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Discovery of Novel Pyridine Derivatives as Anti-Cancer Agents

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Discovery of Novel Pyridine Derivatives as Anticancer Agents

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Abstract

The International Agency for Research on Cancer indicated that cancer burden was raised in 2012 to 14.1 million incidence cases and 8.2 mortality cases. Consequently, novel approaches are urgently required for further improvement in existing cancer therapies. Synthesis of the pyridine ring system and its derivatives occupy an important place in the realm of synthetic organic chemistry, due to their therapeutic and pharmacological properties. The current study investigated the anti-cancer activity of novel pyridine derivatives. Twenty novel Pyridine Derivatives were screened using WST-1 assay on several cell lines to assess their toxicity and to determine whether the effect is tumor or cell type specific. Dose response curves were obtained for compound 9a for further investigations. Detection of apoptosis and cell cycle checkpoint analysis was done using flow cytometry and expression of several apoptotic and anti-apoptotic proteins was carried out by Western Blot. Initial screening revealed that 100μM treatment with pyridine derivatives for the above mentioned cell lines for 24 hours suppressed the viability as follows: compound 9a reduced the viability by 40% in MCF-7 cells and by 45% in HCT-116, compound 7b reduced the viability in SKOV-3 by 35% and finally compound 11 suppressed the viability of SKOV-3 by 44%. Compound 9a induced growth inhibition in MCF-7 cells and resulted in the induction of apoptosis with an IC_{50} of 20μM. It upregulated the expression of p53, Bax and Caspase-3 in MCF-7 cells. In addition, it caused significant down-regulation of Bcl-2, Mdm-2 and Akt. Compound 9a exhibited less toxicity on non-tumorigenic breast epithelial cell line MCF-12a. Findings of the study indicated that compound 9a possesses potent anti-proliferative activity against MCF-7 cells and could be a promising chemotherapeutic agent with less toxicity on non-tumorigenic cells. Further screening and investigating molecular mechanisms of this potent agent on other cancer cell lines is highly required.

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LIST OF ABBREVIATIONS

Abbreviation	Explanation
AFP	Alpha Feto Protein
DMSO	Dimethyl Sulfoxide
FBS	Feto Bovine Serum
DMEM	Dulbecco's Modified Eagle's Medium
IAP	Inhibitor of Apoptosis
IARC	International Agency of Cancer Research
RPMI	Roswell Park Memorial Institute Medium
PBS	Phosphate Buffered Saline
PI	Propidium Iodide
TTBS	Tris-Buffered Saline
TPEN	(N,N,N′,N′-Tetrakis (2-pyridylmethyl) ethylenediamine)
WHO	World Health Organization
K-S test	Kolmogorov-Smirnov
SPSS	Statistical Package for the Social Sciences

Declaration

I declare that this dissertation is my own original work has not been previously submitted for any degree in any other university. All the sources I have used or quoted have been indicated and acknowledged.

Mona O. M. Mohsen

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1. Introduction:

Cancer is the second leading cause of death worldwide. The International Agency for Research on Cancer indicated that the burden in 2012 was raised to 14.1 million incidence cases and 8.2 mortality cases (Siegel, 2013) . In the State of Qatar cancer accounts for 10% of all deaths (Qatar Cancer Registry, 2014). Qatar has a young population at present, whilst in 2010 the proportion of the population over 60 was just 2%, by 2050 this will increase to 20%. An ageing and growing population mean the incidence of cancer in Qatar will double by 2030 (Qatar Cancer Registry, 2014). The National Cancer Strategy was launched in 2011 with the goal that Qatar would become a world-renowned center of excellence in cancer research and care. Best practice in cancer treatment is constantly evolving as new research breakthroughs, this means the development of novel therapies that bring new treatment options to Qatar patients. Consequently, novel approaches are urgently required for further improvement in existing cancer therapies and for treating those cancers for which there are as yet no effective therapies. Synthesis of the pyridine ring system and its derivatives occupy an important place in the realm of synthetic organic chemistry, due to their therapeutic and pharmacological properties (Srivastava et al., 2007). They have emerged as integral backbones of over 7000 existing drugs. In the last few years, particular interest has been focused on 3-cyano-2(1H)-pyridone and its derivatives due to their wide range of practical uses as medicinal compounds. 3-cyanopyridines with different alkyl or aryl/heteroaryl groups were found to possess anti-cancer activity (Davari, 2014). Careful literature survey revealed that incorporation of alkoxy substituents (methoxy and/or aryloxy moieties) results in significant enhancement of several biological activities due to magnification of compounds' lipophilicity (Deslandes et al., 2012).

Hypothesis:

Based on previous studies of anticancer activities of pyridine derivatives, the current study attempts to identify novel and potent pyridine derivatives with minimal toxicity to non-tumorigenic counterparts.

Accordingly, this research aims to:

- Screen novel pyridine derivatives for anti-cancer activity against different cancer cell lines and select effective derivatives
- Test the effective derivative against non-tumorigenic counterparts for cytotoxicity
- Investigate the apoptotic and molecular mechanism of the potent derivatives

2. Literature Survey:

2.1.Cancer Background

Cancer is defined as the uncontrolled growth and metastasis of abnormally dividing cells beyond their boundaries; however, it is a complex class of disease triggered by expression and structural abnormalities of the coding and non-coding genes (Hanahan, 2011).

Hanahan and Weinberg (2011) argued that cancer shares some hallmarks acquired during the development of human neoplasms. The first six biological capabilities of tumor cells that rationalize the complexity of cancer are sustaining proliferative signaling, irresponsiveness to negative signaling, evading apoptosis, inducing angiogenesis, replicative immortality and the ability to invade and metastasize to other tissues. Three emerging hallmarks were added to the existing ones: genome instability is responsible for the genetic diversity in cancer; the reprogramming of the metabolic profile of cancerous cells; and the ability to evade the immune system. In addition to the above listed hallmarks, the ability of tumor cells to create a special environment called "Tumor Environment" adds to the complexity of cancer. "Recognition of the widespread applicability of these concepts will increasingly affect the development of new means to treat human cancer" (Hanahan, 2011).

2.2. Cancer Statistics

2.2.1. Worldwide Statistics

The International Agency for Research on Cancer (IARC) published on December 12, 2013 the latest data on cancer incidence and mortality for 28 types of cancers in about 184 countries worldwide .These statistics offer a comprehensive overview of the worldwide cancer burden. The burden in 2012 was raised to 14.1 million incidence cases and 8.2 deaths from cancer compared with 12.7 million and

7.6 million in 2008 respectively (American Cancer Society, 2014). Lung cancer was the most commonly diagnosed cancer worldwide accounting for (1.8 million, 13% of the total). Followed by breast cancer (1.7 million 11.9%) of total and colon-rectum (1.4 million 9.7%). Lung cancer caused the highest mortality (1.6 million, 19.4% of the total) followed by liver cancer (0.8 million, 9.1%) and finally stomach cancer (0.7 million, 8.8%). American Cancer Society estimated a substantive increase in the number of new cancer cases to 19.3 million yearly by 2025 and 24 million by 2035. This is due to the growth and aging of the global population (American Cancer Society, 2014).

About 1.7 million women were diagnosed with breast cancer in 2012, the incidence of breast cancer has increased by more than 20% while mortality has also increased by 14%, in 2012 about 522,000 deaths were recorded. Breast cancer is the most frequently diagnosed cancer among women in about 140 countries, representing one in four of all cancers in women. The incidence of breast cancer has increased in most world regions but remains higher in more developed regions however the mortality rate is higher in less developed countries due to lack of early detection and less treatment facilities Approximately 232,670 new cases of invasive carcinoma in situ are expected to be diagnosed in the United States of America in addition to about 62,570 new cases of carcinoma in situ only in 2014 (Siegel et al., 2013).

Colorectal cancer is the third most common cancer in both men and women. It is estimated that 96,830 colon cancer and 40,000 rectal cancer cases will be diagnosed in the United States of America in 2014 (American Cancer Society, 2014).

Ovarian cancer is a fetal cancer ranked the eighteenth most common cancer worldwide and the seventh among women, In 2012 about 239, 000 cases were recorded account for about 4% of all new cancer cases. The risk of ovarian cancer occurs usually before menopause and increases slowly after that. It is usually asymptomatic at the early stages, therefore it is usually diagnosed at advanced stages, with approximately low survival rates 30-50% (Siegel et al., 2013).

2.2.2. Qatar Cancer Registry

The "Cancer Incidence in Five Continents; volume X (CI5-X)" published by the International Agency for Research on Cancer (IARC) announced that cancer is considered the third cause of death in Qatar after , it is expected that the burden will nearly triple over the next 20 years (Qatar Cancer Statistics, 2014).

The Supreme Council of Health has estimated that 30% of cancer related deaths are caused by preventable risk factors. Furthermore, the incidence of cancer has increased by 22% in 2012 compared to 2011 with about 7.1 cases per 100,000 population. Cancer incidence among Qataris was 10.8 cases per 10,000 population while for non-Qataris it was 6.5 with a higher incidence in females than males. The most common cancers in the State of Qatar were breast cancer accounting for 18.9% of the total cancer cases, followed by bone marrow cancer by 8% and colo-rectal cancer by 7.3%. Breast cancer cases in the State of Qatar has increased dramatically by 41% since 2011. Table 2.2.2 represents total cancer cases in the State of Qatar in 2012 (Supreme Council of Health, 2012).

Table 2.2.2: Cancer cases in the State of Qatar 2012

	Total	Per 1,000 Population
Total	1,310	7.1
Male	703	5.2
Female	607	12.7
Qatari	281	10.8
Male	117	9.1
Female	164	12.5
Non-Qatari	1,029	6.5
Male	586	4.8
Female	443	12.8

The above measures were age standardized to reflect the average age-structure of all populations compared over the period of use; this would remove the effects of famine and historical events on the composition of age population. WHO new World Standard Population measures the percentage of the population in each 5-year age group (Qatar Cancer Statistics, 2014).

This standard will be adopted widely for global comparison to facilitate the analysis of international comparison of sets of age-specific epidemiological and demographic rates across populations with different age composition, and would reduce the confusion among data users (Jemal et al., 2011).

The incidence of cancer does not drop over the past years with most complicated cases in the developing countries, it is estimated that deaths of cancer will overcome the deaths of cardiovascular diseases (Siegel et al., 2013).

2.3. Chemotherapeutic Agents

Chemotherapy refers to a group of agents act by damaging-DNA, and inducing programmed cell death or what is called apoptosis (Palmieri, 2013). The origin of chemotherapy goes back to 1943 upon the bombing of the American warship in Bari, Italy. This incidence led to the release of a large cloud of mustard gas, thousands of people died, however survivors suffered from bone marrow cells depletion and severe anemia (Weinberg, 2014). Research on mustard gas had begun earlier in 1942 at Yale University when Gustaf and Gilman discovered that intravenous doses of mustard gas could cause significant regression in lymphoma. Furthermore, other alkylating agents were successful in treating glioblastomas (Weinberg, 2014).

Chemotherapeutic agents can be classified according to the mode of action. For example, antimetabolites such as Methotrexate, 5-Flourouracil, Gemcitabine and Clardibine interfere with the synthesis of purines and pyrimidines in the cell and compete with the synthesis of DNA or RNA. Another group is the alkylating agents which form covalent bonds with different cellular components such as DNA. Cyclophosphamide, ifosphamide, melphalan and temozolamide are examples of alkylating agents (Siegel et al., 2013). Microtubule inhibitors disrupt the process of mitosis by interfering with the mitotic spindles and the equilibrium between polymerization and de-polymerization. Taxanes and Vinca alkaloids are examples of this category. The last category is the cytotoxic antibiotics such as Doxorubicin, Dactinomycin, Epirubicin and Bleomycin (Siegel et al., 2013).

The European Organization for Research and Treatment of Cancer has graded and scored the side effects of the chemotherapeutic agents into acute and chronic toxicity. The acute toxicity includes

nausea, vomiting, diarrhea, bone marrow suppression, mucositis, alopecia, peripheral neuropathy, gastrointestinal disturbance, thrombocytopenia and anemia. The chronic toxicity involves pulmonary fibrosis, infertility, cardiac failure and secondary cancer (Palmieri, 2013).

The development of Acute Myeloid Leukemia and lymphoblastic leukemia are the most common cancers developed after the treatment with chemotherapeutic agents. It has been found that alkylating agents would increase the risk of leukemia after 2 years of treatment; the leukemic cells tend to exhibit specific mutations with poor prognosis (Palmieri, 2013).

"In addition to their cytotoxicity, these agents are highly mutagenic, leaving the genomes of exposed cells with hundreds if not thousands of point mutations. This reveals another side of a number of anti-cancer treatments. In addition to their effects in reducing or eliminating tumors, x-rays and certain cytotoxic agents are also carcinogenic and their short-term success in producing clinical remissions may be counterbalanced by the appearance years later of independently arising, second site tumors that are consequences of their mutagenic actions" (Weinberg, 2015). Hannun (1997) has argued that the effectiveness of chemotherapeutic agents is significantly limited by the terrible side effects or their effect on normal cells.

2.4. Chemotherapeutic Agents and Apoptosis

Previous research on the chemotherapeutic agents have shown that these agents exert their anti-tumor activity mainly in three phases. The first phase is the interaction between the agent and its target in the cell through which it has been designed to exert its effect (Hannun, 1997).

The second phase includes the signaling transduction pathways and the third phase is the programmed cell death or apoptosis as illustrated in (Figure 2.4.1.). (Hannun, 1997).

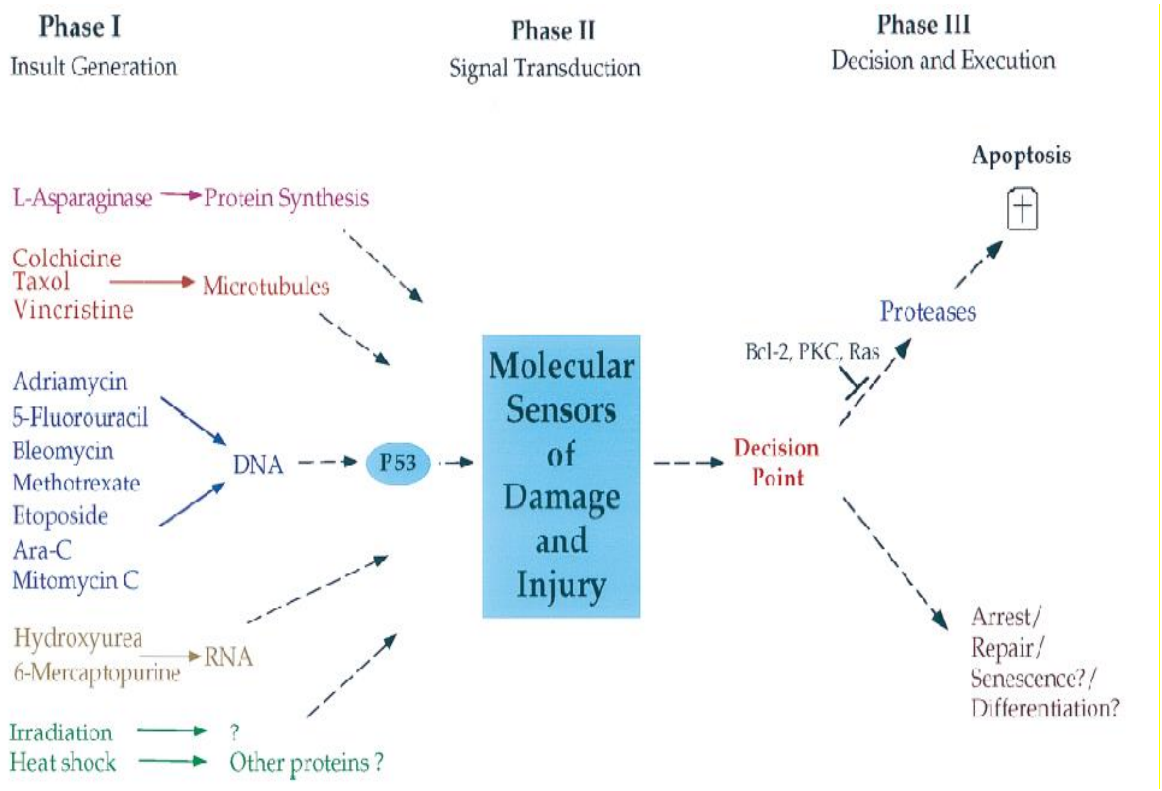

Figure 2.4.1. Pathway of Chemotherapeutic agents in inducing apoptosis through three phases.
Phase I "Insult Generation" by which each chemotherapeutic agent exerts its effect– Phase II "Signal Transduction" due to the molecular sensors of damage and injury and finally Phase III "Decision Execution" by inducing apoptosis.
(Hannun, 1997)

Apoptosis is a general process that occurs in an astonishing rate in the body, billions of cells go into apoptosis in the bone marrow and intestine every hour and more than half of the nerve cells die after formation in the nervous system. The cells in the developing nervous system die by apoptosis to adjust the number of the nerve cells to match the target cell number required for innervation (Mattern, 2004).

Apoptosis has distinctive morphological features including shrinkage of the cell, condensation of the chromatin and blebbing of the plasma membrane. The most common characteristic of apoptosis is pyknosis a result of irreversible condensation of the chromatin. The apoptotic cell appears as a round or oval mass with condensed nuclear chromatin and dark eosinophilic cytoplasm. Blebbing of the plasma membrane without lysis occurs next followed by karyorrhexis, a process of cellular degeneration involving the fragmentation of the nucleus (Wyllie, 2010).

The apoptotic bodies consist of tightly packed cytoplasmic organelles with or without nuclear materials, bound by a membrane. The plasma membrane in apoptotic cells remain intact presenting several epitopes (Wyllie, 2010). These epitopes attract phagocytes and macrophages, which will digest the apoptotic cells and prevent the initiation of inflammatory reaction or secondary necrosis (Elmore, 2007).

The intracellular machinery of apoptosis depends on a family of proteases that have cysteine in their active site and they cleave their protein targets at specific aspartic acid residues. These proteases are called caspases. Caspases are initially synthesized in the cell as pro-caspases or inactive precursors and activated by cleavage at aspartic acids or by different caspases (Golstein, 1997).

This process results in activation of other pro-caspases ending in an amplifying proteolytic cascade. The activated cascades will then activate other proteins found in the cell such as nuclear lamina causing irreversible breakage of the nuclear lamina, it also cleaves a protein that holds DNA-degrading enzyme, allowing the cell to dismantle itself neatly and quickly. This protease cascade is a non-destructive, self-amplifying and irreversible as well (Golstein, 1997).

Pro-caspases activated in the cell by binding to an adaptor protein, all nucleated cells contain pro-caspases require an activation signal to initiate the destruction of the cell and apoptosis, the caspase activity is tightly regulated by the cell to control the programmed death until needed (Hotta et al., 2003).

The activation of pro-caspases is triggered by different adaptor proteins, these adaptor proteins act by aggregating the initiator pro-caspases in a complex. The protease activity of the initiator pro-caspases is small but aggregation will trigger their activation and cleave each other or cause a conformational change that will initiate the pro-caspase. The activated caspase within moments will act to cleave the downstream pro-caspase to amplify the death signal and spread out to the entire cell (Fulda, 2015).

Furthermore, the activation of pro-caspase triggered from outside of the cell by activating specific cell surface receptors known as death receptors (Figure 2.4.2.). For example, killer lymphocytes can induce apoptosis when producing a specific protein called Fas ligand, this protein binds to the cell death receptor. Fas receptor protein on the surface of the target cell will act to recruit intracellular adaptor proteins bind and aggregate to pro-caspase 8 molecule, activating a downstream pro-caspases and inducing apoptosis (Fulda, 2015).

It is known that some damaged or stressed cells will produce Fas receptor and ligand to kill themselves and trigger the intracellular caspase cascade. Also when cells are under stress or damage they can kill themselves by triggering the aggregation and activation of pro-caspases within the cell “the Intrinsic Pathway” as illustrated in (Figure 2.4.2.). This is mainly done when the mitochondria is induced to

release cytochrome c -which is the electron carrier protein- into the cytosol, this activity will result in the activation of an adaptor protein called Apaf-1 (Fulda, 2015).

This pathway of activating the pro-caspases recruited in most forms of apoptosis leading to an amplifying and accelerating caspase cascade. Caspase-3 is a vital cysteine protease in the intrinsic apoptosis execution phase, when apoptosis is triggered caspase-3 acts by cleaving ADP-ribose polymerase PARP and SREBP (Fulda, 2015).

p53 is activated when DNA damage occurs in the cell, p53 will activate transcription genes that encode several proteins that belong to the Bcl-2 family and promote and release cytochrome c from the mitochondria (Fulda, 2015).

Bcl-2 family proteins and IAP proteins are considered the main intracellular regulators of apoptosis and the cell death program as they regulate pro-caspases, however, some members of the Bcl-2 family such as Bcl-2 itself and Bcl-X_L inhibit apoptosis as they block the release of cytochrome c from mitochondria. Other members of the Bcl-2 family will induce apoptosis by promoting pro-caspase cell death and activation, they are not considered as death inhibitors. An example of these promoters of cell death is Bad, which binds and inactivates the death inhibiting members of Bcl-2 family, while Bax and Bak will function by stimulating the release of cytochrome c from the mitochondria, Bax and Bak are crucial in inducing apoptosis in the cells, a mutation in the genes encoding these proteins will cause resistance to most apoptosis-inducing stimuli. Bax and Bak activated by Bid, an apoptosis-promoting member of the Bcl-2 family (Fulda, 2015).

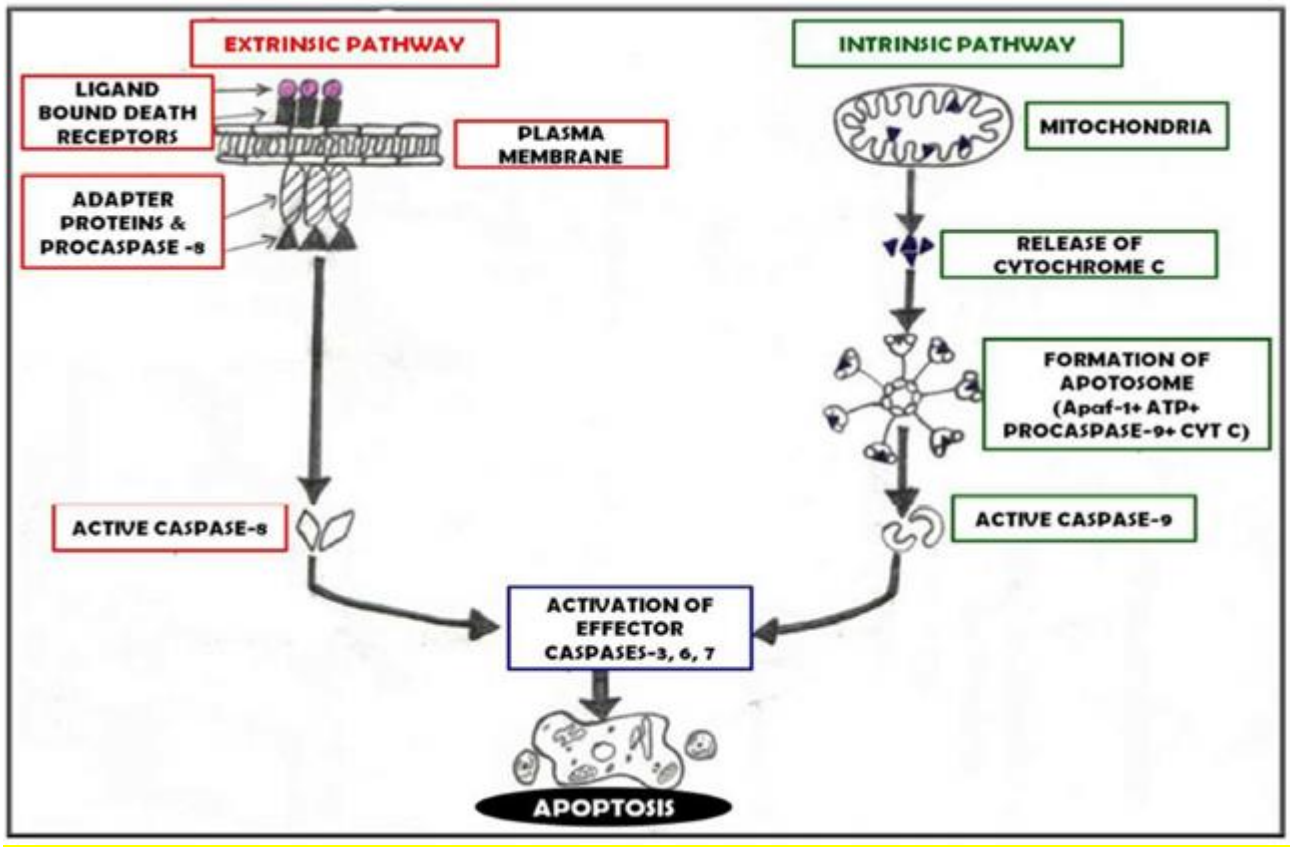

Figure 2.4.2.: Apoptotic Pathway, Intrinsic and Extrinsic Pathways regulating Apoptosis
Induction of apoptosis can be initiated through the intrinsic or extrinsic pathways
(Jain, 2014)

IAP (Inhibitor of Apoptosis) is another important family of the intracellular regulators, IAP were discovered originally as proteins produced by insect viruses; the viruses use them to prevent the infected cell from going into apoptosis and therefore give the virus enough time to replicate. They function to inhibit apoptosis in two ways: either by preventing the activation of by binding to some caspases or by binding to caspases to inhibit their activity (Fulda, 2015).

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The mitochondria releases cytochrome c which will activate Apaf-1, it also releases a protein which will block IAPs; this will increase the efficiency of the process of death activation. Intracellular cell

death program can also be regulated by extracellular signals can inhibit or activate apoptosis, these extracellular signals act by regulating the activity or level of Bcl-2 and IAP family members (Labi, 2015).

2.5. Pyridine

2.5.1. Chemical Properties

Pyridine "azabenzene" an aromatic compound with the chemical formula C_5H_5N is a heterocyclic organic compound –figure 2.5.1., related to the benzene by replacing one methane group (=CH-) with more electronegative nitrogen, this will allow denoting a shift of electron density from the ring towards the nitrogen atom (Dorigo et al., 1993).

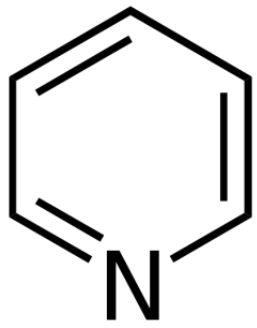


Figure 2.5.1: Pyridine Ring.
Pyridine Derivatives have the chemical formula C_5H_5N
(Dorigo et al., 1993).

Pyridine ring found in several compounds such as Azines (six-membered heterocyclic compounds group contains one or more nitrogen atoms in the ring and vitamin niacin, pyridoxal (vitamin B6) and in highly toxic alkaloids such as nicotine shows the evidence of the potent activity of the Pyridine (Abdel-Aziz et al., 2005).

In 1849 the Scottish chemist Thomas Anderson discovered Pyridine as a constituent of bone oil and coal tar, two years later he was able to isolate pure pyridine by the fractional distillation of the oil (Li et al., 1999). Korner and Dewar recognized that the cyclic nature of Pyridine in 1869 plays an essential role in catalyzing both their chemical and biological systems. Pyridine is the prosthetic nucleotide (NADP) which is involved in different oxidation-reduction processes (Abdel-Aziz et al., 2005)

2.5.2. Pyridine Derivatives

Pyridine derivatives obtain by acylation, alkylation, oxidation and nucleophilic substitution. Acyl chlorides or acid anhydrides react with pyridine to form N-acylpyridinium salts can be used as valuable transcyclating agents in alcohol. Pyridine used as a reagent and solvent. Pyridine also reacts with alkylating agents to form N-alkylpyridinium salts which can be isolated as crystalline solids, these compounds are much stable than their equivalents N-acylpyridinium (Srivastava et al., 2007).

Researchers frequently use Pyridine N-oxides to facilitate the electrophilic substitution ; this type of reaction has a balance between electron withdrawal and release in the oxygen atom in the opposite direction. Pyridine also reacts with nucleophiles and the attack usually occurs at C-2(6) and/or C-4 by inductive effect, another classic reaction for Pyridine is Chichibabian amination which will lead to 2-aminopyridine (Kim et al., 2010). Several substitutes attached to the pyridine ring have the similar chemical behavior of the corresponding groups in benzene, the most commonly encountered Pyridine derivatives are: Methylpyridines (Picolines), Pyridones (Hydroxypyridines), Aminopyridines and Pyridine salts (Abadi et al., 2009).

Pyridine ring system and its derivatives have been found to be an important and versatile nucleus in the pharmaceutical field and considered as the panacea and solution for the control and management of

various diseases, it forms more than 7000 existing drugs in this industry. Recent research have found that pyridine and its derivatives have large and wide biological activities which include anticancer, antiviral, ant diabetic and antimicrobial etc (Allen et al., 2006). 4-(phenylamino) thieno[2,3-b]pyridine derivatives showed a potent inhibitory activity against Herpes Simplex Type I, while 4-(phenylamino)-1H-pyrazolo [3,4-b] pyridine has shown different biological activities against HIV and Vaccı´nia virus. A well known pharmaceutical company synthesized 1-(β-D-ribofuranosyl)-pyridine-2-thiones to treat HIV infectious diseases, while another company synthesized imidazo [1,2-a]pyrrolo [2,3-c] pyridine series as an active agent against the swine fever virus (Bernardino et al., 2012).

Pyridine derivatives have also shown antichagasic activity against Chagas' disease, a parasitic infection caused by Trypanosoma cruzi, this parasite is widely spread in central, and South America. It is estimated about 20 million people are chronically infected with T. cruzi and about 100 million are at risk (Dorigo et al., 1993). Dorigo (1993) synthesized a pyridine derivative, which has anti chagasic activity.

Researchers have also discovered antioxidant activity such as Thiazolo [3,2-a] pyridine derivatives which was synthesized by Feng shi et al., this derivative scavenges free radicals. In addition pyridine derivatives has an antiviral and antifungal activity, Ivachtchenko synthesized 2-imino-5-hydroxymethyl-8-methyl-2H-pyrano[2,3-c] pyridine-3-(N-aryl) carboxamides which is a novel drug has an anti-viral and anti-fungal activities (Dorigo et al., 1993).

Pyridine derivatives show anti-cancer activity as shown in figure 1.4, Epothilones with a nitrogen atom at ortho position and methyl substitution on pyridine ring exhibited a cytotoxic properties against

several types of human cancel cell lines. 2,6-diaryl substituted pyridine showed cytotoxic and topisomerase I inhibitory activity against different human cancer cell lines (Wang, 2005).

Heyakawa has synthesized a series of pyridine derivative called (imidazo[1,2-a]pyridine derivatives), thiazole derivative showed potent inhibition against p110a and strong selectivity for p110a over PI3K isoforms. It has also inhibited tumor growth by inhibiting PI3Kp110a which is now considered as a potential target in anti-cancer treatment (Cocco et al., 2005).

2-amino-3-(3',4',5'-trimethoxybenzoyl)- 6-substituted-4,5,6,7-tetrahydrothieno [2,3-c]pyridine derivatives was synthesized by Romagnoli. This compound showed potential and promising anti-tumor and anti-proliferative activity by affecting the cell cycle and inhibiting tubulin polymerization (Cocco et al., 2005).

This derivative was tested against a panel of four different cell lines, which interacts strongly with colchicines site and tubulin. Furthermore, a novel oral indoline sulfonamide was synthesized by *Lowe et al* based on the disruption of the microtubules and thus exhibit a potent activity against cancer in human both in vitro and in vivo (Lowe, 1993).

Recently Pyridine and its different derivatives showed strong cytotoxicity against several human cancer cell lines (Attia, 2003). Some Pyridine derivatives such as Pyrazolo[4,3-*d*]pyrimidine analogue and pyrazolo[1,5-*a*] pyrimidine showed potent inhibition of tyrosine kinase and (CDK) cycline-dependent kinases which are known to be involved in cellular events and transmission of mitogenic signals in the cell which will lead to uncontrolled proliferation, differentiation, immune response and metabolism. This pyridine derivative is responsible for blocking proliferation of different type of

cancer cell lines (Ruebsam et al., 2009). A novel study showed that 8-methyl-2-(morpholine-4yl)-7-(pyridine-3-methoxy)-4H-1,3-benzoxacine-4-one (LTU27) has the ability to sensitize cancerous cells specifically lung cancer and colon cancer to radiation effect, treating the cells with both radiation and LTU27 increased the apoptosis and inhibited the auto-phosphorylation of DNA-PK and AKT1, LTU27 acts by inhibiting DNA-PK promoting apoptosis by delaying DNA repair (Radhamani et al., 2014).

Another Pyridine derivative with potent anti-proliferating effect is 4-thiazolidinone-, pyridine and piperazine-based conjugates. This compound exhibited good activity against human leukemic cell lines. The mechanism by which this compound worked is by inducing cell cycle arrest and depolarization of the potential of the mitochondrial membrane (Hazra et al., 2013).

2.5.3. Cyanopyridine

In the last few years, particular interest has been focused on cyanopyridine due to its diverse pharmaceutical activity (Lin et al., 2005), it has also been employed in the industrial production of nicotinic acid, nicotinamide and in several organic synthesis (Hazra et al., 2013). Cyanopyridine can fuse to different functional groups to form compounds with broad spectrum of pharmacological activity (Hazra et al., 2013)

One of the most potent cyanopyridine derivatives is 2-oxo-3cyanopyridine a nucleus analogous to alkaloid Ricinine considered as the first alkaloid with cyano group. This compound has captured the researcher's attention after the synthesis and study of the Milrinone a non-Glycosidiccardiotonic agent is an inhibitor of Dipyridinephosphodiesterase (Hazra et al., 2013).

Another important cyanopyridine derivative is 3-cyano-2 Pyridone. This derivative has shown promising and potent anti-cancer activity which is known to be due to its interference with PDE3, P1M1, Kinase and Survivin proteins. The 3-cyanopyridines compounds with different alkyl or aryl/heteroaryl groups were found to possess anti-tubercular, antimicrobial, anti-cancer, antihypertensive, anti- inflammatory as well as antiviral activity against herpes virus and human immunodeficiency virus (Radhamani et al., 2014).

Careful literature survey revealed that incorporation of alkoxy substituents (methoxy and/or aryloxy moieties) results in significant enhancement of several biological activities due to magnification of compounds' lipophilicity (Radhamani et al., 2014). In addition to 3-cyano-2(1H)-pyridone, pyridine-2-thione and pyrazolopyrimidine derivatives received considerable attention due to their activity as anti-cancer, anti-inflammatory, anti-mycobacterial, anti-fungal and anti -viral drugs (Radhamani et al., 2014).

2.6. Pyridine Derivatives and Apoptosis

Apoptosis is not associated with the activation of the immune response; considered a desirable feature in anti-cancer drugs. The intrinsic apoptotic pathway or the mitochondrial pathway activated upon cellular stress signals resulting from DNA damage, loss of cell survival factors and cell cycle failure (Sharath Kumar et al., 2014).

Research has found that micronuclei can be induced following the exposure to clastogenic or aneugenic agents and can be used as a sensitive tool in assessing genotoxicity; studies have shown that micronucleated cells can be eliminated by the apoptotic process involving caspase 9, 8 and 3. A study

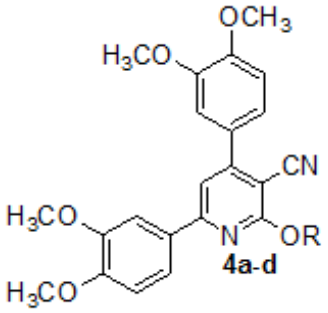
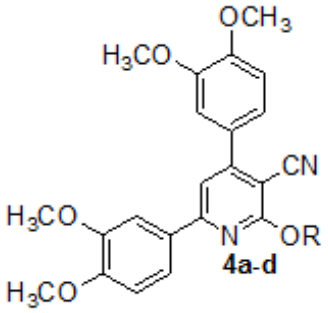
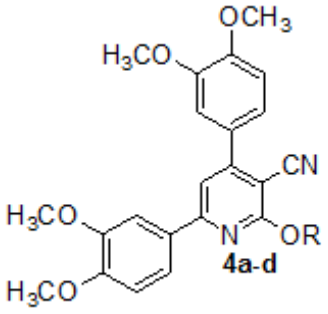
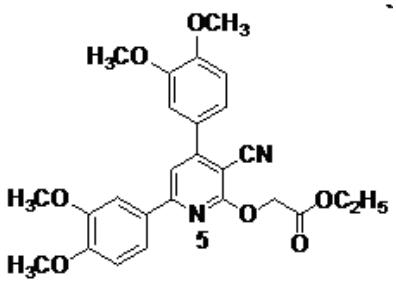
entitled (synthesis and biological evaluation of novel 3-alkylpyridine marine alkaloid analogs with promising anti-cancer activity) in 2014 has shown that novel 3-APA -3 alkylpyridine alkaloid have a promising anti-cancer activity by inducing apoptosis in tumor cell lines and altering the cellular actin cytoskeleton of colon cancer cell lines (Deslandes et al., 2012). Phosphodiesterases such as PDE3, PDE4 and PDE5 are over-expressed in cancerous cells when compared to normal cells, therefore inhibiting phosphodiesterases will lead to the inhibition of tumor proliferation and angiogenesis (Deslandes et al., 2012).

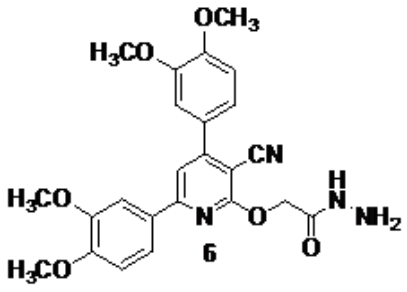
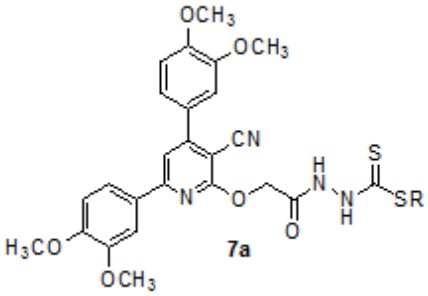
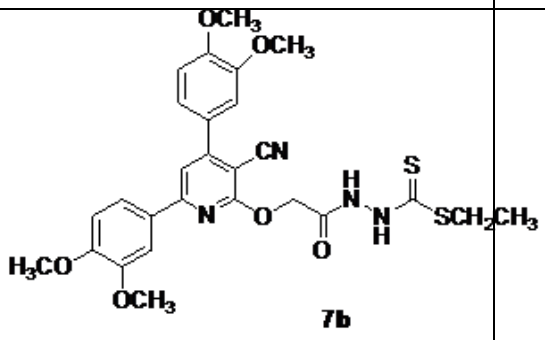
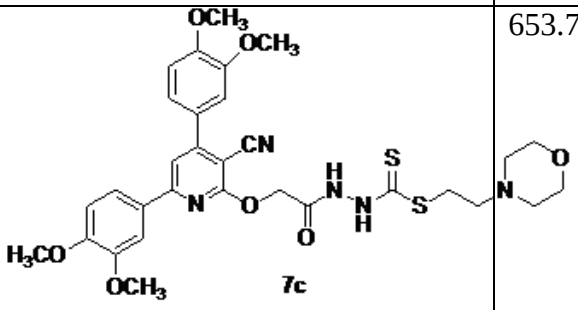
It has been found that inhibition of PDE3 inhibited the uncontrolled growth of HeLa squamous cell carcinoma cell line and HSG cell lines. Cilostamide, which is another pyridine derivative has a selective PDE3 inhibitor was found to have synergism action to anti apoptotic activity of PDE4 inhibitors in leukemic cells. It has been reported that PDE5 inhibitors would increase the sensitivity of certain cancer types to the convention chemotherapeutic drugs (Abadi et al., 2009).

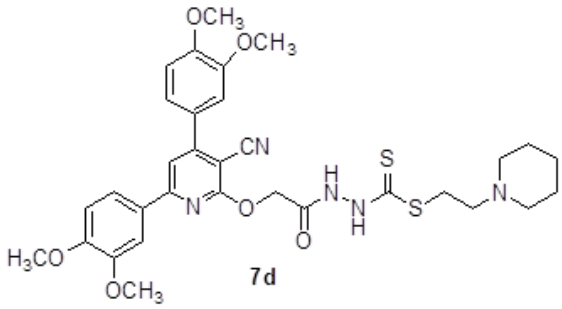
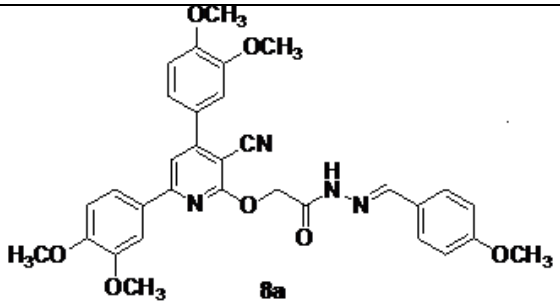
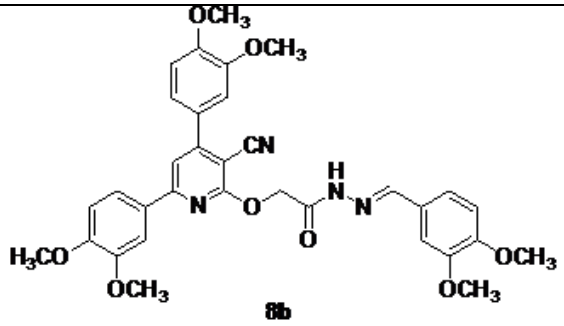
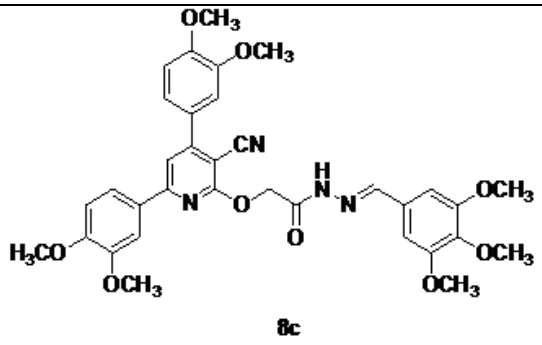
2-oxo-3-cyanopyridine derivative, 4,6-diaryl-2-oxo-1,2-dihydropyridine-3-carbonitriles has shown significant inhibition to PIM-1 (Proto-oncogenic encodes for serine/ threonine kinase) which is over expressed in variety of cancers cells (Lin et al., 2005). Furthermore, 2-oxo-3-cyanopyridine can inhibit Survivin which is a protein encoded by BIRC5 gene and which is known to be over expressed in some cancers, Survivin is also a member of IAP, increase levels of Survinin in tumor cells is associated with poor prognosis and low survival rates (Gundugola et al., 2010).

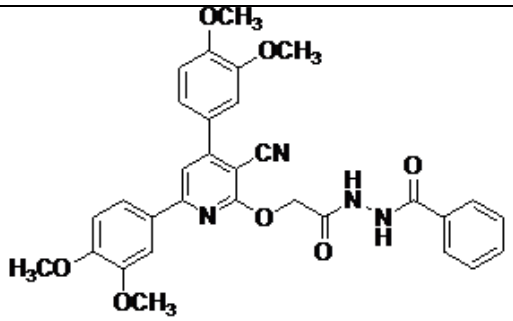
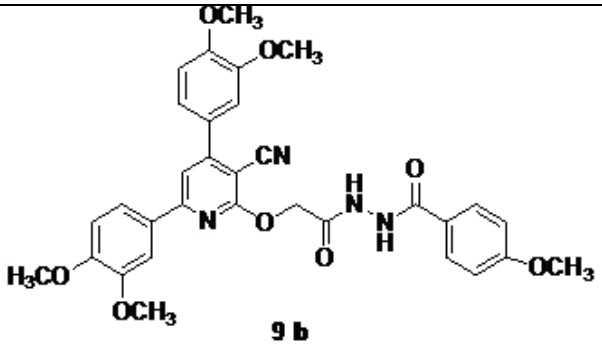
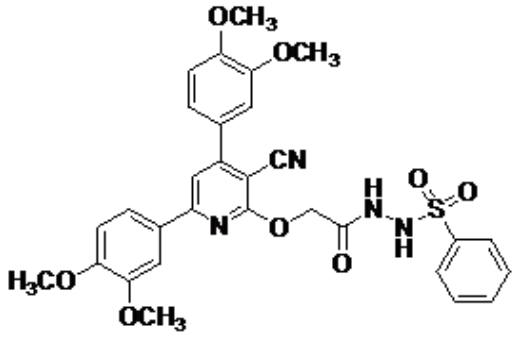
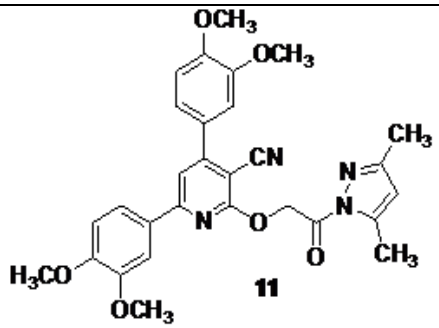
3. Materials:

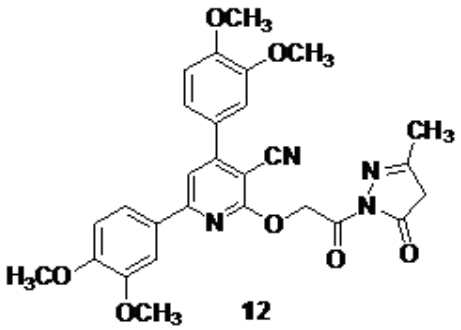
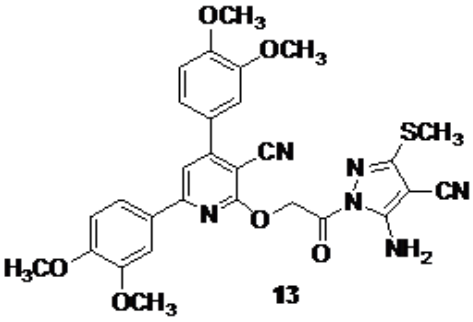
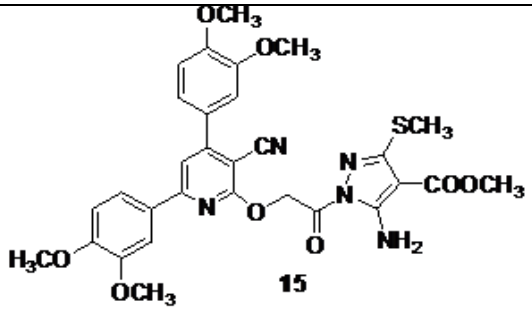
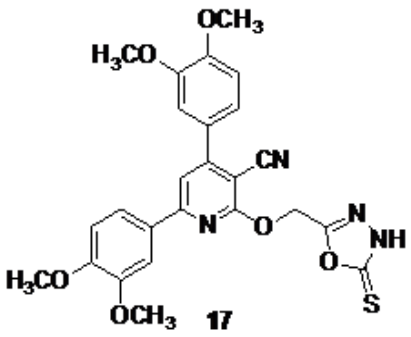
3.1. Pyridine Derivatives:

Compound No.	Structure	Molecular Weight	R Group
4a		406.43	CH3
4b		420.46	C2H5
4d		448.47	CH2COCH3
5		478.49	-

Compound No.	Structure	Molecular Weight	R Group
6		464.47	-
7a		554.64	CH3
7b		568.66	C2H5
7c		653.77	Morpholinoethyl

Compound No.	Structure	Molecular Weight	R Group
7d		651.8	-
8a		582.6	4-OCH3C6H4
8b		612.63	3,4-OCH3C6H3
8c		642.66	3,4,5-OCH3C6H2

Compound No.	Structure	Molecular Weight	R Group
9a	 9a	568.6	C6H5
9b	 9 b	568.58	4-OCH3C6H4
10	 10	604.63	-
11	 11	528.5558	-

Compound No.	Structure	Molecular Weight	R Group
12		530.5286	-
13		586.6183	-
15		619.645	-
17		506.5304	-

3.2. Cell Lines

No	Cell Lines	ATCC
1	MCF-7	HTB-22
2	MDA-MB-231	HTB-26
3	HCT-116	CCL-247
4	SK-OV-3	HTB-77
5	OVCAR-3	HTB-161
6	MCF-12A	CRL-10782

3.3. Chemicals used for cell culture

No	Reagent	Company
1	DMSO	SIGMA
2	Ethanol 95%	-
3	Feto Bovine Serum (FBS)	-
4	Dulbecco's Phosphate Bufferred Saline (PBS)	SIGMA-ALDRICH, Co.
5	Dulbecco's Modified Eagle's Medium (DMEM)	SIGMA-ALDRICH, Co.
6	RPMI medium (1640) 1X	Gibco – Life Technologies
7	Penicillin/Streptomycin 1%	ThermoFisher Scientific
8	Trypsin 1x	-
9	WST-1	Roche Diagnostics GmbH

3.4. Chemicals used for Cell Proliferation Assay

No	Reagent	Company
1	WST-1	Roche Diagnostics GmbH

3.5. Chemicals used for Flow Cytometry Analysis

No	Reagent	Company
1	RNase	SIGMA-ALDRICH, Co.
2	PI “Propidium Iodide”	SIGMA-ALDRICH, Co.
3	Annexin V-FITC Apoptosis Detection Kit	SIGMA-ALDRICH, Co.

3.6. Chemicals used for Western Blot

No	Reagent	Company
1	P53 Monoclonal Antibody	ABCAM
2	Bcl-2 Monoclonal Antibody	ABCAM
3	PBS	SIGMA-ALDRICH, Co.
4	P21 monoclonal Antibody	ABCAM
5	Bax Monoclonal Antibody	ABCAM
6	Mdm-2 Monoclonal Antibody	ABCAM
7	Akt Monoclonal Antibody	ABCAM
8	Pro-Caspase-3 Monoclonal Antibody	ABCAM
9	Caspase-3 Monoclonal Antibody	ABCAM
10	30% Acrylamide Mix	Sigma
11	1.5M Tris (pH 8.8)	-
12	10% SDS	Life Technologies
13	10% APS	Life Technologies
14	TEMED	Sigma
15	10% Protease Inhibitor	Sigma
16	PMSF (100mM)	Sigma
17	RIPA Lysing Buffer	Sigma
18	Chemiluminescence Reagent	Life Technologies
19	Secondary Anti-Rabbit Ig	Sigma

4. Methods:

4.1. Cell Line and Cell Culture:

Studying different cell lines is important in determining if the effect of pyridine derivatives is cell or tumor-type specific, tumor type specific. The following cell lines were used for the screening stage, obtained from the American Type Culture Collection (ATCC, Menassas, Virginia, USA):

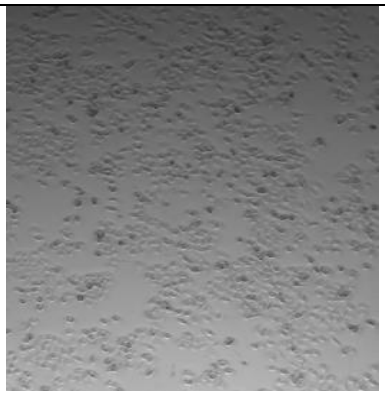
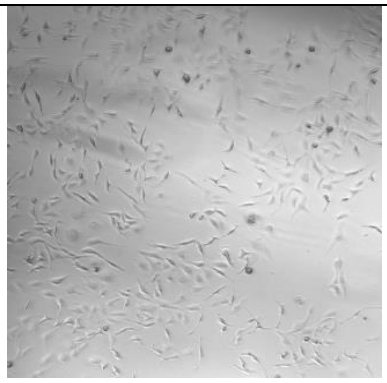
- MDA-MB-231 (ATCC HTB-26) is an adherent epithelial mammary/gland breast cells derived from metastatic sites of pleural effusion from a 51 years female adult with adenocarcinoma.
- MCF-7 (ATCC HTB-22), adherent epithelial cells of the mammary gland/breast from metastatic site of pleural effusion but derived from 69 years old Caucasian female adult.
- HCT-116 (CCL-247) is adherent epithelial cells derived from an adult male with colorectal carcinoma; this cell line has a mutation in RAS proto-oncogene in codon 13.
- SK-OV-3 (HTB-77) is adherent ovarian epithelial cells derived from 64 years old Caucasian female with adenocarcinoma
- OVCAR-3 (HTB-161) is adherent ovarian epithelial cells derived from 60 years old Caucasian female respectively with adenocarcinoma and is a suitable ovarian cell model to study drug resistance in ovarian cancer.
- MCF-12A (ATCC CRL-10782) is adherent epithelial mammary cells derived from 60 years old Caucasian female.

Sub-culturing of the above cell lines was conducted in 75cm^2 flasks, the old culture media was removed and discarded after assessing the confluency of the cells, the cells were rinsed with 2-3 ml diluted Trypsin 1x (Trypsin/EDTA solution consist of 0.025% trypsin and 0.01% EDTA in (PBS),) for

about 5 minutes for complete detachment. The cells were observed under the inverted microscope until the cell layer is completely dispersed. 75cm^2 flasks were prepared and 16 ml of DMEM media for MCF-7, MDA-MB-231, T-47D, SK-OV-3 and OVCAR-3 (DMEM 4500mg Glucose) and RPMI media for HCT-116 were added to each flask, the cells were aspirated gently and added to the flasks. DMEM 4500 mg/L high glucose with 10% FBS and 5% Streptomycin and Penicillin, RPMI 1640 was prepared by adding 5% heat-inactivated FCS and 2 mM glutamine. DMEM was also used for MCF-12A cells with the addition of human epidermal growth factor 20ng/ml, insulin 0.01 mg/ml and hydrocortisone 500ng/ml.

The cells were incubated for 3 days before propagation to treatment at 37°C with 5% CO_2 and 95% air for stabilization. The medium was renewed 2-3 times a week. Further sub-culturing were carried out at a ratio of 1:3 to 1:6 according to the confluency of the cells. Usually it was done when cells reached 75-85% confluence after 3 days. For splitting the medium was aspirated and cells were incubated for 5 minutes with trypsin, cells were then cultured in the desired dilution into new flasks, growing cells were used for all experiments.

Cell Line Culture	Image
a. MCF-7	

b. MDA-MB-231	
c. HCT-116	
d. OVCAR-3	
e. SK-OV-3	

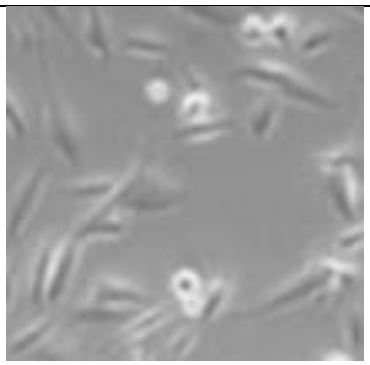
f. F. MCF-12A	

Figure 4.1. Selected Cancer Cell Lines Screened in the Study. a. MCF-7, b. MDA-MB-231, c. HCT-116, d. OVCAR-3 & e. SK-OV-3

4.2. Cell Count using Hemacytometer:

Hemocytometer is a counting chamber device used to perform cell count by measuring the concentration of cells in a suspension. The central area of the instrument is defined by a set of grooves that form "H" shape, two counting areas with ruled grids are separated by a horizontal groove of the "H" each counting area consist of 9 squares (1.0 mm X 1.0 mm) covered by a glass cover-slip at 0.1mm above the surface of the counting area. Cell count is down by adding 10 µL of cell suspension to the counting chamber in each slide; in WST-1 Assay a cell count of about 50,000 cells/mL was achieved.

4.3. Drug Stock Preparation:

- Twenty Pyridine Derivatives compounds numbered {4a – 4b – 4c – 4d – 5 – 6 – 7a – 7b- 7d- 8a – 8b – 8c – 9a – 9b – 10 – 11- 12- 13 -15 - 17} were provided to investigate their role as novel anti-cancer compounds.
- Compounds as illustrated in the material section were prepared for further assays.
- The next step was optimization of the compounds, pyridine derivatives are organic highly hydrophobic compounds, and therefore DMSO was used as the best organic solvent. A

concentration of 0.4% DMSO was used in all experiment after ensuring 100% viability of the cell lines with this concentration. 25mM stock concentration of all compounds were prepared for further studies and analysis.

- TPEN (N,N,N′,N′-Tetrakis(2-pyridylmethyl)ethylenediamine) was used as positive control in tests. TPEN is a lipid soluble compound with high affinity to heavy metal including (Zn^{2+}-Fe^{2+}-Mn^{2+}), TPEN permeate plasma membranes and induce apoptosis through the activation of caspase-3 (Santa Cruz Biotechnology, 2015).

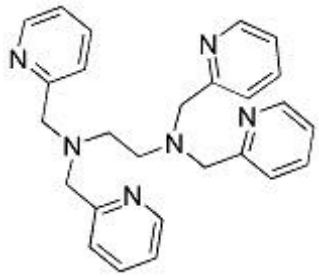


Figure 4.2. TPEN (N,N,N′,N′-Tetrakis(2-pyridylmethyl)ethylenediamine) Structure
http://www.scbt.com/datasheet-200131-tpen.html

4.4. Cell Viability Assay (WST-1 Assay):

WST-1 is a clear, slightly red and ready to use cell proliferation reagent, the reagent contains WST-1 and electron reagent diluted in phosphate buffered saline and filtered for use. WST-1 assay has several applications including the analysis of cytotoxicity by anti-cancer drugs and other types of pharmaceutical compounds. The assay principle is based on cleaving Tetrazolium salts to Formazan by the cellular enzymes of viable cells; an increased number of viable cells indicates an increased activity of mitochondrial dehydrogenases. Therefore, the increase in Formazan dye formation can directly be linked to the number of metabolically viable active cells in the culture. WST-1 has recently show better results and is easier to conduct than MTT Assay in which the MTT reagent will be cleaved

to water insoluble Formazan crystals and therefore requires solubilization after cleavage, while WST-1 yields water-soluble products can be measured directly without the need for extra solubilization step.

WST-1 was carried out as follows: the cells were trypsinized and put in 15ml falcon tube, the cells were centrifuged for 3 min at 1000rpm. 1x Trypsin is decanted using a sterile pipette and then the pellet is loosened by flicking the tube few times, 5-10 mL warm DMEM or RPMI were added to the falcon tube and the pellet is re-suspended. Cell count was done next by taking 10uL of the suspension into the cell counting slide and read under 10X cells/ml. A cell stock of 50,000 cells/ml was prepared for the 96 wells. 100uL of cells added to each well using a sterile pipette each time to ensure consistency. The experiment was planned ahead of time using excel sheet, 3-6 wells were reserved as media blank while 3-6 wells for vehicle (DMSO). The plate incubated for 24 hours for drug treatment step.

DMEM media was used to prepare different dilutions of the compounds. The old DMEM media was removed from the wells using sterile pipette in the hood, the media was replaced with 100 uL media with each compound. The plate was incubated for 24 hours. After 24 hours incubation WST-1 reagent was added to each well and then the plate was incubated for 1 hour before reading using spectrophotometer (VICTOR Multilabel Plate Reader).

The absorbance of this colored solution can be quantified by measuring at a certain wavelength (usually between 500 – 600 nm), in this experiment the first reading was conducted at 540 nm and the second reading at 690 nm for the reference wavelength.

4.5. Morphological Examination:

Morphological examination was performed as described previously; DMA-MB-231, MCF-7, HCT-116, SK-OV3 and OVCAR-3 cells were plated on 96 well plates at $5*10^4$ cells/well; and allowed to reach 75-85% confluence. The compounds were added to the medium, untreated cells were used as negative control, in addition to blank cells (medium) and vehicle which consist of (DMSO 0.4% + cells), TPEN was used as a positive control. After the treatment all the cells were incubated for 24 hours in atmosphere containing 5% CO2 for 24h. After 2 hours of treatment, the morphological changes of the cells were examined and photographs were taken using an inverted microscope at 400X magnification microscopy.

4.6. Dose Response Curve:

The Dose Response Curve relationship is essential to describe the change in the cell lines by different levels of doses after a certain time exposure; it is often referred to as a "graded" response because the measured effect is continuous over a range of doses. Studying and developing the dose response curve is essential to determine the safe and hazardous levels of the drug, in addition it identifies the acute toxic effect of the drug and potential pollutants. Several guidelines and reports on dose response modeling were developed by U.S. Environmental Protection Agency. Dose response curve was performed on compound 9a on MCF-7 cell lines as follows, several doses of compound 9a were prepared; 0.5 µM, 1 µM, 5 µM, 20 µM, 50 µM & 100 µM. MCF-7 cell line passage 30 were seeded in $75cm^2$ flask, after being confluent the cells were trypsinized and kept in 15 ml falcon tube, centrifuged for 3 min at 1000rpm. The supernatant was decanted by glass pipette and the pellet was re-suspended in 8 ml DMEM. Cell count was carried out using the cell counter, a cell stock of 50,000 cells/ml was prepared and 100ul of the cells were seeded in 96 wells plate as planned.

The dose response curve was planned ahead using Excel sheet to include a triplet of each of the following doses 0.5µM, 1µM, 5µM, 20µM, 50µM and 100µM. TPEN was also used as a control at the same range of doses. The cells were treated with the planned doses and incubated for 24h incubation, WST-1 reagent was used and the plate was read by VICTOR Multilabel Plate Reader at two wavelengths as explained earlier. Negative and vehicle controls were also added to the experiment.

4.7. Flow Cytometry:

3.7.1. Cell Cycle Analysis

This assay depends on assessing plasma membrane integrity of the treated cells using (PI). PI is taken by cells, which loose its plasma membrane integrity, and thus allows the uptake of the PI dye; this essay does not distinguish between apoptosis or necrosis but only distinguish between dead and alive cells. However, apoptosis cause the shrinkage of the cells but not necrosis, so a combination of reduced FSC and the uptake of PI can be used to decide the mode of cell death. MCF-7 cells were seeded in 25cm^2 flask until reaching the desirable confluence of 1 million cells; media was changed to media containing compound 9a (20µM), after 24hours incubation the cells were harvested by Trypsinization to detach the cells without affecting the integrity of the cell membrane.

The cells have been incubated with PI for 5 min at room temperature, wash twice with PBS and fixed with ice-cold 70% Ethanol while vortexing. Then the cells were washed and re-suspended in PBS with 5 g/ml RNase A and 50 g/ml PI for analysis by flow cytometry. Cell cycle analysis by using FACScan Flow Cytometer and as per the manufacturer protocol.

4.7.2. Apoptotic Assay

This assay is rapid and sensitive protocol to detect early and late apoptosis, and to differentiate if the treated cells have gone apoptosis or necrosis process. When the cells go under apoptosis, they translocate phophatidylserine (PS) from the inner plasma membrane to the outer plasma membrane "the cell surface". This will allow the detection of apoptotic cells when the fluorescent conjugate Annexin V (FITC) binds to PS on the cell surface.

Annexin-V is a protein with high affinity to phosphatidyleserine. This assay can be performed on live cells and the procedure takes about 10 min to accomplish without fixation. The procedure also contains PI to distinguish between viable cells (FITC negative/ PI negative), early (FITC positive/PI negative) or necrosis/late apoptosis (FITC positive/PI positive. FITC is excited by the blue laser at 488 nm while PI by the green laser at 552 nm.

The procedure was performed as following: MCF-7 cells were cultured in 75cm^2 flask until reaching 75-85% confluence, the cells were then trypsinized with 1x Trypsin. The cells were centrifuged to discard the trypsin and the pellet was re-suspended in 5ml DMEM media. Cell count was performed and 2.5X10^5 cells were then cultured in 6 well plate for further treatment. The test was done in triplicate for compound 9a, unstained cells were used as a negative control, cells with DMSO 0.4% was also tested and cells treated with TPEN was used as a positive control. When MCF-7 cells were confluent, treatment was carried out with compound 9a at 20µM in triplicate. The cell were incubated for 24 hours to be propagated for flow cytometry apoptosis detection. The media in the 6 well plate was aspirated to 15ml conical tube and placed on ice immediately as this media contain a number of detached cells during the death process.

The adherent cells were then washed out using PBS, the PBS was removed to be trypsinized then with 0.5x trypsin and released firmly. The cells were re-suspended in 1x cold binding buffer to 1 x 106 cells/ml and 0.5 ml of the suspension to microfuge tubes, 10µl of the media binding reagent and 1.25ul of Annexin V-FITC were added to the tubes, the tubes were incubated for 15 minutes at RT in the dark. The tubes were then centrifuged for 5 minutes and the media was removed.

The pellet was then resuspended in 0.5ml of cold 1x binding buffer, followed by adding 10µl PI, the tubes were placed in ice in dark before flow cytometry analysis. Flow Cytometry analysis was performed at 488 nm using the blue laser and 552 nm using the green laser.

4.8. Western Blot:

Western blot analysis is used to detect specific proteins in a sample. The proteins are separated by gel electrophoresis and then transferred electrophoretically to a PVDF membrane. Antibodies are used to detect the specific proteins that are immobilized on the membranes. Prior to blotting an SDS-PAGE gel was stained with coomassie blue to provide visual confirmation that equivalent amounts of various samples were being analyzed. For analysis of Bcl-2, p53, caspase-3, p21, mdm-2 and Akt proteins, MCF-7 cells were collected and lysed in RIPA lysis buffer after 24 hours of treatment. Membranes were re-probed with β-actin protein antibody to confirm equal loading of protein samples. Protein Expressions was tested by using monoclonal antibodies and measuring the total cellular protein by western blot. The sample and the loading dye were normalized, then the sample was heated to 95°C for about 5 min and then the protein was separated on SDS-PAGE. After that, the protein was incubated with primary antibodies overnight at a 1:500 dilution in 5% PBS, a three-time wash was carried out before the incubation with the secondary antibodies.

5. Results:

5.1. Cell Viability:

Measurement of cell viability and proliferation forms the basis for numerous in vitro assays of a cell population's response to external factors. The reduction of tetrazolium salts is now widely accepted as a reliable way to examine cell proliferation. WST Assay was performed to evaluate the effect of 20 Pyridine Derivatives 4a – 4b – 4d – 5 – 6- 7a – 7b – 7c – 7d - 8a – 8b – 8c - 9a – 9b – 10 – 11 – 12 – 13 – 15 and 17 on MCF-7, MD-MB-231, HCT-116, SK-OV-3 and OVCAR-3. The cell lines were first treated first with 100 µM of the compounds for 24 hours. Samples were normalized to untreated control.

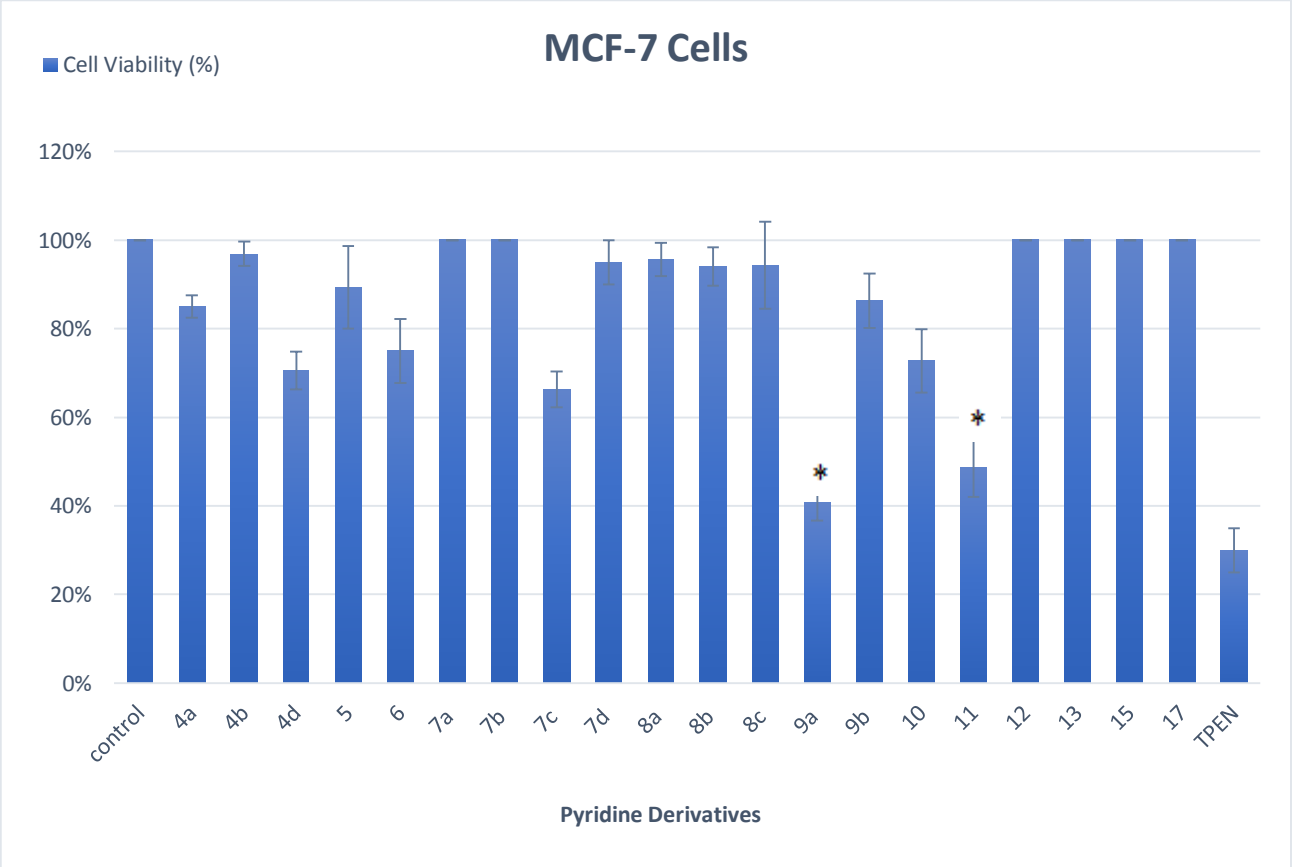


Figure 5.1.1 Cell Viability Assay for MCF-7 cells. The percentage of viability after treatment with 100 µM (4a-4b-4d-5-6-7a-7b-7c-7d-8a-8b-8c-9a-9b-10-11-12-13-15-17 for 24h) using WST-1 Assay. Each graph is an average of results from one experiment performed in triplicate and presented as M ± SD. Cells without Pyridine Derivatives treatment were used as control, TPEN 100 µM was used as a positive control using WST-1 Assay.

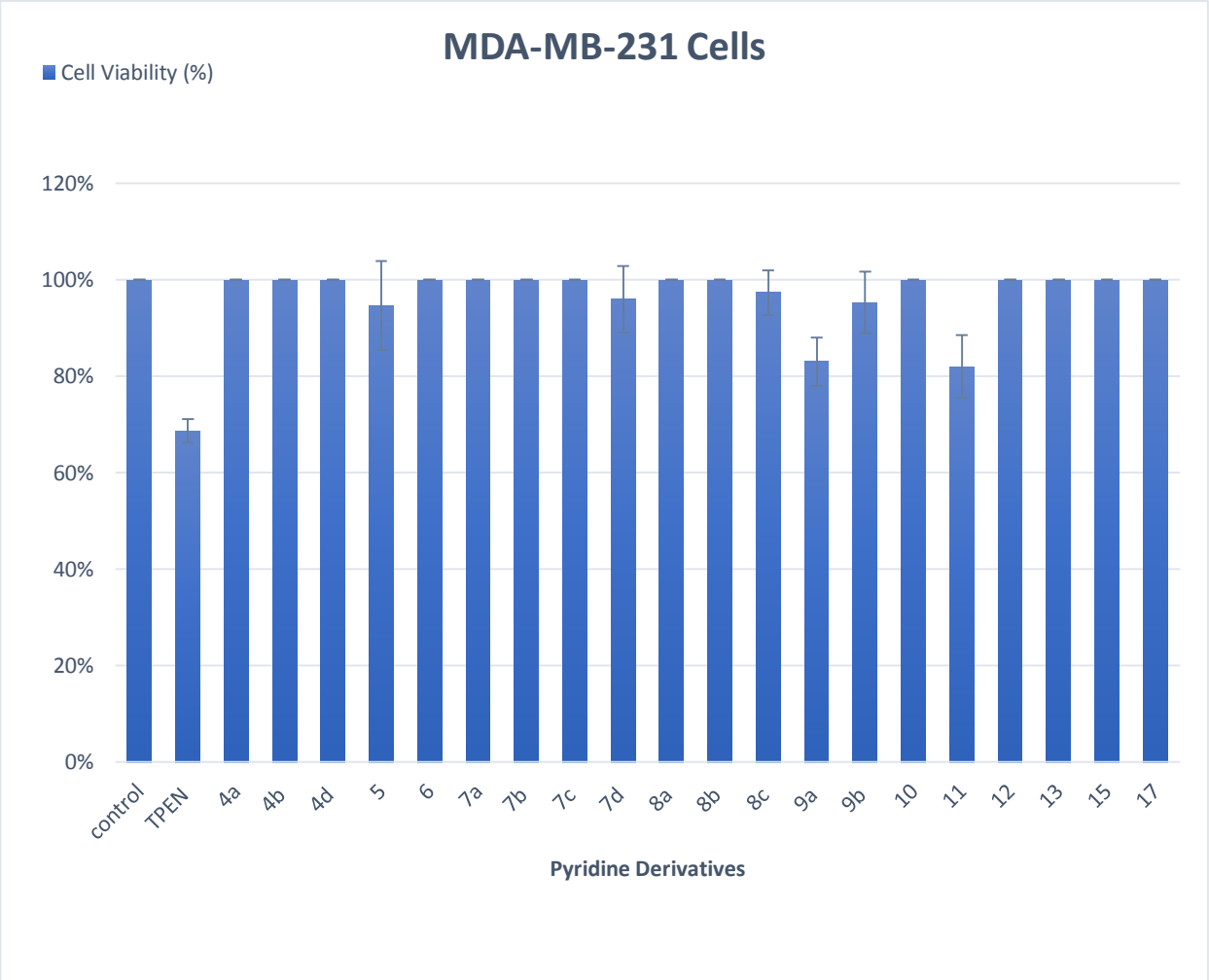


Figure 5.1.2. Cell Viability Assay for MDA-MB-231 cells. The percentage of viability after treatment with 100 µM (4a-4b-4d-5-6-7a-7b-7c-7d-8a-8b-8c-9a-9b-10-11-12-13-15-17 for 24h) using WST-1 Assay. Each graph is an average of results from one experiment performed in triplicate and presented as M ± SD. Cells without Pyridine Derivatives treatment were used as control, TPEN 100 µM was used as a positive control using WST-1 Assay.

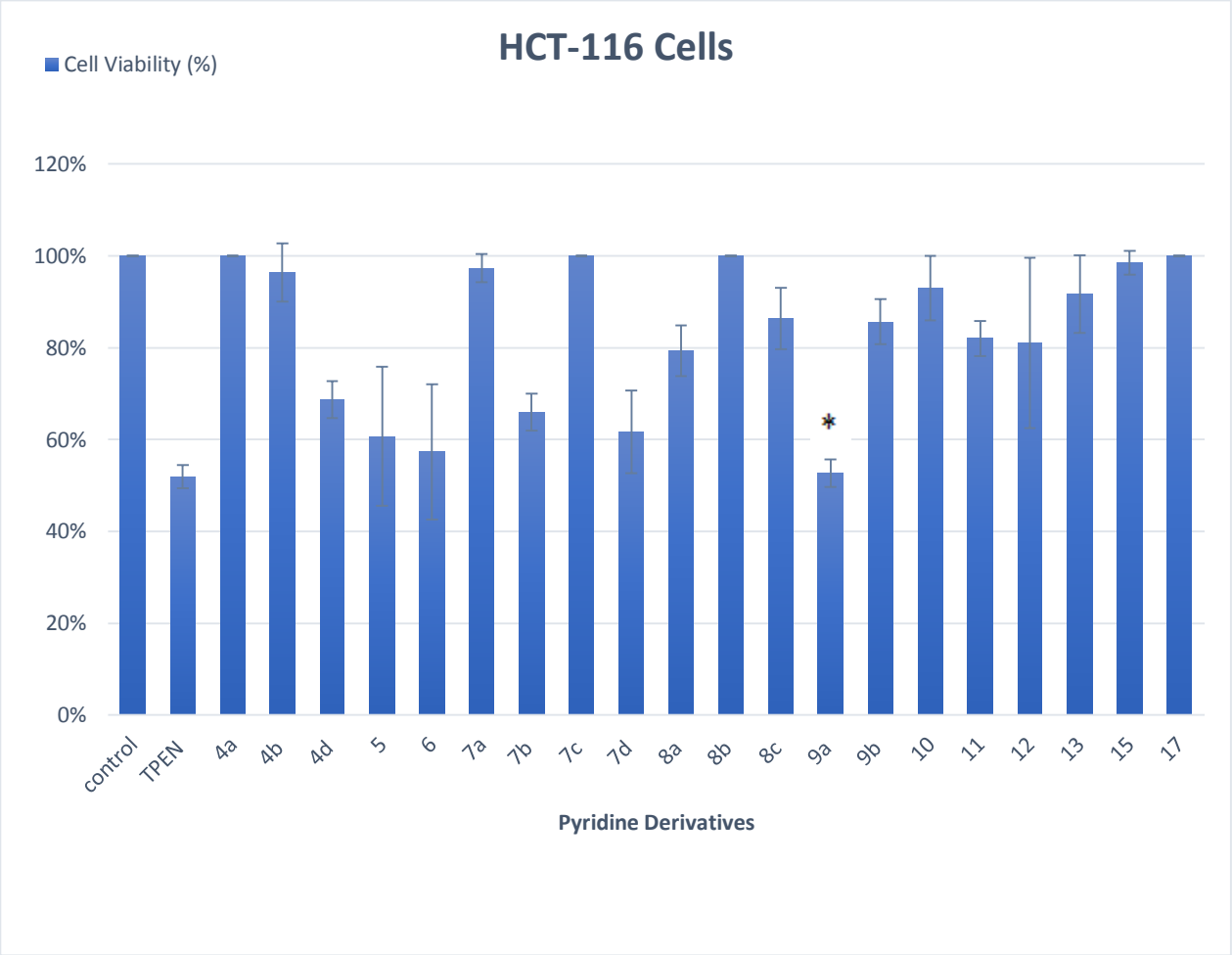


Figure 5.1.3. Cell Viability Assay for HCT-116 cells. The percentage of viability after treatment with 100 µM (4a-4b-4d-5-6-7a-7b-7c-7d-8a-8b-8c-9a-9b-10-11-12-13-15-17 for 24h) using WST-1 Assay. Each graph is an average of results from one experiment performed in triplicate and presented as M ± SD. Cells without Pyridine Derivatives treatment were used as control, TPEN 100 µM was used as a positive control using WST-1 Assay.

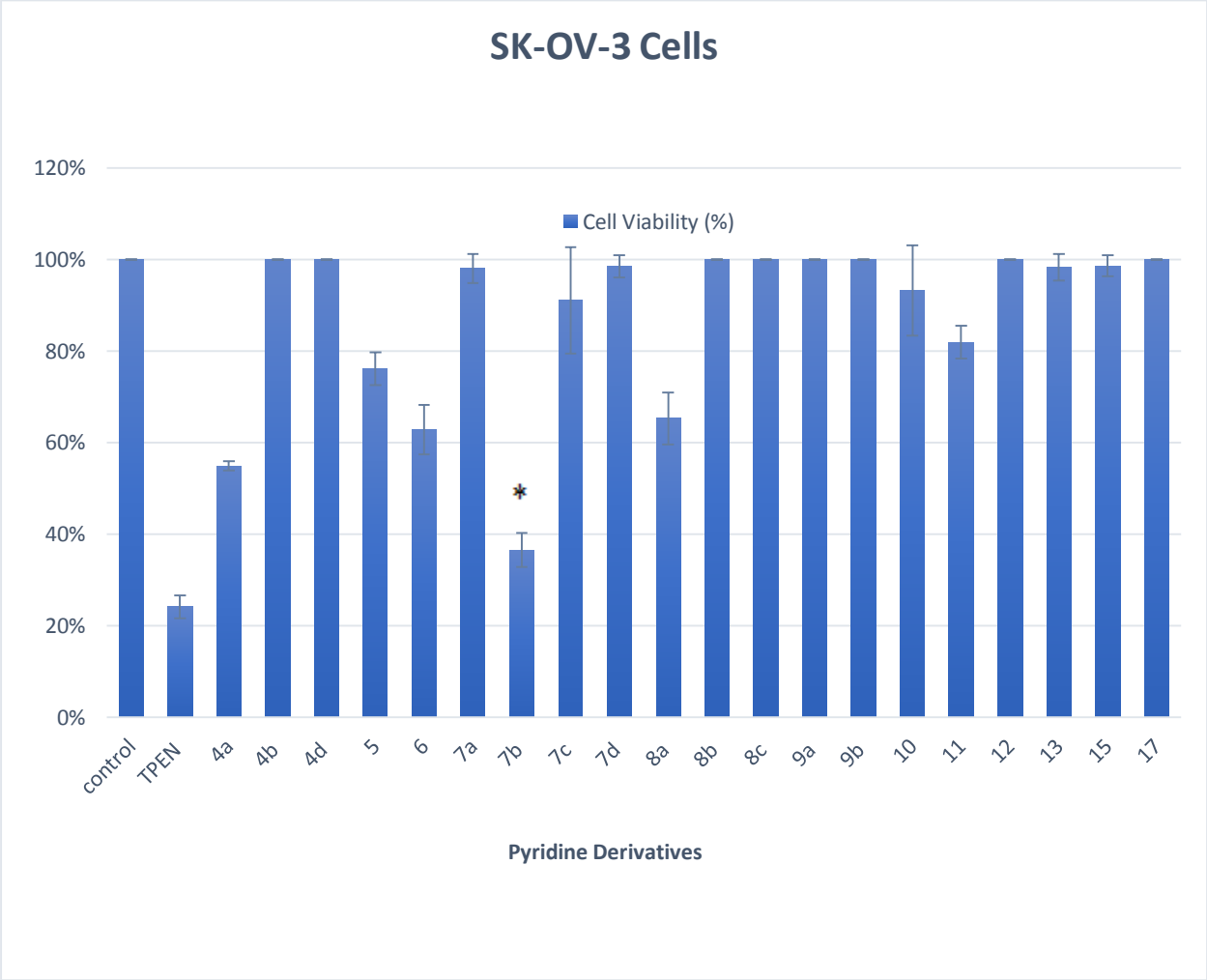


Figure 5.1.4. Cell Viability Assay for SKOV-3 cells. The percentage of viability after treatment with 100 µM (4a-4b-4d-5-6-7a-7b-7c-7d-8a-8b-8c-9a-9b-10-11-12-13-15-17 for 24h) using WST-1 Assay. Each graph is an average of results from one experiment performed in triplicate and presented as M ± SD. Cells without Pyridine Derivatives treatment were used as control, TPEN 100 µM was used as a positive control using WST-1 Assay.

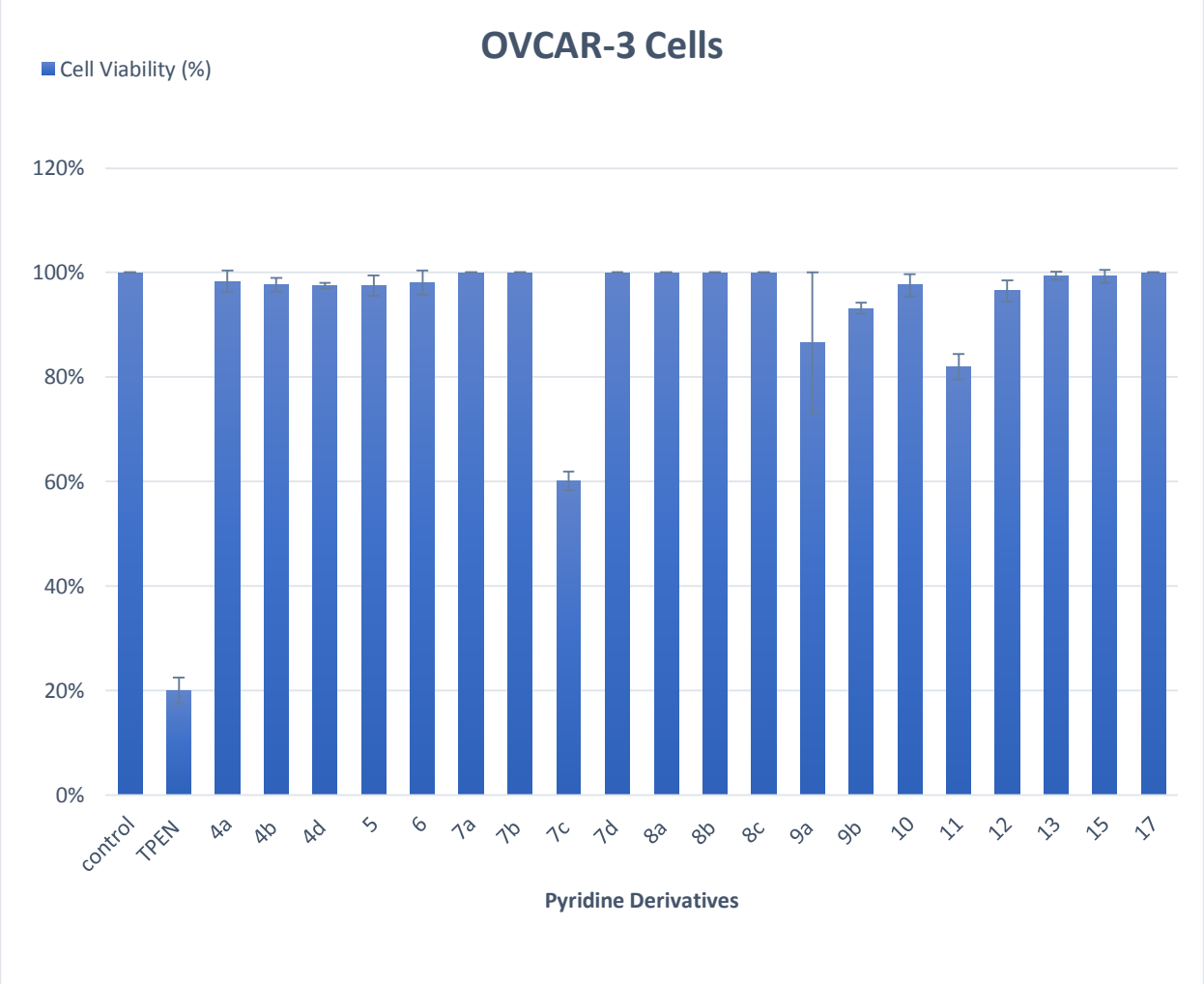


Figure 5.1.5. Cell Viability Assay for OVCAR-3 cells. The percentage of viability after treatment with 100 µM (4a-4b-4d-5-6-7a-7b-7c-7d-8a-8b-8c-9a-9b-10-11-12-13-15-17 for 24h) using WST-1 Assay. Each graph is an average of results from one experiment performed in triplicate and presented as M ± SD. Cells without Pyridine Derivatives treatment were used as control, TPEN 100 µM was used as a positive control using WST-1 Assay.

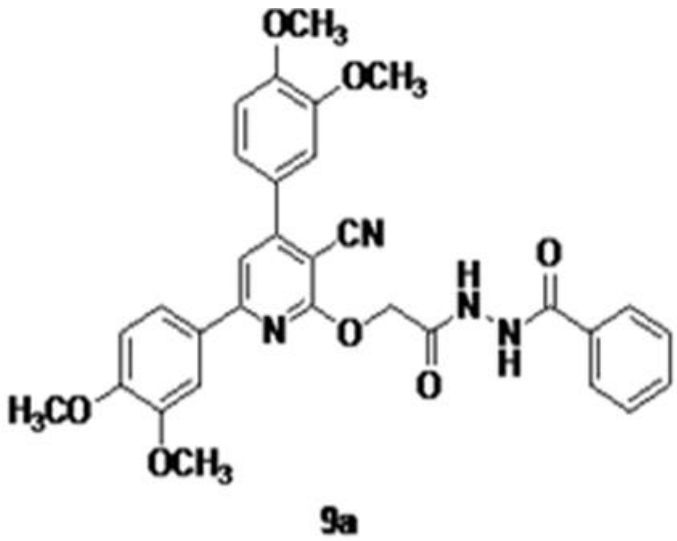
5.2. Potent Pyridine Derivatives

The potent derivatives that reduce cell viability by 50% were summarized for further investigations as shown in in table 5.1.

Table 5.1 Potent Pyridine Derivatives

Compound	Cell Line	Viability %	Concentration
9a	MCF-7	40%	100 µM
9a	HCT-116	45%	100 µM
7b	SKOV-3	35%	100 µM
11	MCF-7	44%	100 µM

5.3. Compound 9a and MCF-7 cells



9a

Figure .3.1. Compound 9a Structure with the chemical formula $C_{30}H_{28}N_4O_7$

Compound 9a is a Pyridine Derivative, an organic aromatic compound miscible with organic solvents. Compound 9a is a weak base due to the presence of lone pairs of electrons at the nitrogen center. Pyridine is protonated when reacting with acids. Compound 9a is soluble in DMSO "dimethyl sulfoxide" an organosulfur colorless solvent.

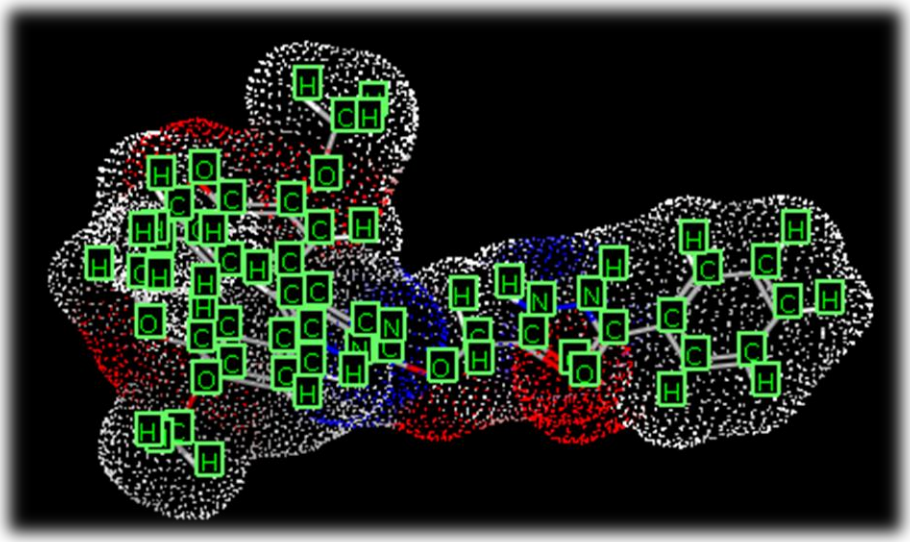


Figure 5.3.2. 3D Structure of Compound 9a

5.4. Morphological Changes of MCF-7 cells after 24h treatment with different concentration of compound 9a

The morphological changes in MCF-7 cells were followed up after the treatment with different concentrations of compound 9a (0.5, 1, 5, 20, 50 and 100 µM). At 0.5, 1 and 5 µM no morphological changes were observed, however upon treating the cells with higher concentrations of 20, 50 ad 100µM apoptotic features were observed including marked apoptotic bodies and cell shrinkage in a concentration dependent manner. Untreated cells were used as a negative control. Treatment of MCF-7 cells with DMSO 0.4% did not show any death of the cells.

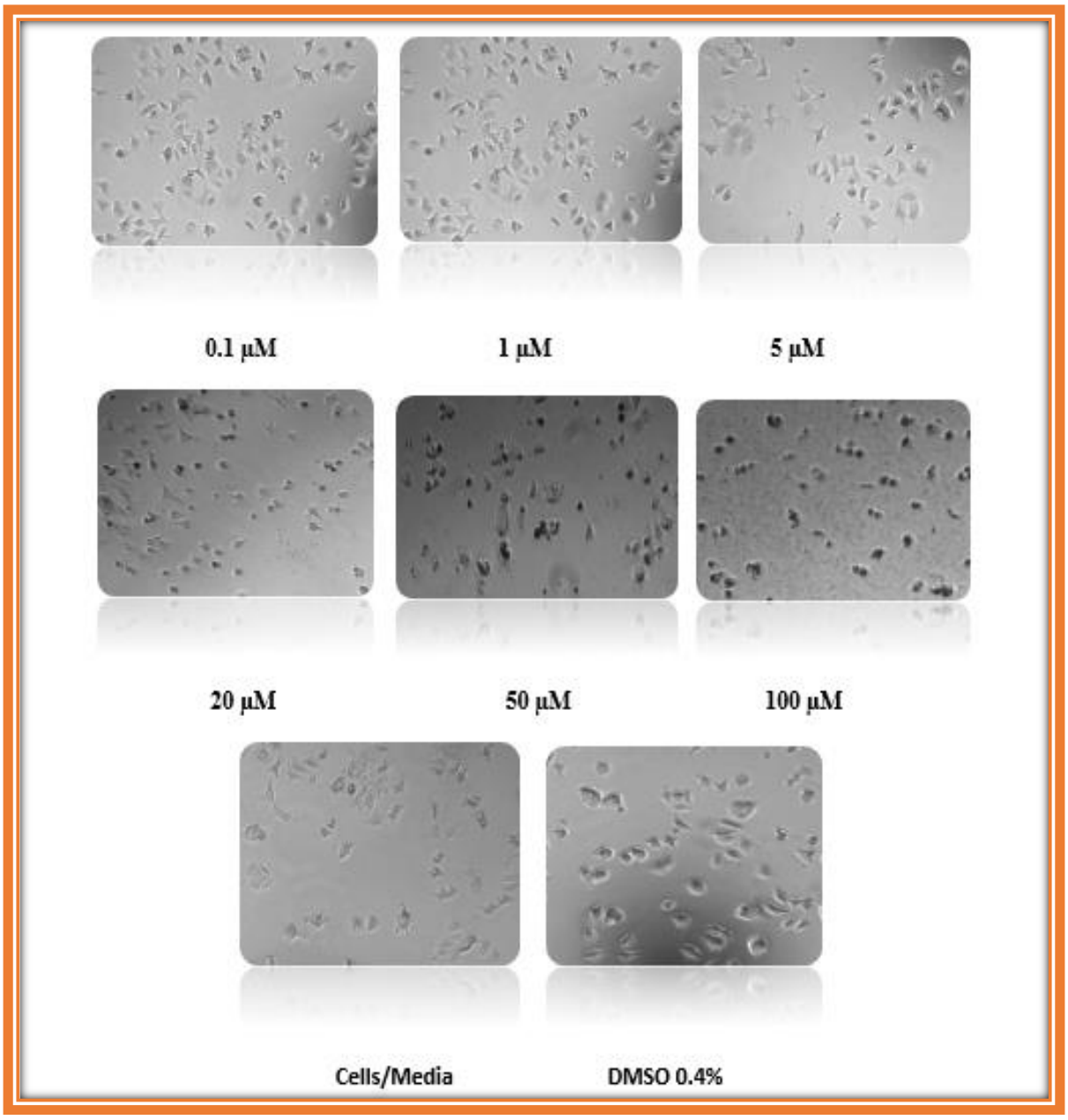


Figure 5.4.1. Effect of 9a on morphological appearance of MCF-7 cells. The morphological appearance of MCF-7 cells after treatment with 0.5, 1, 5, 20, 50 and 100µM of compound 9a for 24h. MCF-7 IC_{50} (20µM) induces apoptotic programmed cell death including marked apoptotic bodies and cell shrinkage in a concentration dependent manner. Untreated cells were used as a negative control. Treatment of MCF-7 cells with DMSO 0.4% did not show any death of the cells.

The morphological changes were also observed in MCF-7 using TPEN as a positive control. TPEN IC_{50} of 5µM was capable of inducing apoptosis as shown hereunder. The apoptotic effect of TPEN increased dramatically with higher concentrations.

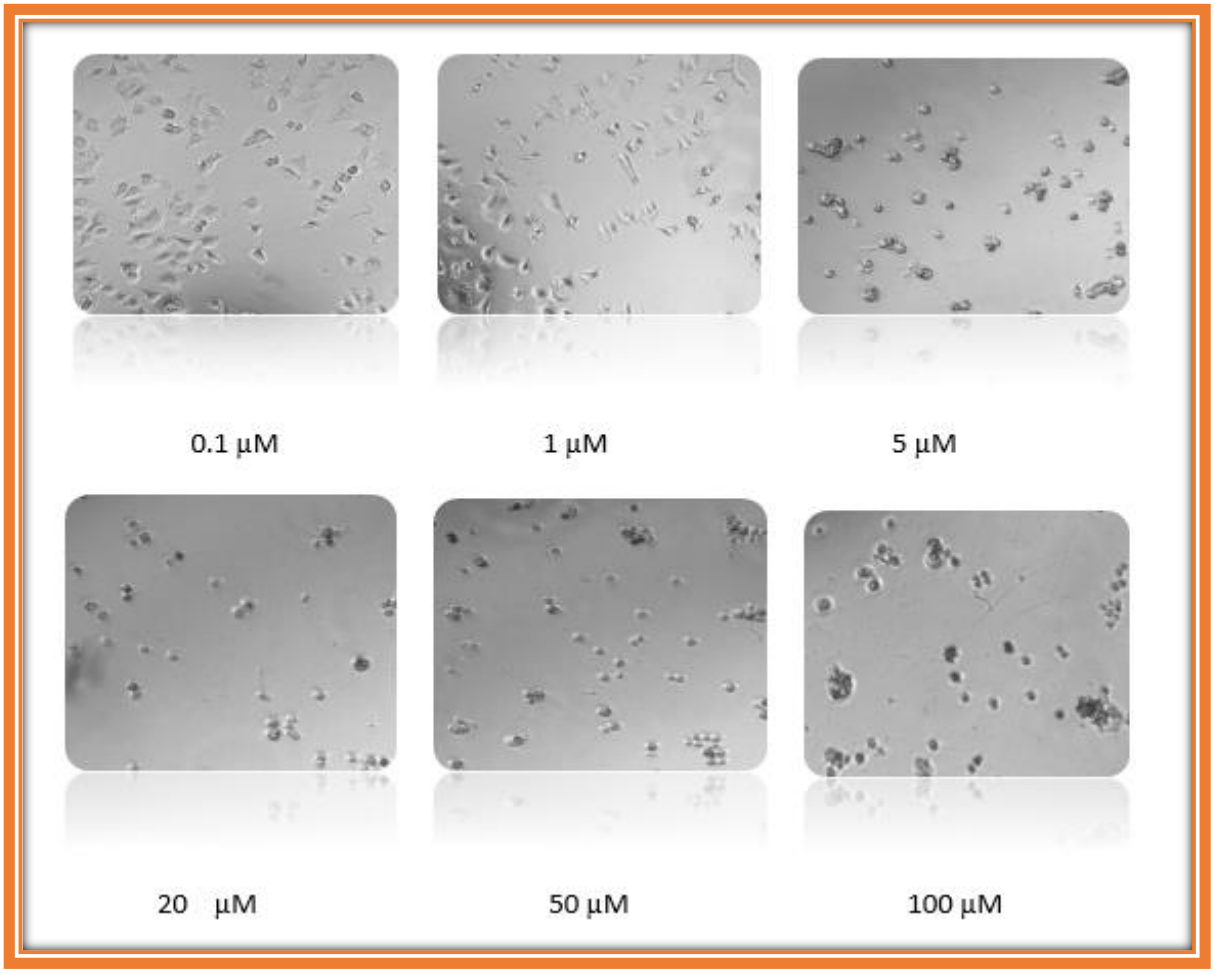


Figure 5.4.2. The morphological appearance of MCF-7 cells after treatment with 0.5, 1, 5, 20, 50 and 100µM of TPEN for 24h. MCF-7 IC_{50} (20µM) induces apoptotic programmed cell death including marked apoptotic bodies and cell shrinkage in a concentration dependent manner. Untreated cells were used as a negative control. Treatment of MCF-7 cells with DMSO 0.4% did not show any death of the cells.

5.5. Dose Response Curve & IC_{50}

For further experiments, dose response curve was constructed, it is essential to describe the changes in cell viability using different doses after a certain time of exposure. The dose response curve is referred as the graded response because the measured effect is continuous over the range of different doses. Therefore, several doses of compound 9a were prepared ranging from; 0.5 µM, 1 µM, 5 µM, 20 µM, 50 µM & 100 µM and the IC_{50} was calculated as 20 µM.

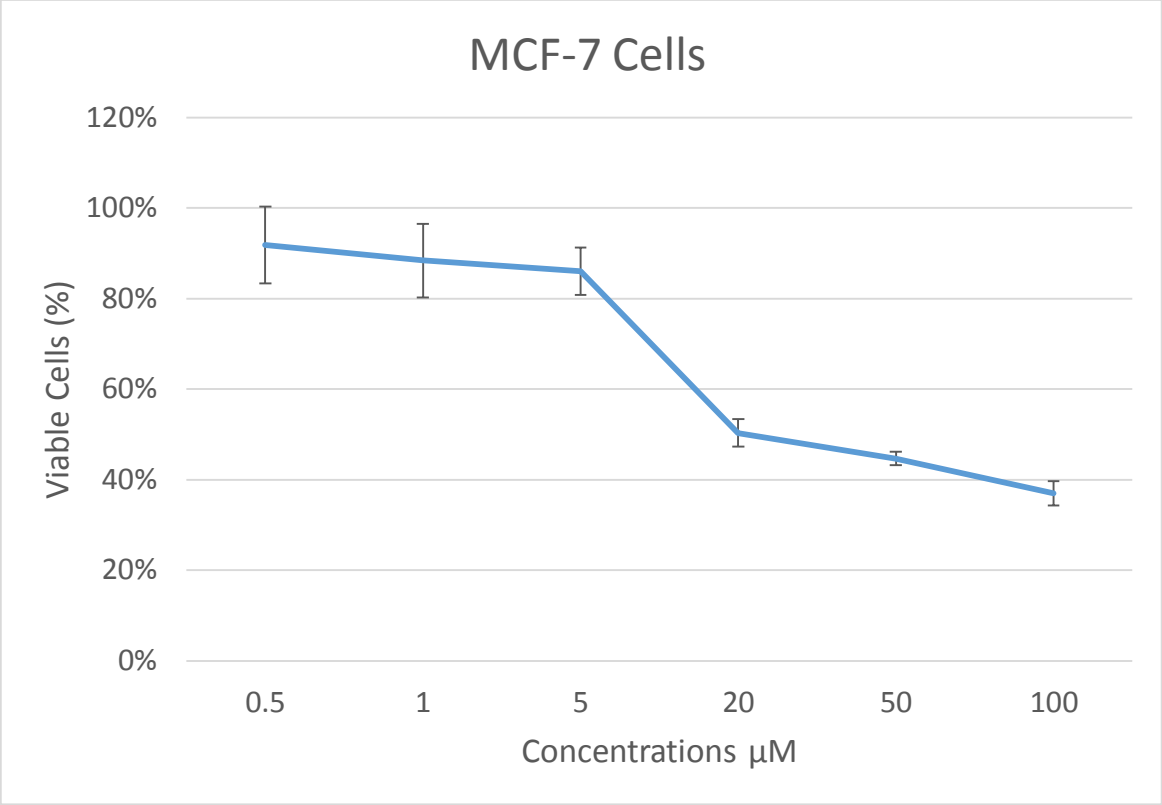


Figure 5.5.1. Dose response curve of compound 9a in MCF-7 cells. Different concentrations of compound 9a were used ranging from 0.5µM to 100µM and blotted against the cell viability as a percentage.

The dose response curve was also constructed for the positive control TPEN (the positive control) as illustrated in figure 5.5.2.

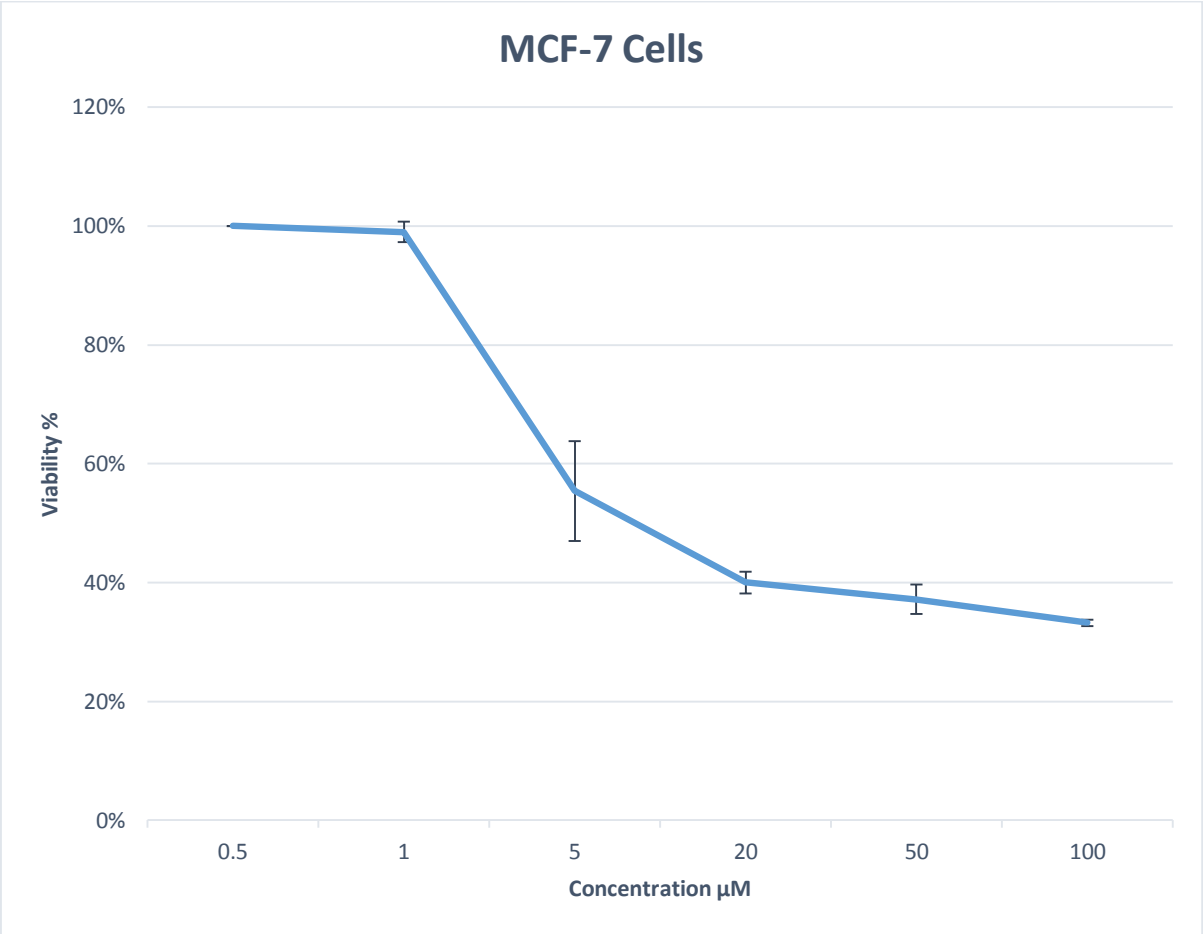


Figure 5.5.2. Dose response curve of TPEN in MCF-7 cells. Different concentrations of TPEN were used ranging from 0.5µM to 100µM and blotted against the cell viability as a percentage.

5.6. Cytotoxicity of compound 9a on non-tumorigenic breast epithelial cells MCF-12a & Statistical Analysis

In order to assess the level of cytotoxicity of compound 9a in non-tumorigenic breast epithelial cells, MCF-12a were treated with 20 µM for 24hours and WST-1 assay was carried out as described in section of materials and methods. The viability of MCF-12a cells were blotted on bar graph in comparison with MCF-7 after the same treatment. The significance of viability between MCF-7 and 12a was calculated SPSS non-parametric analysis of two independent sample test as explained hereunder.

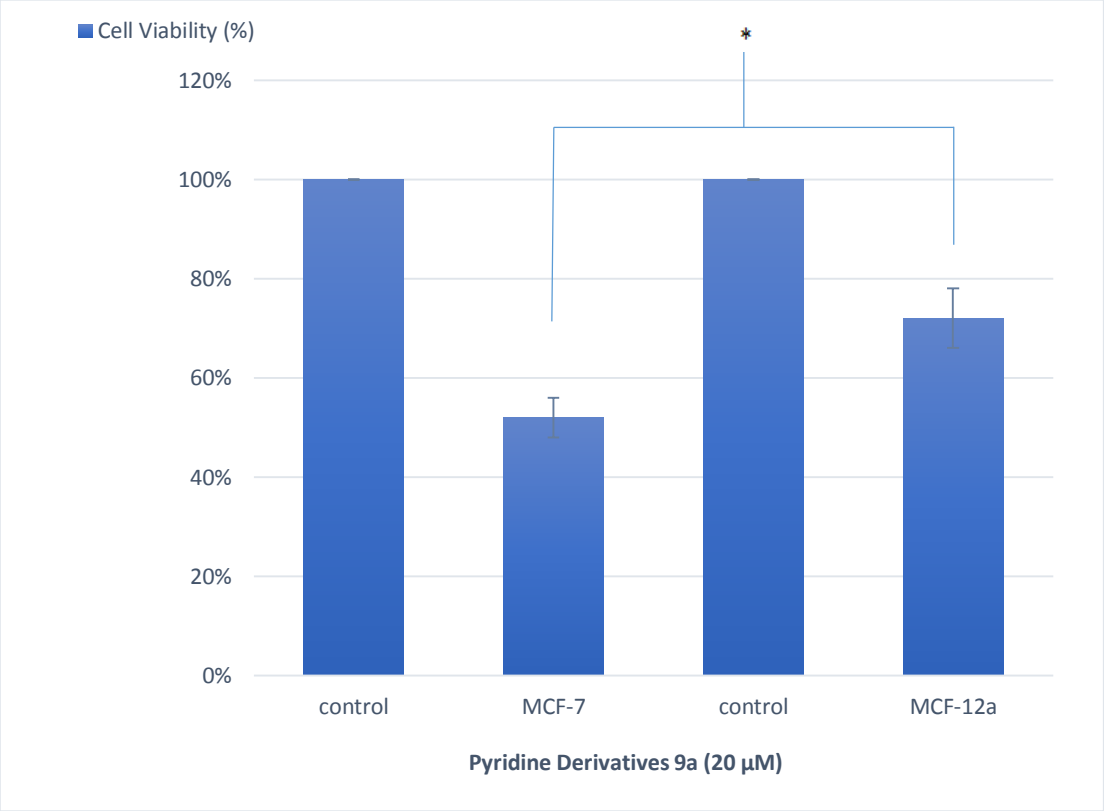


Figure 5.6.1. Cytotoxicity of compound 9a on non-tumorigenic breast epithelial cells MCF-12a. WST-1 Assay was performed on MCF-7 and MCF-12a after treatment with compound 9a 20 µM for 24 hours. The viability in MCF-12a was about 75% in comparison with MCF-7 48%. Each data point was an average of results from three independent experiments performed in triplicate and presented as M ± SD.

Statistical Analysis to calculate the Statistical Significance of viability in MCF-7 and MCF-12A:

Normality for MCF-7 and MCF-12A was tested using SPSS software by two methods: Kolmogorov-Smirnov (K-S test) with specified mean and variance and Shapiro-Wilk with unspecified mean and variance. K-S test is a sensitive useful nonparametric test method used to compare the probability distribution of continuous samples, by measuring the empirical distribution function between two samples, in this case MCF-7 and MCF-12A based on the null hypothesis that MCF-7 and MCF-12A are drawn from same distribution (Zaiontz, 2014). Shapiro-Wilk is another widely used nonparametric test for normality, the significance in both methods was 0.004 as illustrated in table 5.3., as the obtained *p* value was < 0.05 then the null hypothesis should be rejected which indicates that the obtained data for MCF-7 and MCF-12A were deviated from normal distribution. Q-Q Plot was also performed to determine the normality graphically beside numerically, however the data points were not distributed closely to the diagonal line, did not present a linear fashion ("Testing for Normality using SPSS Statistics," 2014).

Table 5.6.1. Testing Normality in MCF-7 and MCF-12a using SPSS

Descriptive

			Statistic	Std. Error
Treats	Mean		.7041	.04983
	95% Confidence Interval for Mean	Lower Bound	.6017	
		Upper Bound	.8065	
	5% Trimmed Mean		.7109	
	Median		.7200	
	Variance		.067	
	Std. Deviation		.25891	
	Minimum		.27	
	Maximum		1.00	
	Range		.73	
	Interquartile Range		.53	

	Skewness	-.204	.448
	Kurtosis	-1.395	.872

Table 5.6.2. Testing Normality in MCF-7 and MCF-12a using SPSS "Kolmogorov-Smirnov" and "Shapiro-Wilk"

Tests of Normality

	Kolmogorov-Smirnov[a]			Shapiro-Wilk		
	Statistic	df	Sig.	Statistic	Df	Sig.
Treats	.207	27	.004	.876	27	.004

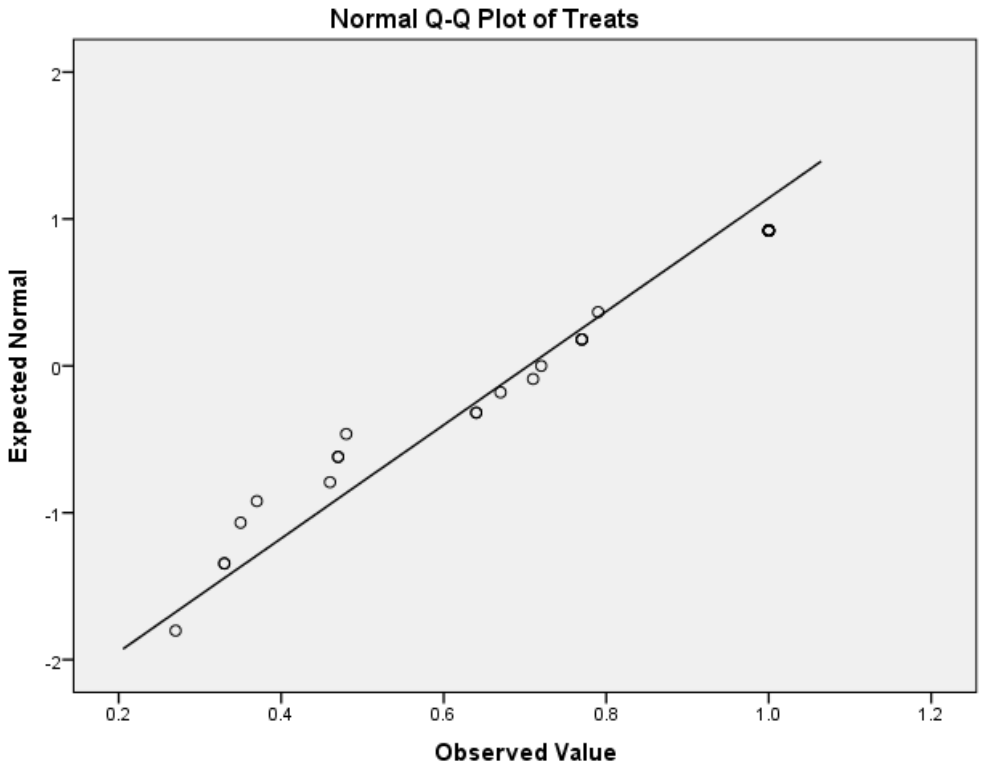


Figure 5.6.2 Q-Q plot for MCF-7 and MCF-12A

Since the viability does not satisfy the normality assumption and due to small sample size, the Non-parametric approach has been used to assess the viability significance between MCF-7 and MCF-12a after treatment with 20µM of compound 9a for 24 hours:

- Kruskal-Wallis Test was used to compare the three independent groups (control – MCF-7 and MCF-12A), kruskal-Wallis is a nonparametric method for testing more than two samples to evaluate whether they originate from the same distribution. It is an equivalent test to the parametric test ANOVA for the one-way analysis of variance (McDonald, 2014). The results of this test has shown statistical significance of p value 0.001 as indicated in table 5.4.

Table 5.4 Statistical Significance for three groups (MCF-7, MCF-12a and control) using Kruskal Wallis method

Ranks

	Groups	N	Mean Rank
Treats	1.00	9	5.00
	2.00	9	14.00
	3.00	9	23.00
	Total	27	

Test Statistics[a,b]

	Treats
Chi-Square	24.076
Df	2
Asymp. Sig.	.001

a. Kruskal Wallis Test

b. Grouping Variable: groups

- Mann-Whitney was also carried out to compare both groups (treated MCF-7 cells and MCF-12A). Mann-Whitney is a nonparametric test for comparing two independent groups, based on the null hypothesis which indicates that both groups come from the same population. This test has shown higher efficacy than t-test on data with non-normal distribution ("Mann-Whitney U

Test using SPSS," 2014). Rejecting the null hypothesis indicates that viability in MCF-7 and MCF-12A is different. The results showed statistical significance of *p* value 0.001.

Table 5.5 Statistical Significance for two groups (MCF-7, MCF-12a) using Mann-Whitney U method

Ranks

	Groups	N	Mean Rank	Sum of Ranks
Treats	1.00	9	5.00	45.00
	2.00	9	14.00	126.00
	Total	18		

Test Statistics[a]

	Treats
Mann-Whitney U	.000
Wilcoxon W	45.000
Z	-3.589
Asymp. Sig. (2-tailed)	.001
Exact Sig. [2*(1-tailed Sig.)]	.001[b]

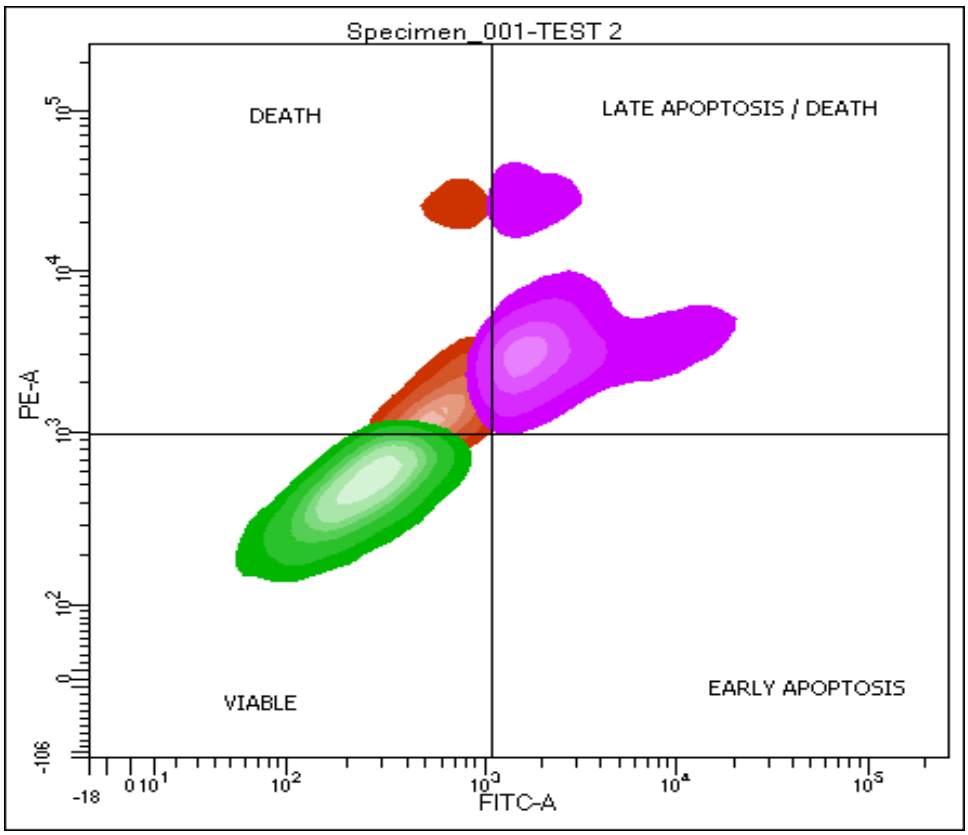
a. Grouping Variable: Groups

b. Not corrected for ties.

Using both non-parametric statistical analysis methods: Mann-Whitney U and Kruskal-Wallis Test, the results show statistical significance of *p* value 0.001 in viability between MCF-7 and MCF-12a after treatment with 20µM of compound 9a.

5.7. Apoptosis Detection Assay Annexin-V FITC

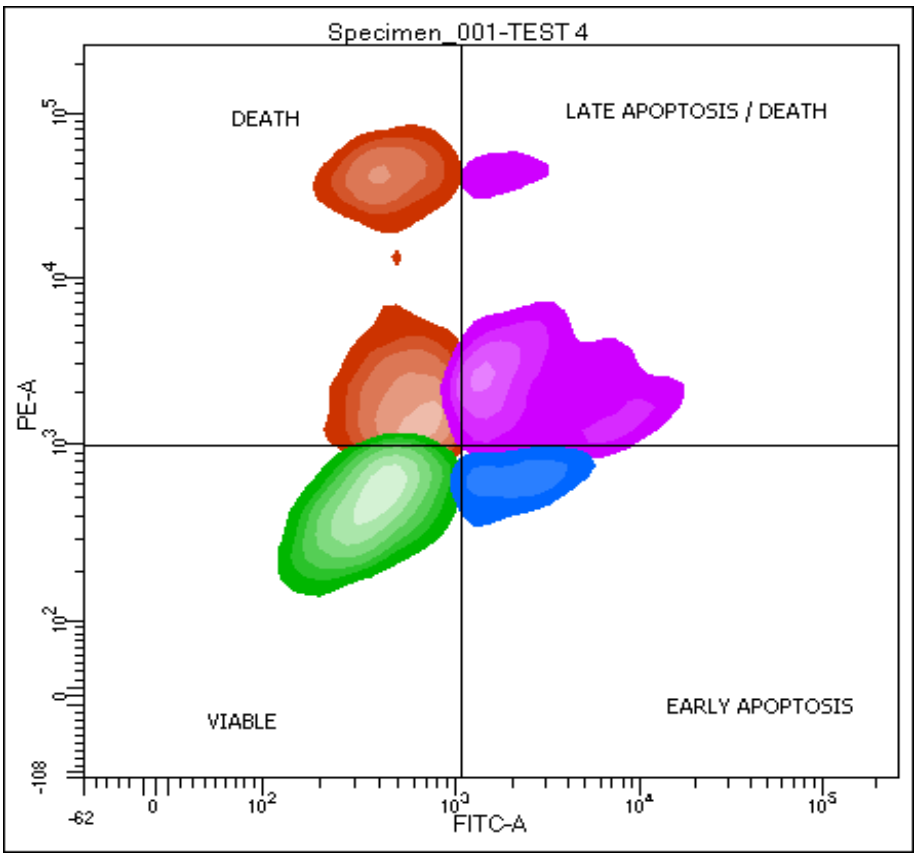
This assay was used to detect apoptosis in MCF-7 cells after treatment with IC_{50} (20 µM) compound 9a for 24hours, the results showed that about 51.7% of the total population were viable cells, 48.3% represents cells in early apoptosis, late apoptosis and dead cells. The apoptosis detection assay was also performed for the positive control TPEN.



Tube: TEST 2

Population	#Events	%Parent	%Total
All Events	10,000	####	100.0
P1	2,505	25.0	25.0
NOT(P1)	7,495	75.0	75.0
DEATH	1,566	20.9	15.7
LATE APOPTOSIS / DEATH	2,607	34.8	26.1
VIABLE	3,260	43.5	32.6
EARLY APOPTOSIS	62	0.8	0.6
P4	527	7.0	5.3
P5	2,515	33.6	25.2

Figure 5.7.1. Compound 9a induces apoptosis in MCF-7. Viable cells constitute 51.7% of the total population, cells in early apoptosis were 1.3%, cells in late apoptosis are 42.4% and dead cells are 3.1%. The data represented is an average of three independent experiments performed in triplicate. MCF-7 cells were treated by compound 9a for 24h before analysis.



Tube: TEST 4

Population	#Events	%Parent	%Total
All Events	10,000	####	100.0
P1	2,635	26.4	26.4
NOT(P1)	7,365	73.6	73.6
DEATH	2,502	34.0	25.0
LATE APOPTOSIS / DEATH	1,869	25.4	18.7
VIABLE	2,556	34.7	25.6
EARLY APOPTOSIS	438	5.9	4.4
P4	575	7.8	5.8
P5	2,477	33.6	24.8

Figure 5.7.2. TPEN induces apoptosis in MCF-7. Viable cells constitute 34.7% of the total population, cells in early apoptosis were 5.9%, cells in late apoptosis are 25.4% and dead cells are 34%. MCF-7 cells were treated by TPEN for 24h before analysis “as a positive control”.

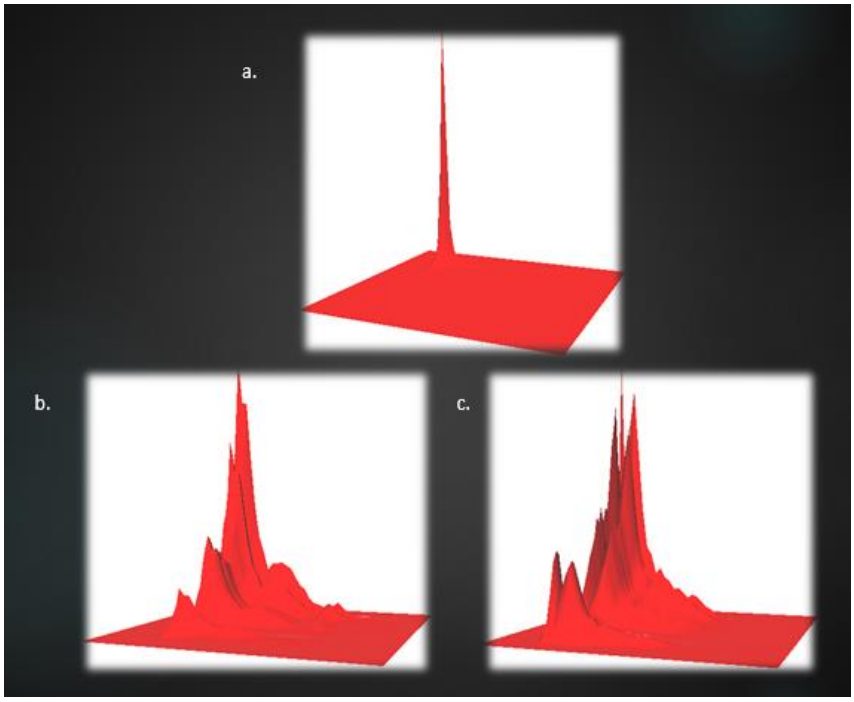


Figure 5.7.3. 3D Orientation Structure of Apoptotic Detection Assay by Flow Cytometry presenting the different populations of viable cells, MCF-7 cells in early apoptosis, MCF-7 cells in late apoptosis and dead MCF-7 cells . a. 3D structure of unstained MCF-7 cells. b. 3D structure of untreated MCF-7 with the vehicle DMSO 0.4%. c. 3D structure of MCF-7 cells treated with TPEN "+ve control". d. 3D structure of MCF-7 cells treated with compound 9a IC_{50} "20µM"for 24hours.

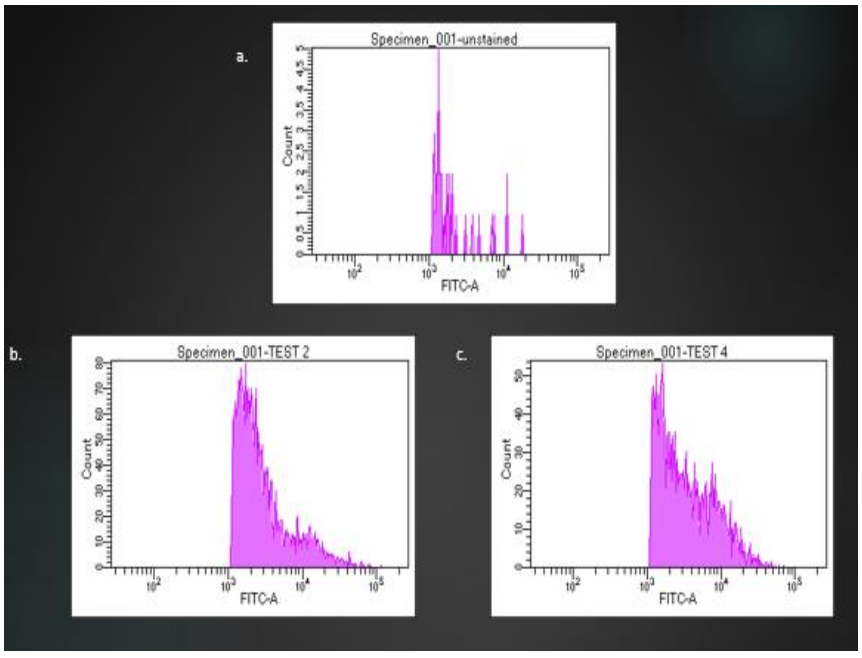
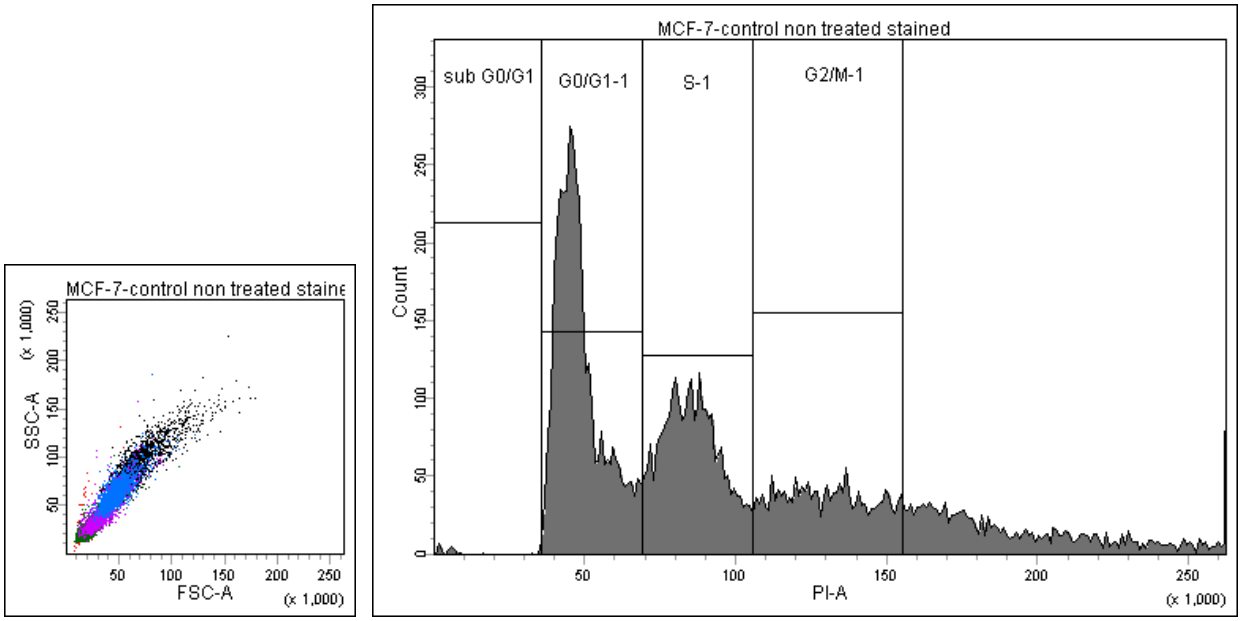


Figure 5.7.4. Histogram representing the Late Apoptotic Phase by Flow Cytometry. a. Histogram of untreated MCF-7 cells. b. Histogram of MCF-7 cells treated with compound 9a for 24hours. c. Histogram of MCF-7 cells treated with TPEN for 24hours.

5.8. Cell Cycle Analysis

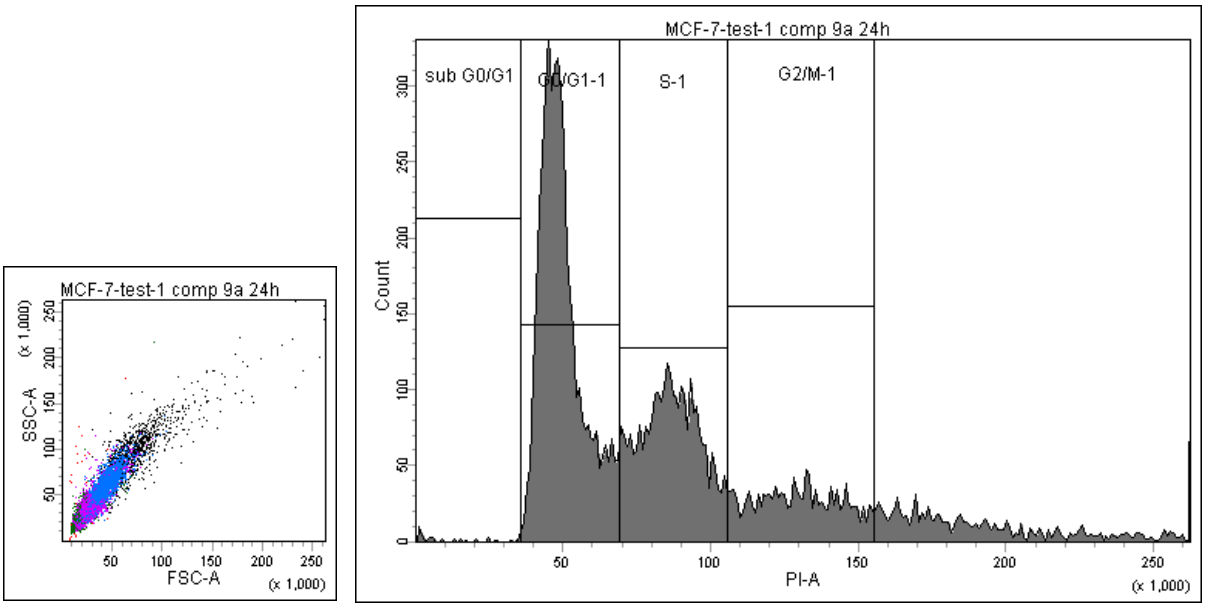
The analysis of the cell cycle checkpoints revealed no significant differences between the untreated and treated MCF-7 cells as illustrated in figures 5.8.1 and 5.8.2. A slight reduction of about 5% in G2/M phase was observed in MCF-7 cells treated with compound 9a (20 µM).



Tube: control non treated stained

Population	#Events	%Parent	%Total
All Events	10,000	####	100.0
sub G0/G1	55	0.5	0.5
G0/G1-1	3,722	37.2	37.2
S-1	2,574	25.7	25.7
G2/M-1	1,791	17.9	17.9

Figure 5.8.1. Cell Cycle Analysis of total 10,000 events of untreated MCF-7 cells. 37.2% of the total cell population was in G0/G1 phase, 25.7% in Phase S and 17.9 in G2/M phase.



Tube: test-1 comp 9a 24h

Population	#Events	%Parent	%Total
All Events	10,000	####	100.0
sub G0/G1	79	0.8	0.8
G0/G1-1	4,532	45.3	45.3
S-1	2,708	27.1	27.1
G2/M-1	1,367	13.7	13.7

Figure 5.8.2. Cell Cycle Analysis of total 10,000 events of MCF-7 cells treated with 20 µM of compound 9a for 24 hours. 45.3% of the total cell population was in G0/G1 phase, 27.1% in Phase S and 13.7 in G2/M phase.

5.9. Impact of 9a on Apoptotic and Survival Signals

Several key downstream components are involved in the regulation of cell cycle. p53, "the Guardian of The Genome", is an important protein in cell cycle and apoptosis, loss of its function will result in cancer. p53 trans-activates other tumor suppressor down-stream genes involved in cell cycle arrest and apoptosis such as p21 and Bax. To elucidate the role of p53, p21 and Bax in the anti-tumor activity of compound 9a, MCF-7 cells were treated with IC_{50} (20 µM) of compound 9a for 24 hours compared with untreated cells serving as a control. The lysates of both treated and untreated cells were prepared and processed for western blot analysis to study the changes in the levels of p53, p21 and Bax in order

to evaluate the effects of compound 9a on these pathways. In this study, compound 9a increased the expression of p53, p21 and Bax in MCF-7 in treated cells compared to untreated cells.

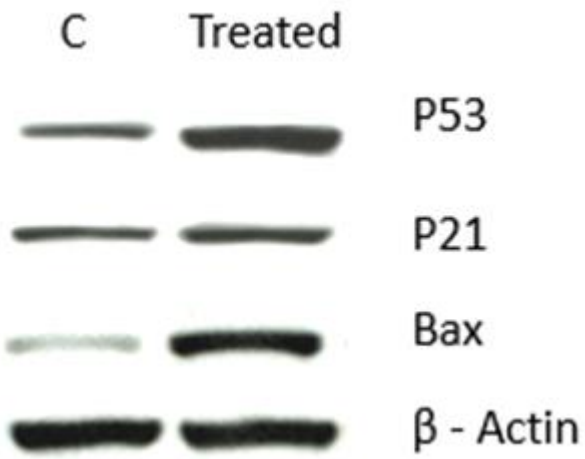


Figure 5.9.1. Compound 9a upregulates p53, p21 and Bax levels in MCF-7 Human Breast Cancer Cells. Cells were treated with 20 µM for 24 hours, cells were lysed and total protein was collected and subjected to Western Blot Analysis to determine p53, p21 and Bax proteins expression levels in treated and non-treated control cells. β-actin was used as a loading control.

MDM2 and Akt are negative regulators of p53 and the apoptotic process. Akt regulates cell survival, growth and glycogen metabolism, there are 3 isoforms of Akt; Akt1 which has a wide tissue distribution, Akt2 in muscles and adipose tissues and Akt3 in testes and brain. Akt is activated when growth hormones and cytokines bind to its receptor RTK. Mouse Double Minute 2 Homolog or MDM2 is a protein encoded by MDM2 gene and function as E3 ubiquitin ligase able to recognize the N-terminal of p53 and inhibits its transcriptional activation.

To elucidate the role of Bcl-2, Akt and MDM-2 in the anti-tumor activity of compound 9a, MCF-7 cells were treated with IC_{50} (20 µM) of compound 9a for 24 hours compared with untreated cells serving as a control. The lysates of both treated and untreated cells were prepared and processed for

western blot analysis to study the changes in the levels of Bcl-2, Akt and MDM-2 in order to evaluate the effects of compound 9a on these pathways. In this study, compound 9a inhibits the expression of Bcl-2, Akt and MDM-2 in MCF-7 treated cells compared to control.

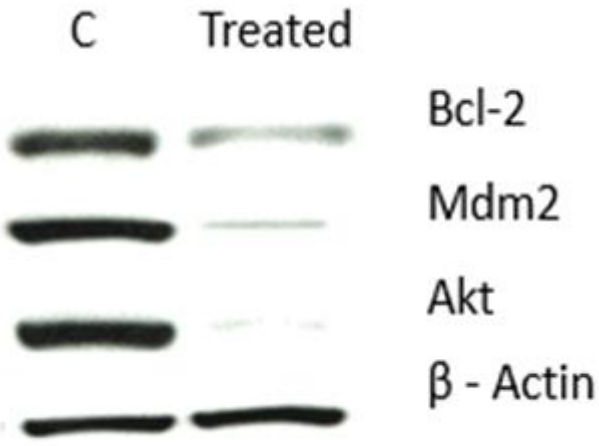


Figure 5.9.2. Compound 9a down-regulates Bcl-2, MDM-2 and Akt levels in MCF-7 Human Breast Cancer Cells. Cells were treated with 20 µM for 24 hours, cells were lysed and total protein was collected and subjected to Western Blot Analysis to determine Bcl-2, MDM-2 and AKT proteins epression levels in treated and non-treated control cells. β-actin was used as a loading control.

Apoptosis execution pathway depends mainly of caspase 3, caspase 3 is a cysteine protease cleave ADP-ribose polymerase and activate SREPBs. Caspase 3 also activates other caspases such as 6, 7 and 9. Caspase 3 is activated by pro caspase 3, therefore the pro form and the active form of caspase 3 constitute a useful biomarker to confirm apoptosis.

To elucidate the role of caspase 3 in the anti-tumor activity of compound 9a, MCF-7 cells were treated with IC_{50} (20 µM) of compound 9a for 24 hours compared with untreated cells serving as a control. The lysates of both treated and untreated cells were prepared and processed for western blot analysis to study the changes in the levels of pro-caspase 3 and caspase 3 in order to evaluate the effects of

compound 9a on these pathways. In this study, compound 9a up-regulated the expression of caspase 3 in MCF-7 treated cells compared to control.

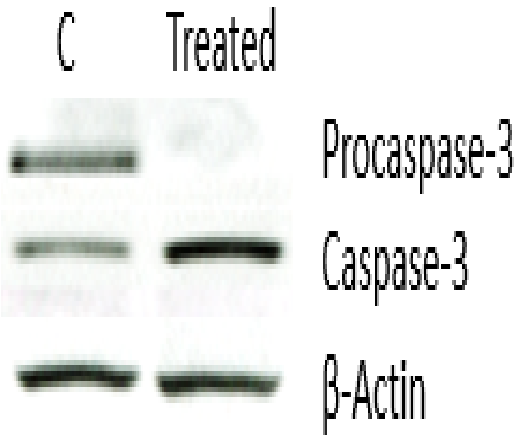


Figure 5.9.3. Compound 9a u-regulate the levels of Caspase-3 in MCF-7 Cells. Cells were treated with 20 µM for 24 hours, cells were lysed and total protein was collected and subjected to Western Blot Analysis to determine Pro-caspase and Caspase-3 proteins expression levels in treated and non-treated control cells. β-actin was used as a loading control.

6. **Discussion**:

The discovery of novel chemotherapeutic analogs with improved safety profile for the treatment of cancer is highly warranted. Pyridine and its derivatives have occupied an important realm in synthetic organic chemistry due to their therapeutic and pharmaceutical properties. Pyridine derivatives have emerged as the backbone of over than 7,000 existing drugs, pyridine ring is an integral part of antimicrobial, anticancer and antiviral agents (Cocco et al., 2005). They have also emerged as potent anti-cancer agents, Epothilones with a nitrogen atom at ortho position and methyl substitution on pyridine ring exhibited anti-cancer activity against several cancer cell lines (Wang, 2005). Furthermore Heyakawa et al., was successful in synthesizing a series of pyridine derivatives with potent inhibition activity against tumor cells by inhibiting PI3K p110a (Heyakawa et al., 2000).

In the present study, the effect of twenty novel pyridine derivatives were screened for their anti-cancer activity against different cell lines MCF-7, MDA-MB-231, HCT-116, SKOV-3 and OVCAR-3. To assess if their effect is cell or tumor type specific. Our data revealed that treating the above mentioned cancer cell lines with an initial concentration of 100µM for 24 hours suppressed the viability as follows: compound 9a reduced the viability by 40% in MCF-7 cells and by 45% in HCT-116, compound 7b reduced the viability in SKOV-3 by 35% and finally compound 11 suppressed the viability of MCF-7 by 44%.

The initial screening of the novel pyridine derivatives indicated that these derivatives are selective in their anti-cancer activity, i.e. not tumor type specific nor generic agents but rather cell type specific. For instance, compound 9a was effective against MCF-7 cell lines but not MDA-MB-231 cell lines, despite the fact that both are adherent epithelial mammary cells derived from metastatic

adenocarcinoma. However, MCF-7 cell lines are responsive to hormone therapy, while MDA-MB-231 is not and is more resistant to treatment and drug therapy than MCF-7. Guisado reported that Resveratrol, a chemotherapeutic agent is able to induce apoptosis in MCF-7 but failed to do so in MDA-MB-231.

Furthermore, MCF-7 and MDA-MB-231 differ in the expression of p53 protein, MCF-7 exhibits the wild type p53 while MDA-MB-231 has a mutation in the arginine amino acid replaced by lysine at position 280 (Gurtner, 2010). This mutation renders the protein in MDA-MB-231 unstable, these cells also exhibit large amounts of phospholipase D which plays a role in the survival and sustention of the mutated p53 (Hui, 2006). Compound 9a was also effective against HCT-116 cell lines, expressing the wild type p53. In addition, compound 7b reduced the viability of SKOV-3 cell lines by 35%, however in OVCAR-3 cell line the viability was 100%, both SKOV-3 and OVCAR-3 are adherent ovarian epithelial cells derived from Caucasian female with adenocarcinoma. OVCAR-3 is a well-studied model of drug resistance in ovarian cancers (ATCC, 2014).

Compound 9a was chosen to investigate the mechanism of action of this novel pyridine derivative against MCF-7 cell lines. The focus on MCF-7 was due to the dramatically increased number of breast cancer cases worldwide, breast cancer is the most frequently diagnosed cancer among women in about 140 countries globally, representing one in four of all cancers in women. The incidence of breast cancer has increased intensely in developed countries, however the mortality rate is much higher in developing countries due to lack of early detection of the disease (Jemal et al., 2011). In the State of Qatar specifically, breast cancer remains the top cause of death among women accounting for 36.3% of the total cases of cancer (Qatar Cancer Registry, 2014).

The study has revealed that compound 9a has anti-proliferative activity against MCF-7 cells with an IC_{50} of 20+/-0.268 µM. Previous researches in this field have supported the premise of using pyridine derivatives as anti-cancer agents against MCF-7 cell lines. Davari has published the anti-cancer activity of4-(1-benzyl-2-ethylthio-5-imidazolyl)-6-(4-bromophenyl0-2-imino-1,2-dihydropyridine-3-carbonitrile against MCF-7 cells with an IC_{50} of 50.18+/- 1.11 µM (Davari et al., 2014). This anti-proliferative activity against MCF-7 cells has also been detected by isatin-pyridine hybrid agents with IC_{50} of 6.3+/-0.79 (Eldehna et al, 2014).

Studying the mechanism of which this novel derivative inhibits the proliferation of MCF-7 is central to build a conceptual framework at the cellular and molecular level assisting the rational approaches towards cancer treatment. Several pieces of evidences support that chemotherapeutic agents affect tumor cells through the biochemically regulated process of apoptosis or programmed cell death. This process received significant attention upon the discovery of inter-nucleosomal DNA breakdown that is different from necrosis. Apoptosis usually results in typical morphological and biochemical characteristics including condensation of the chromatin, shrinkage of the cytoplasm and blebbing of the plasma membrane.

Apoptotic cells present several epitopes attracting macrophages and phagocytic cells and preventing secondary inflammation or the unfavorable initiation of inflammation. Harmlessly disposing of cancer cells is one of the main considerations in chemotherapy and consequently induction of apoptosis is an important strategy in anticancer drug research and development. Initially, in this study treating MCF-7 cells with compound 9a for 24 hours resulted in observed apoptotic morphological features including the appearance of marked apoptotic bodies in concentration dependent manner.

The induction of apoptosis was further confirmed using Annexin-V Apoptotic Assay; a rapid and sensitive assay to detect different phases of apoptosis. The translocation of phosphatidylserine from the inner plasma membrane to the outer surface membrane is a sensitive way to detect cells undergoing apoptosis. Annexin-V conjugated to FITC binds phosphatidylserine on the cell surface; PI was used to detect cells in late apoptosis. The results of this assay indicated that approximately 46.8% of MCF-7 cells treated with IC_{50} "20 µM" of compound 9a for 24 hours are dying by the programmed cell death "apoptosis", 46.8% of the total population represented cells in early apoptosis, late apoptosis and death stage.

Testing the cytotoxicity of any chemotherapeutic agent on non-tumorigenic cells is an essential step in assessing the effectiveness of this novel chemotherapeutic agent, in this study the cytotoxicity of compound 9a was examined and compared in both MCF-7 cells and MCF-12a cell lines after treating the cells with IC_{50} of 20µM for 24hours.

MCF-12a cell lines are adherent epithelial mammary cells derived from 60 years old Caucasian female. Statistical analysis using non-parametric methods "Mann-Whitney Test" of the obtained viability % for both cells indicated that compound 9a is less toxic towards MCF-12a with a statistical significance of 0.001.

The cell cycle checkpoints analysis of MCF-7 cells treated with 20 µM of compound 9a for 24 hours did not reveal any significant changes when compared with the untreated MCF-7 cells. However, a slight increase in G1 phase of about 9% was indicated. Arresting the treated cell in different checkpoints is highly correlated to the amount of p21 protein; this correlation was demonstrated in a study of colon cancer cell lines. Treating colon cancer cells harboring the wild-type p21 with ɤ-

irradiation underwent cell cycle arrest, while cells lacking p21 were unsuccessful in arresting and instead proceeded to apoptosis (Manish, 2001). The study of p21 expression by Western blot indicated a slight change in the expression level of this protein, which may explain arresting MCF-7 cells in G1 phase.

In order to investigate the molecular pathway of compound 9a, the expression of various essential proteins in cell cycle and apoptosis have been studied using Western blot analysis. The obtained data indicated that compound 9a up-regulated p53 in MCF-7 cells treated with 20 µM for 24 hours. p53 the tumor suppressor protein plays an essential role in both regulating the cell cycle arrest and inducing apoptosis, cell lines harboring a mutation in p53 are resistant to most chemotherapeutic agents as indicated earlier in MDA-MB-231 cell line. Another study has revealed that neoplastic cells acquired different mutations including a mutation in p53 are highly resistant to apoptosis (Manish, 2001). MCF-7 cells express the wild type p53 and therefore have the capability to undergo programmed cell death and cell cycle growth arrest. p53 is the most studied tumor suppressor and can apparently be activated by different chemotherapeutic agents including pyridine derivatives.

Oren and his coworkers were the first to investigate the role of p53 in controlling apoptosis upon transfecting myeloid leukemia deficient cells with wild type p53 (Yonish et al., 1991).

Later on, several pieces of evidence supported the role of p53 as a major regulator of programmed cell death using p53 knockdown thymocytes (Clarke et al., 1993). Interestingly, p53 has also been shown to contribute to chemotherapy-induced cell death, on the other hand loss of p53 is a major cause of chemotherapy resistant (Lowe et al., 1993). Up-regulation of p53 activates the apoptotic pathway by controlling several pro-apoptotic proteins of the Bcl-2 family including Bax.

Bax acts as a down-stream regulator to initiate apoptosis, several studies have indicated that the disruption of Bax level in HCT116 cells results in defect in the apoptotic pathway (Zhang et al, 2000). Our current data has revealed that compound 9a can significantly up-regulates Bax expression and thus initiates the apoptotic pathway.

Furthermore, the effect of this potent pyridine derivative compound has also been investigated for important anti-apoptotic proteins such as Bcl-2. Bcl-2 is a strong anti-apoptotic signaling protein of the Bcl-2 family that has been significantly down-regulated in MCF-7 cells upon treatment with compound 9a 20µM for 24hours. Bcl-2 binds to Apaf-1 and thus inhibits the activation of the caspases and execution of the cell, however when the levels of Bax increase, the binding will be disturbed allowing the activation of the apoptotic pathway.

Mdm-2 has also been down-regulated by compound 9a, Mdm-2 is the mouse double minute 2 protein regulated by Mdm-2 gene and was originally discovered in fibroblast 3T3 cells. Mdm-2 is highly associated with p53 and evidence has shown that this protein is capable of controlling the tumor suppressor p53 in the cell. Both proteins are linked to each other by the negative feedback loop that aims to regulate the expression level of p53 and thus protecting species from excess apoptosis and neurodegenerative diseases (Ute .M, 2003).

Mdm-2 is an E3-ubiquitin ligase that inhibits p53 by mainly two mechanisms, by binding to the transactivation domain in p53 thus inhibiting p53-dependent transcription, or by the ubiquitination of p53 and targeting it to proteasome degradation. Induction of p53 and inhibition of Mdm-2 relationship

in the cell will result in the accumulation of p53 and the activation of the apoptotic pathway. Mdm-2 has recently becomes a focus for much research and a target of many cancer therapies (Fulda, 2015).

Compound 9a has also down-regulated the expression of Akt protein (protein-kinase B) in MCF-7 cells after 24hour treatment, the serine-threonine kinase plays an essential role in various cellular processes including apoptosis and cell survival and proliferation (Ute .M, 2003).

In order to investigate whether compound 9a induces caspase-dependent or independent cascade we have also studied the expression of caspase-3. Caspase-3 is an essential member of the cysteine-aspartic acid protease encoded by Casp3 gene and plays a vital role in the execution of cells undergo apoptosis. Caspase-3 exists in an inactive form called “pro-caspase-3” which undergoes proteolytic processes at specific aspartic residues to generate the large and small domains forming the active enzyme. Caspase-3 can be activated it by different caspases 8, 9 and 10. The results of the current study has revealed that compound 9a up-regulates the expression of Caspase-3 in MCF-7 cells treated for 24 hours with the IC_{50} of 20 µM, which indicates that compound 9a induces apoptosis in MCF-7 cells through the caspase-dependent cascade (Fulda, 2015).

6.1. Diagram of Discussion:

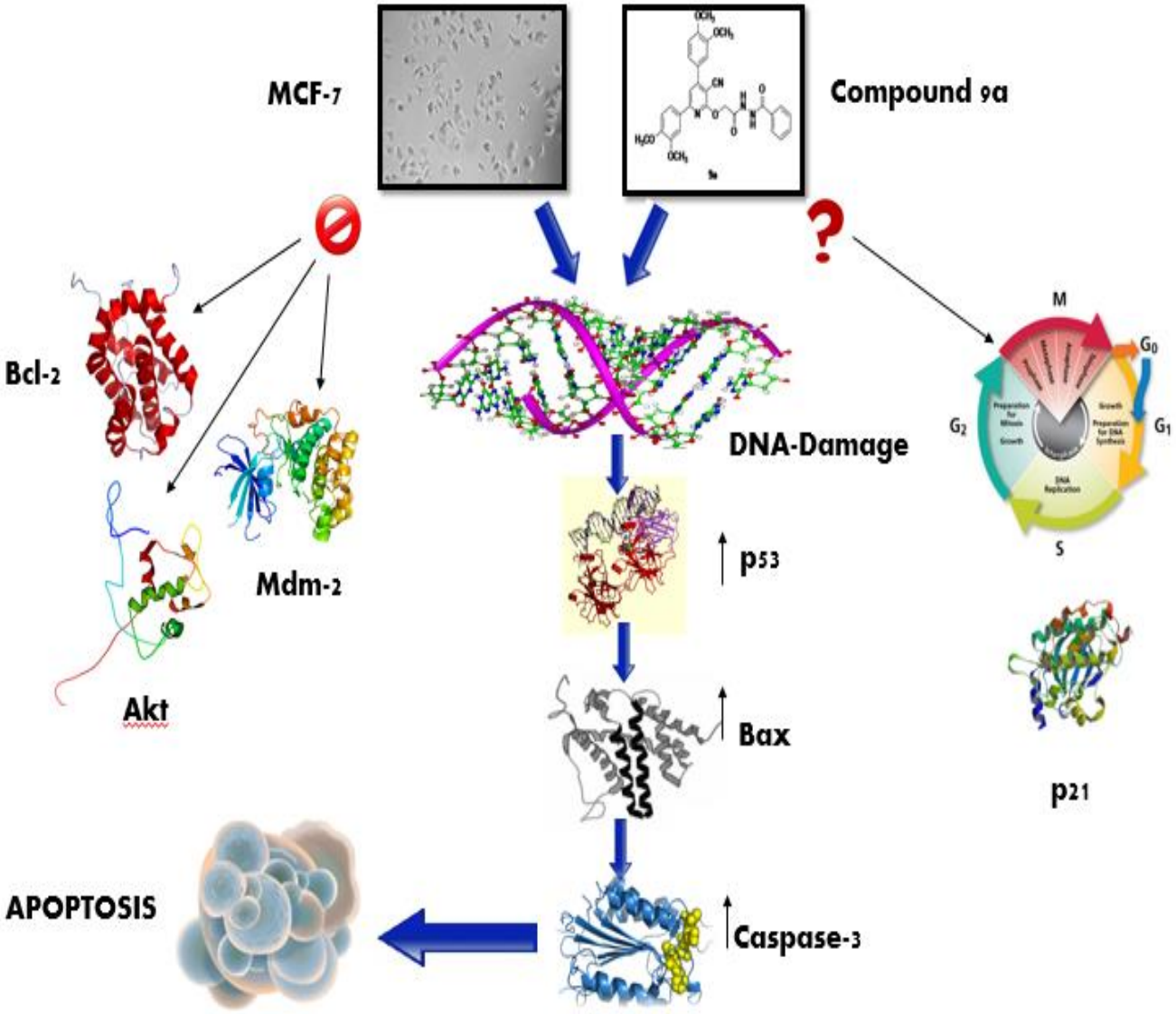


Figure 6.1. Summary of Impact of 9a on MCF-7 cells. Compound 9a induced apoptosis by up-regulating the expression levels of p53, Bax and Caspase-3 and down-regulating Bcl-2, Akt and Mdm-2 proteins. Compound 9a slightly arrested MCF-7 cells in G1 phase correlated with the slight increase in the expression of p21.

7. Conclusion and Recommendation:

Discovering novel chemotherapeutic agents with improved safety profile and less toxicity to non-tumorigenic cells is highly warranted due to the dramatically increasing cancer incidence and mortality. Pyridine derivatives have proven to be potent and effective agents in treating different types of cancer. In the present study, compound 9a revealed anti-proliferation activity against MCF-7 cells and resulted in the induction of apoptosis. Compound 9a up-regulated the expression of p53, Bax and Caspase-3 proteins and down-regulated the following proteins: Bcl-2, Mdm-2 and Akt. Compound 9a exhibited less toxicity on non-tumorigenic breast epithelial cells MCF-12a with a statistical significance of p value < 0.05.

Findings of this study indicated that compound 9a possessed anti-proliferative activity against MCF-7 cells and may be a promising chemotherapeutic agent with less toxicity to non-tumorigenic breast epithelial cells MCF-12a. However, screening novel derivatives on other cancer cell lines is highly required. In addition, mechanistic examinations are also necessary to evaluate the exact role of compound 9a against MCF-7 cells. In-vivo studies are necessary to investigate the mechanism and impact of 9a on physiological processes.

Although the aims of the study were addressed, there were a few limitations to the study. Assessment of ploidy and doubling time of used cell lines were not conducted due to time limitation. More mechanistic investigations are necessary to evaluate the exact molecular mechanism of compound 9a. Finally, screening in different cancer cell lines and their non-tumorigenic counterparts is essential; however the present research is limited with the available cell lines.

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9. **Arabic Abstract and Summery:**

اكتشاف مركبات جديدة من مجموعة مركبات البيريدين لعلاج اورام السرطان

مقدمة من
منـــــى عمـــــر محمـــــود محســـــن
بكالوريوس علوم حيوية طبية، جامعة قطر
للحصول على درجة الماجستير
في العلوم الحيوية الطبية

لجنة المناقشة والحكم: التوقيع

د/ أحمد محمد مالكي (ممتحن داخلي)
استاذ مشارك ومنسق الدراسات العليا في برنامج العلوم الحيوية الطبية
جامعة قطر .....

بروفيسور/ خالد مشاقه (ممتحن خارجي)
بروفيسور في العلوم الفسيولوجية والفيزياء البيولوجية
عميد مشارك لشؤون البحوث
كلية طب وايل كورنيل
الدوحة – قطر .....

دكتور/ حسان عبد العزيز (ممتحن داخلي)
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عميد مساعد في كلية العلوم
جامعة قطر .....

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جامعة قطر .....

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بروفيسور أمراض المناعة والأورام
رئيس المركز الوطني لعلاج وأبحاث السرطان – قطر .....

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جامعة قطر

التوقيع .....

بروفيسور/ خالد مشاقه
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عميد مشارك لشؤون البحوث
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الدوحة – قطر .....

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أستاذ مشارك ورئيس قسم العلوم الحيوية الطبية
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جامعة قطر .....

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بروفيسور أمراض المناعة والأورام
رئيس المركز الوطني لعلاج وأبحاث السرطان – قطر .....

اكتشاف مركبات جديدة من مجموعة مركبات البيريدين لعلاج اورام السرطان

ملخص البحث

خلفية البحث: يعتبر السرطان المسبب الثاني للوفيات عالميا حيث تشير احصائيات المنظمة العالمية لأبحاث السرطان أن خطر الإصابة بمرض السرطان ازداد دراماتيكيا في عام 2012 حيث وصلت الاحصائيات لما يقارب 14 مليون حالة جديدة وما يقارب 8 مليون حالة وفاة. ويتوقع زيادة هذه النسب الى ما يقارب 19 مليون حالة في عام 2025 و24 مليون في عام 2035 م، وتعود هذه التوقعات الى الزيادة المتوقعة في نسبة الأعمار بين الشعوب. كما تمثل حالات السرطان في دولة قطر 10% من مجمل الوفيات، وتعتبر هذه النسبة عالية نسبيا بالنسبة لمرض منفرد، كما ويتوقع أن يتضاعف هذا العدد بدرجة عالية في عام 2030. وعليه، تتسم الحاجة الى اكتشاف علاجات جديدة من شأنها المساهمة في علاج هذا المرض الخبيث ولتحسين نوعية العلاجات المتوفرة. لقد حظيت مركبات البريدين بشهرة واسعة في مجال الكيمياء العضوية لما أثبتته من فعالية كمركبات ذات خصائص علاجية وكيميائية، وتمثل حلقة البريدين حلقة أساسية في العديد من المركبات المستخدمة ضد مختلف الأحياء الدقيقة والفيروسات والاورام المختلفة.

التجارب العملية: في بحثنا الحالي فقد قمنا بدراسة مفصلة لعشرين مركب جديد من فصيلة مركبات البيريدين Pyridine Derivatives على أكثر من نوع من الخلايا السرطانية ومنها (سرطان الثدي، سرطان الرحم وسرطان القولون) لمعرفة تأثير هذه المركبات ان كان عاما ضد مختلف أنواع السرطانات أم ذا تأثير معين. وتم دراسة التركيز التثبيطي النسبي للمركب 9أ باعتباره مركب فعال ضد خلايا الثدي السرطانية، كما تم دراسة الموت المبرمج للخلايا عن طريق تجارب معينة مثل Annexin-V FITC ومراحل دورة الخليه باستخدام Flow Cytometry. كما تمت دراسة المسار الجزيئي للخلايا بعد معالجتها بمركب 9أ عن طريق استخدام Western Blo، بالإضافة الى دراسة تأثير المركب على الخلايا الطبيعية للثدي.

النتائج: أوضحت الدراسة على مركبات البيريدين عند استخدام تركيز 100µM ومعالجة مختلف الخلايا السرطانية لمدة 24 ساعة وجود 4 مركبات فعاله كما يلي: المركب 9أ يثبط خلايا MCF-7 بما يعادل 40% وخلايا HCT-116 بنسبة 45%، كما يقوم المركب 7ب بتثبيط خلايا SKOV-3 بنسبة 35% ومركب 11 يقوم يتثبيط ذات الخلايا بنسبة 44%. وقد تركزت التجارب اللاحقة على مركب 9أ حيث أثبتت التجارب أن مركب 9أ يعمل من خلال تحفيز الخلايا على الموت المبرمج بتركيز تثبيطي نسبي يعادل 20µM. كما قام المركب 9أ بزيادة تركيز البروتين p53, Bax and Caspase-3 في خلايا MCF-7 بعد علاجها بالمركب لما يعادل 24 ساعة، وفي المقابل قام ذات المركب بتثبيط البروتينات Bcl-2, Mdm-2 and Akt. أخيرا فقد أثبتت التجاربة أن التأثيرات السمية على خلايا الثدي الطبيعية كانت أقل عند مقارنتها احصائيا بالخلايا السرطانية.

حصيلة البحث: تدل النتائج النهائية للبحث أن مركب 9أ يمثل مركبا فعالا ذا تأثيرات تثبيطية للخلايا السرطانية MCF-7 وبالتالي قد يمثل مركبا مهما ذا تأثير أقل على الخلايا الطبيعية وبالتالي تجبن التأثيرات الجانبية العلاجات الكيمائية. ومن الضروري استكمال الأبحاث على هذا المركب لمعرفة تأثيره على أنواع أخرى من الخلايا السرطانية ولمعرفة كيفية عمل هذا المركب الفعال وطريقة تثبيطه للخلايا MCF-7، بالإضافة الى ضرورة عمل دراسات على الفئران.

الموجز العربي للبحث

موضوع البحث: اكتشاف مركبات جديدة من مجموعة مركبات البيريدين لعلاج اورام السرطان

مقدمة البحث:

يعتبر السرطان المسبب الثاني للوفيات عالميا حيث تشير احصائيات المنظمة العالمية لأبحاث السرطان أن خطر الإصابة بمرض السرطان ازداد دراماتيكيا في عام 2012 حيث وصلت الاحصائيات لما يقارب 14 مليون حالة. كما تمثل حالات السرطان في دولة قطر 10% من مجمل الوفيات، وتعتبر هذه النسبة عالية نسبيا بالنسبة لمرض منفرد، كما ويتوقع أن يتضاعف هذا العدد بدرجة عالية في عام 2030. وقد حظيت أبحاث السرطان في دولة قطر برعاية سمو الشيخة موزة بنت ناصر المسند انطلاقا من مقولتها الشهيرة " إن محاربة السرطان تعد سبباً رئيساً في توحد المجتمعات بكافة المناطق حول العالم وقد تكون قطر دولة صغيرة من حيث عدد السكان، لكن لدى مجتمعها رغبة كبيرة في أن تلعب دوراً ريادياً في مجال البحث وتطوير العلاجات واستحداث أشكال جديدة للرعاية الصحية، لذا نسعى جاهدين لتحقيق أفضل النتائج لدى مرضى السرطان ومن يهتم برعايتهم.".

الهدف من البحث:

استنادا الى ما تقدم، تتسم الحاجة الى اكتشاف علاجات جديدة من شأنها المساهمة في علاج هذا المرض الخبيث، وفي بحثنا الحالي فقد قمنا بدراسة مفصلة لعشرين مركب جديد من فصيلة مركبات البيريدين Pyridine Derivatives على أكثر من نوع من الخلايا السرطانية ومنها (سرطان الثدي، سرطان الرحم وسرطان القولون) لمعرفة تأثير هذه المركبات ان كان عاما ضد مختلف أنواع السرطانات أم ذا تأثير معين. حيث تم فحص العشرين مركب بتركيز أولي يبلغ 100 µM على كافة أنواع الخلايا السرطانية سابقة الذكر، ومن ثم انتقاء المركبات الاكثر فعالية والتي تثبط الخلايا السرطانية بما يعادل 50%. ومن ثم دراسة المركبات الفعالة فقط لمعرفة طريقة عملها على المستوى البيولوجي للخلايا.

الاختبارات العملية:

لقد أثبتت نتائج الفحص الأولية للمركبات العشرين بتركيز 100µM وجود أربعة مركبات فعالة ضد مختلف الخلايا السرطانية كما يلي: مركب 7ب اثبت فعاليته ضد خلايا SKOV-3 بنسبة 35% وهي نوع من أنواع سرطانات الرحم، مركب 9أ أثبت فعاليته ضد خلايا سرطان الثدي MCF-7 بنسبة 40% وخلايا سرطان الكبد بنسبة 45%، وأخيرا المركب 11 أثبت أيضا فعاليته ضد خلايا SKOV-3 كما الحال لدى المركب 7ب ولكن بنسبة أقل تعادل 44%.

ومن ثم قمنا باختيار المركب 9أ لعدة تجارب والتي تبين الطريقة البيولوجية لعمل هذا المركب على خلايا MCF-7 كما يلي:

- ***التغيرات الشكلية في خلايا MCF-7 وقياس قيمة التثبيط التركيزي النصفي للمركب 9أ:***

تم علاج خلايا MCF-7 بتركيزات مختلفة تدريجية من المركب 9 أ (100 µM – 50 – 20 – 5 – 1 – 0.5) وملاحظة التغييرات الشكلية للخلايا، لم تظهر أية تغييرات الشكلية على الخلايا عند المعالجة بالتركيزات (5 µM – 1 – 0.5) بينما بدأت الخلايا تتغير شكليا الى ما يسمى الموت المبرمج للخلايا (أو الموت المقدر) عند التركيزات الأعلى ابتداء من 20 µM . وتتمثل هذه التغييرات الشكلية بما يلي: انكماش الخلايا وظهور فقاعات او ما يسمى blebs، تغير النواه تغير دراماتيكي متمثل في تكثف الكروماتين، كما تحتفظ هذه الخلايا بالعضيات الداخلية، وأكثر ما يميز هذا النوع من موت الخلايا أنه يتم التهامها من قبل أنواع مختلفة من الخلايا المناعية أو الخلايا المجاورة دون استثارة الجهاز المناعي في الجسم. كما تم قياس التركيز التثبيطي النصفي للمركب 9أ برسم العلاقة البيانية بين النسبة المئوية للتثبيط والتركيز المثبط للنمو، وعليه فقد تم احتساب التركيز التثبيطي النصفي بما يقارب 20uM.

- ***دراسة الموت المبرمج للخلايا عند معالجة خلايا سرطان الثدي MCF-7 بالمركب 9أ:***

ولتأكيد أن المركب 9أ يثبط الخلايا السرطانية MCF-7 عن طريق الموت المبرمج للخلايا فقد قمنا باجراء ما يسمى (فحص الموت المبرمج للخلايا Annexin-V FITC) لدراسة التغييرات التي تطرأ على الغشاء الخلوي لخلايا MCF-7 والتي تتمثل أساسا في تغير موقع phosphatidylserine PS من الطبقة الداخلية للغشاء الى الطبقة الخارجية. ويعد هذا التغيير حدث مهم في الموت المبرمج للخلايا، وعليه يقوم المركب Annexin-V والذي يرتبط بالمركب FITC بالارتباط بالدهون الفسفورية PS وبالتالي تتبع الخلايا التي

تخضع للموت المبرمج. وقد اثبتت التجارب أن ما يقارب من 48.4% من خلايا MCF-7 قد خضعت للموت المبرمج بعد علاجها بالمركب 9µMأ بتركيز 20 لما يقارب 24 ساعة.

- ***دراسة تأثير المركب 9أ على مراحل انقسام الخلايا ومراقبة الجودة في خلايا MCF-7:***

كما تم دراسة تأثير المركب 9أ على مراحل انقسام الخلايا MCF-7 ومراقبة الجودة ، الا أن نتائج البحث لم تظهر تغييرات جوهرية تثبت تأثير المركب 9أ على مراحل انقسام الخلايا MCF-7 عند معالجتها تركيز 20 لما يقارب 24 ساعة. ومن خلال الاطلاع على العديد من الأبحاث السابقة في هذا المجال فان من صفات بعض المركبات ذات التاثير الكيماوي على الخلايا السرطانية أن تقوم بالتأثير على مراحل نمو هذه الخلايا و دفعها الى الموت المبرمج معا، الا أن بعض المركبات تثبط الخلايا السرطانية باتباع احدى هذه الطرق فقط وليس كليهما. ولتكيد هذه المعلومة فقد قمنا بإعادة التجربة المذكورة باستخدام تركيز أعلى من المركب 9أ (50 µM)

- ***دراسة تأثير المركب 9أ على المسار الجزيئي في خلايا MCF-7:***

تعمقا في دراسة كيفية عمل المركب 9أ فقد قمنا بدراسة مفصلة للمسار الجزيئي بعد معالجة خلايا MCF-7 بتركيز 20µM لما يقارب 24 ساعة. وقد أوضحت النتائج زيادة ذات دلالة إحصائية في البروتينات التالية p53, Bax and Caspase-3 والتي تلعب دورا مهما واساسيا في الموت المبرمج للخلايا، كما لوحظ وجود انخفاض واضح في البروتينات المثبطة للموت المبرمج للخلايا ما يسمى زمرةBcl-2 ، كما تم تثبيط بروتينات أخرى منها Mdm-2 التي تلعب دورا حيويا في ابطال مفعول بروتين p53. وكذا كان التأثير على بروتين Akt والذي يلعب دورا فعالا في زيادة انقسام الخلايا ونموها. أما بالنسبة لبروتين p21 فلم توضح النتائج ما يثبت زيادة معدلها وهذا يتوافق مع النتائج السابقة والدالة على عدم تأثير المركب 9أ على دورة انقسام الخلايا MCF-7.

النتائج:

أوضحت الدراسات على مركبات البيريدين وجود أربع مركبات فعالة ضد خلايا سرطانية مختلفة مما يبين أن تأثير هذه المركبات هو محدد لنوع الخلايا السرطانية وليس مركب عام لعلاج جميع أنواع الأورام السرطانية. وقد تمت دراسة المركب 9أ لفعاليته ضد خلايا سرطان الثدي MCF-7، ولقد أوضحت الدراسة المعملية أن المركب 9أ له تأثير مضاد لنمو هذه الخلايا من خلال تجزئة DNA وتحفيز الخلايا للموت المبرمج أو الموت الاضطراري، وتم تأكيد هذه النتائج بعمل اختبارات أخرى تؤكد هذا التأثير ومنها فحص الموت المبرمج للخلايا باستخدام Annexin-V ودراسة المسار الجزيئي والتحليل الكمي لمستوى بروتينات متعددة. وقد تبين أن المركب 9أ يقوم بزيادة ذا دلالة إحصائية للبروتينات p53, Bax & caspase -3 والتي تلعب دورا أساسيا في الموت المبرمج للخلايا، في حين يقوم ذات المركب بخفض مستوى Bcl-2 البروتين المضاد لموت الخلايا. بالإضافة الى خفض مستوى بعض البروتينات الأخرى مثل Mdm-2 & Akt.

محصلة البحث:

نستنتج من هذه الدراسة أن مركب 9أ له تأثير مضاد لسرطان الثدي نوع MCF-7 ، ويعمل هذا المركب من خلال تحفيز الموت المبرمج لهذه الخلايا تتمثل في عدة دلالات منها: التغيرات المورفلوجية في شك الخلايا بعد علاجها بالمركب بتركيز IC_{50} ، تتبع التغيرات الفسيولوجية في الغشاء البلازمي لهذه الخلايا بعد العلاج وأخيرا من خلال دراسة المسار الجزيئي والذي أوضح زيادة معدلات البروتينات الأساسية في هذا النوع من الموت المبرمج مثل p53, Bax and Caspase-3 وخفض معدلات البروتينات المثبطة مثل Bcl-2. كما أوضح التحليل الكمي لمستوى البروتين Mdm-2 انخفاضا واضحا يتوافق مع الزيادة المطردة للبروتين p53. هذا إضافة الى خفض مستوى بروتين Akt والذي يلعب دورا حيويا في نشاط الخلايا السرطانية وزيادة سرعة انتشارها. لم تبين تجارب دراسة مراحل انقسام الخلية ومتابعة الجودة أي تأثير للمركب 9أ وهو ما تم تأكيده من خلال دراسة مستوى بروتين p21، وهذا يؤكد أن المركب 9أ يثبط خلايا MCF-7 من خلال الموت المبرمج للخلايا وليس بالتأثير على دورة انقسام الخلية.

التوصيات:

يتوجب مستقبلا عمل دراسات أخرى على أنواع مختلفة من الخلايا السرطانية مثل خلايا سرطان الرئة والجلد والدم لمعرفة تأثير هذا المركب عليها، كما يتوجب دراسة تأثيره أيضا على خلايا الكبد السرطانية حيث اثبتت التجارب الأولية فعاليته بتثبيط خلايا HCT-116 بنسبة 45% والتي تحتوي على البروتين p53. كما يتوجب عمل دراسات معينه على الجرذان لمعرفة تأثير المركب 9أ على الأورام الداخلية.