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Profiling of FDA-Approved and Clinical Trial Drugs Revealed Shared Cytotoxicity And Collateral Sensitivity In Resistant (H69ar) And Non-Resistant (H69) Small Cell Lung Cancer Cells. (Drug Repurposing in Cancer Chemotherapy)

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PROFILING OF FDA-APPROVED AND CLINICAL TRIAL DRUGS REVEALED SHARED CYTOTOXICITY AND COLLATERAL SENSITIVITY IN RESISTANT (H69AR) AND NON-RESISTANT (H69) SMALL CELL LUNG CANCER CELLS. (DRUG REPURPOSING IN CANCER CHEMOTHERAPY)

BY

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DISSERTATION ACCEPTANCE PAGE
PIUS REYDERG AGYEMANG

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

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This dissertation is dedicated to my mother, Theresah Pomaah.

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TABLE OF CONTENTS

ABBREVIATIONS

7-AAD	7-aminoactinomycin D
ABC	ATP Binding Cassette
ALK	Anaplastic Lymphoma Kinase.
Apaf1	Apoptosis Activating Factor 1.
Bak	B-cell lymphoma associated protein K.
BSO	L-Buthionine Sulfoximine
Caspase	Cysteine-dependent Aspartate-directed Proteases.
CS	Collateral Sensitivity
DISC	Death inducing Signaling Complex.
DMSO	Dimethyl Sulfoxide.
$E_2$17βG	Estradiol Glucuronide
Erl	Erlotinib
EGFR	Epidermal Growth Factor Receptor.
FADD	Fas-associated Death Domain
FGFR1	Fibroblast Growth Factor Receptor 1 gene.
Flud	Fludarabine phosphate
GPX4	Glutathione Peroxidase 4
GSH	Glutathione
GST	Glutathione S Transferase
KRAS	Kirsten Rat Sarcoma gene.
LTC_4	Cysteinyl Leukotriene
MET	Mesenchymal Epithelial Transition factor gene.
MOMP	Mitochondrial Outer Membrane Permeabilization
MRP1	Multi-drug Resistance Protein 1
MYC	Myelocystomatosis
NAC	N-acetylcysteine
NBD	Nucleotide Binding Domains

NSCLC	Non-Small Cell Lung Cancer Cell.
pRb	Retinoblastoma
PTPM1	Protein Tyrosine Phosphate Mitochondrial 1
Pyri	Pyrimethamine
ROS	Reactive Oxygen Species
SCLC	Small Cell Lung Cancer Cell.
TP53	Tumor Protein Gene

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

PROFILING OF FDA-APPROVED AND CLINICAL TRIAL DRUGS REVEALED SHARED CYTOTOXICITY AND COLLATERAL SENSITIVITY IN RESISTANT (H69AR) AND NON-RESISTANT (H69) SMALL CELL LUNG CANCER CELLS. (DRUG REPURPOSING IN CANCER CHEMOTHERAPY)

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Some cancers are capable of "spitting out" drugs being fed to them, metaphorically speaking, becoming resistant to what were previously effective chemotherapeutics. In small-cell lung cancer (SCLC), an overexpression of a membrane protein (MRP1) and its transport activity can lead to chemotherapy failure. However, this study showed that certain drugs are selectively cytotoxic (exhibit collateral sensitivity) to MRP1-overexpressed SCLC (H69AR) cells. In this study, three drugs (Erlotinib, Pyrimethamine, Fludarabine) were identified to exhibit a dose-dependent collateral sensitivity on H69AR with IC_{50} values of ~3.5 μM, ~2 μM, and ~20 μM respectively. Halting the transport activity of the MRP1 with 25 μM MK-571 or 5 μM reversan increased the percent cell viability of MRP1 cells that were treated with erlotinib and pyrimethamine. Thus, the collateral sensitivity induction by these drugs disappeared. Also, the collateral sensitivities of erlotinib and azd1480 were shown to depend on caspase ($P < 0.005$) when the pan caspase inhibitor (Q-VD-OPh) was used to inhibit caspase activation. In a GSH-depletion fluorescence assay using 40uM dibromobimane, the collateral sensitivities of erlotinib and azd1480 again

were shown to deplete total thiols (GSH) when 30uM of the drug is added to cells that have been treated with 50 μM N-acetylcysteine (NAC) for 48hrs. In another experiment, three non-oncology drugs were identified to exhibit shared cytotoxicity on both H69 and H69AR. Using MTT cytotoxicity assay, alexidine HCl, ouabain and cetrimonium bromide (the three non-oncology drugs) induced a shared cytotoxicity on both H69 and H69AR with ic50 of ~1 μM, ~8 nM, and ~1 μM, respectively. Employing annexin V apoptosis assay, alexidine HCl, and cetrimonium bromide induced apoptosis in both H69 and H69AR. Ouabain on the other hand was necrotic on H69 but induced apoptosis in H69AR. These investigations unveiled the cytotoxic mechanisms and targets that could lead to a possible way to accelerate the development of new cancer drugs or repurpose an existing drug to treat cancer.

CHAPTER 1

1.1. Background

Lung cancer is the leading cause of cancer-related death, part of the reason being their ability to remain alive through certain adaptations which has led to poor prognosis. Each year, more people die of lung cancer than colon, prostate, and breast cancer combined. Thus, lung cancer accounts for 13.2% of all new cancers and 25.9% of all cancer death (Cancer Stat Facts, n.d.; Siegel et al., 2021). The overall five-year survival rate of lung cancer is lowest compared to most other cancers (Cancer Stat Facts, n.d.; Siegel et al., 2021). In many cases, cancer-causing substances in the air are inhaled and cause cell damage that later becomes cancer. The various types of lung cancers can be classified as either being a small-cell lung cancer (SCLC) or non-small cell lung cancer (NSCLC). NSCLC constitutes about 85% of all lung cancer cases but it is less aggressive as compared to SCLC which constitutes about 15% of lung cases (Cancer Stat Facts, n.d.; Siegel et al., 2021). The various types of lung cancers are shown in Figure 1.

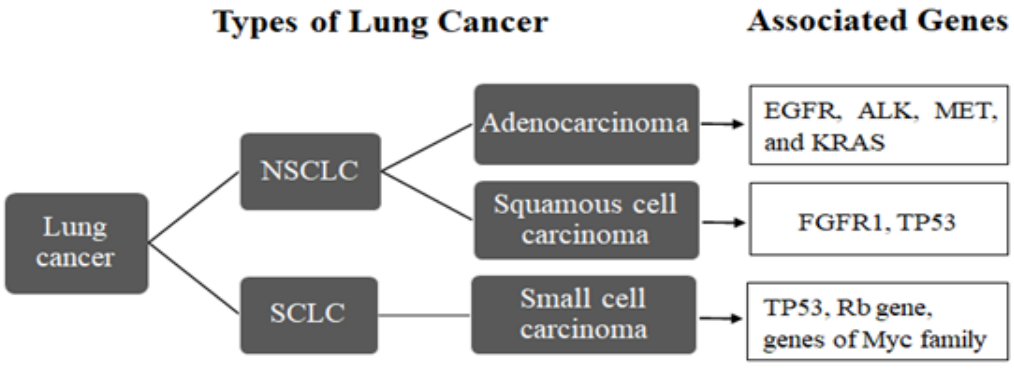


Figure 1**.** The various types of lung cancer and their associated proto-oncogenes.

Certain genetic changes have been associated with lung cancer. A mutation or gene amplification which leads to an activation of a proto-oncogene, or deactivation of tumor suppressor gene, may lead to cancer. Proto-oncogenes are normal genes which help in cell growth and development by producing growth factors, growth-factor receptors, transcription factors and signal transduction proteins. Proto-oncogenes associated with lung cancer includes Kirsten rat sarcoma gene (KRAS), mesenchymal epithelial transition factor gene (MET), epidermal growth factor receptor gene (EGFR), anaplastic lymphoma kinase gene (ALK), fibroblast growth factor receptor 1 gene (FGFR1), and myelocystomatosis gene (MYC) (Antoniou et al., 2013). Tumor-suppressor genes are genes responsible for preventing the excessive growth of cells. Tumor-suppressor genes act as gate keepers, which directly suppress growth, or care takers, which maintain the overall genetic stability of the cells. Tumor-suppressor genes associated with lung cancer include tumor protein gene (TP53) and retinoblastoma gene (pRb) (Kumar et al., 2014). Although abnormal functioning of these genes has been associated with lung cancer, certain risk factors like smoking and pollution have been shown to play a part in causing cells to become cancerous. These risk factors can cause genetic changes in both small cells and non-small cells of the lungs, leading to uncontrolled growth of cells and hence tumor formation. Tumors of lung cancer could start from cells lining the bronchi, bronchioles, or alveoli of the lungs and spread through one side of the chest, which is referred to as the limited stage, or the whole chest and other parts of the body, which is referred to as the extensive stage (American Cancer Society [ACS], n.d). The SCLC type of lung cancer spreads (metastasize) relatively faster, and for this reason, about 70% of people with SCLC will have lung cancer that has already spread at the time they are diagnosed (American

Cancer Society [ACS], n.d.). In terms of treatment, SCLC responds very well to chemotherapy than any other form of therapy due to its high rate of metastasis. (American Cancer Society [ACS], n.d.)

Chemotherapy in cancer treatment is where an anti-cancer drug may be injected into a vein or taken by mouth. These drugs travel through the bloodstream and reach most parts of the body. For limited stage SCLC, chemotherapy is often given at the same time with radiation therapy (this is the use of high energy rays to kill cancer cells). This is termed as concurrent chemoradiation (American Cancer Society [ACS], n.d.). Drugs that are commonly used to treat SCLC are cisplatin and carboplatin. Either of these drugs is given with etoposide or irinotecan in a combinatorial regimen (American Cancer Society [ACS], n.d.). Cisplatin causes ferroptosis (a type of programmed cell death that is dependent on iron by inhibiting glutathione peroxidase 4 (GPX4) protein which is responsible for reducing cytotoxic lipid peroxide (a marker of ferroptosis) to corresponding alcohols (Siddik, 2003). Etoposide and irinotecan inhibit topoisomerases (enzymes involved in DNA replication) and hence interfering with DNA synthesis (Beauchesne et al., 1997). Although chemotherapy is by extension the most effective treatment form of SCLC, certain adaptations of some cancer cells have led to chemoresistance which accounts for about 90 percent of chemotherapy failure. Mutation of drug receptors or transporters, mutation of target sites of drugs, and enhanced drug efflux caused by increased expression of ATP binding cassettes (ABC) membrane transporters such as P-glycoprotein (P-gp/ABCB1), multi-drug resistance protein (MRP/ABCC1) 1 and breast cancer resistance protein (BCRP/ABCG2) have been associated with chemoresistance in cancer cells. These ABC membrane transporters have been involved in multi-drug resistance (MDR) which accounts

for about 90 percent of chemotherapy failure. Long exposure of doxorubicin (a conventional oncology drug) and its related anthracycline compounds to H69 (a small cell lung cancer cell line) causes these cells to overexpress multi-drug resistance protein (MRP) 1 responsible for their chemoresistance (Cole et al., 1992)

Bioinformatic analysis of the open reading frame of MRP1 mRNA suggests its membership in the ATP Binding Cassette (ABC) superfamily transmembrane transporters with MRP polypeptide weighing approximately 171kDa when it is not modified (Ambudkar et al., 1999). Like many vertebrates, members of the ABC superfamily MRP contain two nucleotide binding domains (NBDs), each preceded by multi-spanning transmembrane region (TMD) as shown in Figure 2.

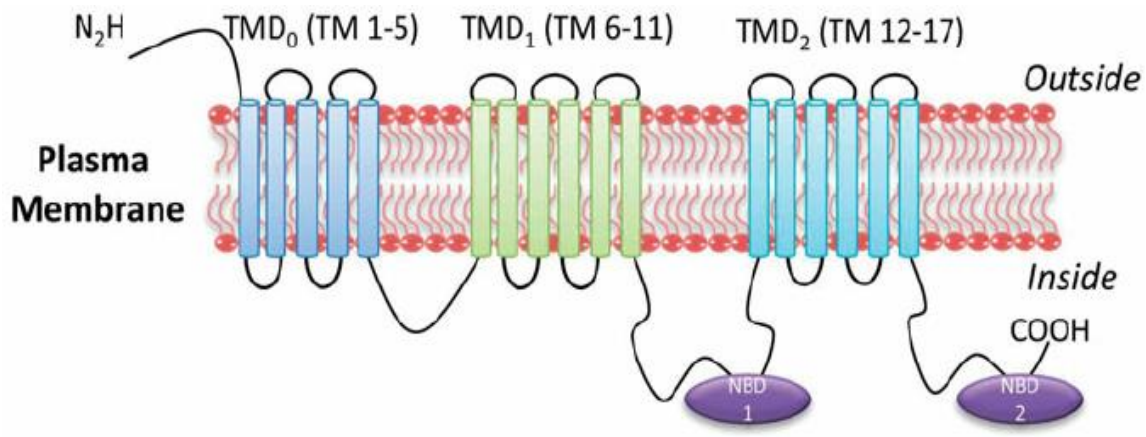


Figure 2. The orientation and topology of MRP1. Lu, Pokharel, and Bebawy (2015).

The NBDs are involved in ATP hydrolysis during transport of substances out of the cell. In the human body, MRP1 is primarily expressed in the basolateral membrane of

epithelial cells of organs such as the lung, gastrointestinal tract, kidney, pancreas, testis, placenta, bladder, and adrenal gland (Flens et al., 1996). MRP1 governs the absorption and disposition of a wide variety of endogenous and exogenous substrates across organs, serving as physiological barriers in the nervous cells and protecting tissues from toxic molecules (Schinkel et al., 2003; Chen et al., 2016). MRP1 can also transport organic anions such as cysteinyl leukotriene (LTC_4), estradiol glucuronide ($E_2$17βG), glutathione (GSH), and cobalamin (Leier et al., 1996; Jedlitschky et al., 1996; Beedholm-Ebsen et al., 2010), which happen to be its physiological substrates. MRP1 effluxes conventional cytotoxic anti-cancer agents such as doxorubicin, etoposide, vincristine, and methotrexate (Cole, 2014). MRP1 can also affect the bioavailability of various types of antivirals, antimalarials, and antibiotics (Cole, 2014). This ability of the MRP1 to develop cross resistance for other compounds makes it a key player of multi-drug resistance. Multi-drug resistance (MDR) be it inherent or acquired is inevitable, and it is characterized by the simultaneous resistance to drugs that differ both structurally and mechanistically.

Apart from the resistance from cancer cells which leads to chemotherapy failure, there are other factors like time and money spent on manufacturing of novel oncology drugs and afflictive side effects of many oncology drugs, which tend to dawdle chemotherapy. These problems in chemotherapy are what this project is intended to provide solutions to. Among many of the solutions is drug repurposing, where an FDA-approved or clinical trial drug is used as a viable alternative in the treatment of a particular disease. In recent years, drug repurposing in chemotherapy has gained popularity as a strategy to accelerate the development of a new cancer drug. Researchers have discovered that drugs used for treating diabetes, alcoholism and arthritis can also kill cancer cells in

the lab (Corsello et al., 2020). A typical example of a drug that has been repurposed for chemotherapy is disulfiram which was originally designed for the treatment of chronic alcoholism. This drug was later reported to inhibit proteasome in some cancer cells leading to their death (Lövborg et al., 2006; Chen et al., 2006). Because these FDA-approved drugs have already been assessed for safety and efficacy, there is low risk of failure and low overall cost from bench to bedside than if a new drug is being tested. As a solution to chemoresistance, drug repurposing can provide a safe alternative for the treatment of cancer cells that have accrued genetic alterations leading to the resistance to what were previously effective chemotherapeutics. This work will therefore achieve the drug re-purposing purpose through three aims:

1. Investigate the anticancer properties of selected FDA-approved and clinical trial drugs on both resistant (H69AR) and non-resistant (H69) small cell lung cancer (SCLC) cells.
2. Investigate the cytotoxic mechanism of non-oncology drugs on both H69 and H69AR.
3. Investigate the mechanism and targets of collateral sensitivity induced by FDA-approved drugs.

CHAPTER 2

INVESTIGATING THE ANTICANCER PROPERTIES OF SELECTED FDA-APPROVED AND CLINICAL TRIAL DRUGS ON BOTH RESISTANT (H69AR) AND NON-RESISTANT (H69) SMALL CELL LUNG CANCER (SCLC) Cells.

2.1. Introduction

In most cancer cells, either an oncogene is activated, or a tumor suppressor gene is deactivated, causing cells to grow uncontrollably leading to tumor formation. Cell growth and division start with replication of DNA and synthesis of organelles. Several pathways that lead to growth and division of cells have been exploited by chemotherapeutics to kill cancer cells. These include, but is not limited to, targeting key enzymes such as aurora kinase in cell division, topoisomerase or telomerase in DNA replication, proteosome, glutathione peroxidase 4, and cytoskeletons like microtubules which aid in mitosis. Suffice it to say that a drug which can inhibit any of the key pathways mentioned above could be cytotoxic to cancer cells.

In this investigation, 118 FDA- approved and clinical trial drugs were screened for their cytotoxicity on H69 and H69AR cells. Some of which were oncology drugs and are expected to exploit one of the above-mentioned targets to kill the cancer cells.

2.2. Materials and Methods

2.2.1. Chemicals

FDA-approved and clinical trial drugs were procured from Selleckchem (Houston, TX), dimethyl sulfoxide (DMSO), thiazolyl blue tetrazolium bromide (MTT) and sodium dodecyl sulfate were procured from Sigma-Aldrich (St. Louis, MO).

2.2.2. Cell lines and cell culture

H69 and H69AR cells were procured from ATCC (Manassas, VA). H69 and H69AR cell lines were cultured in RPMI 1640 (ATCC) media supplemented with 10% fetal bovine serum. H69AR cells were challenged monthly with 0.8 μM doxorubicin and cultured drug-free for one week before use. Cells were grown in a humidified incubator maintaining 5% CO_2 at 37 °C.

2.2.3. Cytotoxicity assay

Cell sensitivity to drugs was analyzed using the MTT colorimetric assay. 250,000 cells in 100 μL RPMI 1640 media are seeded in a 96-well plate (CellBIND®, Corning). Plates are incubated at 37^{o}C for 24hrs. The next day, cells were treated with 15 μM drugs and incubated for 96 hours. DMSO concentration was maintained at 0.15% as a negative control. In a dose-dependent cytotoxicity test, a serial dilution by half was done to obtain eight different concentrations, usually the highest concentration is 50 μM and the lowest is 0.391 μM. DMSO concentration is maintained at 0.5% for all eight concentrations. At the end of the incubation period, 100 μL of culture medium was carefully removed and cells were treated with 0.5 mg/mL of MTT dye (3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide) for 4hrs. The formazan crystals were dissolved by the

addition of 120 µL 15% SDS containing 0.01%v/v HCl and absorbance at 570 nm was recorded using a Hidex Sense Beta Plus plate reader (Turku, Finland).

2.3. Results and Discussion

The MTT dye was reduced to insoluble formazan crystals by NAD(P)H-dependent oxidoreductase enzymes present in viable cells. The formazan crystals were dissolved using solubilization solution as described in the method. At wavelength of 570 nm, the dissolved formazan crystals have maximum absorbance which give an optical density (OD) value that corresponds to the number of viable cells. The higher the OD value the higher the number of viable cells and vice versa. The OD values of the drugs were normalized with that of 0.15% DMSO and expressed as percentage as shown below. This normalization was done because the drugs contain 0.15% of DMSO as solvent.

$$\text{Percent cell viability} = \frac{\text{OD of drug}}{\text{OD of 0.15\% DMSO}} \text{ x } 100$$

After the initial screening, drugs were categorized into three phenotypes based on their relative cytotoxicity on H69 and H69AR. These phenotypes are Phenotype A (Resistance), Phenotype B (Shared cytotoxicity) and Phenotype C (Collateral Sensitivity).

2.3.1. Phenotype A – Resistance

Drugs in this phenotype exhibited selective cytotoxicity towards the parental cell (H69) with little or no effect on the resistant cells (H69AR) hence the phenotype name resistance. These drugs are trifluridine (antiviral), defarasirox (iron chelating agent),

vinblastine (oncology drug), dipyridamole (platelet inhibitor), JNK-IN-8 (oncology), and chloroxine (antibacterial) listed in table 1. As shown in Figure 3, there is a large difference in percent cell viability (with $p < 0.05$) between H69 and H69AR for all the six drugs. The 0.15% DMSO which was set as a control did not show toxicity on both cells. H69AR cells showed relatively high resistance in JNK-IN-8 and chloroxine with percent cell viability greater than 100. On the other hand, H69 was very sensitive to JNK-IN-8 and chloroxine with relatively low percent cell viability. According to Susan and Cole (2014), MRP1 can affect the bioavailability of antiviral and antibacterial drugs due to the resistance towards these drugs. In this work, it was also shown that MRP1-overexpressed cells (H69AR) are resistant to different classes of drugs including the oncology drugs, iron chelating agents, antiviral, antibacterial and platelet inhibitors, this evident the multi-drug resistance of MRP1. Iron chelating agents are useful in inhibiting ferroptosis (a kind of programmed cell death induced by iron), and platelet inhibitors are useful in conditions where there is an excessive blood clotting. The resistance of the MRP1-overexpressed cells demonstrated in this work towards these drugs is also an advisory notice that the drugs cannot be used to achieve their functions in cells that overexpress MRP1.

The rest of the work focused on the other two phenotypes which were part of the underpinning goals of this project, thus drug repurposing in chemotherapy, hence no further experiment was performed with drugs in the phenotype A. However, the results from this phenotype complete and validate the ideologies of the other two phenotypes which is shared cytotoxicity and collateral sensitivity.

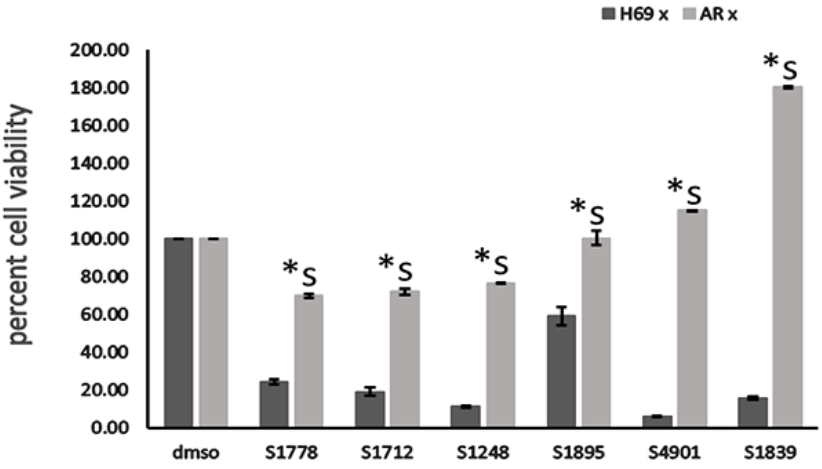


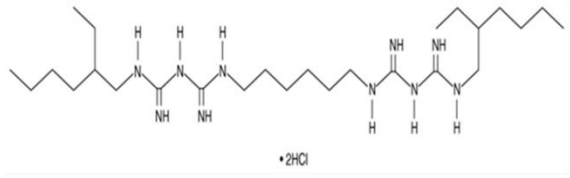
Figure 3. Viability of SCLC cells showing resistance in H69AR on exposure to 15 µM drugs for 96 hrs. Viability was assessed by MTT assay and drug effect was normalized with 0.15% DMSO. Error bars represent standard errors of the mean of two experiment each done in triplicate. *S denotes cell viability of H69AR significantly different from H69 with $p < 0.05$ using Student T-test.

Table 1. Phenotype A drugs descriptions.

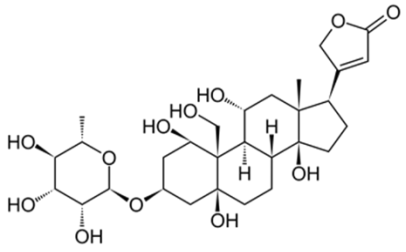
Drug code	Drug name	Drug class
S1778	Trifluridine	Antiviral
S1712	Defarasirox	Iron chelating agent
S1248	Vinblastine	Oncology
S1895	Dipyridamole	Platelet inhibitor
S4901	JNK-IN-8	Oncology
S1839	Chloroxine	Antibacterial

2.3.2. Phenotype B – Shared Cytotoxicity.

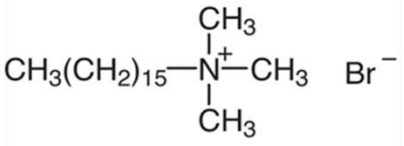
The term shared cytotoxicity was used to describe drugs in this phenotype because of their ability to kill greater number (about 80 percent) of both H69 and H69AR with minimal or no significant difference between the percent cell viabilities of the two cell lines. Three non-oncology drugs namely alexidine HCl, ouabain and cetrimonium bromide were found to exhibit shared cytotoxicity. The bar graph from the initial screening is shown in Figure 5, and in this Figure, the percent cell viabilities of both H69 and H69AR for all the drugs are below 20 which demonstrate their shared cytotoxicity. The first drug in this category is alexidine HCl which is an alkyl bisbiguanide (Figure 4). Bisbiguanides are known for their bactericidal properties and alexidine HCl has been found to have antimicrobial functions and it is used in mouthwash (Zorko & Jerala, 2008). This non-oncology drug has also been found to inhibit the mitochondrial phosphatase PTPMT1 and induces apoptosis in some cancer cell lines (Doughty-Shenton et al., 2010).



Alexidine HCl



Ouabain



Cetrimonium bromide

Figure 4. chemical structure of phenotype B drugs.

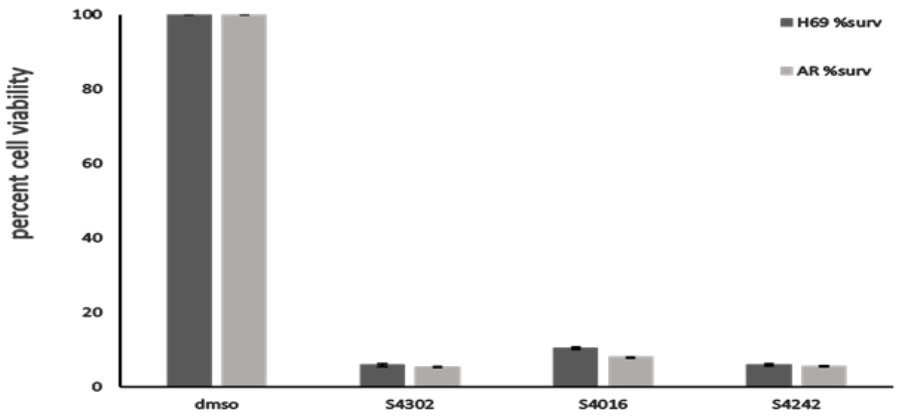


Figure 5. Viability of H69 and H69AR cells on exposure to 15 µM drugs for 96hrs. Viability was assessed by MTT assay and drug effect was normalized with 0.15% DMSO. Error bars represent standard errors of the mean of two experiments done in triplicate.

Exposing both cell lines to 15 µM of alexidine HCl (S4302) for 96 hours, the drug exhibited a shared cytotoxicity with percent cell viabilities less than 10 on both cell lines as seen in Figure 5. In a dose-dependent cytotoxicity test, where varying concentrations of a drug were used instead of a single concentration, it was found that the cytotoxicity of alexidine HCl on both H69 and H69AR is dose dependent as shown in Figure 6 (a), with half maximal inhibitory concentration (IC_{50}) value of ~1 µM each for both H69 and H69AR. Dose-dependent test is usually employed in cytotoxicity tests to find out whether the effect of a drug depends on how much given, and also to determine the half maximal inhibitory concentration (IC_{50}). IC_{50} in this experiment is defined as the concentration of drug that kills half of the cells. It is also a measure of the potency of the drugs in killing the cells. The low IC_{50} values for both cell lines indicate that alexidine HCl is very potent in killing the small-cell lung cancer cells. Also, the relative cytotoxicity of alexidine HCl on both cell lines can be deduced from the steepness or the positions of the sigmoidal curves

of the H69 and H69AR relative to each other. At very low concentrations of alexidine HCl, the sigmoidal curve of H69AR lies below that of the H69 and significant differences are seen in the percent cell viabilities of the H69 and H69AR, thus the drug kills more H69AR cells than H69. As the concentration increases near the IC_{50}, the extent of cytotoxicity becomes almost equal for both cell lines hence the shared cytotoxicity.

The second drug in this phenotype is ouabain, a cardiac glycoside which consist of a sugar molecule bound to a modified cholesterol by a glycosidic linkage (Figure 4). Ouabain is toxic to cells because it can inhibit sodium potassium pump which is found in the plasma membrane of almost every human cell and causes depolarization of neurons leading to osmolysis or calcium necrosis in brain tissues (Wang et al., 2004). However, at lower concentrations, ouabain can be used medically to treat hypotension and arrhythmias (Wang et al., 2004). In this work, the shared cytotoxicity of 15 µM ouabain (S4016) on both cell lines was demonstrated and shown in Figure 5. The dose-dependent cytotoxicity test of the drug on both cell lines was also shown in Figure 6(b), with an IC_{50} value of 7.9 nM for H69 and 9.1 nM for H69AR. These low IC_{50} values inform the high potency of this drug on both cell lines. The sigmoidal curve of H69 lies below that of the H69AR indicating that although the drug is toxic to both cell lines, it kills more H69 than H69AR at all concentrations, especially the lower concentrations. As the concentration increases pass 32 nM, the gap between the two curves narrows, which means that the drug at higher concentrations exhibits similar potency on both cell lines. This low concentration of ouabain can be considered medically to treat small cell lung cancer with minimal or no effect on normal cells.

The third non-oncology drug in this phenotype is cetrimonium bromide, an amine based cationic quaternary surfactant (Figure 4). The hexadecyltrimethylammonium cation is an effective antiseptic agent against bacteria and fungi. Some topical antiseptics including cetrimonium bromide have been shown to have effect on cancer cells in vitro (Sopata et al., 2008). Cetrimonium bromide exhibits anticancer cytotoxicity against several HNC (head and neck cancer) cell lines with minimal effects on normal fibroblasts, a selectivity that exploits cancer-specific metabolic aberrations (Ito et al., 2009). In this work, cetrimonium bromide also exhibited cytotoxicity on small cell lung cancer cell lines, the H69 and its resistant H69AR. At 15 μM of the drug, a shared cytotoxicity was seen on both cell lines as shown in Figure 5. Also, the dose-dependent cytotoxicity of this drug was demonstrated on H69 and H69AR cells with IC_{50} values of 1.1 μM and 2 μM, respectively as shown in Figure 6 (c). At very low concentrations, the sigmoidal curve of the H69AR falls below that of the H69, an indication of higher sensitivity exhibited by the H69AR towards the drugs at these concentrations. After about 630 nM, the sigmoidal curve of the H69 falls below that of the H69AR until about 8 μM where the curves converge. This means that between 630 nM and 8 μM, the drug kills relatively more H69 than H69AR, and after 8 μM, the drug shows similar efficacy on both cell lines.

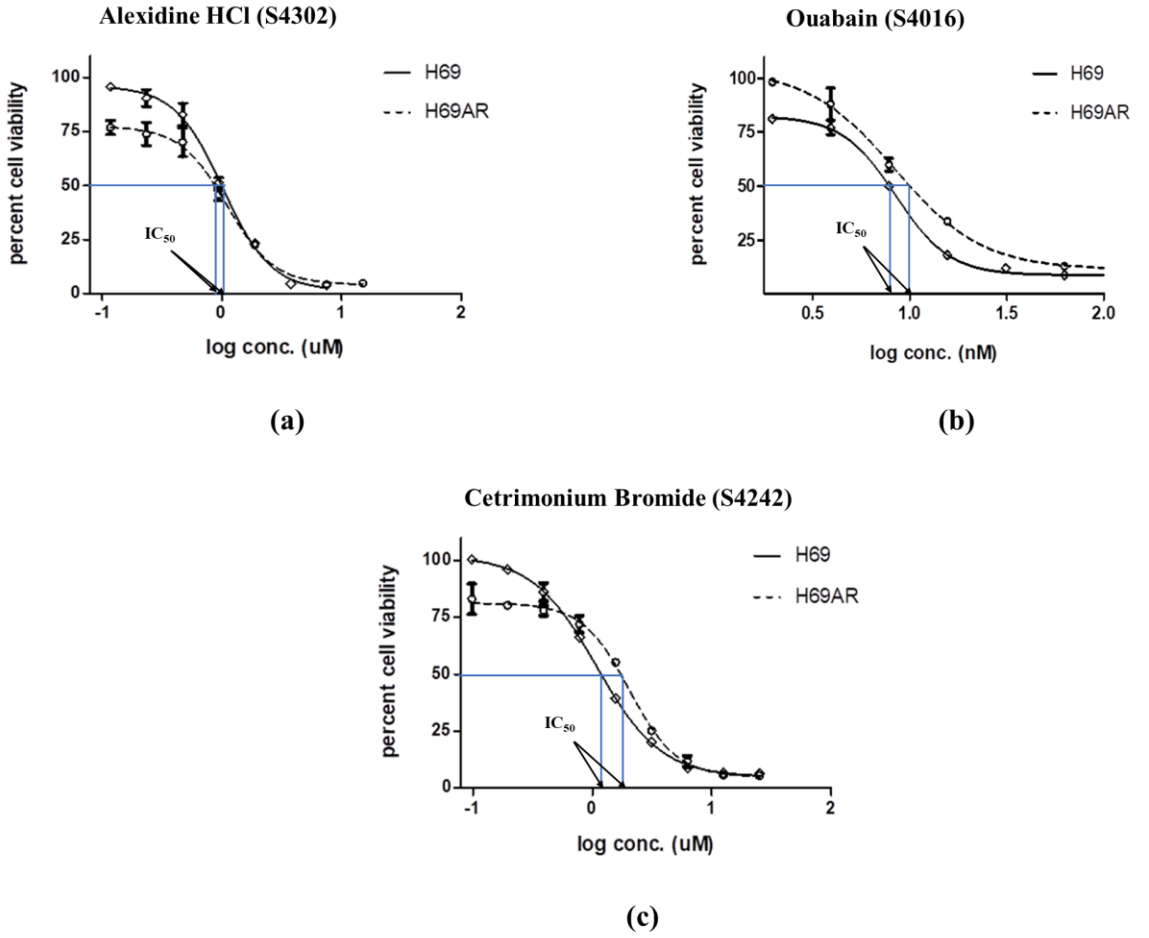


Figure 6**.** Dose-dependent cytotoxicity on SCLC cells from a 0.5-degree serial dilution of (a). Alexidine HCl (S4302) with starting concentration of 15 µM. (b). Ouabain (S4016) with starting concentration of 250 nM. (c). Cetrimonium bromide (S4242) with starting concentration of 25 µM. Cells were incubated for 96hrs, and viability was assessed by MTT assay. Drug effect was normalized with 0.15%, 0.05%, and 0.25% DMSO for alexidine HCl, ouabain, and cetrimonium bromide, respectively. Error bars represent standard errors of the mean of two experiments done in triplicate.

Table 2**:** Phenotype B drugs descriptions and their IC_{50} values for H69 and H69AR

Drug code	Drug name	Drug class	H69 (Mean ± SEM)/uM	H69AR (Mean ± SEM)/uM
S4302	Alexidine	Antimicrobial	(1.1200 ± 0.0072)	(1.1000 ± 0.0080)
S4016	Ouabain	Cardiac glycoside	(1.100 ± 0.020)	(2.000 ± 0.031)
S4242	Cetrimonium bromide	Surfactant	(7.900 ± 0.053)	(9.10 ± 0.83)

2.3.3. Phenotype C – Collateral Sensitivity

The term collateral sensitivity is used to describe the selective cytotoxicity of the drugs in this phenotype towards the resistant cells. It represents the fitness cost for the acquisition of the multi-drug resistance phenotype which involves complex genetic and/or transcriptional adaptations in the resistant cells. Drugs found in this phenotype were erlotinib, AZD1480, pyrimethamine, and fludarabine. These drugs would be referred to as collateral sensitizers. H69AR is resistant to oncology anthracycline drug such as doxorubicin and exhibits cross-resistance towards some non-oncology drugs as seen in Figure 3. On the contrary, the collateral sensitizers from the initial screening consistently exhibited selective cytotoxicity towards H69AR as seen in Figure 8. The difference in percent cell viability between H69 and H69AR was very large for erlotinib and AZD1480 as compared to fludarabine and pyrimethamine. The reason for this can be attributed to the mechanism of action of fludarabine and pyrimethamine. These drugs are both antimetabolites which bear structural resemblance (as seen in Figure 7) to nucleotide bases used in DNA replication. The structural resemblance to the nucleotide bases enables them to compete with the natural nucleotide bases and curtail DNA replication. This competitive mechanism would require large amount of the competitors to overcome the natural nucleotide bases, which is why the 15 μM used in the initial screening did not show large differences in cell viabilities between the H69 and H69AR. On the other hand, erlotinib targets epidermal growth factor receptor (EGFR) and inhibits tyrosine kinase which eventually reduces the growth of cancer cells (Cohen et al., 2005) whiles azd1480 inhibits Janus kinase which is responsible for cell signaling leading to growth and development (Hedvat et al., 2009). These mechanisms of action of erlotinib and azd1480 are targeted

and would not require large concentrations of the drugs, hence the 15 µM used in the initial screening was very effective. However, the reason for the selective cytotoxicity of these drugs towards the resistant cells with minimal cytotoxicity to the parental cell, cannot be explained with the data shown in Figure 8. We therefore performed further experiments to investigate the mechanism of the collateral sensitivity of these drugs which would be discussed later.

When different concentrations of the collateral sensitizers were used to determine the dose-dependent cytotoxicity, pyrimethamine and fludarabine were found to exhibit dose-dependent cytotoxicity on both cell lines. Three parameters can be deduced from the graphs to explain collateral sensitivity or the selective cytotoxicity. The first and the most important parameter is the position of the sigmoidal curves of the two cell lines. The curve of the H69AR must lie below that of the H69. The second parameter is the gap between the two sigmoidal curves. The wider the gap, the more selective the cytotoxicity of the drug towards the resistant cells. The third parameter is the difference in IC_{50} values of the two cell lines. For a drug to exhibit collateral sensitivity, it should have low IC_{50} value for the resistant and high IC_{50} value for the parental. The difference in the IC_{50} values of the two cell lines also generate a parameter called fold resistance which is used to describe how potent the parental cells resist the drug compared to the resistant cells. Fold resistance is calculated as: $H69_{\mathbf{IC50}}/H69AR_{\mathbf{IC50}}$.

As shown in Figure 9 (a), the higher the concentration of pyrimethamine, the lesser the percent cell viability and vice versa for both H69 and H69AR. Also, the sigmoidal curve of the H69AR lies further below that of the H69 until the concentration increases pass 32 µM. After 32 µM, the drug exhibits similar strength of cytotoxicity towards the

parental and the resistant cells. Some drugs are toxic to cells at higher concentrations regardless of acquired resistance, which is why pyrimethamine at higher concentrations exhibited shared toxicity on both cell lines. Considering the gap between the two curves, it is widest at about 4 µM, and at this concentration, only 40 percent of the resistant cells survived compared to about 95 percent of the parental cells. Also, the IC_{50} value for H69AR is lower than that of the H69, yielding a fold-resistant value of 5 which implies that five times the concentration of pyrimethamine which kills 50 percent of H69AR would be required to kill 50 percent of H69. All these evident the selective cytotoxicity of pyrimethamine.

The next drug in this phenotype is fludarabine phosphate, a purine analog which works by interfering with DNA replication. It is a chemotherapy medication used in the treatment of leukemia and lymphoma (Chun et al, 1991). As shown in Figure 9 (b), the nature of the two sigmoidal curves of fludarabine phosphate signifies a dose-dependent cytotoxicity exhibited by the drug, the sigmoidal curve of the H69AR lies below that of the H69, which indicates the selective cytotoxicity of fludarabine phosphate towards H69AR. A fold-resistance value of 6 was obtained from the IC_{50} values of the two cell lines, this means that six times the concentration needed to kill 50 percent of H69AR, would be required to kill 50 percent of H69. We could also deduce the widest gap between the two curves at about 63 µM, at this concentration, about 75 percent of the H69AR would be killed compared to about 5 percent of H69 which will be killed by the same concentration. We also found that concentrations higher than 3 mM exhibited shared cytotoxicity on both cell lines as seen in the Figure 9 (b).

The next drug in this phenotype is erlotinib, under the brand name Tarceva, is a target chemotherapy medication used to treat NSCLC and pancreatic cancer (Cohen et al., 2005). Erlotinib targets the epidermal growth factor receptor of the cell and has been shown to be effective in patients with or without EGFR mutations but happens to be more effective in NSCLC patients with EGFR mutations (Qi et al., 2012). In this work, erlotinib exhibited a dose-dependent cytotoxicity towards the resistant cells only with an IC_{50} value of 3.5 µM as shown in Figure 9 (c). This dose-dependent cytotoxicity of erlotinib was not seen with the parental cells, these cells were highly insensitive to erlotinib to the extent that about 65 percent of the cells survived 600 µM of the erlotinib (Figure 9 (d)). For this reason, we were unable to obtain a sigmoidal curve for the parental cells. A deduction was therefore that since 600 µM of erlotinib could not kill 50 percent of the parental cells, the IC_{50} value will lie somewhere above 600 µM. This yields a very high fold-resistance value which is greater than 170. As mentioned earlier, erlotinib has been reported to be more effective in NSCLC patients with EGFR mutations. The H69 cells used in this work do not have mutated EGFR (EGFR-negative) and hence high resistance. On the other hand, H69AR also EGFR-negative but expresses MRP1, was sensitive to erlotinib. This could mean that the presence of the MRP1 in H69AR, would be the paramount contributing factor for the sensitivity of the H69AR. Till date, erlotinib has not been reported to kill small cell lung cancer cells (H69) and the results obtained in this work proved that erlotinib is not effective in killing H69 cells, however, if the H69 cells acquire multi-drug resistance by expressing multi-drug resistant protein 1, then erlotinib will be very effective in killing them, as shown in Figure 9 (c).

The last drug in this phenotype is AZD1480, a Janus kinase inhibitor that can suppress growth in solid tumor. In this work, 15 µM of the drug exhibited selective cytotoxicity towards H69AR as shown in Figure 8. Also, the drug exhibited a dose-dependent cytotoxicity towards H69AR but not on the H69. The cytotoxicity towards H69 was anomalous and that made it difficult to settle on an IC_{50} value for H69 and to make comparative cytotoxicity as it was done for other three collateral sensitizers. The dose-dependent data for the AZD1480 was not shown. Since 15 µM of the drug exhibited selective cytotoxicity or collateral sensitivity, the drug was included in further experiments to investigate its mechanisms of collateral sensitivity.

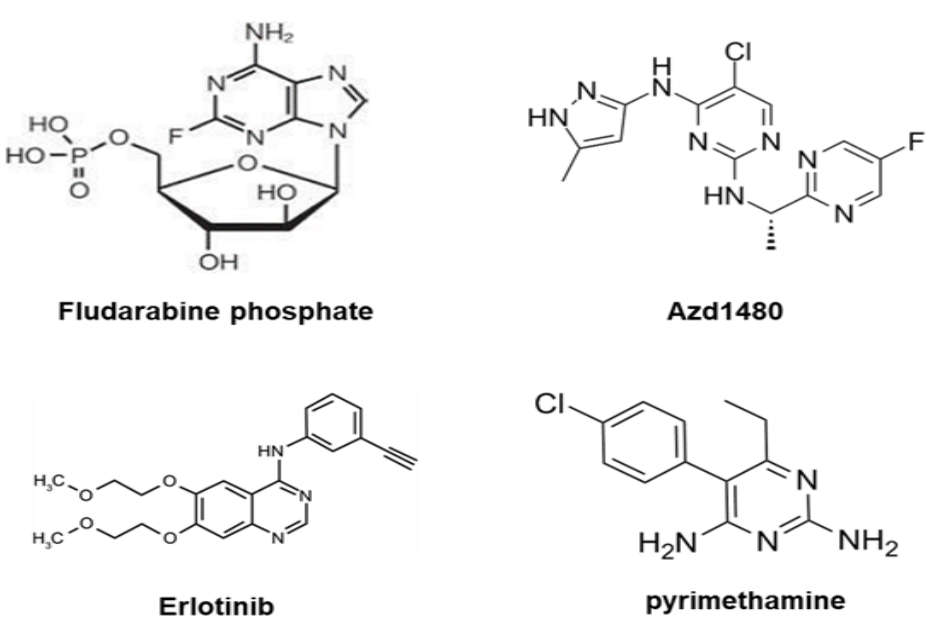


Figure 7. Chemical structure of phenotype C drugs.

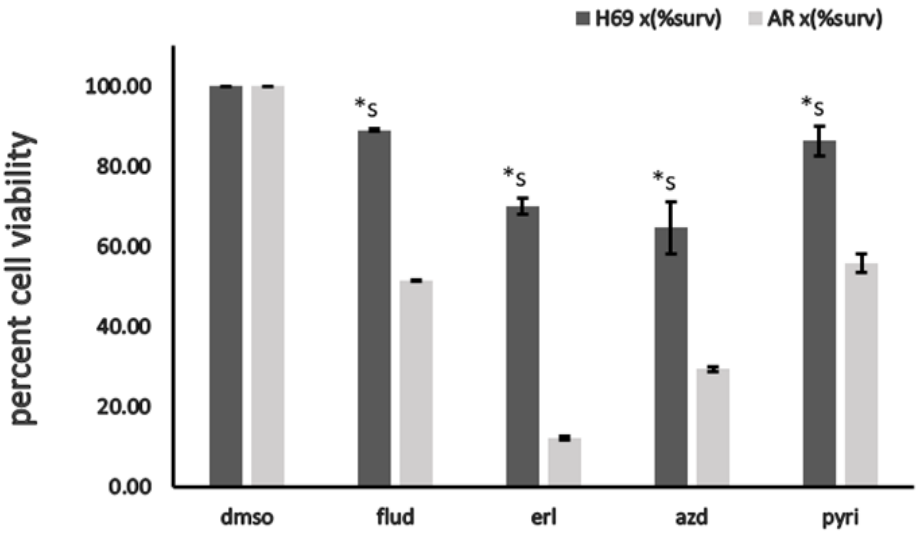


Figure 8. Viability of SCLC cells showing collateral sensitivity in H69AR on exposure to 15 μM drugs for 96 hours. Viability was assessed by MTT assay and drug effect was normalized with 0.015% DMSO. Error bars represent standard errors of the mean of two experiment each done in triplicate. *S denotes cell viability of H69 significantly different from H69AR with $p < 0.05$ using student T-test.

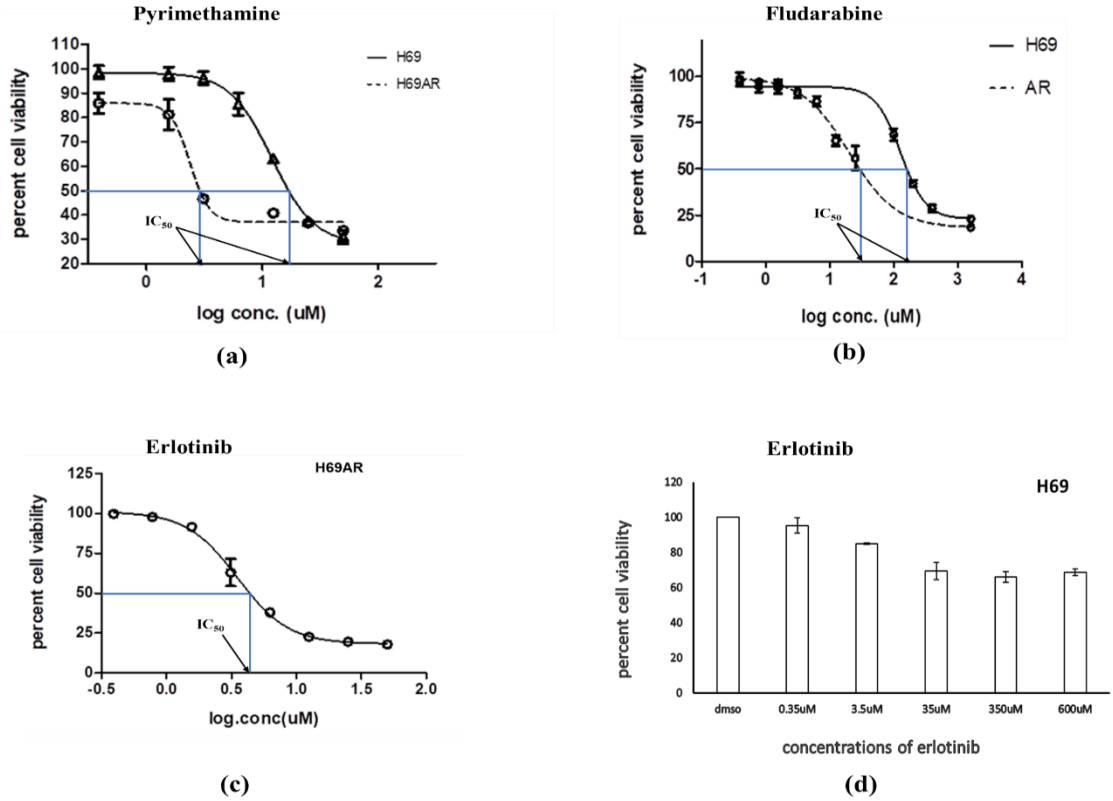


Figure 9: Dose dependent cytotoxicity on SCLC cells from a 0.5-degree serial dilution of (a). Pyrimethamine with starting concentration of 50 µM for both H69 and H69AR. (b). Fludarabine with starting concentration of 1600 µM for both H69 and H69AR. (c). Erlotinib with starting concentration of 50 µM for H69AR (d). Erlotinib with starting concentration of 600 µM for H69. Cells were incubated for 96hrs, and viability was assessed by MTT assay. Drug effect was normalized with 0.05% DMSO. Error bars represent standard errors of the mean of two experiments done in triplicate.

Table 3. Description of collateral sensitizers and their IC_{50} values for H69 and H69AR

Drug name	Drug class	H69 (Mean ± SEM)/uM	H69AR (Mean ± SEM)/uM	Fold resistance
Pyrimethamine	Antimalarial	(12.15 ± 0.32)	(2.4 ± 0.027)	5
Fludarabine	Oncology	(128.4 ± 2.4)	(20.7 ± 2.9)	6
Erlotinib	Oncology	> (600 ± 1.35)	(3.52 ± 0.411)	> 170
AZD1480	Oncology	-	-	-

CHAPTER 3

INVESTIGATE THE CYTOTOXIC MECHANISM OF NON-ONCOLOGY DRUGS ON BOTH H69 AND H69AR

3.1. Introduction

Three non-oncology drugs: alexidine HCl, ouabain and cetrimonium bromide from the previous experiment were found to exhibit shared cytotoxicity which is dose dependent. Alexidine is an alkyl bis(biguanide) antiseptic which has been used in mouthwashes to eliminate plaque forming microorganisms (Zorko & Jerala, 2008). It binds to lipopolysaccharide and lipoteichoic acid and inhibits fungal phospholipase B (Coburn et al., 1978). Alexidine also inhibits the mitochondrial phosphatase PTPMT1 and induces apoptosis in cancer cell lines. Ouabain also known as g-strophanthin, is a plant derived toxic substance that was traditionally used as an arrow poison in eastern Africa for both hunting and warfare. Ouabain is a cardiac glycoside and in lower doses, can be used medically to treat hypotension and some arrhythmias (Wang et al., 2004). It acts by inhibiting the Na/K-ATPase, also known as the sodium-potassium ion pump. Cetrimonium bromide (CTAB) is a quaternary ammonium surfactant. It is also an effective antiseptic agent against bacteria and fungi. CTAB exhibits anticancer cytotoxicity against several HNC (head and neck cancer) cell lines with minimal effects on normal fibroblasts, a selectivity that exploits cancer-specific metabolic aberrations (Ito et al., 2009).

In this investigation, annexin V/7-aminoactinomycin D (7-AAD) apoptosis assay was employed to find the mode of cell death induced by these three non-oncology drugs.

In chemotherapeutics, drugs that induce apoptosis are preferred to those that do not. This is because it is easier to comprehend the mechanisms of these drugs to help in the manufacturing of novel drugs. Apoptosis is a programmed cell death mechanism that many organisms utilize to selectively eliminate cells which show either deleterious reactivities to the host or which have not received a full complement of activation or survival signals. Most chemotherapeutic and targeted cancer therapies kill tumor cells through the generation of pro-death signaling that initiates the intrinsic apoptotic pathway of programmed cell death. The point of no return in the apoptotic cascade is mitochondrial outer membrane permeabilization (MOMP); once it has occurred, it leads to the formation of an apoptosome which facilitates caspase activation and subsequently triggers the other hallmarks of apoptotic cell death. Annexin V and 7-AAD fluorochromes can be used to investigate early and late apoptosis. Annexin V mature molecule is a 320 amino-acid residue, folded into a planar cyclic arrangement of four repeats with each repeat composed of five alpha-helical segments (Demchenko, 2013). it has selective affinity for negatively charged phospholipids. Under defined salt and calcium concentrations, Annexin V is predisposed to bind phosphatidylserine over most other phospholipid (Demchenko, 2013). Phosphatidyl serine and phosphatidyl ethanolamine are the two major lipid components found on the inner side of the lipid bilayer. An alteration in the membrane asymmetry as part of the physiological changes seen in apoptotic cells will result into exposure of the phosphatidyl serine to the exterior. These are thought to be early events during the apoptotic process that culminate in cell death. Annexin V can selectively bind to cells with a compromised membrane phospholipid asymmetry and this property has been exploited to identify populations of cells undergoing apoptosis. During programmed cell death, loss

of cell membrane integrity is a very late event usually preceded by the destructive action of endogenous cellular enzymes (Kanduc et al., 2002; Leist & Nicotera, 1997). The fluorescent DNA intercalator, 7-AAD, would enter a nucleus with compromised nuclear membrane and bind to DNA (Holm et al., 1998). The spectral shifts of these two fluorochromes upon binding to their respective targets make them suitable to be employed in flow cytometry to detect early and late apoptosis induced by apoptotic agents.

3.2. Apoptosis Assay

3.2.1. Materials and Methods

3.2.1.1. Chemicals

FDA-approved drugs (Alexidine HCl, Ouabain, Cetrimonium bromide) were procured from Selleckchem (Houston, TX), APC-Annexin V, Annexin binding buffer and 7-AAD were procured from BD Biosciences (San Jose, CA) and sodium dodecyl sulfate was procured from Sigma-Aldrich (St. Louis, MO).

3.2.1.2. Cell lines and cell culture

H69 cells were procured from ATCC (Manassas, VA). H69 and H69AR cell lines were cultured in RPMI 1640 (ATCC) media supplemented with 10% fetal bovine serum. Cells were grown in a humidified incubator maintaining 5% CO_2 at 37 °C.

3.2.1.3. Annexin V/7-AAD apoptosis assay

Apoptosis stages were analyzed with annexin V and 7-AAD fluorometric assay. This assay can also account for cells that undergo necrosis. 250,000 cells in 100 μL RPMI

1640 media are seeded in 6-well plate. Plate is incubated at 37^{o}C for 24 hrs. The next day, cells are treated with 15 μM of drugs and incubated at 37^{o}C for 48 hrs. After the incubation period, cells are removed and centrifuged at 300 rpm for 5mins. Supernatant is discarded and the pellet is redissolved in phosphate buffer saline (PBS) for a second round of centrifugation to wash debris and residual media from cells. Annexin V (1x) binding buffer is added to cells prior to the addition of annexin V and 7-AAD. Cell plate is covered with foil to provide a dark environment for about 30mins incubation. Fluorometric reading is done with BD Accuri flow cytometry reader.

3.3. Results and Discussion

One of the early morphological changes in cells undergoing apoptosis is the exposure of phosphatidyl serine on the surface of the cell membrane. This is followed by the later stage of apoptosis, where the nuclear membrane becomes compromised, allowing the entry of substances. Nuclear components begin to disintegrate together with other organelles after the late phase and form apoptotic bodies. These apoptotic bodies are engulfed by macrophages in a process known as efferocytosis which clears dead and dying cells to promote homeostasis and marks the completion of apoptosis. However, an alternative and largely underestimated outcome of apoptosis is secondary necrosis, an autolytic process of cell disintegration with release of cell components that occurs when there is no intervention of macrophages. This is mostly seen in tissue culture.

This investigation employed flow cytometric analysis of cells undergoing apoptosis when exposed to the non-oncology drugs. Two fluorescent conjugated dyes annexin V-

APC and 7-AAD, were used to monitor the stages of apoptosis of the cells. The flow cytometer then sorts cells into four quadrants. The first quadrant is Lower left (LL) quadrant which has cells that are negative for both dyes. Cells in this quadrant are not undergoing apoptosis and therefore have their cell membrane and nuclear membrane intact and hence negative for both dyes. Cells present in the LL quadrant are live cells. The second quadrant is the Lower right quadrant, cells in this quadrant are positive for annexin V but negative for 7-AAD. Cells here have their phosphatidyl serine exposed but an intact nuclear membrane. Annexin V can therefore bind to the exposed phosphatidyl serine and cause fluorescence. These cells are thought to be in the early phase of apoptosis. The third quadrant is the upper right (UR) quadrant which has cells that are positive for both dyes. This designates the late phase of apoptosis because both the cell and nuclear membranes are compromised and hence their positive response to both dyes. The last quadrant is the upper left (UL) quadrant which may contain disintegrated cells from apoptosis (secondary necrosis) or cells that underwent necrosis (primary necrosis). These cells are negative for annexin V but positive for 7-AAD.

In Figure 10 showing alexidine, a significant difference ($p < 0.005$) was seen in the percentages between drug-treated cells and the control at the early and late phases. More cells were seen in the lower right quadrant or the early phase than the late phase for both H69 and H69AR. Alexidine has been reported to induce apoptosis in some other cancer cell lines (Zorko et al., 2008). In this investigation, alexidine HCl has again been shown to induce apoptosis in small cell lung cancer cells (H69 and H69AR).

The results from ouabain in Figure 11(a) showed no H69 cells in the lower right quadrant or early phase and upper right quadrant or late phase. But there is quite a

significant number ($p < 0.005$) of cells seen in the upper left quadrant. As mentioned earlier, cells in the upper left quadrant may represent debris from apoptosis (secondary necrosis) or actual necrosis. In the case of ouabain effect on H69, where no cells are found in the quadrants that represent apoptosis, thus the lower right and upper right, ouabain effect is said to be necrotic (primary or actual necrosis). With H69AR, ouabain is thought to induce apoptosis, as shown in Figure 11(b), there is a significant difference ($p < 0.05$) between the control and the drug-treated cells. Ouabain according to these results, is therefore necrotic to H69, and apoptotic to H69AR. One possible explanation to this could stem from the poisonous nature of ouabain. Ouabain at high concentrations such as the one used in this experiment is toxic to cells. H69AR which happens to overexpress MRP1 that defends the cell against toxic xenobiotics, will pump the drug out of the cell. This pumping mechanism could lead to the cells dying in a programmed manner. H69 on the other hand, does not express MRP1 for defense and will therefore succumb to the toxic action of ouabain.

The results from cetrimonium bromide according to Figure 12 indicate apoptosis induction by the drug in both H69 and H69AR. Significant number ($p < 0.05$) of cells were seen in the lower right quadrants or the early phases for both cell lines. Also, Figure 12(c) showed a significant number ($p < 0.05$) of cells in the upper left quadrant which represent debris or disintegrated apoptotic bodies from the final stage of apoptosis (secondary necrosis). This is not uncommon in tissue culture due to the absence of macrophages to scavenge the apoptotic bodies. Disintegrated cells in this region will bind to 7-AAD and fluoresce.

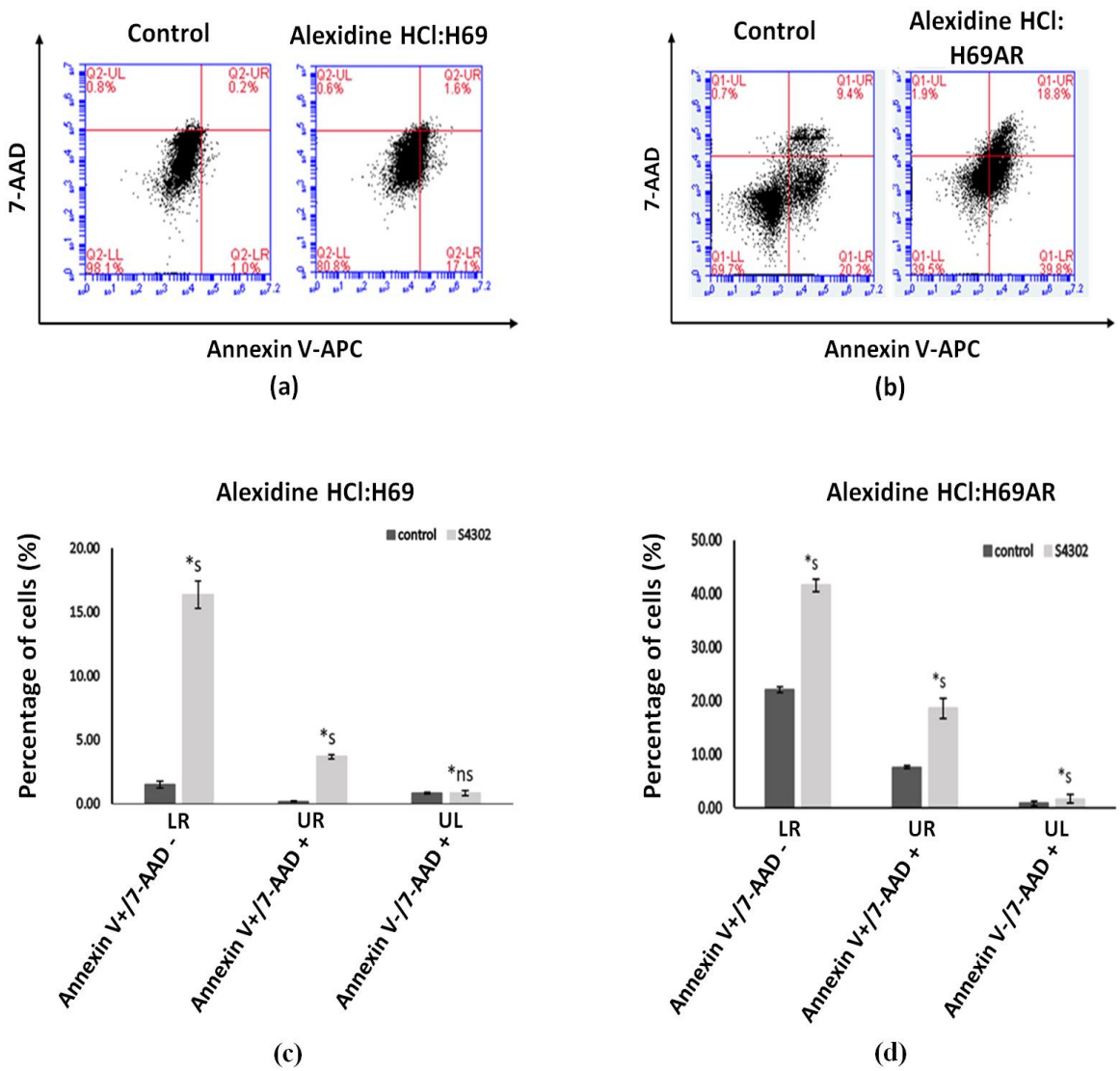


Figure 10. Flow cytometric analysis of the apoptotic effect of Alexidine HCl on (a). H69 and (b). H69AR apoptosis. Cells were treated with 15 μM Alexidine HCl and incubated for 48hrs. Some of the cells were treated with 0.15% DMSO as a control. Annexin V-APC and 7-AAD dyes were used to detect cells undergoing apoptosis. 10,000 cells were counted. (c). and (d). show the percentages of H69 and H69AR cells respectively in the different stages of apoptosis. Error bars represent standard errors of the mean of two experiment each done in triplicate. *s and *ns denote significant difference ($p < 0.05$) and non-significant difference ($p > 0.05$) respectively between drug treated cells and the control, using student T-test.

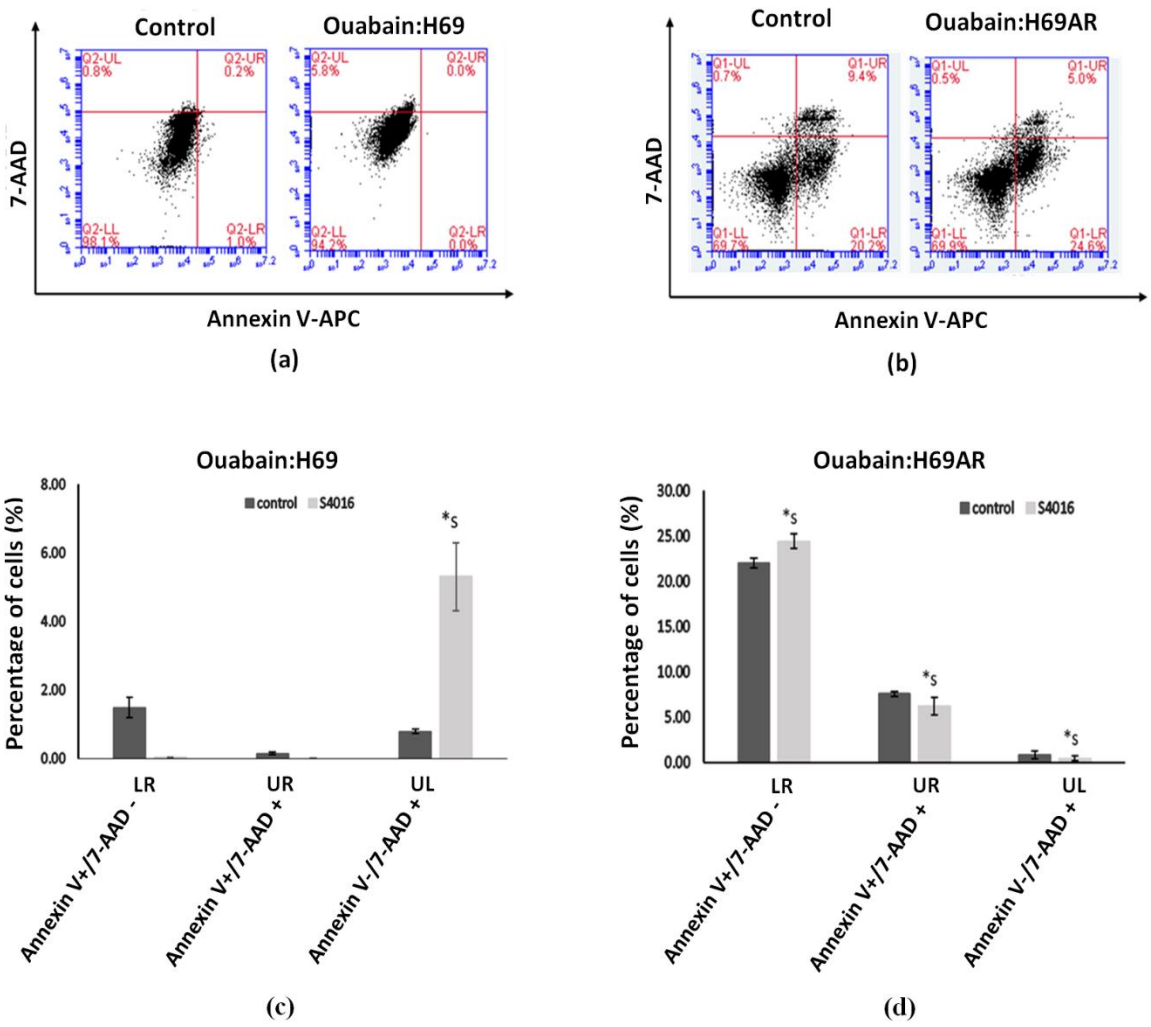


Figure 11. Flow cytometric analysis of Ouabain. (a). H69 (b). H69AR apoptosis. Cells were treated with 15 μM Ouabain and incubated for 48hrs. Some of the cells were treated with 0.15% DMSO as a control. Annexin V-APC and 7-AAD dyes were used to detect cells undergoing apoptosis. (c). and (d). show the percentages of H69 and H69AR cells respectively in the different stages of apoptosis. Error bars represent standard errors of the mean of two experiment each done in triplicate. *s and *ns denote significant difference ($p < 0.05$) and non-significant difference ($p > 0.05$) respectively between drug treated cells and the control, using student T-test.

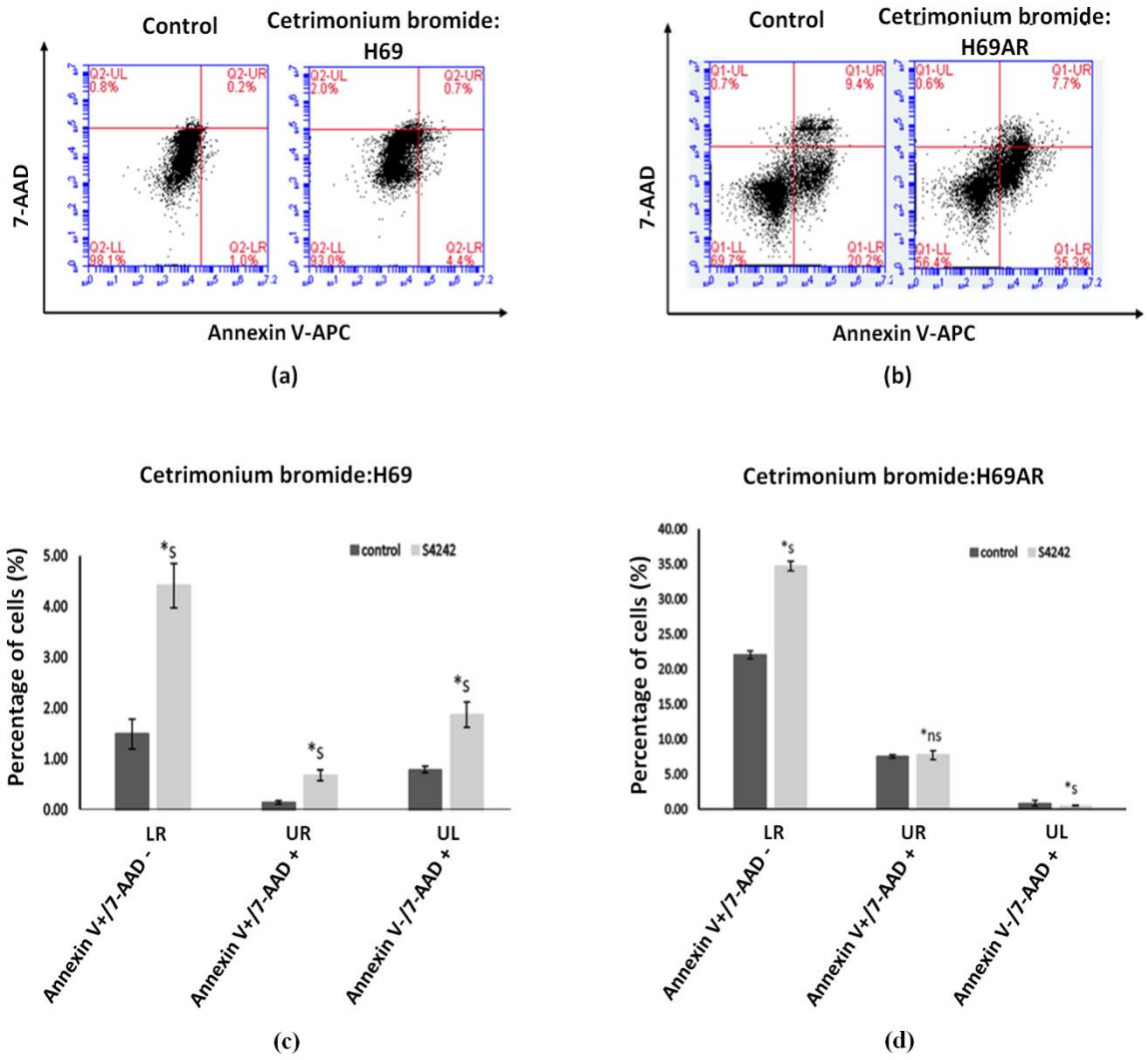


Figure 12. Flow cytometric analysis of Cetrimonium bromide. (a). H69 and (b). H69AR apoptosis. Cells were treated with 15 µM Cetrimonium bromide and incubated for 48hrs. Cells treated with 0.15% DMSO is used as a control. Annexin V-APC and 7-AAD dyes were used to detect cells undergoing apoptosis. (c). and (d). show the percentages of H69 and H69AR cells respectively in the different stages of apoptosis. Error bars represent standard errors of the mean of two experiment each done in triplicate. *s and *ns denote significant difference ($p < 0.05$) and non-significant ($p > 0.05$) between drug treated cells and the control, using student T-test.

CHAPTER 4

INVESTIGATING THE MECHANISM AND TARGETS OF COLLATERAL SENSITIVITY INDUCED BY FDA-APPROVED DRUGS ON MRP1-OVEREXPRESSED CELLS

4.1. Introduction

Although MRP1 can transport a broad spectrum of drugs out of the cell leading to chemotherapy failure, certain drugs or agents can expose the Achille's heel (weakness) of these MRP1-overexpressed cells leading to their death. This phenomenon where certain agents are selectively cytotoxic towards the MRP1-overexpressed cells (resistant cells) is referred to as collateral sensitivity. Collateral sensitivity (CS) is a synthetic lethargy wherein the genetic alteration accrued while developing resistance towards one agent is accompanied by the development of hypersensitivity towards a second agent. The term CS was first described qualitatively by Szybalski and Bryson in 1952 after observation that drug resistant Escherichia coli displayed hypersensitivity to unrelated agents, thus acquiring a potential exploitable weakness because of the drug selection process. There have been purported findings which seem to explain the complex mechanism of collateral sensitivity, each supported by limited experimental evidence. These hypotheses attempt to explain CS by the ability of CS agents to: 1. produce reactive oxygen species (ROS) via futile hydrolysis of ATP (Lövborg et al., 2006), 2. exploit energetic sensitivities (Cole & Deeley, 2006), and 3. extrude endogenous substrate which are essential for cell survival (Cole & Deeley, 2006). These afore-mentioned putative mechanisms of collateral

sensitivity have been summarized in Figure 13 below. According to these putative mechanisms, when a collateral sensitizer for example drug A enters a MRP1-overexpressed cell, it may trigger inflammation and/or undergo phase II metabolism. It is known that MRP1-overexpressed cells must conjugate certain drugs in phase II detoxification metabolism aided by glutathione S transferase (GST) with reduced glutathione (GSH) for their transport (Cole & Deeley, 2006). Glutathione is a tripeptide antioxidant made up of the amino acid glycine, cysteine, and glutamic acid. Glutathione is the most abundant antioxidant among the class of thiols found in the body and it alleviates oxidative stress caused by reactive oxygen species (ROS) such as hydrogen peroxide, superoxide, hydroxyl radical, and singlet oxygen, which are generated during mitochondrial oxidative metabolism as well as in cellular response to xenobiotics, cytokines, and bacterial invasion. In Figure 13 below, the GSH conjugated collateral sensitizer (A-GSH) is being transported out of the cell by the MRP1 which utilizes the hydrolysis of ATP (Adenosine triphosphate). On the other hand, when the collateral sensitizer triggers inflammation, leukotriene C_4 (LTC_4) which is a pro-inflammatory mediator will be synthesized by the addition of GSH to leukotriene A_4 (LTA_4) by LTC_4 synthase (Jakobsson et al., 1996). The LTC_4-GSH formed is an endogenous substrate of the MRP1, which is transported out of the cell by the MRP1 by utilizing the hydrolysis of ATP. These two mechanisms deplete glutathione as well as the energy bank (ATP) of the cell and generate oxygen radicals. To summarize this, drugs which happen to induce collateral sensitivity on H69AR cells could be involved in depletion of glutathione either through phase II metabolism or cause an inflammation by producing reactive oxygen species (ROS) which will result into oxidative stress and hence apoptosis.

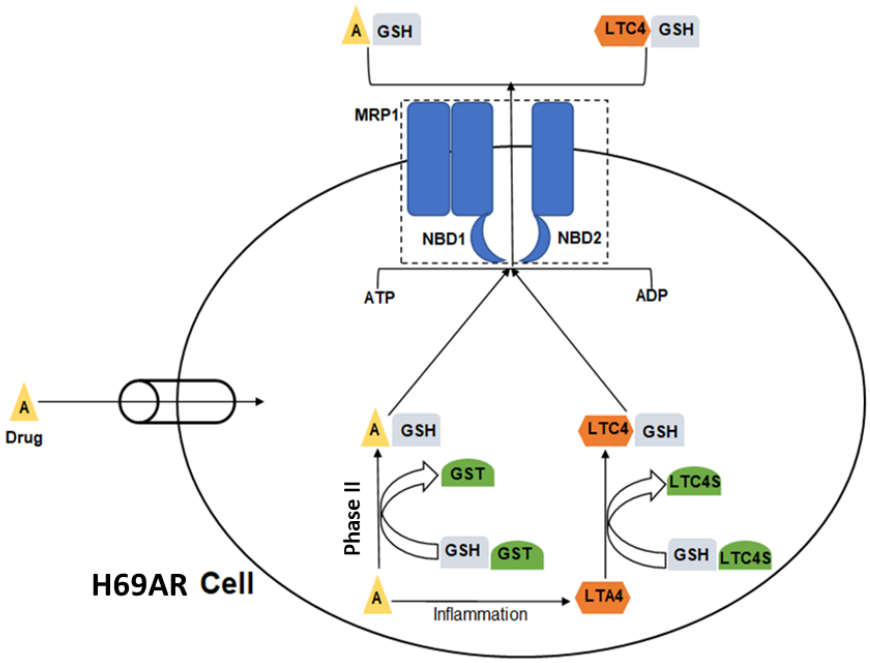


Figure 13. Putative mechanism of collateral sensitivity.

In the initial screening of the FDA-approved drugs, H69AR showed hypersensitivity towards erlotinib, AZD1480, fludarabine, and pyrimethamine. Thus, these drugs exhibited collateral sensitivity towards H69AR. This collateral sensitivity could result from the overexpression of MRP1. As mentioned earlier, MRP1-overexpressed cells respond to certain xenobiotics and inflammation by pumping out glutathione, which is vital for the cell's defense against oxidative stress. This can result into apoptosis of the cell. In this investigation, three mechanistic approaches would be used to validate the previously described putative mechanisms of collateral sensitivity, and to find the targets of the cytotoxicity induced by these drugs.

The first approach is to employ MRP1 dependent assay where the efflux activity of the MRP1 will be halted prior to addition of the collateral sensitizers. Stopping the efflux activity means that considerable amount of GSH may always be present in the cytosol to alleviate oxidative stress and hence survival of the cell.

In the second approach, caspase dependent assay would be used to investigate the mechanism of cytotoxicity. Apoptosis and necroptosis are both caspase-dependent programmed cell death. There are 13 known mammalian caspases classified either as initiator or executor of apoptosis. Caspases 8, 9 and 10 are examples of initiator caspases whiles 3, 6 and 7 are examples of effector caspases. Inhibition of these caspases, especially the initiator caspases will prevent programmed cell death. In a caspase dependent assay, addition of pan caspase inhibitor Q-VD-Oph irreversibly inhibits caspase 1,3,8 and 9 (Keoni & Brown, 2015). Intrinsic apoptotic pathway is initiated through caspase 9 while extrinsic pathway is initiated through caspase 8. This means that Q-VD-Oph can inhibit both intrinsic and extrinsic pathways leading to apoptosis. A collateral sensitizer that induces apoptosis through caspases, will show an increase in cell viability when the caspases are inhibited and vice versa. Most oncology drugs trigger apoptosis via production of ROS. The damage done to proteins, lipid membranes, nucleic acids and other organelles by ROS could lead to activation of programmed cell death by invoking caspases. Irreversible inhibition of caspases in both the intrinsic and extrinsic pathways should show a significant increase in cell viability when cells are treated with apoptotic agents.

The last approach would employ a fluorometric total thiol-depletion assay where total thiols in the cytosol will be measured after addition of collateral sensitizers. The fluorogenic dye dibromobimane, is widely used to detect various thiol-containing

biomolecules such as glutathione in cells. It is a unique fluorogenic cross-linking reagent for thiols because it is unlikely to fluoresce until both of its alkylating groups have reacted (Moore et al., 1995). Glutathione happens to be the most abundant of the total thiols in cells. Depletion of total thiols encompasses depletion of glutathione which could be used as a marker of collateral sensitivity.

4.2. MRP1 Dependent Collateral Sensitivity Assay

4.2.1. Materials and Methods

4.2.1.1. Chemicals

FDA-approved drugs (Erlotinib, azd1480, Fludarabine, pyrimethamine) were procured from Selleckchem (Houston, TX). Thiazolyl blue tetrazolium bromide (MTT) and Q-VD-Oph were procured from ApexBio (Houston, TX). MK-571, reversan and sodium dodecyl sulfate were procured from Sigma-Aldrich (St. Louis, MO).

4.2.1.2. Cell lines and cell culture

H69 and H69AR cells were procured from ATCC (Manassas, VA). H69 cell lines were cultured in RPMI 1640 (ATCC) media supplemented with 10% fetal bovine serum. H69AR cells were challenged monthly with 0.8 µM doxorubicin and cultured drug-free for one week before use. Cells were grown in a humidified incubator maintaining 5% CO_2 at 37°C.

4.2.1.3. Cytotoxicity assay

Cell sensitivity to drugs was analyzed using the MTT colorimetric assay. 250,000 cells in 100 ul RPMI 1640 media are seeded in 96-well plate (CellBIND®, Corning). Plate is incubated at 37^{o}C for 24 hrs. The next day, cells were treated with 25 µM of MK-571 or 5 µM of reversan (potent MRP1 inhibitors) for 90 mins in incubation before 15 µM of collateral sensitivity drugs were added and incubated for 96 hrs. DMSO concentration was maintained at 0.035%. At the end of the incubation period, 100 µL of culture medium was carefully removed and cells were treated with MTT (0.5 mg/mL) for 4 hrs. The formazan crystals were dissolved by the addition of 120 ul of 15% SDS containing 0.01%v/v HCl, and absorbance at 570 nm was recorded using a Hidex Sense Beta Plus plate reader (Turku, Finland).

4.3. Results and Discussion

As previously stated, MRP1 effluxes conventional cytotoxic oncology and non-oncology agents. This efflux action of the MRP1-overexpressed cells may play a role in the collateral sensitivity induced by certain agents. In this investigation, two potent MRP1 inhibitors MK-571 and Reversan, were used to inhibit MRP1 prior to the addition of collateral sensitizers. MK-571 is a selective, orally active leukotriene D4/E4 receptor antagonist for the treatment of bronchoconstriction (Manning et al., 1990, Amirey et at., 1991). Reversan is one of the most potent pyrazolopyrimidine when used in combination with either vincristine or etoposide to treat neuroblastoma in vivo (Burkhart et al., 2008).

Recall from Figure 13, transport activity of the MRP1 can cause depletion of GSH and ATP. By stopping the transport activity of the MRP1 with a potent inhibitor such as MK-571 or reversan, the transport of GSH and energy utilization would be eliminated, and cells would now have enough GSH to mitigate oxidative stress and hence cell survival. In this investigation, inhibition of MRP1 by MK-571 reversed the collateral sensitivity induced by erlotinib. In other words, more cells survived ($p < 0.05$) when MK-571 and erlotinib (indicated as erlMK on the bar graph) were added to cells as compared to adding only erlotinib (indicated as erl on the bar graph) to cells as shown in Figure 14(a). This collateral sensitivity reversal did not occur with the remaining three drugs since there were no significant differences ($p > 0.05$) in cell viability between inhibited and non-inhibited MRP1. On the other hand, inhibiting with reversan showed that the collateral sensitivities of all four drugs were dependent on the MRP1. This is because there is significant difference ($p < 0.05$) in cell viabilities between inhibited and non-inhibited MRP1 for all four drugs as shown in Figure 14 (b). However, the differences seen with AZD 1480 and fludarabine are relatively smaller than those of erlotinib and pyrimethamine. Transport of drugs by MRP1 out of the cell could take either one of these forms, transport of a free drug or transport of a drug with GSH. All these forms of transport out of the cell may require different forms of binding to the MRP1 (Gottesman et al., 2009), these different forms of binding could arise to different inhibitory mechanisms. In Figure 14 (b), reversan probed more drugs than MK-571 which is a clear indication that both inhibitors have different inhibitory mechanisms, which could be attributed to the different forms of binding of the MRP1. The collateral sensitivity of erlotinib disappeared with the inhibition by both MK-571 and reversan, meaning that the collateral sensitivity induced by erlotinib is responsive

to the different forms of inhibition by MK-571 and reversan. Inferring from Figure 14(b), the collateral sensitivity of azd1480 and fludarabine to some extent (even though not as prominent as seen in erlotinib and pyrimethamine) is dependent on the efflux activity of the MRP1 since there was significant difference ($p < 0.05$) in the percent viabilities of the reversan-treated cells and untreated cells.

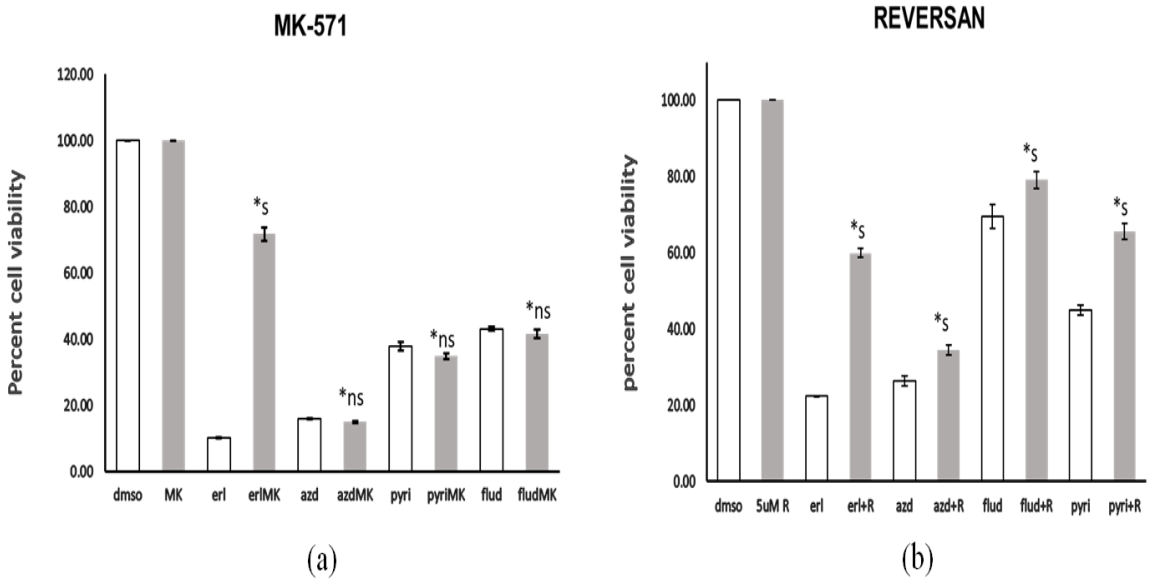


Figure 14. Viability of H69AR cells showing the effect of inhibiting MRP1 prior to the addition of collateral sensitizers. Cells were treated with (a) 25 μM of MK-571, (b) 5 μM of reversan (both of which are MRP1 inhibitors) and incubated for 90 mins before addition of 15 μM drugs for 96hrs (for MK-treated cells) and 72hrs (for reversan-treated cells). Viability was assessed by MTT assay and drug effect was normalized with 0.035% DMSO. Error bars represent standard errors of the mean of two experiments each done in triplicate. *s and *ns denote significant and non-significant difference respectively with $p < 0.05$ for *s and $p \geq 0.05$ for *ns, using student T-test.

4.4. Caspase Dependent Collateral Sensitivity Assay

4.4.1. Materials and Methods

4.4.1.1. Chemicals

FDA-approved drugs (Erlotinib, Azd1480, Fludarabine and Pyrimethamine) were procured from Selleckchem (Houston, TX). Thiazolyl blue tetrazolium bromide (MTT) and Q-VD-Oph were procured from ApexBio (Houston, TX), APC-Annexin V, Annexin binding buffer and 7-AAD procured from BD Biosciences (San Jose, CA) and sodium dodecyl sulfate was procured from Sigma-Aldrich (St. Louis, MO).

4.4.1.2. Cell lines and cell culture

H69 and H69AR cells were procured from ATCC (Manassas, VA). H69 cell lines were cultured in RPMI 1640 (ATCC) media supplemented with 10% fetal bovine serum. H69AR cells were challenged monthly with 0.8 μM doxorubicin and cultured drug-free for one week before use. Cells were grown in a humidified incubator maintaining 5% CO_2 at 37 °C.

4.4.1.3. Cytotoxicity assay

Cell sensitivity to drugs was analyzed using the MTT colorimetric assay. 250,000 cells in 100 μL RPMI 1640 media are seeded in 96-well plate (CellBIND®, Corning). Plate is incubated at 37 ^{o}C for 24hrs. The next day, cells were treated with 25 μM of Q-VD-Oph (pan caspase inhibitor) and incubated for 90 min, before 15 μM of non-oncology drugs were added and incubated for 96 hrs. DMSO concentration was maintained at 0.15%. At the end of the incubation period, 100 ul of culture medium was carefully removed and cells

were treated with MTT (0.5 mg/mL) for 4 hrs. The formazan crystals were dissolved by the addition of 120 µL 15% SDS containing 0.01%v/v HCl and absorbance at 570 nm were recorded using a Hidex Sense Beta Plus plate reader (Turku, Finland).

4.5. Results and Discussion

Programmed cell death such as apoptosis and necroptosis involve procaspase (cysteine-dependent aspartate-directed proteases) activation. Two major forms of caspases exist for apoptosis to take place, the initiator caspases and the effector or executioner caspases. Inhibition of caspases, more importantly the initiator caspases, could prevent apoptosis from taking place. In this investigation, a potent pan caspase inhibitor Q-VD-Oph which has being found to inhibit caspase 1, 3, 8, and 9 is added prior to the addition of collateral sensitivity drugs. Agents that induce caspase-dependent apoptosis do that through a death receptor pathway (extrinsic pathway) or intrinsic pathway as shown in Figure 15 below.

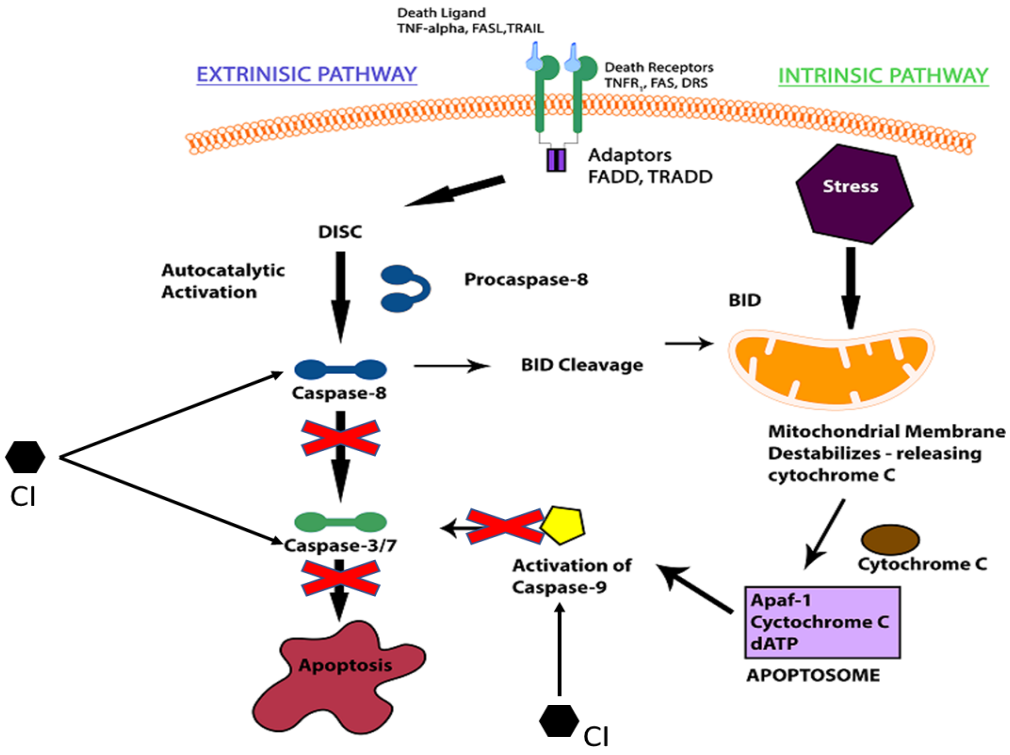


Figure 15. Inhibitory effect of a caspase inhibitor (Q-VD-Oph) on extrinsic and intrinsic pathways leading to apoptosis.

When conditions in the extracellular environment determine that a cell must die, extrinsic pathway will occur. For the extrinsic pathway, death ligands, such as FasL, bind to the death receptor Fas. This receptor-ligand binding, together with Fas-associated death domain (FADD), generate death-inducing signaling complex (DISC), which in turn recruits and assembles initiator caspase-8. This caspase activates the effector caspase-3 and 7, resulting in nuclear protein cleavage and apoptosis. For the intrinsic pathway, stress in a form of DNA damage induces p53 to activate Bax (B-cell lymphoma associated protein x) and Bak (B-cell lymphoma associated protein k), or bid is converted to tbid by caspase 8 which activates Bax and Bak. Bax and Bak act on mitochondria and cause the release of

cytochrome C which then combines with Apaf1 (apoptosis activating factor 1) and in turn activates pro-caspase 9 to caspase 9. Caspase 9 then activates effector caspase 3 and 7 resulting in apoptosis. Both extrinsic and intrinsic pathways converge at the effector caspases which initiate the actual apoptosis process.

The caspase inhibitor (CI), (Q-VD-Oph), used in this experiment inhibits the intrinsic initiator caspase-9, extrinsic initiator caspase-8 and the effector or the executioner caspase-3 as shown Figure 15. This inhibitory function of Q-VD-Oph is such that both intrinsic and extrinsic pathways leading to apoptosis would be blocked prior to the addition of an apoptotic agent.

As shown in Figure 16, a significant difference ($p < 0.05$) in cell viability between inhibited and non-inhibited cells was seen with erlotinib and AZD 1480. This significant difference is evidence of their caspase dependent collateral sensitivity. Erlotinib has been found to inhibit EGFR, and AZD 1480 has also been found to inhibit Janus kinase. The functions of these drugs result in the compromise of downstream signaling that leads to the growth or development of cells. Cells are programmed to die when their growth or development is compromised. In this experiment, the pro-caspase activation pathways that were induced by erlotinib and AZD1480 were blocked by the pan caspase inhibitor Q-VD-Oph hence the significant differences. It is therefore evident in this investigation that the cytotoxicity of erlotinib and AZD1480 depend on caspase. Pyrimethamine and fludarabine on the other hand did not show significant difference ($p > 0.05$) in cell viability between inhibited and non-inhibited cells. Both drugs are antimetabolite and indirectly affect ATP production. Cells can pull energy in the form of ATP from different metabolic pathways. Therefore, the cytotoxic effect of antimetabolite may not depend directly on caspase.

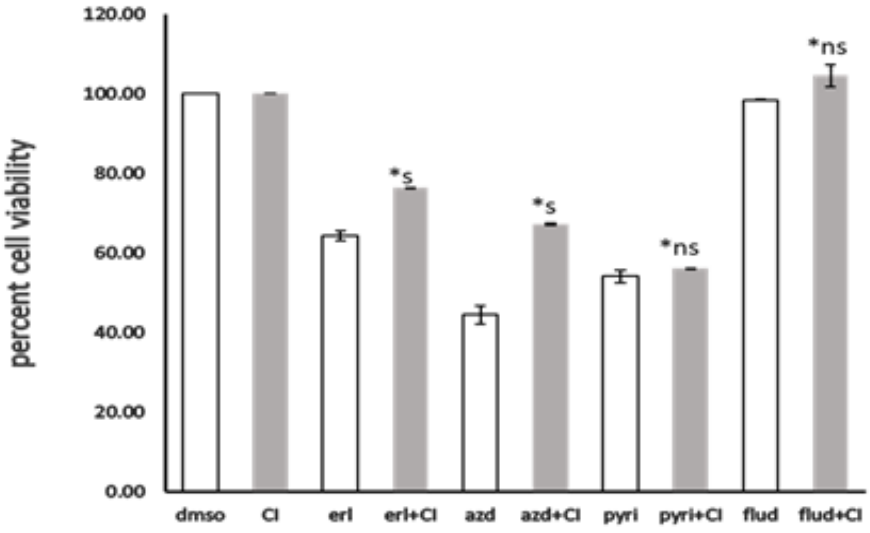


Figure 16. Viability of H69AR cells showing the effect of inhibiting pan caspase prior to the addition of collateral sensitivity drugs. Cells were treated with 25 μM Q-VD-Oph (a pan caspase inhibitor (CI)) and incubated for 90 mins before addition of 15 μM drugs for 4 hours. Viability was assessed by MTT assay and drug effect was normalized with 0.015% DMSO. Error bars represent standard errors of the mean of two experiments each done in triplicate. *s and *ns denote significant and non-significant difference respectively with $p < 0.05$ for *s and $p > 0.05$ for *ns.

4.6. Total Thiol (Glutathione) Depletion

4.6.1. Materials and Methods

4.6.1.1. Chemicals

FDA-approved drugs (Erlotinib, azd1480, Fludarabine, pyrimethamine) were procured from Selleckchem (Houston, TX). Dibromobimane, and N-acetylcysteine (NAC) were procured from ApexBio (Houston, TX). Sodium dodecyl sulfate was procured from Sigma-Aldrich (St. Louis, MO).

4.6.1.2. Cell lines and cell culture

H69 and H69AR cells were procured from ATCC (Manassas, VA). H69 cell lines were cultured in RPMI 1640 (ATCC) media supplemented with 10% fetal bovine serum. H69AR cells were challenged monthly with 0.8 µM doxorubicin and cultured drug-free for one week before use. Cells were grown in a humidified incubator maintaining 5% CO_2 at 37 °C.

4.6.1.3. Thiol depletion assay

Total thiol depletion by drugs was analyzed using dibromobimane fluorescence assay. 250,000 cells in 100 µL RPMI 1640 media are seeded in 96-well plate (NUNC®). Plate is incubated at 37 ^oC for 24hrs. The next day, cells were treated with 50 µM of N-acetylcysteine (NAC) or 50 µM L-buthionine sulfoximine (BSO) for 48 hours in incubation, before 30 µM of collateral sensitivity drugs were added and incubated for 12 hours. DMSO concentration was maintained at 0.035%. A 20 uM dibromobimane was added to cells and incubated for an hour. Fluorescence reading was done with cytation3 reader at excitation and emission wavelengths of 393 nm and 490 nm, respectively.

4.7. Results and Discussion

GSH depletion as discussed earlier, is anticipated to result from the transport of a drug out of the cell or production of the inflammatory mediator LTC_4. In this investigation, N-acetylcysteine (NAC) is first added to cells to stimulate GSH production above basal levels before addition of collateral sensitizers. Cells without NAC were used as control to monitor basal GSH production. Dibromobimane is then added to react with the thiols left

after addition of collateral sensitizers. The reaction of bromobimanes mostly occurs by S_N2 displacement of bromide ion by a thiolate anion from cysteine residue as shown below. The oxidized complex formed can now fluoresce with excitation and emission wavelengths in the uv-vis range. Dibromobimane will not fluoresce until both akylating groups have reacted. This means that two molecules of thiol-containing compounds (GSH) are required to cause dibromobimane to fluoresce.

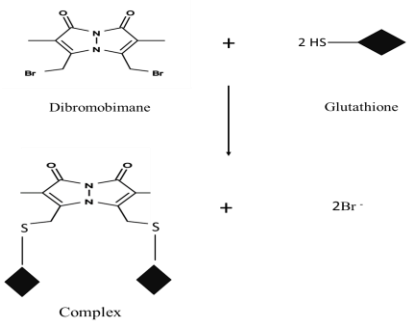


Figure 17. S_N2 displacement of bromide ions on dibromobimane by thiolate anion on cysteine residues.

In the cell, γ-glutamylcysteine synthetase, a key enzyme in glutathione biosynthesis converts NAC into glutathione which led to an increase in relative fluorescence unit (RFU) as shown in Figure 18. L-buthionine sulfoximine (BSO), also used in this experiment, induces experimental glutathione deficiency by inhibiting γ-glutamylcysteine synthetase (Akan et al., 2005). BSO added with NAC to the cells at the same time was able to inhibit this enzyme and therefore showed a significant decrease ($p < 0.05$) in total thiol (GSH) hence a lower RFU (Figure 18). Vincristine which is used to treat leukemia and other types of cancers, including small cell lung cancer, has been reported to exhibit a co-transportation with GSH by MRP1 (Loe et al., 1998; The American Society of Health-System

Pharmacists, n.d.). In this work, vincristine showed a significant decrease ($p < 0.05$) in RFU corresponding to depletion in total thiols (GSH).

Erlotinib and AZD1480 also depleted total thiols (GSH) significantly ($p < 0.005$) showing lower RFU values as seen in Figure 18. The depletion caused by these two drugs reveals the role of GSH in the collateral sensitivity induced by the drugs.

Fludarabine phosphate and pyrimethamine did not show significant decrease ($p > 0.05$) in RFU as seen in Figure 18. Both fludarabine and pyrimethamine are antimetabolite which can exploit the energetic sensitivity of MRP1-overexpressed cells such as H69AR. Although both drugs did not deplete GSH, they could induce their collateral sensitivity through exploitation of the energetic sensitivity of these cells leading to their death.

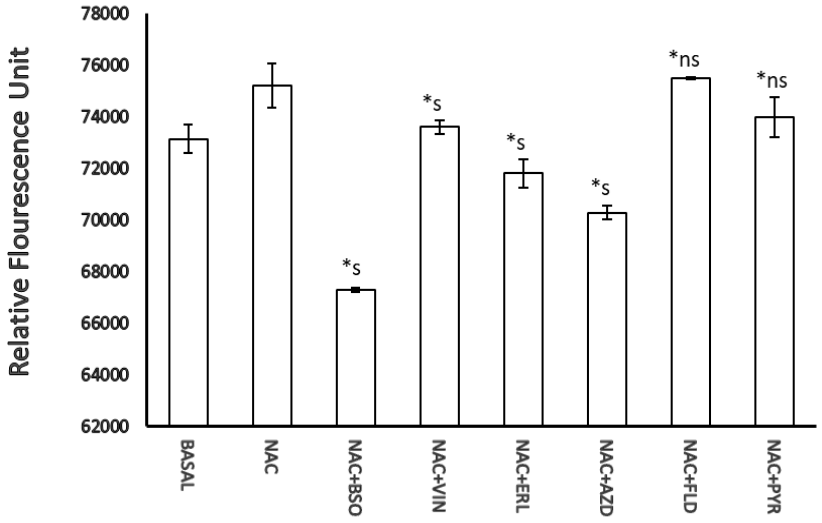


Figure 18. Depletion of total thiols induced by collateral sensitivity drugs. Cells were treated with 50 μM NAC and incubated for 48hrs before addition of 30 μM drugs for 12 hours. Vincristine and BSO are used as control. Cells were treated with dibromobimane dye, and fluorescence were taken at excitation and emission wavelength of 393 nm and 490 nm, respectively. Error bars represent standard errors of the mean of two experiments each done in triplicate. *s and *ns denote significant and non-significant difference from NAC respectively with $p < 0.05$ for *s and $p > 0.05$ for *ns.

CHAPTER 5

SUMMARY, CONCLUSIONS, AND RECOMMENDATIONS

5.1 Summary

FDA-approved and clinical trial drugs were screened for their cytotoxicity towards small cell lung cancer cells (H69 and H69AR) using MTT cytotoxicity assay. H69AR happens to be the resistant form of the H69, with overexpression of multidrug resistance protein 1 (MRP1). After the screening, drugs were grouped based on their relative cytotoxic effect on both cell lines. The first group called the "resistance", showed selective toxicity towards H69 (the parental cell line), but did not show significant toxicity towards H69AR (the resistance). The second group called "shared cytotoxicity", showed almost equal toxicity towards both cell lines with cell viability percentage less than 30. Drugs in this group were mostly non-oncology drugs. The last group called "collateral sensitivity" showed selective toxicity towards the resistant cell line (H69AR). Per the goal of this work, which is to lend addendum to cancer chemotherapy, further experiments were performed to investigate the mechanism of the cytotoxicity of drugs in the last two groups. Further experiments started with finding the half maximal inhibitory concentration of these drugs or what is commonly referred to as IC_{50} in a dose-dependent manner using MTT cytotoxicity assay.

The mechanism of the cytotoxicity of the non-oncology drugs was investigated by employing annexin V and 7-AAD fluorometric apoptosis assay to find out if the cytotoxicity of these drugs is in a programmed manner.

Several experiments were performed to investigate the mechanism of the cytotoxicity of collateral sensitivity drugs. These include:

1. Finding whether the collateral sensitivity of these drugs is dependent on the pumping action of the MRP1, by inhibiting the MRP1 in MTT cytotoxicity assay.
2. Finding whether the cytotoxicity of these drugs is programmed, by employing caspase dependent assay.
3. Finding whether these collateral sensitivity drugs deplete glutathione in the cell.

Drugs in the first group did not fit into the goal of this project and therefore no further experiment was performed with those drugs.

5.2. Conclusions

The two groups "shared cytotoxicity" and "collateral sensitivity", that were of interest included seven drugs. Drugs in the shared cytotoxicity group were alexidine HCl, ouabain, and cetrimonium bromide. All three are non-oncology drugs and showed very low IC_{50} values for both H69 and H69AR. These low IC_{50} values inform high potency in killing both H69 and H69AR. The cytotoxicity of these drugs was also found to be concentration dependent, thus, the higher the concentration, the greater the percentage of cell death. Further experiments were performed to investigate the mechanisms of cell death induced by these drugs. Employing the traditional flow cytometry technique for apoptosis, alexidine HCl and cetrimonium bromide were found to induce apoptosis in both H69 and H69AR. Most cells were found in the early phase of apoptosis after these cells have been treated with 15 μM of the drugs for 48hrs. On the other hand, the effect of ouabain on H69 was

necrotic and on H69AR was apoptotic under the same drug concentration and conditions. It is worth knowing that drugs that exert their cytotoxicity in a programmed manner mostly would have a lead in therapeutics as compared to those that do not. Alexidine HCl and cetrimonium bromide would therefore be good candidates for drug repurposing in cancer chemotherapy.

In the collateral-sensitivity group, four drugs, namely erlotinib, AZD1480, fludarabine, and pyrimethamine were found. Drugs in this group exhibited selective toxicity towards H69AR, which was concentration dependent in a exception of AZD1480. Erlotinib gave an IC_{50} value for H69 which is 170 times more than that of the H69AR. This large-fold difference informs that H69 cells are very insensitive to the drug. Pyrimethamine and fludarabine also gave significant differences, five and six times respectively, indicating their selective toxicity towards H69AR. AZD1480 at 15uM showed collateral sensitivity, but at varying concentrations, its toxicity towards H69 was anomalous. Thus, at one point it exhibited shared cytotoxicity, at another point its exhibited collateral sensitivity. This made it difficult for AZD1480 to be placed this category. In an experiment to investigate the dependency of collateral sensitivity on MRP1, MK-571 and reversan both stopped the collateral sensitivity induced by erlotinib. Also, the collateral sensitivity induced by pyrimethamine was stopped by reversan. These results conclude that the collateral sensitivity of erlotinib and pyrimethamine depends on the pumping action of the MRP1. The reversal seen in AZD1480 and fludarabine using reversan were although significant but too minimal to make a decisive conclusion without performing further experiments with different MRP1 inhibitors.

Drugs that induce caspase activation exhibit cytotoxicity in a programmed manner. In an investigation into the cytotoxicity mechanisms of the collateral-sensitivity drugs, erlotinib and AZD1480 showed a significant increase in cell viability percentage upon inhibition of caspase. Fludarabine and pyrimethamine on the other hand did not show a significant increase in cell viability. This concludes that erlotinib and AZD1480 exhibit their cytotoxicity in a programmed manner whiles fludarabine and erlotinib do not induce caspase activation in their cytotoxic mechanisms.

The last part of the work featured drugs in the collateral sensitivity group. An investigation that was meant to reveal the correlation between GSH depletion and collateral sensitivity. Erlotinib and AZD1480 depleted GSH significantly. This informs that GSH plays a role in the collateral sensitivities of erlotinib and AZD1480. Thus, they deplete the cell's antioxidant (GSH) and make cells susceptible to oxidative stress resulting into death.

5.3. Recommendations

The main goal of this project is to join the train of drug repurposing in chemotherapy. Drugs that were used in this project are already FDA-approved drugs and would be relatively easier to be accepted for drug repurposing. Erlotinib selectively kills H69AR in a programmed manner which involves caspase activation and depleting GSH. However, erlotinib cannot be effective on H69AR if the MRP1 of H69AR is blocked. Erlotinib would therefore be recommended to join the train of drug repurposing to treat small cell lung cancer cells (H69) which has overexpressed MRP1. But in situations where

combinatorial regimen would be applicable, erlotinib should not be combined with a drug that will block MRP1.

One of the problems of cancer chemotherapeutics is the afflictive side effects. Therefore, a drug would be worth recommending if it is a non-oncology drug and has cytotoxicity potential on cancer cells. Alexidine HCl, ouabain, cetrimonium bromide, and pyrimethamine used in this work are non-oncology drugs and were cytotoxic to H69 and/or H69AR. Playing a role of surfactant, cetrimonium bromide could be used in a palliative procedure for lung cancer patients with breathing difficulties. Pyrimethamine, a non-oncology antimetabolite, would be recommended for two reasons: 1). It would possess little side effect as a non-oncology drug. 2). It selectively kills H69AR by depending on the pumping mechanism of the MRP1. Again, pyrimethamine should not be combined with a drug that blocks MRP1, in a combinatorial regimen.

5.4. Future Work

Although the drugs used in this project were tested only on small-cell lung cancer cells, their cytotoxic mechanisms identified in this work could give an insight to mechanistic investigations on different cancer cells. Future work could focus more on the collateral-sensitivity mechanisms of non-oncology antimetabolite drugs on different cancer cells that overexpresses MRP1. Future work could also focus on screening drugs for MRP1 inhibitors to identify a potential unacceptable chemotherapy combinatorial regimen.

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