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APOPTOTIC DOSE-RESPONSE OF BQ'S ON TUMOR-CANCEROUS CELL LINE

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DEDICATORY

To my Mother,
Who has made love sacrifices.

To Jose Carro and Eneida Luna,
They taught me the meaning of real love.

To Christian Vélez and José Marengo;
For becoming my substitute brothers,
my greatest strength
through this entire year and a half journey.

The CHEMTOX was and will be my second family.

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RESUMEN

El desarrollo de nuevos agentes anticancerosos ha aumentado debido a la resistencia a los tratamientos convencionales. Compuestos con características planares-catiónicas y los grupos amino o nitro hacen que las Sales de Benzazolo[3,2-*a*]quinolinium (BQ's) sean candidatos para drogas anticáncer. Estudios previos indican citotoxidad y capacidad mutagénica de los BQ's en células A431 aunque no existe información sobre la modalidad de la muerte celular. Este estudio evalúa la citotoxicidad, potencia, fluorescencia e inducción apoptótica en A431. Las células fueron expuestas a las concentraciones de IC_{50} y contadas a las 24, 48 y 72 horas. Fotos de las células tratadas muestran menor confluencia al compararlas con el Control Negativo. Los datos cualitativos revelan que los ABQ's son mas potentes que los NBQ's para inhibición de crecimiento celular. Observaciones microscópicas permitieron la detección de autoflorescencia en Ellipticina y ABQ's pero no en los Controles Negativos, NBQ' y BQ $_{108.}$ En la Ellipticina y los ABQ's se observaron estructuras subcelulares fluorescentes indicando que los ABQ's y Ellipticina muestran selectividad a los organelos mitocondriales y también podrían interactuar con el retículo endoplásmico. La capacidad de inducción apoptótica fue evaluada utilizando la permeabilización de la membrana mitocondrial con el tinte MITO PT™, la activación de Caspasas 3-7 con el tinte Magic Red™ y la activación de la Caspasa 8 con el tinte FLICA™. Las células tratadas con el IC_{50} a 48 horas fueron teñidas por una hora. Microscopía de fluorescencia cualitativa y análisis de florimetría mostraron que la membrana mitocondrial fue permeabilizada por todos los compuestos. Células tratadas con los ABQ's activaron las Caspasas 3-7 en una intensidad similar a la de Cisplatin y dos veces mas que los NBQ's. No se detectó activación de la Caspasa 8. Se concluye que el IC_{50} de los BQ's contribuyen a la inhibición del crecimiento celular mayormente por medio de apoptosis. Las células tratadas con los BQ's inician el proceso de apoptosis permeabilizando la membrana mitocondrial y activando las Caspasas ejecutoras 3-7. El mecanismo de los BQ's predomina la ruta intrínseca al no involucrar la activación de la Caspasa 8.

ABSTRACT

The development of new anticancer agents has increased in recent years due to resistance to conventional treatments. Characteristics such as planar, cationic, and the nitro or amino species make the Benzazolo[3,2-*a*]quinolinium salts (BQ's) potential candidates for anticancer drugs. Previous studies indicated the cytotoxic and mutagenic capacity of BQ's in A431 cells, proving cell-drug interaction but no specific information regarding cell death modality. This study evaluated the cytotoxicity, potency, fluorescence, and apoptotic induction capacity of the BQ's in cancer cells. A431 cells were exposed to BQ's at the IC_{50} concentrations and then counted 24, 48, and 72 hours. Photos of BQ treated cells show lower confluence compared to the Negative Control. Qualitative data revealed that ABQ's were more potent than NBQ's on growth inhibition of cells. Microscopical observations allowed the detection of cell-drug auto-fluorescence on Ellipticine and ABQ's but not on Negative control, NBQ's or BQ_{108} Ellipticine and ABQ's were observed in similar fluorescent sub-cellular structures, indicating that ABQ's and Ellipticine selectively bind to mitochondria and might also interact with the endoplasmic reticulum. The apoptotic induction capacity was assessed using the permeabilization of the mitochondrial membrane and activation of Caspases 3, 7 and 8 by fluorescence analysis with MITO PT™, Magic Red™, and FLICA™ fluorescent dyes, respectively. Cells treated with BQ at the IC_{50} concentrations for 48 hours were stained with all three different dyes for an hour. Qualitative fluorescence microscopy and quantitative fluorimetry analyses showed that mitochondrial membrane was permeabilized with all four BQ's drugs at 72 hours. At 48 hours, Caspases 3 and 7 were positively activated, while Caspases 8 was negatively activated. ABQ treated cells activated Caspases 3 and 7 similarly to Cisplatin and almost double NBQ's. Apoptosis was the cell death mechanism that mainly contributes to the overall cell growth inhibition caused by BQ's IC_{50}. BQ treated cells initially trigger apoptosis by damaging the mitochondrial membrane, activating downstream execution of Caspases 3 and 7. BQ's mechanisms are mainly intrinsic due to the lack of involvement of Caspase 8.

CHAPTER I

INTRODUCTION

Problem background

Cancer is one of the most significant health burdens in the United States, and it's the second cause of death of Hispanic/Latin adults (American Cancer Society [ACS], 2004), causing one in every four deaths (Jemal et al., 2004). Age is related to cancer incidence, with seventy-six percent (76%) of cancers diagnosed to subjects over 55 years old (National Institutes of Health [NIH], 2003). Five to ten percent (5%-10%) of cancers are hereditary, and the rest ninety to ninety-five percent (90%-95%) is due to nonhereditary causes (National Cancer Institute [NCI], 2003). Tobacco smoke and sunlight exposure (ultraviolet rays) have been identified as substantial risk factors to cancer incidence (United States Department of Health and Human Services [USDHHS], 2006; Hu et al., 2004). The trend of the last 60 years has demonstrated cancer prevalence as evidence that cancer is difficult to eradicate. Cancer trend behavior through the years had a statistically low growth but continuous on the United States (US) and Puerto Rico (PR) (Edwards, B.
K. et al, 2005; ACS, 2004; ACS, 2005; ACS, 2006). National Center for Health Statistics reported a general decrease of point eight percent (0.8%) per year in the incidence rate for all cancers combined from 1990 to 1997 (Wilson et al. 2002).

Advances in cancer detection have also contributed to a higher number of cancers reported influenced cancer statistical data. The continuous increment in the worldwide population has also affected the number of reported new cases. Yearly cancer reports demonstrate the estimated new cases and deaths. The top emerging types of cancer for men are prostate, lung-bronchus, and colon-rectum. Women are breast, lung-bronchus, and colon-rectum, respectively (Jemal et al. 2004).

These statistics also reveal a significant vulnerable population that includes children, minorities, women, and the poor (Wilson et al., 2002). On the other hand, in several different countries, the population with high survival rates have been linked to higher income, education, occupation, and health insurance status (American Cancer Society (ACS), 2005; Boyd, J. et al., 1999).

Treatments for specific types of cancer have been integrated into each line of research to produce a comprehensive understanding of the disease (Solomon, 2006). Research analysis on malignant tissue is also necessary for clinical trials and studies that continue to be a challenge. The government and academia assign budgets specifically forthe development of anticancer treatments. Sometimes funding gets cut off, affecting advances in drug development. On the other hand, the private sector (pharmaceutical companies) has more resources, equipment, and experts to develop drugs. They have played an essential role in discovering anticancer drugs by developing research with fewer budget limitations (Solomon, 2006).

Problem under study:

Although advances have been achieved in cancer treatment, no definitive cure has been discovered for all cancer types. Efforts need to be employed to characterize cancer risk factors better. In this way, prevention of exposure to carcinogens could decrease cancer incidence. Prevention of exposure has also had shown to help reduce the increment of cancer cases (ACS, 2005). Changing lifestyle factors, the use of preventive vaccines, protection from sun exposure, reduction of environmental contaminants, and protective equipment in the workplace where carcinogenic materials are handled can reduce a substantial amount of people diagnosed with cancer (ACS, 2005). Prevention implicates fewer cancer death cases and savings on the yearly budget invested in treatment by the public and private sectors.

Cancer risks factors

Exposure to a wide variety of natural and man-made substances in the environment accounts for at least two-thirds of all cancer cases (NIH, 2003). Anthropogenic causes can be attributed to people's lifestyles. Most cancers are related to known lifestyle factors, and their choices increase the probability of risk of occurrence (ACSH, 1995). Activities such as cigarette smoking, excessive alcohol consumption, poor diet, lack of exercise, excessive sunlight exposure, sexual behavior, and exposition to certain viruses (ex. H.P.V.) have been identified as leading factors for cancer incidence(NCI, 2003). Other factors include the use of specific medical drugs, hormones, exposure to radiation, viruses, bacteria, and environmental chemicals present in the air, water, food, and workplace (NIH, 2003). The cancer risks associated with many environmental chemicals have been identified through studies of occupational groups with higher exposures to these chemicals than the general population. The 10th Report on Carcinogens, published in December 2002, lists 228 substances that are either known to cause or are suspected of causing cancer. It also describes where they are found and the scientific evidence that they cause cancer (NIH, 2003).

Cancer consequences

Consequently, exposure to carcinogenic agents results in mutation of deoxyribonucleic acid (DNA), damaging it in a way that would cause uncontrolled cell growth. Because cancer results from a series of genetic alterations in the genes that control cell growth, division, and differentiation (Vogelstein et al., 1988; Hanahan & Weinberg, 2000; Kinzler & Vogelstein, 2002), cells escape their standard growth control; developing into tumors. Cancer complexity and varied mechanism have caused many different approaches to the treatment or diagnostics depending on its localization.

Tumor development results from the accumulation of various mutations in cells. It can take multiple years for a tumor to be developed. Also, It has been estimated that after the incidental and or cumulative exposure to carcinogens (cancer-causing agents), it can take up to 20 to 30 years for cancer to be diagnosed (NIH, 2003).

Cells have different mechanisms to repair damaged DNA or demeans by apoptosis when mutations occur (Shapiro & Harper, 1999). Tumors can be benign or malignant and result from the mutation in cancer responsible genes, thus activating oncogenes and overexpressing or suppressing proto-oncogenes. The main problem with tumors is that they are challenging to treat because they propagate quickly and have shown resistance to cancer therapy (Höckel & Vaupel, 2001).

Cancer therapy

Cancer can be treated with a greater rate of success if detected early. Initial treatment for cancer therapy had revealed that forty-three percent (43%) of all patients had surgery, seven-point nine percent (7.9%) received radiotherapy, three-point two percent (3.2%) received chemotherapy, point one percent (0.1%) received treatment with hormones, twenty-one point eight (21.8%) received combined therapy and the remaining twenty-four percent (24%) were not treated (NCI, 2005).

Once found, there are a variety of treatments such as radiotherapy, chemotherapy, surgery, monoclonal antibodies in front to the receptor of growth receptor factor, retinoic acid, tyrosine kinase inhibitors, and proteasome inhibitors (Brown D et al., 2003, Lopez M et al., 2005) that can either suppress the tumor growth and or decrease the tumor size. Hypoxia (absence of oxygen) is a feature of some tumors (Hodgkiss R J, 1991; Jain R & Forbes, N, 2001). The extent of hypoxia in tumors influences the success of different cancer treatments, requiring the use of up to three times the dose (radiotherapy) needed to kill cancerous cells in comparison to healthy cells where oxygen is present (Wardman, 2001).

The chemical properties of each agent used as a treatment will be responsible for an individual or specific mode of action affecting the tumor. Researchers have studied cell viability and cell death mode by treating cancer cell lines with different anticancer agents to test their effectiveness. The results provide the dose of the drugs needed to kill the cells and also recreates the behavior of a specific physiological condition & body location (body part and if tissue is cancerous or not). The dose-

response curves generated have pointed out the particular concentrations observed effects. The IC_{50} concentrations generally found on toxicological tests show the concentrations in the dose-response turn. The subjects under study (cells, animals, humans) are inhibited on growth or killed by fifty percent (50%). In studies where small mammals have been used (rats & mice), extrapolation on the dose-response curves obtained helps estimate the dose needed to treat a person. Oncologic toxicology preclinical studies identify safe and effective drug doses, minimize patients treated with ineffective doses, and assist in designing human dosing regimens and escalation schemes (Clark et al., 1999).

Anticancer drug therapy's objective is to activate cell death in cancer cells through apoptosis (Hong & Yi-min, 2006), necrosis, or the inhibition/interruption of uncontrolled growth (Jing, 2006) to control the formation of tumors. To overcome hypoxic tumors therapy's resistance (Gray L H, 1953; Churchill-Davidson E, 1966; Hodgkiss R J, 1991), researchers took an alternative approach not only to inhibit or interrupt this abnormal cell growth but also to target only the malignant cells without damaging healthy cells using hypoxia to its advantage.

Anticancer drug selectivity

Selectivity of cancer cell death induction can be obtained by enhancing cellular oxidative stress, activation of reductase enzymes, and oxygen-reduction chemistry in hypoxic cells (Hodgkiss, 1991). Amine compounds (including polyamines) and their analogs have been proved in recent research to have a significant anti-tumor activity for solid tumors (Chol-Ha et al., 1997). Nitro-aromatic compounds have shown the capability of binding by intercalation, specifically in the nuclear region of the cell, due to their affinity to DNA (Hodgkiss et al., 1991). Baez et al., 1983 had defined benzazolo quinolinium salts (BQ's) as a new member of anti-cancer drugs whose activity can be attributed to binding to DNA to interfere with its function as a template for nucleic acid synthesis in susceptible cells (Waring et al., 1981). Nitro benzazolo quinolinium salts (NBQ's); are reductively activated by enzymatic reducing agents' hypoxanthine (or

xanthine)/xanthine oxidase and nicotinamide adenine dehydrogenase (NADH) in a hypoxic environment (Alegría et al., 2003). NBQ's have a nitroarene's chemical type-structure, proving its potential as an anticancer drug (Trocher J H, 1997). This selective toxicity upon solid tumors is favored under hypoxic conditions by being bio-reduced by various reductases (Wardman et al. 2001; Brown et al., 2000).

BQ's as an anticancer drug

BQ's stands for bezazolo [3-2a] quinolinium salts and have been synthesized by Dr. Osvaldo Cox since 1982. They have been studied for anticancer drug development. They have proven to target topoisomerase II (an enzyme that controls, maintains, and modifies the structure of DNA), inhibiting it by arresting it in a covalent cleavage complex (Vivas-Mejia et al., 1997a). Some cause cytotoxic effects in human carcinoma cell lines, where A431 was included as one of the most sensitive and at concentrations similar to those required for m-AMSA (a current anti-tumor drug used for cancer treatment) (Vivas-Mejia et al., 1997b).

From the family of BQ compounds that have been synthesized, five were selected for the development of this study: NBQ_{38}, NBQ_{95}, BQ_{108}, $ABQ_{38,}$ and ABQ_{95}. (Figure 1). All BQ's share similar arranges in a chemical structure, having four (4) interconnected rings and two side chains. The difference lies in the terminal group of two opposite side chains. NBQ_{38} and $NBQ_{95,}$ the terminal group, are nitro, while ABQ_{38} and ABQ_{95} have an amino-terminal group. These differences in structure allow us to evaluate if those drugs containing the amino group will be more ready to be available for reduction/oxidation and, therefore, for potential binding capacity with mitochondrial or nuclear DNA than those with the nitro group.

Cytotoxicity

Cytotoxic and mutagenic properties have already been evaluated on five different BQ's, including four of the BQ's proposed to be studied in this assay (Arroyo et al., unpublished data). For cytotoxicity determination, the A431 epidermoid carcinoma cell line was exposed to NBQ_{38}, NBQ_{95}, $ABQ_{38,}$ and ABQ_{95} at different concentrations for 48

hours. Dose-response curves were generated, revealing the cell viability using Trypan Blue exclusion assay (differential indicator for dead/alive cell). NBQ_{95} had the lowest IC_{50} (28uM), thus the most potent agent (more cytotoxic), following ABQ_{38} with 32uM and NBQ_{38} & $ABQ_{95,}$ both with 36uM. Inferring that apoptosis is involved as the primary cell death mechanism, then the potencies of cytotoxicity will be positively related to the proportion of apoptotic cells intended to be found in this study.

Reduction

In hypoxic conditions, intermediate active compounds can be produced through reduction by the activity of a reductase enzyme (xanthine oxidase [XO]). This statement was supported by studies where other anticancer drug metabolites intermediates have been generated upon this enzymatic decomposition because of the activated redox chemistry by activating reductase enzymes (Wardman, 2001) and reducing the nitro group from a NO_2 to an NH_2 (six oxygen singlets removed in total). The reduction makes it a more capable compound to bind upon intercalation to the DNA. This characteristic has made BQ ideal for anticancer drugs since it blocks DNA and RNA synthesis, interfering with protein production. Moreover, it can impact cellular respiration interrupt adenosine triphosphate (ATP) production leading to cell cycle arrest. Inhibition of topoisomerase II has also been identified, concluding that DNA replication and transcription are interrupted.

Oxidation

Ellipticine is a compound that has a structure similar to the BQ's. Its natural source is from the leaves of the evergreen tree *Ochrosia elliptical* Labill (Apocynaceae) (Goodwin *et al.*, 1959). In the presence of oxygen, the enzymes responsible for generating intermediate drug metabolites through oxidation are the cytochrome P450 (CYP1A1) peroxidases (Stiborova et al., 2006). This compound has been shown to be an intercalating agent (Kohn, K. W. et al., 1975), a Topoisomerase II activity inhibitor (Monnot et al. 1991; Chu & Hsu, 1992), andon are p53 protein phosphorylation inhibition in several human cancer cell lines. This also leads to uncoupling mitochondrial oxidative

phosphorylation and disrupts the energy balance of cells (Aimova & Stiborova, 2005). Considering the Ellipticine mechanism of action, we expect the potential anticancer drugs presented in this study to be like Ellipticine.

BQ's mechanisms of action within the human cancer cells are not explicitly known. In hypoxic conditions, adducts have been found; now, in an oxidative environment,we will develop different essays to elucidate its partial mechanism route in the presence of oxygen. This could be achieved by identifying the product of BQ's oxidationby analyzing the mass spectrum of adducts. A more exhaustive essay will need to be performed using CYP 450 specific enzymes to determine the metabolites produced. This subject will not be covered in this dissertation. As a future project, that will need to be investigated and if apoptosis and adduct formation are positively identified.

In general, the results showed that BQ's were interacting with those cells treated, causing cytotoxicity at different exposure concentrations (Arroyo, 2007 unpublished data). BQ analogs have also been tested for mutagenicity in Drosophila DNA and showed negative results for this parameter, suggesting that their application in normal human tissues might not affect DNA (Pérez-Chiesa et al., 1991). However, mutagenicity in epidermoid carcinoma human cells exposed to BQ's presented mutagenicity's at different mutation frequencies (Arroyo, V. et al., 2006).

BQ's adducts with deoxyguanosine

It is known that many chemicals can form covalent adducts with DNA, directly, in some cases and following metabolic activation, in others (Josephy, 1997). The chemical Characterization of adducts clarifies the nature of the activated intermediates which reacted with DNA. For example, xenobiotics that undergo biotransformation have helped elucidate the mechanism of metabolic activation (Josephy, 1997). Asbestos (aka xenobiotic) has been shown to induce DNA mitochondrial damage and cell dysfunction, linked to the development of apoptosis (Shukla A, 2003).

Study's justification

Cell proliferation and programmed cell death are mandatory for maintaining homeostasis in normal tissues, and alterations in the level or rate are essential elements of the carcinogenic process (EPA, 2003). A balance between the two directly affects the survival and growth of initiated cells and pre-neoplastic and tumor cell populations (i.e., increase in cell proliferation or decrease in cell death) (EPA, 2003). Identifying new agents that can selectively kill cancer cells becomes crucial in clinical treatment. The aim is to kill cancer cells without damaging healthy cells selectively. The most used chemotherapy for eliminating cancer can be achieved by activating the mitochondrial apoptotic pathway (Diaz-Rubio; Jing, 2006). Measurements of these events contribute to the evidence for the mode of action of understanding proliferation (EPA, 2003) as a tool of cancer risk management.

When applying information from studies on cell proliferation and apoptosis to risk assessment, it is crucial to identify the tissues and target cells involved, measure effects in both normal and neoplastic tissue, distinguish between apoptosis & necrosis, and determine the dose that affects these processes. DNA adducts have recently been used as biomarkers for exposure (De Flora S, 1996). They might also work as a biomarker for treatment showing the extent of their reach in anticancer drug development.

The fact that BQ's have been shown to kill human cells in vitro (Arroyo, 2007 unpublished data) demonstrate that they are penetrating the cell, making them future potential candidates for cell disruption. Since DNA is present in the nucleus and mitochondrion (Lewis, 2006), we understand that the BQ's bind to genomic DNA in an excellent probability. To evaluate if adduct formation with DNA was possible, calf thymus (CT) bovine DNA was used as standard and mixed with different exposure concentrations of BQ's. This sample was digested with three enzymes (Nuclease P-1, Phosphodiesterase I, and Alkaline Phosphatase). Elutes were analyzed with HPLC-ESI MS/MS. Adducts were found bounded by intercalation at the 2 deoxyguanosine to

CT-DNA nucleosides (Figures 5 & 7) (Zayas, B. et al., 2007).This indicated that BQ's could penetrate the nuclear and mitochondrial membrane, inducing the possibility of binding to the DNA contained in it. Since substances that bind or react with DNA have the potential to disrupt cell functions and lead to cell death(Luo et al., 2005; Esterbauer et al., 1991; Kehrer & Biswal, 2000) BQ's might show this characteristic if adducts from genomic DNA of treated cells are detected.

BQ's undone work

We propose to help describe the potential interaction of BQ's on A431 treated cells and provide a more straightforward path for the mechanism using the IC_{50} concentrations. There was no experimental data regarding treated cells with these compounds in the presence of oxygen for shorter/more extended exposure periods (24 and 72 hours) with the IC_{50} concentrations. Qualitative and quantitative cytotoxicity evidence at 24, 48, and 72 hours will establish the drug potency and if the cells line shows resistance to these drugs.

No data has been collected relating cell drug interaction using cell auto-fluorescence as an indicator, mitochondrial membrane damage, and intrinsic/extrinsic caspase activation; as a result, target for exposure to BQ's in vitro. Qualitative and quantitative changes in the fluorescence of the treated cells will evidence cell drug interaction. Qualitative and quantitative data on the permeabilization of the mitochondrial membrane and the activation of caspases 3 and 7 not only will serve as indirect pivotal biochemical indicators of apoptosis but also will help us to visualize the difference at which the order of these events occurs and how it changes with each drug from drug to drug.

Apoptosis detection will point out if these cells have been induced to die using the permeabilization of the mitochondrial membrane as an early apoptotic event and caspases 3 and 7 activations as late effector indicators. Mitochondria will be stained with a cationic dye that preferentially gets retained in the nondisturbed membranes forming an aggregate and showing a red color. Disturbed mitochondria will have their membrane

permeabilized, and the dye will escape to the cytoplasm showing its monomeric form showing a green color. This works as an indicator of early apoptosis without involving the measurement of Caspases intervention. Cytotoxicity has been tested but for three time periods to see if there is resistance to the drug on those cells treated (V. Arroyo, unpublished data)

Genomic DNA extraction from treated cells will allow future analysis on the possible adduct formation using a mass spectrum obtained from an HPLC-ESI MS/MS. DNA adduct evidence will help clarify whether nuclear and or mitochondrial DNA is the specific treatment target of this family of drugs. These steps will allow us to differentiate possible mechanisms of action that BQ's induce on human epithelial cancer cells as anticancer treatments.

Investigative inquiries

- Are some BQ's more cytotoxic than others at different exposure times in A431exposed cells?
- Do BQ's induce cell-drug auto-fluorescence in A431 exposed cells?
- Is mitochondrial membrane permeabilization involved in the cell death and apoptotic induction of BQ exposed A431 cells?
- Are caspases 3, 7, and 8 involved in the cell death and apoptotic induction ofBQ exposed A431 cells?

Goal

Analyze extended cytotoxicity effects, cell-drug auto-fluorescence, and apoptotic induction pathway in A431 treated with BQ's IC_{50} concentrations by comparing:

- Permeabilization of the mitochondrial membrane
- Activation of caspases 3, 7 and 8

Objectives

1) Qualitatively and quantitatively determine BQ's cell drug cytotoxicity and potency of BQ's IC_{50} concentrations on A431 at 24, 48, and 72 hours.
2) Qualitatively determine cell drug uptake viewing differences in cell

fluorescence as an indirect indicator of the extent of drug cell interactions.

3) Determine apoptosis induction pathway of BQ's IC_{50} concentrations on A431 using:
    a. Qualitative and quantitative mitochondria membrane permeabilization
    b. Qualitative and quantitative caspases 3 and 7 activation
    c. Qualitative caspases 8 activation

CHAPTER II

LITERARY REVISION

"Cellular immortality is a basic trait of cancer. The unlimited proliferative potential of transformed cells enables many of the most insidious characteristics of cancerous diseases. Disrupting the mechanism by which aberrant cells escape mortality offers a significant opportunity for chemotherapy" (Lambert T H & Danishefsky S J, 2006). Recently anticancer drug development has been modified to induce growth inhibition through various pathways. Many of the compounds under study as anti-tumor agents act at multiple steps in the cell cycle. Their effects may be cytostatic (cell-cycle inhibitors) or cytotoxic (inhibit the proliferation of cancerous cells) depending on the cell cycle status of the target cells. The molecular interactions involved may suggest ways to sensitize cells to the effects of these compounds. Combinations of drugs, applied in a specific sequence, may be used to maneuver a tumor cell population into a state where it is most susceptible to the cytotoxic effects of novel or chemotherapeutic agents (Shapiro1 G I, Harper J W, 1999).

The practical applications of these techniques are helpful to determine whether a proposed anticancer drug might be a valuable resource for potential cancer treatment. To save time for researchers, synthesized drugs are tested first in vitro to check their potency to induce cell growth inhibition using specific cancerous cell lines.

Historical background

The Cancer Fact & Figures report from 1999 to 2005 expressed estimated cancer trend continuance over the years. Women's cancer cases predominate breast, lung, and colon cancers from 1992 to 2000. For men, more than half of the new cancer cases and deaths are from prostate, lung, and colon cancers (EPA, 2003). In the last 40 years, cancer had a slight but constant increase in cancer cases (Fisher, A.C. et al.

1995).

Cancer treatment costs

Cancer treatment spending has risen but remains stable in proportion to total US treatment spending (NCI, 2005). Between 1995 and 2004, the overall costs of treating cancer increased by seventy-five percent (75%) (NCI, 2005). Anticancer therapy constituted the most significant component of cancer-related drug costs accounting for sixty-seven percent (67%) and increased to seventy-six percent (76%) from 1995 to 1998 (Halbert et al., 2002). Most charges were incurred in agents administered by injection to treat solid tumors (Halbert et al., 2002). The financial costs of cancer treatment are a burden to people diagnosed with cancer, their families, and society. By 1998 the average cost of cases and deaths per person for anticancer drug therapy was about two thousand dollars ($2,000) in a year (Halbertet al, 2002). Medicare does not cover certain cancer care expenses, such as oral medicines commonly used to treat breast and prostate cancer (NCI, 2005).

Direct medical expenditures are only one component of the total economic burden of cancer. The indirect costs include time and economic productivity losses resulting from cancer-related illness and death. Cancer treatment costs increased dramatically between 1963 and 2004 (NCI, 2005). The total economic burden of cancer in 2004 is estimated to have been one hundred and ninety billion dollars ($190,000,000,000) (NCI, 2005). By 2006 the cost had risen to two hundred and nine point nine billion dollars ($209,900,000,000) (ACS, 2006). Additionally, cancer screening spending was estimated to have a cost an additional ten to fifteen billion dollars ($10,000,000,000 to $15,000,000,000) in 2004. Still, the proportion of these costs to all health care expenditures had remained stable (NCI, 2005). It is expected that cancer costs may increase faster than overall medical expenditures (NCI, 2005). As the population ages, the absolute number of people treated for cancer will increase faster than the overall population (NCI, 2005). Cancer cases will increase relative to other disease categories, even if cancer incidence rates remain constant or decrease

somewhat (NCI, 2005). Costs also are likely to increase at the individual level as new, more advanced, and more expensive treatments are adopted as standards of care (NCI, 2005). NCI has been monitoring cancer medical costs for cancer care, and in the last 30 years, the cost percentage has been constant (NCI, 2005).

Among the four most common cancers, the first-year costs for lung and colorectal cancer are higher because screening is not as commonly used to detect these cancers (NCI, 2005). The highest incidence of cancer in Puerto Rico identifies the mouth, pharynx, esophagus, stomach, and cervix (Martínez et al., 1975). The lack of health insurance and other healthcare barriers prevent many cancer patients from receiving optimal medical care (ACS, 2006). This statement explains why there is a higher incidence of cancer mortality in underdeveloped countries and low-income areas.

Cancer in women as an example

Minority women in the US (including Puerto Ricans) are less likely to have health insurance and more likely to be underinsured. They lack a regular health care source (Glanz et al., 2003, OHRIA, 2004). They are further disadvantaged by barriers like long distances to health clinics, language differences, and a lack of culturally sensitive health care (Glanz et al., 2003, OHRIA, 2004). The most frequent cancer in this group is lung cancer (1st) and breast cancer (2nd) (Jemal A et al., 2004). The cancer incidence in Puerto Rican women in the US could be attributed to thirty-point three percent (30.3%), the 2nd highest among minority groups. They also have a twenty-eight to thirty-nine percent (28%-39%) obesity as a cause of high animal fat intake, being the 8th and amongst the lowest on minority groups (Glanz K, 2002). Puerto Rican women on the island had cancer of the cervix ranked as number one (1). The incidence of malignant tumors of the upper alimentary tract (esophagus and stomach) in this group was higher than that of the lower organs (colon and rectum) (Martínez et al., 1975).

Mortality rates from breast cancer have been increasing for at least 40 years in most Latin American Countries (Robles S C & Galanis E, 2002). Among women, lung cancer death rates increased from 1995 through 2002, but lung cancer incidence rates

stabilized from 1998 through 2002 (Edwards, B. K. et al., 2005). Socioeconomic development and consequent changes in reproductive behaviors over the past 50 years have contributed to the increased risk of breast cancer (Robles, S. C. & Galanis, E., 2002). Socioeconomic development has also increased women's health awareness and, therefore, the demand for quality services. In industrialized countries, screening and widely available, high-quality treatment protocols are being implemented as the primary strategy for breast cancer control (Robles S C & Galanis E, 2002). The use of chemotherapy with surgery has been screened as a possible cancer control in women, specifically epithelial ovarian cancer (Morrison, J. et al., 2007). *Morrison and others* tried to determine if chemotherapy would improve the surviving quality if performed pre-surgery compared to post-surgery and revealed no significant difference. This confirms that although surgery is the preferred treatment method, chemotherapy still needs to be developed and improved to be used in combination with surgery.

Conceptual frame

Cancer, solid hypoxic tumors

Cancer is a generic term for a group of more than 100 diseases that can affect any part of the body (WHO-World Health Organization). In humans, accelerated excessive/abnormal cell growth has lost its functions: it can invade nearby tissues and spread through the bloodstream and lymphatic system to other body prats. Cancer cells divide through proliferation; normal cells differ by only growing.

There are several main types of cancer. Carcinoma begins in the skin or tissues that line or cover internal organs. Sarcoma is cancer in bone, cartilage, fat, muscle, blood vessels, or other connective or supportive tissue. Leukemia is cancer that starts in blood-forming tissue such as the bone marrow and causes large numbers of abnormal blood cells to be produced and enter the bloodstream. Lymphoma and multiple myeloma are cancers that begin in the cells of the immune system (National Cancer Institute, 2006). In specific reference to this study, the cancer cell line under investigation is representative of the squamous cell epithelium. This is the part of the tissue in the outer

layer of the skin.

These processes are multistage (Yuspa S H, 1994). They are characterized by the capability of destroying non-infected tissue (infiltration), invading, and sending cells to different zones of the organism, where they can grow originating new tumors and replicate. These stages (cancer pathogenesis) are initiation (T1); promotion (T2); pre-malignant progression (T3); and malignant conversion (T4) (Yuspa S H, 1994) (U.S. EPA, 2003). Some scientists have defined these stages upon DNA changes; six cellular alterations, or hallmarks, collectively drive a population of normal cells to become cancer. The six hallmarks are (i) self-sufficiency in growth signals (SG), (ii) insensitivity to antigrowth signals (IA), (iii) evasion of apoptosis (EA), (iv) limitless replicative potential (LR), (v) sustained angiogenesis (SA), and (vi) tissue invasion and metastasis (Hanahan and Weinberg, 2000). Genetic instability is an enabling characteristic that facilitates the acquisition of other mutations due to defects in DNA repair. These hallmarks from a candidate set of rules that underlie the transformation of normal tissue to a cancerous one (Spencer SL, 2006) when multi-genic (various genes implicated) and multi-factorial (various factors involved) develop in a long period by successive accumulation of these genetic mutations (SEB, 2000).

Because cancer results from a series of genetic alterations in the genes that control cell growth, division, and differentiation (Vogelstein et al., 1988; Hanahan and Weinberg, 2000; Kinzler and Vogelstein, 2002), the ability of an agent to affect genotype (and hence gene products) or gene expression is of obvious importance in evaluating its influence on the carcinogenic (or anti-carcinogenic) process (U. S. EPA, 2003). Cells that escape their standard growth control; develop into tumors. Two types of genes encode proteins that govern cell cycle speed—proto-oncogenes (cause unregulated growth) and Oncogenes (slow down cell growth).

Oncogene p53 (in chromosome 17) prevents various tumors in the human body and triggers apoptosis in cells with DNA damage (Levine A J, 1997; Lowe et al., 1993). Mutation of p53 disables the inhibition of cell growth (Hertzog J, 2003, Garbe J, 1999),

promotes apoptosis (Yu et al., 1996, Yu et al. 1997, Spillare et al. 1999), decreases DNA repair (Hertzog, J., 2003; Bowman, K. K., 2000, Ford J M, 1997) and diminishes catalase activity (Hertzog J, 2003; Hoffschir, F, 1993). Those cells with expression -/- of p53 will show resistance to drugs whose target is to activate apoptosis, following malignant cell division undisrupted and cancer proliferation.

Apoptosis and mitochondria as the target of anticancer drugs

Cells regulate their growth naturally through apoptosis. Apoptosis is a morphologically distinct form of cell death (Padanilam P J, 2003) in damaged cells proliferating in an organism without damaging surrounding cells and tissue (Philchenkov A, 2004), counterbalancing the excess during mitosis. It is an integral part of maintaining the immune system's regular sculpting of the early embryo (Padanilam P J, 2003, Gluksmann A et al., 1951; Ranganath R M et al., 2001). Suppression of apoptosis can result in autoimmune diseases and cancer (Padanilam PJ, 2003; Ferreira CG et al., 2002; Kerr J F et al. 1972).

Cancer cells avoid growth control and or apoptosis by overriding regular cell cycle checkpoints and proliferating faster than normal cells forming tumors. Then the activation of the apoptosis pathway is a crucial mechanism to kill tumor cells (Debatin, 2004). Lately, researchers have developed therapies that include the use of anticancer drugs whose main objective is to activate apoptosis or the inhibition/interruption of uncontrolledcell growth on cancer cells (Vance K, 2005).

Apoptosis has many stages, and membrane permeabilization in mitochondria is one of the first (Petit, P.X. et al., 1996; Zamzami et al. 1996a; Zamzami, N et al. 1996b; Marchetti P et al., 1996, Singh et al. 1999). Mitochondria are the site of the tri-carboxylic acid cycle and oxidative phosphorylation, which comprise cellular respiration; needed to produce ATP (Cereghetti GM & Scorrano L, 2006). Since all mammalian cells require energy in the form of ATP (Adenosine triphosphate) to perform all essential functions, mitochondria supply normal cells' ATP and are involved in energy-dependent apoptosis control. Now, if a neoplastic transformation has been shown to induce functional

changes in morphology and functionality of mitochondria (Pedersen P L, 1978; Lincon V et al. 1980), its dysfunction has offered to participate in the induction of apoptosis and has even been suggested to be central to the apoptotic pathway. (Ly JD, 2003). This has made the mitochondrial organelle a therapeutic target for anticancer treatment.

Cytotoxic drugs have been shown to lead to activation of the intrinsic mitochondrial pathway of apoptosis, contributes to the sensitivity of tumor cells towards cytotoxic treatment (Debatin, 2004), and in cultured cells exposed to toxins and chemicals (Martin L J, 2001), including dyes (ex. EDKC) (Oseroff A R et al., 1986). Recently four different mechanisms have been proposed by which anticancer drugs might induce mitochondrial toxicity and unchain apoptosis: (1) inhibiting the electron transport chain, (2) uncoupling or inhibiting mitochondrial oxidative phosphorylation, (3) induction of mitochondrial oxidative stress (4), and interruption of normal protein synthesis/biogenesis (Chan et al. 2005). Amplifying mitochondria-drug interaction can also imply that apoptosis is the preferential cell death response upon treatment.

Biochemical apoptosis stages

Apoptosis could be activated by two primary mechanisms (Kuo, Y. C. et al. 2006); the intrinsic pathway, which involves mitochondria-related apoptosis, and the extrinsic pathway, which involves the death-inducing signaling complex (DISC) (Green and Reed, 1998; Sheikh and Huang, 2004). Many apoptotic stimuli that induce metabolic stress in cell organelles include fragmentation of the endoplasmic reticulum and remodeling the cristaceae as early positive signals for intrinsic apoptosis. However, most of all, apoptotic signaling eventually converges on the mitochondria apoptotic death pathway (Philchenkov A, 2004), leading the cell finally to die due to its dysfunction. Not all apoptotic events necessary involves the activation of the caspases for its execution, but it does compromise the permeabilization of the mitochondrial membrane as the key biochemical indicator and initial hallmark of activation (Constantini P et al., 2000; Green, D. R., and Reed, J. C., 1998; Skulachev, 1996).

Mitochondria have been pointed out to be centrally responsible as an early

indicator of apoptosis on the intrinsic apoptotic pathway. Various inducers of apoptosis can directly or indirectly influence disruption of the organelle affecting the permeability of the outer mitochondrial membrane, ultimately leading to the completion of apoptosis. Before cell lysis, permeabilization of both mitochondrial membranes and the loss of inter-transmembranal potential (ITP) is the first response for disruption in normal and cancerous cells (Constantini P et al., 2000). The dissipation in the ITP ($\Delta\Psi_m$) causes the permeabilization of mitochondrial membranes (Constantini P, 2000; Xiang J, 1996; Pastorno J G, 1998; Hirsch T, 1997; Brunet C L, 1998; Berndt C, 1998, Zamazami N., 1996; Vander Heiden M. G., 1997; Bossy-Wetzel G., 1998) pursued by the liberation of apoptogenic proteins such as cytochrome c (Shinohara Y. et al. 2002), apoptosis induction factor (AIF) (Constantini P., 2000; Li J. D. et al., 2003) and SMAC/DIABLO (Karbowski M. et al., 2003). The liberation provokes a domino effect leading to the apoptosis cascade by activating other pro-apoptotic proteins and inactivating anti-apoptotic proteins (Letai A. et al., 2002; Karbowski M. et al., 2003).

Death receptors localized on the cell's exterior (such as Fas, TNF, and TNFR1) act as upstream activators of the extrinsic pathway. Opposed to TNF, Fas ligand-receptor is the most straightforward, direct, and specific apoptotic extrinsic upstream activator that subsequently triggers the caspases cascade (Dash, P., 2007; Timmer et al., 2002).

Caspases are a family of proteins from the cysteine proteases that become activated upon apoptosis (Dash, P., 2007). They break down cellular components that cause fragmentation of nuclear DNA into nucleosomal units upon activation of caspase-activated DNase (CAD) (Dash, P., 2007). The apoptotic caspases cascade is a network of interconnected initiators and effectors that order the main event of execution. The most important and revised caspases are the initiator caspases 8, 9, and 10 and the e3 and 7. Caspases 8 and 10 activate through the Death Inducing Signaling Complex (DISC) (Fas ligand). Caspase 9 becomes activated through the permeabilization of the mitochondrial membrane, which releases cytochrome *c*, Apaf-1, and pro-caspase 9.

(Scorrano L, 2005). Subsequently, Caspase 9 could activate caspase 3 and 7 downstream.

Caspases 3 and 7 become activated through DISC, resulting in a permeabilized mitochondrial membrane. Also, Caspases 8 and 10 could converge with the intrinsic mitochondrial pathway, starting apoptosis parallel to the extrinsic and downstream activating caspases 3 and 7. Both intrinsic and extrinsic pathways include activating the mitochondria apoptotic-dependent (intrinsic) death pathway by permeabilizing the inter-membranes leading to cytoplasmic efflux of cytochrome *c* as key events (Philchenkov A, 2004).

Case study

BQ's and selective toxicity

Fluorescent dyes and anticancer drugs selectivity

Some anticancer drugs have been found in nature. Berberine is present on several *Berberis* genera (Mantena S. K. et al., 2006) and Ellipticine on *Oschrosia elliptica* (Kuo, Y. C., et al. 2005; Goodwin *et al.*, 1959). As opposed to Berberine, Ellipticine works as a fluorescent-specific probe on isolated mitochondria organelles in single living cells (Sureau et al., 1993). Berberine and Ellipticine are potential anticancer drugs with a structure similar to BQ's (Figure 1). Both compounds are apoptotic agents (Kuo, Y.C. et al. 2006; Mantena, S. K. et al., 2006) with somewhat similar mechanisms of action.

Ellipticine's mechanism of action involves the induction of apoptosis through the extrinsic pathway activating the Fas death-ligand, which converges in the initiation of the intrinsic mitochondrial pathway (Kuo, Y.C. et al. 2006). Berberine is significantly selective toxic for cancer cells (A431) when compared to normal cells, initiates apoptosis activating caspases 3 and 9 and disrupting the mitochondrial membrane in a dose-dependent manner (Mantena, S. K. et al., 2006). At the DNA level, Ellipticine is an intercalating agent, Topoisomerase II activity inhibitor (Kohn, K. W. et al., 1975; Stiborova, 2006) and restores its tumor suppressor transcription function on mutant and

wild type p53 allowing the execution of apoptosis (Kuo, Y.C. et al. 2006; Monnot et al. 1991; Chu & Hsu, 1992). DNA adduct formation by ellipticine has been attributed to induce CYP1A1 and or CYP1A2 oxidative activation (Stiborova et al., 2006) to metabolites that generated two major DNA adducts in human and rat livers (Aimová, D et al. 2007).

Dyes such as Rhodamine 123 had been demonstrated to identify mitochondria from the rest of the cell organelles without causing cytotoxic effects to different cells lines at physiological pH (=7) (Lincon V et al., 1980; Oseroff A R et al., 1986). This is possible due to its cationic charge (positive); allowing this compound to be attracted to the highly negative electric potential across the mitochondrial membrane (Lincon, V. et al., 1980; Haaker, H. et al., 1972; Oseroff, A. R., et al., 1986). Its fluorescence allowed selective localization of the mitochondrion organelle (Davis, S. et al. 1985; Lincon, V. et al., 1980). Mitochondria has lower dye uptake in normal cells disrupted with chemicals (FCCP) (Fossati, G. et al., 2003). Using this dye with carcinoma cell mitochondrion has shown a higher uptake concentration and a longer retention time when compared to normal cells (Davis, S. et al. 1985; Summerhayes, I. C., 1982; Bernal, S. D., 1982; Chen, L. B., 1983; Oseroff, A. R., 1986). Different cancer cell lines treated with chemotherapy showed dye retention as a signal that mitochondria were successfully damaged (Davis, S., 1985; Bernal, S. D., 1983, Oseroff, A. R., 1986).

JC-1 is another dye used as a fluorescent mitochondrial indicator that also has lower retention of the dye in mitochondrion in normal cells disrupted with chemicals (Fossati G et al., 2003). This dye works like a differential stain allowing visual confirmation and measurement of the dissipation of the inter transmembranal potential (Johnson, L.V et al., 1980; Chen, L. B. 1989). JC-1 shows a red stain in nondisrupted mitochondrial membrane versus green in the disrupted mitochondrial membrane on cells treated with Valinomicyn (Inai Y. et al., 1997; Nomura Y., 2004; Shinohara Y. et al. 2002). Valinomycin has been used widely as a positive control to induce apoptosis by disrupting the mitochondrial membrane and comparing it with others with cationic

preferential dyes just as this one.

The loss of retention of these different selective mitochondrial dyes directly indicated that the mechanism in which the uptake of dye was lessened because of the loss of the inter transmembrane potential that led cells to undergo apoptosis (Inai Y. et al., 1997; Nomura Y., 2004; Green D et al., 1980; Constantini P et al. 2000). Now considering that mitochondria preferentially retain substances with cationic properties in the membrane (Johnson L V, 1980; Chen L B, 1982; Davis S, 1985; Oseroff A R, 1986, Powers S K, 1987) suggesting that certain anticancer drugs that exhibit this characteristic could behave similarly to certain selective dyes in term of selective binding to the mitochondrial membrane.

Mitochondrial Selectivity and Apoptosis Development

Cancer cells have disrupted mitochondrial function that includes a higher membrane potential. This higher potential results from a higher negative charge on the membrane when compared to mitochondria in normal cells (Power S K, 1987). The higher negative charge in cancer mitochondrial membrane enhances cationic charged chemotherapeutic treatments (Johnson L V, 1980; Chen L B, 1982; Davis S, 1985; Oseroff A R, 1986, Powers S K, 1987) such as those on BQ's. Induction of functional and structural damages to this organelle by cationic charged chemotherapeutics has been demonstrated to cause cell death by reducing the intermembrane potential (Divi, R.L. et al. 2004), leading ultimately to apoptosis. BQ's cationic charge presents promising selectivity potential towards the damage of mitochondrial membrane potential upon treatment of cancer cells. The positive charge in the basic BQ's structure might give them the same properties as other cationic substances (Rhodamine 123) to be retained preferentially by the mitochondrion and later to negatively charged DNA contained in it (Oseroff A R et al. 1986; Detty M R, 1987; Foley J W, 1987; Chen L B 1987, Power K P, 1987). Also, the flat hydrophobic structure of aromatic amines and polycyclic aromatic amines make them excellent candidates as DNA intercalators; specifically selective for the guanine C-8 (Josephy, 1997)

Metabolism changes and selectivity for apoptosis development

In tumors, adenosine triphosphate (ATP) synthesis is dependent on O_2 pressure, and it is delivered to neoplastic cells is reduced by structural abnormalities of tumor and disturbed circulation. Otto Warburg investigated cancer cells' metabolism and stated in his theory that these cells switch from OXPHOS to the glycolytic pathway to produce ATP (Mathulapa E.J. et al., 2004). OXPHOS involves the generation of ROS while the glycolytic pathway does not. ROS production in mitochondria works as an indicator of oxidative stress as partially responsible for cell death (Luo J, 2005) and the inhibition and or over-expression of gene p53 (Bensaad & Voudsen, 2007).

Oxidative Phosphorylation Pathway

Mitochondria are organelles that regulate the energy-producing more than ninety percent (90%) of their metabolic energy in eukaryotic cells and transform organic material into energy in the form of ATP by oxidative phosphorylation (OXPHOS) and produce ROS in physiological conditions (Balaban et al., 2005; Luo J et al., 2005).

Several lines of evidence support a role for ROS as a mediator of apoptosis. Oxidative stress occurs in cells when there is an imbalance between the rate of generation and removal of ROS. The gene expression pathway studied by Bensaad & Vousden has shown a relationship with the expression/suppression of the p53 gene and the amount of ROS. There is also the relationship of ROS and p53 as it regulates the expression of antioxidant sestrin proteins which help to regenerate the factors involved in the breakdown of hydrogen peroxide (H_2O_2) (Budanov, A. V. et al., 2004).

One electron reduction of oxygen produces superoxide, whereas two-electron reductions produce hydrogen peroxide (H_2O_2) (Fridovich I, 1986). In mammalian cells, the conversion of H_2O_2 to H_2O is also accomplished by the reaction with glutathione catalyzed by glutathione peroxidases. H_2O_2 is considered a ROS product and had demonstrated to induce apoptosis upon treatment in various cell lines. (Simizu, S., 1998; Hockenbery, D. M. 1993, Gardner, A M, 1997) ROS production has been detected in significantly higher levels when other cell lines had been exposed to other substances

(Luo, J. et al., 2005). In tumor-derived mutations, the altered expression on p53 has shown that it initiates the apoptotic pathway or shows resistance to drugs whose objective is to induce apoptosis (Volgestein et al., 2000).

Glycolytic Pathway

Most tumor cells acquire energy from the metabolism of glucose to lactic acid even in conditions where there is oxygen available (Ramon, B. and Caro, J., 2007, Kim, J. W., Gardner, L. B., and Dang, C. V., 2005). This aerobic glycolysis is different from anaerobic glycolysis because it occurs as a normal adaptation response to hypoxia. There is still a debate on aerobic glycolysis; some argue it results from an adaptive response to hypoxia, others from cancer cell alterations (Kim, J. W., Gardner, L. B. and Dang, C.V., 2005). However, tumors present hypoxic conditions due to abnormal vascularization (supply of oxygen and nutrients) making this metabolic route essential for survival.

Recent findings have suggested that forcing cancer cells into mitochondrial metabolism (OXPHOS) efficiently suppresses cancer growth (Ristow, M. 2006). This approach has integrated the function of p53 as the regulator between the switch of glycolysis and OXPHOS (Rozan and El-Deiry, 2007; Matoba, S. et al., 2006). Mataboa and his coworkers found in 2006 that the ratio of ATP produced by glycolysis versus ATP produced by aerobic respiration increased with p53 deficiency. There is also the DNA damage that could be induced by chemotherapeutic agents, where p53 promotes cell death via the intrinsic pathway (Rozan and El-Deiry, 2007). When p53 is expressed, it promotes OXPHOS over glycolysis; when mutant or null, it promotes glycolysis over OXPHOS, as explained earlier by Otto Warburg in 1956. Ideally, a cancerous cell treated with a chemotherapeutic treatment could be induced to switch to OXPHOS due to a reversed mutation on p53 by DNA chemotherapeutic damage. The result could be ROS production as a by-product of reduction-oxidation, affecting the permeability of the mitochondrial membrane. This could finally lead to the intrinsic activation of the apoptotic pathway.

The use of nitro-aromatic compounds has been suggested to identify hypoxic cells. Bio-reduction of the nitro group makes the nitro group a more fluorescent one. This is because the nitro group quenches the fluorescence on the aromatic ring system. In the absence of oxygen, bio-reduction of nitro-imidazoles is known to lead to the binding of bio-reductive metabolites to cellular macromolecules (ex: DNA), which provides a mechanism for binding the fluorescent moiety to hypoxic cells. (Hodkiss, 1991 &1994). This is possible because the nitro-imidazoles are inhibited by oxygen. This biological reductive activation modality targets killing cells with limited amount of oxygen content (Alegría, 2003), hypoxic tumor cells (Wardman, 2001), and cells that have a preferred metabolic pathway that favors reduction (aerobic glycolysis).

Nitro benzazolo quinoline salts (NBQ's) is reductively activated by the enzymatic reducing agent's hypoxanthine and or xanthine oxidase and NADH dehydrogenase (Alegría, 2003). Its chemical type-structure (nitroarene) has proven its potential as an anticancer drug (Trocher, 1997) because is selectively toxic to solid tumors when is bio-reduced by various reductases, favored under hypoxic conditions (Wardman, 2001; Brown, 2000).

Another reducing enzyme that might be involved in the bio-activation of BQ's is NQO1. NQO1 is found in various human tissues throughout the body, in epithelial and endothelial cells (Winski, S. H., 2002). Levels of NQO1 in human tumors can be 5 five to twenty-five-fold greater than other reductive enzymes (Han, Y. et al. 2003; Smitskamp, W. et al., 1995) and its' frequently elevated or overexpressed in human tumors (Winski, S. H., 2002) when compared to normal tissue (Han, Y. et al. 2003).

The principal function of NQO1 is the detoxification of quinone xenobiotics and their derivates by two-electron reduction and probably contributes to the reductive activation of cancer chemotherapeutic agents (Han, Y. et al. 2003; Pink, J. et al. 1999). NQO1 involvement activity was tested against 69 human tumor cell lines from the NCI, National Institutes of Health for MMC. Data suggested that NQO1 was a critical activator of MMC by enhancing its antitumor effects on the cell lines.

Mitomycin C and doxorubicin (antitumor agents) are also quinones, and they are known to be activated or inactivated by a two-electron reduction catalyzed by NAD(P)H:quinone oxidoreductase-1 (NQO1) or by a one-electron reduction catalyzed by NAD(P)H:cytochrome P450 reductase (P450 reductase) (Han, Y. et al. 2003). NQO1 is known to utilize NAD(P)H as an electron donor, which generates NAD(P) (Gaiwak A, 2001). The reaction products of any of this metabolism are toxic intermediates N-hydroxy arylamines (Arlt et al., 2003). Both Amino and Nitro BQ's could be reduced or oxidized down to N-hydroxy arylamines (Zayaset al, 2007; Fan, Y., 2007, Jorga K, et al. 1999). N-hydroxy arylamine has been hypothesized to be the intermediate capable of forming covalent adducts with DNA (Zayas et al., 2007). An imbalance in the donation of electrons by NAD(P)H: NAD(P) upon redox of BQ's species could generate ROS.

The proposed reduction of BQ's is obtained by gaining 6 electrons to become the corresponding amine (Zayas et al., 2007). The proposed N-hydroxy arylamines reactive intermediate is obtained upon gaining four electrons from NBQ's or losing two from ABQ's. This suggests that the governing metabolic pathway could downregulate the direction of redox.DNA adducts and selective toxicity

DNA adducts and selective toxicity

DNA adducts are the products of the covalent reaction of electrophilic molecules with DNA (De Flora S., 1996). Many chemicals can form covalent adducts with DNA, directly in some cases and following metabolic activation in others. In most cases, however, drugs must undergo intracellular bioactivation-generating reactive metabolites, which bind to cellular proteins to form adducts (Svensson, C. K., 2003; Roychowdhury S. et al., 2005). The chemical characterization of adducts clarifies the nature of the activated intermediates, which reacted with DNA to form them. In the case of xenobiotics which undergo biotransformation, this helps elucidate the mechanism of metabolic activation (Josephy P D, 1997). Nuclear adducts are intermediate biomarkers evaluating the molecular dose and the biologically effective dose at the molecular level (De flora S. 1996). If a substance binds or reacts with DNA it will potentially disrupt cell

functions and lead to cell death (Luo J, 2005; [Esterbauer et al., 1991; Kehrer and Biswal,2000]).

Mitochondria self-replicate and have their DNA (Lewis W, 2006), but when exposed to certain drugs, they interfere with mitotic replication (Divi R L, 2004). Lately, attention has been given to the study of mtDNA (Wiesner, R. J. 1991). The mtDNA of different cell types and organisms is more prone to oxidative injury than the nDNA (Hertzog J, 2003; [Yakes F M, 1997; Salazar J J, 1997; Ballinger S W, 1999; Deng G, 1999; Mandavilli B, 2000; Santos J H, 2002]) and is subject to mutation tenfold higher than nuclear DNA (Wiesner R J, 1991; [Wallace, 1989]). It was demonstrated that depending on the intensity of the damage (dose and time of exposure), nuclear and mitochondrial genomes are differentially damaged and repaired (Hertzog J, 2003). This higher vulnerability of mtDNA when compared to nDNA is due to lack of compact nucleosome structure, limited mitochondrial repair pathways and the proximity of the mtDNA to the primary source of ROS generation (Hertzog J, 2003; Yakes F M, 1997; Sawyer D E, 1999).

Fluorescent metabolites can bind by intercalation, specifically to the nuclear region of the cell due to its affinity with DNA (Hodgkiss, 1991 & 1994). DNA in humans is present in nucleus and mitochondrion (Lewis W, 2006). Considering that certain BQ's are fluorescent drugs, we believe that one of the 13 polypeptides encoded in mtDNA that involves the function of the electron transport chain (Hetzog J, 2003) could get interrupted (García-Ruiz C, 1996) thus affecting ATP production finally leading to apoptosis. Also, a higher mitochondrial membrane charge of cancer cells over normal cells (Powers, S.K., 1987) could enhance binding selectivity of BQ's on cancer cells over normal cells.

Zayas and his colleagues in 2007 found that NBQ_{38} potentially bounded to DNA under anaerobic conditions in the presence of xanthine oxidase. Although the literature supports the possible binding capacity of BQ's to DNA, scientists should not overlook that mtDNA is less easily repaired and more prone to oxidative damage than nuclear

DNA, enhancing the possibility that adducts detected might come from genomic DNA due to this fact. DNA-adducts can be detected with the help of an HPLC-ESI MS/MS. The chromatogram peaks and the mass spectrum can then identify the presence of this adduct; this is possible due to the known molecular weight of the drug and DNA bases (Adenine, Guanine, Cytosine and Thymine). Genomic A431 DNA from exposed and unexposed cells could be analyzed using this technology.

Research cell lines

A431 is one of the many cell lines used to investigate possible drug interactions in the human body. This cancer cell line comes from the squamous cell (epithelium) of an 85-year-old female. Its growth is adherent, and it has been shown to form rapidly growing subcutaneous tumors in mice and immuno-suppressed mice and colonies in agar (ATCC Product Information Sheet CRL-1555). Employing this study, they might probably show what has been proved with other analogous drugs at the cellular level; that DNA intercalating drugs inhibit cell growth by inducing apoptosis (cell death). Not all drugs have the same mechanism; therefore, all must be tested prior to their in vivoexperimental research.

Legal frame

Drugs

In the U.S., the Food and Drug Administration (FDA) must approve a substance as a drug before it can be marketed (FDA). The approval process of a new drug that is being developed involves four principal phases: 1) research and development (Pre-Clinical); 2) the conversion of organic and natural substances into bulk pharmaceutical substances or ingredients through fermentation, extraction, and chemical synthesis (Clinical); 3) the formulation of the final pharmaceutical product; (Research Review) and Post Marketing Surveillance (EPA, 1997).

Mainly this study is still on a Pre-Clinical Test, meaning that it has not yet reached stage two. At this stage, we will seek to understand what this cancerous cell does to the NBQ's and ABQ's molecule (pharmacokinetic) and what the molecule does

to the cancerous cells (pharmacodynamic) (Silva H, 2006). This has begun since BQ's has shown to be promising in isolated laboratory tests. These drugs will continue their development to ensure that is biologically active and safe for normal cells.

After completing this pre-clinical test and before testing the drug in humans, an application is filed with FDA known as an Investigational New Drug Application (IND). The application must show the results of this pre-clinical testing and detail the plans for human clinical tests. It must also contain information about the chemical structure of the compound and a general description as to how the compound is manufactured (EPA, 1997).

Biomedical wastes

Biomedical wastes used to be covered on the Resource Conservation and Recovery Act of 1976 (RCRA) under Subtitle C for hazardous wastes. Puerto Rico participated in the 2-year demonstration program that extended from November 1988 to November 1991. After that period, Subtitle J expired and any biomedical waste that was generated, transported, and or disposed was subject to state regulation.

Since 1997 the Environmental Quality Board (in Spanish JCA) in Puerto Rico has tracked the generation, transportation, and disposition of biomedical wastes as of today under the Regulation for Non-Hazardous Waste Management, Chapter V, rules 580 thru 586. They include any solid waste generated upon the diagnostic, treatment, or immunization of human beings and or animals through investigation, a product of research or as a result of biological products as of in the embalming of human bodies (JCA, 1997). Biomedical wastes are delimitated as culture, stock, and biological products; pathological wastes; human blood and or derived products from blood; sharp wastes; animal wastes; and quarantine (JCA, 1997). Generation, segregation, packing, labeling, storage, treatment, management, transportation, disposition, and documentation are covered under this rule (JCA, 1997).

All experiments that generated biomedical wastes at the ChemTox Lab in Universidad Metropolitana were carried out from August 2006 to December 2007. This

was done out of the 1988 to 1991 period, and wastes were subject to 1997 state generator regulations. The amendment of December 17 of 1997 on the Regulation for Non-Hazardous Waste Management states that the generator is responsible for keeping track of the waste generated through a manifest until it is finally disposed of.

Waste from cell lines and materials in contact was handled as non-hazardous/special wastes and defined as Regulated Biomedical Waste (DBR in Spanish). Chapter IV of this Thesis contains the Biomedical Wastes Management and Handling Protocol describing a methodological procedure that includes treatment and disposal and or packaging of regulated biomedical wastes as defined on page 16 of the Non-Hazardous Solid Waste Management Rule. The Institutional generator number details for regulated cell and mixed wastes comply with rules 580 thru 582. A contracted third-party company (Biomedical VIP) complied with rules 583 thru 586 for the transportation and final disposal of DBR generated on the experiments.

Chemical wastes:

Most of the chemical reagents used are not considered hazardous wastes. All products purchased were accompanied by its Material Safety Data Sheet (MSDS). Upon arrival, the MSDS was read, signed to verify complete knowledge of the safety precautions needed, and always archived in the MSDS binder available for revision in the laboratory.

Other regulation that might indirectly cover this investigation includes Federal Agencies that are responsible for establishing acceptable levels of exposure to chemical substances in the general environment, home, and workplace, and in food, water, and medical drugs are:

- Consumer Product Safety Commission (CPSC) is responsible for reducing the risks of injuries and deaths associated with consumer products.
- The Environmental Protection Agency (EPA) government regulatory agency is charged with protecting human health and safeguarding the

natural environment.

- The Food and Drug Administration (FDA) helps safe and effective products reach the market quickly and monitors the products for safety after they are in use.
- The Agency for Toxic Substances and Disease Registry (ATSDR) regulates and lists all toxic substances and the hazardous conditions that exhibit a substance at a given concentration.

CHAPTER III

METHOD

Objectives:

1) **Qualitatively and quantitatively determine BQ's cell drug cytotoxicity and potency of BQ's IC_{50} concentrations on A431 at 24, 48, and 72 hours.**
2) **Qualitatively determine cell drug uptake viewing differences in cell fluorescence as an indirect indicator of the extent of drug cell interactions.**
3) **Determine apoptosis-induction pathway of BQ's IC_{50} concentrations on A431 using:**
    a. **Qualitative and quantitative mitochondria membrane permeabilization**
    b. **Qualitative and quantitative caspases 3 and 7 activation**
    c. **Qualitative caspases 8 activation**

Design overview

We analyzed the probable mechanism of action of BQ's as anticancer drugs to induce cell death through apoptosis. The methodology of this essay contains Objective 1 cell drug cytotoxicity and potency of the drugs, Objective 2 cell drug uptake, Objective 3 apoptosis activation using the permeabilization of the mitochondrial membrane, the activation of caspases 3, 7, and 8 (Figure 2). Our findings helped construct the probable mechanism of action for BQ's. This partially completes the preclinical phase of anticancer drug approval as required by the Federal Drug Administration.

Experimental cell population

Cell lines are used broadly in studies such as dose-response as the control variable. We selected A-431 cells in our study to test the activity of the BQ's understudy. This specific cell line comes from an 85-year-old female subject and the origin of the source of the tissue corresponds to the squamous cell epithelium that had cancer.

Cells were kept in an incubator that controls temperature, gas, and humidity. It is one

of the most important pieces of equipment since it will recreate the physiological conditions of the human body of the temperature and carbon dioxide (37^{o}C and 5% CO_2). It will also keepcells in a sterile environment free of contamination.

Methodological procedures

Experimental set-up

Sanitation

Personal preparation always included a lab coat and gloves. Hands were washed, and all areas were sanitized with ethanol 70%, Sporicidin and or Lysol. Media, PBS, and trypsin must be warmed previously to its use in a water bath until solutions temperature reaches 37^{o}C to avoid cell shock temperature. All cell handling tasks were performed inside a laminar flow hood to control air flux, preventing contamination. Hood surface sanitation and ultraviolet irradiation were performed for ten minutes prior any experiment. Materials, hood surfaces and the technician's hands were sprayed every time entering the sterile area.

Cell culture

Immortalized cell line A431 was purchased from the commercial cell provider American Type Culture Collection (known as A.T.C.C.) by UMET's ChemTox Lab to develop the initial subculture of cells. A431 is a continuous cell line from the epithelium of the vulva of an 85-year-old female tissue donor. The source of the tissue is relevant to this study since it provides a representation of one of the highest groups exposed to cancer, considering the sex, age, and type of tissue cancer has historically affected.

Cell line A-431 was sub-cultivated and or trypsinized every 48 to 72 hours, depending on the growth rate. Most cultured cell lines show a lag phase (non-growth) in the first 24 hours until they stabilize and reach the log phase (exponential growth) upto its maximum carrying capacity. Another indicator of maximum capacity is cell confluence. Cell viability was determined with a hemacytometer. In order to better understand the A-431 cell line behavior we developed a small experiment in which we examined its growth. We determined the ideal cell numbers that could recover from

subculture and growth in a short period before they were exposed to the drugs. Two experiments of duplicate plates of A431 cells were seeded on 25cm^2 t-flasks with 2.5 x 10^6 and 5 x 10^6 cells in a 9mL final volume of modified RPMI 1640. This ideal number of cells was determined for 25cm^2 t-flasks plates using ATCC and other publication recommendations. Results were graphed as a cell growth curve to find the lag and log phase.

Subculture, maintenance, and monitoring

To perform the complete assay, a continuous source of living cells was always available. This parental culture was kept in the same growth conditions as experimental cells. If there was a low or high cell concentration, cells were trypsinized and concentrated on a pellet to sub-cultivate it. If confluence was high, cells were distributed on different flasks.

Continuous monitoring of the cell subculture was necessary to check for possible sources of contamination and the maintenance of optimal conditions. All flasks were checked throughout the week using the fluorescence microscope. This procedure helped in the identification of cell integrity and prevention of contamination. All procedures for subculture and maintenance are included detailed as protocols on the Annexes.

Drug stock solutions

Stock solutions of 3mM were prepared for each drug (following the Exposure protocol). Some of the drugs are photosensitive; thus, we kept the vials sealed with parafilm in scintillation vials at 4°C in the fridge and covered with foil paper. This helped in avoiding evaporation and photo-degradation of the stock solutions.

Experimental design

Concentration exposure

Exposure of epidermoid carcinoma cells at the IC_{50} concentration revealed cell death or cell drug resistance for each specific BQ and the changes in the 72-hour period where samples were checked every 24 hours. Results generated a time response curve, signaling how the cell population varied with exposure time.

We selected the BQ's IC_{50} as the experimental concentrations since the dose-response curve was already explored (V. Arroyo et al., unpublished data). All samples had a 0uM exposure concentration as a control to keep track of the results obtained on non-exposed cells. Experimental concentrations were used as follows:

1) NBQ_{38} IC_{50}: 36uM

2) NBQ_{95} IC_{50}: 28uM

3) ABQ_{38} IC_{50}: 32uM

4) ABQ_{95} IC_{50}: 36uM

5) BQ_{108} IC_{50}: 4uM

Time exposure

Three times of exposure (24, 48, and 72 hours) were suggested to verify inhibition and or resistance on cell growth for Objective 1, 2, and 3. For certain parts of Phase II and III, we used only 48 hours. The design of this study needed the employment of different times of exposure on a specific BQ concentration (IC_{50}) to see the cell drug uptake.

Experimental procedures

Objective 1): Qualitatively and quantitatively determine BQ's cell drug cytotoxicity and potency of BQ's IC_{50} concentrations on A431 at 24, 48 and 72 hours.

Introduction

Cytotoxicity and potency were determined qualitatively and quantitatively by exposing cell line A431 to the drugs at the IC_{50} concentrations. Cells were stained following the Trypan Blue dye exclusion; they were counted with a hemacytometer and microscopefor quantitative cytotoxicity.

This method has been widely accepted as a useful and accurate way to evaluate cytotoxicity on mammalian cells when exposed to xenobiotics agents (Elia et al., 1993). Still, the application of this dye must be limited to no more than five minutes since it can show false results (Jones KH, 1985). The principle of the dye is that living cells will not

take up the dye while dead cells do. In this way, we can count living cells. The reactivityof trypan blue is based on the chromophore being negatively charged and does not interact with the cell unless the membrane is damaged. Consequently, all the cells that excluded the dye were viable.

Experimental procedure

Two experiments of duplicate plates of cells were seeded to the nearest 5 x 10^6 cells on 25cm^2 t-flasks. Final volumes on each plate were 9mL of modified RPMI 1640 that contained the cell aliquot, media, and drug. Treatment was administered at the IC_{50} concentrations: NBQ_{38}: 36uM, NBQ_{95}: 28uM BQ_{108}: 4uM, ABQ_{38}: 32uM, and ABQ_{95}: 36uM. Cells were incubated for 24, 48 & 72 hours at 37° C and 5% CO_2. As a negative control, we used non-treated cells. At the end of the treatment time, cells were washed with PBS, detached with 5% trypsin enzyme, and centrifuged. Cell viability was measured qualitatively with an Olympus inverted fluorescent microscope cells were observed and photographed every 24 hours using 10X objective. Quantitative cytotoxicity was performed by staining cells with trypan blue. Cells aliquots were counted with a hemocytometer by microscopy. The percentage of surviving cells was calculated and plotted against exposition time in a graph using excel.

Protocols used in this experiment

1) Exposure Protocol (Appendix 1.)
2) Trypsinization Protocol (Appendix 2.)
3) Pelleting Protocol (Appendix 3.)
4) Cell Viability Protocol (Appendix 4.)
5) Culture Count Protocols and Formulas (Appendix 5.)
6) Biohazard Wastes Management and Disposal Protocol (Appendix 9.)

Objective 2): Qualitatively determine cell drug uptake viewing differences in cell fluorescence as an indirect indicator of the extent of drug cell interactions.

Introduction

Cell drug uptake was determined using the cell auto-fluorescence co-localization as an indicator of the cell drug interaction to determine cell drug interaction. Cells were exposed from 24 to 72 hours to each BQ at the IC_{50} concentrations. BQ's delocalized positive charge could be preferentially retained to selectively negative charged membranes such as mitochondria.

Experimental procedure

Two experiments of duplicate plates of cells were seeded to the nearest 5 x 10^6 cells on 25cm^2 t-flasks. The final volume on each plate was 9mL of modified RPMI 1640 that contained the cell aliquot, media, and drug. Treatment was administered at the IC_{50} concentrations: NBQ_{38}: 36uM, NBQ_{95}: 28uM BQ_{108}: 4uM, ABQ_{38}: 32uM, ABQ_{95}: 36uM, Negative Control: 0uM and Ellipticine Positive Control: 0.5uM. Cells were incubated for 72 hours at 37° C and 5% CO_2. Ellipticine was used as the positive control and non-treated cells as the negative control for cell auto-fluorescence observations.At the end of the treatment time, cells were observed with an inverted fluorescent microscope.

Flasks were observed with the 40X objective and photographed at 13-17 seconds of exposure in an Olympus inverted fluorescent microscope and Q Capture Software. Auto-fluorescent cells were distinguished from non-auto-fluorescent ones using the negative and positive controls as reference.

Protocols used in this experiment

1) Exposure Protocol (Appendix 1.)
2) Trypsinization Protocol (Appendix 2.)
3) Pelleting Protocol (Appendix 3.)
4) Cell Viability Protocol (Appendix 4.)
5) Culture Count Protocols and Formulas (Appendix 5.)
6) Biohazard Wastes Management and Disposal Protocol (Appendix 9.)

Objective 3): Determine apoptosis induction pathway of BQ's IC_{50} concentrations on A431 using:

a) Qualitative and quantitative mitochondrial membrane permeabilization

Introduction

Apoptosis was verified as the primary cell death mechanism using the permeabilization of the mitochondrial membrane as a positive or negative indicator after exposing cells with the IC_{50} of BQs for 48 hours. Cells were then stained with the Mito PT™ dye following the manufacturer's instructions with slight modifications. This dye has a cationic preferential charge for negatively charged mitochondrial membrane. The MitoPT™ reagent allows the dye to penetrate cells and healthy mitochondria. Once inside a healthy non-apoptotic cell, the lipophilic MitoPT™ reagent, bearing a delocalized positive charge, enters the negatively charged mitochondria where it aggregates and fluoresce red (Cossarizza et al., 1993). These aggregates are called J- aggregates (Jelley, 1936). When the mitochondrial $\Delta\Psi$ collapses in apoptotic cells, the MitoPT™ reagent no longer accumulates inside the mitochondria. Instead, it is distributed throughout the cell. When dispersed in this manner, the MitoPT™ reagent assumes a monomeric form, which fluoresces green (Reer M et al., 1991). The use of the MitoPT™ kit allows the distinction between non-apoptotic red fluorescent cells and apoptotic green fluorescent cells. In this way, disrupted mitochondria would not retain the dye and dissipate inside the cell on its monomeric form, fluoresced on a green color. Undisrupted cells fluoresce red, a product of the aggregation of the dye to the mitochondrial membrane.

Literature indicates that Valinomycin dissipates the membrane potential of cells and mitochondria and induces apoptosis (In-Ja, 2006, Inai et al., 1996; Nomura, 2004). This is due to the release of mitochondrial cytochrome *c* in a permeability transition (PT)-independent manner (Shinohara Y et al., 2002; In-Ja R, 2006), making Valinomycin a useful positive control for this assay.

Experimental procedure

Two experiments of duplicate plates of cells were seeded to the nearest 5 x 10^6 cells on 25cm^2 t-flasks. The final volume on each plate was 9mL of modified RPMI 1640 that contained the cell aliquot, media, and drug. Treatment was administered at the IC_{50}

concentrations: NBQ_{38}: 36uM, NBQ_{95}: 28uM BQ_{108}: 4uM, ABQ_{38}: 32uM, ABQ_{95}: 36uM, Negative Control: 0uM, Valinomycin Positive Control: 5uM. Cells were incubated for 48 hours at 37° C and 5% CO_2. Valinomycin was used as the positive control and non-treated cells as the negative control for apoptosis induction capability. At the end of the treatment time, cells were washed with PBS, detached with 5% trypsin enzyme, and centrifuged. Cells were counted, and aliquots were stained for an hour with the Mito PT™dye solution.

Aggregated and monomeric forms of the dye were qualitatively verified by mounting the cells on glass slides with coverslips and photographed at 7 seconds of exposure time in an Olympus inverted fluorescent microscope and Q Capture Software. Apoptotic cells (green) were distinguished from non-apoptotic (reddish-orange) using the negative and positive controls as reference using the 40 X Objective.

Apoptotic cells were estimated by capturing the fluorescent signal that emits the monomers and the J-aggregates. Monomers emit at 527nm and J-aggregates at 590nm. The Blue filter of the Modulus Fluorimeter captures emission ranges from 515-570 nm (monomeric structure emits at 527 nm), capturing the apoptotic cells (green) fluorescence intensity revealing damaged mitochondria structures. The green filter captures the emission range of 580-640 nm (dye j-aggregates structure emits at 590 nm), capturing the non-apoptotic cells (red) fluorescence intensity revealing healthy mitochondria.

Since ABQ's weakly emit fluorescence near the emission wavelengths of the dyes, we measured stained and unstained aliquots fluorescence on both filters of the Modulus fluorimeter. Data were calculated and double normalized by subtracting the non-stained cell aliquots from their duplicate stained and the negative control. The percentage of apoptotic cells was calculated and plotted against drugs in a graph using excel.

Protocols used in this experiment

1) Exposure Protocol (Appendix 1.)

2) Trypsinization Protocol (Appendix 2.)
3) Pelleting Protocol (Appendix 3.)
4) Cell Viability Protocol (Appendix 4.)
5) Culture Count Protocols and Formulas (Appendix 5.)
6) Immunochemistry's Apoptosis (Mito PT™) Adjusted Staining Protocol (Appendix 5.)
7) Biohazard Wastes Management and Disposal Protocol (Appendix 9.)

b) Qualitative and quantitative caspases 3 and 7 activation

Introduction

During apoptosis, zymogens located on the lysosomes are cleaved, becoming DEVDases, the active enzyme of Caspases 3 and 7 (Belloc, F. et al. 2000). Zymogens are inactive enzyme precursors that require biochemical change (cleavage) to reveal their active site (Biagiotti, E. et al. 2000; Lee, B. W., 2003). DEVD (aspartylglutamylalanylaspartic acid) is the common four amino acid target sequence of Caspases 3 and 7 enzymes (Belloc, F. et al. 2000).

The use of the Magic Red™ kit allowed the identification of active Caspases 3 and 7. The active fluorophore is cresyl violet coupled via two amide linkage bi-substituted to two DEVD sequence groups becoming MR-$[DEVD]_2$ = Magic Red. When one or both MR-$[DEVD]_2$ aspartic acids amide linkage sites were cleaved by hydrolysis, cresyl violet fluoresced red (Reed, J. C., 2000). Therefore, activation of Caspases 3 and 7 could be monitored using DEVDase mediated hydrolysis production as an indirect indicator. Undisrupted cells will not fluoresce while active Caspases 3 and 7 fluoresces red, indicating negative or positive apoptosis.

Apoptosis was verified as the primary cell death mechanism using Caspases 3 and 7 activations as a positive or negative indicator after exposing cells with the IC_{50} concentration of BQ's for 48 hours. Cells were then stained with the Magic Red™ dye following the manufacturer's instructions with slight modifications. The Magic Red™ dye

detects active intracellular cleavage of DEVD target; Caspases 3 and 7 DEVDease specific enzyme target. The lipophilic reagent penetrates the cell membranes and organelles in a non-fluorescent state. When Caspases 3 and 7 become active, the Magic Red™ dye fluoresces.

Literature indicates that Staurosporine and Cisplatin induce apoptosis with the involvement of Caspases 3 and 7 activations (Zhang, X. D. et al. 2004; Blanc, C. et al. 2000; Lee, B. W. et al. 2003; Fuertes, M. A., et al. 2003). Making Cisplatin and Staurosporine proper positive controls for this assay.

Experimental procedure

Two experiments of duplicate plates of cells were seeded to the nearest 5 x 10^6 cells on 25cm^2 t-flasks. The final volume on each plate was 9mL of modified RPMI 1640 that contained the cell aliquot, media, and drug. Treatment was administered at the IC_{50} concentrations: NBQ_{38}: 36uM, NBQ_{95}: 28uM, BQ_{108}: 4uM, ABQ_{38}: 32uM, ABQ_{95}: 36uM, Negative Control: 0uM, Ellipticine: 5uM, and Cisplatin: 3uM. Cells were incubated for 48 hours at 37° C and 5% CO_2. Cisplatin and Ellipticine were used as positive controls and non-treated cells as the negative control for apoptosis induction capability. At the end of the treatment time, cells were washed with PBS, detached with 5% trypsin enzyme and centrifuged. Cells were counted and aliquots were stained for an hour with the Mito PT™

Active caspases 3 and 7 were monitored qualitatively by photographing cells at 9 seconds of exposure with an Olympus fluorescent inverted microscope and Q Capture Software with a 40 X Objective in the G field. Apoptotic cells were distinguished from non-apoptotic comparing photos fluorescent with light photos and co-localizing those cells with red fluorescence. Apoptotic cells (red) were distinguished from non-apoptotic (non-fluorescent) using the negative and positive controls as reference.

Apoptotic cells were estimated by capturing the red fluorescent signal that emits cleaved MR-$[DEVD]_2$ at >610nm. The Red filter of the Modulus Fluorimeter captures emission signals from 640nm. Since ABQ's weakly emit fluorescence near the emission

wavelengths of the dyes, we measured stained and unstained aliquots fluorescence on the Red filter. Data were calculated and double normalized by subtracting the non-stained cell aliquots from duplicate stained and the negative control. The percentage of apoptotic cells was calculated and plotted against drugs in a graph using excel.

Protocols used in this experiment

1) Exposure Protocol (Appendix 1.)
2) Trypsinization Protocol (Appendix 2.)
3) Pelleting Protocol (Appendix 3.)
4) Cell Viability Protocol (Appendix 4.)
5) Culture Count Protocols and Formulas (Appendix 5.)
6) Immunochemistry's Qualitative and Quantitative Caspase 3 and 7 Evaluation Protocol (Appendix 7.)
7) Biohazard Wastes Management and Disposal Protocol (Appendix 9.)

c) Qualitative and quantitative caspases 8 activation

Introduction

Apoptotic activation, as discussed earlier, could be achieved via two classic pathways: an extrinsic pathway with Caspase 8 being the initiator or an intrinsic pathway where the Mitochondrial Membrane becomes permeabilized, liberating Apaf-1 and cytochrome c; activating Caspase 9 (Kaur, M. et al. 2005). Reports have evidenced that the activation of Caspase 8 is through the external surface receptor Fas (Węglarczyk, K et al. 2005).

Both pathways (intrinsic and extrinsic) could converge at two different points through apoptosis: in the mitochondrial membrane permeabilization through the cleavage of Bid and or the activation of Caspases 3 and 7 for the final execution (Hengartner, M., 2000). Thus, the importance of identifying the involvement of Caspase 8 activation and its relationship with the status of the mitochondrial membrane and the activation of Caspase 3 and 7.

Apoptosis was verified as the primary cell death mechanism using Caspase 8 activation as a positive or negative indicator after exposing cells with the IC_{50} concentration of BQ's for 48 hours. Cells were stained with the FLICA™ dye following the manufacturer's instructions with slight modifications. FLICA™ is a green fluorescent label inhibitor, FAM-VAD-FMK, which is a carboxyfluorescein (FAM) derivative of valyl alanyl aspartic acid (VAD) fluoromethyl ketone (FMK), a potent inhibitor of Caspase 8 activity (Ekert, P. G., et al. 1999). FAM-VAD-FMK penetrates the cell membranes and organelles in a non-fluorescent state when Caspase 8 is inactive (zymogen). When apoptosis activates Caspase 8 and the FLICA™ reagent penetrates the cell membrane and covalently binds to a reactive cysteine residue. That residue is on the large subunit of the active Caspase 8 heterodimer (Ekert, P. G. et al. 1999). Non-active Caspase 8 cells will not fluoresce while active Caspases 8 cells fluoresce green indicating negative or positive apoptosis.

Literature indicates that Staurosporine and Cisplatin induce apoptosis with the involvement of Caspase 8 activation (Zhang, X. D. et al. 2004; Blanc, C. et al. 2000; Fuertes, M. A., 2003) Making Cisplatin and Staurosporine useful positive controls for this assay.

Experimental procedure

Two experiments of duplicate plates of cells were seeded to the nearest 5 x 10^6 cells on 25cm^2 t-flasks. The final volume on each plate was 9mL of modified RPMI 1640 that contained the cell aliquot, media, and drug. Treatment was administered at the IC_{50} concentrations: NBQ_{38}: 36uM, BQ_{108}: 4uM, ABQ_{38}: 32uM, ABQ_{95}: 36uM, Negative Control: 0uM, Ellipticine: .5uM, Cisplatin: 3uM and Staurosporine 1uM. Cells were incubated for 48 hours at 37° C and 5% CO_2. Cisplatin, Ellipticine, and Staurosporine were used as the positive control and non-treated cells as the negative control for apoptosis induction capability. At the end of the treatment time, cells were washed with PBS, detached with 5% trypsin enzyme, and centrifuged. Cells were counted, and aliquots were stained for an hour with the Mito PT™

Active Caspase 8 was monitored qualitatively by photographing cells at 7 seconds of exposure with an Olympus fluorescent inverted microscope, a 40 X objective, and Q Capture Software. The microscope had a filter (B field) that captured the fluorescent signal emitted at 515-535nm. Apoptotic cells were distinguished from non-apoptotic comparing photos fluorescent with light photos and co-localizing those green fluorescence cells. Apoptotic cells (green) were distinguished from non-apoptotic (non-fluorescent) using the negative and positive controls as reference.

Protocols used in this experiment

1. Exposure Protocol (Appendix 1.)
2. Tripzinization Protocol (Appendix 2.)
3. Pelleting Protocol (Appendix 3.)
4. Cell Viability Protocol (Appendix 4.)
5. Culture Count Protocols and Formulas (Appendix 5.)
6. Immunochemistry's Qualitative and Quantitative Caspase 8 Evaluation Protocol (Appendix 8.)
7. Biohazard Wastes Management and Disposal Protocol (Appendix 9)

CHAPTER IV

RESULTS AND DISCUSSION

A431 Growth curve

Previous to the execution of experiments using A-431, two different concentrations of initial cells were seeded on 25cm2 t-flasks at 2.5 x 10^6 cells and 5 x 10^6 cells without a drug. This was done in order to determine the best initial concentration of cells. Cells were monitored for attachment for 12 hours. Most of the cells were attached to the matrix of the t-flask at approximately four hours upon seeding. Both concentrations of initial seeding presented a continuous growth from 0 to 72 hours. The graph does not plateau at 72 hours, indicating that cells can grow without changing the media for the length of the experimental procedure (Figure 3).

Objective 1): Qualitatively and quantitatively determine BQ's cell drug cytotoxicity and potency of BQ's IC_{50} concentrations on A431 at 24, 48, and 72 hours.

BQ's anticancer drugs were evaluated using the epidermoid carcinoma cell line A-431. BQ's tested included two drugs with nitro terminal groups (NBQ_{38} and NBQ_{95}), two drugs with amino-terminal groups (ABQ_{38} and ABQ_{95}), and one with a cloro terminal group (BQ_{108}) (Figure 1). A431 was used to test these drugs using the IC_{50} concentration as previously determined (V. Arroyo et al., unpublished data). BQ's were tested for cytotoxicity and drug potency, using the IC_{50} concentration in 72 hours with 24-hour increments.

a) Qualitative cell cytotoxicity and potency

Nitro (NBQ's) and Amino's (ABQ's) drugs showed suppression of cell growth compared to the control group. Photos of cell confluence revealed that cells treated with all BQ's positively inhibited growth at 48 hours compared to the Negative Control except for BQ108 (Figures 4, 7 and 8). This observation was consistent and increased in the 48

and 72 hours time-lapse (Figures 4, 7, and 8). ABQ_{38} and ABQ_{95} had the least confluent cell pictures than NBQ_{38} and NBQ_{95} (Figures 7 and8).

b) Quantitative Cell Cytotoxicity and Potency

All drugs inhibited cell growth from 35% to 61% compared with the negative control at 24 hours (Table 1(a), Figures 9 and 10). The first difference in the intensity of inhibition between the two types of drugs was observed at 48 hours and this difference widened at 72 hours (Table 1(a), Figures 9, 11, and 12). At 48 hours ABQ's suppressed cell growth from 70% to 83%, three times more than those cells treated with NBQ's that had 9% to 38% (Table 1(a), Figures 9 and 11). At 72 hours a more significantdifference in cell growth inhibition was observed (Table 1(a), Figures 9 and 12). ABQ's inhibited almost 4 times more cell growth than NBQ's (Table 1(b), Figures 9 and 13). The average potency of ABQ's was 2.5 times stronger in cell growth inhibition over the NBQ's in the entire treatment time (0 to 72 hours) (Table 1(b), Figure 13).

Comparing treated cells with the Negative Control we found that the percentage on growth inhibition revealed had the following order: 24 Hours; ABQ_{38} (61%) > ABQ_{95} (47%) > NBQ_{95} (44%) > NBQ_{38} (35%) > Negative Control (0%) > BQ_{108} (-10%.) (Figure 10). 48 Hours*:* ABQ_{95} (83%) > ABQ38 (70%) > NBQ_{95} (38%) > NBQ_{38} (9%) > BQ_{108} (7%) > Negative Control (0%) (Figure 11). 72 Hours; ABQ_{95} (89%) > ABQ_{38} 79%) > NBQ_{38} (28%) > NBQ_{95} (14%) > BQ_{108} (14%) > Negative Control (0%) (Figure 12). This marked difference in cytotoxicity could result from cell drug resistance to the NBQ's after 48 hours of exposure (Table 1(b), Figure 13).

The drug's potency was evaluated, and data revealed that ABQ's were stronger than NBQ's in the following order: 24 Hours; 1.4 times, 48 Hours; 3.3 times and 72 Hours; 3.9 times (Table 1(b) and Figure 13). This indicates that ABQ's overall 2.4 times more potent on inhibiting A431 cells with their IC_{50} concentrations than NBQ's (Table 1(b) and Figure 13).

Objective 2): Qualitatively determine cell drug uptake viewing differences in cell fluorescence as an indirect indicator of the extent of drug cell interactions.

Previous to the execution of experiments, published IC_{50} concentrations (1uM by Sureau, F. et al., 1993 and 4.1uM by Kuo, Y. et al., 2006) of Ellipticine were tested. Experimentally cells were mainly killed. Thus, we proceeded to evaluate its cytotoxicity and determine the IC_{50} concentration.

a) Ellipticine Cytotoxicity

Cells were seeded on 25cm^2 t-flasks at 5 x 10^6 cells and exposed for 48 Hours. Photos of cell confluence revealed that all concentrations of Ellipticine inhibited cell proliferation when compared to the Negative Control (Table 2(a), Figure 6). The average percentage of surviving cells was plotted against dose concentrations revealing that cytotoxicity of Ellipticine was dependent on dose concentration at 48 Hours (Table 2(a), Figure 5). The nearest data point to inhibit 50% of the cells on the plot was the .5uM dose, reflecting 40% of surviving cells (Table 2(a), Figure 5). A linear equation ($y=mx + b$) was obtained from the plot using excel (Figure 5). By substituting a 50% (.50) in the y variable, we obtained x. The IC_{50} value of Ellipticine in A431 resulted in .57 $\pm$ .03uM (Table 2(b)). We decided to use .5uM of Ellipticine for experiments and have enough cells to perform cell auto-fluorescence and caspases staining.

b) Qualitative Cell Drug Uptake

A431 exposed cells to BQ's were thoroughly observed using the IC_{50} concentration for 72 hours. NBQ's and ABQ's drugs exhibited a peculiar difference; ABQ's fluorescently stained A431 cells while NBQ's and BQ_{108} did not.Photos revealing cell auto-fluorescence were captured for ABQ's and Ellipticine at 72 hours of exposure (Figure 14). No images of cell auto-fluorescence were detected earlier than this.

Co-localization of Ellipticine has been BQ's positively identified at similar cytosolic structures (Figure 14). This observation is consistent with previous reports on

Ellipticine, where has been identified as a fluorescent-specific probe on isolated mitochondria organelles on single living cells (Sureau et al., 1993). Sureau also found that Ellipticine behaves as a fluorescent probe of mitochondrial pH, higher in mitochondria than in chromatin.

Ellipticine has a binding affinity for circular (mtDNA) and linear DNA (Kohn et al. 1975), forming DNA adducts (Stiborova, M. et al., 2004; Stiborova, M. 2006). Some NBQ's (NBQ_{38}) could bind to DNA forming covalent adducts in vitro with Calf Thymus DNA in the presence of xanthine oxidase (Zayas, B. et al., 2007). Still, preferential binding has not been evaluated without mitochondria of living cells. Considering the selective higher binding affinity of Ellipticine towards mitochondria,ABQ's could primarily bind to mitochondria and then to genomic DNA/RNA. On the otherhand, BQ's has shown that induce DNA forward mutation on the HPRT gene (Arroyo, L. et al., unpublished data). DNA damage could not only be exclusive to the HPRT gene. It might also be affecting other genes such as p53.

112 Ellipticine derivatives have been tested in the NCI anticancer drug discovery program. Ellipticine anticancer activity narrowed some of this derivate more active against p53 mutant cell lines than against p53 wild type (Weinstein, 1997; Shi, L. M. et al., 1998). ABQ's and Ellipticine are alkaloids and structural analogs demonstrating similar fluorescence co-localization and binding activity thus in A431 (p53 mutant cell line) (Reiss et al., 1992; Choi et al., 2000). In the same way that the HPRT was mutated by BQ's; p53 function could be rescued by transcriptionally activated, reverting the mutant status of this gene on A431.

The restoration of the p53 function has been involved as a significant switch of the mitochondrial metabolism by regulating glucose metabolism (Bensaad, K., and Voudsen, K. H., 2007). This could ultimately lead to ROS generation and cause oxidative damage to the mitochondrial membrane, preferentially activating the intrinsic apoptotic pathway.

Objective 3): Determine apoptosis induction pathway of BQ's IC_{50} concentrations on A431

Apoptosis was quantified using three different parameters: (A) permeabilized mitochondrial membrane with Mito PT™ (JC-1) apoptotic mitochondria, (B) active Caspases 3 & 7 with Magic Red™, and (C) active Caspase 8 with FLICA™.

a) Qualitative mitochondrial membrane permeabilization

For apoptosis, we used the selective fluorescent cationic dye Mito PT (JC-1) to stain BQ-treated cells. Valinomycin (apoptotic reagent) was used as positive controls and non-treated cells as a negative control. Photos allowed qualitative distinction on those cells with permeabilized mitochondrial membrane as an early indicator of apoptosis. Observations were based on the shifting of the red color (non-apoptotic) to the green color (apoptotic) as a function of time. A single curvette fluorimeter with two filters captured the fluorescence intensity on apoptotic and non-apoptotic cells stained.

The Mito PT™ dye dually stained mitochondria allowing the differentiation of the state of the permeability of the membrane. As exposure time progressed in this experiment, exposed cells swiftly shifted from a bright orange (BO) to an intermediate yellow (IY), finally leading to a bright green (BG). Categories of these three stages were made to understand better the transition of the healthy to the damaged mitochondrial membrane. Bright orange represented healthy mitochondria (Figure 15), yellow represented an intermediate state of permeabilization, and green a complete permeabilized mitochondrial membrane (PMM) (Figure 15).

Photos at 24 Hours indicated that NBQ_{38} and ABQ_{38} presented a majority of BO, NBQ_{95}, and ABQ_{95} had IY-BG, indicating the initiation of a PMM (Table 3; Figures 16 and 18). At 48 Hours, NBQ_{38} and ABQ_{38} had BO, IY, and BG, indicating a diverse representation of a heterogeneous population of cells through the three stages (Table 3; Figures 16 and 18). NBQ_{95} had IY-BG as a more advanced stage of the PMM (Table 3; Figure 17). ABQ_{95} was the only drug that at 48 hours presented BG; almost complete PMM (Table 3; Figure 19). All cells exposed to BQ's at 72 hours presented BG, a

complete PMM compared to Valinomycin (Table 3; Figures 15-18 and 19). ABQ_{95} was the earliest drug that PMM at 24 hours, followed by NBQ_{95}, ABQ_{38} and NBQ_{38} (Table3).

b) Quantitative mitochondrial membrane permeabilization

BQ's treated apoptotic cells were detected after 48 hours of exposure. Valinomycin was used to establish an ideal 100% apoptotic index and determine the apoptotic background of A431. Results showed that 35% of the cells in A-431 resulted in Cisplatin-related apoptosis.

After 48 Hours, all BQ's treatments presented positive PMMs compared to the control group and confirmed the findings of the qualitative results (Table 4; Figure 20). NBQ's presented the strongest PMM but not farther than ABQ's (Figure 20). BQ's mitochondrial damage resulted to be nearly as strong as Valinomycin in the following order: Valinomycin; 65 $\pm$ 6% > NBQ_{95}; 51 $\pm$ 10% > NBQ_{38}; 48 $\pm$ 11% > ABQ_{95}; 45 $\pm$ 8% > BQ_{108}; 39 $\pm$ 20% > ABQ_{38}; 36 $\pm$ 8% and Negative Control; 0 $\pm$ 2% (Figure 20). NBQ_{95} presented higher preferential mitochondrial damage at 48 hours of exposure (Table 4; Figure 17 and 20).

c) Qualitative Caspases 3 and 7 activations

To assess caspases 3 and 7 activations, we used the Magic Red™ dye to stain BQ treated cells with the IC_{50} concentrations of BQ's for 48 hours. Magic Red™ was used as a fluorescent probe that identifies cells with induced apoptosis, using DEVDase activity as an indicator of the activity of Caspases 3 & 7.

Cisplatin (anti-cancer drug Positive Control), Ellipticine (structural analog), and Staurosporine (Caspase 3 and 7 Positive Control) were used as positive controls and non-treated cells as Negative Control (Table 5; Figures 21 and 22). Photos allowed qualitative identification on the relative activity of Caspases 3 and 7 on those cells treated with BQ's (experimental) when compared to those untreated (Negative Control).

Observations were based on the presence (apoptotic) or absence (non-apoptotic) of the red color (Table 5; Figures 21).

Cells resulted in positive Caspases 3 and 7 after exposing them to BQ's IC_{50} for 48 hours. NBQ_{38} NBQ_{95} and BQ_{108} revealed similar fluorescence intensity to Ellipticine (Table 5; Figures 22, 23, and 24). ABQ_{38} and ABQ_{95} had a more intensefluorescent color than the other drugs and were similar to Cisplatin and Staurosporine (Table 5; Figure 21, 22, 25, and 26). This indicated that Caspases 3 and 7 are involved in executing apoptosis as early as 48 hours.

d) Quantitative Caspases 3 and 7 activations

The intensity of the emitted fluorescence measured by the fluorimeter reflected the intensity of activated Caspases 3 and 7 of the cells. BQ's treated apoptotic cells were detected after 48 hours of exposure. Results were normalized, subtracting the Caspases 3 and 7 background activity from the negative control. Cisplatin was used to establish an ideal positive control for a 100% Caspases 3 and 7 apoptotic indexes and to determine the apoptotic background of the A431 cell line. Results showed that 49% of the cells in A431 resulted in apoptosis naturally or due to the experimental method.

BQ's treated samples revealed higher caspases activity when compared the Negative Control (Table 6; Figure 27). ABQ's and Cisplatin had similar Caspases 3 & 7 activation values and almost double when compared to NBQ's (Table 6; Figure 27). Caspases 3 and 7 activation was found in the following order; Cisplatin 51 $\pm$ 13% = ABQ_{38}; 51 $\pm$ 1% > ABQ_{95}; 46 $\pm$ 6% > BQ_{108}; 27 $\pm$ 5% > NBQ_{38}; 22 $\pm$ 7% and Negative Control; 0 $\pm$ 1% (Table 6; Figure 27). ABQ_{38} and ABQ_{95} presented to be the strongest Caspases 3 and 7 apoptotic inducers (Table 6; Figure 27). Quantitative analysis on NBQ_{95} was not performed due to limitations on the drug synthesis. This quantitative analysis is also consistent with the qualitative observations on the activation of Caspases 3 and 7 validating that both ABQ's might preferentially rely on this pathway for the final execution when compared to NBQ_{38} and BQ_{108} (Table 5 and 6; Figure 27).

e) Qualitative Caspase 8 activation

To assess Caspase 8 activation, we used the FLICA™ dye to stain BQ treated cells with the IC_{50} concentrations for 48 hours. FLICA™ was used as a fluorescent probe that identifies cells with induced apoptosis, using Caspase 8 green fluorescence activity.

Cisplatin (anti-cancer drug Positive Control), Ellipticine (structural analog), and Staurosporine (Caspase 3 and 7 Positive Control) were used as positive controls and non-treated cells as Negative Control (Table 5; Figures 28 and 29). Photos allowed qualitative identification on the relative activity of Caspase 8 on those cells treated with BQ's (experimental) when compared to those untreated (Negative Control). Observations were based on the presence (apoptotic) or absence (non-apoptotic) of the green color.

Cells resulted in negative activation of Caspase 8 after exposing them to BQ's IC_{50} for 48 hours. NBQ_{38}, BQ_{108} nor ABQ's revealed any fluorescence related to Caspase 8 activation (Table 5; Figures 30-32 and 33). Since Ellipticine is a fluorescent probe, the fluorescence emission was mainly due to its natural cell-drug auto-fluorescence characteristic, as reported earlier (Sureau et al., 1993) (Table 5; Figure 29). Cisplatin and Staurosporine had a strong fluorescence emission as an indicator of Caspase 8 apoptotic activation (Table 5; Figures 28 and 29) confirming the reliability of the method. This result confirms that Caspase 8 is not actively involved in executing apoptosis on A431 cells treated with BQ's IC_{50} 48 hours. Execution of quantitative analysis was not needed due to negative results on the activation of Caspase 8.

Integral Discussion

Comparing the overall results of BQ's to Ellipticine, it activates mainly the intrinsic apoptotic pathway through the permeabilization of the mitochondrial membrane, activation of Caspases 3 and 7 (Kuo, Y. et al., 2006) but not Caspase 8. Certain apoptotic anti-cancer agent inducers had also revealed a similar intrinsic preferential pathway behavior. *O*6MeG (an anti-cancer agent) activates apoptosis through The intrinsic pathway by permeabilizing the mitochondrial membrane, activation of Caspases

3, 7, and 9 but without activation of Caspase 8 (Ochs, K. and Kaina, B. 2000).

Another possible source of apoptotic triggering could be attributed to stress-induced by BQ's to the endoplasmic reticulum (Hägg, M. et al., 2004). When the endoplasmic reticulum senses stress, it transmits signals to mitochondria, leading to the opening of the Mitochondrial Permeability Transition Pore (PTP) (Hägg, M. et al, 2004; Green, D. R. and Reed, J. C., 1998). Opening of the PTP allows non-specific molecules of $\leq$ 1.5kD including water and solutes to enter (Green, D. R. and Reed, J. C., 1998). This event uncouples the respiratory chain by dissipating the H^+ gradient across the inner membrane (Green, D. R., and Reed, J. C., 1998). It also allows the release of pro-apoptotic proteins (ex: Apaf-1, cytochrome c) that ultimately lead to the activation of Caspase 9 and finally Caspases 3 and 7 completing the apoptotic process.

CHAPTER V

CONCLUSIONS AND RECOMMENDATIONS

In this study, we have identified BQ's as an apoptotic agent inhibiting cell growth when compared to alkaloids Berberine and Ellipticine (structural analogs) which have been used to treat cancer cell lines (Kuo, Y. et al., 2006; Sudheer, K. et al. 2006). In studies of cell death mechanisms, Ellipticine activates apoptosis. It is mainly triggered by specifically DNA intercalation, inhibition of topoisomerase 2, covalent alkylation of macromolecules, and generation of cytotoxic free radicals (Shi, L. M. et al., 1998).

For the Objective 1 of this study, we determined BQ's cytotoxicity and potency. BQ's are dose-dependent cancer cell growth inhibitors (Arroyo, V. et al., unpublished data). Cytotoxicity was also found to be dependent on time of exposure. At shorter exposure times, BQ's activities seem to overlap in cytotoxicity. As exposure time increased, ABQ's treated cells' cytotoxicity increased, whereas NBQ's treated cells presented certain resistance to the treatment. Average cytotoxic of BQ's IC_{50} for the complete exposure time presented that this difference was 2.5 times greater for ABQ's when compared to NBQ's. This difference in cytotoxicity of ABQ's could be attributed to its amino terminal group. The step of Amine oxidation to a hydroxylamine involves less gain/loss of electrons that Nitro reduction. Re-administration of NBQ's every 48 hours could be an option to inhibit cell growth more aggressively.

For Objective 2, we qualitatively determined cell drug uptake by observing fluorescence of the cell as indicator of the extent of the penetration of the drug. ABQ's and Ellipticine fluorescently stained similar sub-cellular structures in A431 cells. This strongly suggests that ABQ's and Ellipticine could have equal preferential binding to mitochondria and/or DNA. As supported through the literature, cationic properties of BQ's allowed to be attracted and bound or retained the highly negative electric potential

across the mitochondrial membrane (Lincon V et al., 1980; Haaker H et al., 1972; Oseroff A R et al., 1986). The amount of BQ's bound and unbound was not quantitative assessed, but once BQ's were inside the cell, they interfered with the inner trans-membrane potential leading cells to undergo apoptosis (Inai Y. et al, 1997; Nomura Y., 2004; Green D et al., 1980; Constantini P et al 2000).

For the Objective 3, we found that BQ's induces apoptosis through the permeabilization of the mitochondrial membrane as an upstream event. This event was positively detected as early as 24 hours on NQB_{95} and ABQ_{95}. Both drugs with Sulfur and Cloro in the same position appeared to have a quicker response on permeabilizing the mitochondrial membrane. Although quantitative intensities of mitochondrial damage state a similar response independently to the terminal groups, the qualitative analysis states that positive response upon treatment is quicker for drugs with Cloro and Sulfur (NBQ_{95} and ABQ_{95}). Longer exposure times still present almost complete permeabilization of the mitochondrial membrane as a definitive response to BQ's exposure. It has been demonstrated that the permeabilization of the mitochondrial membrane is involved in the liberation of pro-apoptotic proteins leading to activation Caspases 9,3 and 7. (Scorrano, L. et al., 2005). Then BQ's involvement in the induction of apoptosis relies on the permeabilization of the mitochondrial membrane which enhances the downstream effector Caspases 3 and 7.

As an integral part of apoptosis induction by BQ's, downstream activation of Caspases 3, 7 and upstream Caspases 8 were verified. BQ's presented positive activation of Caspases 3 and 7; and negative activation of Caspases 8. Qualitatively, ABQ's had almost two times more activation of Caspases 3 and 7 at 48 Hours than NBQ's. Also, ABQ's intensity of the activation of Caspases 3 and 7 is similar to that of Cisplatin. The source for the activation of Caspases 3 and 7 downstream activated could be triggered by the permeabilization of the mitochondrial membrane.

BQ's reflected a negative response of Caspases 8 activation. This lack of activation of Caspases 8 indicates that BQ's are neither intimately involved as upstream initiators activation through the DISC involvement. The elimination of the involvement of death receptors in BQ's apoptosis cell death mechanism simplifies and confirms that the intrinsic pathway governs the execution of apoptosis as the cell death in BQ treated cells.

Finally, we conclude that apoptosis is the cell death mechanism of action that mainly contributes to the overall cell growth inhibition caused by BQ's IC_{50}. Apoptosis on BQ's treated cells is initially triggered by damaging the mitochondrial membrane, activating downstream execution of Caspases 3 and 7. BQ's mechanisms are mainly intrinsic due to the lack of involvement of Caspase 8.

Limitations

Cell culture management can sometimes be tedious and time-consuming. Inappropriate sanitation of materials or lack of detailed monitoring of the cell culture can lead to contamination. During the experiment, cell cultures became contaminated, which led the repetition of experiments, delaying the overall progress of the project.

Recommendations

1) In order to distinguish if BQ's selectively induce apoptosis in cancer cells preferentially and additional study on normal cells must be performed and compared with the results here obtained.
2) Additional tests that can identify how BQ's influence the expression of p53 could clarify the mechanism of action of BQ's from a molecular perspective as indicators of specific DNA selective damage to compare results with other drugs that are structural analogs such as Ellipticine.

3) A new cytotoxicity test on BQ_{108} should be reconsidered in order to adjust its IC_{50} since it revealed poor cell growth inhibition at 4uM dose concentration.
4) Ellipticine and BQ's are structural analogs with similar physical properties; therefore, the experiments should be conducted to evaluate stress-induced to the endoplasmic reticulum.

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TABLES

(a) Percentage of Cell Growth and Inhibition of A431 Cells Treated with BQ's IC_{50}

Drugs	Time of Exposure (Hours)											
	0			24			48			72		
	Growth	Inhibition	Error	Growth	Inhibition	Error	Growth	Inhibition	Error	Growth	Inhibition	Error
NBQ_{38}	100.0%	0.0%	0%	65.2%	34.8%	7%	90.9%	9.1%	9%	71.5%	28.5%	6%
ABQ_{38}	100.0%	0.0%	0%	39.2%	60.8%	2%	29.8%	70.2%	2%	21.0%	79.0%	3%
NBQ_{95}	100.0%	0.0%	0%	55.9%	44.1%	7%	62.0%	38.0%	8%	85.5%	14.5%	7%
ABQ_{95}	100.0%	0.0%	0%	53.2%	46.8%	3%	16.8%	83.2%	2%	11.4%	88.6%	2%
BQ_{108}	100.0%	0.0%	0%	110.2%	-10.2%	3%	92.9%	7.1%	4%	87.9%	12.1%	16%
Negative Control	100.0%	0.0%	0%	100.0%	0.0%	8%	100.0%	0.0%	7%	100.0%	0.0%	6%

(b) Comparison of the Average Percentage of Cell Growth Inhibition on NBQ_{38} & NBQ_{95} (Nitro) vs. ABQ_{38} & ABQ_{95} (Amino).

Drugs	Time of Exposure (Hours)			Overall %
	24	48	72	
NBQ_{38} & NBQ_{95} (Amino)	39.4%	23.6%	21.5%	28.2%
ABQ_{38} & ABQ_{95} (Nitro)	53.8%	76.7%	83.8%	71.4%
Strength amino vs. nitro (x times)	1.4	3.3	3.9	2.5

Table 1. (a) Percentage of Cell Growth and Inhibition of A431 Cells Treated with BQ's IC_{50}. (b) Comparison of the Average Percentage of Cell Growth Inhibition on NBQ_{38} & NBQ_{95} (Nitro) vs. ABQ_{38} & ABQ_{95} (Amino).

(a) A431 Ellipticine Cytotoxicity

Elipticine [uM]	*Experiment 1*		*Experiment 2*		*% Average Living Cells*	*% Error*
	A	*B*	*A*	*B*		
0	100%	100%	100%	100%	100%	0%
0.5	48%	40%	28%	44%	40%	4%
1	48%	28%	22%	38%	34%	6%
1.5	55%	29%	6%	27%	29%	10%
2	2%	1%	0%	2%	1%	0%
3	0%	0%	0%	0%	0%	0%
4.1	0%	0%	0%	0%	0%	0%
4.9	0%	0%	0%	0%	0%	0%

Table 2. (a). A431 Ellipticine Cytotoxicity. A431 was tested for the cytotoxicity of Ellipticine in two experiments of duplicates. Different Ellipticine concentrations were used including previously published IC_{50} concentrations (1uM by Sureau, F. et al., 1993 and 4.1uM by Kuo, Y. et al., 2006). Experimental results reflected lower percentage of cell survival than those published.

(b) Ellipticine IC_{50}

Linear Equation	***y = -0.1574x + 0.5899***
IC_{50} =	(y-b)/m
50%=	(.50-.5899)/-.1574
IC_{50}=	0.57uM

Table 2. (b). Ellipticine IC_{50}. Results of Table 3 (a) were plotted and the linear equation (y=mx + b). Substituting 50% in the y variable, we obtained x; the IC_{50} value of Ellipticine in A431 was .57 $\pm$ .03uM (Standard error of the IC_{50} was obtained calculating the average of the standard error of the experiment).

State of the Mitochondrial Membrane in A431 Cells Treated with BQ's

Drugs	*Stage*	*Time of Exposure (Hours)*		
		24 Hours	*48 Hours*	*72 Hours*
NBQ$_{38}$	BO	X	X	
	IY		X	
	BG		X	X
NBQ$_{95}$	BO			
	IY	X	X	
	BG	X	X	X
ABQ$_{38}$	BO	X	X	
	IY	X	X	
	BG		X	X
ABQ$_{95}$	BO			
	IY	X		
	BG	X	X	X

Table 3. Qualitative Permeabilization of the mitochondrial membrane in A431 cells treated with BQ's IC_{50}. Three categories were created to assess visual permeabilization of the mitochondrial membrane (refer to the legend below). All BQ-treated cells completely permeabilized the mitochondrial membrane upon treatment with BQ's IC_{50} at 72 hours.

Legend:
BO = Bright Orange; Healthy Mitochondrial Membrane
IY = Intermediate Yellow; Partially Permeabilized Mitochondrial Membrane
BG = Bright Green; Permeabilized Mitochondrial Membrane

Healthy and Apoptotic A431 Mitochondria Treated with BQ's IC_{50} for 48 Hours

Drugs	*% Apoptotic Mitochondria*	*% Healthy Mitochondria*	*% Error*
NBQ_{38}	48%	52%	11%
NBQ_{95}	51%	49%	10%
BQ_{108}	39%	61%	20%
ABQ_{38}	36%	64%	8%
ABQ_{95}	45%	55%	8%
Negative Control	0%	100%	2%
Valinomycin Positive Control	65%	35%	6%

Table 4. Quantitative Healthy and Apoptotic A431 Mitochondria Treated with BQ's IC_{50} for 48 Hours. A431 was tested for the apoptotic mitochondria using the permeabilization of the mitochondrial membrane as indicator. Two experiments of duplicates plates were exposed for each BQ's IC_{50} for 48 Hours. Valinomycin was used as Positive Control. Experimental results were normalized to correct the apoptotic background of the cell line (35%). BQ treated cells resulted in permeabilized mitochondrial membrane.

Activation of Caspases 3, 7, and 8 in A431 Treated with BQ's IC_{50} for 48 Hours

Drugs	*Caspase 3 and 7*		*Caspase 8*	
	Active	*Non-Active*	*Active*	*Non-Active*
Negative Control		X		X
Staurosporine	X		X	
Ellipticine	X		n/d	
Cisplatin	X		X	
NBQ_{38}	X			X
NBQ_{95}	X			X
BQ_{108}	X			X
ABQ_{38}	X			X
ABQ_{95}	X			X

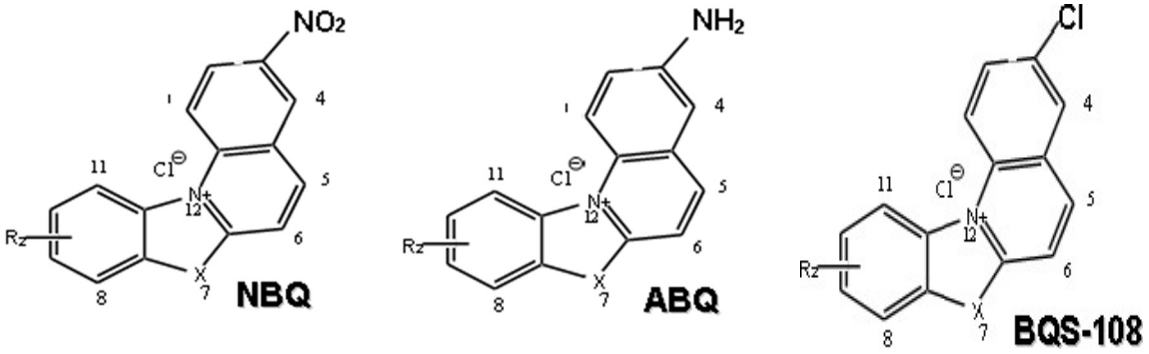
Table 5. Qualitative Activation of Caspases 3, 7, and 8 in A431 Treated with BQ's IC_{50} for 48 Hours. Samples were stained with Magic Red TM for Caspases 3 and 7 and with FLICA for Caspase 8 for one hour after exposure with BQ's. Cells were mounted in glass slides observed in regular and fluorescent light. Using Positive and Negative Controls as indicators samples were catalogued as Active (fluorescence-activated Caspases) or Non-active (No fluorescence). Controls responded accordingly, Ellipticine activated Caspases 3 and 7 but Caspase 8 could not be determined due to fluorescence overlapping. Cisplatin activated both Caspases while all BQ drugs only activated Caspases 3 and 7 without activating Caspase 8.

Activation of Caspases 3 and 7 in A431 Treated with BQ's IC_{50} for 48 Hours

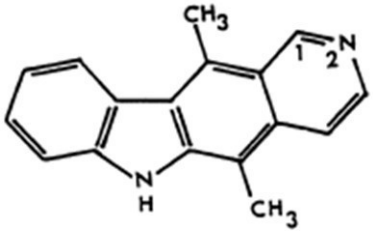
Drug	*% Activated Caspases 3 and 7*	*% of Error*
NBQ_{38}	22%	7%
NBQ_{108}	27%	5%
ABQ_{38}	51%	1%
ABQ_{95}	46%	6%
Cisplatin	51%	13%
NE	0%	1%

Table 6. Quantitative Apoptotic Activation of Caspases 3 and 7 in A431 Treated with BQ's IC_{50} for 48 Hours. A431 was tested for the apoptotic activation of Caspases 3 and 7 by staining exposed cells with Magic Red™ . Two experiments of duplicates plates were exposed for each BQ's IC_{50} for 48 Hours. Cisplatin was used as Positive Control. Experimental results were normalized to correct Caspases 3 and 7 apoptotic background cell line activation. BQ treated cells resulted in active Caspases 3 and 7 activations. ABQ's IC_{50} activated Caspases 3 and 7 similarly to Cisplatin's IC_{50} and almost doubled NBQ's IC_{50}. The order of Caspases 3 and 7 activations are Cisplatin; 51 $\pm$ 13% = ABQ_{38}; 51 $\pm$ 1%) > ABQ_{95}; 46 $\pm$ 6% > BQ_{108}; 27 $\pm$ 5% > NBQ_{38}; 22 $\pm$ 7% and Negative Control; 0 $\pm$ 1%. ABQ_{38} presented the highest Caspases 3 and 7 activations at 48 hours of exposure.

FIGURES



Compound	X	R1	R2	MW (g/mol)	IC50
BQS-108	S	4-Cl	10-CH3	320.23	4uM
NBQ-38	NC2H5	3-NO2	H	327.07	36uM
ABQ-38	NC2H5	3-NH2	H	297.78	32uM
NBQ-95	S	2-Cl; 3-NO2	10-CH3	365.25	28uM
ABQ-95	S	2-Cl; 3-NH2	10-CH3	335.95	36uM



Ellipticine

Figure 1. BQ's chemical structure and Ellipticine (structural analog). NBQ's have a nitro terminal group (NO_2), ABQ's have an amino-terminal group (NH_2), and BQS_{108} has a cloro terminal group (Cl). NBQ's are NBQ_{38}; NBQ_{95} and ABQ's are ABQ_{38} and ABQ_{95}. Each compound has its molecular weight and IC_{50}'s of each compound was determined previously (Arroyo, V., unpublished data). All BQ's are novel proposed anticancer compounds, and Dr. Osvaldo Cox has synthesized them.

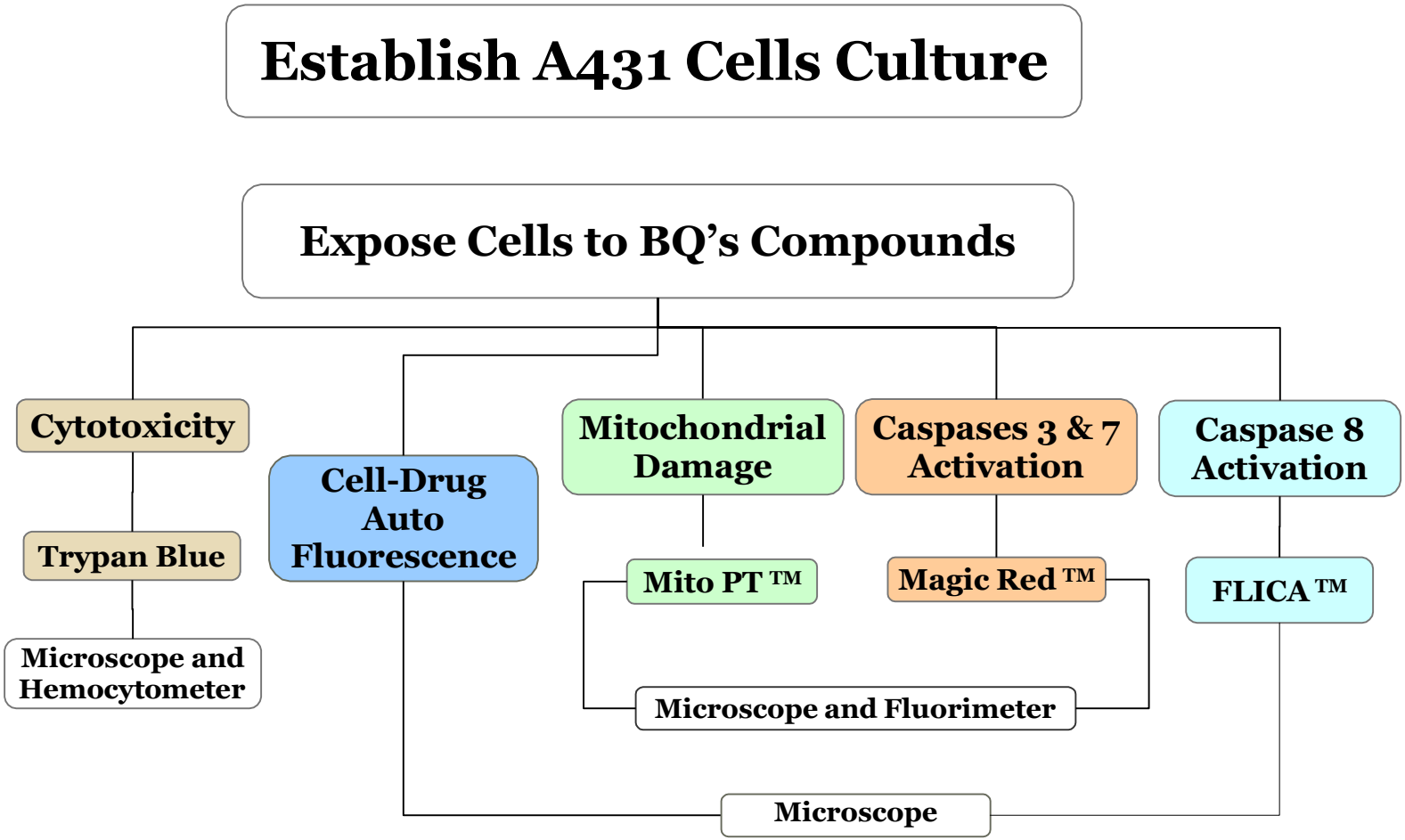


Figure 2. Experimental Methodology. A continuous source of cells was available after establishing the cell line. Cellswere exposed to BQ's and five (5) different experiments were performed.

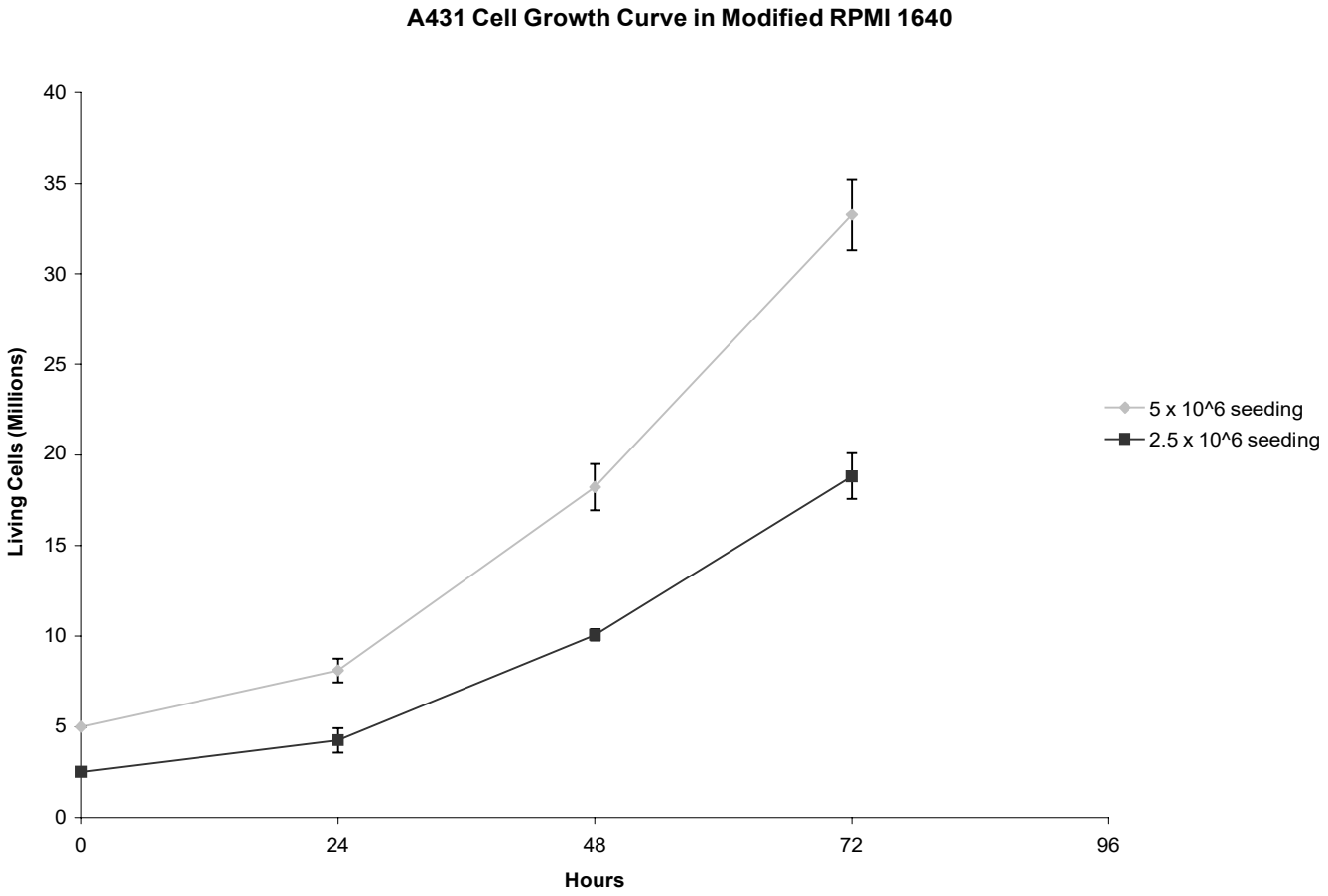
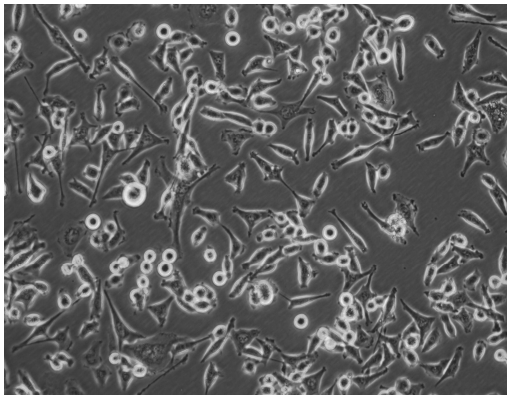
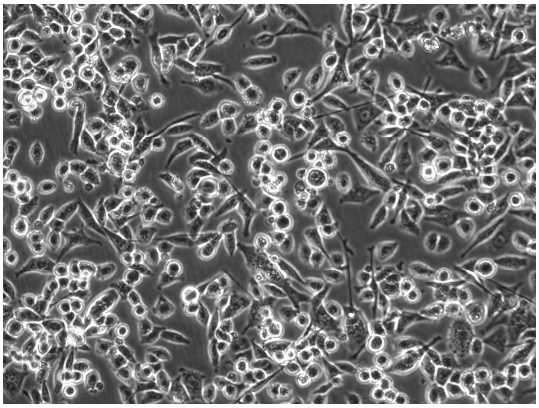


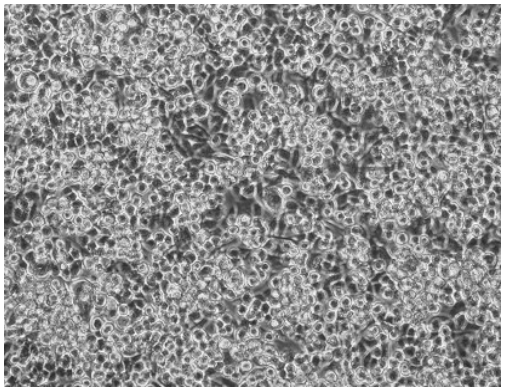
Figure 3. Growth curve values of A431 in modified RPMI 1640. Two different numbers of cells were seeded on 25cm2 t-flasks to determine the best initial concentration of cells. Both amounts show a continuous growth from o to 72 hours. The graph does not plateau at 72 hours indicating that cells are capable of growing without changing the media for 72 hours. 24 Hours



24 Hours

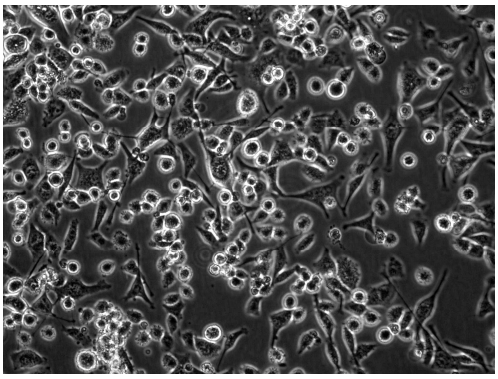


48 Hours

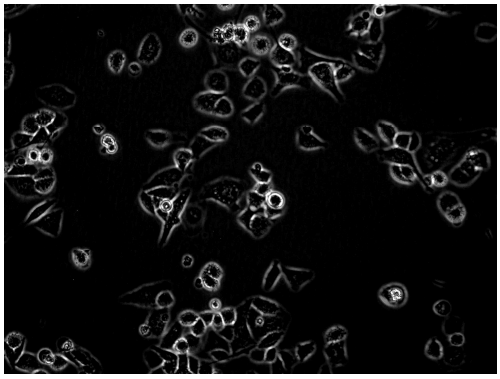


72 Hours

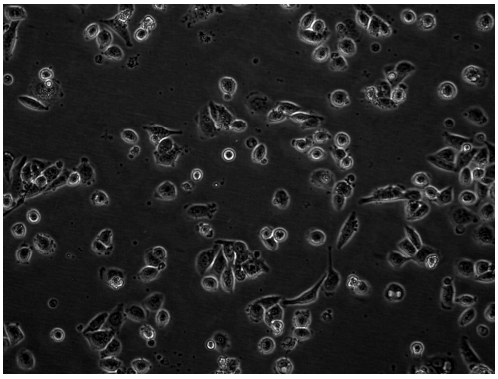
Figure 4. Photos from the Growth Curve of A431 in modified RPMI 1640 media. (Objective 10X).



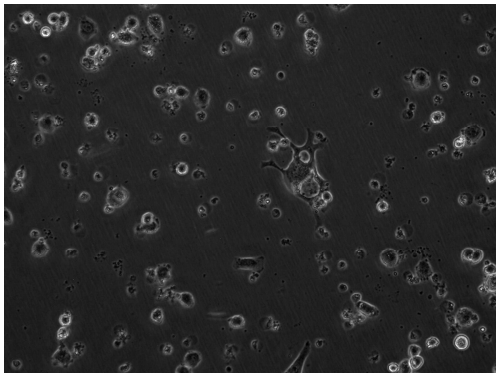
Negative Control



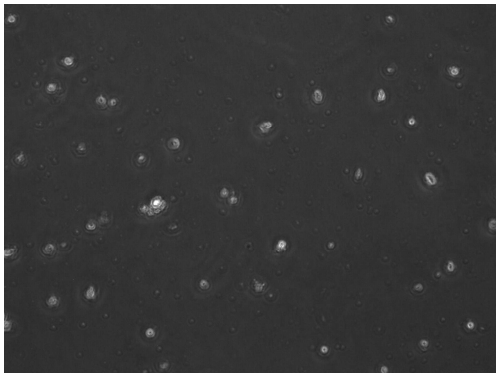
[.5uM]



[1uM]



[2uM]



[3uM]

Figure 5. Photos of A431 cell line exposed to Ellipticine at different concentrations for 48 hours to determine its IC_{50} value = 57 $\pm$ .03 (Objective 10X). Different concentrations of Ellipticine were used to determine the IC_{50} experimental value for A431. All concentrations of Ellipticine inhibited cell proliferation when compared to the Negative Control. Growth inhibition was dependent on Ellipticine concentration.

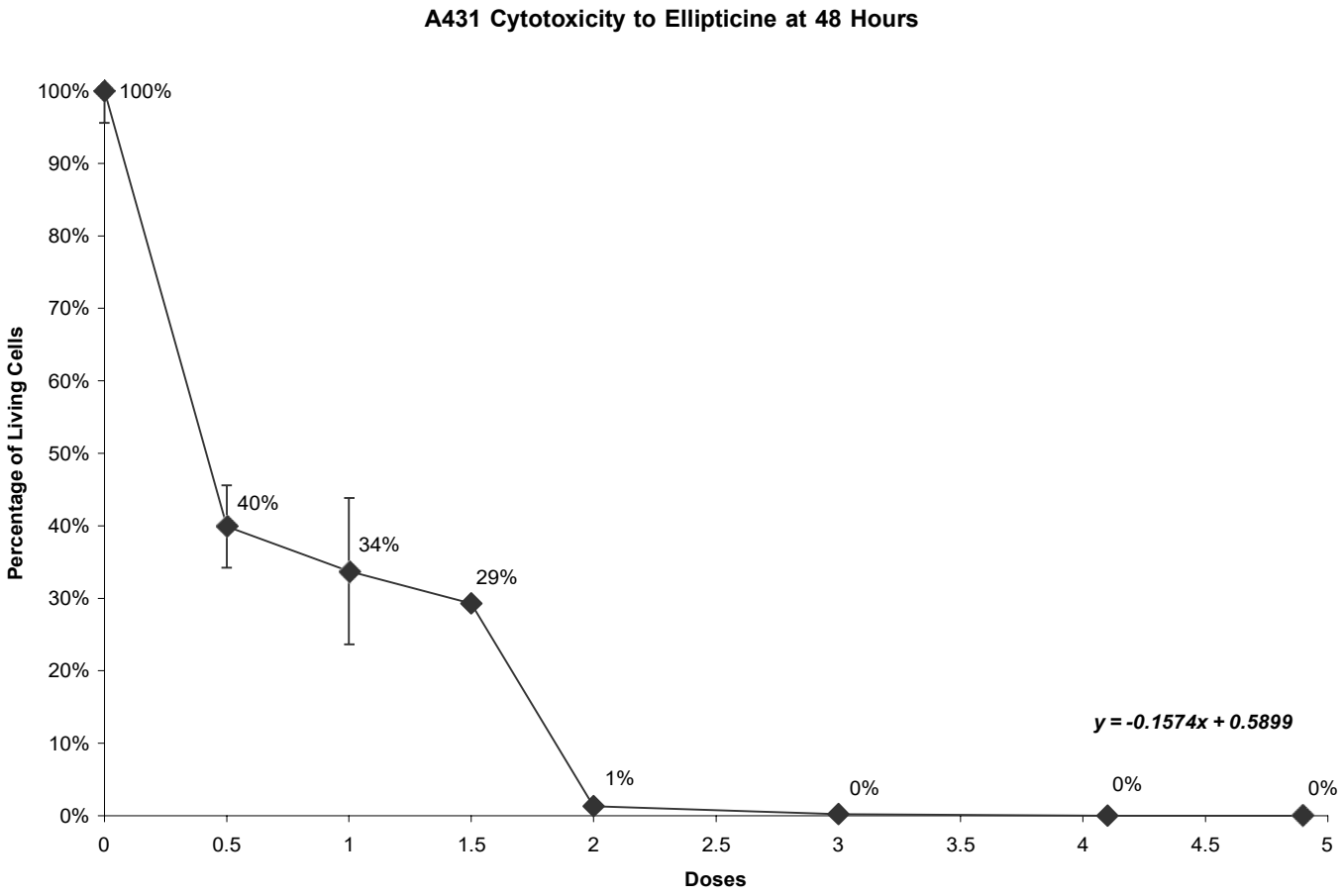
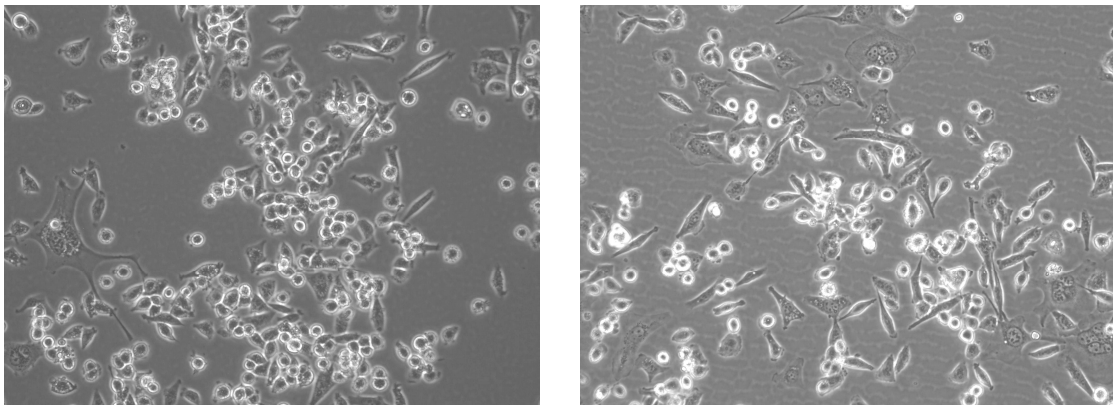
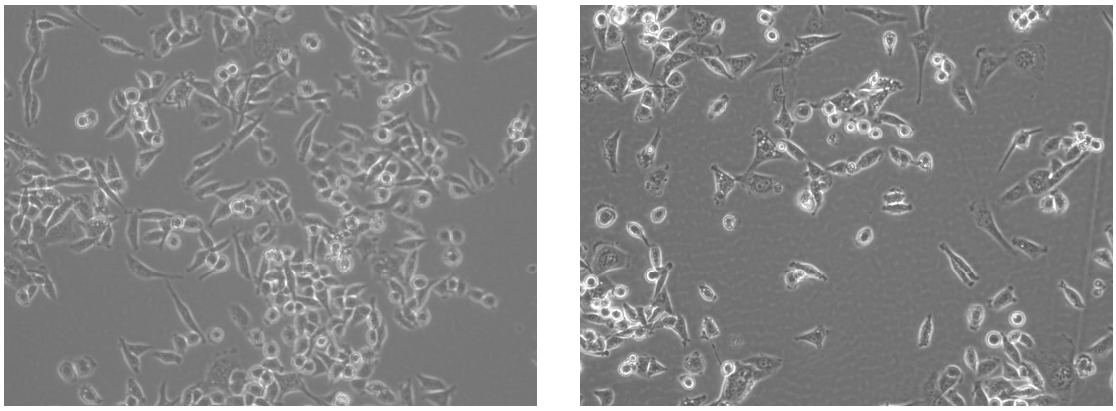


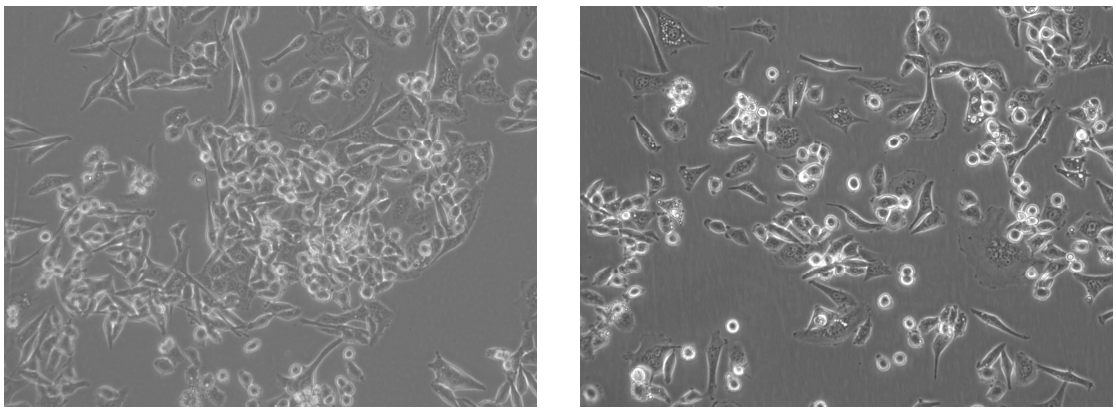
Figure 6. Graph of A431 cell line exposed to Ellipticine at different concentrations for 48 hours to determine its IC_{50} value = 5.7 $\pm$ .03; calculated in table 3 (b). The average percentage of surviving cells was plotted against dose concentrations revealing that cytotoxicity of Ellipticine is dependent on dose at 48 Hours. The nearest data point in this plot is the .5uM dose; reflecting 40% of surviving cells.



NBQ_{38} 24H **ABQ_{38} 24H**

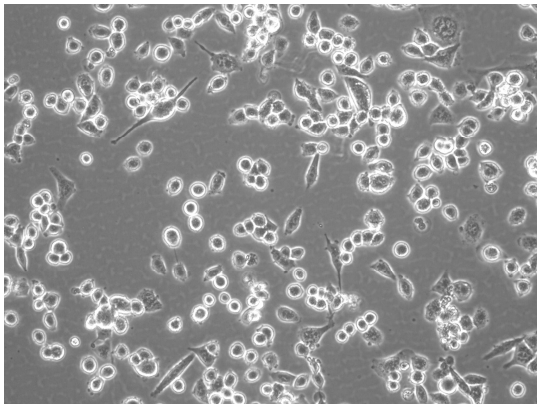


NBQ_{38} 48H **ABQ_{38} 48H**

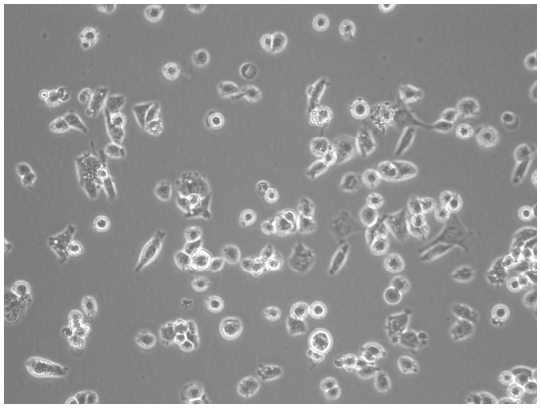


NBQ_{38} 72H **ABQ_{38} 72H**

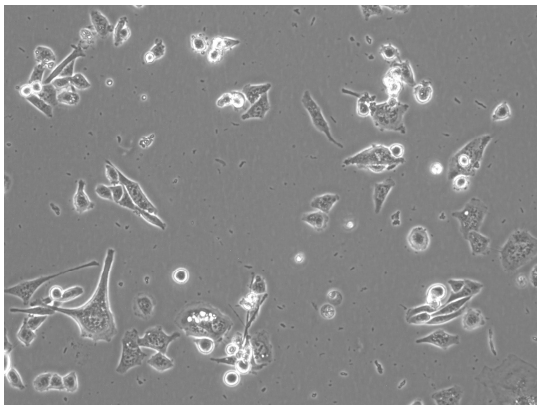
Figure 7. Cell confluence photos of NBQ_{38} and ABQ_{38} (Objective 10X). NBQ's and ABQ's inhibited cell growth in a time dependent manner when compared to control.



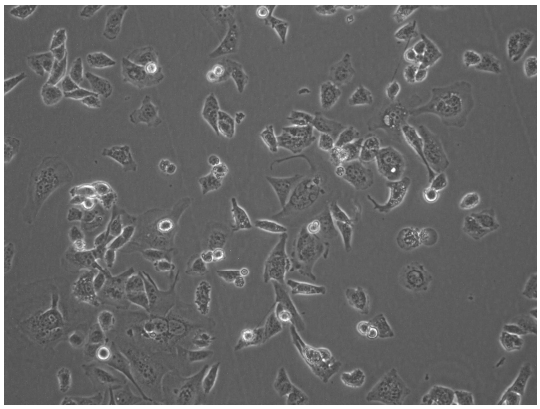
NBQ_{95} 24H



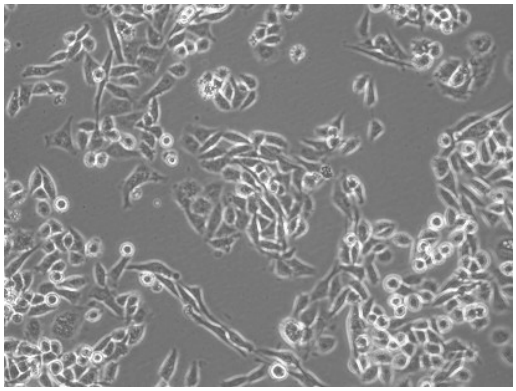
ABQ_{95} 24H



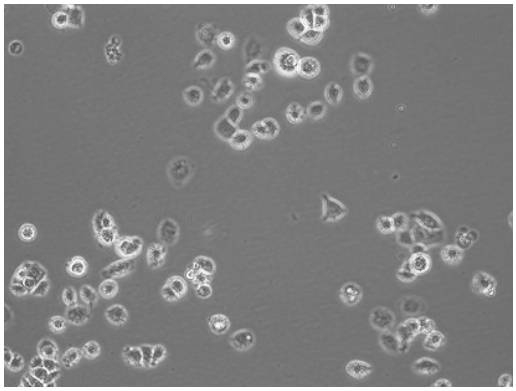
NBQ_{95} 48H



ABQ_{95} 48H



NBQ_{95} 72H



ABQ_{95} 72H

Figure 8. Cell confluence photos of NBQ_{95} and ABQ_{95} (Objective 10X). NBQ's and ABQ's inhibited cell growth in a time-dependent manner when compared to control.

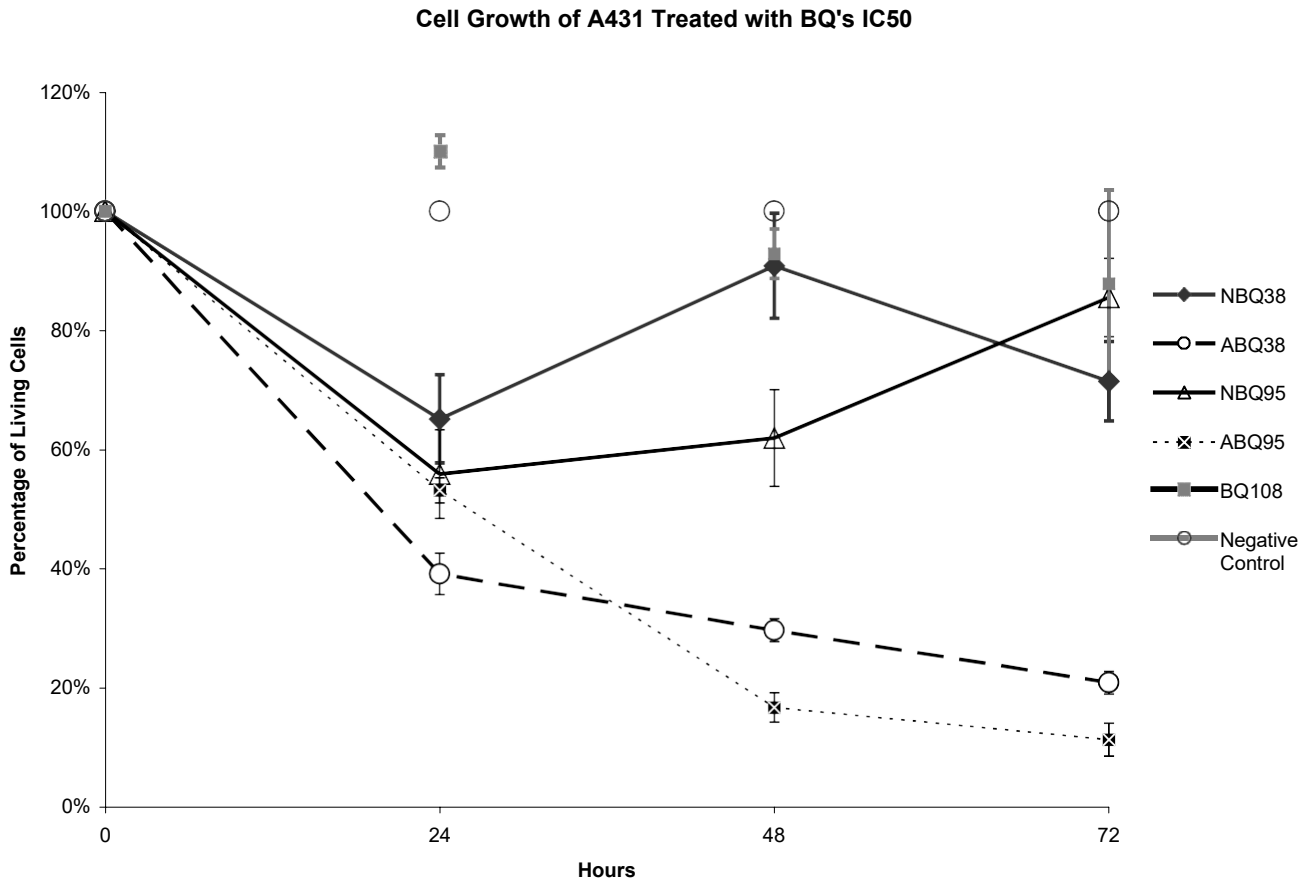


Figure 9. Graph for the Percentage of Cell Growth of A431 Treated with BQ's IC_{50}. The percentage of A431 living cells decreased with an increase in exposure time. ABQ's predominantly inhibited more aggressively cell growth at 72 hours than NBQ's. Both ABQ's behave similarly by continuously decreasing living cells. However NBQ's did not exhibit superior growth inhibition than ABQ's. IC_{50} values of four of the five drugs tested might need to be reconsidered for adjustment.

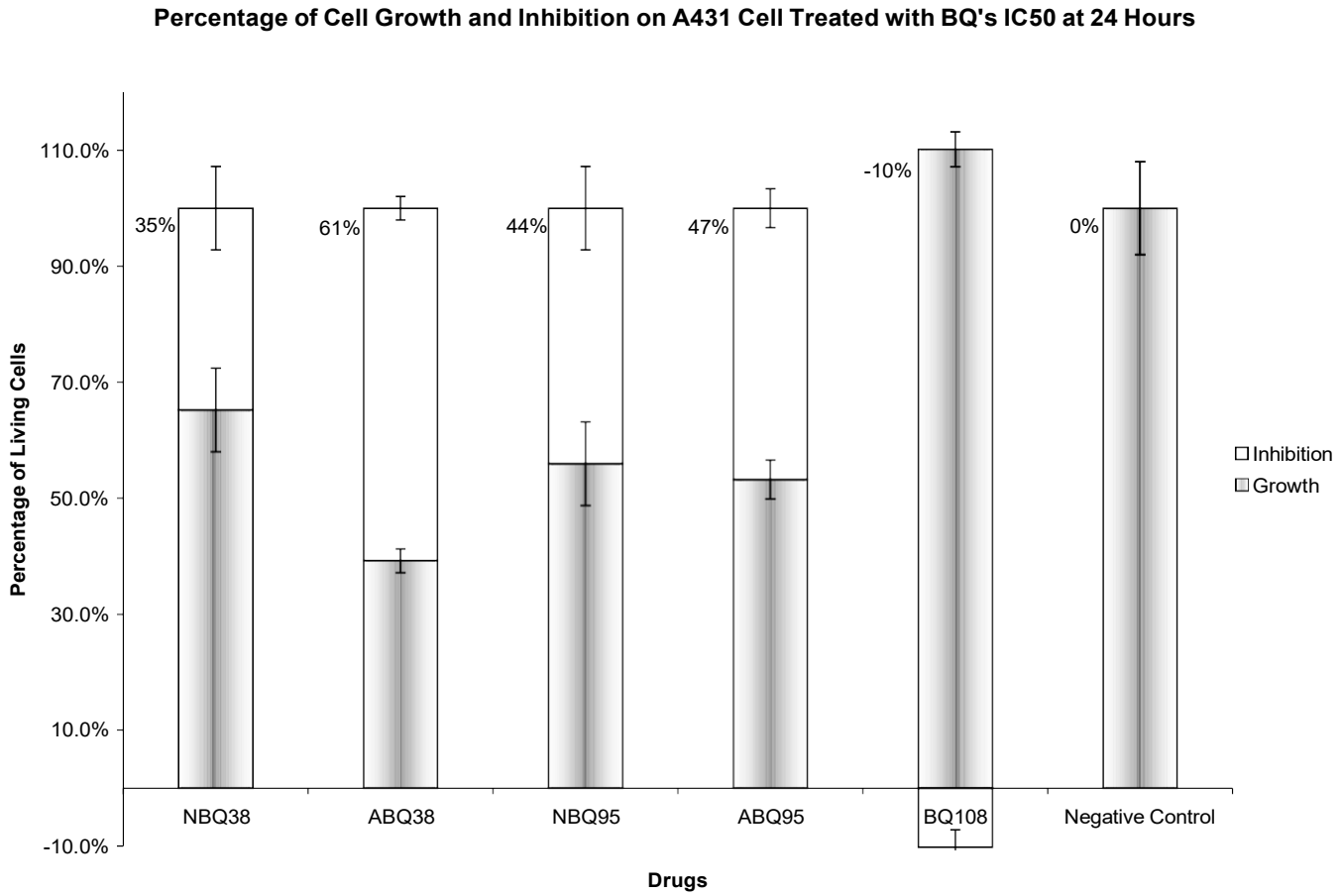


Figure 10. Graph of the Percentage of Cell Growth Inhibition on A431 Cells Treated with BQ's IC_{50} at 24 Hours. Bars on top are the percentage of cell growth inhibited versus the percentage of cells that grew on the bars at the bottom. Amino and Nitro terminal drugs inhibited cell growth from 35% to 61%. Order of potency of the inhibitors at 24 hours was: ABQ38 (61%) > ABQ_{95} (47%) > NBQ_{95} (44%) > NBQ_{38} (35%) > Negative Control (0%) > BQ108 (-10%.).

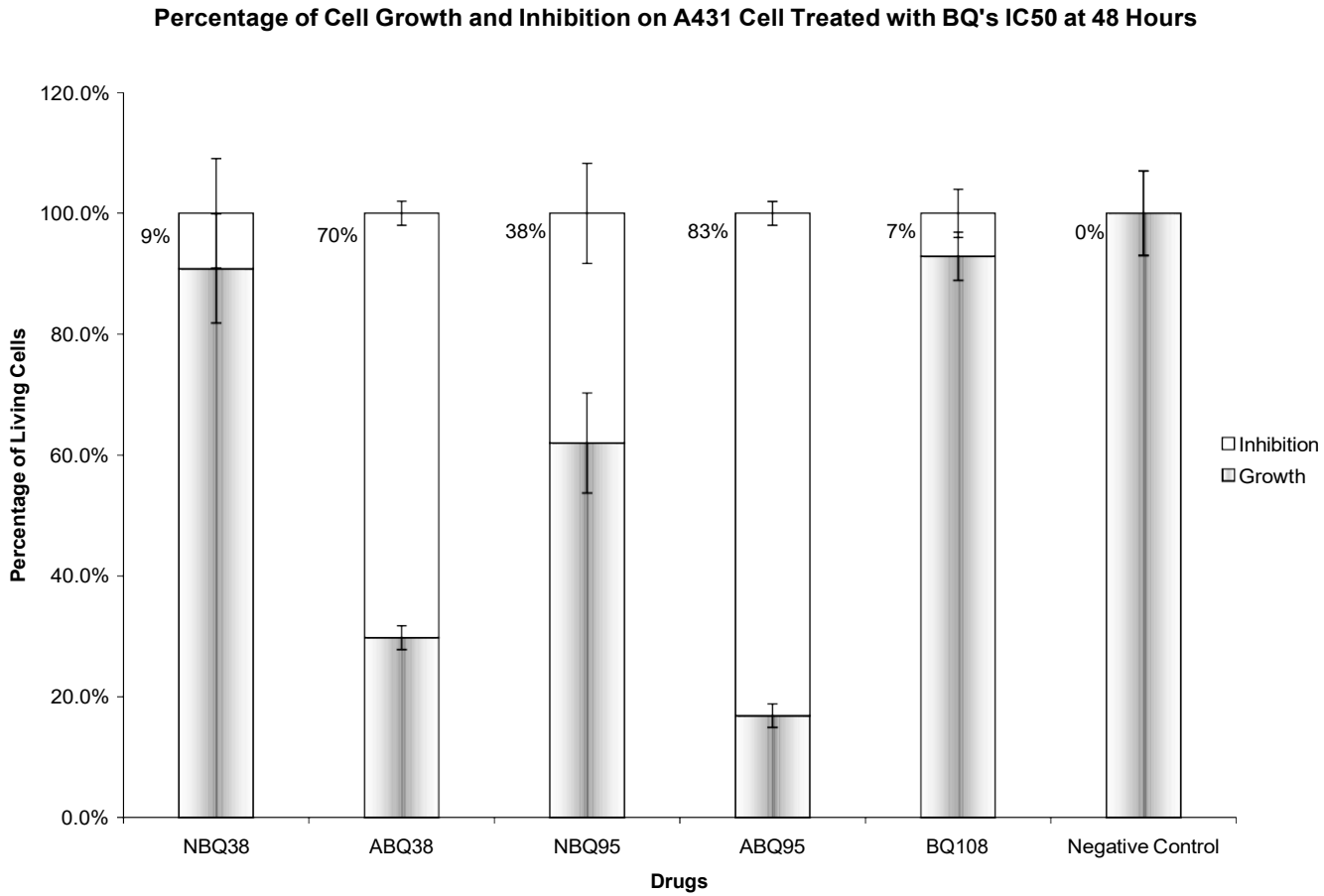


Figure 11. Graph of the Percentage of Cell Growth Inhibition on A431 Cells Treated with BQ's IC_{50} at 48 Hours. Bars on top are the percentage of cell growth inhibited versus the percentage of cells that grew on the bars at the bottom. Amino and Nitro terminal drugs inhibited cell growth from 9% to 83%. Order of potency of the inhibitors at 48 hours was: ABQ_{95} (83%) > ABQ38 (70%) > NBQ_{95} (38%) > NBQ_{38} (9%) > BQ108 (7%) > Negative Control (0%).

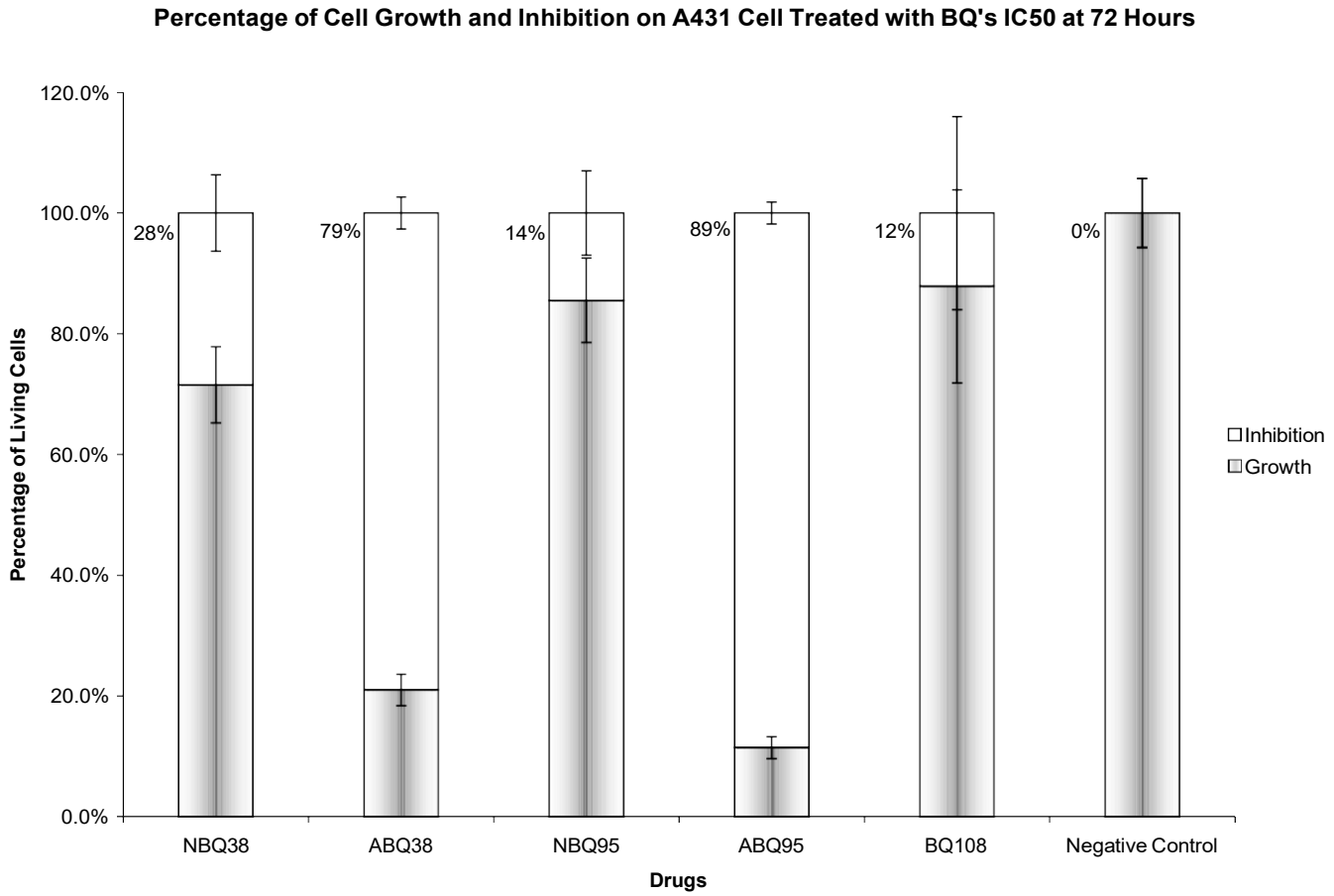


Figure 12. Graph of the Percentage of Cell Growth Inhibition on A431 Cells Treated with BQ's IC_{50} at 72 Hours. Bars on top are the percentage of cell growth inhibited versus the percentage of cells that grew on the bars at the bottom. Amino and Nitro terminal drugs inhibited cell growth from 14% to 89%. Order of potency of the inhibitors at 72 hours was: ABQ_{95} (89%) > ABQ38 79%) > NBQ_{38} (28%) > NBQ_{95} (14%) > BQ_{108} (14%) > Negative Control (0%)

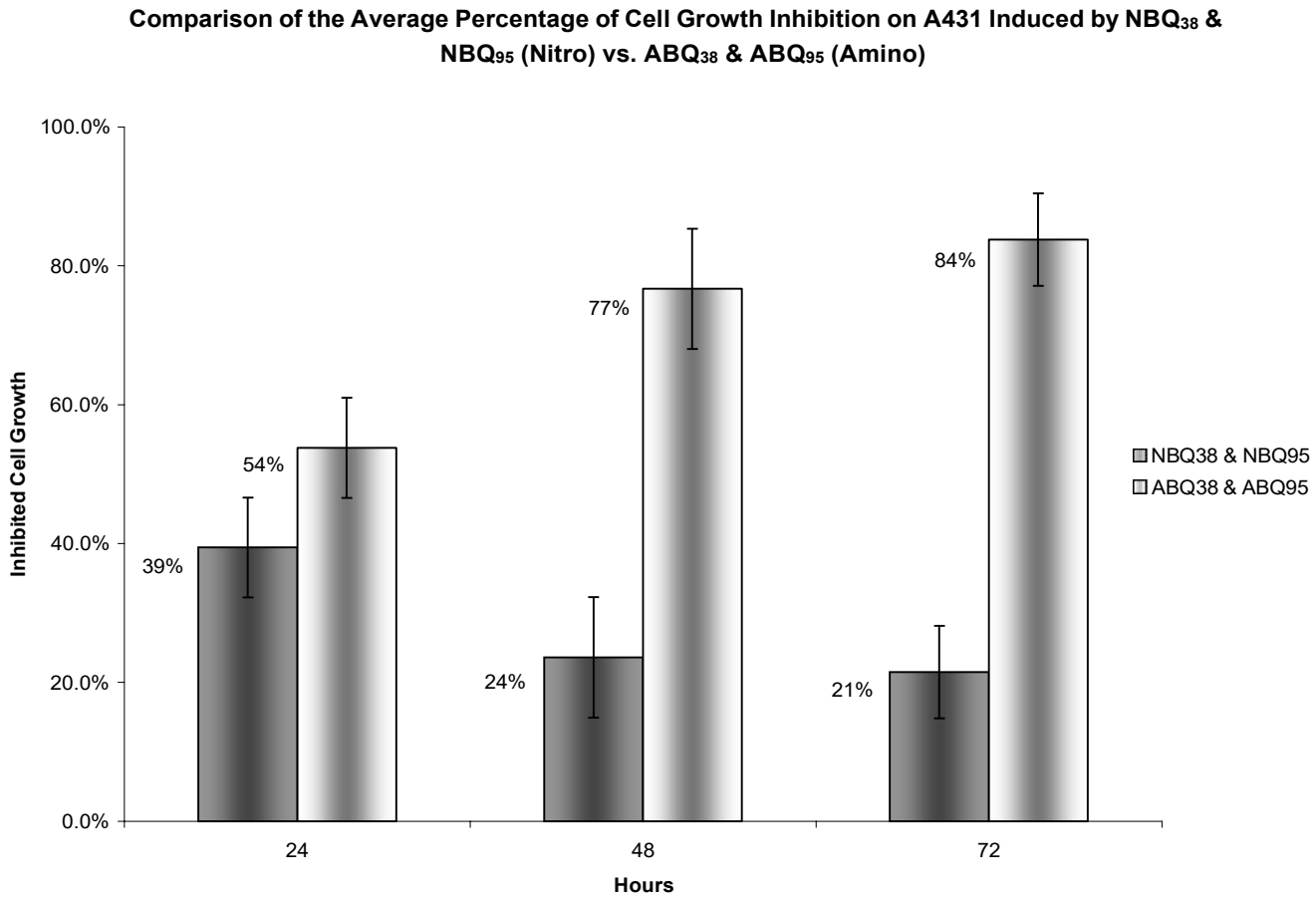
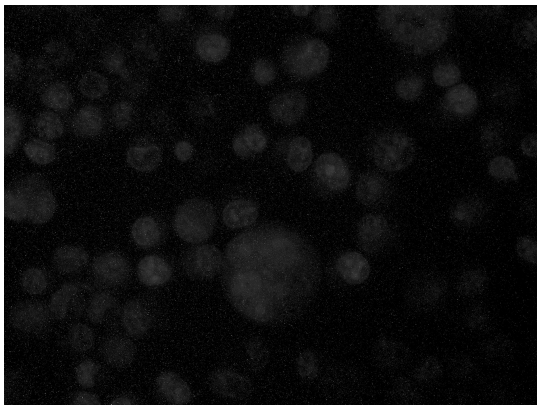
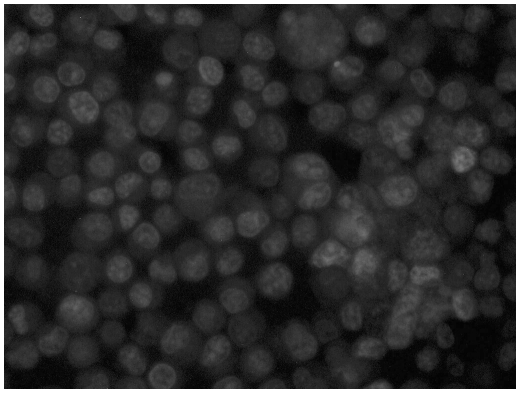


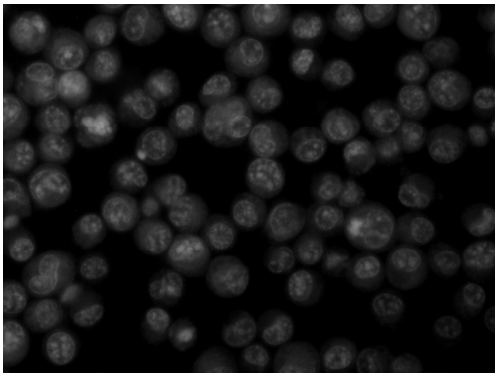
Figure 13. Graph for the Comparison of the Average Percentage of Cell Growth Inhibition on A431 Induced by NBQ_{38} & NBQ_{95} (Nitro) vs. ABQ38 & ABQ_{95} (Amino). Nitro compounds are represented by dark grey and Amino compounds by light grey. Amino and Nitro terminal drugs inhibited cell growth from 22% to 84%. Order of Potency by time of exposure was: 24 Hours; Amino (54%) > Nitro (39%), 48 Hours; Amino (77%) > Nitro (24%) and 72 Hours; Amino (84%) > Nitro (21%).



ABQ_{38} IC_{50} 72 Hours

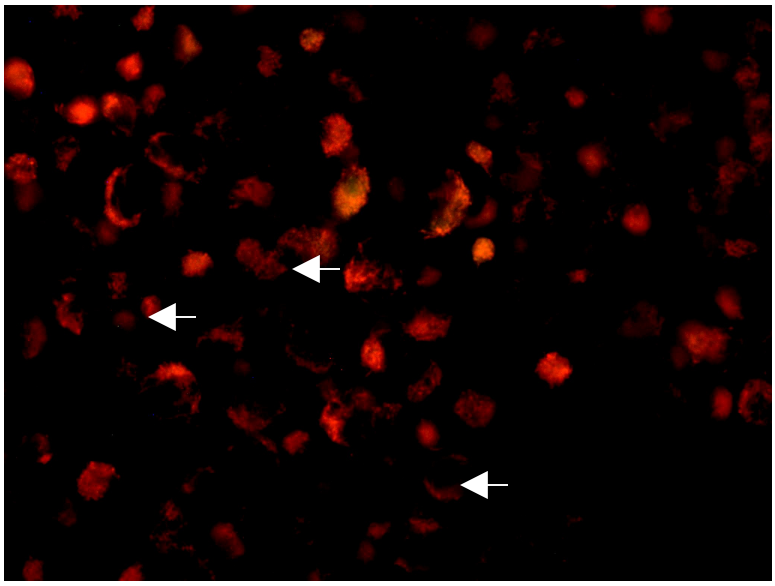


ABQ_{95} IC_{50} 72 Hours



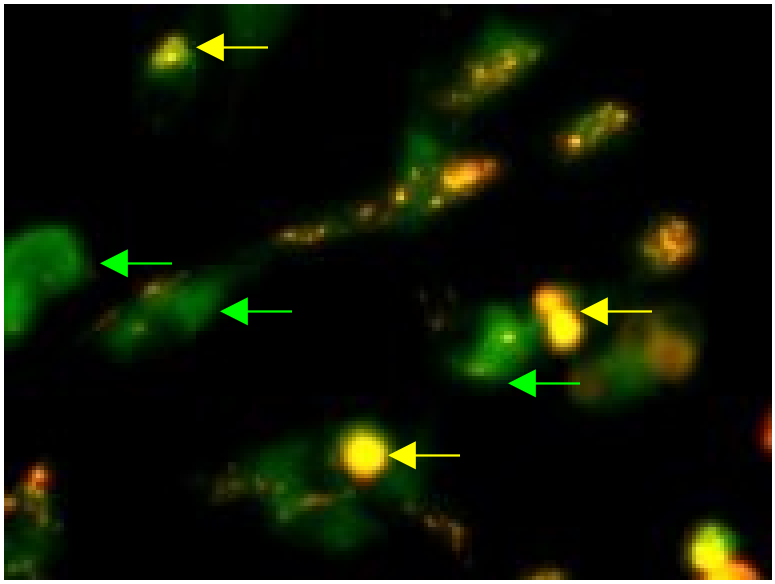
Ellipticine IC_{50} 72 Hours

Figure 14. Photos of ABQ's cell auto-fluorescence and positive control Ellipticine (40X Objective). A431 was exposed from 24 to 72 hours at the IC_{50} concentrations of BQ's. Negative control and NBQ's did not exhibit cell auto-fluorescence (images not shown), while ABQ's did. A similar sub-cellular fluorescent structure co-localizes the drug. It is known that Ellipticine selectively binds to mitochondria and endoplasmic reticulum due to its negative charge.



Bright Orange

Negative Control

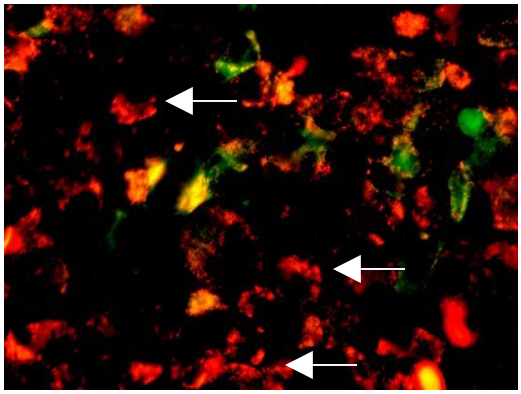


Bright Green

Intermediate Yellow

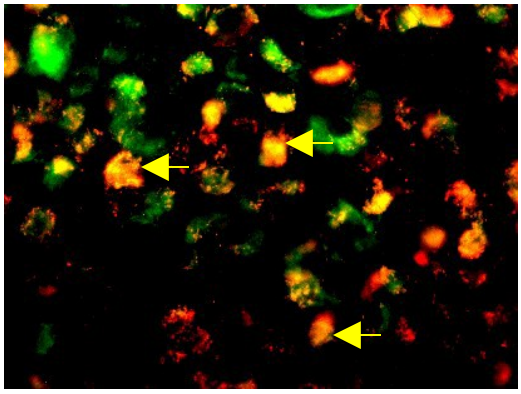
Positive Control (Valinomycin)

Figure 15. Photos of Mito PT™ Mitochondrial Permeability Negative and Positive Controls. After 48 hours, Controls were stained with Mito PT for 1 hour, and cells were mounted in glass slides with coverslips to detect permeabilization on the mitochondrial membrane. Pictures revealed distinctive colors; Bright Orange (non-permeabilized membrane) in Negative Control and Intermediate Yellow and Bright Green (partial and complete permeabilized membrane) in Positive Control (Valinomycin). White arrows identify some of the "healthy mitochondria" (BO). Yellow arrows are Intermediate Yellow (IY), and green arrows are Bright Green (BG), identifying the transition and completion of "damaged mitochondria"



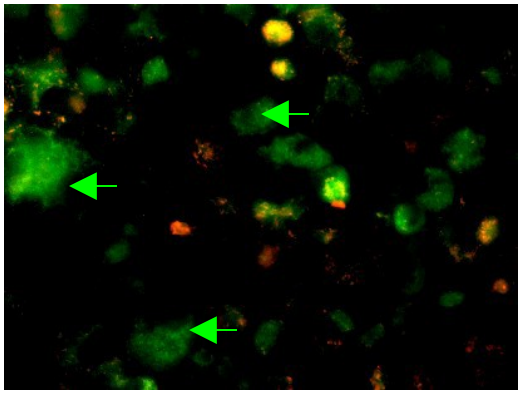
Bright Orange

NBQ_{38} IC_{50} 24 Hours



Intermediate Yellow

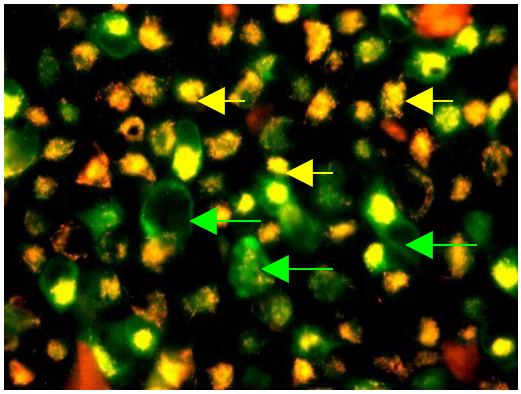
NBQ_{38} IC_{50} 48 Hours



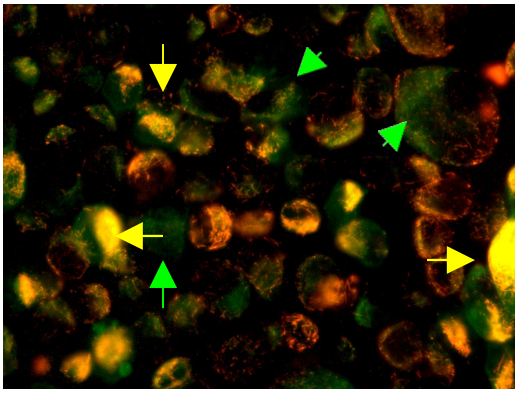
Bright Green

NBQ_{38} IC_{50} 72 Hours

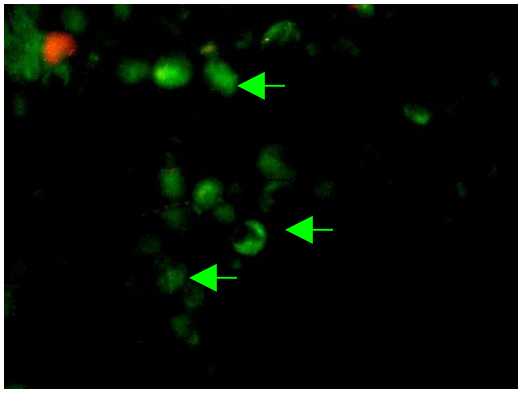
Figure 16. Photos of Mito PT™ Mitochondrial Permeability NBQ_{38} IC_{50}. After exposure to NBQ_{38}, samples were stained with Mito PT for 1 hour, and cells were mounted in glass slides with coverslips to detect permeabilization on the mitochondrial membrane. Pictures revealed that NBQ_{38} had predominant “healthy mitochondria” (BO) at 24 Hours. At 48 hours, most of the cells had an intermediate permeabilized membrane (IY). At 72 hours cells have permeabilized membrane (BG) predominantly as an indication of damaged mitochondria.



NBQ_{95} IC_{50} 24 Hours

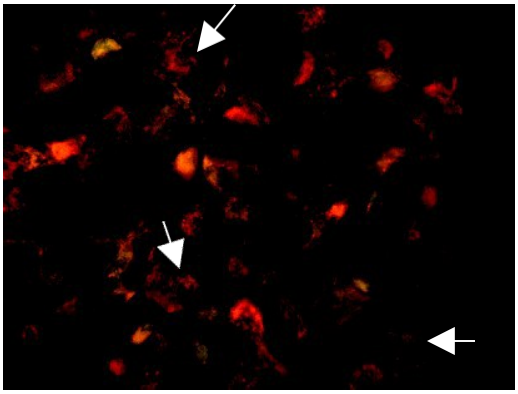


NBQ_{95} IC_{50} 48 Hours



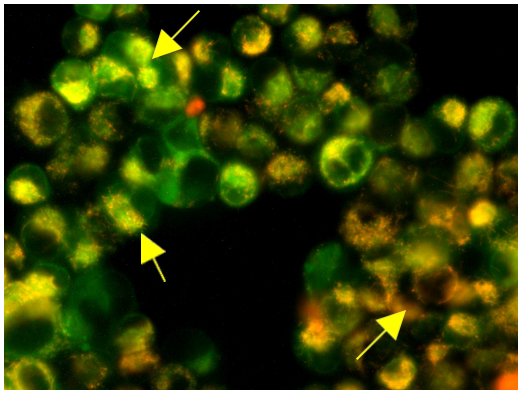
NBQ_{95} IC_{50} 72 Hours

Figure 17. Photos of Mito PT™ Mitochondrial Permeability NBQ_{95} IC_{50}. After exposure to NBQ_{95}, samples were stained with Mito PT for 1 hour, and cells were mounted in glass slides with coverslips to detect permeabilization on the mitochondrial membrane. At 24 and 48 hours, pictures revealed that NBQ_{95} had a mixed population of cells with intermediate permeabilized membrane (IY) and permeabilized membrane (BG). At 72 Hours cells have permeabilized membrane (BG) predominantly as an indication of damaged mitochondria.



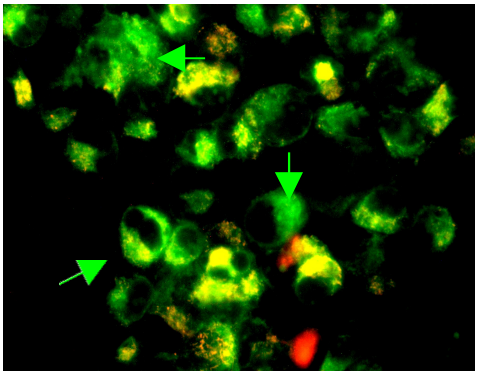
Bright Orange

ABQ_{38} IC_{50} 24 Hours



Intermediate Yellow

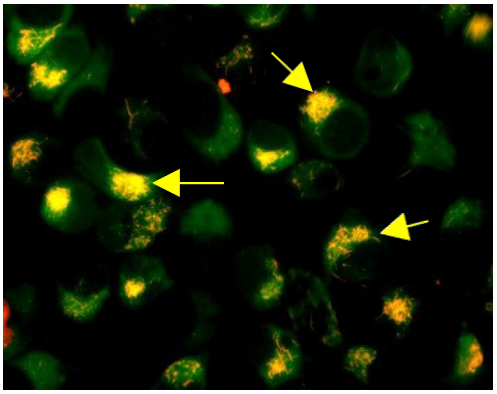
ABQ_{38} IC_{50} 48 Hours



Bright Green

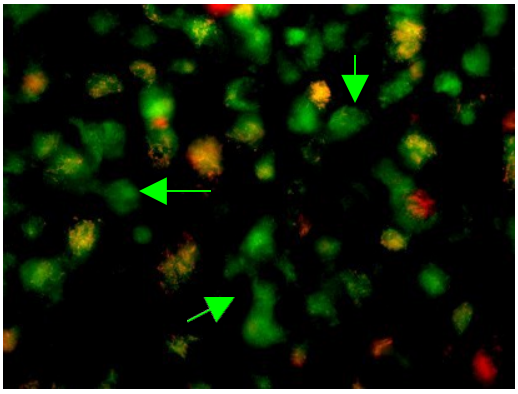
ABQ_{38} IC_{50} 72 Hours

Figure 18. Photos of Mito PT™ Mitochondrial Permeability ABQ_{38} IC_{50}. After exposure to ABQ_{38}, samples were stained with Mito PT™ for 1 hour, and cells were mounted in glass slides with coverslips to detect permeabilization on the mitochondrial membrane. At 48 hours, pictures revealed that ABQ_{38} had a mixed population of cells with the intermediate permeabilized membrane (IY) and permeabilized membrane (BG). At 72 hours cells have permeabilized membrane (BG) predominantly as an indication of damaged mitochondria.



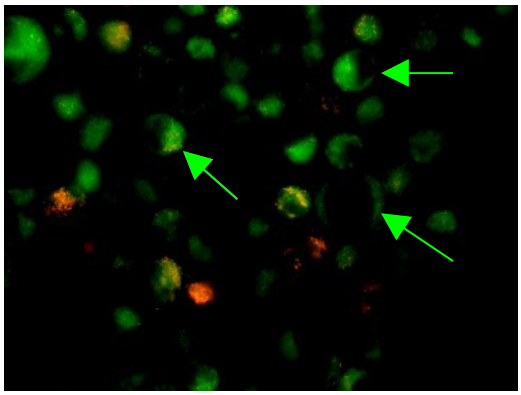
Intermediate Yellow

ABQ_{95} IC_{50} 24 Hours



Bright Green

ABQ_{95} IC_{50} 48 Hours



Bright Green

ABQ_{95} IC_{50} 72 Hours

Figure 19. Photos of Mito PT™ Mitochondrial Permeability ABQ_{95} IC_{50}. After exposure to ABQ_{95}, samples were stained with Mito PT for 1 hour, and cells were mounted in glass slides with coverslips to detect permeabilization on the mitochondrial membrane. At 24 hours, pictures revealed that ABQ_{95} had a mixed population of cells with the intermediate permeabilized membrane (IY) and permeabilized membrane (BG). At
48 and 72 Hours cells have permeabilized membrane (BG), predominantly indicating damaged mitochondria.

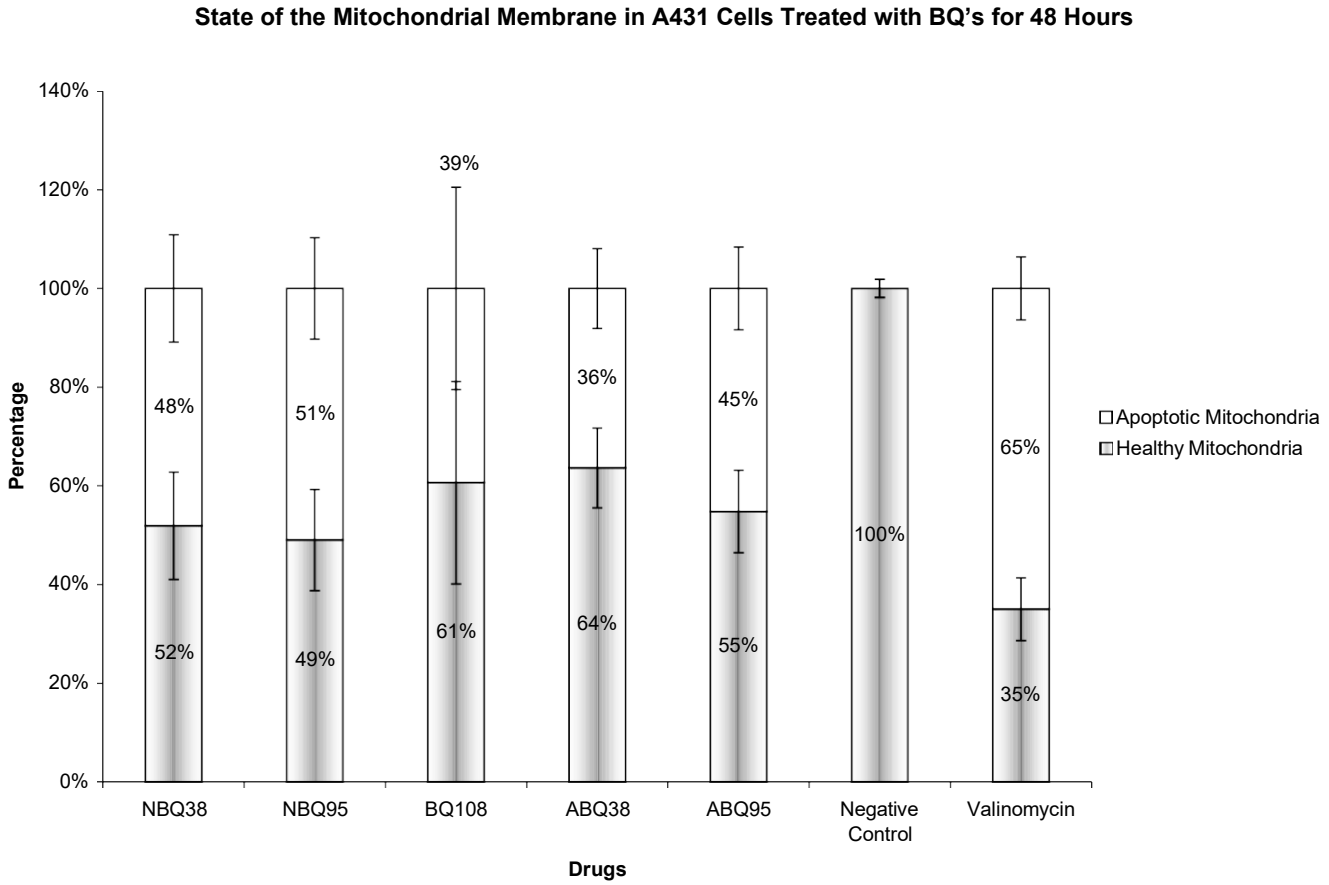
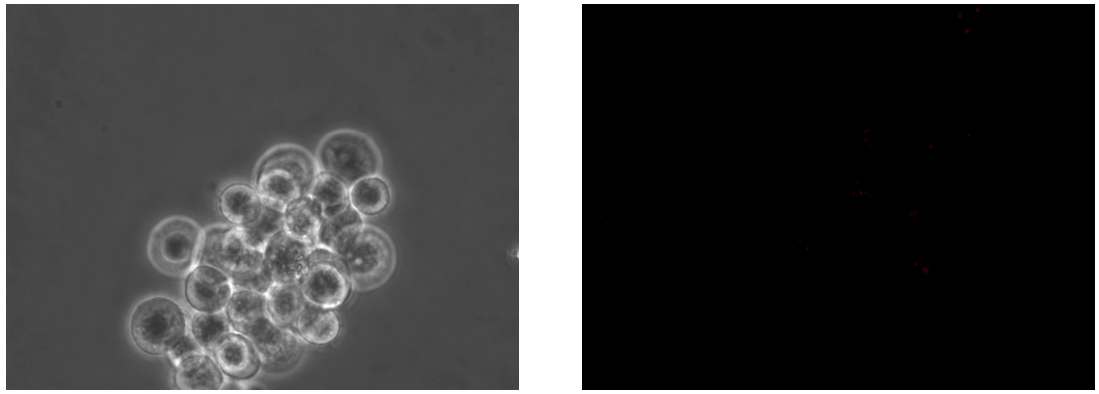
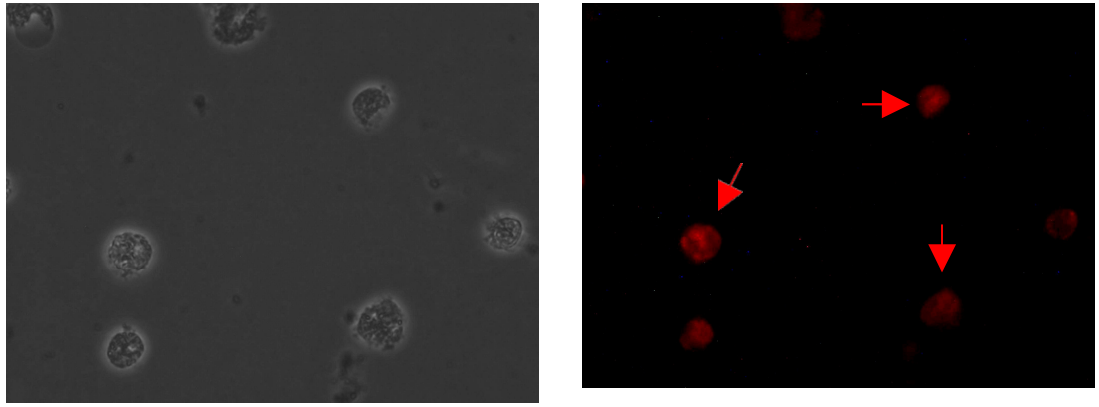


Figure 20. Graph for the State of the Mitochondrial Membrane in A431 Cells Treated with BQ's for 48 Hours. Healthy mitochondria are represented by the dark grey and damaged mitochondria in white. Experimental values were normalized by subtracting the A431 damaged mitochondrial value (35 $\pm$ 2%). All BQ's showed permeabilization of the mitochondrial membrane at 48 hours. The order of permeabilization of the mitochondrial membrane was: Valinomycin; 65 $\pm$ 6% > NBQ_{95}; 51 $\pm$ 10% > NBQ_{38}; 48 $\pm$ 11% > ABQ_{95}; 45 $\pm$ 8% > BQ_{108}; 39 $\pm$ 20% > ABQ_{38}; 36 $\pm$ 8% and Negative Control; 0 $\pm$ 2%. NBQ_{95} presented higher preferential mitochondrial damage at 48 hours of exposure.



Non-Active
Caspases 3 and 7

Negative Control 48 Hours



Active Caspases 3
and 7

Positive Control (Staurosporine 1uM) 48 Hours

Figure 21. *Photos* of Magic Red™ Caspases 3 and 7 Negative and Positive Controls. Contrasting images from Positive Control and Negative Control allows distinction between active (red arrows) and non-active Caspases 3 and 7.

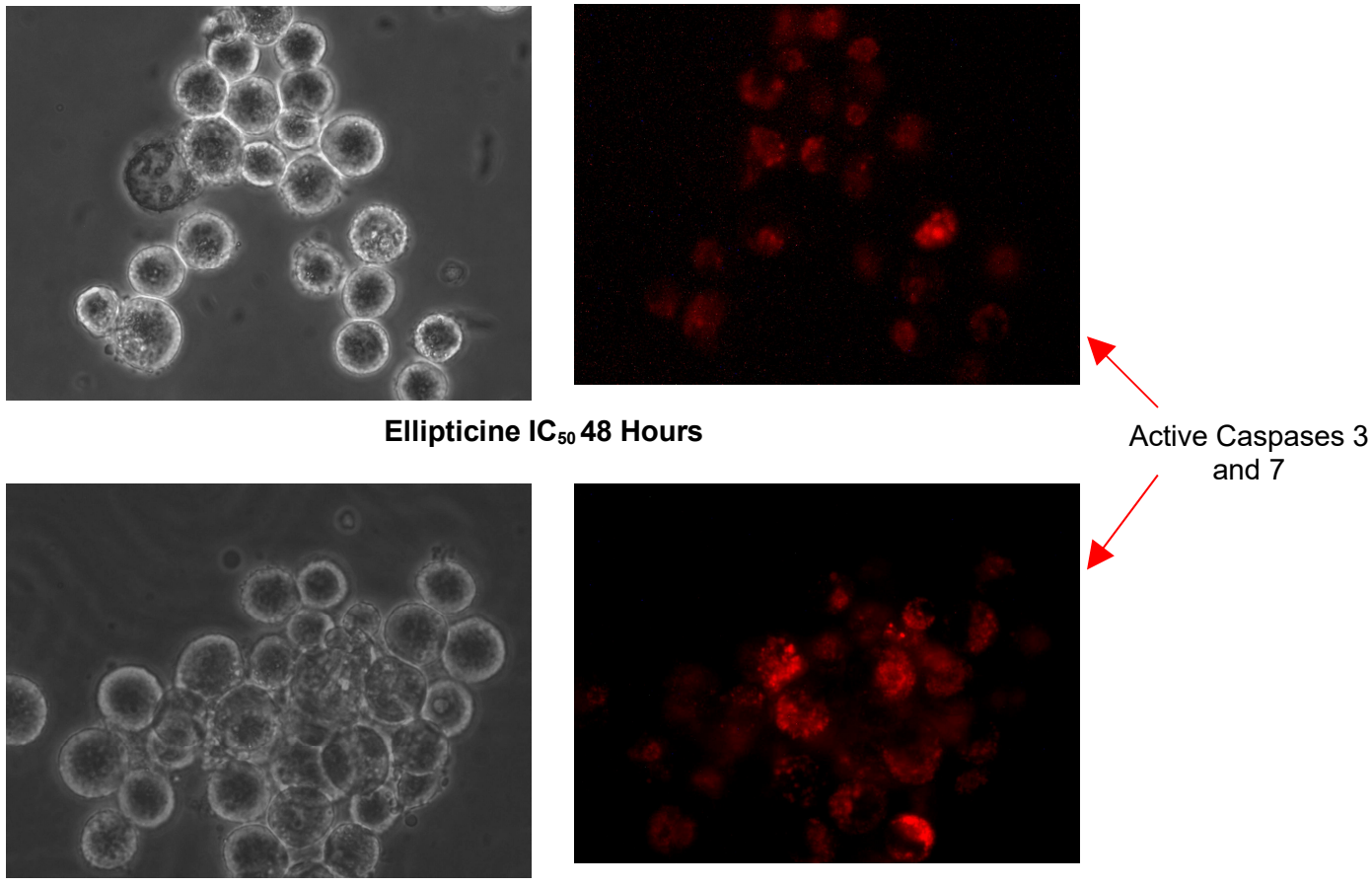


Figure 22. Photos of Magic Red™ Caspases 3 and 7 Structural Analog (Ellipticine) and Anti-cancer/Positive Control (Cisplatin). Both pictures show intense red fluorescent spots as an indicator of active Caspases 3 and 7 (40X Objective).

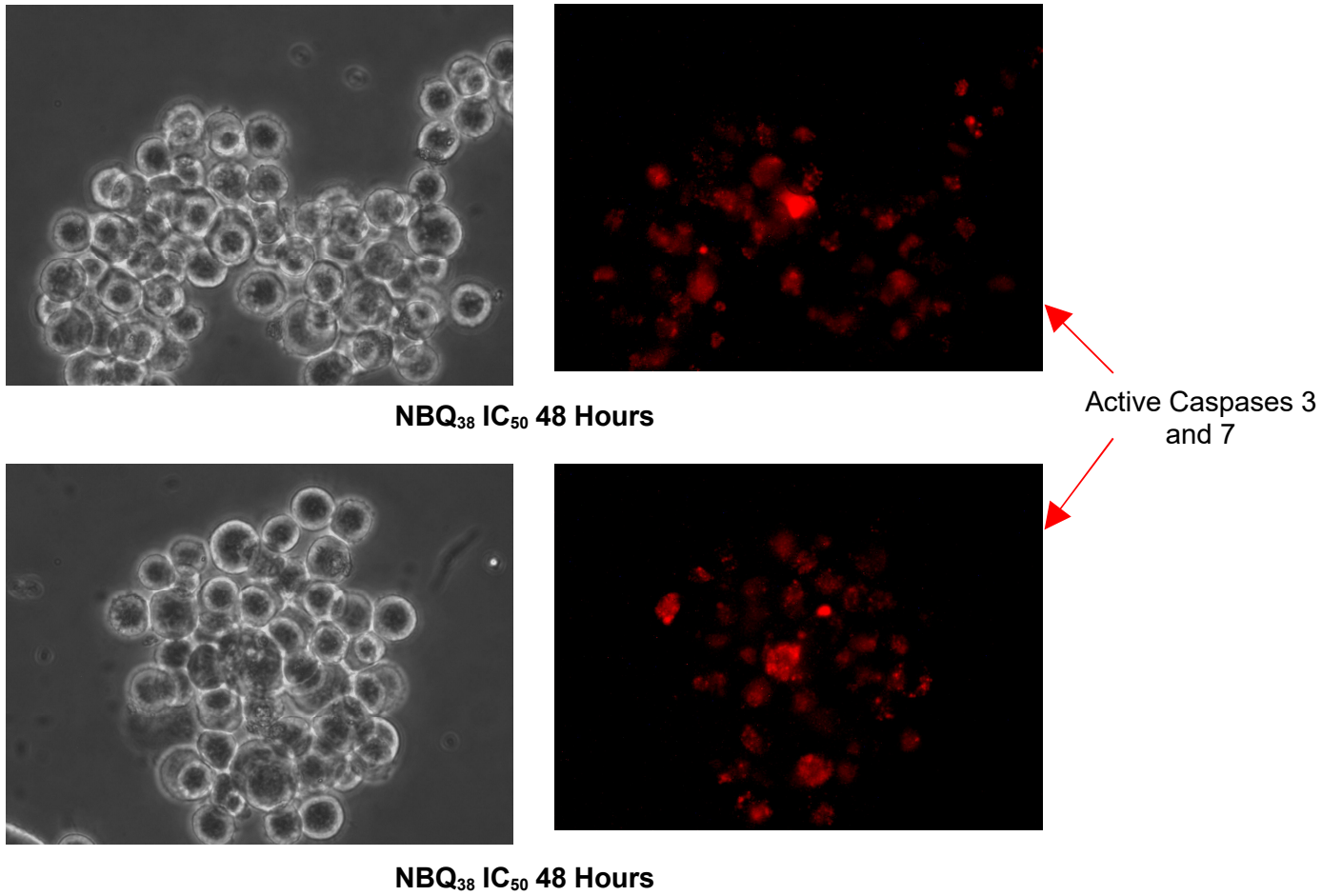


Figure 23. Photos of Magic Red™ Caspases 3 and 7 NBQ_{38} IC_{50} 48 Hours. Both pictures show intense red fluorescent spots as an indicator of active Caspases 3 and 7 (40X Objective).

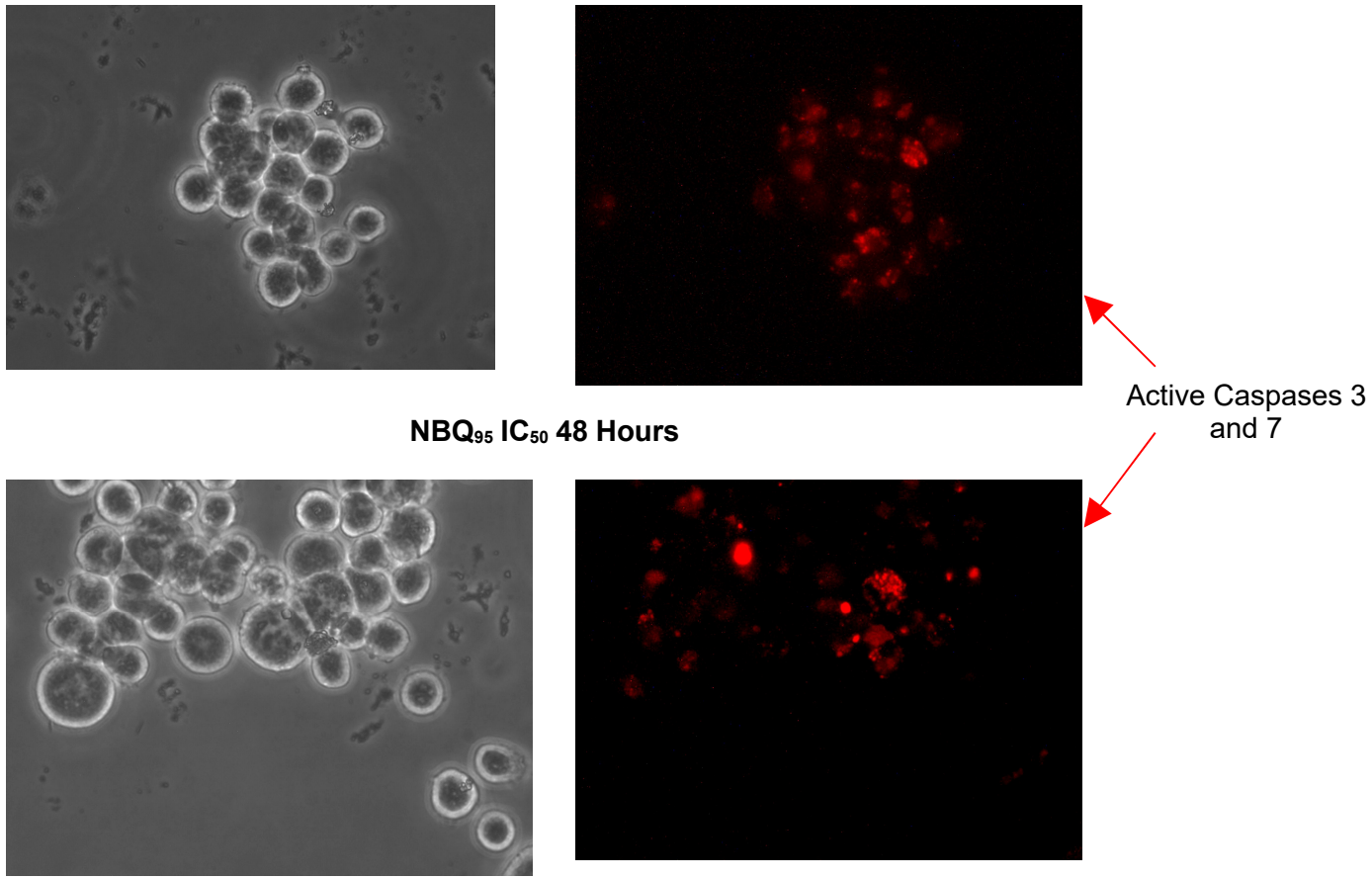


Figure 24 Photos of Magic Red™ Caspases 3 and 7 NBQ_{95} IC_{50} 48 Hours. Both pictures show intense red fluorescent spots as an indicator of active Caspases 3 and 7 (40X Objective).

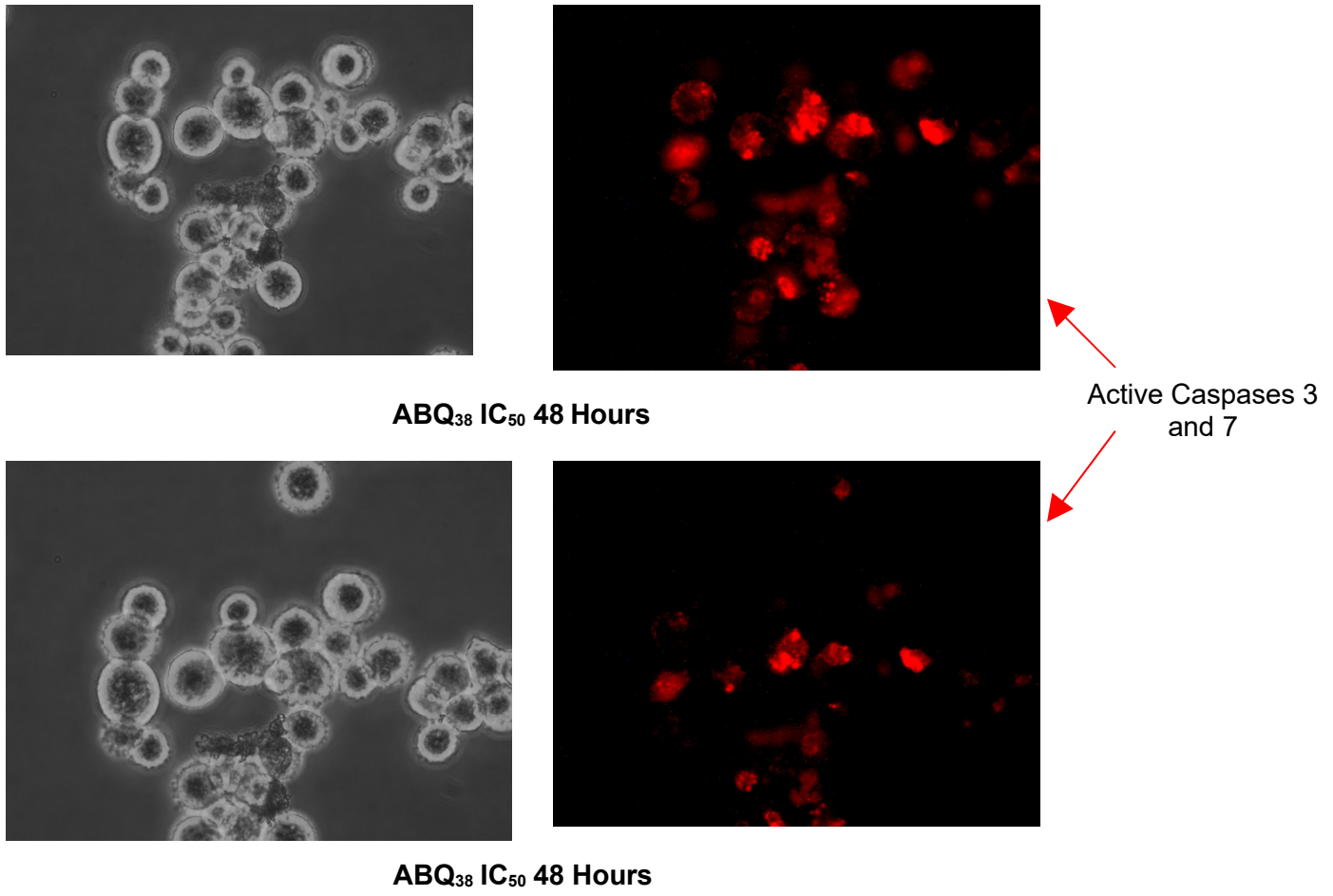


Figure 25. Photos of Magic Red™ Caspases 3 and 7 ABQ_{38} IC_{50} 48 Hours. Both pictures show intense red fluorescent spots as an indicator of active Caspases 3 and 7 (40X Objective).

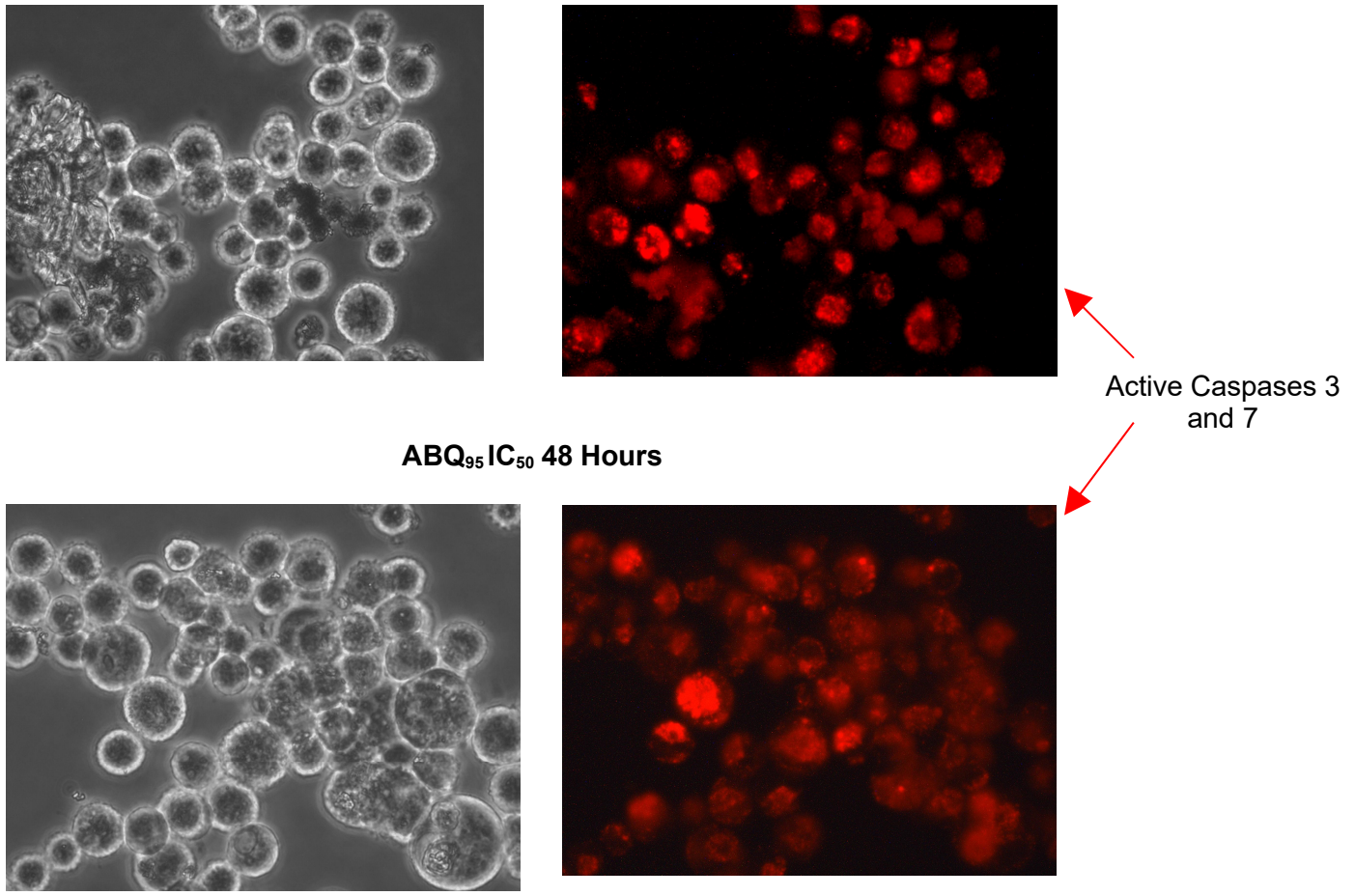


Figure 26. Photos of Magic Red™ Caspases 3 and 7 ABQ_{95} IC_{50} 48 Hours. Both pictures show intense red fluorescent spots as an indicator of active Caspases 3 and 7 (40X Objective).

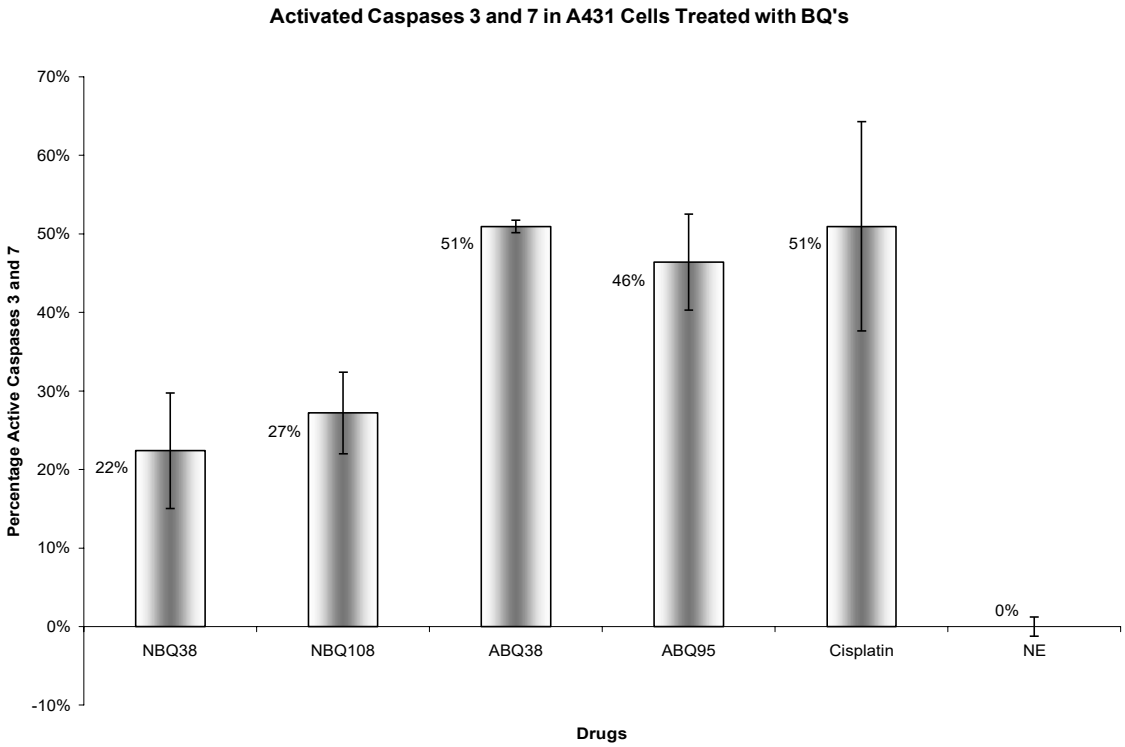
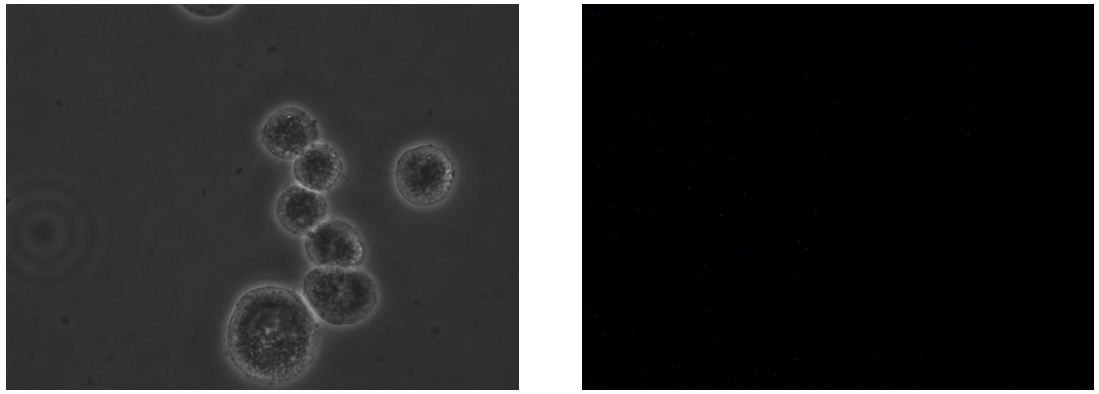
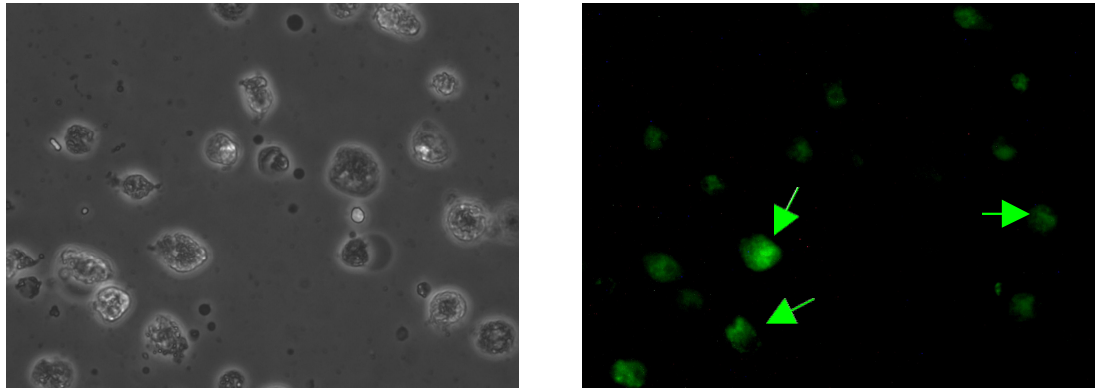


Figure 27. Graph of Activated Caspases 3 and 7 in A431 Cells Treated with BQ's IC_{50}'s for 48 Hours. Non-active caspases 3 and 7 are represented by NE. Experimental values were normalized by subtracting A431 active caspases 3 and 7 values. All BQ's showed activation of Caspases 3 and 7 at 48 hours. ABQ's had almost double the activation compared to NBQ's and were as potent as Cisplatin. The order of Caspases 3 and 7 activation are Cisplatin; 51 $\pm$ 13% = ABQ_{38}; 51 $\pm$ 1%) > ABQ_{95}; 46 $\pm$ 6% > BQ_{108}; 27 $\pm$ 5% > NBQ_{38}; 22 $\pm$ 7% and Negative Control; 0 $\pm$ 1%. ABQ_{38} presented the highest Caspases 3 and 7 activations at 48 hours of exposure.



Non-Active Caspase 8

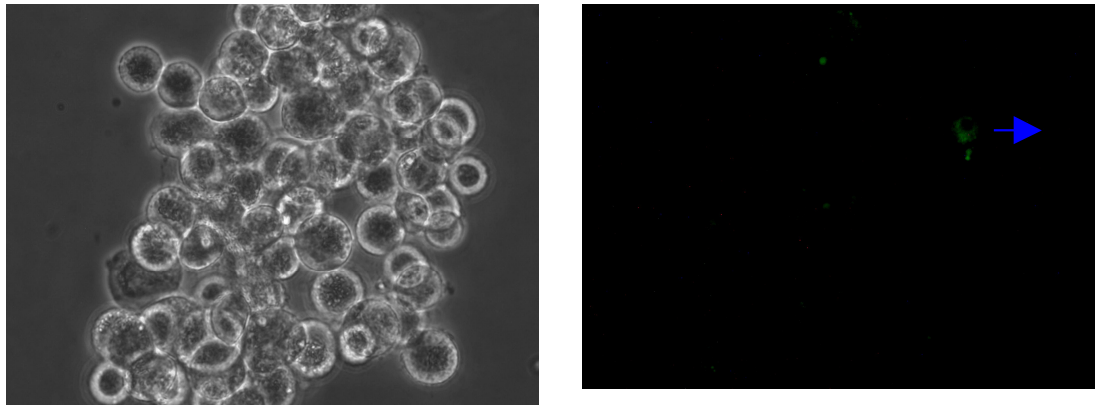
Negative Control 48 Hours



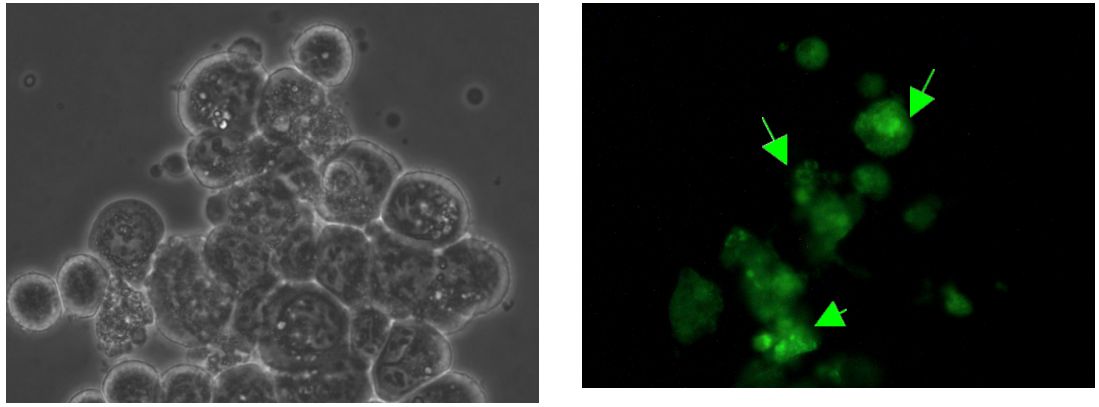
Active Caspase 8

Positive Control (Staurosporine 1 uM) 48 Hours

Figure 28. Photos of FLICA™ Caspase 8 Negative and Positive Controls. Contrasting images from Positive Control and Negative Control allows distinction between active (green arrows) and non-active Caspase 8.



Ellipticine IC_{50} 48 Hours



Cisplatin IC_{50} 48 Hours

Figure 29. Photos of FLICA™ Caspase 8 Structural Analog (Ellipticine) and Anti-cancer/Positive Control (Cisplatin). Ellipticine fluorescence can not be attributed to Caspase 8 activation. The emission of active Caspase 8 and the natural fluorescence emission from cells exposed to Ellipticine have wavelength overlapping. Cisplatin IC_{50} activates Caspase 8 (green arrows) at 48 hours.

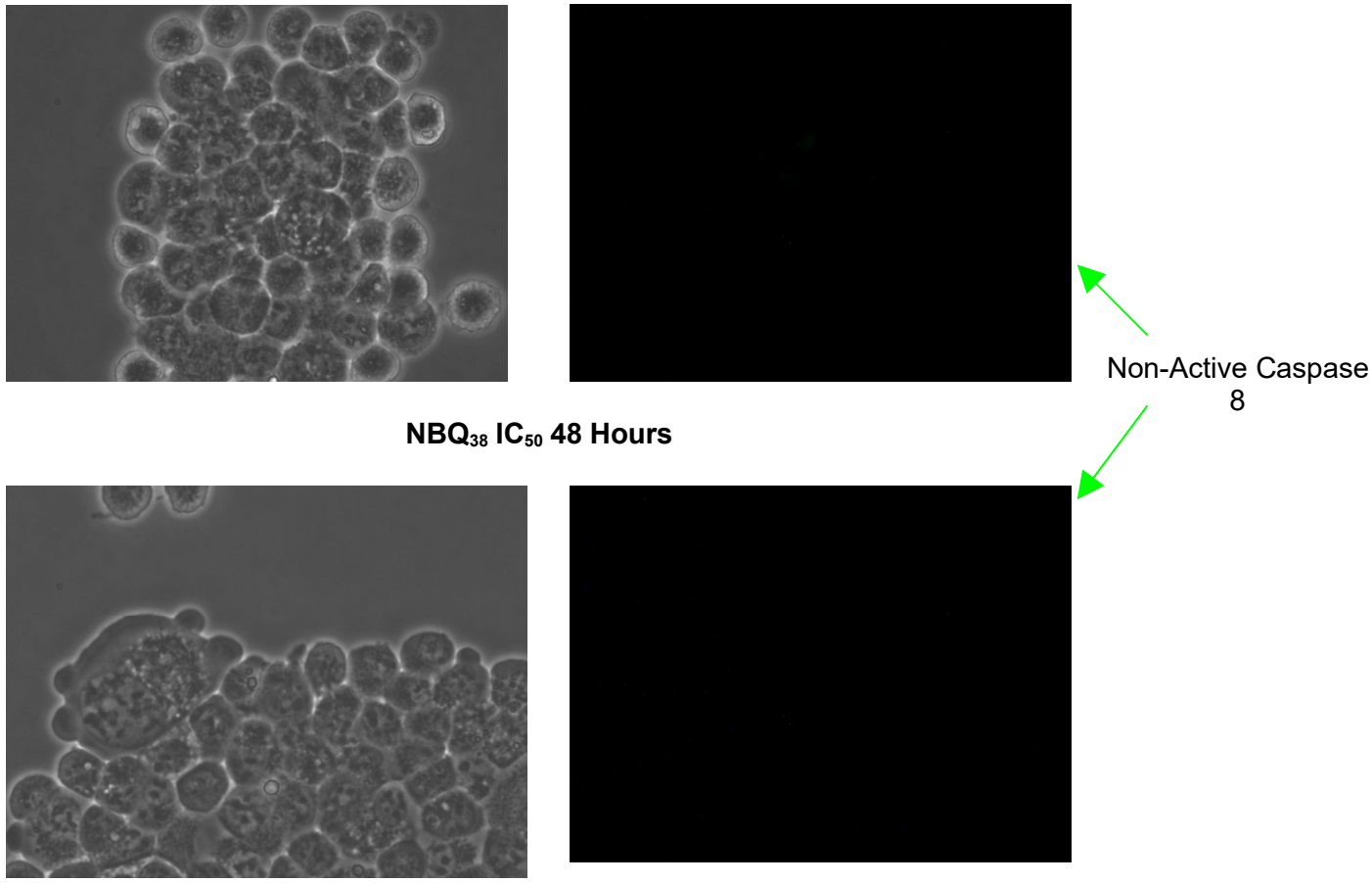


Figure 30. Photos of FLICA™ Caspase 8 NBQ_{38} IC_{50} 48 Hours. Both pictures show no color or green fluorescent spots as an indicator of non-active Caspases 8 (40X Objective).

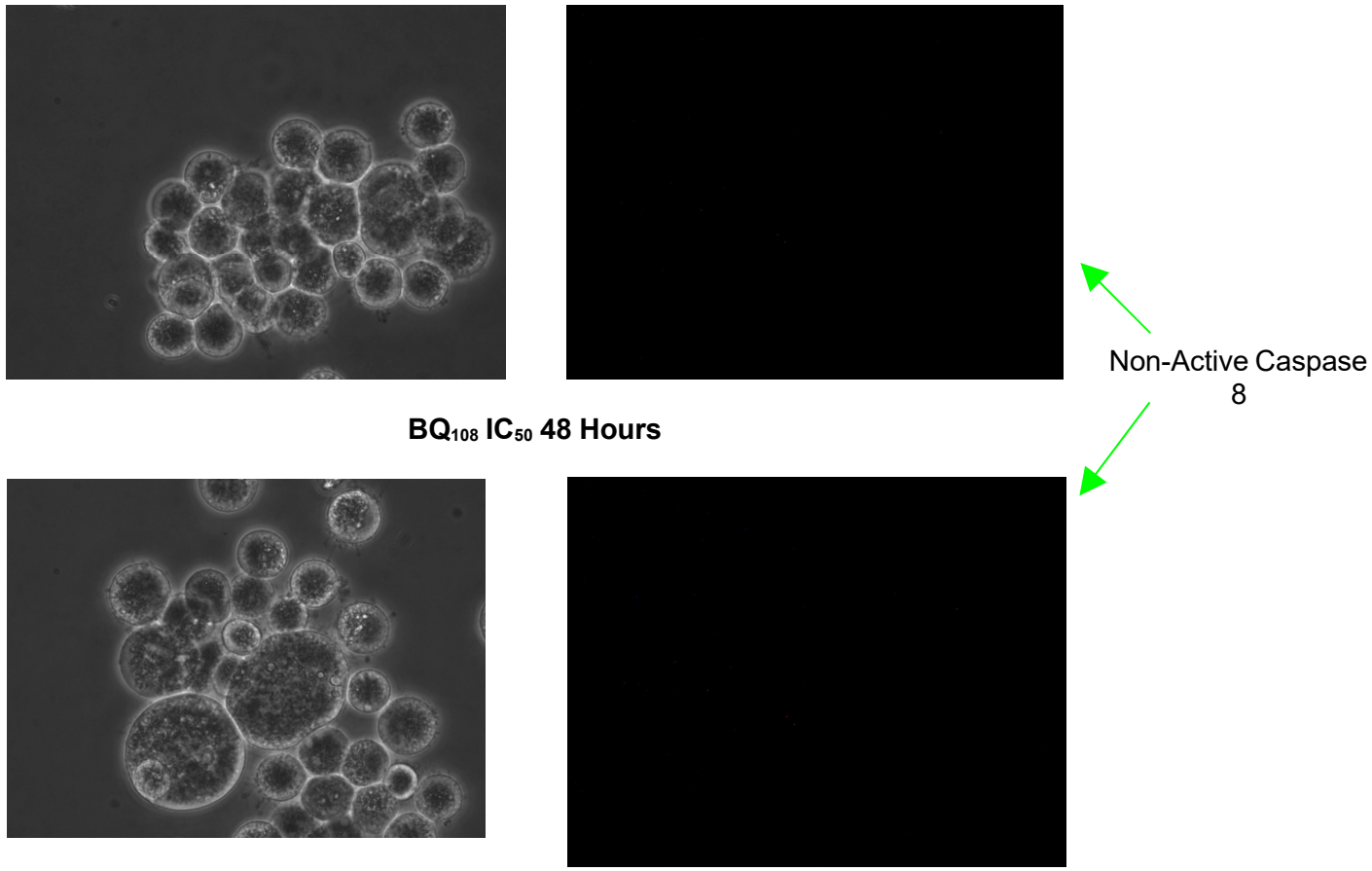


Figure 31. Photos of FLICA™ Caspase 8 BQ_{108} IC_{50} 48 Hours. Both pictures show no color or green fluorescent spots as an indicator of non-active Caspases 8 (40X Objective).

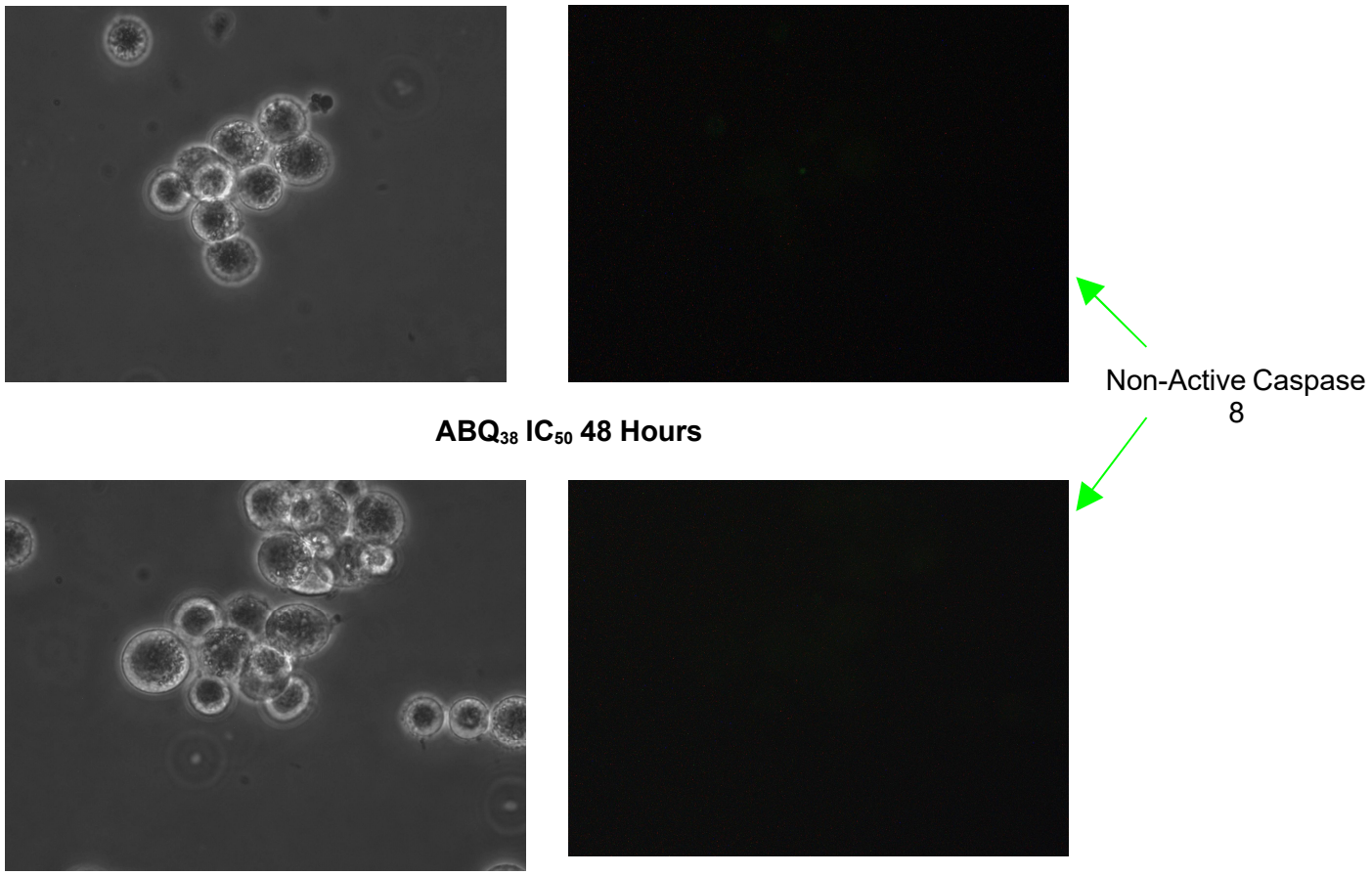


Figure 32. Photos of FLICA™ Caspase 8 ABQ_{38} IC_{50} 48 Hours. Both pictures show no color or green fluorescent spots as an indicator of non-active Caspases 8 (40X Objective).

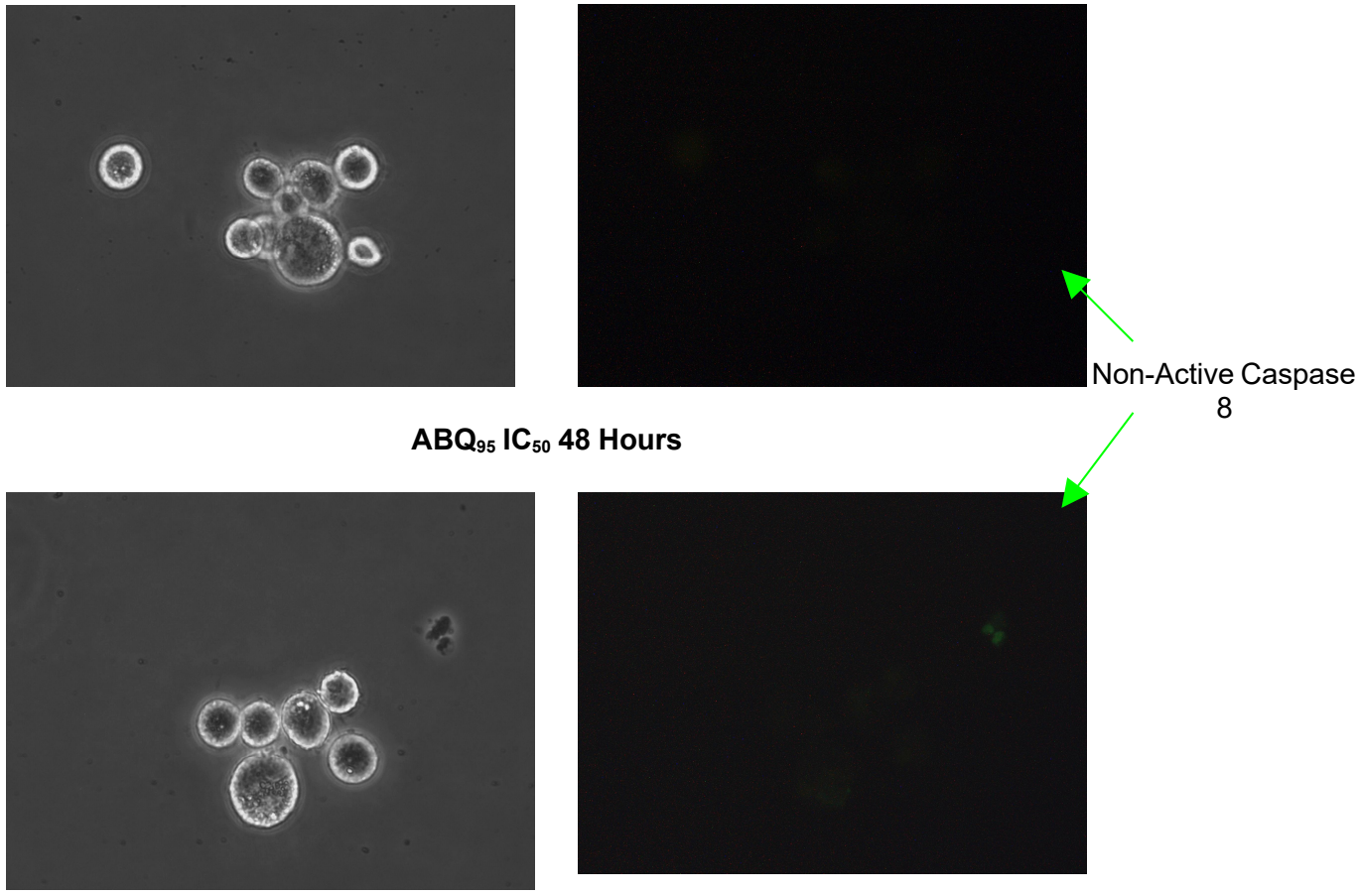


Figure 33. Photos of FLICA™ Caspase 8 ABQ_{95} IC_{50} 48 Hours. Both pictures show no color or green fluorescent spots as an indicator of non-active Caspases 8 (40X Objective).

ANNEXES

PROTOCOLS

Appendix 1. Exposure Protocol

A) NBQ's Solution Preparation and Calculation Protocol

BQx	***Molecular weight***
NBQ_{38}	292.10
NBQ_{95}	329.02
ABQ_{38}	335.25
ABQ_{95}	297.78
BQ_{108}	320.23

(3×10^{-3} mol/L) (MW g/mol) = mg/mL

i. NBQ_{38} (3×10^{-3} mol/L) (292.10 g/mol) = .8763 mg/mL

ii. NBQ_{95} (3×10^{-3} mol/L) (329.02 g/mol) = .9871mg/mL

iii. ABQ_{38} (3×10^{-3} mol/L) (335.25 g/mol) = 1.006 mg/mL

iv. NBQ_{95} (3×10^{-3} mol/L) (297.78 g/mol) = .8933 mg/mL

v. BQ_{108} (3×10^{-3} mol/L) (320.23 g/mol) = .9607 mg/mL

BQ's were prepared in 3mM solutions by dissolving the solid in sterile water. The formula at the bottom was used to calculate the needed volume to obtain the experimental dose concentrations.

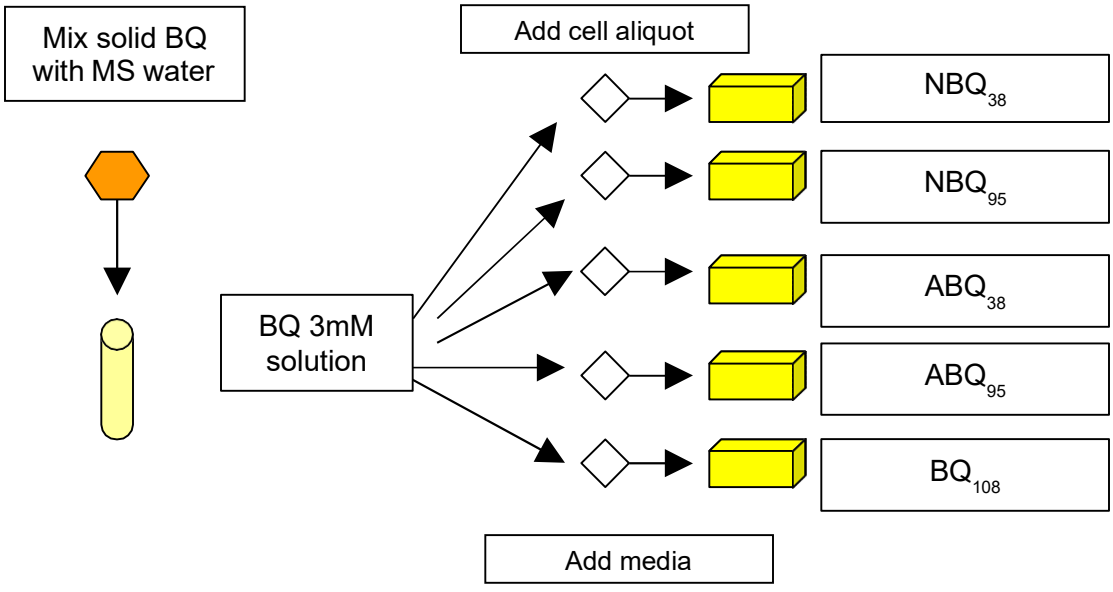
$$\textbf{Vol Sol } BQ_X \textbf{ (uL)} = \frac{[BQ_X \textbf{ Dose (uM)}] \textbf{ X } \{\textbf{Total t-flask Vol (9mL)}\}}{\textbf{3 mM } BQ_X \textbf{ (uL)}}$$

A) Administration

1. Fill culture flasks with 8mL of media.

2. Calculate the required volume of cell aliquot to seed the nearest amountof $5x10^6$ cells per flask. (Volumes of cell aliquots vary in every experiment).
3. Wait at least 4 hours to allow cells to adhere to the flask.
4. Add the calculated cell aliquot volume (uL), the volume of 3mM drug solution andmedia in order to have a final volume of 9mL per 25 cm^2 flask.

Dose [uM] IC_{50} BQ_X	***Volume of 3mM BQ Solution (uL)***
0 Control (0uM)	100 sterile water
NBQ_{38} (36uM)	108
NBQ_{95} (28uM)	84
ABQ_{38} (32uM)	96
ABQ_{95} (36uM)	108
BQ_{108} (4uM)	12



Prepared by: Sujey Carro

Appendix 2. Trypsinization Protocol:

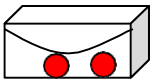
Step	*Flask 12.5 cm^2*	*Flask 25 cm^2*	*Flask 75 cm^2*
Remove media	4.5mL	9mL	20mL
Wash with PBS (2 times)	1.5mL	3mL	5mL
Add trypsin	.5mL	1mL	2mL
Add media	1.25mL	2.5mL	5mL

The table above shows all three different flask sizes used thought all essays here performed. Quantities of reactants vary upon the size of the flask.

Steps:

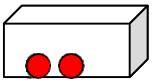
1. Remove media using a pipette.
2. Wash twice with PBS and move the flask as if you were drawing an eight shape.
3. Add trypsin and incubate for 10 minutes in an incubator at 37° C 5% CO_2.
4. Remove from the incubator and check with the microscope that cells are detached or floating. If there are still adherent, tap the flask softly with the fingers avoiding that the flask's contents splash the top or the sides of the container. Keep on checking until most of the cells become detached and float.
5. Add media to stop trypsinization action.
6. Transfer with a pipette the contents of the flask into a 15mL conical tube.
7. Add PBS with a pipette to rinse the flask and transfer it to the 15mL conical tube.
8. Proceed with the pelleting protocol.

1



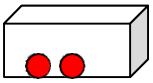
Remove media

2



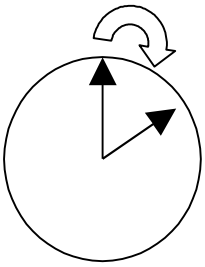
Wash PBS (2X)

3



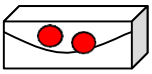
Add trypsin

4



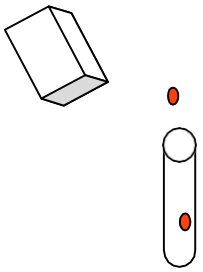
Incubate for 10 minutes

5



Add media

6

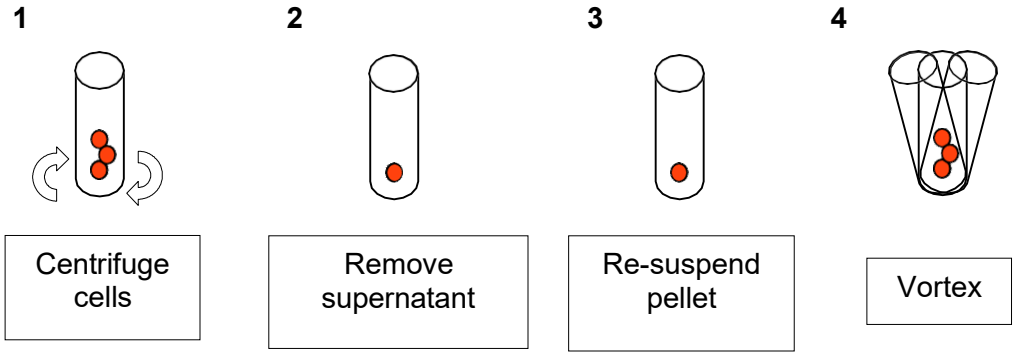


Transfer cells

Prepared by: Sujey Carro

Appendix 3. Pelleting Protocol:

1. Place conical tubes in the centrifuge at 4,000 rpm for 1 minute.
2. Remove from the centrifuge and check for a semitransparent pellet at the bottom of the tube. If there is no visible pellet on the bottom, centrifuge again at 4,000 rpm for 30 seconds.
3. Carefully remove the media (supernatant) with a pipette without disturbing the pellet.
4. Add 1mL of PBS to the pellet without disturbing the pellet. This step is used to wash any residue of old media and or trypsin.
5. Re-suspend pellet with *XmL of media. Some cell pellets will need moremedia volume to dissolve the pellet uniformly.
6. Vortex the re-suspended pellet until it's visually dissolved.
7. Count the cells in the aliquot following the cell viability protocol.

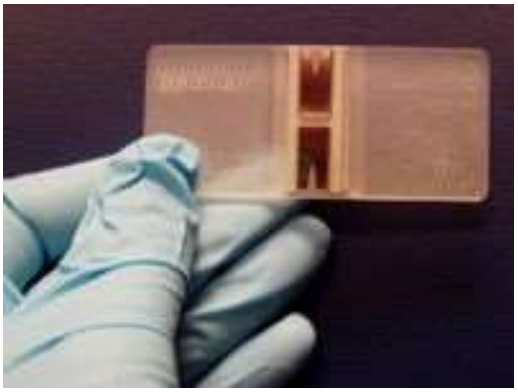


* = The amount of media used for the re-suspension might vary depending on the size of the pellet. Note that the equation for calculating the amount of cells in the flask has to be adjusted accordingly by substituting the correct amount used for re-suspension.

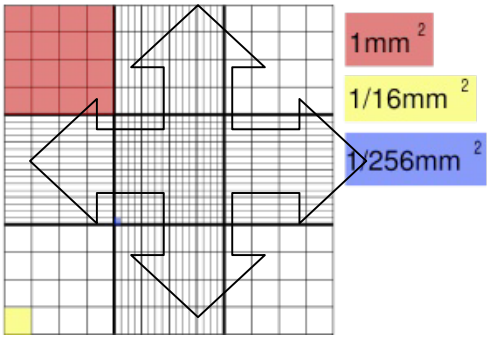
Prepared by: Sujey Carro

Appendix 4. Cell Viability Protocol (Trypan Blue Cell Exclusion)

1. Tryptinize and pellet cells (following trypsinization and pelleting protocols).
2. Transfer with a pipette 100 ul of the cell suspension to a well of a 96 well plate.
3. Add 100ul of Trypan Blue stain to the same well.
4. Incubate at 37°C and 5% CO_2 for 2 minutes.
5. Wash the hemocytometer and the coverslip with methanol and dry it with a Kimwipe. Transfer 10 ul of the trypan blue-cell mixture to each side of the hemocytometer. This is done by carefully touching the edge of the cover-slip with the pipette tip and filling the chamber with capillary action. Do not overfill or underfill the chambers.



Hemocytometer



Hemocytometer grid

Image provided by Wikipedia

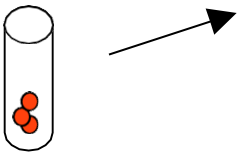
6. Count the viable cells (non-viable cells stain blue, viable cells will remain unstained) in each of the nine 1mm squares of the hemocytometer.
7. There are two nine 1mm squares per each side, count both sides and estimate the mean using the following equations:
    - **Mean of cells counted in the hemocytometer =**
        i. **(Mean of side one + Mean of side two)/*2***
8. From the result of the previous equation, determine then the cell population that was initially in the flask, and it is being currently counted on the conical tube:

    - **Cells in the aliquot =**

    i. **[(Mean of cells) x (*10^4*) x (*2*) x (*X* * mL)]**

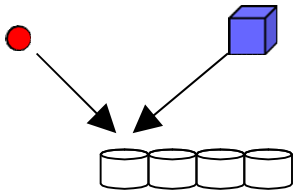
* = Amount might change depending on the volume used to resuspend the aliquot

1



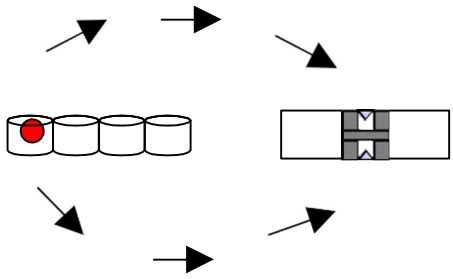
Get 100 uL cell aliq

2



Add 100 uL of Trypan Blue
to the 100 uL cell aliq

3



Add 10 uL of the Trypan
Blue cell aliq mixture

Prepared by: Sujey Carro

Appendix 5. Culture Count Protocol and Formulas

1. Tryptinize cells following trypsinization protocol
2. Pellet cells following pelleting protocol.
3. Determine cell viability following the trypan blue cell viability protocol.
4. Substitute in the following formula to determine the number of cells in the conical tube:

Mean counted cells in hemocytometer $x\ 10^4\ x\ 2\ x$ **Volume used for final pellet resuspension**

5. Substitute in the equation the values calculated on the cell viability protocol and calculate the volume of the aliquot that will be needed to seed the desired amount of cells for the experiment.

$$\frac{\text{Number of desired cells to be seeded}}{\text{Final number of cells in the aliquot}} \times \text{Volume of media used for pellet re-suspension} \times 1000^*$$

$$\frac{\text{Concentration 1}}{\text{Volume 1}} = \frac{\text{Concentration 2}}{\text{Volume 2}}$$

* = This number changes the final value from mL to uL.

Prepared by: Sujey Carro

Appendix. 6. Immunochemistry's Apoptosis (Mito PT™) Adjusted Staining Protocol

1) Day ONE:

   a) Reagents Pre-Preparation and Storage:

      i) Mix 500uL DMSO with the dry concentrate of the dye of the kit and vortex to dissolve it.

      ii) Store the reconstituted dye in aliquots of 10uL, cover them with foil paper and keep them frozen for future use. Each aliquot will be good to work with two samples. (This is done only once and it will last up to six months without thawing them more than twice).

      iii) Store the 10 X Buffer solution in aliquots of 2mL. Each aliquot will be good to work with four samples.

   b) Cell Culture Preparation:

      i) Triptinize, pellet and count cells following trypsinization, pelleting and cell viability protocols.

      ii) Seed cells and put samples in the incubator at 37°C and 5% CO_2 for 4 hours to allow cell adherence.

      iii) Remove from the incubator and expose cells to BQ's following the exposure protocol.

2) Day THREE:

   a) Positive Control Preparation:

      i) Thirty minutes before the beginning of the staining procedure, add 1uL of Valinomycin to the t-flask that will be labeled as positive control and incubate for 20 minutes at 37°C and 5% CO_2.

   b) Reagent Preparation Procedure:

   Perform all of the following **without light and protect reagents with foil paper**.

i) Warm the 10X Buffer solution at 37°C in a water bath.

ii) Mix the warmed 10X Buffer solution with DI water to dilute it 1/10.(This will now be the 1X buffer stock solution).

(1) Ex: 2mL 10X Buffer solution in 18mL deionized (D.I.) water to form a final 20mL 1X Buffer solution that will work for four samples.

iii) Keep the 1X Buffer solution warm at 37°C in a water bath.

iv) In a small Eppendorf vial, mix 10uL of Mito PT dye with 990uL of the 1X Buffer solution. This will be ready to use Mito PT dye and it will be good to stain two samples.

v) Centrifuge the contents at 1,000 rpm for 15 minutes. This will prevent bubbles and will mix the buffer and dye evenly.

vi) Vortex the ready to use Mito PT dye until completely dissolved.

c) <u>Staining the cells:</u>

i) Triptinize, pellet, and count cells following trypsinization, pellet, and cell viability protocols.

ii) Once the amount of cells of the exposed aliquots is estimated, calculate the volume needed to prepare aliquots of $5x10^5$ cells following the culture count protocol. For each exposed aliquot, two aliquots of $5x10^5$ will be prepared; one will be stained, and the other one will not be stained.

iii) Add 500uL of the ready to use Mito PT dye to each stained sample and 495uL of a 1X Buffer solution with 5uL of DI water to the non-stained samples.

iv) Keep the rest of the unused buffer warm since it will be used now for a series of washes.

v) Incubate samples for one hour at 37°C and 5%CO_2.

vi) Add to each conical 2mL of the 1X buffer vortex, and centrifuge at 1000 rpm for five minutes. Remove and discard the buffer without disturbing the pellet. Repeat a second wash with 2mL and a third wash with 1mL. Discard the supernatant without disturbing the pellet.

d) <u>Qualitative Fluorescence Microscope Procedure:</u>

i) Ten minutes before the incubation time, turn on the microscope mercury lamp.

ii) Re-suspend the stained cells with 25uL of 1X Buffer solution.

iii) Place the 25uL over a glass slide with a coverslip.

iv) To observe dual fluorescence, select the BF field (U filter)

v) Open the Q Capture software and preview the image by adjusting it to 7 seconds of exposure.

vi) Take pictures and store them as jpeg files.

e) <u>Quantitative Fluorescence Procedure:</u>

i) Ten minutes before the end of the incubation time, turn the Modulus fluorimeter with the Green and or the Blue filter adapters. The **Green** filter will capture the fluorescence of healthy cells while **Blue** filter will capture fluorescence of apoptotic cells.

ii) Access the settings menu to perform three measurements in 1/3 of asecond each time.

(1) To Measure fluorescence on the mini-cell curvette adaptor (250uL-300uL working volume):

(a) Place the mini-cell adapter inside the cavity of the filter used.

(b) Re-suspend stained and non-stained cells with 275uL of 1X Buffer solution and transfer the contents of each sample to individual glass mini-cell curvettes.

(c) Place the glass mini-cell curvettes in the mini-cell adaptor and measure fluorescence.

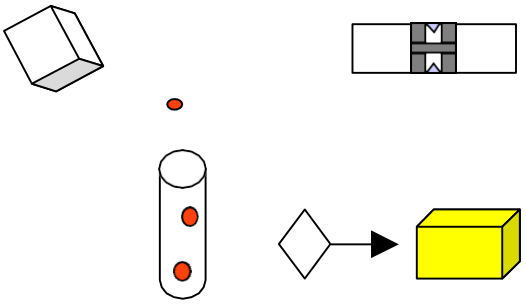
(2) To Measure fluorescence on a 10 x 10mm curvette (remember that the working volume will be 2mL)

(a) Load 1mL of pre-warmed 1X Buffer solution to each methacrylate curvette that will be used. Transfer the stained cell pellet by adding 1mL of 1 X Buffer solution and mixing it with the other 1mL in the curvette. Mix slowly by pipetting up and down the contents.

(b) Place the curvettes in the filter and measure fluorescence.

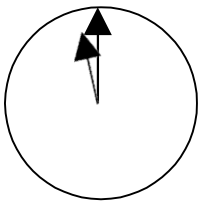
iii) Once all samples had been measured in one of the before mentioned channels, turn on the Modulus, switch the filter adapters (Green and or Blue), and turn on the equipment. Wait ten minutes and record sample readings.

1



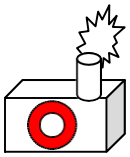
Tryptinize, count, seed and expose cells for 48 hours

2



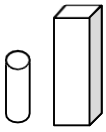
Tryptinize, count and stain cells for and hour with the dye

3



Photograph cells with fluorescent microscope for qualitative apoptosis analysis

4



… or Measure qualitative apoptosis with any of the curvette sizes in the fluorimeter

Prepared by: Sujey Carro

Appendix 7. Immunochemistry's Adjusted Qualitative and Quantitative Caspases 3 & 7 Evaluation Protocol

Preparation of cell cultures:

1. Day ONE:
    a. ***Reagents Pre-Preparation and Storage:***
        i. Reconstitute the Magic Red dye by adding 200 uL of culture grade DMSO to the vial. Vortex to mix properly and store in aliquots of 10uL (5 samples each aliquot). Aliquots will be suitable for up to 6 months.
    b. ***Cell Culture Preparation:***
        i. Triptinize, pellet, and count cells following trypsinization, pellet, andcell viability protocols.
        ii. Once the amount of cells in the aliquots is estimated, calculate the volume of aliquots needed to seed $5x10^6$ cells following the culture count protocol.
        iii. Seed cells and put samples in the incubator at 37°C and 5% CO_2 for 4 hours to allow cell adherence.
        iv. Remove from the incubator and expose cells to BQs following the exposure protocol.
2. Day THREE:
    a. ***Reagent Preparation Procedure:***

Perform all of the following without light and protect reagents with foil paper.

i. Warm the 10uL of concentrated Magic Red dye at 37°C in a water bath.

ii. Mix the warmed 10uL Magic Red dye with 40uL of MS-Water and vortex for 2-5 seconds to dilute it. (This will now be ready to use Magic Red solution).

iii. Vortex the ready to use Magic Red solution until completely dissolved.

iv. Centrifuge the contents at 1,000g for 15 minutes. This will prevent bubbles and will mix the solution evenly.

v. Let it warm for 10 minutes and vortex again until the mixture becomesevenly distributed. This will work for four samples.

vi. Prepare an aliquot of 50 mL of PBS and keep it warm at 37^{o}C in a water bath.

b. <u>Staining the cells:</u>

i. Triptinize, pellet, and count cells following trypsinization, pellet, andcell viability protocols.

ii. Once the amount of cells of the exposed aliquots is estimated, calculate the volume needed to prepare aliquots of 1x10^{6} cells following the culture count protocol. For each exposed aliquot, two aliquots of 2x10^{6} will be prepared; one will be stained, and the other one will not be stained.

iii. Centrifuge at max speed (6,000rpm) for 5 minutes and remove the supernatant.

iv. Add 300uL to each one of the Eppendorf tubes and vortex for 2-5 second to distribute the cells evenly.

v. Add 10uL of the diluted dye to the samples and vortex again for 2-5 seconds.

vi. Incubate the samples for an hour—vortex samples every 20 minutes to ensure that the dye is well distributed.

vii. If dual staining is being performed, remove samples from the incubator and add 1.6uL of Hoechst stain to each one of the samples. Vortex for 2-5 seconds and return samples to the incubator.

viii. Once the staining is completed, vortex and centrifuge at maximum speed (13,400 rpm) for one minute, remove and discard the supernatant.

ix. Add 300uL of pre-warmed PBS and vortex each sample for 2-5 seconds. Centrifuge at maximum speed (13,400 rpm) for one minute. Remove and discard the supernatant. Repeat this step once more.

c. Qualitative Fluorescence Microscope Procedure:

i. Ten minutes before the incubation time, turn on the microscope mercury lamp.

ii. Re-suspend the stained cells with 25uL of PBS.

iii. Place the 25uL over a glass slide with a coverslip.

iv. To observe fluorescence, select the G field (Red filter)

v. Open the Q Capture software and preview the image by adjusting it to 7-9 seconds of exposure.

vi. Take pictures and store them as jpegs files.

d. Quantitative Fluorescence Procedure:

Measure the fluorescence of the Magic Red (Caspases 3 & 7) in the Modulus Flourimeter using the Red filter. Ensure that the readings we performed three consecutive times by selecting continuous sampling from theMain Menu.

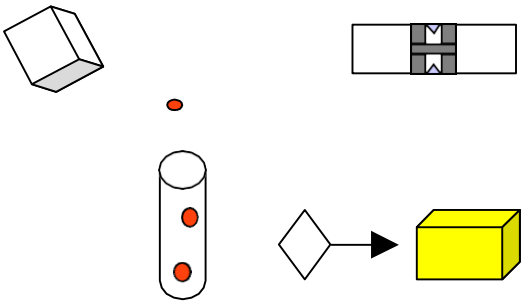
i. **To Measure fluorescence on the mini-cell curvette adaptor (250uL-300uL working volume):**

    1. Add 300uL of PBS and mix well in the Eppendorf by slowly pipetting up and down. Transfer the contents to the mini-cell curvettes and place the mini-cell adaptor on the Red Filter. Measure continuously each sample three times.

ii. **To Measure fluorescence on a 10 x 10mm curvette remember that the working volume will be 2mL.**

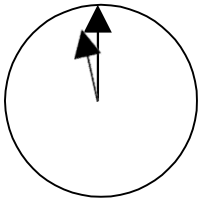
    1. Add 1mL of pre-warmed PBS to each methacrylate curvette that will be used. Transfer the stained cell pellet by adding 1mL of PBS and mixing it with the other 1mL in the curvette. Mix slowly by pipetting up and down the contents.

1



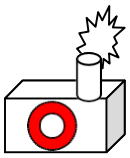
Tryptinize, count, seed and expose cells for 48 hours

2



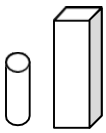
Tryptinize, count and stain cells for and hour with the dye

3



Photograph cells with fluorescent microscope for qualitative apoptosis analysis

4



… or Measure qualitative apoptosis with any of the curvette sizes in the fluorimeter

Prepared by: Sujey Carro

Appendix 8. Immunochemistry's Apoptosis FLICA Caspases 8 Modified Protocol

1. Day ONE:
    a. ***Reagents Pre-Preparation and Storage:***
        i. Reconstitute each 25 sample vial of 150 X concentrated powder stock with 50 uL of Culture Grade DMSO. Each sample requires 2uL of the 150 X *FLICA* Reagent. Aliquots of 12uL of 150 X *FLICA* stains up to 6 samples.
        ii. We prepared 15 Eppendorf tubes of 150 X *FLICA* reagent suitable forsix samples each and only one Eppendorf with 20uL good for ten samples.
        iii. 10 X Buffer were aliquoted to handle six samples at a time.
    b. ***Cell Culture Preparation:***
        i. Triptinize, pellet, and count cells following trypsinization, pellet, andcell viability protocols.
        ii. Seed cells and put samples in the incubator at 37oC and 5% CO2 for 4 hours to allow cell adherence.
        iii. Remove from the incubator and expose cells to BQs following the exposure protocol.
2. Day THREE:
    a. ***Reagent Preparation Procedure:***
        i. Before use, dilute 12uL of 150 X *FLICA* reagent with 48uL of PBS. This will now become the 10 X Buffer solution.
        ii. Add 600uL of 10 X Buffer solution to 5.4mL of DI Water. This will now become the 1 X Buffer solution. Each sample requires 6mL of adiluted buffer.

iii. Keep the 1X Buffer solution warm at 37°C in a water bath.

iv. At the end of the administration period, trypsinized, pellet, and countfollowing the protocols.

b. <u>Staining the cells:</u>

i. Triptinize, pellet, and count cells following trypsinization, pellet, andcell viability protocols.

ii. Once the amount of cells of the exposed aliquots is estimated, calculate the volume needed to prepare aliquots of $5x10^5$ cells following the culture count protocol. For each exposed aliquot, two aliquots of $5x10^5$ will be prepared; one will be stained, and the other one will not be stained.

iii. Centrifuge samples at 4,000 rpm and remove supernatant.

iv. 10uL of 1X Buffer solution to the non-stained samples.

v. Keep the rest of the unused buffer warm since it will be used for a series of washings.

vi. Incubate samples for one hour in an incubator at 37°C and 5%CO_2.

vii. Once the staining is completed, vortex and centrifuge at maximum speed (13,400 rpm) for one minute, remove and discard the supernatant.

viii. Add 2mL of 1X Buffer solution and vortex each sample for 2-5 seconds—centrifuge at maximum speed (13,400 rpm) for one minute. Remove and discard the supernatant. Repeat this step once more.

c. Qualitative Fluorescence Microscope Procedure:

i. Ten minutes before the incubation time ends, turn on the microscope mercury lamp.

ii. Re-suspend the stained cells with 25uL of 1X Buffer solution.

iii. Place the 25uL over a glass slide with a coverslip.

iv. To observe fluorescence, select the B field (Green filter)

v. Open the Q Capture software and preview the image by adjusting it to 7-9 seconds of exposure.

vi. Take pictures and store them as jpegs files.

d. Quantitative Fluorescence Procedure:

Measure the fluorescence of the FLIICA (Caspase 8) in the Modulus Fluorimeter using the Blue filter. Ensure that the readings are performed three consecutive times by selecting continuous sampling from the Main Menu.

i. To Measure fluorescence on the mini-cell curvette adaptor (250uL-300uL working volume):

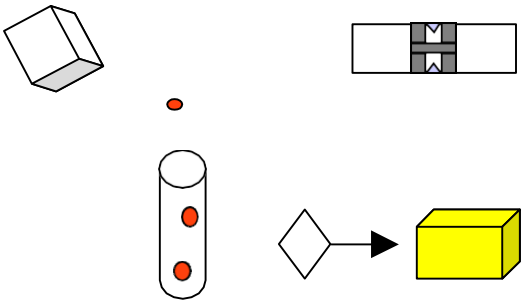
1. Add 300uL of 1X Buffer Solution and mix well in the Eppendorf by slowly pipetting up and down. Transfer the contents to the mini-cell curvettes and place the mini-cell adaptor on the Blue Filter. Measure continuously each sample three times.

ii. To Measure fluorescence on a 10 x 10mm curvette remember that the working volume will be 2mL.

1. Add 1mL of pre-warmed 1X Buffer solution to each methacrylate curvette that will be used. Transfer the stained cell pellet by adding 1mL of 1X Buffer solution and mixing it

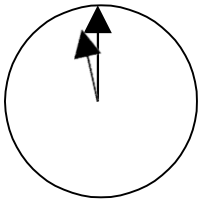
With the other 1mL that is in the curvette. Mix slowly bypipetting up and down the contents.

1



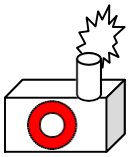
Tryptinize, count, seed and expose cells for 48 hours

2



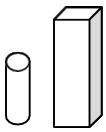
Tryptinize, count and stain cells for and hour with the dye

3



Photograph cells with fluorescent microscope for qualitative apoptosis analysis

4



… or Measure qualitative apoptosis with any of the curvette sizes in the fluorimeter

Prepared by: Sujey Carro

Appendix 9. Biohazard Wastes Management and Disposal Protocol

JCA in Puerto Rico has regulated the Management of Regulated Biomedical Disposal since 1997. Specifically, Chapter V rules 580 thru 582 cover generators' responsibilities before transporting this waste.

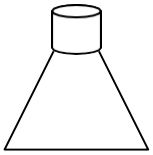
Cancer cell line A431 wastes and the materials that were in contact with it were considered as Regulated Treated and Regulated Destroyed Biomedical Wastes under this rule. Disposable tips, flasks, tubes and pipettes were considered as Mixed Regulated Biomedical Wastes. In order to comply with the law we followed the following procedures:

1. University had a valid generator number as required by JCA (#DBR- OC065-93-09-0053-RN-09).
2. Cell by-products and cell liquid waste were treated with 10% bleach solution for 5 minutes, and contents were flushed down the drain with copious amounts of water.
3. All areas, including the hood were sanitized with 70% ethanol, Sporicidin, Lysol and or LPH before and after its use. The hood was irradiated for ten (10) minutes before and after use.
4. Disposable materials such as tips, flasks, tubes, and pipettes were deposited and sealed on an orange biohazard plastic bag.
    a. Twice a month, bags were collected by a third-party private waste management company contracted to handle this according to state regulation.
    b. This company operates by complying as required by JCA with rules 583 thru 586 of the Management of Regulated Biomedical Disposal.

c. When the third-party private waste management company completed the transportation and disposition of the wastes, written confirmation was received at the laboratory.

5. Other non-disposable materials such as glass beakers were rinsed with 10% bleach solution for 5 minutes, and contents were flushed down the drain with copious amounts of water. A second rinse with Alconox was done before and stored.
6. A graduate lab technician was in charge of training personnel involved in experiments execution. Training and protocols were explained, read and signed before experiments began. Records are kept under custody and are available for revision.

Biohazard bags



10% Bleach solution