CHARACTERIZATION OF VR118 QUINACRINE DERIVATIVE AS A POTENTIAL ANTICANCER AGENT

by

Danah Almnayan

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Thesis Examiners/Examinateurs de thèse:

Dr. Hoyun Lee
(Supervisor/Directeur de thèse)

Dr. Amadeo Parissenti
(Committee member/Membre du comité)

Approved for the School of Graduate Studies

Dr. Mazen Saleh   Approuvé pour l'École des études supérieures
(Committee member/Membre du comité)   Dr. David Lesbarrères
                                         M. David Lesbarrères

Dr. Ingeborg Zehbe   Director, School of Graduate Studies
(External Examiner/Examinatrice externe) Directeur, École des études supérieures

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ABSTRACT

The discovery and development of effective chemotherapeutic agents in the past few decades have immensely enhanced the treatment and management of human cancer. However, because these drugs are associated with adverse side effects, high genotoxicity, risk for secondary cancers and devastating effects on the patients' immune system; the need for developing more effective anticancer agents remains. A priority Research shows that 9-aminoacridine (9AA) derivatives have substantial anticancer properties. The pharmacological properties of this agent are well characterized and this scaffold has been widely used to treat different diseases for decades. Quinacrine is a 9AA derivative, which was first discovered as an antimalarial compound in 1930's and since then had been widely used in treating a variety of parasitic infections and demonstrated potential for cancer treatment. Importantly, the polypharmacology of Quinacrine makes it an attractive drug to treat a variety of cancers. Quinacrine acts by specifically targeting cellular signaling pathways that play an important role in cell survival. Given the distinctive cancer treating abilities of Quinacrine by specifically targeting cellular signaling pathways, it was the objective of this study to develop a compound that has similar properties as Quinacrine but has better efficacy and selectivity in targeting tumor cells. Therefore, for this project we created derivatives of 9AA compound using hybrid pharmapore approach and examined one of the derivatives of Quinacrine compound named VR118. After performing a series of experiments to test the efficacy and selectivity of the Quinacrine derivative VR118, I came to the conclusion that VR118 is highly effective in treating cancer cells and have the potential to selectively target cancer cells without causing severe harm to normal cells at concentrations applicable for malignant cell lines. This report discusses the efficacy and selectivity of VR118 compound in targeting cellular signaling pathways and the mechanisms through which VR118 kills cancer cells.

ACKNOWLEDGEMENT

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LIST OF ABBREVIATIONS

^{0}C   degree in Celsius

cc   cubic centimeter

cm   centimeter

DMEM   Dulbecco's Modified Eagle Medium

DNA   deoxyribonucleic acid

DSBs   double-stranded breaks

ECL   enhanced chemiluminescence

EGF   epidermal growth factor

FACS   fluorescence-activated cell sorting

FBS   fetal bovine serum

fig   figure

GAPDH   glyceraldehyde 3-phosphate dehydrogenase

HDAC1   histone deacetylase 1

h   hour

QC   Quinacrine

IC$_{50}$ 50% Inhibitory concentration (dose which inhibits 50% of cell proliferation)

M   molar

MDM2   murine double minute 2

μM   micromolar

mM   millimolar

μl   microliter

μg   microgram

min	minute
mL	milli liter
mRNA	messenger RNA
mTOR	mammalian target of rapamycin
NF-κB	nuclear factor kappa-light-chain-enhancer of activated B cells (signaling molecule
nm	nanometer
PBS	phosphate buffered saline
PI	propidium iodide
PLA2	phospholipase A2
PMSF	phenylmethanesulfonylfluoride
PTEN	phosphatase and tensin homolog
RCC	renal cell carcinoma
RIPA	radioimmunoprecipitation assay buffer
RNA	ribonucleic acid
Rnase	ribonuclease
RPMI	Roswell Park Memorial Institute medium
SDS-PAGE	sodium dodecyl sulfate-polyacrylamide gel electrophoresis
SRB	sulforhodamine B
TBS	tris buffered saline
TBST	tris buffered saline tween20
TCA	trichloroacetic acid
TUNEL	terminal uridine nick-end labeling
v/v	volume/volume
w/v	weight/volume

Chapter 1

1   INTRODUCTION

1.1   Cancer Epidemiology

Cancer is an extremely heterogeneous disease that occurs due to series of genetic changes and molecular events that fundamentally alter the normal properties of cells. Therefore, cancer is a generic term that is generally used to describe a large group of malignant tumors and neoplasms affecting any part of the body (Does et al., 2013). Normal cells in a multicellular organism function in accordance with the normal rules that govern the basic function of cells such as cell growth, reproduction, and apoptosis (programmed cell death) (Does et al., 2013). However, cancer cells do not function normally and, therefore, proliferate despite the presence of signals that normally inhibit cell growth and proliferation.

Many intracellular changes can cause cells to develop new characteristics; such as changes in cell structure, cell adhesion, and the production of new enzymes that lead to the loss of contact inhibition, loss of the apoptotic pathway, failure to mature and differentiate, and finally developing the ability to metastasize (Schneider, 2001). Metastasis is a critical step in the progression of cancer and is a major cause of death in cancer patients. When malignant cancer cells invade other tissues and organs, they may alter the cellular signaling pathways within these cells. This leads to the loss of function and efficiency of cellular signaling pathways that are required to control particular functions within these cells. Given the complex changes in cell behavior of metastatic cells along with the unpredictable nature of cancer cells in tissues where they invade, cells' response to a cancer therapy varies from patient to patient and case-by-case depending on the site and organ affected (Schneider, 2001).

Despite advances in treatment modalities in recent decades, cancer still remains largely a fatal disease. Globally, cancer was the leading cause of death in 2008 accounting for 7.6 million deaths (Globocan, 2008). The World Health Organization (WHO) estimates that cancer will account for approximately 13.1 million deaths worldwide by 2030 (WHO, 2013). The American Cancer Society (ACS) estimates that approximately 1,660,290 new cancer cases will be diagnosed in the US in 2013 and about 580,350 cancer patients will die in the same year (ACS,

2013a). Although international cancer organizations and healthcare systems in many countries are implementing effective preventative measures to reduce the burden of cancer by controlling for risk factors such as; tobacco use, alcohol use, unhealthy diet and physical inactivity, the effort has not proven very successful (WHO, 2013). Therefore, radiation and chemotherapy are still the main stay of reducing cancer burden.

1.2 Anticancer Therapies

Currently, various treatment options are available to treat cancer. However, the preference for a specific treatment depends on the type and stage of cancer, patient's response to a treatment, patient's overall health and preferences and possible side effects of the treatment. Common treatment options available for cancer include; chemotherapy, radiation therapy, hormone therapy and surgery. These treatments are often administered in combinations and have proven successful to an extent in eradicating cancer cells. Chemotherapy is a systematic treatment, which involves in the use of a single drug or a combination of drugs. Unlike radiation and surgery, which are local treatments, chemotherapeutic drugs travel throughout the body to destroy cancer cells wherever they are (Gullatte and Gaddis, 2004). Over the past few decades, scientific advances have resulted in the development of new chemotherapy drugs, which vary widely in their chemical composition and are useful in treating specific forms of cancer. Currently available chemotherapeutic drugs can be divided into several groups based on their chemical structures and modes of action. Different groups of chemotherapeutic drugs include (but are not limited to) alkylating agents, platinum-based compounds, anti-metabolites, anti-tumor antibiotics, topoisomerase inhibitors, mitotic inhibitors, and miscellaneous chemotherapy drugs (Freter and Perry, 2008).

1.3 Mechanism of Action of Chemotherapeutic Drugs

Watson and Crick revealed the DNA structure in 1953 (Watson and Crick, 1953). Cancer occurs due to mutations in DNA causing abnormal growth. Moreover, lack of DNA repair machinery in tumor cells due to certain gene mutations (e.g., checkpoint genes) results in the deregulation of DNA replication control mechanism, causing further DNA damage. These findings made DNA the primary target in developing anticancer drugs as it became very clear that DNA mutation is the main cause of tumorigensis (Gurova, 2009). However, advancements in cancer research in

the past few decades led to the development of chemotherapeutic drugs that kill cancer cells by blocking or inhibiting cell cycle progression without causing DNA damage.

The majority of anticancer chemotherapeutic drugs used to treat cancer patients are DNA-damaging agents. Some of the established groups of drugs that target DNA include: antimetabolites, alkylation agents and intercalators. Antimetabolites damage DNA by acting on nucleotides, alkylating agents such as nitrogen mustard cause direct DNA damage, while intercalators such as actinomycins damage DNA by binding to DNA and inhibiting the activity of many enzymes that use DNA as substrate (Malina et al., 2005; Trudi et al., 1991). Similarly, recently developed anticancer agents such as inhibitors of topoisomerases I and II, mitomycin C and platinum compounds as well as γ-irradiation also cause DNA damage to kill cancer cells (Finlay et al., 1989; Gurova, 2009).

Both alkylating agents and platinum-based drugs, such as nitrogen mustard and cisplatin target rapidly dividing cells by attacking nucleophilic sites. These drugs attach to the alkyl or alkyl-like group onto the guanine base of DNA causing cross-linking, thus damaging DNA and interfering with replication and transcription (Freter and Perry, 2008). Antimetabolites such as 5-fluorouracil (5-FU) and 6-mercaptopurine (6-MP) destroy tumor cells by interferring in DNA and RNA metabolism. Drugs 5-FU and 6-MP target rapidly diving cells by inhibiting specific enzymatic steps in the DNA synthesis pathway. For example, nucleoside analogues incorporate into DNA in the place of nucleotides and act as terminators, inhibiting further DNA synthesis (Freter and Perry, 2008).

Another anti-metabolic agent, dihydrofolatereductase (DHFR) inhibitor, inhibits the DHFR enzyme and prevents the reduction of folic acid into tetrahydrofolic acid, thus disrupting the synthesis of thymidine, adenine and guanine (Gullatte and Gaddis, 2004). Another class of chemotherapy drugs such as anti-tumor antibiotics (also called anthracyclines) interfere with enzymes involved in DNA replication (Freter and Perry, 2008). Though these chemotherapeutic agents have proven to be effective in reducing cell division and killing malignant cells; treatment with these compounds can not only effect normal cell physiology but also cause molecular alterations within cancer cells leading to the development of resistance (Gullatte and Gaddis, 2004).

Chemotherapeutic agents that do not cause DNA damage destroy cancer cells by inhibiting cell cycle progression and cell proliferation. Certain groups of chemotherapeutic drugs induce programmed cell death in cells by inhibiting protein kinases or by overstabilizing microtubules within cells. Flavopiridol is one such novel derivative of cyclin-dependent kinase inhibitors (CDKIs) that has the ability to induce apoptosis in neoplastic cells by inhibiting the activity of a number of protein kinases necessary for cell proloferation in cancer cells (Wang and Ren, 2010). Another group of chemotherapeutic agents called taxanes inhibit cell division in cancer cells by overstabilizing microtubles thus disrupting mitosis (Zhou, and Giannakakou, 2005).

1.4   Need for New Therapies

Although DNA damaging agents have proven successful in treating many cancers, there are a number of limitations associated with this approach. Firstly, DNA damaging agents are not the best agents to cure cancer because they can cause long-term damage to the bone marrow (Gurova, 2009). Secondly, they are limited to use due to their adverse side effects. Thirdly, the high genotoxicity associated with DNA-damaging agents increases the risk for secondary cancers (Gurova, 2009). In addition, chemotherapeutic agents also have a devastating effect on the patients' immune system and overall health. The undesirable side effects associated with chemotherapeutic drugs coupled with drug resistance and lowering immunity are the main cause of treatment failure in cancer patients. Similar many targeted therapies available currently exhibit considerable side effects though less severe and different from that of chemotherapeutic drugs.

Thus, there is the need to develop novel therapies that will rely on the combination of different inhibitors to prevent the emergence of resistant sub-population. Therefore, researchers are trying to develop new anticancer therapies that target both cancer cell-specific pathways and specific proteins that are directly involved in the neoplastic process without causing side effects. A desirable way to control tumor without damaging DNA, without developing resistant sub population and without lowering immunity is through the development of compounds that specifically target cancer cells by altering cellular signaling pathways that favor the induction of programmed cell death in tumor cells without causing any harm to normal cells.

1.5   Targeted Cancer Therapies

After decades of research, recent advances in science help in a better understanding of the complex intracellular processes of cancer cells. Researchers found that cancer cells differ significantly from normal cells in terms of metabolic properties. Cancer cells depend on aerobic glycolysis for energy production and have abnormal metabolic characteristics such as increased rate of fatty acid synthesis and glutamine metabolism (Zhao et al., 2013). Moreover published data suggest that increased fatty acid synthesis causes membrane biogenesis of tumor cell lipids fostering increased growth and survival (Pandey et al., 2012). Similarly, increased glutamine metabolism in tumor cells releases amino-acid precursors as byproducts, which are necessary for rapidly proliferating cells (Erickson and Cerione, 2010). Moreover, emerging evidence shows that cancer treatments can be improved by targeting cellular metabolism. Increased drug resistance in patients receiving chemotherapy coupled with the high failure rates of cancer treatments motivated researchers to develop cancer drugs that are targeted to work specifically on cancer cells and not normal, healthy cells (Zhao et al., 2013).

Targeted cancer drugs also referred to as 'molecularly targeted drugs' or 'molecularly targeted therapies' are substances that interfere with specific proteins or molecules that promote tumor growth and progression in cancer cells; thereby blocking the growth and metastasis of cancer cells (Pandey et al., 2012). Because cancer targeted therapies mainly target molecular changes within cells and are capable of inhibiting proteins that are highly expressed in cancer cells when compared with normal cells, they are more effective than other therapies such as DNA damaging chemotherapy and radiation treatment. In addition, normal cells are less affected in cancer-targeted therapies and have less serious side effects, compared to chemotherapy drugs and radiation.

Targeted therapies kill cancer cells by interfering with the ability of the cell to divide, grow and repair. Targeted therapies primarily involve the use of humanized monoclonal antibodies and small molecules that can alter the normal functioning of cells causing them to die (ACS, 2013b; NIH, 2013). Some of targeted therapies available today include:

a) Signal transduction inhibitors: These targeted therapies block specific enzymes and growth factor receptors involved in cancer cell proliferation. Signal transduction inhibitors include:

Imatinib Mesylate (protein-tyrosine kinase inhibitor), Genefitinib (epidermal growth factor receptor tyrosine kinase inhibitor - EGFR-TK), Cetuximab (epidermal growth factor receptor), Lapatinib (epidermal growth factor receptor (EGFR) and human epidermal receptor type 2 (HER2) tyrosine kinase inhibitor (ACS, 2013b; NIH, 2013).

b) Targeted therapies that modify the function of proteins that regulate gene expression and other cellular functions. Ex: Vorinostat (Zolinza®), Romidepsin (Istodax®), Bexarotene (Targretin®), Alitretinoin (Panretin®), Tretinoin (Vesanoid®) (NIH, 2013).

c) Targeted therapies where monoclonal antibodies deliver toxic molecules to cancer cells specifically. Ex: Tositumomab and 131I-tositumomab (Bexxar®) (NIH, 2013).

d) Anti-angiogenesis drugs: These drugs block the growth of blood vessels that supply blood and nutrients to tumors. Ex: Bevacizumab (Avastin®), Ziv-aflibercept (Zaltrap®) (NIH, 2013).

e) Targeted therapies that act by helping the immune system to destroy cancer cells. Ex: Rituximab (Rituxan®), Alemtuzumab (Campath®), Ofatumumab (Arzerra®), Ipilimumab (Yervoy™) (NIH, 2013).

In addition, cancer vaccines and gene therapies are also considered as targeted therapies. Although targeted therapies have the ability to specifically target cancer cells without causing any harm to normal cells they cannot be considered as a replacement for traditional therapies but can be used in combination with traditional therapies. Despite the advantages of targeted therapies in specifically killing cancer cells without damaging normal cells, they still cause considerable side effects (ACS, 2013b; NIH, 2013).

Basic function of cells such as cell growth, cell division, cell movement, cell responses to specific external stimuli and normal apoptotic process are governed by complex communication systems through numerous cell signaling pathways (Erickson and Cerione, 2010). As targeted cancer therapies mainly focus on proteins that are involved in cell signaling pathways, blocking signals that govern basic cellular functions and activities in cancer cells that grow and divide uncontrollably will help to stop cancer progression by inducing tumor cell death. In addition, cancer targeted drugs can also be used in combination with other cancer treatments such as

chemotherapy and radiation therapy. Quinacrine is one such potential compound that targets multiple cell signaling pathways and can be used as a potential anticancer agent.

1.6   Quinacrine as a Therapeutic Agent

Quinacrine (QC) (also known as atabrine or mepacrine) is a 9AA derivative that has favorable pharmacological and toxicological properties and is widely used in medicine. Atabrine was first discovered in German laboratories in 1930's during a research performed on biologically active dyes (Ciak et al., 1967).

Quinacrine is a bright yellow crystalline powder that has a heterocyclic three-ring structure as shown in Figure 1. The acridine compound is a derivative of 9AA through the use of a technique called hybrid pharmaphore approach (Ciak et al., 1967). The IUPAC name of Quinacrine is "4-N-(6-chloro-2-methoxyacridin-9-yl)-1-N,1-N-diethylpentane-1,4-diamine". During the Second World War Quinacrine was rediscovered as "American Atabrine" in American laboratories (Greenwood, 1995). The compound is 'readily available as Quinacrine dihydrochloride' (Ehsanian et al., 2011).

Since, historical times, Quinacrine has been mainly used to treat malaria and giardiasis (Gardner and Hill, 2001). Quinacrine hydrochloride pellets were used as a possible agent for non-surgical female sterilization in the 1960s and early 1970s by Jaime Zipper (Bashir, 1993). 9AAs are well known DNA intercalators. Although 9AA and Quinacrine have similar characteristics, the former has not been used in medicine. Quinacrine has generally been prescribed as an anti-inflammatory drug and used to treat lupus erythematosus and sporadic Creutzfeldt-Jakob disease (Toubi et al., 2006; Wallace, 1989). Further, Quinacrine had been used as an intrapleural sclerosing agent in patients with high rate of recurrence of pleural effusion or pneumothorax, to prevent recurrence (Larrieu et al., 1979; Taylor et al., 1977).

Figure 1: Structure of Quinacrine

Also known as: Mepacrine, Atabrine, Erion, Acrinamine, Antimalarina, Haffkinine, Quinactine, Acriquine, Akrichin (Figure adapted from NCBI, 2013).

Molecular Formula: $C_{23}H_{30}ClN_3O$

Molecular Weight: 399.9568

IUPAC Name: {4-[(6-chloro-2-methoxyacridin-9-yl)amino]pentyl}diethylamine
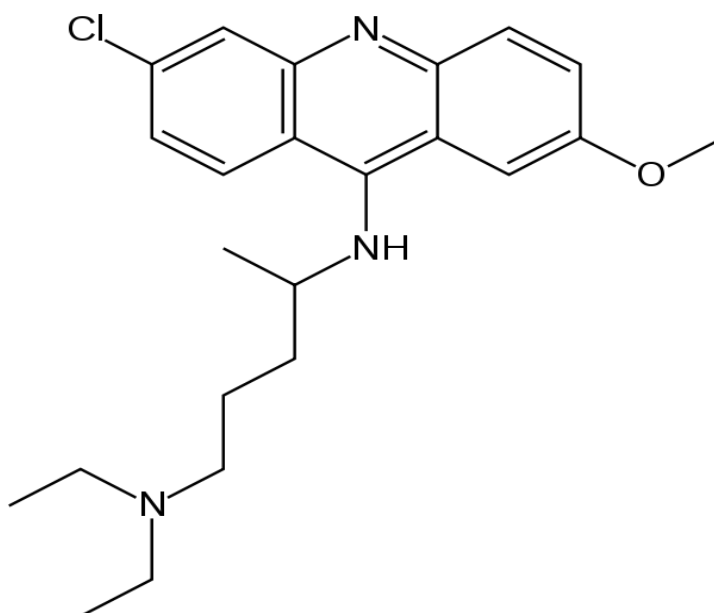
1.7 Anticancer Properties of Quinacrine

The anticancer properties of Quinacrine have been well characterized in various tumors. Published data suggests that Quinacrine intercalates into DNA bases with high affinity owing to the presence of the acridine ring in its structure. This interaction with DNA leads to selective activation and inhibition of specific cellular signaling pathways that can activate cell-suicidal programs, resulting in the reduction of tumors (Neznanov et al., 2009; Zipper et al., 1995). However, only a few studies established this association. In a study performed to examine the effects of Quinacrine in head and neck cancer patients, researchers found that Quinacrine restored the sensitivity to cisplatin in squamous cell carcinomas of head and neck with wild-type p53 (Friedman et al., 2007).

A study performed in rats showed that Quinacrine elicits an innate immune response, which could be involved in the elimination of experimental glioma in rats (Reyes et al., 2001; Sotelo et al., 2000). Moreover, researchers report that Quinacrine killed breast cancer cells through the inhibition of topoisomerase activity (Finlay, 1989). In a study performed to investigate the anticancer properties of Quinacrine on breast cancer tumors, researchers found that Quinacrine decreased the growth of breast cancer cells by inducing apoptosis through the activation of proapoptotic proteins Bax, PARP and p53 and by downregulating of antiapoptotic proteins Bcl-xL and Nuclear Factor-κB (NF-κB) in MCF7 cells (Preet et al., 2012). In addition, 9AA compounds or Quinacrine have been shown to strongly activate p53 and inhibit NF-κB in renal cell carcinoma tumor cells. In this study Quinacrine destroyed tumor cells by apoptosis without causing genotoxicity (Gurova et al., 2005).

Further studies showed that Quinacrine also acts as a chemosensitizer when combined with other chemotherapeutic agents. It is well known that death receptor-5 (DR5) mediates cell death induced by TNF-related apoptosis-inducing ligand (TRAIL) in most tumor cells. However, three hepatocellular carcinoma cell lines (HepG2, Hep3B and Huh7) are resistant to TRAIL. Therefore, there is a necessity to overcome TRAIL resistance for an effective TRAIL-targetting therapy in hepatocellular carcinoma patients resistant to a TRAIL therapy. Quinacrine helps in overcoming the resistance of hepatocellular carcinoma cells to TRAIL. A study performed to test the chemosensitizing effect of Quinacrine showed that 10-20 μM of Quinacrine treatment for 1-2

days alone did not effectively kill hepatocellular carcinoma cells including the HepG2, Hep3B, and Huh7 cell lines. However, when Quinacrine is given in combination with TRAIL and chemotherapeutic agents, hepatocellular cancer cells were killed effectively because Quinacrine significantly increased the levels of DR5, a pro-apoptotic death receptor of TRAIL (Wang et al., 2011).

1.8   Apoptosis

There are a number of cellular signals that determine whether a cell will undergo apoptosis (Elmore, 2007). However, the cellular pathways that induce the process of apoptosis are often disrupted in cancer by mutations, leading to uncontrolled cell proliferation (McDonnel et al., 1989). There are at least three different types of cell death: apoptosis, autophagy and necrosis. Necrosis is caused by external trauma or injury to the cells, while both apoptosis and autophagy are processes initiated from within the cell. In multicellular organisms, the number of cells in the body is regulated to maintain tissue homeostasis. This process is not only controlled by genes regulating the rate of cell division, but also by controlling programmed cell death. Normally, apoptosis causes cell death in response to environmental and developmental signals (Wyllie et al., 1980). The Fas/CD95 receptor normally controls cell numbers in the immune system by eliminating certain cells through apoptosis. However, when this mechanism is disrupted due to mutations in cancer-related genes, a cancer may develop. Morphologically, the process of apoptosis includes cell shrinkage, membrane blebbing, and chromatin condensation (Duprez et al., 2009). The apoptotic process is triggered by either internal stimuli or external stimuli. Once apoptotic pathway signals are activated, a series of events takes place within the cell until the process of cell death is completed. The mechanism of apoptosis involves the activation and function of a group of proteases known as the caspases. Two types of apoptotic pathways help in the activation of caspases: the death receptor or extrinsic and mitochondrial or intrinsic pathways (Earnshaw, 1995).

The intrinsic pathway is activated by various stimuli including radiation, starvation, heat, and DNA damage, and acts through the mitochondria, which are controlled by the Bcl-2 family of proteins. The anti-apoptotic Bcl-2 family of proteins maintains the integrity of mitochondria in homeostatic conditions by preventing the pro-apoptotic Bcl-2 family members such as Bax and

Bak from causing mitochondrial damage. However, under stress conditions the Bcl-2-homology 3 (BH3)-only proteins are activated, counteracting the anti-apoptotic effect of Bcl-2 family members, relieving the inhibition of Bax and Bak. This causes the activation of Bax and Bak, which translocates from cytoplasm to mitochondria, and increases the mitochondrial membrane permeability by opening the mitochondrial membrane pore complex through which Cytochrome c is released into the cytosol. Once Cytochrome c enters the cytoplasm it associates with the adaptor protein Apaf-1 and several pro-caspase-9 proteins, forming apoptosomes. The activated pro-caspase-9 then cleaves and activates the executioner caspases -3, -6 and -7, which initiate the execution of apoptosiss. In addition to the caspases -3, -6, and -7, several other pro-apoptotic proteins are released from the mitochondria to trigger the cellular suicide mechanism (Riedl and Salvesen, 2007).

The extrinsic pathway is mediated by external stimuli such as cytokines and growth factors, and involves the activation of TNFR family receptors such as TNFR, TRAIL and Fas. Activation of these receptors induces a variety of cellular responses leading to formation of a death-inducing signaling complex (DISC) which, inturn, activates initiator caspases-8 and/or -10 via homotypic death domain interactions (Peter and Krammer, 2003; Wilson et al., 2009). This process takes place through the formation of two complexes. Complex I is formed at the plasma membrane and consists of TNFR1, TNFR-associated death domain (TRADD), TRAF2, RIP1, cIAP1 and cIAP2. Complex II includes TRADD, FADD, and caspase-8 and/or -10. Once the death initiator caspase-8 and/ -10 are activated, this leads to the activation of downstream executioner caspases such as caspase-3 (Green and Reed, 1998). Caspase-3 is a major player in the execution of apoptosis as it cleaves other caspases and essential cell proteins such as poly (ADP-ribose) polymerase (PARP) and retinoblastoma proteins. In addition, the activation of caspase-8 also activates the death receptor-induced cell death program by activating the mitochondrial pathway of apoptosis (Martin and Green, 1995; Peter and Krammer, 2003; Wilson et al., 2009). In contrast to this pathway, another extrinsic pathway was discovered in experiments using treatment with TNF and Smac mimetics. However, this pathway is totally dependent on kinase active RIP1 and involves autodegradation of cIAP1 and cIAP2 by Smac mimetics, leading to release of RIP1 from the receptor complex to form a caspase-8 activating platform consisting of RIP1, FADD, and caspase-8 (Wang et al., 2008).

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1.9 Ratio of Anti-Apoptotic and Pro-Apoptotic Factors Decides the Fate of a Cell

Quinacrine can affect cancer cells via a number of different mechanisms, however the most exciting and promising property of Quinacrine is its ability to induce apoptosis in cancer cells. Studies show that Quinacrine significantly inhibits cell growth and induces cell death. Reed (1998) reported that Bcl-2 and Bcl-xL are anti-apoptotic or death suppressers, whereas Bax protien is a death promoter (Reed, 1998). Moreover, published data show that the release of pro-apoptotic Bax resulted in increased permeability of the mitochondrial membrane, leading to the release of Cytochrome c triggering the apoptotic pathway. On the contrary, when anti-apoptotic Bcl-2 was released, it decreased the permeability of mitochondrial membrane and prevented the release of Cytochrome c; thereby preventing apoptosis (Duprez et al., 2009; Hockenbery et al., 1990; McDonnel et al., 1989). These data suggest that the fate of a cell depends on the ratio between anti-apoptotic and pro-apoptotic signals within cells.

Furthermore, studies have also shown a correlation between the stages of the cell cycle and the levels of Bcl-2 expression. Gui et al. (2005) demonstrated that cells in G1 phase had high expression of Bcl-2, and were more resistant to the induction of apoptosis as compared to the cells in S-phase of the cell cycle where the expression of Bcl-2 proteins is very low, increasing the chance of apoptosis (Gui et al., 2005).

1.10 Pharmaco-dynamics of Quinacrine

In addition to the pharmacological properties of Quinacrine as an antimalarial drug and as an antibiotic, published data suggests that Quinacrine is also a potential anti-cancer agent. The bio-molecular binding property of Quinacrine with nucleic acids and phospholipids makes it a suitable drug for the treatment of anti-viral and anti-bacterial infections (Wallace, 1989). Moreover, published data suggests that Quinacrine is also an effective anti-prion drug. Although, prions do not have nucleic acids, Quinacrine seemed to be the most promising compound for immediate application for the treatment of prion diseases such as Creutzfeldt-Jakob disease and kuru in humans (Ghaemmagham et al., 2009). Korth et al. (2001) tested the efficacy of Quinacrine on prions in cell culture models and found that Quinacrine induced the clearance of the pathogenic and protease-resistant PrPSc isoform (PrPSc) (Korth et al., 2001). However, Quinacrine did not appear to be effective on prion diseases *in vivo*. Researchers attribute the

translational gap in Quinacrine's effects to the differences in pharmacodynamic and pharmacokinetic properties *in vitro* and *in vivo* (Ghaemmagham et al., 2009).

According to a study on the effects of Quinacrine in *Escherichia* coli, Quinacrine blocks DNA synthesis, and inhibits the syntheses of RNA and protein at a concentration of 8×10^{-4} moles per liter. But, when the concentration was reduced to 2×10^{-4} moles per liter, the drug only partially inhibited the syntheses of protein and DNA, but not at all RNA synthesis. This finding showed that Quinacrine acts by inhibiting DNA replication, transcription, and protein synthesis by intercalating into DNA and RNA (Ciak et al., 1967; McCarroll et al., 1981; Whitehouse and Boström, 1965). A study performed to investigate the tumor-killing effects with gastric cancer cells showed that Quinacrine significantly inhibited cancer cell (SGC-7901) proliferation by inducing apoptosis. Moreover, the same authors also revealed that Quinacrine acted on shifting the ratio of Bax/Bcl-2 in favoring apoptosis by significantly increasing the levels of proapoptotic proteins, cytochrome c, Bax, and p53, and by concomitantly decreasing the levels of antiapoptotic protein Bcl-2 (Wu et al., 2012).

1.11 P53 and NF-κB Pathways are often deregulated in Cancer Cells

The pro-apoptotic p53 and anti-apoptotic NF-κB pathways are two major stress response pathways. Under normal conditions, p53 and NF-κB are inactive in the cytoplasm due to binding with specific negative regulators. However, under stress conditions, the negatively regulating factors dissociate from p53 and NF-κB in the cytoplasm, resulting in the translocation of p53 and NF-κB to the nucleus and bind to several DNA sites. Activation of NF-κB promotes cell survival and cell growth, while the activation of p53 promotes cell growth inhibition, temporary arrest of cell cycle, irreversible arrest (senescence), or apoptosis. In normal cells, p53 and NF-κB pathways negatively regulate each other mutually through simultaneous activation and inhibition. However, in tumor cells the p53 and the NF-κB pathways are deregulated, resulting in the inhibition of p53 pathway and the activation of NF-κB pathway (Gudkov et al., 2011; Lane and Levine, 2012). According to Gurova and colleagues, Quinacrine suppresses NF-κB and activates p53 signaling, causing apoptosis in cells (Gurova et al., 2005).

1.12   Quinacrine Inhibits NF-κB

Nuclear factor-κB (NF-κB), the major regulator of inflammation consists of a family of transcription factors that help cells to acclimatize and respond to environmental changes. The NF-κB transcription factors play a crucial role in basic cell responses such as; inflammation, immunity, cell proliferation, differentiation, and survival (Zhong et al., 2002). Activation of NF-κB is a commonly acquired characteristic of tumor cells. The NF-κB dimers are generally retained in inactive form in the cytosol by interacting with IkB (Hayden and Ghosh, 2004). However, phosphorylation of IkB leads to its degradation, promoting the translocation to the NF-κB nucleus, where it induces transcription of target genes. The NF-κB pathway is activated by many external stimuli and is regulated by IkBa, p105, or A20 which are NF-κB dependent; suggesting the involvement of auto-regulatory feedback loop in the NF-κB response (Hayden et al., 2006).

NF-κB-mediated gene transcription is induced during viral or bacterial infection as well as by TNF-α and IL-1β pro-inflammatory kinases. Once the NF-κB is activated, it presents itself as trans-activator and leads to the increased expression of inhibitors of apoptotic proteins (Hayden et al., 2006). However, in the presence of Quinacrine, the phosphorylation of NF-κB is inhibited thus making it a trans-repressor. This transcriptionally inactive state of NF-κB binds to the histone deacetylase (HDAC1). This complex of NF-κB and HDAC1 bound to DNA negatively regulates NF- κB-dependent gene transcription (Magnaghi-Jaulin et al., 1998). Hence, Quinacrine converts NF-κB from a transactivator to a trans-repressor. In a study performed to understand the potential mechanism of Quinacrine *in vivo*, Gorbachev et al. (2007) found that contact hypersensitivity response to mice skin cells was mainly because of NF-κB (Gorbachev et al., 2007). In addition, Gorbachev and colleagues reported that Quinacrine reduced contact hypersensitivity response by inhibiting NF-κB activation and as well as the production of cytokines (TNFα, IL-1β, and CCL21) by NF-κB. However, the same group could not identify the exact mechanism through which Quinacrine inhibited the activation of NF-κB as well as cytokine production (Gorbachev et al., 2007).

1.13   Quinacrine Activates P53

Activation of the tumor suppressor protein p53 induces apoptosis in cells via the mitochondrial pathway (Ehsanian et al., 2011).   A potential problem is that the upregulation of NF-κB can reduce the tumor suppressor activity of p53, resulting in inflammatory responses. Quinacrine can induce a strong p53 response and initiates the process of apoptosis in tumor cells as it can simultaneously inactivate NF-κB, making it a very promising drug. Quinacrine stabilizes p53 protein by blocking its ubiquitination without phosphorylation, thereby activating p53-dependent apoptosis in tumor cells, which is independent of the DNA damage repair pathway. In addition, Quinacrine induces p53-dependent cell death by increasing the release of Bax, which is a key cell death inducer in both the intrinsic and extrinsic apoptotic pathways (Wang et al., 2005). Gurova et al. (2005) reported that Quinacrine activates p53 pathway in renal carcinoma cancer cells without inducing DNA damage (Gurova et al., 2005).

1.14   Quinacrine Inhibits AKT Signaling Pathway

The PI3K-PKB/Akt signaling pathway is a key pathway in cell survival and is involved in the NF-κB and p53 issignaling pathways. Akt/PKB is a serine/threonine kinase that plays a key role in multiple cellular processes and basic cellular functions such as glucose metabolism, cell survival and cell growth. Akt sits at the centre of signaling networks that connects many nodes. Each of the targets from these nodes ultimately favors the activation of cell survival pathways, thus, contributing to tumorigenesis (Feng and Levine, 2010; Hemmings and Restuccia, 2012). However in tumor cells, the Akt pathways receive continuous signals that favor growth and metabolism, which leads to tumorigensis. In this process Akt phosphorylates the NF-κB subunit p65 and MDM2. Phosporylation of these Akt substrates induces translocalization of MDM2 into the nucleus, where it can bind to p53 and promote its degradation. Moreover, nuclear MDM2 can transport p53 from the nucleus to the cytoplasm, where it suppresses the p53 activity thereby inhibiting apoptosis (Fresno Vara et al., 2004).

Akt is considered as a principal anti-apoptotic signaling protein involved in the suppression of p53 in many different cancers including glioblastomas, pancreatic cancer, renal cell carcinoma, breast cancer, and gastric carcinoma. When Quinacrine is administered, it inhibits the Akt activity and thus promotes apoptosis through the activation of p53 (Fresno Vara et al., 2004).

This property of Quinacrine has been demonstrated by Guo et al. (Guo et al., 2009). Guo et al. found that 9AA inhibits Akt activity by preventing its phosphorylation at Ser473. In addition, the authors showed that the inhibition is because of the reduced activity of mTOR and not by the downregulation of the Akt activity *per se*. From this, it can be inferred that there is a feedback loop between AKT and mTOR, and Quinacrine successfully intereferes with this loop (Feng and Levine, 2010; Guo et al., 2009). The effect of Quinacrine on Akt pathway was reaffirmed in a study performed to understand the role of the arachidonic acid pathway and epidermal growth dactor in neurotensin induced prostate cancer. Hassan and Carraway found that Quinacrine inhibited neurotensin and, to a lesser extent, EGF-stimulated phosphorylation of AKT (Hassan and Carraway, 2006).

1.15   Quinacrine Disrupts Arachidonic Acid Pathway

The arachidonic acid pathway promotes the growth of tumor cells in prostate, gastrointestinal, lung, esophageal, and breast cancers. Quinacrine has been proven to effectively disrupt the arachidonic acid pathway by the inhibition of phospholipase A2 (PLA2). The inhibition of PLA2 by Quinacrine leads to a wide range of effects. Firstly, Quinacrine binds to membrane phospholipids, primarily phosphatidyl-ethanolamine and intercalates into the cell membrane, causing inhibition of membrane binding activity of PLA2. Once PLA2 is blocked, it decreases production of arachidonic acid, which in turn results in the inhibition of leukotrienes, prostanoids and eicosanoids (Abdel-Latif et al., 1983; Ahmed et al., 1992). Thus, Quinacrine reduces the progression of tumor cells through cell cycle by blocking the arachidonic acid pathway.

1.16   Pharmacokinetics of Quinacrine

Quinacrine can be administered through oral, intralesional or paralesional, intramuscular, rectal, intravenous, transcervical, and interstitial routes. Although intravenous administration is believed to be most rapid way through which Quinacrine can be delivered, it is typically administered through oral route. When orally administered, the drug is proven to be much more effective as it is absorbed rapidly through the gastrointestinal tract. Studies show that when the drug is administered through the oral route, the plasma levels of Quinacrine significantly increased within 2–4 hours after administration, reaching a peak within 8–12 hours (Wallace, 1989).

Besides oral and intravenous routes, Quinacrine is also absorbed rapidly after intrapleural, intralesional/paralesional, and intrauterinal administration (Laufe et al., 1996).

Once within the body, 80–90% of the drug is bound to plasma proteins. The highest concentrations of Quinacrine are found in the liver, spleen, lungs and adrenal glands, along with skin, fingernails and hair. Low concentrations of Quinacrine are found in the brain, heart and skeletal muscle. The half-life of Quinacrine is five to fourteen days depending on the dosing regimen. Although bile, sweat and saliva release a small amount of Quinacrine, the major route of Quinacrine elimination is through the renal system (Goodman and Gilman, 1954).

1.17   Adverse Effects of Quinacrine

Although Quinacrine was found to be effective against prostate, gastrointestinal, lung, esophageal and breast cancers, it produces potential toxic side effects. The normal dosage of Quinacrine in giardia patients is 100 mg/kg, three times a day for over 5 to 7 days in adults and 6 mg/kg/day in three divided doses over 5 to 7 days for children (Lerman and Walker, 1982). At a dose of 100 mg daily, Quinacrine causes persistent abdominal cramping, diarrhoea, headache, dizziness, and other gastrointestinal symptoms such as anorexia and nausea in patients (Lerman and Walker, 1982). Quinacrine is also believed to cause other ill effects such as restlessness, vertigo, insomnia, nightmares, hyperirritability, psychosis and convulsions at doses ranging between 200 to 1,200 mg daily for ten days (Evans et al., 1984).

In addition, studies also reported that administration of 100 mg of Quinacrine each day for about two and a half years during World War II increased the risk for toxic psychosis and aplastic anemia. Studies performed on soldiers that took part in World War II showed that the incidence of aplastic anemia was as low as 0.003% and incidence of toxic psychosis was 0.4% when they were administered 100 mg of Quinacrine daily for about two and a half years (Custer, 1946; Gaskill and Fitz-Hugh, 1945). However, to date the use of Quinacrine has not been contraindicated since side effects can be largely reduced or prevented by a decrease in the dosage of Quinacrine. Alhough Quinacrine has proven to be effective in treating cancer cells; it requires a high dosage regimen (cell arrest at doses less than 5 μM and apoptosis at higher doses (10–20μM) to cure cancer. In addition, high dosages of Quinacrine cause considerable side effects. As a consequence, the application of Quinacrine as a novel chemotherapeutic agent is not

justifiable and, therefore, requires the need for further research to enhance the efficacy of Quinacrine by eliminating its side effects.

1.18   Quinacrine Derivatives as Anticancer Agents

In order to enhance the efficacy of Quinacrine as an anticancer drug, novel derivatives were developed based on the 9AA scaffold using a hybrid pharmaphore approach in Dr. Lee's lab at the Northeast Cancer Center in Sudbury, Ontario. The functionality of 9AA derivatives was studied using both malignant and non-malignant cells. A handful of those derivatives preferentially induced cell death in malignant cells over normal cells. Preliminary data from Dr.Lee's laboratory indicated that these new compounds inhibit malignant cell proliferation with greater efficacy and also exhibited reduced toxicity towards normal cells as compared to the parent compound Quinacrine. One of the derivatives selected for further investigation based on its efficacy and low toxicity is VR118.

1.19   Specific Aims of This Study

This particular study investigated the novel VR118 Quinacrine derivative as a potential anticancer agent. This study has two specific aims,

A) To study and evaluate the effects of this novel derivative drug on different human cancer cell lines.

B) To identify the mechanism of action of VR118 *in vitro.*

Chapter 2

2 MATERIALS AND METHODS

The following section describes the general methods and routine protocols employed in performing the experiments, to test the effectiveness of the compound VR118.

2.1 Cell Lines

All the cell lines used in the experiments were purchased from ATCC and include:

i) HeLa S3 (Human Cervical Carcinoma Cells) and MCF7 (breast adenocarcinoma, estrogen receptor positive) cell lines.

ii) MDA-MB231 (estrogen negative, p53-/-, k-ras mutant breast cancer cell lines)

iii) MDA-MB468 (PTEN negative, RB1 negative, SMAD4, p53 mutant, estrogen receptor-negative metastasis-derived breast cancer cell line).

iv) 184B5 (Chang et al., 2006) and MCF10A non-malignant breast cell lines (Gratzner et al., 1975).

2.2 Culture Media and Reagents Used

The following is a list of media and medium supplements used in the routine culture of the above cell lines:

i) RPM-I 1640 medium

ii) 10% (v/v) fetal bovine serum (FBS)

iii) 100 μg/ml streptomycin

iv) 100 units/ml penicillin

v) Dulbecco's modified Eagle's medium (DMEM) medium

vi) 100 μg/ml cholera toxin

vii) 10 μg/ml insulin, 20 μg/ml EGF (epidermal growth factor)

viii) 0.5 μg/ml hydrocortisone, 10% (v/v)

ix) 10% equine serum

2.3   Maintenance of Cell Lines

The following culture protocols were used to maintain the cells under laboratory conditions:

i)   HeLa and MDA-MB231 cells were maintained in RPMI 1640 medium supplemented with 10% (v/v) fetal bovine serum (FBS), 100 μg/ml streptomycin, and 100 units/ml penicillin.

ii)   MCF7 and MDA-MB468 cells were maintained in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% (v/v) FBS, 100 μg/ml streptomycin and 100 units/ml penicillin.

iii)   184B5 and MCF10A cells were grown in DMEM F12 medium supplemented with 100 μg/ml cholera toxin, 10 μg/ml insulin, 20 μg/ml epidermal growth factor (EGF), 0.5 μg/ml hydrocortisone, 10% (v/v) 10% equine serum, 100 μg/ml streptomycin and 100 units/ml penicillin.

2.4   Drug Treatment

The experimental design involved dividing the cell samples into test and control groups. The VR118 compound is dissolved in Dimethyl sulfoxide (DMSO). The cells in the test group are treated with VR118 compound dissolved in DMSO, whereas the cells in control group (sham treated cells) are only treated with a volume of DMSO equal to the test sample. In experiments where varying concentrations of VR118 compound are used, cells in the control group are treated with a concentration of DMSO equal to the highest concentration of VR118 compound used in that experiment. Another control group included in the experiment is non-treated group, which are grown in a complete media and not treated with either VR118 compound, or DMSO, unlike the test group and sham treated cells. Similar to the drug treated samples and the sham treated samples; the non-treated samples were collected at the same point of time in every experiment. Moreover, the positive control groups varied with each experiment.

2.5   Clonogenic Assay

Clonogenic assays are cell survival assays that are generally performed to determine the ability of a single cell to grow into a large colony that can be visualized with naked eye in semi-solid medium. In this experiment, the protocol by Franken et al. (2006) was adopted:

i)   Cells are seeded out in appropriate dilutions and allowed to grow.

ii)   After 24 h of post plating, cells were collected, counted and predetermined numbers of cells (10,000/ml) were plated in T25 flasks with 5 ml medium.

iii)   MCF7 cells in each T25 flask were treated with increasing concentrations of VR118 starting from 0.31 μM, and gradually increasing to 1.5 μM, 6.25 μM, 25 μM and 100 μM, resulting in exposure of the cells to different concentrations of the drug for 24 h.

iv)   On completion of the drug exposure for 24 h, the treated cells were washed, trypsinized, and re-suspended in 1 ml of medium and later suspended in 0.4% agarose kept in a water bath at 37°C.

v)   The medium is mixed thoroughly using a 3cc syringe equipped with a 16-gauge needle.

vi)   Later 1 ml of sample was dispensed into each well of a six-well plate and set aside for 5 min for agar to solidify

vii)   Upon solidification of agarose, 500 μl of medium was added on the top of agar, followed by incubation at 37°C and 5% CO_2 (Franken et al. 2006).

viii)   After 12 days of incubation, colonies were counted from 10 random fields of each sample (Franken et al. 2006).

ix)   The average number of colonies per field was plotted against the concentration of the cytotoxic agent being tested and only those colonies containing more than 50 cells/colony were counted as viable colonies.

x)   Clonogenic assay Also was performed without agrose for MDA-MB231, and 184B5 cells (Preet et al). For this cells were seeded in 10 cm plates and grown for 24 hr

xi)   Then treated with different concentrations of VR118 (0.5–100 μM) for next 24 hr.

xii)   Thereafter, cells recounted and medium was replaced with fresh medium, and plate was returned to the incubator for (10-12) days.

xiii)   After colony formation, medium was removed and plate was whashed, air dried and stained with 0.2% crystal violet (made in 25% methanol and stored at room temperature).

xiv)   Then, the plates were washed twice with distilled water, and colonies were counted.

2.6   Sulphorodhamine B (SRB) Assay

SRB assays were performed to determine the cell density based on the cellular protein and nucleic acids content. The protocol by Vichai and Kritikara (2006) was adopted for SRB assays:

i)   Each of the 96-well plates were incubated for 24 h after plating them with the following number of cells:

   ➢ 4,000/ml for MCF7 cells and 184B5 cells

   ➢ 3,000/ml for HeLa, MDA-MB231 and MDA-MB468 cells

ii)   After 24 h, the growth medium in each plate was replaced with a medium containing different concentrations of VR118 compound and incubated for additional 48 h.

iii)   After 48 h, cells in each plate were fixed with 50% ice-cold TCA (trichloro acetic acid) and incubated at 4ºC for 1 h, followed by repeated washing in running water and air drying.

iv)   Cells were then stained with 0.1% (w/v) SRB solution (400 mg of Sulphorodhamine B in 100 ml of 1% acetic acid in water) for 30 min at room temperature, and were later re-suspended in 200 μl of 10 mM Tris buffer (pH 10.5). Absorbance was recorded at a wavelength of 530 nm using a plate reader (Molecular devices, Spectra max 340 PC) (Vichai and Kritikara, 2006).

v)   For positive control, mock-treated cells were used; and for negative control, 50% TCA treated cells were used.

vi)   The IC_{50} values were calculated using a sigmoidal dose-response curve (variable slope) using Graph Pad Prism V 4.02 (Graph Pad Software, Inc.).

vii)   The following formula was used to normalize the data:

   (Where, Tp is mean O.D. of positive control and Tn is mean O.D. of negative control)

   (Mean OD sample - Tn) / Tp- Tn

2.7   Cell Proliferation

Cell proliferation assays are generally employed to evaluate the response of a cell population to external factors. For cell proliferation experiments, the protocol was adopted from Chang et al. (Chang et al., 2006)

i)   Exponentially growing cells were trypsinized, cell numbers were determined, and later a fixed number of these cells (30–40% confluence) were plated on a 10-cm plate and incubated at 37ºC for 24 h.

ii)   After incubation, these cells were treated with either the IC_{50} range or higher concentration (2/4/6/8/10 µM) of VR118.

iii)   Then cells were collected, and pellets were briefly re-suspended in PBS at pH 7.4 and later counted after treating with 15 µl of 1% Trypan blue (Chang et al., 2006).

iv)   Cells were counted using a hemocyrometer under the microscope in 1 x 1mm squares of one chamber, and the average number of cells per square was determined.

v)   Counting of cells was performed each day for a replica of four days. Only those cells, which excluded Trypan blue, were counted and plotted.

vi)   Each sample and numbers of cells counted each day was plotted using a GraphPad computer program.

2.8   Detection of Apoptosis by Acridine Orange/Ethidium Bromide

To examine the cellular morphology of MDA-MB231 and MCF7 cells with or without treatment with DMSO and/or VR118, the following acridine orange staining method was used.

i)   MDA-MB231and MCF7 cells were grown on glass cover slips overnight at 37ºC to allow for adherence of cells.

ii)   Cells were then exposed to DMSO and/or VR118 for 24 and 48 h. For this experiment, cells were also treated with 6 µg/ml of camptothecin as a positive control for apoptosis.

iii)   Each sample was then stained with 100 µg/ml acridine orange (Sigma-Aldrich) for 5 min and then stained with 100 µg/ml of ethidium bromide (Sigma-Aldrich) for 5 min.

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iv)   Finally, the cover slips were washed with 1X PBS and were then gently placed onto a glass slide and sealed with clear nail polish. Each sample was done one at a time for the cells to be fixed. Pictures were taken using a confocal microscope as described by Pearce et al. (2001).

2.9   Analysis of Cell Cycle Distribution

This approach helps to detect apoptotic cells with a subG1 DNA content and distribution of cells in three major phases of the cell cycle (G1 vs S vs G2/M). The following protocol was used to analyze the cell cycle distribution:

i)   MCF7, MDA-MB231 and 184B5 cells were trypsinized after exposure to various compounds at different time intervals, ranging from 24 h to 72 h. Cells were washed twice in 1X PBS and fixed overnight in 75% ice-cold ethanol at -20ºC.

ii)   This was followed by centrifugation of cells, which are later re-suspended for 1 h in propidium iodide (PI) staining solution (1X PBS, 0.3% Nonidet P-40, 100 µg/ml RNase A, and 100 µg/ml propidium iodide).

iii)   Re-suspended samples were then analyzed by flow cytometry using an Epics Elite Flow Cytometer (Beckman Coulter) according to the protocol by Gratzner et al. (Gratzner et al., 1975).

iv)   Later ten thousand events were gated to accurately measure PI intensity. PI fluoresces at 623 nm when excited and single parameter displays were obtained using the flow cytometeric data acquisition software, and then FL3 fluorescence signals were recorded.

2.10   TUNEL Assay

TUNEL (terminal uridine nick-end labeling) assay is one of the most widely used methods to detect apoptotic cells that undergo DNA degradation during the late stage of apoptosis. The method is based on detecting DNA strand breaks by enzymatically labeling the free 3'-OH termini with fluorescent nucleotides using an In Situ Cell Death Detection Assay Kit (Roche, CA, USA). This kit provides complete components including positive and negative control cells for conveniently detecting DNA fragmentation by fluorescence microscopy.

i) Firstly, cells grown on cover slips were fixed with 4% paraformaldehyde (PFA) in PBS (pH 7.4) for 10 min at room temperature.

ii) Immediately, cover slips were washed and permeabilized with 0.1% Triton X-100 in 0.1% PBS for 5 min. A solution was then applied to the samples (50 μl enzyme solution in 450 μl label solution), according to the protocol adapted from (Nunez, 2001).

iii) Lastly, cells were incubated at 37^0C in a dark humidified atmosphere for 1 h. Samples were then analyzed by confocal fluorescence microscopy using the wavelength at 450-500 nm (green).

2.11 Protein Extraction and Quantification

For the purpose of protein extraction and quantification, the following protocol by Santi and Lee (2009) was employed:

i) Cells were plated on 10 cm plates (Sarstedt) and incubated at 37ºC for 24 h before exposing them to 2 μM concentration of VR118.

ii) Post VR118 treatment, exposed cells were collected at different time points (0, 24, 48, and 72 h).

iii) Later whole cell extracts were prepared in RIPA buffer (1X PBS, 1% Nonidet P-40, 0.5% sodium deoxycholate, 0.1% SDS) supplemented with 1 μM PMSF, a protease inhibitor cocktail tablet (Roche Diagnostics), 2 μM sodium orthovanadate, and 10 μM sodium fluoride.

iv) After lysing the cells in RIPA buffer and ice for 10 min, the cell lysates were cleared by centrifugation (Beckman Coulter, microfuge 22R) at 4ºC for 15 min at 14,000 g (Santi and Lee, 2009).

v) Finally, protein quantification was carried out using a BCA Assay kit (Pierce) and samples were read at 562 nm using a Multiscan MCC/340 plate reader. The concentration of unknown proteins was analyzed against the standard curve using the Graph Pad Prism version 4.03.

2.12   SDS-PAGE

For performing sodium dodecyl sulfate (SDS) - Polyacrylamide gel electrophoresis (PAGE), protocols by Laemmli and Towbin were used:

i)   Samples were prepared by adding 5X loading buffer (0.1 M Tris-HCl, 10% [w/v] SDS, 40% [v/v] glycerol, 10% bromophenol blue), and heated for 5 min to denature.

ii)   25 μg or 50 μg of protein sample was loaded onto 6–15% polyacrylamide gel, and proteins were seperated by electrophoresis in 1X Running buffer (10X Running buffer: 0.25 M Trizma base, 1.92 M glycine, 1% SDS). Run apparatus at 120 volts for 2-3 h

iii)   Proteins in the gel were then transferred onto a PVDF membrane (GE Health Care), which had been immersed in Transfer buffer (48 mM Tris, 39 mM glycine, 20% [v/v] methanol, 0.037% [w/v] SDS), using a semidry transfer apparatus at 12 volts for 1 h (Laemmli, 1970; Towbin, 1979).

2.13   Western Blotting

This is an important technique used in cell and molecular biology to identify specific proteins from a complex mixture of proteins extracted from cells. For Western blotting:

i)   The membrane to which electrophoresed proteins were transferred was "blocked" for 1 h by incubating it in Blocking buffer (1X TBS with 0.1% Tween, 50 mM Tris-HCl, pH 7.4, 150 M NaCl, 0.1 % [v/v] Tween 20 with 5% Carnation non-fat skim milk).

ii)   The membrane was washed with TBST before incubating it with primary antibodies overnight (diluted in 5% carnation non-fat skim milk, dissolved in 0.1% TBS solution) at 4ºC. The titer of antibody used varied and was according to the manufacturer's recommended protocol

iii)   After that, the blot was washed three times in 0.1% TBST, and incubated in a secondary antibody (diluted in 0.1% TBST with 5% non-fat skim milk) for 1 h at room temperature.

iv)   This was followed by two more washes with 0.1% TBST, and once with 0.1% TBS. The target proteins were visualized using ECL kit (Enhanced Chemiluminescense, GE Health care).

2.14   Densitometric Analysis

Protein band intensity was determined by densitometry using Alphaease Fluorochem (FC) 8900 version 4.0.1 software, and graphed using Graph Pad Prism V 4.02 (Graph Pad Software Inc). GAPDH or tubulin was used as a loading control. Normalization for loading differences was achieved by dividing the densitometry values for individual bands, with the densitometry values for loading control (or total protein) in the same lane.

2.15   Statistical Analysis

For analysis of each treatment group, data was reported as a mean plus or minus the standard error of the mean (SEM). IC_{50} values were calculated using a sigmoidal-dose response curve, which was generated by GraphPad Prism V.402. Values are means of two or three independent experiments. Statistical analysis One-way ANOVA was used where $p < 0.05$ was considered to be statistically significant.

3 RESULTS

3.1 VR118 Inhibits the Proliferation of Malignant Cell Lines

To determine the anti-proliferative effect and IC_{50} of VR118 on malignant and non-malignant cell lines, the SRB and clonogenic assays were performed. Findings from both the assays revealed that VR118 exhibits significant anti-proliferative effect on malignant cells. The SRB assay showed significant anti-proliferative activity on many different breast cancer and HeLa cell lines. VR118 compound decreased the cell viability and proliferation of MCF7 cells and was found to be very effective. The SRB assay showed that IC_{50} concentrations of VR118 compound were 3.1 μM, 6.2 μM, 3.5 μM and 1.5 μM for MCF7, MDA-MB231, HeLa and MDA-MB468 cell lines, respectively. Table 1 summarizes the IC_{50} concentrations of VR118 compound on cells lines as per SRB assay.

In addition to the cytotoxicity of VR118 by the SRB assays, data from clonogenic assay also showed that VR118 could significantly inhibit colony-forming capacity of malignant cells. The experimental results revealed that VR118 compound was very effective on MCF7, MDA-MB231, MDA-MB468 and HeLa cell lines compared to the controls. Moreover, VR118 showed dose-dependent effects on cell growth and colony forming abilities of cancer cells. The data from clonogenic assay were consistent with those from the SRB assay. Table 2 summarizes the IC_{50} concentrations of VR118 determined by clonogenic assays.

Figure 2: The effects of VR118 on MCF7, MDA-MB468, HeLa, MCF10A and 184B5 cells as determined by SRB assays

The SRB assays were performed to determine the ant-proliferating activity of VR118. As stated in the Materials and Methods, MDA-MB468, HeLa, MCF10A and 184B5 cells were seeded onto 96-well cell culture plates and treated with seven different concentrations of VR118 for 48 h. The number of cells was plotted against the varying concentrations of VR118 using a GraphPad Prism sigmoidal dose response curve. Represented graphs are means of three independent experiments. Error bars represent 95% confidence intervals.
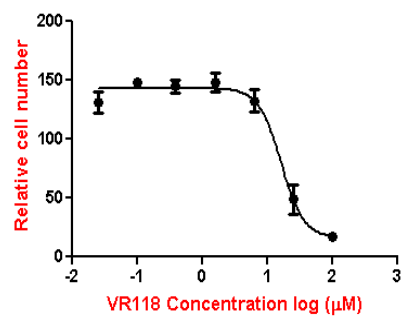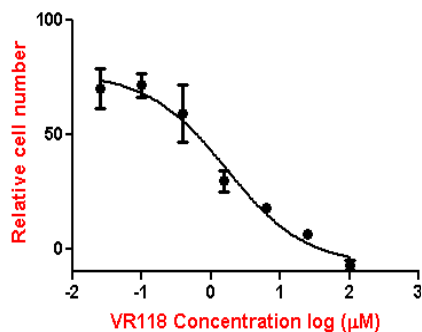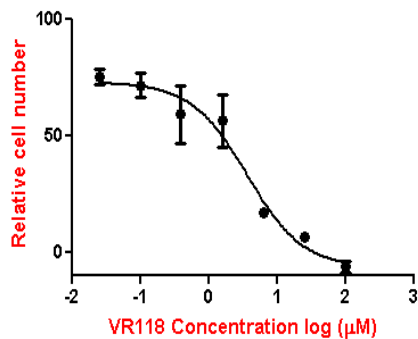
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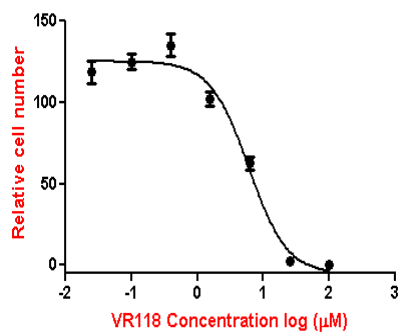
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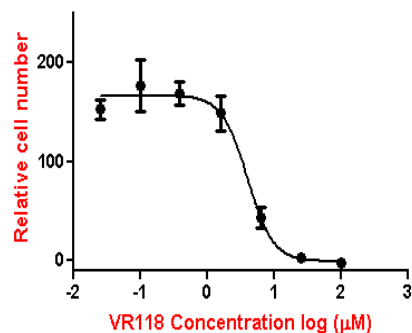
Effect of VR118 on 184B5

Effect of VR118 on MDA-MB468

Effect of VR118 On HeLa

Effect of VR118 on MCF10A
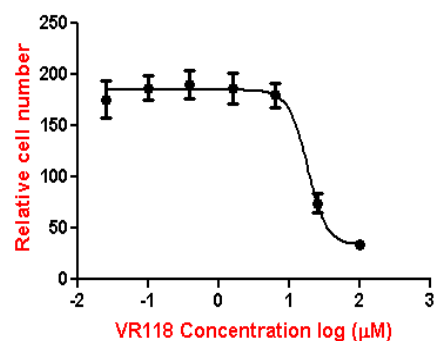
Table 1: IC$_{50}$ of VR118 on MCF7 cells based on data from SRB assays

IC$_{50}$ represents the concentration of a drug that is required for 50% inhibition of cell growth compared to non-treated controls. IC$_{50}$ values were calculated using the GraphPad Prism sigmoidal dose response curve. Each experiment was performed in triplicate and each value represents the mean ± SD of three independent experiments. $p < 0.05$ vs. Non-cancer cells.

	IC50 (µM) for cancer cells				IC50 (µM) Non-cancer cells	
Compound	MCF7	MDA-MB231	HeLa	MDA-MB468	184B5	MCF-10A
Quinacrine	4.2±0.2	6.0±1.2	6.1±3.1	5.6±0.4	4.0 ± 0.2	5.0 ± 0.1
VR118	3.1± 3.5	6.2±2.0	3.5±0.3	1.5 ± 1.0	16.4 ± 0.8	18.2 ± 2.5
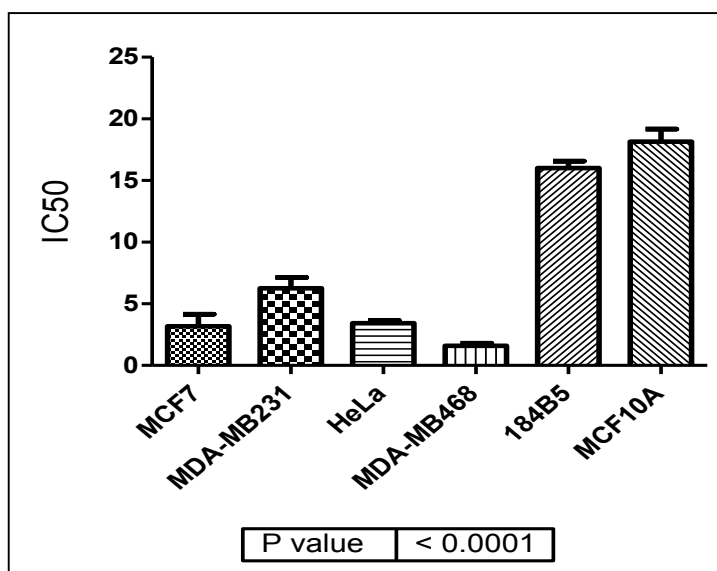
Figure 3: Surviving fraction of MCF7, MDA-MB468 and HeLa cells determined by their ability to form colonies after 12 days.

Figure 3 shows representative samples of clonogenic assays for different cell lines exposed to the concentrations of VR118 in an IC_{50} range (3.5 µM). Cells were exposed to different concentrations of VR118 (0.7, 1.5, 3.5, 6.25, 12.5, 25, 50 and 100 µM), along with a sham-treated control. The surviving number of colonies was counted after 12 days as described in Materials and Methods.
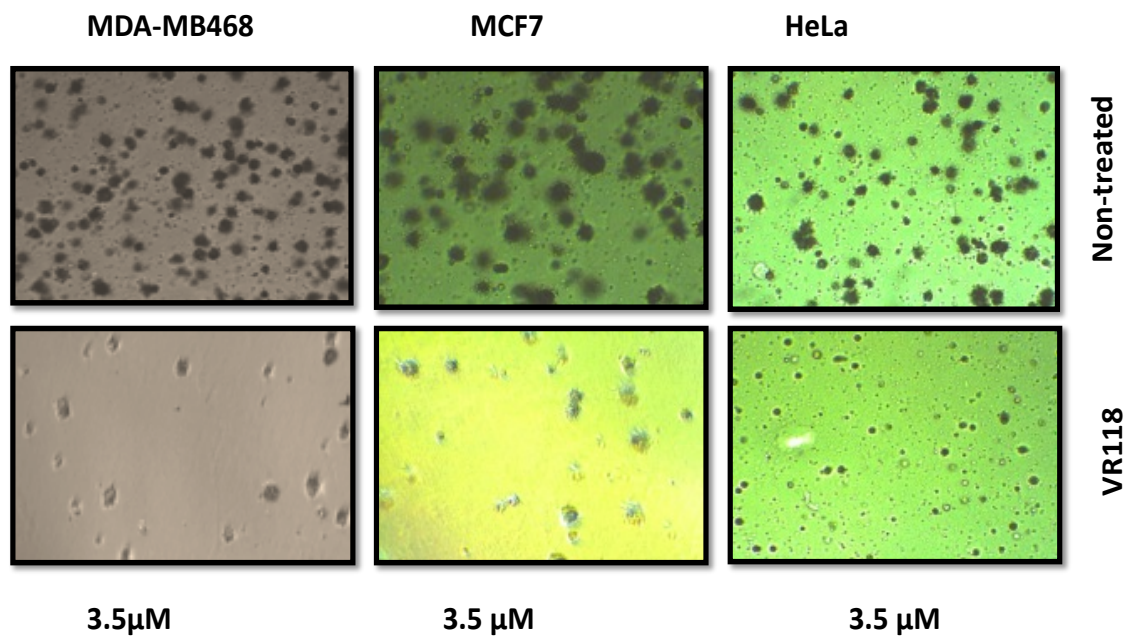
Figure 4: Dose-response curves of VR118 determined by a clonognic assay

MCF7 cells

MDA-MB231 cells

MDA-MB468 cells

HeLa cells

184B5 cells
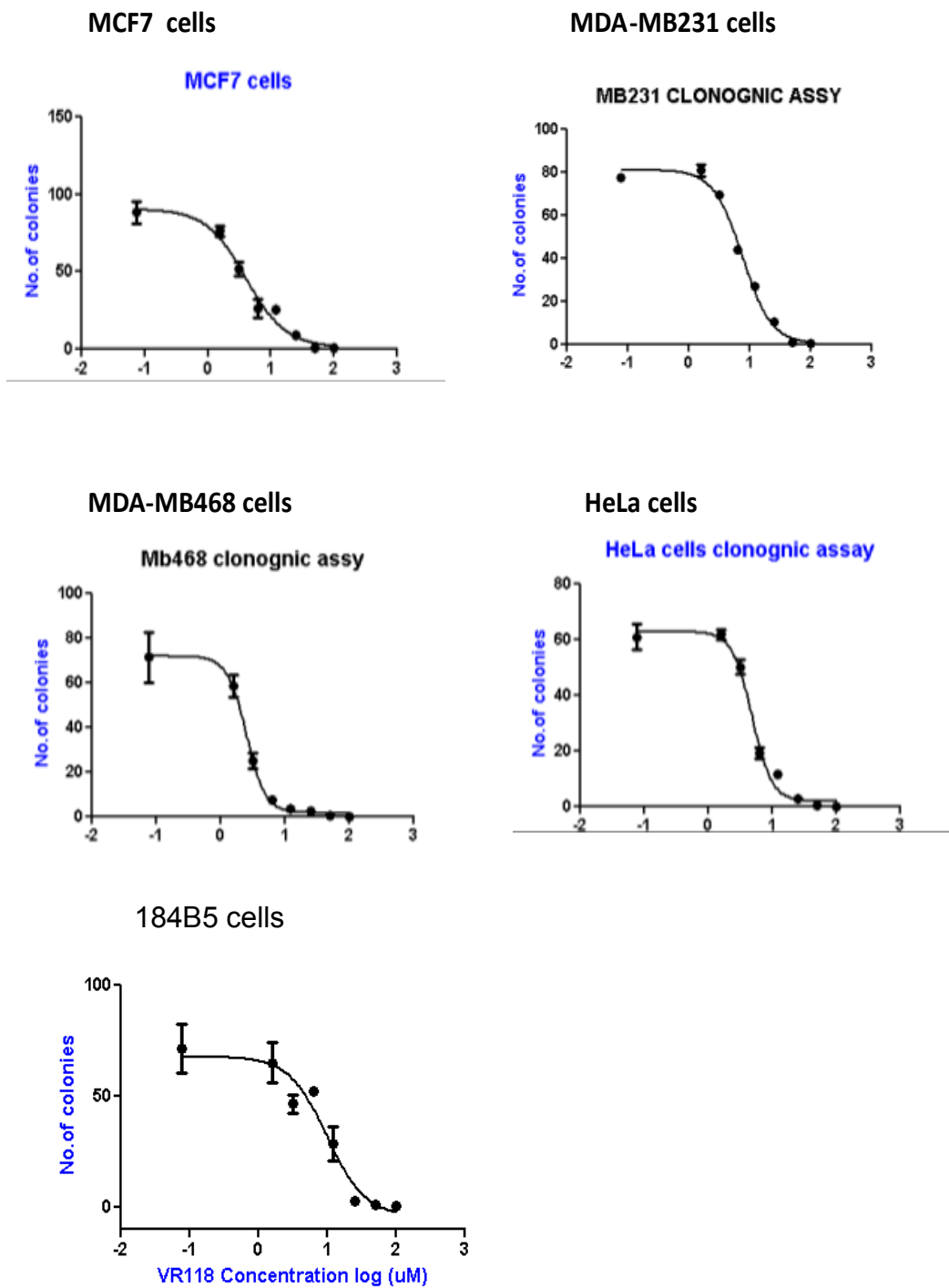
Table 2: IC$_{50}$ of VR118 on MCF7, MDA-MB231, HeLa, and MDA-MB468 cells as obtained from clonogenic assay.

IC$_{50}$ represents the concentration of a drug that is required for 50% reduction of colony forming ability compared to non-treated controls. IC$_{50}$ values were calculated using the GraphPad Prism sigmoidal dose curves (variable slope). Each experiment was performed in triplicate and each value represents the mean ± SD of three independent experiments. $p < 0.05$ vs. Non-cancer cells.

	IC50(µM) for cancer cells				IC50(µM) Non-cancer cells
Compound	MCF7	MB231	HeLa	MB468	184B5
Quinacrine	5.9±0.2	7.5±1.0	8.1±3.2	6.6±0.3	4.6±0.21
VR118	4.1± 1.0	6.5±3.0	4.0±0.32	2.5 ± 1.0	10.0 ± 0.70
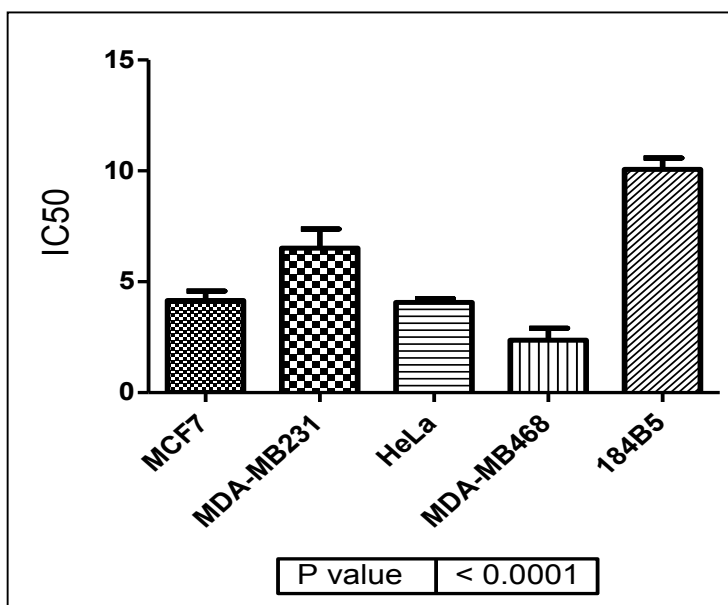
Data from (Table 1 and Table 2), (Fig. 2 and Fig. 3) suggest that VR118 decreases proliferating activity of malignant cells. Observations from the cell proliferation assays further demand the need to investigate the cytotoxic property of VR118 compound to differentiate between malignant and non-malignant cells.

3.2   VR118 is more Cytotoxic to Malignant Cells than Non-malignant cells

To examine whether VR118 shows selective toxicity to cancer cells, anti-proliferative activity of VR118 on malignant and non-malignant cells was analyzed using a Trypan blue exclusion assay. The result showed that VR118 compound is much less toxic to non-malignant (184B5, MCF10A) cells. Unlike Quinacrine, which did not differentiate between malignant and non-malignant cells, VR118 was capable of differentiating between malignant and non-malignant cells while killing. In addition, 184B5 cells did not show any changes in their cell cycle progression when treated with the same dose as malignant cells. Data revealed that VR118 did not increase plasma membrane permeability in non-malignant breast epithelial cells (184B5 and MCF10A cell lines), but had increased plasma membrane permeability in malignant breast cancer cells (MCF7, MDA-MB231, MDA-MB468 and HeLa cell lines) (Fig. 3 and Fig. 4). When 184B5 cells were treated with the IC_{50} dose of VR118 for malignant cells, they were not as greatly affected as cancer cells (Fig. 5). Similar findings were reported in the SRB assays, which showed that VR118 is less toxic to non-cancer cell lines (184B5, MCF10A) (Fig. 2, $p < 0.05$).Results from the Trypan blue exclusion assay clearly demonstrates the cancer cell-specific targetting nature of VR118.

Figure 5: Anti-proliferative activity of VR118 on different cell lines.

VR118 showed strong anti-proliferative activity on many different breast cancer and HeLa cell lines, but not on non-cancer cell lines (184B5, MCF10A). As described in Material and Methods, cells on 10 cm plates were grown in medium containing 2–10 µM concentration of VR118. Cells were stained with Trypan blue and counted using a hemocytometer to measure viable cell numbers. VR118 significantly decreased the viable MCF7 cell numbers after 24 h, whereas sham-treated controls exhibited significant proliferation. Moreover, data shows that VR118 is cancer cell specific at low concentration, and the non-cancer cell lines were less affected at 2–6 µM ranges. The data shows representative plots of three replicates. There were no discernable differences among the three independent experiments. Trypan blue is used to stain dead cells. Live cell count (not uptaking Trypan blue cells). Total cell count including those stained with Trypan blue.

A — Effect of VR118 on MCF7 cells
B — Effect of VR118 on MDA-MB231cells
C — Effect of VR118 on HeLa cells
D — Effect of VR118 on MDA-MB468 cells
E — Effects of vr118 on MCF10A cells
F — Effects of vr118 on184B5 cells
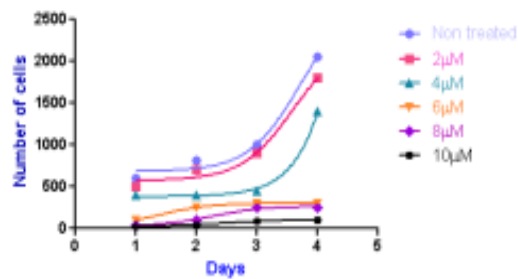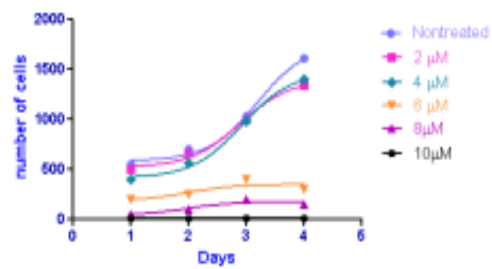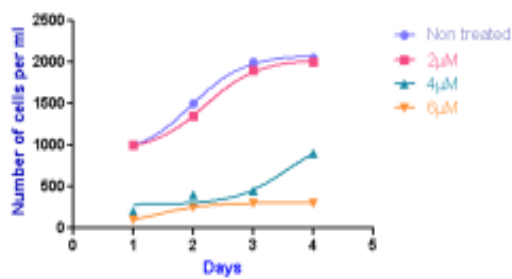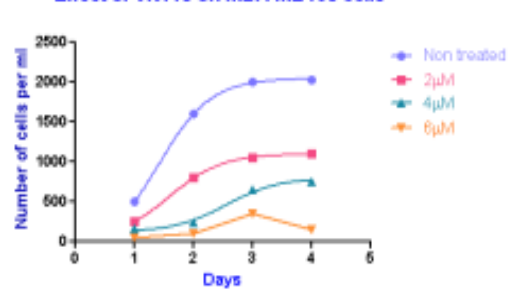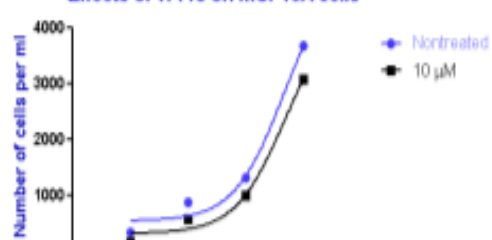
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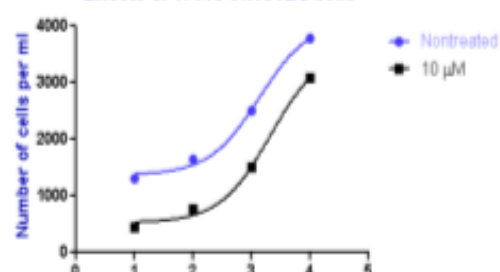
Figure 6: VR118 did not affect the cell cycle progression in 184B5 non-malignant breast cell lines.

To examine noticeable changes in the cell cycle progression of 184B5 cells in response to VR118, 184B5 cells were exposed to various concentrations of VR118 and were later analyzed by flow cytometry. The resultant data did not reveal any sign of cell cycle arrest or apoptosis in response to 2–6 μM of VR118. A total of 10,000 events were analyzed by flow cytometry using PI (propidium iodide) staining. Results are expressed as the percentage of total cells in each phase of the cell cycle. Sub-G1 displays a cell with lower DNA content than an intact cell, which indicates that the cells likely died by apoptosis or other mechanisms resulting in cells possessing a subG1 level of DNA content. G1 peak corresponds to the 2N content of DNA. G2/M peak corresponds to 4N, S phase represents >2N and <4N content of DNA. Plots shown are representative of three independent experiments. From this it can be inferred that VR118 treatment of 184B5 cells does not generate cells of a sub G1 DNA content, nor does the drug induce an accumulation of cells at a particular phase in the cell cycle. This suggests very little effect of VR118 on cell progression through the cell cycle.

184B5 cells treated with VR118
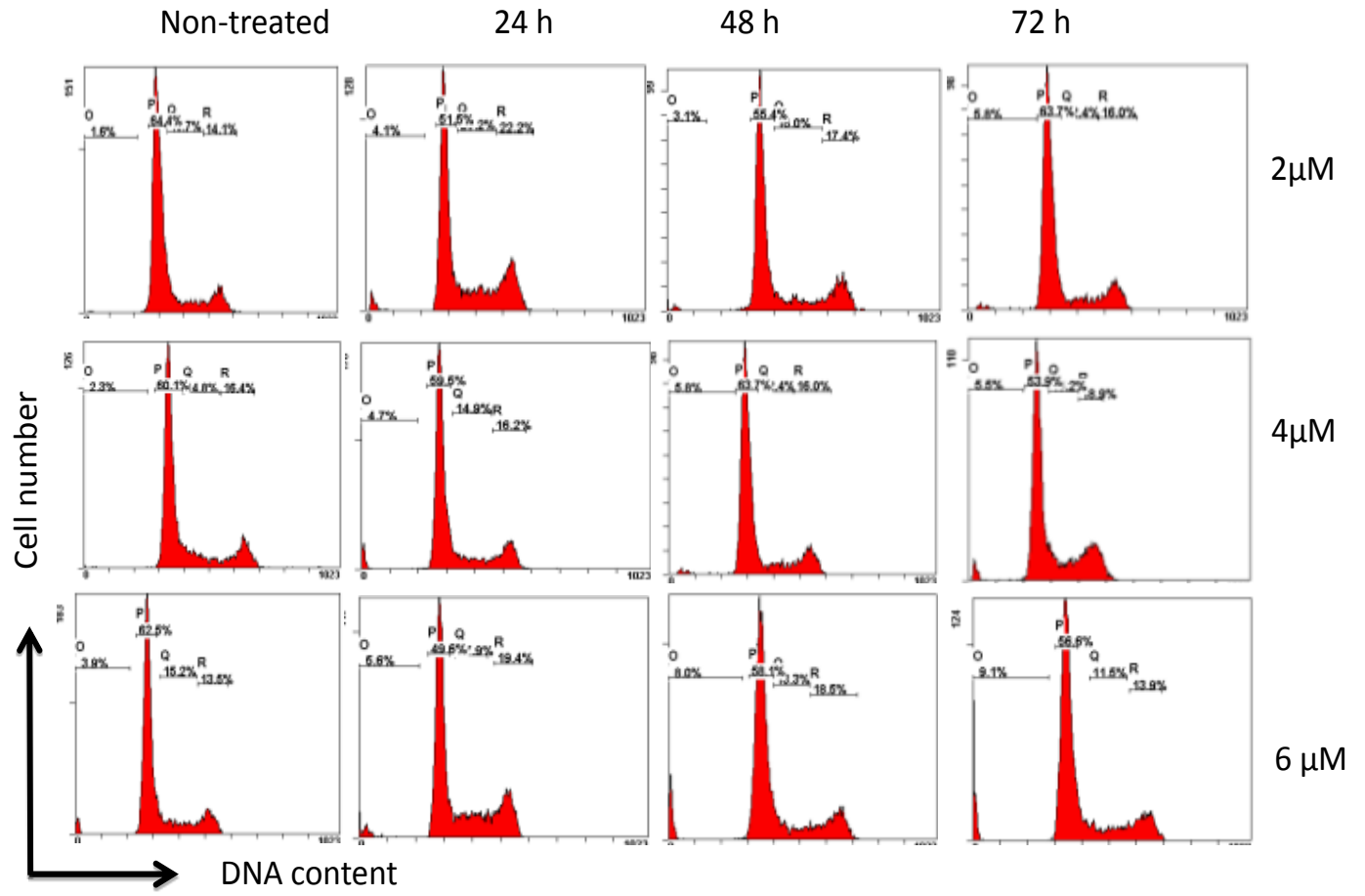
3.3   VR118 Causes Apoptosis in Breast Cancer Cells

After confirming that VR118 has the property of killing cells in a cancer-specific manner, I decided to study the possible mechanism of VR118 in killing cells and inhibiting growth. The induction of apoptosis and cell cycle arrest are both anti-proliferative responses, which likely contribute to the anti-neoplastic action of Quinacrine derivatives on cancer cells. To evaluate whether VR118 can cause apoptosis, three experiments were performed namely: Acridine orange/Ethidium bromide staining, Flow cytometry and a TUNEL assay.

Acridine orange and Ethidium bromide staining experiments were performed to determine if MDA-MB231 and MCF7 cells undergo apoptosis when treated with VR118. Acridine orange staining helps to determine cell morphology, cell size and refractive properties of cells treated with VR118. The rationale behind Acridine orange/Ethidium bromide staining experiment is that normal cell membranes are permeable to acridine orange but not to ethidium bromide stain. However, when cell membranes lose their structural integrity, they are permeable to ethidium bromide. Results from the double staining experiment revealed that malignant cancer cells underwent chromation condensation and extensive membrane blebbing which are hall marks of apoptosis, while sham-treated cells did not demonstrate any visible damage to the nuclear or cell membrane. Data in(Fig.7 and Fig. 8) clearly show that MDA-MB231 and MCF7 cells treated with VR118 have lost their structural integrity after 48 h with numerous blebbings. These findings are consistent with the Trypan blue assays. This demonstrates that these cells may be at a late stage of apoptosis. The presence of many vacuoles (bright yellow spans) suggests that these cells may also undergo autophagy (Fig. 7A).

A decrease in cell proliferation can be a result of either altered cell cycle progression or cell death. Therefore, I investigated the effect of this novel compound on cell cycle progression by flow cytometry. Flow cytometry results showed that VR118 induces apoptosis in MDA-MB231 and MCF7 cells as substabntial amounts of cells were observed in a dose and time dependent manner (Fig. 9 and Fig. 10). However, MCF7 data showed that number of cells undergoing apoptosis was slower (Fig. 10).

Data suggest that VR118 induces apoptosis in cancer cells by causing an increase in sub-G1 cell population which is suggestive of apoptosis. Microscopy findings revealed that MDA-MB231 cells treated with VR118 s showed chromatin condensation and nuclear fragmentation clearly indicating that they are undergoing apoptosis. MCF7 cells treated with VR118 showed morphologic characteristics similar to apoptosis, including cell shrinkage and membrane blebbing. Though VR118 induced apoptosis was immediately visible in MDA-MB231 cell lines, the process was delayed in MCF7 cell lines. This data clearly demonstrate the apoptosis inducing nature of VR118 in malignant cells.

I further used TUNEL staining to investigate DNA damage caused by VR118. The TUNEL assay is based on the fact that TdT enzyme can adda labelled deoxyuridine triphosphate to the free end (3'-termini) of damaged DNA. Data from TUNEL assay revealed that over 40% of MDA-MB231 treated with 6 μM VR118 underwent apoptosis (Fig. 11). Late stage apoptosis was clearly evident in TUNEL assay with significant delay in MCF7 cells compared with MDA-MB231 cells (Fig. 11 and Fig. 12). VR118 induced apoptosis was immediately visible in MDA-MB231 cell lines; whereas, the process was significantly delayed in MCF7 cell lines. These findings correlate with data obtained from Acridine orange staining (Fig. 8). Together data from the Acridine orange/Ethidium bromide staining, flow cytometry and TUNEL assays clearly demonstrate that VR118 causes apoptosis in cancer cells.

Figure 7: VR118-induced apoptotic cell death was observed by Acridine orange/Ethidium bromide staining.

MDA-MB231 cells grown on cover slips were treated with 6 μM of VR118 for 24 h. Cells were incubated with 100 μg/ml acridine orange and 100 μg/ml of ethidium bromide for 5 min. The cover slips were then analyzed by confocal microscopy. **A)** The cells showed chromatin condensation and fragmentation, clearly indicating that they are undergoing apoptosis. Extensive membrane blebbing was also evident. Magnification is 400X. Representative images from three independent experiments are shown. **B)** 100 cells were counted from randomly selected field. Representative graph is the mean from three dependent experiments.

Cells undergoing apoptosis are more granular than cells that are not. Live cells have a normal nucleus (green); early apoptotic cells have (bright green nucleus) shows condensed or fragmented chromatin and belbbing membrain; late apoptotic cells display condensed and fragmented (red or orange) chromatin.

A)

	Ethidium bromide	Acridine orange	Bright	Merge
Non-treated				
VR118				
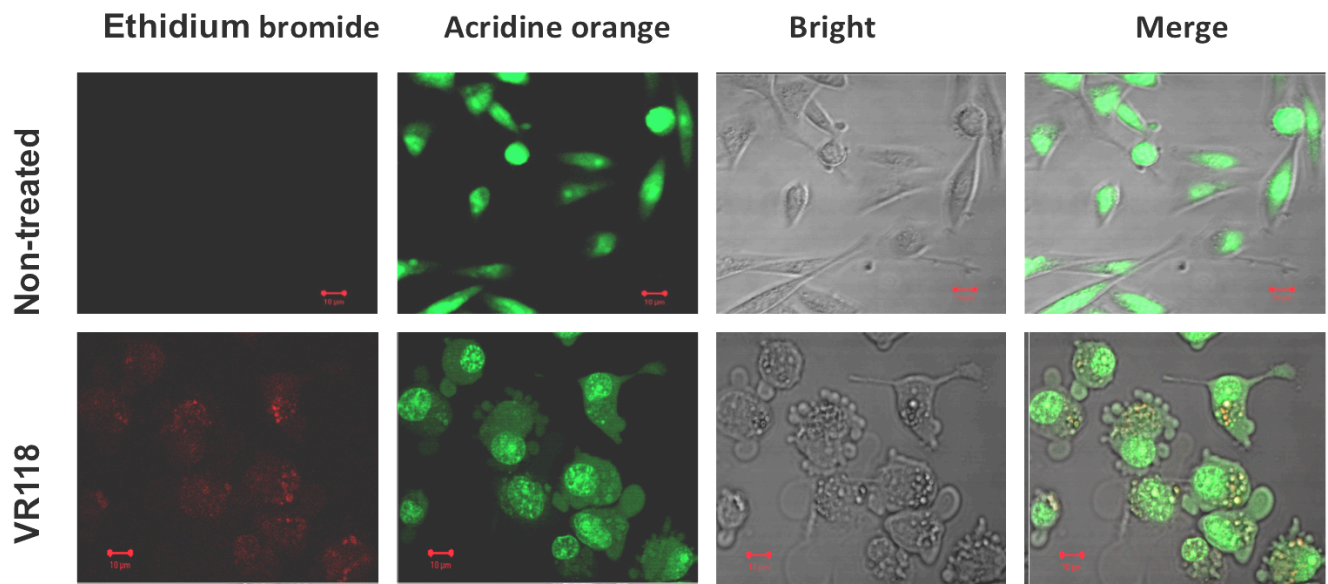
B)
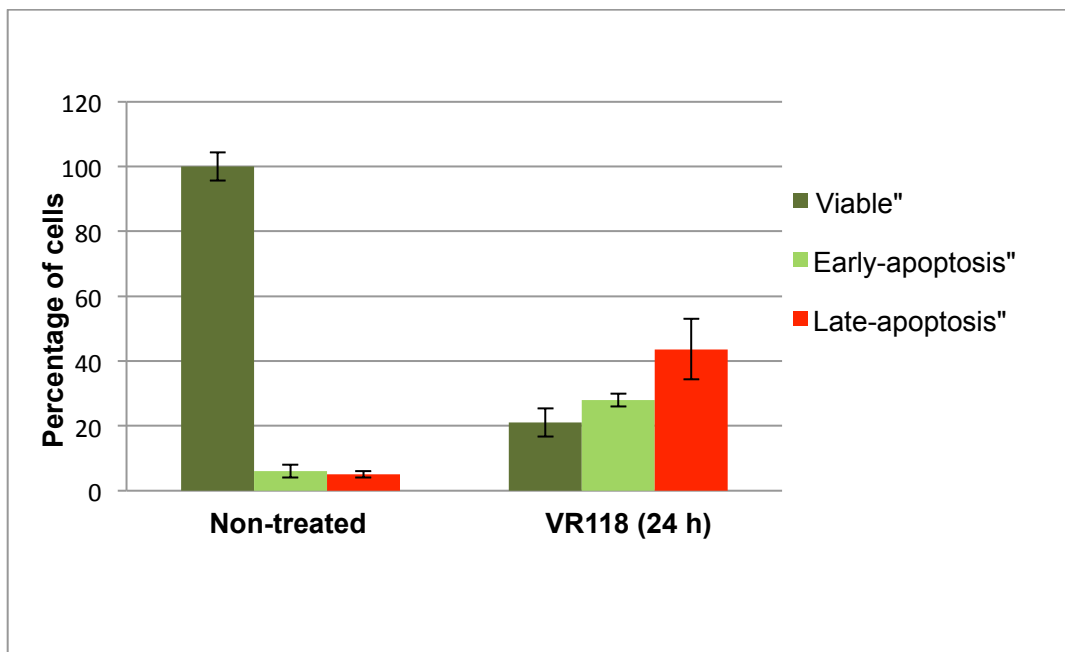
Figure 8: Exposure to the VR118 shows a sign of apoptotic cell death in MCF7 cells.

MCF7 cells grown on glass cover slips were treated with VR118 for 24 h and followed by staining with acridine orange/ethidum bromide. **A)** MCF7 cells treated with VR118 (4 μm) showed morphology characteristics to apoptosis, including cell shrinkage and membrane blebbing. MCF7 cells exposed to VR118 for 48 h showed chromatin degradation in the cells, compared to non-treated samples. **B)** 100 cells were counted from randomly selected field. Representative graph is the mean of three dependent experiments.

A)
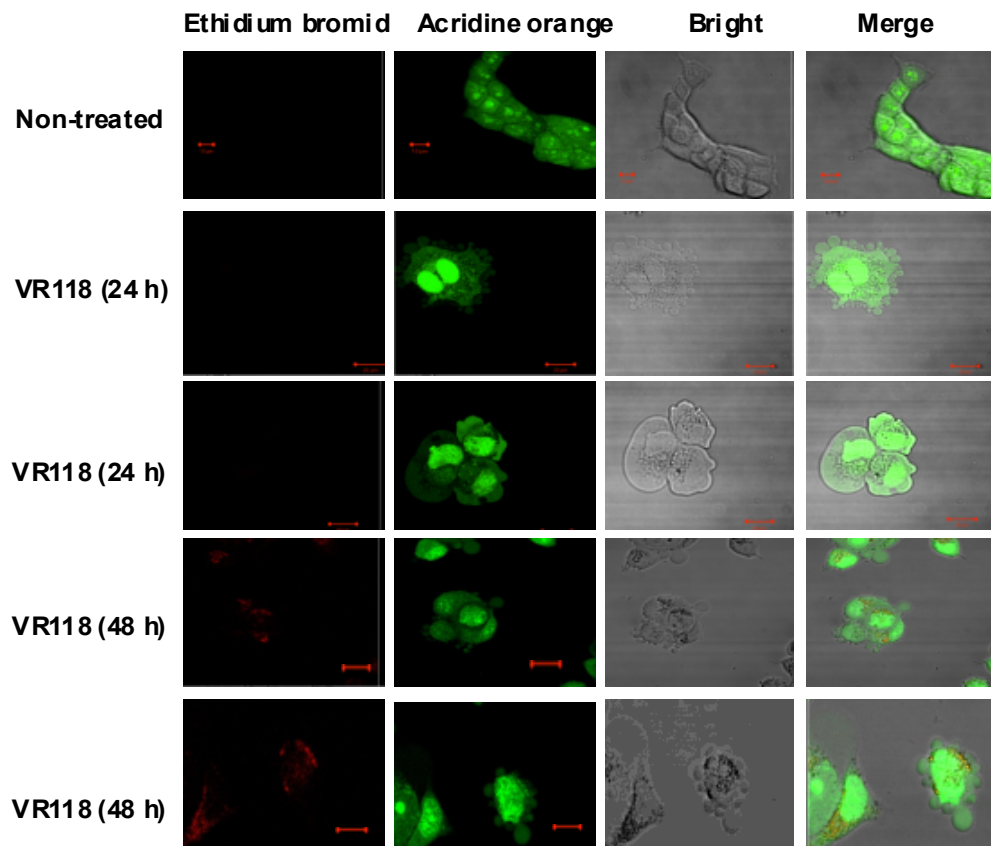
B)
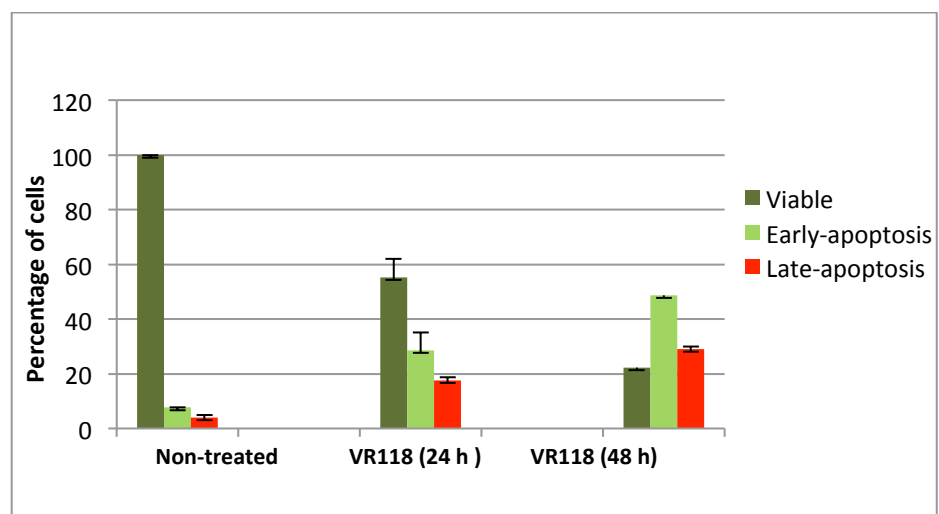
Figure 9: Cell cycle analysis of MDA-MB231 cells exposed to VR118.

Data shown is flow cytometry profiles of MDA-MB231 cells treated with 4 µM or 6 µM or 10 µM of VR118. Cells were collected at scheduled timepoints, post-exposure to VR118, fixed overnight in 75% ethanol, and then stained with PI solution. Exposure to high concentrations of VR118 resulted in an increase of sub-G1 cell population indicating that VR118 induces apoptosis. The percentage for the cell cycle distributions of cells was estimated by gating for the fluorescent intensity corresponding to the amount of DNA in each event. Gates were adjusted with respect to non-treated cells. X-axis represents the DNA content stained with PI. Y-axis corresponds to the number of cells. The amount results shown are representative of three independent experiments.

MDA-MB231 cells treated with VR118
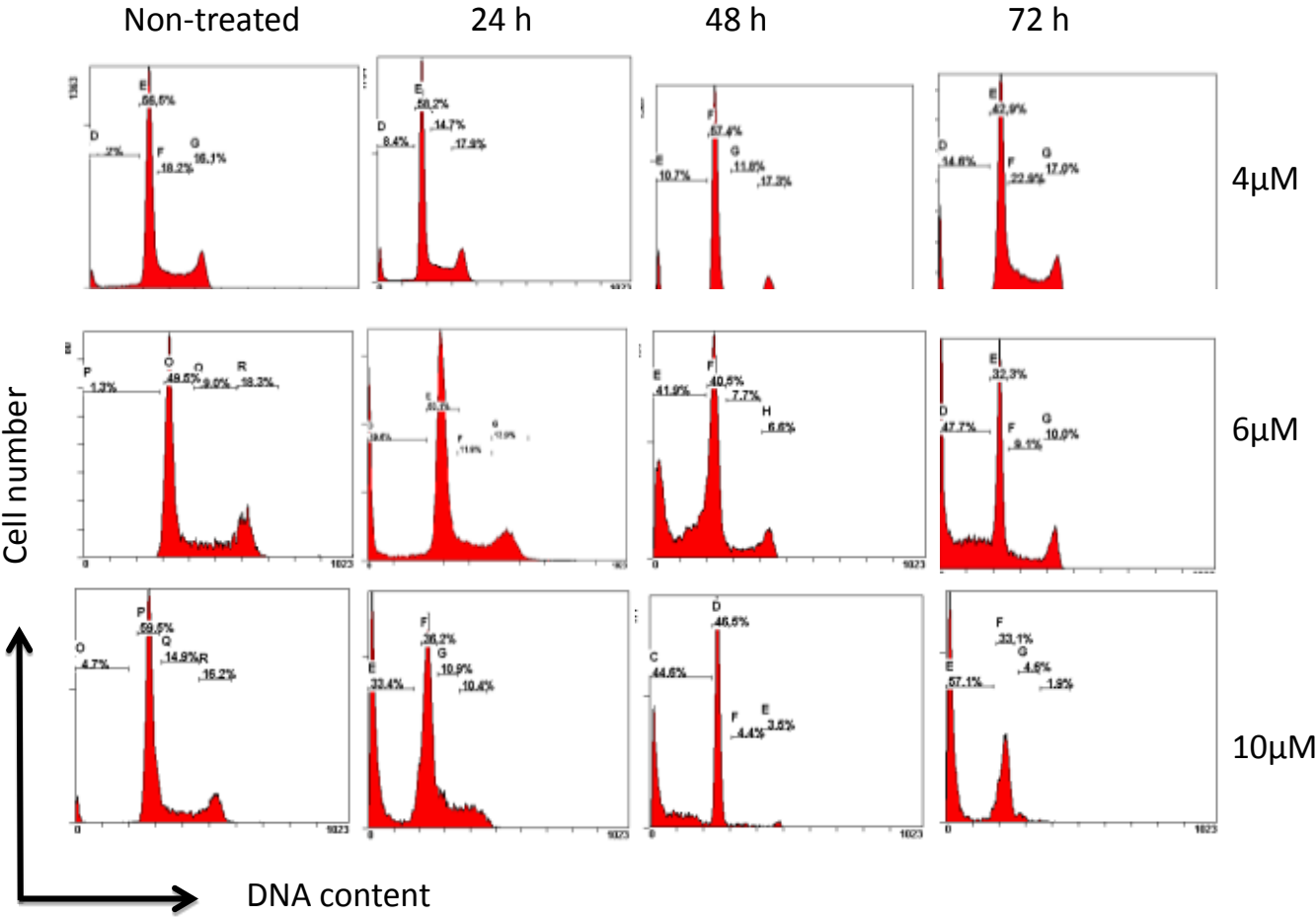
Figure 10: The effects of VR118 and Quinacrine on the MCF7 cell cycle progression.

Data shown is flow cytometry profiles of MCF7 cells, treated with IC_{50} concentration of VR118 and Quinacrine. Cells were harvested for 72 h post-exposure to VR118, fixed overnight in 75% ethanol, and then stained with PI solution for the analysis of DNA content. Exposure to VR118 resulted in the increase of sub-G1 population, indicating that the compound induces apoptosis in MCF7 cells. In contrast, Quinacrine did not induce apoptosis but resulted in cell cycle arrest in S phase. The percentage shows cell cycle distributions of cells for each plot as estimated by the gating for the fluorescent intensity corresponding to the concentration of DNA in each event. Gates were adjusted with respect to sham-treated cells. X-axis represents the DNA content stained with PI. Y-axis corresponds to the number of cells. Data shown are representative of the mean of three independent experiments.

MCF7 treated with VR118 and QC at 4 µM at different time point
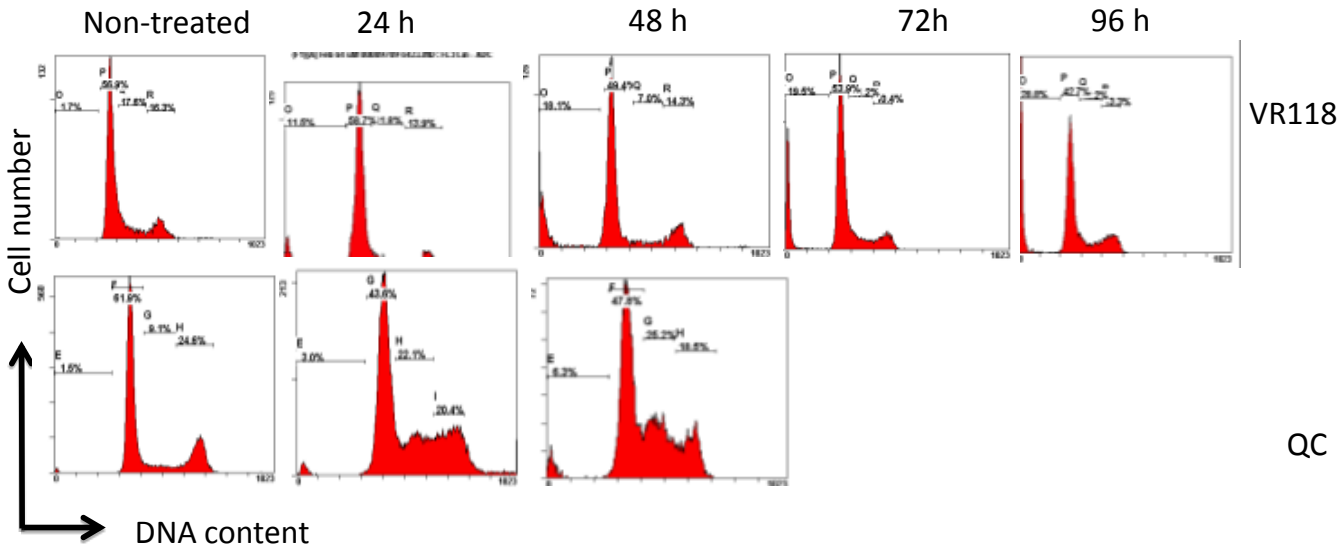
Figure 11: Detection of DNA fragmentation in MDA-MB231 treated cells by a TUNEL assay.

A TUNEL assay measures and quantifies apoptosis by labeling and detecting DNA strand breaks in individual cells. A TUNEL assay was carried out by an In Situ cell death Detection kit, supplied Fluorescien. The first row shows untreated control cells. The second row shows recombinant DNase I (3 μ/ml) treated sample used as positive controls. The third row shows MDA-MB231 cells treated with VR118 at 6 μM. Both treated cells and control samples were analyzed for the induction of apoptosis after 24 h. MDA-MB231 cells treated with VR118 underwent rapid apoptosis (35%) compared with untreated control cells (2%). A) The images of TUNEL positive cells were captured by a confocal microscope (×400). B) 100 cells were counted for each sample and triplicate samples were used.

A)

	Tunel	Bright	Merg
Non-treated			
Positive control DNase I recombinant RNase-free			
VR118			
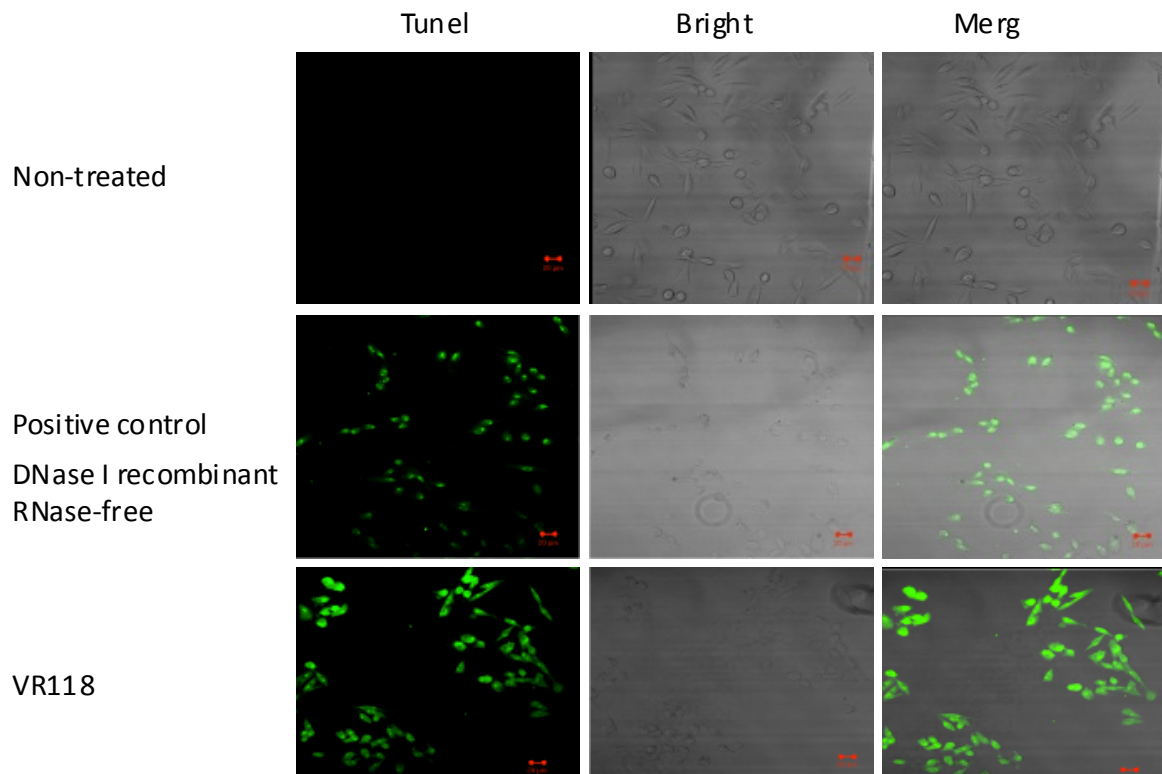
B)
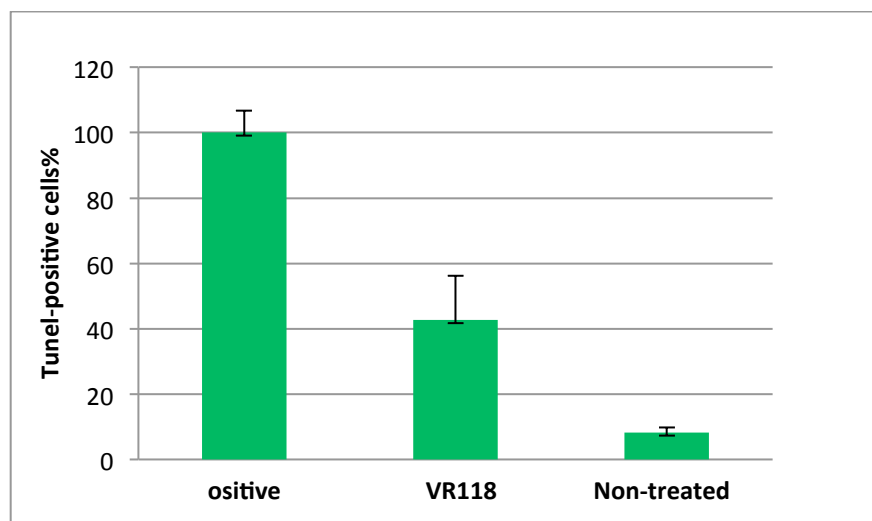
Figure 12: Induction of DNA fragmentation in MCF7 cells after 48 h post-treatment with VR118 (4 μM).

MCF7 cells were treated with VR118 as described in Material and Methods. Cells were examined with a TUNEL assay kit to determine apoptosis. Apoptotic cells were visualized by confocal microscopy. Triplicate samples were used for both treated and untreated control cells. Findings from the TUNEL assay revealed a delay in the process of apoptosis in MCF7 cells. A) The images of TUNEL positive cells were captured by a confocal microscope (×400). B) 100 cells were counted for each sample and triplicate samples were used.

A)

	Tunel	Bright	Merg
Non-tr eated			
Positiv e control DNase I recom binant RNase-free			
VR118			
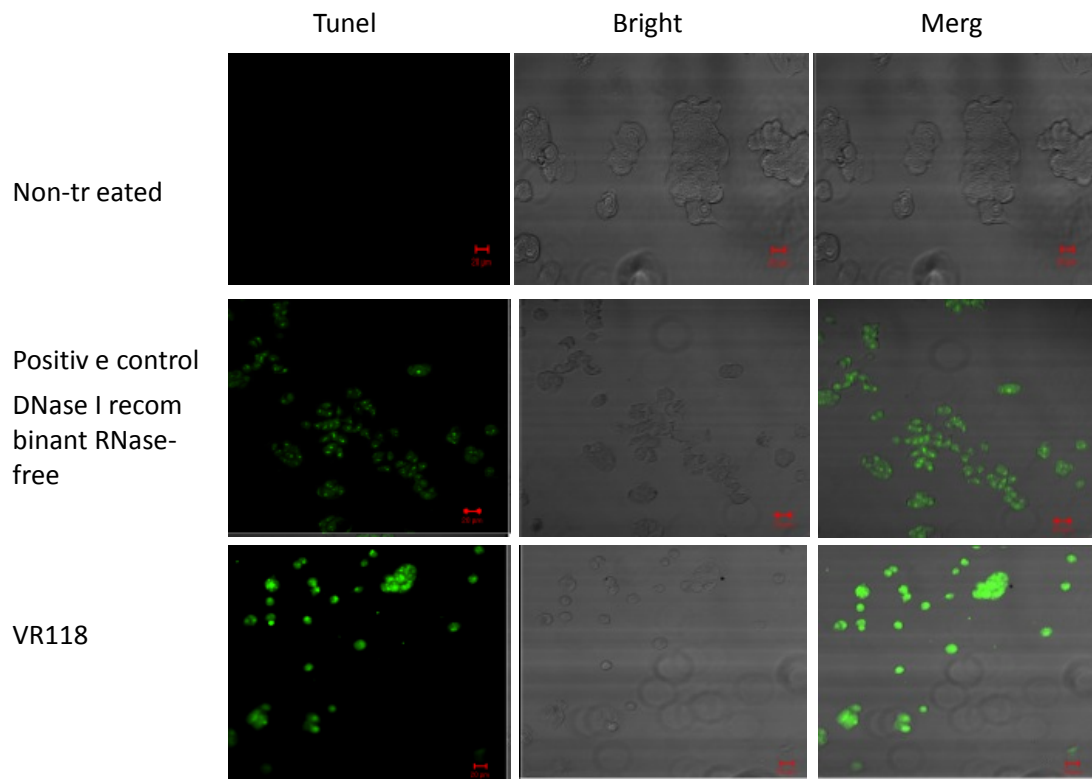
B)
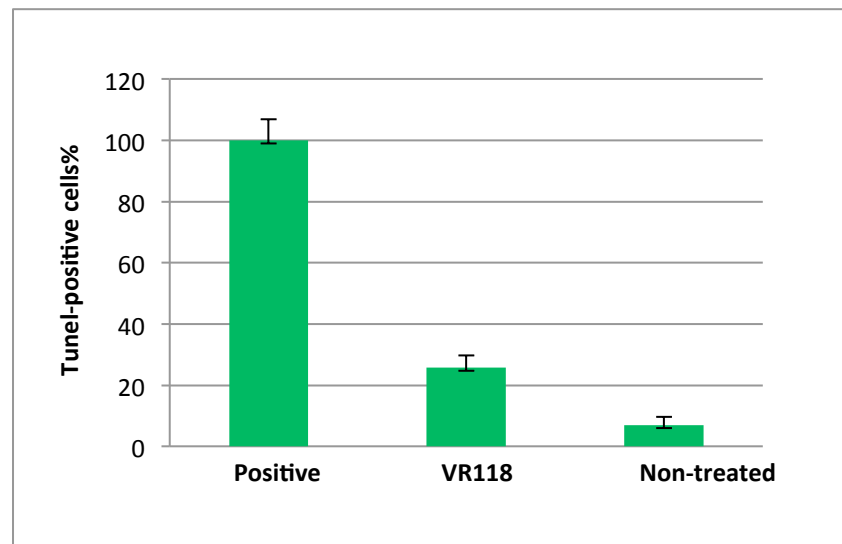
Molecular Pathways Involved in VR118 Mediated Apoptosis

To further understand the underlying molecular mechanisms through which VR118 induces apoptosis, Western blotting analysis was performed to analyze important molecular markers responsible for apoptosis. Findings revealed that apoptosis is dependent on the mitochondria-dependent signaling pathway. Findings also revealed that the expression levels of pro-apoptotic markers such as Bad and Bax increased significantly in cells undergoing apoptosis. Further findings also revealed that VR118 releases cytochrome c and activates PARP to induce apoptosis in cancer cells.

Figure 13: VR118 induced apoptosis by downregulating anti-apoptotic proteins and upregulating pro-apoptotic proteins in MDA-MB231 cells.

Whole cell lysates were prepared after treating MDA-MB231 cells with 6 μM of VR118 for 24 h, 48 h, and 72 h. The levels and cleavage status of proteins were analyzed by Western blotting using antibodies specific to Bcl-2, Bcl-xL, Bad, Bax, cytochrome C and PARP-1. A) Expression levels of the anti-apoptotic proteins Bcl-2 and Bcl-xL were significantly decreased. However, that of cytochrome C, Bad, and Bax pro-apoptotic proteins increased in a time-dependent manner. B) PARP-1 plot shows the full-length PARP (116 kDa) and the larger fragment (85kDa) of apoptotically cleaved products. GAPDH and β-tublin were used as a loading control. Results are representative of two separate experiments.
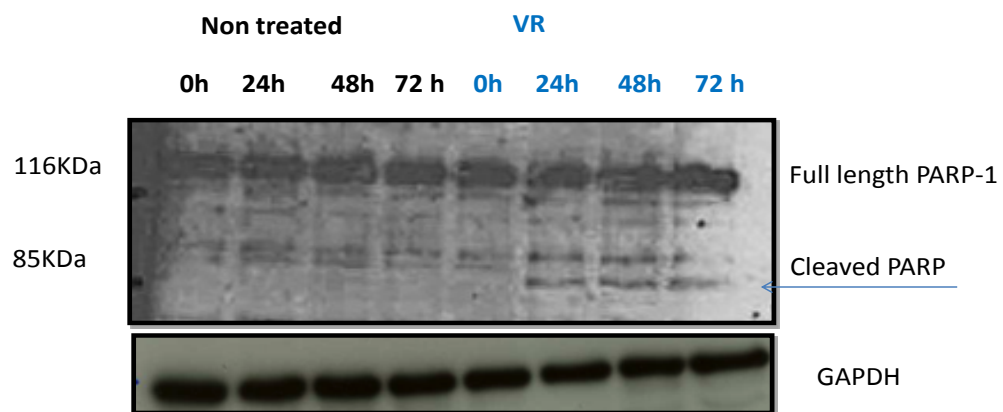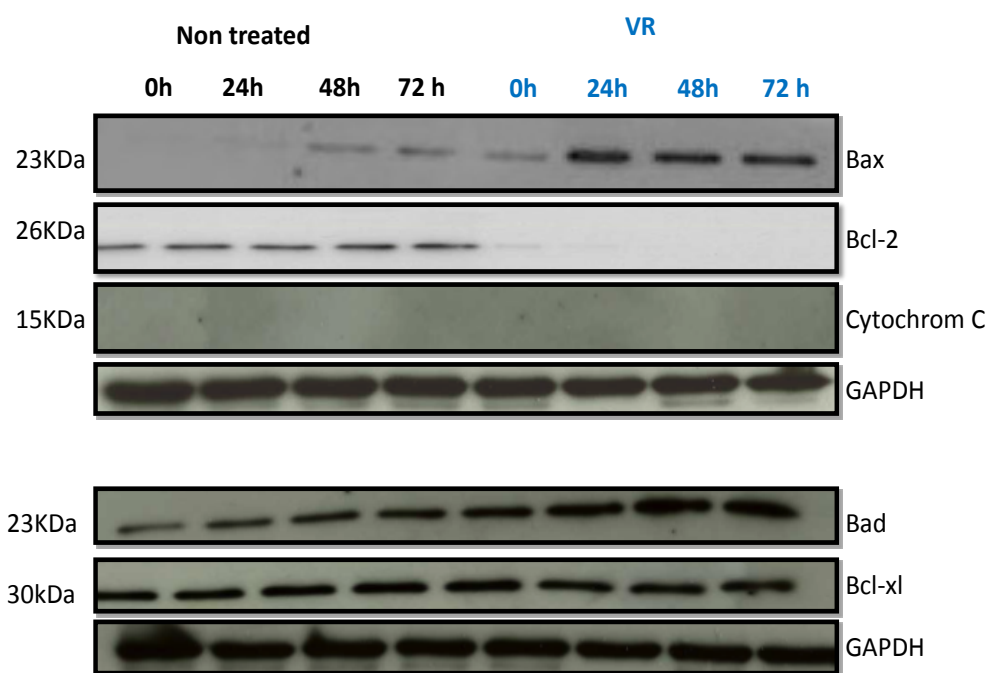
Figure 14: VR118 reduced the level of the anti-apoptotic proteins, and increased pro-apoptotic proteins in MCF7 cells.

MCF7 cells (70% confluent) were treated with two different concentrations of VR118 for 24 h. Cells were harvested and cell lysate was prepared at 24 or 48 h timepoint. Proteins, separated in 10% SDS–PAGE, were transferred to a PVDF membrane. The membrane was probed with anti-Bax, anti-Bcl-2, anti Bad, and anti-p53 antibodies according to the manufacturer's protocol. The GAPDH served as a control. Data are the representative loading of two different experiments.
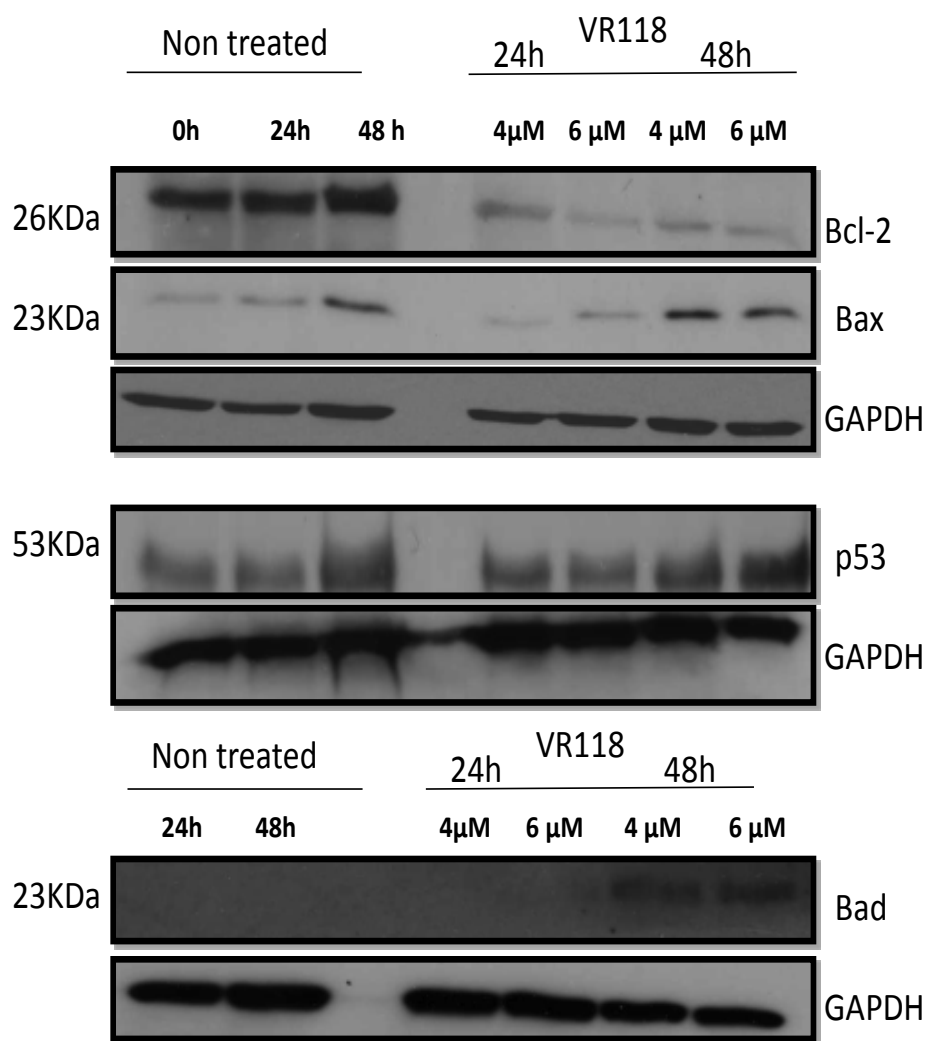
Figure 15: MCF7 cells treated with VR118 released cytochrome c and activated PARP.

MCF7 cells were treated with IC_{50} value (4µM) of VR118. Cells were harvested and cell lysate was prepared at 24, 48, 72 h timepoint. Proteins, separated in 8% SDS–PAGE, were transferred to a PVDF membrane. The membrane was probed with anti-PARP-1, anti-cytochrome c. The GAPDH or β-tubulin used as loading control. Data are the representative loading of two different experiments.

Chapter 4
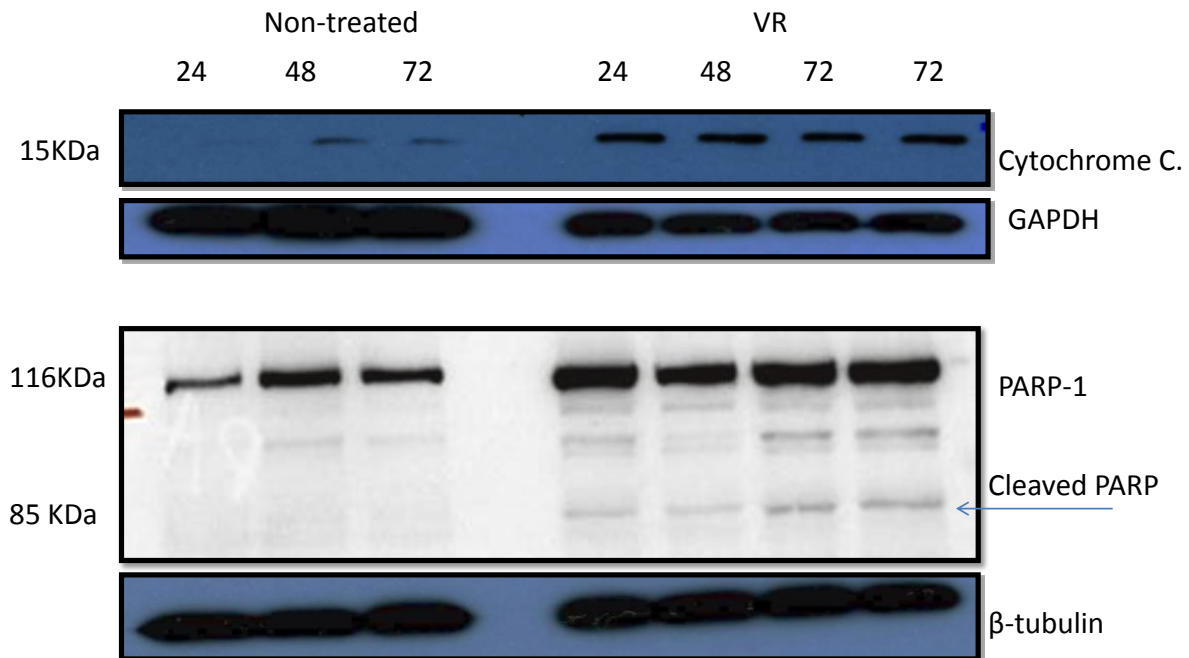
4   DISCUSSION

Given the heterogeneous nature of cancer and the complex processes involved at cellular and molecular levels, the need for effective cancer cell specific targeted therapy remains. Moreover, targeting cell signaling pathways that can alter cellular functions in cancer cells is an ideal area of target for the development of new anticancer drugs. Current research shows that Quinacrine is one such drug that has the capability to kill cancer cells by inducing apoptosis through selective activation and inhibition of cell signaling pathways. Recent studies showed that 9AA derivatives have proven to be effective against renal carcinoma cancer cells and are reported to affect two cellular pathways, by selective inhibition and activation of NF-κB and p53 pathways, respectively (Gurova et al., 2005). Although Quinacrine has proven to effectively kill cancer cells by targeting specific pathways, studies *in vitro* showed that it lacks the ability to differentiate between malignant and non-malignant cells (Table 1) (Custer, 1946; Gaskill and Fitz-Hugh, 1945). Given the advantages and disadvantages of Quinacrine, there is a need for developing a compound that has low levels of drug toxicity and cancer cell specificity in inducing apoptosis.

Therefore, to develop a potent and cancer-specific drug, Quinacrine was modified by a hybrid pharmacophore approach in Dr.Lee's lab. This study is based on the hypothesis that the anticancer properties of VR118 would be better than the lead compound Quinacrine. The present study was performed to examine the effectiveness of the compound Quinacrine. I indeed found that compound VR118 more selectively targets cancer cells, compared to its parent compound Quinacrine.

In order to justify the cancer cell-specific targeting nature of VR118, I executed a wide range of experiments on malignant cancer cells lines (MCF7, MDA-MB231, MDA-MB468, and HeLa) and non-malignant cell lines (184B5 and MCF10A), which led me to make a conclusion that VR118 is potentially better than Quinacrine as an anti-cancer agent. In this study, I systematically investigated the antiproliferative potential of VR118 in malignant cell lines and clarified the mechanism of action through which VR118 kills cancer cells. First, I evaluated the

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anticancer effect of VR118 on malignant cells. I then confirmed the cytotoxic activity of VR118 and Quinacrine on malignant and non-malignant cells. Further, I examined the possible mechanisms involved in cancer cell killing process of VR118 such as p53 level, cytochrome c, and Bax/Bcl-2 ratio and studied the proteins involved in the regulation of apoptosis. Based on the findings, I came to the conclusion that VR118 induced apoptosis in malignant cells by causing upregulation of p53, resulting in increase in Bax/Bcl-2 ratio, triggering the release of cyctochrome c from the mitochondria favoring apoptosis. VR118 exhibited the following cancer cell-specific killing properties: a) Anti-proliferative activity and cytotoxicity in a cancer-specific manner; b) the major mode of cell killing is by apoptosis; and c) apoptosis is induced by altering the Bcl-2/Bax ratio.

4.1   VR118 suppresses tumor cell proliferation

One of the principle characteristics of cancer cells is their ability to proliferate uncontrollably, leading to tumorigensis. Although Quinacrine exhibited significant anti-proliferative activity against cancer cells, its derivative VR118 exhibited generally higher anti-proliferative activity on malignant cells with different genetic backgrounds. In this study, I systematically investigated the antiproliferative effect of VR118 on malignant cells and noticed that VR118 significantly inhibited the proliferation of cancer cells. This effect of VR118 has been demonstrated by both SRB and clonogenic assays. VR118 showed significant anti-proliferative effect on MCF7, MDA-MB468 and HeLa cells (Fig. 2 and Fig.3).

Moreover, Clonognic ssays revealed that VR118 exhibited a dose dependent relationship with the rate of colony formation decreasing with an increase in concentration of VR118 (Fig.4). The inhibition of many different cell lines with different genetic background suggests that VR118 affects a common mechanism in malignant cell lines that is required for their growth and proliferation. Hence, it can be inferred that VR118 exhibits significant anti-proliferative effect on malignant cells in a dose dependent manner and has the potential to be a potent anti-cancer drug.

4.2   VR118 is More Cytotoxic to Malignant Cells than Non-Malignant cells

The most important criteria for any compound to qualify as a potent drug is the ability to prevent toxic effects or side effects on the normal cells of the body. Although Quinacrine has proven

successful to interfere with the ability of cancerous cells to divide and reproduce; it causes significant damage to normal healthy cells along with cancer cell lines. My observations from the study not only showed that VR118 exhibits significant efficacy in killing cancer cells, but also showed that it does not have any noticeable effect on non-cancer cells. One of the negative aspects of Quinacrine is its toxic effect on non-cancerous cells. In this analysis non-malignant breast cancer cells lines namely 184B5 and MCF10A cell lines underwent stress and toxic effects when treated with Quinacrine at concentrations applicable for malignant cell lines. In comparison, VR118 clearly indicated that it was non-toxic on non-malignant cell lines such as 184B5 and MCF10A, at least at lower concentrations (Fig.2, Table.1 $p < 0.05$ vs. Non-cancer cells).

This showed that VR118 clearly differentiates malignant cells from non-malignant cells *in vitro* and demonstrated significant anti-proliferative activity specific to malignant cell lines with different genetic backgrounds. The experiment findings also showed that the antiproliferative effect of VR118 is partly influenced by the concentration of the drug and the characteristics of the cell lines examined. This definitely qualifies VR118 as a potential anti-cancer therapeutic drug; since it meets the basic requirement of an ideal drug that is; non-toxicity to non-cancer cells. In addition, flow cytometry results revealed that low concentrations of VR118 had no significant effect on cell cycle progression in the non-malignant cell line 184B5, but had substantial effect on the cell cycle progression in cancer cells. This cancer specific property of VR118 without causing substantial harm to normal cells makes it an ideal drug for cancer treatment. Although VR118 demonstrated little effect on cell cycle progression and proliferation of normal cells *in vitro*, the true capability of VR118 to selectively kill cancer cells with no cytotoxicity towards normal cells will only come from experiments in animal systems. Testing the efficacy and specificacy of VR118 *in vivo* is important because VR118 could possibly have physiological effects in living systems that cannot be adequately assessed using cell lines.

4.3   VR118 Increases Sub-G1 Cell Population in Cancer Cells

The control of the cell cycle is a key regulatory mechanism for cell growth and proliferation (Goranav et al., 2009). Therefore, targeting the cell cycle is one of the key driving forces behind the development of new anticancer drugs. Normal human cells respond to DNA damage by

activating cell-cycle checkpoints where cells try to repair DNA by temporarily arresting DNA replication or cell division (King and Cidlowski, 1998; Pellegata et al. 1996). Temporary arresting of cell cycle progression is important for maintaining the genetic integrity (Thompson, 1995). In circumstances where the DNA damage is irreversible, cells may undergo apoptosis to prevent the development of mutated DNA. Therefore, to understand the mechanism through which VR118 induced apoptosis, I performed a series of experiments on breast cancer cells and non-malignant cells.

My experimental findings not only supported the cancer cell specificity of VR118, but also clarified the mechanism of action through which VR118 induces apoptosis in different two malignant cell lines. The apoptotic function in normal mammalian cells is generally regulated by p53, a tumor suppressor. However, in tumor cells, the p53 is often mutated. When MCF7 cancer cells are treated with VR118, results showed that it induced apoptosis in cancer cells by causing upregulation of p53 slightly. On the contrary, anticancer therapies such as chemotherapeutic drugs and radiation therapy kill tumor cells by causing cell cycle arrest at the G0/G1, S, or G2/M phase often in a p53 independent manner (Chau and Figg 2009; Murray, 2004; Torres and Horwitz, 1998). Cell death independent of p53 is not a perfect process because the arrest of cell cycle may cause side effects including miss repair of DNA damage. Therefore, chemotherapeutic drugs causing cell cycle arrest may result in mutations (Pietenpol and Stewart, 2003). Nonetheless, existing evidence suggests that new generation of drugs can induce apoptosis through the inhibition of specific cell signaling pathways (Hsu et al., 2005). Moreover, experimental findings revealed that VR118 does not cause arrest of cell cycle progression prior to the induction of apoptosis.

To determine the apoptosis inducing effect of VR118 and Quinacrine, a series of experiments studying the cell cycle and apoptosis morphology were performed using Acridine orange/Ehtium bromide staining. In the present study, Quinacrine seemed to induce apoptosis in MCF7 cells through cell cycle arrest in S phase (Fig. 10). Similar mechanism of action of parent compound Quinacrine was reported by Preet et al. (2012) in breast cancer cells (MCF7) and by Wu et al. (2012) in gastric cancer cells (SGC-7901), where Quinacrine exhibited cell cycle arrest by significantly decreasing the cell populations in G0/G1 and G2/M phases and inducing apoptosis

(Preet et al., 2012; Wu et al., 2012). Contrary to these findings, a study by Gurova et al. showed that Quinacrine activated the p53 pathway in renal carcinoma cancer cells without inducing DNA damage at low concentrations. Moreover, Gurova et al. (2005) reported that Quinacrine stabilized the p53 protein by preventing its ubiquination without phosphorylation suggesting apoptosis induction by activating p53-dependent signaling pathways in tumor cells (Gurova et al., 2005). Although Quinacrine seemed to cause cell cycle arrest in my experiments, further analysis is required to study if the mechanism through which Quinacrine induces apoptosis in a dose-dependent manner in malignant cells.

Unlike Quinacrine, MCF7 cells treated with VR118 showed a sub-G1 peak indicating that the compound induces apoptosis in MCF7 cells without causing cell cycle arrest. This shows that VR118 may not induce activation of a damage-mediated signaling pathway, unlike its parent compound and other chemotherapeutic agents (Gorbachev et al., 2007; Rosenzweig et al., 1997; Vainio et al., 1997). Instead, VR118 seems to induce apoptosis through the activation of cell signaling pathways. In normal cells, the stability of a genome is maintained, in part, by p53 (also known as tumor suppressor). When a normal cell is subject to stress, p53 senses DNA damage and determines if the cell should undergo DNA repair by temporarily arresting the cell cycle in G1 phase or trigger apoptosis if the damage is irreversible. However, in tumor cells the genetic stability can be lost due to defective p53. Once the tumor suppressing function of p53 is lost, it leads to loss of the G1 checkpoint where critical DNA repair can be activated to avoid further damage by continuous DNA replication (Pellegata et al. 1996). DNA G1 phase is an ideal phase for inducing apoptosis by p53 as it increases the expression of pro-apoptotic proteins (Bax) in this phase favoring apoptosis (Pellegata et al. 1996). VR118 induced apoptosis in G1 phase appears to be independent of cell cycle arrest (Piazza et al., 1997). These findings indicate VR118 may be a much safer anticancer drug than Quinacrine.

Further, the analysis of the FACS data clearly showed that VR118 increased the percentage of cells undergoing apoptosis in MDA-MB231 and MCF7 cells in a dose-dependent manner. According to the observations made from Acridine/Ehtium bromide staining experiments, the activation of apoptosis in MCF7 cells by VR118 is substantially delayed, compared to that in MDA-MB231. For example, the substantial amount of sub G(1) peak was visible only after 48-

96 h in MCF7 cells treated with VR118. Although morphological markers for apoptosis like shrinkage and membrane blebbing were evident in MCF7 cells within the first 24 h of post exposure to VR118, chromatin degradation was not in pace with that of MDA-MB231 cells. Similar findings were observed in the TUNEL assay, where MCF7 cells demonstrated delayed DNA fragmentation to at least 48 h post-VR118 (Fig.12).

The delay in apoptosis in MCF7 cells can be attributable to the fact that MCF7 cells lack caspase 3, which is an essential component for most apoptotic signaling pathways (Janicke et al., 1998). In MCF7 cells, the functional 47-bp inside the exon 3 of the CASP-3 gene, which is very important to perform the function of apoptosis, is deleted (Janicke et al., 1998). However, a few studies report that the apoptotic process in MCF7 cells is independent of caspase 3, because MCF7 cells undergo apoptosis through the formation of apoptotic bodies through biophysical alterations. Kawaga et al performed a study to investigate the role of caspase-3 in Bax-induced apoptosis using parental MCF7 cells deficient of caspase-3 and cells transfected with the caspase-3 gene (MCF7/Casp3). Findings from the study showed parental MCF7 cells deficient of caspase-3 failed to undergo morphological nuclear and DNA fragmentation, whereas clones transinfected with caspase-3 demonstrated intact nuclear dismantling and DNA fragmentation (Kagawa et al., 2001). Researchers of the study also reported that deficiency of caspase-3 did not prevent the cells from undergoing Bax-induced apoptosis, but aided in blocking Bax-mediated nuclear fragmentation (Kagawa et al., 2001). To further understand the process of apoptosis in caspase-3 deficient MCF7 cells, reseachers Liang et al. (2001) treated both mock- and bcl-2-transfected MCF-7 cells with DNA-cleaving antimitotic agent, neocarzinostatin (NCS). Researchers noticed that MCF7 cells underwent apoptosis through release of cytochrome c from the mitochondria resulting in decreased levels of Bcl-2 and increased levels of Bax. To further understand the process, researchers used caspase inhibitors with overlapping specificities and found that MCF7 cells underwent apoptosis through sequential activation of caspases 9, 7 and 6 (Liang et al., 2001). Similar findings were reported by Janicke et al in 1998 that apoptosis inducers such as transforming growth factor-β1, Fas, and TNF or staurosporine activate caspases in MCF cells (Janicke et al., 1998).

Furthermore, in a study performed to examine the anticancer effects of dracorhodin perchlorate

(DP), Yu et al. (2013) reported that the treatment of MCF7 cells with DP induced apoptosis through translocation of apoptosis inducing factor (AIF) from the mitochondria to the cytoplasm. AIF causes the release of cytochrome c from mitochondrion which further activates caspase 3 inducing apoptosis. However, Yu et al. state that apoptosis in MCF7 cells is independent of caspase-3, but dependent on AIF (Yu et al., 2013). Although researchers attribute different mechanisms responsible for inducing apoptosis in caspase-3 lacking MCF7 cells, further analysis is required to fully understand the mechanism through which VR118 and Quinacrine induce apoptosis in MCF7 cells. Moreover, there is scarcity of published data to understand the effect of Quinacrine and its derivatives on cell-cycle progression.

It can be inferred from these observations that VR118 mediated apoptosis is caspase 3 independent, as MCF7 cells underwent apoptosis. From these observations, it can also be stated that VR118 mediated apoptosis in MCF7 cells is p53 dependent. Additionally, it provides conclusive evidence that VR118 induces apoptosis without any fascinations for any particular stage of the cell cycle. The process of apoptosis therefore deserves much attention due to obvious reasons of efficacy of such a drug capable of inducing it. Further, available literature suggests that Quinacrine induces apoptosis in breast cancer cells through upregulation of p53.

4.4 VR118 Activates Apoptosis by Upregulating Pro-apoptotic Signals and Downregulating Anti-apoptotic Signals

Having determined that VR118 can induce apoptosis by specifically targeting cancer cells, I next focused on understanding the cellular mechanism through which VR118 induces apoptosis. Apoptosis is a major thrust area of anticancer therapy, and experimental findings revealed that VR118 is capable of inducing the process. However, understanding the underlying molecular mechanisms through which VR118 induces apoptosis is important. To gain insight into the molecular mechansim, I examined several proteins involved in the regulation of apoptosis. Data from the experiments showed that VR118 causes upregulation of pro-apoptotic protein Bax resulting in the release of cytochtome c from the mitochondria. At the same time, VR118 significantly downregulated anti-apoptotic proteins Bcl-2 and Bcl-xL. PARP protein was also cleaved in response to VR118.

The change in ratio between Bax and Bcl-2 caused by VR118 possibly stimulates the release of

cytochrome c from mitochondria into the cytoplasm, similarly to Quinacrine (Duprez et al., 2009; Hockenbery et al., 1990; Orzáez et al., 2009). The release of cytochrome c from the mitochondria can occur either by the death-receptor dependent or extrinsic apoptotic pathway, and a death-receptor independent or intrinsic apoptotic pathway (Schuler et al., 2000). Cytochtrome c released into the cytosol then interacts with apoptotic protease activating factor (APAF-1) in the presence of ATP and leads to the activation of caspase-3, and PARP which is an important activator of caspase independent apoptotis (Schuler et al., 2000; Tsann-Long et al., 1995). These experimental findings further support the key role, p53 plays in VR118 induced apoptosis; however, there may be other mechanisms through which VR118 induces apoptosis.

4.5 Summary and Conclusions

In this project, I have investigated the anticancer properties of compound VR118 which is derived from Quinacrine using a hybrid pharmacophore approach. In summary, it is clearly evident from the experimental findings that the Quinacrine derivative VR118 can be a potent anticancer therapeutic drug given its idealistic characteristics such as reduced toxicity on non-malignant cells; and control on cell proliferation and induction of apoptosis in a substantially tumor-specific manner. Given the advantages of VR118 in specifically targeting cancer cells, further research and development are warranted.

4.6 Future Direction

Although evidence from the experiments clearly indicates that VR118 induces apoptosis in cancer cells by acting on the mitochondrial pathway; there appears to be another mechanism through which VR118 induces apoptosis in MCF7 cells. Therefore, further studies on caspases 9, 7 and 6 may need to clarify the VR118-induced apoptotic pathway. Furthermore, examination of its efficacy should be verified by in vitro studies.

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