2008

PROMOTION OF TUMOR CELL DEATH THROUGH THE INDUCTION OF

Tuyen Nguyen
Virginia Commonwealth University

PROMOTION OF TUMOR CELL DEATH THROUGH THE INDUCTION OF AUTOPHAGY IN HCT-116 COLON CANCER CELLS BY MICROTUBULE POISON, JG-03-14

A Thesis submitted in partial fulfillment of the requirements for the degree of M.S. in Pharmacology and Toxicology at Virginia Commonwealth University.

by

TUYEN NGUYEN
B.S. Biology, Virginia Commonwealth Univeristy, 2006

Director: DR. DAVID GEWIRTZ
PROFESSOR, PHARMACOLOGY AND TOXICOLOGY

Virginia Commonwealth University
Richmond, Virginia
August 2008

Acknowledgement

The first person I would like to express my gratitude for is my advisor and director, Dr. David Gewirtz. He was willing to take me under his wing and taught me about research which I had no knowledge of. His patience and guidance are truly priceless and without them I would not have succeeded.

I would like to thank my parents for their support and continued motivation in all my endeavors. They have taken an arduous journey to provide the opportunity with which I have received. They have strived to bring the best out of me and have always showed me that there are no limits in life. I would also like to thank them for my strength and perseverance, my courage to initiate in life, and for believing in me.

The third but not last person that I am grateful for is my other half, Tim. It has been a rocky journey as he struggled to support us financially in addition to dealing with my attitude and demands. He has delivered me laughter and joy in times when I am down, has kept me focus when things got cloudy, and has kept me on par in times of despair.

Table of Contents

List of Figures

Page

Abstract

PROMOTION OF TUMOR CELL DEATH THROUGH THE INDUCTION OF AUTOPHAGY IN HCT-116 COLON CANCER CELLS BY MICROTUBULE POISON, JG-03-14

By Tuyen Nguyen, B.S. Biology

A Thesis submitted in partial fulfillment of the requirements for the degree of Master of Science at Virginia Commonwealth University.

Virginia Commonwealth University, 2008

Major Director: Dr. David Gewirtz
Professor, Pharmacology and Toxicology

Microtubule poisons have proven to be effective in the treatment of a variety of malignancies. Although taxol-based derivatives promote microtubule stabilization, there is continuing interest in compounds that, like colchicines, act as microtubule destabilizing agents. Previous work from this laboratory showed that the novel microtubule poison, JG-03-14, was active against breast tumor cells, promoting autophagic cell death. In the current work, we studied the influence of JG-03-14 on p53 wild type HCT116 colon carcinoma cells. A crystal violet sensitivity assay indicated that JG-03-14 induced growth

inhibition, with 75% suppression of growth evident at a concentration of 500 nM. Time course studies of drug effects on cell viability indicated that JG-03-14 also produced cell killing. FACS analysis demonstrated that the HCT-116 cells arrested in the G2/M stage; furthermore, there was evidence of a hyperdiploid population that would be consistent with failure of the cells to divide despite completion of DNA synthesis. Finally, there was evidence of a small sub G0/G1cell population, indicating that the cells were not dying primarily by apoptosis, and suggesting that JG-03-14 induces an alternative mode of cell death. In contrast to cell shrinkage and nuclear fragmentation that was evident after treatment with taxol (a positive control for apoptosis), DAPI staining of HCT-116 cells treated with JG-03-14 showed intact and enlarged nuclei, again consistent with the absence of apoptosis. Furthermore, there was no evidence of mitotic catastrophe (micronuclei in binucleated cells). Based on previous studies in MCF-7 and MDA-MB231 cell lines that demonstrated a substantial population of autophagic cells, HCT-116 cells were subjected to staining with acridine orange and monodansylcadaverine after treatment with JG-03-14. While control cells tended to show a single large autophagic vesicle closely associated with the cell nucleus, treatment with JG-03-14 resulted in extensive distribution of small acidic vesicles within the cytoplasm, indicative of autophagy. GFP-LC3 transfected cells incubated with JG-03-14 showed punctuated patterns that were also consistent with the promotion of autophagy. Finally, activation of the DNA damage response pathway was ruled out by the lack of induction of p53 and p21 in cells treated with JG-03-14. In summary, our studies indicate that the JG-03-14 induces both growth arrest and autophagic cell death in HCT116 colon carcinoma cells. The possibility of an alternative mode of cell

death induced by JG-03-14 makes it a potentially usefull candidate as a chemotherapeutic drug that could be used to treat cancers resistant to apoptosis. Our result also suggested that JG-03-14 failed to induce bone marrow toxicity adding to its potential for clinical use.

CHAPTER 1 Introduction

I. Colon Cancer

In the US, cancer accounts for one in every four deaths, with colorectal cancer as the second leading cause of cancer death (American Cancer Society, 2008). Death from colorectal cancer is attributed to the metastatic form of the disease rather than the primary tumor (Delaunoit et al, 2005). Drug resistance accounts for greater than 90% of treatment failure in patients with metastatic disease, with colon cancer being completely refractory to currently approved microtubule targeting agents, such as taxanes (Fojo et al, 2007; Sampath et al, 2006). The use of chemotherapy in the treatment of colon cancer would be facilitated by the development of chemotherapeutic agents that have high potency, efficacy in destroying the tumor cells, selectivity for the tumor target versus normal host cells and refractory to the common resistance mechanisms in colon cancer.

II. Anti-tubulin compound for chemotherapy

Microtubules are crucial to cellular metabolism, intracellular transport, and cell division (Nogales, 2001). A variety of natural compounds and their derivatives have been shown to disrupt microtubule function (Xiao et al, 2005). These compounds fall into two categories: microtubule stabilizers (polymerization) or microtubule destabilizers (depolymerization); the latter group can be further subdivided into those compounds that bind to the colchicine binding site of tubulin

or the vinca alkaloid binding domain (Kuo et al, 2004). The microtubule stabilizers such as paclitaxel (taxol), discodermolide, and epothilones stimulate the assembly of purified tubulin whereas the destabilizers disrupt the assembly of purified tubulin. Combretastatin A-4, podophyllotoxin and 2-methoxyestradiol (2ME2) are microtubule destabilizers that bind to the colchicine binding site. Drugs that inhibit microtubule dynamics can ultimately hinder normal mitotic spindle function, leading to mitotic arrest and initiation of apoptosis or autophagy (Pasquier et al, 2008; Arthur et al, 2007).

III. Response to drug treatment

A. Apoptosis

Apoptosis is a form of programmed cell death that includes the extrinsic pathway, which is activated by proapoptotic receptors at the cell surface and/or the intrinsic pathway, which is activated by mitochondrial signals, where both pathways converge on the executioner caspase, caspase 3, that signals to effectors leading to cell death (Song et al, 2008; Wang et al, 2008; Ashkenazi and Herbst 2008). In addition, apoptosis has also been shown to occur via a caspase-independent mechanism (Cummings et al, 2004). Apoptosis is characterized by membrane blebbing that pinches off into small apoptotic bodies, condensation and fragmentation of the nucleus and DNA (Taylor et al, 2008). Activation of apoptosis can occur via the DNA damage response protein p53 or by a p53 independent mechanism (Gulbins et al, 2000). Resistance to chemotherapy that is associated

with the p53 dependent induction of apoptosis is often related to mutations in p53 and evasion of the p53 signaling pathway (Chendil et al, 2000).

B. Autophagy

Autophagy is characterized by the sequestration of proteins and organelles into a double membrane acidic vesicle that eventually becomes degraded (**Figure C)** (Gozuacik and Kimchi, 2004). The process begins with a double membrane that enwraps around a portion of the cytosol, containing organelles and proteins, to form the autophagosomes. The autophagosome then fuses with the lysosome, forming an autolysosome, where the contents are degraded. This process is conserved from yeast to human but differs in the site of induction, with pre-autophagosomal structures (PAS) next to the vacuole in yeast but with multiple sites in mammalian cells (Jahreiss et al. 2008; Qadir et al, 2007). There are three types of autophagy, microautophagy, macroautophagy, and chaperone-mediated autophagy, but these will not be distinguished for the purpose of this study. It has been shown that autophagy can be activated by the PI3K-Akt-mTor pathway, nutrient deprivation, ceramide, and Atg proteins (Lavieu et al, 2007; Mizushima et al, 2008). Chemotherapeutics drugs have also been shown to promote autophagy in tumor cells (Cui et al, 2007).

The process of autophagy under nutrient deprivation is under the control of the PI3-K-Akt-mTor pathway (Ravikumar et al, 2004). When nutrients are abundant, the mTor pathway is activated and inhibits autophagy; however, under conditions of nutrient depletion, mTor becomes inactivated and autophagy is no

longer suppressed. Though the process of autophagy by nutrient deprivation is well characterized, the role of autophagy in response to chemotherapeutic drugs requires extensive investigation.

A widely used method for detection of autophagy is to monitor the levels of autophagy associated proteins . The yeast Atg8 homologue MAP-light chain 3(LC3) appears to have a crucial role in the expansion and completion of autophagosome formation (Kouno 2005; Kabeya et al. 2000). MAP-LC3 is cleaved by a cysteine protease Atg4 and activated by Atg7 to produce LC3-I, found in the cytosol (Sou et al. 2006). After its generation, LC3-I is converted to LC3-II via the actions of E1 and E2 (both are ubiquitin like proteins, Atg7 and Atg3 respectively) and locates to the autophagic membrane. The target of LC3-II has been identified to be phosphatidylethanolamine (PE), found on the autophagosome membrane. This tight binding between LC3-II and PE is thought to promote autophagosome maturation and then fusion of the autophagosome with the lysosome to complete autophagy (Asanuma et al, 2003; Sou et al, 2006; Tanida et al, 2002).

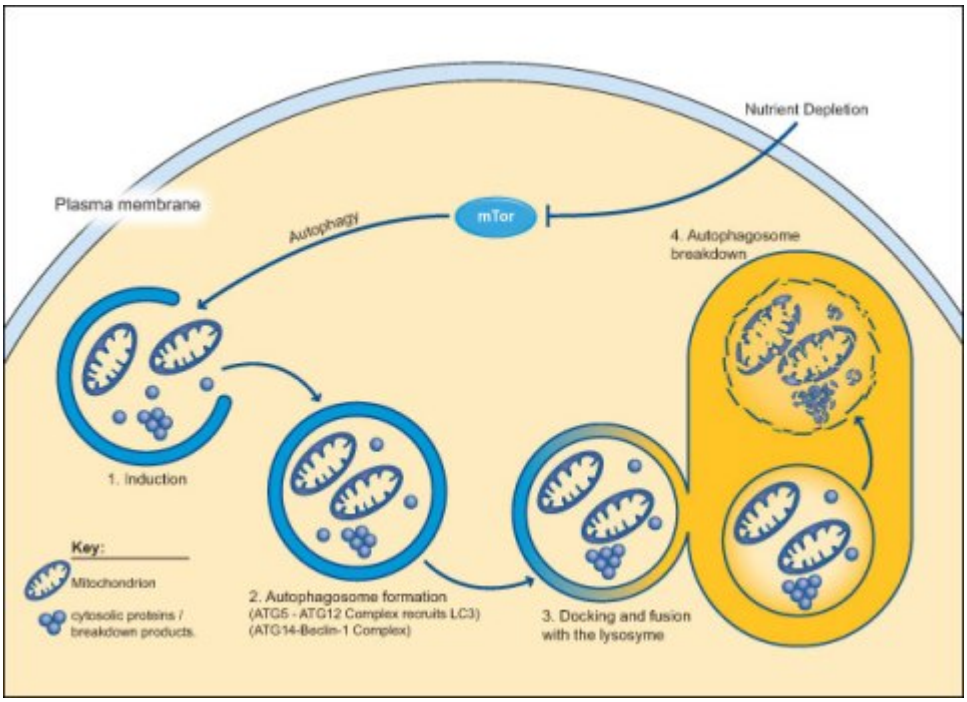


Figure C – Autophagy pathway

IV. Drug Resistance

The clinical success of cancer chemotherapy is often limited by the challenge of resistance to antitumor drugs by cancer cells. Some relatively frequent mechanism of resistance includes decreased drug accumulation in tumor cells, drug inactivation in tumor cells, increased DNA repair, and amplification or mutation of drug target genes (Eastman and Schulte, 1988; Lai et al, 1988; Cazin et al, 1992). Some of the enzymes responsible for drug inactivation includes glutathione S-transferase and aldehyde dehydrogenase (Hoban et al, 1992; Hilton, 1984) Drug resistance pumps are often responsible for the decrease in drug accumulation in cancer cells and can involve p-glycoprotein, a membrane pump

that can extrude a wide range of antitumor drugs, as well as other multidrug resistant protein pumps (Harris et al, 1992; Baines et al, 1992; Lizano et al, 1993 .) Chemotherapeutic drug resistance is a cause of frequent treatment failure in colon cancer (Fojo et al, 1987; Hengstler et al, 1998). Resistance to drugs that are widely used in the treatment of colon cancer such as the antimetabolite 5-fluorouracil, the topoisomerase I inhibitor irinotecan, and the DNA damaging agent, oxaliplatin, have been reported (Boyer et al, 2004; Oliver et al, 2002). Loss of response to apoptosis mediated cell death can often be attributed to a defect in p53 status (Violette et al, 2002). As a result, drug development requires the identification of compounds that are poor substrates for the multidrug efflux pumps. In addition, drug combination treatments represent a strategy to evade the intrinsic drug resistance mechanisms of tumor cells (Hiro et al, 2008).

V. Substituted pyrroles as anti-tumor drugs

Pyrroles are components of a more complex macrocycle that is made up of a substituted aromatic ring structure **Figure A**. They are components of the complex macrocycles such as porphyrin of heme and the chlorines and bacteriochlorins of chlorophyll (Katsiaouni et al, 2008; Wu et al, 1985). Pyrrole compounds such as CC-1065, distamycin and duocarmycins are a natural class of antibiotics that have antitumor activities (Shinohara et al, 2006; Nagamura et al, 1996; Hiraku et al, 2002). Prompted by the readily available natural material and the ability to make major or minor alterations to the natural structure has led

researchers to screen for more natural products. Lamellarins are another class of pyrroles that have been obtained from a natural source, the marine prosobranch mollusk, and which have been shown to have antitumor activity as well as the capacity to reverse multidrug resistance (Quesada et al, 1996; Davis et al, 1999). It was observed that lamellarins along with many marine derived pyrroles possess a 3,4-diarylated pyrrole 2-carboxylic acid ester or amide moiety (Fan et al, 2007). The oxygenated 3,4-diaryl substitution appears to be associated with the MDR reversal behavior while the phenolic hydroxyl groups are associated with the cytotoxic behavior (Gupton 2006).

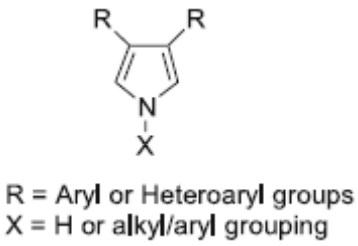


Figure A – Structure of a pyrrole

The discovery of drug templates derived from natural sources, where modifications to maximize pharmacokinetics and pharmacodynamic properties of the compound are easily performed, has led to the generation of versatile synthetic analogs.

A recent class of substituted pyrrole compounds is based on a 3,4-diarylated pyrrole 2-carboxylic acid ester **Figure B.** Out of the group of brominated pyrroles, JG-03-14 has been shown to bind at the colchicine binding

site of tubulin between the alpha and beta interfaces on purified tubulin (Mooberry et al, 2007). Studies have indicated that JG-03-14 is cytotoxic at a concentration of 500nM and it has been demonstrated to initiate both apoptosis and autophagy in MCF-7 and MDA-MB-231 breast tumor cells (Arthur et al, 2007). While some studies in the literature suggest that functional p53 is required for autophagy (Abida and Gu, 2008) JG-03-14 also induced autophagy in p53 mutant MDA-MB-231 breast cancer cells. The mechanisms by which JG-03-14 induces autophagy in MCF-7 and MDA-MB-231 breast cancer cells and the role of functional p53 requires further elucidation.

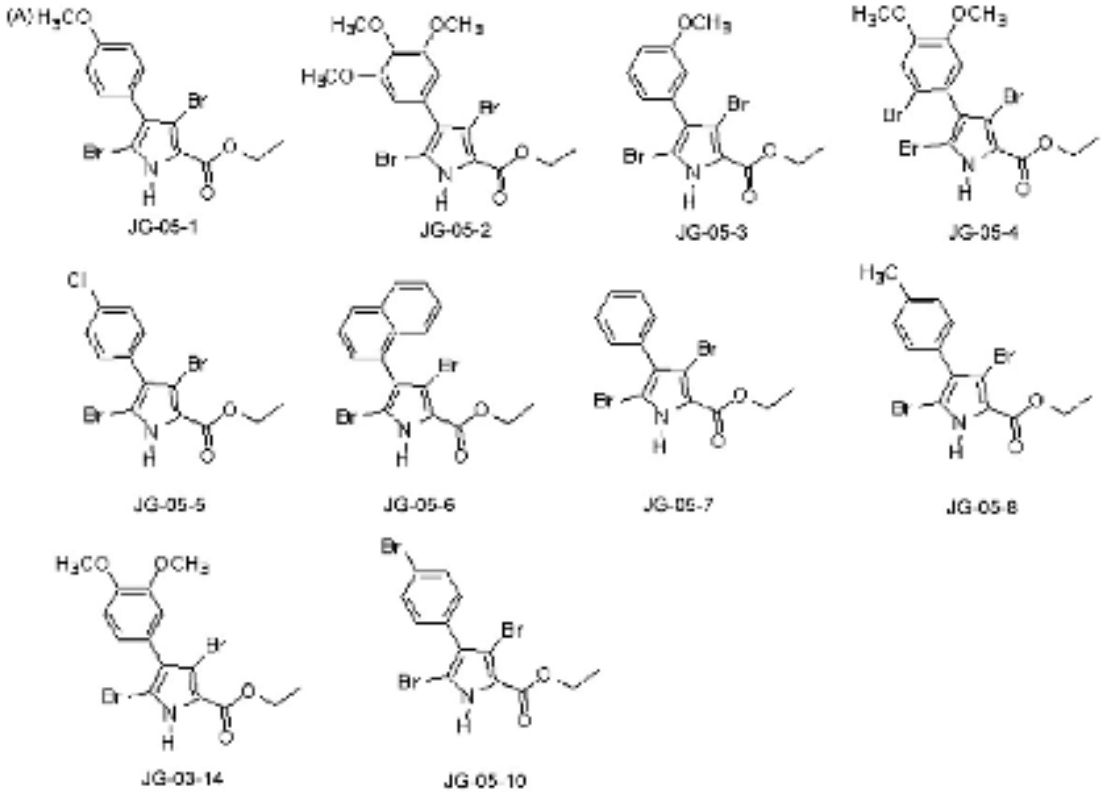


Figure B- Strucutres of the substituted pyrroles. JG-03-14 is used in this study.

The aim of our study was to determine if JG-03-14 might also have antiproliferative and or cytotoxic effects in HCT116 colon carcinoma cells, the mode(s) of cell death is induced by JG-03-14, and whether the compound activates p53 proteins and/or p21Waf1/Cip1.

CHAPTER 2 Materials and Methods

Cell Culture

HCT116 p53 wild type colon carcinoma cells were obtained from ATCC and kept frozen in 10% DMSO (Sigma Chemical, St. Louis, MO) with Fetal Bovine Serum (FBS)(GIBCO Life Technologies, Gaithersburg, MD) until use. Cells were quickly thawed and cultured in a T75 flask (Cellstar) in basal RPMI 1640 medium supplemented with 5% fetal bovine serum, 5% bovine calf serum, 2 mM L-glutamine, and penicillin/streptomycin 0.5 ml/100 ml medium(10,000units/ml penicillin and 10 mg/ml streptomycin; GIBCO Life Technologies, Gaithersburg, MD) at 37^0 C under a humidified, 5% CO_2 atmosphere. Cells were passaged at 80% confluency by washing one time with 1X PBS(GIBCO), harvested with trypsin-EDTA 0.25 %(GIBCO) and then deactivated with RPMI medium, collected and centrifuged at 15,000 rpm for 3 minutes. Media was aspirated and fresh media was added to the cell pellet; cells were re-suspended in media and 300 µl of the cell resuspension was placed into a fresh T75 flask with 10mL RPMI medium. For all culture experiments, cells were plated and permitted to adhere overnight before drug treatment. All treatment involved continuous drug exposure.

MTT assay

Cells were prepared as described above. Using a 96 well plate, 5,000 cells/150uL media were plated per well and allowed to adhere overnight before drug treatment. After 72 hours, 100 µl of the MTT solution (2mg/mL PBS prepared in the dark) was added to each well and incubated for 3 hours at 37^0 C The MTT solution was removed and 100µl of DMSO was added to each well, and the blue dye was allowed to dissolve for 5 minutes. An absorbance reading was taken at 490 nm (KC Junior software, EL800 Universal Microplate Reader).

Crystal Violet Assay

Cells were prepared as described above. The crystal violet assay, as described by Wosikowski et al, (1993), was used to determine drug effects on cell viability. In a 48 well plate, 20,000 cells /500 µl media were plated per well and allowed to adhere overnight. After 72 hours of treatment, the drug was aspirated and the cell monolayer was fixed with 100% methanol for 10 minutes. The methanol was aspirated and a crystal violet 0.5% solution in 25% methanol was used to stain the cells for 10 minutes. The stain was removed and cells were washed 4 times to remove excess dye. Fixed cells were left to air dry overnight. The next day, cells were solubilized in 1 ml 0.1 M sodium citrate in 50% ethanol and placed on a shaker for 10 minutes. Absorbance readings were taken at 540nm.

Time Course of Drug Effects on Viable Cell Number

Cells were prepared as described above. Cells were plated at 1X10^6 cells per 96 mm Petri plate (CellStar) and allowed to adhere overnight. At the various time points, media containing the drug was aspirated and cells were washed once using 1X PBS. Cells were then harvested and centrifuged at 15,000 rpm for 3 minutes and media was removed. The cell pellet was resuspended in fresh media and 10µl of the cell resuspension was added to 90µl of 0.4% trypan blue exclusion dye (1:9; trypan blue: PBS). 10µl of the mixture was placed onto a hemocytometer and viable cells (those that excluded the dye) were counted using the phase contrast microscopy.

DAPI Staining for Apoptosis and Mitotic Catastrophe

Cells were prepared and treated as in the time course studies, above. The assay used was from Gavrieli et al, 1992. At the various time points, adherent and non-adherent cells were harvested and centrifuged at 15,000 rpm for 3 minutes. The media was aspirated and fresh media was added to resuspend the pellet. Viable cell numbers was determined using trypan blue exclusion dye as described previously. A dilution of 20,000 cells in 200 µl of 1X PBS per slide was prepared and cells were spun at 10,000 rpm for 5 minutes (Shandon Cytospin 4, Thermal Electron Corp). Slides were refrigerated until ready for staining. Cells were fixed with 4% formaldehyde in PBS for 10 minutes at room temperature, and then washed with PBS twice for 5 minutes at room temperature. A 1:2 dilution of acetic

acid and ethanol was used to fix the cells at 20^0 C for 5 minutes. The slides were then washed twice with PBS for 5 minutes at room temperature. A 1:1000 dilution was prepared for Vectashield :Dapi and each slide was mounted with 10µl of the solution. Coverslips were sealed using clear nail polish and photographs were taken at 10X using a Nikon fluorescent microscope and an Olympus camera. Slides were stored at 4^0 C , and three field per condition were evaluated.

Flow Cytometry

Cells were plated at 3 million cells per 96 mm Petri plates for each time point and allowed to adhere overnight. Setup was prepared for day 5 and worked backward so that all treatments would complete on the same date. Drug treatment was initiated the following day and drug was left on the cells for continuous exposure. Upon the completion of treatment, media was aspirated and cells were washed one time with 1X PBS. Cells were harvested using trypsin and deactivated with 10ml media, then centrifuged at 15,000 rpm for 3 minutes. Media was aspirated and the cell pellet was resuspended in fresh media. Viable cell number was determined by trypan blue exclusion dye. At least 1 million cells per condition were used in the cell cycle analysis. 1.5 ml of Propidium iodide stain with RNAse was added to each million cells. The cell suspension was filtered through a 70 micron mesh 2 times. All samples were covered in aluminum foil and refrigerated until the next day. FACs acquisition was performed using the EPICS 753 flow cytometer (Coulter Electronics, Hialeah, FL) using a 488 nm argon laser

and standard optical emission filters. A minimum of 13,000 events were collected per sample. Cellular DNA distributions were analyzed at the various stages of the cell cycle using Cytologic Software (Coulter Electronics).

Acridine Orange staining for autophagy

As a marker for autophagy, the acridine orange staining method described by Paglin et al, (2001) was used. Cells were plated at 2 X 10^5 per 6 well plate and allowed to adhere overnight. Drug was added the following day and left on for continuous exposure. At the final time point, the drug was removed and cells were washed one time with 1X PBS. Cells were then stained with acridine orange (1ug/ml) for 15 minutes. .

Monodansylcadaverine

Monodansylcadaverine(MDC) is another marker for autophagy that stains the autophagic membrane (Niemann et al, 2000). Cells were treated as described above. MDC(Sigma) was dissolved in DMSO for a stock concentration of 100 mM. At selected time points, cells were stained with 0.5 mM monodansylcadaverine for 10 minutes. The stain was removed and cells were washed with 1X PBS four times before fresh media was added to the wells. Pictures were taken with the inverted Nikon fluorescent microscope and an Olympus camera at 20X magnification

Transfection with GFP-LC3

As a marker for autophagy, LC3 tagged GFP was used to detect the processing of the LC3 proteins in the autophagosomes (Kabeya et al, 2000). Cells were prepared from frozen stock and passaged twice at least 2 days before nucleofection. 1 X 10^6 cells were centrifuged and suspended in a pellet. 100 µl of the Nucleofector V (Nucleofector Kit V, Amaxa) mix was added to resuspend the cells. The resuspension was added to 1 µg of the GFP-LC3 vector and the entire suspension was transferred to the cuvette. The cuvette was placed in the Nucleofector device and program D-032 was used to transfect the cells. 500 µl warm medium was added to the transfected solution and the total suspension was transferred to a Petri plate. These steps were repeated for the positive and negative controls using 2 µg of empty vector-GFP, and 2 µg pmax-GFP.

Western Blotting

After the indicated times, cellular proteins were extracted from treated cells using 100 to 200 µL of 1X tris lysis buffer (1 M Tris(pH 6.8), 10% SDS, and dH_2O) containing protease inhibitors with boiling for 5 minutes. Protein concentration was determined with a Lowry protein assay and 10 µg of total cell lysates were separated using 12% SDS-PAGE. Proteins were transferred onto nitrocellulose membrane for 1 hour and blocked in TBS-Tween 20 buffer containing 5% nonfat dry milk. Membranes were then immunoblotted with the respective primary antibodies overnight in 20^0 C. The following day, the membrane was incubated

with horseradish peroxidase-conjugated secondary antibody for 1 hour. Signals were detected using an enhanced chemilluminescence detection reagents from Pierce (Rockford, IL).

Assessing Bone Marrow Toxicity

Two C57BL6 mice were sacrificed by cervical-cranial dislocation. The femur and tibia of both mice were removed and all exo-skeletal parts were removed. All muscle tissue was scraped off with a scalpel and bones were placed in 1X PBS. The femur was cut at both ends to expose the hollow bone and fresh 1X PBS was used to wash out the bone marrow cells using a 23 G needle. The same process was repeated for the other femur and tibias after which the cell suspension was spun down at 10,000 rpm for 3 minutes. The pellet was washed with 1X PBS and re-centrifuged. To lyse red blood cells, red cell lysis buffer was added to the pellet and incubated at 37^0 C for 5 mins. Cells were centrifuged and washed with 1X PBS. The bone marrow cell pellet was resuspended in 1 ml RPMI medium supplemented with 30% serum. Cells were counted and plated at 2X10^5 cells/0.5ml medium. Drug was added directly to the cell suspension with incubation for 72 hours. Cell viability was evaluated using Alamar Blue dye (10% dissolved medium) and absorbance of 570 nm and 600 nm was taken after 30 minutes with the KC Junior microplate reader.

Statistical Analysis

Statistical analysis was performed using a two-tailed student's T test; groups with $p \leq 0.05$ were considered to be significantly different than vehicle controls.

CHAPTER 3 Results

I. Effects of JG-03-14 on the DNA damage response pathway in MCF-7 breast tumor cells.

In previous studies, (Mooberry et al., 2007) the novel substituted pyrrole compound, JG-03-14, was shown to inhibit the polymerization of purified tubulin in vitro; evidence was also presented that JG-03-14 bound to the colchicine binding site on tubulin. In a separate report utilizing molecular modeling, it was determined that JG-03-14 was likely to form a very stable complex with high affinity to tubulin, suggesting a promising role for this compound as a chemotherapeutic agent (Tripathi et al, 2008). Studies within our laboratory demonstrated that JG-03-14 promoted cell death in both p53 wild-type MCF-7 and p53 mutant MDA-MB-231 breast tumor cells; furthermore, JG-03-14 retained activity in multi-drug resistant cells (Arthur et al., 2007; Mooberry et al, 2007).

Although both of the studies cited above supported the concept that JG-03-14 acts as a microtubule poison, it was important to test and eliminate the possibility that JG-03-14 might also act through the induction of DNA damage. Since DNA damaging agents generally induce p53 and p21 (Lanni and Jacks, 1998), Western blotting was performed using antibodies against p53 and p21 after exposure of MCF-7 cells to JG-03-14. **Figure 1** shows that 10 Gy of irradiation (a positive control) induced p53 at 1h and 2h and p21 at 2h and 4h. JG-03-14 failed

to induce p53 or p21, indicating that there was no DNA damage response to JG-03-14 in the p53 wild type MCF-7 cells.

II. Effects of JG-03-14 and other microtubule poisons on viability of HCT-116 colon cancer cells

Due to JG-03-14's ability to cause cytotoxicity in cells expressing the multi-drug resistant phenotype, we chose to analyze its potential to interfere with growth and or cause cell death in colon tumor cells as these cancers are notorious for acquiring resistance against drugs such as colchicine, vinblastine, lapatinib and mitomycin (Incles et al., 2003; Martin et al., 2008). We selected p53 wild type HCT-116 cells because of the availability of isogenic derivative cells lines where p53 or p21 function have been silenced (for future work).

Sensitivity of HCT-116 colon tumor cells to JG-03-14 was determined using the Crystal Violet Assay (Wosikowski et al. 1993). Cells were treated with the compound by continuous exposure for 72 hours prior to fixation and staining. **Figure 2A** indicates that JG-03-14 demonstrated concentration-dependent growth inhibition; at concentrations of 250 nM and 500nM, substantial growth inhibition was observed compared to the untreated controls. Treatment with adriamycin at 100nM was used as a positive control. All subsequent studies were performed using JG-03-14 at a concentration of 500 nM.

For comparative purposes, we also assessed sensitivity of HCT-116 cells to two other microtubule poisons, colchicine and taxol. Colchicine causes

microtubule disruption, like JG-03-14, while taxol is a microtubule stabilizer. **Figure 2B** shows that after 72 hours of continuous drug treatments, the three compounds all reduced viable cell number by approximately equivalent amounts, with a reduction of 75% by JG-03-14, 82.5% by colchicine and 67% by taxol.

III. Cell death of HCT-116 cells after exposure to JG-03-14 and other microtubule poisons

The crystal violet studies do not distinguish between the effects of drugs on cell growth arrest or viability. Consequently, we monitored the effects of JG-03-14 on cell viability over a period of 5 days**. Figure 3A** shows a time dependent decline in viable cell number when HCT116 cells were treated by continuous exposure to JG-03-14 at 500nM, a concentration that we have previously found to be effective in promoting breast tumor growth arrest and cell death (Arthur, 2007). All the microtubule poisons produced a similar time-dependent decline in viable cell number (**Figure 3B**).

IV. Assessment of apoptosis in HCT-116 cells after treatment with JG-03-14 and other microtubule poisons

The possibility that the cell death observed in HCT-116 in a time dependent manner might be due to apoptotic events was assessed using 4',6-diamino-2-phenylindole (DAPI), a blue fluorescent nucleic acid stain that binds to double stranded DNA. **Figure 4** shows the untreated vehicle control with a clearly

defined intact nucleus; in contrast, our positive control, treatment with 10 μM taxol, shows nuclear fragmentation characteristic of apoptosis. The nuclei of cells treated with JG-03-14 treated were intact, similar to the untreated cell and vehicle controls; however, nuclei of cells treated with JG-03-14 were significantly enlarged.

Since the positive taxol 10 μM control was not a pharmacologically relevant concentration, we also assessed the effects of colchicine (100nM) and taxol (100nM) on nuclear integrity. Dapi staining showed that the majority of nuclei were fragmented by both treatments after three days (**Figure 4).**

V. Cell cycle effects of JG-03-14 and other microtubule poisons on HCT-116 tumor cells

Microtubule poisons generally produce G2/M arrest in tumor cells (Tseng et al, 2002). JG-03-14 also produced a G2 arrest in the p53 wild type MCF-7 cells (Arthur et al, 2007). The next series of experiments were designed to evaluate the nature of growth arrest by JG-03-14 as well as to verify the absence of apoptosis. **Figure 5** presents FACS analysis of cells treated with JG-03-14 at various time points. JG-03-14 transiently arrested the p53 wild type HCT-116 tumor cells in the G2/M cell phase after the first day of treatment, consistent with its microtubule destabilizing abilities. This G2/M block persisted for 72 hours. Of particular interest was the ability of p53 wild type HCT-116 cells to continue to replicate following the G2/M arrest, resulting in a hyperdiploid population. This effect was

observed after the first day of treatment and the hyperdiploid cell population had more than doubled after 72 hours, where 21 % of the cells were polyploid. FACS analysis confirmed the absence of cells in a sub G1 population both at 24 and 72 hours following the incubation with 500 nM JG-03-14, confirming the absence of apoptosis in HCT-116 p53 wild type cells.

We also evaluated the effects of the microtubule destabilizer, colchicine, on cell cycle distribution. At a concentration of 100nM, where cell growth is inhibited by 90% (**Figure 3**), approximately half the population arrested in the G1 phase and the other half accumulated in G2/M after 24 hours (**Figure 5**). This was shifted to a 49% G2/M arrest and 4.5% in G1 after 72 hours. A similar cell cycle distribution was observed in cells treated with JG-03-14 after 72 hours, where 63% were arrested in G2/M and 4.5% were in G1. Colchicine also induced polyploidy in the HCT-116 cells. 2% of the population were hyperdiploid after 24 hours and 14.7% was hyperdiploid after 72 hours, coinciding with the characteristic of microtubule destabilizers (Morrison and Rieder, 2004). Approximately 34% of the cell population consisted of sub G1 cells after 24 h, indicating that approximately one-third of the cells were undergoing apoptosis, a characteristic consistent with colchicine treatments in other studies (Bonfoco et al, 1995; Wang et al, 1999). This finding is also consistent with the DAPI staining studies shown in **Figure 4**.

In contrast to the effects of the microtubule destabilizing agents, colchicine and JG-03-14, the microtubule stabilizing agent, taxol, at 100nM, arrested the cells primarily in the G1/S phase. Approximately 42% and 60% of the cells were in G1

after 24 and 72 hours, respectively. A significant fraction of the cell population (20 %) was in the sub G1 phase after 24 hours, with an increase to 30% after 72 hours. Again, the capacity of taxol to generate a sub G1 cell population confirmed the presence of apoptosis in HCT-116 cells that was indicated by the DAPI studies.

VI. Assesment of mitotic catastrophe in HCT-116 cells after JG-03-14 treatment

We concluded that our p53 wild type HCT-116 cells were not succumbing to death via apoptosis after treatment with JG-03-14, based on our Dapi staining and FACS analysis. Due to the presence of enlarged cells and a hyperdiploid cell population we considered the possibility of mitotic catastrophe being the mode of cell death in HCT-116 tumor cells. However, **Figure 4** failed to demonstrate bi-nucleated cells and micronuclei that are characteristics of mitotic death (DeMasters et al, 2006).

VII. Induction of autophagy by JG-03-14 in colon cancer cells.

In our previous work, the p53 wild type MCF-7 and p53 mutant MDA-MB-231 breast tumor cells demonstrated essentially similar sensitivity to JG-03-14; however, their response was different with regard to the modes of cell death. MCF-7 cells were not dying through apoptosis based on the FACS analysis and TUNEL assay, but rather through autophagy. MDA-MB231 cells demonstrated

both apoptosis and autophagy. Since the MDA-MB231 cells are p53 mutant cancer cells, both apoptotic death and autophagy can be independent of the p53 pathway.

Our studies suggested that the death of the p53 wild type HCT-116 colon cancer cells was not triggered through apoptosis after treatment with JG-03-14 based on the DAPI staining and FACS analysis. The lack of micronuclei and binucleated cells in **Figure 4** also ruled out the possibility of mitotic death. Since JG-03-14 was shown in our previous work to promote autophagy in both MCF-7 and MDA-MB231 breast tumor cells, we also assessed this possibility in the HCT-116 colon carcinoma cells using acridine orange staining. **Figure 6** shows that the untreated control had a uni-vesicular stain that was situated near the nucleus. In dramatic contrast, the cells treated with JG-03-14 exhibited a more punctuate staining that was distributed throughout the cytoplasm. The cells treated with JG-03-14 were also quite enlarged, nearly double the size of the untreated cells, likely due to the increased DNA content.

We also evaluated the capacity of the microtubule poisons, colchicine and taxol, to promote autophagy, **Figure 7**. Surprisingly, a similar pattern of punctuated stained vacuoles were also exhibited in cells treated with colchicine and taxol, both at 100 nM concentrations. This was not expected due to the large apoptotic population observed by FACS analysis and the fragmented nuclei evident with DAPI staining.

VIII. Confirmation of the induction of autophagy by JG-03-14

To confirm the promotion of autophagy, we used an additional approach, staining of autophagic vesicles with monodansylcadaverine. This is a selective stain that binds to the lipid membrane of the autophagosomes, and which would be more sensitive to the presence of autophagosomes, unlike the acridine orange, which primarily detects the acidic pH of the autophagic vacuoles (Niemann et al., 2000; Biederbick et al., 1995). **Figure 8** compares the cells treated with JG-03-14 and controls. After day 3, a substantial amount of monodansylcadaverine staining of the autophagic vacuoles is evident in the treated cells compared to the control, which showed a single stain that was centered on a uni vacuole, similar to what was present in the acridine orange stains. The monodansylcadaverine positive stained vacuoles also showed a highly distributed punctuate pattern throughout the cytoplasm. The staining with both acridine orange and monodansylcadaverine confirmed the presence of autophagosomes in HCT-116 cells treated with 500 nM JG-03-14. The presence of autophagosomes was also detected in cells incubated with either 100 nM taxol or colchicine (**Figure 7 and 8**).

IX. Assessment of LC3-II protein levels in HCT-116 cells tested with JG-03-14

In efforts to further confirm that autophagy may be the mechanism by which JG-03-14 is inducing cell death, we performed western blotting to detect conversion of LC3-I to LC3-II at various time points. LC3-I conversion to LC3-II is

required for autophagosome maturation and is found both inside and outside the vesicle (Amer et al, 2005). We attempted multiple times using both other tumor cell lines and cells transfected with GFP-LC3-II via lipofection and nucleofection but failed to obtain a clear and reproducible western blot with LC3-II. Our blots showed stains that were just background noise, irrelevant to our interest. We tried using three different antibodies from three different companies that also failed to produce anything close to clean blots (data not included).

X. Detection of LC3-II punctate in HCT-116 cells with JG-03-14 treatments

LC3-II is a cleaved product of LC3-I and is involved in the formation of autophagosomes (Kabeya et al, 2000). We attempted to confirm that autophagy was occurring by detecting the presence of LC-3 II in the treated cells. HCT-116 colon tumor cells were transfected with a GFP-LC3 vector via nucleofection and the cells were observed under immunofluorescence after 72 hours with 500 nM JG-03-14. After 72 hours of drug treatment, the transfected cells showed a punctuate distribution of LC-3 II proteins in the cytoplasm (**Figure 9**) whereas cells transfected with the empty vector lacked any punctuated stains. Due to the low efficiency of transfection and the relatively short life of the GFP fluorescent dye, there were very few cells remaining which exhibited the fluorescence after 72 hours.

XI. Effects of autophagic inhibitors on the response to JG-03-14 treatment in HCT-116 cells

There is continuing controversy in the literature as to whether autophagy is a cytoprotective mechanism or an actual mode of cell death(Mori et al, 2007; Boya et al, 2005). In a further attempt to confirm the mode of cell death caused by JG-03-14 and to elucidate the ambiguous role of autophagy, we sought to block autophagy. In efforts to address this question, cells were treated with 3-**methyladenine** (3-MA), which has been shown to inhibit autophagy (Seglen and Gordon, 1982). HCT-116 cells were incubated with both 25mM 3-MA and 500 nM JG-03-14, after which cells were stained with acridine orange at the various time points. Our untreated controls exhibited one acidic vesicle situated near the nucleus as shown in **Figure 10**. Cells exposed to JG-03-14 showed distributed acidic vesicles that were throughout the cytoplasm. 3-MA however, did not inhibit or decrease the generation of acidic vesicles by JG-03-14. Cells were still enlarged and highly punctuated with autophagosomes (**Figure 10**). There was no noticeable difference evident between cells treated with JG-03-14 alone and those that were co-treated with 3-MA, suggesting that it was necessary to identify an alternative means to interfere with autophagy.

Bafilomycin A-1 is a macrolide antibiotic that was shown to inhibit autophagy at high concentrations (Shacka et al., 2006). Using the MTT assay, contradictory results were obtained in terms of the cytotoxicity of bafilomycin-A1(data not shown). Due to the conflicting results of the experiments using the

MTT assay, we reverted to using the crystal violet assay to test for sensitivity. Using this assay, Bafilomycin-A1 at 100 nM did not show any growth inhibition. When cells were treated with Bafilomycin-A1 in combination with JG-03-14 at 500 nM, there was no reduction in the cytotoxic effects of JG-03-14 (**Figure 11A**). We then went on to test the inhibitory effect of Bafilomycin-A1 on autophagy. Cells were co-incubated with JG-03-14 (500nM) and Bafilomycin-A1 (100 nM) with continuous exposure prior to acridine orange staining. **Figure 11B** showed that there was no difference in the extent of autophagy with JG-03-14 alone and with JG-03-14 combined with the autophagy inhibitor, Bafilomycin-A1 (**Figure 8)**.

XII. Effects of JG-03-14 on the DNA damage stress response activation pathway in HCT-116 cells

Although we appear to have ruled out activation of the DNA damage response pathway by JG-03-14 in the MCF-7 cell studies (**Figure 1**) it remained possible that JG-03-14 might be activating this pathway in the HCT-116 cells. To examine this possibility western blotting was performed at 4 and 8 hours to detect levels of p53 and p21. **Figure 12** indicates that JG-03-14 alone did not induce p53 at 4 and 8 hours similar to the untreated controls. However, the treatment with taxol or colchicine showed induction of p53 or p21 at 4 and 8 hours. Irradiation at 10 Gy was also used as a positive control.

XIII. Evaluation of the impact of JG-03-14 on bone marrow toxicity

Bone marrow toxicity is a major concern with cancer chemotherapy. In order for JG-03-14 to be clinically successful, it should not express this mode of toxicity. Figure 13 showed the effects of JG-03-14 on hematopoietic cell viability using Alamar Blue staining. JG-03-14 showed no suppression of bone marrow cell growth while taxol and adriamycin produced small effects.

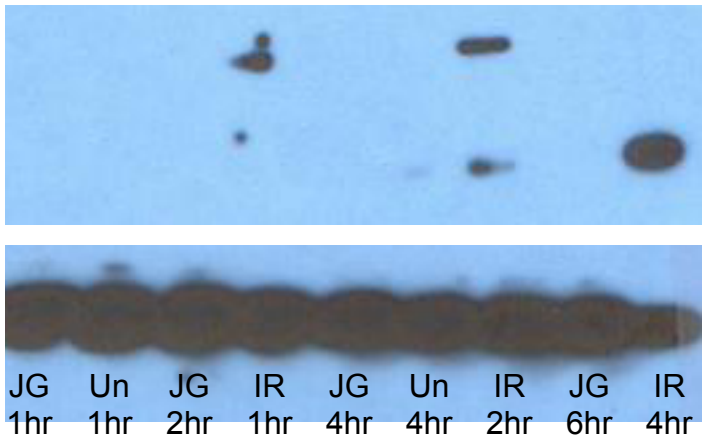
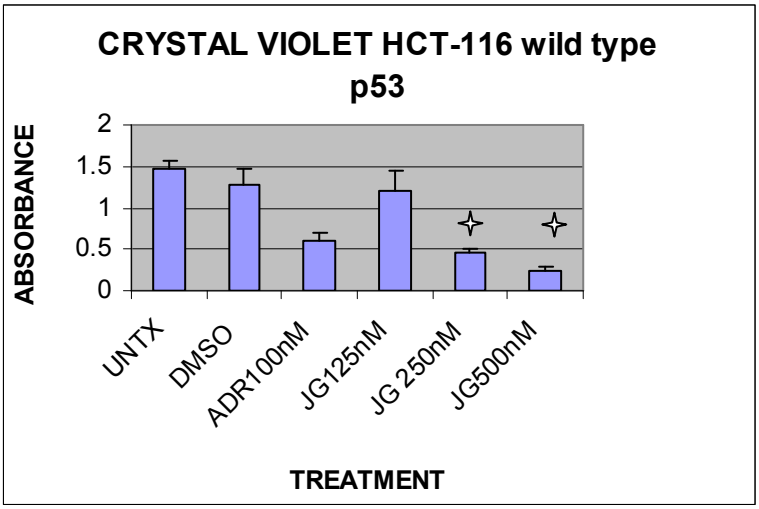


Figure 1- Lack of induction of p53 and p21 by JG-03-14 in MCF-7 breast tumor cells. MCF-7 cells were incubated with JG-03-14 (500nM) for the time periods indicated before harvesting and subjecting cell lysates to western blotting. Irradiation (10 Gy) was used as a positive control and beta actin was the loading control. p53 and p21 were induced by irradiation (lanes 4, 7, and 9).

A.



B.

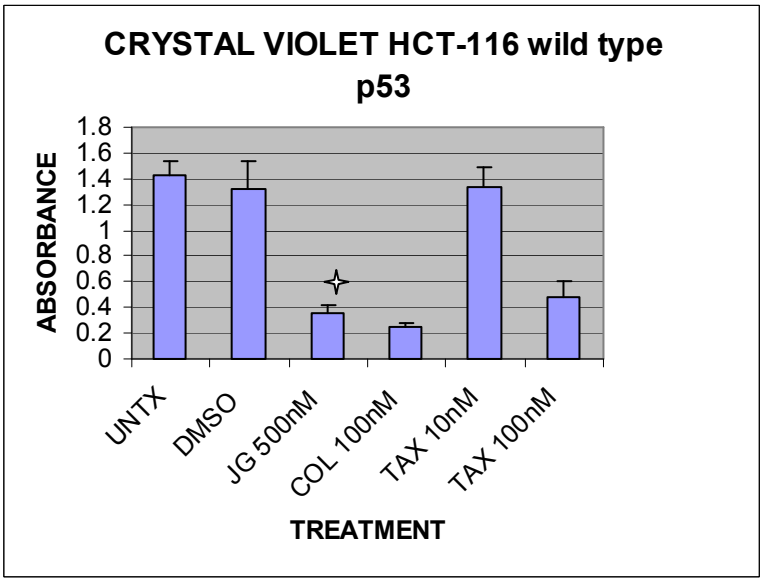
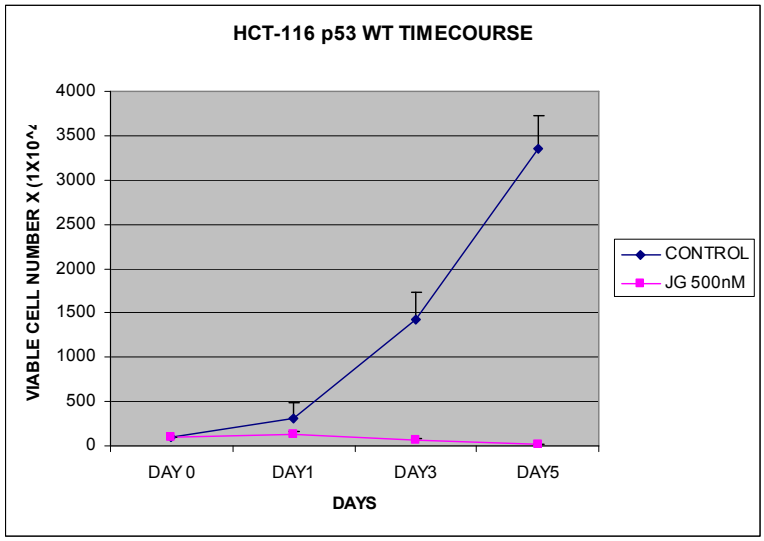


Figure 2 – Drug sensitivity of HCT-116 colon carcinoma cells by the Crystal Violet assay. **A.** Sensitivity to JG-03-14. **B.** Sensitivity to other microtubule poisons. Cells were seeded in a 48-well plates and incubated with the indicated compounds at the various concentration for 72 h. DMSO was a negative control and Adriamycin was used as a positive control. Values shown represent means ± standard errors for three replicate experiments. Growth inhibition at 250 nM and 500 nM JG-03-14 was statistically significant compared to vehicle controls with $p \leq 0.05$ for HCT-116 cells.

A.



B.

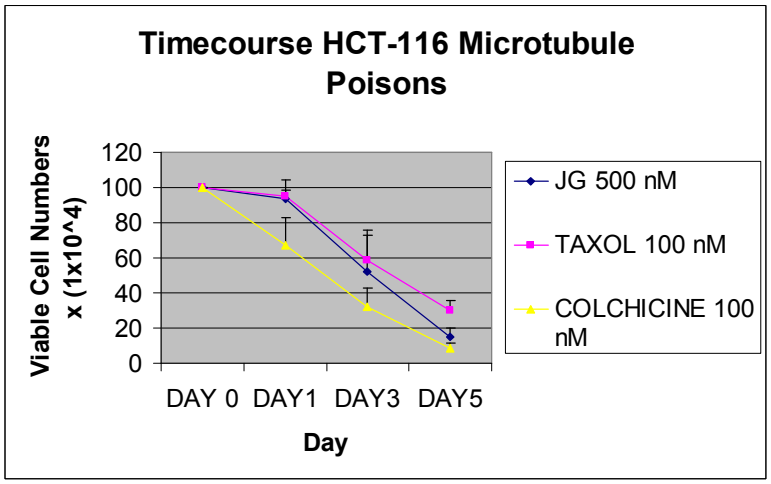


Figure 3 – Growth arrest and cell death in HCT-116 cells. Cell death was evaluated in response to a continuous 5 day exposure to 500 nM JG-03-14, 100nM colchicine or 100nM taxol. Cell viability was monitored using trypan blue exclusion. Studies were independent of each other. **A.** Time course response of HCT-116 cells to JG-03-14. Adriamycin (100 nM)was used as our positive control (not shown). **B.** Time course response of HCT-116 cells to JG-03-14 500nM, colchicines 100nM and taxol 100nM. Values shown represent means ± standard errors for three replicate experiments.

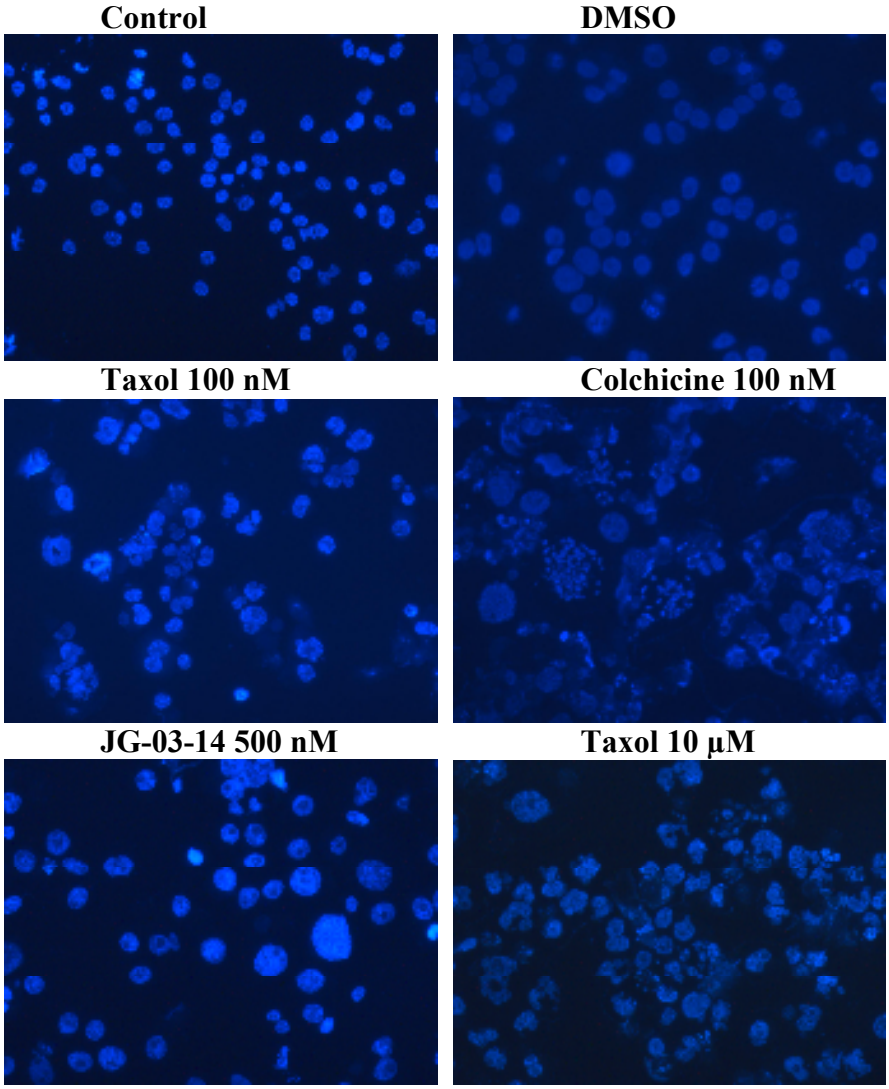


Figure 4– Assessment of effects of JG-03-14 on nuclear integrity of HCT-116 cells. HCT-116 cells were treated with the various drugs by continuous exposure. After 72 h, slides were prepared with adherent and non-adherent cells, fixed, and stained. Cells treated with JG-03-14 showed very minimal nuclear perturbations compared to the pronounced nuclear fragmentation evident in treatments with taxol and colchicine.

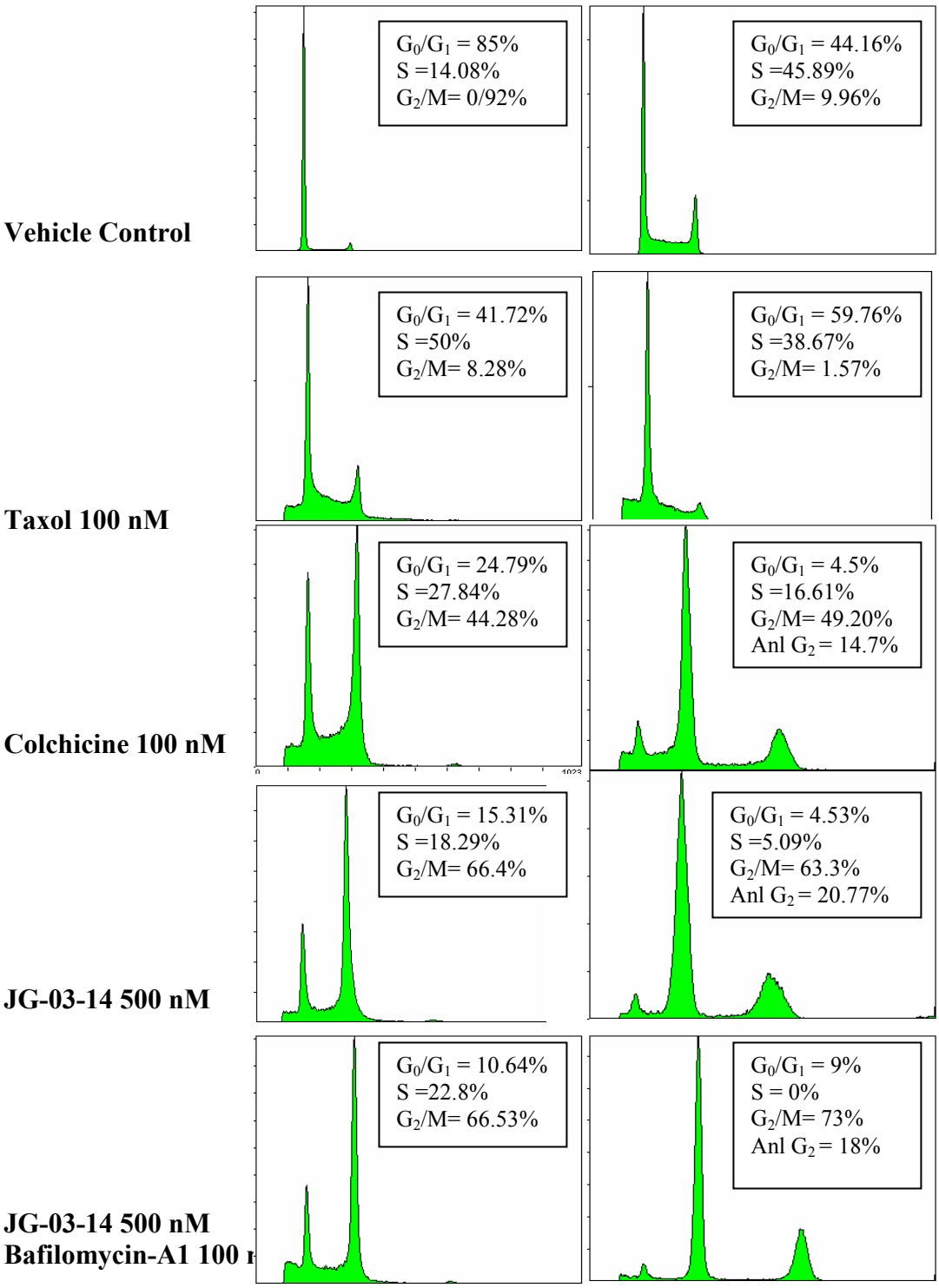
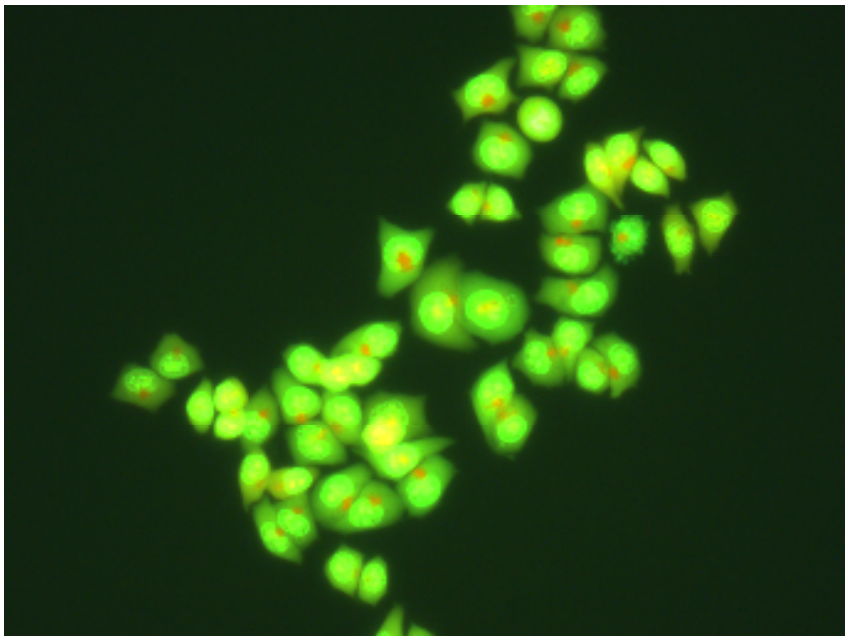


Figure 5- Influence of JG-03-14 on cell cycle progression of HCT-116 cells. Cell cycle distribution was determined by flow cytometry of HCT-116 cells exposed to the various microtubule compounds. Cells were exposed for 72 h, at which time were harvested and stained with propidium iodide staining (PIF) solution. Flow cytometry PIF voltages were altered for the treatment groups compared to the control to accommodate the increasing polyploidy observed following drug treatment at 24h and 72 h.

Control



JG-03-14

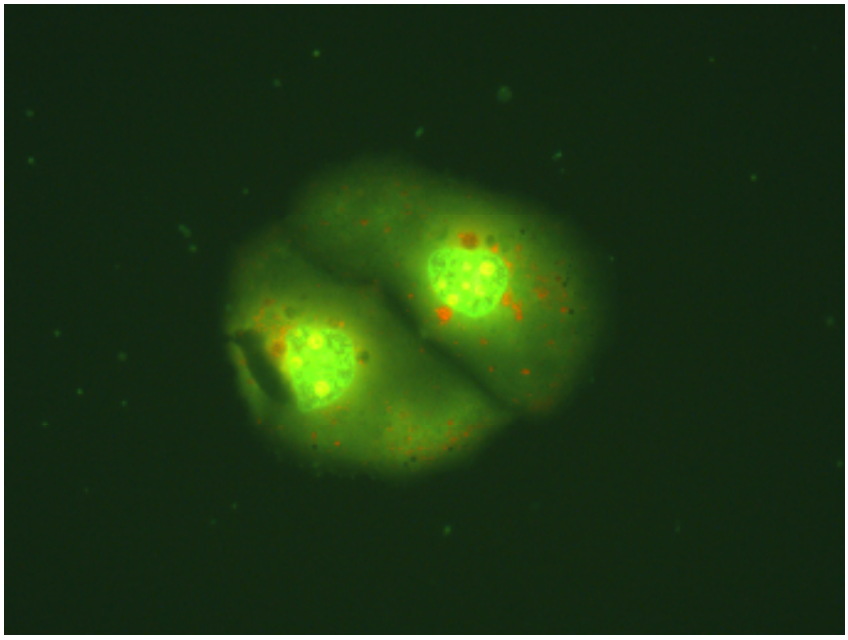


Figure 6- Determination of autophagy by acridine orange staining. HCT-116 cells were incubated with JG-03-14 at 500 nM for 3 days, stained, washed, and observed by fluorescence microscopy. Green stain reflects the background while bright orange staining occurs in acidic (autophagic) vacuoles. Although the control showed one acidic vacuole , it was localized to the nucleus and is different from the accumulation of the distributed autophagic vacuoles seen in treatment with JG-03-14. All photos are taken at 20X.

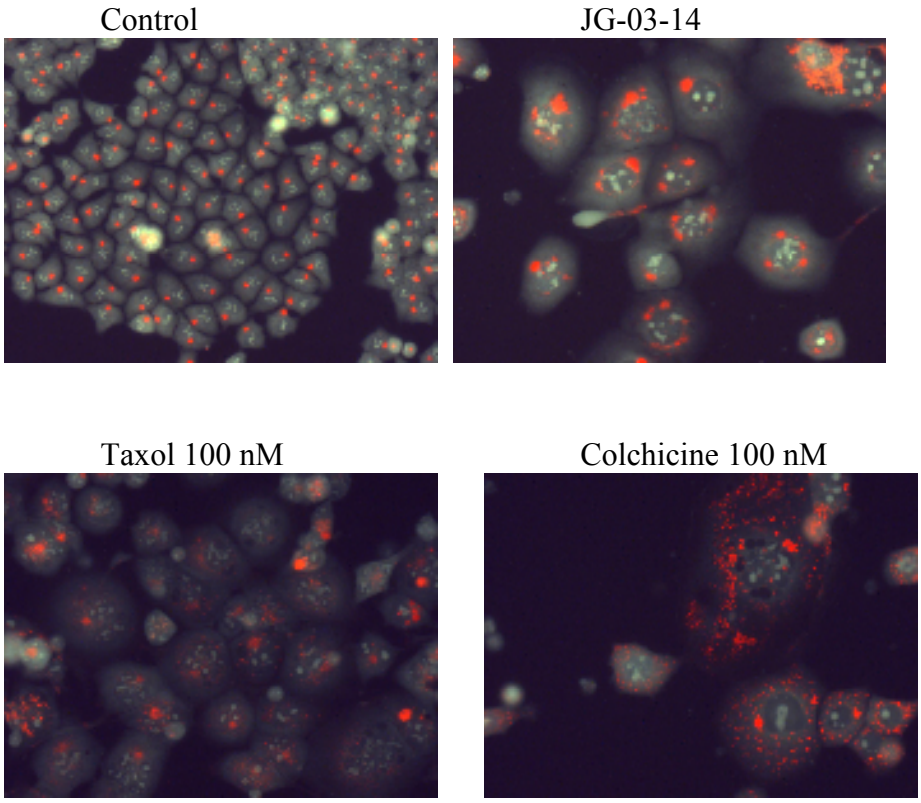


Figure 7 - Comparing autophagy by microtubule poisons in HCT-116 cells using acridine orange staining. HCT-116 cells were incubated with the different treatments for 3 days, stained, washed, and observed by fluorescence microscopy. These photos were taken without the green background which better differentiates the staining. Bright orange staining occurs in acidic (autophagic) vacuoles. Although the control showed one acidic stained vacuole, it was localized to the nucleus and is different from the accumulation of the distributed autophagic vacuoles seen in our treatment with JG-03-14. All photos were taken at 20X.

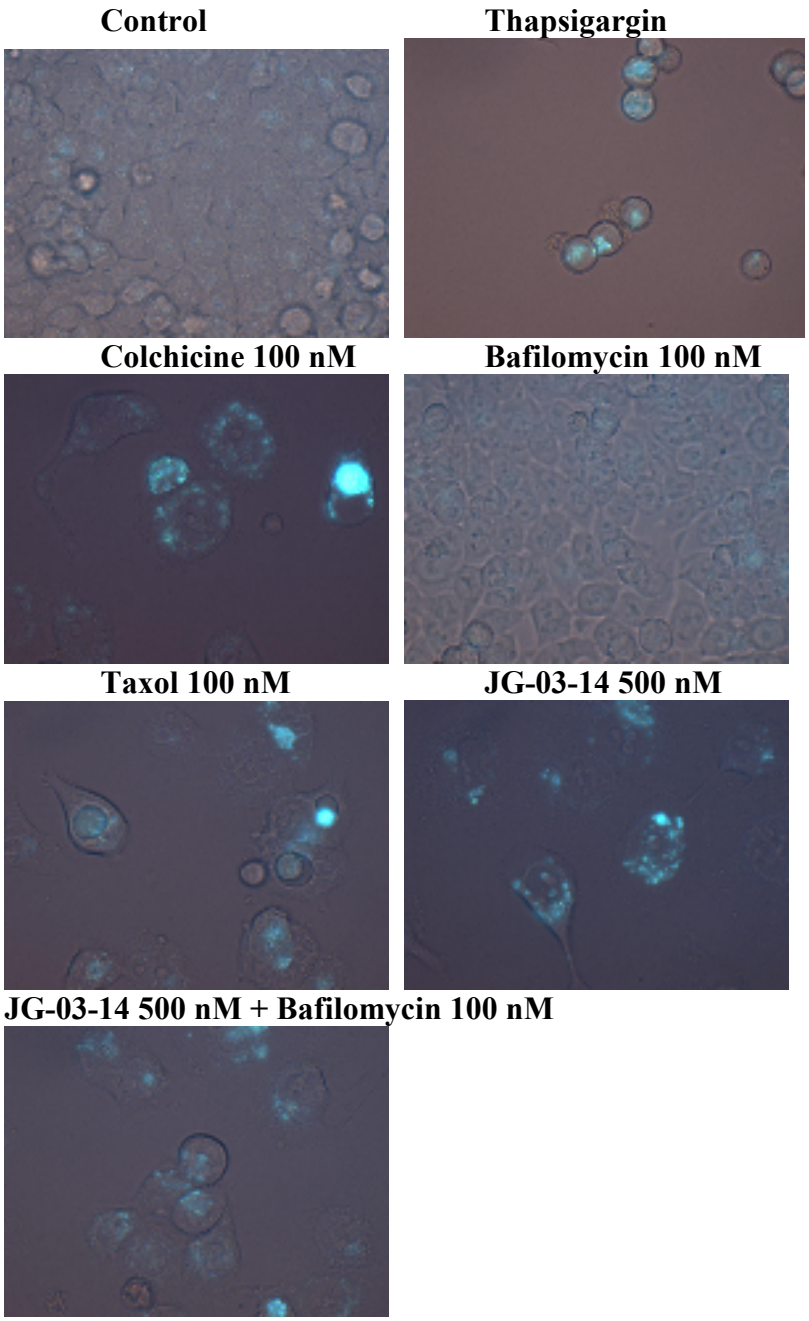


Figure 8– Determination of autophagic vacuole formation using monodansylcadaverine (MDC) staining. HCT-116 cells were incubated with the different microtubule poisons for 3 days, stained, washed, and observed by flurescence microscopy. Bright blue staining occurs in the autophagic membrane Thapsigargin 0.5 uM was used as our positive control. All photos were taken at 20X.

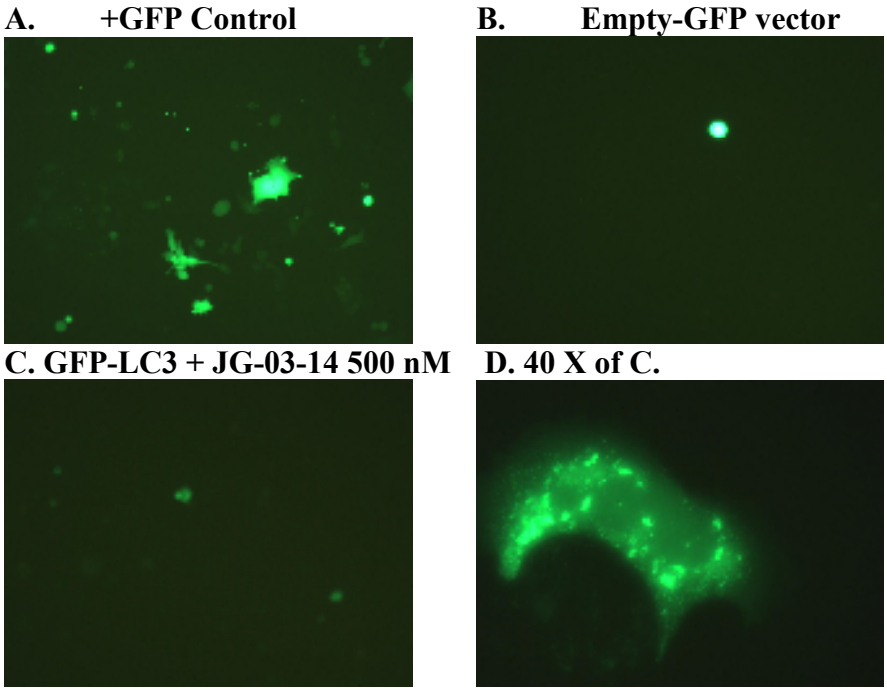
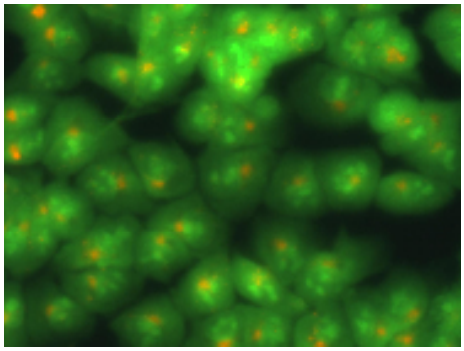
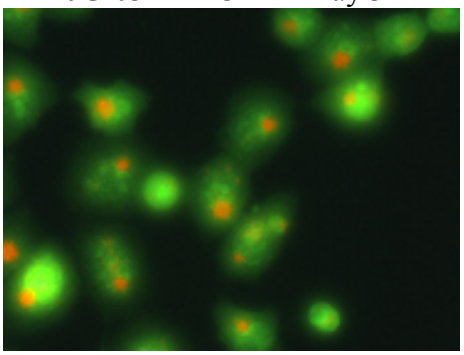


Figure 9– Detection of the LC3-II protein in cells treated with JG-03-14. HCT-116 cells were transfected with (A) GFP (B) empty-GFP vector (C) GFP-LC3-II vector and treated the following day with JG-03-14 (500 nM, continuous exposure for 72 h). Photos were taken at 10 X for (A)-(C) and 40X for (D).

Control



JG-03-14 + 3-MA Day 3



JG-03-14 + 3-MA Day 5

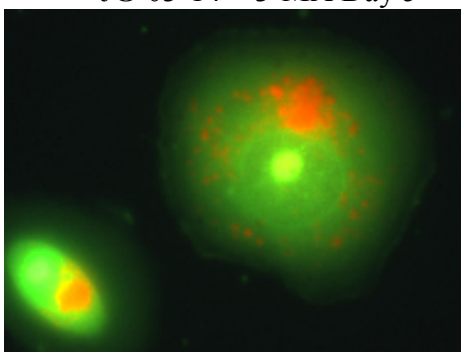
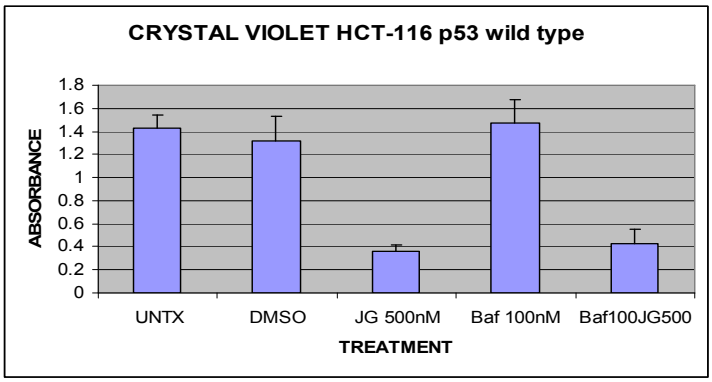


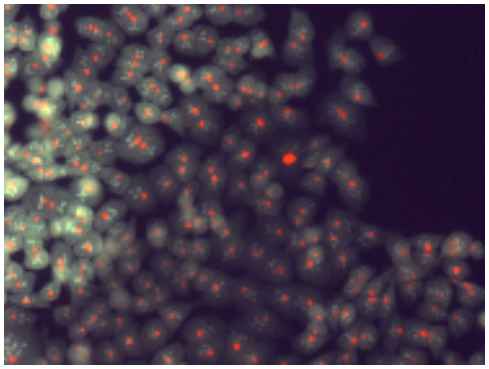
Figure 10–Effects of the autophagy inhibitor, 3-methyladenine (3-MA) on the response of HCT-116 cells to JG-03-14. HCT-116 cells were co-incubated with JG-03-14 (500 nM) and 3-MA (25 mM) by continuous exposure for 3 and 5 days, stained with acridine orange, washed, and observed by fluorescence microscopy. Acidic stained vacuoles were still present even after day 5. All photos were taken at 20X

A.

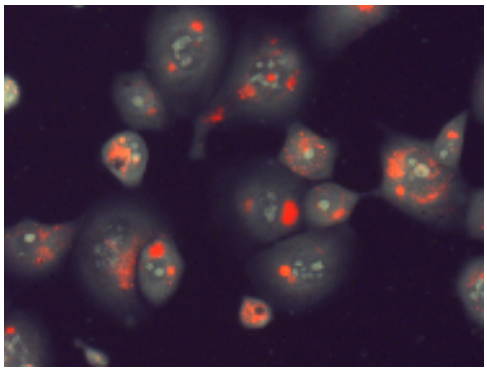


B.

Control



JG-03-14 500 nM



JG-03-14 500 nM + Bafilomycin-A1 100 nM

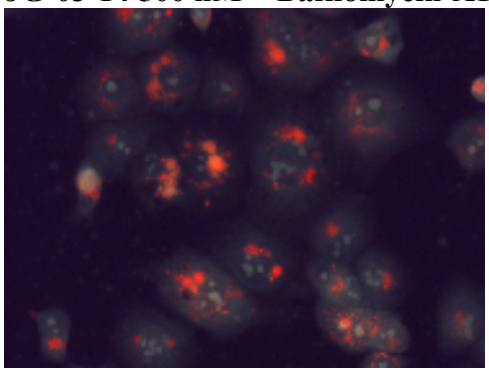


Figure 11- (A) Growth inhibition with the combination treatment was assessed using the crystal violet assay. Cells were seeded in a 48-well plates at 20,000 cells per well and incubated with the indicated compounds for 72h. The combination of JG-03-14 500 nM and Bafilomycin-A1 100 nM was added for continuous exposure. DMSO was our negative control. Values shown represent means ± standard errors for three replicate experiments. (B) Treatment of HCT-116 cells using the autophagy inhibitor, Bafilomycin-A1. HCT-116 cells were plated in a 6-well plate and co-incubated with JG-03-14 (500 nM) and Bafilomycin-A1 (100 nM) continuous exposure for 3 days, stained with acridine orange, washed, and observed by fluorescence microscopy. Acidic stained vacuoles were still detected after treatment with Bafilomycin. All photos were taken at 20X.

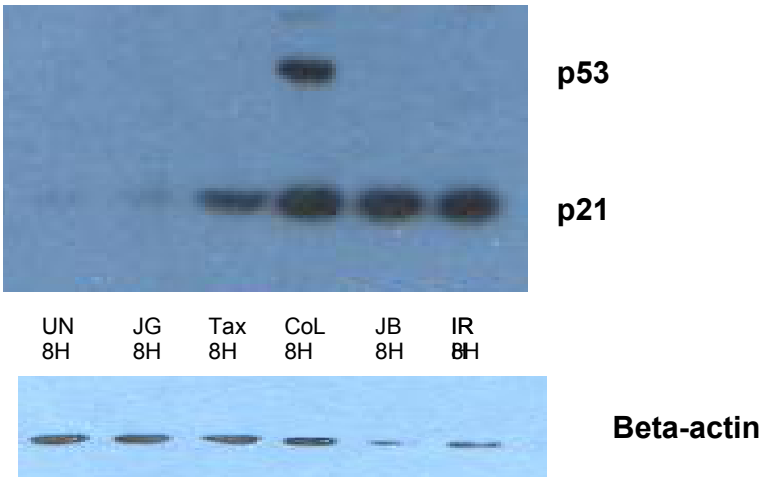


Figure 12– Assessment of DNA damage response protein expression in HCT-116 cells. HCT-116 cells were treated with either irradiation or the indicated drugs and the expression of p53 and p21 was assessed by western blotting at the indicated time points. JB=JG-03-14 (500 nM) + Bafilomycin-A1 (100) nM; Tax=taxol (100nM); Col=colchicines (100 nM); Un= untreated control; JG=JG-03-14 (500 nM); IR=irradiation (10 Gy).

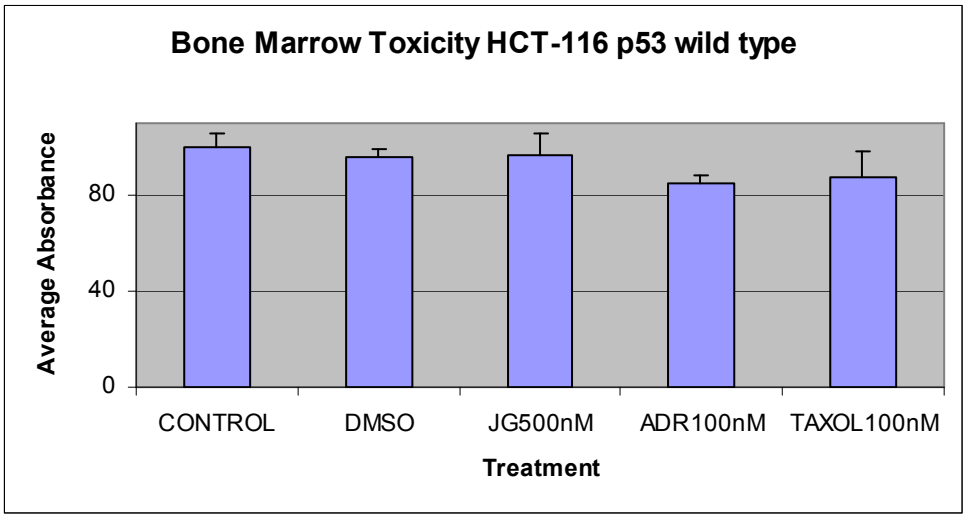


Figure 13– Assessment of bone marrow suppression by JG-03-14 in HCT-116 cells. Bone marrow cells were obtained from C57BL6 mice and seeded in a 24-well plates at 2 x 10^5 cells per well. Cells were incubated for 72 h with the drug treatments. Viable cell numbers were assessed using the Alamar Blue assay. DMSO solvent was used as a control.

CHAPTER 4 Discussion

Colorectal cancer remains the second leading cause of cancer morbidity in the US. There were approximately 98,200 new cases diagnosed with about 48,100 deaths in 2001 (Greenlee et al, 2001). Colon cancer is a heterogeneous disease which evolves through two pathways: the major genetic pathway is a consequence of chromosomal instability and accounts for 70-85% of colon cancer cases while the non-genetic pathway, which accounts for 15% of cases, is characterized by a loss of DNA mismatch repair system (MMR) and MSI(microsatellite instability) (Popat et al, 2005; Jass et al, 2002; Zlobec et al, 2008). Surgery is the primary treatment, with radiotherapy given before or after surgery to prevent local recurrence(Mainprize et al, 1998). Although colon cancer can be successfully treated with surgery when detected before it has metastasized out of the colon, it has been shown that about half of the resected patients develop recurrent disease(Sorensen et al, 2008). Colon cancer is also notoriously unresponsive to chemotherapy. Current chemotherapeutic treatments for colorectal cancer involve 5-fluorouracil, monoclonal antibody therapy, and inhibitors of EGFR and VEGF (Shia, 2008;Gallegos-Arreola et al, 2008; Zrieki et al, 2008; Schwartz RN 2008; Igbal and Lenz, 2004) . However, patient survival is generally limited due to the development of drug resistance and metastatic disease.

The primary reasons that colon cancer is thought to be resistant to most conventional chemotherapies includes mutation of p53, constitutive receptor activation, Kras overexpression, multidrug resistance pumps, overexpression of antiapoptotic molecules in the BcL family, silencing of DNA methylation proteins, and constitutive activity of NFkB (Zlobec et al, 2008; Sumitomo et al, 1999; Szakacs et al, 2006).

We have previously found that the substituted pyrrole, JG-03-14, is able to promote cell death in both p53 wild type and p53 mutant breast tumor cells through both autophagy and apoptosis (Arthur et a, 2007). Autophagy is the primary mode of cell death in the MCF-7 cells while both autophagy and apoptosis were observed in the MDA-MB-231 cells. We reasoned that if one of the reasons for colon cancer resistance to therapy is its capacity to evade apoptosis, then a drug that promotes autophagy might be effective.

We performed studies using the HCT-116 colon cancer cell line. One reason for choosing this line is that isogenic lines with disruption of p53 and p21 are available for comparative studies (although these studies will be performed in the future). HCT-116 cells were sensitive to JG-03-14 at 250 nM and 500 nM concentrations; at the 500 nM concentration, JG-03-14 demonstrated growth inhibition of 75% compared to the untreated control. This was also the concentration that was effective against MCF-7, MCF-7/caspase-3, and MDA-MB-231 cells in previous studies from this laboratory (Arthur et al, 2007). The

microtubule stabilizer, taxol, and the microtubule destabilizer, colchicine, similarly inhibited growth of HCT-116 cells, although at concentrations of 100nM.

The majority of the population of cells treated with 500 nM JG-03-14 were arrested in G2/M, a finding which correlates with the effects of other microtubule destabilizers (Azeddine et al., 1998). HCT-116 cells treated with colchicine also arrested in the G2/M phase after day 3 of treatment, a finding that was different than the response to taxol in which the population was arrested in the G1 phase. This finding suggest that there may be a difference in the way microtubule destabilizers such as colchicine and JG-03-14 act on the cell cycle compared to the microtubule stabilizer, taxol.

It is likely that the difference between the microtubule stabilizer and destabilizer is in the way these compounds interact with the spindle assembly checkpoint. The spindle assembly checkpoint is activated in the presence of unattached kinetochores or the absence of tension between paired kinetochores (Xia et al, 2004). Since centrioles organize the spindle apparatus that is required for chromosome segregation, any defect in it or its kinetochores would activate the spindle assembly checkpoint (Rudner et al, 1996). It has been observed by Sudo et al, (2004) that taxol, which binds on beta tubulin, stabilizes the microtubules and interfere with the dynamics of the spindle apparatus formation and therefore activate the spindle assembly checkpoint to arrest the cells at mitosis. This may explain why we observe G1 cell cycle arrest in the HCT-116 cells treated with taxol. On the other hand, the microtubule destabilizers, colchicines and JG-03-14,

interfere with the formation of the spindle apparatus and perhaps may abolish the formation of the spindle kinetochores completely. If it is in fact the case, then the replicating cells lacking centrioles thus will also lack kinetochores or spindle apparatus; without the spindle apparatus the spindle assembly checkpoint will not be activated. Thus, the cells treated with JG-03-14 and colchicine which presumably lack a spindle assembly checkpoint to promote arrest in G1 instead undergo mitotic slippage and continue to re-replicate without division. However, it is more likely that the formation of the spindle apparatus may occur before the microtubule depolymerizers JG-03-14 and colchicine take a complete toll on breaking down all microtubule structures (centrioles, spindles, kinetochores); consequently, in the case of colchicines we observe a population of cell arrested in G1 (24.79 %) and in G2 (44.28%). Since the mitotic block triggered by the spindle assembly checkpoint is not permanent, cells can undergo mitotic slippage and re-replicate, a reason why we see the population of cells treated with colchicine and JG-03-14 in the G2 cell cycle arrest after the first 24 hours (Weaver et al, 2005). Once the depolymerizing agents continue to destroy the integrity of the microtubules, the spindle assembly becomes dysfunctional; hence the cell will lack a spindle assembly checkpoint to halt the cell cycle progression and will continue to re-replicate and become hyperdiploid, as evident after 72 hours with JG-03-14 or colchicines treatment.

Normally, activation of p53 is triggered by DNA damage, spindle disruption, hypoxia, radiation, or chemotherapeutics(Das et al, 2001;Amundson et al, 1998;

Lowe 1999) Although JG-03-14 treated cells failed to demonstrate induction of either p53 or p21, this finding is different from other studies using microtubule poisons showing activation of p53 and p21. When p53 is induced, this generally arrests the cells in the G1 phase of the cell cycle by activating the cdk2-cyclin-B inhibitor p21/WAF1/Cip1; p53 can also activate the DNA damage repair response if there is any DNA damage that requires repair before the cell can resume the normal cell cycle. In cases where DNA damage is beyond repair, p53 may activate the apoptotic pathway(Barboule et al, 1997; De Leonardo et al, 1997; Khan et al, 1998). This checkpoint pathway was found to function in colchicine and taxol studies showing the induction of p53 and p21 that eventually lead to apoptosis, which is similar to our finding(Tao et al, 2005; Taylor et al, 1997;Masuda et al, 2003). Concurrent with the checkpoint activation, taxol at 100 nM induced a G1/S arrest with a population of cells evident in sub G1 even after three days of treatment, indicating that taxol kill cells in part through the apoptotic pathway (Wagenknecht et al., 1998; Blajeski et al., 2001). JG-03-14 failed to arrest cells in a G1, which correlated with a lack of induction of p53 and p21 in our studies.

Although there was no indication of a DNA damage response to JG-03-14, we sought to determine if cell death might be occurring through checkpoint independent apoptosis (Shi et al, 2008). However, Dapi staining showed that JG-03-14 treatment did not cause DNA fragmentation that would be indicative of apoptosis (Notterman, et al, 1998). The difference in the induction of p53 and p21

and the large population of sub G1 cells found after colchicine treatment compared to the relatively small population and the lack of p53 and p21 induction by JG-03-14 raised the question of how microtubule depolymerizing agents that bind to the same site on tubulin may activate different signaling cascades and different cell death pathway.

With reference to the previous discussion on spindle assembly checkpoint, the study by Chan et al, (2008) demonstrated that the functional spindle assembly checkpoint and p53 are required for the postmitotic G1 checkpoint. Consequently, in order for the spindle assembly checkpoint to be activated, the cells must be actively replicating with a functional spindle assembly. In regards to the kinetics of microtubule assembly, microtubules grow toward the (+) end of the beta tubulin, in which tubulin-GTP addition is coupled directly to the hydrolysis of a longitudinally adjacent tubulin-GTP also known as the Lateral Cap Model (Vandecandelaere et al, 1999). The microtubules will grow with the addition of one tubulin-GTP and will shrink at the rate of hydrolysis of one tubulin-GTP at a steady state; the affinity of tubulin-GTP for a site at the end of the microtubule is highest when tubulin-GTP content at the site is high The polymerization of tubulin-GTP to the (+) beta end is found to stabilize the microtubule structure, where lack of such structure would make the ends unstable and rapidly depolymerize whereas the (-) alpha end tends to be stable when exposed and would resume polymerization (Desai et al, 1997). In addition, microtubule associated proteins (MAP) are found to act with tubulin on the alpha and beta interface to stabilize the microtubules and phosphorylation of

MAPs can lead to increased microtubule turnover (Rodionov et al, 1990; McNally 1996). Now, recall that colchicine binds on the beta tubulin where it prevents beta (+) tubulin-GTP from being polymerize, or added to the microtubule whereas; JG-03-14 binds on the alpha and beta interface of tubulin (Jayanarayan et al, 2002). Due to this binding, JG-03-14 may have a more profound impact on the rate at which microtubules depolymerize because: (1) by binding to the alpha and beta interface, it can block the polymerization of tubulin at the (+) beta end as well as prevent the (-) alpha end from resuming polymerization and (2) it could lead to displacement of MAPs and enhance the turnover rate of microtubules. This rapid turnover of microtubules induced by JG-03-14 may explain why the treated cells failed to exhibit any cell cycle arrest in the premitotic G1 phase and a lack of p53 activation because (1) the cell cycle arrest in premitotic G1 requires the activation of the spindle assembly checkpoint; (2) since JG-03-14 may bind on a site that would enhance rapid turnover of microtubules, there would be no spindle formation to activate the spindle assembly checkpoint; and (3) since both the functional spindle assembly checkpoint and p53 are required for the postmitotic G1 checkpoint, this may explain why we did not see induction of p53 in HCT-116 tumor cells. However, further work is required to ascertain whether these hypothesis can be validated.

We observed a hyperdiploid population after treatment with JG-03-14 and colchicine, due to the ability of cancer cells to undergo mitotic slippage out of the G2/M arrest and re-replicate their DNA (Gross et al., 1995). This process

involves DNA replication without cell division where 4N cells re-replicate to form 8N and is called endoreduplication. However, this condition, which promotes polyploidy, where cells continue to synthesize DNA but fail to undergo cytokinesis, is thought to exist primarily in cells lacking a functional p53; where p53 is functional, the DNA damage response acts to arrest cells in G1 post-mitotic slippage (Stewart et al., 1999; Elhajouji et al, 1998). Studies which indicate that p53 is required to prevent DNA reduplication are not consistent withour findings (Aylon et al., 2006). In fact, our previous studies showed that MDA-MB-231 mutant p53 breast tumor cells, but not p53 wild type MCF-7 cells, undergo endoreduplication(Arthur et al, 2007). The ability of the wild type p53 HCT-116 colon cancer cells to undergo mitotic slippage indicates that tumor cells can become polyploid independent of functional wild type p53. By inducing a rapidly activated cell death response that is independent of functional p53 (and presumably p21) JG-03-14 may therefore bypass the common classical pathway of acquired resistance to antineoplastic drugs through loss of checkpoint function. However, the role of functional p53 and p21 in JG-03-14 action remains to be fully elucidated with studies using HCT-116 cells where p53 or p21 are silenced.

Another question that arises is the basis for the difference in the mode of cell death between microtubule destabilizers JG-03-14 and colchicine in HCT-116 cancer cells. Studies have shown that although cancer cells can undergo mitotic slippage and become hyperdiploid, this tetraploidy is still regulated by p53, and that p53 can eventually arrest and abort the polyploidy cells through apoptosis.

This was confirmed from our cell cycle analysis and the induction of p53 and p21 upon colchicine treatment (Cross et al., 1995; Vogel et al, 2004). However, this was not evident in cells treated with JG-03-14, suggesting that there may be a delayed apoptosis since we did see a small population of sub G1 cells. Alternatively, the cells may die through autophagy.

Autophagy is a process that involves the formation of double membrane acidic vesicles, the autophagosomes, which fuse with lysosoms to complete protein degradation. In acridine orange-stained cells, the cytoplasm and nucleus fluoresce bright green and dim red respectively, whereas the acidic compartments fluoresces bright red during autophagy (Paglin et al., 2001). Our observations of autophagic vacuoles stained using acridine orange in JG-03-14 treated cells indicates the presence of autophagosomes. The untreated control is also stained with acridine orange; however, there is only one acidic compartment per control cell, situated near the nucleus. Similar to the treatment with JG-03-14, taxol and colchicine also caused distributed acridine orange stained autophagic vacuoles throughout the cytoplasm. The literature does indicate that in some cases autophagy is an alternative mode of cell death when apoptosis is defective due to p53 mutation or its inhibition (Kim et al., 2006; Yu et al., 2006). Since our cells are not p53 deficient, this explanation would not apply. However, other work has shown that autophagy may contribute to the tumor suppression function of p53, (Jin 2005; Zeng et al., 2007). There was also a suggestion that p53 may be involved in the induction of autophagy (Abida et al, 2008).

The positive monodansylcadaverine stain confirms the presence of autophagosomes; this is a more reliable stain for autophagic vacuole because of its interaction with the autophagic membrane and not just the acidic compartment of the autophagosome (Biederbick et al., 1995).

It appears that cell death in HCT-116 cells treated with JG-03-14 is due to induction of autophagy, as there was only a very small population of sub G1 cells that would suggest the occurrence of apoptosis. We also failed to detect evidence of mitotic catastrophe, although this mode of cell death would be consistent with cell enlargement. Although we do not know the exact mechanism of autophagic death induced by JG-03-14, the literature suggests that it could be a consequence of selective protein degradation, specifically catalase degradation causing an accumulation of ROS or degradation of other essential proteins that would eventually kill the cells (Yu et al., 2006; Onodera and Ohsumi, 2004). Future studies using reactive oxygen species (ROS) inhibitors and measurement of catalase could help to clarify this question.

Drugs that have been used in suppressing autophagy include 3-methyladenine(3-MA), bafilomycin-A1, and chloroquine (Shacka et al, 2006; Boya et al., 2005). Phosphatidylinositol 3-kinase (PI3K) is shown to be involved in the autophagic pathway in tumor cells but has only been shown to be activated through growth factor or amino acids (Blume-Jensen and Hunter, 2001; Vivanco and Sawyer, 2002). 3-MA acts through inhibition of the PI3K pathway to inhibit autophagy only during nutrient depletion but may not be a specific inhibitor for JG-

03-14 induction of autophagic death, since the autophagy promoted by JG-03-14 is not due to nutrient deprivation that would otherwise be activated by PI3K-Akt-mTor (Reggiori and Klionsky, 2002). Attempts to use 3-MA for inhibition were unsuccessful, as acidic stained vacuoles were still present when cells were co-incubated with JG-03-14 at 500 nM. The persistent cell death observed may be due to the failure of 3-MA to inhibit autophagy.

We also attempted to inhibit autophagy using Bafilomycin-A1, a vacuole ATP-ase inhibitor that interferes with the pH of lysosomes. Bafilomycin-A1 is shown to prevent the fusion of lysosomes to autophagosomes, thereby inhibiting completion of autophagy (Yamamoto et al, 1998). Cells treated with Bafilomycin-A1 at 100 nM and JG-03-14 500 nM were enlarged and showed acridine orange staining similar to the JG-03-14 treated cells, with continued cell death.
If autophagy is inhibited by Bafilomycin-A1 with JG-03-14 treatment, why do we continue to see the acidic stains and cell death? Bafilomycin-A1 has the demonstrated ability to induce accumulation of autophagic vacuoles concomitant with apoptotic death at concentrations exceeding 10 nM (Boya et al., 2005). With 100 nM Bafilomycin-A1 treatment in combination with JG-03-14, Dapi staining showed an abundance of nuclear damage that was not detected with JG-03-14 treatment alone. This could indicate that the co-incubation induced an alternate mechanism of cytotoxicity and cell death, specifically apoptosis; however we would need to assess nuclear fragmentation to clarify this question.

Since Bafilomycin-A1 acts at the level of interfering with fusion and not down regulation of the autophagosome formation, this may be a reason why autophagosomes are detected after this treatment (Shacka et al., 2006). When autophagosomes sequester protein for degradation, they may selectively sequester essential proteins. When this occurs, and autophagy is inhibited by Bafilomycin A-1, the cells can revert to another mode of death.

LC3-II is a proteolytic product of LC3-I protein that is required for the formation of autophagosomes and is a known marker of autophagy. Before the induction of autophagy, GFP-LC3 shows a diffuse localization of green within the cytoplasm; once autophagy is induced, GFP-LC3 will appear as a punctuated dot that is distributed in the cytoplasm (Zeng et al., 2007). Our GFP-LC3-II punctuate pattern within the cytoplasm is consistent with autophagic activity after incubation with JG-03-14 500 nM. Unfortunately, since we failed to include an untreated control(Niemann et al., 2000), this assay is not fully confirmatory of autophagy.

In summary, our studies lead to a number of conclusions. Although the response to JG-03-14 was found to occur at a higher concentration than colchicine and taxol, JG-03-14 did not show bone marrow toxicity and thus may have a higher therapeutic index compared to the other two agents. Colchicine is not used in the clinic because it has been shown to be a substrate for the p-glycoprotein MDR pump as well as the glutathione S-transferase found in resistant cells (Ruiz-Gomez et al, 2000). Colchicine has also been shown to suppress bone marrow function (Liu et al, 2008; Harris et al, 2000).

Different microtubule targeting agents can cause either a G1 or G2 arrest, and the arrest may be p53-dependent or p53 independent. The fact that JG-03-14 induced a G2 arrest and there was no induction of p53 indicates that JG-03-14 is acting through a p53 independent mechanism. This is significant in that cancers that acquire resistance via evasion of the p53-dependent mechanism may be sensitive to the alternative mechanism of cell cycle arrest which could ultimately lead to an alternative death signal. Since adjuvant radiotherapy is often used in preventing colon cancer recurrence, a G2/M block induced by JG-03-14 could also stage the colon cancer cells for radio sensitization (Jorgensen et al, 2007).

The results presented above have potentially important implications for JG-03-14 as a clinically effective agent due to its ability to promote autophagy in colon cancer cells. By targeting autophagic induction of tumor regression, JG-03-14 may be able to bypass colon cancer's high metastatic rate and resistance to apoptosis; furthermore, JG-03-14 is not a substrate for the multidrug resistant pump.

Drugs that target microtubules are among the most effective in treating childhood and adult tumors. Though drugs such as combretastatin A-4 and 2ME2 bind to the same site on tubulin, they have different effects on tumor vasculature. 2ME2 has anti-angiogenic actions while combretastatin A-4 has antivascular activities that lead to a rapid collapse in tumor vasculature (Mooberry et al, 2003, Griggs et al, 2001). The ability of JG to bind to tubulin and its action as a destabilizer generated interest in its potential effect on tumor vasculature.

Angiogenesis begins with the recruitment of angioblasts and other extracellular matrix proteins. Subsequently, maturation into endothelial cells is crucial for vascular tubule formation (Tozer et al, 1999). Unpublished studies by our collaborator, Dr. Edward Schwartz, showed that JG-03-14 at 100 nM was able to inhibit endothelial cell tube formation, attachment, and migration, which indicates that JG-03-14 may have potential as a chemotherapeutic drug for cancer.

Another reason why JG-03-14 has potential as a chemotherapeutic is that it may not induce bone marrow toxicity. Bone marrow toxicity has been an issue with many chemotherapeutics such as colchicine and taxol, which limits their use in cancer treatment (Liu et al, 2008; Cardinali et al, 1961).

Literature Cited

Literature Cited

Abida, W. M., & Gu, W. (2008). p53-dependent and p53-independent activation of autophagy by ARF. *Cancer Research, 68*(2), 352-357.

Amer, A. O., & Swanson, M. S. (2005). Autophagy is an immediate macrophage response to legionella pneumophila. *Cellular Microbiology, 7*(6), 765-778.

Amundson, S. A., Myers, T. G., & Fornace, A. J.,Jr. (1998). Roles for p53 in growth arrest and apoptosis: Putting on the brakes after genotoxic stress. *Oncogene, 17*(25), 3287-3299.

Andreassen, P. R., Lohez, O. D., Lacroix, F. B., & Margolis, R. L. (2001). Tetraploid state induces p53-dependent arrest of nontransformed mammalian cells in G1. *Molecular Biology of the Cell, 12*(5), 1315-1328.

Arthur, C. R., Gupton, J. T., Kellogg, G. E., Yeudall, W. A., Cabot, M. C., Newsham, I. F., et al. (2007). Autophagic cell death, polyploidy and senescence induced in breast tumor cells by the substituted pyrrole JG-03-14, a novel microtubule poison. *Biochemical Pharmacology, 74*(7), 981-991.

Asanuma, K., Tanida, I., Shirato, I., Ueno, T., Takahara, H., Nishitani, T., et al. (2003). MAP-LC3, a promising autophagosomal marker, is processed during the differentiation and recovery of podocytes from PAN nephrosis. *The FASEB Journal : Official Publication of the Federation of American Societies for Experimental Biology, 17*(9), 1165-1167.

Ashkenazi, A., & Herbst, R. S. (2008). To kill a tumor cell: The potential of proapoptotic receptor agonists. *The Journal of Clinical Investigation, 118*(6), 1979-1990.

Aylon, Y., Michael, D., Shmueli, A., Yabuta, N., Nojima, H., & Oren, M. (2006). A positive feedback loop between the p53 and Lats2 tumor suppressors prevents tetraploidization. *Genes & Development, 20*(19), 2687-2700.

Baines, P., Cumber, P., & Padua, R. A. (1992). Multidrug resistance in leukaemia. *Bailliere's Clinical Haematology, 5*(4), 943-960.

Barboule, N., Chadebech, P., Baldin, V., Vidal, S., & Valette, A. (1997). Involvement of p21 in mitotic exit after paclitaxel treatment in MCF-7 breast adenocarcinoma cell line. *Oncogene, 15*(23), 2867-2875.

Biederbick, A., Kern, H. F., & Elsasser, H. P. (1995). Monodansylcadaverine (MDC) is a specific in vivo marker for autophagic vacuoles. *European Journal of Cell Biology, 66*(1), 3-14.

Blajeski, A. L., Phan, V. A., Kottke, T. J., & Kaufmann, S. H. (2002). G(1) and G(2) cell-cycle arrest following microtubule depolymerization in human breast cancer cells. *The Journal of Clinical Investigation, 110*(1), 91-99.

Blume-Jensen, P., & Hunter, T. (2001). Oncogenic kinase signalling. *Nature, 411*(6835), 355-365.

Boger, D. L., & Johnson, D. S. (1995). CC-1065 and the duocarmycins: Unraveling the keys to a new class of naturally derived DNA alkylating agents. *Proceedings of the National Academy of Sciences of the United States of America, 92*(9), 3642-3649.

Bonfoco, E., Ceccatelli, S., Manzo, L., & Nicotera, P. (1995). Colchicine induces apoptosis in cerebellar granule cells. *Experimental Cell Research, 218*(1), 189-200.

Boya, P., Gonzalez-Polo, R. A., Casares, N., Perfettini, J. L., Dessen, P., Larochette, N., et al. (2005). Inhibition of macroautophagy triggers apoptosis. *Molecular and Cellular Biology, 25*(3), 1025-1040.

Boyer, J., McLean, E. G., Aroori, S., Wilson, P., McCulla, A., Carey, P. D., et al. (2004). Characterization of p53 wild-type and null isogenic colorectal cancer cell lines resistant to 5-fluorouracil, oxaliplatin, and irinotecan. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research, 10*(6), 2158-2167.

Cao, T. M., Durrant, D., Tripathi, A., Liu, J., Tsai, S., Kellogg, G. E., et al. (2008). Stilbene derivatives that are colchicine-site microtubule inhibitors have antileukemic activity and minimal systemic toxicity. *American Journal of Hematology, 83*(5), 390-397.

CARDINALI, G., CARDINALI, G., & AGRIFOGLIO, M. F. (1961). The colchicine method in the study of bone marrow cell proliferation. *Blood, 18*, 328-335.

Cazin, J. L., Gosselin, P., Cappelaere, P., Robert, J., & Demaille, A. (1992). Drug resistance in oncology: From concepts to applications. *Journal of Cancer Research and Clinical Oncology, 119*(2), 76-86.

Chan, Y. W., On, K. F., Chan, W. M., Wong, W., Siu, H. O., Hau, P. M., et al. (2008). The kinetics of p53 activation versus cyclin E accumulation underlies the relationship between the spindle-assembly checkpoint and the postmitotic checkpoint. *The Journal of Biological Chemistry, 283*(23), 15716-15723.

Chendil, D., Oakes, R., Alcock, R. A., Patel, N., Mayhew, C., Mohiuddin, M., et al. (2000). Low dose fractionated radiation enhances the radiosensitization effect of paclitaxel in colorectal tumor cells with mutant p53. *Cancer, 89*(9), 1893-1900.

Cross, S. M., Sanchez, C. A., Morgan, C. A., Schimke, M. K., Ramel, S., Idzerda, R. L., et al. (1995). A p53-dependent mouse spindle checkpoint. *Science (New York, N.Y.), 267*(5202), 1353-1356.

Cui, Q., Tashiro, S., Onodera, S., Minami, M., & Ikejima, T. (2007). Oridonin induced autophagy in human cervical carcinoma HeLa cells through ras, JNK, and P38 regulation. *Journal of Pharmacological Sciences, 105*(4), 317-325.

Cummings, B. S., Kinsey, G. R., Bolchoz, L. J., & Schnellmann, R. G. (2004). Identification of caspase-independent apoptosis in epithelial and cancer cells. *The Journal of Pharmacology and Experimental Therapeutics, 310*(1), 126-134.

Das, G. C., Holiday, D., Gallardo, R., & Haas, C. (2001). Taxol-induced cell cycle arrest and apoptosis: Dose-response relationship in lung cancer cells of different wild-type p53 status and under isogenic condition. *Cancer Letters, 165*(2), 147-153.

Davis, R. A., Carroll, A. R., Pierens, G. K., & Quinn, R. J. (1999). New lamellarin alkaloids from the australian ascidian, didemnum chartaceum. *Journal of Natural Products, 62*(3), 419-424.

Delaunoit, T., Alberts, S. R., Sargent, D. J., Green, E., Goldberg, R. M., Krook, J., et al. (2005). Chemotherapy permits resection of metastatic colorectal cancer: Experience from intergroup N9741. *Annals of Oncology : Official Journal of the European Society for Medical Oncology / ESMO, 16*(3), 425-429.

Demasters, G., Di, X., Newsham, I., Shiu, R., & Gewirtz, D. A. (2006). Potentiation of radiation sensitivity in breast tumor cells by the vitamin D3 analogue, EB 1089, through promotion of autophagy and interference with proliferative recovery. *Molecular Cancer Therapeutics, 5*(11), 2786-2797.

Desai, A., & Mitchison, T. J. (1997). Microtubule polymerization dynamics. *Annual Review of Cell and Developmental Biology, 13*, 83-117.

D'Hondt, V., Symann, M., & Machiels, J. P. (2001). Chemoprotection and selection by chemotherapy of multidrug resistance-associated protein-1 (MRP1) transduced cells. *Current Gene Therapy, 1*(4), 359-366.

Di Leonardo, A., Khan, S. H., Linke, S. P., Greco, V., Seidita, G., & Wahl, G. M. (1997). DNA rereplication in the presence of mitotic spindle inhibitors in human and mouse fibroblasts lacking either p53 or pRb function. *Cancer Research, 57*(6), 1013-1019.

Ding, W. X., Ni, H. M., Gao, W., Hou, Y. F., Melan, M. A., Chen, X., et al. (2007). Differential effects of endoplasmic reticulum stress-induced autophagy on cell survival. *The Journal of Biological Chemistry, 282*(7), 4702-4710.

Dylla, S. J., Beviglia, L., Park, I. K., Chartier, C., Raval, J., Ngan, L., et al. (2008). Colorectal cancer stem cells are enriched in xenogeneic tumors following chemotherapy. *PLoS ONE, 3*(6), e2428.

Eastman, A., & Schulte, N. (1988). Enhanced DNA repair as a mechanism of resistance to cis-diamminedichloroplatinum(II). *Biochemistry, 27*(13), 4730-4734.

Elhajouji, A., Cunha, M., & Kirsch-Volders, M. (1998). Spindle poisons can induce polyploidy by mitotic slippage and micronucleate mononucleates in the cytokinesis-block assay. *Mutagenesis, 13*(2), 193-198.

Fan, H., Peng, J., Hamann, M. T., & Hu, J. F. (2008). Lamellarins and related pyrrole-derived alkaloids from marine organisms. *Chemical Reviews, 108*(1), 264-287.

Fojo, A. T., Ueda, K., Slamon, D. J., Poplack, D. G., Gottesman, M. M., & Pastan, I. (1987). Expression of a multidrug-resistance gene in human tumors and tissues. *Proceedings of the National Academy of Sciences of the United States of America, 84*(1), 265-269.

Fojo, T., & Menefee, M. (2007). Mechanisms of multidrug resistance: The potential role of microtubule-stabilizing agents. *Annals of Oncology : Official Journal of the European Society for Medical Oncology / ESMO, 18 Suppl 5*, v3-8.

Folkman, J. (1997). Angiogenesis and angiogenesis inhibition: An overview. *EXS, 79*, 1-8.

Fruehauf, S., Wermann, K., Buss, E. C., Hundsdoerfer, P., Veldwijk, M. R., Haas, R., et al. (1998). Protection of hematopoietic stem cells from chemotherapy-induced toxicity by multidrug-resistance 1 gene transfer. *Recent Results in Cancer Research.Fortschritte Der Krebsforschung.Progres Dans Les Recherches Sur Le Cancer, 144*, 93-115.

Fujiwara, K., Daido, S., Yamamoto, A., Kobayashi, R., Yokoyama, T., Aoki, H., et al. (2008). Pivotal role of the cyclin-dependent kinase inhibitor p21WAF1/CIP1 in apoptosis and autophagy. *The Journal of Biological Chemistry, 283*(1), 388-397.

Gallegos-Arreola, M. P., Peralta-Leal, V., Morgan-Villela, G., & Puebla-Perez, A. M. (2008). Frequency of TS1494del6 polymorphism in colorectal patients from west of mexico. [Frecuencia del polimorfismo TS 1494del6 en pacientes con cancer colorrectal del occidente de Mexico] *Revista De Investigacion Clinica; Organo Del Hospital De Enfermedades De La Nutricion, 60*(1), 21-30.

Gavrieli, Y., Sherman, Y., & Ben-Sasson, S. A. (1992). Identification of programmed cell death in situ via specific labeling of nuclear DNA fragmentation. *The Journal of Cell Biology, 119*(3), 493-501.

Gozuacik, D., & Kimchi, A. (2004). Autophagy as a cell death and tumor suppressor mechanism. *Oncogene, 23*(16), 2891-2906.

Greenlee, R. T., Hill-Harmon, M. B., Murray, T., & Thun, M. (2001). Cancer statistics, 2001. *CA: A Cancer Journal for Clinicians, 51*(1), 15-36.

Gulbins, E., Jekle, A., Ferlinz, K., Grassme, H., & Lang, F. (2000). Physiology of apoptosis. *American Journal of Physiology.Renal Physiology, 279*(4), F605-15.

Gupton, J. T., Burham, B. S., Byrd, B. D., Krumpe, K. E., Stokes, C., Shuford, J., et al. (1999). The cytotoxicity and mode of action of 2,3,4-trisubstituted pyrroles and related derivatives in human Tmolt4 leukemia cells. *Die Pharmazie, 54*(9), 691-697.

Harris, A. L., & Hochhauser, D. (1992). Mechanisms of multidrug resistance in cancer treatment. *Acta Oncologica (Stockholm, Sweden), 31*(2), 205-213.

Harris, R., Marx, G., Gillett, M., Kark, A., & Arunanthy, S. (2000). Colchicine-induced bone marrow suppression: Treatment with granulocyte colony-stimulating factor. *The Journal of Emergency Medicine, 18*(4), 435-440.

Hengstler, J. G., Bottger, T., Tanner, B., Dietrich, B., Henrich, M., Knapstein, P. G., et al. (1998). Resistance factors in colon cancer tissue and the adjacent normal colon tissue: Glutathione S-transferases alpha and pi, glutathione and aldehyde dehydrogenase. *Cancer Letters, 128*(1), 105-112.

Hilton, J. (1984). Role of aldehyde dehydrogenase in cyclophosphamide-resistant L1210 leukemia. *Cancer Research, 44*(11), 5156-5160.

Hiraku, Y., Oikawa, S., & Kawanishi, S. (2002). Distamycin A, a minor groove binder, changes enediyne-induced DNA cleavage sites and enhances apoptosis. *Nucleic Acids Research.Supplement (2001), (2)*(2), 95-96.

Hiro, J., Inoue, Y., Toiyama, Y., Miki, C., & Kusunoki, M. (2008). Mechanism of resistance to chemoradiation in p53 mutant human colon cancer. *International Journal of Oncology, 32*(6), 1305-1310.

Hoban, P. R., Robson, C. N., Davies, S. M., Hall, A. G., Cattan, A. R., Hickson, I. D., et al. (1992). Reduced topoisomerase II and elevated alpha class glutathione S-transferase expression in a multidrug resistant CHO cell line highly cross-resistant to mitomycin C. *Biochemical Pharmacology, 43*(4), 685-693.

Impens, F., Van Damme, P., Demol, H., Van Damme, J., Vandekerckhove, J., & Gevaert, K. (2008). Mechanistic insight into taxol-induced cell death. *Oncogene,*

Incles, C. M., Schultes, C. M., Kelland, L. R., & Neidle, S. (2003). Acquired cellular resistance to flavopiridol in a human colon carcinoma cell line involves up-regulation of the telomerase catalytic subunit and telomere elongation. sensitivity of resistant cells to combination treatment with a telomerase inhibitor. *Molecular Pharmacology, 64*(5), 1101-1108.

Iqbal, S., & Lenz, H. J. (2004). Angiogenesis inhibitors in the treatment of colorectal cancer. *Seminars in Oncology, 31*(6 Suppl 17), 10-16.

Jahreiss, L., Menzies, F. M., & Rubinsztein, D. C. (2008). The itinerary of autophagosomes: From peripheral formation to kiss-and-run fusion with lysosomes. *Traffic (Copenhagen, Denmark), 9*(4), 574-587.

Jass, J. R., Whitehall, V. L., Young, J., & Leggett, B. A. (2002). Emerging concepts in colorectal neoplasia. *Gastroenterology, 123*(3), 862-876.

Jayanarayan, K. G., & Dey, C. S. (2002). Microtubules: Dynamics, drug interaction and drug resistance in leishmania. *Journal of Clinical Pharmacy and Therapeutics, 27*(5), 313-320.

Jin, S. (2005). p53, autophagy and tumor suppression. *Autophagy, 1*(3), 171-173.

Jorgensen, T. J., Tian, H., Joseph, I. B., Menon, K., & Frost, D. (2007). Chemosensitization and radiosensitization of human lung and colon cancers by antimitotic agent, ABT-751, in athymic murine xenograft models of subcutaneous tumor growth. *Cancer Chemotherapy and Pharmacology, 59*(6), 725-732.

Kabeya, Y., Mizushima, N., Ueno, T., Yamamoto, A., Kirisako, T., Noda, T., et al. (2000). LC3, a mammalian homologue of yeast Apg8p, is localized in autophagosome membranes after processing. *The EMBO Journal, 19*(21), 5720-5728.

Katsiaouni, S., Dechert, S., Brinas, R. P., Bruckner, C., & Meyer, F. (2008). Schiff base macrocycles containing pyrroles and pyrazoles. *Chemistry (Weinheim an Der Bergstrasse, Germany), 14*(16), 4823-4835.

Kaur, G., Hollingshead, M., Holbeck, S., Schauer-Vukasinovic, V., Camalier, R. F., Domling, A., et al. (2006). Biological evaluation of tubulysin A: A potential anticancer and antiangiogenic natural product. *The Biochemical Journal, 396*(2), 235-242.

Kaushik, S., Massey, A. C., Mizushima, N., & Cuervo, A. M. (2008). Constitutive activation of chaperone-mediated autophagy in cells with impaired macroautophagy. *Molecular Biology of the Cell, 19*(5), 2179-2192.

Keiko Yagi1 , Kazufumi Kunitomo1, Kunio Ii and Seiki Tashiro. (1997). Relationship of P-glycoprotein and p53 protein to chemosensitivity in colorectal cancer. *International Journal of Clinical Oncology, 2*(2), 81.

Kerr, J. F., Wyllie, A. H., & Currie, A. R. (1972). Apoptosis: A basic biological phenomenon with wide-ranging implications in tissue kinetics. *British Journal of Cancer, 26*(4), 239-257.

Kerssemakers, J. W., Munteanu, E. L., Laan, L., Noetzel, T. L., Janson, M. E., & Dogterom, M. (2006). Assembly dynamics of microtubules at molecular resolution. *Nature, 442*(7103), 709-712.

Khan, S. H., & Wahl, G. M. (1998). p53 and pRb prevent rereplication in response to microtubule inhibitors by mediating a reversible G1 arrest. *Cancer Research, 58*(3), 396-401.

Khawli, L. A., Hu, P., & Epstein, A. L. (2008). Cytokine, chemokine, and co-stimulatory fusion proteins for the immunotherapy of solid tumors. *Handbook of Experimental Pharmacology, (181)*(181), 291-328.

Kim, K. W., Mutter, R. W., Cao, C., Albert, J. M., Freeman, M., Hallahan, D. E., et al. (2006). Autophagy for cancer therapy through inhibition of pro-apoptotic proteins and mammalian target of rapamycin signaling. *The Journal of Biological Chemistry, 281*(48), 36883-36890.

Klionsky, D. J., & Emr, S. D. (2000). Autophagy as a regulated pathway of cellular degradation. *Science (New York, N.Y.), 290*(5497), 1717-1721.

Kondo, Y., Kanzawa, T., Sawaya, R., & Kondo, S. (2005). The role of autophagy in cancer development and response to therapy. *Nature Reviews.Cancer, 5*(9), 726-734.

Kouno, T., Mizuguchi, M., Tanida, I., Ueno, T., Kominami, E., & Kawano, K. (2004). 1H, 13C, and 15N resonance assignments of human microtubule-associated protein light chain-3. *Journal of Biomolecular NMR, 29*(3), 415-416.

Kuo, C. C., Hsieh, H. P., Pan, W. Y., Chen, C. P., Liou, J. P., Lee, S. J., et al. (2004). BPR0L075, a novel synthetic indole compound with antimitotic activity in human cancer cells, exerts effective antitumoral activity in vivo. *Cancer Research, 64*(13), 4621-4628.

Lai, G. M., Ozols, R. F., Smyth, J. F., Young, R. C., & Hamilton, T. C. (1988). Enhanced DNA repair and resistance to cisplatin in human ovarian cancer. *Biochemical Pharmacology, 37*(24), 4597-4600.

Lanni, J. S., Lowe, S. W., Licitra, E. J., Liu, J. O., & Jacks, T. (1997). P53-independent apoptosis induced by paclitaxel through an indirect mechanism. *Proceedings of the National Academy of Sciences of the United States of America, 94*(18), 9679-9683.

Lavieu, G., Scarlatti, F., Sala, G., Levade, T., Ghidoni, R., Botti, J., et al. (2007). Is autophagy the key mechanism by which the sphingolipid rheostat controls the cell fate decision? *Autophagy, 3*(1), 45-47.

Lefevre, J. H., Rondelli, F., Mourra, N., Bennis, M., Tiret, E., Parc, R., et al. (2008). Lumboaortic and iliac lymphadenectomy for lymph node recurrence of colorectal cancer: Prognostic value of the MSI phenotype. *Annals of Surgical Oncology,*

Lefranc, F., Facchini, V., & Kiss, R. (2007). Proautophagic drugs: A novel means to combat apoptosis-resistant cancers, with a special emphasis on glioblastomas. *The Oncologist, 12*(12), 1395-1403.

Li, W., Lam, M. S., Birkeland, A., Riffel, A., Montana, L., Sullivan, M. E., et al. (2006). Cell-based assays for profiling activity and safety properties of cancer drugs. *Journal of Pharmacological and Toxicological Methods, 54*(3), 313-319.

Liu, W. C., Chuang, W. L., Tsai, M. L., Hong, J. H., McBride, W. H., & Chiang, C. S. (2008). Cordyceps sinensis health supplement enhances recovery from taxol-induced leukopenia. *Experimental Biology and Medicine (Maywood, N.J.), 233*(4), 447-455.

Lizano Soberon, M., & Barrera Rodriquez, R. (1993). Multiple drug resistance: A problem in cancer chemotherapy. [Resistencia multiple a drogas: un problema en la quimioterapia de cancer] *Revista De Investigacion Clinica; Organo Del Hospital De Enfermedades De La Nutricion, 45*(5), 481-492.

Logarinho, E., Bousbaa, H., Dias, J. M., Lopes, C., Amorim, I., Antunes-Martins, A., et al. (2004). Different spindle checkpoint proteins monitor microtubule attachment and tension at kinetochores in drosophila cells. *Journal of Cell Science, 117*(Pt 9), 1757-1771.

Lowe, S. W. (1999). Activation of p53 by oncogenes. *Endocrine-Related Cancer, 6*(1), 45-48.

Lowe, S. W., Bodis, S., McClatchey, A., Remington, L., Ruley, H. E., Fisher, D. E., et al. (1994). P53 status and the efficacy of cancer therapy in vivo. *Science (New York, N.Y.), 266*(5186), 807-810.

Lowe, S. W., Ruley, H. E., Jacks, T., & Housman, D. E. (1993). P53-dependent apoptosis modulates the cytotoxicity of anticancer agents. *Cell, 74*(6), 957-967.

Luna, E. J., & Hitt, A. L. (1992). Cytoskeleton--plasma membrane interactions. *Science (New York, N.Y.), 258*(5084), 955-964.

Mainprize, K. S., Mortensen, N. J., & Warren, B. F. (1998). Early colorectal cancer: Recognition, classification and treatment. *The British Journal of Surgery, 85*(4), 469-476.

Martin, A. P., Miller, A., Emdad, L., Rahmani, M., Walker, T., Mitchell, C., et al. (2008). Lapatinib resistance in HCT116 cells is mediated by elevated MCL-1 expression, decreased BAK activation, and not by ERBB receptor mutation. *Molecular Pharmacology,*

Masuda, A., Maeno, K., Nakagawa, T., Saito, H., & Takahashi, T. (2003). Association between mitotic spindle checkpoint impairment and susceptibility to the induction of apoptosis by anti-microtubule agents in human lung cancers. *The American Journal of Pathology, 163*(3), 1109-1116.

Mizushima, N., Levine, B., Cuervo, A. M., & Klionsky, D. J. (2008). Autophagy fights disease through cellular self-digestion. *Nature, 451*(7182), 1069-1075.

Mizushima, N., Yamamoto, A., Hatano, M., Kobayashi, Y., Kabeya, Y., Suzuki, K., et al. (2001). Dissection of autophagosome formation using Apg5-deficient mouse embryonic stem cells. *The Journal of Cell Biology, 152*(4), 657-668.

Mooberry, S. L. (2003). Mechanism of action of 2-methoxyestradiol: New developments. *Drug Resistance Updates : Reviews and Commentaries in Antimicrobial and Anticancer Chemotherapy, 6*(6), 355-361.

Mooberry, S. L., Weiderhold, K. N., Dakshanamurthy, S., Hamel, E., Banner, E. J., Kharlamova, A., et al. (2007). Identification and characterization of a new tubulin-binding tetrasubstituted brominated pyrrole. *Molecular Pharmacology, 72*(1), 132-140.

Moretti, L., Yang, E. S., Kim, K. W., & Lu, B. (2007). Autophagy signaling in cancer and its potential as novel target to improve anticancer therapy. *Drug Resistance Updates : Reviews and Commentaries in Antimicrobial and Anticancer Chemotherapy, 10*(4-5), 135-143.

Mori, S., Sawada, T., Okada, T., Ohsawa, T., Adachi, M., & Keiichi, K. (2007). New anti-proliferative agent, MK615, from japanese apricot "prunus mume" induces striking autophagy in colon cancer cells in vitro. *World Journal of Gastroenterology : WJG, 13*(48), 6512-6517.

Morrison, C., & Rieder, C. L. (2004). Chromosome damage and progression into and through mitosis in vertebrates. *DNA Repair, 3*(8-9), 1133-1139.

Nagamura, S., Kobayashi, E., Gomi, K., & Saito, H. (1996). Synthesis and antitumor activity of duocarmycin derivatives: A-ring pyrrole analogues of duocarmycin B2. *Bioorganic & Medicinal Chemistry, 4*(8), 1379-1391.

Nakamura, N., Matsuura, A., Wada, Y., & Ohsumi, Y. (1997). Acidification of vacuoles is required for autophagic degradation in the yeast, saccharomyces cerevisiae. *Journal of Biochemistry, 121*(2), 338-344.

Niemann, A., Baltes, J., & Elsasser, H. P. (2001). Fluorescence properties and staining behavior of monodansylpentane, a structural homologue of the lysosomotropic agent monodansylcadaverine. *The Journal of Histochemistry and Cytochemistry : Official Journal of the Histochemistry Society, 49*(2), 177-185.

Niemann, A., Takatsuki, A., & Elsasser, H. P. (2000). The lysosomotropic agent monodansylcadaverine also acts as a solvent polarity probe. *The Journal of Histochemistry and Cytochemistry : Official Journal of the Histochemistry Society, 48*(2), 251-258.

Nogales, E. (2001). Structural insight into microtubule function. *Annual Review of Biophysics and Biomolecular Structure, 30*, 397-420.

Notterman, D., Young, S., Wainger, B., & Levine, A. J. (1998). Prevention of mammalian DNA reduplication, following the release from the mitotic spindle checkpoint, requires p53 protein, but not p53-mediated transcriptional activity. *Oncogene, 17*(21), 2743-2751.

Oliver, L., Cordel, S., Barbieux, I., LeCabellec, M. T., Meflah, K., Gregoire, M., et al. (2002). Resistance to apoptosis is increased during metastatic dissemination of colon cancer. *Clinical & Experimental Metastasis, 19*(2), 175-180.

Onodera, J., & Ohsumi, Y. (2004). Ald6p is a preferred target for autophagy in yeast, saccharomyces cerevisiae. *The Journal of Biological Chemistry, 279*(16), 16071-16076.

Otrubova, K., Lushington, G., Vander Velde, D., McGuire, K. L., & McAlpine, S. R. (2008). Comprehensive study of sansalvamide A derivatives and their structure-activity relationships against drug-resistant colon cancer cell lines. *Journal of Medicinal Chemistry, 51*(3), 530-544.

Paglin, S., Hollister, T., Delohery, T., Hackett, N., McMahill, M., Sphicas, E., et al. (2001). A novel response of cancer cells to radiation involves autophagy and formation of acidic vesicles. *Cancer Research, 61*(2), 439-444.

Pasquier, E., & Kavallaris, M. (2008). Microtubules: A dynamic target in cancer therapy. *IUBMB Life, 60*(3), 165-170.

Patschan, S., Chen, J., Polotskaia, A., Mendelev, N., Cheng, J., Patschan, D., et al. (2008). Lipid mediators of autophagy in stress-induced premature senescence of endothelial cells. *American Journal of Physiology.Heart and Circulatory Physiology, 294*(3), H1119-29.

Popat, S., & Houlston, R. S. (2005). A systematic review and meta-analysis of the relationship between chromosome 18q genotype, DCC status and colorectal cancer prognosis. *European Journal of Cancer (Oxford, England : 1990), 41*(14), 2060-2070.

Qadir, M. A., Kwok, B., Dragowska, W. H., To, K. H., Le, D., Bally, M. B., et al. (2008). Macroautophagy inhibition sensitizes tamoxifen-resistant breast cancer cells and enhances mitochondrial depolarization. *Breast Cancer Research and Treatment,*

Quesada, A. R., Garcia Gravalos, M. D., & Fernandez Puentes, J. L. (1996). Polyaromatic alkaloids from marine invertebrates as cytotoxic compounds and inhibitors of multidrug resistance caused by P-glycoprotein. *British Journal of Cancer, 74*(5), 677-682.

Ravikumar, B., Vacher, C., Berger, Z., Davies, J. E., Luo, S., Oroz, L. G., et al. (2004). Inhibition of mTOR induces autophagy and reduces toxicity of polyglutamine expansions in fly and mouse models of huntington disease. *Nature Genetics, 36*(6), 585-595.

Reggiori, F., & Klionsky, D. J. (2002). Autophagy in the eukaryotic cell. *Eukaryotic Cell, 1*(1), 11-21.

Rieder, C. L., & Maiato, H. (2004). Stuck in division or passing through: What happens when cells cannot satisfy the spindle assembly checkpoint. *Developmental Cell, 7*(5), 637-651.

Romagnoli, R., Baraldi, P. G., Pavani, M. G., Tabrizi, M. A., Preti, D., Fruttarolo, F., et al. (2006). Synthesis and biological evaluation of 2-amino-3-(3',4',5'-trimethoxybenzoyl)-5-aryl thiophenes as a new class of potent antitubulin agents. *Journal of Medicinal Chemistry, 49*(13), 3906-3915.

Roth, W., Wagenknecht, B., Grimmel, C., Dichgans, J., & Weller, M. (1998). Taxol-mediated augmentation of CD95 ligand-induced apoptosis of human malignant glioma cells: Association with bcl-2 phosphorylation but neither activation of p53 nor G2/M cell cycle arrest. *British Journal of Cancer, 77*(3), 404-411.

Rudner, A. D., & Murray, A. W. (1996). The spindle assembly checkpoint. *Current Opinion in Cell Biology, 8*(6), 773-780.

Ruiz Gomez, M. J., Gil, L., Souviron, A., & Martinez Morillo, M. (2000). Multidrug resistance increment in a human colon carcinoma cell line by colchicine. *Journal of Physiology and Biochemistry, 56*(1), 33-38.

Ruiz-Gomez, M. J., Souviron, A., Martinez-Morillo, M., & Gil, L. (2000). P-glycoprotein, glutathione and glutathione S-transferase increase in a colon carcinoma cell line by colchicine. *Journal of Physiology and Biochemistry, 56*(4), 307-312.

Sampath, D., Greenberger, L. M., Beyer, C., Hari, M., Liu, H., Baxter, M., et al. (2006). Preclinical pharmacologic evaluation of MST-997, an orally active taxane with superior in vitro and in vivo efficacy in paclitaxel- and docetaxel-resistant tumor models. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research, 12*(11 Pt 1), 3459-3469.

Schmoranzer, J., & Simon, S. M. (2003). Role of microtubules in fusion of post-golgi vesicles to the plasma membrane. *Molecular Biology of the Cell, 14*(4), 1558-1569.

Schwartz, R. N. (2008). Management of early and advanced colorectal cancer: Therapeutic issues. *American Journal of Health-System Pharmacy : AJHP : Official Journal of the American Society of Health-System Pharmacists, 65*(11 Suppl 4), S8-14.

Seglen, P. O., & Gordon, P. B. (1982). 3-methyladenine: Specific inhibitor of autophagic/lysosomal protein degradation in isolated rat hepatocytes. *Proceedings of the National Academy of Sciences of the United States of America, 79*(6), 1889-1892.

Shacka, J. J., Klocke, B. J., & Roth, K. A. (2006). Autophagy, bafilomycin and cell death: The "a-B-cs" of plecomacrolide-induced neuroprotection. *Autophagy, 2*(3), 228-230.

Shacka, J. J., Klocke, B. J., Shibata, M., Uchiyama, Y., Datta, G., Schmidt, R. E., et al. (2006). Bafilomycin A1 inhibits chloroquine-induced death of cerebellar granule neurons. *Molecular Pharmacology, 69*(4), 1125-1136.

Shi, J., Orth, J. D., & Mitchison, T. (2008). Cell type variation in responses to antimitotic drugs that target microtubules and kinesin-5. *Cancer Research, 68*(9), 3269-3276.

Shia, J. (2008). Immunohistochemistry versus microsatellite instability testing for screening colorectal cancer patients at risk for hereditary nonpolyposis colorectal cancer syndrome. part I. the utility of immunohistochemistry. *The Journal of Molecular Diagnostics : JMD, 10*(4), 293-300.

Shinohara, K., Bando, T., Sasaki, S., Sakakibara, Y., Minoshima, M., & Sugiyama, H. (2006). Antitumor activity of sequence-specific alkylating agents: Pyrolle-imidazole CBI conjugates with indole linker. *Cancer Science, 97*(3), 219-225.

Shintani, T., & Klionsky, D. J. (2004). Autophagy in health and disease: A double-edged sword. *Science (New York, N.Y.), 306*(5698), 990-995.

Sissung, T. M., Mross, K., Steinberg, S. M., Behringer, D., Figg, W. D., Sparreboom, A., et al. (2006). Association of ABCB1 genotypes with paclitaxel-mediated peripheral neuropathy and neutropenia. *European Journal of Cancer (Oxford, England : 1990), 42*(17), 2893-2896.

Song, J. H., Kandasamy, K., & Kraft, A. S. (2008). ABT-737 induces expression of the death receptor 5 and sensitizes human cancer cells to TRAIL-induced apoptosis. *The Journal of Biological Chemistry,*

Sorensen, N. M., Schrohl, A. S., Jensen, V., Christensen, I. J., Nielsen, H. J., & Brunner, N. (2008). Comparative studies of tissue inhibitor of metalloproteinases-1 in plasma, serum and tumour tissue extracts from patients with primary colorectal cancer. *Scandinavian Journal of Gastroenterology, 43*(2), 186-191.

Sosin, M., & Handa, S. (2003). Low dose methotrexate and bone marrow suppression. *BMJ (Clinical Research Ed.), 326*(7383), 266-267.

Sou, Y. S., Tanida, I., Komatsu, M., Ueno, T., & Kominami, E. (2006). Phosphatidylserine in addition to phosphatidylethanolamine is an in vitro target of the mammalian Atg8 modifiers, LC3, GABARAP, and GATE-16. *The Journal of Biological Chemistry, 281*(6), 3017-3024.

Stewart, Z. A., Mays, D., & Pietenpol, J. A. (1999). Defective G1-S cell cycle checkpoint function sensitizes cells to microtubule inhibitor-induced apoptosis. *Cancer Research, 59*(15), 3831-3837.

Stewart, Z. A., & Pietenpol, J. A. (1999). Cell cycle checkpoints as therapeutic targets. *Journal of Mammary Gland Biology and Neoplasia, 4*(4), 389-400.

Sudo, T., Nitta, M., Saya, H., & Ueno, N. T. (2004). Dependence of paclitaxel sensitivity on a functional spindle assembly checkpoint. *Cancer Research, 64*(7), 2502-2508.

Sumitomo, M., Tachibana, M., Nakashima, J., Murai, M., Miyajima, A., Kimura, F., et al. (1999). An essential role for nuclear factor kappa B in preventing TNF-alpha-induced cell death in prostate cancer cells. *The Journal of Urology, 161*(2), 674-679.

Szakacs, G., Paterson, J. K., Ludwig, J. A., Booth-Genthe, C., & Gottesman, M. M. (2006). Targeting multidrug resistance in cancer. *Nature Reviews.Drug Discovery, 5*(3), 219-234.

Tanida, I., Nishitani, T., Nemoto, T., Ueno, T., & Kominami, E. (2002). Mammalian Apg12p, but not the Apg12p.Apg5p conjugate, facilitates LC3 processing. *Biochemical and Biophysical Research Communications, 296*(5), 1164-1170.

Tao, W., South, V. J., Zhang, Y., Davide, J. P., Farrell, L., Kohl, N. E., et al. (2005). Induction of apoptosis by an inhibitor of the mitotic kinesin KSP requires both activation of the spindle assembly checkpoint and mitotic slippage. *Cancer Cell, 8*(1), 49-59.

Taylor, R. C., Cullen, S. P., & Martin, S. J. (2008). Apoptosis: Controlled demolition at the cellular level. *Nature Reviews.Molecular Cell Biology, 9*(3), 231-241.

Taylor, S. S., & McKeon, F. (1997). Kinetochore localization of murine Bub1 is required for normal mitotic timing and checkpoint response to spindle damage. *Cell, 89*(5), 727-735.

Tozer, G. M., Prise, V. E., Wilson, J., Locke, R. J., Vojnovic, B., Stratford, M. R., et al. (1999). Combretastatin A-4 phosphate as a tumor vascular-targeting agent: Early effects in tumors and normal tissues. *Cancer Research, 59*(7), 1626-1634.

Traganos, F., & Darzynkiewicz, Z. (1994). Lysosomal proton pump activity: Supravital cell staining with acridine orange differentiates leukocyte subpopulations. *Methods in Cell Biology, 41*, 185-194.

Tripathi, A., Fornabaio, M., Kellogg, G. E., Gupton, J. T., Gewirtz, D. A., Yeudall, W. A., et al. (2008). Docking and hydropathic scoring of polysubstituted pyrrole compounds with antitubulin activity. *Bioorganic & Medicinal Chemistry, 16*(5), 2235-2242.

Tseng, C. J., Wang, Y. J., Liang, Y. C., Jeng, J. H., Lee, W. S., Lin, J. K., et al. (2002). Microtubule damaging agents induce apoptosis in HL 60 cells and G2/M cell cycle arrest in HT 29 cells. *Toxicology, 175*(1-3), 123-142.

Vakifahmetoglu, H., Olsson, M., & Zhivotovsky, B. (2008). Death through a tragedy: Mitotic catastrophe. *Cell Death and Differentiation, 15*(7), 1153-1162.

Vandecandelaere, A., Brune, M., Webb, M. R., Martin, S. R., & Bayley, P. M. (1999). Phosphate release during microtubule assembly: What stabilizes growing microtubules? *Biochemistry, 38*(25), 8179-8188.

Violette, S., Poulain, L., Dussaulx, E., Pepin, D., Faussat, A. M., Chambaz, J., et al. (2002). Resistance of colon cancer cells to long-term 5-fluorouracil exposure is correlated to the relative level of bcl-2 and bcl-X(L) in addition to bax and p53 status. *International Journal of Cancer.Journal International Du Cancer, 98*(4), 498-504.

Vivanco, I., & Sawyers, C. L. (2002). The phosphatidylinositol 3-kinase AKT pathway in human cancer. *Nature Reviews.Cancer, 2*(7), 489-501.

Vogel, C., Kienitz, A., Hofmann, I., Muller, R., & Bastians, H. (2004). Crosstalk of the mitotic spindle assembly checkpoint with p53 to prevent polyploidy. *Oncogene, 23*(41), 6845-6853.

Wang, H. J., Tashiro, S., Onodera, S., & Ikejima, T. (2008). Inhibition of insulin-like growth factor 1 receptor signaling enhanced silibinin-induced activation of death receptor and mitochondrial apoptotic pathways in human breast cancer MCF-7 cells. *Journal of Pharmacological Sciences, 107*(3), 260-269.

Wang, H. M., Chen, L. H., Zheng, X. K., Wu, X. Y., & Xia, Y. F. (2008). The role of cell cycle arrest in radiosensitization of nasopharyngeal carcinoma cell line CNE1 by inhibiting ATM expression. *Ai Zheng = Aizheng = Chinese Journal of Cancer, 27*(5), 466-470.

Wang, L. G., Liu, X. M., Kreis, W., & Budman, D. R. (1999). The effect of antimicrotubule agents on signal transduction pathways of apoptosis: A review. *Cancer Chemotherapy and Pharmacology, 44*(5), 355-361.

Wang, Y., Wright, S. C., & Larrick, J. W. (2003). Synthesis and preliminary cytotoxicity of nitrogen mustard derivatives of distamycin A. *Bioorganic & Medicinal Chemistry Letters, 13*(3), 459-461.

Watanabe, T., Wu, T. T., Catalano, P. J., Ueki, T., Satriano, R., Haller, D. G., et al. (2001). Molecular predictors of survival after adjuvant chemotherapy for colon cancer. *The New England Journal of Medicine, 344*(16), 1196-1206.

Weaver, B. A., & Cleveland, D. W. (2005). Decoding the links between mitosis, cancer, and chemotherapy: The mitotic checkpoint, adaptation, and cell death. *Cancer Cell, 8*(1), 7-12.

Woods, C. M., Zhu, J., McQueney, P. A., Bollag, D., & Lazarides, E. (1995). Taxol-induced mitotic block triggers rapid onset of a p53-independent apoptotic pathway. *Molecular Medicine (Cambridge, Mass.), 1*(5), 506-526.

Wosikowski, K., Kung, W., Hasmann, M., Loser, R., & Eppenberger, U. (1993). Inhibition of growth-factor-activated proliferation by anti-estrogens and effects on early gene expression of MCF-7 cells. *International Journal of Cancer.Journal International Du Cancer, 53*(2), 290-297.

Wu, S. M., & Rebeiz, C. A. (1985). Chloroplast biogenesis. molecular structure of chlorophyll b (E489 F666). *The Journal of Biological Chemistry, 260*(6), 3632-3634.

Xia, G., Luo, X., Habu, T., Rizo, J., Matsumoto, T., & Yu, H. (2004). Conformation-specific binding of p31(comet) antagonizes the function of Mad2 in the spindle checkpoint. *The EMBO Journal, 23*(15), 3133-3143.

Xiao, Z., Xue, J., Semizarov, D., Sowin, T. J., Rosenberg, S. H., & Zhang, H. (2005). Novel indication for cancer therapy: Chk1 inhibition sensitizes tumor cells to antimitotics. *International Journal of Cancer.Journal International Du Cancer, 115*(4), 528-538.

Yamamoto, A., Tagawa, Y., Yoshimori, T., Moriyama, Y., Masaki, R., & Tashiro, Y. (1998). Bafilomycin A1 prevents maturation of autophagic vacuoles by inhibiting fusion between autophagosomes and lysosomes in rat hepatoma cell line, H-4-II-E cells. *Cell Structure and Function, 23*(1), 33-42.

Yazbeck, V. Y., Buglio, D., Georgakis, G. V., Li, Y., Iwado, E., Romaguera, J. E., et al. (2008). Temsirolimus downregulates p21 without altering cyclin D1 expression and induces autophagy and synergizes with vorinostat in mantle cell lymphoma. *Experimental Hematology, 36*(4), 443-450.

Yu, L., Wan, F., Dutta, S., Welsh, S., Liu, Z., Freundt, E., et al. (2006). Autophagic programmed cell death by selective catalase degradation. *Proceedings of the National Academy of Sciences of the United States of America, 103*(13), 4952-4957.

Zaboikin, M., Srinivasakumar, N., & Schuening, F. (2006). Gene therapy with drug resistance genes. *Cancer Gene Therapy, 13*(4), 335-345.

Zeng, X., Overmeyer, J. H., & Maltese, W. A. (2006). Functional specificity of the mammalian beclin-Vps34 PI 3-kinase complex in macroautophagy versus endocytosis and lysosomal enzyme trafficking. *Journal of Cell Science, 119*(Pt 2), 259-270.

Zeng, X., Yan, T., Schupp, J. E., Seo, Y., & Kinsella, T. J. (2007). DNA mismatch repair initiates 6-thioguanine--induced autophagy through p53 activation in human tumor cells. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research, 13*(4), 1315-1321.

Zhang, C. C., Yang, J. M., White, E., Murphy, M., Levine, A., & Hait, W. N. (1998). The role of MAP4 expression in the sensitivity to paclitaxel and resistance to vinca alkaloids in p53 mutant cells. *Oncogene, 16*(12), 1617-1624.

Zlobec, I., Baker, K., Terracciano, L. M., & Lugli, A. (2008). RHAMM, p21 combined phenotype identifies microsatellite instability-high colorectal cancers with a highly adverse prognosis. *Clinical Cancer Research : An Official Journal of the American Association for Cancer Research, 14*(12), 3798-3806.

Zrieki, A., Farinotti, R., & Buyse, M. (2008). Cyclooxygenase inhibitors down regulate P-glycoprotein in human colorectal caco-2 cell line. *Pharmaceutical Research,*

VITA

Tuyen Nguyen is 27 years old and was born in Vietnam in 1981. She graduated from Meadowbrook High School in 2000 and completed her undergraduate studies in biology at Virginia Commonwealth University in June 2006.