.

I would also like to acknowledge the sincere effort of my advisory committee members especially Dr. Ashu Bhan Tiku and Prof. Paulraj Rajamani for critically evaluating my Ph.D. work presentations and providing input and comments.

My sincere regards to my work collaborator, Prof. Amir Azam, for the synthesis of chemical compound(s). I can't miss to express my gratitude to him for all the warm hospitality I received whenever I visited him.

I am thankful to the Dean, Prof. K. Nataranjan (present time) and past, of our department under whom I carried out my work. I sincerely thank all the technical and clerical staff for their kind help in doing my experiments and paper works. I thank to the animal ethical committee, JNU, for providing me the space to do my animal experiments.

I am thankful to the Dean, Prof. Pawan K. Dhar (School of Biotechnology, JNU) for critical suggestion and scientific inputs during my work.

My very special thanks go to all my lab mates for their encouragement, constructive suggestion, support and love. To Mr. Guru Prasad Sharma and Mr. Kunwar Somesh Vikramdeo for introducing me to the lab-work and basic experiment techniques when I joined the lab. I have been really happy company of Sumiran and Sangeeta. Working with really happy to have a wonderful experience.

My acknowledgements will never be complete without mention of Mr. Lokesh nigam, help made me relax my mind whenever I was tensed because of failed experiments and other issues and supporting me as a big brother. Their enthusiastic efforts patience and encouragement has played an important role in completing this work successfully.

Special thanks to Mr. Bittu Sharma and Mr. Balwant Singh, who played an important role in maintaining the lab in its proper condition when I was busy creating mess during my experiments.

I also thank my dearest classmates Anshuman, Atanu, Rishi, Rajkumar, Tarun, Sunil, Sandeep, etc. I still miss the starting times in JNU when all of us used to wander together and visit all the beautiful places in Delhi. Almost inevitable is mention of those friends who have helped me even in the direst situation and supported me in every circumstances.

To my dear seniors, Mr. Siddharth Manvati, Mr. Mohd. Hafiz Ahmad, Mr. Amit Shrivastava, Mr. Kailash, Mr. Sandeep Sir, Hitesh Sir, Nitin Sir, Anand Sir etc. the long time spent with you people was mesmerizing and the support you provided has been really great. I specially thank Mr. Siddharth Manvati for critical suggestion and scientific inputs during my work and supporting me as big brother.

I would like to acknowledge all the M.Sc. batch mates at utkal university, BBSR, when I was there in Utkal University. To Chinmay, Tanmoy, Raju, Poonam, Pamchui and Bhanu. I really had a great time with you all. I specially thank Sarkar for providing critical suggestion and scientific inputs during my Ph.D and supporting me as younger brother (Dada)

My very special thanks go to all my juniors, Dhakaram, Devbrat, Manoj, Sudhist, Neha, Anjali, Pankaj, Aakash, Shuree, Shivani, Preeti etc. for their encouragement, constructive suggestion, support and love. Working with really happy to have a wonderful experience.

My acknowledgements will never be complete without mention of my friends Sonu Kumar, Vikas Singh, Pramod Sagar, Sanjeev Prabha, Neha singh, Vinod Singh, Monika, Pragya Tripathi, Rakhee Rajput, Prachi, Danny, Vivek Yadav and Vikas etc. whose help made me relax my mind whenever I was tensed.

On the special note, I would like to mention our weekly get-togethers "every Saturday night", without which I couldn't have had relaxed my mind.

Funding for this project was provided in part by the UPE-II, DBT-PURSE-II, DST-FIST and UGC-RNW. I was financially supported by the Rajiv Gandhi National fellowship (RGNF).

Finally, and the most important heartfelt thanks to Papaji, Maa, my brother's Mr. Manoj Gautam, Mr. Munish Gautam and Mr. Amit Gautam and sister's

Sonam Gautam and Khushaboo Gautam for their love affection and undemanding support. I hope I can keep up to their faith in me.

Finally, I would like to thank each and everyone who have been the part of the success of this thesis and express my sincere apology for not mentioning them personally.

SUMIT KUMAR GAUTAM

ABBREVIATIONS

Ab	Antibody
APS	Ammonium persulphate
ER	Estrogen Receptor
PR	Progesterone Receptor
HER-2	Human Epidermal Growth Factor Receptor-2
TNBC	Triple Negative Breast Cancer
ADME	Absorption, Distribution, Metabolism, and Excretion
BSA	Bovine Serum Albumin
Bcl-2	B-cell lymphoma-2
CA	Carbonic anhydrase
CDK	Cyclin Dependent Kinase
CAK	Cdk Activating Kinase
CKI	Cdk Kinase Inhibitor
Caspase	Cysteine-aspartic proteases, cysteine aspartases
CAT	Catalase
DAPI	4', 6-Diamidino-2-phenylindone
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
DRE	Digital Rectal Exam
DCF-DA	Dichloro-dihydro-fluorescein diacetate
DTNB	5,5’-Dithiobis-(2-Nitrobenzoic acid)
DTT	Dithiothreitol
EDTA	Ethylene diamine tetraacetic acid
FBS	Fetal Bovine Serum
FIU	Fluorescence Intensity Unit
G	Gap, a phase in cell cycle
gm	Gram
GSH	Glutathione reductase
IFA	Immunofluorescence Assay
M	Molar

mg	Milligrams
ml	Millilitres
mM	Millimolar
µg	Microgram
µl	Microlitre
µM	Micromolar
MMP	Mitochondrial membrane potential
MDA	Malondialdehyde
NBT	Nitro blue tetrazolium chloride
NEM	N–Ethylmaleimide
NADPH	Nicotinamide adenine dinucleotide phosphate
O.D.	Optical Density
OPT	1,10-Phenanthroline monohydrate
PBS	Phosphate buffer saline
PMS	Phenazine methosulphate
P/S	Penicillin Streptomycin
PARP	Poly (ADP-ribose) polymerase
RNA	Ribonucleic acid
rpm	Revolution per minute
ROS	Reactive Oxygen Species
SDS	Sodium dodecyl sulphate
SOD	Superoxide dismutase
SDS-PAGE	Sodium dodecyl sulpahte polyacrylamide gel electrophoresis
TEMED	N,N,N’,N’-Tetramethylethylenediamine
TBE	Tris-Borate-EDTA
TBARS	Thiobarbituric acid reactive substance
TBA	Thiobarbituric acid
TCA	Trichloroacetic acid
WB	Western Blot
hrs.	Hours

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CHAPTER-1

REVIEW OF LITERATURE

1.1.Introduction of cancer

Cancer is a group of diseases, in which cells exhibit uncontrolled growth and spreading of the abnormal cell from one place to another place via lymph and blood circulation (metastasis). If metastasis is uncontrolled, it can be led to death. Causative factors of cancer can be intrinsic such as inherited mutation, immune condition and hormones or extrinsic such as chemicals, infectious radiation, tobacco and organisms. These factors may act either in synergy or in sequence initiating or promoting different stages of cancer eventually increasing cancer incidences. Treatments of cancer include chemotherapy, hormonal therapy, radiation, surgery and targeted therapy.

Cancer is a leading cause of death worldwide, approximate 18.1 million new cases are reported and about 9.6 million people are dying due to the various type of cancers in worldwide(Bray et al., 2018; Siegel et al., 2018). The frequency and number of people affected by cancer are increasing especially in developing countries like India. Most of the common cancers such as breast, lung, oral, cervical and colorectal cancer are found in India. Continuous research is going on all over the world to develop an effective treatment to cure different types of cancers such as hormonal, radiation, chemotherapy, etc.

Cancers are of different types. Classification of cancerous cells are done either based on the type of tissues affected such as carcinoma, sarcoma, leukemia, and lymphoma or on the basis of organs affected such as breast, lung, cervical and stomach.

When cancer originates from the epithelial cell of the tissue called as carcinomas and about 85% all of the cancers. It is two type; squamous cell carcinomas and adenocarcinomas. Squamous cell carcinoma originates from epithelial cells called squamous cells, which forms the outer lining of skin, windpipe and esophagus. Adenocarcinomas arise in the specialized epithelial cells that secrete some protective substances in the cavities lined by them.

Nearly, 1% of all the cancers diagnosed are non-epithelial cancers, sarcoma and are usually divided into two important types, osteosarcoma and soft tissue sarcomas. Leukemia's and Lymphomas constitute about 7% of the total cancer cases originate from blood-forming cells.

Based on the organ of origin cancer can also be classified. Some of the examples are breast cancer, cervical cancer, lung cancer, pancreatic cancer, prostate cancer, thyroid cancer, leukemia, non-Hodgkin lymphoma, melanoma, and many more.

1.2. Stages of cancer

Different stages of cancers can be described based on the extent of spread of the disease. Tumor Node Metastasis (TNM) is one of the most common method which is used for classified of the cancer into stages(DENOIX),which divides stages of cancer based on three parameters namely degree of growth in tumor, degree of invasiveness in lymph node and degree of spread to distant organs and tissues i.e. metastasis. Based on the progression of cancer, it is broadly classified under the following stages:

1.2.1. Stage 0: It represents that anomalous cells are present only *in situ* i.e. where they developed.

1.2.2. Stage I: Confinement of the tumor cells to the organ of origin, without showing any sign of invasiveness.

1.2.3. Stage II and III: The transformed cancerous cells are no longer confined to the primary site. The cancerous cells have invaded neighboring lymph nodes or organs and tissues.

1.2.4. Stage IV: This stage indicates that cancer has advanced from the primary site to remote organs or distant lymph nodes. It is the metastatic stage.

Breast cancer is a highly malignant form of cancer found in females worldwide. Rarely, about 1% breast cancer cases are also found in male.

1.3. About the breast

The breast is a build up by different kinds of fatty and dense tissues. Each tissue is a network of lobes and per lobe made up by lobules that contain milk glands. The very fine ducts are connected to lobe, lobules and milk gland which is carrying the milk from lobe to the nipple. The nipple is located in the center of a dark area of the breast called an areola. The whole breast is nourishing by blood vessel and lymph vessel also present which is carrying the waste product. Lymph vessels are connected to the lymph node. It is a canter of the interaction of antigens and the immune system.

1.4. About the breast cancer

Breast cancer is a transform of normal breast cells to cancer cells due to the uncontrolled cell growth, forming a cell's mass called a tumor. Based on the malignancy properties tumor divided into two categories such as a malignant and benign tumor. In the case of malignant tumor, breast cancer cells are showing uncontrolled growth and migrate one place to another place via blood circulation or lymph vessels in breast cancer patients. A benign tumor, breast

cancer cell showing uncontrolled cell growth and don't spread one place to another place in breast cancer patients.

Based on the progression of breast cancer, which is divided into fourth stages "I, II, III and IV". When breast cancer cells are present in a localized area or lymph nodes of the breast in the early stages (I-III stages). In the advanced stage, breast cancer cells migrate from lymph nodes to bones, lung, liver, brain and other organs of the breast cancer patients. This is called stage IV or metastatic breast cancer.

1.5. Type of breast cancer

Breast cancer divided into two categories based on malignancy properties called as "invasive" and "non-invasive". Invasive breast cancer, cells are migrate into surrounding tissues. "Non-invasive" breast cancer, cells don't spread and present at the surrounding area of origin places such as milk duct or lobules. When breast cancer started at duct or lobes called ductal and lobular carcinoma.

1.5.1. Ductal carcinoma: It is most abundant breast cancer which started at cell lining of milk ducts of the breast and divided into two categories.

1.5.1.1. Ductal carcinoma in situ (DCIS): It is started at duct of milk gland.

1.5.1.2. Invasive ductal carcinoma (IDC): It is metastasize from the milk duct.

1.5.2. Lobular carcinoma: It is started in the lobules of the breast and divided further into two types.

1.5.2.1. Lobular carcinoma in situ (LCIS): It is founded in lobules of the breast but it is not a considerable factor for developing of breast cancer. It can act as a risk factor for spreadingthe breast cancer cells in both of the breast.

1.5.2.2. Invasive lobular carcinoma: It can spread outside of the lobules in the breast.

Other less common breast cancers are present such as a medullary, mucinous, tubular, metaplastic, papillary and inflammatory.

1.6. Breast cancer subtypes

Breast cancer further divided into three main subtypes based on the "hormonal receptors" and "human epidermal growth factor receptor 2 (HER 2)" or extra copies of the *HER2* gene.

1.6.1. Luminal breast cancer: Breast cancer cells are expressing hormonal receptors (HR) such as "estrogens", "progesterone" receptor and "human epidermal growth factor receptor 2 (HER2)". It is almost 84% of all the breast cancer and it is further divided into two main categories.

1.6.1.1. Luminal A (HR+/HER2-): Breast cancer cells are expressing the hormonal receptor (HR) but lack the expression of "human epidermal growth factor receptor 2 (HER2)". It is about 71% of all the luminal breast cancer and showing slow growth rate or lesser aggressive as compared to other "luminal breast cancer". Hormonal positive breast cancers occur in any age of women but most common in menopause stage of women. It is controlled by hormonal therapies.

1.6.1.2. Luminal B (HR+/HER2+): Breast cancer cells have both of the receptors. It is approximate 12% all of the luminal breast cancer and expresses the huge amount of Ki67 (a proliferation marker). It is showing a high growth

rate with less poor prognosis as compared to the luminal A. It can be controlled by either hormonal or anti-HER2 therapies and both of them.

1.6.2. Human epidermal growth factor receptor-2 positive (HER2+)breast cancer: Cancer cells have overexpression or amplification of HER2 oncogene. It is almost 5% of all of the breast cancer. It is controlled by anti-HER2 therapies.

1.6.3. Triple negative breast cancer (HR-/HER2-): Breast cancer cells are not express hormonal and HER2 receptors, called "triple negative breast cancer (TNBC)". It is approximate 12% all of the breast cancer with a more aggressive type of nature as compared to the other breast cancer because of lack of the treatment. It is common cancer in younger women's due to the frequent mutation in "*BRCA1" or "BRCA2"* genes.

1.7. Breast cancer: Incidence and Statistics

Approximate 11.6 million new cases are reported and about 6.6 million people are dying due to breast cancer, therefore, it is a second leading cause of deaths due to breast cancer in both sex (Bray et al., 2018; Siegel et al., 2018). It is the most highlighted cancer diagnosed in women, worldwide approximate 8.6 million new cases were reported in 2018. About 4.2 million women's are dying due to the breast cancer, therefore, it is first leading cause of death due to cancer in women worldwide(Bray et al., 2018; Siegel et al., 2018). The cases of breast cancer are increasing rapidly in both less and more developed countries.

1.8. Breast cancer in India

Breast cancer is one most common cancer in women worldwide. Breast cancer is curable when detected in the early stages. On a global scale, there is an increase in 1 million new cases of breast cancer annually. According to world cancer report, 8.6 million cases might be

diagnosed in 2018. In India, breast cancer is one of the most diagnosed cancers in women (Bray et al., 2018; Siegel et al., 2018). Breast cancer cases are increasing in mostly in metropolitan cities. At present 1, 62,468 new cases are diagnosed and 87,090 breast cancer patients are dying in India according to Globocan 2018, IARC. This continuous increase in breast cancer cases and deaths resulting from it is making it a major health concern in India. Therefore, it demands a more drastic and refined approach to tackle it.

1.9. Risk factor contributing to breast cancer

The main risk factors of breast cancer are female sex and older ages. Other potential risks factors such as genetics, a higher level of certain hormones, dietary patterns and obesity are responsible for the increased incidence of breast cancer.

Tobacco smoking causes an increased incidence of cancer risk. But prolonged smoking can multiply the risk for breast cancer from 35% to 50% (Johnson et al., 2011). Lack of physical activity also accounts for the increased risk of 10% breast cancer. According to the "abortion-breast cancer hypothesis", abortion also accounts for the increased breast cancer risk. Other dietary patterns like high level of fat (Blackburn and Wang, 2007) and cholesterol diet (Kaiser, 2013), minimal iodine diet, drinking alcohol (Boffetta et al., 2006) are also responsible for the increasing the risk of breast cancer. Some pesticides, organic solvents (Brody et al., 2007), hydrocarbons and radiation also increased breast cancer risk.

Mutation in "BRCA-1" and "BRCA-2" genes are the major common risk factors for increasingthe risk of breast cancer up to 60-80%. Other genes such as p53, PTEN, STK11, ATM, etc. also account increased breast cancer risk (Gage et al., 2012). Diabetes mellitus also increase breast cancer risk in diabetic patients (Anothaisintawee et al., 2013).

1.10. Cell cycle and its role in cancer progression

The cell cycle is a piece of highly coordinate and regulatory machinery, in which each cell are divided into two daughter cell with the help of cyclins and “CDKs (cyclin-dependent kinases)”. The whole cell cycle is completed with four phases such as “G1, S, G2 and M”. Each phase has a unique function for cell division. In the G_1 phase of cell cycle, the cell is synthesized proteins and size increased in cell organelles, therefore, it is called a growth phase. In S phase of the cell cycle, DNA replication (formation of a second copy of the DNA) occurs. Next phase is the G_2 phase, in which cell is synthesized proteins for M phase (Mitosis Phase). M phase is the last phase of cell cycle, in which segregation of nuclear content into two daughter cells occurs. The error-free and efficient cell cycle is the preserve of genomic integrity of the cell.

Many of the proteins are involved in cell cycle progression, which is mention below.

1.10.1. Cyclins and cyclin-dependent kinases (CDKs)

Cyclins are a group of proteins, which are essential for the progression of cell cycle via “CDKs (cyclin-dependent kinases)” activation. Each cyclin has interactive domain called as a cyclin box, which is build-up by 100 residues and it interacts with particular CDKs lead to form cyclin-CDK complex therefore cyclin is play as a major role in cell cycle progression (Fisher, 1997; Horton and Templeton, 1997; Johnson and Walker, 1999; Morgan, 1997). This complex is activated different proteins and factors for cell cycle progress from one phase to the next phase in cell division.

CDKs (cyclin-dependent kinases) are common proteins which are activated by particular cyclins lead to form cyclin-CDK complex then activate certain proteins and factors, essential for cell cycle progression from one phase to next phase (Arellano and Moreno, 1997). In the

absence of cyclin, CDKs is found in inactivated form but during the activation, they are interacting with specific cyclins and form a partial activating cyclin-CDK complex. This complex is fully activated by interaction of Cdks activating kinase (CAK) in humans (Kaldis, 1999; Kaldis et al., 1998).

At the start point of the cell cycle, there is interaction between cyclin D and CDK 4/6 resulting in a complex formation which transcribes the essential genes responsible for entry of the cell into the cell cycle by overcoming the restriction point, that movement is confirming the cells to not revert back until completion of the cell cycle (Blagosklonny and Pardee, 2002). That signal is responsible for the activity of "cyclin E-CDK-2 complex", which commits the cell to continue the cell cycle. This commitment confirm by overexpression of cyclin D act as pro-oncogene in many types of cancer (Fu et al., 2004). While high activity of "cyclin E-CDK-2 complex" is required for transition into G1 phase to S phase due to the overexpression of cyclin E protein and mRNA in many types of cancer cells (Cassia et al., 2003; Courjal et al., 1996; Hwang and Clurman, 2005; Molendini et al., 1998; Müller-Tidow et al., 2001; Schraml et al., 2003; Wołowiec et al., 1996).

After finished the G1 phase to S phase transition, CDK-2 is dissociated from the cyclin E and interact with cyclin A led to form "cyclin A-CDK-2 complex", which is essential for transcription of certain genes, which is committed for normal cell cycle progression from S phase. "Cyclin A-CDK-2 complex"maintains the genomic integrity via inhibiting the twice DNA replication in the same cell cycle (Yam et al., 2002). Overexpression of cyclin A occurs in some cancer that is promoting of anchorage-independent cancer cell growth (Barboule et al., 1998; Chao et al., 1998; Chetty and Simelane, 1999; Handa et al., 1999; Hauser et al., 1998).

At the early G2 phase, cyclin A dissociates from CDK-2 and interact with CDK-1, which stabilize the cyclin B and promote the formation of cyclin B-CDK-1 complex. Cyclin B-CDK-1 complex is triggered for the degradation of cyclin A and this complex is crucial for G_2 phase to M phase transition (Faha et al., 1992). Overexpression of cyclin B also responsible for different cancer cell growth (Nozoe et al., 2002; Soria et al., 2000; Suzuki et al., 2007).

1.11. Cell cycle regulation

In cancer cells are showing uncontrolled cell division by overexpression of cyclins and activity of CDKs at a different cell cycle phases, therefore, cyclins and CDKs are the major target for cancer prevention.

1.11.1. Cyclin-dependent kinase inhibitors (CKI)

For initiation and progression of cell cycle,Cyclin-dependent kinases (CDKs) are crucial proteins, therefore, CDKs arethe most common target to control the high rate of cell proliferation in cancer therapies. The cell has evolved CKI proteins (cyclin-dependent kinase inhibitors), which are inhibiting the CDKs and regulate cancer cell growth (Lloyd, 1998; Sherr and Roberts, 1995). Based on the specificity of CKI proteins are classified into two major types. First CKI proteins are called as INK4 family proteins, which is made up by p16INK4, p15INK4b, p18INK4c and p19INK4d. These proteins are inhibiting the activities of CDK-4/6 which results in cell cycle arrest at G_1 phase (Hirai et al., 1995; Ortega et al., 2002; Pavletich, 1999).

Second CKI proteins are called as Cip/Kip family proteins, which is made up by p21cip1/waf1, p27kip1 and p57kip2. These proteins are also inhibiting cell cycle phase-independent activity of CDK-4/6 which leads to cell cycle arrest at different phases. p57kip2

cause cell cycle arrest at “G1 phase” due to the inhibition of CDK-4/6 activity and p21 also arrest the cell cycle at G1 phase follow as the same pathway but cyclins are translocated in the nucleus (Alt et al., 2002) therefore p21 can positively regulate of cyclin D1. p21 has the ability to interact with “proliferating cell nuclear antigen (PCNA)”, which is clamp protein and plays an essential role in replication of DNA. Therefore interaction of p21 with PCNA results in replication blockage in cell cycle (Cayrol et al., 1998).

1.11.2. Role of p53 in cell cycle regulation

The p53 is a “tumor suppressor protein”, which is plays an essential role in regulation of cell cycle. In normal condition, cells have a low level of p53 due to the interaction with Mdm-2, which is a ubiquitin ligase that is degraded of p53 protein via proteasome (Momand et al., 1992). During the stress condition (DNA damages occurred in the cell) p53 protein is accumulated in the cell due to the phosphorylation of p53 protein via DNA damage responsive (DDR) kinase such as ATM/ATR and Chk1/Chk2 and lead to p53 protein dissociate from Mdm-2 resulting cell cycle arrest and allow the cell to commence DNA repair (Bunz et al., 1998; Smith et al., 2000). In case of a fault in DNA repair, p53 cause apoptotic cell death due to activation of caspases families and other pro-apoptotic proteins (Haupt et al., 2003; Nakano and Vousden, 2001; Schuler and Green, 2001). In most of the cancer cases, p53 protein is mutated resulting controlled cell proliferation of the cancer cell (Greenblatt et al., 1994; Hollstein et al., 1991; Muller and Vousden, 2013; Rodrigues et al., 1990).

1.11.3. Role of Retinoblastoma protein (Rb) in cell cycle regulation

Retinoblastoma (Rb) also tumor suppressor protein, which belongs to the family of the proteins called pocket proteins. Rb protein is negatively regulated of the cell cycle via bind to

transcription factor (E2F), which is essential for entry into the cell cycle (Hurford et al., 1997). During cell cycle initiation, cyclin D-CDK4 complex phosphorylate to Rb and lead to dissociate of Rb from E2F, therefore, E2F is found in the free state which is transcribed certain essential genes for further cell cycle progression. That is the main reason, Rb act as a checkpoint for G_1 phase in the cell cycle.

1.12. Reactive Oxygen Species (ROS) and Cancer

Intracellular ROS is an essential player of cellular homeostasis and under normal conditions, ROS homeostasis is tightly regulated. Cellular exposure to chemotherapeutic agents and ionizing radiation causes water radiolysis and results in the release of free superoxide ($O_2^{\bullet-}$) and perhydroxyl ($HO_2^{\bullet}$) radicals collectively called as ROS (Azzam et al., 2012). These highly reactive free radicals interact with biomolecules like DNA causing damage and oxidative stress (Azzam et al., 2012). As compared to normal cells, cancer cells have elevated ROS level which are considered to be one of the major sources of oncogenesis (Panieri and Santoro, 2016; Schumacker, 2006; Wang and Yi, 2008). Cancer cells not only high ROS level but they also have large amount of antioxidant proteins like GSH, catalase, “superoxide dismutase (SOD)”, etc. that can quench excess ROS (Liou and Storz, 2010) and thus maintain ROS homeostasis. Most of the cancer therapies target this ROS level of cancer cells which is either downregulated by means of antioxidants and other phytochemicals (Ahmad and Mukhtar, 2013; Fuchs-Tarlovsky, 2013)or unregulated by means of chemotherapeutic compounds (Pelicano et al., 2004; Trachootham et al., 2009) and physical agents like radiation (Azzam et al., 2012).

1.12.1. Reactive Oxygen Species (ROS) and its role in signaling pathways

The high level of ROS in cancer cells as compare to normal cells considered to be one of the major sources of oncogenesis (Panieri and Santoro, 2016; Schumacker, 2006; Wang and Yi, 2008).H_2O_2 is a well-known secondary messenger and play a major role in various cell signaling pathways.

MAPK/Erk1/2 is a well know signaling pathway, which essential for cell survival and that is controlled by upstream activators such as K-ras and $p90^{RSK}$. ROS can modulate these types of upstream activators lead to activate Erk1/2 signaling in oncogenic cells, resultant high rate of cell proliferation and metastasis (Chan et al., 2008; Lander et al., 1997; McCubrey et al., 2006).

Another most common signaling is the Akt/PKB pathway, which is essential for cell survival and inhibits the pro-apoptotic proteins. ROS also affected this signaling and lead to hyperactivate signaling process resultant oncogenesis. The hydrogen peroxide (H_2O_2) can be capable for activation of Akt/PKB signaling pathway by inhibiting PTEN protein which is act as a negative regulator of Akt. The inhibited PTEN can also promote more ROS generation by suppressing of various antioxidant enzymes such as super oxide dismutase (SOD).

ROS can also increase the high rate of proliferation of tumor cells growth by modulating the mRNA levels of several cyclins, which is involved in cell cycle progression.

1.12.2. Enhancement of ROS level used in anticancer therapies

As above the statement, higher ROS generation is essential for the high rate of cell proliferation and cell survival rate in cancer cells which is act as major sources of

oncogenesis (Panieri and Santoro, 2016; Schumacker, 2006; Wang and Yi, 2008). The huge production of ROS is not beneficial for the cancer cells resultant causing irreversible damages in a cell by failed defense system (antioxidants) such as GSH, SOD and Catalase, etc.

This ROS level of cancer cells targeted by most cancer therapies in which either downregulated via antioxidants and other phytochemicals (Ahmad and Mukhtar, 2013; Fuchs-Tarlovsky, 2013)or unregulated by chemotherapeutic agents and physical agents such as radiation (Azzam et al., 2012; Pelicano et al., 2004; Trachootham et al., 2009).

1.13. Breast cancer: Treatment Strategies

In the initial stages, the tumor may be treated by electrocoagulation, cryotherapy and surgery. Invasive breast cancers are treated either by surgery or radiation, or chemotherapy or in combination.

1.13.1. Surgery

In cancer patients, surgery is the most common method to remove cancer cells tissues at the localized area but this is mainly effective in early stage of cancer. In case, cancer cells metastasized to another place in cancer patients via the bloodstream, it's become so very difficult to cure of cancer patients by surgery. At this moment, cancer patients may be cured by surgery with other anticancer treatment strategies so that can control the metastatic potential of cancer cells.

1.13.2. Immunotherapy

Immunotherapy act as a provides a boost for cancer patients resultant generate natural defense against cancer cells i.e. activate the immune system. Currently, two approaches are using for immunotherapy. First is called passive immunity, in which cytokines are transferred

into cancer patients lead to activate the immune system against cancer cells (Rescigno et al., 2007). In the second approach, provide antibodies such as Trantuzumab and Imatinib to cancer patients which are targeted specific receptors and enzymes. Some types of breast cancer cells show over-expression of Her2+ receptor. Trantuzumabtarget is Her2+ receptor, therefore it results in suppression of Her2+ receptor signaling in breast cancer patients (Boekhout et al., 2011). Imatinib is another antibody which is designed against Bcr-Abl tyrosine kinase resultant control the hyperactivity of Bcr-Abl tyrosine kinase in CML patients.

1.13.3. Radiation therapy

This is the most common method to treat cancer patients via ionizing and non-ionizing radiation (Barcellos-Hoff et al., 2005; Brooks, 1998). Radiation therapy hasa drawback, because some residual cells are left in core of tumor during the radiation therapy resultant after the few time passes tumors are formed again in cancer patients. Nowadays cancer patients can be treated via radiation therapy with surgery or chemotherapy and cure different cancer such as breast, melanoma and leukemia (Dudley et al., 2005).

1.13.4. Chemotherapy

Generally, after surgical treatment, cancer patients have some residual and undetectable cancer cells, which are responsible for further development of a secondary tumor. Chemotherapy is used to remove these residual cells. These chemical agents target the rapidly dividing cancer cell and inhibit cancer cell growth by interfering in the progression of cell cycle which results in cell cycle arrest at different phases in the cell cycle profile of cancer cells. These agents also have several side effects such as myelosuppression / immunosuppression, mucositis and alopecia (Chabner, 1982; Foye, 1995; Longo and

Chabner, 1996), which is due to the fact that human body also contains highly proliferating cells in normal tissues compartment's such as bone marrow, urinary tract and inner linings of the gut.

1.13.4.1. History of cancer chemotherapy

In the early 20thcentury, drugs were used for the cancer treatment but originally it was not proposed for this purpose."Mustard gas" was in use during World War I and II as a chemical warfare agent. Later on, people of military operation were diagnosed with very low white blood cells counts who were exposed to mustard gas during the war. It was assumed that the agent might have an anti-carcinogenic effect as it reduced the white blood cell count. In the 1940s, several advanced lymphomas patients were treated with that agent intravenously. This led to extensive research for analogous compounds against cancer. Consequently, for treatment of cancer several other drugs were developed. Therefore this drug development resulted in a "multibillion-dollar industry", but still there are many limitations to chemotherapy which remains to be resolved (Archives; Hersh, 1968).

1.13.4.2. Principal of chemotherapy

Cancer is the result of dysregulation in cell cycle progression, therefore cells are showing uncontrolled cell proliferation rate than leading to generate metastatic potential and migrate one place to another place via lymph or blood circulation in the cancer patient. Interaction between genetic susceptibility and environmental toxins causes cancer i.e. depends on multiple factors for its manifestation. Most of the cytotoxic drugs control cancer cell growth via inhibiting the cell cycle of the fast-dividing cells. Chemotherapy target highly proliferative cells in the cancer patient. Depending on the rate of division and growth, some cancers respond better than the others. For example, tumor with aggressive growth such as

lymphoma, Hodgkin's disease and acute myelogenous leukaemia are more susceptible to chemotherapy when compared to the slow-growing indolent lymphomas. The effectiveness of chemotherapy also depends on the size of the tumor. Small, younger tumors respond better to the chemotherapy as compared to the larger size solid tumors as the drug does not get to the core of the tumor. These core cells result in the relapse of cancer. These type of tumor are treated with radiation therapy and surgery. Recently, small pumps have been determined present on the cancer cell surface that actively moves chemotherapeutic agents from outside to the inside of the cell. For increasing the effectiveness of chemotherapy, trials for inhibiting the activity of p-glycoprotein function are undergoing (DeVita, 1978; MARSHALL, 1964; Steel, 1968; Tannock, 1983).As the chemotherapeutic agents target the highly proliferative cells and these agents also affect the normal dividing cells, therefore, these drugs have several side effects such as hair loss, low count in white blood cells and inflammation in the inner lining of the digestive tract.

Chemotherapy is given either solely or in combination with other therapies. The combinatorial method, in which drugs are used with other therapy such as radiation, surgery has now become common in use. In a combination of chemotherapy, more than one drug is used for cancer treatment, in which each drug has a different pharmacokinetic effect. Combinatorial therapies are intended to minimize resistivity against any of the administered drugs (DeVita and Schein, 1973; ELION et al., 1954; Nathanson et al., 1969; POTTER and SIMONSON, 1951; Sartorelli, 1965). “Neoadjuvant chemotherapy” is a type of preoperative treatment, in which preliminary treatment is designed to reduce the primary tumor with less destruction and more effectiveness. “Adjuvant chemotherapy” is the type of postoperative treatment, which is effective when newly tumoris rapidly growing and very susceptible to

treating agents. Palliative chemotherapy is provided without curative intent, which is responsible for decreased tumor load and increased life expectancy.

1.13.4.3. Role of chemotherapy in Breast Cancer

Breast cancer treatment is depended on the type and the stage of breast cancer. In spite of the fact that breast tumor is controlled by surgery or radiation therapy, there is always a chance for re-occurrence because there may be some residual cancer cells left in the body that may give rise to new tumor later on. To avoid this risk of a recurrence via chemotherapy.

Chemotherapy is the method of cancer treatment with anticancer agents. Chemotherapy is often used in combination with surgery and radiotherapy. Chemotherapy is also performed to shrink tumor size prior to surgery or radiotherapy is known as neoadjuvant chemotherapy. In the later stages after breast cancer has spread throughout the body of the patient, chemotherapy is used to slow down its progression. Chemotherapeutic drug entering via blood and lymph circulation spread throughout the body and might kill cancer cells at all their sites.

Different chemotherapeutic drugs are used to treat breast cancer, and the specific drug regimen may vary among patients. Frequency of medication depends on the type of chemotherapy regimen recommended to the patient. Drugs like cisplatin, paclitaxel, 5-FU, hydroxyurea and ifosfamide are most commonly used for breast cancer. Each of these drugs has severe side effects as along with the cancer cells it also affects the normal cells.

Different approaches are performed in chemotherapy

- Monotherapy (uses the single drug)
- Combination chemotherapy (includes a couple of drugs which act together)
- Chemotherapy with other treatments such as surgery and radiotherapy

Some of the drugs used for treating breast cancer are mentioned below.

1.14. Types of Anti-cancer agents

Anticancer agents are either inhibitors or poisons, thus cytotoxic in nature. Based on the mode of action these anti-cancerous agents are classified into different types such as anthracyclines, antimetabolites, alkylating agents, topoisomerase inhibitors, plant alkaloids and other antitumor agents. Some of the drugs like etoposide, camptothecin, etc. directly interfere in DNA function in the cancerous cell. Other agents like inhibitors of tyrosine kinase and monoclonal antibodies do not interfere directly with DNA but specifically target molecules. Drugs like tamoxifen, raloxifene, etc., modulate tumor cells behavior without directly attacking these cells. These drugs compete with the ligand, bind to the receptor, and thus block the cell proliferation and growth of the tumor. Anti-cancerous activities of the drugs targets parameters like high rate of proliferation (Uncontrolled cell division in the population of those cells dividing rapidly with respect to the whole population) and multitude doubling time of cancer cell in breast tumor (the time taken to double in size by tumors). Dividing cells show more sensitivity towards the cytotoxic effect of the drug as compared to dormant cells. Tumors with shorter mass doubling timeare more susceptible than larger mass doubling time to drug treatment. Unfortunately, normal cells/tissues such as bone marrow, oral, intestinal mucosa and hair follicles, etc., also are dividing faster and thus damaged by anticancer agents. Uncontrolled cell division of cancer cell is major targeted by anti-cancerous agents. Cell during S phase are most sensitive to the anti-cancer agents because at that time they are undergoing “DNA synthesis”. Other agents are targeted different phases of cell cycle such as G_1 and G_2 resulting cell cycle arrest than finally, cells go into the cell death process. The G_0 phase cells are resistant to chemotherapy. In large tumors where some of the

cells are in the non-dividing stage, are not damaged by most of the anticancer agents and these cells are responsible for re-establishment of the tumor mass resulting in disease recurrence. Thus, cell cycle non-specific drugs are used to address this problem. Drug resistance is one of the biggest challenges of chemotherapy.

Drug resistance may develop due to followings:

- Drug efflux decrease drug level within the cell thus decreasing efficacy of the drug. Example Dactinomycin, epidopodophyllotoxins, vinca alkaloids and anthracyclines.
- Drug inactivation. Example "alkylating agents", "antimetabolites" and "bleomycin".
- The less active form of drug. Example "antimetabolites"(5-FU, 6-MP, etc.) During the therapeutic activity observation, these agents first converted into nucleotide.
- Alteration in amount or mutation of the targeted enzymes or receptors.

Some of the anticancer agents used for treating triple negative breast cancer are mentioned below.

1.14.1. Taxanes

Taxanes are most common chemotherapeutic drugs of this era. It acts as hydrophobic mitotic poisons, which inhibit the depolymerization of microtubules process in cell division of cancer cell. These agents are used in treatment of many cancers for example breast, lung, ovarian, prostate (KHAN et al., 2003; Tannock et al., 2004), head and neck (Nabell and Spencer, 2003) and gastric, pancreas (Di Cosimo et al., 2003; Roth and Ajani, 2003). Taxanes are divided into two main groups such as Paclitaxel (Taxol), Docetaxel (Taxotere) and taxanes homologs, which are obtained from natural sources. Paclitaxel is derived from "*TaxusBrevifolia"*(Wani et al., 1971), whereas Docetaxel which is a semisynthetic analog ofesterified derivative of "10 deacetylbaccatin-III (10-DAB)" obtained from

"*TaxusBaccata"*(Bissery et al., 1991). They have several side effect such as neuropathy or destruction of sensory nervesand developed resistivity in patients.

1.14.1.1. Effect on cell growth, proliferation and differentiation

Taxanesact by suppressing proliferation and hence, growth of cancer cells due to the inhibition of cell division through chromatid separation. Taxanes inhibit microtubule depolymerization in rapidly dividing cells, thus termed as "mitotic poison". Docetaxel is a more dominant cytotoxic agent to cancer cell as compared to the taxol(Braakhuis et al.; Vanhoefer et al., 1997). The main target of these agents are microtubules, which play a major role in transport of the cell, division of cell, transcription, post-translation modification and various other cellular processes (Nogales, 2001; Zhou and Giannakakou, 2005). Both Paclitaxel and Docetaxel work by either causing excessive microtubule polymerization or depolymerization in the cell. These agents strongly bind with β-tubulin (Rao et al., 1999; Snyder et al., 2001)and cause stabilization of microtubules during the elongation phase of polymerization, cause high rate of microtubule nucleation and growth(Derry et al., 1995; Jordan and Wilson, 1998; Yvon et al., 1999)leading to "mitotic cell cycle arrest". These agents show different effects on cancer cells depending on their concentration. Higher concentration of taxanes caused bundles formation due to microtubules rearrangement (Schiff and Horwitz, 1980; SCHIFF et al., 1979), while lower concentrations suppresses and stabilizes microtubule dynamics without alteringpolymer mass formation(Derry et al., 1995; Jordan et al., 1993, 1996; Yvon et al., 1999) and their lowest concentration cause inhibition of cell proliferation with no mitotic arrest (Giannakakou et al., 2001). These agents have different cytotoxic and anti-proliferative effect on different cancer cells depending on their concentration and time(Chan et al., 2002; Geng et al., 2003; Giannakakou et al., 2001; Guo et

al., 2002; Ilgar and Arıcan, 2009; Liebmann et al., 1993; Okano et al., 2007; Torres and Horwitz, 1998; Wani et al., 1971; Zhou et al., 1999).

1.14.1.2. Induction of various genes

Taxanes inhibit cell proliferation, angiogenesis, cancer cell growth and induce apoptosis, inflammation and transcriptional pathway in cancer cells (Moos and Fitzpatrick, 1998a, 1998b; Perera et al., 1996) by activating of some genes such as cytokines (Lee et al., 1997), interleukins and "tumor necrosis factors (TNF-α)". Taxanes such as taxol, taxotere and taxane homologs have varying efficacies to activate various genes but have a common mechanism of action. These agents induce apoptotic pathway by enhancing "Bax/Bcl-2" ratio with the help of certain proapoptotic genes such as "Yama protease (CPP32β)"(Ibrado et al., 1996), P^{21}WAF-1, c-raf-1, P^{53} (Shah and Schwartz, 2001), cdc-like kinase, activates P^{34}cdc-2, other "cyclin-depended kinases (CDKs)"(Ilgar and Arıcan, 2009; Moos and Fitzpatrick, 1998a, 1998b) and isoform of "protein kinase C (PKC)" leading to apoptotic cell death. Cancer cells also develop the resistivity against these agents by activating of some antiapoptotic genes such as Bcl-2 family proteins and p27 (Brown et al., 2004). A combination of these chemotherapeutic agents can be a possible way to resolve this problem. Taxanes translocated the "NF-κB (Nuclear Factor-Kappa B)" from the cytosol to nucleus and activate certain proapoptotic genes such as TNF-α and interleukins IL-1 and IL-6 (Perera et al., 1996). Taxol also activates caspases families due to some genes such as CHUK (Perera et al., 1996) and "NF-κB (Nuclear Factor-Kappa B)" gene which are involved in inflammatory, adhesive, invasive, acute phase properties of the cell. These agents enhanced the expression of certain cytokine genes like crg-2 and COX-2 (Moos and Fitzpatrick, 1998a) which induced inflammation, amelioration of ERK2 kinase inhibition and CDK4 led to induction of

apoptotic cell deaths (Ilgar and Arıcan, 2009; Moos and Fitzpatrick, 1998b; Shah and Schwartz, 2001).

In briefly, taxanes induced apoptotic genes, prevent cancer cell growth and inhibit resistivity against chemotherapy in the cancer cell.

In briefly, taxanes induced apoptotic genes, prevent cancer cell growth and inhibit resistivity against chemotherapy in the cancer cell.

1.14.1.3. Cell cycle and apoptosis

In several past decades, numerous methods are used to suppress cancer cell growth via inducing apoptotic cell deaths. Taxanes also kill cancer cells via activating an apoptotic process in in-vitro and in-vivo condition. Taxanescauses induction of G_2/M phase arrest of cell cycle (Shah and Schwartz, 2001) by inhibiting the depolymerization process of microtubules and cells are halted in transition from pro-metaphase to metaphase (Cunha et al., 2001)which activates apoptotic programmed cell death (Fabbri et al., 2006; Lowe and Lin, 2000). These agents activate the whole cascade of apoptotic pathways by enhancing the expression of proapoptotic genes, therefore, useful in cancer management. Activation of proapoptotic genes such as apoptotic Bcl-2 family genes induced cytotoxicity via phosphorylation of Bcl-xl and Bcl-2/Bax proteins (Moos and Fitzpatrick, 1998b; Pienta, 2001) and inhibited development of resistivity in cancer cells (Chun and Lee, 2004) and play a major role in breast cancer(Callagy et al., 2006; Noguchi, 2006) as well as prostate (Haldar et al., 1996; Yoshino et al., 2006) cancer treatment. Recent studies have found that Bcl-2 gene enhances taxanes induction of chemo-sensitivity (Ferlini et al., 2009) in solid tumors, thus,taxanes are used in cancer treatment. Taxanes induced as a result of high level of “ROS

(Reactive Oxygen Species)" generation (Geng et al., 2003). The high level of ROS regulates c-Raf-1 kinase (Moos and Fitzpatrick, 1998a; Torres and Horwitz, 1998), which upregulates the wild-type p53 as well as p21WAF1 proteins (Blagosklonny et al., 1995; Chang et al., 2006)and downregulates the protooncogene c-myc protein (el Khyari et al., 1997; Yim et al., 2004). These agents induced cell death via inhibiting the MAPK pathway and activating the ERK, JNK and P38 kinases (McDaid and Horwitz, 2001; Okano et al., 2007; Wang and Wieder, 2004). Taxanes also cause DNA fragmentation (Torres and Horwitz, 1998) when in prolonged contact with DNA. Taxanes induced apoptosis via activation of caspase-3, which is the main executioner of programmed cell deaths (Mahaffey et al., 2007; Moos and Fitzpatrick, 1998b; Torres and Horwitz, 1998). Activated caspase-3 induces "PARP (Poly-ADP Ribose Polymerase)" cleavage with the help of other ortholog caspases such as 8 and 9 in different cancers (Mahaffey et al., 2007; Solomon et al., 2008), thyroid (Meng et al., 2008), prostate and breast (Wang and Wieder, 2004) cancer cells.Taxanes are also capable of activating "caspase-independent lysosomal cell death"(Mediavilla-Varela et al., 2009). Taxanes induced apoptotic enhancers such as "ATRA (all-trans retinoic acid)", d-limonene(non-nutrient dietary component), anti-oestrogens, octreotide conjugates, cyclosporine-A and several others (Bishayee and Rabi, 2009; Bryan et al., 2011; Ferlini, 1998; Kim et al., 2006; Kucukzeybek et al., 2008; Nakahara et al., 2003; Nehmé et al., 2001; Sun et al., 2007; Wang and Wieder, 2004), therefore enhanced the therapeutic strategy of cancer treatment. Taxanes alone or in combination with another form of taxanes are capable of inducing apoptosis, which is detected with the help of elevated "apoptosis serum biomarkers" such as cyt-c, nucleosomal DNA and "caspase-cleaved cytokeratin 18 (CK18-

Asp396)"(Kramer et al., 2006). This may aid in detecting resistivity towards the drug and thus will help in assisting therapeutic strategy for cancer treatment.

1.14.1.4. Inhibitors of angiogenesis

The angiogenesis is the main process for tumor growth and cancer cell metastasis due to the new blood vessels formation in cancer patients. Taxanes inhibit the angiogenesis by regulating angiogenic cytokines "VEGF (vascular endothelial growth factor)", "bFGF (basic fibroblast growth factor)", MMP-9, MMP-2, IL-8, by upregulating E-cadherin and nm23, and decreasing MVD (intra-tumor micro-vessel density) in cancer cells. Therefore taxanes act as angiogenic inhibitors when used as monotherapy or in combination therapy. These agents regulate the VEGF and bFGF in combination with pro-apoptotic agents (Chan et al., 2002), endogenous estrogens (Klauber et al., 1997), angiogenic inhibitors (Inoue et al., 2003), tyrosine kinase inhibitors (Naumova et al., 2006), anti-Erb-2 (Klos et al., 2003), thalidomide (Zhang et al., 2005), "bevacizumab"(Fujita et al., 2007)and angiostatin gene (Galaup et al., 2003) in different tumors such as breast, bladder, ovarian, lung, liver and prostate tumors. Taxotere is a major inhibitor of migration, invasion, angiogenesis and proliferation of endothelial cell as compared to the taxol in "*in-vivo"* and "*in-vitro"* condition (Grant et al., 2003; Hotchkiss et al., 2002; Pölcher et al., 2010).

1.14.2. Cisplatin

Cisplatin is one of the heavy metal based cancer chemotherapeutic compound that has a range of unique biological effects. The chemical name of cisplatin is "*Cis*-(II) platinum diaminedichloride (*cis*-DDP)". Michele Peyrone in 1845 described this compound and this was known as "Peyrone's salt" for a long time. This was discovered by Alferd Werner, Barnett Rosenberg and his colleagues (Rosenberg et al., 1969; ROSENBERG et al., 1965).

Cisplatin is an established very efficient drug for treating bladder carcinoma, testicular, ovarian carcinoma, and neck cancer. The anti-cancerous property of cisplatin is observed to be best in aqueous solution. The platinum has tetravalent valances in cisplatin structure, two of which binds to chloride ions and the other two binds with NH_3 group. Only the cis-dichloro structure of cisplatin act as an active antitumor agent. Inability to form stable cross-link with DNA, this form of the compound lacks cytotoxic effect (Filipski et al., 1980). In an aqueous medium, the chloride atom of cisplatin is displaced and platinum bind with a water molecule and form an aqua-complex $[PtCl(H_2O)(NH_3)_2]^+$. This type of reaction is fast in low concentration of chloride ions environment such as inside the cell or in the urine. This active aqua-complex interacts and forms bi-functional covalent bonds via nucleophilic reaction with biomolecules e.g. DNA, RNA or protein which is similar to alkylation reaction. This aqua complex prefers guanine and form $[PtCl\ (guanine\text{-}DNA)(NH_3)_2]^+$. Cisplatin can form complex with DNA in several different ways and ultimately resulting in DNA damage (Cohen et al., 1979; Scovell and O'Connor, 1977; Zwelling and Kohn). The DNA repair machinery is activated but due to the cross-linking unable to perform repair. This activates apoptotic pathway by which the affected cells are killed. The cell cycle inhibited by cis-DDP is poorly understood. In G1 phase (intermitotic) of the cell cycle, some of the cells are sensitive to cis-DDP because DNA synthesis rate is less as compared to the S phase (Glover et al., 1984). Thiourea, sodium thiosulfate and diethyl dithiocarbamate may prevent formation of DNA cross links. An elevated level of intracellular "glutathione (GSH)" or metallothionine (thiol-rich protein) was observed which might be responsible for the inhibition of the repair pathway.

These drugs have several side effects such as neurotoxicity, nausea, vomiting, low white and red blood cell counts, hair loss, etc. So there is a need to develop the anti-cancer compound, which hasa novel structure with a new mechanism of action that can specifically target cancerous cell and have the minimized effect on normal cells. Keeping this objective in mind, the present study was performed in the direction of discovering a potentially effective drug. Large number of sulfonamide compounds are available in the Pubchem but their anti-cancerous activity has not been investigated yet. We used CID-6861424 which is one such derivative of sulfonamide, is known for exerting“antimicrobial” as well as “anti-inflammatory” activities(Kia et al., 2009)but nothing is known about its anti-cancer activity. It is synthesized by the group of Prof Amir Azam, “Department of Chemistry”, “JamiaMiliaIslamia University”, New Delhi.

The structures of CID-6861424 for the present work are as follows:

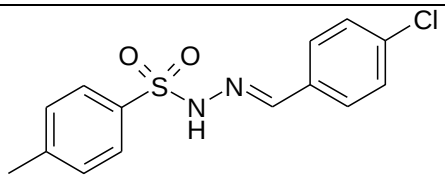
Name of Synthetic Compound	**Exact structure.**	**Molecular Weight**
CID-6861424		308.79

Table No. 1 Structure of CID-6861424 Synthetic compound used in the study.

1.15. Research Objectives

Chemotherapy has been used totreat breast cancer from long time but its major challenge is toxicity towardnon-cancerous, rapidly proliferating cells such as those of bone marrow. Over the course of time the cancer cells have also begin to show resistance towards available drugs which results in multidrug resistance. These are the two most glaring problems associated with the use of chemotherapy. Our objective was to address these problems and find out a way by which specific toxicity towards breast cancer cells could generated.We also wanted to find a compound which can inhibit the growth of breast cancer cells and could also sensitize breast cancer cells to low dose of γ-radiation which could limit the toxicity to non-cancerous cells. Keeping these in mind, following objectives were designed for this study:

1. To evaluate the effect of synthetic compound(s) on the cell viability in breast cancer cell lines.

2. To evaluate the effect of synthetic compound(s) on cell cycle profile and related proteins.

3. To elucidate the mechanism(s) responsible for possible anticancer effects of synthetic compound(s).

Chapter 2

MATERIAL AND METHODS

CHAPTER-2

MATERIAL AND METHODS

2.1 Cell line and reagents

"MCF-7, MDA-MB-231 and NIH3T3" cells was obtained from "American Type Culture Collection (Manassas, VA)" and grown in DMEM and Leibovitz L-15 medium (Invitrogen) supplemented with 10% heat-inactivated FBS (Invitrogen) and 5% penicillin-streptomycin (Invitrogen) at 5% CO_2, 37°C in a "humidified chamber" for "MCF-7 and NIH3T3" cells. "MDA-MB-231" cells were grown without CO_2 at 37°C in a "humidified chamber". Antibodies used from "Cell Signaling Technology (CST)" and "Santa Cruz Biotechnology (SCBT)": cyclin B1 (#4138), cyclin D1 (#2922), CDK-1 (#9116), p21 (#2947), PTEN (#9559), p-Akt (#4058), Akt (#9272), p-GSK-3β (#9323), GSK-3β (#9832), p-ERK (#9101), ERK (#4695), Bax (#5023), Vimentin (#5741), CDK-4 (sc-260), CDK-6 (sc-177), p53 (sc-126), Bcl-2 (sc-7382), γ-H2AX (sc-517348), Survivin (sc-17779), β-actin (sc-47778),secondary anti-rabbit & anti-mouse antibody (#7074S & #7076S). FITC labelled secondary antibody was obtained from (Invitrogen). "Rhodamine123 (RH-123)" (546054) and "2',7'-Dichlorofluorescein diacetate (DCF-DA) (D6883)", "Propidium Iodide (PI)" (P4170), Trypan blue dye (C.I. 23850) and Agarose (A9539) were obtained from Sigma Aldrich. Low melting agar (36601) was obtained from SRL Chemical India.Nylon mesh filter (70μM) were purchased from Corning (cat-1995-065), 10cc syringes and 23G needles were purchased from BD Biosciences (cat-309604 and 305145). Chemicals purchased from HI media were; "anhydrous tetra-Sodium pyrophosphate (cat-GRM7515)", "Trichloroacetic acid (cat- 0219605780)", "Nitroblue tetrazolium chloride (NBT) (cat-RM578)", "N–

Ethylmaleimide(NEM) (cat-RM1498)", "2-Thiobarbituric acid (TBA) (cat-RM1594)", "NADPH (cat-RM393)", "5,5'-Dithiobis (2-nitrobenzoic acid) (DTNB) (cat- GRM1677)", "Glutathione reductase (GSH) (cat- 210068901)" and "Phenazinemethosulphate (PMS) (cat-MB206)".All the reagents were prepared as per suppliers instruction using respective solvents and triple distilled water ("Millipore"). Other usual chemicals/reagents were obtained in their commercially available highest purity grade.

2.2 Synthesis of CID-6861424

The CID-6861424 was synthesized through reaction of p-Tosylhydrazine and 4-chlorobenzaldehyde by our collaborators Prof. Amir Azam as followed the same protocol Reza Kia *et al*. (Kia et al., 2009).

2.3 Irradiation of cancerous and non-cancerous cells

Cells were irradiated using ^{60}Co in a gamma-chamber (240 TBq^{60}Co Model 4000A) obtained from the isotope division of the Bhabha Atomic Research Centre (BARC), Mumbai, India. The dose rate was described by Fricke's dosimetry and was 3rad/sec.

2.4 Cell viability assay

Cells were seeded in 2ml medium in 35mm^2 culture dishes and exposed toindicated concentrations of CID-6861424 or 2Gy-γ-radiation alone or combination of 2Gy-γ-radiation and 25µM-CID-6861424 for specific time as indicated at 5% CO_2,37 ºC. After specific time interval, cells were harvested, washed with 1XPBS and collected in their respective tubes. To 100µl of cell suspension, 10µl of "0.5% trypan blue" dye was mixed and pipetted well and then left at room temperature for 5-10 minutes. For cell counting, 12µl of stained cell suspension was taken and loaded on hematocytometer. Live (unstained) and Dead (blue

stained) cells were counted using an inverted microscope and the procedure was repeated for every set of each sample. Every sample was counted in triplicate and total cell number of viable & dead cells was determined

Percentage of viable cells: **No. of viable cells (unstained)/Total no. of the cells X 100**.

2.5 Cell morphology study

Cells were seeded in 35mm^2 culture dish and were treated with specific concentration of CID-6861424 or 2Gy-γ-radiation alone or combination of 2Gy-γ-radiation and 25μM-CID-6861424 for 72hours. Morphological changes were studied using bright field microscopy. All images were captured at 20X magnification (NIKON ECLIPSE Ti-S, Tokyo, Japan) and analysed (NIS Elements D, Tokyo, Japan).

2.6 Wound-healing assay

Cells were seeded in a 35mm^2 culture dish and grown up to 80% confluency.Wound was formed by scratching the monolayer using a 200μl sterile tip. Cells were then exposed toindicated concentration of CID-6861424 or 2Gy-γ-radiation alone or combination of 2Gy-γ-radiation and 25μM-CID-6861424 for 72hours. Wound widths were measured at 0 hour and post 72 hour using bright field microscope (NIKON ECLIPSE Ti-S, Tokyo, Japan) and analysed (NIS Elements D, Tokyo, Japan).

2.7 Clonogenic survival assay

Cells were plated in 35mm^2 culture dishes and were treated with specific concentration of CID-6861424 or 2Gy-γ-radiation alone or combination of 2Gy-γ-radiation and 25μM-CID-6861424 for 72hours. Post incubation, cells were harvested and ~400 to 600 cells per well

were seeded in a 6-well culture plate and 60mm^2 culture dishes with 5mL of medium per well. The plates/culture dishes were incubated at 5% CO_2, 37°C or 37°C in a humidified chamber for ~14days. Number of the colonies were observed post 14 days by fixing in chilled methanol and staining with 5 % crystal violet.

2.8 Comet assay by single gel electrophoresis

Cells were seeded in 35mm^2 culture dishes and treated with 25µM-CID-6861424 or 2Gy-γ-radiation alone or in combination for 72hours. ~20,000 trypsinized cells were used for single gel electrophoresis. Comet assay was carried out as per the standard protocol (Dhawan et al.).Slides were observed under the fluorescence microscope ("NIKON ECLIPSE Ti, Tokyo, Japan").“NIS Elements AR version-3.000 software” was used for analysis of the fluorescence intensity of the cells.

2.9 Cell cycle analysis

Cells were seeded in 35mm^2 culture dish and treated with specific concentration of CID-6861424 or 2Gy-γ-radiation alone or combination of 2Gy-γ-radiation and 25µM-CID-6861424 for different time intervals. Cells were trypsinized, washed with 1XPBS, fixed in 70% chilled ethanol, treated with RNaseA enzyme and stained with “propidium iodide (PI)”. DNA content of “10,000 events/cells” was measured using “flow cytometer” (BD Bioscience) and analysed by “modFit LT (verity software house)” software.

2.10 Reactive Oxygen Species (ROS) generation assay

ROS generated was measured by flow cytometry following staining with “DCFH-DA” dye. Cells (4x 10^4/ml) were seeded in 35mm^2 culture dishes and after overnight incubation, cells

were treated with different concentration of CID-6861424 or 2Gy-γ-radiation alone or combination of 2Gy-γ-radiation and 25µM-CID-6861424 for 72 hours. After that cells were stained with 8 µM DCFH-DA dye at 37^0C for 30 min, and washed twice with 1X PBS, then cells pellets were finally suspended in 300µl 1X PBS. Fluorescence measurement was done by flow cytometry (BD Bioscience).

2.11 Mitochondrial membrane potential (Δψm) assay

Δψm depletion in control and cells treated with CID-6861424 or 2Gy-γ-radiation alone or combination of 2Gy-γ-radiation and 25µM-CID-6861424 treated cells were measured by flow cytometry by staining with Rhodamine 123 dye. Briefly, cells (4x 10^4/ml) were plated in 35mm^2 culture dishes and after overnight incubation, cells were treated with different concentration of CID-6861424 or 2Gy-γ-radiation alone or combination of 2Gy-γ-radiation and 25µM-CID-6861424 for 72 hrs. Cells were then stained with 10 µM Rhodamine 123 dye for 30 min at 37^0C, and washed twice with 1X PBS & cell pellets were finally suspended in 300µl 1X PBS. Fluorescence was measured by flow cytometry (BD Bioscience).

2.12 Apoptosis assay

Apoptosis was quantified in CID-6861424 treated breast cancer cells (MCF-7 & MDA-MB-231 cells) with FITC annexin V/7AAD kit from BD Biosciences. Cells (4x 10^4 cells/ml) were plated in 35mm^2 culture dishes and after the overnight incubation, cells were exposed to different concentration of CID-6861424 for 72hrs. Cells were collected and processed

according to the protocol specified by manufacturer and apoptotic cells were quantified by flow cytometry (BD Biosciences).

2.13 Immunofluorescence assay

Around 8,000 cells were plated in 35mm^2 culture dish above the coverslip and then treated with indicated concentration of CID-6861424 or 2Gy-γ-radiation alone or combination of 2Gy-γ-radiation and 25μM-CID-6861424 for 72hours. After 72hours of incubation, cells were processed and incubated with γ-H2AX, Cyclin B1, CDK-1, β-tubulin and p53 primary antibody (1:50, overnight, 4°C) and FITC labelled secondary antibody (1:10,000, room temperature, 1hour). DAPI (1:1000) was used to counterstain the nucleus. Coverslips were mounted using antifade (sigma) over the slides. Slides were observed under 60X under a fluorescence microscope (NIKON ECLIPSE Ti, Tokyo, Japan). Fluorescence intensities from images of randomly selected microscopic fields of cells were semi-quantitatively analyzed by NIS Elements AR version-3.000 software. For every set of data, more than 50 cells were quantified.

2.14 Western blot analysis

Expression level of indicated proteins were analyzed by Western blot. ~80,000 cells/ml were plated in 100mm^2 culture dishes and exposed to indicated concentration of CID-6861424 or 2Gy-γ-radiation alone or combination of 2Gy-γ-radiation and 25μM-CID-6861424 for indicated time points. Cells were harvested and lysed in lysis buffer (50mM TrisCl-pH 8, 300mM NaCl, 10% glycerol, 1% NP-40, and 2mM EDTA) at 4ºC. Protein quantification was done by Bradford assay and then quantified protein extract was run on SDS-PAGE. The

proteins were transferred to PVDF membrane and probed with particular primary and secondary antibodies to detect the protein of interest by manual “ECL method”. The images were captured in “X-ray films” and all results were analyzed with respect to “loading control (β-Actin)”.

2.15 Animals

4-6 weeks old and randomly bred female BALB/c mice with 25±2.0 g of weight were used for the study. They were maintained in “animal facility at Jawaharlal Nehru University (JNU), New Delhi” with prescribed conditions of “ad libitum pellet-diet”, tap water and alternate per day cycles of light and darkness. Their care was in compliance with the “Committee for the Purpose of Control and Supervision of Experiments on Animals (CPCSEA).” All experimental protocols were approved by “institutional Animal Ethics Committee” (IAEC code-22/2015 dated November 19, 2015). Animals were randomly grouped into three separate sets, with four mice per set and all the treatments were carried out by intraperitoneal dose. Set-I (Control) animals were administered DMSO, vehicle of the CID-6861424. Set-II, and Set-III were treated with a dose of 5mg/kg and 10mg/kg of animal body weight of CID-6861424 respectively, the treatment was aseptically given for 30 alternate days.

2.16 Isolation of bone marrow cells

The bone marrow cell were isolated under aseptic conditions as per Xiaoyu Liu et al., (Liu and Quan, 2015). Briefly, after dissecting out tibia and femur, their ends were sliced off. Then using a 10cc syringe with 23 gauge needle filled with chilled HBSS buffer, the bone

marrow cells were flushed on to a nylon mesh filter (70µM) attached to a 50 ml falcon tube. The cells were then centrifuged at 1500 rpm for 10 min at 4°C. The pellets were resuspended

in RBC lysis buffer, incubated for 5 min at room temperature and neutralized by 5 ml FBS. The cells were then centrifuged at 1500 rpm for 10 min at 4°C and the pellets were resuspended in DMEM media with 10% FBS and stored at 4°C for further processing for comet assay.

2.17 Preparation of blood hemolysate and tissue homogenate

After the treatment of CID-6861424 for 30 days, ~300µl of blood was sampled by retro-orbital sinus bleeding and collected in vials containing 10 units of heparin. Blood hemolysates were used for SOD and Catalase, by preparing 20% hemolysates in chilled distilled water. Blood related experiments were carried out on the day of blood isolation. Animal euthanization and dissection was carried out next day. Liver perfusion was done by chilled normal saline and was isolated followed by isolation of kidney and spleen. Liver, kidney and spleen homogenate (1gm/mL) was prepared in 0.1M phosphate buffer, pH 7.4.

2.18 Determination of DNA damage in blood and bone marrow cells by comet assay

Diagnosis of DNA damage was carried out by comet assay as per Singh et al., (Singh et al., 1988; Tice et al., 2000) with slight modification. Blood and bone marrow cells in with approximately 20, 000 cells were mixed with 75µl of 1% “low melting agarose (LMA) (prepared in ion-free PBS, pH 7.5)” and was smeared to 0.5% “normal melting agar” pre-

coated slide by using coverglass. Once LMA solidified, another LMA layer was coated and cells were then lysed with the help of alkaline lysis buffer ("2.5M NaCl, 100mM EDTA, 10mM Tris (pH 10), freshly added 10% DMSO and 1% Triton X-100") at 4ºC in dark for 3 hours. The slides were then placed in electrophoresis buffer ("1mM Na_2EDTA and 300mM NaOH, pH >13") for half hour at room temperature in dark and electrophoresis was performed for 30 min at 24 volts and 300 milliamperes current. The slides were then washed twice with chilled neutralization buffer ("0.4M Tris, pH 7.5"), stained with 75µl of 1X EtBr and comets were counted microscopically using a fluorescence microscope (Olympus).

2.19 Protein estimation in blood and tissue samples

Total protein in blood and tissue samples was estimated as per method described by Bradford (Bradford, 1976) at 650 nm wavelength, and protein content was calculated against standard concentrations of "Bovine serum albumin (BSA)". Blood samples were centrifuged at 500 rpm, 4°C for 10 minutes, supernatant was then centrifuged at 12000 rpm, 4°C for 15 minutes and protein was estimated. Protein content from 0.2 mL of 20% of homogenates was obtained by overnight precipitation in "0.2 mL of TCA" and centrifuged at 2000 rpm for 10 minutes at 4°C. Pellets were dissolved in "0.2 mL of 0.1N NaOH" and used for protein estimation.

2.20 Estimation of reactive oxygen species

Blood and tissue ROS was described as per Socci et al., (Socci et al., 1999) using "2',7'-dichlorofluorescein diacetate (DCFDA)" dye, which upon oxidation produces fluorescence measured at 488 nm excitation and 525 nm emission wavelengths . 50µl of whole blood

and 1% tissue homogenates were mixed to “40mM of Tris-HCL buffer”, pH 7.4. To this 40µl of DCFDA was added. The mixture was incubated for 15 min, 37°C and fluorescence was measured and ROS content was defined as “DCF formed/min” per mL of RBC or mg tissue.

2.21 Measurement of Thiobarbituric acid (TBA) reactive substances for lipid peroxidation

Lipid peroxidation in samples was estimated by determination of TBA as described by Dwivedi et al., (Dwivedi et al., 2014) at 535 nm and expressed as nanomoles of “MDA/mL blood or µg/g tissue samples”. Blood TBA was measured by mixing 100µl of hemolysate and “0.2 mL of 8% SDS”, to this “1.5 mL 20% acetic acid” and “1.5 mL 0.8% freshly prepared TBA” and 0.7 mL of “distilled water (dH_2O)” was added sequentially. The contents were boiled for 60 minutes, and a characteristic “pink colour” was observed. The mixture was cooled and 1mL cold “distilled water (dH_2O)” was added, centrifuged (6000rpm, 4°C for 15 minutes) and absorbance of supernatant was measured. Tissue TBA was measured by mixing, 0.5 mL of “20% homogenate” with “0.5 mL of 0.15 M KCl” and incubated at 37°C, 30 minutes. To this, 1 mL 10% TCA was added and “centrifuged” at 4000 rpm for 15 min. Supernatant (500µl) was mixed with 0.5 mL of TBA and the contents were initially boiled for 60 minutes. Tissue samples were then processed as blood samples.

2.22 Determination of reduced Glutathione (GSH)

Blood “glutathione (GSH)” measurement was carried out as per Ellman et al., (Ellman, 1959), with few modifications at 412nm and expressed as mg/mL of samples. 0.2 mL of blood was mixed with 1.8 mL of “distilled water (dH_2O)” and "4% sulfosalicylic acid”,

centrifuged at 2500 g, 15 minutes. 1 mL of supernatant was then mixed “2 mL phosphate buffer” and “0.4 mL DTNB” and absorbance was measured. Tissue GSH was measured by slightly modified protocols described by “Hissin and Hilf” (Hissin and Hilf, 1976) and “Dwivedi et al” (Dwivedi and Flora, 2011). 0.375 mL of “20% tissue homogenate” was mixed with “1.125 mL of phosphate–EDTA buffer” and 1 mL of “25% HPO3” and “centrifuged” at 3000 rpm, 4 °C, 10 min for protein precipitation. The mixture “centrifuged” at 4°C, 10,000 g for 30 min. “0.2 mL of supernatant” was mixed with “1.8 mL of phosphate–EDTA buffer” and “0.1 mL of OPT”. The reaction mixture was incubated (in dark) for 15 minutes at 37°C. Absorbance was measured at 420 nm, and GSH levels in mg/g of tissue sample were calculated.

2.23 Catalase assay

Blood and tissue catalase was estimated as per Sinha et al., (Sinha, 1972). 0.25 mL of 20% tissue homogenates was mixed with “0.75 mL of phosphate buffer”, centrifuged at 2500 rpm, 4°C for 10 minutes. 0.1 mL of supernatant and hemolysate (from blood) were then separately mixed with 1 mL of phosphate buffer, 0.4 mL distilled water and 0.5 mL of H_2O_2 and incubated for 1 min at 37°C. A control reaction mixture without samples was also setup. Reactions were stopped by 2mL of acetic acid with dichromate mixture (“5% potassium dichromate and glacial acetic acid in 1:3 ratios”). The mixtures were boiled for 15 min, cooled, and absorbance was measured at 570 nm.

2.24 Superoxide dismutase (SOD) assay

Tissue SOD activity was evaluated by as per Kakkar et al., (Kakkar et al., 1984), while blood SOD, was evaluated as per Weinterbour et al., (Winterbourn et al., 1975) with slight modifications. 0.3 mL hemolysate was mixed with "0.3 mL of PMS" and NBT along with 0.8 mL of distilled water and "0.2 mL NADH". 0.25 mL 20% homogenate was mixed with "0.75 mL of phosphate buffer" and centrifuged at 10,000 rpm, 4°C for 10 minutes. 0.2 mL of supernatant was then mixed with "1.2 mL of sodium pyrophosphate" and "0.3 mL of PMS", resulting in violet colour mixture. To this "0.3 mL of NBT", 0.8 mL of distilled water and "0.2 mL of NADH" was added. A control reaction mixture without samples was also setup. Reaction mixtures were then incubated at 37°C for 90 seconds and reactions were stopped by adding of "1 mL of acetic acid" and absorbance was measured at 560nm.

2.25 Statistical analysis

All experiments were minimally carried out for three times. Statistical analysis done by "Graph Pad Prism software". Experimental data are expressed as means ± S.D. and the significance of differences was analyzed by one-way ANOVA test. Tukey test for cell viability with different concentrations of CID-6861424 and Dunnett test for 25μM-CID-6861424, 2Gy-γ-radiation respectively or in combination treated MCF-7, MDA-MB-231 and NIH3T3 cells for different experiments. The t-test for different experiments of CID-6861424 treated breast cancer cell lines. Difference with a value of $P < 0.05$ were considered statistically significant.Western blotting data quantified by Image J software.

Chapter 3

CID-6861424 INDUCES APOPTOTIC CELL DEATH IN MCF-7 ER-POSITIVE BREAST CANCER CELL LINE VIA INHIBITION OF AKT CELL PROLIFERATION AND ERK CELL SURVIVAL PATHWAY

CHAPTER-3

CID-6861424 INDUCES APOPTOTIC CELL DEATH IN MCF-7 ER-POSITIVE BREAST CANCER CELL VIA INHIBITION OF Akt CELL PROLIFERATION AND ERK CELL SURVIVAL PATHWAY

3.1 Introduction

Worldwise every year almost 18.1 million new cancer cases are reported and the mortality rate due to various type of cancer is around 9.6 million (Bray et al., 2018; Siegel et al., 2018). The number of cancer affected people are increasing all over the world. Mortality rate due cancers such as breast, lung, oral, cervical and colorectal cancer are high in India. Continuous research for better hormonal, radiation, chemotherapy is going on all over the world to develop an effective treatment to cure different types of cancers.

Women are affectd by breast cancer worldwide and is major cause of death accounting to almost 15% of death caused due to cancer among women in 2018 (Bray et al., 2018; Siegel et al., 2018). It is an economic liability for the country. Estrogen receptor (ER) plays an important role in cellular signaling as well as breast cancer development. Out of the total number of breast cancer cases, almost 80% cases are ER positive (Konecny et al., 2003). Various features like cell type, nuclear and cytoplasmic localization, factors and cofactors modulate the ER signaling pathway. Endocrine therapy like tamoxifen competes with estrogen for binding to ER and blocks the signal transduction pathway leading to blockage of cell growth and proliferation of breast cancer cells. While it acts as an antagonist in the

hjbreast cell, it act as estrogen in other cells like uterus and endometrium cell activates cellular signaling for cell proliferation leading to in development of endometrium cancer, one of the major side effect of tamoxifen. Moreover due to intrinsic and acquired resistance the The effectiveness of most of the available drugs are reduced (Fan et al., 2015). Resistance towards the drugs is one of the biggest challenge and there is continuous search for effective drugs with lower cytotoxicity and side effect for breast cancer.

Sulfonamide compounds having functional sulfonamide group affects diverse biological activities. They show antibacterial, diuretic, hypoglycemia, anti-carbonic anhydrase, including anti-cancerous activities (casini et al., 2002). Large number of these compounds are displayed in the Pubchem but their antitumor property has not been looked into yet. CID-6861424 is one such derivative of sulfonamide, which is known to exert antimicrobial and anti-inflammatory activities (Kia et al., 2009) but nothing is known about its anti-cancer activity. We checked the anti-cancerous activity of the compound on breast cancer MCF-7 cell line. Here we show that CID-6861424 inhibited MCF-7 cell division. The inhibition is more with the increase in dose and time. In MCF-7 cells. IC_{50} value of 50μM was observed. Exposer to CID-6861424 resulted in downregulation of cyclin D1-CDK4/6 and G1 cell cycle block. 50μM compound disrupted **Δψm,** increased ROS and DNA damage. It upregulated p53, p21 and γ-H2AX protein expression levels. 50μM CID-6861424 resulted in upregulation of Bax/Bcl-2 ratio, PTEN and downregulation of phosphorylated Akt, GSK-3β and ERK protein expression and mitochondrial-mediated apoptotic cell death via inhibition of Akt cell proliferation and ERK cell survival pathway in MCF-7 cell. Our results show that CID-6861424 can be used as an anticancer agent to improve chemotherapy of breast cancer.

3.2 Results

3.2.1 CID-6861424 inhibit cell proliferation of MCF-7 cell

By Trypan blue exclusion assay, we checked the MCF-7 cell viability post 24, 48 and 72 hours CID-6861424 treatment. Different concentration of CID-6861424 were used as indicated **(Figure 3.1; Table No. 3.1)**. With the increase in time and concentration, the cell viability decreased. Post 72 hour CID-6861424 treatment IC_{50} value of ~ 50µM was observed.

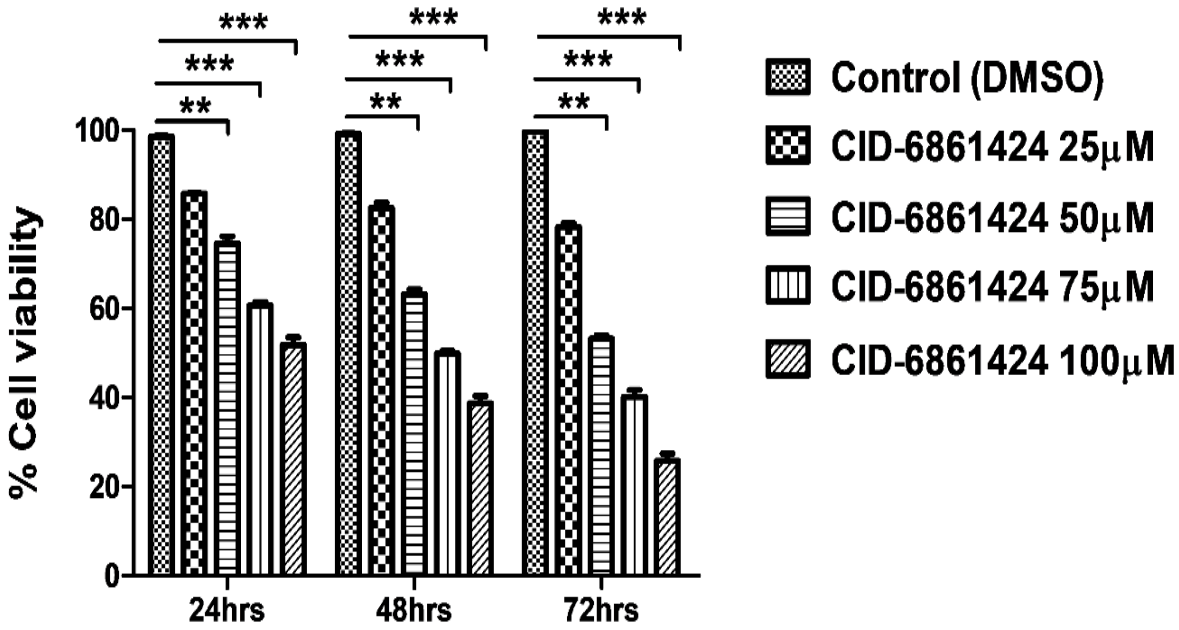


Figure. 3.1 CID-6861424 effect on cell viability of MCF-7 cell shown by bar diagram at indicated concentrations and time by Trypan Blue assay. Mean ± SD value (Data represented was calculated for three independent experiments). **P<0.01 and ***P<0.001.

Time after treatment	Treatments	MCF-7
24hrs.	CONTROL (DMSO)	98.55 ± 0.36
	CID-6861424 25µM	85.78 ± 0.23
	CID-6861424 50µM	74.65 ± 2.73
	CID-6861424 75µM	60.73 ± 1.24
	CID-6861424100µM	51.90 ± 2.77
48hrs.	CONTROL (DMSO)	99.23 ± 0.30
	CID-6861424 25µM	82.55 ± 2.05
	CID-6861424 50µM	63.17 ± 1.96
	CID-6861424 75µM	49.90 ± 1.00
	CID-6861424100µM	38.70 ± 2.84
72hrs.	CONTROL (DMSO)	99.83 ± 0.16
	CID-6861424 25µM	78.30 ± 1.43
	CID-6861424 50µM	53.19 ± 1.27
	CID-6861424 75µM	40.22 ± 2.52
	CID-6861424100µM	25.75 ± 2.99

Table No 3.1 CID-6861424 inhibit the cell proliferation of "MCF-7" cell with the increase in concentration and time points by trypan blue assay.

Compared to the control set, the cell number reduced drastically with increasing concentration of CID-6861424 at 72 hours **(Figure 3.2)**.

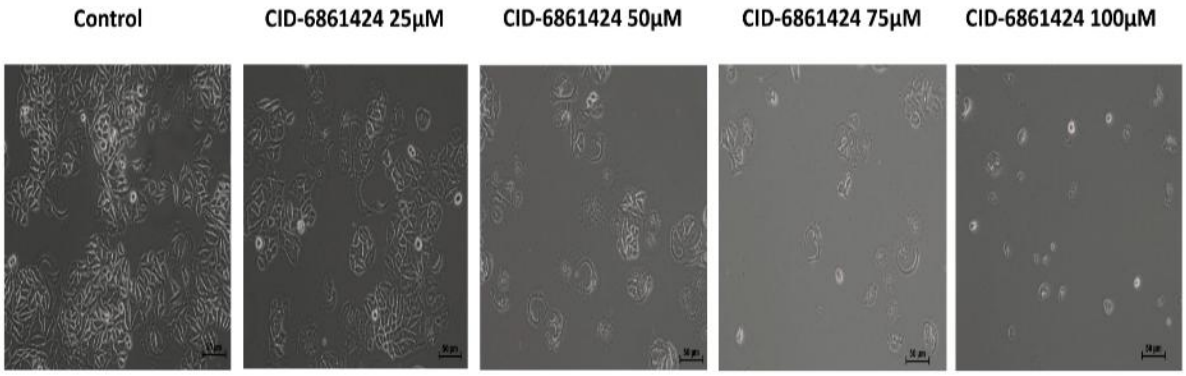


Figure. 3.2 Changes in cell morphology, using a bright field microscope at 20X, was observed post 72hours treatment with indicated concentrations of CID-6861424.

Using Bright field microscopy changes in cell morphologies at indicated concentration of CID-6861424 was observed. At 50µM CID-6861424, cells became elongated flattened with dense cytoplasm and rough cell membrane **(Figure 3.2**). With increase in concentration, changes became more prominent. At 75µM and 100µM, all cells displayed completely distorted morphology suggesting cell death **(Figure 3.2**). With the help of β-tubulin staining cytoskeletal structural changes were observed when exposed to 50µM CID-6861424 for 72 hours **(Figure 3.3**).

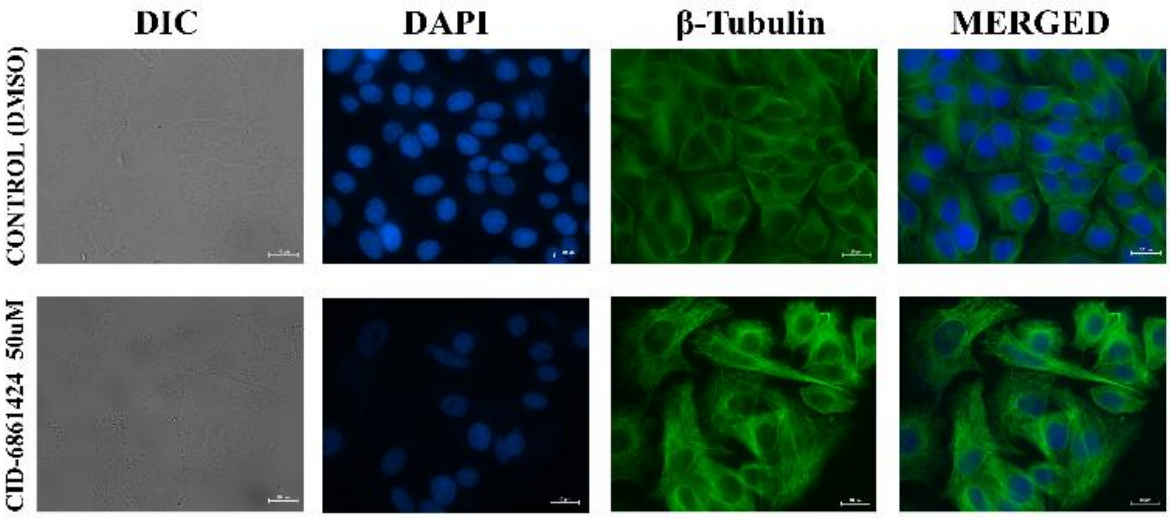


Figure. 3.3 Changes in cell cytoskeleton visualized by β-tubulin post 72hours 50µM CID-6861424 exposure using Fluorescence microscope at 60X magnification.

Pre-wound and post-wound widths were measured after 72hours of treatment with either 25µM or 50µM CID-6861424 for the scratch assay. Wound-healing capacity decreased incrementally with a corresponding increase in CID-6861424 concentration, ranging from ~80% wound-filling for control cells to ~40% of wound closure observed in 50µM CID-6861424 treated cells. 25µM CID-6861424 treatment resulted in ~ 60% of wound closure **(Figure 3.4 (a) and 3.4 (b); Table No. 3.2)**.

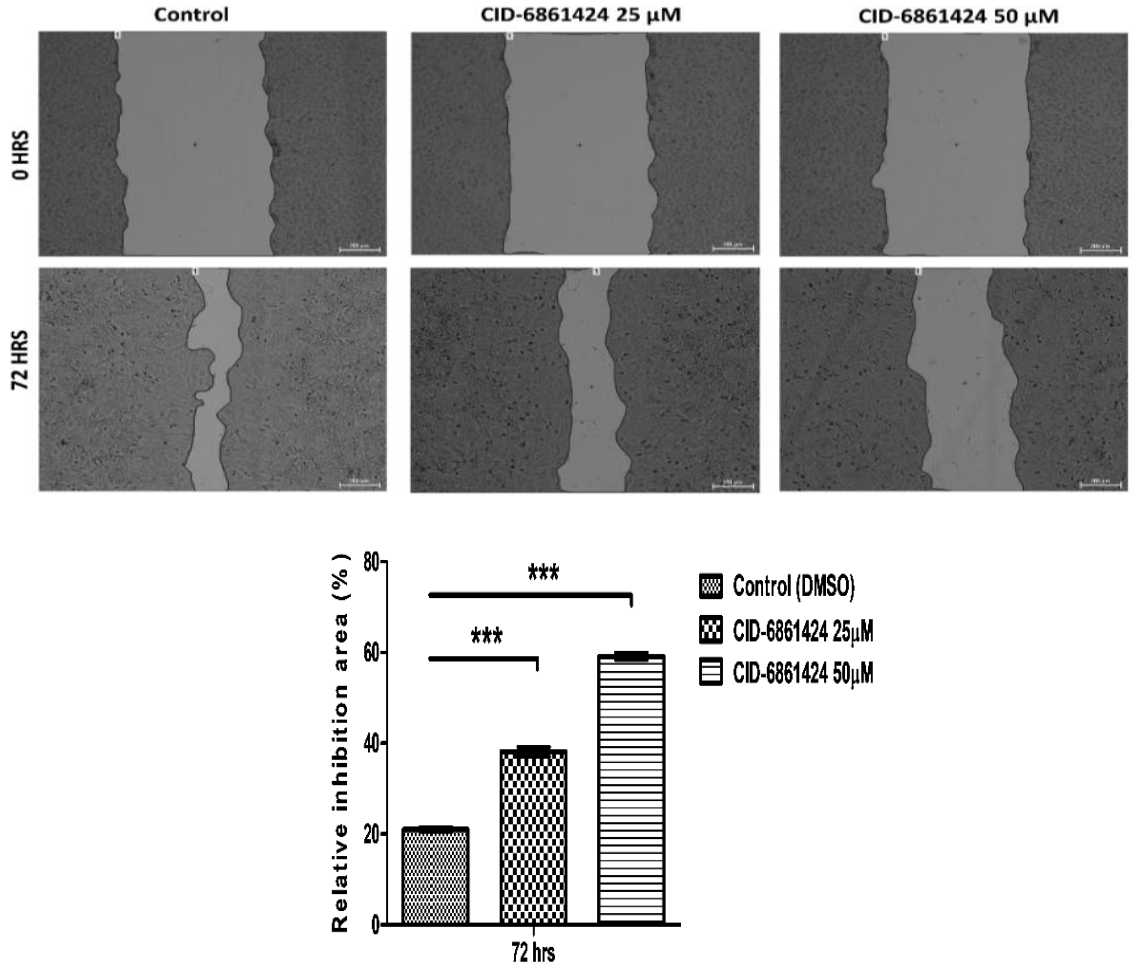


Figure. 3.4 (a) CID-6861424 inhibit the cell migration potential. (b) bar-diagram representation of relative inhibition of wound healing area of MCF-7 cell. Mean ± SD value (Data represented was calculated for three independent experiments). ***P<0.001.

Cell Line	Time Point	Control	CID-6861424 25µM	CID-6861424 50µM
MCF-7	0 Hrs	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
	72 Hrs	20.95 ± 1.31	38.10 ± 1.88	59.11 ± 1.36

Table No. 3.2 CID-6861424 inhibit the cell migration potential of MCF-7 cell at 72 hours.

Colony forming assays were performed to check clonogenic potentials. Cells were treated at 25µM and 50µM CID-6861424 for 72hours followed by harvesting. Around 600 harvested cells were seeded in fresh culture plates and incubated for ~14 days. Post incubation the number of colonies were compared for each set. The result showed that CID-6861424 significantly decreased the colony forming ability of MCF-7 cells and it is dose dependent. Decline in colony forming ability was noticeable in 50µM as compared to the 25µM CID-6861424 treated and control set **(Figure 3.5)**.

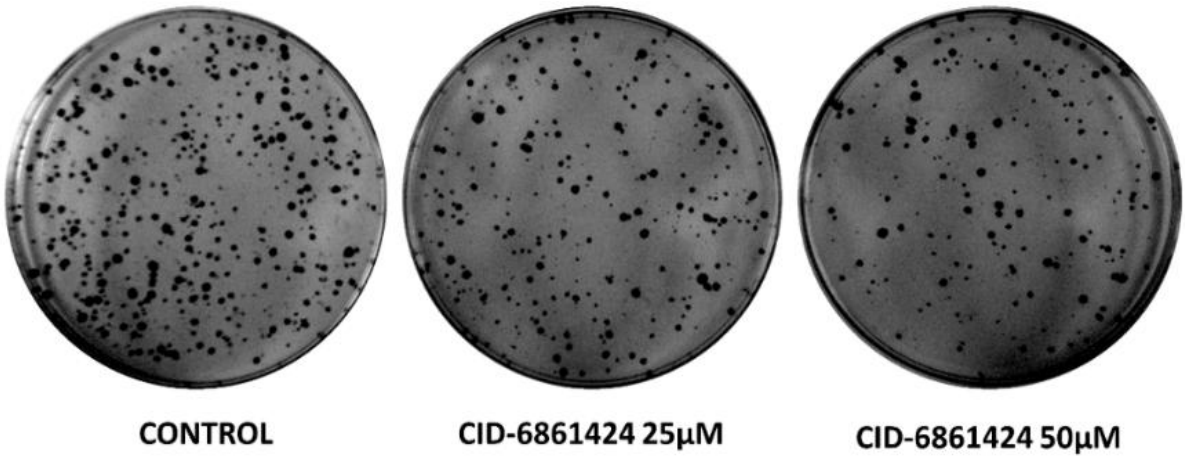


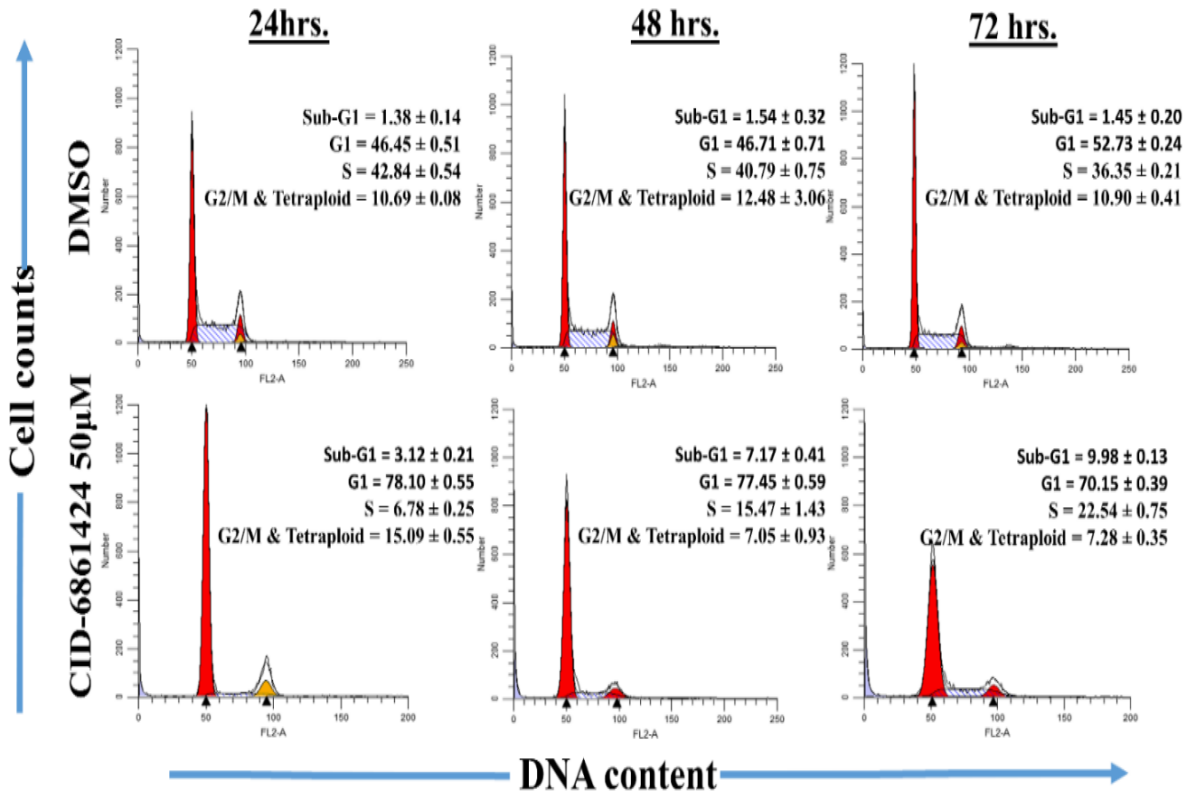
Figure. 3.5 The reduced number of the MCF-7 cell colonies in the presence of CID-6861424 treatment.

These results suggested CID-6861424 significantly inhibits the MCF-7 cell viability, cell counts, cell migration and colony formation potential.

3.2.2 CID-6861424 induces cell cycle arrest at G_1 phase in MCF-7 cell

MCF-7 cell cycle profile was studied by measuring cellular DNA content using flow cytometry. 50µM CID-6861424 treatment was carried out for 24, 48 and 72 hours. Cell cycle data indicated that 50µM CID-6861424 resulted in ~78%, ~77%, and ~70% G_1 phase

cell accumulation as compared to ~46%, ~46% and ~52% in control set at 24, 48 and 72 hours, respectively. G_1 phase arrest accompanies a corresponding reduction in the other respective phases of the cell cycle population **(Figure 3.6 (a) and 3.6 (b); Table No. 3.3)**. Increase in a "sub-G_1 population" representing dead cells was also highest (~10%) in 50µM CID-6861424 as compared to control set which was around ~1.4% of total cell population at 72hours **(Figure 3.6 (a); Table No. 3.3)**.



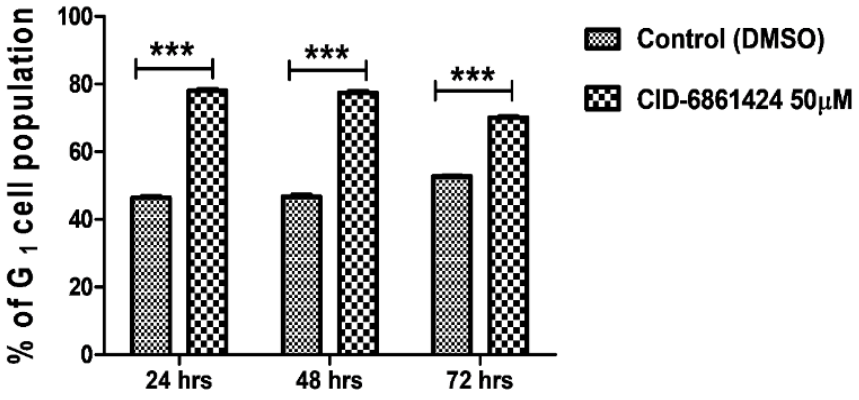
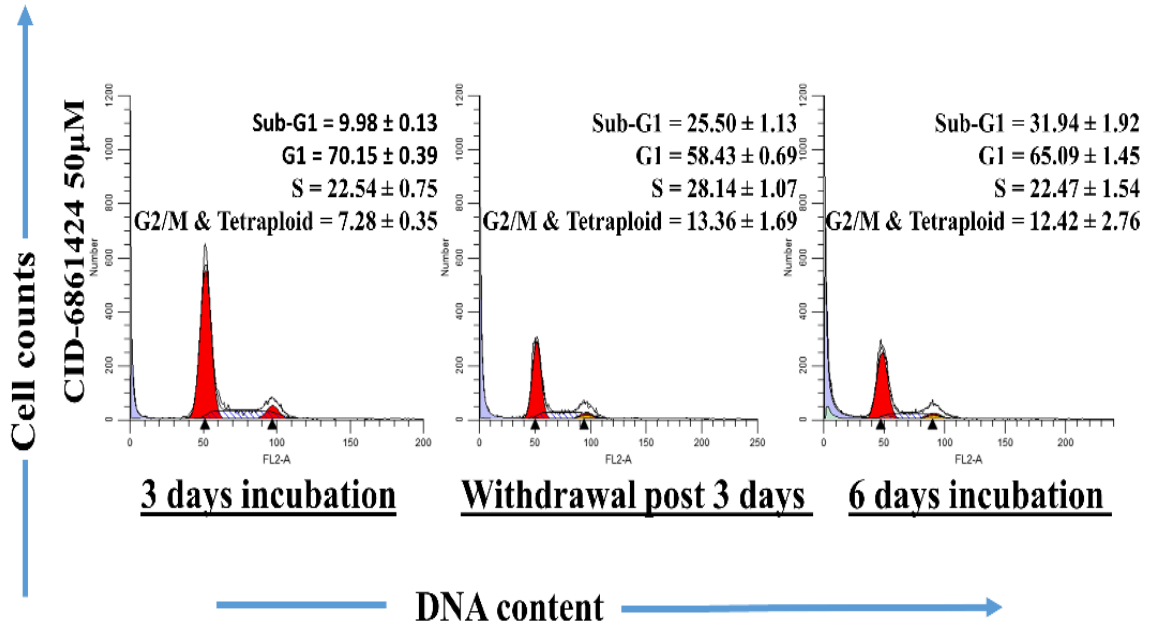


Figure 3.6 (a) 50µM CID-6861424 induces G_1 phase arrest in MCF-7 cell with using flow cytometry at indicated time intervals. (b) Bar-diagram representation of respective G_1 phase arrest. Mean ± SD value (Data represented was calculated for three independent experiments). ***P<0.001.

Next, the question was asked whether the cells recovered after the removal of the compound from the medium. For withdrawal experiment, two sets of 50µM CID-6861424 treatment were used. In one set, cells were exposed to 50µM CID-6861424 for six days continuously. In the second set, first cells were incubated with 50µM CID-6861424 for three days and then mediu was replaced without adding the compound. Cells exposed to 50µM CID-6861424 for three days, served as the reference. Cell cycle data indicated that sub-G_1 population was increased in both the sets, in withdrawal set ~2.5 fold and in continuous set ~3.0 fold as compared to 3days of 50µM CID-6861424 treated set **(Figure 3.7 (a) and 3.7 (b))**. The detailed cell cycle distribution upon treatment of all the sets is given in **Table No. 3.3.**



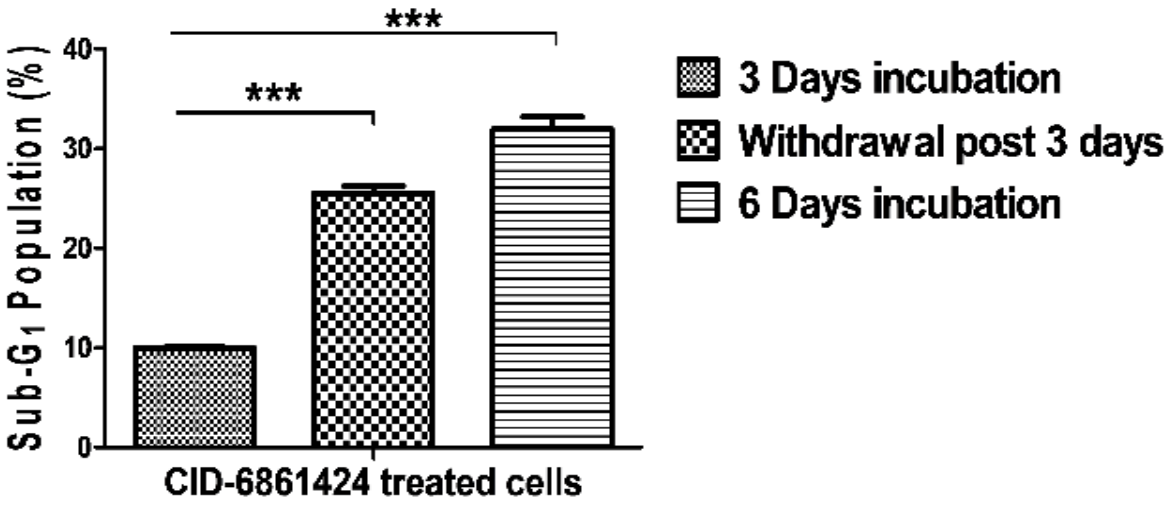


Figure 3.7 (a) 50µM-CID-6861424 increased sub-G_1 population in MCF-7 cells at 6 days continuous or 3 days withdrawal set as compared to 3 days incubation set by flow cytometry. (b) Bar-diagram representation of the respective enhanced sub-G1 population. Mean ± SD value (Data represented was calculated for three independent experiments). ***P<0.001.

	Time Point	Treatment	Cellular distribution of total cells in cell cycle process				G2M + T	Sub-G 1 Cells
			G 1	S	G 2	Tetraploid (T)		
MCF-7 Cell line	24 hrs.	Control	46.45 ± 0.51	42.84 ± 0.54	7.76 ± 0.00	2.93 ± 0.08	10.69 ± 0.08	1.38 ± 0.14
		CID-6861424 50µM	78.10 ± 0.55	6.78 ± 0.25	7.38 ± 0.04	7.71 ± 0.60	15.09 ± 0.55	3.12 ± 0.21
	48 hrs.	Control	46.71 ± 0.71	40.79 ± 0.75	7.60 ± 0.03	4.88 ± 0.42	12.48 ± 3.06	1.54 ± 0.32
		CID-6861424 50µM	77.45 ± 0.59	15.47 ± 1.43	6.39 ± 0.87	0.66 ± 0.07	7.05 ± 0.93	7.17 ± 0.41
	72 hrs.	Control	52.73 ± 0.24	36.35 ± 0.21	7.74 ± 0.03	3.16 ± 0.44	10.90 ± 0.41	1.45 ± 0.20
		CID-6861424 50µM	70.15 ± 0.39	22.54 ± 0.75	6.73 ± 0.44	0.55 ± 0.22	7.28 ± 0.35	9.98 ± 0.13
	Withdrawal post 3 Day	CID-6861424 50µM	58.43 ± 0.69	28.14 ± 1.07	7.67 ± 0.07	5.69 ± 1.62	13.36 ± 1.69	25.50 ± 1.13
	6 Day incubation	CID-6861424 50µM	65.09 ± 1.45	22.47 ± 1.54	7.61 ± 0.24	4.81 ± 3.00	12.42 ± 2.76	31.94 ± 1.92

Table No 3.3 Cell cycle distribution of various cellular populations after exposure to 50µM CID-6861424 at indicated time points.

Expression profiles of cyclin D1, CDK-4 and CDK-6 via western blotting supported the cell cycle data. Cyclin D1 play a major role in progression of the cell cycle from "G_1 to S phase" with the help of CDKs partners, CDK-4 and 6. 50µM CID-6861424 treated samples show significant reduction in Cyclin D1 and corresponding CDK partner CDK 4/6 with increasing treatment time **(Figure 3.8)**.

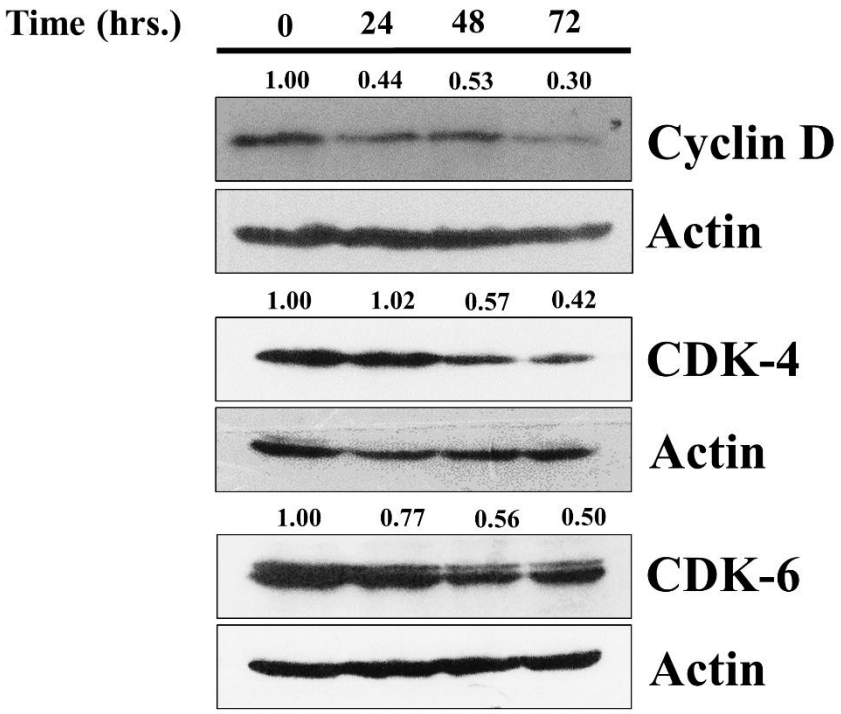


Figure 3.8 50µM-CID-6861424 exposure resulted in decrease in cyclin D1 and CDK4/6 expression and quantified with Image J software.

These results suggested that 50µM CID-6861424 treatment resulted in "MCF-7" cells halting at "G_1 phase" of cell cycle progression with increasing in sub-G_1 population.

3.2.3 CID-6861424 induces reactive oxygen species (ROS) generation and mitochondrial membrane potential (Δψm) depletion in MCF-7 cell

Cancer cells have elevated levels of ROS as compared to normal cells making them vulnerable to additional increase in ROS levels. Post 72 hour CID-6861424 treatment, we analysed ROS-generation by flow cytometry using Dichloro-dihydro-fluorescein diacetate (DCFH-DA) dye. Samples exposed to 25µM & 50µM CID-6861424 caused a considerable increase in ROS generation with respect to control. In this experiment, Doxorubicin was taken as a "positive control". ROS generation in the 25µM treated sample was ~1.3 times higher as compared to control, whereas 50µM treatment showed ~2 times higher ROS generation **(Figure 3.9 (a) and 3.9 (b); Table No. 3.4)**.

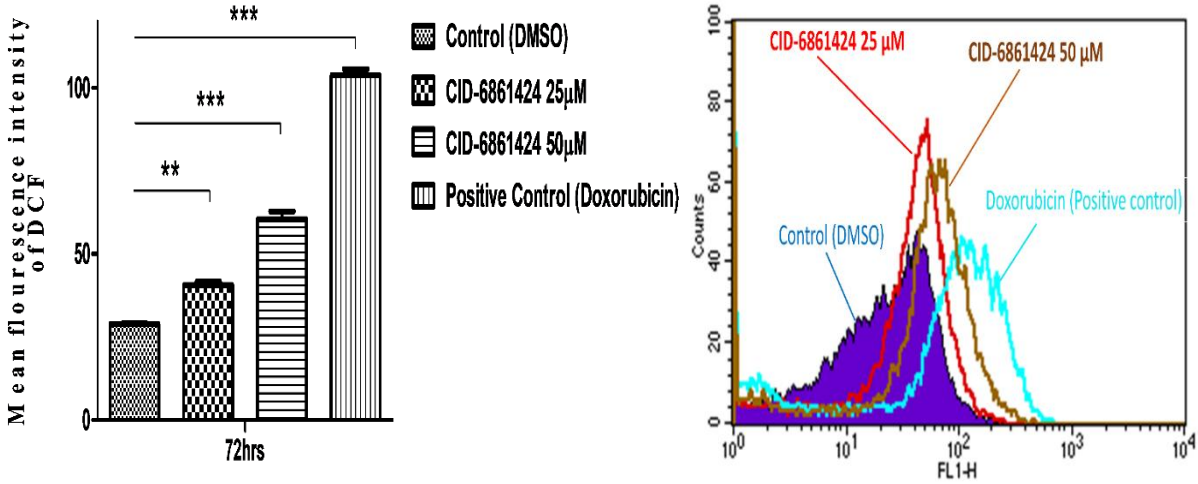


Figure 3.9. CID-6861424 exposure resulted in induction of ROS generation in MCF-7 cell. Bar-diagram and histogram show enhanced (a) and (b) ROS generation shown by elevated DCF fluorescence intensity post 72hours CID-6861424 treatment. Mean ± SD value (Data represented was calculated for three independent experiments). **P<0.01, and ***P<0.001.

Cell Line	Treatments	72hrs
MCF-7	CONTROL	28.85 ± 0.57
	CID-6861424 25uM	40.66 ± 1.73
	CID-6861424 50uM	60.56 ± 3.95
	Positive Control (Doxorubicin)	103.89 ± 3.18

Table No 3.4 Post 72hours CID-6861424 treatment elevated ROS production level in MCF-7 cell shown by higher DCF fluorescence intensity.

With cationic fluorescent dye Rhodamine 123, we next investigated the effect of CID-6861424 on mitochondrial membrane potential (**Δψm**) using flow cytometry after 72 hours CID-6861424 treatment. Doxorubicin was again used as the positive control. The treatment of 25µM and 50µM CID-6861424 resulted in approximately ~1.5 and ~2.2 fold disruption in **Δψm** as compared to the control (DMSO), respectively **(Figure 3.10 (a) and 3.10 (b); Table No. 3.5)**.

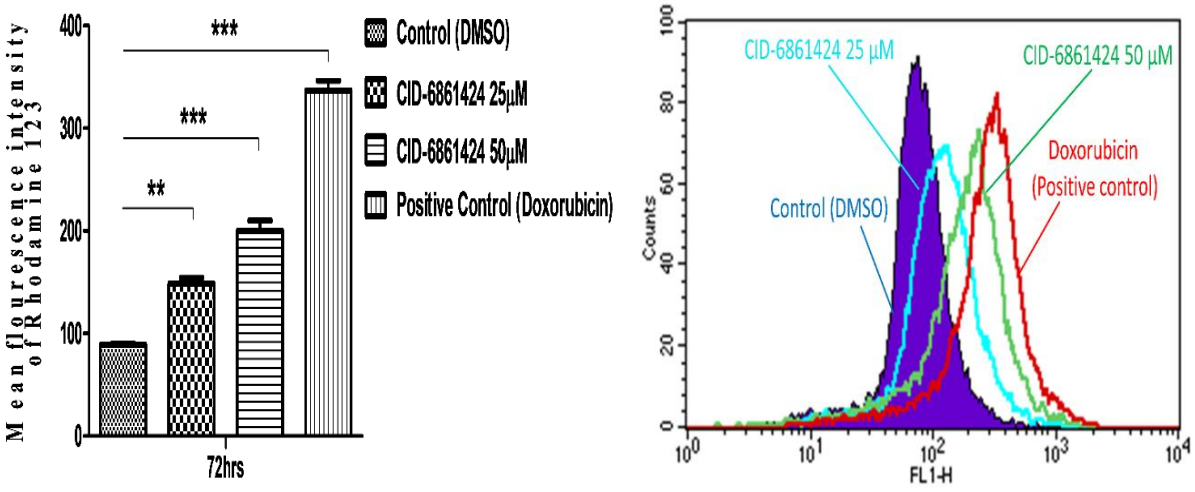


Figure 3.10. CID-6861424 increases Δψm dissipation in MCF-7 cell. Bar-diagram and histogram show elevated (a) and (b) Δψm dissipation sensed by Rhodamine123-dye in post 72hours CID-6861424 treated MCF-7 cells. Data represented as the mean ± SD of three independent experiments. **P<0.01, and ***P<0.001.

Cell Line	Treatments	72hrs
MCF-7	CONTROL	89.29 ± 2.05
	CID-6861424 25uM	148.54 ± 10.31
	CID-6861424 50uM	200.12 ± 17.30
	Positive Control (Doxorubicin)	336.92 ± 16.66

Table No 3.5 Post 72hours CID-6861424 treatment enhances dissipation of Δψm in MCF-7 cell shown by elevated Rhodamine123-dye fluorescence intensity.

These results suggested that 50μM CID-6861424 significantly increased ROS generation and depletion of mitochondrial membrane potential as compared to 25μM CID-6861424 and control set is a more lethal concentration for the MCF-7 cells.

3.2.4 CID-6861424 enhances DNA damage and apoptotic cell deaths in MCF-7 cell

The cell cycle data showed that the cells treated with IC_{50} value of CID-6861424 caused ~7 fold increase in "sub-G1 population" as compared to the control at 72 hours **(Figure 3.6 (a))**. Elevated ROS levels and **Δψm** disruption indicate DNA damage leading to decreased cell viability in 50μM CID-6861424-treated cells. To confirm the DNA damage, immunofluorescence of γ-H2AX, an indicator of DNA double strand breaks was used. 50μM CID-6861424-treated cells exhibited ~1.8 fold increase in "γ-H2AX" fluorescence

intensity with respect to control set as shown by bar-diagram **(Figure 3.11 (a) and 3.11 (b); Table No. 3.6)**. Immunofluorescence data supported by expression levels of γ-H2AX protein as determined by western blot where 50μM CID-6861424-treated cells demonstrated increase of γ-H2AX with increase in treatment time **(Figure 3.13 (a))**.

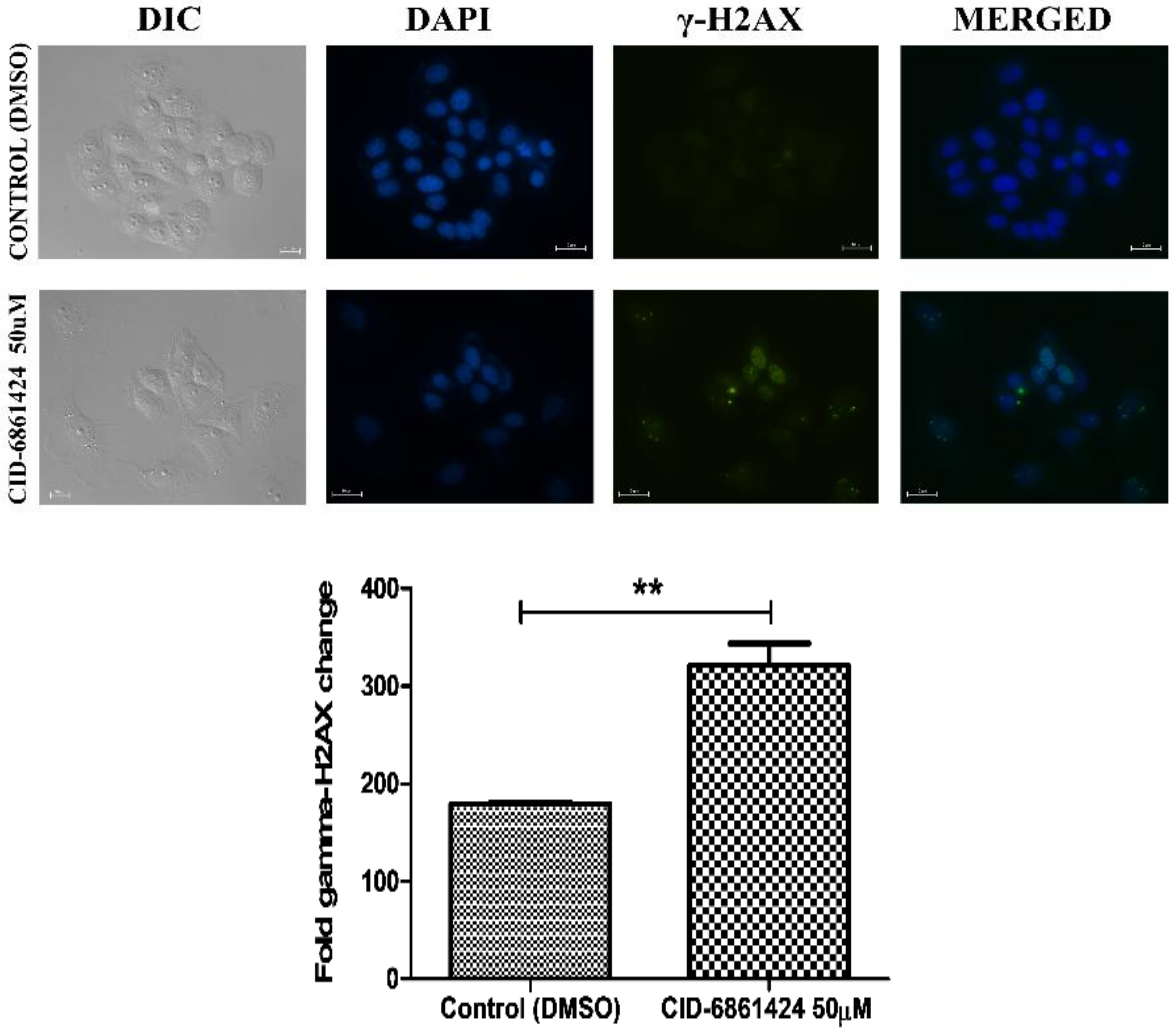


Figure 3.11. 50μM CID-6861424 induced DNA damage in MCF-7 cells at 72 hours. (a) induced γ-H2AX foci formation. (b) The bar-diagram representation of increased fluorescence intensity of γ-H2AX in MCF-7 cell. Mean ± SD value (Data represented was calculated for three independent experiments). **P<0.01.

Cell Line	Treatments	72hrs
MCF-7	CONTROL	179.76 ± 2.42
	CID-6861424 50uM	321.90 ± 34.14

Table No 3.6 50µM CID-6861424 induced DNA damage in MCF-7 cell at 72 hours.

DNA damages were further confirmed by expression level of p53, a tumor suppressor protein and transcription factor, which is activated due to the DNA damages leading to cells being halted at different phases of the "cell cycle" for repair. 50µM CID-6861424-treated cells showed increase in p53 with increase in treatment time **(Figure 3.13 (a))**.

The p21 is a "cyclin-dependent kinase inhibitor (CKI)", capable of inhibiting "cyclin/CDK complexes" and a major target of p53 activity, itself associated with "deoxyribonucleic acid (DNA)" damages leading to the "cell cycle" arrest for repair. 50µM CID-6861424 treated samples show significant upregulation of p21 with increasing in time was observed **(Figure 3.13 (a))**. Elevated level of ROS, depletion in **$\Delta\psi m$** and extensive DNA damages in cells induces apoptotic cell death. This was confirmed by apoptosis assay with annexin V and flow cytometry-based 7-Amino Actinomycin D (7AAD) dye kit after 72 hours CID-6861424 treatment. In comparison to control, cells exposed to 50µM and 100µM CID-6861424 caused a significant elevation in late apoptotic cells. as compared to the untreated set, ~1.5 fold enhancement in late apoptotic cells were detected when exposed to 50µM CID-6861424, while almost 3.5 fold increase was detected when treated with 100µM CID-6861424 (**Figure 3.12 (a) and 3.12 (b); Table No. 3.7**).

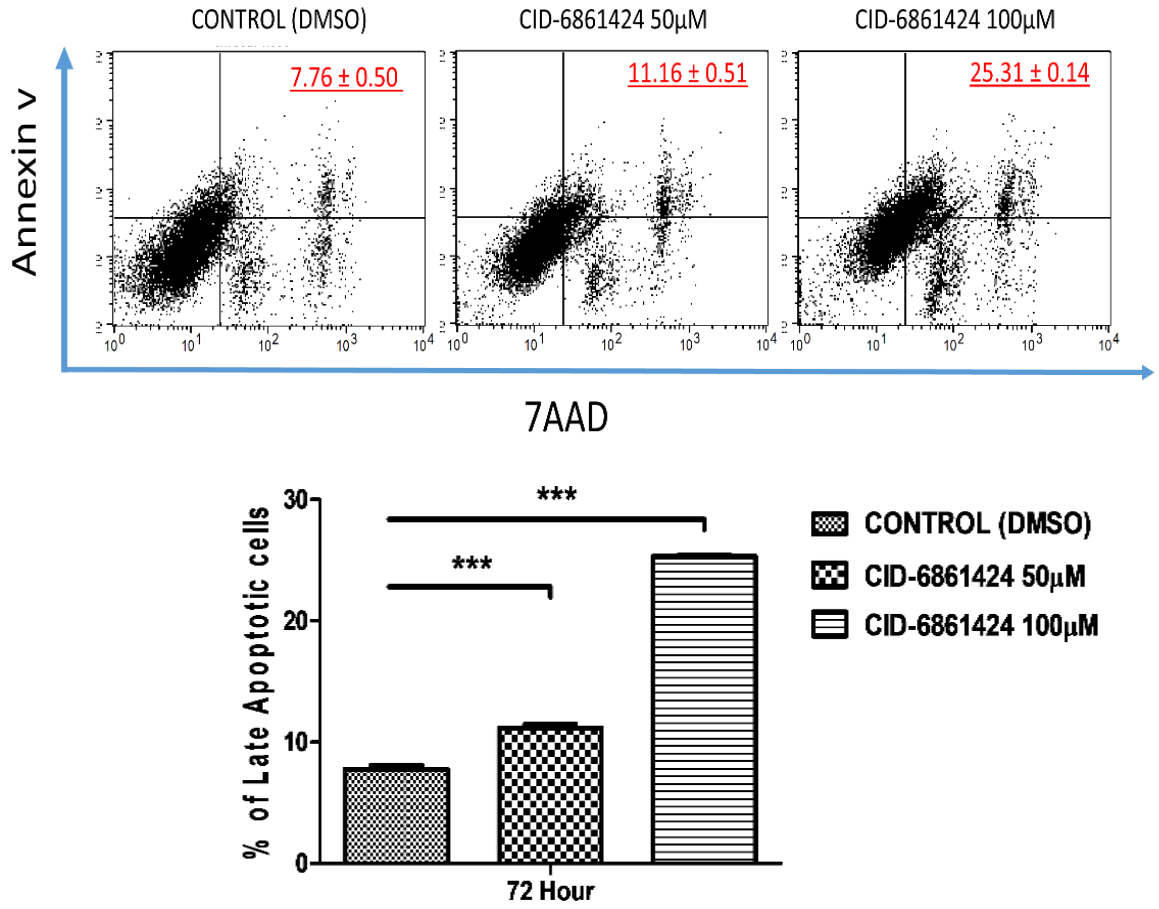


Figure 3.12. (a) CID-6861424 induced late apoptotic cell deaths by Annexin V / 7AAD kit (BD Biosciences) using flow cytometry and (b) The bar-diagram representation of increased late apoptotic cell deaths with increasing concentration of CID-6861424. Mean ± SD value (Data represented was calculated for three independent experiments). ***P<0.001.

Groups	Damaged Cells	Late Apoptosis	Viable Cell	Early Apoptosis
Control (DMSO)	9.85 ± 0.35	7.76 ± 0.50	73.85 ± 0.85	8.53 ± 0.03
50µM CID-6861424	12.16 ± 0.67	11.16 ± 0.51	71.56 ± 1.41	5.10 ± 0.23
100µM CID-6861424	19.83 ± 0.60	25.31 ± 0.14	46.40 ± 0.59	8.46 ± 0.08

Table No 3.7 CID-6861424 induced late apoptotic cell deaths in MCF-7 cell at 72 hours.

Western blot data of apoptotic related proteins further confirmed the Annexin V / 7AAD apoptotic data. We checked the expression levels of apoptotic cell death-related proteins such as Bcl-2, an "anti-apoptotic protein", and Bax, a "pro-apoptotic protein" by western blotting. 50µM CID-6861424-treated cells showed increased Bax and decreased Bcl-2 levels with increase in treatment time. This in turn resulted in increase in Bax/Bcl-2 ratio with time. **(Figure 3.13 (a) and 3.13 (b)**.

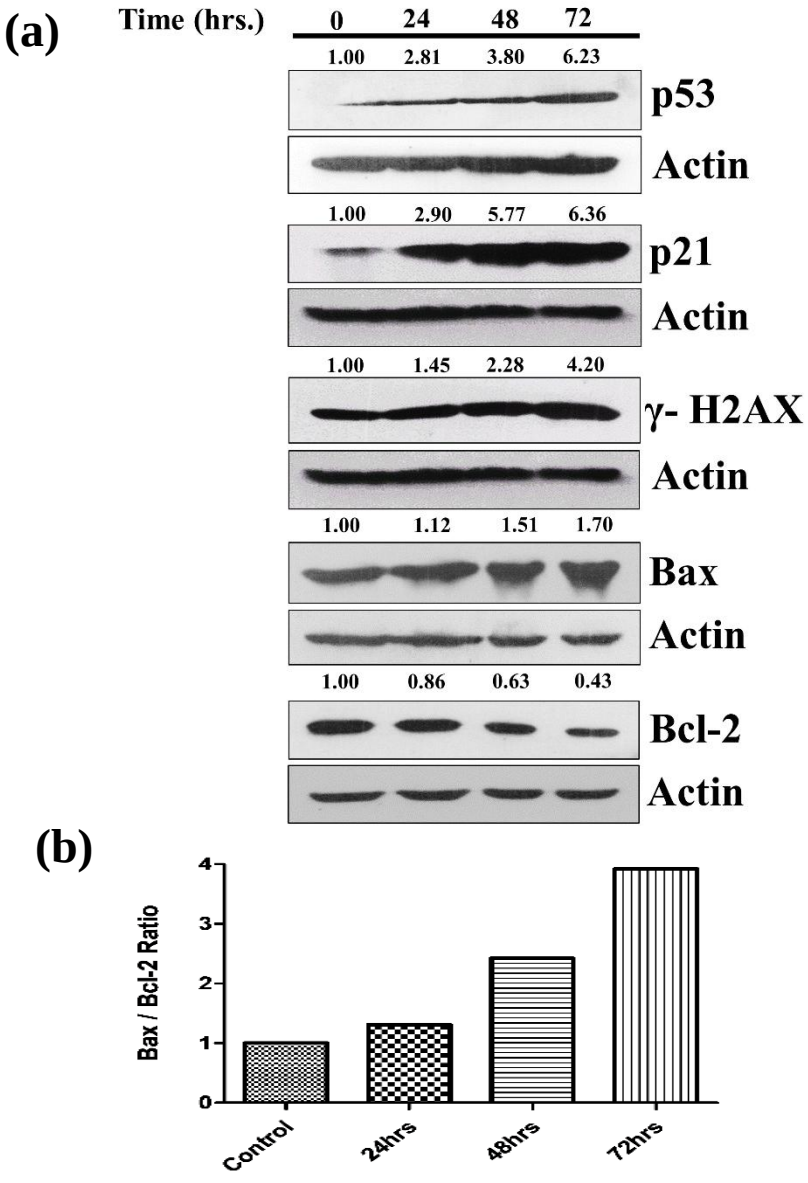


Figure 3.13. (a) Western blot data shows upregulation of p53, p21, γ-H2AX, Bax and downregulation of Bcl-2 proteins expression in presence of 50μM CID-6861424 for the indicated time and quantified with Image J software. (b) The bar-diagram representation of increasing Bax/Bcl-2 ratio in a time dependent manner.

These results suggested that, 50μM CID-6861424-treated cells have enhanced ROS generation, disrupted **Δψm**, extensive DNA damages, magnified late apoptotic cell population and elevated Bax/Bcl-2 ratio leading to induction of mitochondria-dependent apoptotic cell death in MCF-7 cell.

3.2.5 Inhibition of Akt cell proliferation and ERK cell survival pathway in MCF-7 cell by 50μM CID-6861424

PIP3/Akt pathway is a "signal transduction pathway" that promotes cell survival and growth in the response to "extracellular signals". "Phosphatase and tensin homolog (PTEN)" acts as a "tumor suppressor protein". PTEN has phosphatase activity, which regulates the "Akt signaling pathway" via dephosphorylation of "phosphatidylinositol (3,4,5)-triphosphate (PIP_3)" to PIP_2. 50μM CID-6861424-treated cells showed upregulation of PTEN with increase in time **(Figure 3.14)**. Akt a "serine/threonine kinase" when activated interacts with secondary messenger "phosphatidylinositol (3,4,5)-triphosphate (PIP_3)" on the plasma membrane. Activated Akt phosphorylates several downstream proteins responsible for cell growth and division, "cell survival", cell migration and angiogenesis. 50μM CID-6861424-treated cells showed decrease in phosphorylation of Akt and downstream proteins GSK-3β. The decrease in phosphorylation became more prominent with increase in treatment time **(Figure 3.14)**.

The “extracellular signal regulated kinase (ERK)” pathway is a major signaling pathway of “mitogen activated protein kinase (MAPK)” signaling pathway, activated by various stimulations such as extracellular agents, growth factors, hormones and cellular stress, which is responsible for cell proliferation and differentiation of the cells. Raf family members such as Raf-1, A-Raf and B-Raf are the main components of “MAPK/ERK kinase kinase (MEKK)” pathway, that is phosphorylating on serine residues of “MAPK/ERK kinase (MEK)” components ERK1/2, which is responsible for activation of certain downstream proteins led to transcribe genes which are essential for mainly cell proliferation, angiogenesis and epithelial-mesenchymal transition (EMT). 50µM CID-6861424 treated cells showed downregulation of phosphorylated ERK **(Figure 3.14)**. These results suggests inhibition of ERK cell survival pathway in 50µM CID-6861424 treated MCF-7 cell.

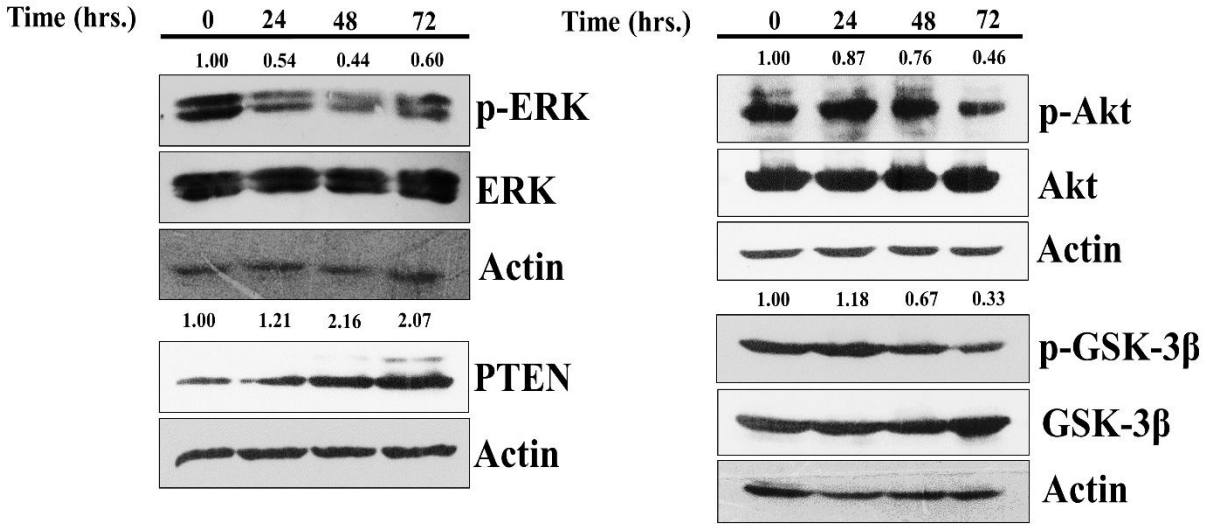


Figure 3.14. CID-6861424 treated MCF-7 cells exhibited inhibition of Akt cell proliferation and ERK cell survival pathway. Western blot data showing upregulation of PTEN and downregulation of phosphorylated Akt, GSK-3β & ERK proteins expression in presence of 50µM CID-6861424 for the indicated time and quantified with Image J software.

These results suggests that 50µM CID-6861424 treatment results in inhibition of Akt cell proliferation and ERK cell survival pathway in MCF-7 cell.

3.3 Discussion

Breast cancer morbidity has been increasing since the 1970s (Bielawski et al., 2002; Chun et al., 2015). Breast cancer is one of the most aggressive form of cancers and it might be due to the imbalance of hormonal signaling thus making its treatment challenging. In case of chemotherapy, most of the anticancer drugs used for treatment of breast cancer e.g. doxorubicin, epirubicin, capecitabine, gemcitabine, etoposide, paclitaxel and docetaxel, etc., have several side effects such as gastrointestinal toxicities, cardiotoxicity, thrombocytopenia, erythrodysesthesia, diarrhea, neuropathy, etc. Side-effects of anticancer drugs are largely due to the presence of multiple targets in cellular processes such as microtubule polymerization, DNA synthesis and transcription in cancerous as well as non-cancerous cells (Ambros et al., 2014; Blackstein et al., 2002; Mauri et al., 2010; Piccart-Gebhart et al., 2008; Pusztai et al., 1998). In light of this, developing efficient and low toxic compounds could be crucial for breast cancer chemotherapy. In the present study, we worked on the anti-cancerous activity of sulfonamide derivative, CID-6861424. Cell viability results showed that CID-6861424 considerably reduced MCF-7 cell survival. Cell viability decreased with the increase in concentration and time of treatment. Morphology of MCF-7 also distorted when exposed to CID-6861424. Alteration in morphology increased at higher concentrations. 50µM, IC_{50} value of the compound, treatment resulted in decrease in MCF-7 cell division distorted cell morphology such as elongated, dense cytoplasm and

distorted cellular membrane. Features like dense cytoplasm and membrane protrusions can be indicators of cellular stress and apoptosis (Decoster et al., 2010; Hacker, 2000). Aggressiveness of cancerous cells often predicted by its migratory potential. We investigate the migratory potential by wound healing assay at 25µM and 50µM concentrations. In presence of either of the concentrations, CID-6861424 considerably blocked wound closure, though at 50µM CID-6861424 it is more effective. Cancer cells can multiply indefinitely and to determine their post treatment clonogenic potentials, colony formation assay was performed (Franken et al., 2006). In the present study, 50µM CID-6861424 significantly reduced the clonogenic ability of MCF-7 cells. The "cell viability", "morphology", "wound healing" and "colony formation assay" results suggested that 50µM CID-6861424 can be an effective recipe for killing MCF-7 cells. To evaluate the underlying biological interventions leading to cell death, we analysed the effects of 50µM CID-6861424 on the "cell cycle" profile of MCF-7 cells. The cell cycle is a complicated and highly regulated process, which mandates cellular replication. Unrestrained cell cycle actions are the hallmark of cancer cells, forcing the cell for cellular division even under unfavorable conditions. The cell cycle data suggests treatment of MCF-7 cells with IC_{50} value of CID-6861424 resulted in G_1 phase arrest with a significant elevation in "sub-G_1 cell population" as compared to untreated cells. The cyclin D1 makes active complex with CDK4/6 and hypophosphorylates "retinoblastoma protein", which required for "cell cycle progression" in G_1 phase (Alao, 2007). The "cell cycle" data was further supported by the decrease in expression of "cyclin D1" and CDK4/6 proteins detected via western blotting. The results indicated that at 50µM CID-6861424 treatment reduced cyclin D1-CDK4/6 complex formation. We observed that when treated longer treatments (for 6days), either by removal of CID-6861424 at 72 hours

or continuous treatment for 6 days, significantly larger Sub-G0/G1 population in continuously treated cells were observed as compared to the 72hour treatment set. These results indicated that, in both conditions MCF-7 cells failed to return to normal cell cycle or revive back. ROS homeostasis plays a vital role in cellular signaling. It is tightly regulated. Cellular exposure to chemotherapeutic drugs can cause water radiolysis, resulting in release of free superoxide ($O_2^{\bullet-}$) and perhydroxyl ($HO_2^{\bullet}$) radicals, collectively known as ROS (Manuscript and Magnitude, 2013). These "free radicals" are highly reactive and interact with biomolecules including "DNA", causing damage and oxidative stress (Manuscript and Magnitude, 2013). Compared to normal cells, cancerous cells have elevated levels of ROS. If ROS homeostasis is not regulated properly and can be a major cause of cancer formation (Panieri and Santoro, 2016; Schumacker, 2006a; Wang and Yi, 2008a). Additionally, antioxidant proteins such as GSH, catalase, SOD, etc., capable of quenching excess ROS, are also highly expressed in cancerous cells (Liou and Storz, 2010b) helping maintain ROS homeostasis, though at elevated levels. Major cancer therapies target elevated ROS levels of cancer cells either by decreasing the level by using antioxidants and other phytochemicals (Ahmad and Mukhtar, 2013a), or by deregulating it by means of chemotherapeutic compounds (Pelicano et al., 2004; Trachootham et al., 2009a) and physical agents like radiation (Manuscript and Magnitude, 2013). Consequently, we analysed cellular ROS production post 72 hours of CID-6861424 treatment. 25µM CID-6861424 elevated ROS levels, however much higher levels of ROS generation were detected in presence of 50µM CID-6861424. The relatively lower levels of ROS generated in 25µM CID-6861424 as compared to 50µM CID-6861424 can attributed to the much stronger quenching effects of the antioxidant system, resulting in higher cell survival rates. The effectiveness of the

antioxidant system in maintaining ROS levels, is however, curtailed at 50µM CID-6861424, the IC_{50} value, resulting in increased cell mortality. Disruptions in mitochondrial membrane potentials (**Δψm**) (Banki et al., 1999; Fonseca-Silva et al., 2011; Johnson et al., 1996) due to elevated ROS levels result in mitochondrial-mediated cellular apoptosis among mammalian cells (Reed et al., 1998). Hence, the effects of CID-6861424 on **Δψm** in MCF-7 cells was studied. 50µM CID-6861424 caused significantly greater disruptions in **Δψm** than 25µM CID-6861424. Excessive ROS induces DNA damage due to oxidative stress. γ-H2AX, a phosphorylated histone H2A variant, is associated with the repair of "DNA double strand breaks (DSBs)", as the first protein recruited for further recruitment and localization of DNA repair molecules. Additionally, γ-H2AX is overexpressed during DNA DSBs, and is unanimously considered as a marker for DNA DSBs (Dickey et al., 2009). In the present study, treatment of 50µM CID-6861424 resulted in increased expression of γ-H2AX in a time-dependent manner, hinting at CID-6861424 induced DNA damage. After 72 hours of treatment, microscopic investigation of γ-H2AX foci clearly demonstrated significantly higher DNA damage in 50µM treated sample compared to control, based on comparative intensities of γ-H2AX foci. Western blot analysis of "γ-H2AX" expression clearly confirmed upregulation and hence, an indication of higher DSBs in MCF-7 cells in 50µM CID-6861424. p53 is a tumor suppressor protein, and is upregulated in response to increased DNA damage due to chemotherapeutic drugs, oxidative stress, etc. (Hientz et al., 2017). It is a well-studied protein, and has a well-documented role in protection against DNA damage via regulation of downstream genes that are involved in "cell cycle arrest", "DNA repair" and "apoptosis" (Bargonetti and Manfredi, 2002a; Chiang et al., 2013; Klein and Vassilev, 2004; Ko and Prives, 1996; Levine, 1997; SMITH et al., 2003). The p21 is a cyclin-

dependent kinase inhibitor (CKI), inhibits all cyclin/CDK complex formations as a result of "DNA damage", leading to "cell cycle arrest" at different phases (Piccolo and Crispi, 2012). Overexpression of p21 induced by p53 protein occurs in DNA damaged cells, leading to cell cycle arrest (Piccolo and Crispi, 2012). Our results clearly suggested that, cells were exposure to CID-6861424 caused upregulation of p53 and p21 proteins level in MCF-7 cells with increasing time interval.

Elevated level of ROS, disruption in **Δψm** and massive DNA damages results in "apoptotic cell death" (Banki et al., 1999; Fonseca-Silva et al., 2011; Johnson et al., 1996; Reed et al., 1998). The cell cycle analysis showed cell death that increase with increase in CID-6861424 exposure time. Apoptosis was confirmed via annexin-V/7AAD assay using flow cytometry. Similarly, cells treated with 50μM and 100μM CID-6861424 had significantly higher proportions of "late apoptotic cells" as compared to the DMSO control. Bcl-2 and Bax, a set of "anti-apoptotic" and "pro-apoptotic" proteins, respectively, These proteins are intimately connected with mitochondrial apoptosis. Bcl-2 is localized in the outer mitochondrial membrane and blocks "mitochondrial membrane permeability transition pores" oppening (Zamzami, 1996) through perpetuation of NADPH (Esposti et al., 1999; Kowaltowski et al., 2000). This results in oxidative attenuation of proteins involved in its opening (Kowaltowski et al., 2001) and blocking of apoptogenic proteins under apoptosis inducing circumstances (Daugas et al., 1996; Kluck, 1997; Yang et al., 1997; Zamzami, 1996). Bcl-2 also reduces oxidative stress by blocking lipid peroxidation (Hockenbery et al., 1993). In case of 50μM treated cells, ratio of Bax/Bcl-2 significantly increased with increase in time and induced mitochondrial-mediated apoptosis. Thus, prolonged G_1 phase arrest due to inhibition of cyclin D1 & CDK4/6 complex, overproduction of ROS leading to DNA damage and **Δψm**

disruption followed by upregulation of p53, p21, γ-H2AX, Bax, and downregulation of Bcl-2 leads to induction of mitochondrial-mediated apoptosis in 50µM CID-6861424-treated MCF-7 cells.

The PI3K/Akt pathway is an one of the essential pathway for "cell survival" and cell growth regulating responses (Khan et al., 2013). PTEN along with p53 "tumor suppressor protein", is overexpressed in response to "DNA damage" (Ming and He, 2012). High expression levels of PTEN inhibits PI3K/Akt pathway via dephosphorylation of phosphatidylinositol (3,4,5)-triphosphate (PIP_3) to PIP_2 (Khan et al., 2013). Akt, a "serine/threonine kinase", phosphorylates various downstream proteins accountable for cell growth and division, "cell survival", "cell migration" and "angiogenesis" (Luo et al., 2003). Increased phosphorylation of Akt has been observed in metastatic and hormone resistance breast carcinoma cells resulting in continuous activation of protein causing blocking of apoptosis and stimulation of cell division and survival (Carmona et al., 2016; Hernandez-Aya and Gonzalez-Angulo, 2011; Pérez-Tenorio et al., 2002). GSK-3β is one of the key downstream effector where convergence of various signaling pathway takes place. It controls cell death machinery in response to cellular stress and exposure to chemotherapeutic agents (Chiara and Rasola, 2013). Tan et al. have shown that inhibition of GSK-3β resulted in activation of Bax and induction of apoptosis (Tan et al., 2005). IC_{50} value of CID-6861424 inhibits Akt mediated cell proliferation pathway in MCF-7 cells as confirmed by Western blot revealing downregulation of phosphorylated Akt and downstream GSK-3β. Hence, 50µM CID-6861424 induced mitochondrial-mediated apoptotic cell death in MCF-7 cells, again due to the inhibition of Akt cell proliferation pathway.

"Extracellular signal related kinases (ERK)", is a most common member of "MAPK signaling pathway", which is essential for cell proliferation, differentiation, cell survival, angiogenesis and high rate of "epithelial-mesenchymal transition (EMT)" (Bartholomeusz et al., 2012; McCawley et al., 1999; Miglietta et al., 2006). Raf family members are the main component of ERK signaling pathway, which phosphorylate ERK1/2 on serine residues results in transcribing certain genes and leads to a high rate of "cell proliferation" and "cell survival" rate in cancer cells. Activated MAPK signaling in breast cancer promotes, cancer cells proliferation and differentiation leading to metastasis into lymph nodes (Adeyinka et al., 2002). The results shows that ERK cell survival pathway is also blocked in 50µM CID-6861424 treatment MCF-7 cell.

3.4 Conclusion

In conclusion, CID-6861424 inhibiting cell growth, suppressing cell migration potential, promoting G_1 phase arrest, elevated ROS generation, depletion of mitochondrial membrane potential, increased DNA damage, leading to mitochondrial mediated apoptotic cell deaths via inhibition of Akt cell proliferation and ERK cell survival pathway with downregulation of phosphorylated Akt, GSK-3β and ERK proteins expression level in MCF-7 cells. Based on results a model is proposed **(Figure 3.15)**. The CID-6861424 can be used as an anticancer agent to improve chemotherapy for breast cancer.

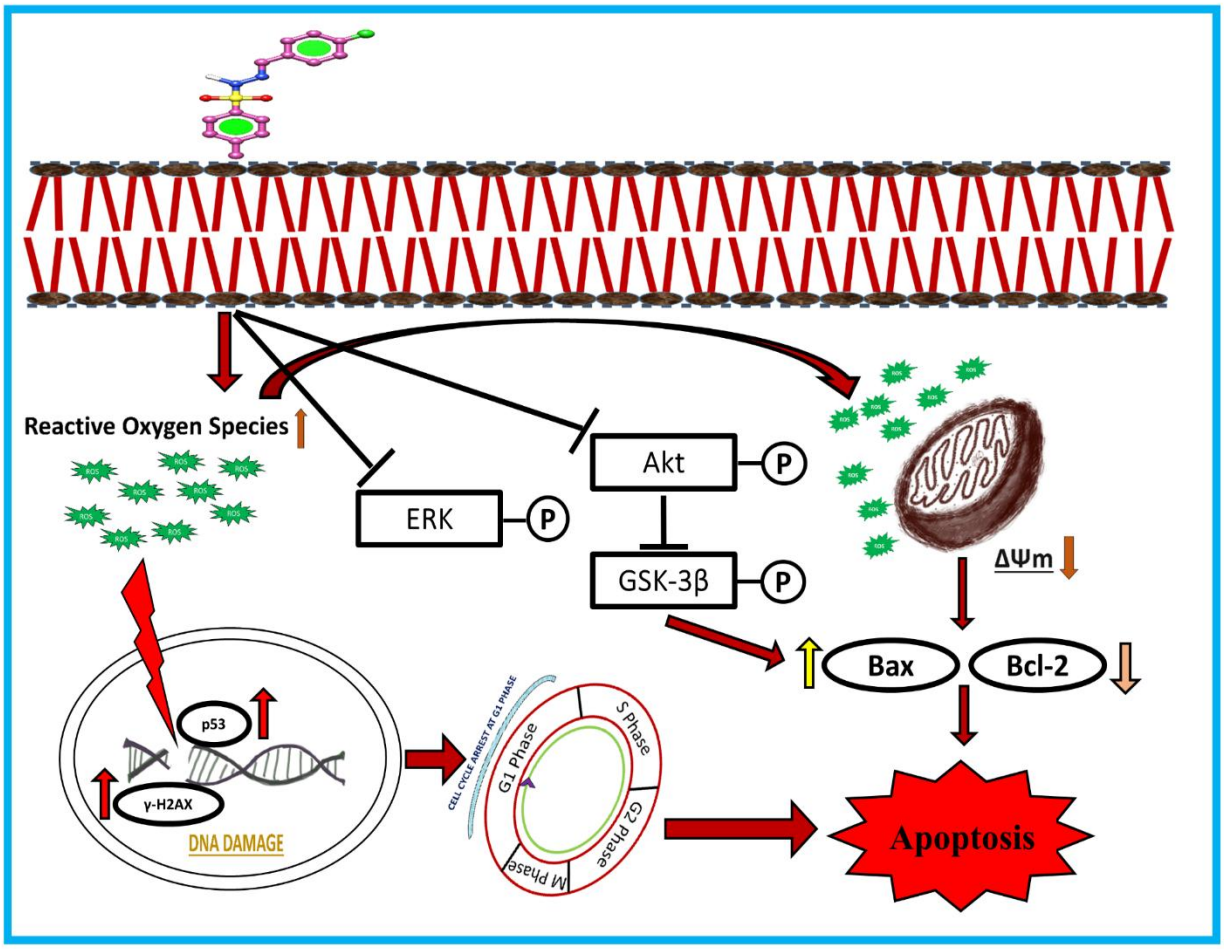


Figure 3.15. Proposed model for the mechanism of action of CID-6861424 in MCF-7 cell.

Chapter 4

CID-6861424 INDUCES APOPTOTIC CELL DEATH IN MDA-MB-231 TRIPLE NEGATIVE BREAST CANCER CELL LINE VIA INHIBITION OF ERK CELL SURVIVAL PATHWAY

CHAPTER-4

CID-6861424 INDUCES APOPTOTIC CELL DEATH IN MDA-MB-231 TRIPLE NEGATIVE BREAST CANCER CELL LINE VIA INHIBITION OF ERK CELL SURVIVAL PATHWAY

4.1 Introduction

About 20% of all breast cancer is“triple negative breast cancer (TNBC)”. TNBC cells do not express any of these three receptors such as “estrogen receptor (ER)”, “progesterone receptor (PR)” and “human epidermal growth factor receptor (HER 2)”. Therefore, TNBC patients do not respond to hormonal therapy. This is one of the most common reasons that TNBC is the most aggressive type of breast cancer, thus making its treatment challenging.

Most of the anti-cancer drugs against TNBC available in the market have strong side effects. Drugs such as paclitaxel and docetaxel, which target microtubule polymerization, are associated with mylagia, gastrointestinal toxicities, epiphora and neuropathy (Mauri et al., 2010). Anthracyclines like doxorubicin and epirubicin, which intercalate with DNA and RNA and inhibit replication and transcription are also reported to exhibit alopecia, vomiting, cardiotoxicity and neutropenia (Piccart-Gebhart et al., 2008). Similarly, antimetabolites of DNA synthesis like capecitabine, gemcitabine and etoposide (which inhibits type II topoisomerase) are associated with severe adverse effects including erythrodysesthesia, thrombocytopenia, diarrhea, gastrointestinal and blood related abnormalities(Ambros et al., 2014; Blackstein et al., 2002; Pusztai et al., 1998).In light of this, developing efficient and low toxic compound could be crucial for “triple negative breast cancer (TNBC)”, MDA-MB-231.

Sulfonamides have several biological activities including antibacterial, anti-carbonic anhydrase, diuretic, hypoglycemia, anti-thyroid, anti-protease and antitumor activities(casini et al., 2002). Sulphonyl derivates like sulphonyl carbonic anhydrase including acetazolamide, ethoxzolamide and methazolamide,etc are known to exhibit anti-cancerous properties. Similarly, other sulphonyl derivates including chloroquinoxaline sulfonamide and sulfonylureas are also reported to have anti-cancerous properties in a variety of cancer including leukemia, melanoma, lung cancer, ovarian cancer, renal cancer,etc(casini et al., 2002). Besides, many new sulphonyl derivatives have also been reported to exert anticancer properties in pilot studies(Kachaeva et al., 2018; Okolotowicz et al., 2018). All derivatives have a common chemical motif of aromatic/heterocyclic sulfonamide. CID-6861424 is one such derivative of sulfonamide, which is known to exert "antimicrobial' and "anti-inflammatory" activities(Kia et al., 2009). Since sulfonamides have been reported to have antitumor activity, we checked whether this compound can inhibit the proliferation of MD-MBA 231, a TNBC cell lineand can it be used as a chemotherapeutic agent.

Our results show that CID-6861424 inhibits the proliferation of "MDA-MB-231" cell in a dose and time dependent manner. The IC_{50} value of CID-6861424 on "MDA-MB-231" cell is 50μM, inducing cell cycle arrest at "G_2/M phase" by blocking the formation of "Cyclin B-CDK1 complex" due to the downregulation of Cyclin B & CDK1 and upregulation of p21 proteins expression. 50μM CID-6861424 treatment showed increase oxidative stress, disruption in **Δψm**leading to the DNA damage with upregulation of p53 and γ-H2AX protein expression level. 50μM CID-6861424 resulted in upregulation of "Bax/Bcl-2" ratio and downregulation of phosphorylated ERK, vimentin and survivin proteins expression leading to mitochondrial-mediated apoptotic cell death via inhibition of epithelial

mesenchymal transition (EMT) and ERK signaling pathway in "MDA-MB-231" cell. Our results clearly show that CID-6861424 can be used as an anticancer agent to improve chemotherapy of triple negative breast cancer (TNBC).

4.2 Results

4.2.1 CID-6861424 inhibit cell proliferation of MDA-MB-231 cell

Cell viability was evaluated on MDA-MB-231 cell via Trypan blue exclusion assay for 24, 48 and 72 hours at different concentrations of CID-6861424 **(Figure 4.1; Table No. 4.1)**. The result suggested that cell viability decreased with the increase in concentration and time. At 72 hour, CID-6861424 showed an IC_{50} value of ~ 50µM.

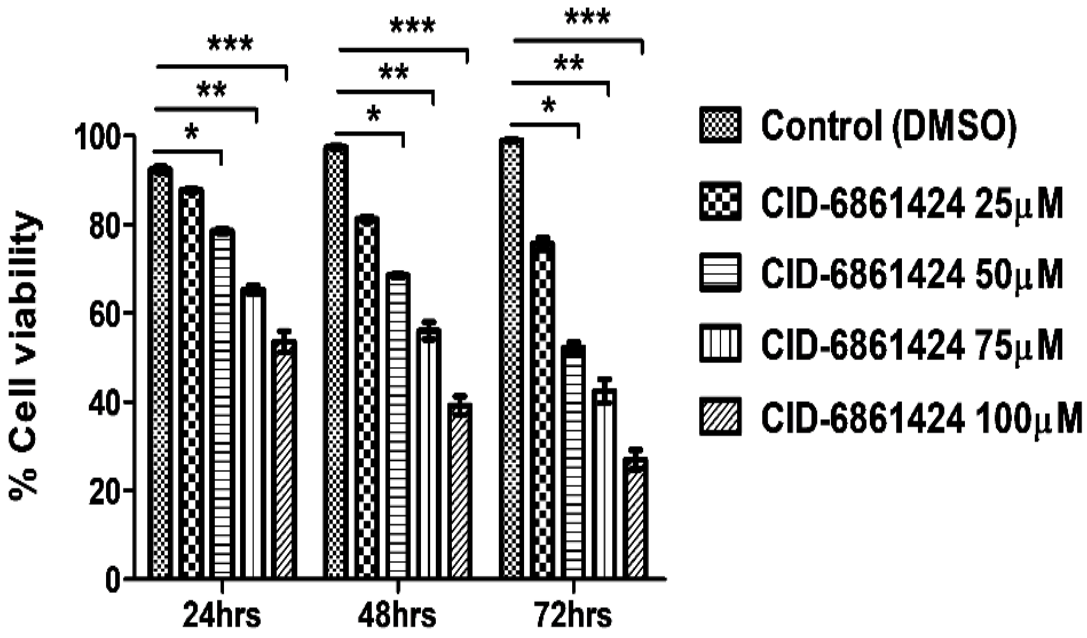


Figure. 4.1 Bar diagram showing effect of CID-6861424 on MDA-MB-231 cell viability at indicated concentrations and time points by Trypan Blue assay.Data are represented as the mean ± SD of three independent experiments. *P<0.05, **P<0.01, and ***P<0.001.

Time after treatment	Treatments	MDA-MB-231
24hrs.	CONTROL	92.45 ± 1.14
	CID-6861424 25µM	87.78 ± 0.66
	CID-6861424 50µM	78.59 ± 0.88
	CID-6861424 75µM	65.30 ± 0.97
	CID-6861424 100µM	53.60 ± 4.11
48hrs.	CONTROL	97.46 ± 0.59
	CID-6861424 25µM	81.25 ± 0.89
	CID-6861424 50µM	68.51 ± 0.60
	CID-6861424 75µM	56.05 ± 1.96
	CID-6861424 100µM	39.19 ± 3.57
72hrs.	CONTROL	99.06 ± 0.47
	CID-6861424 25µM	75.63 ± 2.31
	CID-6861424 50µM	52.27 ± 2.06
	CID-6861424 75µM	42.46 ± 2.63
	CID-6861424 100µM	26.98 ± 3.84

Table No 4.1 CID-6861424 inhibit the cell proliferation of MDA-MB-231 cell with the increase in concentration and time by trypan blue assay.

Further, cell morphology in the presence ofindicated concentrations of the compound was examined under a bright field microscope. At 72 hours, the cell count decreased drastically with increasing concentration as compared to the control set **(Figure 4.2)**. Change in cell morphology became more prominent with the increase in concentration. The cell morphology data at50µM concentration showed small and roundish cells as compared to the control, with dense cytoplasm and irregular cell membrane **(Figure 4.2)**. At 75µM and 100µM all cells exhibited completely deformed morphology suggesting cell death **(Figure 4.2**).

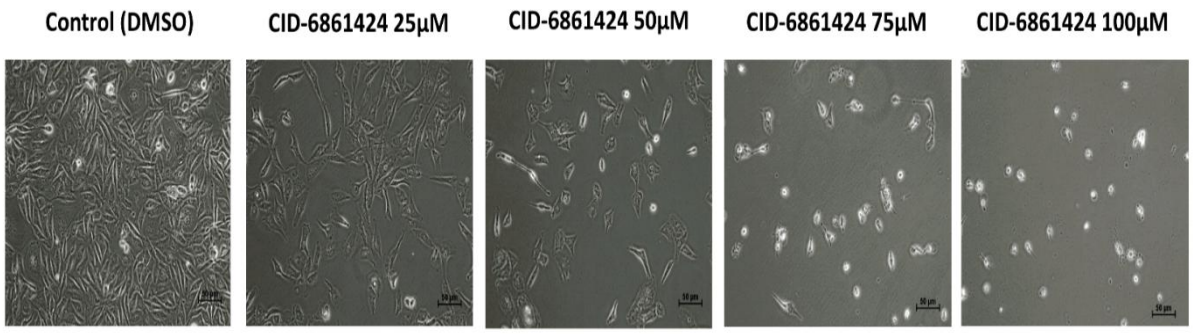


Figure. 4.2 Changes in MDA-MB-231 cell morphology, using a bright field microscope at 20X, was observed post 72 hours treatment with indicated concentration of CID-6861424.

Wound-healing assay was performed by measuring pre and post wound widths after 72 hours of exposure to 25µM & 50µM CID-6861424 treatments. The untreated cells migrated and rapidly filled ~ 80% of the wound, while ~ 50% of wound closure was observed in cells treated with 25µM CID-6861424. 50µM CID-6861424 treatment was most effective resulting in only ~ 25% of wound closure **(Figure 4.3 (a) and 4.3 (b); Table No. 4.2)**.

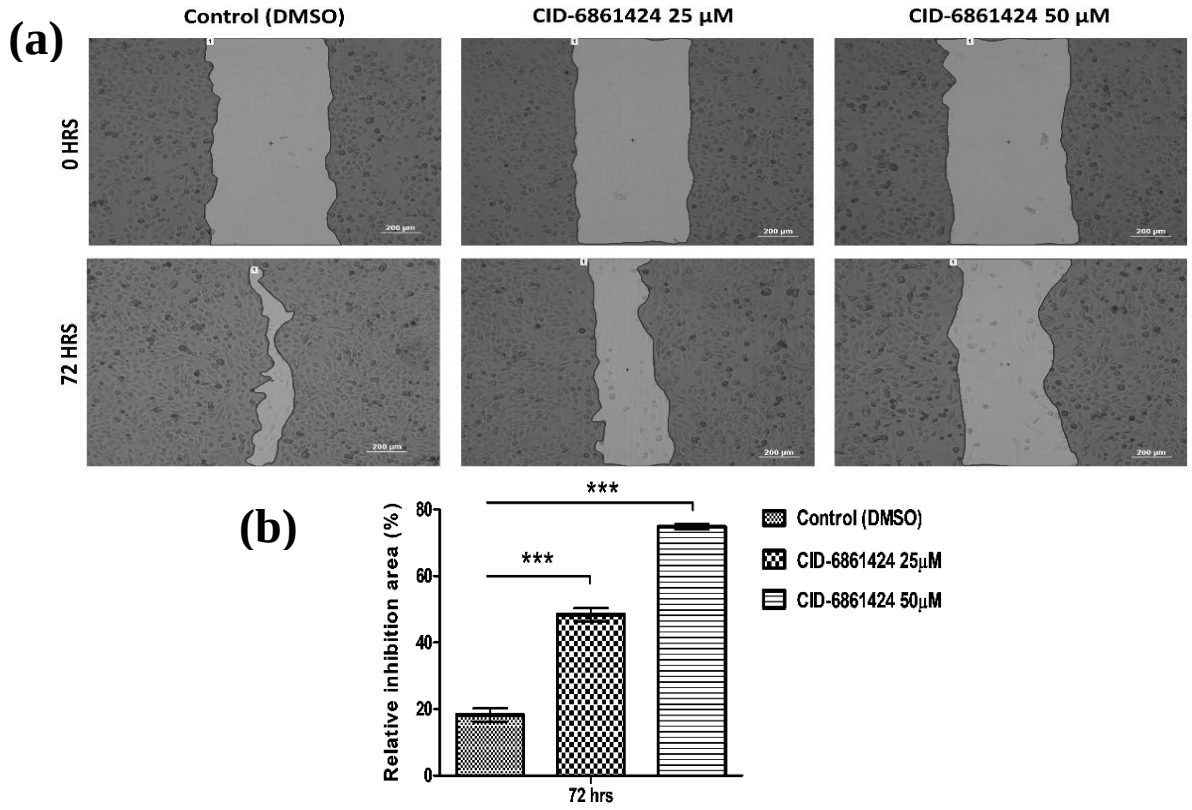


Figure. 4.3 (a) CID-6861424 inhibits MDA-MB-231 cell migration potential. (b) bar-diagram representation of relative inhibition of wound healing area of MDA-MB-231cell.Mean ± SD value (Data represented was calculatedfor three independent experiments). ***P<0.001.

Cell Line	Time Point	Control	CID-6861424 25µM	CID-6861424 50µM
MDA-MB-231	0 Hrs	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
	72 Hrs	18.24 ± 3.46	48.41 ± 3.40	74.82 ± 1.41

Table No. 4.2 CID-6861424 inhibitscell migration potential of MDA-MB-231 cell post 72 hours treatment.

For the colony formation assay, 72 hours post treated cells were harvested and ~ 400 cells were seeded in fresh cell culture dish from each set and incubated for ~14 days. Post incubation, comparison of number of colonies showed that 50µM CID-6861424 treated set formed significantly less number of colonies as compared to the 25µM CID-6861424 treated set as well as control set **(Figure 4.4).**

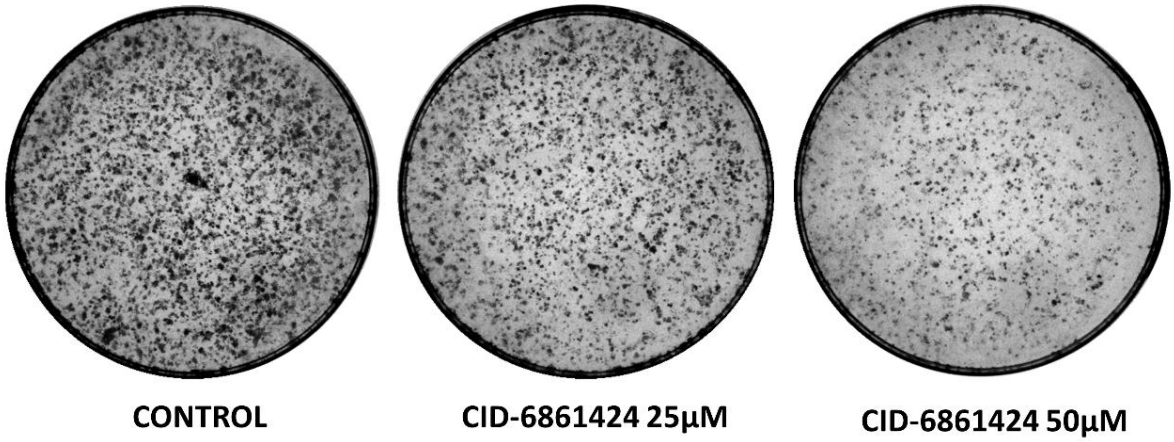


Figure. 4.4 Reduced in the colony count of MDA-MB-231 cells in presence of CID-6861424 treatment.

These results showed that, at the IC_{50} Value, CID-6861424 significantly inhibited the MDA-MB-231 cell viability, cell counts, cell migration and colony formation potential as compared to control set.

4.2.2 CID-6861424 induces cell cycle arrest at the G_2/M phase in MDA-MB-231 cell

The effect of CID-6861424 on cell cycle profile of MDA-MB-231 was studied by assessing the cellular DNA content using flow cytometrypost 24hrs, 48hrs and 72hrs. The cell cycle data indicated that cells treated with 25µM CID-6861424 resulted in ~25%, ~26% and ~33% G_2/M phase accumulation as compared to ~18%, ~18% and ~23% in control set at 24hrs, 48hrs and 72hrs respectively. This is accompanied with a corresponding reduction in the other respective phases of cell cycle population **(Figure 4.5 (a) and 4.5 (b); Table No. 4.3)**.

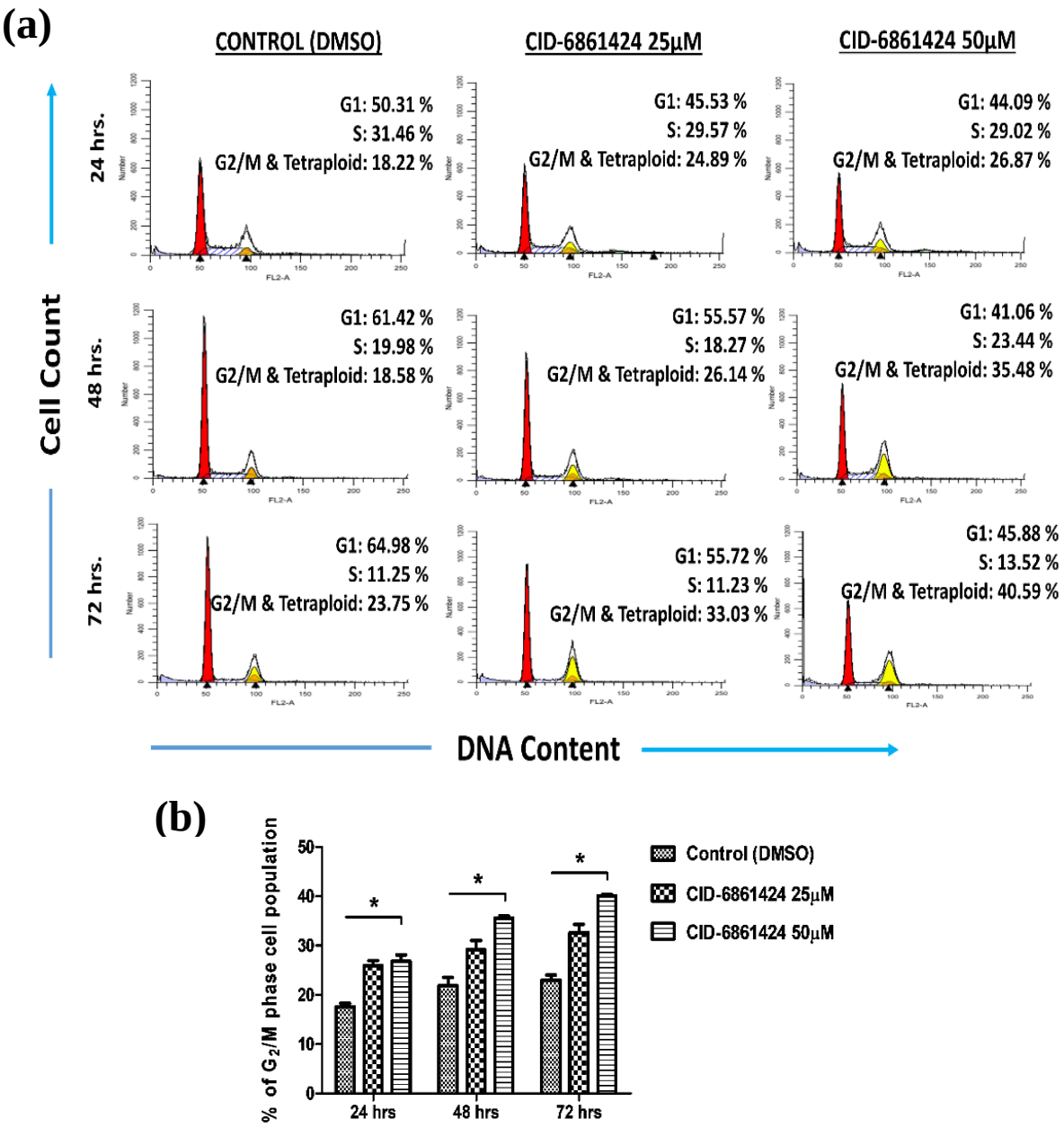


Figure 4.5(a) Flow cytometry analysis indicatingCID-6861424 induces G_2/M phase arrest in "MDA-MB-231" cell at indicated time. (b)Bar-diagram representation of respective G_2/M phase arrest.Mean ± SD value (Data represented was calculatedfor three independent experiments).*P<0.05.

Cells when exposed to the 50µM CID-6861424, resulted in accumulation of ~26%, ~35% and ~40% of the cell in G_2/M phase, which is ~8%, ~8% and ~17% higher than the untreated sample at 24hrs, 48hrs and 72hrs accompanied with a corresponding reduction in G1 phase of cell cycle population. The S phase was slightly increased in the 50µM treated sample compared to the control set **(Figure 4.5 (a) and 4.5 (b); Table No. 4.3)**. The detailed cell cycle distribution upon treatment of all the sets is given in **Table No.4.3**.

Cell Line	Time	Treatments	G1 PHASE	S PHASE	G2/M PHASE
MDA-MB-231	24hrs	CONTROL	50.97 ± 1.32	31.40 ± 2.22	17.61 ± 1.15
		CID-6861424 25µM	43.37 ± 3.65	30.73 ± 1.80	25.88 ± 1.85
		CID-6861424 50µM	41.15 ± 5.13	31.29 ± 3.40	26.82 ± 2.24
	48hrs	CONTROL	60.86 ± 0.89	17.26 ± 2.57	21.85 ± 2.85
		CID-6861424 25µM	54.37 ± 3.14	17.63 ± 0.75	29.16 ± 3.20
		CID-6861424 50µM	41.37 ± 0.40	23.05 ± 0.52	35.56 ± 0.71
	72hrs	CONTROL	65.71 ± 1.67	11.26 ± 0.10	23.00 ± 1.76
		CID-6861424 25µM	56.30 ± 2.02	11.05 ± 0.95	32.62 ± 2.92
		CID-6861424 50µM	44.00 ± 2.15	15.87 ± 2.59	40.10 ± 3.93

Table No 4.3 Cell cycle distribution of various cellular populations after exposure to CID-6861424 at indicated time points.

Cyclin B is responsible for the progression of the cell cycle from "G_2 phase" to "M phase" with the help of CDK-1. The p21 is a "cyclin-dependent kinase inhibitor (CKI)", whichinhibitscyclin/CDK complex.50µM CID-6861424 treated samples showeda significant

reduction of Cyclin B1 and slight decrease in corresponding CDK-1. The treatment also resulted in slight upregulation of p21 with increasing in the treatment time**(Figure 4.6)**.

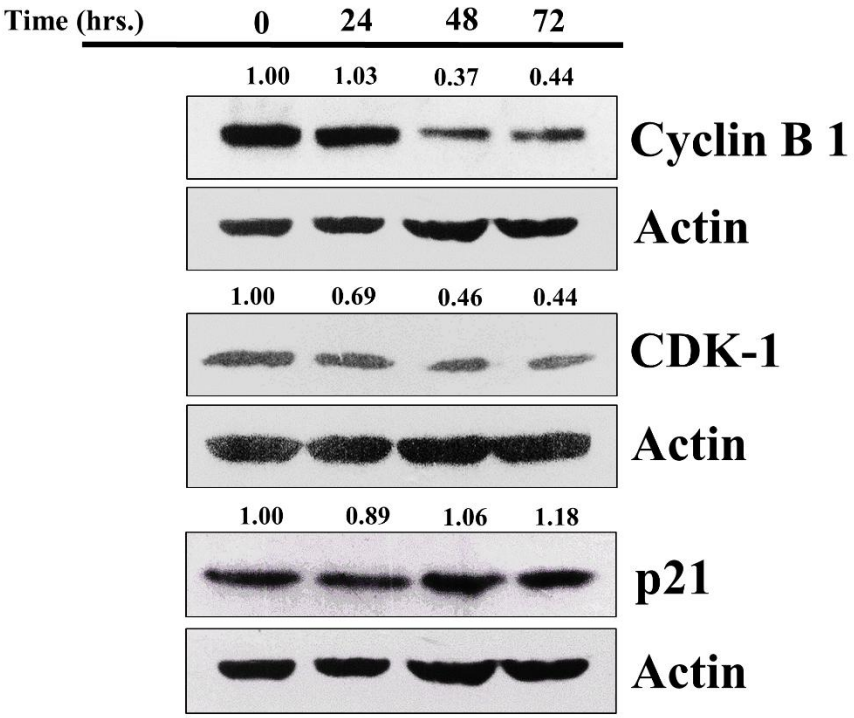


Figure 4.6 50µM-CID-6861424treatment resulted in downregulation of cyclin B1, CDK-1 and upregulation of p21 protein expression at the indicated time quantified with Image J software.

These results suggested that 50µM CID-6861424 treatment resulted in halting of MDA-MB-231 cells at G_2/Mphase of cell cycle progression.

4.2.3 CID-6861424 induces reactive oxygen species (ROS) generation and mitochondrial membrane potential(Δψm)depletion in MDA-MB-231 cell

Cancer cells have high ROS levels than normal cells and hence, are susceptible to a further increase in the levels of ROS. ROS production was analysed by flow cytometry using DCFH-DA dye in MDA-MB-231 cells post 72 hour CID-6861424 treatment.Samples

treated with 25µM & 50µM CID-6861424 caused a significant increase in ROS generation with respect to control. Doxorubicin was used as a positive control in this experiment. In contrast, ROS generation in 25µM treated samples, was ~ 1.0 times higher as compared to the control (DMSO) whereas ~ 1.5 higher ROS generation was observed in 50µM treated sample as compared to the control set **(Figure 4.7 (a) and 4.7 (b); Table No. 4.4)**.

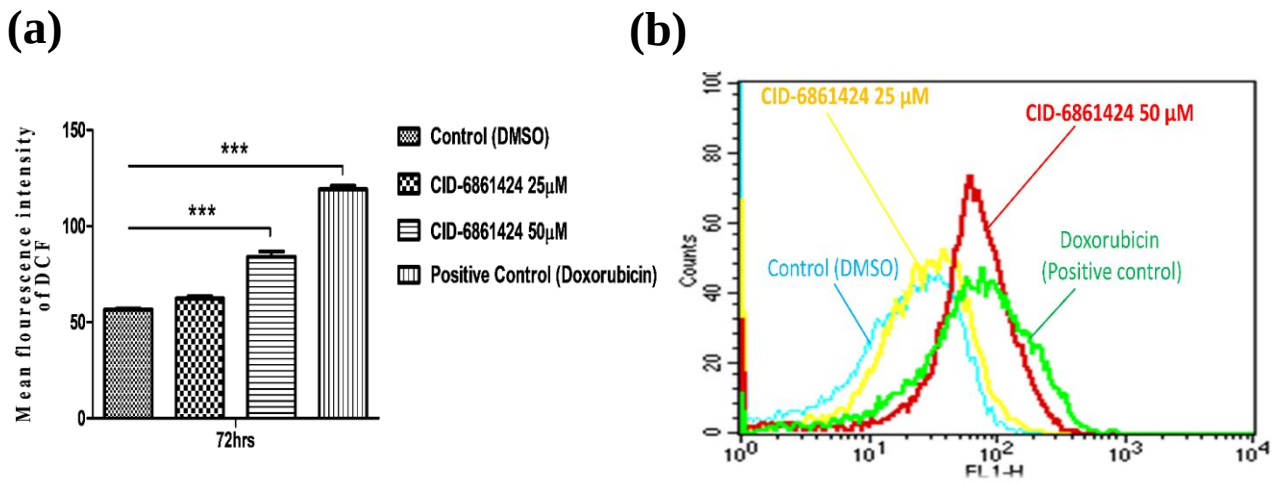


Figure 4.7 CID-6861424 induced ROS generationin MDA-MB-231 cell: Bar-diagram and histogram show enhanced (a) and (b). ROS generation indicated by increased DCF fluorescence intensity post 72hours CID-6861424treatment.Mean ± SD value (Data represented was calculatedfor three independent experiments). ***P<0.001.

Cell Line	Treatments	72hrs
MDA-MB-231	CONTROL	56.57 ± 0.86
	CID-6861424 25uM	62.29 ± 1.97
	CID-6861424 50uM	84.09 ± 4.61
	Positive Control (Doxorubicin)	119.21 ± 2.92

Table No 4.4 CID-6861424 induced ROS generation in "MDA-MB-231" cell indicated by increased DCF-DA fluorescence intensity post 72hrs treatment.

We further checked the mitochondrial membrane potential (**Δψm**) with cationic fluorescent dye Rhodamine 123 using flow cytometry after 72 hours of treatment as a measure of disruption in**Δψm.** Doxorubicin was again used as a positive control in this experiment. The treatment of 25µM resulted in approximately ~1.4 fold increase in disruptionof**Δψm** as compared to the control. Whereas 50µM treatment resulted in almost ~1.7 fold enhansment of **Δψm**disruptionas compared to control set **(Figure 4.8 (a) and 4.8 (b); Table No. 4.5)**.

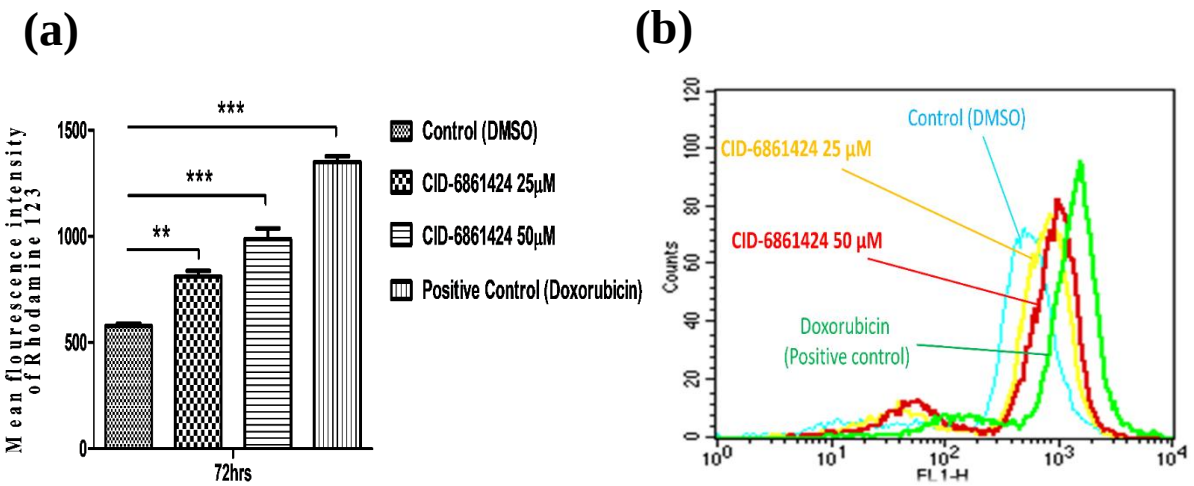


Figure 4.8CID-6861424 enhances dissipation of Δψmin MDA-MB-231 cell: Bar-diagram and histogram show enhanced (a) and (b)Δψmdissipation detected by Rhodamine123-dye in "MDA-MB-231" cell post 72hours treatment with CID-6861424.Mean ± SD value (Data represented was calculatedfor three independent experiments). **P<0.01, and ***P<0.001.

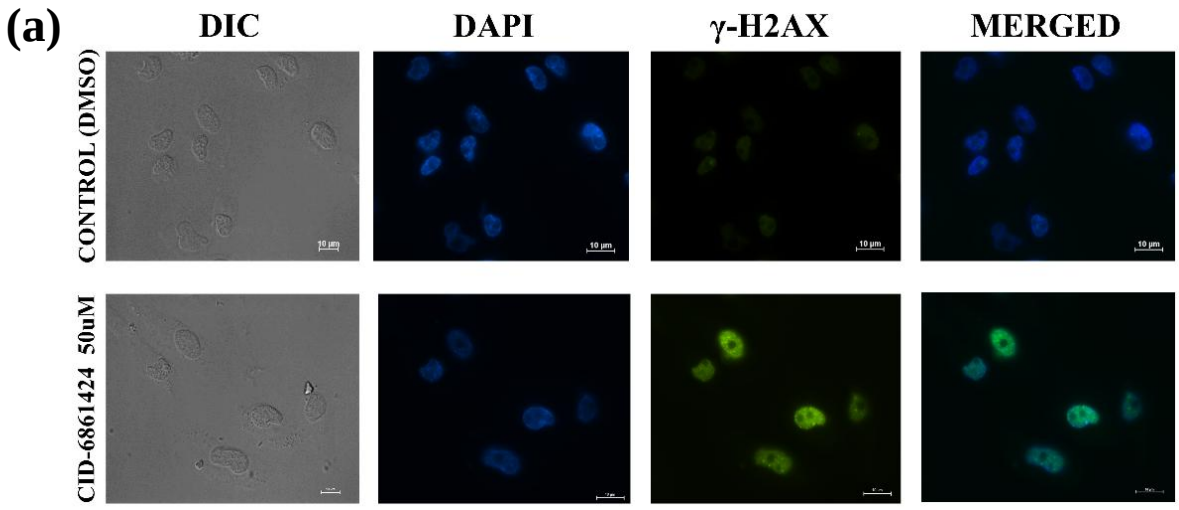
Cell Line	Treatments	72hrs
MDA-MB-231	CONTROL	578.33 ± 20.10
	CID-6861424 25uM	810.00 ± 49.32
	CID-6861424 50uM	170.61 ± 13.62
	Positive Control (Doxorubicin)	1350.70 ± 48.23

Table No 4.5 CID-6861424 enhances dissipation of Δψm in MDA-MB-231 cell indicated by increased Rhodamine123-dye fluorescence intensity post 72hrs treatment.

These results suggested that 50µM CID-6861424 is a more lethal concentration for the cells which significantly increases ROS generation and causes depletion of the **Δψm**of MDA-MB-231 cells as compared to 25µM CID-6861424 treated set as well as the control set.

4.2.4 CID-6861424 induces DNA damage and apoptotic cell deaths in MDA-MB-231 cell

The elevated levels of ROS, disruption in **Δψm** indicated that treatment of 50µM CID-6861424might be inducing DNA damage leading to decreased cell viability. Further, DNA damage was confirmed by immunofluorescence intensity of γ-H2AX, an indicator of DNA double strand break.50µM CID-6861424 treated cells exhibited ~ 5 fold increase in "γ-H2AX" intensity with respect to control set are shown in the immunofluorescence panels and are bar-diagram **(Figure 4.9 (a) and 4.9 (b); Table No. 4.6)**. Immunofluorescence data is further supported by expression levels of γ-H2AX protein as determined by western blot. Cells treated with 50µM demonstratessignificant upregulation of γ-H2AX with increasing in the time **(Figure 4.12 (a))**.



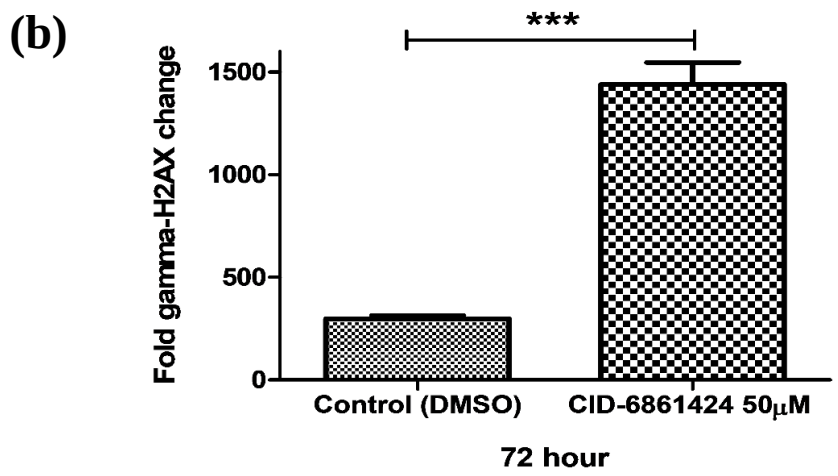


Figure 4.9. 50μM CID-6861424 induced DNA damage in "MDA-MB-231" cell at 72 hours. 4.9 (a) 50μM CID-6861424 treatment resulted in γ-H2AX foci formation and 4.9 (b) The bar-diagram representation of increased fluorescence intensity of γ-H2AX in MDA-MB-231 cell. Mean ± SD value (Data represented was calculatedfor three independent experiments). ***P<0.001.

Cell Line	Treatments	72hrs
MDA-MB-231	CONTROL	298.42 ± 24.45
	CID-6861424 50uM	1438.72 ± 852.52

Table No 4.6 50μM CID-6861424 induced DNA damage in "MDA-MB-231" cell at 72 hours.

DNA damage could be further confirmed by immunofluorescence of p53. The p53 is a tumor suppressor protein, which is also activated due to the DNA damages, which tanscribes p21 resulting in p21 protein expression and leading to cell cycle block and initiation of repait of DNA. The treatment of 50μM CID-6861424 resulted in ~3.5 fold increase innuclear p53 intensity as compared to control set respectively as shown by bar-diagram **(Figure 4.10 (a) and 4.10 (b); Table No. 4.7)**. The p53 immunofluorescence data was further confirmed by western blot analysis. 50μM treated cells demonstrated an upregulation

of p53 with increasein treatment time **(Figure 4.12 (a))**. The upregulation of nuclear p53 protein expression indicate cell cycle arrest leading to cell deaths.

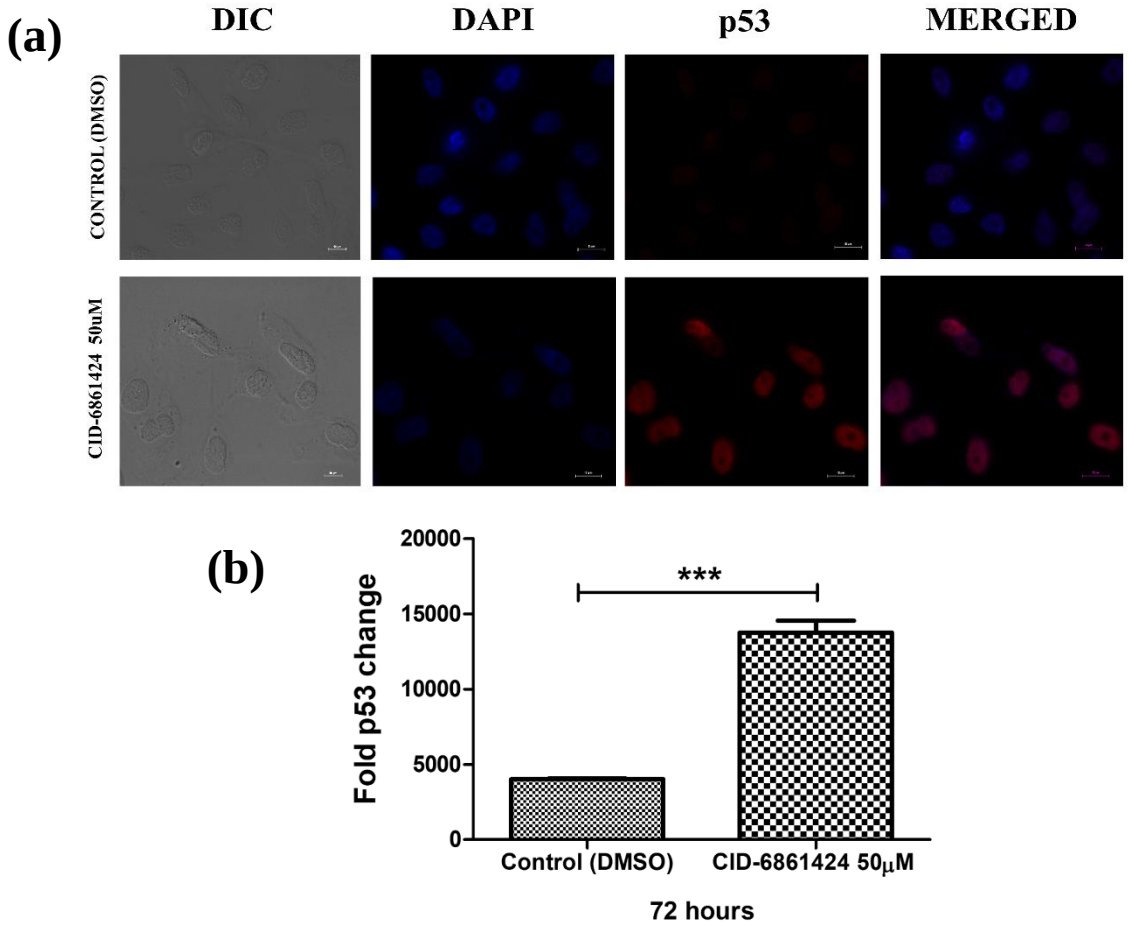
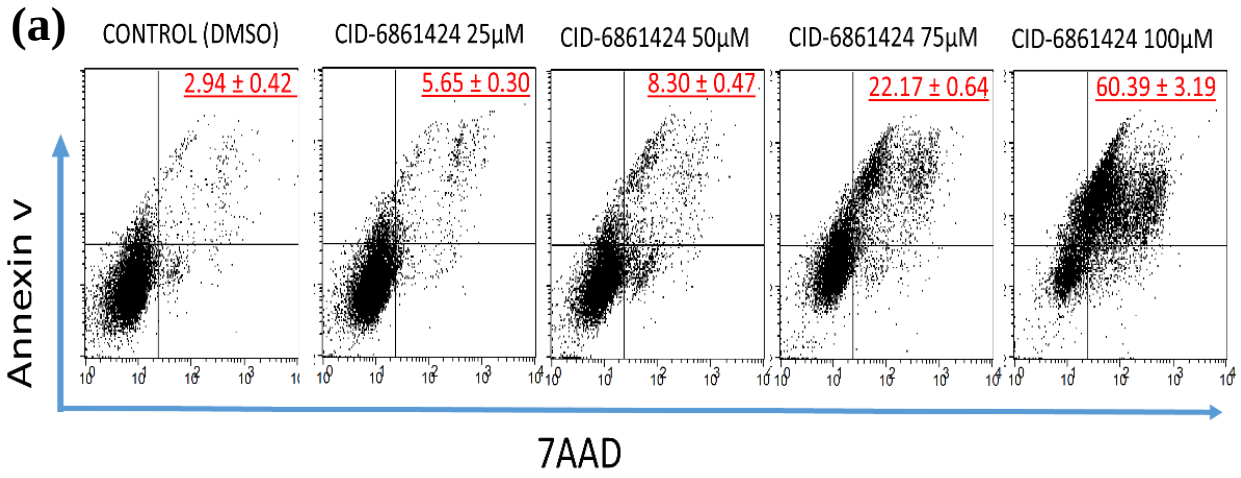


Figure 4.10(a) 50μM CID-6861424 induced p53 protein accumulation in "MDA-MB-231" cell nucleus. (b) The bar-diagram representation of increased fluorescence intensity of p53 in the nucleus of "MDA-MB-231" cell. Mean ± SD value (Data represented was calculatedfor three independent experiments). ***P<0.001.

Protein Expression Profile	Treatments	72hrs
p53	CONTROL	4000.11 ± 540.01
	CID-6861424 50uM	13784.89 ± 4154.56

Table No 4.7 50µM CID-6861424 induced DNA damage in "MDA-MB-231" cell which is confirm by enhanced fluorescence intensity of p53 protein in the "MDA-MB-231" cell nucleus.

This data further supported by apoptosis assay with annexin V and 7AAD dye based kit using flow cytometry after 72 hours of treatment. Samples treated with different concentration of CID-6861424, ranging from 25µM to 100µM, caused significantly increased late apoptotic cells with increasing concentration as compared to the control. The treatment of 50µM CID-6861424 resulted in ~1.7 fold increase in late apoptotic cells as compared to the control. Whereas 75µM and 100µM treatment resulted in almost 4.5 and 9 fold increase in late apoptotic cells as compared to the control set respectively (**Figure 4.11 (a) and 4.11 (b); Table No. 4.8**).



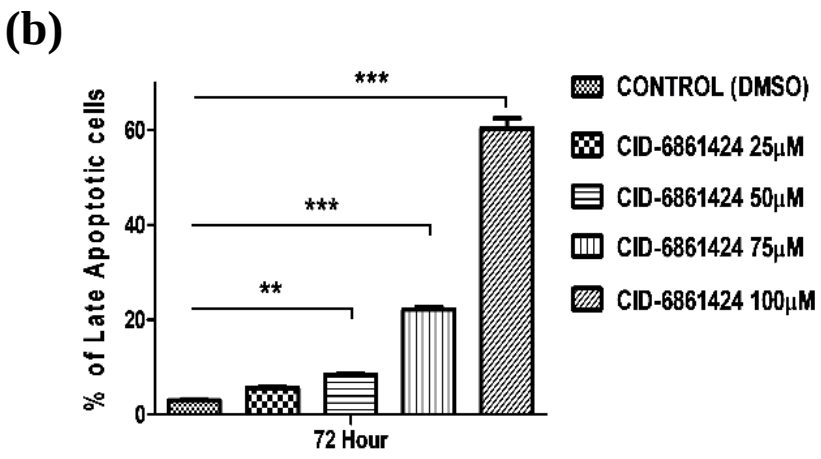


Figure 4.11. (a) CID-6861424 induces late apoptotic cell deaths dectectedby Annexin V / 7AAD kit (BD Biosciences) using flow cytometry and (b) The bar-diagram representation of increased late apoptotic cell deaths with increasing concentration of CID-6861424 in "MDA-MB-231" cell at 72 hours.Mean ± SD value (Data represented was calculatedfor three independent experiments). **P<0.01, and ***P<0.001.

Groups	Damaged Cells	Late Apoptosis	Viable Cell	Early Apoptosis	Total Apoptosis
Control (DMSO)	2.16 ± 0.19	2.94 ± 0.42	89.56 ± 0.62	5.33 ± 0.25	8.27
CID-6861424 25µM	2.34 ± 0.24	5.65 ± 0.30	85.15 ± 0.61	6.85 ± 0.47	12.5
CID-6861424 50µM	5.76 ± 0.22	8.30 ± 0.47	79.65 ± 0.40	6.41 ± 0.06	14.71
CID-6861424 75µM	3.11 ± 0.56	22.17 ± 0.64	59.59 ± 0.72	15.12 ± 0.90	37.29
CID-6861424 100µM	5.77 ± 1.67	60.39 ± 3.19	22.32 ± 1.46	11.42 ± 0.27	71.81

Table No 4.8 CID-6861424 induced late apoptotic cell death in "MDA-MB-231" cell at 72 hours.

This data was further confirmed by expression of apoptotic cell death-related proteins such as Bcl-2 "anti-apoptotic protein" and Bax "pro-apoptotic protein" as determined by western blot data. 50µM CID-6861424 treated cells demonstrated upregulation of Bax and downregulation of Bcl-2 leading to the enhanced the ratio of Bax/Bcl-2 with increasing treatment time **(Figure 4.12 (a) and 4.12 (b))**.Apoptosis data was further confirmed by western blot dat of survivin protein expression.Survivin acts as an anti-apoptotic protein, which inhibits caspase activity and block cell deaths due to the overexpression of survivin protein in a cancer cell. Decrease in survivin protein expression due to 50µM CID-6861424 treatment was detedted**(Figure 4.12 (a)).**

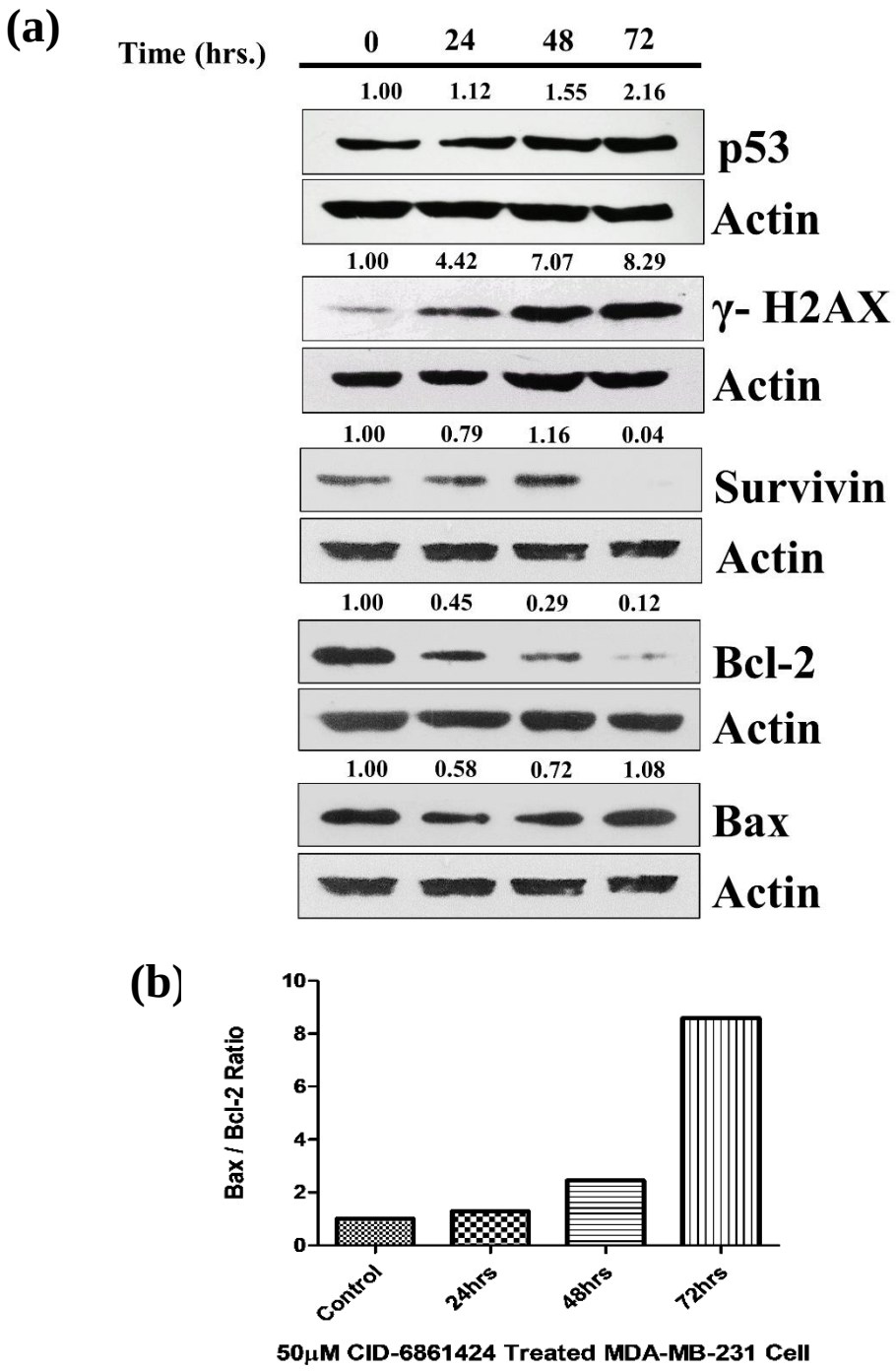


Figure 4.12. (a)The upregulation of p53, γ-H2AX, Bax and downregulation of Bcl-2, survivin proteins expression in presence of 50μM CID-6861424 for the indicated time was observed by western blotting and quantified with Image J software and (b) The bar-diagram representation of increasing Bax/Bcl-2 ratio with time dependent manner.

These results suggested that 50µM CID-6861424 cells enhance ROS generation, cause disruption in **Δψm**, induces excessive DND damage and upregulate Bax/Bcl-2 ratio leading to induction of mitochondrial dependent apoptotic cell death.

4.2.5 50µMCID-6861424 block the epithelial-mesenchymal transition (EMT) via inhibiting the ERK cell survival pathway in MDA-MB-231 cell

The "extracellular signal regulated kinase (ERK)" pathway is a major signaling pathway of "mitogen activated protein kinase (MAPK)" signaling pathway, activated by various stimulations such as extracellular agents, growth factors, hormones and cellular stress, which is responsible for cell proliferation, and differentiation of the cell. Raf family members such as Raf-1, A-Raf and B-Raf are the main components of "MAPK/ERK kinase kinase (MEKK)" pathway, that is phosphorylating on serine residues of "MAPK/ERK kinase (MEK)" components ERK1/2, which is responsible for activation of certain downstream proteins led to transcription of genes essential for mainly cell proliferation, angiogenesis and epithelial-mesenchymal transition (EMT). 50µM CID-6861424 treated cells shows downregulation of phosphorylated of ERK protein expression with increasing in treatment time **(Figure 4.13)**.

The ERK signaling pathway is responsible for inducing epithelial-mesenchymal transition (EMT) via downregulation of epithelial cell junction proteins such as "E-cadherin" and upregulation of mesenchymal cytoskeleton proteins such as "vimentin". This in turn resulting in high rate of malignancy properties in TNBC patients. 50µM CID-6861424 treated cells shows downregulation of vimentin protein expression with increasing treatment time**(Figure 4.13)**.

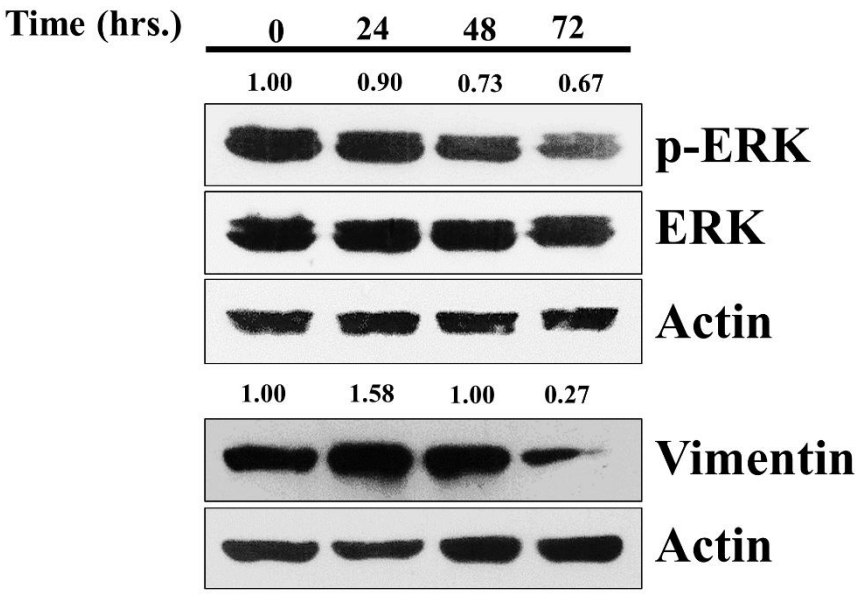


Figure 4.13.50µM CID-6861424 block the ERK cell survival pathway in "MDA-MB-231" cell and epithelial mesenchymal transition (EMT) due to the downregulation of phosphorylated ERK and vimentin proteins expression at the indicated time andquantified with Image J software.

These results suggested that, treatment of 50µM CID-6861424 induces mitochondria mediated cellular apoptotic cell deaths by inhibition ofepithelial-mesenchymal transition (EMT) and ERK cell survival pathway in MDA-MB-231 cell.

4.3 Discussion

Breast cancer is affecting the women all over the world and morbidity of breast cancer has been increasing since the 1970s(Bielawski et al., 2002; Chun et al., 2015).Majority of breast cancer cases are associated with deregulation of hormone receptors. Certain breast cancers lack hormone receptors and are thus called as "hormone-receptor negativebreast cancer". "Triple negative breast cancer (TNBC)" is one such type of cancer which exhibits

aggressive nature and frequently develops resistivity against drugs used in such pathophysiology. Moreover, prolonged treatment and or overdosing of “anti-cancer drugs” are often accompanied with strong toxic side effects. The aggressive and resistant behavior of TNBC is further ameliorated by mutations inp53 and BRCA1 proteins which are otherwise common chemotherapeutic targets, making its treatment challenging. In light of this, developing the efficient and low toxic compound, could be crucial for breast cancer chemotherapy. In the present study, we investigated on a sulfonamide derivative and found it to have potential anti-cancerous property in “MDA-MB-231”cells.The cell viability data suggest that, CID-6861424 significantly inhibit the MDA-MB-231 cell growth with doses and time dependent manner. MDA-MB-231 cell morphology also changed in the presence of an increasing concentration of CID-6861424. Cellular morphology is often used to characterize cellular health. IC_{50} value of this compound is 50µM and significantly inhibited the “MDA-MB-231”cell growth and also changed the cell morphology with abnormal physiology such assmall and roundish as compared to the control with dense cytoplasm and irregular cell membrane. Further, features like dense cytoplasm and membrane protrusions can be an indicator of cellular stress and apoptosis(Decoster et al., 2010; Hacker, 2000).The aggressive nature of cancer cells is often reciprocated by their strong migratory potential. Thus, to evaluate the effect of CID-6861424, we performed wound healing assay and it was observed that the 50µM satisfactorily inhibited cell migration. Cancer cells can multiply indefinitely and to determine their post treatment clonogenic potentials, colony formation assay is performed(Franken et al., 2006). In the present study, the 50µM significantly reduced the clonogenic ability of MDA-MB-231cells. The data from cell viability, morphology, “wound healing” and colony formation assays indicated that 50µM CID-

6861424 successfully inhibiting MDA-MB-231 cell growth. We analyzed the effects of CID-6861424 on cell cycle profile of MDA-MB-231 cells to unravel the mechanism underling. The cell cycle is a complex and tightly regulated process. Unrestrained and irregular cell cyce regulation resulting in uncontrolled cell division is one of the hallmark of cancer. The cell cycle data suggest that, treatment of "MDA-MB-231" cells with CID-6861424 resulted in the significantly G_2/Mphase arrest as compared to the control set. Cyclin B is form an active complex with CDK-1 after it gets dephosphorylated by phosphatase family members such as Cdc25, which is essential for G2 phase to M phase cell cycle progression (Fisher et al., 2012; Gavet and Pines, 2010; Jang et al., 2016). The results indicated that, the IC_{50} value of CID-6861424 disturb the "cyclin B-CDK-1 complex" formation due to the downregulation of cyclin B and CDK-1protein expression. The p21 is a "cyclin-dependent kinase inhibitor (CKI)", that inhibits"cyclin/CDK complexes" formation after DNA damage leading to cell cycle arrest at different phases (Cazzalini et al., 2010; Piccolo and Crispi, 2012). The cell cycle data was supported by upregulation of p21 protein expression.DNA damage activates p53 which in turn transcribes p21 leading to cell cycle arrest (Piccolo and Crispi, 2012). One of the regulator of cellular homeostais is intracellular ROS which is strictly regulated in the normal cells.Cellular exposure to chemotherapeutics drugs causes water radiolysis and results in release of free superoxide ($O_2^{\bullet-}$) and perhydroxyl ($HO_2^{\bullet}$) radicals collectively called ROS (Azzam et al., 2012). These "free radicals" are highly reactive and interact with biomolecules including DNA causing damage and oxidative stress (Azzam et al., 2012). Cancer cells comparatively have higher ROS levels and is one of the major causative factor for oncogenesis (Panieri and Santoro, 2016; Schumacker, 2006; Wang and Yi, 2008). Apart from generation of high levels of ROS,

cancer cells generate high levels of antioxidant proteins such as GSH, catalase, SOD etc. that can quench excess ROS (Liou and Storz, 2010) thus maintain ROS homeostasis. Most of the cancer therapies target the ROS level of cancer cells which is either downregulated by means of antioxidants and other phytochemicals (Ahmad and Mukhtar, 2013)or unregulated by means of chemotherapeutic compounds (Pelicano et al., 2004; Trachootham et al., 2009)and physical agents like radiation (Azzam et al., 2012). Thus, we analysed the generation of cellular ROS post 72 hour treatment. The treatment of 25µM CID-6861424 increased ROS levels, however a significant increase in levels of ROS was observed in 50µM CID-6861424 treated set as compared to control set. Due to high level of antioxidant expression in cancer cells, ROS generated after 25µM CID-6861424 treatment may have neutralized ROS and the net charge in ROS level show was not high. In case of 50µM treatment, ROS generation might have been very hight and even the high level of antioxidants available in cancer cells failed to neutralize it leading to detection of significant change ROS level. In mammalian cells elevated levels of ROS can lead to disruption of **Δψm**(Banki et al., 1999; Fonseca-Silva et al., 2011; Johnson et al., 1996) resulting in mitochondrial mediated cellular apoptosis (Reed et al., 1998). Thus, to investigate mitochondrial membrane potential status, we further studied the effects of the CID-6861424 treatment on **Δψm** in MDA-MB-231 cells. 25µM CID-6861424 treated MDA-MB-231 cells caused disruption in **Δψm**, while their 50µM comparatively caused significant disruption in **Δψm**. Over production of ROS can also induce DNA damage due to oxidative stress. γ-H2AX is associated with the repair of double strand (ds) DNA breakage/damage induced by exposure to chemotherapeutic drugs, oxidative stress, radiation, etc. and is considered as a marker of ds DNA breakage (Dickey et al., 2009). In the present study, treatment of 50µM

CID-6861424 resulted in increased expression of γ-H2AX with increasing treatment time. Further, at 72 hours post treatment, microscopic investigation of γ-H2AX foci clearly demonstrates that the 50µM treatment leads to excessive DNA damage as compared to DMSO control. The western blot and microscopic analysis of γ-H2AX clearly indicate that 50µM CID-6861424 induced ds DNA breaks in "MDA-MB-231" cells. The p53 is a tumor suppressor protein, which is overexpressed due to the excessive DNA damage by exposure to chemotherapeutic drugs and oxidative stress etc. (Hientz et al., 2017). It is a well-known protein that guards gainst"DNA damage" via cell cycle arrest, DNA repair and apoptosis (Bargonetti and Manfredi, 2002; Chiang et al., 2013; Klein and Vassilev, 2004; Ko and Prives, 1996; Levine, 1997; SMITH et al., 2003). Our results show that cell on exposure to CID-6861424 caused upregulation of p53 protein level in "MDA-MB-231" cells with increasing treatment time and these result were further confirmed by immunofluorescence data. Increased ROS, depletion in **Δψm** due to the DNA damages can be induce the cell death caused due to apoptosis. The apoptosis confirms via annexin V/ 7AAD assay using flow cytometry."MDA-MB-231" cells treated with increasing concentration of CID-6861424 induced significantly increased late apoptotic cells as compared to the DMSO control. The upregulation of p53 protein level in the cell, indicates activation of mitochondrial depended apoptotic cell deaths and enhance the ratio of pro-apoptotic/anti-apoptotic proteins level in the cell (Chipuk and Green, 2004). Bcl-2 and Bax an anti-apoptotic and apoptotic proteins, respectively are closely linked with mitochondrial apoptosis. Localized in the outer mitochondrial membrane, Bcl-2 is reported to inhibit opening of "mitochondrial membrane permeability transition pores"(Zamzami, 1996)through perpetuation of NADPH(Esposti et al., 1999; Kowaltowski et al.,

2000),leading to decrease in proteins accountable for its opening(Kowaltowski et al., 2001). It also results in blocking of release of apoptogenic proteins in apoptosis inducing environment(Daugas et al., 1996; Kluck, 1997; Yang et al., 1997; Zamzami, 1996). Besides, Bcl-2 is also reported to quench oxidative stress by inhibiting lipid per-oxidation (Hockenbery et al., 1993).In case of 50µM treated cells, ratio of Bax/Bcl-2 level was significantly high with increase in treatment time and induced mitochondrial mediated apoptosis. These results were further confirmed by downregulation of survivin protein expression with increasing time, which is observed by western blotting. Survivin protein act as anti-apoptotic protein, which is overexpressed in the cancer cell and inhibit apoptotic cell deaths by blocking the caspases activity (Mittal et al., 2015). Thus, prolonged G_2/Mphase arrestdue to inhibition of "cyclin B-CDK-1 complex" formation, overproduction of ROS resulted in DNA damage and disruption in **$\Delta\psi m$** followed by upregulation of p53, p21, γ-H2AX, Bax, and downregulation of Bcl-2&survivin leading to mitochondrial mediated apoptosis in 50µM CID-6861424 treated sample.

In TNBC Patients, "mitogen activated protein kinase (MAPK)" signaling pathway is a well-known activated pathway. "Extracellular signal related kinases (ERK)", is a one of the important member of "MAPK signaling pathway", which is essential for cell proliferation, differentiation, cell survival, angiogenesis and high rate of "epithelial-mesenchymal transition (EMT)"(Bartholomeusz et al., 2012; McCawley et al., 1999; Miglietta et al., 2006).

Raf family members are the main component of ERK signaling pathway, which is phosphorylated of ERK1/2 at serine residues resulting in activation of ERK and leads to

high rate of cell proliferation and cell survival. Activated MAPK signaling in TNBC promote, cancer cells are metastasis into lymph nodes (Adeyinka et al., 2002).

TNBC is a more aggressive type of breast cancer and difficult to treat due to lack of hormonal based therapies. TNBC has a high rate of epithelial-mesenchymal transition (EMT) withdownregulation of epithelial cell junction proteins such as "E-cadherin" and upregulation of mesenchymal cytoskeletal proteins such as "Vimentin" resulting in metastasis(Arias, 2001; Thompson and Newgreen, 2005).

IC_{50} value of CID-6861424 inhibit of ERK signaling pathway and "epithelial-mesenchymal transition (EMT)"was confirmed by western blotting data, downregulation of phosphorylated ERK and vimentin proteins expression in "MDA-MB-231" cell was observed. The 50µM CID-6861424 induced mitochondrial mediated apoptotic cells death in "MDA-MB-231" cell due to the inhibition of epithelial-mesenchymal transition (EMT) and ERK cell survival pathway.

4.4 Conclusion

CID-6861424 inhibits cell growth, suppresses cell migration potential, promotes G_2/M phase cell cycle arrest, elevates ROS generation, deplets of mitochondrial membrane potential and increases DNA damage. This resulted in mitochondrial mediated apoptotic cell death via downregulation of phosphorylated ERK protein expression level resulting in inhibition of ERK cell survival pathway. The treatment also inhibit epithelial-mesenchymal transition (EMT) in MDA-MB-231 cells. Based on results a model is proposed **(Figure 4.14)**CID-6861424 can be used as an anticancer agent to improve chemotherapy for TNBC.

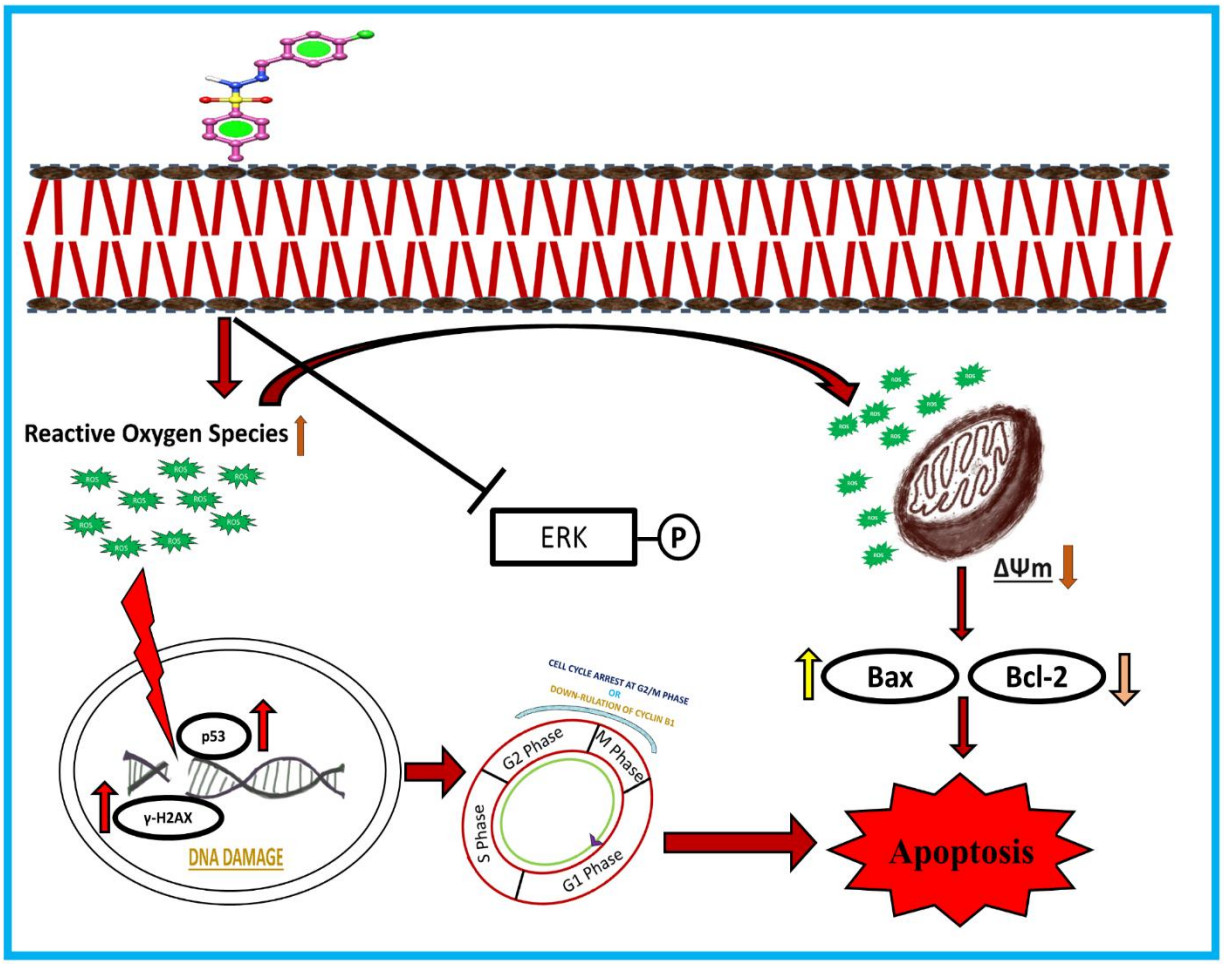


Figure 4.14. Proposed model for the mechanism of action of CID-6861424 in MDA-MB-231 cell.

Chapter 5

CID-6861424 IS NON-TOXIC IN IN-VITRO AS WELL AS IN-VIVO STUDIES

CHAPTER-5

CID-6861424 IS NON-TOXIC IN IN-VITRO AS WELL AS IN-VIVO STUDIES

5.1 Introduction

Chemotherapy is one of the most common choices in the treatment of cancer, particularly in the early stage. Chemotherapeutics are also administered as adjuvants along with radiotherapy and/or immunotherapy. Mechanistically most anti-cancer drugs are cytotoxic in nature and thus also show cytotoxicity to healthy cells. The cancer cells are normal cells harboring cellular insults and thus chemotherapeutics often fail to differentiate and also induce toxicity to healthy cells. Pharmacovigilance is a branch where evaluation and assessment of benefits and risks of chemotherapeutics is carried out (Meyboom et al., 1999). Almost all commercial drugs are screened for their adverse effects, yet many of them show prominent side effects. Many anti-cancerous drugs like “Doxorubicin”, “Paclitaxel”, “Cisplatine”, “Anthracyclines”, “Tamoxifen” etc. show side effects like anaemia, thrombocytopenia, nausea, diarrhea, myelopathy, neuropathy, hypersensitivity, cardiac, hepatic and renal toxicity etc (GB. McDonald, 1984; McGowan et al., 2017; Phillips, 2001; Remesh, 2012). Besides, studies have shown that many novel drugs fail in clinical trials due to their severe side effects (Hwang et al., 2016). Small chemical drug like molecules are potent but the two major concerns regarding their use is the toxicity of the parent compound and their acquired toxicities and drug resistance post metabolism (Akhdar et al., 2012; Filipa Reis-Mendes et al., 2015; Hrynchak et al., 2017). Thus, evaluation of side effects of anti-cancer drugs is required for the risk management of such drugs.

The present study concurs the requirement of pharmacovigilance of novel compounds and thus presents a systematic evaluation of such canonical parameters often used in pharmacovigilance. The study aims to evaluate the “in-vitro” and “in-vivo” effects of CID-6861424 in NIH3T3 cells and female “BALB/c” mice. Using “in-vitro” cell based assay like cell viability, cell morphology, cell migration, cell cycle and levels of ROS and changes in **Δψm**, we have determined the effects of CID-6861424 in comparison to control (DMSO). Also, analysis of “DNA damage” by comet assay in whole blood and bone marrow cells in CID-6861424 treated female BALB/c mice was carried out. Besides, effects of CID-6861424 treatment on the routine parameters like ROS, lipid peroxidation, GSH, SOD and catalase as indicative of oxidative stress were also evaluated in blood, liver, kidney and spleen of female BALB/c mice.

5.2 Results

5.2.1 In vitro evaluation of toxic effects of CID-6861424 in NIH3T3 cells

In previous chapters, we have shown that CID-6861424 alone at 50µM induces apoptosis in MCF-7 and MDA-MB-231 breast cancer cells by targeting Akt cell proliferation and ERK cell survival pathway**(The detail is provided in the section of chapter-3 and 4)**. Thus, to evaluate the effects of CID-6861424 at 25µM and 50µM CID-6861424 in non-cancerous fibroblast cells (NIH3T3) following studies were performed.

5.2.1.1 Effect of CID-6861424 on NIH3T3 cell viability and morphology

In previous chapters treatment of CID-6861424 at 50µM induced apoptosis in breast cancer cell lines at 24 hours, 48 hours and 72 hours of treatment, thus NIH3T3 cells were also treated with 25µM and 50µM of CID-6861424 and no significant effect on cell viability was observed at indicated time interval**(Figure 5.1; Table 5.1)**.Also in comparison to control (DMSO), no significant changes in cell morphology was observed post 72 hours of treatment of CID-6861424 25µM and 50µM **(Figure 5.2)**.

The cell viability and morphology data demonstrates that CID-6861424 at 25µM and 50µMtreatment is non-toxic to normal cells

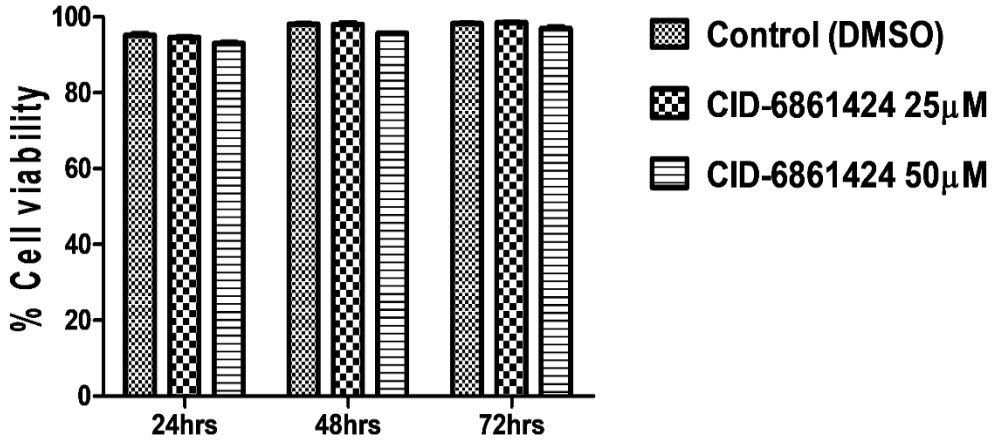


Figure 5.1 Cell viability of NIH3T3 cell at 24 hours, 48 hours and 72 hours of treatment of CID-6861424 at 25µM and 50µM in comparison to control (DMSO) treated cells. Each bar-diagram represents the calculated values of percentage of viable NIH3T3 cells and are mean ±SD with n=3.

Time after treatment	Treatments	NIH3T3
24 hrs	CONTROL	95.21±0.64
	CID-6861424 25µM	94.60±0.33
	CID-6861424 50µM	93.03±0.63
48 hrs	CONTROL	98.27±0.31
	CID-6861424 25µM	98.04±0.75
	CID-6861424 50µM	95.67±0.05
72 hrs	CONTROL	98.27±0.11
	CID-6861424 25µM	98.53±0.19
	CID-6861424 50µM	96.95±0.65

Table 5.1 Percentage of viable NIH3T3 cells at 24 hours, 48 hours and 72 hours of treatment of CID-6861424 25µM and 50µM in comparison to control (DMSO).

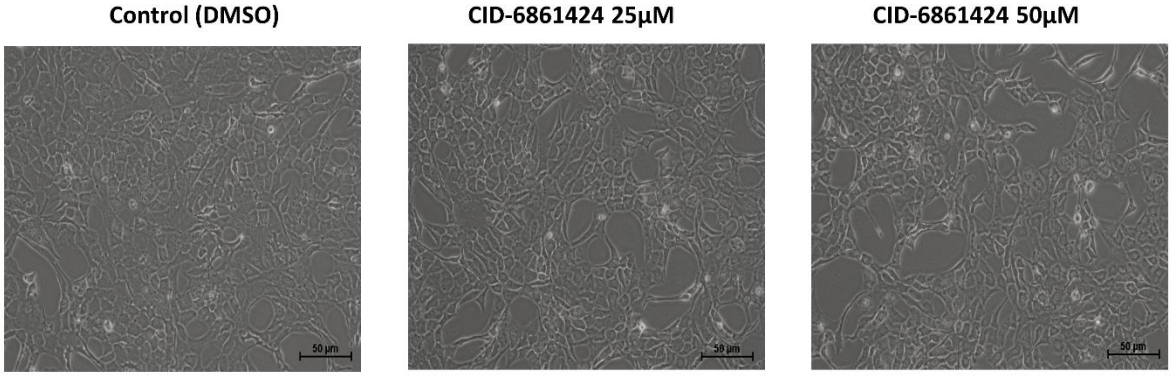


Figure 5.2 Cell morphology of NIH3T3 cell line post 72 hour of treatment of CID-6861424 at 25µM and 50µM, in comparison to control (DMSO) treated cells at 20X magnification.

5.2.1.2 Effect of CID-6861424 on NIH3T3 cell migration potential

In previous chapters, treatment of CID-6861424 significantly inhibited migration of breast cancer cells. To study their effects in NIH3T3 cells, cell migration study was carried out. After 72 hour of treatment of CID-6861424 alone at 25µM and 50µM did not inhibited cell migration and the length of the scratch was almost similar to control (DMSO) **(Figure 5.3)**.

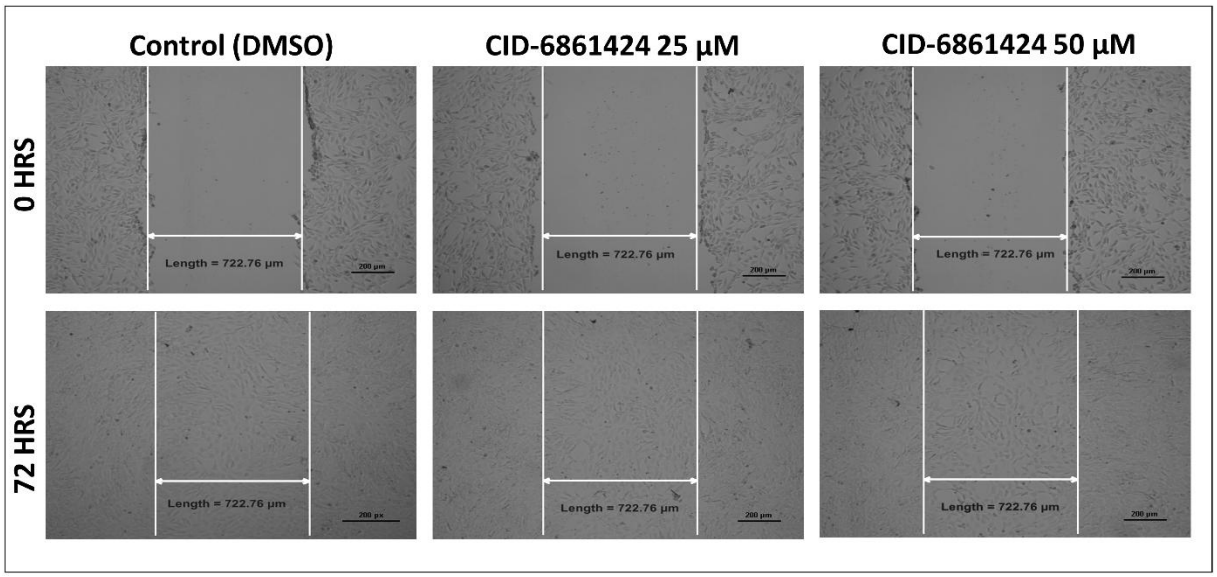


Figure 5.3 Cell-migration at 20X magnification in control (DMSO), CID-6861424 at 25µM and 50µM NIH3T3 cell line at 72 hour.

5.2.1.3 Effect of CID-6861424 on NIH3T3 cell cycle profile

CID-6861424 induces cell cycle arrest in breast cancer cell line. Thus, cell cycle analysis was carried out in NIH3T3 cells to assess the effects of CID-6861424 post 72 hour of treatment. The cell cycle data shows that CID-6861424 at 25µM and 50µM did not affected NIH3T3 cell cycle and the cell cycle profile of control (DMSO) treated cells was similar to that of CID-6861424 25µM and 50µM treated cells and no significant changes were observed **(Figure 5.4)**. A detail analysis of different cell cycle stages of NIH3T3 cells in

response to control (DMSO), CID-6861424 25µM and CID-6861424 50µM treatment at 72 hour is described in **Table 5.2**

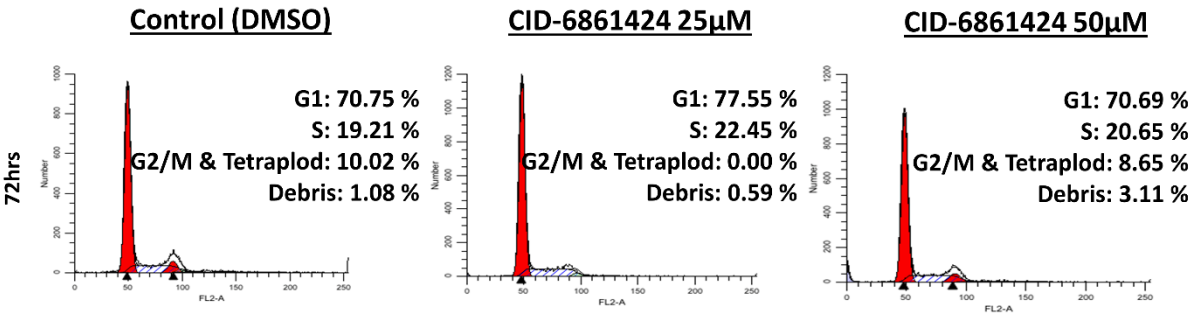


Figure 5.4 Cell cycle profile of NIH3T3 cells post 72 hour of treatment with CID-6861424 25µM and 50µM with respect to control (DMSO).

Cell Line	Time	Treatments	G1 PHASE	S PHASE	G2/M PHASE
NIH3T3	72hrs	CONTROL (DMSO)	71.49±1.04	19.26±0.07	9.22±1.12
		CID-6861424 25µM	76.09±2.06	20.77±2.36	3.12±4.41
		CID-6861424 50µM	71.34±0.92	20.22±0.60	8.42±0.32

Table 5.2 Different phases of NIH3T3 cell cycle post 72 hour of treatment with CID-6861424 25µM and 50µM with respect to control (DMSO).

5.2.1.4 Effect of CID-6861424 on generation of reactive oxygen species and disruption of mitochondrial membrane potential (Δψm) in NIH3T3 cell

In our previous studies on breast cancer cell lines, CID-6861424 enhanced intracellular ROS production leading to disruption of **Δψm** and causing apoptosis. Thus, ROS generation and changes in **Δψm** of NIH3T3 cells in response to CID-6861424 treatment was instigated using DMSO as control and doxorubicin as positive control. The ROS

production was measured by changes in fluorescence of DCF (2', 7' –dichlorofluorescein); an oxidized form of DCFDA (2',7' –dichlorofluorescein diacetate). There was no significant change in fluorescence data in control (DMSO) and CID-6861424 treated sets (25µM and 50µM), while the doxorubicin positive control set showed significantly higher fluorescence indicating generation of ROS **(Figure 5.5 (a) and 5.5 (b); Table 5.3)**. Similarly, **Δψm** is measured by change in fluorescence of rhodamine 123 dye, where the more stable the **Δψm**, less is the fluorescence. Treatment of NIH3T3 cells with control (DMSO) and CID-6861424 treated sets (25µM and 50µM) showed similar fluorescence of rhodamine 123, while the doxorubicin positive control set showed positive shift in fluorescence peak indicating disruption of **Δψm (Figure 5.6 (a) and 5.6 (b), Table 5.4)**. Thus, the generation of ROS and disruption of **Δψm** data clearly shows that CID-6861424 at 25µM and 50µM has no effect on ROS production and **Δψm** disruption in NIH3T3 cells.

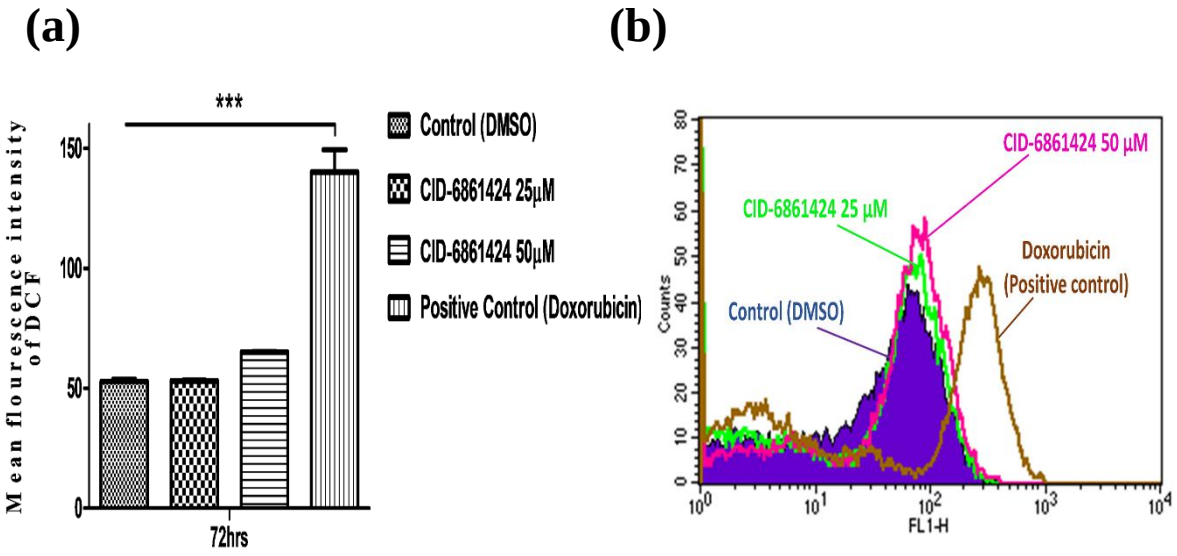


Figure 5.5 (a)Analysis of mean fluorescence intensity of DCF in NIH3T3 cell in response to treatment of control (DMSO), CID-6861424 25µM, CID-6861424 50µM and doxorubicin. (b)ROS production profile as a measure of change in fluorescence intensity of DCF in

response to control (DMSO) (blue peak), CID-6861424 25µM (green peak), CID-6861424 50µM (magenta peak) and doxorubicin (brown peak) treatment in NIH3T3 cells after 72 hour. Each histogram represents the calculated value of mean fluorescence intensity of DCF.Mean ± SD value (Data represented was calculatedfor three independent experiments).*p<0.001.**

Cell Line	Treatments	Mean fluorescence intensity (DCF)
NIH3T3	CONTROL	52.90 ± 1.37
	CID-6861424 25uM	53.07 ± 0.33
	CID-6861424 50uM	65.07 ± 0.41
	Positive Control (Doxorubicin)	140.25 ± 15.97

Table 5.3 Mean fluorescence intensity of DCF in NIH3T3 cell in response to treatment of control (DMSO), CID-6861424 25µM, CID-6861424 50µM and doxorubicin.

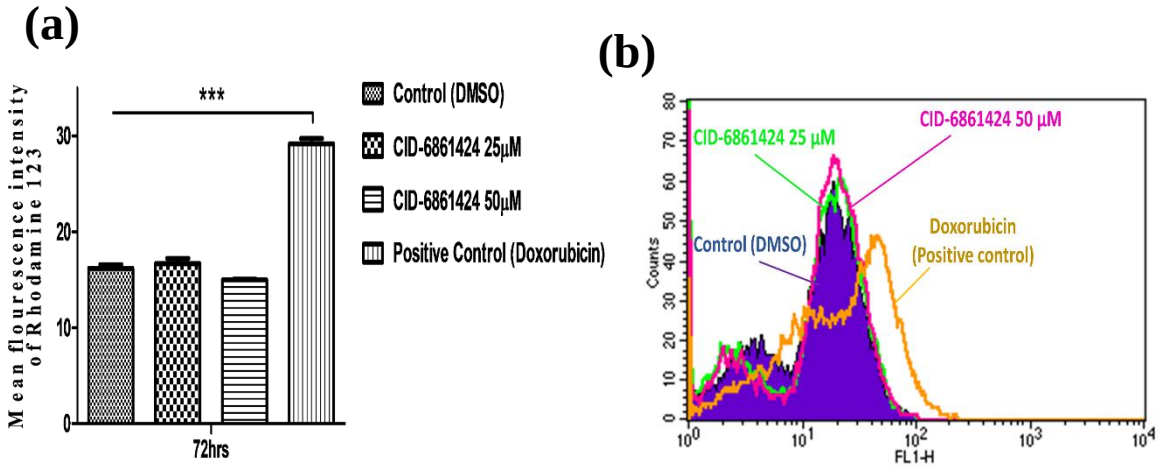


Figure 5.6 (a)Analysis of mean fluorescence intensity of rhodamine 123 dye in NIH3T3 cell in response to treatment of control (DMSO), CID-6861424 25µM, CID-6861424 50µM and doxorubicin.(b)Analysis of Δψmdisruption profile as a measure of change in fluorescence intensity of rhodamine 123 dye in response to control (DMSO) (blue peak), CID-6861424

25µM (green peak), CID-6861424 50µM (magenta peak) and doxorubicin (brown peak) treatment in NIH3T3 cells after 72 hour. Each histogram represents the calculated value of mean fluorescence intensity of Rhodamine 123 dye.Mean ± SD value (Data represented was calculatedfor three independent experiments).*p<0.001.**

Cell Line	Treatments	Mean fluorescence intensity (Rhodamine 123)
NIH3T3	CONTROL	16.16 ± 0.71
	CID-6861424 25uM	16.66 ± 0.93
	CID-6861424 50uM	14.96 ± 0.15
	Positive Control (Doxorubicin)	29.15 ± 0.97

Table 5.4 Mean fluorescence intensity of Rhodamine 123 in NIH3T3 cell in response to treatment of control (DMSO), CID-6861424 25µM, CID-6861424 50µM and doxorubicin.

5.2.2 In vivo evaluation of toxic effect of CID-6861424 in female BALB/c mice

The *in vitro* effects of CID-6861424 were evaluated in NIH3T3 cells using different cell culture based assays. Further, to evaluate the effects of CID-6861424 in mice model, following parameters were evaluated to study the toxic effects of CID-6861424.

5.2.2.1 CID-6861424 treatment does not induce significant change in body weight of female BALB/cmice

Chemotherapy may increase or decrease body weight and such changes are induced due to altered metabolism upon treatment. Thus, we monitored the body weights of control (DMSO) and CID-6861424 (5mg/kg and 10mg/kg) treated female BALB/c mice. The initial mean body weight for each set i.e. Set-1 control (DMSO), Set-2 (5mg/kg body

weight) and Set-3 (10mg/kg body weight) of CID-6861424 was approximately 25±2.0 g. No significant change in body weight was found during the course of treatment of 30 days **(Figure 5.7)**. During the study, initially the body weight in all the groups declined to 15 to 20 g, but as the study proceeded there was stability in the body weights amongst all the groups from day 5 to day 12. The body weight of animals in all the groups then increased and further no decline in body weight was observed from day 13 onwards. The comparative body weight data clearly shows that treatment of CID-6861424 does not induces any significant change in the body weights in both Set-2 and Set-3 as compare to the Set-1 control (DMSO).

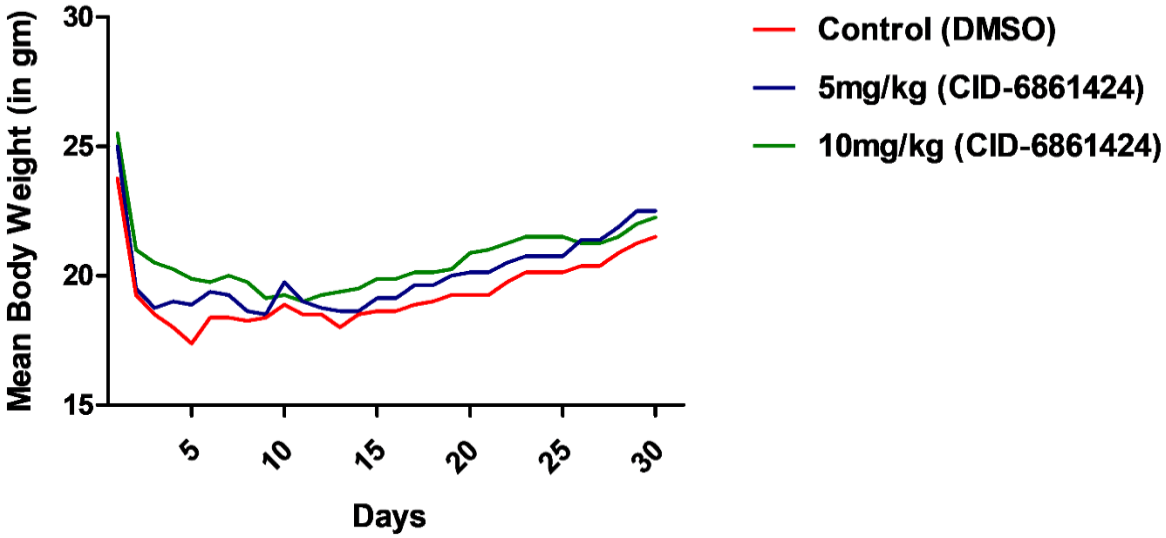


Figure 5.7 Comparative body weight graphs amongst the three treated groups with n=4: Set-1 control (DMSO) in red, Set-2 (5mg/kg body weight) in blue and Set-3 (10mg/kg body weight) in green.

5.2.2.2 CID-6861424 treatment does cause DNA damage in blood and bone marrow of female BALB/cmice

To study the effect of CID-6861424 treatment on DNA, comet assay in whole blood and bone marrow cells was performed in all the three treated groups. Alkaline comet assay is a

strong and standard technique used to analyze DNA damage indicated by formation of comet like appearance, where the distortion of nucleus (comet head) and tail lengths represent extension of DNA damage. The alkaline "comet assay" is based on the principal that DNA when damaged will lose its structural integrity and in response charge the fragmented DNA will travel more than the non-fragmented DNA. Thus the fragmented DNA appears as a smear called as "comets" when observed under fluorescence microscope upon ethidium bromide staining. Treatment of CID-6861424 does not show any significant "DNA damage" in the whole blood and bone marrow cells. The comet assay data from whole blood cells clearly shows that the nucleus in all the three treated groups is uniform showing smooth boundaries without forming any tail **(Figure 5.8)**.

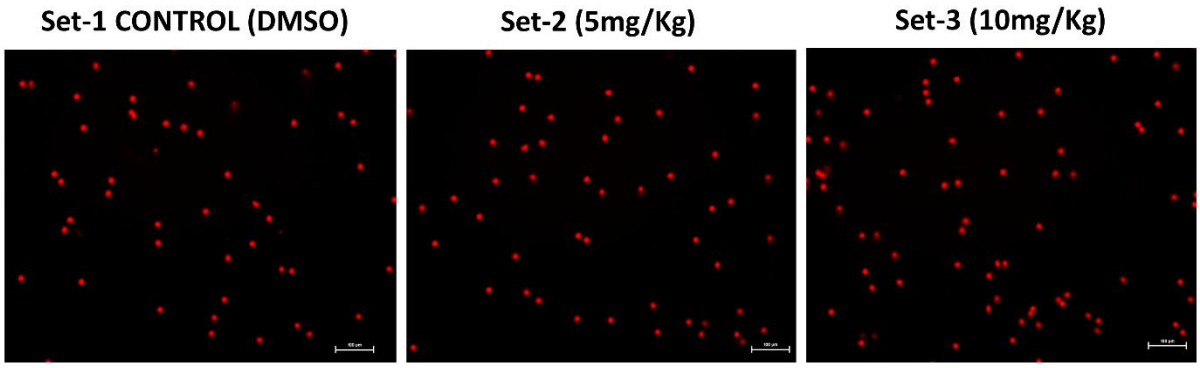


Figure 5.8 Comparative analysis of DNA damage by comet formation in whole blood cells in all the three treated group: Set-1 control (DMSO), Set-2 (5mg/kg body weight) and Set-3 (10mg/kg body weight) treated cells.

The comet assay data from bone marrow cells in all three treated group shows similar morphologies of comets with few cells forming comets and few cells showing prominent circular morphological features having smooth boundaries. The similarities of comets amongst all three treated groups in bone marrow cells show that CID-6861424 treatment does not induce in bone marrow cells **(Figure 5.9)**.

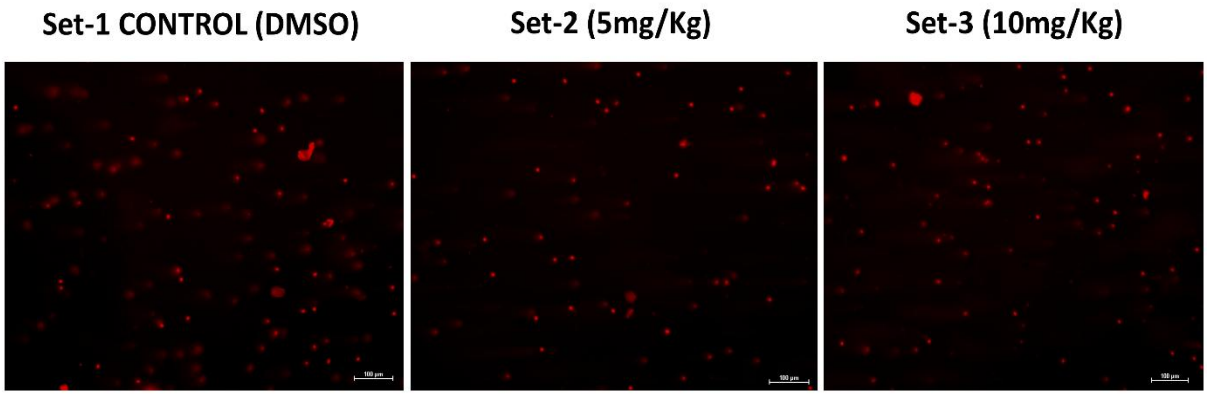


Figure 5.9 Comparative analysis of "DNA damage" by comet formation in bone marrow cells in all the three treated groups: Set-1 control (DMSO), Set-2 (5mg/kg body weight) and Set-3 (10mg/kg body weight) treated cells.

5.2.3 Analysis of canonical oxidative stress parameters in CID-6861424 treated blood, liver, kidney and spleen of female BALB/cmice with n=4

5.2.3.1 Evaluation of CID-6861424 on reactive oxygen species (ROS)

Reactive oxygen species are used as markers of oxidative stress which causes damage to DNA by free radicals. Thus, we determined the levels of ROS generated in response to CID-6861424 treatment in female BALB/c mice. The blood ROS levels in Set-1 (control (DMSO) treated group) was 2.53±0.27 which was similar to CID-6861424 treated Set-2 (5mg/kg body weight) and Set-3 (10mg/kg body weight) treatments with mean values of 2.39±0.12 and 2.22±0.22 respectively. The liver ROS mean values for Set-1, Set-2 and Set-3 were also in the same range with 12.38±0.13 for Set-1, 12.45±0.05 for Set-2 and 12.36±0.21 for Set-3 respectively. The mean value for ROS levels in kidney was 13.27±0.70 for Set-1, 13.17±0.74 for Set-2 and 13.25±0.55 for Set-3. Similarly there were no significant changes in spleen ROS values between control and CID-6861424 treated groups with 4.92±1.05 for Set-1, 5.06±0.26 for Set-2 and 4.82±0.45 for Set-3 **(Figure 5.10 & Table 5.5)**.

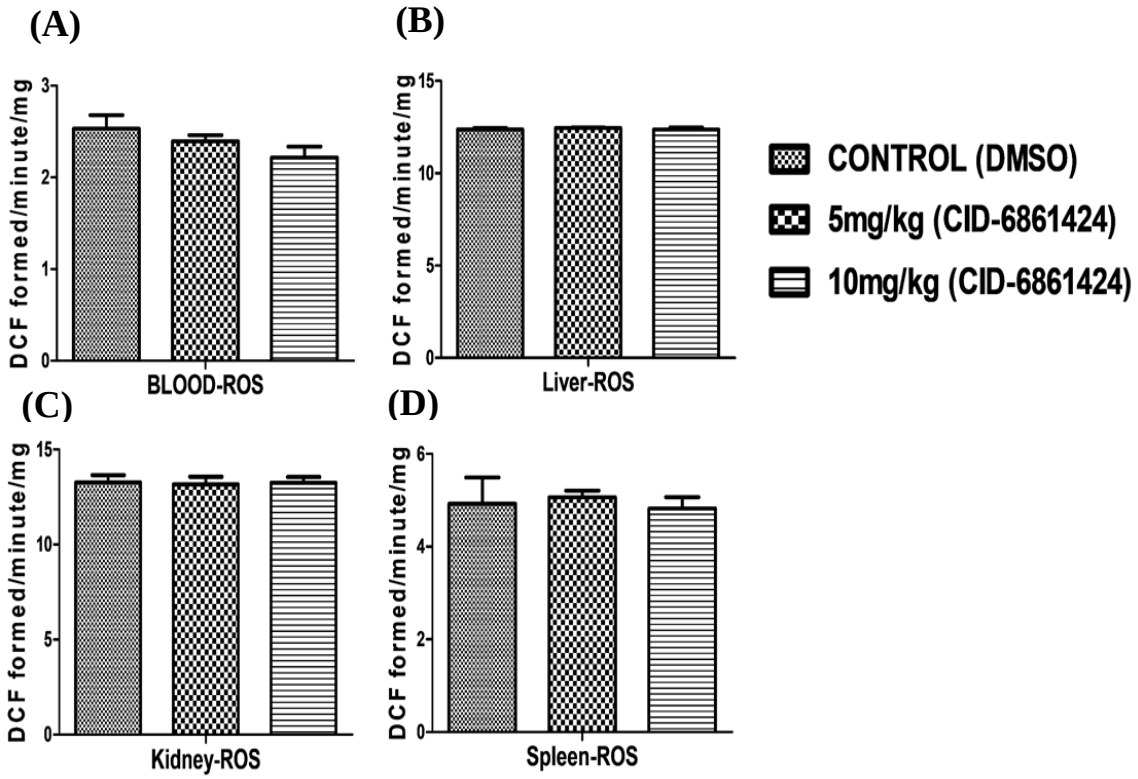


Figure 5.10The amount of ROS measured as active DCF formed/minute/ml of RBCs or per mg tissue and its fluorescent intensity was determined in (A) blood, (B) liver, (C) kidney and (D) spleen of female BALB/c mice upon CID-6861424 treatment: Control (DMSO) (Set-1), treatment of 5mg/kg body weight of CID-6861424 (Set-2) and treatment of 10mg/kg body weight of CID-6861424 (Set-3). Each histogram represents the calculated value of ROS and are mean ±SD with n=4.

Tissue/Organ	**Set-1** Control (Vehicle only)	**Set-2** 5 mg/kg body wt (alternatively for 30 days)	**Set-3** 10 mg/kg body wt (alternatively for 30 days)
Blood	**2.53 ± 0.27**	**2.39 ± 0.12**	**2.22 ± 0.22**
Liver	**12.38 ± 0.13**	**12.45 ± 0.05**	**12.36 ± 0.21**
Kidney	**13.27 ± 0.70**	**13.17 ± 0.74**	**13.25 ± 0.55**
Spleen	**4.92 ± 1.05**	**5.06 ± 0.26**	**4.82 ± 0.45**

Table 5.5 Levels of ROS in blood, liver, kidney and spleen in CID-6861424 treated female BALB/c mice (n=4)

5.2.3.2 Evaluation of CID-6861424 on lipid peroxidation by measurement of Thiobarbituric acid reactive substances (TABARS)

Lipid peroxidation is also used as a marker of oxidation induced cellular stress. Increase in lipid peroxidation leads to generation of ROS and thus increase in lipid peroxidation can be used as marker of DNA damage by oxidizing agents (Niki, 2008). Lipid upon peroxidation produce malondialdehyde (MDA) as one of the by-products, which when reacts with thiobarbituric acid (TBA) is used as an index of lipid peroxidation (Del Rio et al., 2005). The level of lipid peroxidation upon CID-6861424 treatment was thus evaluated in female BALB/c mice by TBARS assay. The blood lipid peroxidation levels in Set-1 (control (DMSO) treated group) was 284.20±10.86 which was similar to CID-6861424 treated Set-3 (10mg/kg body weight) 269.09±16.68. However the values of lipid peroxidation in CID-6861424 treated Set-2 (5 mg/kg body weight) was significantly low with mean value of 226.91±45.43. The liver lipid peroxidation mean values for Set-1, Set-2 and Set-3 were in the same range with 103.49±13.48 for Set-1, 107.11±16.71 for Set-2 and 109.33±7.13 for Set-3 respectively. The mean value for lipid peroxidation levels in kidney was 123.46±4.85 for Set-1, 114.20±0.20 for Set-2 and 13.25±0.55 for Set-3. Similarly there were no significant changes in spleen lipid peroxidation between control and CID-6861424 treated groups with and the calculated mean TBARS values were 4.92±1.05 for Set-1, 5.06±0.26 for Set-2 and 4.82±0.45 for Set-3 **(Figure 5.11 & Table 5.6)**.

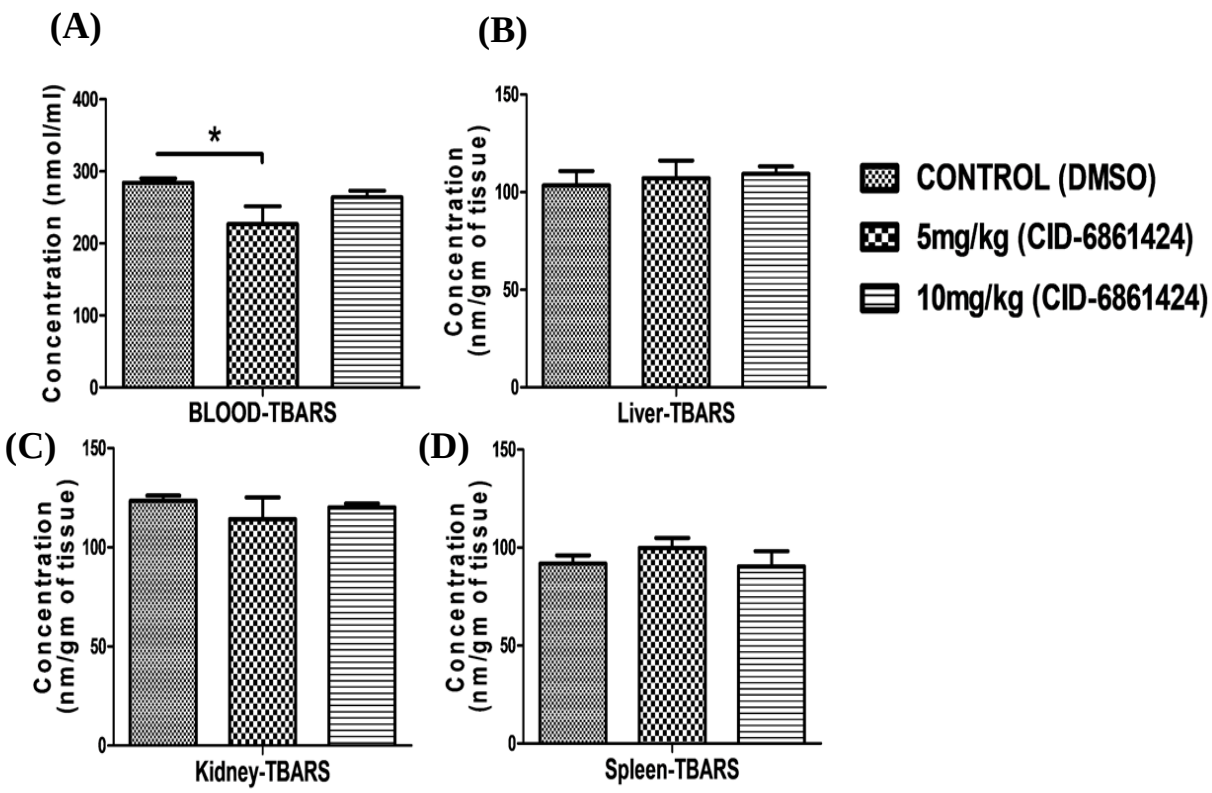


Figure 5.11The amount of TBARS formed and measured as concentration in nm/ml of blood or per mg of tissue as an indicator of lipid peroxidation in (A) blood, (B) liver, (C) kidney and (D) spleen of female BALB/c mice upon CID-6861424 treatment: Control (DMSO) (Set-1), treatment of 5mg/kg body weight of CID-6861424 (Set-2) and treatment of 10mg/kg body weight of CID-6861424 (Set-3). Each histogram represents the calculated value of TBARS and are mean ±SD with n=4.

Tissue/Organ	**Set-1** Control (Vehicle only)	**Set-2** 5 mg/kg body wt (alternatively for 30 days)	**Set-3** 10 mg/kg body wt (alternatively for 30 days)
Blood	**284.20 ± 10.86**	**226.91 ± 45.43**	**264.09 ± 16.68**
Liver	**103.49 ± 13.48**	**107.11 ± 16.71**	**109.33 ± 7.13**
Kidney	**123.46 ± 4.85**	**114.20 ± 20.38**	**120.21 ± 3.46**
Spleen	**91.85 ± 7.79**	**99.84 ± 9.33**	**90.45 ± 14.29**

Table 5.6 Levels of TBARS in blood, liver, kidney and spleen in CID-6861424 treated BALB/c mice (n=4)

5.2.3.3 Evaluation of CID-6861424 on reduced glutathione tri-peptide GSH for assessment of reducing equivalence

Cells encounters number of injuries in response to free radicals and GSH a small non-protein sulfhydryl comprising of three amino acid sequence Gly-Ser-His is the key player which neutralizes free radicals and repair cellular injuries by oxidation-reduction catalysis. Besides it also reduces the toxic effects of anti-neoplastic drugs (Arrick and Nathan, 1984). The level of GSH upon CID-6861424 treatment was thus evaluated in female BALB/c mice. The blood GSH levels in all the three groups was almost same with values of 0.53±0.0 for Set-1 (control (DMSO) treated group), 0.49±0.07 and 0.50±0.05 for CID-6861424 treated Set-2 and Set-3. The liver GSH mean values for Set-1, Set-2 and Set-3 were also in the same range with 42.81±0.46 for Set-1, 43.46±0.21 for Set-2 and 43.69±0.76 for Set-3 respectively. The mean GSH value in kidney was 95.81±0.50 for Set-1, 94.08±1.46 for Set-2 and 95.76±0.80 for Set-3. Similarly there were no significant changes in spleen mean GSH values between control and CID-6861424 treated groups with 62.67±1.15 for Set-1, 61.62±0.54 for Set-2 and 62.51±0.59 for Set-3 respectively **(Figure 5.12 & Table 5.7)**.

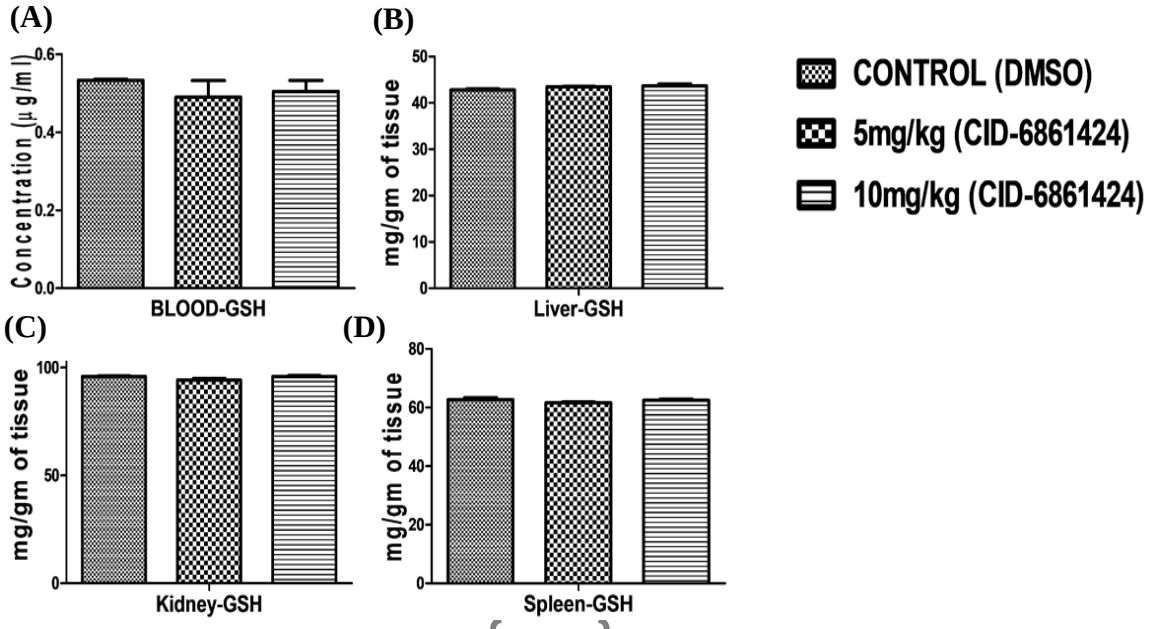


Figure 5.12The amount of GSH measured as concentration in µg/ml in blood and mg/gm of tissue samples in (A) blood, (B) liver, (C) kidney and (D) spleen of female BALB/c mice upon CID-6861424 treatment: Control (DMSO) (Set-1), treatment of 5mg/kg body weight of CID-6861424 (Set-2) and treatment of 10mg/kg body weight of CID-6861424 (Set-3). Each histogram represents the calculated value of GSH and are mean ±SD with n=4.

Tissue/Organ	**Set-1** Control (Vehicle only)	**Set-2** 5 mg/kg body wt (alternatively for 30 days)	**Set-3** 10 mg/kg body wt (alternatively for 30 days)
Blood	**0.53 ± 0.00**	**0.49 ± 0.07**	**0.50 ± 0.05**
Liver	**42.81 ± 0.46**	**43.46 ± 0.21**	**43.69 ± 0.76**
Kidney	**95.81 ± 0.50**	**94.08 ± 1.46**	**95.76 ± 0.80**
Spleen	**62.67 ± 1.15**	**61.62 ± 0.54**	**62.51 ± 0.59**

Table 5.7 Levels of GSH in blood, liver, kidney and spleen in CID-6861424 treated BALB/c mice (n=4)

5.2.3.4 Evaluation of CID-6861424 on superoxide dismutase (SOD)activity for assessment of free radical neutralization

Superoxide dismutase (SOD) is a group of enzymes which converts superoxides into hydrogen peroxide activity and thereby reduces the harmful effects of by converting highly reactive anions into stable form. These enzymes contain iron, copper and manganese ions which serve as chelating agents and quench free radicals resulting in cellular repair (Johnson and Giulivi, 2005). Thus we evaluated SOD activity in CID-6861424 treated female BALB/c mice. The blood SOD activity levels in all the three groups was almost same with values of 1.59±0.17 for Set-1 (control (DMSO) treated group), 1.61±0.26 and 1.52±0.18 for CID-6861424 treated Set-2 and Set-3. The mean values of SOD activity in liver for Set-1, Set-2 and Set-3 were also in the same range with 0.46±0.01 for Set-1,

0.47±0.02 for Set-2 and 0.46±0.01 for Set-3 respectively. The mean values of SOD activity in kidney was 0.55±0.02 for Set-1, 0.56±0.02 for Set-2 and 0.55±0.01 for Set-3. Similarly there were no significant changes in spleen mean values of SOD activity between control and CID-6861424 treated groups with 0.58±0.02 for Set-1, 0.61±0.03 for Set-2 and 0.58±0.02 for Set-3 respectively **(Figure 5.13 & Table 5.8)**.

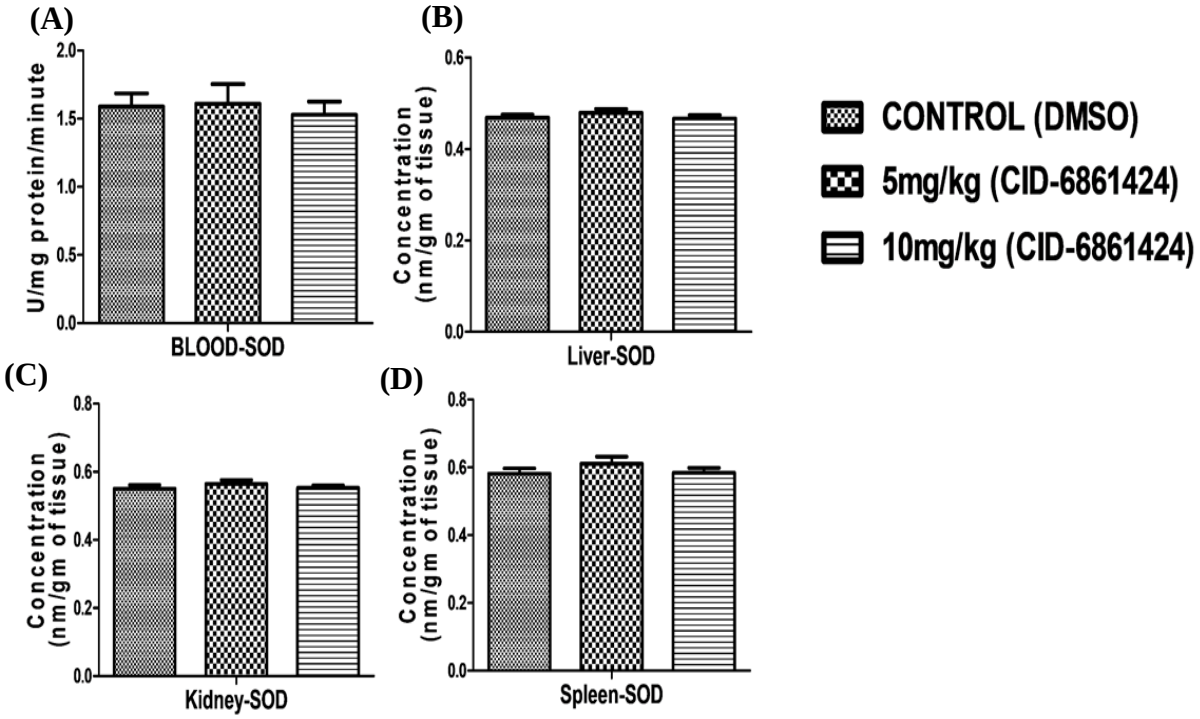


Figure 5.13Measurement of SOD activity as Unit of SOD/mg of protein/min in blood samples and concentration of SOD in nm/gm of tissue in (A) blood, (B) liver, (C) kidney and (D) spleen of female BALB/c mice upon CID-6861424 treatment: Control (DMSO) (Set-1), treatment of 5mg/kg body weight of CID-6861424 (Set-2) and treatment of 10mg/kg body weight of CID-6861424 (Set-3). Each histogram represents the calculated value of SOD and are mean ±SD with n=4.

Tissue/Organ	**Set-1** Control (Vehicle only)	**Set-2** 5 mg/kg body wt (alternatively for 30 days)	**Set-3** 10 mg/kg body wt (alternatively for 30 days)
Blood	**1.59 ± 0.17**	**1.61 ± 0.26**	**1.52 ± 0.18**
Liver	**0.46 ± 0.01**	**0.47 ± 0.01**	**0.46 ± 0.01**
Kidney	**0.55 ± 0.02**	**0.56 ± 0.02**	**0.55 ± 0.01**
Spleen	**0.58 ± 0.02**	**0.61 ± 0.03**	**0.58 ± 0.02**

Table 5.8 Levels of SOD activity in blood, liver, kidney and spleen in CID-6861424 treated BALB/c mice (n=4)

5.2.3.5 Evaluation of CID-6861424 on catalase activity for assessment of hydrogen peroxide catabolism

Catalase is an enzyme that cleaves hydrogen peroxide into water and oxygen and mediates the final step of neutralizing free radicals (David and F., 1938). Thus, catalase activity was also evaluated in CID-6861424 treated female BALB/c mice. The means blood catalase activity values were slightly higher in Set-1 (control (DMSO) treated group) with values of 777.21 ± 56.58 while they were insignificantly lower in CID-6861424 treated Set-2 and Set-3 with corresponding values of 662.96 ± 98.47and 697.14 ± 74.98 respectively. The mean values of catalase activity in liver for Set-1, Set-2 and Set-3 were almost in the same range with 178.30 ± 3.93 for Set-1, 186.92 ± 8.35 for Set-2 and 178.55 ± 5.50 for Set-3. The mean values of catalase activity in kidney were 166.89 ± 8.64 for Set-1, 174.07 ± 6.78 for Set-2 and 165.42 ± 2.24 for Set-3. Similarly there were no significant changes in spleen mean values of catalase activity between control and CID-6861424 treated groups with 25.45 ± 1.03 for Set-1, 28.92 ± 1.91 for Set-2 and 30.77 ± 7.96 for Set-3 respectively**(Figure 5.14&Table 5.9)**.

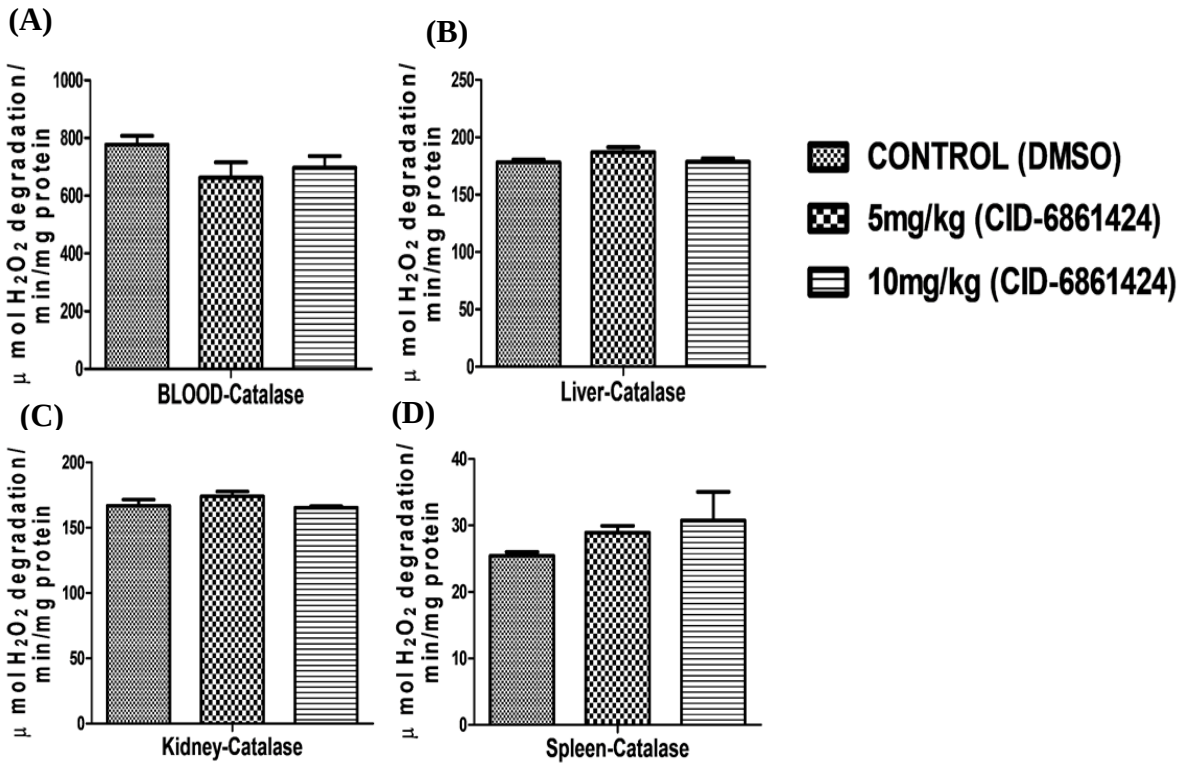


Figure 5.14Measurement of Catalase activity as µM of H_2O_2 degradation/min/mg/protein in blood and per mg of tissue sample in (A) blood, (B) liver, (C) kidney and (D) spleen of female BALB/c mice upon CID-6861424 treatment: Control (DMSO) (Set-1), treatment of 5mg/kg body weight of CID-6861424 (Set-2) and treatment of 10mg/kg body weight of CID-6861424 (Set-3). Each histogram represents the calculated value of Catalase and are mean ±SD with n=4.

Tissue/Organ	**Set-1** Control (Vehicle only)	**Set-2** 5 mg/kg body wt (alternatively for 30 days)	**Set-3** 10 mg/kg body wt (alternatively for 30 days)
Blood	**777.21 ± 56.58**	**662.96 ± 98.47**	**697.14 ± 74.98**
Liver	**178.30 ± 3.93**	**186.92 ± 8.35**	**178.55 ± 5.50**
Kidney	**166.89 ± 8.64**	**174.07 ± 6.78**	**165.42 ± 2.24**
Spleen	**254.45 ± 1.03**	**28.92 ± 1.91**	**30.77 ± 7.96**

Table 5.9 Levels of Catalase activity in blood, liver, kidney and spleen in CID-6861424 treated BALB/c mice (n=4)

5.3 Discussion

Cancer is a leading cause of "death" worldwide and approximately 18.1 million new cases and 9.6 million cancer associated deaths are reported in 2018 (Bray et al., 2018; Siegel et al., 2018). Chemotherapy is one of the most commonly employed mode in treatment of cancers and may also be used as adjuvants (Ak and Aydiner, 2019). Many anti-cancer drugs show prominent effects against cancer but also pose severe side effects (Grigorian and O'Brien, 2014; Remesh, 2012). Thus, pharmacovigilance is one of the prerequisite requirements in the field of drug discovery.

The present study conducts *in vitro* and *in vivo* evaluation of toxic effects of CID-6861424 in fibroblast cells (NIH3T3) and female "BALB/c" mice. The "*in-vitro"* studies showed that CID-6861424 at 25µM and 50µM did not induce any significant cell cytotoxicity in NIH3T3 cells as indicated by cell viability and cell morphology. Also, the scratch assay data showed no change in the migratory potentials of NIH3T3 cells by treatment of CID-6861424 at 25µM and 50µM. The cell morphology and scratch assay data clearly indicated that CID-6861424 at 25µM and 50µM did not altered physiology and physical properties of NIH3T3 cells indicating that it does not induced cellular stress. Cell viability and normal physical properties of a cell indicates normal cell homeostasis and proliferation. The cell proliferation status can be determined by evaluation of different phases of "cell cycle" where prominent G1 and S phase status indicates healthy cells (Bertoli et al., 2013). In previous chapters **(chapter 3 and 4)**, treatment of CID-6861424 induced prominent cell cycle arrest in G1 and G2/M phase arrest in "MCf-7" and "MDA-MB-231" breast cancer cells. Thus we evaluated cell cycle phase status in NIH3T3 cells upon treatment of CID-6861424 and it was found that most of the cells were in G1 and S phase indicating that they

were healthy and proliferative. Cancer cells have higher ROS in comparison to normal cells and elevated ROS helps in cancer cell proliferation (Liou and Storz, 2010; Sosa et al., 2013). However, increase in endogeneous levels of ROS is one of the most common mode of action used by various anticancer therapies (Liu and Wang, 2015). Our previous studies in breast cancer cells "MCF-7" and "MDA-MB-231" **(chapter 3 and 4)** showed that, CID-6861424 increased cellular ROS. Thus, we also evaluated levels of ROS in CID-6861424 treated NIH3T3 cells and no significant changes in ROS levels was found, indicating that CID-6861424 does not alters ROS in non-cancerous cells. In cell, mitochondria play important role in generating energy, besides it also serves as apoptotic checkpoint where depending on the nature of signals, mitochondria can initiate apoptosis or cell survival (Kroemer et al., 2007). The disruption of **Δψm** is used as marker of apoptosis (Lizard et al., 1995; Lugli et al., 2005) which was observed by treatment of CID-6861424 in breast cancer cells **(chapter 3 and 4)**. However, in NIH3T3 cells, treatment of CID-6861424 does not altered **Δψm** indicating that it does not induce apoptosis in NIH3T3 cells and thus, does not induce cytotoxicity in such cells.

In vitro data from different cell based assays established that CID-6861424 does not induce any lethal effects in NIH3T3 cells, to further evaluate its *in vivo* effects; studies on female BALB/c mice were conducted. Treatment of anti-cancerous compounds generally alters and deregulates homeostasis of an organism. These disturbances can lead to severe damage to the vital organs and thus can induce severe systemic effects and subsequently, loss in body weight. The preliminary examination of relative body weights amongst all treated groups with control (DMSO), CID-6861424 "5mg/kg" and "10 mg/kg" body weight

showed no significant differences suggesting that the animals were healthy during the course of treatment.

One of the most common effects of drug induced toxicity is deregulated redox homeostasis. Altered redox status can enhance ROS levels leading to DNA damage by inducing mutations. The most common of which is transversion mutation caused by nucleophilic attack of $^{\bullet}OH$ on guanine nucleotide, leading to oxidation of guanine and formation of 8-hydroxydeoxyguanosine (8-OHdG) (Barzilai and Yamamoto, 2004). The 8-OHdG type transversion mutations are most common in breast cancers (Reuter et al., 2010). ROS can not only amputate DNA integrity, it can also effect protein machinery by inducing formation of protein adducts (Trachootham et al., 2008). Thus, to analyse effects of CID-6861424, intraperitoneal dosing of 5mg/kg and 10 mg/kg body weight of CID-6861424 in female BALB/c micewas carried outfor 30 days and the doses were administered every alternate day.

Blood is one of the most common sources of pathological analysis. It can be used to detect myriads of biochemical changes that occur in response to a disease or drug. Most importantly sampling of blood is easy and any abnormal change can be analyzed from whole blood. The bone marrow contains stem cells which get differentiated into different cell types in response to particular stimuli. Any abnormality particularly mutations in DNA arising due to excessive “DNA damage” in bone marrow cells may be lethal for an organism. Thus, we analyzed “DNA damage” in blood and bone marrow cells as well, in response to treatment of CID-6861424. The analysis of “DNA damage” by comet assay in whole blood and bone marrow cells showed no significant differences with respect to control indicating that CID-6861424 does not induce DNA damage. More often DNA

damage is a consequence of elevated ROS, thus evaluation of ROS in blood, liver, kidney and spleen of female “BALB/c” mice was carried out and no significant changes in ROS levels were observed in respective organs in comparison to control (DMSO) treated mice indicating that CID-6861424 does not increase ROS levels in healthy tissues. These findings are in concurrence with our in vitro data, where CID-6861424 did not increased ROS in fibroblast cells.

ROS also causes lipid peroxidation, a chain reaction like process, where more lipid molecules are subsequently oxidized by oxidized lipid molecules. The end products of lipid peroxidation, particularly “malondialdehyde (MDA)” and “4-hydroxy-2-nonenal (HNE)” are highly reactive to DNA and proteins leading to alterations in DNA and protein (Trachootham et al., 2007). Treatment of CID-6861424 “5mg/kg” and “10 mg/kg” body weight showed no significant changes in lipid peroxidation as indicated by estimation of MDA contents in blood, liver, kidney and spleen of female BALB/c mice. Analysis of DNA damage by comet assay, ROS and MDA contents shows that CID-6861424 treatment does not increase ROS, lipid peroxidation and subsequently DNA damage in healthy organism.

The cell contains defensive mechanism to counteract excessive ROS production and associated lipid peroxidation by virtue of antioxidants that are produced endogenously and can quench ROS either by direct interactions or by intermediate steps. These antioxidants include “glutathione (GSH)”, “superoxide dismutases (SOD)”, “Catalase (CAT)”, “alpha-lipoic acid”, “peroxiredoxins (PRXs)”, “metallothionein” etc. which rescue cells from oxidative stress.

The tri-peptide GSH (Gly-Ser-His) is one of the most potent antioxidant which can quench free radicals and peroxides in a hydrophilic environment by oxidation reaction carried out mainly by glutathione peroxidases (GPx) (Boots et al., 2008). Endogeneous GSH also interacts with carcinogens and other toxic metabolites produced from pro-drugs and eliminate them from the cells (Reuter et al., 2010). Cytosolic GSH also reduces cytochrome c (Tait and Green, 2010) while nuclear GSH circumvents DNA modifications by oxidative species and inhibits apoptosis (Trachootham et al., 2008). Similarly, SOD and catalase are two essential anti-oxidative enzymes that catalyze reduction of free radical and reduce intracellular oxidative stress (David and F., 1938; Johnson and Giulivi, 2005). The GSH, SOD and catalase homeostasis is therefore essential and requires monitoring under such studies. The levels of GSH upon treatment of CID-6861424 in blood, liver, kidney and spleen were in the range and in concurrence to that of control indicating that, CID-6861424 induces no prominent effect on GSH homeostasis. Similarly, levels of SOD and catalase were also in the normal range as control treated mice, indicating that CID-6861424 does not adversely effects anti-oxidative defense mechanism of the treated animals.

5.4 Conclusion

The present study conducts "*in-vitro"* and "*in-vivo"* toxicological evaluation of CID-6861424 in NIH3T3 cells and female "BALB/c" mice. The aim of the study was to find out severe toxic/side effects of CID-6861424 if any? The data obtained from "*in-vitro"* and "*in-vivo"*studies of different parameters like cell viability, cell cycle analysis, **Δψm** and other oxidative stress parameters shows that CID-6861424 at sub-lethal concentration (25μM) and 50μM concentrations does not induce toxic effects.

Chapter 6

CID-6861424 INCREASES SENSITIVITY OF BREAST CANCEROUS CELL LINES WITH LEAST EFFECT ON NON-CANCEROUS CELL LINE TO LOW DOSE OF ϒ-RADIATION

CHAPTER-6

CID-6861424 INCREASES SENSITIVITY OF BREAST CANCEROUS CELL LINES WITH LEAST EFFECT ON NON-CANCEROURS CELL LINE TO LOW DOSE OF γ-RADIATION

6.1 Introduction

Breast cancer is one of the most common cancer in women and one of the leading causes of female mortality throughout the world(Bray et al., 2018a; Siegel et al., 2018a). “Estrogen receptor (ER), Progesterone receptor (PR) and Human epidermal growth factor receptor (HER-2)” play major roles in the progression and development of breast cancer. About 80% of breast cancer are hormone-receptor-positive, signifying the expression of these receptors in breast cancer cells (Konecny et al., 2003). About 20% of breast cancer cases do not express all the three receptors and hence are called as “Triple negative breast cancer (TNBC)” (Bauer et al., 2007; Kim et al., 2006b). Majority of breast cancer cases are associated with deregulation of hormone receptors. Triple negative breast cancer (TNBC) is one of the most aggressive kind. TNBC patients do not respond to hormonal therapy and frequently develops resistance against drugs used in such pathophysiology. Moreover, prolonged treatment and/or overdosing of anti-cancer drugs are often accompanied with strong toxic side effects(Guestini et al., 2016). The aggressive and resistant behavior ofTNBC is further ameliorated by mutations inp53 and BRCA1 proteins which are otherwise common chemotherapeutic targets andmaybe one of the reasons for radiotherapy being less effective in inhibiting TNBC progression (Dent et al., 2007; Sekine et al., 2009). Prolonged radiotherapy may also lead to escalation in resistance and relapse of cancer due to

radiation induced “bystander effect”(Marín et al., 2014; Najafi et al., 2014). “MDA-MB-231” cells particularly are reported to be recalcitrant to radiotherapy and instead they become more aggressive and invasive (Paquette et al., 2011, 2013) as compared to all other types of breast cancer. More recent mode of treatment of hormone-receptor negative breast cancer involves sensitization by certain adjuvants followed by low doses of radiation. Many such adjuvants are of chemical origin and pose cytotoxicity to non-cancerous cells. Exploration and development of efficient and low toxicity drugs that can sensitize the cancer cells to low dose of radiation are crucial for breast cancerradiotherapy.

Sulfonamide derivative CID-6861424 inhibited the proliferation of breast cancer cell lines, “MCF-7 and MDA-MB-231”. Our results showed that CID-6861424 inhibits the proliferation of both the aforementioned cell lines in a dose- and time-dependent manner and arrested them at G_1 and G_2/M phase of the cell cycle respectively with minimal effect on non-cancerous cell line, NIH3T3 (**details provided in chapter no. 3, 4 and 5**). We investigated whether it can sensitize the breast cancer cell lines to low dose of γ-radiation and how the combination of CID-6861424 and γ-radiation affects the non-cancerous cell line, NIH3T3. Several studies carried out in the past show that cancer cells are most sensitive in the G_2/M phase and most resistant in S phase against radiation(Pawlik and Keyomarsi, 2004).

High dose of chemotherapy or radiation therapy leads to a lot of side effects and systemic toxicity in cancer patients. Instead of using a high dosage of chemo- or radiotherapy, a low dose of the combination of radiation and drug might be better. Keeping this as focus, evaluation of CID-6861424 was done at sub-cytotoxic concentration 25μM either alone or in combination with a low dose, 2Gy-γ-radiation, on “MCF-7, MDA-MB-231 and NIH3T3”

cells.We found that combinatorial treatment of 25μM sub-cytotoxic concentration of CID-6861424 and 2Gy-γ-radiation suppressed "MCF-7 and MDA-MB-231" cell proliferation and arrested them at G_2/M phase of the cell cycle with least effect on NIH3T3 cell. It also increased oxidative stress, depletion in **Δψm**, γ-H2AX upregulation indicating dsDNA break and cell death. Our results show that 25μM-CID-6861424 is more sensitizing the TNBC, MDA MB-231 cells to low dose of γ-radiation comparatively more than MCF-7 cells with least effect on NIH3T3 cells.In future, it can be used asa sensitizer for breast cancer radiotherapy.

6.2 Results

6.2.1 Combination of 25µM CID-6861424 and 2Gy-γ-radiation selectively inhibit cell proliferation of breast cancer cell lines

Assessment of cell viability by trypan blue exclusion indicated that at 72 hours, approximately ~75% cells were viable when treated with either 25µM of CID-6861424 or 2Gy-γ-radiation alone. While ~50% of viable cells were observed when treated in combination with 25µM of CID-6861424 and 2Gy-γ-radiation in "MCF-7 and MDA-MB-231" cells with least cytotoxic effect in NIH3T3 cell **(Figure 6.1 (a), 6.1 (b) and 6.1 (c); Table No. 6.1).**

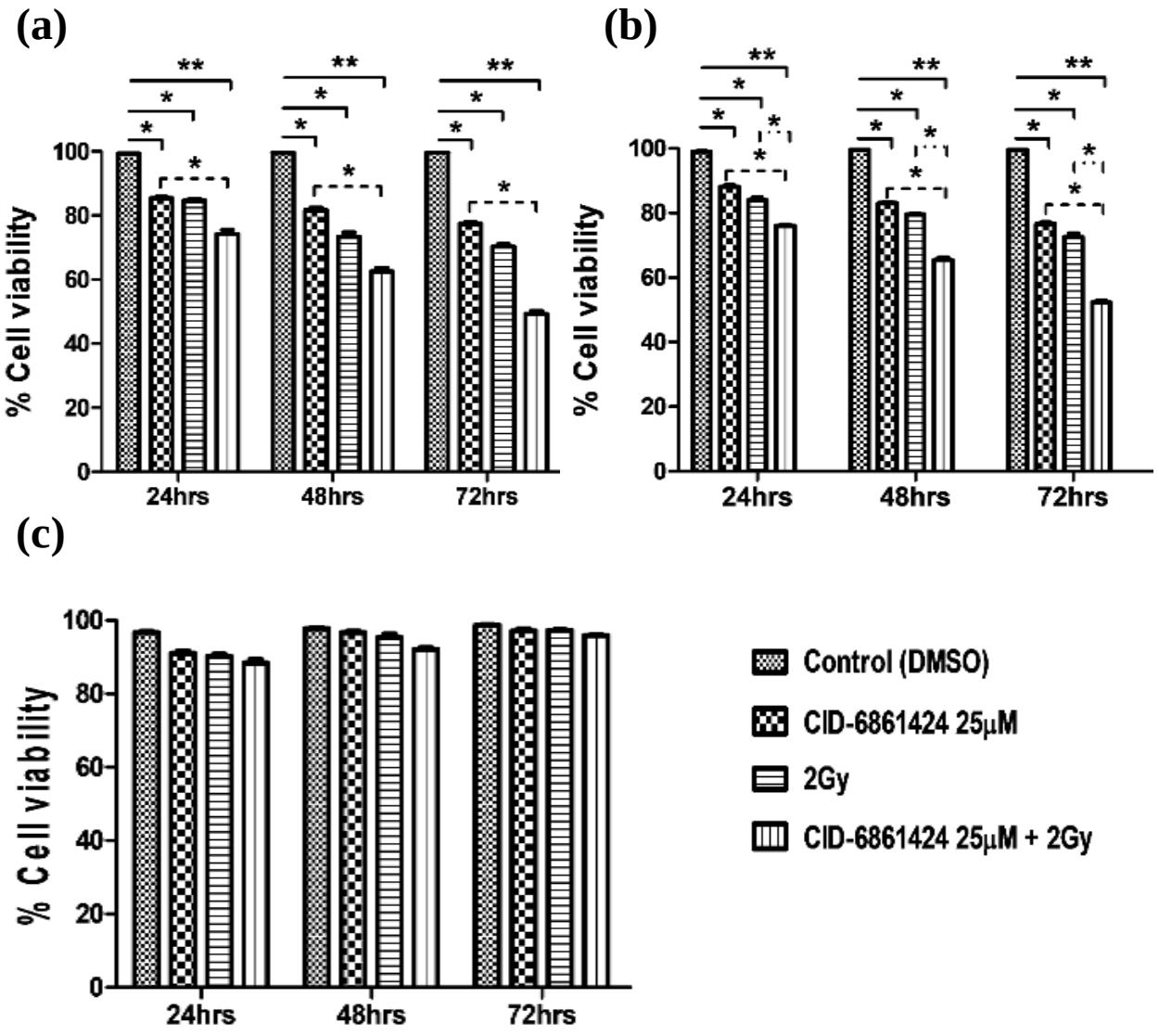


Figure. 6.1 Cell viability determined by trypan blue assay at 24, 48 and 72 hours. Bar diagram-showing effect of 25µM CID-6861424 or 2Gy-γ-radiation alone or in combination on (a) "MCF-7, (b) MDA-MB-231 and (c) NIH3T3" cells. Mean ± SD value (Data represented was calculatedfor three independent experiments). *P<0.05 and **P<0.01.

Time after treatment	Treatments	MCF-7	MDA-MB-231	NIH3T3
24hrs.	CONTROL	99.28 ± 0.02	98.98 ± 0.19	96.68 ± 0.68
	CID-6861424 25µM	85.39 ± 0.65	88.13 ± 0.86	91.03 ± 1.07
	2Gy	84.63 ± 0.76	83.98 ± 1.36	90.17 ± 1.12
	CID-6861424 25µM+ 2Gy	74.22 ± 2.23	76.02 ± 0.58	88.47 ± 1.54
48hrs.	CONTROL	99.51 ± 0.10	99.54 ± 0.11	97.76 ± 0.51
	CID-6861424 25µM	81.59 ± 1.55	83.06 ± 0.39	96.73 ± 0.69
	2Gy	73.26 ± 2.56	79.51 ± 0.53	95.38 ± 1.55
	CID-6861424 25µM+ 2Gy	62.48 ± 1.93	65.48 ± 1.24	92.11 ± 0.90
72hrs.	CONTROL	99.66 ± 0.00	99.64 ± 0.090	98.78 ± 0.13
	CID-6861424 25µM	77.38 ± 1.02	76.61 ± 0.92	97.10 ± 1.09
	2Gy	70.27 ± 1.31	72.54 ± 1.85	97.26 ± 0.40
	CID-6861424 25µM+ 2Gy	49.30 ± 1.57	52.25 ± 1.20	95.89 ± 0.38

Table No 6.1 25µM CID-6861424 or 2Gy-γ-radiation alone or in combination inhibit the cell proliferation of "MCF-7 and MDA-MB-231" cell with least effect in NIH3T3 cell by trypan blue method.

Further, cell morphology, cell migration and colony formation potentials were determined after treatment with either 25µM CID-6861424 or 2Gy-γ-radiation alone or in combination in both cancer cell lines for 72 hours. Post-treatment, cell morphology and count were examined under a bright field microscope. Combined treatment led to altered cell morphology. Cells were elongated, flattened, dense cytoplasm with irregular cell membrane and decreased cell population as compared to the control as well as alone treated sets. In case of NIH3T3 cell, either 25µM CID-6861424 or 2Gy-γ-radiation alone or in combination

treatment did not show significant change in morphology as well as cell numbers with respect to the control set **(Figure 6.2)**.

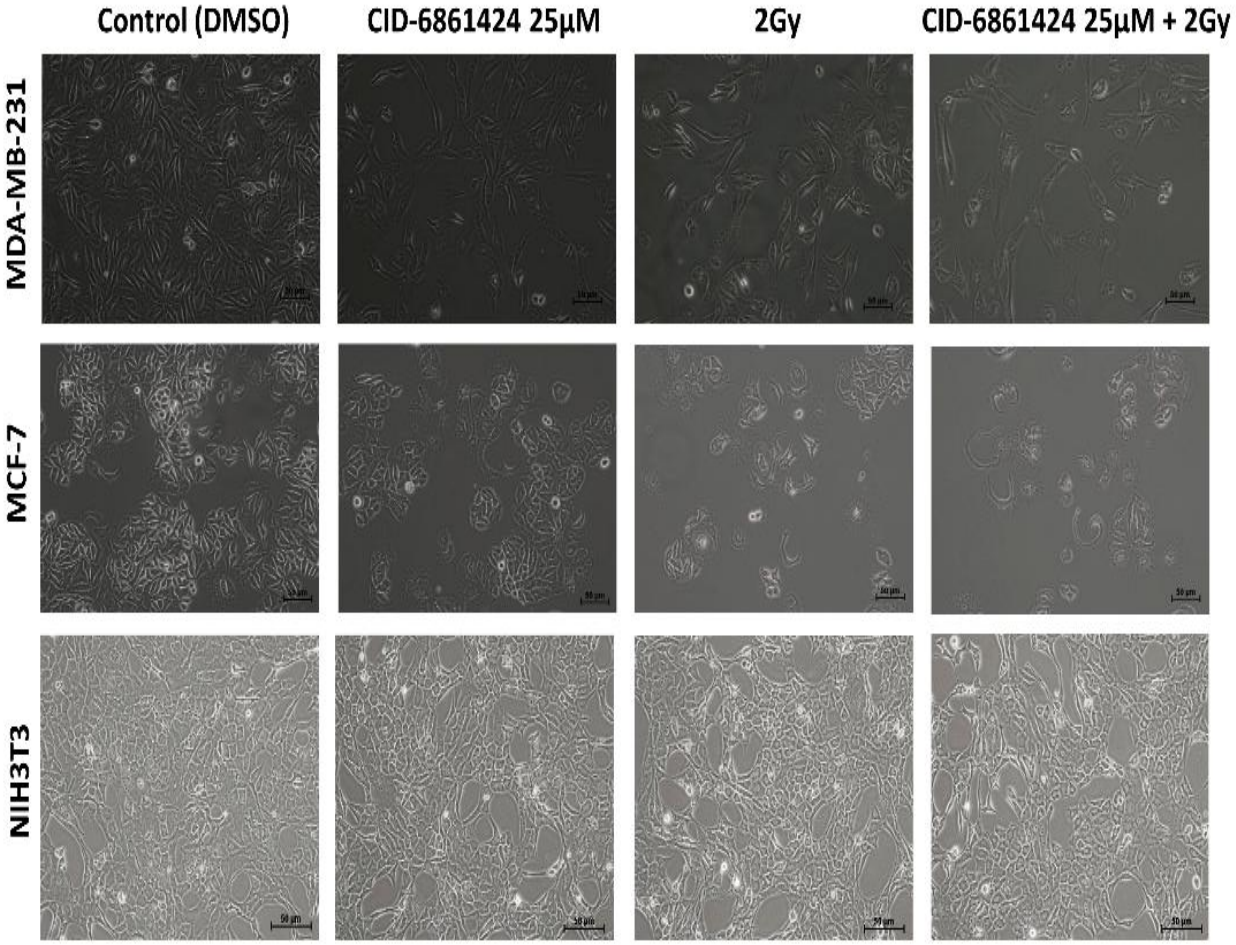


Figure. 6.2 "MDA-MB-231, MCF-7 and NIH3T3" cells morphology were visualized in presence of 25µM CID-6861424 or 2Gy-γ-radiation alone or in combination after 72hours using bright field microscope at 20X.

Wound-healing assay was performed by measuring pre and post wound widths after 72 hours of exposure to all three sets of treatments. In case of MCF-7, the untreated cells migrated and rapidly filled ~75% of the wound, while ~65% and ~70% of wound closure was observed in cells treated with 25µM CID-6861424 or 2Gy-γ-radiation alone. The combination treatment was most effective resulting in only ~40% of wound closure **(Figure 6.3 (a) and 6.3 (b); Table No. 6.2)**.

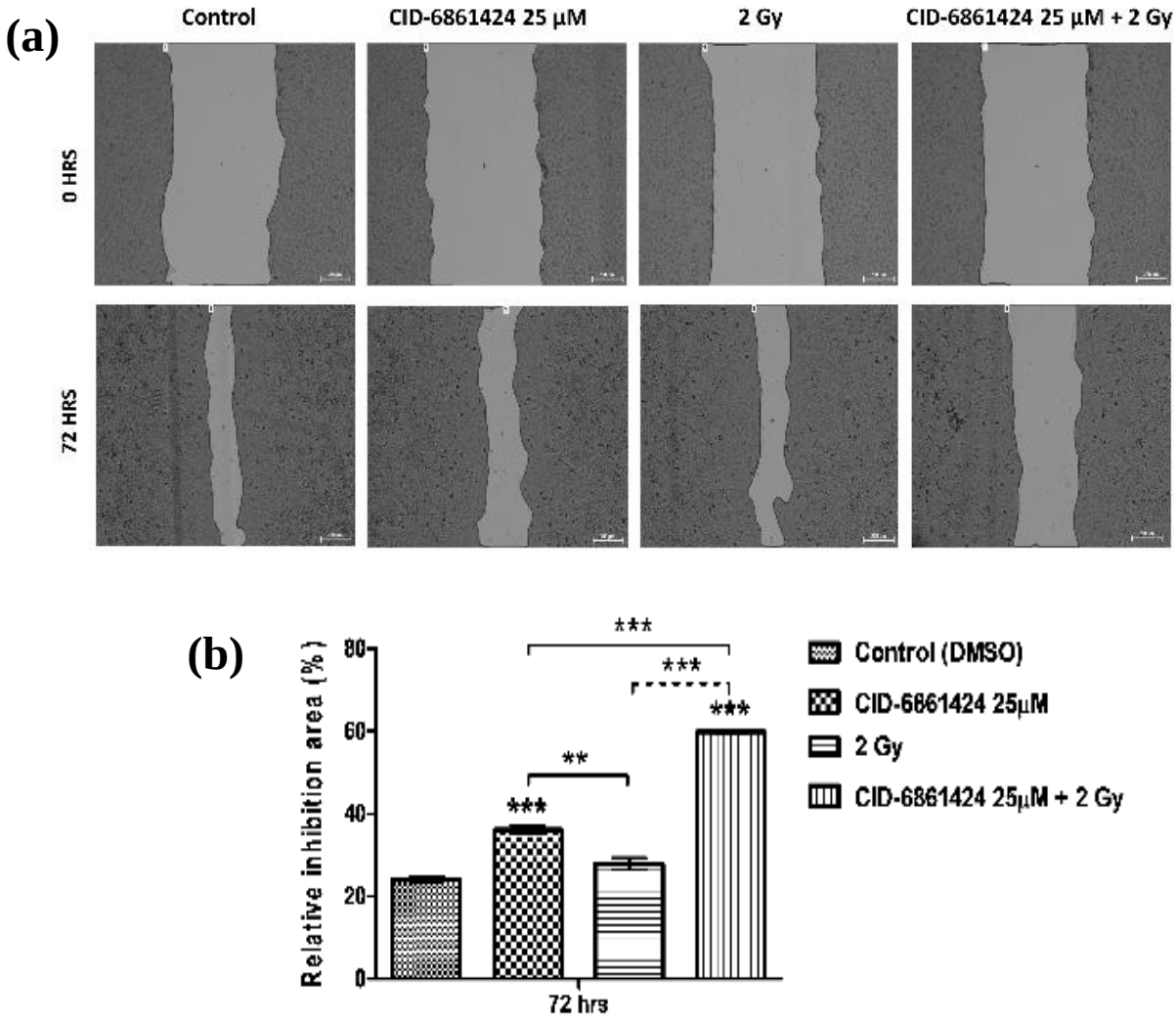


Figure. 6.3 (a) 25µM CID-6861424 or 2Gy-γ-radiation alone or in combination inhibited the MCF-7 cell migration potential. (b) Bar-diagram representation of relative inhibition area of MCF-7cell in presence of 25µM CID-6861424 or 2Gy-γ-radiation alone or in combination treatment.Mean ± SD value (Data represented was calculatedfor three independent experiments). **P<0.01 and ***P<0.001.

In case of MDA-MB-231, the untreated cells migrated and rapidly filled ~85% of the wound, while ~50% of wound closure was observed in cells treated with either 25µM CID-6861424 or 2Gy-γ-radiation alone. The combined treatment was most effective resulting in only ~25% of wound closure **(Figure 6.4 (a) and 6.4 (b); Table No. 6.2)**.

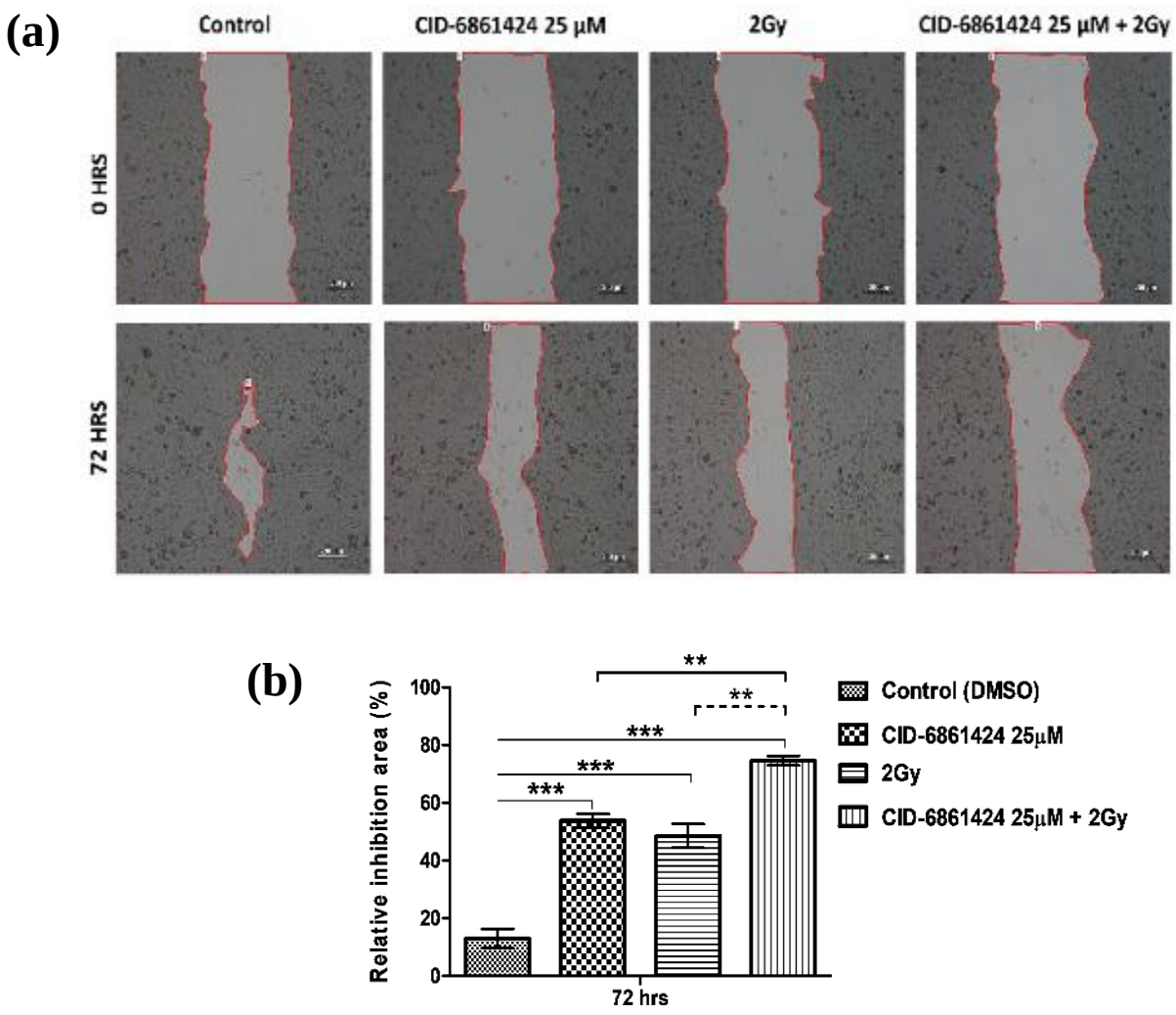


Figure. 6.4 (a) 25µM CID-6861424 or 2Gy-γ-radiation alone or in combination inhibited the MDA-MB-231 cell migration potential. (b) Bar-diagram representation of relative inhibition area of MDA-MB-231 cell in presence of 25µM CID-6861424 or 2Gy-γ-radiation alone or in combination treatment.Mean ± SD value (Data represented was calculatedfor three independent experiments). **P<0.01 and ***P<0.001.

Cell Line	Time after treatment	Control (DMSO)	CID-6861424 25µM	2 Gy	CID-6861424 25µM + 2Gy
MCF-7	0 Hrs	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
	72 Hrs	24.18 ± 1.05	36.08 ± 1.78	27.93 ± 2.39	59.81 ± 0.68
MDA-MB-231	0 Hrs	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00	100.00 ± 0.00
	72 Hrs	13.02 ± 5.87	53.75 ± 4.18	48.57 ± 7.09	74.58 ± 2.89

Table No. 6.2 25µM CID-6861424 or 2Gy-γ-radiation alone or in combination inhibits the cell migration potential of "MCF-7 and MDA-MB-231" cell at 72 hours.

In NIH3T3 cells, the wound closer in presence of 25µM CID-6861424 or2Gy-γ-radiation alone or combination treatment showed similar results and are comparable with the control set **(Figure 6.5)**.

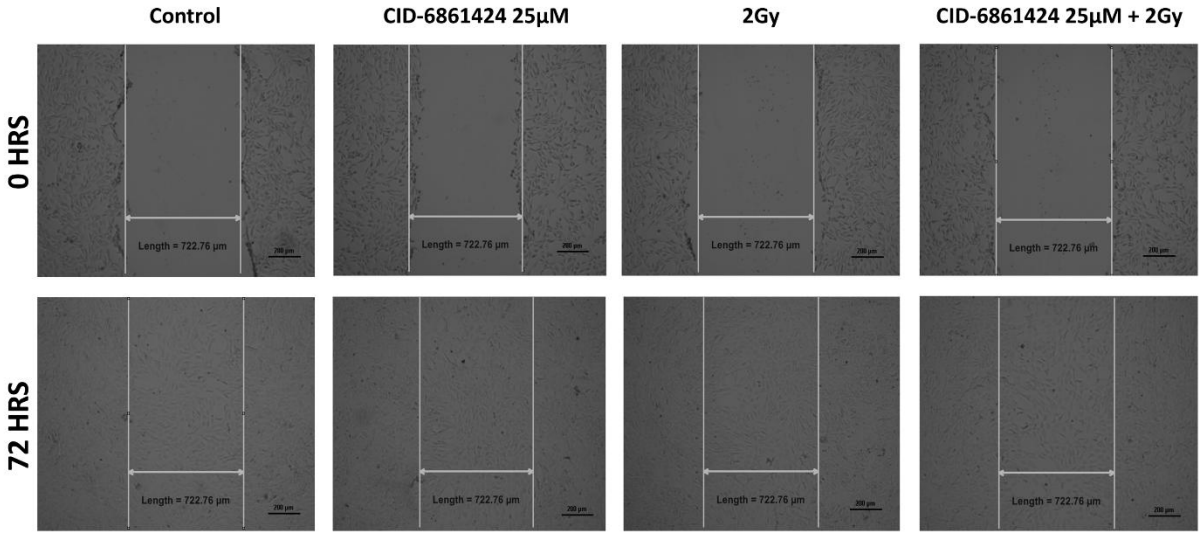


Figure. 6.5 Effect of 25µM CID-6861424 or 2Gy-γ-radiation alone or in combination on cell migration potential of NIH3T3 cell.Mean ± SD value (Data represented was calculatedfor three independent experiments).

These results showed that a combination of 25µM-CID-6861424 and 2Gy-γ-radiation significantly inhibited the breast cancer cells viability, cell counts and cell migration potential as compared to alone treated sets as well as the control set.

6.2.2 Combination of 25µM CID-6861424 and 2Gy-γ-radiation induced cell cycle arrest in G_2/M phase in breast cancer cells with least effect on non-cancerous cell

The effect of 25µM CID-6861424 and 2Gy-γ-radiation alone or in combination on cell cycle profile of "MCF-7, MDA-MB-231 and NIH3T3" cells was carried out by flow cytometrypost 72 hours of treatment. In case of MCF-7 cell, cell cycle data indicated that combined treatment resulted in ~35% G_2/M phase arrest as compared to ~30% in 2Gy-γ-radiation and ~18% in compound treated and control set accompanied with a corresponding reduction in the other respective phases of cell cycle population **(Figure 6.6 (a) and 6.6 (b); Table No. 6.3)**.

Cell cycle data of "MDA-MB-231"indicated that combination resulted in ~50% G_2/M phase arrest as compared to ~35% in the compound and in 2Gy-γ-radiation treated sets or ~22% in control set accompanied with a corresponding reduction in the other respective phases of cell cycle population **(Figure 6.6 (a) and 6.6 (c); Table No. 6.3)**.Increase in a "sub-G_1 population" representing dead cells was also highest (~13%) in combination as compared to alone treated samples (~8-9%) and control set which was around 4% of the total cell population **(Figure 6.6 (a) and 6.6 (e); Table No. 6.3)**.

In case of NIH3T3 cell, cell cycle data indicated that combined treatment resulted in ~6% G_2/M phase arrest as compared to ~2% in 2Gy-γ-radiationtreated set and ~3% in the compound and control set accompanied with a no significant reduction in the other respective phases of cell cycle population **(Figure 6.6 (a); Table No. 6.3).** The detailed cell cycle distribution upon treatment of all the sets is given in **Table No. 6.3**.

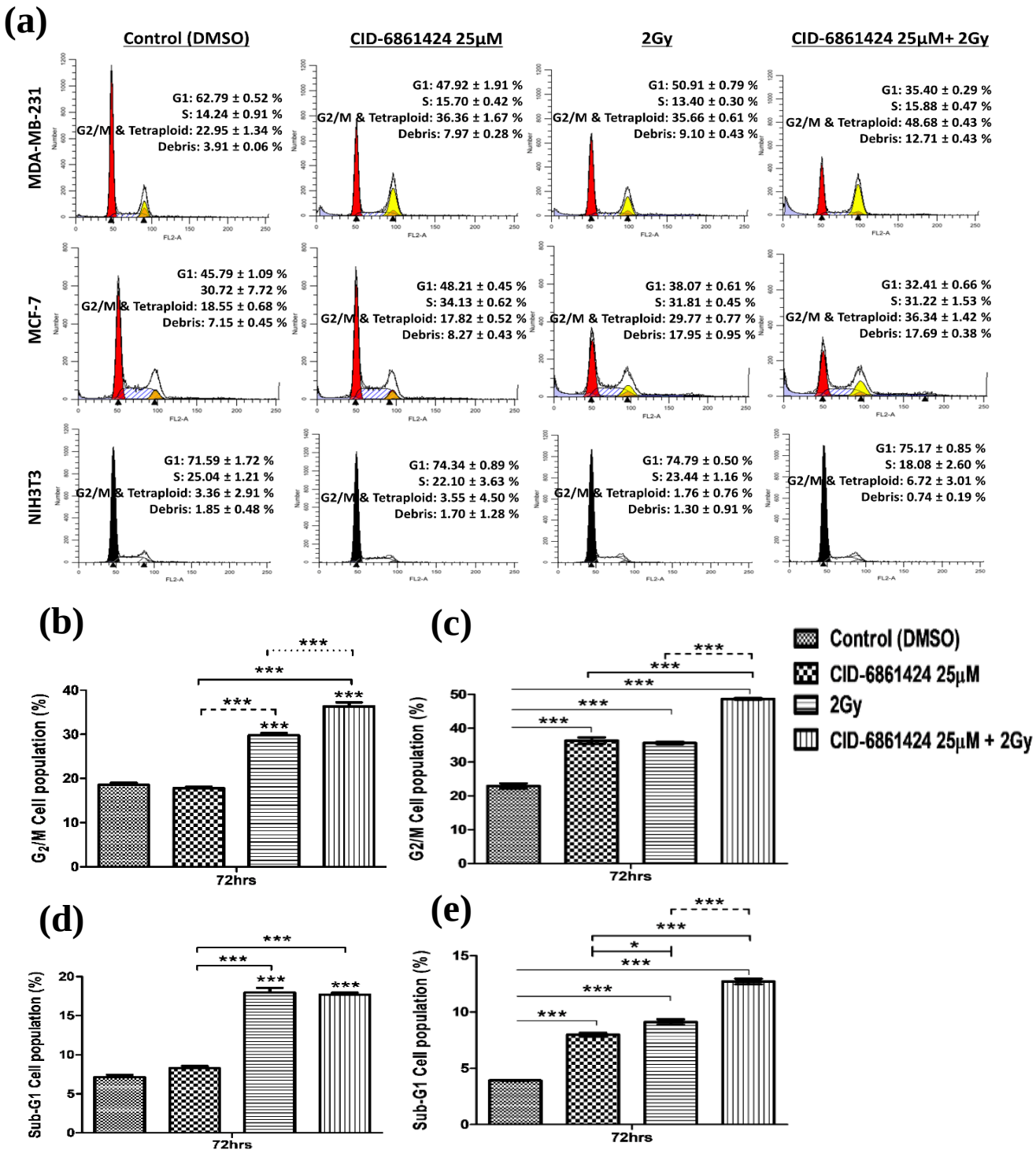


Figure 6.6(a) 25µM CID-6861424 and 2Gy-γ-radiation alone or in combination induces G_2/M phase arrest more effectively in MDA-MB-231 than MCF-7 cells with least effect in NIH3T3 cell by flow cytometry at 72hours. (b) and (c) Bar-diagram representation of respective G_2/M

phase arrest in (b)"MCF-7" and (c)"MDA-MB-231" cells. (d) and (e) The Bar-diagram representation of respective Sub-G_1 population in (d) "MCF-7" and (e) "MDA-MB-231" cells.Mean ± SD value (Data represented was calculatedfor three independent experiments). *P<0.05 and ***P<0.001.

Time	Cell Line	Treatments	G1 PHASE	S PHASE	G2/M PHASE	Sub G1 Phase
72 hrs	MDA-MB-231	CONTROL	62.79 ± 0.52	14.24 ± 0.91	22.95 ± 1.34	3.91 ± 0.06
		CID-6861424 25µM	47.92 ± 1.91	15.70 ± 0.42	36.36 ± 1.67	7.97 ± 0.28
		2 Gy	50.91 ± 0.79	13.40 ± 0.30	35.66 ± 0.61	9.10 ± 0.43
		CID-6861424 25µM+ 2 Gy	35.4 ± 0.29	15.88 ± 0.47	48.68 ± 0.43	12.71 ± 0.43
	MCF-7	CONTROL	45.79 ± 1.09	30.72 ± 7.72	18.55 ± 0.68	7.15 ± 0.45
		CID-6861424 25µM	48.21 ± 0.45	34.13 ± 0.62	17.82 ± 0.52	8.27 ± 0.43
		2 Gy	38.07 ± 0.61	31.81 ± 0.45	29.77 ± 0.77	17.95 ± 0.95
		CID-6861424 25µM+ 2 Gy	32.41 ± 0.66	31.22 ± 1.53	36.34 ± 1.42	17.69 ± 0.38
	NIH 3T3	CONTROL	71.59 ± 1.72	25.04 ± 1.21	3.36 ± 2.91	1.85 ± 0.48
		CID-6861424 25µM	74.34 ± 0.89	22.10 ± 3.63	3.55 ± 4.50	1.70 ± 1.28
		2 Gy	74.79 ± 0.50	23.44 ± 1.16	1.76 ± 0.76	1.30 ± 0.91
		CID-6861424 25µM+ 2 Gy	75.17 ± 0.85	18.08 ± 2.60	6.72 ± 3.01	0.74 ± 0.19

Table No6.3 Cell cycle distribution of various cellular populations after exposure to 25µM CID-6861424 and 2Gy-γ-radiation alone or in combination on "MCF-7, MDA-MB-231 and NIH3T3" cells at 72 hours.

Since the cells were arrested in G_2/M phase, the expression profile of cyclin B1 protein was analyzed by western blotting. Western blot data indicates that MDA-MB-231 cells treated with 25µM CID-6861424 and 2Gy-γ-radiation alone or in combination significantly reduced the expression of G_2/M phase cyclin B1 at 72 hours as compared to 2 hours as well as control set **(Figure 6.7 (a))**. When compared among the treated samples, post 72 hours, the decrease in expression of cyclin B1 is more prominent in combination treated sample.

In case of MCF-7 cells, 25µM CID-6861424 combined with 2Gy-γ-radiation significantly reduced the expression of G_2/M phase cyclin B1 at 72 hours as compared to the alone treated samples and control set **(Figure 6.7 (b))**.

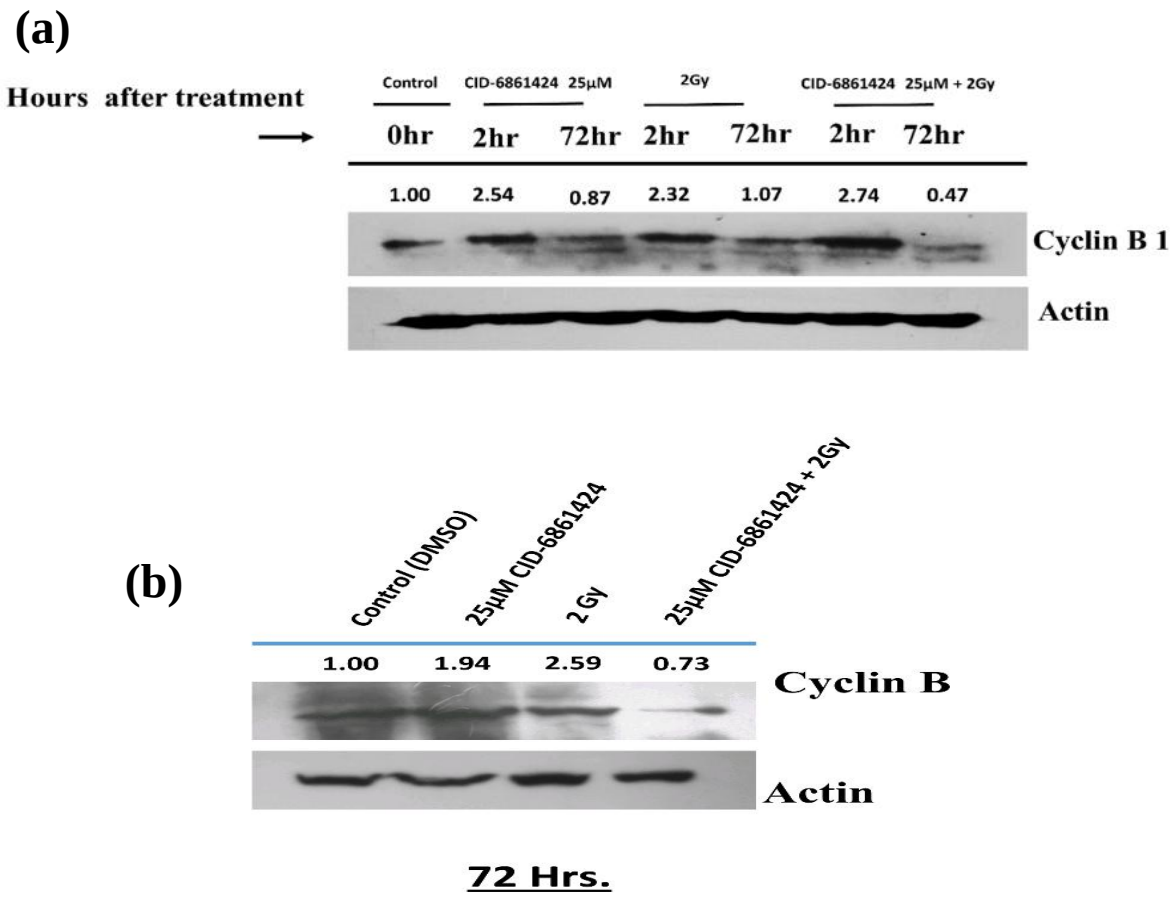


Figure 6.7 The downregulation of cyclin B1 protein expression in combination of 25µM-CID-6861424and 2Gy-γ-radiation for 72hour treatment was observed by western blotting and quantified with image J software in (a)"MDA-MB-231" cell and (b) "MCF-7" cell.

These results suggested that combinatorial treatment of 25µM CID-6861424 and 2Gy-γ-radiation more effectively induced G_2/M cell cycle arrest with the reduced expression level of cyclin B1 in both of cancer cell lines.

6.2.3 Combination of 25µM CID-6861424 and 2Gy-γ-radiation induced ROS generation and mitochondrial membrane potential (Δψm)depletion in breast cancer cells more effectively than a non-cancerous cell

Cancer cells have a high level of "reactive oxygen species (ROS)" then normal cells and hence are susceptible to a further increase in the levels of ROS. ROS-production was analysed by flow cytometry using DCFH-DA dye (8µM) in "MCF-7, MDA-MB-231 and NIH3T3" cell lines post 72 hours. MCF-7 cells treated with compound and 2Gy-γ-radiation showed about 1.5 & 1.7 times higher ROS-generation as compare to the control set. In contrast, ROS generation in combination treated samples was about 1.5 times higher as compared to the samples treated with either the compound or 2Gy-γ-radiation. 2.5 times higher ROS-generation was observed in combination as compared to the control (DMSO)**(Figure 6.8 (a) and 6.8 (b); Table No. 6.4)**.

In the case of "MDA-MB-231", samples treated with either the compound or 2Gy-γ-radiation radiation caused an insignificant increase in ROS-generation as compared to the control (DMSO). In contrast, ROS generation in combination treated samples, was almost 5 times higher as compared to the samples treated with either the compound or 2Gy-γ-radiation alone as well as control**(Figure 6.8 (c) and 6.8 (d); Table No. 6.4)**.

NIH3T3 cells sample treated with 25µM CID-6861424 and 2Gy-γ-radiation alone or in combination caused an insignificant increase in ROS generation as compared to the control. Doxorubicin was used as a positive control in this experiment **(Figure 6.8 (e) and 6.8 (f); Table No. 6.4)**.

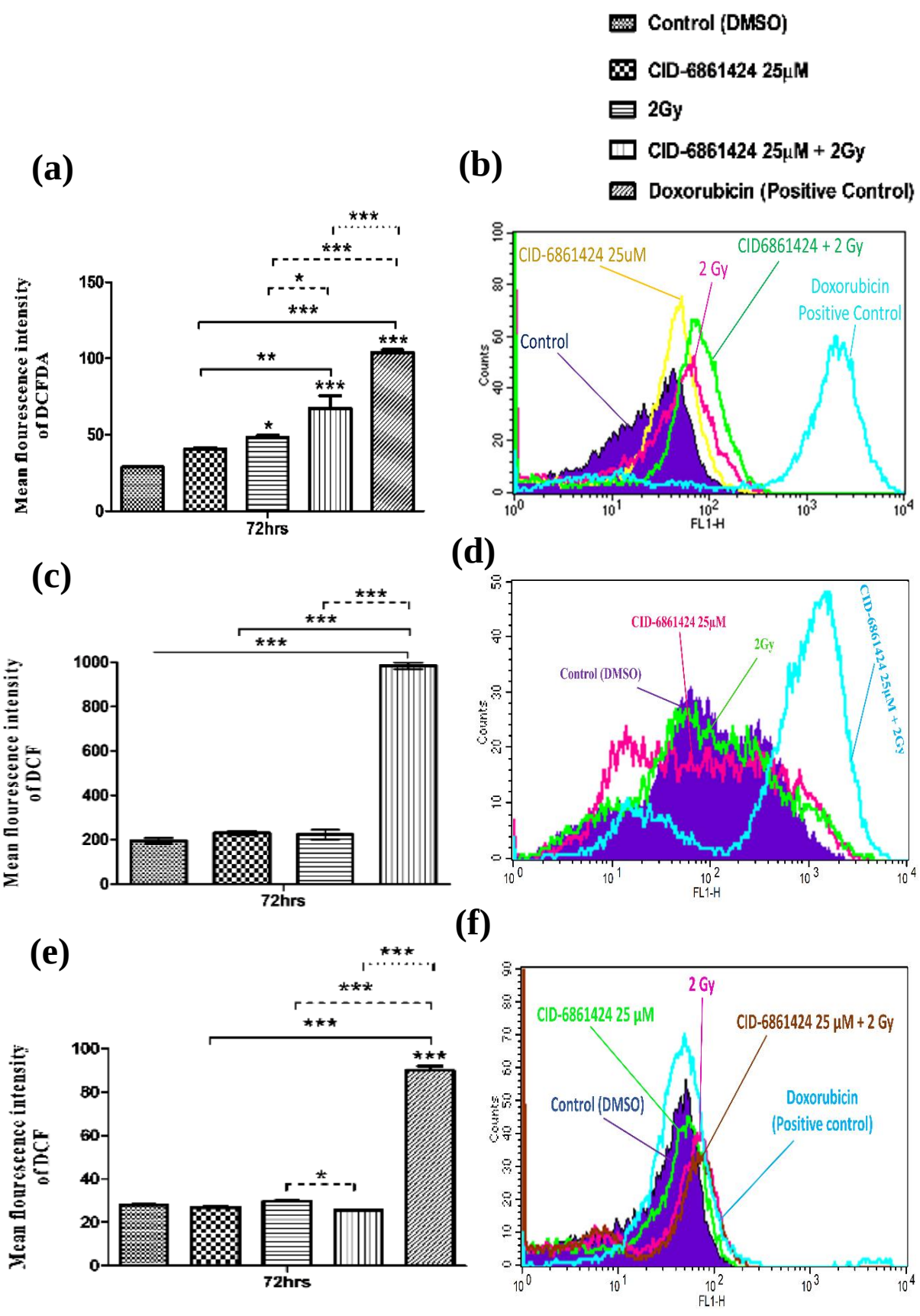

(a)
(b)
(c)
(d)
(e)
(f)
Control (DMSO)
CID-6861424 25µM
2Gy
CID-6861424 25µM + 2Gy
Doxorubicin (Positive Control)
Mean flourescence intensity of DCFDA
Mean flourescence intensity of DCF
72hrs
CID-6861424 25uM
2 Gy
CID6861424 + 2 Gy
Doxorubicin
Positive Control
Control
Counts
FL1-H
CID-6861424 25µM
2Gy
Control (DMSO)
CID-6861424 25µM + 2Gy
CID-6861424 25 µM
CID-6861424 25 µM + 2 Gy
Doxorubicin
(Positive control)

Figure 6.8Combination of 25µM CID-6861424 and 2Gy-γ-radiation induces ROS generation more effectively in "MCF-7 and MDA-MB-231" than "NIH3T3" cells: (a), (c) and (e). Bar-diagram representation of enhanced ROS generation in "MCF-7 and MDA-MB-231" than "NIH3T3" cells indicated by increased DCF-DA fluorescence post 72 hours treatment with either 25µM CID-6861424 or 2Gy alone or in combination. (b), (d) and (f). Histogram shows enhanced ROS generation in "MCF-7 and MDA-MB-231" than "NIH3T3" cells.Mean ± SD value (Data represented was calculatedfor three independent experiments). *P<0.05 and ***P<0.001.

Time	Treatments	MC-7	MDA-MB-231	NIH3T3
72hours	CONTROL	28.85 ± 0.05	194.82 ± 24.43	27.97 ± 0.27
	CID-6861424 25µM	40.66 ± 1.73	230.78 ± 15.91	26.89 ± 0.46
	2Gy	48.09 ± 2.58	223.59 ± 37.12	29.72 ± 0.32
	CID-6861424 25µM + 2Gy	67.46 ± 13.93	984.54 ± 25.55	25.57 ± 0.39
	Positive Control (Doxorubicin)	101.50 ± 2.82	-	90.06 ± 3.07

Table No6.4 CID-6861424 sensitizes and induces over-production of ROS in "MCF-7, MDA-MB-231" than "NIH3T3" cells upon exposure to 2Gy-γ-radiation at 25µM-CID-6861424.

We further checked the mitochondrial membrane potential (**Δψm**) with cationic fluorescent dye Rhodamine 123 using flow cytometry after 72 hours of treatment as a measure of **Δψm** disruptionin "MCF-7, MDA-MB-231 and NIH3T3" cells. The individual treatment of either 25µM CID-6861424 or 2Gy-γ-radiation resulted in approximately 1.5 fold disruption in **Δψm** as compared to the control. Whereas combined treatment resulted in almost 2.0 fold disruption in **Δψm** as compared to the control set was observed in both "breast cancer cell lines" **(Figure 6.9 (a), 6.9 (b), 6.9 (c) and 6.9 (d); Table No. 6.5)**.

NIH3T3 cell sample treated with 25µM CID-6861424 and 2Gy-γ-radiation alone or in combination caused insignificant disruption in **Δψm** as compared to the control was observed and doxorubicin here used as a positive control **(Figure 6.9 (e) and 6.9 (f); Table No. 6.5)**.

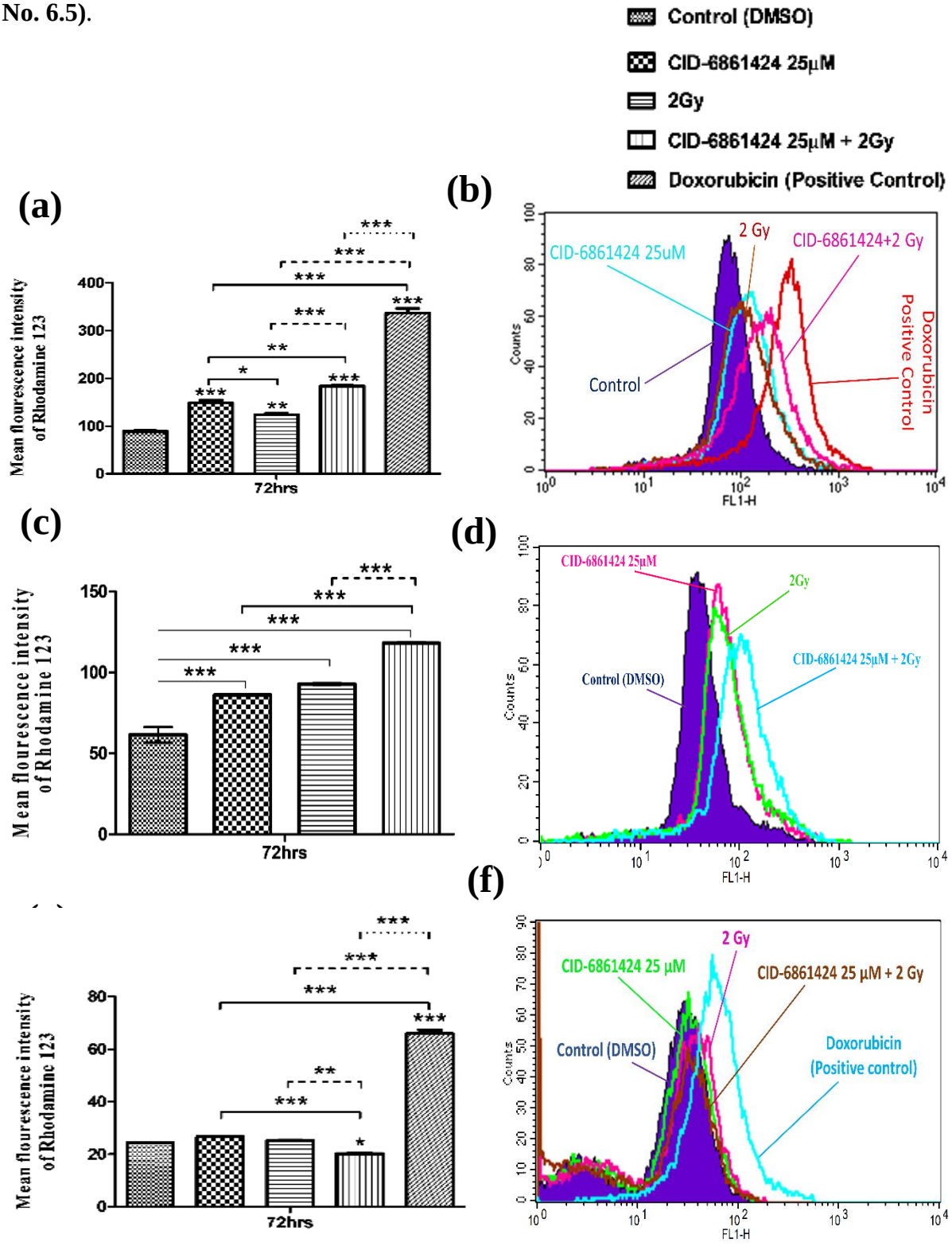


Figure 6.9Combination of 25µM CID-6861424 and 2Gy-γ-radiation enhances dissipation of Δψm more effectively in "MCF-7 and MDA-MB-231 than NIH3T3" cells: (a), (c) and (e). Bar-diagram representation Δψmdissipation in "MCF-7 and MDA-MB-231" than NIH3T3 cells detected by Rhodamine123-dye post 72hours treatment with either 25µM CID-6861424 or 2Gy alone or in combination. (b), (d) and (f). Histogram shows depleted Δψm in "MCF-7 and MDA-MB-231" than NIH3T3 cells.Data are represented as the mean ± SD of three independent experiments. *P<0.05 and ***P<0.001.

Time	Treatments	MC-7	MDA-MB-231	NIH3T3
72hours	CONTROL	89.29 ± 2.05	61.54 ± 8.32	24.36 ± 0.21
	CID-6861424 25µM	148.54 ± 10.31	86.14 ± 0.97	26.64 ± 0.45
	2Gy	123.86 ± 4.60	92.84 ± 0.95	25.14 ± 0.38
	CID-6861424 25µM + 2Gy	183.74 ± 2.49	118.22 ± 0.70	20.22 ± 0.52
	Positive Control (Doxorubicin)	336.92 ± 16.66	-	65.91 ± 2.51

Table No6.5 CID-6861424 sensitizes and induces depletion of Δψm in "MCF-7, MDA-MB-231" than NIH3T3 cells upon exposure to 2Gy-γ-radiation at 25µM-CID-6861424.

These results suggested that combinatorial treatment of 25µM CID-6861424 and 2Gy-γ-radiation more effectively increased ROS levels and **Δψm** disruption as compared to either compound or 2Gy radiation treated samples as well as the control set with least effect in NIH3T3 cells.

6.2.4 Combination treatment of 25µM CID-6861424 and 2Gy-γ-radiation increased DNA damage in breast cancer cells

The cell cycle data showed that, "MCF-7 and MDA-MB-231" cells treated with a combination of 25µM CID-6861424 and 2Gy-γ-radiation caused significant increase in sub-

G1 population as compared to alone treated sets and control set **(Figure 6.6 (d) and 6.6 (e)**). The elevated levels of ROS indicated that treatments of 25µM CID-6861424 and 2Gy-γ-radiation either alone or in combination might be inducing DNA damage leading to decreased cell viability. To confirm the DNA damage, comet assay by single gel electrophoresis was performed in MDA-MB-231 cells and tail lengths as an indicator of DNA damage was compared post 72 hours of treatment. In combination treated cells, enlarged tail lengths of comets were observed as compared to the cells which were treated with either the compound or 2Gy-γ-radiation as well as control set **(Figure 6.10**).

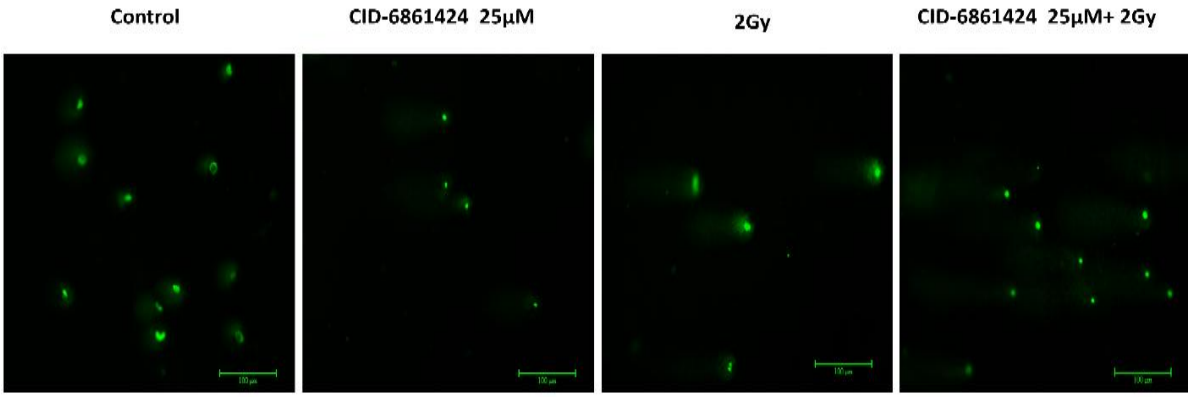
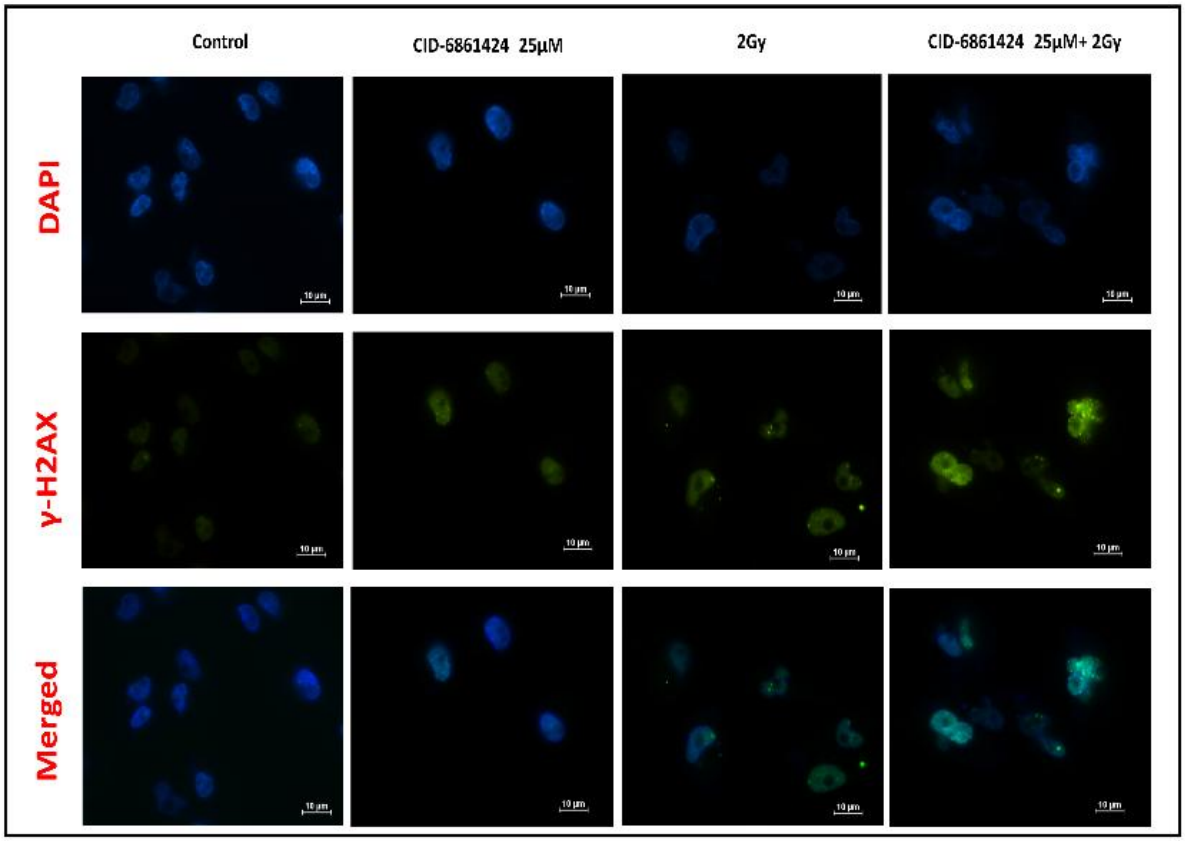


Figure 6.10Combination of 25µM CID-6861424 and 2Gy-γ-radiation increased DNA damage due to increasing the genotoxicity inMDA-MB-231 cells indicated via tail length of comet as compare to alone treatment as well as control panel at 72 hours.

The enlarged tail length indicated that DNA damage increased in the compound and 2Gy-γ-radiation combination treated set as compared to the alone as well as the control set. Further, DNA damage was analyzed by immunofluorescence of γ-H2AX in MDA-MB-231 cell, an indicator of DNA double-strand break. Cells, when treated with a combination of the compound and the radiation exhibited 5.0 fold increase in γ-H2AX intensity, while only ~2 folds increase in samples treated with either the compound or radiation with respect to control set **(Figure 6.11 (a) and 6.11 (b); Table No. 6.6)**. The quantified relative intensities

of γ-H2AX in all the treatment sets and control are shown by bar-diagram **(Figure 6.11 (b); Table No. 6.6)**.

(a)



(b)

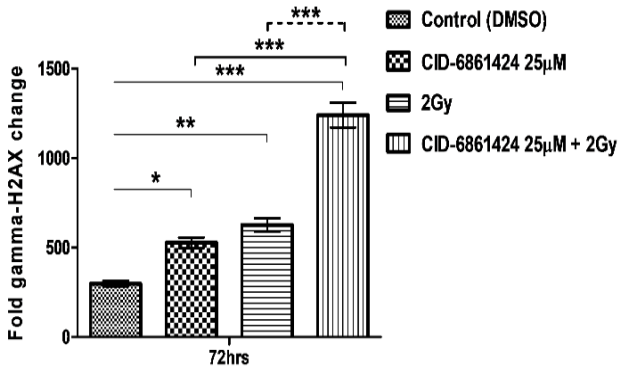


Figure 6.11 (a) Combination of 25µM CID-6861424 and 2Gy-γ-radiation induces γ-H2AX foci formation in "MDA-MB-231" cell and (b) The bar-diagram representation of fluorescence intensity of γ-H2AX in MDA-MB-231 cell at 72 hours. *P<0.05 and ***P<0.001.

Cell Line	Treatments	72hrs
MDA-MB-231	CONTROL	298.42 ± 24.45
	CID-6861424 25uM	527.53 ± 45.71
	2Gy	626.93 ± 58.43
	CID-6861424 25uM+ 2Gy	1240.98 ± 107.48

Table No6.6 CID-6861424 sensitizes and induces "DNA damage" in MDA-MB-231 cells upon exposure to 2Gy-γ-radiation at 25μM CID-6861424 indicated by increased intensity of γ-H2AX foci formation.

The result was further supported by western blot data. 25μM-CID-6861424 or 2Gy-γ-radiation alone showed an insignificant increase in γ-H2AX expression, but combination treatment resulted in almost ~4.0 fold increasein "MDA-MB-231" cells **(Figure 6.12)**. While MCF-7 cells treated with either compound or radiation alone caused increase in almost ~2.5 or 1.5 fold γ-H2AX expression as compared to the control (DMSO). In case of combination treatment γ-H2AX expression increased almost ~3.5 fold in as compared to the control set **(Figure 6.12)**.

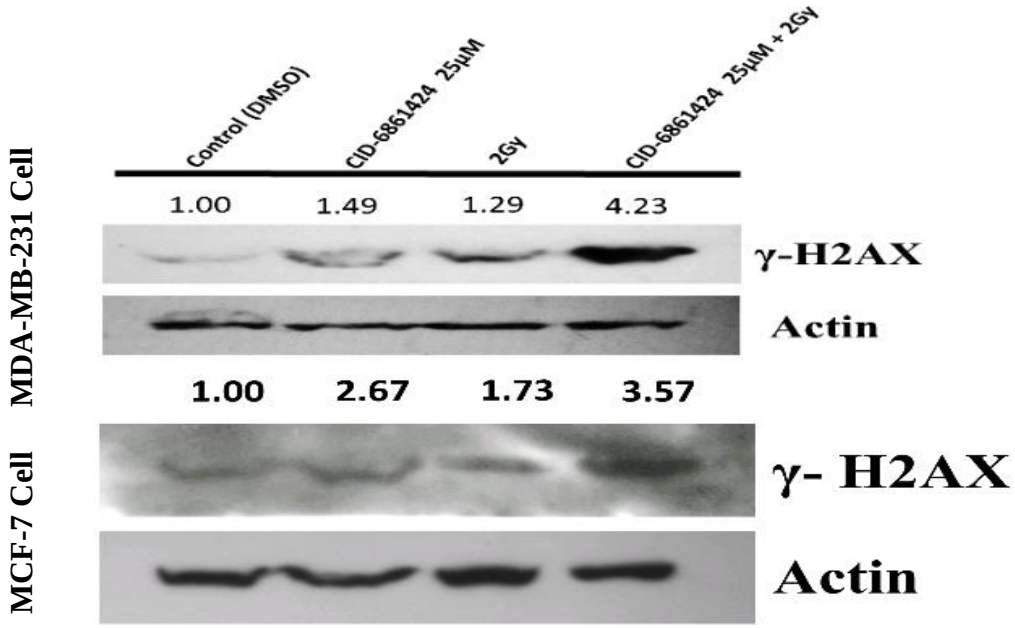


Figure 6.12 Combination of 25µM CID-6861424 and 2Gy-γ-radiation increased expression of γ-H2AX and quantified with image J software in "MCF-7 and MDA-MB-231" cells.

Results clearly show that combination treatment increased cells sensitivity causing enhanced dsDNA breaks as compared to alone treatment as well as control in both breast cancer cells.

6.3 Discussion

The cell viability data suggest that sub-cytotoxic concentration of 25µM CID-6861424 or low dose of 2Gy-γ-radiation does not significantly inhibit the cell growth.Whereas the combination treatment of 25µM CID-6861424 and 2Gy-γ-radiation significantly inhibited cell growth indicating that the sub-cytotoxic concentration of 25µM CID-6861424 with 2Gy-γ-radiation induces inhibition of "MCF-7 and MDA-MB-231" cells growth. NIH3T3 cells were least effected. Cellular morphology is employed frequently to describe cellularcondition. Cells with abnormal physiology such as elongated, flattened, dense cytoplasm and irregular cell membrane. Further, features like dense cytoplasm and membrane protrusions can be an indication of cellular stress and apoptosis (Decoster et al., 2010; Hacker, 2000). The combination of CID-6861424 (25µM) and 2Gy-γ-radiation not only reduced cell numbers but also showed change in morphological features like elongated, flattened, membrane protrusions and dense cytoplasmic bodies in both cancer cells as compared to non-cancerous cells. The aggressive nature of cancer cells often reciprocated by their migration potential and breast cancer cells exhibit strong migratory properties. Thus, to evaluate the effect of CID-6861424 and γ-radiation alone and in combination, we performed wound healing assay and we observed that the combination treatment satisfactorily inhibited

cell migration in both cancer cell lines. The data from cell viability, morphology and wound healing assay indicated that the combination of 25µM CID-6861424 and 2Gy-γ-radiation could be an effective recipe for killing "MCF-7 and MDA-MB-231" cells. To further evaluate the underlying biological interventions resulting in cell death, we analysed the effects of CID-6861424 and 2Gy-γ-radiation alone or in combination on cell cycle profile of "MCF-7, MDA-MB-231 and NIH3T3" cells. The cell cycle is a complicated and firmlyregulated process. Unregulated cell cycle events are hallmark of cancer cells, which directs cell cycle for cell division even under uncongenialcircumstances.

In the present study, combination of γ-radiation and CID-6861424 arrested cell cycle at G_2/M phase in "MDA-MB-231 and MCF-7" cells. 25µM CID-6861424 or2Gy-γ-radiation alone or combination treatment showed no significant change of cell cycle profile of NIH3T3 cell as compared to the control set. γ-radiation is known to arrest cell cycle at G_2/M phase in both p53 positive (Naderi et al., 2002) and p53 null cells (Strasser-Wozak et al., 1998). Similarly, in our study, irradiation of both breast cancer cells harboring p53 positive (MCF-7 cell) and mutated p53 (MDA-MB-231 cell) resulted in "G_2/M phase" arrest. Further, the co-treatment of CID-6861424 and γ-irradiated increased G_2/M arrest suggesting that CID-6861424 increases the radiosensitivity of "MDA-MB-231" cells. The effect of combination treatment was not that significant in "MCF-7" cells. These findings are in accordance with the earlier study where pretreatment of nocodazole sensitizes cells against a low dose of gamma radiation and resulted in elongated "G_2/M arrest" in p53 null cells (Ning and Knox, 1999). Cyclin A2 and cyclin B1 are believed to be cooperatively associated with early mitotic events like chromatin condensation and nuclear envelope breakdown and are thus crucial for cell division (Gong and Ferrell, 2010). It is also reported that radiation

induced G_2/M arrest is associated with downregulated cyclin B1 protein expression (Bernhard et al., 1995). Thus, we next evaluated the expression profile of cyclin B1 in both breast cancer cell lines with all three treatment. Western blot analysis reveals that both γ-radiation and CID-6861424 alone downregulated expression of cyclin B1. However, the combination of compound and γ-radiation resulted in maximum downregulation of cyclin B1 leading to higher cell population in G_2/M phase arrest (Bernhard et al., 1995)in "MDA-MB-231" cell.Even though maximum downregulation of cyclin B1 protein expression was observed in combination than alone treatment in MCF-7 cells, cell cycle data did not show that significant change specially, between γ-radiation and combination treated sets. Prolonged G_2/M arrest leads to cellular apoptosis (Ning and Knox, 1999). The combinatorial treatment significantly lower down the expression of cyclin B1 leading to increased G_2/M arrest, consequently the sub-G1 cell population also significantly increased in the combination set as compared to the other sets specially, in "MDA-MB-231"cells. The cell cycle almost remained unchanged in "NIH3T3" cells.

Intracellular ROS is an essential player of cellular homeostasis and under normal conditions, ROS homeostasis is tightly regulated. Cellular exposure to ionising radiations causes water radiolysis and results in the release of free superoxide ($O_2^{\bullet-}$) and perhydroxyl ($HO_2^{\bullet}$) radicals collectively called as ROS (Azzam et al., 2012). These "free radicals" are highly reactive and interact with biomolecules including "deoxyribonucleic acid (DNA)" causing damage and oxidative stress (Azzam et al., 2012). Cancer cells in contrast to normal cells have higher levels of ROS which are considered to be one of the major sources of oncogenesis (Panieri and Santoro, 2016; Schumacker, 2006b; Wang and Yi, 2008b). Beside generation of high levels of "reactive oxygen species (ROS)", cancer cells also express high

levels of “antioxidant” proteins such as GSH, catalase, SOD, etc. that can quench excess ROS (Liou and Storz, 2010b) thus maintain ROS homeostasis. Most of the cancer therapies target this ROS level of cancer cells which is either downregulated by means of antioxidants and other phytochemicals (Ahmad and Mukhtar, 2013b) or unregulated by means of chemotherapeutic compounds (Pelicano et al., 2004; Trachootham et al., 2009a) and physical agents like radiation (Azzam et al., 2012). Thus, we analysed the generation of cellular ROS post 72hours treatment. Individual treatment of 25µM CID-6861424 and 2Gy-γ-radiation increased ROS levels, however a significant increase in levels of ROS was observed in combination treatment set in breast cancer cell lines. The relatively lower ROS generation upon individual treatments of 25µM CID-6861424 and 2Gy-γ-radiation as compared to the combinatorial treatment might be due to the overexpression of antioxidant proteins, which might have neutralized most of the ROS generated during individual treatments. While in case of combinatorial treatment the flux of generated ROS might be so strong that the antioxidant system of “MCF-7 and MDA-MB-231” cells failed to neutralize it resulting in this difference. In case of NIH3T3 cells, generation of cellular ROS in individual and combination treatments showed no significant increase in ROS generation, which might be due to the strong antioxidant system of NIH3T3 cell. In mammalian cells elevated levels of ROS can lead to disruption of **Δψm**(Banki et al., 1999; Fonseca-Silva et al., 2011; Johnson et al., 1996) resulting in mitochondrial mediated cellular apoptosis (Reed et al., 1998). Thus, to investigate mitochondrial membrane potential status, we further investigated the effects of the compound and γ-radiation alone or in combination treatment on **Δψm** disruption in breast cancer cells and non-cancerous cells. Independent treatment 25µM CID-6861424 and 2Gy-γ-radiationindependent treatment on “MCF-7 and MDA-MB-

231" cells caused disruption in **Δψm**, while their combination caused significant disruption of **Δψm**, higher then when individual treatment was given. In case of NIH3T3 cell, treated with either 25µM CID-6861424 or 2Gy-γ-radiation alone or in combination caused insignificant change in disruption of **Δψm** as compare to the control set. The results indicating that the compound might be selectively sensitizing the breast cancer cells and thus combination treatment of the compound and 2Gy-γ-radiation is comparatively more lethal than the individual treatments.

Overproduction of ROS can also induce DNA damage due to oxidative stress. Comet assay is used to determine nuclear DNA damage upon exposure to oxidative stress, where the length of comets is directly related to breaks in nuclear DNA (Collins, 1999). Thus, comet assay was performed on post treated samples of MDA-MB-231 cells. Individual treatments of 25µM CID-6861424 and 2Gy-γ-radiation resulted in elongated comet tails as compared to DMSO control, however combination treatment of 25µM CID-6861424 and 2Gy-γ-radiation resulted in comparatively longer comet tails in MDA-MB-231 cell. Longer comet tails are indicative of excessive DNA damage and thus, it explains the relatively higher TNBC cell populations in sub-G1 phase. The results suggested that the cells are getting radio-sensitized in presence of sub-cytotoxic concentration of CID-6861424. The γ-H2AX is associated with the repair of double-strand (ds) DNA breakage/damage induced by exposure to radiation, chemotherapeutic drugs, oxidative stress, etc. Thus, γ-H2AX is overexpressed upon ds DNA break and is unanimously considered as a marker of ds DNA breakage (Dickey et al., 2009). In the present study, individual treatment of 25µM CID-6861424 and 2Gy-γ-radiation resulted in increased expression of γ-H2AX after 72 hours of exposure. The overexpression was maximum in the combination set at 72 hours time point indicating that although both

CID-6861424 at 25µM and 2Gy-γ-radiation individually induced DNA damage, but the maximum DNA damage occurred in combination of 25µM CID-6861424 and 2Gy-γ-radiation treatment in "MCF-7 and MDA-MB-231" cells. Further, at 72 hours post treatment, microscopic investigation of γ-H2AX foci clearly demonstrates that the combinatorial treatment led to excessive DNA damage as compared to individual treatments and DMSO control as assessed by comparative intensities of "γ-H2AX" foci in MDA-MB-231 cell.

Results clearly show that combination treatment increased cells sensitivity causing enhanced dsDNA breaks as compared to alone treatment as well as the control set.

6.4 Conclusion

Due to compromised cell cycle checkpoints, TNBC cells are less radiosensitive as compare to the ER-positive breast cancer cells. Our data shows that 25µM sub-cytotoxic concentration of CID-6861424 enhanced the sensitivity of TNBC MDA-MB-231 to a low dose of 2Gy-γ-radiation. MCF-7 cell were not that effectively radiosensitized by the compound. NIH3T3 cells remained unaffected by the treatments. Based on results a model is proposed **(Figure 6.13)**. Elevated ROS generation due to combination treatment caused increased oxidative stress, dissipated **$\Delta\psi m$** and ds-DNA-break. Cells enter the G_2/M phase even with dsDNA-break resulting in cell death. Our results provide a mechanistic rational for using CID-6861424 as a sensitizer in the presence of a low dose of γ-radiation to improve radiotherapy for breast cancer cells.

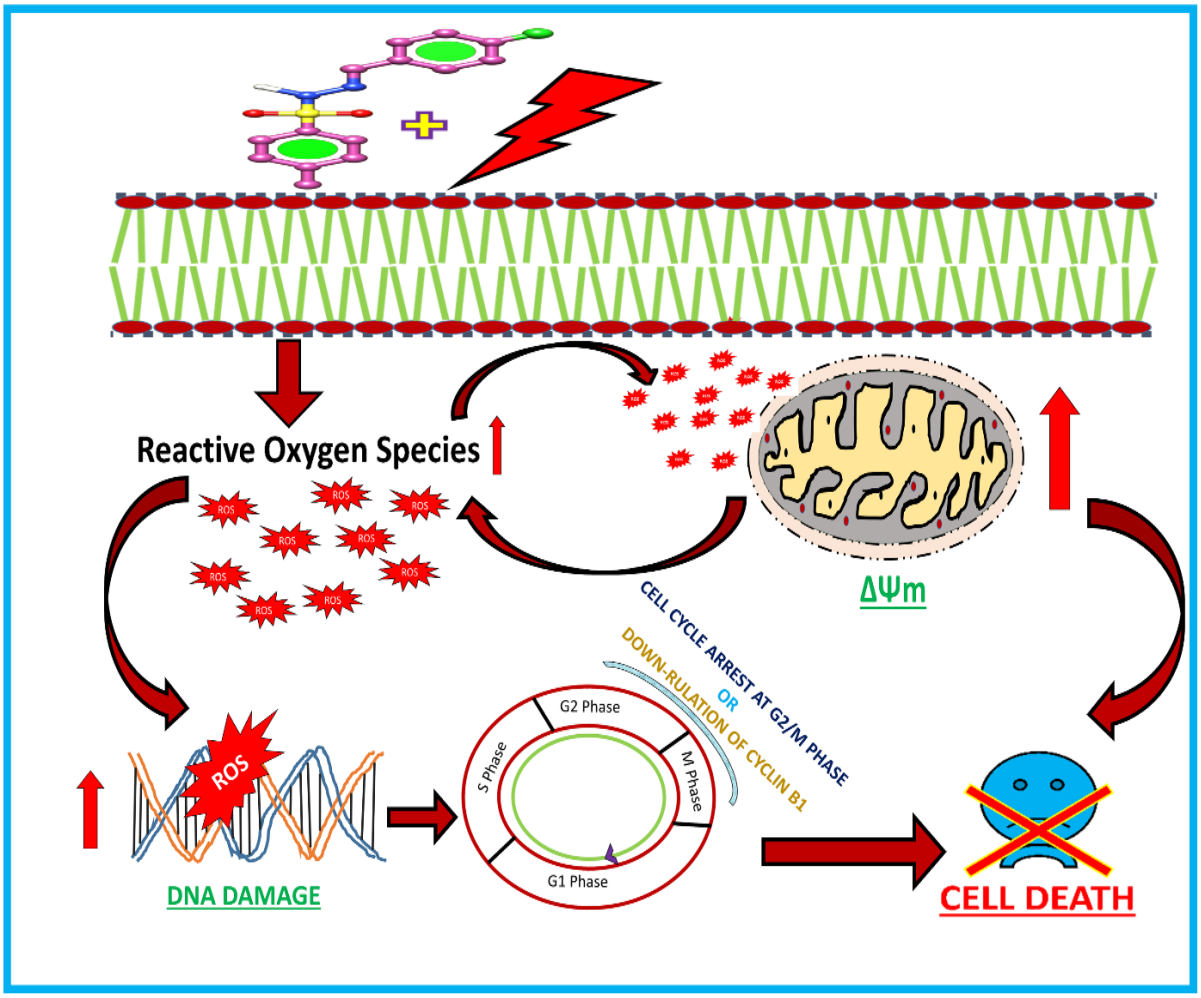


Figure 6.13Proposed model for mechanism of action of CID-6861424 and 2Gy-γ-radiation in combination in breast cancer cell line.

CHAPTER-7

SUMMMARY

Cancer is alteration of cellular proliferative properties in response to cellular insults involving DNA damage and altered cell signaling. Breast cancer represents the most common and prominent form of female malignancy and is a major cause of worldwide female mortality (Torre et al., 2015). Recent GLOBCAN 2018 data shows that out of total cancer incidences, 11.6% incidences are of breast cancer and it leads to a total of 6.6% mortality all over the world (Bray et al., 2018a). The common cause of breast cancer includes hereditary lineage, smoking, alcoholism, improper diet, lack of exercise, menopause and life style etc. (Ataollahi et al., 2015). The clinical segregation of breast cancer types is based on the expression or silencing of three cell surface receptors namely "estrogen receptor (ER), progesterone receptor (PR) and human epidermal receptors (HER)" which in combination or as a unit contribute to the molecular and phenotypic attributes of a particular breast cancer. These receptors mainly function in transducing cell signals triggered in response to specific hormone/signaling molecule leading to expression of several downstream proteins that either promote angiogenesis, cell growth and proliferation or inhibit apoptosis. ER and PR are the prime regulator of female reproductive system which regulates cell proliferation and differentiation during different phases of female reproductive cycle and pregnancy (Conneely et al., 2017; Jabbour et al., 2006). They belong to a family of nuclear receptors, which primarily function by binding to specific response elements (REs), "estrogen response elements (EREs)" (Klinge, 2001) and "progesterone response element (PREs)" (Bain et al., 2007) localized in the promoter region of the target gene. Amongst all different breast cancers, 80% of them express ER and are thus termed as ERpositive (ER+ve)

(Konecny et al., 2003). In post-menopausal femalesER+vebreast cancers are associated with poor prognosis (Ruder et al., 1989). Unlike “receptor positive breast cancers”, “receptor negative breast cancers” (RNBC) are more aggressive and lead to poor prognosis particularly in pre-menopausal stages (Jitariu et al., 2017). Amongst RNBC, “triple-negative breast cancers” (TNBC) are the most aggressive type with increased invasiveness and stemness (Honeth et al., 2008; Li et al., 2017; Neve et al., 2006). More importantly, TNBC cells do not respond to endocrine therapies and thus, require many different regimens for their treatment, which also may be non-responsive under late stages leading to surgery as the only last option.

Endocrine or hormone therapies with molecules like goserelin, fulvestrant and tamoxifen etc. are commonly used for the treatment of breast cancers with ER+ve status (Engel and Schally, 2007; Puhalla et al., 2012). Chemotherapeutics like methotrexate, doxorubicin, paclitaxel etc. are also used in the treatment of both ER+ve and “TNBC” however,TNBCs respond less to chemotherapy (Holliday and Speirs, 2011). Moreover these chemotherapeutics often show serious adverse effects to heart (Bomzer et al., 2014) and liver (Grigorian and O’Brien, 2014) along with toxicities causing vomiting, nausea and hair loss (Teven et al., 2017).

Radiotherapy is another mode of treatment of cancers including breast cancers. Clinically, use of ionizing radiations particularly γ-radiation routinely practiced for the treatment of breast cancers. The basic mechanism by which these radiations kill cancer cells is by inducing DNA damage in the tumors. However systemic effect is one of the most common issuesof radiotherapy and thus, it also induces DNA damage to nearby healthy tissues, a phenomenon commonly called as by-stander effect (Mothersill and Seymour, 2001). Also, there are many reports showing that females are more vulnerable to radiation induced by stander effect and develop other secondary cancers in response to frequent and high doses of radiotherapy (Armstrong et al., 2007; Bhatia

and Sklar, 2002). Thus, to minimize the harmful effects of radiotherapy, clinical treatments often use a regimen of low dose radiation in adjunct with chemotherapy.

In the present study, considering the fact that there is a constant need to find more potent chemical drug with least possible side effects, sulfonamide derivative CID-6861424, was evaluated and was found to be effective in heterogeneous breast cancer cell population and can also be used as adjuvant to radiation therapy and does not show any significant toxic effects. The treatment of different concentrations of CID-6861424 in "MCF-7 and TNBC cell MDA-MB-231" was carried out and it was found that the IC_{50} value for CID-6861424 was 50µM at 72 hours in both cell lines. Also at 50µM, CID-6861424 significantly affected the morphologies, migratory and clonogenic properties of these cell lines. In "MCF-7 cell lines", CID-6861424 at 50µM induced G1 phase arrest, while in "MDA-MB-231 cell lines", it induced G_2/M phase arrest indicated by analysis of their "cell cycle profile" and significant downregulation of corresponding cell cycle cyclins and cdks, Cyclin D1 and CDK4/6 in MCF-7 andcyclin B and CDK-1 in MDA-MB-231. Besides, we also found that treatment of CID-6861424 also upregulated p21 in "MDA-MB-231 cell lines". CID-6861424 at 50µM also enhanced ROS production, $\Delta\boldsymbol{\psi m}$ disruption and subsequently DNA damage leading to apoptosis in both the cell lines as indicated by upregulation of p53, p21, γ-H2AX and Annexin V/7AAD profiling. We tried to decipher the underlying mechanism by which 50µM CID-6861424 affected "MCF-7 and TNBC MDA-MB-231" cells. In "MCF-7 cells", CID-6861424 downregulated phosphorylation of ERK/Akt signaling pathway, while in "MDA-MB-231", it downregulated phosphorylation of ERK signaling pathway consecutively leading to upregulation of "Bax/Bcl-2 ratio" and downregulation of phosphorylated GSK-3β.

The toxicological studies in cell culture based assays in NIH3T3 cells demonstrated non-toxic nature of CID-6861424 at 25µM and 50µM. Cell viability, morphology and migratory properties remain unperturbed after the treatment and comparable to the untreated set. Moreover, "cell cycle profile", levels of ROS and $\Delta\psi m$ of NIH3T3 cells remain unaffected further supporting its non-toxic nature to normal cells. In female BALB/c mice, CID-6861424 treatment at 5mg/kg and 10 mg/kg of body weight for 30 days showed no adverse effects as indicated by analysis of body weight, DNA damage and oxidative stress parameters like ROS, TBARS, GSH, SOD and catalase in blood, liver, spleen and kidneys of female BALB/c mice which confirmed its non-toxic nature.

As CID-6861424 was found effective against "MCF-7 and triple negative breast cancer cell MDA-MB-231", we also determined if it can enhance radio-sensitivity of the cell lines. In this study, adjunct effects of sub-cytotoxic concentration of CID-6861424 (25µM) along with low dose of γ-radiation (2Gy) were evaluated on "MCF-7"breast cancer cells and triple negative breast cancer cells"MDA-MB-231". Also the effects of this adjunct therapy were evaluated on non-cancerous human fibroblast NIH3T3 cells. In this study, cell viability data showed that, the IC_{50} of the adjunct treatment was at 72 hours. However, at this IC_{50}, the NIH3T3 cells remained ineffective to the treatment. Similarly, the adjunct treatment showed prominent effects on the morphologies and migration of "MCF-7 and MDA-MB-231" cells, while the morphologies and migratory properties of NIH3T3 cells remained normal. The adjunct treatment also reduced clonogenic properties of "MCF-7 and MDA-MB-231" cells and induced G_2/M phase arrest in them while no effects were observed on cell cycle of NIH3T3 cells as indicated by cell cycle analysis and reduced expression of cyclin B1. Also, the adjunct treatment of sub-cytotoxic concentration of CID-6861424 (25µM) along with low dose of γ-radiation (2Gy) increased

intracellular ROS, disrupted Δψm and induced DNA damage as indicated by upregulation expression of γ-H2AX, while no such effects were observed in case of NIH3T3 cells upon adjunct treatment of sub-cytotoxic concentration of CID-6861424 (25μM) along with low dose of γ-radiation (2Gy).

These findings suggests that CID-6861424 can be a potential candidate for anti-cancer therapy which can not only selectively kill ER+ve and TNBC breast cancer cells, but can also increase their sensitivity towards radiotherapy without causing side effects to normal healthy cells and tissues.

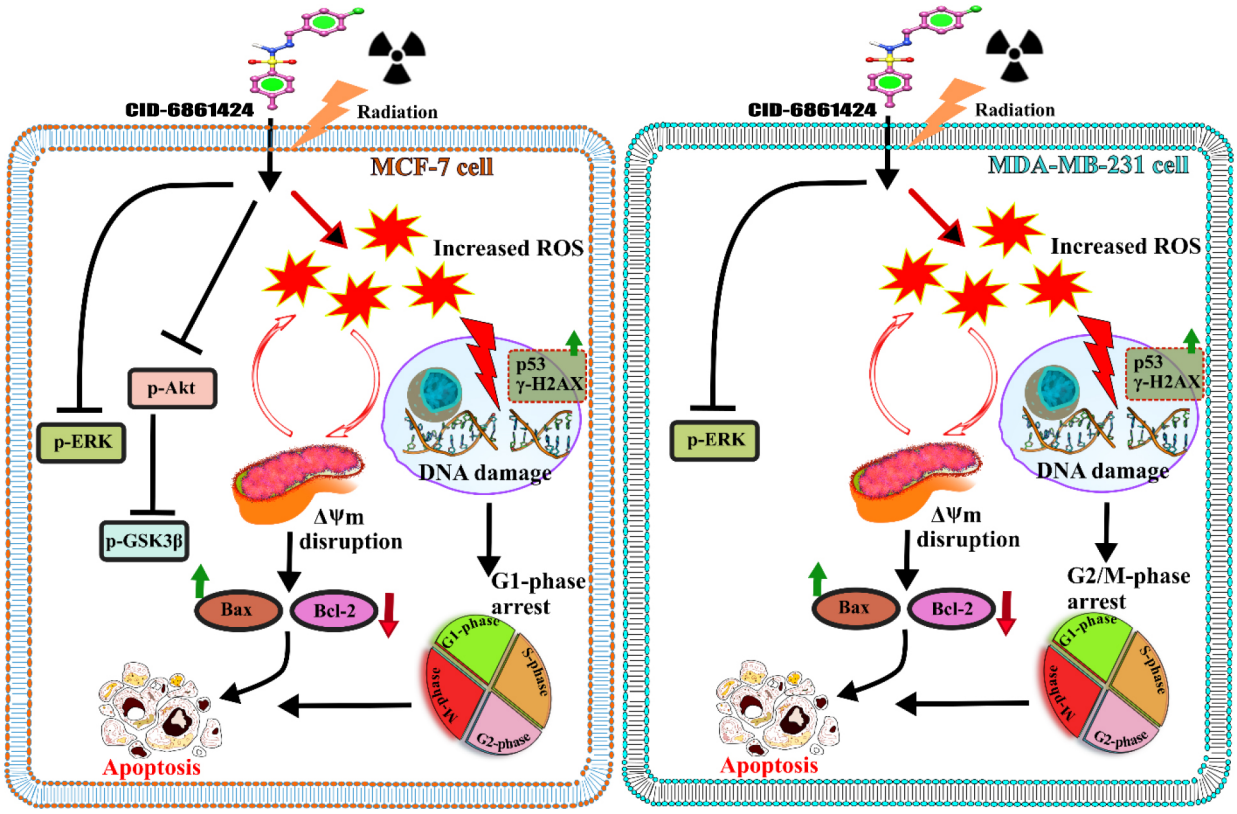


Figure: Mechanism of CID-6861424 induced cell death in MCF-7 & MDA-MB-231. Also mechanism of cell death in response to adjunct treatment of CID-6861424& 2Gy-γ-radiation is represented (in red arrows only).

REFERENCES

REFERENCES

- Adeyinka, A., Nui, Y., Cherlet, T., Snell, L., Watson, P.H., and Murphy, L.C. (2002). Activated mitogen-activated protein kinase expression during human breast tumorigenesis and breast cancer progression. Clin. Cancer Res. *8*, 1747–1753.
- Ahmad, N., and Mukhtar, H. (2013a). Antioxidants meet molecular targets for cancer prevention and therapeutics. Antioxid. Redox Signal. *19*, 85–88.
- Ahmad, N., and Mukhtar, H. (2013b). Antioxidants Meet Molecular Targets for Cancer Prevention and Therapeutics. Antioxid. Redox Signal. *19*, 85–88.
- Ak, N., and Aydiner, A. (2019). Systemic Treatment Drugs and Regimens BT - Breast Cancer : A Guide to Clinical Practice. A. Aydiner, A. Igci, and A. Soran, eds. (Cham: Springer International Publishing), pp. 587–608.
- Akhdar, H., Legendre, C., Aninat, C., and More, F. (2012). Anticancer Drug Metabolism: Chemotherapy Resistance and New Therapeutic Approaches. In Topics on Drug Metabolism, pp. 137–170.
- Alao, J.P. (2007). The regulation of cyclin D1 degradation: roles in cancer development and the potential for therapeutic invention. Mol. Cancer *6*, 24.
- Alt, J.R., Gladden, A.B., and Diehl, J.A. (2002). p21 ^{Cip1} Promotes Cyclin D1 Nuclear Accumulation via Direct Inhibition of Nuclear Export. J. Biol. Chem. *277*, 8517–8523.
- Ambros, T., Zeichner, S.B., Zaravinos, J., Montero, A.J., Ahn, E., Aruna, M., Kronish, L., Mahtani, R.L., and Vogel, C.L. (2014). A retrospective study evaluating a fixed low dose capecitabine monotherapy in women with HER-2 negative metastatic breast cancer.

Breast Cancer Res. Treat. *146*, 7–14.

- Anderson, M.A., Deng, J., Seymour, J.F., Tam, C., Kim, S.Y., Fein, J., Yu, L., Brown, J.R., Westerman, D., Si, E.G., et al. (2016). The BCL2 selective inhibitor venetoclax induces rapid onset apoptosis of CLL cells in patients via a TP53-independent mechanism. Blood *127*, 3215–3224.
- Anothaisintawee, T., Wiratkapun, C., Lerdsitthichai, P., Kasamesup, V., Wongwaisayawan, S., Srinakarin, J., Hirunpat, S., Woodtichartpreecha, P., Boonlikit, S., Teerawattananon, Y., et al. (2013). Risk Factors of Breast Cancer. Asia Pacific J. Public Heal. *25*, 368–387.
- Archives, N. Stewart F . Alexander , " Final Report of Bari Mustard Casualties " Stewart F . Alexander , " Final Report of Bari Mustard Casualties ." 1–4.
- Arellano, M., and Moreno, S. (1997). Regulation of CDK/cyclin complexes during the cell cycle. Int. J. Biochem. Cell Biol. *29*, 559–573.
- Arias, A.M. (2001). Epithelial mesenchymal interactions in cancer and development. Cell *105*, 425–431.
- Armstrong, G.T., Sklar, C.A., Hudson, M.M., and Robison, L.L. (2007). Long-Term Health Status Among Survivors of Childhood Cancer: Does Sex Matter? J. Clin. Oncol. *25*, 4477–4489.
- Arrick, B.A., and Nathan, C.F. (1984). Glutathione Metabolism as a Determinant of Therapeutic Efficacy: A Review. Cancer Res. *44*, 4224 LP – 4232.
- Ataollahi, M.R., Sharifi, J., Paknahad, M.R., and Paknahad, A. (2015). Breast cancer and associated factors: a review. J. Med. Life *8*, 6–11.
- Azzam, E.I., Jay-Gerin, J.-P., and Pain, D. (2012). Ionizing radiation-induced metabolic

oxidative stress and prolonged cell injury. Cancer Lett. *327*, 48–60.

- Bain, D.L., Heneghan, A.F., Connaghan-Jones, K.D., and Miura, M.T. (2007). Nuclear Receptor Structure: Implications for Function. Annu. Rev. Physiol. *69*, 201–220.
- Banki, K., Hutter, E., Gonchoroff, N.J., and Perl, A. (1999). Elevation of mitochondrial transmembrane potential and reactive oxygen intermediate levels are early events and occur independently from activation of caspases in Fas signaling. J. Immunol. *162*, 1466–1479.
- Barboule, N., Baldin, V., Jozan, S., Vidal, S., and Valette, A. (1998). Increased level of p21 in human ovarian tumors is associated with increased expression of CDK2, cyclin A and PNCA. Int. J. Cancer *76*, 891–896.
- Barcellos-Hoff, M.H., Park, C., and Wright, E.G. (2005). Radiation and the microenvironment – tumorigenesis and therapy. Nat. Rev. Cancer *5*, 867–875.
- Bargonetti, J., and Manfredi, J.J. (2002a). Multiple roles of the tumor suppressor p53. Curr. Opin. Oncol. *14*, 86–91.
- Bargonetti, J., and Manfredi, J.J. (2002b). Multiple roles of the tumor suppressor p53. Curr. Opin. Oncol. *14*, 86–91.
- Bartholomeusz, C., Gonzalez-Angulo, A.M., Liu, P., Hayashi, N., Lluch, A., Ferrer-Lozano, J., and Hortobagyi, G.N. (2012). High ERK Protein Expression Levels Correlate with Shorter Survival in Triple-Negative Breast Cancer Patients. Oncologist *17*, 766–774.
- Barzilai, A., and Yamamoto, K.-I. (2004). DNA damage responses to oxidative stress. DNA Repair (Amst). *3*, 1109–1115.
- Bauer, K.R., Brown, M., Cress, R.D., Parise, C.A., and Caggiano, V. (2007). Descriptive analysis of estrogen receptor (ER)-negative, progesterone receptor (PR)-negative, and

HER2-negative invasive breast cancer, the so-called triple-negative phenotype: A population-based study from the California Cancer Registry. Cancer *109*, 1721–1728.

- Bernhard, E.J., Maity, A., Muschel, R.J., and McKenna, W.G. (1995). Effects of ionizing radiation on cell cycle progression. Radiat. Environ. Biophys. *34*, 79–83.
- Bertoli, C., Skotheim, J.M., and de Bruin, R.A.M. (2013). Control of cell cycle transcription during G1 and S phases. Nat. Rev. Mol. Cell Biol. *14*, 518–528.
- Bhatia, S., and Sklar, C. (2002). Second cancers in survivors of childhood cancer. Nat. Rev. Cancer *2*, 124.
- Bielawski, K., Bielawska, A., and Wolczynski, S. (2002). Synthesis, DNA-Binding Activity and Cytotoxicity of Carbamate Derivatives of Hoechst 33258 in Breast Cancer MCF-7 Cells. Biol. Pharm. Bull. *25*, 916–919.
- Bishayee, A., and Rabi, T. (2009). *d* -Limonene sensitizes docetaxel-induced cytotoxicity in human prostate cancer cells: Generation of reactive oxygen species and induction of apoptosis. J. Carcinog. *8*, 9.
- Bissery, M.C., Guénard, D., Guéritte-Voegelein, F., and Lavelle, F. (1991). Experimental antitumor activity of taxotere (RP 56976, NSC 628503), a taxol analogue. Cancer Res. *51*, 4845–4852.
- Blackburn, G.L., and Wang, K.A. (2007). Dietary fat reduction and breast cancer outcome: results from the Women's Intervention Nutrition Study (WINS). Am. J. Clin. Nutr. *86*, 878S-881S.
- Blackstein, M., Vogel, C.L., Ambinder, R., Cowan, J., Iglesias, J., and Melemed, A. (2002). Gemcitabine as First-Line Therapy in Patients with Metastatic Breast Cancer: A Phase II Trial. Oncology *62*, 2–8.

- Blagosklonny, M. V., and Pardee, A.B. (2002). The Restriction Point of the Cell Cycle. Cell Cycle *1*, 102–109.
- Blagosklonny, M. V, Schulte, T.W., Nguyen, P., Mimnaugh, E.G., Trepel, J., and Neckers, L. (1995). Taxol induction of p21WAF1 and p53 requires c-raf-1. Cancer Res. *55*, 4623–4626.
- Boekhout, A.H., Beijnen, J.H., and Schellens, J.H.M. (2011). Trastuzumab. Oncologist *16*, 800–810.
- Boffetta, P., Hashibe, M., La Vecchia, C., Zatonski, W., and Rehm, J. (2006). The burden of cancer attributable to alcohol drinking. Int. J. Cancer *119*, 884–887.
- Bomzer, C.A., Care, A.C., and Care, A.H. (2014). Cardiovascular Toxicity of Common Chemotherapy Drugs Used to Treat Breast Cancer : An Overview Cardiovascular Toxicity of Common Chemotherapy Drugs Used to Treat. J Patient Cent Res Rev. *1*, 133–136.
- Boots, A.W., Haenen, G.R.M.M., and Bast, A. (2008). Health effects of quercetin: From antioxidant to nutraceutical. Eur. J. Pharmacol. *585*, 325–337.
- Braakhuis, B.J., Hill, B.T., Dietel, M., Kelland, L.R., Aapro, M.S., Zoli, W., and Lelieveld, P. In vitro antiproliferative activity of docetaxel (Taxotere), paclitaxel (Taxol) and cisplatin against human tumour and normal bone marrow cells. Anticancer Res. *14*, 205–208.
- Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R.L., Torre, L.A., and Jemal, A. (2018a). Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA. Cancer J. Clin. *68*, 394–424.
- Bray, F., Ferlay, J., Soerjomataram, I., Siegel, R.L., Torre, L.A., and Jemal, A. (2018b).

Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries. CA. Cancer J. Clin.

- Brody, J.G., Rudel, R.A., Michels, K.B., Moysich, K.B., Bernstein, L., Attfield, K.R., and Gray, S. (2007). Environmental pollutants, diet, physical activity, body size, and breast cancer. Cancer *109*, 2627–2634.
- Brooks, C. (1998). Radiation Therapy. Physiotherapy *84*, 387–395.
- Brown, I., Shalli, K., McDonald, S.L., Moir, S.E., Hutcheon, A.W., Heys, S.D., and Schofield, A.C. (2004). Reduced expression of p27 is a novel mechanism of docetaxel resistance in breast cancer cells. Breast Cancer Res. *6*, R601.
- Bryan, M., Pulte, E.D., Toomey, K.C., Pliner, L., Pavlick, A.C., Saunders, T., and Wieder, R. (2011). A pilot phase II trial of all-trans retinoic acid (Vesanoid) and paclitaxel (Taxol) in patients with recurrent or metastatic breast cancer. Invest. New Drugs *29*, 1482–1487.
- Bunz, F., Dutriaux, A., Lengauer, C., Waldman, T., Zhou, S., Brown, J.P., Sedivy, J.M., Kinzler, K.W., and Vogelstein, B. (1998). Requirement for p53 and p21 to sustain G2 arrest after DNA damage. Science *282*, 1497–1501.
- Callagy, G.M., Pharoah, P.D., Pinder, S.E., Hsu, F.D., Nielsen, T.O., Ragaz, J., Ellis, I.O., Huntsman, D., and Caldas, C. (2006). Bcl-2 Is a Prognostic Marker in Breast Cancer Independently of the Nottingham Prognostic Index. Clin. Cancer Res. *12*, 2468–2475.
- Carmona, F.J., Montemurro, F., Kannan, S., Rossi, V., Verma, C., Baselga, J., and Scaltriti, M. (2016). AKT signaling in ERBB2-amplified breast cancer. Pharmacol. Ther. *158*, 63–70.
- casini, A., Scozzafava, A., Mastrolorenzo, A., and Supuran, C. (2002). Sulfonamides and

Sulfonylated Derivatives as Anticancer Agents. Curr. Cancer Drug Targets *2*, 55–75.

- Cassia, R., Moreno-Bueno, G., Rodríguez-Perales, S., Hardisson, D., Cigudosa, J.C., and Palacios, J. (2003). Cyclin E gene (*CCNE*) amplification and *hCDC4* mutations in endometrial carcinoma. J. Pathol. *201*, 589–595.
- Cayrol, C., Knibiehler, M., and Ducommun, B. (1998). p21 binding to PCNA causes G1 and G2 cell cycle arrest in p53-deficient cells. Oncogene *16*, 311–320.
- Cazzalini, O., Scovassi, A.I., Savio, M., Stivala, L.A., and Prosperi, E. (2010). Multiple roles of the cell cycle inhibitor p21CDKN1A in the DNA damage response. Mutat. Res. Mutat. Res. *704*, 12–20.
- Chabner, B. (1982). Pharmacologic principles of cancer treatment (Saunders).
- Chan, D.C., Earle, K.A., Zhao, T.L.M., Helfrich, B., Zeng, C., Baron, A., Whitehead, C.M., Piazza, G., Pamukcu, R., Thompson, W.J., et al. (2002). Exisulind in combination with docetaxel inhibits growth and metastasis of human lung cancer and prolongs survival in athymic nude rats with orthotopic lung tumors. Clin. Cancer Res. *8*, 904–912.
- Chan, D.W., Liu, V.W.S., Tsao, G.S.W., Yao, K.-M., Furukawa, T., Chan, K.K.L., and Ngan, H.Y.S. (2008). Loss of MKP3 mediated by oxidative stress enhances tumorigenicity and chemoresistance of ovarian cancer cells. Carcinogenesis *29*, 1742–1750.
- Chang, J.T., Chang, G.-C., Ko, J.-L., Liao, H.-Y., Liu, H.-J., Chen, C.-C., Su, J.-M., Lee, H., and Sheu, G.-T. (2006). Induction of tubulin by docetaxel is associated with p53 status in human non small cell lung cancer cell lines. Int. J. Cancer *118*, 317–325.
- Chao, Y., Shih, Y.L., Chiu, J.H., Chau, G.Y., Lui, W.Y., Yang, W.K., Lee, S.D., and Huang, T.S. (1998). Overexpression of cyclin A but not Skp 2 correlates with the tumor

relapse of human hepatocellular carcinoma. Cancer Res. *58*, 985–990.

- Chen, J., Jin, S., Abraham, V., Huang, X., Liu, B., Mitten, M.J., Nimmer, P., Lin, X., Smith, M., Shen, Y., et al. (2011). The Bcl-2/Bcl-XL/Bcl-w Inhibitor, Navitoclax, Enhances the Activity of Chemotherapeutic Agents In Vitro and In Vivo. Mol. Cancer Ther. *10*, 2340–2349.
- Chetty, R., and Simelane, S. (1999). p53 and Cyclin A Protein Expression in Squamous Carcinoma of the Oesophagus. Pathol. Oncol. Res. *5*, 193–196.
- Chiang, M.-F., Chou, P.-Y., Wang, W.-J., Sze, C.-I., and Chang, N.-S. (2013). Tumor Suppressor WWOX and p53 Alterations and Drug Resistance in Glioblastomas. Front. Oncol. *3*, 43.
- Chiara, F., and Rasola, A. (2013). GSK-3 and mitochondria in cancer cells. Front. Oncol. *3*, 16.
- Chipuk, J.E., and Green, D.R. (2004). Cytoplasmic p53: bax and forward. Cell Cycle *3*, 429–431.
- Chun, E., and Lee, K.-Y. (2004). Bcl-2 and Bcl-xL are important for the induction of paclitaxel resistance in human hepatocellular carcinoma cells. Biochem. Biophys. Res. Commun. *315*, 771–779.
- Chun, J., Li, R.-J., Cheng, M.-S., and Kim, Y.S. (2015). Alantolactone selectively suppresses STAT3 activation and exhibits potent anticancer activity in MDA-MB-231 cells. Cancer Lett. *357*, 393–403.
- Cohen, G.L., Bauer, W.R., Barton, J.K., and Lippard, S.J. (1979). Binding of cis- and trans-dichlorodiammineplatinum(II) to DNA: evidence for unwinding and shortening of the double helix. Science *203*, 1014–1016.

- Collins, A.R. (1999). Oxidative DNA damage, antioxidants, and cancer. BioEssays *21*, 238–246.
- Conneely, O.M., Lydon, J.P., De Mayo, F., and O'Malley, B.W. (2017). Reproductive Functions of the Progesterone Receptor. J. Soc. Gynecol. Investig. *7*, S25–S32.
- Di Cosimo, S., Ferretti, G., Fazio, N., Silvestris, N., Carlini, P., Alimonti, A., Gelibter, A., Felici, A., Papaldo, P., and Cognetti, F. (2003). Docetaxel in advanced gastric cancer--review of the main clinical trials. Acta Oncol. *42*, 693–700.
- Courjal, F., Louason, G., Speiser, P., Katsaros, D., Zeillinger, R., and Theillet, C. (1996). Cyclin gene amplification and overexpression in breast and ovarian cancers: Evidence for the selection ofcyclin D1 in breast andcyclin E in ovarian tumors. Int. J. Cancer *69*, 247–253.
- Cunha, K.S., Reguly, M.L., Graf, U., and de Andrade, H.H. (2001). Taxanes: the genetic toxicity of paclitaxel and docetaxel in somatic cells of Drosophila melanogaster. Mutagenesis *16*, 79–84.
- Daugas, E., Geuskens, M., and Kroemer, G. (1996). Bcl-2 Inhibits the Mitochondrial Release o f an A p o p t o g e n i c Protease. *184*.
- David, K., and F., H.E. (1938). On the mechanism of the decomposition of hydrogen peroxide by catalase. Proc. R. Soc. London. Ser. B - Biol. Sci. *124*, 397–405.
- Decoster, M.A., Maddi, S., Dutta, V., and Mcnamara, J. (2010). Microscopy and image analysis of individual and group cell shape changes during apoptosis. Microsc. Sci. Technol. Appl. Educ. 836–843.
- DENOIX, P. [Not Available]. Bull. Inst. Natl. Hyg. *1*, 12–17.
- Dent, R., Trudeau, M., Pritchard, K.I., Hanna, W.M., Kahn, H.K., Sawka, C.A., Lickley,

L.A., Rawlinson, E., Sun, P., and Narod, S.A. (2007). Triple-negative breast cancer: Clinical features and patterns of recurrence. Clin. Cancer Res. *13*, 4429–4434.

- Derry, W.B., Wilson, L., and Jordan, M.A. (1995). Substoichiometric Binding of Taxol Suppresses Microtubule Dynamics. Biochemistry *34*, 2203–2211.
- DeVita, V.T. (1978). The Evolution of Therapeutic Research in Cancer. N. Engl. J. Med. *298*, 907–910.
- DeVita, V.T., and Schein, P.S. (1973). The Use of Drugs in Combination for the Treatment of Cancer. N. Engl. J. Med. *288*, 998–1006.
- Dhawan, A., Mahima Bajpayee, Mnas., Kumar Pandey, A., and Parmar, D. ITRC: THE SCGE/ COMET ASSAY PROTOCOL PROTOCOL FOR THE SINGLE CELL GEL ELECTROPHORESIS / COMET ASSAY FOR RAPID GENOTOXICITY ASSESSMENT.
- Dickey, J.S., Redon, C.E., Nakamura, A.J., Baird, B.J., Sedelnikova, O.A., and Bonner, W.M. (2009). H2AX: Functional roles and potential applications. Chromosoma *118*, 683–692.
- Dudley, M.E., Wunderlich, J.R., Yang, J.C., Sherry, R.M., Topalian, S.L., Restifo, N.P., Royal, R.E., Kammula, U., White, D.E., Mavroukakis, S.A., et al. (2005). Adoptive Cell Transfer Therapy Following Non-Myeloablative but Lymphodepleting Chemotherapy for the Treatment of Patients With Refractory Metastatic Melanoma. J. Clin. Oncol. *23*, 2346–2357.
- ELION, G.B., SINGER, S., and HITCHINGS, G.H. (1954). Antagonists of nucleic acid derivatives. VIII. Synergism in combinations of biochemically related antimetabolites. J. Biol. Chem. *208*, 477–488.

- Engel, J.B., and Schally, A. V (2007). Drug Insight: clinical use of agonists and antagonists of luteinizing-hormone-releasing hormone. Nat. Clin. Pract. Endocrinol. &Amp; Metab. *3*, 157.
- Esposti, M., Hatzinisiriou, I., and McLennan, H. (1999). Bcl-2 and mitochondrial oxygen radicals. J. Biol. … *274*, 29831–29837.
- Fabbri, F., Carloni, S., Brigliadori, G., Zoli, W., Lapalombella, R., and Marini, M. (2006). Sequential events of apoptosis involving docetaxel, a microtubule-interfering agent: a cytometric study. BMC Cell Biol. *7*, 6.
- Faha, B., Ewen, M.E., Tsai, L.H., Livingston, D.M., and Harlow, E. (1992). Interaction between human cyclin A and adenovirus E1A-associated p107 protein. Science *255*, 87–90.
- Fan, W., Chang, J., and Fu, P. (2015). Endocrine therapy resistance in breast cancer: current status, possible mechanisms and overcoming strategies. Future Med. Chem. *7*, 1511.
- Ferlini, C. (1998). The synergistic anti-tumour activity of ICI 182,780 in combination with docetaxel is mediated by P-glycoprotein inhibition. Endocr. Relat. Cancer *5*, 315–324.
- Ferlini, C., Cicchillitti, L., Raspaglio, G., Bartollino, S., Cimitan, S., Bertucci, C., Mozzetti, S., Gallo, D., Persico, M., Fattorusso, C., et al. (2009). Paclitaxel Directly Binds to Bcl-2 and Functionally Mimics Activity of Nur77. Cancer Res. *69*, 6906–6914.
- Filipa Reis-Mendes, A., Sousa, E., de Lourdes Bastos, M., and Marisa Costa, V. (2015). The Role of the Metabolism of Anticancer Drugs in Their Induced-Cardiotoxicity. Curr. Drug Metab. *17*, 75–90.

- Filipski, J., Kohn, K.W., and Bonner, W.M. (1980). The nature of inactivating lesions produced by platinum(II) complexes in phage lambda DNA. Chem. Biol. Interact. *32*, 321–330.
- Fisher, R.P. (1997). CDKs and cyclins in transition(s). Curr. Opin. Genet. Dev. *7*, 32–38.
- Fisher, D., Krasinska, L., Coudreuse, D., and Novak, B. (2012). Phosphorylation network dynamics in the control of cell cycle transitions. J. Cell Sci. *125*, 4703–4711.
- Fonseca-Silva, F., Inacio, J.D.F., Canto-Cavalheiro, M.M., and Almeida-Amaral, E.E. (2011). Reactive oxygen species production and mitochondrial dysfunction contribute to quercetin induced death in Leishmania amazonensis. PLoS One *6*.
- Foye, W.O. (1995). Cancer chemotherapeutic agents (American Chemical Society).
- Franken, N.A.P., Rodermond, H.M., Stap, J., Haveman, J., and van Bree, C. (2006). Clonogenic assay of cells in vitro. Nat. Protoc. *1*, 2315–2319.
- Fu, M., Wang, C., Li, Z., Sakamaki, T., and Pestell, R.G. (2004). Minireview: Cyclin D1: Normal and Abnormal Functions. Endocrinology *145*, 5439–5447.
- Fuchs-Tarlovsky, V. (2013). Role of antioxidants in cancer therapy. Nutrition *29*, 15–21.
- Fujita, K., Sano, D., Kimura, M., Yamashita, Y., Kawakami, M., Ishiguro, Y., Nishimura, G., Matsuda, H., and Tsukuda, M. (2007). Anti-tumor effects of bevacizumab in combination with paclitaxel on head and neck squamous cell carcinoma. Oncol. Rep. *18*, 47–51.
- Gage, M., Wattendorf, D., and Henry, L.R. (2012). Translational advances regarding hereditary breast cancer syndromes. J. Surg. Oncol. *105*, 444–451.
- Galaup, A., Opolon, P., Bouquet, C., Li, H., Opolon, D., Bissery, M.-C., Tursz, T., Perricaudet, M., and Griscelli, F. (2003). Combined effects of docetaxel and angiostatin

gene therapy in prostate tumor model. Mol. Ther. *7*, 731–740.

- Gavet, O., and Pines, J. (2010). Progressive Activation of CyclinB1-Cdk1 Coordinates Entry to Mitosis. Dev. Cell *18*, 533–543.
- GB. McDonald, N.T. (1984). Intestinal and Liver Toxicity of Antineoplastic Drugs. West J Med *140*, 250–259.
- Geng, C.-X., Zeng, Z.-C., and Wang, J.-Y. (2003). Docetaxel inhibits SMMC-7721 human hepatocellular carcinoma cells growth and induces apoptosis. World J. Gastroenterol. *9*, 696.
- Giannakakou, P., Robey, R., Fojo, T., and Blagosklonny, M. V (2001). Low concentrations of paclitaxel induce cell type-dependent p53, p21 and G1/G2 arrest instead of mitotic arrest: molecular determinants of paclitaxel-induced cytotoxicity. Oncogene *20*, 3806–3813.
- Glover, D., Glick, J.H., Weiler, C., Yuhas, J., and Kligerman, M.M. (1984). Phase I trials of WR-2721 and cis-platinum. Int. J. Radiat. Oncol. *10*, 1781–1784.
- Gong, D., and Ferrell, J.E. (2010). The roles of cyclin A2, B1, and B2 in early and late mitotic events. Mol. Biol. Cell *21*, 3149–3161.
- Grant, D.S., Williams, T.L., Zahaczewsky, M., and Dicker, A.P. (2003). Comparison of antiangiogenic activities using paclitaxel (taxol) and docetaxel (taxotere). Int. J. Cancer *104*, 121–129.
- Greenblatt, M.S., Bennett, W.P., Hollstein, M., and Harris, C.C. (1994). Mutations in the p53 tumor suppressor gene: clues to cancer etiology and molecular pathogenesis. Cancer Res. *54*, 4855–4878.
- Grigorian, A., and O'Brien, C.B. (2014). Hepatotoxicity secondary to chemotherapy. J.

Clin. Transl. Hepatol. *2*, 95–102.

- Guestini, F., McNamara, K.M., Ishida, T., and Sasano, H. (2016). Triple negative breast cancer chemosensitivity and chemoresistance: current advances in biomarkers indentification. Expert Opin. Ther. Targets *20*, 705–720.
- Guo, W., Zeng, C., Dong, F., and Lei, W. (2002). Paclitaxel-induced apoptosis in osteosarcoma cell line U-2 OS. Chin. Med. J. (Engl). *115*, 1796–1801.
- Hacker, G. (2000). The morphology of apoptosis. Cell Tissue Res. 2000 *301*, 5–17.
- Haldar, S., Chintapalli, J., and Croce, C.M. (1996). Taxol induces bcl-2 phosphorylation and death of prostate cancer cells. Cancer Res. *56*, 1253–1255.
- Handa, K., Yamakawa, M., Takeda, H., Kimura, S., and Takahashi, T. (1999). Expression of cell cycle markers in colorectal carcinoma: Superiority of cyclin A as an indicator of poor prognosis. Int. J. Cancer *84*, 225–233.
- Haupt, S., Berger, M., Goldberg, Z., and Haupt, Y. (2003). Apoptosis - the p53 network. J. Cell Sci. *116*, 4077–4085.
- Hauser, P.J., Agrawal, D., and Pledger, W.J. (1998). Primary keratinocytes have an adhesion dependent S phase checkpoint that is absent in immortalized cell lines. Oncogene *17*, 3083–3092.
- Hernandez-Aya, L.F., and Gonzalez-Angulo, A.M. (2011). Targeting the phosphatidylinositol 3-kinase signaling pathway in breast cancer. Oncologist *16*, 404–414.
- Hersh, S.M. (1968). Chemical and biological warfare; America's hidden arsenal, (Indianapolis,: Bobbs-Merrill).
- Hientz, K., Mohr, A., Bhakta-Guha, D., and Efferth, T. (2017). The role of p53 in cancer

drug resistance and targeted chemotherapy. Oncotarget *8*, 8921–8946.

- Hirai, H., Roussel, M.F., Kato, J.Y., Ashmun, R.A., and Sherr, C.J. (1995). Novel INK4 proteins, p19 and p18, are specific inhibitors of the cyclin D-dependent kinases CDK4 and CDK6. Mol. Cell. Biol. *15*, 2672–2681.
- Hockenbery, D.M., Oltvai, Z.N., Yin, X.M., Milliman, C.L., and Korsmeyer, S.J. (1993). Bcl-2 functions in an antioxidant pathway to prevent apoptosis. Cell *75*, 241–251.
- Holliday, D.L., and Speirs, V. (2011). Choosing the right cell line for breast cancer research. Breast Cancer Res. *13*, 1–7.
- Hollstein, M., Sidransky, D., Vogelstein, B., and Harris, C.C. (1991). p53 mutations in human cancers. Science *253*, 49–53.
- Honeth, G., Bendahl, P.O., Ringnér, M., Saal, L.H., Gruvberger-Saal, S.K., Lövgren, K., Grabau, D., Fernö, M., Borg, Å., and Hegardt, C. (2008). The CD44+/CD24-phenotype is enriched in basal-like breast tumors. Breast Cancer Res. *10*, 1–12.
- Horton, L.E., and Templeton, D.J. (1997). The cyclin box and C-terminus of cyclins A and E specify CDK activation and substrate specificity. Oncogene *14*, 491–498.
- Hotchkiss, K.A., Ashton, A.W., Mahmood, R., Russell, R.G., Sparano, J.A., and Schwartz, E.L. (2002). Inhibition of endothelial cell function in vitro and angiogenesis in vivo by docetaxel (Taxotere): association with impaired repositioning of the microtubule organizing center. Mol. Cancer Ther. *1*, 1191–1200.
- Hrynchak, I., Sousa, E., Pinto, M., and Costa, V.M. (2017). The importance of drug metabolites synthesis: the case-study of cardiotoxic anticancer drugs. Drug Metab. Rev. *49*, 158–196.
- Hurford, R.K., Cobrinik, D., Lee, M.H., and Dyson, N. (1997). pRB and p107/p130 are

required for the regulated expression of different sets of E2F responsive genes. Genes Dev. *11*, 1447–1463.

- Hwang, H.C., and Clurman, B.E. (2005). Cyclin E in normal and neoplastic cell cycles. Oncogene *24*, 2776–2786.
- Hwang, T.J., Carpenter, D., Lauffenburger, J.C., Wang, B., Franklin, J.M., and Kesselheim, A.S. (2016). Failure of investigational drugs in late-stage clinical development and publication of trial results. JAMA Intern. Med. *176*, 1826–1833.
- Ibrado, A.M., Huang, Y., Fang, G., and Bhalla, K. (1996). Bcl-xL overexpression inhibits taxol-induced Yama protease activity and apoptosis. Cell Growth Differ. *7*, 1087–1094.
- Ilgar, N.N., and Arıcan, G.O. (2009). Induction of apoptosis and cell proliferation inhibition by paclitaxel in FM3A cell cultures. African J. Biotechnol. *8*, 547–555.
- Inoue, K., Chikazawa, M., Fukata, S., Yoshikawa, C., and Shuin, T. (2003). Docetaxel enhances the therapeutic effect of the angiogenesis inhibitor TNP-470 (AGM-1470) in metastatic human transitional cell carcinoma. Clin. Cancer Res. *9*, 886–899.
- Jabbour, H.N., Kelly, R.W., Fraser, H.M., and Critchley, H.O.D. (2006). Endocrine regulation of menstruation. Endocr. Rev. *27*, 17–46.
- Jang, S.H., Kim, A.-R., Park, N.-H., Park, J.W., and Han, I.-S. (2016). DRG2 Regulates G2/M Progression via the Cyclin B1-Cdk1 Complex. Mol. Cells *39*, 699–704.
- Jitariu, A.-A., Cîmpean, A.M., Ribatti, D., and Raica, M. (2017). Triple negative breast cancer: the kiss of death. Oncotarget *8*, 46652–46662.
- Johnson, D.G., and Walker, C.L. (1999). CYCLINS AND CELL CYCLE CHECKPOINTS. Annu. Rev. Pharmacol. Toxicol. *39*, 295–312.
- Johnson, F., and Giulivi, C. (2005). Superoxide dismutases and their impact upon human

health. Mol. Aspects Med. *26*, 340–352.

- Johnson, K.C., Miller, A.B., Collishaw, N.E., Palmer, J.R., Hammond, S.K., Salmon, A.G., Cantor, K.P., Miller, M.D., Boyd, N.F., Millar, J., et al. (2011). Active smoking and secondhand smoke increase breast cancer risk: the report of the Canadian Expert Panel on Tobacco Smoke and Breast Cancer Risk (2009). Tob. Control *20*, e2–e2.
- Johnson, T.M., Yu, Z.X., Ferrans, V.J., Lowenstein, R.A., and Finkel, T. (1996). Reactive oxygen species are downstream mediators of p53-dependent apoptosis. Proc. Natl. Acad. Sci. U. S. A. *93*, 11848–11852.
- Jordan, M.A., and Wilson, L. (1998). Microtubules and actin filaments: dynamic targets for cancer chemotherapy. Curr. Opin. Cell Biol. *10*, 123–130.
- Jordan, M.A., Toso, R.J., Thrower, D., and Wilson, L. (1993). Mechanism of mitotic block and inhibition of cell proliferation by taxol at low concentrations. Proc. Natl. Acad. Sci. U. S. A. *90*, 9552–9556.
- Jordan, M.A., Wendell, K., Gardiner, S., Derry, W.B., Copp, H., and Wilson, L. (1996). Mitotic block induced in HeLa cells by low concentrations of paclitaxel (Taxol) results in abnormal mitotic exit and apoptotic cell death. Cancer Res. *56*, 816–825.
- Kachaeva, M. V, Hodyna, D.M., Semenyuta, I. V, Pilyo, S.G., Prokopenko, V.M., Kovalishyn, V. V, Metelytsia, L.O., and Brovarets, V.S. (2018). Design, synthesis and evaluation of novel sulfonamides as potential anticancer agents. Comput. Biol. Chem. *74*, 294–303.
- Kaiser, J. (2013). Cholesterol Forges Link Between Obesity and Breast Cancer. Science (80-.). *342*, 1028–1028.
- Kaldis, P. (1999). The cdk-activating kinase (CAK): from yeast to mammals. Cell. Mol.

Life Sci. C. *55*, 284–296.

- Kaldis, P., Russo, A.A., Chou, H.S., Pavletich, N.P., and Solomon, M.J. (1998). Human and Yeast Cdk-activating Kinases (CAKs) Display Distinct Substrate Specificities. Mol. Biol. Cell *9*, 2545–2560.
- Khan, K.H., Yap, T.A., Yan, L., and Cunningham, D. (2013). Targeting the PI3K-AKT-mTOR signaling network in cancer. Chin. J. Cancer *32*, 253–265.
- KHAN, M.A., CARDUCCI, M.A., and PARTIN, A.W. (2003). The Evolving Role of Docetaxel in the Management of Androgen Independent Prostate Cancer. J. Urol. *170*, 1709–1716.
- el Khyari, S., Bourgarel, V., Barra, Y., Braguer, D., and Briand, C. (1997). Pretreatment by tubulin agents decreases C-MYC induction in human colon carcinoma cell line HT29-D4. Biochem. Biophys. Res. Commun. *231*, 751–754.
- Kia, R., Fun, H.-K., and Kargar, H. (2009a). (*E*)- *N* ′-(4-Chlorobenzylidene)- *p* -toluenesulfonohydrazide 0.15-hydrate. Acta Crystallogr. Sect. E Struct. Reports Online.
- Kia, R., Fun, H.-K., and Kargar, H. (2009b). (*E*)- *N* ′-(4-Chlorobenzylidene)- *p* -toluenesulfonohydrazide 0.15-hydrate. Acta Crystallogr. Sect. E Struct. Reports Online *65*, o1119–o1120.
- Kim, C.H., Yoo, J.S., Lee, C.-T., Kim, Y.W., Han, S.K., Shim, Y.-S., and Yoo, C.-G. (2006a). FHIT protein enhances paclitaxel-induced apoptosis in lung cancer cells. Int. J. Cancer *118*, 1692–1698.
- Kim, M.J., Ro, J.Y., Ahn, S.H., Kim, H.H., Kim, S.B., and Gong, G. (2006b). Clinicopathologic significance of the basal-like subtype of breast cancer: a comparison with hormone receptor and Her2/neu-overexpressing phenotypes. Hum. Pathol. *37*,

1217–1226.

- Klauber, N., Parangi, S., Flynn, E., Hamel, E., and D'Amato, R.J. (1997). Inhibition of angiogenesis and breast cancer in mice by the microtubule inhibitors 2-methoxyestradiol and taxol. Cancer Res. *57*, 81–86.
- Klein, C., and Vassilev, L.T. (2004). Targeting the p53-MDM2 interaction to treat cancer. Br. J. Cancer *91*, 1415–1419.
- Klinge, C.M. (2001). Estrogen receptor interaction with estrogen response elements. Nucleic Acids Res. *29*, 2905–2919.
- Klos, K.S., Zhou, X., Lee, S., Zhang, L., Yang, W., Nagata, Y., and Yu, D. (2003). Combined trastuzumab and paclitaxel treatment better inhibits ErbB-2-mediated angiogenesis in breast carcinoma through a more effective inhibition of Akt than either treatment alone. Cancer *98*, 1377–1385.
- Kluck, R.M. (1997). The Release of Cytochrome c from Mitochondria: A Primary Site for Bcl-2 Regulation of Apoptosis. Science (80-.). *275*, 1132–1136.
- Ko, L.J., and Prives, C. (1996). p53: puzzle and paradigm. Genes Dev. *10*, 1054–1072.
- Konecny, G., Pauletti, G., Pegram, M., Untch, M., Dandekar, S., Aguilar, Z., Wilson, C., Rong, H.-M., Bauerfeind, I., Felber, M., et al. (2003). Quantitative Association Between HER-2/neu and Steroid Hormone Receptors in Hormone Receptor-Positive Primary Breast Cancer. JNCI J. Natl. Cancer Inst. *95*, 142–153.
- Kowaltowski, a J., Castilho, R.F., and Vercesi, a E. (2001). Mitochondrial permeability transition and oxidative stress. FEBS Lett *495*, 12–15.
- Kowaltowski, A.J., Vercesi, A.E., and Fiskum, G. (2000). Bcl-2 prevents mitochondrial permeability transition and cytochrome c release via maintenance of reduced pyridine

nucleotides. Cell Death Differ *7*, 903–910.

- Kramer, G., Schwarz, S., Hägg, M., Havelka, A.M., and Linder, S. (2006). Docetaxel induces apoptosis in hormone refractory prostate carcinomas during multiple treatment cycles. Br. J. Cancer *94*, 1592–1598.
- Kroemer, G., Galluzzi, L., and Brenner, C. (2007). Mitochondrial Membrane Permeabilization in Cell Death. Physiol. Reveiw 99–163.
- Kucukzeybek, Y., Gul, M.K., Cengiz, E., Erten, C., Karaca, B., Gorumlu, G., Atmaca, H., Uzunoglu, S., Karabulut, B., Sanli, U.A., et al. (2008). Enhancement of docetaxel-induced cytotoxicity and apoptosis by all-trans retinoic acid (ATRA) through downregulation of survivin (BIRC5), MCL-1 and LTbeta-R in hormone- and drug resistant prostate cancer cell line, DU-145. J. Exp. Clin. Cancer Res. *27*, 37.
- Lander, H.M., Hajjar, D.P., Hempstead, B.L., Mirza, U.A., Chait, B.T., Campbell, S., and Quilliam, L.A. (1997). A molecular redox switch on p21(ras). Structural basis for the nitric oxide-p21(ras) interaction. J. Biol. Chem. *272*, 4323–4326.
- Lee, L.F., Haskill, J.S., Mukaida, N., Matsushima, K., and Ting, J.P. (1997). Identification of tumor-specific paclitaxel (Taxol)-responsive regulatory elements in the interleukin-8 promoter. Mol. Cell. Biol. *17*, 5097–5105.
- Levine, A.J. (1997). p53, the cellular gatekeeper for growth and division. Cell *88*, 323–331.
- Li, W., Ma, H., Zhang, J., Zhu, L., Wang, C., and Yang, Y. (2017). Unraveling the roles of CD44/CD24 and ALDH1 as cancer stem cell markers in tumorigenesis and metastasis. Sci. Rep. *7*, 1–15.
- Liebmann, J.E., Cook, J.A., Lipschultz, C., Teague, D., Fisher, J., and Mitchell, J.B.

(1993). Cytotoxic studies of paclitaxel (Taxol) in human tumour cell lines. Br. J. Cancer *68*, 1104–1109.

- Liou, G.-Y., and Storz, P. (2010a). Reactive oxygen species in cancer. Free Radic. Res. *44*, 479–496.
- Liou, M.-Y., and Storz, P. (2010b). Reactive oxygen species in cancer.
- Liu, J., and Wang, Z. (2015). Increased Oxidative Stress as a Selective Anticancer Therapy. Oxid. Med. Cell. Longev. *2015*, 1–12.
- Liu, X., and Quan, N. (2015). Immune Cell Isolation from Mouse Femur Bone Marrow. Bio-Protocol *5*, e1631.
- Lizard, G., Fournel, S., Genestier, L., Dhedin, N., Chaput, C., Flacher, M., Mutin, M., Panaye, G., and Revillard, J.-P. (1995). Kinetics of plasma membrane and mitochondrial alterations in cells undergoing apoptosis. Cytometry *21*, 275–283.
- Lloyd, A.C. (1998). Ras versus cyclin-dependent kinase inhibitors. Curr. Opin. Genet. Dev. *8*, 43–48.
- Longo, D.L. (Dan L., and Chabner, B. (1996). Cancer chemotherapy and biotherapy : principles and practice (Philadelphia: Lippincott-Raven Publishers).
- Lowe, S.W., and Lin, A.W. (2000). Apoptosis in cancer. Carcinogenesis *21*, 485–495.
- Lugli, E., Troiano, L., Ferraresi, R., Roat, E., Prada, N., Nasi, M., Pinti, M., Cooper, E.L., and Cossarizza, A. (2005). Characterization of cells with different mitochondrial membrane potential during apoptosis. Cytom. Part A *68A*, 28–35.
- Lumachi, F. (2015). Current medical treatment of estrogen receptor-positive breast cancer. World J. Biol. Chem. *6*, 231.
- Luo, J., Manning, B.D., and Cantley, L.C. (2003). Targeting the PI3K-Akt pathway in

human cancer: rationale and promise. Cancer Cell *4*, 257–262.

- Mahaffey, C.M., Davies, A.M., Lara, P.N., Pryde, B., Holland, W., Mack, P.C., Gumerlock, P.H., and Gandara, D.R. (2007). Schedule-dependent apoptosis in K-ras mutant non-small-cell lung cancer cell lines treated with docetaxel and erlotinib: rationale for pharmacodynamic separation. Clin. Lung Cancer *8*, 548–553.
- Manuscript, A., and Magnitude, S. (2013). NIH Public Access. *31*, 1713–1723.
- Marín, A., Martín, M., Liñán, O., Alvarenga, F., López, M., Fernández, L., Büchser, D., and Cerezo, L. (2014). Bystander effects and radiotherapy. Reports Pract. Oncol. Radiother. *20*, 12–21.
- MARSHALL, E.K. (1964). HISTORICAL PERSPECTIVES IN CHEMOTHERAPY. Adv. Chemother. *13*, 1–8.
- Mauri, D., Kamposioras, K., Tsali, L., Bristianou, M., Valachis, A., Karathanasi, I., Georgiou, C., and Polyzos, N.P. (2010). Overall survival benefit for weekly vs. three-weekly taxanes regimens in advanced breast cancer: A meta-analysis. Cancer Treat. Rev. *36*, 69–74.
- McCawley, L.J., Li, S., Wattenberg, E. V, and Hudson, L.G. (1999). Sustained activation of the mitogen-activated protein kinase pathway. A mechanism underlying receptor tyrosine kinase specificity for matrix metalloproteinase-9 induction and cell migration. J. Biol. Chem. *274*, 4347–4353.
- McCubrey, J.A., Steelman, L.S., Abrams, S.L., Lee, J.T., Chang, F., Bertrand, F.E., Navolanic, P.M., Terrian, D.M., Franklin, R.A., D'Assoro, A.B., et al. (2006). Roles of the RAF/MEK/ERK and PI3K/PTEN/AKT pathways in malignant transformation and drug resistance. Adv. Enzyme Regul. *46*, 249–279.

- McDaid, H.M., and Horwitz, S.B. (2001). Selective potentiation of paclitaxel (taxol)-induced cell death by mitogen-activated protein kinase kinase inhibition in human cancer cell lines. Mol. Pharmacol. *60*, 290–301.
- McGowan, J. V, Chung, R., Maulik, A., Piotrowska, I., Walker, J.M., and Yellon, D.M. (2017). Anthracycline Chemotherapy and Cardiotoxicity. Cardiovasc. Drugs Ther. *31*, 63–75.
- Mediavilla-Varela, M., Pacheco, F.J., Almaguel, F., Perez, J., Sahakian, E., Daniels, T.R., Leoh, L.S., Padilla, A., Wall, N.R., Lilly, M.B., et al. (2009). Docetaxel-induced prostate cancer cell death involves concomitant activation of caspase and lysosomal pathways and is attenuated by LEDGF/p75. Mol. Cancer *8*, 68.
- Meng, Z., Mitsutake, N., Nakashima, M., Starenki, D., Matsuse, M., Takakura, S., Namba, H., Saenko, V., Umezawa, K., Ohtsuru, A., et al. (2008). Dehydroxymethylepoxyquinomicin, a Novel Nuclear Factor-κB Inhibitor, Enhances Antitumor Activity of Taxanes in Anaplastic Thyroid Cancer Cells. Endocrinology *149*, 5357–5365.
- Meyboom, R.H.B., Egberts, A.C.G., Gribnau, F.W.J., and Hekster, Y.A. (1999). Pharmacovigilance in Perspective. Drug Saf. *21*, 429–447.
- Miglietta, A., Bozzo, F., Bocca, C., Gabriel, L., Trombetta, A., Belotti, S., and Canuto, R.A. (2006). Conjugated linoleic acid induces apoptosis in MDA-MB-231 breast cancer cells through ERK/MAPK signalling and mitochondrial pathway. Cancer Lett. *234*, 149–157.
- Ming, M., and He, Y.-Y. (2012). PTEN in DNA damage repair. Cancer Lett. *319*, 125–129.

- Mittal, R., Jaiswal, P., and Goel, A. (2015). Survivin: A molecular biomarker in cancer. Indian J. Med. Res. *141*, 389.
- Molendini, L., Benassi, M.S., Magagnoli, G., Merli, M., Sollazzo, M.R., Ragazzini, P., Gamberi, G., Ferrari, C., Balladelli, A., Bacchini, P., et al. (1998). Prognostic significance of cyclin expression in human osteosarcoma. Int. J. Oncol. *12*, 1007–1011.
- Momand, J., Zambetti, G.P., Olson, D.C., George, D., and Levine, A.J. (1992). The mdm-2 oncogene product forms a complex with the p53 protein and inhibits p53-mediated transactivation. Cell *69*, 1237–1245.
- Moos, P.J., and Fitzpatrick, F.A. (1998a). Taxane-mediated gene induction is independent of microtubule stabilization: induction of transcription regulators and enzymes that modulate inflammation and apoptosis. Proc. Natl. Acad. Sci. U. S. A. *95*, 3896–3901.
- Moos, P.J., and Fitzpatrick, F.A. (1998b). Taxanes propagate apoptosis via two cell populations with distinctive cytological and molecular traits. Cell Growth Differ. *9*, 687–697.
- Morgan, D.O. (1997). CYCLIN-DEPENDENT KINASES: Engines, Clocks, and Microprocessors. Annu. Rev. Cell Dev. Biol. *13*, 261–291.
- Mothersill, C., and Seymour, C. (2001). Radiation-induced bystander effects: past history and future directions. Radiat. Res. *155*, 759–767.
- Müller-Tidow, C., Metzger, R., Kügler, K., Diederichs, S., Idos, G., Thomas, M., Dockhorn-Dworniczak, B., Schneider, P.M., Koeffler, H.P., Berdel, W.E., et al. (2001). Cyclin E is the only cyclin-dependent kinase 2-associated cyclin that predicts metastasis and survival in early stage non-small cell lung cancer. Cancer Res. *61*, 647–653.

- Muller, P.A.J., and Vousden, K.H. (2013). p53 mutations in cancer. Nat. Cell Biol. *15*, 2–8.
- Nabell, L., and Spencer, S. (2003). Docetaxel with concurrent radiotherapy in head and neck cancer. Semin. Oncol. *30*, 89–93.
- Naderi, S., Hunton, I.C., and Wang, J.Y.J. (2002). Radiation dose-dependent maintenance of G(2) arrest requires retinoblastoma protein. Cell Cycle *1*, 193–200.
- Najafi, M., Fardid, R., Hadadi, G., and Fardid, M. (2014). The mechanisms of radiation-induced bystander effect. J. Biomed. Phys. Eng. *4*, 163–172.
- Nakahara, C., Nakamura, K., Yamanaka, N., Baba, E., Wada, M., Matsunaga, H., Noshiro, H., Tanaka, M., Morisaki, T., and Katano, M. (2003). Cyclosporin-A enhances docetaxel-induced apoptosis through inhibition of nuclear factor-kappaB activation in human gastric carcinoma cells. Clin. Cancer Res. *9*, 5409–5416.
- Nakano, K., and Vousden, K.H. (2001). PUMA, a Novel Proapoptotic Gene, Is Induced by p53. Mol. Cell *7*, 683–694.
- Nathanson, L., Hall, T.C., Schilling, A., and Miller, S. (1969). Concurrent combination chemotherapy of human solid tumors: experience with a three-drug regimen and review of the literature. Cancer Res. *29*, 419–425.
- Naumova, E., Ubezio, P., Garofalo, A., Borsotti, P., Cassis, L., Riccardi, E., Scanziani, E., Eccles, S.A., Bani, M.R., and Giavazzi, R. (2006). The Vascular Targeting Property of Paclitaxel Is Enhanced by SU6668, a Receptor Tyrosine Kinase Inhibitor, Causing Apoptosis of Endothelial Cells and Inhibition of Angiogenesis. Clin. Cancer Res. *12*, 1839–1849.
- Nehmé, A., Varadarajan, P., Sellakumar, G., Gerhold, M., Niedner, H., Zhang, Q., Lin,

X., and Christen, R.D. (2001). Modulation of docetaxel-induced apoptosis and cell cycle arrest by all- trans retinoic acid in prostate cancer cells. Br. J. Cancer *84*, 1571–1576.

- Neve, R.M., Chin, K., Fridlyand, J., Yeh, J., Baehner, F.L., Fevr, T., Clark, L., Bayani, N., Coppe, J.-P., Tong, F., et al. (2006). A collection of breast cancer cell lines for the study of functionally distinct cancer subtypes. Cancer Cell *10*, 515–527.
- Niki, E. (2008). Lipid peroxidation products as oxidative stress biomarkers. BioFactors *34*, 171–180.
- Ning, S., and Knox, S.J. (1999). G 2 /M-Phase Arrest and Death by Apoptosis of HL60 Cells Irradiated with Exponentially Decreasing Low-Dose-Rate Gamma Radiation. Radiat. Res. *151*, 659.
- Nogales, E. (2001). Structural Insights into Microtubule Function. Annu. Rev. Biophys. Biomol. Struct. *30*, 397–420.
- Noguchi, S. (2006). Predictive factors for response to docetaxel in human breast cancers. Cancer Sci. *97*, 813–820.
- Nozoe, T., Korenaga, D., Kabashima, A., Ohga, T., Saeki, H., and Sugimachi, K. (2002). Significance of cyclin B1 expression as an independent prognostic indicator of patients with squamous cell carcinoma of the esophagus. Clin. Cancer Res. *8*, 817–822.
- Okano, J.-I., Nagahara, T., Matsumoto, K., and Murawaki, Y. (2007). The growth inhibition of liver cancer cells by paclitaxel and the involvement of extracellular signal-regulated kinase and apoptosis. Oncol. Rep. *17*, 1195–1200.
- Okolotowicz, K.J., Dwyer, M., Ryan, D., Cheng, J., Cashman, E.A., Moore, S., Mercola, M., and Cashman, J.R. (2018). Novel tertiary sulfonamides as potent anti-cancer agents. Bioorg. Med. Chem. *26*, 4441–4451.

- Ortega, S., Malumbres, M., and Barbacid, M. (2002). Cyclin D-dependent kinases, INK4 inhibitors and cancer. Biochim. Biophys. Acta *1602*, 73–87.
- Panieri, E., and Santoro, M.M. (2016). ROS homeostasis and metabolism: a dangerous liason in cancer cells. Cell Death Dis. *7*, e2253.
- Paquette, B., Therriault, H., Desmarais, G., Wagner, R., Royer, R., and Bujold, R. (2011). Radiation-enhancement of MDA-MB-231 breast cancer cell invasion prevented by a cyclooxygenase-2 inhibitor. Br. J. Cancer *105*, 534–541.
- Paquette, B., Therriault, H., and Wagner, J.R. (2013). Role of Interleukin-1β in Radiation-Enhancement of MDA-MB-231 Breast Cancer Cell Invasion. Radiat. Res. *180*, 292–298.
- Pavletich, N.P. (1999). Mechanisms of cyclin-dependent kinase regulation: structures of cdks, their cyclin activators, and cip and INK4 inhibitors. J. Mol. Biol. *287*, 821–828.
- Pawlik, T.M., and Keyomarsi, K. (2004). Role of cell cycle in mediating sensitivity to radiotherapy. Int. J. Radiat. Oncol. Biol. Phys. *59*, 928–942.
- Pelicano, H., Carney, D., and Huang, P. (2004). ROS stress in cancer cells and therapeutic implications. Drug Resist. Updat. *7*, 97–110.
- Perera, P.Y., Qureshi, N., and Vogel, S.N. (1996). Paclitaxel (Taxol)-induced NF-kappaB translocation in murine macrophages. Infect. Immun. *64*, 878–884.
- Pérez-Tenorio, G., Stål, O., and Southeast Sweden Breast Cancer Group (2002). Activation of AKT/PKB in breast cancer predicts a worse outcome among endocrine treated patients. Br. J. Cancer *86*, 540–545.
- Phillips, D.H. (2001). Understanding the genotoxicity of tamoxifen ? Carcinogenesis *22*, 839–849.

- Piccart-Gebhart, M.J., Burzykowski, T., Buyse, M., Sledge, G., Carmichael, J., Lück, H.-J., Mackey, J.R., Nabholtz, J.-M., Paridaens, R., Biganzoli, L., et al. (2008). Taxanes Alone or in Combination With Anthracyclines As First-Line Therapy of Patients With Metastatic Breast Cancer. J. Clin. Oncol. *26*, 1980–1986.
- Piccolo, M., and Crispi, S. (2012). The Dual Role Played by p21 May Influence the Apoptotic or Anti-Apoptotic Fate in Cancer. J. Can. Res. Updates 189–202.
- Pienta, K.J. (2001). Preclinical mechanisms of action of docetaxel and docetaxel combinations in prostate cancer. Semin. Oncol. *28*, 3–7.
- Pölcher, M., Rudlowski, C., Friedrichs, N., Mielich, M., Höller, T., Wolfgarten, M., Kübler, K., Büttner, R., Kuhn, W., and Braun, M. (2010). In vivo intratumor angiogenic treatment effects during taxane-based neoadjuvant chemotherapy of ovarian cancer. BMC Cancer *10*, 137.
- POTTER, V.R., and SIMONSON, H. (1951). Sequential blocking of metabolic pathways in vivo. Proc. Soc. Exp. Biol. Med. *76*, 41–46.
- Puhalla, S., Bhattacharya, S., and Davidson, N.E. (2012). Hormonal therapy in breast cancer: A model disease for the personalization of cancer care. Mol. Oncol. *6*, 222–236.
- Pusztai, L., Walters, R.S., Valero, V., Theriault, R.L., and Hortobagyi, G.N. (1998). Daily Oral Etoposide in Patients With Heavily Pretreated Metastatic Breast Cancer. Am. J. Clin. Oncol. *21*.
- Rao, S., He, L., Chakravarty, S., Ojima, I., Orr, G.A., and Horwitz, S.B. (1999). Characterization of the Taxol binding site on the microtubule. Identification of Arg(282) in beta-tubulin as the site of photoincorporation of a 7-benzophenone analogue of Taxol. J. Biol. Chem. *274*, 37990–37994.

- Reed, J., Jurgensmeier, J., and Matsuyama, S. (1998). Bcl-2 family proteins and mitochondria. Biochim. Biophys. Acta - Bioenerg. *1366*, 127–137.
- Remesh, A. (2012). Toxicities of anticancer drugs and its management. IJBCP Int. J. Basic Clin. Pharmacol. *1*, 2–12.
- Rescigno, M., Avogadri, F., and Curigliano, G. (2007). Challenges and prospects of immunotherapy as cancer treatment. Biochim. Biophys. Acta *1776*, 108–123.
- Reuter, S., Gupta, S.C., Chaturvedi, M.M., and Aggarwal, B.B. (2010). Oxidative stress, inflammation, and cancer: How are they linked? Free Radic. Biol. Med. *49*, 1603–1616.
- Del Rio, D., Stewart, A.J., and Pellegrini, N. (2005). A review of recent studies on malondialdehyde as toxic molecule and biological marker of oxidative stress. Nutr. Metab. Cardiovasc. Dis. *15*, 316–328.
- Roberts, A.W., and Huang, D. (2017). Targeting BCL2 With BH3 Mimetics: Basic Science and Clinical Application of Venetoclax in Chronic Lymphocytic Leukemia and Related B Cell Malignancies. Clin. Pharmacol. Ther. *101*, 89–98.
- Rodrigues, N.R., Rowan, A., Smith, M.E., Kerr, I.B., Bodmer, W.F., Gannon, J. V, and Lane, D.P. (1990). p53 mutations in colorectal cancer. Proc. Natl. Acad. Sci. U. S. A. *87*, 7555–7559.
- Rosenberg, B., VanCamp, L., Trosko, J.E., and Mansour, V.H. (1969). Platinum compounds: a new class of potent antitumour agents. Nature *222*, 385–386.
- ROSENBERG, B., VAN CAMP, L., and KRIGAS, T. (1965). Inhibition of Cell Division in Escherichia coli by Electrolysis Products from a Platinum Electrode. Nature *205*, 698–699.
- Roth, A.D., and Ajani, J. (2003). Docetaxel-based chemotherapy in the treatment of

gastric cancer. Ann. Oncol. Off. J. Eur. Soc. Med. Oncol. *14 Suppl 2*, ii41-4.

- Ruder, A.M., Lubin, F., Wax, Y., Geier, A., Alfundary, E., and Chetrit, A. (1989). Estrogen and progesterone receptors in breast cancer patients. Epidemiologic characteristics and survival differences. Cancer *64*, 196–202.
- Sartorelli, A.C. (1965). Approaches to the combination chemotherapy of transplantable neoplasms. Prog. Exp. Tumor Res. *6*, 228–288.
- Schiff, P.B., and Horwitz, S.B. (1980). Taxol stabilizes microtubules in mouse fibroblast cells. Proc. Natl. Acad. Sci. U. S. A. *77*, 1561–1565.
- SCHIFF, P.B., FANT, J., and HORWITZ, S.B. (1979). Promotion of microtubule assembly in vitro by taxol. Nature *277*, 665–667.
- Schraml, P., Bucher, C., Bissig, H., Nocito, A., Haas, P., Wilber, K., Seelig, S., Kononen, J., Mihatsch, M.J., Dirnhofer, S., et al. (2003). Cyclin E overexpression and amplification in human tumours. J. Pathol. *200*, 375–382.
- Schuler, M., and Green, D.R. (2001). Mechanisms of p53-dependent apoptosis. Biochem. Soc. Trans. *29*, 684–688.
- Schumacker, P.T. (2006a). Reactive oxygen species in cancer cells: Live by the sword, die by the sword. Cancer Cell *10*, 175–176.
- Schumacker, P.T. (2006b). Reactive oxygen species in cancer cells: Live by the sword, die by the sword. Cancer Cell *10*, 175–176.
- Scovell, W.M., and O'Connor, T. (1977). Interaction of aquated cis-[NH3)2Ptii]with nucleic acid constituents. 1. Ribonucleosides. J. Am. Chem. Soc. *99*, 120–126.
- Sekine, I., Shimizu, C., Nishio, K., Saijo, N., and Tamura, T. (2009). A literature review of molecular markers predictive of clinical response to cytotoxic chemotherapy in

patients with breast cancer. Int. J. Clin. Oncol. *14*, 112–119.

- Shah, M.A., and Schwartz, G.K. (2001). Cell cycle-mediated drug resistance: an emerging concept in cancer therapy. Clin. Cancer Res. *7*, 2168–2181.
- Sherr, C.J., and Roberts, J.M. (1995). Inhibitors of mammalian G1 cyclin-dependent kinases. Genes Dev. *9*, 1149–1163.
- Siegel, R.L., Miller, K.D., and Jemal, A. (2018a). Cancer statistics, 2018. CA. Cancer J. Clin. *68*, 7–30.
- Siegel, R.L., Miller, K.D., and Jemal, A. (2018b). Cancer statistics, 2018. CA. Cancer J. Clin. *68*, 7–30.
- Smith, M.L., Ford, J.M., Hollander, M.C., Bortnick, R.A., Amundson, S.A., Seo, Y.R., Deng, C.X., Hanawalt, P.C., and Fornace, A.J. (2000). p53-mediated DNA repair responses to UV radiation: studies of mouse cells lacking p53, p21, and/or gadd45 genes. Mol. Cell. Biol. *20*, 3705–3714.
- SMITH, N.D., RUBENSTEIN, J.N., EGGENER, S.E., and KOZLOWSKI, J.M. (2003). The p53 Tumor Suppressor Gene and Nuclear Protein: Basic Science Review and Relevance in the Management of Bladder Cancer. J. Urol. *169*, 1219–1228.
- Snyder, J.P., Nettles, J.H., Cornett, B., Downing, K.H., and Nogales, E. (2001). The binding conformation of Taxol in -tubulin: A model based on electron crystallographic density. Proc. Natl. Acad. Sci. *98*, 5312–5316.
- Solomon, L.A., Ali, S., Banerjee, S., Munkarah, A.R., Morris, R.T., and Sarkar, F.H. (2008). Sensitization of ovarian cancer cells to cisplatin by genistein: the role of NF-kappaB. J. Ovarian Res. *1*, 9.
- Soria, J.C., Jang, S.J., Khuri, F.R., Hassan, K., Liu, D., Hong, W.K., and Mao, L. (2000).

Overexpression of cyclin B1 in early-stage non-small cell lung cancer and its clinical implication. Cancer Res. *60*, 4000–4004.

- Sosa, V., Moliné, T., Somoza, R., Paciucci, R., Kondoh, H., and LLeonart, M.E. (2013). Oxidative stress and cancer: An overview. Ageing Res. Rev. *12*, 376–390.
- Steel, G.G. (1968). CELL LOSS FROM EXPERIMENTAL TUMOURS. Cell Prolif. *1*, 193–207.
- Strasser-Wozak, E.M., Hartmann, B.L., Geley, S., Sgonc, R., Böck, G., AJ Santos, Hattmannstorfer, R., Wolf, H., Pavelka, M., and Kofler, R. (1998). Irradiation induces G2/M cell cycle arrest and apoptosis in p53-deficient lymphoblastic leukemia cells without affecting Bcl-2 and Bax expression. Cell Death Differ. *5*, 687–693.
- Sun, M.L., Wei, J.M., Wang, X.W., Li, L., Wang, P., Li, M., and Yi, C.H. (2007). Paclitaxel-octreotide conjugates inhibit growth of human non-small cell lung cancer cells in vitro. Exp. Oncol. *29*, 186–191.
- Suzuki, T., Urano, T., Miki, Y., Moriya, T., Akahira, J., Ishida, T., Horie, K., Inoue, S., and Sasano, H. (2007). Nuclear cyclin B1 in human breast carcinoma as a potent prognostic factor. Cancer Sci. *98*, 644–651.
- Tait, S.W.G., and Green, D.R. (2010). Mitochondria and cell death: outer membrane permeabilization and beyond. Nat. Rev. Mol. Cell Biol. *11*, 621–632.
- Tan, J., Zhuang, L., Leong, H.-S., Iyer, N.G., Liu, E.T., and Yu, Q. (2005). Pharmacologic Modulation of Glycogen Synthase Kinase-3β Promotes p53-Dependent Apoptosis through a Direct Bax-Mediated Mitochondrial Pathway in Colorectal Cancer Cells. Cancer Res. *65*, 9012–9020.
- Tannock, I.F. (1983). Biology and tumor growth. Hosp. Pract. (Off. Ed). *18*, 81–93.

- Tannock, I.F., de Wit, R., Berry, W.R., Horti, J., Pluzanska, A., Chi, K.N., Oudard, S., Théodore, C., James, N.D., Turesson, I., et al. (2004). Docetaxel plus Prednisone or Mitoxantrone plus Prednisone for Advanced Prostate Cancer. N. Engl. J. Med. *351*, 1502–1512.
- Teven, C.M., Schmid, D.B., Sisco, M., Ward, J., and Howard, M.A. (2017). Systemic Therapy for Early-Stage Breast Cancer: What the Plastic Surgeon Should Know. Eplasty *17*, 71–86.
- Thompson, E.W., and Newgreen, D.F. (2005). Carcinoma Invasion and Metastasis: A Role for Epithelial-Mesenchymal Transition? Cancer Res. *65*, 5991.1-5995.
- Torre, L. a, Bray, F., Siegel, R.L., Ferlay, J., Lortet-tieulent, J., and Jemal, A. (2015). Global Cancer Statistics, 2012. *00*, 1–22.
- Torres, K., and Horwitz, S.B. (1998). Mechanisms of Taxol-induced cell death are concentration dependent. Cancer Res. *58*, 3620–3626.
- Trachootham, D., Lu, W., Ogasawara, M.A., Valle, N.R., and Huang, P. (2007). Mechanisms of Cell Death in Oxidative Stress. Antioxid. Redox Signal. *9*, 49–89.
- Trachootham, D., Lu, W., Ogasawara, M.A., Valle, N.R., and Huang, P. (2008). Redox Regulation of Cell Survival. Antioxid. Redox Signal. *10*, 1343–1374.
- Trachootham, D., Alexandre, J., and Huang, P. (2009a). Targeting cancer cells by ROS-mediated mechanisms: A radical therapeutic approach? Nat. Rev. Drug Discov. *8*, 579–591.
- Trachootham, D., Alexandre, J., and Huang, P. (2009b). Targeting cancer cells by ROS-mediated mechanisms: a radical therapeutic approach? Nat. Rev. Drug Discov. *8*, 579–591.

- Vanhoefer, U., Cao, S., Harstrick, A., Seeber, S., and Rustum, Y.M. (1997). Comparative antitumor efficacy of docetaxel and paclitaxel in nude mice bearing human tumor xenografts that overexpress the multidrug resistance protein (MRP). Ann. Oncol. Off. J. Eur. Soc. Med. Oncol. *8*, 1221–1228.
- Wang, J., and Yi, J. (2008a). Cancer cell killing via ROS: To increase or decrease, that is a question. Cancer Biol. Ther. *7*, 1875–1884.
- Wang, J., and Yi, J. (2008b). Cancer cell killing via ROS: To increase or decrease, that is the question. Cancer Biol. Ther. *7*, 1875–1884.
- Wang, Q., and Wieder, R. (2004). All-trans retinoic acid potentiates Taxotere-induced cell death mediated by Jun N-terminal kinase in breast cancer cells. Oncogene *23*, 426–433.
- Wani, M.C., Taylor, H.L., Wall, M.E., Coggon, P., and McPhail, A.T. (1971). Plant antitumor agents. VI. Isolation and structure of taxol, a novel antileukemic and antitumor agent from Taxus brevifolia. J. Am. Chem. Soc. *93*, 2325–2327.
- Wołowiec, D., Mekki, Y., Ffrench, P., Manel, A.M., Bertrand, Y., Rimokh, R., Philippe, N., Bryon, P.A., and Ffrench, M. (1996). Differential expression of cell proliferation regulatory proteins in B- and T-lineage acute lymphoblastic leukaemias. Br. J. Haematol. *95*, 518–523.
- Yam, C.H., Fung, T.K., and Poon, R.Y.C. (2002). Cyclin A in cell cycle control and cancer. Cell. Mol. Life Sci. *59*, 1317–1326.
- Yang, J., Liu, X., Bhalla, K., Kim, C.N., Ibrado, A.M., Cai, J., Peng, T.I., Jones, D.P., and Wang, X. (1997). Prevention of apoptosis by Bcl-2: Release of cytochrome c from mitochondria blocked. Science (80-.). *275*, 1129–1132.

- Yim, E.-K., Bae, J.-S., Lee, S.-B., Lee, K.-H., Kim, C.-J., Namkoong, S.-E., Um, S.-J., and Park, J.-S. (2004). Proteome analysis of differential protein expression in cervical cancer cells after paclitaxel treatment. Cancer Res. Treat. *36*, 395–399.
- Yoshino, T., Shiina, H., Urakami, S., Kikuno, N., Yoneda, T., Shigeno, K., and Igawa, M. (2006). Bcl-2 Expression as a Predictive Marker of Hormone-Refractory Prostate Cancer Treated with Taxane-Based Chemotherapy. Clin. Cancer Res. *12*, 6116–6124.
- Yvon, A.-M.C., Wadsworth, P., and Jordan, M.A. (1999). Taxol Suppresses Dynamics of Individual Microtubules in Living Human Tumor Cells. Mol. Biol. Cell *10*, 947–959.
- Zamzami, N. (1996). Mitochondrial control of nuclear apoptosis. J. Exp. Med. *183*, 1533–1544.
- Zhang, Z.-L., Liu, Z.-S., and Sun, Q. (2005). [Anti-tumor effect of thalidomide and paclitaxel on hepatocellular carcinoma in nude mice]. Chin. Med. J. (Engl). *118*, 1688–1694.
- Zhou, J., and Giannakakou, P. (2005). Targeting microtubules for cancer chemotherapy. Curr. Med. Chem. Anticancer. Agents *5*, 65–71.
- Zhou, X., Xu, L., Zhu, W., Zhang, T., Li, X., Jin, A., and He, K. (1999). Effects of Taxol on three different types of lymphoma cell lines. Chinese J. Cancer Res. *11*, 105–110.
- Zwelling, L.A., and Kohn, K.W. Mechanism of action of cis-dichlorodiammineplatinum(II). Cancer Treat. Rep. *63*, 1439–1444.

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Induction of G_1 Phase Cell Cycle Arrest and Apoptosis in Breast Cancer MCF-7 Cells by Sulphonamide Derivative CID-6861424

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Abstract

Breast cancer is most frequently detected and leading cause of cancer death in women worldwide. 80% of the breast cancer are estrogen receptor positive and the presently available drugs are ineffective either due to intrinsic resistance or due to acquired resistance. Sulfonamide compounds are a class of compounds showing activities like, antibacterial, antiviral including antitumor. In the present study we have investigated the anti-cancerous activity of sulfonamide derivative CID-6861424 on breast cancer cell line, MCF-7. Our data shows inhibition of MCF-7 cell viability by CID-6861424 in a concentration and time dependent manner. 50μM was the IC_{50} value of CID-6861424 on MCF-7 cell. The compound downregulated cyclin D1 and CDK 4/6, induced G_1 phase cell cycle arrest and apoptotic cell death. 50μM CID-6861424 upregulated ROS generation, disrupted mitochondrial membrane potential ($\Delta\psi m$), increased DNA damage and upregulated tumor suppressor protein, p53 in MCF-7 cells. The compound reduced phosphorylation of Akt and GSK-3β, downregulated Bcl-2 and upregulated Bax indicating apoptotic cell death. These results suggest CID-6861424 as a potential anticancer agent against breast cancer.

Keywords: *CID-6861424; MCF-7 cell, cell cycle, double strand DNA break, apoptosis.*

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INTRODUCTION

Breast cancer is one of the most common cancer affecting women all over the world and accounted for approximately 15% of all cancer deaths among women in 2018 [1, 2]. It is an economic burden for both developed as well as developing country. Approximately 80% of the breast cancers are estrogen receptor positive (ER +ve) [3]. ER signal transduction depends on various aspects including factors, co-factors, localization and cell type. Endocrine therapy, targeting the ER is the first choice of treatment, which includes, drugs like tamoxifen. The effectiveness of the drugs is reduced due to either intrinsic resistance or acquired resistance developed due to prolonged usage [4]. The endocrine resistance remains a big challenge and there is continuous search for better drugs for breast cancer therapy.

Sulfonamide compounds are a class of compounds that have functional sulfonamide group. They have shown various biological functional activities including antibacterial, anti-carbonic anhydrase, diuretic, hypoglycemia, anti-thyroid, anti-protease as well as antitumor activities [5]. These compounds have been modified, reformulated and synthesized with improved bioavailability and effectiveness and low toxicity. Navitoclax, a sulfonamide compound inhibits Bcl-2, Bcl-Xl and Bcl-w [6]. Another compound, Venetoclax is BH-3 mimetic and inhibits Bcl-2 resulting in apoptotic cell death in chronic lymphocytic leukemia [7, 8].

Large number of sulfonamide compounds are available in the Pubchem but their anti-cancerous activity has not been investigated yet. CID-6861424 is one such derivative of sulfonamide, which is known to exert antimicrobial and anti-inflammatory activities [9] but nothing is known about its anti-cancer activity. We checked the anti-cancerous

activity of the compound on breast cancer MCF-7 cell line. In the present study, we have shown that CID-6861424 inhibited MCF-7 cell proliferation in a dose and time dependent manner. IC_{50} value of CID-6861424 on MCF-7 cell was 50μM. It induced G_1 phase cell cycle arrest. We observed downregulation of Cyclin D1 and CDK4/6 in a time dependent manner in presence of 50μM CID-6861424. The compound inhibited phosphorylation of Akt and GSK-3β. The treatment also showed increase in ROS generation and disruption in **Δψm.** Exposure to 50μM CID-6861424 resulted in DNA damage shown by upregulation of γ-H2AX and p53 expression level. It upregulated pro-apoptotic protein Bax and downregulated Bcl-2 indicating apoptotic cell death. Our results suggest that CID-6861424 can be used as an anticancer agent against breast cancer.

MATERIAL AND METHODS

Cell Line and Reagents

MCF-7 cell line was obtained from American Type Culture Collection (Manassas, VA) and cultured in DMEM medium (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Invitrogen) and 5% penicillin-streptomycin (Invitrogen) at 5% CO_2 humidified chamber. Antibodies used from Cell Signaling and Santa Cruz: cyclin D1 (#2922), p-Akt (#4058), Akt (#9272), p-GSK-3β (#9323), GSK-3β (#9832), Bax (#5023), CDK4 (sc-260), CDK6 (sc-177), p53 (sc-126), Bcl-2 (sc-7382), γ-H2AX (sc-517348), β-actin (sc-47778), secondary anti-rabbit & anti-mouse antibody (#7074S & #7076S). FITC labelled secondary antibody was obtained from (Invitrogen). Rhodamcine123 (546054) and 2`,7`-Dichlorofluorescein diacetate (DCF-DA) (D6883), Propidium iodide (P4170), Trypan blue dye (C.I. 23850) were obtained from Sigma Aldrich. Triple distilled water prepared by "Millipore" was used in making the reagents. Other routine chemicals were obtained in their commercially available highest purity grade.

Synthesis of CID-6861424

The CID-6861424 was synthesized by the reaction between of p-Tosylhydrazine and 4-chlorobenzaldehyde following the Reza Kia *et al*. protocol [9].

Cell Viability Assay

Cells were plated in 2ml DMEM medium in 35mm^2 culture dishes in the presence of indicated concentrations of CID-6861424 for 24hr, 48hr and 72hr at 37°C. At the end of the stipulated time, cells were harvested, washed twice with 1x PBS and collected in separate tubes. For viability assay, 100μl volume of the homogeneous cell suspension was transferred to micro-centrifuge tube and 10μl of 0.5% trypan blue dye was added. The suspension was mixed well and left for 5-10 minutes at room temperature. 12μl of stained cell suspension was loaded on each chamber of the hemocytometer. Unstained cells were counted as live and blue stained cells as dead cell in an inverted microscope and the procedure was repeated for each set of all samples. Each sample was counted in triplicate and total cell number of live & dead cells was determined.

Percentage of Viable Cells: **No. of Viable Cells (unstained)/Total No. of the Cells X 100**.

Cell Morphology Study

Cells were plated in 35 mm^2 culture dish and cells and treated with indicated concentration of CID-6861424 for 72 hr. Bright field microscopy was used for studying the morphology in response to the drug treatment. All images were captured at 20X magnification using bright field microscope (NIKON ECLIPSE Ti-S, Tokyo, Japan) and analysed (NIS Elements D, Tokyo, Japan).

Wound-Healing Assay

Cells were plated in a 35mm^2 culture dish and grown up to 80% confluency. After creating wound by scratching the monolayer with a 200μl sterile tip cells were exposed to 25μM and 50μM CID-6861424 for 72 hr. Wound widths at pre and post 72 hour treatment were measured using bright field microscope (NIKON ECLIPSE Ti-S, Tokyo, Japan) and analysed (NIS Elements D, Tokyo, Japan).

Clonogenic Survival Assay

Cells were seeded in a 35 mm^2 culture dishes and treated with 25μM & 50μM CID-6861424 for 72hr, harvested and 600 cells were seeded in 60mm^2 culture dishes in 5ml of medium. The plates were incubated at 5% CO_2, 37°C

under a humidified chamber for 14 days. Cells were fixed in chilled methanol and stained with 5% crystal violet and colonies were counted.

Cell Cycle Analysis

Cells (4×10^4/ml) were seeded in 35mm^2 culture dish and exposed to 50µM CID-6861424 for 24hr, 48hr and 72hr. Cells were harvested at stipulated time, washed with chilled 1XPBS, fixed in to chilled 70% ethanol, stained with propidium iodide. The cellular DNA content of 10,000 events was measured using flow cytometer (BD Bioscience) and analysed by using modFit LT (verity software house) software.

Reactive Oxygen Species (ROS) Generation Assay

ROS generated in control and CID-6861424 treated cells was measured by flow cytometry following staining with DCFH-DA. Briefly, cells were seeded (4×10^4/ml) in 35 mm^2 culture dishes and after overnight incubation, cells were exposed to 25 µM and 50 µM CID-6861424 for 72 hr. Cells were then stained with 8 µM DCFH-DA dye for 30 min at 37°C, and washed twice with 1x PBS and cells pellets were dissolved in 300 µl 1x PBS. Fluorescence was measured by flow cytometry (BD Bioscience).

Mitochondrial Membrane Potential (Δψm) Assay

Δψm depletion in control and CID-6861424 treated cells was measured by flow cytometry following staining with Rhodamine 123 dye. Briefly, cells were seeded (4×10^4/ml) in 35mm^2 culture dishes and after overnight incubation, cells were exposed to 25µM and 50µM CID-6861424 for 72hr. Cells were then stained with 10 µM Rhodamine 123 dye for 30 min at 37°C, washed twice with 1X PBS and cell pellets were dissolved in 300µl 1x PBS. Fluorescence was measured by flow cytometry (BD Bioscience).

Apoptosis Assay

Apoptosis was quantified in CID-6861424 treated MCF-7 cell with FITC annexin V/7AAD kit from BD Biosciences. 4×10^4cells/ml cells were seeded in 35mm^2 culture dishes and incubated overnight. Cells were exposed to 50µM and 100µM CID-6861424 for 72hr. Cells were collected and processed as per the protocol given by manufacturer and apoptotic cells were determined by flow cytometry (BD Biosciences).

Immunofluorescence Assay

Approximately 8,000 cells were seeded in 35mm^2 culture dish over the cover slip and treated with 50µM CID-6861424 for 72hr. Post 72hr incubation, cells were processed and subjected to γ-H2AX and β-tubulin primary antibody (1:50, overnight, 4°C) and FITC labelled secondary antibody (1:10,000, room temperature, 1 hour). DAPI (1:1000) was used to counterstain the nucleus. Coverslips were mounted in antifade (Sigma) over the slides. Slides were observed under 60x under fluorescence microscope (NIKON ECLIPSE Ti, Tokyo, Japan). Fluorescence intensities from images of randomly selected microscopic fields of cells were semi-quantitatively analyzed by NIS Elements AR version-3.000 software. For each set of data, more than 50 cells were quantified.

Western Blot

The expression level of indicated proteins in response to 50µM CID-6861424 was analyzed by Western blot. Briefly, 80,000 cells/ml were seeded in 100mm^2 culture dishes and treated with 50µM CID-6861424 for 24hr, 48hr and 72hr. Cells were harvested and lysed in lysis buffer (50mM TrisCl-pH 8, 300mM NaCl, 10% glycerol, 1% NP-40, and 2 mM EDTA) at 4°C. Protein extracts were quantified by Bradford assay and analyzed on SDS-PAGE. The proteins were transferred to PVDF membrane and probed with specific primary and secondary antibodies to detect the protein of interest by manual ECL method. The images were captured in X-ray films and all results were analyzed with respect to loading control (β-Actin).

Statistical Analysis

Statistical analysis were carried out using Graph Pad Prism software. Experimental data are expressed as means ± S.D. and the significance of differences was analyzed by

one-way ANOVA test. Tukey test for cell viability result with different concentrations of CID-6861424 whereas Dunnett test and t-test for different experiments of CID-6861424 treated MCF-7 cells. Difference with a value of $P< 0.05$ were considered to be statistically significant.

RESULTS

CID-6861424 Inhibit Cell Proliferation and Affects Cell Morphology of MCF-7 Cell

Effect of CID-6861424 (Figure 1a) on cell viability was evaluated on breast cancer cell line, MCF-7 using Trypan blue exclusion assay. Cells were exposed to indicated concentrations of CID-6861424 for 24hr, 48hr and 72hr (Figure 1b). The result suggested that cell viability decreased with the increase in concentration and time. At 72hr, CID-6861424 showed IC_{50} value at 50μM. Cell morphology in presence of indicated concentration of the compound was examined under bright field microscope. At 72hr, the cell count decreased drastically with increasing concentrations as compared to the control set (Figure 1c). Cellular morphology was distorted with elongated, flattened cells, dense cytoplasm and irregular cell membrane with small protrusions (Figure 1c). Further, changes in the cytoskeletal structure were observed in the presence of 50 μM CID-6861424 when stained with β-tubulin (Figure 1d).

CID-6861424 Affects Cell Migration and Reduces Colony Forming Ability of MCF-7 Cell

Cytoskeletal arrangement affects cell migration. We investigated the effect of CID-6861424 on cell migration by performing wound-healing assay. Cells were grown to 80% confluency, wound was introduced, and wound widths was measured pre and post 72 hour of 25μM and 50μM CID-6861424 treatment. The untreated cells migrated and rapidly filled ~80% of wound, while ~60% of wound closure was observed in cells treated with 25μM CID-6861424. 50μM CID-6861424 treatment was more effective resulting in only ~40% of wound closure (Figure 2a and b).

The effect of CID-6861424 on colony forming ability was checked by colony forming assay. MCF-7 cells were treated with the compound for 72hr and the treated cells were plated at a concentration of 600 cells/60 mm^2 cell culture dish and incubated for 14 days in absence of the compound. Post incubation, the plates were processed and colonies were counted. The result showed that CID-6861424 significantly reduced the ability of colony formation of MCF-7 cells and it is dose dependent. Reduction in colony formation was more prominent in 50μM as compared to the 25μM CID-6861424 treated set (Figure 2c).

CID-6861424 Induced Cell Cycle Arrest at G_1 Phase in MCF-7 Cell

The effect of CID-6861424 on cell cycle profile was checked by exposing MCF-7 cells to 50μM of the compound for 24, 48 and 72hr. Cell cycle data indicated that 50 μM CID-6861424 resulted in G_1 phase arrest post 24hr treatment. Approximately 78% of the cells were in the G1 phase in the treated sample as compared to only 46% in the untreated sample, showing almost 32% increase in accumulation of cell in the G_1 phase after CID-6861424 treatment. The result showed increase in sub-G_1 population with the increase in treatment time, from 3% at 24 hour to almost 10% at 72 hour (Figure 3a).

Cyclin and cyclin-dependent kinases (cdk) are one of the important player in cell cycle regulation. Cyclin D1 and CDK4/6 is responsible for progression in G_1 phase of the cell cycle. Since CID-6861424 caused G_1 phase cell cycle arrest, we investigated the effect of the compound on cyclin D1 and CDK4/6 in MCF-7 cells. 50μM CID-6861424 exposure resulted in significant downregulation of cyclin D1. The expression decreased with the increase in time. CDK4/6 expression also decreased with increase in time (Figure 3b). These results suggested that CID-6861424 induced G_1 phase cell cycle arrest in MCF-7 by downregulating cyclin D1 and CDK4/6 expression.

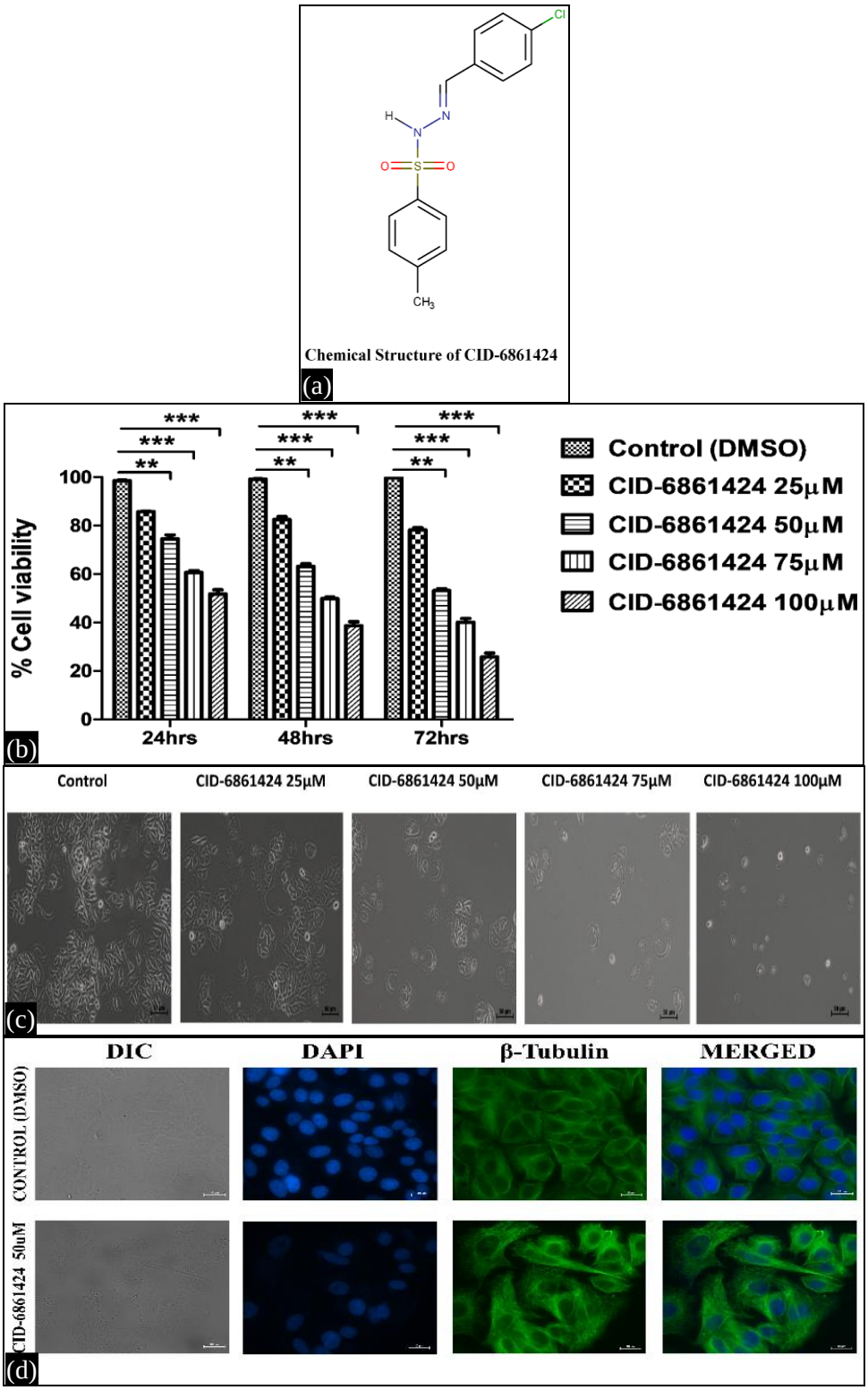


Fig. 1. *CID-6861424 Inhibit Cell Proliferation and Affects cell Morphology of MCF-7 cell. (a). Chemical structure of* ***CID-6861424****. (b). Bar diagram showing effect of* ***CID-6861424*** *on MCF-7 cell viability at indicated concentrations and time points by Trypan Blue assay. (c) Changes in cell morphology, using a bright field microscope at 20x, was observed post 72hr treatment with indicated concentrations of* ***CID-6861424****. (d) Using florescence microscope at 60X, β-tubulin staining post 72hr treatment with 50µM* ***CID-6861424,*** *showed changes in cell morphology. Data are represented as the mean ± SD of three independent experiments. **P<0.01, and ***P<0.001.*

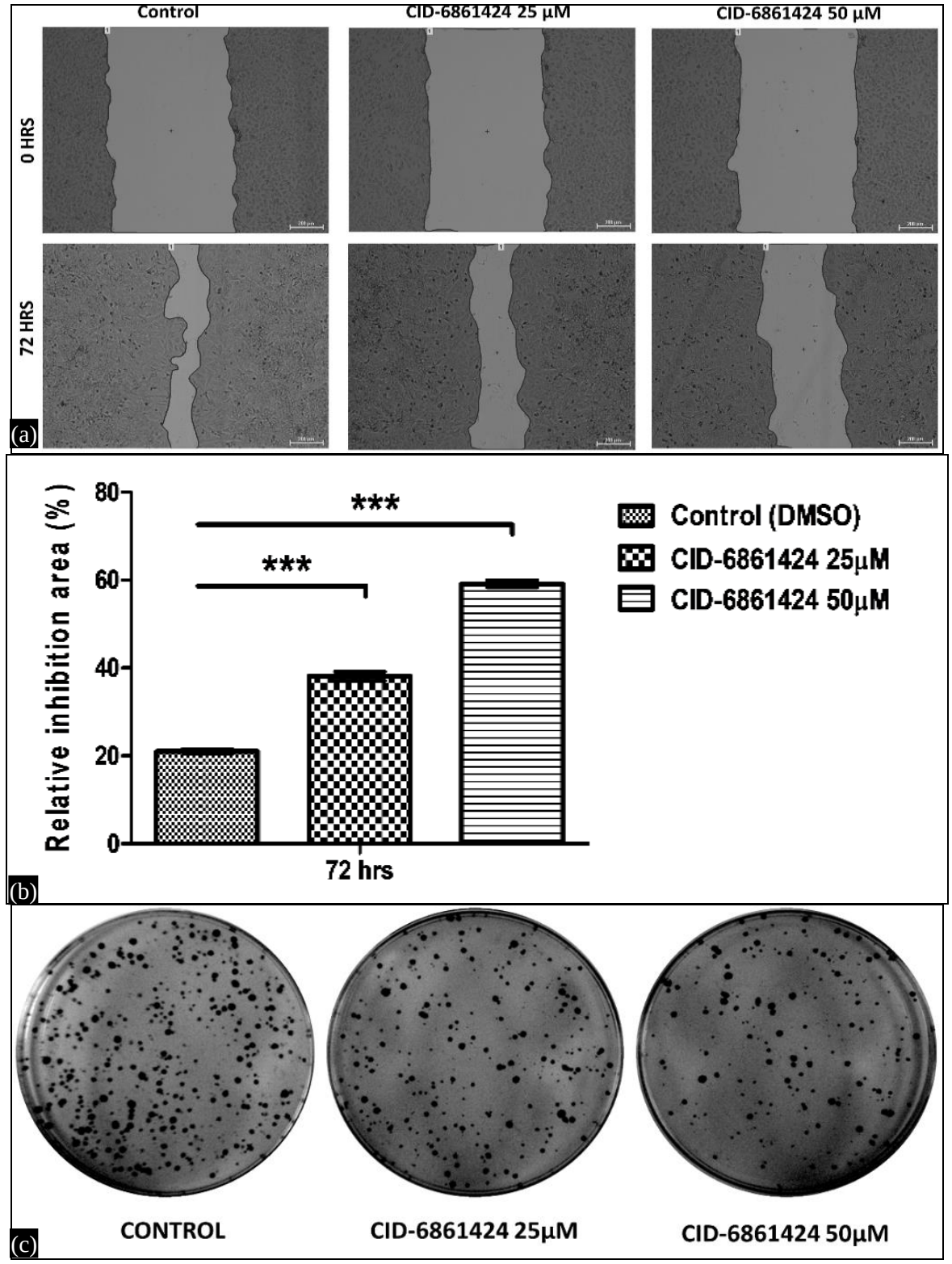


Fig. 2. *CID-6861424 Affects Cell Migration and Reduces Colony Forming Ability of MCF-7 Cell. (a). Post 72hr treatment with CID-6861424 cell migration potential was checked by comparing the wound closer before and after the treatment (b). Bar-diagram representation of relative inhibition of wound healing area of MCF-7 cell. (c). The reduced number of the MCF-7 cell colonies in the presence of CID-6861424 treatment. Data are represented as the mean ± SD of three independent experiments. **P<0.01, and ***P<0.001.*

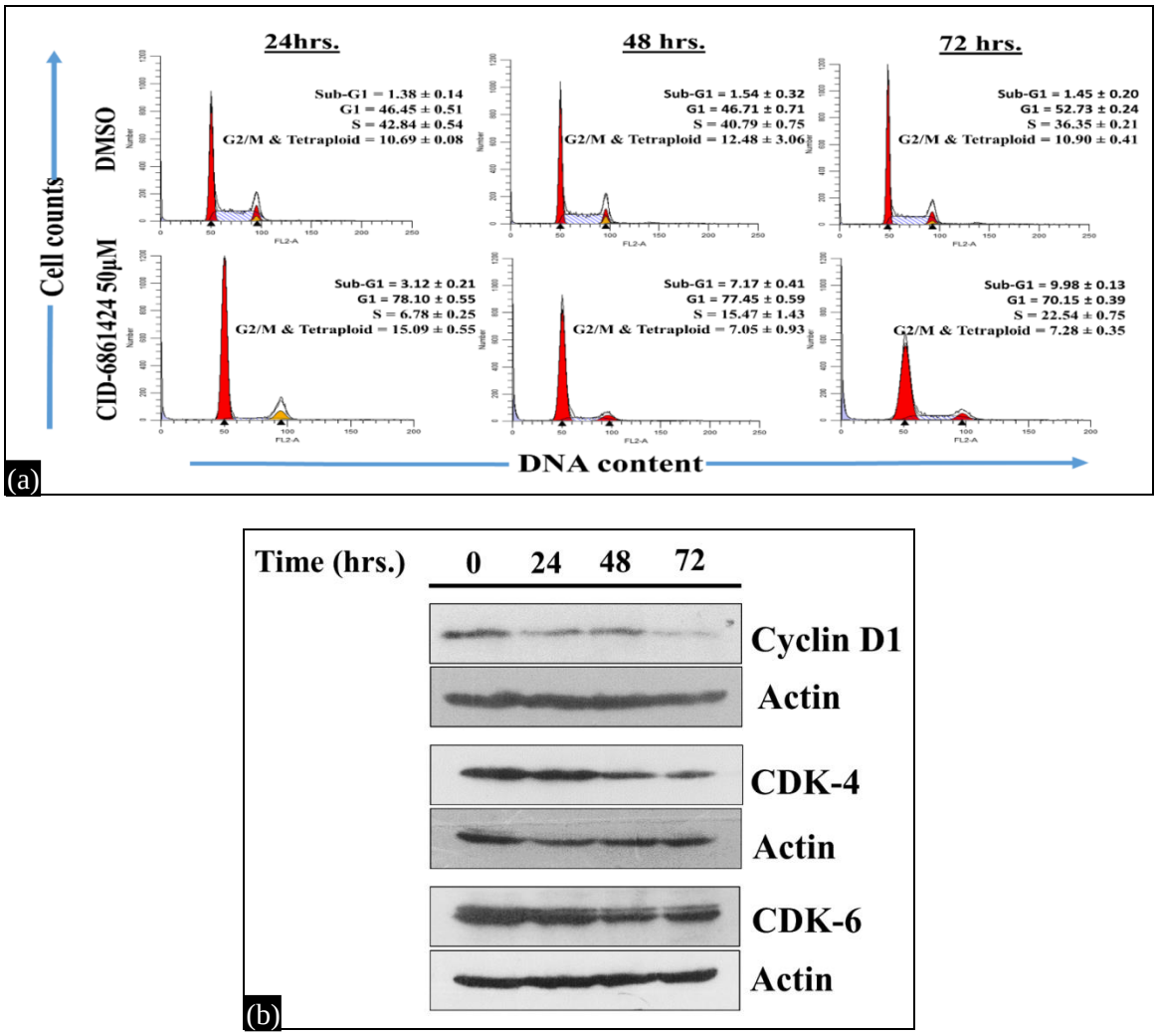


Fig 3. *CID-6861424 Induced Cell Cycle Arrest at G_1 Phase in MCF-7 Cell. (a) Cells were treated with 50 µM-CID-6861424 for 24hr, 48hr and 72hr and cell cycle analysis was done by flow cytometry. (b). The downregulation of cyclin D1 and CDK4/6 proteins expression in presence of 50 µM-CID-6861424 was observed by western blotting. Data are represented as the mean ± SD of three independent experiments.*

CID-6861424 Induced ROS Generation and Δψm Depletion in MCF-7 Cell

Cancer cells have higher ROS levels than normal cells and hence are susceptible to further increase in ROS levels. ROS-production was analysed by flow cytometry using DCFH-DA dye in MCF-7 cells post 72hr exposure to CID-6861424. Samples treated with CID-6861424 showed significant increase in ROS generation as compared to the control. Doxorubicin was used as positive control. ROS generation was approximately 1.3 and 2.0 times higher as compared to control in 25µM and 50µM treated samples respectively (Figure 4a and b). We further checked the Δψm with cationic fluorescent dye Rhodamine 123 using flow cytometry post 72hr of CID-6861424 treatment. Doxorubicin was used as positive control. Compared to control, 25µM CID-6861424 treatment resulted in approximately 1.5-fold disruption of Δψm whereas 50µM treatment resulted in around 2.2 fold Δψm disruption (Figure 4c and d). These results suggested that CID-6861424 significantly increased ROS generation and disruption of Δψm in MCF-7 cells.

CID-6861424 Induced DNA Damage in MCF-7 Cell

Increase in ROS levels causes DNA damage. CID-6861424 treatment resulted in elevated levels of ROS. We next investigated the effect

of the compound on DNA damage. 50μM CID-6861424 treatment showed significant increase in γ-H2AX immunofluorescence intensity as compared to the untreated cells. The increase was around 1.8-fold as shown in the bar diagram (Figure 5a and b). Further, western blot data supported Immunofluorescence data showing upregulation of γ-H2AX expression in 50μM treated sample as compared to the untreated sample (Figure 5c). The DNA damage increases with increase in treatment time. DNA damage results in upregulation of the tumor suppressor protein, p53. 50μM CID-6861424 treatment resulted in upregulation of p53 in a time dependent manner as shown by western blot data (Figure 5c).

CID-6861424 Induced Apoptotic Cell Death in MCF-7 Cells

CID-6861424 exposure resulted in increase in ROS levels and disruption of Δψm in MCF-7 cells. Cell cycle data showed that the cells treated with 50μM CID-6861424 caused increase in sub-G_1 population as compared to the control (Figure 3a) suggesting cell death. Mode of cell death was assessed by annexin V and 7AAD dye using flow cytometry post 72hr CID-6861424 treatment. Samples treated with 50μM and 100μM CID-6861424 showed significantly increased late apoptotic cells as compared to the control. 50μM treatment resulted in ~1.5-fold increase in late apoptotic cells whereas 100μM treatment resulted in almost 3.5-fold increase as compared to the control set (Figure 6a and b). CID-6861424 treatment disrupted Δψm in MCF-7 cells. Δψm disruption affects pro-apoptotic protein, Bax, and anti-apoptotic protein, Bcl-2, and causes apoptotic cell death. We checked the expression levels of Bax and Bcl-2. CID-6861424 treated cells demonstrated upregulation of Bax and downregulation of Bcl-2 with increase in time (Figure 6c) further confirmed the apoptotic cell death.

50μM CID-6861424 Inhibits Akt and GSK-3β Phosphorylation

Akt is a serine/threonine kinase responsible for cell survival, growth, proliferation and cell migration. CID-6861424 treatment resulted in inhibition of MCF-7 cell proliferation and migration and induction of apoptosis, we investigated it effect on Akt phosphorylation. 50μM CID-6861424 treatment resulted in downregulation of Akt phosphorylation. Reduction in phosphorylation of the downstream target GSK-3β was also observed. The downregulation in phosphorylation was more prominent post 72hr treatment (Figure 7).

DISCUSSION

Breast cancer is one of the leading causes of cancer death in women worldwide and accounted for approximately 15% of all cancer deaths among women in 2018 [1, 2]. Either due to intrinsic resistance or due to acquired resistance the presently available drugs are not effective enough. There is a constant search for better drugs for breast cancer treatment. Sulfonamide compounds are a class of compounds showing various biological activities including antibacterial, anti-carbonic anhydrase and anti-cancerous [5, 10]. In the PubChem numerous sulfonamide compounds are available but most of them are not biologically characterized. CID-6861424 is one such sulfonamide compound whose anti-tumorigenic property remained unexplored. In the present study, we investigated the anti-cancer activity of CID-6861424.

The cell viability data suggest that, CID-6861424 significantly inhibit the breast cancer MCF-7 cell viability in a dose and time dependent manner. IC_{50} value of the compound was 50μM. Cellular morphology is often used to characterize cellular health. MCF-7 cell morphology changed in presence of increasing concentration of CID-6861424. 50μM of the compound significantly inhibited the cell proliferation and showed morphological changes like, elongated, and flattened cells with dense cytoplasm and irregular cell membrane. These are indication of cellular stress and apoptosis [11, 12]. The aggressive nature of cancer cells is often reciprocated by their strong migratory potential. To investigate this, we performed wound healing assay at two different concentrations, 25μM and 50μM, by measuring the wound closure. We observed that CID-6861424 significantly inhibited cell

migration at both the concentrations, though at 50μM it was more prominent. One of the hallmarks of cancer cells is that they multiply indefinitely and have the capability to form colony [13]. Colony forming assay was performed to check the clonogenic potential post CID-6861424 treatment. When tested in presence of two different concentration, 25μM and 50μM of the compound, both were able to reduce the clonogenic ability of MCF-7 cells, though 50μM was more significant. The data from cell viability, morphology, wound healing and colony formation assays indicated that CID-6861424 effectively inhibited cell proliferation and viability.

The cell cycle is a complex and tightly regulated process, which mandates cellular replication. Uncontrolled cell cycle events are hallmark of cancer cells which modulates cell cycle towards cellular division even under compromised conditions [14]. The cell cycle analysis data showed that post 24 hour, 50μM CID-6861424 treatment resulted in significant G_1 phase cell cycle arrest with increased sub-G_1 cell population as compared to control set. The cyclin D1 is a proto-oncogene and make an active complex with CDK4/6 and phosphorylate and partially inactivate retinoblastoma protein, which is essential for cell cycle progression in G_1 phase [15]. Exposure to 50μM CID-6861424 resulted in downregulation of cyclin D1 and CDK4/6 expression supporting the G_1 phase cell cycle arrest data.

DNA damage endangers genomic integrity thus cells have developed surveillance mechanism to continuously monitor the DNA integrity and respond by activating checkpoints and DNA repair machinery [16–22] . DNA damage upregulates p53 and γH2AX recognizes double strand breaks [16, 23]. 50μM CID-6861424 treatment resulted in increased DNA damage caused due to double strand break as shown by increased γH2AX. Increase in p53 expression was also observed.

Intracellular ROS are essential players of cellular homeostasis and tightly regulated under normal condition. Cancer cells in contrast to normal cells have higher levels of ROS which is considered to be one of the major sources of oncogenesis [24–26]. Besides generation of high levels of ROS, cancer cells also express high level of antioxidants that can quench excess ROS [27] thus maintain ROS homeostasis albeit at higher level. Most of the cancer therapies target the ROS homeostasis in cancer cells either by downregulating antioxidants [28] or by upregulating ROS levels [29, 30]. Cellular exposure to chemotherapeutics drugs causes increased ROS generation [31, 32]. The free radicals are highly reactive and interact with biomolecules including DNA causing damage and oxidative stress [31]. Since we observed increased DNA damage in presence of CID-6861424, we analysed generation of cellular ROS post 72hr treatment. The treatment of 25μM CID-6861424 increased ROS levels, however a significant increase in ROS levels were observed in 50μM CID-6861424 treated set as compared to control set. The relatively lower ROS generation upon 25μM CID-6861424 treatment could be explained by the expression of antioxidants, which might have neutralized some of the ROS generated during treatment with sub-cytotoxic concentration. In case of 50μM CID-6861424 treatment, the influx of generated ROS might have been so strong that the antioxidant system of MCF-7 cells failed to neutralize it resulting in high expression of ROS levels.

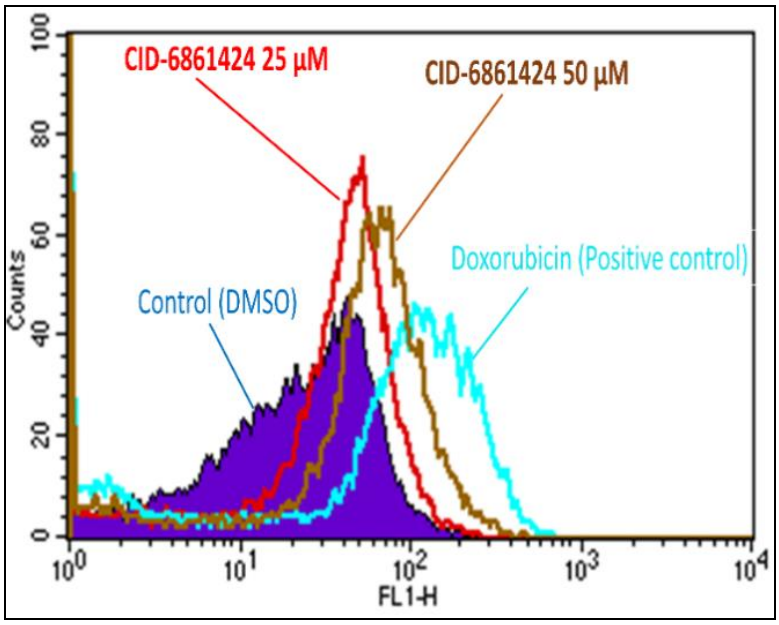
In mammalian cells elevated ROS levels can cause disruption of **Δψm** [33–35] resulting in mitochondrial mediated cellular apoptosis [36]. We checked the effect of CID-6861424 treatment on **Δψm** in MCF-7 cells. A significant **Δψm** disruption was observed in presence of 50μM CID-6861424.

Increased ROS levels, depletion in **Δψm** and extensive DNA damages induces apoptotic cell death [33–36]. The cell cycle data showed cell death which increase with increase in exposure time to CID-6861424. We used annexin V/ 7AAD assay to check the mode of cell death. Flow cytometry data showed that MCF-7 cells treated with CID-6861424 significantly induced late apoptosis as compared to the DMSO control. Bcl-2 and Bax, anti-apoptotic and pro-apoptotic proteins respectively, are

closely linked with mitochondrial apoptosis. Localized in the outer mitochondrial membrane, Bcl-2 is reported to inhibit opening of mitochondrial membrane permeability transition pores [37] through perpetuation of NADPH [38, 39], leading to oxidative attenuation of proteins responsible for its opening [40] and inhibition of release of apoptogenic proteins under apoptosis inducing conditions [41–43]. Besides, Bcl-2 is also reported to quench oxidative stress by inhibiting lipid per-oxidation [44]. 50µM CID-6861424 treated MCF-7 cells showed reduction in Bcl-2 and induction in Bax expression with increase in time and suggesting induction of mitochondrial mediated apoptosis.

The Akt/PKB pathway is an essential pathway for cell survival and cell growths in response to extracellular signals [45]. Akt is a serine/threonine kinase, phosphorylates several downstream proteins and is responsible for cell survival, growth, proliferation, cell migration and angiogenesis [46]. Metastatic as well as endocrine resistance breast cancer cells show increased Akt/PKB phosphorylation leading to sustained activation of the protein resulting in inhibition of apoptosis and induction of cell proliferation and survival [47–49]. GSK-3β is a major downstream effector where various signal transduction pathway converges. It modulates cell death machinery in response to cellular stress and exposure to chemotherapeutic agents [50]. Tan et al. have shown that inhibition of GSK-3β resulted in activation of Bax and induction of apoptosis [51]. MCF-7 cells when exposed to 50µM CID-6861424 showed downregulation of phosphorylated Akt and GSK-3β which in turn might have affected the status of proteins involved in apoptosis, like Bcl-2 and Bax.

Based on our findings, we have proposed a model for the mode of action of CID-6861424 in breast cancer MCF-7 cells (Figure 8). CID-6861424 induces G_1 phase cell cycle arrest due to inhibition of cyclin D1 and CDK4/6 expression. It induces ROS generation leading to DNA damage, upregulation of p53 and disruption of $\Delta\psi m$. CID-6861424 treatment downregulated phosphorylation of Akt/PKB and GSK-3β, upregulated of pro-apoptotic protein Bax and downregulated of anti-apoptotic protein Bcl-2 resulting in induction of mitochondrial mediated apoptosis.



(a)

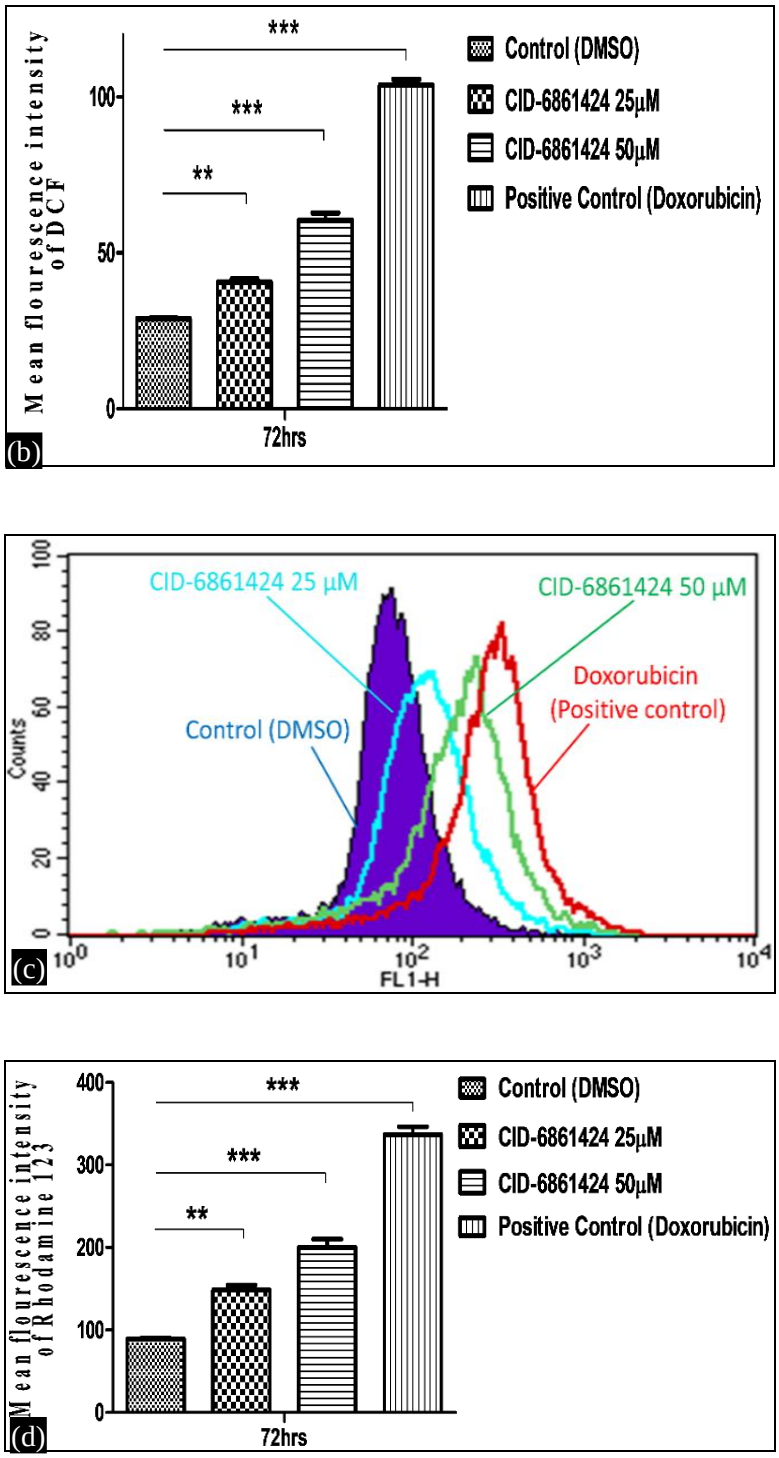


Fig. 4. *CID-6861424 Induced ROS Generation and Mitochondrial Membrane Potential (Δψm) depletion in MCF-7 cell. (a) and (b) Histogram and Bar-diagram show enhanced ROS generation indicated by increased DCF-DA fluorescence in MCF-7 cell post 72hr treatment with 50µM-CID-6861424. (c) and (d) Histogram and Bar-diagram show dissipation of Δψm detected by Rhodamine123-dye in MCF-7 cell post 72hr treatment with 50µM-CID-6861424. Data are represented as the mean ± SD of three independent experiments. **P<0.01, and ***P<0.001.*

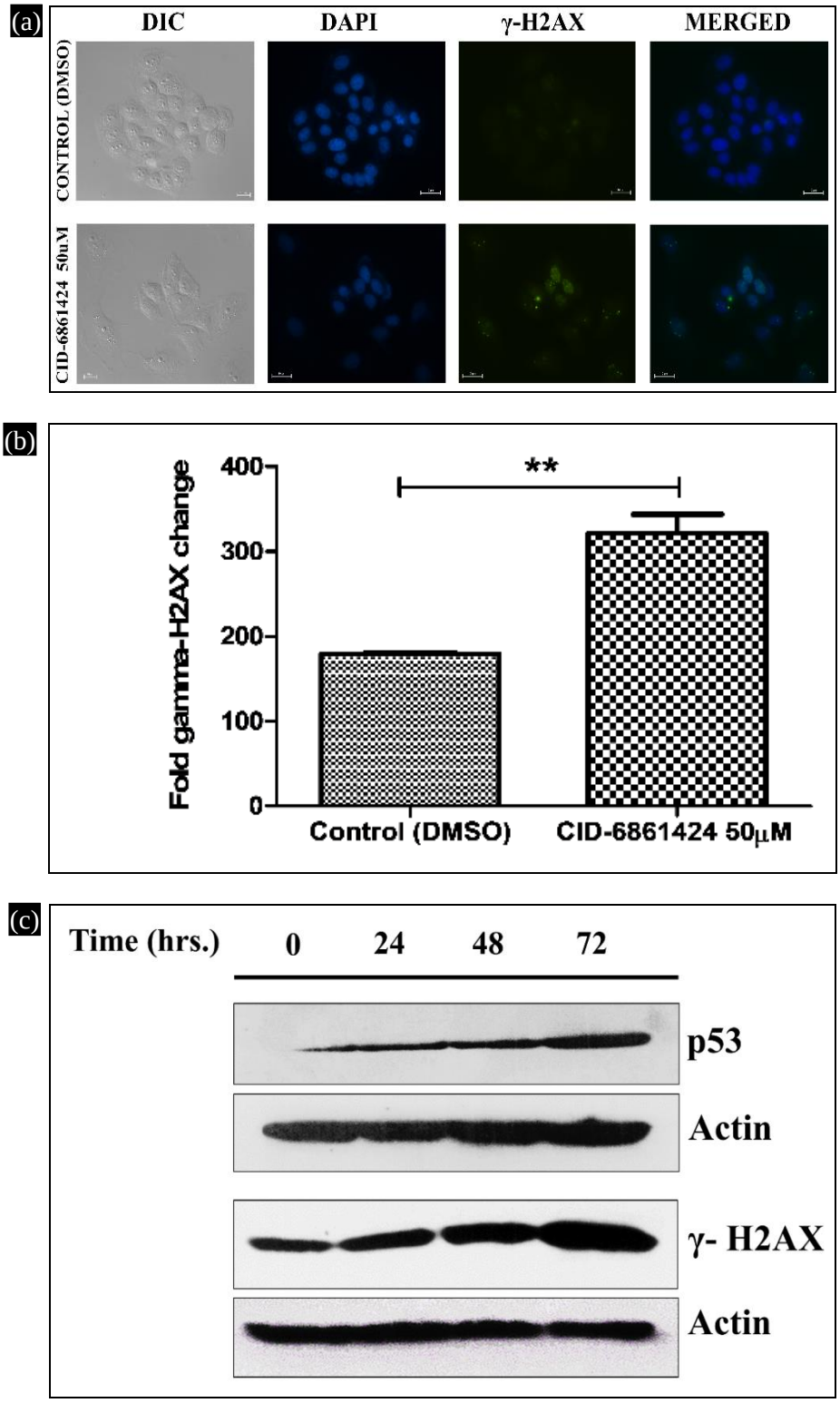


Fig. 5. *CID-6861424 Induced DNA Damage in MCF-7Ccell. (a) Post 72hr treatment with 50μM-CID-6861424 induced γ-H2AX. (b) The bar-diagram representation of increased fluorescence intensity of γ-H2AX in MCF-7 cell. (c) The upregulation of p53 and γ-H2AX proteins expression in presence of 50μM-CID-6861424 was observed by western blotting. Data are represented as the mean ± SD of three independent experiments. **P<0.01.*

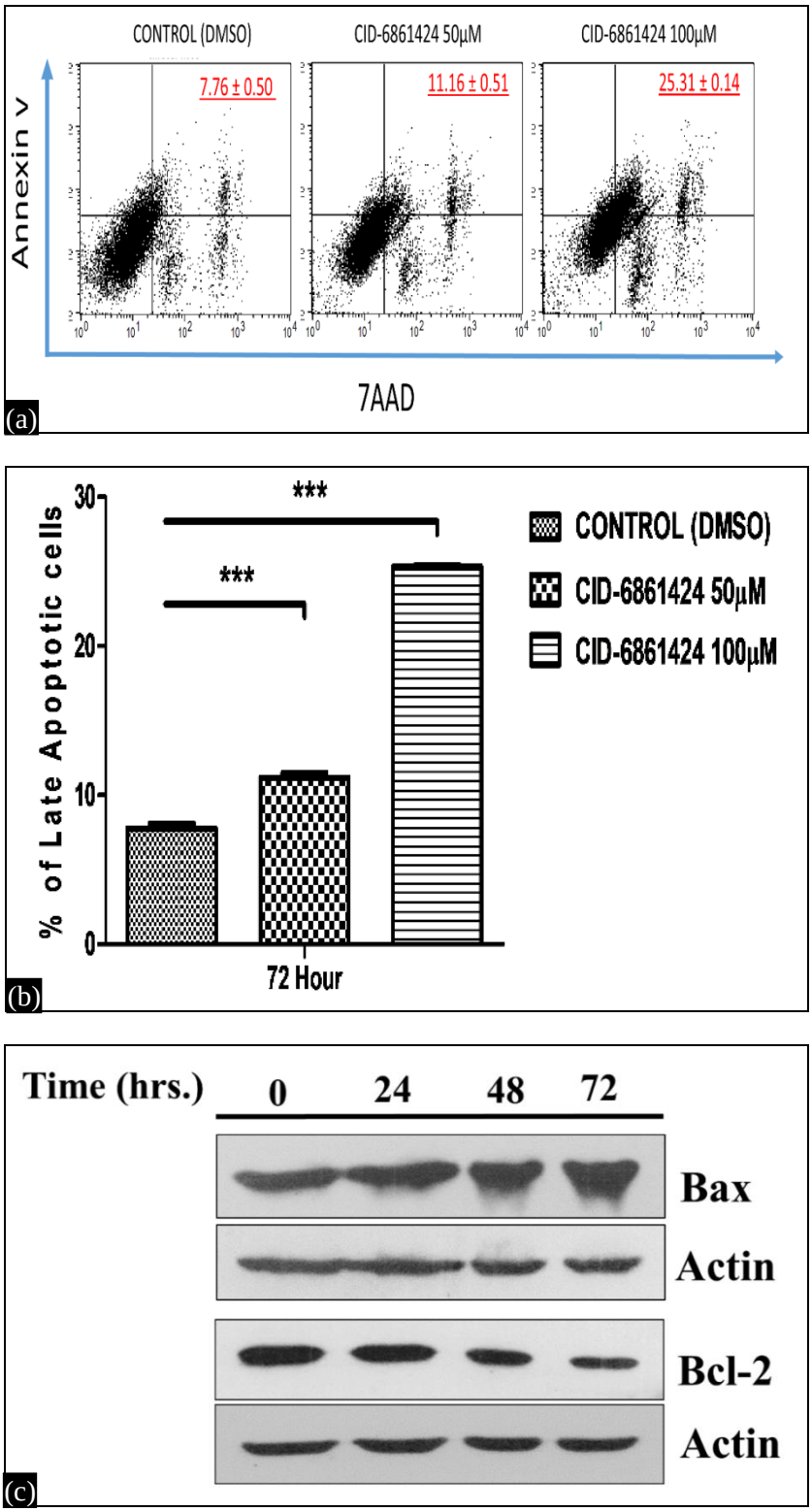


Fig. 6: *CID-6861424 Induced Apoptotic Cell Death in MCF-7 Cell. (a) Detection of late apoptotic cell death by Annexin V/7AAD kit (BD Biosciences) post 72hr CID-6861424 treatment using flow cytometry. (b) The bar-diagram representation of increased late apoptotic cell population with an increasing concentration of CID-6861424. (c) Upregulation of Bax and downregulation of Bcl-2 proteins expression in presence of 50µM-CID-6861424 was observed by western blotting. Data are represented as the mean ± SD of three independent experiments. ***P<0.001*

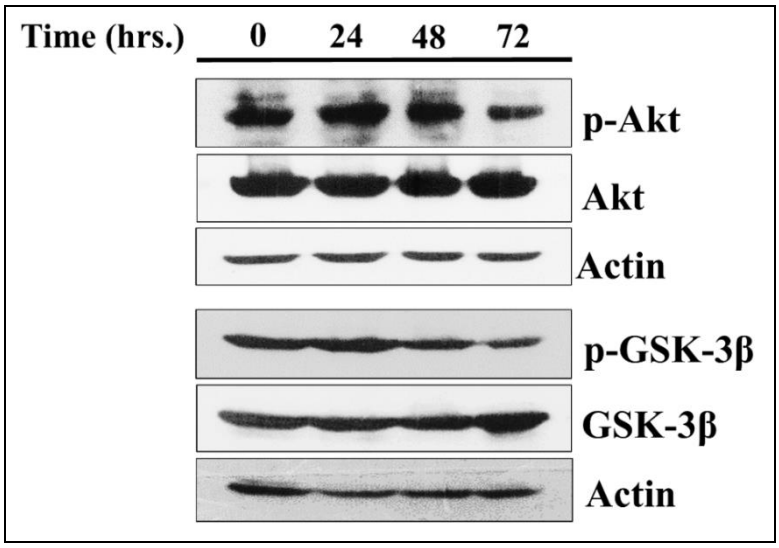


Fig. 7. *50 µM CID-6861424 Inhibits the Akt and GSK-3β Phosphorylation in MCF-7 Cell. The downregulation of phosphorylated Akt and GSK-3β proteins expression in presence of 50µM-CID-6861424 was observed by western blotting.*

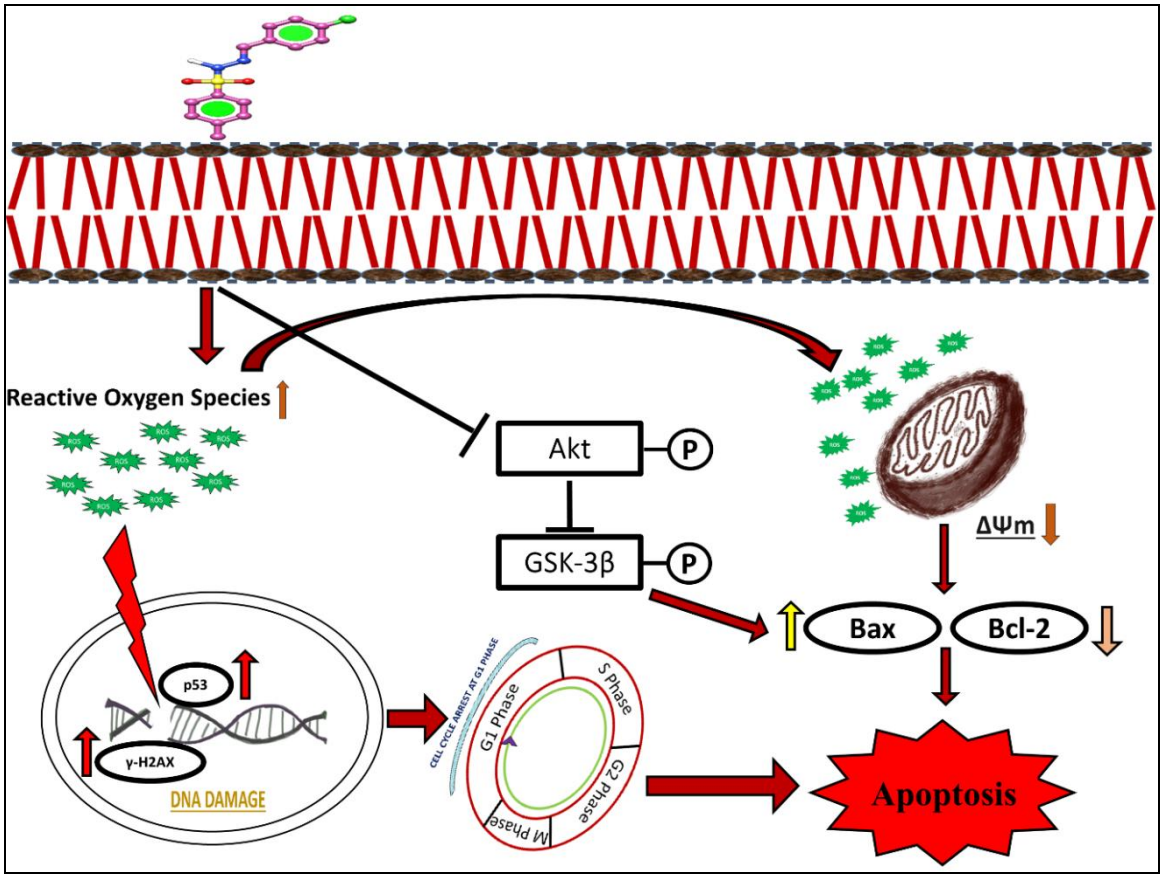


Fig. 8. *Proposed Model for the Mechanism of Action of* ***CID-6861424*** *in MCF-7 Cell.*

CONCLUSION

Breast cancer cases are increasing in women worldwide and is main cause of cancer related death. Approximately 80% of the breast cancers are ER positive. The effectiveness of endocrine therapeutic drugs are reduced due to either intrinsic resistance or acquired resistance developed due to prolonged usage. In this study, we have investigated the anti-cancer activity of CID-6861424, a

Sulfonamide compounds on breast cancer cell line, MCF-7. CID-6861424 inhibited cell proliferation, cell migration and colony forming ability of MCF-7 cells. Exposure to the compound resulted in downregulation of Cyclin D1 and CDK4/6 lead to induce G_1 phase cell cycle arrest. CID-6861424 treatment elevated ROS generation, depleted of **Δψm**, increased DNA damage and apoptotic cell death. The compound also downregulated the phosphorylation of Akt and its effector protein GSK-3β. The results suggest that CID-6861424 is a potential anticancer candidate against breast cancer. Further work is needed before it can be used as a therapeutic agent.

FUNDING

This work was done and supported by UPE-II, DBT Builder, DST-FIST, DST-PURSE-II and UGC RNW (Dr. Neelima Mondal) and Rajiv Gandhi National Fellowship (Sumit Kumar Gautam).

REFERENCES

1. R.L. Siegel, K.D. Miller, A. Jemal, Cancer statistics, 2018, CA. Cancer J. Clin. 68 (2018) 7–30. doi:10.3322/caac.21442.
2. F. Bray, J. Ferlay, I. Soerjomataram, R.L. Siegel, L.A. Torre, A. Jemal, Global cancer statistics 2018: GLOBOCAN estimates of incidence and mortality worldwide for 36 cancers in 185 countries, CA. Cancer J. Clin. (2018). doi:10.3322/caac.21492.
3. G. Konecny, G. Pauletti, M. Pegram, M. Untch, S. Dandekar, Z. Aguilar, C. Wilson, H.-M. Rong, I. Bauerfeind, M. Felber, H.-J. Wang, M. Beryt, R. Seshadri, H. Hepp, D.J. Slamon, Quantitative Association Between HER-2/neu and Steroid Hormone Receptors in Hormone Receptor-Positive Primary Breast Cancer, JNCI J. Natl. Cancer Inst. 95 (2003) 142–153. doi:10.1093/jnci/95.2.142.
4. W. Fan, J. Chang, P. Fu, Endocrine therapy resistance in breast cancer: current status, possible mechanisms and overcoming strategies, Future Med. Chem. 7 (2015) 1511. doi:10.4155/FMC.15.93.
5. A. casini, A. Scozzafava, A. Mastrolorenzo, C. Supuran, Sulfonamides and Sulfonylated Derivatives as Anticancer Agents, Curr. Cancer Drug Targets. 2 (2002) 55–75. doi:10.2174/1568009023334060.
6. [6] J. Chen, S. Jin, V. Abraham, X. Huang, B. Liu, M.J. Mitten, P. Nimmer, X. Lin, M. Smith, Y. Shen, A.R. Shoemaker, S.K. Tahir, H. Zhang, S.L. Ackler, S.H. Rosenberg, H. Maecker, D. Sampath, J.D. Leverson, C. Tse, S.W. Elmore, The Bcl-2/Bcl-XL/Bcl-w Inhibitor, Navitoclax, Enhances the Activity of Chemotherapeutic Agents In Vitro and In Vivo, Mol. Cancer Ther. 10 (2011) 2340–2349. doi:10.1158/1535-7163.MCT-11-0415.
7. [7] A.W. Roberts, D. Huang, Targeting BCL2 With BH3 Mimetics: Basic Science and Clinical Application of Venetoclax in Chronic Lymphocytic Leukemia and Related B Cell Malignancies., Clin. Pharmacol. Ther. 101 (2017) 89–98. doi:10.1002/cpt.553.
8. [8] M.A. Anderson, J. Deng, J.F. Seymour, C. Tam, S.Y. Kim, J. Fein, L. Yu, J.R. Brown, D. Westerman, E.G. Si, I.J. Majewski, D. Segal, S.L. Heitner Enschede, D.C.S. Huang, M.S. Davids, A. Letai, A.W. Roberts, The BCL2 selective inhibitor venetoclax induces rapid onset apoptosis of CLL cells in patients via a TP53-independent mechanism., Blood. 127 (2016) 3215–24. doi:10.1182/blood-2016-01-688796.
9. [9] R. Kia, H.-K. Fun, H. Kargar, (*E*)- *N* ′-(4-Chlorobenzylidene)- *p* -toluenesulfonohydrazide 0.15-hydrate, Acta Crystallogr. Sect. E Struct. Reports Online. (2009). doi:10.1107/S1600536809014512.
10. [10] G.P. Sharma, S. Gurung, A. Inam, L. Nigam, A. Bist, D. Mohapatra, S. Senapati, S. Naidu, A. Azam, N. Mondal, CID-6033590 inhibits p38MAPK pathway and induces S-phase cell cycle arrest and apoptosis in DU145 and PC-3 cells, Toxicol. Vitr. (2019). doi:10.1016/j.tiv.2019.06.003.
11. G. Hacker, The morphology of apoptosis. Cell Tissue Res, 2000. 301 (2000) 5–17.
12. M.A. Decoster, S. Maddi, V. Dutta, J. Mcnamara, Microscopy and image analysis of individual and group cell shape

changes during apoptosis, Microsc. Sci. Technol. Appl. Educ. (2010) 836–843.
13. N.A.P. Franken, H.M. Rodermond, J. Stap, J. Haveman, C. van Bree, Clonogenic assay of cells in vitro, Nat. Protoc. 1 (2006) 2315–2319. doi:10.1038/nprot.2006.339.
14. Y.A. Fouad, C. Aanei, Revisiting the hallmarks of cancer., Am. J. Cancer Res. 7 (2017) 1016–1036. http://www.ncbi.nlm.nih.gov/pubmed/28560055 (accessed June 19, 2019).
15. J.P. Alao, The regulation of cyclin D1 degradation: roles in cancer development and the potential for therapeutic invention, Mol. Cancer. 6 (2007) 24. doi:10.1186/1476-4598-6-24.
16. K. Hientz, A. Mohr, D. Bhakta-Guha, T. Efferth, The role of p53 in cancer drug resistance and targeted chemotherapy., Oncotarget. 8 (2017) 8921–8946. doi:10.18632/oncotarget.13475.
17. C. Klein, L.T. Vassilev, Targeting the p53-MDM2 interaction to treat cancer., Br. J. Cancer. 91 (2004) 1415–9. doi:10.1038/sj.bjc.6602164.
18. N.D. SMITH, J.N. RUBENSTEIN, S.E. EGGENER, J.M. KOZLOWSKI, The p53 Tumor Suppressor Gene and Nuclear Protein: Basic Science Review and Relevance in the Management of Bladder Cancer, J. Urol. 169 (2003) 1219–1228. doi:10.1097/01.ju.0000056085.58221.80.
19. J. Bargonetti, J.J. Manfredi, Multiple roles of the tumor suppressor p53., Curr. Opin. Oncol. 14 (2002) 86–91. http://www.ncbi.nlm.nih.gov/pubmed/11790986 (accessed January 19, 2019).
20. M.-F. Chiang, P.-Y. Chou, W.-J. Wang, C.-I. Sze, N.-S. Chang, Tumor Suppressor WWOX and p53 Alterations and Drug Resistance in Glioblastomas, Front. Oncol. 3 (2013) 43. doi:10.3389/fonc.2013.00043.
21. L.J. Ko, C. Prives, p53: puzzle and paradigm., Genes Dev. 10 (1996) 1054–72. http://www.ncbi.nlm.nih.gov/pubmed/8654922 (accessed January 19, 2019).
22. A.J. Levine, p53, the cellular gatekeeper for growth and division., Cell. 88 (1997) 323–31. http://www.ncbi.nlm.nih.gov/pubmed/9039259 (accessed January 19, 2019).
23. J.S. Dickey, C.E. Redon, A.J. Nakamura, B.J. Baird, O.A. Sedelnikova, W.M. Bonner, H2AX: Functional roles and potential applications, Chromosoma. 118 (2009) 683–692. doi:10.1007/s00412-009-0234-4.
24. J. Wang, J. Yi, Cancer cell killing via ROS: To increase or decrease, that is a question, Cancer Biol. Ther. 7 (2008) 1875–1884. doi:10.4161/cbt.7.12.7067.
25. E. Panieri, M.M. Santoro, ROS homeostasis and metabolism: a dangerous liason in cancer cells, Cell Death Dis. 7 (2016) e2253. doi:10.1038/cddis.2016.105.
26. P.T. Schumacker, Reactive oxygen species in cancer cells: Live by the sword, die by the sword, Cancer Cell. 10 (2006) 175–176. doi:10.1016/j.ccr.2006.08.015.
27. M.-Y. Liou, P. Storz, Reactive oxygen species in cancer, 2010. doi:10.3109/10715761003667554.Reactive.
28. N. Ahmad, H. Mukhtar, Antioxidants meet molecular targets for cancer prevention and therapeutics., Antioxid. Redox Signal. 19 (2013) 85–8. doi:10.1089/ars.2013.5299.
29. H. Pelicano, D. Carney, P. Huang, ROS stress in cancer cells and therapeutic implications, Drug Resist. Updat. 7 (2004) 97–110. doi:10.1016/j.drup.2004.01.004.
30. D. Trachootham, J. Alexandre, P. Huang, Targeting cancer cells by ROS-mediated mechanisms: A radical therapeutic approach?, Nat. Rev. Drug Discov. 8 (2009) 579–591. doi:10.1038/nrd2803.
31. A. Manuscript, S. Magnitude, NIH Public Access, 31 (2013) 1713–1723. doi:10.1109/TMI.2012.2196707.Separate.
32. S.K. Gurung, S. Dana, K. Mandal, P. Mukhopadhyay, N. Mondal, Downregulation of c-Myc and p21 expression and induction of S phase arrest by naphthalene diimide derivative in gastric adenocarcinoma cells, Chem. Biol. Interact. 304 (2019) 106–123. doi:10.1016/j.cbi.2019.02.010.
33. K. Banki, E. Hutter, N.J. Gonchoroff, A. Perl, Elevation of mitochondrial

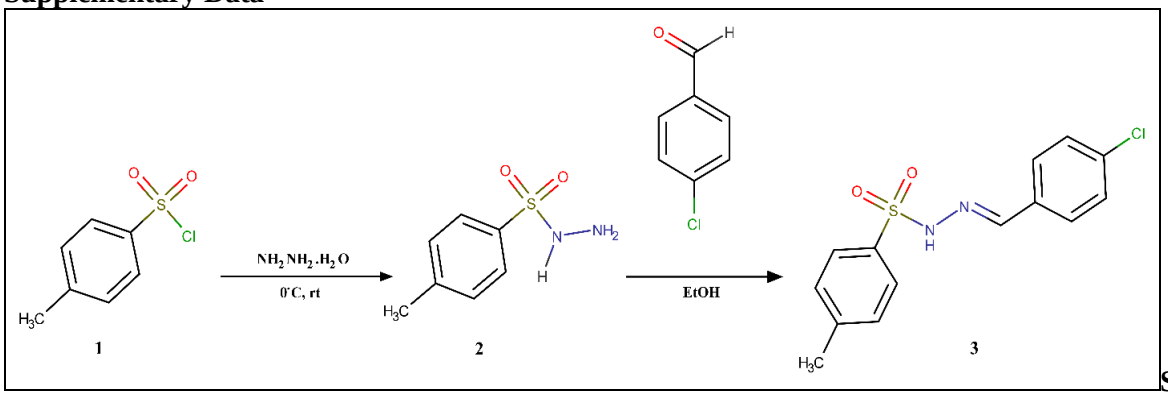
transmembrane potential and reactive oxygen intermediate levels are early events and occur independently from activation of caspases in Fas signaling., J. Immunol. 162 (1999) 1466–79. http://www.ncbi.nlm.nih.gov/pubmed/9973403 (accessed April 4, 2018).

34. T.M. Johnson, Z.X. Yu, V.J. Ferrans, R.A. Lowenstein, T. Finkel, Reactive oxygen species are downstream mediators of p53-dependent apoptosis., Proc. Natl. Acad. Sci. U. S. A. 93 (1996) 11848–52. doi:10.1073/pnas.93.21.11848.
35. F. Fonseca-Silva, J.D.F. Inacio, M.M. Canto-Cavalheiro, E.E. Almeida-Amaral, Reactive oxygen species production and mitochondrial dysfunction contribute to quercetin induced death in Leishmania amazonensis, PLoS One. 6 (2011). doi:10.1371/journal.pone.0014666.
36. J. Reed, J. Jurgensmeier, S. Matsuyama, Bcl-2 family proteins and mitochondria, Biochim. Biophys. Acta - Bioenerg. 1366 (1998) 127–137. doi:10.1016/S0005-2728(98)00108-X.
37. N. Zamzami, Mitochondrial control of nuclear apoptosis, J. Exp. Med. 183 (1996) 1533–1544. doi:10.1084/jem.183.4.1533.
38. M. Esposti, I. Hatzinisiriou, H. McLennan, Bcl-2 and mitochondrial oxygen radicals, J. Biol. …. 274 (1999) 29831–29837. http://www.jbc.org/cgi/content/abstract/274/42/29831%5Cnpapers2://publication/uuid/62F02A13-5CFE-430E-AF12-CA5329EF4048.
39. A.J. Kowaltowski, A.E. Vercesi, G. Fiskum, Bcl-2 prevents mitochondrial permeability transition and cytochrome c release via maintenance of reduced pyridine nucleotides, Cell Death Differ. 7 (2000) 903–910. doi:10.1038/sj.cdd.4400722.
40. a J. Kowaltowski, R.F. Castilho, a E. Vercesi, Mitochondrial permeability transition and oxidative stress, FEBS Lett. 495 (2001) 12–15. doi:S0014-5793(01)02316-X [pii].
41. R.M. Kluck, The Release of Cytochrome c from Mitochondria: A Primary Site for Bcl-2 Regulation of Apoptosis, Science (80-.). 275 (1997) 1132–1136. doi:10.1126/science.275.5303.1132.
42. J. Yang, X. Liu, K. Bhalla, C.N. Kim, A.M. Ibrado, J. Cai, T.I. Peng, D.P. Jones, X. Wang, Prevention of apoptosis by Bcl-2: Release of cytochrome c from mitochondria blocked, Science (80-.). 275 (1997) 1129–1132. doi:10.1126/science.275.5303.1129.
43. E. Daugas, M. Geuskens, G. Kroemer, Bcl-2 Inhibits the Mitochondrial Release o f an A p o p t o g e n i c Protease, 184 (1996).
44. D.M. Hockenbery, Z.N. Oltvai, X.M. Yin, C.L. Milliman, S.J. Korsmeyer, Bcl-2 functions in an antioxidant pathway to prevent apoptosis, Cell. 75 (1993) 241–251. doi:10.1016/0092-8674(93)80066-N.
45. K.H. Khan, T.A. Yap, L. Yan, D. Cunningham, Targeting the PI3K-AKT-mTOR signaling network in cancer, Chin. J. Cancer. 32 (2013) 253–265. doi:10.5732/cjc.013.10057.
46. J. Luo, B.D. Manning, L.C. Cantley, Targeting the PI3K-Akt pathway in human cancer: rationale and promise., Cancer Cell. 4 (2003) 257–62. http://www.ncbi.nlm.nih.gov/pubmed/14585353 (accessed January 19, 2019).
47. L.F. Hernandez-Aya, A.M. Gonzalez-Angulo, Targeting the phosphatidylinositol 3-kinase signaling pathway in breast cancer., Oncologist. 16 (2011) 404–14. doi:10.1634/theoncologist.2010-0402.
48. F.J. Carmona, F. Montemurro, S. Kannan, V. Rossi, C. Verma, J. Baselga, M. Scaltriti, AKT signaling in ERBB2-amplified breast cancer., Pharmacol. Ther. 158 (2016) 63–70. doi:10.1016/j.pharmthera.2015.11.013.
49. G. Pérez-Tenorio, O. Stål, Southeast Sweden Breast Cancer Group, Activation of AKT/PKB in breast cancer predicts a worse outcome among endocrine treated patients. Br. J. Cancer. 86 (2002) 540–5. doi:10.1038/sj.bjc.6600126.
50. F. Chiara, A. Rasola, GSK-3 and mitochondria in cancer cells, Front. Oncol. 3 (2013) 16. doi:10.3389/fonc.2013.00016.
51. J. Tan, L. Zhuang, H.-S. Leong, N.G. Iyer, E.T. Liu, Q. Yu, Pharmacologic Modulation of Glycogen Synthase Kinase-3β Promotes p53-Dependent Apoptosis through a Direct Bax-Mediated

Mitochondrial Pathway in Colorectal Cancer Cells, Cancer Res. 65 (2005) 9012–9020. doi:10.1158/0008-5472.CAN-05-1226.

Cite this Article
Sumit Kumar Gautam, Afreen Inam, Amir Azam, Neelima Mondal. Induction of G1 phase cell cycle arrest and apoptosis in breast cancer MCF-7 cells by sulphonamide derivative CID-6861424. *Research & Reviews: A Journal of Toxicology*. 2019; 9(2): 1–19p.

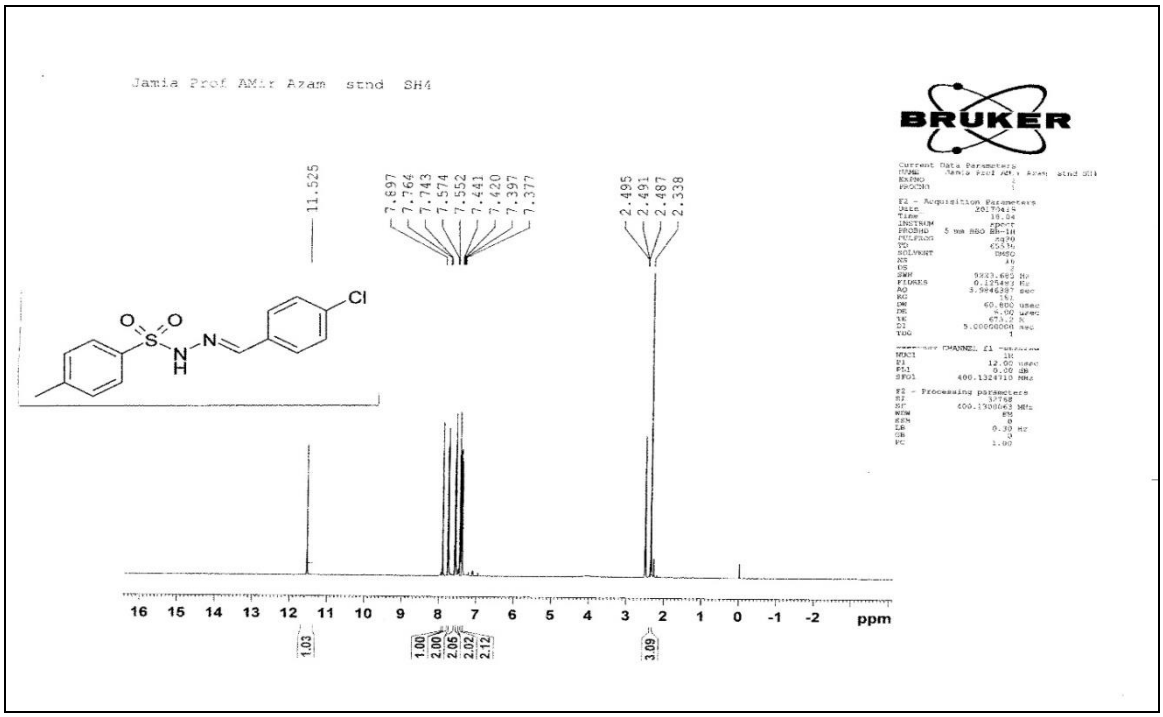
Supplementary Data



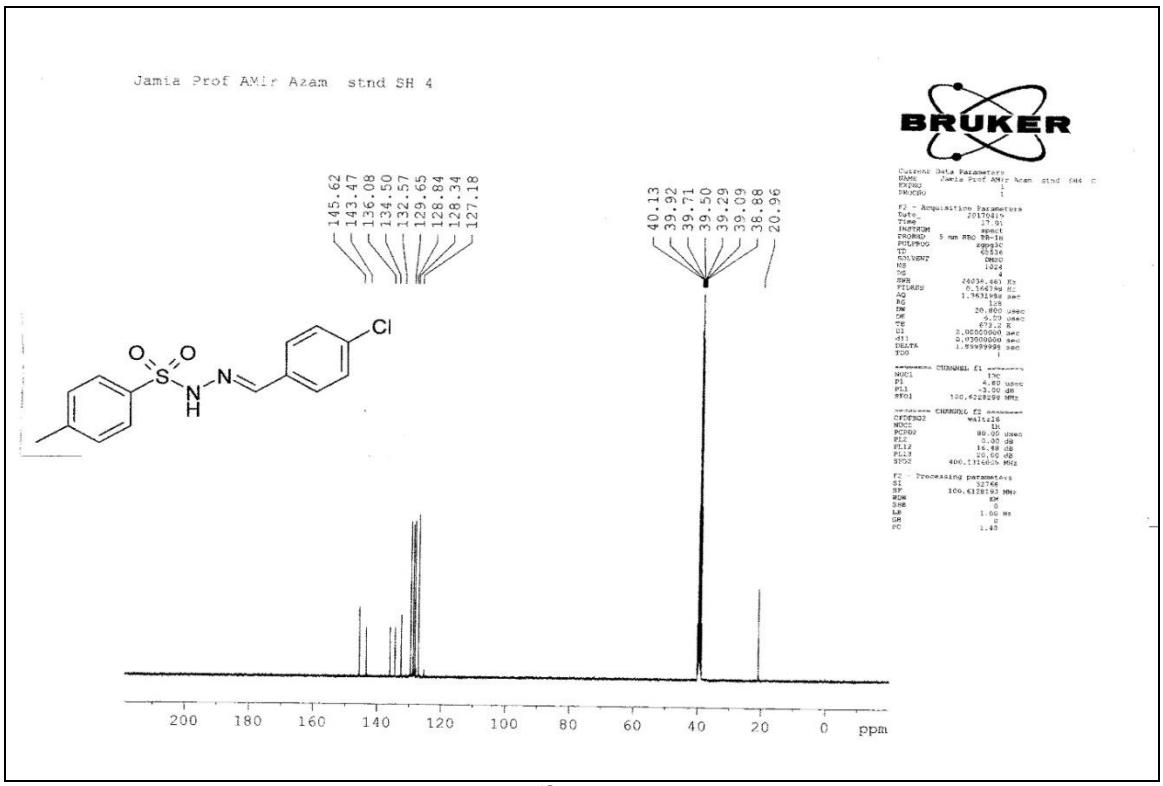
Synthesis of CID-6861424:

Scheme: Synthesis of CID-6861424

N'-[(1*E*)-(4-chlorophenyl) methylene]-4 methylbenzenesulfonohydrazide (**CID-6861424**) was successfully synthesized and confirmed by IR, 1H NMR, ^{13}C NMR analysis. Observation from results of IR, 1H NMR, ^{13}C NMR are described as (Off white solid, Ethanol) Yield: 87 %; mp: 159-160^{o}C; Anal. calc. for $C_{14}H_{13}ClN_2O_2S$: C 54.5, H 4.2, N 9.1, S 10.4%. found: C 54.5, H 4.1, N 9.1, S 10.2%; IR *V*max (cm^{-1}): 3186 (N-H), 2920 (Ar-H), 1598 (C=N), 1495 (C=C), 1363, 1125 (SO_2); ^{1}H NMR (DMSO- d_6) δ (ppm): 2.49 (s, 3H, -CH_3), 7.39 (d, 2H, *J*= 8.0 Hz, Ar-H), 7.44 (d, 2H, *J*= 8.4, Ar-H), 7.57 (d, 2H, *J*= 8.8 Hz, Ar-H), 7.76 (d, 2H, *J*= 8.4 Hz, Ar-H), 7.89 (s, 1H, N=CH), 11.52 (s, 1H, -NH); ^{13}C NMR (DMSO- d_6) δ (ppm): 20.96, 127.18, 128.34, 128.84, 129.65, 132.57, 134.50, 1136.08, 143.47, 145.62; FAB-MS (m/z): 309 (M^+ + 1, 100%).



Supplementary Figure 1 : ^{1}H NMR Spectra of CID-6861424



Supplementary Figure 2: ^{13}C NMR Spectra of CID-6861424

APPENDIX

List I: Publications and Conferences

Publications:

1. **Sumit Kumar Gautam**[1], Afreen Inam[2], Amir Azam[2], Neelima Mondal[1]*. Induction of G1 phase cell cycle arrest and apoptosis in breast cancer MCF-7 cells by sulphonamide derivative CID-6861424. **Research and Reviews: A Journal of Toxicology (2019).** ISSN 2349-1264 (**Accepted**)

2. **Sumit Kumar Gautam**[1], Afreen Inam[2], Amir Azam[2], Neelima Mondal[1]*. CID-6861424 increases sensitivity of Triple Negative Breast Cancer Cell line to low dose of γ-radiation. **Heliyon Journal (2019).** ISSN 2405-8440 (**Under Revision**)

International and National Conferences/Workshops:

1. **Sumit Kumar Gautam**, Amir Azam, Neelima Mondal, Annual Scientific Symposium, (Biospark-2019), School of life sciences, Jawaharlal Nehru university, New Delhi, India, 15-16th March, 2019. **Awarded for best poster presentation**.

2. **Sumit Kumar Gautam**, Amir Azam, Neelima Mondal, International Conference on Mitochondria in Health and Disease, New Delhi, India, 10 – 11 February, 2017.

3. **Sumit Kumar Gautam**, Amir Azam, Neelima Mondal, 83rd Annual Meeting of Society of Biological Chemists (India) and Symposium on Evolution: Molecules to Life", Bhubaneswar, Orissa, India, 18th – 21st December, 2014.

4. **Sumit Kumar Gautam**, Amir Azam, Neelima Mondal, Science and Technology for Specially Abled Persons (National Science Day -2017), New Delhi, India, 28 February, 2017.

5. **Sumit Kumar Gautam**, Amir Azam, Neelima Mondal, Annual Scientific Symposium, (BioEpoch 2017), School of Biotechnology, Jawaharlal Nehru University, New Delhi, India, 23rd-24th March, 2017.

6. **Sumit Kumar Gautam**, Workshop on "Basic & Research Application of Flow Cytometer" Adavanced Instrumentation Research Facility, Jawaharlal Nehru University, New Delhi, India, 30th – 31st August 2016.

7. **Sumit Kumar Gautam**, Training on "Safe Handling of Radioactive in Research Application" School of Life Sciences, Jawaharlal Nehru University, New Delhi, India, 3rd-6th November 2015.

List II: Stock solutions and reagents

All solutions were prepared in deionized, ultra-filtered water from a water purification system (Millipore, USA). All solutions were sterilized by autoclaving at 15 lbs /square inch for 20 min, unless stated otherwise.

RNase A stock solution (1 mg/ml)

10 mg of RNase A was dissolved in 10 ml of autoclaved 10 mM Tris-Cl (pH 8.0). The solution was filter sterilized using 0.2 µm filter. Aliquots were stored at -20^{o}C until needed.

1 M Tris.HCl (pH 8.0)

Dissolved 121.14 g of Trizma™ base (Tris base) in water and pH was adjusted to 8.0 using 6 N HCl and volume was made up to 1000ml with water.

DMEM Incomplete medium

DMEM was dissolved in 900 ml of autoclaved water, $NaHCO_3$ was added to final concentration of, pH was adjusted to 7.2. Volume was made up to 1 liter and media was filtered with 0.22 µm filter before use.

L-15 (Leibovitz) Incomplete medium

L-15 was dissolved in 900 ml of autoclaved water, Penicillin-Streptomycin (pen-strep) was added, pH was adjusted to 7.2. Volume was made up to 1 liter and media was filtered with 0.22 µm filter before use.

Freezing mixture for cell lines

Freezing mixture for cell lines made of 5% DMSO, 20% FBS in DMEM. Filter-sterilize; store frozen.

0.5 M EDTA (pH 8.0)

18.61 gm of EDTA was dissolved in 80 ml water. The pH was adjusted to 8.0 with NaOH pellets. The volume was made up to 100 ml.

6X DNA Gel Loading Dye

Bromophenol blue	0.25%
Xylene cyanole FF	0.25%
Glycerol	30%

Gel Loading Dye was prepared in water, and stored in aliquots at 4^{o}C.

30% Acrylamide solution

29 gm of acrylamide and 1 gm of N, N'- methylene bisacrylamide were dissolved in 90 ml of autoclaved water (29:1). The final volume was made up to 100 ml and the solution was stored in a dark bottle at 4^{o}C.

10% SDS solution

5 gm of SDS was dissolved in 40 ml of autoclaved water. The final volume was made up to 50 ml and stored at room temperature.

10% APS

1 gm of ammonium persulphate was dissolved in 8 ml of autoclaved water. The final volume was made up to 10 ml. Aliquots were stored at -20^{o}C.

1.5 M Tris base (pH 8.8)

18.1 gm of Tris base was dissolved in 80 ml of water and the pH was adjusted to 8.8 with concentrated HCl and the final volume was made up to 100 ml.

1 M Tris base (pH 6.8)

12.1 gm of Tris base was dissolved in 80 ml of water and the pH was adjusted to 6.8 with concentrated HCl and the final volume was made up to 100 ml.

5X SDS PAGE sample loading dye

β-Mercaptoethanol	15%
SDS	15%
Bromophenol blue	0.6%

Glycerol	50%

Electrode buffer

Glycine	192 mM
Tris base	25 mM
SDS	0.1%

The buffer was prepared in autoclaved water.

12% Resolving gel

30% Acrylamide solution	4.0 ml
1.5 M Tris HCl (pH 8.8)	2.5 ml
Autoclaved water	3.35 ml
10% SDS solution	100 µl
10% APS solution	50 µl
TEMED	8 µl

Stacking gel

30% Acrylamide solution	0.65 ml
1 M Tris HCl (pH 6.8)	0.65 ml
Autoclaved water	3.65 ml
10% SDS solution	50 µl
10% APS solution	25 µl
TEMED	6 µl

Staining solution

Coomassie brilliant blue	0.25%
Methanol	40%

Glacial acetic acid	10%

Destaining solution

Methanol	40%
Glacial acetic acid	10%

Transfer buffer (pH 8.3)

Glycine	192 mM
Tris base	25 mM
Methanol	20%

Cell lysis buffer

Tris-Cl (pH-8.0)	50 mM
NaCl	150 mM
EDTA	2mM
Glycerol	10%
NP-40	1%

PBS-Tween 20

0.5 ml of Tween 20 was added to 1000 ml of autoclaved 1X PBS.

Ethidium bromide (10 mg/ml)

0.1 gm of ethidium bromide was dissolved in 8 ml of autoclaved water. The volume was made up to 10 ml. Aliquots were stored in dark at room temperature.

1.25 mM DCFDA

3 mg of DCFDA powder was dissolved in 5 ml of DMSO or methanol. This should be used freshly in the reaction.

10 mM DTNB or Ellman's reagent

40 mg of DTNB powder was dissolved in 10 ml of methanol. This should be used freshly in the reaction**.**

0.2M H_2O_2

741 µl of 30% hydrogen peroxide was dissolved in 9.259 ml of distilled water. This should be used freshly in the reaction

180 µM PMS

3.06 mg of PMS powder was dissolved in 10 ml of double distilled water.

780 µM NADPH

8.0 mg of PMS powder was dissolved in 15 ml of double distilled water.

300 µM NBT

5.0 mg of PMS powder was dissolved in 20 ml of methanol.

5.55 mM OPT

1.0 mg of OPT powder was dissolved in 10 ml of methanol.

LIST-III: List of figures

Chapter-3

Chapter-4

Chapter-5

Chapter-6

LIST-IV: List of tables