

**Natural Product Anticancer Drug Discovery and
Mechanistic Studies of Hapalosin and Silvestrol**

BY

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THESIS

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This dissertation is dedicated to my parents, and my husband, without whom it would never have been accomplished.

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LIST OF ABBREVIATIONS

| | |
|------------------|---|
| ABC | adenosine triphosphate-binding cassette |
| ADP | adenosine diphosphate |
| ASK1 | apoptosis signal-regulating kinase 1 |
| Atg | autophagy-related gene |
| ATP | adenosine triphosphate |
| at-MDR | atypical multidrug resistance |
| BCL-2 | B-cell leukemia/lymphoma 2 |
| BSA | bovine serum albumin |
| DISC | death-inducing signaling complex |
| DMSO | dimethyl sulfoxide |
| DNA | deoxyribonucleic acid |
| EBSS | Earl's balanced salt solution |
| ECL | enhanced chemiluminescence |
| eIF | eukaryotic initiation factor |
| FADD | Fas-associated death domain |
| FITC | fluorescein isothiocyanate |
| HPLC | high-performance liquid chromatography |
| IC ₅₀ | half maximal inhibitory concentration |
| I κ B | Inhibitor of NF- κ B |
| IRGM | immunity-related gtpase M |
| LRP | lung resistance protein |
| MAP1-LC3-II | microtubule associated protein 1-light chain 3-II |

LIST OF ABBREVIATIONS (continued)

| | |
|-------------------|--|
| MDR | multidrug resistance |
| MFI | mean fluorescence intensity |
| MPT | mitochondrial permeability transition |
| MRP | multidrug resistance-associated protein |
| mTOR | mammalian target of rapamycin |
| NCI | National Cancer Institute |
| NE _x T | NCI's Experimental Therapeutics |
| NF- κ B | nuclear factor kappa-light-chain-enhancer of activated B cells |
| NMR | nuclear magnetic resonance |
| PBS | phosphate-buffered saline |
| PCD | programmed cell death |
| PDK | Phosphoinositide dependent kinase |
| P-gp | P-glycoprotein |
| P _i | phosphate ion |
| PIP ₃ | phosphatidylinositol-3,4,5-trisphosphate |
| PI3K | phosphatidylinositol-3 kinase |
| PKB | protein kinase B |
| PVDF | polyvinylidene difluoride |
| Rheb | Ras homolog enriched in brain |
| RNA | ribonucleic acid |
| SDS-PAGE | sodium dodecyl sulfate-polyacrylamide gel electrophoresis |

LIST OF ABBREVIATIONS (continued)

| | |
|---------|--|
| TBST | tris-buffered saline with 0.05 % Tween 20 |
| TNF | tumor necrosis factor |
| topo II | type II DNA topoisomerase |
| TSC | tuberous sclerosis complex |
| TUNEL | terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling |
| UBP | ubiquitin-proteasome pathway |
| Vi | vanadate ion |
| VLB | vinblastine |

SUMMARY

Cancer is one of the major health concerns for people and will be for the foreseeable future. Chemotherapy, as well as surgery and radiation, is an important clinical method to successfully treat cancer. Within the chemotherapeutic agents, natural products and chemicals derived from or related to natural products constitute an important part of the oncologist's armamentarium.

The studies in this thesis focus on screening natural products for chemotherapeutic candidates and clarifying their mechanisms. This thesis contains three parts. The first part describes screening natural products for activity against human cancer cells. The second part is focused on elucidating the mechanism of action of hapalosin and hapalosin B, chemicals capable of reversing multidrug resistance. The third section of the thesis contains a study on the mechanism of anticancer activity of silvestrol, a promising chemotherapeutic candidate.

The collected natural materials, from tropical plants, aquatic cyanobacteria, or filamentous fungi, were tested in human cancer cells to screen for inhibitors of cancer cell growth and proteasome activity. Screening began with evaluation of crude extracts. Active extracts were separated into several fractions the fractions were tested again. The fractionation and bioassay procedure were repeated until active pure compounds were obtained. This process is referred to as bioassay guided fractionation. Hapalosin B and silvestrol were the two most interesting compounds that were evaluated during this project.

Hapalosin B, as well as hapalosin, was isolated from species of the cyanobacterium *Nostoc*, using a bioactivity guided strategy. Its analogue, hapalosin, can reverse multidrug resistance in cancer cells overexpressing P-gp protein, a plasma membrane transporter that

SUMMARY (continued)

pumps a variety of substances, including anti-cancer drugs, out of the cell. Experiments presented here extend these findings by demonstrating that hapalosin B has similar activity as hapalosin: the hapalosins can reverse multidrug resistance in cells that overexpress P-gp. Furthermore, the hapalosins were found to inhibit P-gp activity in a non-competitive manner and not to affect the expression of P-gp protein production.

Silvestrol was isolated from the fruits and twigs of *Aglaia silvestris* (M. Roemer) Merrill and has shown potent (low nM) anticancer activity. It has been reported that it interferes with the assembly of the eIF4F complex, which inhibits initiation of protein synthesis. Studies were conducted to further these findings. Apoptosis is induced by silvestrol through inhibition of the PI3K/AKT pathway; which further suppresses protein synthesis by inhibiting the activity of p70S6K. Silvestrol also induces autophagy and its upstream genes expression.

Chapter 1 Discovery of hapalosin as a weak inhibitor of the proteasome

1.1 Introduction

1.1.1 Natural products as a source for anticancer drugs

Cancer is one of the major health concerns for people and will be for the foreseeable future. Other than surgery and radiation, chemotherapy is an important clinical method to successfully treat some types of solid tumors, lymphomas, and leukemias. Natural sourced compounds are currently widely used in cancer chemotherapy and have a promising future (1). Natural products and chemicals derived from or related to natural products have constituted, and should continue to constitute, a significant number of new drug candidates. Small molecule natural products accounted for about half of the new chemical entities approved from 2000 to 2006 (2). Especially in anticancer drugs, only 24% of the anticancer drugs effectively available to the West and Japan have no relation to natural products: 10% are large peptides or proteins isolated from an organism/cell line or artificially produced in a surrogate host; 14% are natural products. Fully 76% of anticancer drugs are either derived from a natural product (28%), totally synthetic natural product mimics (8%), synthesized based on a natural pharmacophore (11%), natural product mimics with natural pharmacophore made by total synthesis (4%) or vaccines (1%) (2).

1.1.2 Source of natural products used in this project

In the current study, we investigated extracts from tropical plants, aquatic cyanobacteria, and filamentous fungi to maximize the likelihood of discovering effective new cancer drugs.

In recent decades, four major groups of anticancer agents have been derived from

plants including vinca (*Catharanthus*) bisindole alkaloids, the epipodophyllotoxin analogs, the taxanes, and the camptothecins. Tropical rainforests are ideal sources for discovering natural products with medicinal properties since individual organisms must compete for survival in a highly biodiverse environment (1).

Cyanobacteria (blue-green algae) are a well-recognized source of promising and novel bioactive metabolites and two agents (dolastatins 10 and dolastins 15) have been tested in Phase II clinical trials against different types of cancers. Also, there is report that the antineoplastic compounds are widely distributed among cyanobacteria (1). Moreover, it is significantly less likely to rediscover known bioactive compounds among cyanobacteria than other microbial sources with similar hit rates. What is more, cyanobacteria are relatively unexplored as potential anticancer agents (1).

Anticancer activity has been found from a number of previously discovered fungal compounds, including brefeldin A, cytochalasin E, gliotoxin, illudin S, irofulven, the leptomycins, palmarumycin CP1, terreacyclic acid A, and wortmannin, Irofulven and lodamin, which are either derivative from a fungal product or isolated from fungi. (1).

1.1.3 Strategy for discovering anticancer drugs

After initial crude extracts prepared from tropical plants, aquatic cyanobacteria, and filamentous fungi by organic solvent, they are subjected to initial biological activity screening by cytotoxicity assay and cell-based proteasome inhibition assay. Thereafter, the extracts with biological activity will be fractionated and analyze again, until the active pure compounds are discovered.

Human melanoma cell line MDA-MB-435, human lung cancer cell line NCI-H23, and

human colon cancer cell line SW620 are used for screening bioactive natural products. They are all included in the 60 human tumor cell lines panel, which is a tool developed by the Developmental Therapeutics Program of the National Cancer Institute for early stage of drug discovery screening. Also, they have a high sensitivity against bortezomib, which is an FDA-approved anti-cancer agent targeting proteasome (3).

1.1.4 The proteasome is validated target for anticancer drug discovery

Major intracellular processes, including cell cycle, growth, apoptosis and etc., are regulated by protein synthesis, modification, and degradation. It has been reported that protein degradation is critical for cell function and the ubiquitin-proteasome pathway (UBP) is a predominant pathway for intracellular protein degradation (4). Through this pathway, the majority of damaged/misfolded, short-lived (half-lives less than three hours), or long-lived regulatory proteins in the cell get degraded. Proteasomes are key regulators of protein degradation, which regulates multiple cellular functions such as transcription, stress response, cell cycle, ribosome biogenesis, and DNA repair.

Proteasome inhibitors began to be considered as cancer drug candidates when they first were shown to induce apoptosis in leukemia cell lines (4). Furthermore, proteasome inhibitors are more cytotoxic to proliferating malignant cells than to quiescent normal cells (5). The mechanism of action attributed to proteasome inhibitors is that they inhibit the degradation of a number of proteins involved in regulating apoptosis, cell cycle progression and proliferation (4). One of the first proteins known to be affected by proteasome inhibition is I κ B, which binds NF- κ B, thus preventing it from translocating to the nucleus where it acts as a transcription factor that activates the expression of a large constituency of proliferation and anti-apoptotic

genes (6). In addition to I κ B, proteasome inhibitors also disrupt the timely degradation of cyclins and other cell cycle regulatory proteins, which results in cell cycle arrest. Proteasome inhibitors can induce a proapoptotic state in cells by stabilizing proapoptotic proteins such as p53 and Bax while reducing levels of some antiapoptotic proteins, such as Bcl-2 (4).

1.1.5 Hypothesis

Given the history of development of natural products of medicinal value from plants, marine organisms, and microbes, and the fact that many organisms from these life forms have yet to be studied, it is reasonable to propose that there may be more medicinal natural products yet to be discovered from these sources.

1.1.6 Summary

As discussed above, natural products make an excellent candidate pool for novel anticancer drugs. And, within all the natural organisms, plants (especially tropic plants), cyanobacteria, and fungi are excellent sources for novel biologically active natural products.

To screen for anticancer drug candidates, the cell proliferation inhibitors are obviously a choice. Also, inhibitors for proteasome, a validated target for anticancer drug discovery, are an alternative choice. Therefore, the experiments in this chapter were designed to test the hypothesis that isolates from plants, fungi or cyanobacteria would contain novel anticancer drug candidates that could be discovered by monitoring their cytotoxicity and proteasome inhibitory potential.

1.2 Materials and methods

1.2.1 Materials

All substances were provided by the laboratories of our collaborators who included Dr. A. Douglas Kinghorn of the Ohio State University (plant-derived materials), Dr. Jimmy Orjala of the University of Illinois at Chicago (cyanobacterial-derived materials) and Dr. Nicholas Oberlies of the University of North Carolina at Greensboro (fungal-derived materials).

1.2.2 Cells and culture conditions

The human cancer cell lines designated MDA-MB-435 (melanoma), MCF-7 (breast), NCI-H23 (lung), and SW620 (colon) were purchased from the American Type Culture Collection (ATCC; Manassas, VA). All cell lines were cultured in RPMI 1640 medium supplemented with fetal bovine serum (10%, v/v), penicillin (100 units/ml) and streptomycin (100 µg/ml; Life Technologies, Carlsbad, CA). Cell density was maintained between $10^4/\text{cm}^2$ and $10^5/\text{cm}^2$ and were subcultured every 3 to 4 days. Cell lines used in the experiments described herein were subcultured no more than 20 passages at which time frozen stocks were thawed for use. Cell cultures were maintained within the exponential phase of growth in a humidified atmosphere of 95% air and 5% CO₂ at 37 °C.

1.2.3 Cell proliferation assay

Cells in log phase growth were harvested by trypsinization followed by extensive washing to remove all traces of enzyme. A total of 5,000 cells were seeded per well of a 96-well microtiter plate and incubated overnight (37 °C in 5% CO₂). Test substance was then added at the following final concentrations: 25 µg/ml, 20 µg/ml or 2 µg/ml. After a 72-hour incubation, an MTS assay was performed using a commercially available kit according to the manufacturer's instructions (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega Corp, Madison, Wisconsin; MTS: 3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymetho-

xyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inert salt).

1.2.4 Proteasome inhibition assay

Cells in log phase growth were harvested by trypsinization followed by extensive washing to remove all traces of enzyme. A total of 5,000 cells were seeded per well of a 96-well microtiter plate and incubated overnight (37 °C in 5% CO₂). Samples dissolved in DMSO were then added to achieve a final concentration of 20 µg/ml (total volume: 100µL; DMSO: 0.2%). The cells were incubated in the presence of test substance for two hours at 37 °C and evaluated for proteasome activity using a commercial luminescent assay kit according to the manufacture's protocol (Proteasome-Glo™ Chymothypsin-Like, Trypsin-Like and Caspase-Like Cell-Based, Promega Corp, Madison, WI).

1.3 Results

1.3.1 Cell proliferation inhibition

Of 178 extracts and fractions screened in the cell proliferation assay, 44 inhibited cell proliferation by greater than 90% compared to cells exposed to the vehicle (DMSO) alone. Ten samples inhibited cell proliferation by 80 to 89%, and 23 samples caused a decrease of 50 to 79% proliferation at 20 µg/ml. Also, at a 1/10 lower concentration (2 µg/ml), 9 of them can inhibit more than 90% growth; 11 of them can inhibit 80 to 89% growth; 32 of them can inhibit 50 to 79% growth. The details of the results can be found in Appendix I. Also, some pure compounds were obtained and showed significant growth inhibition activity. Their IC₅₀ against each cell line is listed in Table I. Also, for some active pure compounds, the inhibition activity at 25 µg/ml is listed in Table II.

TABLE I CYTOTOXICITY OF PURE COMPOUNDS ISOLATED FROM CYANOBACTERIA BY THE ORJALA LABORATORY

| Sample ID | MDA-MB-435 | MCF-7 | HT-29 |
|-----------------|------------|-------|-------|
| Merocyclophan A | 7.1 | 7.6 | 7.6 |
| Merocyclophan B | 7.2 | 7.8 | 5.3 |
| Hapalosin | 35.0 | 14.5 | 14.5 |
| Hapalosin B | 20.3 | 40.4 | 39.2 |
| 10109-P4 | 6.2 | 5.1 | 3.3 |
| 10109-P5 | 3.1 | 3.0 | 2.1 |
| tolytoxin | 10.7 | 8.8 | 5.6 |

All values are expressed as IC₅₀ (μM)

TABLE II CYTOTOXIC ACTIVITY OF PURE MINUTISSAMIDE DERIVATIVES ISOLATED FROM CYANOBACTERIA BY THE ORJALA LABORATORY

| Sample | % Inhibition |
|-----------------|--------------|
| Minutissamide A | 91 |
| Minutissamide B | 86 |
| Minutissamide C | 93 |
| Minutissamide D | 51 |

All compounds were tested at 25 μg/mL.

1.3.2 Proteasome inhibition

The proteasome is a validated target for screening chemotherapeutic agents. 277 extracts and/or fractions were tested against cancer cells susceptible to bortezomib, which is a first-in-class proteasome inhibitor approved by the Food and Drug Administration for the treatment of multiple myeloma. Of the 277 samples tested at 20 μg/ml, 4 inhibited more than 90% proteasome activity compared to the control group proteasome activity. 4 samples caused 80 to 89% inhibition and 16 samples induced 40 to 79% inhibition. Detailed results are presented in Appendix II. Also, two pure compounds isolated by the Kinghorn Laboratory from plant samples were found to inhibit the proteasome completely (100% inhibition) at 20

µg/ml. Thereafter, they were also tested for cytotoxic activity in a series of cancer cell lines and the IC₅₀ values are shown in Table III.

TABLE III CYTOTOXIC IC₅₀ OF PURE COMPOUNDS PREVIOUSLY DEMONSTRATED TO POSSESS PROTEASOME INHIBITION ACTIVITY

| Cell Line | Sample | | |
|------------|-----------------|-----------------|------------|
| | A06333D3F4-R70A | A06333D3F4-R80A | Bortezomib |
| SW-620 | 2.0 | 2.3 | 6.2 |
| HT-29 | 1.6 | 1.6 | 11.0 |
| NCI-H23 | 3.2 | 1.1 | 10.1 |
| MDA-MB-435 | 2.0 | 1.3 | 5.8 |
| PC-3 | 2.8 | 1.8 | 7.3 |

Data are expressed as IC₅₀ values in µM.

1.4 Discussion:

Based on these results, we can tell that the screening rational is working well: all of the three kinds of organisms are resourceful for anticancer drugs, either for proliferation inhibitors or for proteasome inhibitors. Since, we did discover some pure compounds from the natural source, and some active fractions are promising for isolating more compounds after further fractionation. These hits will be further analyzed in both mechanistic studies and *in vivo* assays.

High-throughput screening of natural products and synthetic compounds in cell-based cytotoxicity assay, known as the conventional anticancer drug discovery, is how most of the commonly used cytotoxic anticancer drugs were obtained. However, this method has its own inherent limitation: most compounds are not very specific and have a low therapeutic index (132). Thereafter, for hits obtained from cytotoxicity assays, we need to figure out their exact mechanisms of action, and their toxicology information. For mechanism clarification, the first information to obtain is the death pathway treated cells undergoes: necrosis, apoptosis, or autophagy. Thereafter, a cell cycle analysis can tell at which cell cycle stage the compound is

working. Thus, a prediction of the action mechanism can be predicted based on this information. For toxicology study, several normal cell lines can be used, such as BALB3T3 cells and WI-38 cells. If the drug candidates have more than twice activity in cancer cells than in normal cells (drug candidates have half IC_{50} in cancer cells of their IC_{50} in normal cells), they can be used for further study.

Evolved from the conventional approach, a more effective and selective approach has been developed: targeting specific molecules thought to be responsible for the development and support of cancer (132). Proteasome is such a target, the elevated activity of which contributes to tumorigenesis (133). Even though what we are doing is a cell-based screening for target specific inhibitors, there still could be false positive hits. To distinguish these false hits, a cell-free proteasome inhibition assay should be performed to confirm the specificity of the compounds toward the target. Since elevated proteasome activity acts through antiapoptotic protection and enhanced ubiquitination of irregular proteins (133), apoptosis assay and immunoblot analysis of ubiquitin protein should follow for further confirmation.

For *in vivo* studies, the hollow fiber assay can be utilized before using the xenograft system to predict activity. This method is to grow human cancer cells in hollow fibers with pores small enough to hold the cancer cells but large enough for compounds to diffuse in. Then, the fibers are implanted into the peritoneum or under the skin of immunodeficient mice, the host can be treated with promising hits and the fibers are subsequently retrieved for analysis of viable cell mass. This can give us a relatively direct idea of their *in vivo* activity of inhibiting cancer cells growth (68).

Other than these routine ways of analyzing drug candidates, we can be flexible on some compounds with special activities, such as hapalosin and hapalosin B, which will be discussed in next chapter. Also, the mechanistic study of a previously discovered compound, silvestrol, will be discussed in the third chapter.

Chapter 2 Hapalosin B

2.1 Introduction

2.1.1 Isolation and structure elucidation

Nostoc is a genus of filamentous cyanobacteria, also known as “blue-green algae” (10), though they have no relationship to the eukaryotic algae (11). Cyanobacteria are one of the largest and most important groups of bacteria and have undergone 3.5 billion years of evolution, which gained them a rich chemical diversity (12). Therefore, cyanobacteria is a well known source for new pharmaceutical lead compounds (13). *Nostoc* consists of more than 1,000 species (10), and is widely distributed in both terrestrial and aquatic habitats, including soil, moist rocks, the bottoms of lakes and springs (both fresh and saltwater), but rarely can be found in marine habitats (14). These photosynthetic bacteria contain photosynthetic pigments in their cytoplasm (15).

Hapalosin B, as well as hapalosin, was isolated from *Nostoc* sp. 4A using a bioactivity guided strategy (16). This *Nostoc* sp. 4A strain was isolated from a rock sample located in Negev Desert, Israel (17). The crude extract of *Nostoc* sp. 4A displays cytotoxic activity against the human colon cancer cell line designated HT29. The five subfractions yielded by different further extractions using methanol and isopropyl alcohol were tested and led to the isolation of the active pure compounds, which were obtained by high-performance liquid chromatography (HPLC). Their structures were elucidated using nuclear magnetic resonance (NMR). As shown in Figure 1, hapalosin and hapalosin B share the same structures except for one group. Literature review of these structures revealed that hapalosin has been isolated and characterized previously. Other than cytotoxicity, hapalosin was known to reverse multidrug

resistance to chemotherapeutic agents such as vinblastine and doxorubicin in cells that overexpress the P-glycoprotein (P-gp) (18, 130, 131).

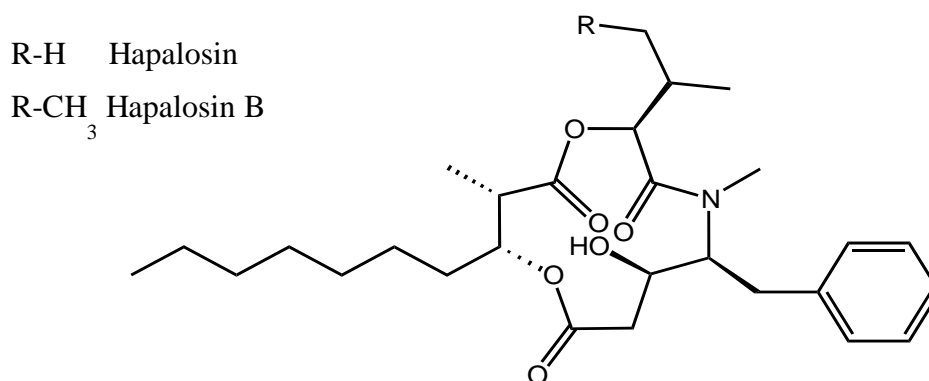


Figure 1. Structures of hapalosin and hapalosin B

2.1.2 Hypothesis

Since the structure of the novel compound hapalosin B is similar with hapalosin, we proposed that hapalosin B might possess similar multidrug resistance reversal activity. We also hypothesized that both hapalosin and hapalosin B could inhibit P-gp mediated drug efflux by direct interaction with the protein.

2.1.3 Multidrug resistance

A major obstacle to successful application of cancer chemotherapy is the occurrence of multiple drug resistance (MDR) in cancer patients (19). MDR is a phenomenon that tumor cells may become not only resistant to the drug originally administered but also to a wide variety of

structurally and mechanistically unrelated drugs (20). Based on the molecular studies on MDR, it has been revealed that cells with multidrug-resistance differ from the drug-sensitive parental cells by (1) reduced accumulation of chemotherapeutic drugs due to decreased drug influx and/or increased drug efflux; (2) altered drug response because of changed expression of certain cellular protein; and (3) physiological changes that alter the intracellular milieu (e.g. pH) (21). MDR due to a reduced accumulation of drugs presents the phenomenon by which mammalian tumor cells selected for resistance to one natural product anticancer agent display a broad cross-resistance to a variety of other natural product drugs of dissimilar structure and action. These agents include Vinca alkaloids, anthracyclines, and epipodophyllotoxins (22). Several intracellular protein overexpression are involved in the reduced accumulation of chemotherapeutic drugs / increased drug efflux, including the multidrug resistance MDR1 gene product P-gp, the multidrug resistance-associated protein (MRP), the lung resistance protein (LRP), and enzymes associated with the glutathione metabolism. Moreover, decreased expression or altered activity of topoisomerase II are claimed to contribute to atypical multidrug resistance (21).

2.1.4 P-glycoprotein

P-gp was discovered as the most prevalent MDR transporter, a correlation between which expression in patient tumor specimens and a poor chemotherapy treatment prognosis has been well established. P-gp is a high molecular weight integral plasma membrane protein, belonging to a large superfamily of adenosine triphosphate (ATP)-binding cassette (ABC) transporters, which predominantly localizes in the plasma membrane at the cell surface of multidrug-resistant cells (23).

P-gp is composed of two homologous halves, each harboring six transmembrane domains and an ATP binding/utilization domain, joined by a flexible linker polypeptide, within which four serine residues (S661, S667, S671, and S683) are phosphorylated by protein A and C kinases and this phosphorylation does not affect transport function (24, 25). P-gp is a transporter is located within the plasma membrane by the glycosylation in the first extracellular loop, which is not required for the transport function of P-gp (23, 25). The P-gp recognizes a certain group of hydrophobic molecules, which possess at least one of these chemical units: (a) Two electron donor groups with a spatial separation of $2.5 \pm 0.3 \text{ \AA}$. (b) Two electron donor groups with a spatial separation of $4.6 \pm 0.6 \text{ \AA}$. (c) Three electron donor groups with a spatial separation of the outer two groups of $4.6 \pm 0.6 \text{ \AA}$ (25). Substrates bind to P-gp drug binding domains, which reside deep within the protein near the transmembrane domains. Once bound, the conformation of P-gp changes such that the protein forms an opening towards the outside of the cell and ejecting the substrate molecule. The entire process is driven by ATP hydrolysis (26). The two ATP binding/utilization domains are capable of hydrolyzing ATP but not simultaneously since the stoichiometry of ATP hydrolysis is 1 mol ATP/mol of P-gp (25).

Overexpression of P-gp, an energy-dependent multidrug efflux pump is the best-studied mechanism of MDR. P-gp confers resistance against a wide spectrum of compounds including hydrophobic, amphipathic natural product drugs (24). Overexpression of class 1 P-gp causes cancer cells to become resistant to a variety of structurally and functionally unrelated anticancer drugs (e.g., vinblastine, vincristine, doxorubicin, daunorubicin, etoposide). These compounds are chemically diverse but are all hydrophobic, thus, they enter cells by passive diffusion (24). These drugs, which are substrates for transport by P-gp, directly bind to

P-gp and are actively effluxed from the cell via a pore or channel formed by the molecule's multiple transmembrane domains using energy derived from P-gp-mediated ATP hydrolysis (23). The ATP hydrolysis leads to a catalytic transition conformation with reduced affinity for substrates, which would result in substrate dissociation toward the extracellular side (27). This hydrolysis is vanadate sensitive. Using orthovanadate (Vi) can overcome this hydrolysis by generating a stable noncovalent ternary complex in the form of the transition state. Vi is similar to phosphate in size and charge, which enables it to behave as an efficient inhibitor of P-gp ATPase activity (25). P-gp confers multidrug resistance to mammalian cells by this drug efflux pump function, which makes increased expression of P-gp a well-characterized mechanism used by cancer cells to evade the cytotoxic action of anticancer drugs (28).

2.1.5 Multidrug resistant cell lines

CCRF-CEM is an established line of human leukemic lymphoblasts that is sensitive to vinblastine. There have been sublines of CCRF-CEM developed with varying degrees of resistance to vinblastine by growth in the presence of sublethal concentrations of drug. The resistance of these sublines to vinblastine ranges from 10.6-fold to more than 2000-fold when compared with the sensitive parent cells (29).

CEM/VLB100 is a well-characterized MDR cell line that was derived from the CEM parent cell line by culturing in increasing concentrations of vinblastine. The resistance of CEM/VLB₁₀₀ to vinblastine is more than 100 ng per ml. The mechanism of resistance overexpression of P-gp, which leads to a diminished uptake and retention of drug and tendency to form large aggregates in stationary suspension culture even at relatively low density (29, 30).

CEM/VM-1-5 cells were also derived from the CEM parent cell line and display “atypical” multidrug resistance (at-MDR) in that they differ the “typical” CEM/VLB₁₀₀ cells in the following aspects. First, CEM/VM-1-5 cells are cross-resistant to a wide variety of natural product antitumor drugs, except the Vinca alkaloids. Second, verapamil and chloroquine, which enhance the cytotoxicity of vinblastine in CEM/VLB₁₀₀ cells, have little or no ability to do so in the CEM/VM-1-5 cells (22). Moreover, CEM/VM-1-5 cells overexpress MRP1 (ABCC1), rather than MDR1. Further mechanistic studies revealed that this resistance results from altered type II DNA topoisomerase (topo II) activity. Topoisomerases are enzymes participating in many aspects of DNA metabolism, including replication, transcription, chromatin assembly, recombination and repair, which make them targets of anticancer drugs. The topo II catalyzes the relaxation, unwinding, decatenation, or catenation of double-stranded DNA. Doxorubicin is a traditional inhibitor of the topo II, which blocks the relegation of the cleaved DNA. The CEM/VM-1-5 cells display significant resistance to doxorubicin, around 1,000-fold decreased cytotoxic concentration. The resistance of CEM/VM-1-5 cells appears to be a result of the reduced stability of DNA-topo II complexes as well as the altered topo II intensity and attenuated distribution (31).

2.1.6 Reversal of MDR

Since MDR is one of the major causes for chemotherapeutic treatment failure in cancer patients, approaches have been explored to circumvent MDR (32). Attempts to circumvent MDR include inhibition of P-gp expression by small interfering RNA (33) and by metformin treatment (34).

A more established method to reverse MDR is by inhibiting the activity of P-gp directly (21). A large number of noncytotoxic compounds, known as chemosensitizers, can sensitize resistant cells to the action of cytotoxic drugs. These chemosensitizers include calcium channel blockers, calmodulin antagonists, steroids, cyclic peptides, and drug analogs (24) and are either substrates for transport by P-gp or modulators not transported by P-gp that may block substrate binding to P-gp (21). Chemosensitizers are divided into three classes based on the effect they exert on P-gp ATPase activity. Class I compounds (e.g., vinblastine, verapamil, and paclitaxel) enhance ATPase activity at low concentrations but inhibit activity at high concentrations (36). Class II compounds (e.g., bisantrene, valinomycin and tetraphenylphosphonium) stimulate ATPase activity in a dose-dependent manner without any inhibition. Class III compounds (e.g., cyclosporine A, rapamycin, and gramicidin D) inhibit both basal and verapamil-stimulated ATPase activity (24). Other compounds have no effect on the basal P-gp ATPase activity but inhibit verapamil-stimulated ATP hydrolysis (e.g., glycyrrhetic acid) (37).

Verapamil, a well-established MDR reversing agent, was originally used in the clinic as coronary vasodilator (38). In 1981, Tsuruo and colleagues studied the ability of verapamil to enhance cytotoxicity of vincristine using leukemia cell line P388 and its subline P388/VCR, which is resistant to vincristine and vinblastine. These investigators observed that verapamil caused the accumulation of vincristine in P388/VCR cells was by inhibiting drug efflux (39). In addition to the *Vinca* alkaloids, verapamil could also potentiate the cytotoxicity of adriamycin toward ovarian resistant cancer cells by inhibiting efflux (40, 41). Furthermore, it was reported that verapamil could directly bind to P-gp and could be actively transported out of the cell by

P-gp, suggesting that verapamil can reverse multidrug resistance by competitively inhibiting drug transport (42).

Cyclosporin A is an undecapeptide natural product originally isolated from cultures of the fungus *Tolypocladium inflatum* (Gams) as an antifungal antibiotic (43). Cyclosporin A also has potent immunosuppressive activity primarily by selective inhibition of T lymphocyte activation and proliferation induced by mitogen or by mixed lymphocyte reaction (44). It has been used clinically since the 1980s for the prevention and treatment of allograft rejection of organ transplantation of kidney, heart, liver, and multiple organ transplantation (45). Cyclosporin A's immunosuppressive activity is based on its binding to endogenous intracellular receptors, the immunophilins, and the resulting complexes inhibit the phosphatase calcineurin, which activates nuclear factor of activated T-cells (NF-AT). Thus, the NF-AT-stimulated transcription of factors necessary for the immune response is inhibited by cyclosporin A (44). Also, the Gupta group have shown that cyclosporine A reverse MDR in cancer cells both *in vitro* and *in vivo* (46, 47). Even though cyclosporin A is a immunosuppressive drug, it is well accepted that immunosuppression activity is not a prerequisite for its MDR activity, since its derivative, PSC833, has little immunosuppression but potent MDR reversing activity (48). To overcome MDR, cyclosporin A binds directly to P-gp (49) in a non-competitive manner (27), thus the recognition of substrate by P-gp and ATP hydrolysis is interfered (24, 27).

2.2 Materials and Methods

2.2.1 Chemicals

Hapalosin and hapalosin B were compounds isolated by the laboratory of our collaborators, Dr. Jimmy Orjala (University of Illinois at Chicago, Chicago, IL). All solvents and chemicals were purchased from Sigma – Aldrich Co. (St. Louis, MO) or Fisher Scientific (Itasca, IL) unless stated otherwise. RIPA Lysis and Extraction Buffer, and Halt Protease and Phosphatase Inhibitor Cocktail were from Thermal Scientific (Rockford, IL). The Quick Start Bradford Dye Reagent was purchased from BioRad Laboratories (Hercules, CA). Rabbit anti-P-gp primary antibody was purchased from Abgent Company (San Diego, CA); mouse anti- β -actin antibody was purchased from Sigma – Aldrich Co. (St. Louis, MO); goat anti-rabbit IgG, HRP-linked antibody and anti-mouse IgG, HRP-linked antibody were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA). The CellTiter 96 Aqueous One Solution Cell Proliferation Assay (MTS) was purchased from Promega Corp. The FluoroPureTM grade Rhodamine 123 was from Life Technologies Corporation (Carlsbad, CA).

2.2.2 Cell lines and culture conditions

The human cell lines employed for these studies were developed at the Children's Cancer Research Foundation from a line designated CCRF-CEM, which was established from a child with the initials C.E.M. who had developed acute leukemia. Beck and colleagues propagated CCRF-CEM cells, hereafter designated CEM, in increasing but sublethal concentrations of vinblastine (VLB). In this manner, CEM/VLB₁₀₀ were gradually selected to proliferate in the presence of VLB (100 ng/mL) at the same rate as CEM cells in the absence of VLB, which exhibits an IC₅₀ of about 2 ng/mL in the parent line (28, 29). Dr. William T. Beck of the University of Illinois at Chicago kindly provided both CEM and CEM/VLB₁₀₀ lines

used in the current studies. The cells were propagated as stationary suspension cultures in MEM supplemented with 10% heat-inactivated FBS in an atmosphere of 5% CO₂ in air and 100% humidity. Cell density was maintained between 10⁵ and 10⁶/mL and cultures were passed twice per week.

2.2.3 Cell Growth Inhibition Assay

In order to determine the sub-lethal dose of hapalosin and hapalosin B, a total of 5,000 CCRF-CEM or CEM/CEM/VLB₁₀₀ cells seeded per well of 96-well clear, flat-bottom plates (Microtest 96[®], Falcon) and incubated overnight (37 °C in 5% CO₂). Hapalosins dissolved in DMSO were then sequentially diluted and added to the appropriate wells (total volume: 100 µL). The cells were incubated in the presence of test substance for 72 hours at 37 °C and evaluated for viability with a commercial absorbance assay (CellTiter 96[®] AQueous One Solution Cell Proliferation Assay, Promega Corp, Madison, WI) that measured the amount of viable cells. Activity was expressed as the percentage of viable cells present relative to the negative (solvent) control. The concentration required to inhibit cell growth by 50% (IC₅₀) was calculated using GraphPad-Prism software.

The MTS assay was also used to assess whether hapalosin or hapalosin B can alter the potency of vinblastine. CCRF-CEM or CEM/VLB₁₀₀ cells were exposed to doses ranging from 1 nM to 10 mM vinblastine sulfate (Sigma–Aldrich, St. Louise, MO) for 72 h, either alone, or in the presence of sub-lethal concentrations of hapalosin and hapalosin B or verapamil hydrochloride (10 µM). The dose of verapamil employed in this study has previously been shown to be sub-toxic in both the CCRF-CEM and CEM/VLB₁₀₀ cell lines. The same assays were performed to measure CEM/VM-1-5 cells susceptibility to doxorubicin as well.

2.2.4 Rhodamine 123 accumulation assays

CEM/VLB₁₀₀ cells in log phase growth were harvested by centrifugation at 250 g for 10 min. A total of 2×10^5 cells were seeded into each 35-mm culture dish in 2 ml medium. The cells were treated with verapamil, hapalosin, or hapalosin B at a final concentration of 10 μ M for 30 min. DMSO served as the negative control. The fluorescent dyes rhodamine 123 (3 μ M) and Hoechst (5 μ g/mL) were then added to the medium. After 30 min incubation, the cells were collected by centrifugation and washed twice with PBS, spread on a microscope slide and imaged using a Zeiss LSM 710 confocal microscope.

For analysis by flow cytometry, log-phase CEM or CEM/VLB₁₀₀ cells (6×10^5 cells per sample in 2 ml) were incubated with vehicle, hapalosin (2.5, 5, or 10 μ M), hapalosin B (2.5, 5, or 10 μ M), or verapamil (10 μ M) for 30 min prior to the addition of rhodamine 123 (3 μ M; Invitrogen, Carlsbad, CA) and incubated for a further 30 min in the dark, at 37 °C. The cells were then harvested and immediately analyzed on a FACScan flow cytometer equipped with a 488 nm argon laser using CellQuest™ software (BD, Sydney, NSW). Rh123 fluorescence was measured by a 530 nm band-pass filter and propidium iodide fluorescence measured with a 585 nm band-pass filter. Gates were set to exclude propidium iodide positive cells and clumps and debris on the basis of forward and side scatter

2.2.5 Protein Extraction and Determination

Whole cell lysates were prepared from CEM/VLB₁₀₀ cells treated with vehicle, hapalosin B (10 μ M), or verapamil (10 μ M) for 72 h. Cells were harvested, washed and resuspended in the RIPA Lysis and Extraction Buffer [25mM Tris-HCl (pH 7.6), 150 mM NaCl, 1% NP-40, 1% sodium deoxycholate, 0.1% SDS; Thermo Fisher Scientific-Pierce

Protein Research Products, Rockford, IL] supplemented with 1 mM β -mercaptoethanol, Halt Protease and Phosphatase Inhibitor Cocktail (Pierce) after washing with PBS. Lysates were incubated on ice for 15 min and vigorously vortexed before centrifugation at 14,000 g for 15 min at 4 °C. The supernatant containing cellular proteins were collected. Protein concentration was determined using Bio-Rad Quick Start Bradford Dye Reagent (BioRad Laboratories, Hercules, CA).

2.2.6 Immunoblot Analysis

Immunoblot was used for semi-quantitative analysis of specific proteins in samples. Total proteins (25 μ g) were separated on 7.5% SDS-PAGE, and blotted onto polyvinylidene difluoride (PVDF) membranes using an Invitrogen iBlot transfer module. After saturating with 5% nonfat dry milk in TBST [20 mM Tris-HCl (pH 7.6), 200 mM NaCl, 0.05% Tween 20] for 1 h at room temperature, the blots were incubated with rabbit polyclonal anti-P-gp (1:60) primary antibody or mouse monoclonal anti- β -actin (1:3000) primary antibody overnight at 4 °C. The blots were then incubated with horseradish peroxidase-conjugated IgG secondary antibody (goat anti-rabbit or anti-mouse IgG, 1:25,000) at room temperature for 1 hour after extensive washing with TBST. Finally, the proteins were visualized using an enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech Inc., Piscataway, NJ) after three washings in TBST.

2.2.7 P-gp ATPase activity assay

The ATPase activity of P-gp was determined using a luminescent ATP detection kit (Pgp-Glo Assay System with P-glycoprotein, Promega, Madison, WI) according to the manufacturers' recommendation. Briefly, 0.5 mg/ml P-gp membranes and 10 mM MgATP

were incubated in the absence or presence of ATPase modulator and/or testing compound at 37 °C for 40 min, and the remaining ATP were detected as a luciferase-generated luminescent signal. Basal P-gp ATPase activities were determined as the difference between the ATP hydrolysis in the presence or absence of sodium orthovanadate. Verapamil-stimulated P-gp ATPase activity was measured in the presence of 100 µM verapamil. Verapamil-stimulated P-gp ATPase activity inhibition was established with cyclosporin A by adding both verapamil and cyclosporin A together at the incubation step. The inhibition effect of hapalosin and hapalosin B toward verapamil-stimulated P-gp ATPase activity was then measured.

2.2.8 Statistical Analysis

Data are expressed as the mean \pm standard error of the mean (SEM). Statistical differences were determined with a one-way analysis of variance (ANOVA) followed by Tukey's multiple comparison tests.

2.3 Results

2.3.1 Determination of sub-lethal dose of hapalosin and hapalosin B to CEM, CEM/VLB₁₀₀, and CEM/VM-1-5 cells

As shown in Table IV and Table V, both hapalosin and hapalosin B have no cytotoxic activity to CEM, CEM/VLB₁₀₀, or CEM/VM-1-5 cells at 5 µM or lower, and minimal activity at 10 µM. Thus, we used concentrations of 10 µM or lower for all subsequent assays.

TABLE IV CELL VIABILITY IN RESPONSE TO HAPALOSIN TREATMENT

| Hapalosin (μM) | Viability (%) | | |
|--|----------------------|------------------------------|-------------------|
| | CEM | CEM/VLB₁₀₀ | CEM/VM-1-5 |
| 20 | 64 | 50 | 100 |
| 10 | 92 | 89 | 100 |
| 5 | 100 | 99 | 100 |
| 2.5 | 100 | 100 | 100 |
| 1.25 | 100 | 100 | 100 |
| 0.625 | 100 | 100 | 100 |
| 0 | 100 | 100 | 100 |

TABLE V CELL VIABILITY IN RESPONSE TO HAPALOSIN B TREATMENT

| Hapalosin B (μM) | Viability (%) | | |
|--|----------------------|------------------------------|-------------------|
| | CEM | CEM/VLB₁₀₀ | CEM/VM-1-5 |
| 20 | 67 | 52 | 71 |
| 10 | 90 | 86 | 100 |
| 5 | 100 | 96 | 100 |
| 2.5 | 100 | 100 | 100 |
| 1.25 | 100 | 100 | 100 |
| 0.625 | 100 | 100 | 100 |
| 0 | 100 | 100 | 100 |

2.3.2 Hapalosins can reverse the resistance of CEM/VLB₁₀₀ cells to vinblastine

Hapalosin B was so named due to its similarity in structure to hapalosin, a depsipeptide previously reported to possess MDR reversal activity in cells that over express P-gp (18). These compounds represent a new class of MDR reversal agents, and as such, warrant further investigation regarding their pharmacology and potential toxicology. To this end, we tested hapalosin and hapalosin B in CEM/VLB₁₀₀ cells, which have been selected to be resistant to

VLB at 100 ng/ml (123 nM). A series of experiments in which a range of vinblastine concentrations were tested at each concentration of either hapalosin or hapalosin B were shown in Figure 2 and summarized in Table VI (Qi Shen). At 5 and 10 μ M, both depsipeptides caused between 50 and over 135 fold lower IC_{50} of vinblastine with some measurable toxicity to the cells. Verapamil (10 μ M) served as positive control and lowered the IC_{50} of vinblastine by over two orders of magnitude (from 297 nM to 2.6 nM).

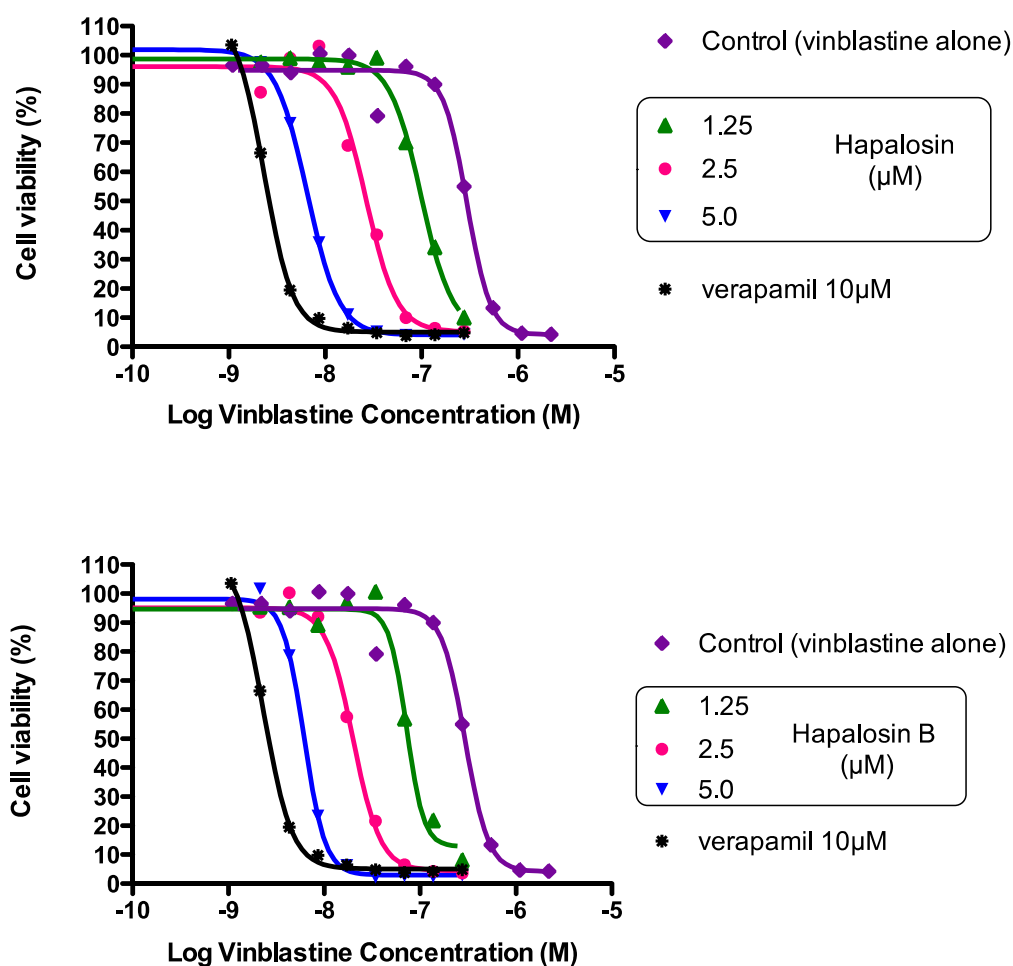


Figure 2. Enhanced cytotoxicity of vinblastine by hapalosin and hapalosin B in CEM/VLB₁₀₀ cells. The CEM/VLB₁₀₀ cells were treated with a series doses of vinblastine (ranges from 1 nM to 10 μM) in presence of either hapalosin or hapalosin B at the concentrations indicated in the figure. These experiments were conducted by Ms. Qi Shen of the Swanson Laboratory.

TABLE VI EFFECTS OF HAPALOSIN AND HAPALOSIN B ON THE CYTOTOXICITY OF VINBLASTIN IN CEM/VLB₁₀₀ CELLS

| Treatment | Concentration (μM) | VBL IC ₅₀ (nM) | Fold decrease In cytotoxic Concentration * |
|-------------|--------------------|---------------------------|--|
| Untreated | 0 | 297.0 | 100 |
| Verapamil | 10 | 2.6 | 113 |
| Hapalosin | 20 | <2.2 | >135 |
| | 10 | <2.2 | >135 |
| | 5 | 6.4 | 47 |
| | 2.5 | 26.4 | 11 |
| | 1.25 | 103.4 | 3 |
| | 0.625 | 143.0 | 2 |
| Hapalosin B | 20 | <2.2 | >135 |
| | 10 | <2.2 | >135 |
| | 5 | 6.1 | 47 |
| | 2.5 | 18.7 | 11 |
| | 1.25 | 73.7 | 3 |
| | 0.625 | 143.0 | 2 |

***Fold decrease:** The fold-decrease in resistance to vinblastine (VBL) was calculated as the quotient of the IC₅₀ of vinblastine in the absence of hapalosin, hapalosin B or verapamil by the IC₅₀ of vinblastine in the presence of these compounds.

2.3.3 Hapalosins have weak effect on CEM/VM-1-5 cells

Even though both hapalosin and hapalosin B have significant effect in reversing drug resistance of vinblastine in CEM/VLB₁₀₀ cells, they have very weak effect in CEM/VM-1-5 cells, which, unlike CEM/VLB₁₀₀ cells, do not over express P-gp (22). As shown in Figure 3 both hapalosin and hapalosin B can decrease the cytotoxic concentration of doxorubicin by no more than 2 fold, while verapamil, a well known multidrug resistance reversing agent could decrease the cytotoxic concentration of doxorubicin by no more than 4 fold. Moreover this effect is not due to the cytotoxic effect of these compounds as shown in Table VII.

These results indicate that hapalosin and hapalosin B may reverse the multidrug resistance of cells by affecting the activity of P-gp, which is an ATP-dependent drug efflux pump overexpressed on the membrane of some drug-resistant cell lines (29, 52).

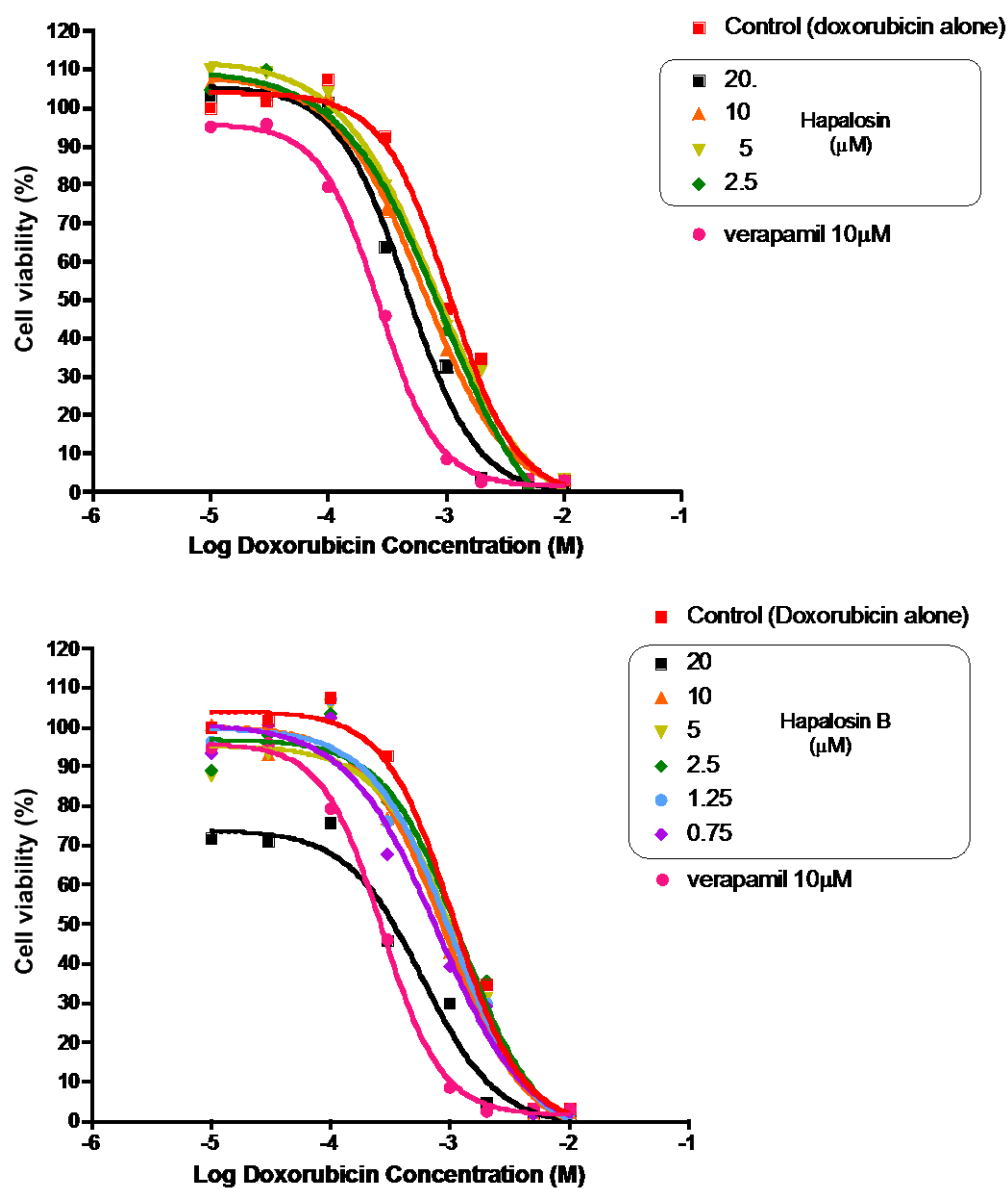


Figure 3. Little enhanced cytotoxicity of doxorubicin by hapalosin and hapalosin B in CEM/VM-1-5 cells. CEM/VM-1-5 cells were treated with

doxorubicin (from 1 nM to 10 μ M) in the presence of either hapalosin or hapalosin B at the concentrations indicated.

TABLE VII EFFECTS OF HAPALOSIN AND HAPALOSIN B ON THE CYTOTOXICITY OF DOXORUBICIN IN CEM/VLB-1-5 CELLS

| Treatment | Concentration (μM) | DOX IC₅₀ (nM) | Fold decrease In cytotoxic Concentration | Viability (%) |
|--------------------|--|---------------------------------|---|----------------------|
| Untreated | 0 | 1061.0 | 1 | 100 |
| Verapamil | 10 | 269 | 3.9 | 95 |
| Hapalosin | 20 | 464.4 | 2.3 | 100 |
| | 10 | 635.4 | 1.7 | 100 |
| | 5 | 800.6 | 1.3 | 100 |
| | 2.5 | 916.4 | 1.2 | 100 |
| | | | | |
| Hapalosin B | 20 | 584.3 | 1.8 | 71 |
| | 10 | 900.1 | 1.2 | 100 |
| | 5 | 1124 | 0.9 | 100 |
| | 2.5 | 1228 | 0.9 | 100 |
| | 1.25 | 1019 | 1.0 | 100 |
| | 0.75 | 773.5 | 1.4 | 100 |
| | | | | |

2.3.4 Hapalosins have no effect on P-gp expression level in CEM/VLB₁₀₀ cells

Our data revealed that hapalosin and hapalosin B are able to reverse resistance of an MDR cell line that overexpresses P-gp (CEM/VLB₁₀₀) but are ineffective in a cell line that has normal levels of P-gp (CEM/VM-1-5). Furthermore, Stratmann and colleagues have reported that hapalosin can reverse MDR in MCF7/ADR and SKVLB1, both of which over express P-gp (18). Also, it has been reported that P-gp expression can be decreased by MDR reversing compounds (34, 53). To test the hypothesis that the depsipeptides work by decreasing P-gp protein expression, immuno blot was performed. According to the calculation conducted by Gottesman group, the half life of P-gp was determined by as ~16 h (54). Also, the Jeong

group reported that after 48 h treatment with metformin, the P-gp expression level in a P-gp overexpression cell line was significantly lower than the untreated control (34). Thus, we concluded that a 72 h treatment would be sufficient to determine if hapalosin or hapalosin B down-regulated P-gp expression. As shown in Figure 4, neither hapalosin nor hapalosin B effected P-gp protein expression in CEM/VLB₁₀₀ cells.

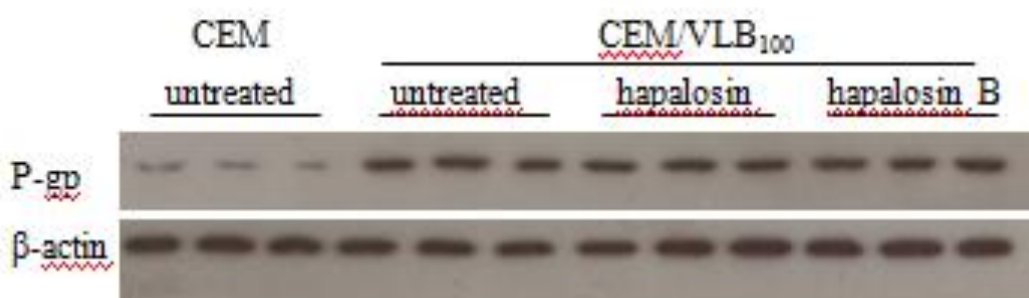


Figure 4. P-gp expression level in CEM cells and CEM/VLB₁₀₀ cells. Proteins of CEM/VLB₁₀₀ cells treated with hapalosin, hapalosin B, or vehicle, and CEM cells were assessed by anti-P-gp immunoblot.

2.3.5 Hapalosins block rhodamine 123 efflux

To further test the hypothesis that the depsipeptides work by inhibiting P-gp, we used a technique of applying fluorescent dyes as molecular probes to study MDR. Rhodamine 123 is a cationic fluorescent dye known to be a substrate for P-gp and to be extruded by P-gp over expressing cells relative to non-resistant parent cell lines (32). Quantification of the relative fluorescence accumulation is also a simple, sensitive, and quantitative tool to assess the

capacity of a drug to inhibit P-gp transporters (55). In these experiments, the depsipeptides were incubated with cells for a half hour to allow time for the P-gp to be affected followed by addition of the fluorescent substrate, rhodamine 123. The negative solvent control contained DMSO while verapamil served as positive control. The photomicrographs presented in Figure 5 demonstrate that both of the depsipeptides can cause accumulation of rhodamine 123 in P-gp over expressing CEM/VLB₁₀₀ cells to a degree similar to that of verapamil. This effect was quantitatively evaluated using FACS analyses (Figure 6). Note that both hapalosin and hapalosin B are capable of eliciting a concentration-dependent accumulation of rhodamine 123 in CEM/VLB₁₀₀ cells and at 10 μ M, the hapalosins can cause a similar amount of rhodamine accumulation in CEM/VLB₁₀₀ cells as 10 μ M verapamil.

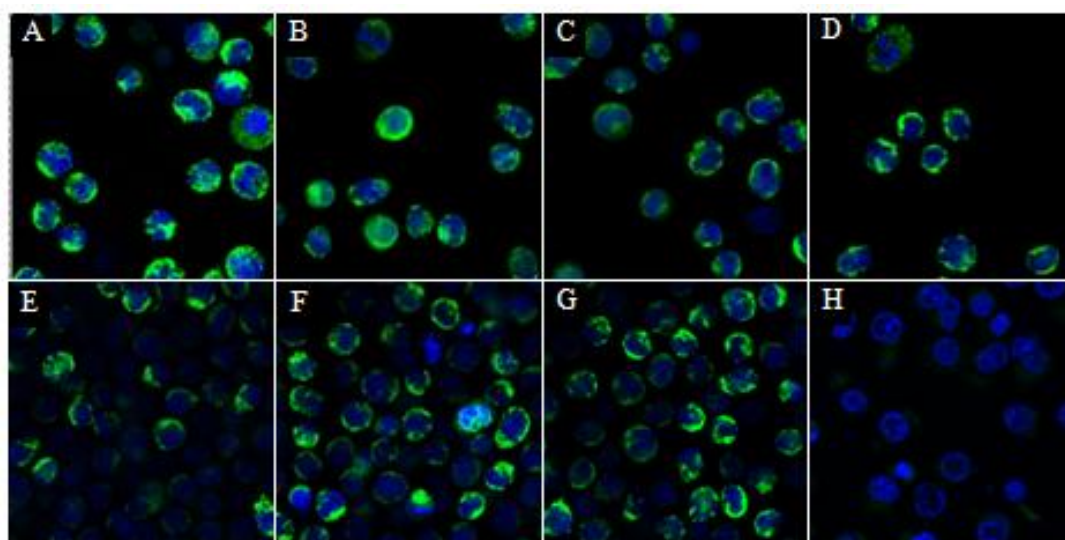


Figure 5. The effect of hapalosin and hapalosin B on intracellular rhodamine-123 accumulation in CEM and CEM/VLB₁₀₀ cells. Images of CEM (A-D) and CEM/VLB₁₀₀ (E-F) cells incubated with rhodamine-123 after pretreatment with 10 μ M hapalosin (A and E), 10 μ M hapalosin B (B and F), 10 μ M verapamil (C and G), or vehicle (D and H). Hoechst was used to stain the nucleus.

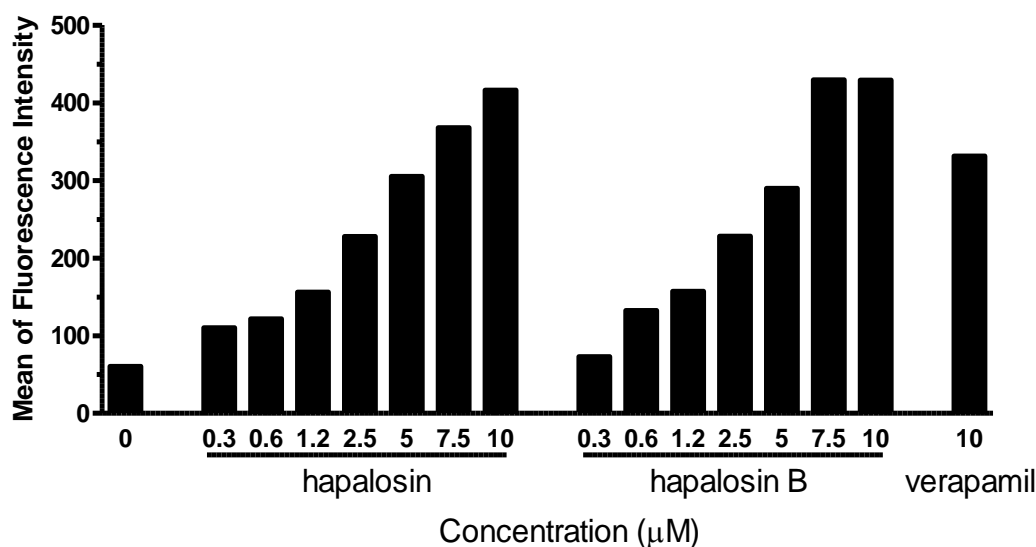


Figure 6. The quantitative effect of hapalosin and hapalosin B on intracellular rhodamine-123 accumulation in CEM/VLB₁₀₀ cells. Flow cytometry assay was performed to measure intracellular rhodamine-123 accumulation in response to hapalosin and hapalosin B treatment at concentrations indicated in the figure. Verapamil was used as positive control.

2.3.6 Analysis of P-gp ATPase activity

The results presented thus far indicate that hapalosin and hapalosin B potentiate the cytotoxic effect of vinblastine in P-gp overexpressing cells without downregulating P-gp protein expression. In contrast, the hapalosins were ineffective at reversing MDR in cells that overexpress MRP1. The hapalosins can affect MDR but not by altering the quantity of P-gp in resistant cells. The next question was whether the hapalosins altered P-gp activity. Since P-gp activity is driven by ATP hydrolysis, an ATPase activity was performed to monitor P-gp activity as a function of hapalosin exposure.

According to Gottesman's categorization, compounds that interact with P-gp can be divided into three classes: (1) compounds that stimulate ATPase activity at low concentrations but inhibit ATPase at higher concentrations; (2) compounds that enhance ATPase activity in a dose-dependent manner without any inhibition; (3) compounds that inhibit both basal and substrate (e.g., verapamil)-stimulated ATPase activity (24). As shown in Figure 7, hapalosin or hapalosin B did not alter the ATPase activity of P-gp on their own; neither stimulating as a P-gp substrate, nor inhibiting as an ATPase inhibitor. Therefore, it would be anticipated that the hapalosins inhibit the verapamil-stimulated ATPase activity only.

Cyclosporin A is known to reverse MDR in cancer cells by retarding both basal and verapamil-stimulated ATPase activity (46). As shown in Figure 8, both hapalosin and hapalosin B can inhibit verapamil-stimulated ATPase activity, which is nearly four times higher than basal level. According to the one-way ANOVA analysis, among the three compounds: cyclosporin A, hapalosin and hapalosin B, the P values between each two are greater than 0.05. Thus, hapalosin and hapalosin B have similar inhibition effect on verapamil-stimulated ATPase activity as cyclosporin A.

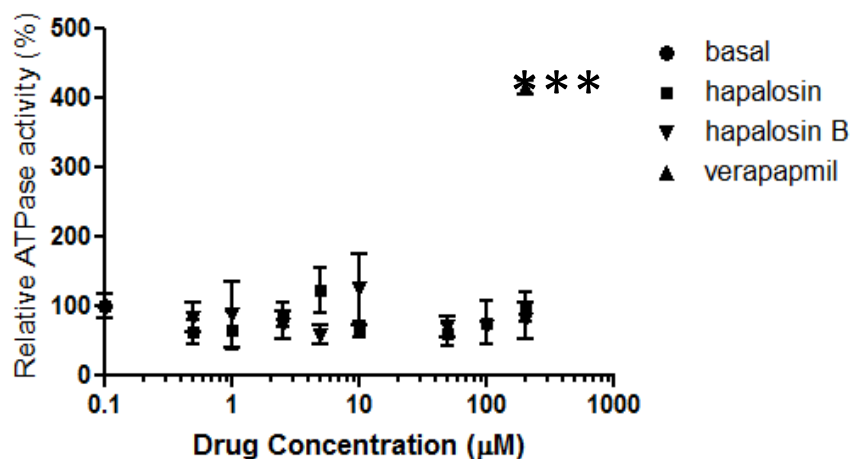


Figure 7. P-gp ATPase activity in response to treatment with hapalosin and hapalosin B. Cell membrane fraction containing P-gp was treated with 200 μ M verapamil or hapalosin B the P-gp ATPase activity was then measured. Results are expressed as the mean \pm SEM (n=3). ***, $p < 0.001$, and others $p > 0.05$, compared with basal level of P-gp ATPase activity.

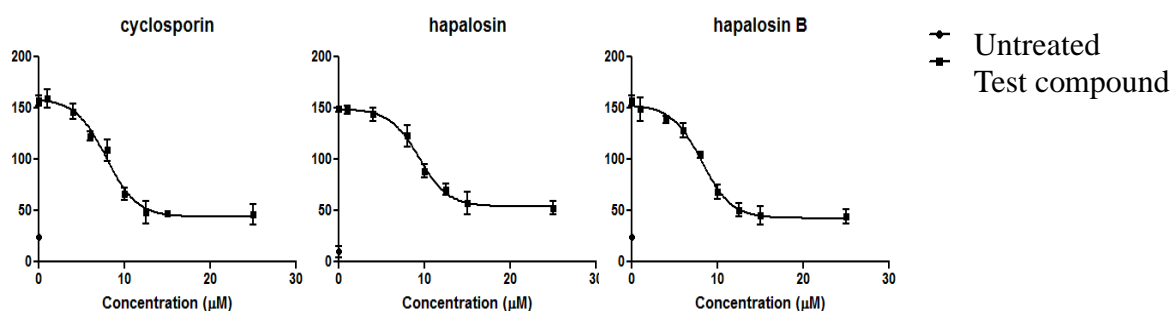


Figure 8. Inhibition of verapamil-stimulated P-gp ATPase activity. Cyclosporine, hapalosin, and hapalosin B were tested at a range of concentrations for its capacity to inhibit 100 μ M verapamil-stimulated P-gp ATPase activity. Results are expressed as the means \pm SEM (n=3).

2.4 Discussion

2.4.1 P-gp inhibitors have yet to show promising clinical outcomes

Because of the close correlation between P-gp over expression in patients' tumors and poor prognosis of chemotherapeutic treatment (23), combating tumor drug efflux to restore sensitivity of cancer cells to chemotherapy has been undergoing intense study during the past 30 years (56). Three distinct generations, classified based on their discovery strategy, of P-gp inhibitors have been tested in the clinic to date. They are established pharmacological agents, modified first generation drugs, drugs developed by structure-activity relationships and combinatorial chemistry, and natural sourced compounds (57).

The first successful pre-clinical study was conducted by the Sakurai et al. By screening the membrane-modifying agents, they found a Ca^{2+} channel blocker could potentiate the cytotoxicity of vincristine and vinblastine in a P388 vincristine-resistant subline at a nontoxic dose *in vivo* and *in vitro* (39). However, the clinical study revealed considerable cardiotoxicity produced at a thousand fold lower concentration than that required by P-gp inhibition (57). The next identified P-gp inhibitor was cyclosporin, also named cyclosporin A (46), which elicited similar toxicity problems as verapamil (57), even though it is one of the most effective first generation MDR modulators (58). In addition, other MDR modulators had the same outcome in clinical trials (57). These disappointing results are ascribed to the high required dose of the chemosensitizers due to their low binding affinities to P-gp (59).

The second-generation compounds were more potent and considerably less toxic than the original ones (58). Their main advantage over the first generation compounds was their high binding affinity. Nevertheless, the second generation compounds yielded disappointing results in clinical trials. Two properties of these compounds contributed to their lack of efficacy: (1) they were substrates for cytochrome P450 isoenzyme 3A4 as the cytotoxic agents, thus the

competition for this enzyme resulted in inhibiting metabolism of chemotherapeutic agents, and thereby the patients were at risk of drug overexposure; (2) they are also substrates for other transporters, particularly ABC family transporters, inhibition of which would reduce the normal cell self-protecting ability from cytotoxic agents and lead to greater adverse effects of anticancer drugs (59).

Based on the information obtained from the first two generations, the third generation of P-gp modulators were developed using combinatorial chemistry and directed structure-activity relationships, in order to increase the potency and selectivity (57). These agents did overcome the limitations of the second-generation: no effect on cytochrome P450 3A4 at relevant concentration, and no inhibition on other ABC transporters. Thus, clinically they circumvented alterations in the pharmacokinetics of the co-administered cytotoxic agents (59). Even though the strengths of the third-generation modulators were a significant advance, there were only few successful clinical trials (57). For example, tariquidar was one of the most promising third-generation P-gp inhibitors, which specifically and noncompetitively bound to P-gp with high affinity and potently inhibiting its activity for a relatively long period (around 20 hours after removal) (59), but had only shown limited clinical activity. Only one out of 17 patients developed an objective tumor response when tariquidar was added to the docetaxel/trastuzumab regimen (60, 61).

2.4.2 Natural-sourced compounds are novel source for P-gp modulators

Cyanobacteria are well known as a promising source for new pharmaceutical lead compounds partially due to their wealth of structurally diverse natural products (13). To date a

large number of chemically diverse and bioactive metabolites have been obtained from cyanobacteria over the last few decades (63).

2.4.3 The mechanism of MDR reversal by the hapalosin

Generations of P-gp modulators have been developed and tested in pre-clinical and clinical studies, it is important to understand the mechanism of the reversing effect of these agents, although there exists a lack of information concerning the P-gp transport mechanism and its drug-handling ability.

From the cytotoxicity assay we can tell that hapalosins can enhance the efficacy of vinblastine in P-gp overexpressing cells but not their parent cells. This is an indication that the hapalosins may inhibit substrate efflux mediated by P-gp. Thereafter, the rhodamine 123 accumulation assay, with the result of enhanced cellular accumulation after 0.5 h hapalosins treatment, suggests that hapalosins may inhibit the activity of P-gp. It is because that the half life of P-gp protein is ~ 16 h (54), and hapalosins can inhibit the majority of P-gp efflux activity within 0.5 h treatment. Then, this assumption is confirmed by western blot assay, which suggest that P-gp expression level is not altered by hapalosins treatment. Finally, the activity of P-gp ATPase was measured to understand the molecular mechanism by which hapalosins reverse MDR. The results suggest that the hapalosins can neither stimulate nor inhibit basal ATPase activity of P-gp, but can inhibit the verapamil-stimulated ATPase activity of P-gp.

Different classes of drugs such as *Vinca* alkaloids, calcium channel blockers, anthracyclines, antiarrhythmics, epipodophyllotoxins, antihypertensives, antibiotics, immunosuppressants, cytotoxic agents, steroid hormones, and HIV protease inhibitors interact with P-gp. They are either hydrophobic or amphipathic molecules and enter cells by passive

diffusion (25). Calcium channel blockers such as verapamil inhibit xenobiotic efflux. Even though verapamil and vinblastine mutually exclusively bind to P-gp, they bind to two different binding sites of P-gp in a non-competitive manner, and a negative allosteric interaction between distant sites may be utilized. It is also claimed that verapamil is a non-competitive inhibitor of the cytotoxins (64, 65). On the other side, cyclosporin A, which is an immunosuppressant, is a competitor of cytotoxins but not a substrate (65). It inhibits both the basal and verapamil-stimulated ATPase activity of P-gp (24).

Even though there is no direct and solid evidence supporting that the substrate of P-gp should stimulate its ATPase activity, this concept is widely accepted (66). All the reported P-gp substrates are P-gp ATPase stimulators, such as verapamil, and vinblastine. Furthermore, substances that are not P-gp substrates but inhibit its activity, such as cyclosporine A (24) and tariquidar (67), display ATPase inhibition activity. Our results with hapalosin and hapalosin B suggest that neither depsipeptide is a substrate for P-gp nor do they affect P-gp's basal ATPase activity. However, both compounds inhibit verapamil-stimulated ATPase activity of P-gp, suggesting that they can inhibit P-gp substrate binding, inhibit ATP hydrolysis by P-gp or both. To confirm that they are not P-gp substrates, a hapalosin (or hapalosin B) uptake assay could be performed using photoanalogs or radioisotopes of the compounds. The difference of uptake levels of hapalosin (or hapalosin B) analogs between P-gp overexpressing cells and their parent cells can tell if the hapalosins are P-gp substrates (42). Moreover, since they do not inhibit the basal ATPase activity, the ATP hydrolysis inhibition possibility may be ruled out.

2.4.4 Future directions

After observing the resistant phenotype reversal modulated by sub lethal dose of hapalosin and hapalosin B, we have already answered the questions that this reversal is accomplished by decreasing cellular drug accumulation, which is fulfilled by inhibiting the substrate-stimulated P-gp ATPase activity.

Other than these conclusions, some questions about the inhibition mechanism are still open. One of them is that we still do not have enough evidence to conclude if they are competitive or non-competitive modulators of P-gp, the later one represents a better inhibitor to P-gp. It is because that, to inhibit P-gp in the competitive manner will require a high dose of the modulators (57).

Also, we want to know if the hapalosins bind to the protein directly. The P-gp protein can be immunoprecipitated by specific antibody from P-gp containing membrane incubated with photolabeled hapalosin (or hapalosin B) and analyzed by SDS-PAGE. The radioactivity measured at 170-180KD can be a strong evidence of direct interaction between hapalosins and P-gp (42). Since most P-gp modulators bind to the protein, the answer is likely to be “yes” (57). If hapalosins interact with P-gp directly, another question will raise: which binding site of P-gp do hapalosins interact with. It has been reported by groups that there exist distinct binding sites of P-gp for different modulators or substrates (65). The function of these binding sites remains ambiguous: it is claimed that the multiple sites may actually be functionally classified as transport or modulatory in nature (66). Also, there is evidence supporting that the modulator, cyclosporin A, can interact with both sites (65). For hapalosins, we want to know that if hapalosins interact preferentially with one site or interact with both; and if hapalosins interact

with the same site as the substrates they inhibit efflux or they interact with the other site and there is a negative allosteric interaction between sites.

The next question we need to answer is whether hapalosins inhibit P-gp specifically and exclusively. A cross inhibition of other MDR proteins (ABCB1, ABCC1 and ABCG2) is often observed from the P-gp modulators. This cross-reactivity is usually blamed as the cause of toxicity to normal cells and tissues, since many of the ABC transporters behave as a protective factor in barrier or secretory tissues by efflux the toxins/drugs from cells (57). According to Figure 3, we can tell that the hapalosins cannot enhance the efficacy of doxorubicin in CEM/VM-1-5 cells, which overexpress MRP1 (ABCC1). This is only a weak evidence to support that hapalosins do not inhibit MRP1. CEM/VM-1-5 also has a mutation at topoisomerase II, which leads to decreased expression of topoisomerase II β . Thus, the reduced cellular topo II activity may also contribute to the resistance to doxorubicin (51). It is because doxorubicin targets on the topoisomerase II and leads to collision of the replication fork (31). Thus, more evidence will be needed.

After answering these mechanism questions, we may be able to describe the inhibition mechanism of hapalosins. With clear mechanism, we may then need to know if they can potentiate the efficacy of chemotherapeutic agents *in vivo* as they do *in vitro*. An *in vivo* hollow fiber assay can be utilized before using the xenograft system to predict activity. This method is to grow human cancer cells in hollow fibers with pores small enough to hold the cancer cells but large enough for compounds to diffuse in. Then, the fibers are implanted into the peritoneum or under the skin of immunodeficient mice, the host can be treated with hapalosin (or hapalosin B) combined with vinblastin and the fibers are subsequently retrieved for analysis

of viable cell mass. This can give us a relatively direct idea of their *in vivo* P-gp inhibition activity (68).

Chapter 3 Silvestrol

3.1 Introduction

3.1.1 The natural product silvestrol is a potential anticancer therapeutic agent

The genus *Aglaia* (Meliaceae) consists of over 100 species of dioecious trees or shrubs with small fragrant flowers indigenous to the tropical rain forests of Indonesia and Malaysia. Previous phytochemical studies on *Aglaia* species have shown that among all the isolates, cyclopenta-[*b*]benzofurans such as rocaglate and rocaglamide derivatives deserve further study due to their unusual carbon skeleton unique to members of the genus *Aglaia*. Moreover, the antileukemic and/or cytotoxic activity of certain rocaglamide and rocaglate derivatives are the result of their ability to block protein synthesis and to induce growth arrest in the G2/M-phase of the cell cycle. Silvestrol was isolated from the fruits and twigs of *Aglaia silvestris* (M. Roemer) Merrill by using bioassay-guided fractionation procedures as part of a program to discover novel anticancer agents of plant origin. Silvestrol exhibited potent *in vitro* cytotoxic activity and it was further evaluated in mice using both the hollow fiber assay and the P-388 lymphocytic leukemia system (69).

3.1.2 Silvestrol inhibits protein synthesis

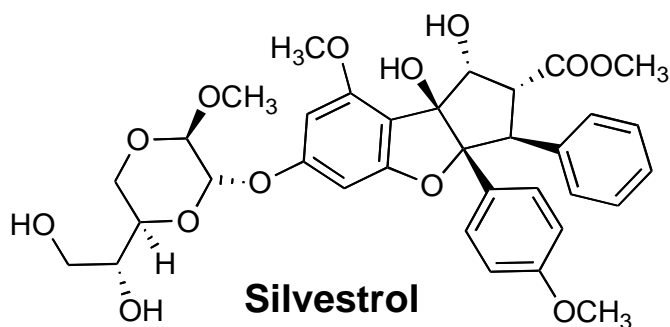


Figure 9. The chemical structure of silvestrol.

Rocaglates are a class of anticancer natural products with exceptional potency and selectivity for cancer cells (70), and silvestrol is one of the most active naturally occurring congener. It has been demonstrated that silvestrol exhibited potent *in vitro* cytotoxic activity against human cell lines derived from breast, prostate and lung cancers. The potency of silvestrol (IC₅₀ 1.2 to 1.5 nM) was similar to that observed for paclitaxel (IC₅₀ 0.7 to 4.7 nM) and camptothecin (IC₅₀ \approx 30 nM). Moreover, the further *in vivo* studies performed in hollow fiber assay model and murine P388 leukemia model showed the *in vivo* activity and selectivity of silvestrol (71).

Cyclopenta[*b*]benzofuran flavaglines such as silvestrol inhibit cap-dependent translation initiation at the eukaryotic initiation factor (eIF) 4F complex (eIF4F) (72). All eukaryotic mRNAs have a cap structure at the 5'ends, which is composed of a terminal 7-methylguanosine residue linked to the first transcribed nucleotide. This structure protects mRNA from degradation and facilitates translation initiation. Translation of most mRNA is exclusively cap-dependent (73). Translation initiation is a complex process in which eIFs can

assemble initiator tRNA (tMet-RNA_i) 40S, and 60S ribosomal subunits into an 80S ribosome at the initiation codon of mRNA. The first step of translation initiation is cap-mediated attachment of 43S pre-initiation complex (40S ribosomal subunit and associated factors) to mRNA, which is mediated by eIF4F and facilitated by eIF4B. eIF4F is a heterotrimeric factor composed of 3 subunits: eIF4E, which binds to the cap structure; eIF4A, a DEAD-box RNA helicase, which unwinds 5' mRNA structure to prepare a ribosome landing loci for 43S pre-initiation complex; and eIF4G, which interacts with 40S-associated eIF3 to facilitate ribosome recruiting (74). As a translation initiation factor, eIF4A exists in both free form and a subunit form as a component of eIF4F complex. The helicase activity of eIF4A is much less efficient in free form than subunit form. Silvestrol acts as a chemical inducer of dimerization to force an engagement between the free form of eIF4A and RNA, which can result in the inhibition of translation initiation (75).

3.1.3 Hypothesis

As it has been reported that silvestrol inhibits protein synthesis and has potent cytotoxic activity toward cancer cells. Our working hypothesis is that inhibition of protein synthesis by silvestrol causes the cell to initiate autophagy, which ultimately leads to apoptosis.

3.1.4 Apoptosis and related upstream pathways

3.1.4.1 Apoptosis

Apoptosis, also called type 1 cell death, is the process of programmed cell death (PCD) that normally occurs during development and aging as a homeostatic mechanism to keep cell populations static. Also, apoptosis can behave as a defense mechanism in autoimmune reactions or when cells sustain injury by disease or noxious agents (76, 77). Apoptosis is

accompanied by many cellular processes, including rounding-up of the cell, cell shrinkage, chromatin condensation, chromosomal DNA fragmentation, little or no ultrastructural modification of cytoplasmic organelles, plasma membrane blebbing, and maintenance of an intact plasma membrane until late stages of the process. While DNA fragmentation or caspase activation are not required for the execution of apoptosis, the measurement of these parameters is often helpful in diagnosing apoptosis (78).

There are two key molecular signaling pathways that lead to apoptosis: the extrinsic or death receptor pathway and the intrinsic or mitochondrial pathway (76). The extrinsic pathway is induced by proapoptotic ligands that activate specialized cell surface death receptors. Once activated, the receptor can recruit the adapter Fas-associated death domain (FADD) and procaspases 8 and 10 to form the death-inducing signaling complex (DISC). Formation of the DISC results in the activation and release of the initiator caspases 8 and 10, which in turn activate the effector 3, 6, and 7 (79). The intrinsic pathway is activated by a plethora of cytotoxic stimuli through members of the B-cell leukemia/lymphoma 2 (BCL-2) protein family, which lead to the opening of the mitochondrial permeability transition (MPT) pore and then the release of pro-apoptotic proteins, such as cytochrome *c*, from the intermembrane space into the cytosol (76, 79, 80). Once released into the cytosol, these proteins trigger the execution of cell death by promoting caspase activation or by acting as caspase-independent death effectors (80).

3.1.4.2 PI3-Kinase/AKT pathway

Phosphatidylinositol-3 kinases (PI3Ks) are a family of intracellular lipid kinases that phosphorylate the 3'-hydroxyl group of phosphatidylinositols and phosphoinositides (81).

Class I PI3Ks are heterodimers composed of a regulatory subunit (p85) and a catalytic subunit (p110) (82). The p110 α subunit is a catalytic subunit of the Class IA PI3Ks. In response to various extracellular stimuli, p110 α is recruited to activated growth factor receptor tyrosine kinases and catalyzes the formation of phosphatidylinositol-3,4,5-trisphosphate (PIP₃) by phosphorylating phosphatidylinositol-4,5-bisphosphate (PIP₂). Once formed, PIP₃ transduces activating signals to the downstream kinases (81).

The serine-threonine kinase AKT, also known as protein kinase B (PKB), is a downstream effector of PIP₃ (82) and is also a key regulator of a wide range of cellular processes including growth, proliferation and survival (83). AKT proteins contain the amino-terminal pleckstrin homology domain, which permits anchorage of proteins to the cell membrane via phospholipid interactions with PIP₃ (84). AKT can be activated by phosphorylation either by PDK1 (3-phosphoinositide-dependent kinase) at threonine 308 or by the mTOR complex 2 (mTORC2) at serine 473 (81).

AKT stimulates cell growth by phosphorylating the tuberous sclerosis complex 2 (TSC2) inhibiting TSC2 binding to TSC1 to form a complex (85). TSC1-TSC2 complex inhibits mTORC1 activity as a functional unit (86). The interaction of TSC1 with TSC2 excludes TSC2 from interacting with the HERC1 ubiquitin ligase and stabilizes TSC2 (87). In response to growth factors, AKT directly phosphorylates TSC2, and this phosphorylation impairs the interaction between TSC1 and TSC2 and thus the stability and activity of the TSC1-TSC2 complex (85). The TSC2 protein contains a domain homologous to GTPase-activating proteins and negatively regulates the small G protein Rheb, (short for 'Ras

homolog enriched in brain') (88). Rheb interacts with the mTOR complex and positively regulates its activity (89).

Mammalian target of rapamycin (mTOR) is a protein kinase with 289kD in size (89). Most mTOR targets are components of the translation machinery, especially those taking part in recruiting the ribosome to the mRNA, which is critical for proper control of cellular growth, proliferation, differentiation, and apoptosis (90, 91). mTOR functions in two distinct and mutually exclusive complexes: the mTOR complex 1 (mTORC1) in association with raptor and G β L and the mTOR complex 2 (mTORC2) in association with rictor and G β L. Most studies have focused on mTORC1, which stimulates translational initiation through the phosphorylation of 4E-BP-1 and p70S6 kinase (92, 93).

In its unphosphorylated state, 4E-BP-1 inhibits binding of eIF-4E to the eIF-4G scaffold, thereby repressing translation. Phosphorylation of 4E-BP-1 promotes its dissociation from eIF-4E, enabling recruitment of the latter into the eIF4F complex and the initiation of gene transcription (89, 92). The p70 S6 kinase is a Ser/Thr protein kinase that plays an important role in cell growth and G1 cell cycle progression. p70 S6 kinase phosphorylates the S6 protein of the 40S ribosomal subunit at Ser235, Ser236, Ser240, Ser244, Ser411, Thr421 and Ser424 resulting in an increase in mRNA translation (127-129).

3.1.4.3 Crosstalk between apoptosis pathways and the PI3K/AKT pathway

The commitment of a cell to apoptosis is determined by the integration of inputs from stress signal pathways and survival pathways (94). PI3K plays a critical role in the promotion of cell survival and inhibition of apoptosis and AKT is the key protein downstream of PI3K that mediates its cell-survival activities. In response to a variety of stimuli from growth factors,

hormones, cytokines, etc., PI3K phosphorylates phosphoinositol 3,4 bisphosphate (PIP₂) to phosphoinositol 3,4,5 triphosphate (PIP₃). This allows AKT to be recruited to the plasma membrane where it binds to PIP₃ at its pleckstrin homology (PH) domain. Once anchored to the plasma membrane, AKT is phosphorylated by phosphoinositide-dependent kinase 1 (PDK1). The protein targets of phosphorylated AKT are many and populate disparate signaling pathways within the cell. In general, phosphorylated AKT promotes cell survival and stimulates cellular proliferation. The role that phosphorylated AKT plays in apoptosis suppression has been thoroughly characterized in the literature. Furthermore, inhibitors of AKT downstream targets such as mTOR inhibit carcinogenesis (95). Finally, activated AKT can block apoptosis at the postmitochondrial level (96) by phosphorylating ASK1 (apoptosis signal-regulating kinase 1), which blunts its apoptosis promoting activity (97). Moreover, phosphorylation of Mdm2 by AKT restrain p53 activity, thereby limiting p53-dependent apoptosis (94).

3.1.5 Autophagy and related upstream pathways

3.1.5.1 Autophagy

Autophagy is a strategy that cells utilize during periods of nutrient stress to switch from an anabolic program when nutrients are plentiful to a catabolic metabolic program in which cellular constituents are degraded for energy production (98). The word ‘autophagy’ is derived from the Greek *autos*, meaning self, and *phagein*, to eat. Autophagy, specifically macroautophagy (99), is a process in which a double membrane vesicle termed the autophagosome is formed in the cytosol and engulfs organelles and bulk cytoplasm. This

autophagosome is then delivered to the lysosome where the contents are degraded and recycled (98, 100).

Autophagy is a process that the cell can invoke when nutrient in the environment require the cell to ratchet down its metabolic activity including protein synthesis, which accounts for nearly half of the energy expenditure of the metabolically active cell. A cell's autophagy program may be induced by limited nutrient availability, or the absence of hormone or growth factor stimulation. Autophagy plays an important role in a number of physiological processes such as development, differentiation, and cancer (101). The presence of growth factors, hormones or sufficient intracellular ATP and amino acids maintains mTOR in the activated state and capable of phosphorylating ribosomal protein S6 kinase and 4E-BP1, which increases cap-dependent translation and inhibits autophagy (102).

Once autophagy is induced in mammalian cells, the first response is the expansion of the pre-autophagosomal membrane by recruiting the mAtg12-mAtg5 protein complex to fuse together and become the organelle. As the membrane expands and upon the induction by the Beclin1/PI3K class III complex, MAP1-LC3-II (Microtubule Associated Protein 1-Light Chain 3-II), which is generated from LC3-I cleavage and phosphatidylethanolamine conjugation, is conjugated to the membrane. Meanwhile, the membrane expands and engulfs the cell material to be enclosed (103). Thus formed, the autophagosome becomes a double membrane vacuole encapsulating a variety of cell material such as organelles, soluble cytosolic proteins, and protein aggregates destined for degradation. Ultimately, the autophagosome fuses with the membrane of a lysosome to form an autolysosome subjecting the contents of the autosome to the lytic action of lysosomal hydrolases (104). By recycling cellular organelles

and other components, free fatty acids and amino acids are generated that can be reused by the cell to maintain the ATP production and protein synthesis vital to cell survival (101).

3.1.5.2 Autophagy, cell death and cancer

Autophagy has dual roles in cell survival: it is a possible mechanism for non-apoptotic death and a survival strategy in times of stress (98). The relation between autophagy and cancer is also complex: it can either suppress cancer development (105) or protect cancer cells during times of extreme stress (106).

Although once intensely debated, it is now accepted that prolonged autophagy can result in autophagic, or type II programmed cell death, which is a form of programmed cell death distinct from apoptosis. In autophagic cell death, there is early degradation of organelles and preservation of cytoskeletal elements until late stages, which is opposite to apoptosis. Also, in certain circumstance of uncontrolled upregulation of autophagy, autophagy can trigger apoptosis (99). However, under most forms of cellular stress, autophagy is critical for maintaining cell viability, since ATG gene knockdown/knockout accelerates rather than delays cell death (99). This is achieved by degradation of cytoplasmic material and generation of both nutrients and energy, which can contribute to the cytoprotection, especially when they are undergoing nutrient shortage (109).

Cancer is a result of dysregulation of cellular proliferation differentiation and survival. Since autophagy can contribute to both cell death and cell survival depending on the circumstances, the role that autophagy in carcinogenesis is complex and has been vigorously debated in the literature (101). On the one hand, there is a strong correlation between defects in autophagy and the progression of tumorigenesis. (110). Defects in specific components of the

autophagy machinery can lead to increased DNA damage and gene amplification, decreased cellular differentiation and protein catabolism, especially during stress (99, 101). On the other hand, there are studies showing that genetic or pharmacological inhibition of autophagy enhances efficacy of cancer chemotherapeutic agents (99). However, there is limited direct evidence that autophagy is a bona fide death pathway in chemotherapy-treated cancer cells (107).

3.2 Materials and Methods

3.2.1 Materials

Silvestrol was the generous gift of Dr. A. Douglas Kinghorn (Ohio State University, Columbus, OH). All solvents and chemicals were purchased from Sigma – Aldrich Co. (St. Louis, MO) or Fisher Scientific (Itasca, IL) unless stated otherwise. HT-29 cells and A549 cells were obtained from the American Type Culture Collection (ATCC) (Manassas, VA). Rabbit anti-PI3K (p110 α), anti-PDK1, anti-phospho-PDK1 (Ser241), anti-AKT, anti-phospho-AKT (Thr308), anti-phospho-AKT (Ser473), anti-TSC2, anti-phospho-TSC2 (Thr1462), anti-mTOR, anti-phospho-TOR (Ser2448), anti-p70S6K, anti-phospho-70S6K (Thr421/Ser424), anti-S6 ribosomal protein, and anti-phospho-S6 ribosomal (Ser235/236) were purchased from Cell Signaling Technology (Danverse, MA). Mouse anti- β -actin antibody was purchased from Sigma – Aldrich Co. (St. Louis, MO). Goat anti-rabbit IgG, HRP-linked antibody and anti-mouse IgG, HRP-linked antibody were purchased from Jackson ImmunoResearch Laboratories, Inc. (West Grove, PA).

3.2.2 Cell Growth Inhibition Assay.

Cytotoxicity assays were performed using human colon cancer cell line HT-29. All cells were propagated in RPMI 1640 medium supplemented with fetal bovine serum (10% v/v), penicillin (100 units/ml) and streptomycin (100 µg/ml). Cells were plated at 2,500 cells per well in 96-well microtiter plates and incubated for 16 h in a humidified, 5% CO₂ atmosphere. Silvestrol was then added to the cells. After a 72-hour incubation, an MTS assay was performed using a commercially available kit according to the manufacturer's instructions (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega Corp, Madison, Wisconsin;

MTS:3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium, inner salt). Bortezomib served as the positive control (IC₅₀ 5 nM). The reading of the negative control is considered as 100% viability and the reading of blank control is considered as 0% viability. The IC₅₀ values were calculated using Prism (GraphPad Software, Inc., La Jolla, CA).

3.2.3 Cell Apoptosis Detection.

Treated cells were fixed with 1% (w/v) paraformaldehyde in PBS (pH 7.4) and stored in 70% ethanol (v/v) at -20 °C until use. An apoptosis detection kit (APO-BRDU kit, BD Biosciences, San Diego, CA) was employed to stain the fixed cells according to the manufacture's protocol. Briefly, cells were resuspended in wash buffer (containing 0.05% sodium azide) and centrifuged twice. Each pellet was resuspended in 50 µl DNA labeling solution consisting of 10 µl reaction buffer (cacodylic acid dimethylarsenic), 0.75 µl TdT Enzyme [200 µg/ml (S.A.= 100,000 U/mg)] in 50% (v/v) glycerol solution), 8 µl Br-dUTP (54.7 µg/ml) and 32.25 µl distilled H₂O at 4 °C for 16h to label the end of broken double-strand

DNA. The cells were rinsed and centrifuged twice and the pellets were resuspended and incubated in the dark for 30 min in antibody staining solution, [5 μ l FITC-labeled anti-BrdU (Isotype: mouse; 1 μ g/test) and 95 μ l rinsing buffer],. Lastly, PI/RNase staining buffer was added to the antibody staining solution and the cells were incubated in the dark an additional 30 min. The stained cells were analyzed by flow cytometry within 3 hours (Flow Cytometry Service, UIC Research Resources Center).

3.2.4 Caspase-3/-7 activity Assay.

Growth phase cells were plated at 2,500 cells per well in a white-walled 96-well microtiter plate and incubated for 16 h in a humidified, 5% CO₂ atmosphere. Silvestrol was then added to the cells to a final volume of 100 μ l. After 72-hour incubation, the activities of caspases 3 and 7 were measured using a commercially available kit according to the manufacturer's instructions (Caspase-Glo[®] 3/7 Assay, Promega Corp, Madison, WI). The kit employs a luminogenic substrate, aminoluciferin, covalently linked to the tetrapeptide sequence DEVE, which is a caspase-3 and -7 selective cleavage target. Cleavage by caspases 3 or 7 releases aminoluciferin, which is a substrate for luciferase. The reagent was added at a 1:1 ratio to the wells containing treated cells. The contents were gently mixed and incubated at room temperature for 1.5 hours. The signal was measured in a plate-reading luminometer.

3.2.5 Protein Extraction and Determination

For tissue cell lysis, the T-PER tissue protein extraction reagent (Pierce Biotechnology, Rockford, IL) was employed. The reagent uses a proprietary detergent in 25 mM bicine, 150 mM sodium chloride (pH 7.6) and was supplemented with Halt protease inhibitor mixture (Pierce, Rockford, IL). Tissue debris was removed by centrifugation for 15 min at 14,000 rpm.

Protein concentration in the supernatant was determined by the Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA) using BSA as a reference standard.

3.2.6 Immunoblot Analysis

Immunoblot was used for semi-quantitative analysis of specific proteins in samples. Total proteins (25 µg) were separated on 7.5% (or 10%) SDS-PAGE, and blotted onto polyvinylidene difluoride (PVDF) membranes using an Invitrogen iBlot transfer module. After saturating with 5% nonfat dry milk in TBST [20 mM Tris-HCl (pH 7.6), 200 mM NaCl, and 0.05% Tween 20] for 1 h at room temperature, the blots were incubated with primary antibodies overnight at 4 °C. The mouse anti-β-actin primary antibody was used at 1:10,000 dilution, and rabbit anti-PARP, rabbit anti-PI3K p110α, rabbit anti-AKT, rabbit anti-AKT (pan), rabbit anti-Phospho-Akt (Ser473), rabbit anti-Phospho-Akt (Thr308), rabbit anti-TSC2, rabbit anti-Phospho-TSC2 (1462), rabbit anti-mTOR, rabbit anti-Phospho-mTOR (2448), rabbit anti-p70S6K, rabbit anti-Phospho-p70S6K (241/242), rabbit anti-S6, rabbit anti-Phospho-S6 (235/236) were used at 1:1,000 dilution. The blots were then incubated with horseradish peroxidase-conjugated IgG secondary antibody (goat anti-rabbit or anti-mouse IgG, 1:25,000) at room temperature for 1 hour after extensive washing with TBST. Finally, the proteins were visualized using an enhanced chemiluminescence (ECL) system (Amersham Pharmacia Biotech Inc., Piscataway, NJ) after three washings in TBST.

3.2.7 Transient protein expression

To visualize autophagosome formation, a GFP-LC3 fusion protein was transiently expressed employing a mammalian expression plasmid containing the human LC3B gene fused at 5' end to the GFP gene (pSELECT-GFP-LC3 plasmid, InvivoGen Corp, San Diego,

CA). A549 cells were plated in 35-mm dishes containing DMEM with 10% heat-inactivated FBS. Once the cells had reached approximately 60% to 80% confluent, they were transfected with the pSELECT-GFP-LC3 plasmid using the lipofection reagent lipofectAMINE 2000 (Life technologies, Grand Island, NY) according to the manufacturer's protocol. The reactions were limited to no more than 4 hours. The cells were then replated onto 8-chamber slides coated with collagen containing DMEM with 10% heat-inactivated FBS and allowed to propagate to 50% to 60% confluency overnight. The transfected cells were washed with PBS, fixed with 3% paraformaldehyde, mounted with VECTASHIELD® Mounting Media for Fluorescence (Vector Laboratories, Burlingame, CA), and sealed with nail oil and air-dried. The following day, the cells were observed using an ECLIPSE E600 microscope and imaged using a NIS-Elements software (Nikon, Japan).

3.2.8 Plasmid amplification and purification

The purchased p-SELECT-GFP-LC3 expression plasmid was transformed into commercially available DH5 α competent cells (Subcloning Efficiency™ DH5 α ™ Competent Cells, Life technologies, Grand Island, NY) using the heat shock transformation method according to the manufacturer's instructions. Then the transformed cells were spread onto pre-warmed Zeocin selective LB plates and incubated at 37 °C overnight. An appropriate amount of bacteria were taken out from a plaque and cultured in 5 ml Zeocin-LB selective medium for 8 hours. The cultured bacteria were then subinoculated into 15 ml fresh medium for further amplification overnight. The following day, the cultured bacteria were harvested and subject to plasmid purification using a commercially available kit according to the manufacturer's instructions (Plasmid Mini Kit for purification of up to 20 μ g transfection grade

plasmid DNA, Qiagen, Valencia, CA). Finally, the concentration of the DNA was determined by Nanodrop 2000 UV-Vis Spectrophotometer (Thermo Scientific, Wilmington, DE).

3.3 Results

3.3.1 Cytotoxicity of silvestrol against human colon cancer cells *in vitro*

The overall goal of the experiments described in this chapter was to study the mechanism of silvestrol induced cancer cell death. Our first experiment was to confirm the cytotoxic efficacy of a new supply of silvestrol against a human colon cancer cell line designated HT29, which we had previously found to be in the single-digit nanomolar range (71). As is shown in Figure 10, the IC_{50} of silvestrol was 2.6 nM against HT29 cells.

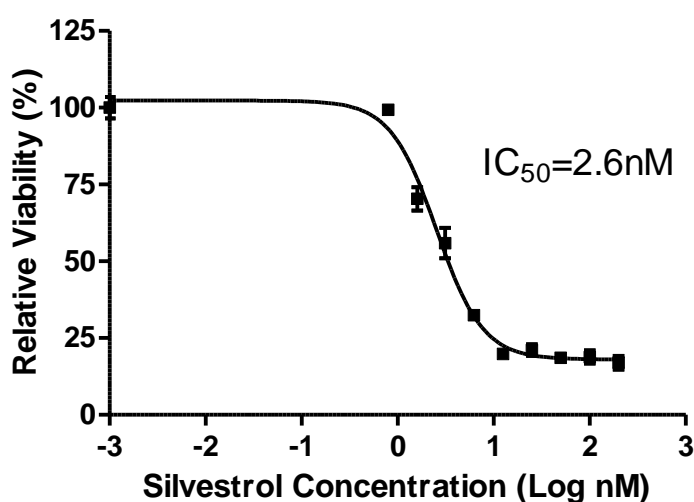
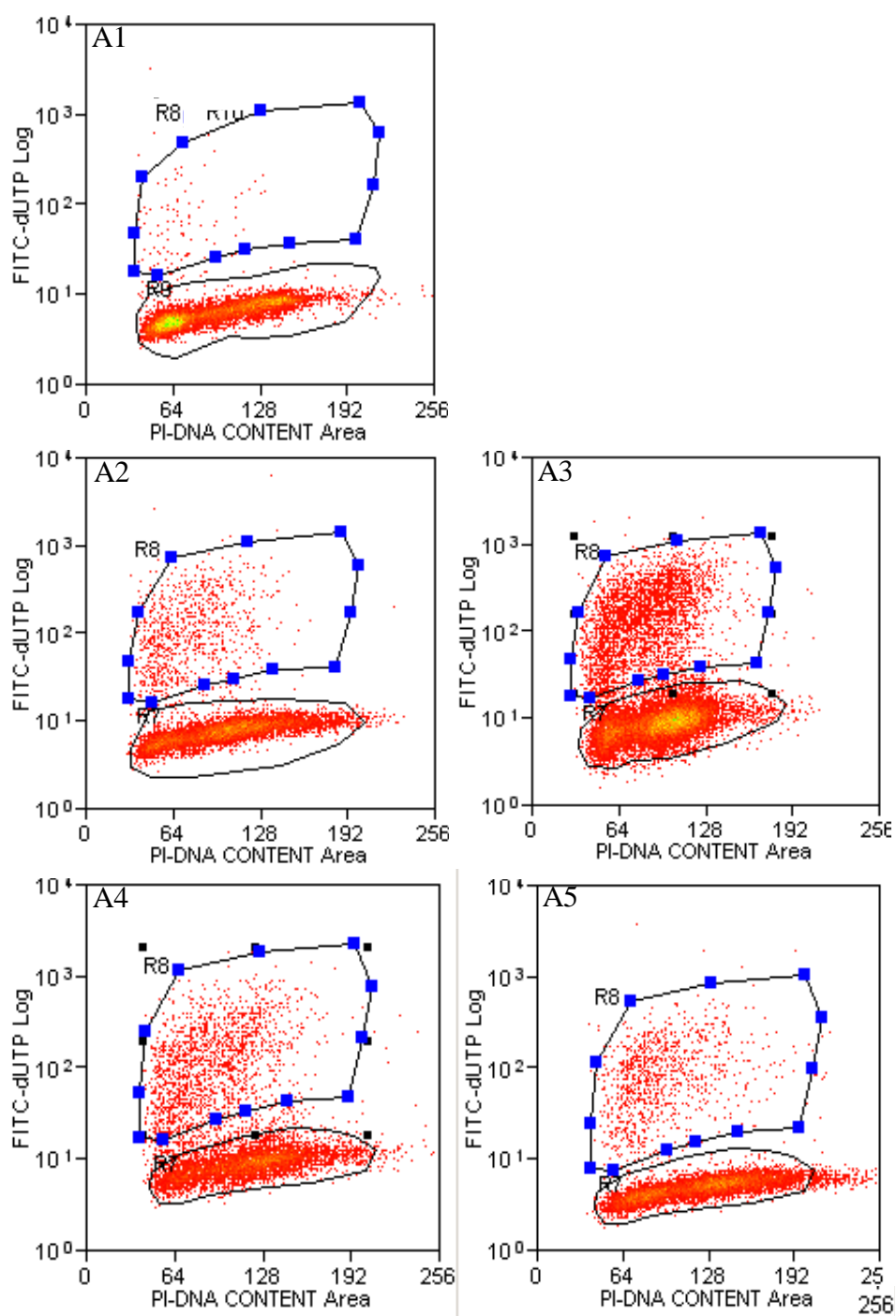


Figure 10. Silvestrol dose response curve.

3.3.2 Silvestrol could induce apoptosis

Anticancer strategies used clinically such as chemotherapy, γ -irradiation, suicide gene therapy or immunotherapy, induce apoptosis through the intrinsic and/or extrinsic pathways (80). Thus, we asked if silvestrol kills cancer cells by inducing apoptosis. To answer this question, three parallel apoptosis detecting assays were performed.

Firstly, cells exposed to silvestrol were evaluated using the TUNEL (terminal deoxynucleotidyl transferase biotin-dUTP nick end labeling) assay, in which the ends of DNA created by the limited nuclease digestion that often accompanies apoptosis are stained and analyzed by fluorescence-based flow cytometry (Figure 11). The DNA end fragments are stained with the fluorescent dye FITC and propidium iodide (PI) is used to stain all the DNA content, including unfragmented DNA. Double-positive cells were counted as 'apoptotic cells', whereas PI single positive cells were considered as 'viable cells'. The flow cytometry results presented in Figure 11 suggest that silvestrol treatment causes DNA fragmentation in a time- and concentration-dependent manner. The TUNEL assay may be subject to false positives from necrotic cells and cells undergoing DNA repair or gene transcription (98). Therefore, we conducted additional experiments to confirm these results.



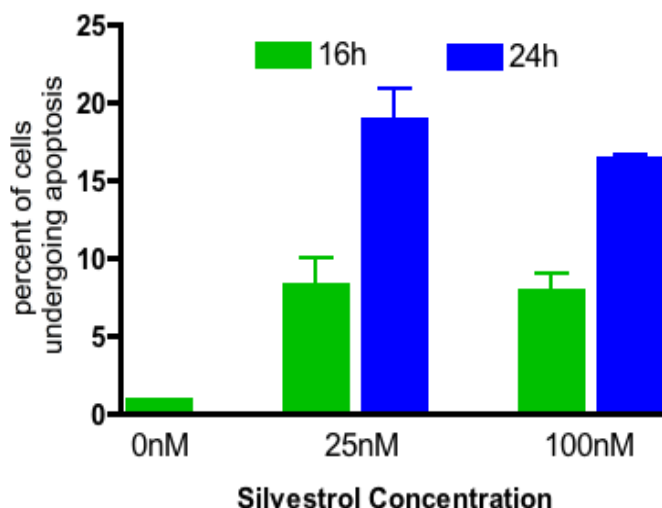


Figure 11. Effect of silvestrol on HT-29 cell apoptosis induction marked by TUNEL staining and assessed by flow cytometry. Each bar represents the mean \pm SEM of 3 replicates.

The second method employed to assess the ability of silvestrol to induce apoptosis in HT-29 cells was analysis of caspases activity. Caspases have proteolytic activity and once activated, apoptosis proceeds irreversibly towards cell death. Within the identified major caspases, the effectors or executioners are caspase-3, caspase-6, and caspase-7 (76). HT-29 cells were exposed to silvestrol and activity of caspases activity was subsequently evaluated using the Caspase-Glo[®] 3/7 Assay (Promega Corp, Madison, WI). Detection of the increased caspase-3/7 activity is directly proportional to luminescence generated by caspases cleavage of a peptide (DEVD) covalently bound to a luciferase substrate (aminoluciferin). The elevated luminescent signal represents the activated caspase-3/7, as well as induced apoptosis (Figure 12). Also, an immunoblot of PARP [Poly (ADP-ribose) polymerase] which is a substrate of

activated caspases (Figure 13), revealed cleavage in response to silvestrol exposure providing a further evidence of activated executive caspases and induced apoptosis.

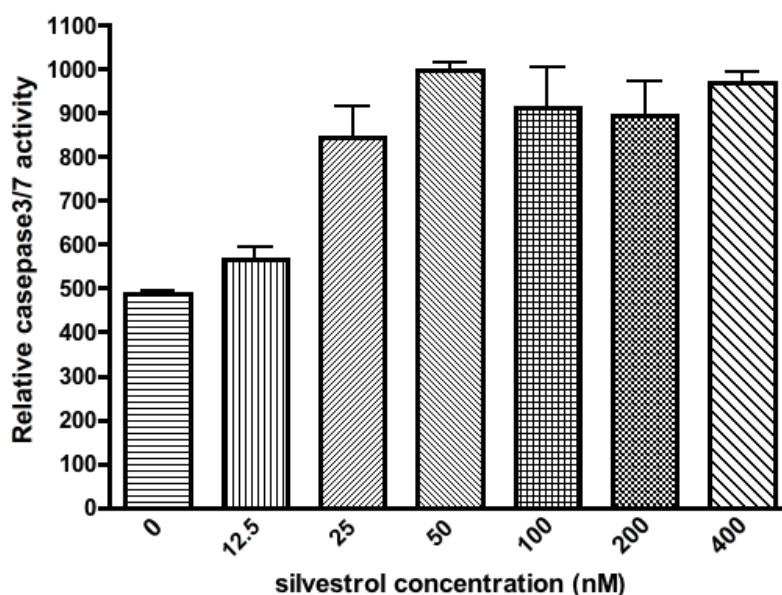


Figure 12. Silvestrol-induced apoptosis of HT-29 cells assessed by caspase-3/-7 activity. HT-29 cells were treated with silvestrol as indicated., Luciferin fluorescence served as the readout for caspase-3/-7 activity. The data are expressed as the mean \pm SEM of three replicates.

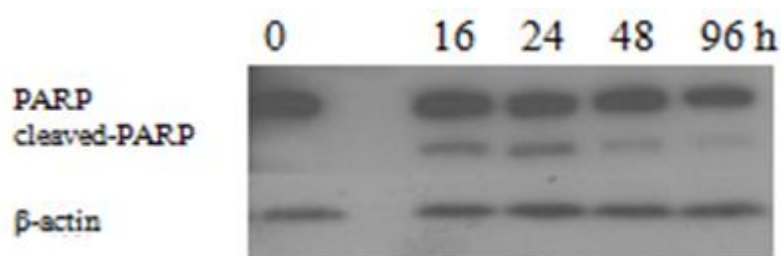


Figure 13. Apoptosis of HT-29 cells assessed by anti-PARP immunoblot. Cells were treated with 100 nM silvestrol for time periods indicated. Equivalent protein loading was assessed by re-probing with anti-β-actin antibody.

3.3.3 Silvestrol regulates AKT/mTOR pathway

Since the AKT/mTOR pathway plays an important role in cell survival and apoptosis regulation, several proteins involved in this pathway were studied to learn if their expression or activity was altered by silvestrol exposure.

As shown in Figure 14, the catalytic subunit of PI3K (p110 α), which is essential for the generation of second messenger PIP₃ (112), became undetectable upon silvestrol treatment. Since PIP₃ recruits AKT to the cellular membrane to mediate its phosphorylation by PDK1 (113), we next measured the phosphorylation status of AKT. As expected, the phosphorylation of AKT was down regulated by silvestrol treatment at both threonine 308 and serine 473 residues, which indicates that the activity of AKT kinase is dampened by silvestrol exposure.

Although AKT has many downstream substrates, one of its main functions is through the mTOR kinase to modulate cell fate. As shown in Figure 14, TSC2 protein level is up regulated as a function of silvestrol treatment. This correlated with loss of AKT phosphorylation, which is significant since activated AKT is known to phosphorylate and destabilize TSC2 (85). TSC2 further negatively regulates mTOR through the G-protein Rheb (89). The activated mTOR functions in two distinct and mutually exclusive complexes. The mTOR complex 1 stimulates translational initiation through the phosphorylation of 4E-BP-1 and p70S6 kinase (92). As illustrated in Figure 14, phosphorylation of both p70S6K and its substrate S6 protein is decreased by silvestrol treatment. Since the S6 ribosomal protein is a component of 40S ribosomal subunit and involved in translational control, it is possible that silvestrol also inhibits protein synthesis due to S6 protein activation inhibition.

Taken together, these data suggest that silvestrol affects the AKT/mTOR pathway by (1)

AKT and mTOR deactivation, which can promote apoptosis and (2) by inactivating p70S6K and S6 ribosomal protein, resulting in protein translation inhibition.

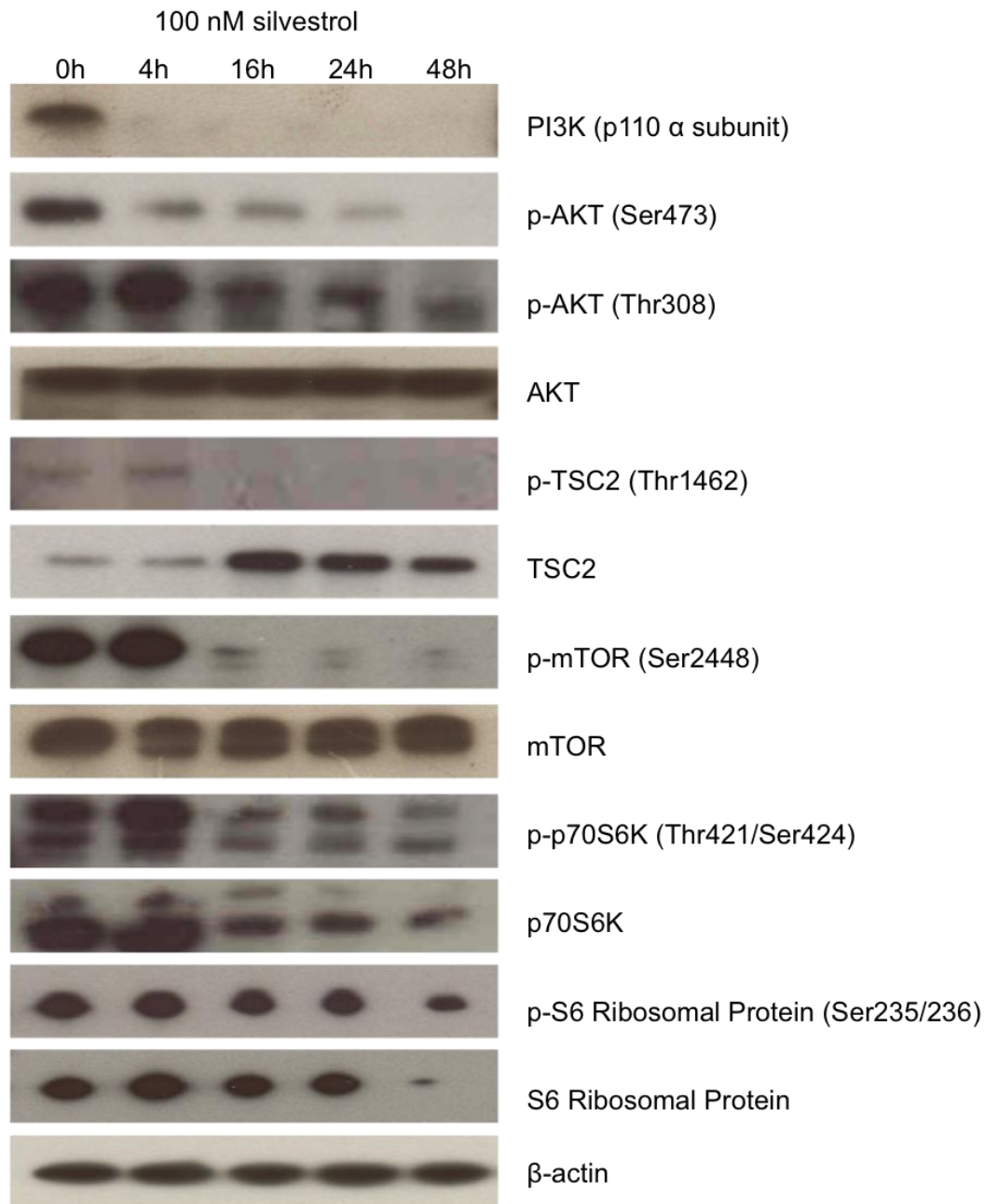


Figure 14. Proteins regulated by silvestrol treatment. HT-29 cells treated with 100 nM silvestrol as indicated. Equivalent protein loading was assessed by re-probing with anti-β-actin antibody.

3.3.4 Silvestrol can induce autophagy

Microtubule-associated protein 1 light chain 3 (LC3), a homolog of yeast Atg8, subcellular location was used as an autophagy marker. In cells undergoing autophagy, LC3 is specifically localized to autophagic structures including the autophagosome and its precursor structure the phagophore and the autolysosome. Under normal, non-stressed conditions, cellular LC3 protein is distributed throughout the cell cytoplasm. In response to stress or other forces that induce autophagy, LC3 is processed and recruited to the autophagosome membrane resulting in a punctate concentration of LC3 on autophagic vacuoles. The GFP-LC3 fusion protein responds to autophagy induction in a manner indistinguishable from that of native LC3 protein yielding cytoplasmic puncta when viewed under fluorescence microscopy (114).

A549 cells were transiently transfected with a plasmid expressing GFP-LC3 fusion protein to study the cell response to silvestrol. As shown in Figure 15, cells starved for 2h in Earl's balanced saline solution (EBSS) (panel A) and cells treated with 200 nM silvestrol for 2h displayed multiple foci per cell where LC3 was concentrated, while cells treated with vehicle lacked such punctuations. These results suggest that autophagy was induced by silvestrol in the cells.

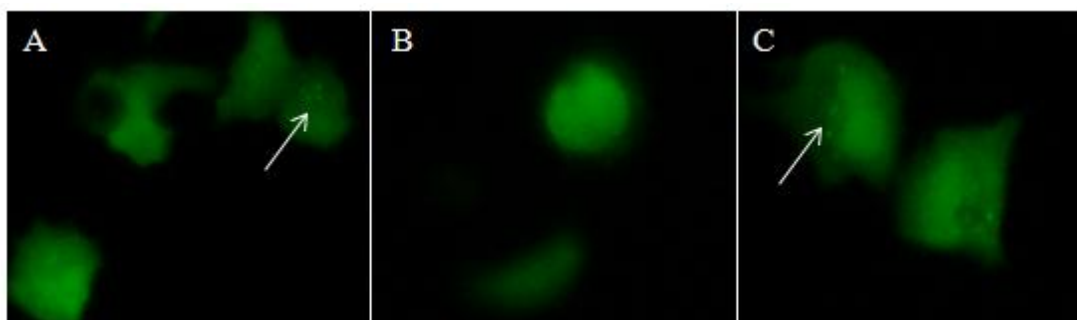


Figure 15. Induction of autophagy by silvestrol. GFP-LC3 transfected cells treated with EBSS/starvation (A), vehicle (B), or 200 nM silvestrol (C) for 2 h. The observed GFP punctuates were imaged and identified by arrows.

3.3.5 Silvestrol induces autophagy-related genes expression

To further study the silvestrol-induced autophagy, a number of autophagy related genes were analyzed. Beclin-1 is one of the critical proteins regulating autophagy induction, which can stimulate autophagy when overexpressed in mammalian cells (115). Beclin-1 regulator 1 binds to Beclin-1 and plays a positive role in the Beclin-1-dependent induction of autophagy (116). The expression of Beclin-1 regulator 1 mRNA is significantly upregulated in response to silvestrol exposure (Figure 16).

Control of autophagy relies on proteins encoded by a set of AuTophagy-related genes (Atg). Many of the early discoveries in the autophagy program were made using yeast model systems. Atg8 is a ubiquitin-like protein involved in yeast autophagy (117). It is cleaved by Atg4, transferred to the E2-like enzyme Atg3, and finally forms the Atg8-phosphatidylethanolamine (Atg8-PE), which is an important component of autophagic vesicles. Subsequently, Atg4 reverses this lipidation reaction releasing Atg8 to be re-used by the cell. In mammalian cells, Atg8 has at least four homologs (GATE-16, GABARAP,

MAP1LC3, and ATG8L), and Atg4 also has four homologs (ATG4A, ATG4B, ATG4C, and ATG4D) (118, 119).

Immunity-related gtpase M (IRGM) also induces autophagy in response to mycobacteria infection, although the exact mechanisms by which it promotes autophagy are not known (120). Autophagy is activated by signaling pathways that also control apoptosis. For example, tumor necrosis factor (TNF) can stimulate autophagic cell death independent of caspase activation (121). Protein kinase R-like endoplasmic reticulum kinase (PERK) regulates hypoxia-increased transcription of the essential autophagy genes microtubule-associated protein 1 light chain 3 β and autophagy-related gene 5 (ATG5), which mediate phagophore expansion and autophagosome formation (122). Also, the AMP activated protein kinase (AMPK), a key energy sensor and metabolism modulator to maintain energy homeostasis, promotes autophagy by directly activating the mammalian autophagy-initiating kinase Ulk1, a homologue of yeast ATG1, while mTOR inhibits its activation (123). All the proteins mentioned above that positively regulate autophagy are up regulated by silvestrol exposure, and the AKT1 and PI3K, which negatively correlate with autophagy, are down regulated by silvestrol treatment (Figure 16, Qi Shen).

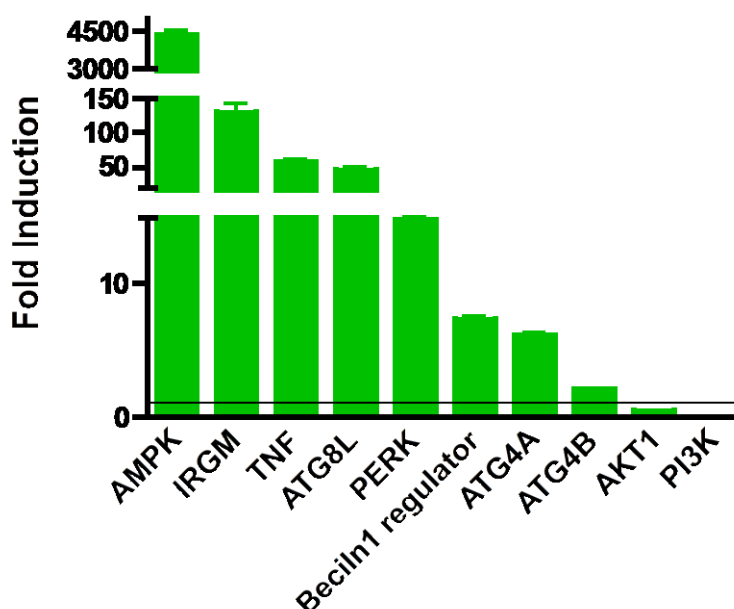


Figure 16. Expression of autophagy related genes induced by silvestrol treatment. HT-29 cells were treated with 25 nm silvestrol for 16 hours and mRNA of cells were evaluated by RT-PCR. Equivalent mRNA loading was assessed by internal control. These experiments were conducted by Ms. Qi Shen of the Swanson Laboratory.

3.4 Discussion

3.4.1 Silvestrol is a promising anticancer drug candidate

The National Cancer Institute (NCI) has established the NExT Program (NCI's Experimental Therapeutics program) to support the most promising new drug discovery and development projects that originate in academia and other not-for-profit organizations to bring improved therapies to cancer patients (124). The NExT program consists of 8 constitutive stages: exploratory screen development, screening/designed synthesis, lead development, candidate seeking, clinical candidate, phase 0, phase I, and phase II (125). Silvestrol is

involved in this NExT program, and undergoing safety analyses at NCI and is currently being evaluated for efficacy and safety in dogs (126).

3.4.2 Silvestrol causes cell death by triggering different mechanisms

The results of the present study suggest that silvestrol, a plant-derived cyclopenta[b]benzofuran, induces both apoptosis and autophagy in human cancer cells.

The TUNEL staining assay results (Figure 11) showed that silvestrol induced DNA fragmentation in a time and concentration dependent manner. This fragmentation is one of the major characteristics of cells undergoing apoptosis. Also, the increased caspase-3/7 activity (Figure 12) and the increased PARP cleavage confirmed the induced apoptosis by silvestrol treatment. Caspase-3 and -7 are downstream apoptosis effectors for both intrinsic and extrinsic pathways. Also, PARP cleavage further confirmed this, since PARP is a classical substrate of caspase-3 and -7.

To study the mechanism of apoptosis induction, the upstream PI3K/AKT pathway cell signal pathways involved in cell survival and apoptosis was studied. As shown in Figure 14, all pro-survival proteins in this pathway, PI3K, AKT, and mTOR, were significantly depressed by silvestrol treatment, which can promote apoptosis. Furthermore, the pathway downstream of PI3K/Akt that regulates protein synthesis was muted by silvestrol treatment.

Even though the IC_{50} of silvestrol in HT29 cells was 2.6 nM, there were still live cells after incubating the cells with 100 nM silvestrol for 72 or even 96 hours. Treatment of cells with up to 40 fold higher concentration of silvestrol than its IC_{50} for more than 48 hours should kill all the cells. When the toxicity assays were conducted, cell viability was measured by the MTS assay, which directly measures NADPH production. Therefore, even if the cells

were scored as “dead” by this assay, they might be still alive, but with minimum metabolic activity. Also, cells exposed to silvestrol assumed a different morphology from that upon exposure to most apoptosis inducers such as bortezomib, which induces cells to round up and detach from the substratum. Cells treated with silvestrol assumed a flattened surface morphology, suggesting that the cells were stressed, but not undergoing the characteristic morphologic transformation indicative of apoptosis. This morphology and the fact that silvestrol is known to inhibit protein synthesis suggested to us that the cells may be entering a state of autophagy. During autophagy, the cellular metabolism is greatly reduced which could result in a level of NADPH production so low as to resemble a dead cell leading to a false negative result in the MTS cytotoxicity assay. This may be why a silvestrol dose much higher than its IC_{50} could not kill all the treated cells. When detecting autophagy, the GFP-LC3 distribution pattern shown in Figure 15 indicated that autophagy was induced by silvestrol treatment. Moreover, the mRNA analysis revealed that the expression of genes that stimulate autophagy were highly upregulated.

3.4.3 The role of autophagy in silvestrol-induced cell death needs further study

As illustrated in the “Introduction”, autophagy has dual roles in normal cell survival: either a possible mechanism for non-apoptotic cell death or a survival strategy in times of stress (98). This dual-role phenomenon helps to explain the complex relationship between autophagy and cancer. In one way, autophagy can suppress cancer development by inducing autophagic cell death, or activating apoptosis (99, 105). Conversely, autophagy can protect cancer cells from apoptosis during stress, such as chemotherapy (99, 106). This protection can decrease efficacy of chemotherapeutic agents.

Given that silvestrol can induce both autophagy and apoptosis, future studies should address the relationship between these two pathways as well as the role of each of them plays in cell death. A widely used method to sort out the relative contributions that these two pathways to silvestrol-induced death is to inhibit one pathway and study the other. For example, we can use Z-VAD-FMK, a pan-caspase inhibitor, to inhibit apoptosis, and study the induction of autophagy and apoptosis-independent cell death. Also, we can use 3-methyladenine, which blocks autophagosome formation via the inhibition of type III phosphatidylinositol 3-kinase, to inhibit autophagy and study the induction of autophagy-unrelated apoptosis induction and cell death.

CITED LITERATURES

1. Kinghorn AD, Carcache de Blanco EJ, Chai HB, Orjala J, Farnsworth NR, et al. 2009. *Pure Appl Chem* 81: 1051-63
2. Newman D, Cragg G. 2007. *J. Nat. Prod.* 70: 461 – 77
3. Holbeck SL, Collins JM, Doroshow JH. 2010. *Mol Cancer Ther* 9: 1451-60
4. Orłowski RZ, Kuhn DJ. 2008. *Clin Cancer Res* 14: 1649-57
5. Chauhan D, Hideshima T, Anderson KC. 2005. *Annu Rev Pharmacol Toxicol* 45: 465-76
6. Weinberg RA. 2007. *The biology of Cancer*: Garland Science
7. Clark AM. 1996. *Pharmaceutical Research* 13: 1133-41
8. Newman DJ. 2003. *J. Nat. Prod.* 66: 1022-37
9. Cragg GM, Newman DJ, Snader KM. 1997. *Journal of Natural Products* 60: 52-60
10. UniProt. Species *Nostoc* sp. (strain PPC 7120/UTEX 2576). UniProt
11. Speer BR. 1995. Life History and Ecology of Cyanobacteria. University of California Museum of Paleontology
12. Speer BR. 1995. Introduction to the Cyanobacteria. University of California Museum of Paleontology
13. Choi H, Engene N, Smith JE, Preskitt LB, Gerwick WH. 2009. *Journal of Natural Products* 73: 517-22
14. Dodds WK, Gudder DA. 1995. *Journal of Phycology* 31: 2-18
15. Gao K, Qiu B, Xia J, Yu A. 1998. *Journal of Applied Phycology* 10: 51-3
16. Chilipala G. 2012. Cyanos Database. University of Illinois at Chicago
17. Friedmann EI. 2008. UTEX The Culture Collection of Algae. University of Texas at Austin: University of Texas at Austin
18. Stratmann K, Burgoyne DL, Moore RE, Patterson GML. 1994. *J. Org Chem* 59: 7219-26
19. Majumder S, Dutta P, Mukherjee P, Datta ER, Efferth T, et al. 2006. *Cancer Lett* 244: 16-23
20. Gillet JP, Gottesman MM. 2012. *Drug Resist Updat* 15: 2-4
21. German UA. 1996. *European Journal of Cancer* 32: 927-44
22. Beck WT, Cirtain MC, Mary K. Danks, Felsted RL, Safa AR, et al. 1987. *Cancer Res* 47: 5455-60

23. Endicott JA, Ling V. 1989. *Annual Review of Biochemistry* 58: 137-71
24. Ambudkar SV, Dey S, Hrycyna CA, Ramachandra M, Pastan I, Gottesman MM. 1999. *Annual Review of Pharmacology and toxicology* 39: 361-98
25. Ambudkar SV, Kimchi-Sarfaty C, Sauna ZE, Gottesman MM. 2003. *Oncogene* 22: 7468-85
26. Goodsell D. 2010. Protein Data Bank. ed. D Goodsell. <http://www.rcsb.org/pdb/101/motm.do?momID=123>
27. Ramachandra M, Ambudkar SV, Chen D, Hrycyna CA, Dey S, et al. 1998. *Biochemistry* 37: 5010-9
28. Holland ML, Panetta JA, Hoskins JM, Bebawy M, Roufogalis BD, et al. 2006. *Biochem Pharmacol* 71: 1146-54
29. Beck WT, Mueller TJ, Tanzer LR. 1979. *Cancer Research* 39: 2070-6
30. Beck WT, Cirtain MC, Danks MK, Felsted RL, Safa AR, et al. 1987. *Cancer Research* 47: 5455-60
31. Beck WT, Morgan SE, Mo YY, Bhat UG. 1999. *Drug Resist Updat* 2: 382-9
32. Palmeira A, Vasconcelos MH, Paiva A, Fernandes MX, Pinto M, Sousa E. 2012. *Biochem Pharmacol* 83: 57-68
33. Wu H, Hait WN, Yang J-M. 2003. *Cancer Res* 63: 1515-9
34. Kim HG, Hien TT, Han EH, Hwang YP, Choi JH, et al. 2011. *Br J Pharmacol* 162: 1096-108
35. Sachs CW, Safa AR, Harrison SD, Fine RL. 1995. *The Journal of Biological Chemistry* 270: 26639-48
36. Orłowski S, Mir LM, JR. JB, Garrigos M. 1996. *Biochemical Journal* 317: 515-22
37. Nabekura T, Yamaki T, Ueno K, Kitagawa S. 2008. *Cancer Chemother Pharmacol* 62: 867-73
38. Kawai C, Konishi T, Matsuyama E, Okazaki H. 1981. *Circulation* 63: 1035-42
39. Tsuruo T, Iida H, Tsukagoshi S. 1981. *Cancer Research* 41: 1967-72
40. Rogan AM, Hamilton TC, Young RC, Klecker RW. 1984. *Science* 224: 994-6
41. 2006.
42. Yusa K, Tsuruo T. 1989. *Cancer Research* 49: 5002-6
43. Dreyfuss M, Harerri E, Hofmann H, Kobel H, Pache W, Tscherter H. 1976. *Eur J Appl Microbiol* 3: 125-33
44. Schreiber SL, Crabtree GR. 1992. *Immunology Today* 13: 136-42

45. Sigal NH, Dumont FJ. 1992. *Annu Rev Immunol* 10: 519-60
46. Slater LM, Sweet P, Stupecky M, Gupta S. 1986. *Journal of Clinical Investigation* 77: 1405-8
47. Slater LM, Sweet P, Stupecky M, Wetzel MW, Gupta S. 1986. *British Journal of Cancer* 54: 235-8
48. Keller RP, Altermatt HJ, Nooter K, Poschmann G, Laissie JA, et al. 1992. *International Journal of Cancer* 50: 593-7
49. Foxwell BMJ, Mackie A, Ling V, Ryffel B. 1989. *Molecular Pharmacology* 36: 543-6
50. Wang Q, Beck WT. 1998. *Cancer Research* 58
51. Chen M, Beck WT. 1995. *Oncology Research* 7: 103-11
52. Pires MM, Emmert D, Hrycyna CA, Chmielewski J. 2009. *Mol Pharmacol* 75: 92-100
53. Muller C, Bailly JD, Goubin F, Laredo J, Jaffrezou JP, et al. 1994. *International Journal of Cancer* 56: 749-54
54. Aleman C, Annereau J-P, Liang X-J, Cardarelli CO, Taylor B, et al. 2003. *Cancer Research* 63: 3084-91
55. Wang E-J, Casciano CN, Clement RP, Johnson WW. 2000. *Drug Metabolism and Disposition* 25: 522-8
56. Szakacs G, Paterson JK, Ludwig JA, Booth-Genthe C, Gottesman MM. 2006. *Nat Rev Drug Discov* 5: 219-34
57. McHugh K, Callaghan R. 2008. *Multidrug Resistance: Biological and Pharmaceutical Advances in Antitumour Treatment*: Research Signpost
58. Krishna R, Mayer LD. 2000. *European Journal of Pharmaceutical Sciences* 11: 265-83
59. Thomas H, Coley HM. 2003. *Cancer Control* 10: 159-65
60. Pusztai L, Wagner P, Ibrahim N, Rivera E, Theriault R, et al. 2005. *Cancer* 104: 682-91
61. Coley HM. 2008. *Cancer Treat Rev* 34: 378-90
62. Deferme S, Gelder JV, Augustijns P. 2002. *Journal of Pharmacy and Pharmacology* 54: 1213-9
63. Chlipala GE, Mo S, Orjala J. 2011. *Current Drug Targets* 12: 1654-73
64. Pascaud C, Garrigos M, Orlowski S. 1998. *Biochemical Journal* 333: 351-8
65. Ayesb S, Shao Y-M, Stein WD. 1996. *Biochimica et Biophysica Acta* 1316: 8-18
66. Martin C, Berridge G, Mistry P, Higgins C, Charlton P, Callaghan R. 1999. *British Journal of Pharmacology* 128: 403-11
67. Fox E, Bates SE. 2007. *Expert Review of Anticancer Therapy* 7: 447-59

68. Mi Q, Pezzuto. JM, Farnsworth NR, Wani MC, Kinghorn AD, Swanson SM. 2009. *J Nat Prod* 72: 573-80
69. Hwang B-Y, Su B-N, Kinghorn AD. 2004. *J. Org Chem* 69: 3350-8
70. Flynn B. 2011. Anticancer drug discovery: synthesis and SAR studies of novel rocaglate analogues using a polycyclisation cascade. Monash University
71. Mi Q, Kim S, Hwang BY, Su B-N, Chai H, et al. 2006. *Anticancer Research* 26: 3349-56
72. Bordeleu M-E, Robert F, Gerard B, Lindqvist L, Chen SMH, et al. 2008. *Journal of Clinical Investigation* 118: 2651-60
73. Kozak M. 1991. *The Journal of Biological Chemistry* 266: 19867-70
74. Pestova TV, Kolupaeva VG, Lomakin IB, Pilipenko EV, Shatsky IN, et al. 2001. *Proc Natl Acad Sci U S A* 98: 7029-36
75. Cencic R, Carrier M, Galicia-Vazquez G, Bordeleau ME, Sukarieh R, et al. 2009. *PLoS One* 4: e5223
76. Elmore S. 2007. *Toxicol Pathol* 35: 495-516
77. Galluzzi L, Maiuri MC, Vitale I, Zischka H, Castedo M, et al. 2007. *Cell Death Differ* 14: 1237-43
78. Kroemer G, El-Deiry WS, Golstein P, Peter ME, Vaux D, et al. 2005. *Cell Death Differ* 12 Suppl 2: 1463-7
79. Ashkenazi A. 2008. *Cytokine Growth Factor Rev* 19: 325-31
80. Fulda S, Debatin KM. 2006. *Oncogene* 25: 4798-811
81. Chalhoub N, Baker SJ. 2009. *Annu Rev Pathol* 4: 127-50
82. Fresno Vara JA, Casado E, de Castro J, Cejas P, Belda-Iniesta C, Gonzalez-Baron M. 2004. *Cancer Treat Rev* 30: 193-204
83. Bozulic L, Hemmings BA. 2009. *Curr Opin Cell Biol* 21: 256-61
84. Tokunaga E, Oki E, Egashira A, Sadanaga N, Morita M, et al. 2008. *Current Cancer Drug Targets* 8
85. Potter CJ, Pedraza LG, Xu T. 2002. *Nat Cell Biol* 4: 658-65
86. Huang J, Manning BD. 2009. *Biochem Soc Trans* 37: 217-22
87. Chong-Kopera H, Inoki K, Li Y, Zhu T, Garcia-Gonzalo FR, et al. 2006. *J Biol Chem* 281: 8313-6
88. Manning B. 2003. *Trends in Biochemical Sciences* 28: 573-6
89. Long X, Lin Y, Ortiz-Vega S, Yonezawa K, Avruch J. 2005. *Curr Biol* 15: 702-13

90. Hay N, Sonenberg N. 2004. *Genes Dev* 18: 1926-45
91. Moerke NJ, Aktas H, Chen H, Cantel S, Reibarkh MY, et al. 2007. *Cell* 128: 257-67
92. Shaw RJ, Cantley LC. 2006. *Nature* 441: 424-30
93. Guertin DA, Sabatini DM. 2007. *Cancer Cell* 12: 9-22
94. Gottlieb TM, Leal JFM, Seger R, Taya Y, Oren M. 2002. *Oncogene* 21: 5
95. Franke TF, Hornik CP, Segev L, Shostak GA, Sugimoto C. 2003. *Oncogene* 22: 8983-98
96. Zhou H, Li X, Meinkoth J, Pittman RN. 2000. *Journal of Cell Biology* 151: 483-94
97. Kim AH, Khursigara G, Sun X, Franke TF, Chao MV. 2001. *Mol Cell Biol* 21: 893-901
98. Edinger AL, Thompson CB. 2004. *Curr Opin Cell Biol* 16(6): 663-9
99. Mizushima N, Levine B, Cuervo AM, Klionsky DJ. 2008. *Nature* 451: 1069-75
100. Gozuacik D, Kimchi A. 2004. *Oncogene* 23: 2891-906
101. Levine B, Yuan J. 2005. *J Clin Invest* 115: 2679-88
102. Lum JJ, DeBerardinis RJ, Thompson CB. 2005. *Nat Rev Mol Cell Biol* 6: 439-48
103. Meijer AJ, Codogno P. 2004. *Int J Biochem Cell Biol* 36: 2445-62
104. Todde V, Veenhuis M, van der Klei IJ. 2009. *Biochim Biophys Acta* 1792: 3-13
105. Edinger AL, Thompson CB. 2003. *Cancer Cell*: 3
106. Maiuri MC, Tasdemir E, Criollo A, Morselli E, Vicencio JM, et al. 2009. *Cell Death Differ* 16: 87-93
107. Suh Y, Afaq F, Khan N, Johnson JJ, Khusro FH, Mukhtar H. 2010. *Carcinogenesis* 31: 1424-33
108. Kroemer G, Levine B. 2008. *Nat Rev Mol Cell Biol* 9: 1004-10
109. Debnath J, Baehrecke EH, Kroemer G. 2005. *Autophagy* 1: 66-74
110. Eskelinen EL, Saftig P. 2009. *Biochim Biophys Acta* 1793: 664-73
111. Hait WN, Jin S, Yang JM. 2006. *Clin Cancer Res* 12: 1961-5
112. Choi Y, Zhang J, Murga C, Yu H, Koller E, et al. 2002. *Oncogene* 21: 12
113. Franke TF, Kaplan DR, Cantly LC. 1997. *Cell* 88: 3
114. Kimura S, Fujita N, Noda T, Yoshimori T. 2009. 452: 1-12
115. Liang XH, Jackson S, Seaman M, Brown K, Kempkes B, et al. 1999. *Nature* 402: 672-6
116. Fimia GM, Stoykova A, Romagnoli A, Giunta L, Di Bartolomeo S, et al. 2007. *Nature* 447: 1121-5

117. Reggiori F, Klionsky DJ. 2002. *Eukaryotic Cell* 1: 11-21
118. Marino G, Uria JA, Puente XS, Quesada V, Bordallo J, Lopez-Otin C. 2003. *J Biol Chem* 278: 3671-8
119. Sou YS, Waguri S, Iwata J, Ueno T, Fujimura T, et al. 2008. *Mol Biol Cell* 19: 4762-75
120. Levine B, Deretic V. 2007. *Nat Rev Immunol* 7: 767-77
121. Codogno P, Meijer AJ. 2005. *Cell Death Differ* 12 Suppl 2: 1509-18
122. Rouschop KM, van den Beucken T, Dubois L, Niessen H, Bussink J, et al. 2010. *J Clin Invest* 120: 127-41
123. Kim J, Kundu M, Viollet B, Guan KL. 2011. *Nat Cell Biol* 13: 132-41
124. 2012 NCI Experimental Therapeutics Program. In: National Cancer Institute
125. 2010 NExT STAGE GATES. In: National Cancer Institute
126. Doroshow, JH. 2009. The NExT Steps in Drug Development at NCI. In: National Cancer Institute
127. Pullen N, Thomas G. 1997. *FEBS Lett.* 410: 78-82
128. Peterson RT, Schreiber SL. 1998. *Curr Biol.* 8(7): R248-50
129. Ferrari S, Bandi HR, Hofsteenge J, Bussian BM, Thomas G. 1991. *J Biol Chem.* 266(33): 22770-5
130. Dinh TQ, Du X, Smith CD, Armstrong RW. 1997. *J Org Chem.* 62:6773-83.
131. O'Connell CE, Salvato KA, Meng Z, Littlefield BA, Schwartz CE. 1999. *Bioorg Med Chem Lett.* 1541-6.
132. Frank MB. 2002. *J Natl Cancer Inst* 94(2): 78-79.
133. Arlt A, Bauer I, Schafmayer C, Tepel J, Brosch M, Mürk öster SS, Röder C, Kalthoff H, Hampe J, Moyer PM, Fölsch RU and Sch äfe H. 2002. *J Natl Cancer Inst* 94(2): 78-79.

APPENDICES

APPENDIX I Cytotoxic screening results

| Institute | Sample ID | Activity (% Inhibition) | | Submission Date (mm/dd/yyyy) |
|-------------------|-----------|-------------------------|---------|---------------------------------|
| | | 20 µg/ml | 2 µg/ml | |
| UNCG Oberlies Lab | 103-sm | 17 | 3 | 02/30/2012 |
| UNCG Oberlies Lab | 103-1 | 33 | 30 | 02/30/2012 |
| UNCG Oberlies Lab | 103-2 | 22 | 10 | 02/30/2012 |
| UNCG Oberlies Lab | 103-3 | 46 | 47 | 02/30/2012 |
| UNCG Oberlies Lab | 103-4 | 59 | 20 | 02/30/2012 |
| UNCG Oberlies Lab | 104-sm | 99 | 83 | 02/30/2012 |
| UNCG Oberlies Lab | 104-1 | 18 | 16 | 02/30/2012 |
| UNCG Oberlies Lab | 104-2 | 98 | 94 | 02/30/2012 |
| UNCG Oberlies Lab | 104-3 | 88 | 15 | 02/30/2012 |
| UNCG Oberlies Lab | 104-4 | 96 | 76 | 02/30/2012 |
| UNCG Oberlies Lab | 105-sm | 36 | 33 | 02/30/2012 |
| UNCG Oberlies Lab | 105-1 | 15 | 5 | 02/30/2012 |
| UNCG Oberlies Lab | 105-2 | 39 | 32 | 02/30/2012 |
| UNCG Oberlies Lab | 105-3 | 0 | 3 | 02/30/2012 |
| UNCG Oberlies Lab | 106-sm | 64 | 15 | 02/30/2012 |
| UNCG Oberlies Lab | 106-1 | 12 | 8 | 02/30/2012 |
| UNCG Oberlies Lab | 106-2 | 88 | 21 | 02/30/2012 |
| UNCG Oberlies Lab | 106-3 | 74 | 15 | 02/30/2012 |
| UNCG Oberlies Lab | 107-sm | 37 | 38 | 02/30/2012 |
| UNCG Oberlies Lab | 107-1 | 16 | 11 | 02/30/2012 |
| UNCG Oberlies Lab | 107-2 | 43 | 25 | 02/30/2012 |
| UNCG Oberlies Lab | 107-3 | 53 | 63 | 02/30/2012 |
| UNCG Oberlies Lab | 107-4 | 58 | 48 | 02/30/2012 |
| UNCG Oberlies Lab | 108-sm | 99 | 97 | 02/30/2012 |
| UNCG Oberlies Lab | 108-1 | 1 | 1 | 02/30/2012 |
| UNCG Oberlies Lab | 108-2 | 99 | 88 | 02/30/2012 |
| UNCG Oberlies Lab | 108-3 | 96 | 99 | 02/30/2012 |
| UNCG Oberlies Lab | 108-4 | 96 | 13 | 02/30/2012 |
| UNCG Oberlies Lab | 109-sm | 88 | 86 | 02/30/2012 |
| UNCG Oberlies Lab | 109-1 | 1 | 2 | 02/30/2012 |
| UNCG Oberlies Lab | 109-2 | 17 | 17 | 02/30/2012 |
| UNCG Oberlies Lab | 109-3 | 93 | 35 | 02/30/2012 |
| UNCG Oberlies Lab | 109-4 | 91 | 94 | 02/30/2012 |
| UNCG Oberlies Lab | 110-sm | 94 | 43 | 02/30/2012 |
| UNCG Oberlies Lab | 110-1 | 28 | 23 | 02/30/2012 |
| UNCG Oberlies Lab | 110-2 | 2 | 0 | 02/30/2012 |
| UNCG Oberlies Lab | 110-3 | 10 | 8 | 02/30/2012 |
| UNCG Oberlies Lab | 110-4 | 97 | 96 | 02/30/2012 |

APPENDIX I (Continued)

| | | | | |
|-------------------|--------|----|----|------------|
| UNCG Oberlies Lab | 111-sm | 90 | 84 | 02/30/2012 |
| UNCG Oberlies Lab | 111-1 | 0 | 8 | 02/30/2012 |
| UNCG Oberlies Lab | 111-2 | 23 | 10 | 02/30/2012 |
| UNCG Oberlies Lab | 111-3 | 14 | 0 | 02/30/2012 |
| UNCG Oberlies Lab | 111-4 | 94 | 94 | 02/30/2012 |
| UNCG Oberlies Lab | 112-sm | 66 | 18 | 02/30/2012 |
| UNCG Oberlies Lab | 112-1 | 0 | 0 | 02/30/2012 |
| UNCG Oberlies Lab | 112-2 | 74 | 55 | 02/30/2012 |
| UNCG Oberlies Lab | 112-3 | 98 | 84 | 02/30/2012 |
| UNCG Oberlies Lab | 112-4 | 50 | 4 | 02/30/2012 |
| UNCG Oberlies Lab | 113-sm | 91 | 23 | 02/30/2012 |
| UNCG Oberlies Lab | 113-1 | 7 | 6 | 02/30/2012 |
| UNCG Oberlies Lab | 113-2 | 87 | 60 | 02/30/2012 |
| UNCG Oberlies Lab | 113-3 | 1 | 1 | 02/30/2012 |
| UNCG Oberlies Lab | 113-4 | 59 | 17 | 02/30/2012 |
| UNCG Oberlies Lab | 115-sm | 98 | 0 | 02/30/2012 |
| UNCG Oberlies Lab | 115-1 | 29 | 24 | 02/30/2012 |
| UNCG Oberlies Lab | 115-2 | 98 | 30 | 02/30/2012 |
| UNCG Oberlies Lab | 115-3 | 35 | 31 | 02/30/2012 |
| UNCG Oberlies Lab | 116-sm | 99 | 56 | 02/30/2012 |
| UNCG Oberlies Lab | 116-1 | 96 | 37 | 02/30/2012 |
| UNCG Oberlies Lab | 116-2 | 99 | 99 | 02/30/2012 |
| UNCG Oberlies Lab | 116-3 | 8 | 23 | 02/30/2012 |
| UNCG Oberlies Lab | 117-sm | 44 | 28 | 02/30/2012 |
| UNCG Oberlies Lab | 117-1 | 0 | 22 | 02/30/2012 |
| UNCG Oberlies Lab | 117-2 | 83 | 17 | 02/30/2012 |
| UNCG Oberlies Lab | 117-3 | 96 | 41 | 02/30/2012 |
| UNCG Oberlies Lab | 117-4 | 79 | 36 | 02/30/2012 |
| UNCG Oberlies Lab | 118-sm | 41 | 6 | 02/30/2012 |
| UNCG Oberlies Lab | 118-1 | 19 | 26 | 02/30/2012 |
| UNCG Oberlies Lab | 118-2 | 94 | 26 | 02/30/2012 |
| UNCG Oberlies Lab | 118-3 | 40 | 11 | 02/30/2012 |
| UNCG Oberlies Lab | 118-4 | 21 | 0 | 02/30/2012 |
| UNCG Oberlies Lab | 119-sm | 98 | 59 | 02/30/2012 |
| UNCG Oberlies Lab | 119-1 | 98 | 89 | 02/30/2012 |
| UNCG Oberlies Lab | 119-2 | 97 | 70 | 02/30/2012 |
| UNCG Oberlies Lab | 119-3 | 82 | 13 | 02/30/2012 |
| UNCG Oberlies Lab | 119-4 | 2 | 1 | 02/30/2012 |
| UNCG Oberlies Lab | 120-sm | 40 | 0 | 02/30/2012 |
| UNCG Oberlies Lab | 120-1 | 2 | 0 | 02/30/2012 |
| UNCG Oberlies Lab | 120-2 | 46 | 25 | 02/30/2012 |
| UNCG Oberlies Lab | 120-3 | -7 | 7 | 02/30/2012 |

APPENDIX I (Continued)

| | | | | |
|-------------------|--------------|-----|-----|------------|
| UNCG Oberlies Lab | 120-4 | -3 | 0 | 02/30/2012 |
| UNCG Oberlies Lab | 121-sm | 93 | 64 | 02/30/2012 |
| UNCG Oberlies Lab | 121-1 | -7 | 7 | 02/30/2012 |
| UNCG Oberlies Lab | 121-2 | 93 | 75 | 02/30/2012 |
| UNCG Oberlies Lab | 121-3 | 93 | 59 | 02/30/2012 |
| UNCG Oberlies Lab | 121-4 | 64 | 38 | 02/30/2012 |
| UNCG Oberlies Lab | 122-sm | 82 | 59 | 02/30/2012 |
| UNCG Oberlies Lab | 122-1 | 43 | 37 | 02/30/2012 |
| UNCG Oberlies Lab | 122-2 | 97 | 43 | 02/30/2012 |
| UNCG Oberlies Lab | 122-3 | 60 | 52 | 02/30/2012 |
| UNCG Oberlies Lab | 122-4 | 47 | 39 | 02/30/2012 |
| UNCG Oberlies Lab | 122-5 | 40 | 11 | 02/30/2012 |
| UNCG Oberlies Lab | 01009-131-1 | 71 | 44 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-2 | 7 | 6 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-3 | 49 | 0 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-4 | 59 | 6 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-5 | 50 | 5 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-6 | 57 | 1 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-7 | 91 | 20 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-8 | 34 | 21 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-9 | 60 | 31 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-10 | 36 | 30 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-11 | 91 | 89 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-12 | 6 | 0 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-13 | 92 | 82 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-14 | 94 | 31 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-15 | 83 | 14 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-16 | 92 | 84 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-17 | 100 | 83 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-18 | 100 | 76 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-19 | 24 | 14 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-20 | 83 | 16 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-21 | 75 | 7 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-22 | 0 | 0 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-23 | 71 | 11 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-24 | 7 | 7 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-25 | 0 | 0 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-26 | 99 | 99 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-27 | 4 | 4 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-28 | 100 | 100 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-29 | 6 | 0 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-30 | 5 | 0 | 03/19/2012 |

APPENDIX I (Continued)

| | | | | |
|-------------------|---------------|-----|----|------------|
| UNCG Oberlies Lab | 01009-131-31 | 6 | 4 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-32 | 41 | 1 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-33 | 0 | 0 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-34 | 0 | 0 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-35 | 77 | 43 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-36 | 13 | 9 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-37 | 63 | 1 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-38 | 94 | 28 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-39 | 0 | 6 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-40 | 0 | 4 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-41 | 36 | 0 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-42 | 78 | 6 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-43 | 10 | 4 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-44 | 0 | 0 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-45 | 0 | 0 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-46 | 0 | 0 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-47 | 0 | 0 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-48 | 0 | 0 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-49 | 5 | 4 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-50 | 29 | 20 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-51 | 7 | 9 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-52 | 2 | 0 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-53 | 0 | 0 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-54 | 31 | 17 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-55 | 46 | 48 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-56 | 45 | 42 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-57 | 12 | 14 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-58 | 7 | 4 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-59 | 8 | 5 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-60 | 100 | 13 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-61 | 5 | 4 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-62 | 0 | 0 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-63 | 4 | 0 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-64 | 0 | 0 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-65 | 59 | 12 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-66A | 94 | 87 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-131-67V | 83 | 60 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-137-sm | 6 | 6 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-137-1 | 2 | 6 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-137-2 | 19 | 3 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-137-3 | 0 | 0 | 03/19/2012 |
| UNCG Oberlies Lab | 01009-137-4 | 5 | 18 | 03/19/2012 |

APPENDIX I (Continued)

| | | | | |
|----------------|------------|------|---|------------|
| UIC Orjala Lab | WI53-P1 | N/A | X | 01/27/2010 |
| UIC Orjala Lab | WI53-P2 | N/A | X | 01/27/2010 |
| UIC Orjala Lab | 369-40-1 | N/A | X | 01/27/2010 |
| UIC Orjala Lab | 369-40-2 | N/A | X | 01/27/2010 |
| UIC Orjala Lab | 10056-70-2 | N/A | X | 01/27/2010 |
| UIC Orjala Lab | 10056-70-3 | N/A | X | 01/27/2010 |
| UIC Orjala Lab | 10045-F3 | 91.9 | X | 01/27/2010 |
| UIC Orjala Lab | 10109-F2 | N/A | X | 01/27/2010 |
| UIC Orjala Lab | 10109-F3 | 92.2 | X | 01/27/2010 |
| UIC Orjala Lab | 10109-F4 | 92.0 | X | 01/27/2010 |
| UIC Orjala Lab | 10109-F5 | N/A | X | 01/27/2010 |
| UIC Orjala Lab | 10109-F6 | N/A | X | 01/27/2010 |
| UIC Orjala Lab | 10109-F7 | N/A | X | 01/27/2010 |
| UIC Orjala Lab | 10109-F8 | N/A | X | 01/27/2010 |

APPENDIX II Proteasome inhibition screening results

| Institute | Sample ID | Activity (% Inhibition) | | Submission Date (mm/dd/yyyy) |
|------------------|-----------|----------------------------|---------|---------------------------------|
| | | 20 µg/ml | 5 µg/ml | |
| OSU Kinghorn Lab | A06424 | 23 | 32 | |
| OSU Kinghorn Lab | A06425 | 21 | 19 | |
| OSU Kinghorn Lab | A06426 | 18 | 25 | |
| OSU Kinghorn Lab | A06427 | 9 | 23 | |
| OSU Kinghorn Lab | A06428 | 11 | 24 | |
| OSU Kinghorn Lab | A06429 | 23 | 16 | |
| OSU Kinghorn Lab | A06430 | 22 | 21 | |
| OSU Kinghorn Lab | A06431 | 14 | 19 | |
| OSU Kinghorn Lab | A06432 | 11 | 16 | |
| OSU Kinghorn Lab | A06433 | 22 | 21 | |
| OSU Kinghorn Lab | A06434 | 8 | 18 | |
| OSU Kinghorn Lab | A06435 | 0 | 4 | |
| OSU Kinghorn Lab | A06436 | 0 | 0 | |
| OSU Kinghorn Lab | A06437 | 8 | 10 | |
| OSU Kinghorn Lab | A06438 | 15 | 26 | |
| OSU Kinghorn Lab | A06439 | 6 | 12 | |
| OSU Kinghorn Lab | A06440 | 12 | 7 | |
| OSU Kinghorn Lab | A06441 | 8 | 0 | |
| OSU Kinghorn Lab | A06442 | 25 | 17 | |
| OSU Kinghorn Lab | A06443 | 19 | 6 | |
| OSU Kinghorn Lab | A06444 | 25 | -3 | |
| OSU Kinghorn Lab | A06445 | 24 | 8 | |
| OSU Kinghorn Lab | A06446 | 15 | 2 | |
| OSU Kinghorn Lab | A06447 | 10 | 8 | |
| OSU Kinghorn Lab | A06448 | 24 | 31 | |
| OSU Kinghorn Lab | A06449 | 13 | 9 | |
| OSU Kinghorn Lab | A06450 | 22 | 12 | |
| OSU Kinghorn Lab | A06451 | 11 | 15 | |
| OSU Kinghorn Lab | A06452 | 19 | 9 | |
| OSU Kinghorn Lab | A06453 | 35 | 31 | |
| OSU Kinghorn Lab | A06454 | 24 | 14 | |
| OSU Kinghorn Lab | A06455 | 21 | 8 | |
| OSU Kinghorn Lab | A06456 | 21 | 27 | |
| OSU Kinghorn Lab | A06457 | 17 | 16 | |
| OSU Kinghorn Lab | A06458 | 4 | 9 | |
| OSU Kinghorn Lab | A06459 | 8 | 15 | |
| OSU Kinghorn Lab | A06460 | 6 | 20 | |
| OSU Kinghorn Lab | A06461 | 4 | 9 | |

APPENDIX II (Continued)

| | | | | |
|------------------|--------|----|----|--|
| OSU Kinghorn Lab | A06462 | 11 | 22 | |
| OSU Kinghorn Lab | A06463 | 30 | 33 | |
| OSU Kinghorn Lab | A06464 | 5 | 0 | |
| OSU Kinghorn Lab | A06465 | 21 | 0 | |
| OSU Kinghorn Lab | A06466 | 26 | 19 | |
| OSU Kinghorn Lab | A06467 | 20 | 8 | |
| OSU Kinghorn Lab | A06468 | 24 | 2 | |
| OSU Kinghorn Lab | A06469 | 14 | 0 | |
| OSU Kinghorn Lab | A06470 | 18 | 0 | |
| OSU Kinghorn Lab | A06471 | 21 | 0 | |
| OSU Kinghorn Lab | A06472 | 18 | 17 | |
| OSU Kinghorn Lab | A06473 | 0 | 6 | |
| OSU Kinghorn Lab | A06474 | 0 | 3 | |
| OSU Kinghorn Lab | A06475 | 2 | 4 | |
| OSU Kinghorn Lab | A06476 | 22 | 9 | |
| OSU Kinghorn Lab | A06477 | 47 | 19 | |
| OSU Kinghorn Lab | A06478 | 30 | 30 | |
| OSU Kinghorn Lab | A06479 | 36 | 36 | |
| OSU Kinghorn Lab | A06480 | 39 | 28 | |
| OSU Kinghorn Lab | A06481 | 21 | 13 | |
| OSU Kinghorn Lab | A06482 | 6 | 33 | |
| OSU Kinghorn Lab | A06483 | 14 | 30 | |
| OSU Kinghorn Lab | A06484 | 18 | 24 | |
| OSU Kinghorn Lab | A06485 | 33 | 17 | |
| OSU Kinghorn Lab | A06486 | 8 | 13 | |
| OSU Kinghorn Lab | A06487 | 8 | 0 | |
| OSU Kinghorn Lab | A06488 | 0 | 0 | |
| OSU Kinghorn Lab | A06489 | 4 | 20 | |
| OSU Kinghorn Lab | A06490 | 10 | 22 | |
| OSU Kinghorn Lab | A06491 | 27 | 29 | |
| OSU Kinghorn Lab | A06492 | 41 | 17 | |
| OSU Kinghorn Lab | A06493 | 43 | 16 | |
| OSU Kinghorn Lab | A06494 | 0 | 21 | |
| OSU Kinghorn Lab | A06495 | 6 | 23 | |
| OSU Kinghorn Lab | A06496 | 0 | 28 | |
| OSU Kinghorn Lab | A06497 | 0 | 38 | |
| OSU Kinghorn Lab | A06498 | 0 | 46 | |
| OSU Kinghorn Lab | A06499 | 17 | 39 | |
| OSU Kinghorn Lab | A06500 | 21 | 0 | |
| OSU Kinghorn Lab | A06501 | 37 | 3 | |
| OSU Kinghorn Lab | A06502 | 16 | 13 | |
| OSU Kinghorn Lab | A06503 | 10 | 3 | |

APPENDIX II (Continued)

| | | | | |
|------------------|--------|----|----|--|
| OSU Kinghorn Lab | A06504 | 0 | 0 | |
| OSU Kinghorn Lab | A06505 | 15 | 2 | |
| OSU Kinghorn Lab | A06506 | 6 | 5 | |
| OSU Kinghorn Lab | A06507 | 11 | 0 | |
| OSU Kinghorn Lab | A06508 | 17 | 0 | |
| OSU Kinghorn Lab | A06509 | 17 | 0 | |
| OSU Kinghorn Lab | A06510 | 5 | 3 | |
| OSU Kinghorn Lab | A06511 | 8 | 7 | |
| OSU Kinghorn Lab | A06512 | 1 | 0 | |
| OSU Kinghorn Lab | A06513 | 0 | 0 | |
| OSU Kinghorn Lab | A06514 | 0 | 5 | |
| OSU Kinghorn Lab | A06515 | 0 | 0 | |
| OSU Kinghorn Lab | A06516 | 1 | 6 | |
| OSU Kinghorn Lab | A06517 | 4 | 11 | |
| OSU Kinghorn Lab | A06518 | 0 | 0 | |
| OSU Kinghorn Lab | A06519 | 0 | 4 | |
| OSU Kinghorn Lab | A06520 | 12 | 0 | |
| OSU Kinghorn Lab | A06521 | 20 | 4 | |
| OSU Kinghorn Lab | A06522 | 3 | 0 | |
| OSU Kinghorn Lab | A06523 | 6 | 7 | |
| OSU Kinghorn Lab | A06524 | 3 | 9 | |
| OSU Kinghorn Lab | A06525 | 12 | 9 | |
| OSU Kinghorn Lab | A06526 | 0 | 0 | |
| OSU Kinghorn Lab | A06527 | 0 | 0 | |
| OSU Kinghorn Lab | A06528 | 6 | 5 | |
| OSU Kinghorn Lab | A06529 | 1 | 4 | |
| OSU Kinghorn Lab | A06530 | 0 | 0 | |
| OSU Kinghorn Lab | A06531 | 0 | 0 | |
| OSU Kinghorn Lab | A06532 | 12 | 0 | |
| OSU Kinghorn Lab | A06533 | 13 | 9 | |
| OSU Kinghorn Lab | A06534 | 9 | 9 | |
| OSU Kinghorn Lab | A06535 | 1 | 0 | |
| OSU Kinghorn Lab | A06536 | 0 | 0 | |
| OSU Kinghorn Lab | A06537 | 7 | 0 | |
| OSU Kinghorn Lab | A06538 | 14 | 15 | |
| OSU Kinghorn Lab | A06539 | 3 | 0 | |
| OSU Kinghorn Lab | A06540 | 3 | 0 | |
| OSU Kinghorn Lab | A06541 | 21 | 5 | |
| OSU Kinghorn Lab | A06542 | 2 | 12 | |
| OSU Kinghorn Lab | A06543 | 0 | 11 | |
| OSU Kinghorn Lab | A06544 | 0 | 0 | |
| OSU Kinghorn Lab | A06545 | 0 | 4 | |

APPENDIX II (Continued)

| | | | | |
|------------------|--------|-----|----|------------|
| OSU Kinghorn Lab | A06546 | 3 | 0 | |
| OSU Kinghorn Lab | A06547 | 0 | 16 | |
| OSU Kinghorn Lab | A06548 | 8 | 2 | |
| OSU Kinghorn Lab | A06549 | 10 | 5 | |
| OSU Kinghorn Lab | A06550 | 19 | 9 | |
| OSU Kinghorn Lab | A06551 | 0 | 12 | |
| OSU Kinghorn Lab | A06552 | 0 | 5 | |
| OSU Kinghorn Lab | A06553 | 7 | 6 | |
| OSU Kinghorn Lab | A06554 | 43 | 8 | |
| OSU Kinghorn Lab | A06555 | 13 | 12 | |
| OSU Kinghorn Lab | A06556 | 29 | 20 | |
| OSU Kinghorn Lab | A06557 | 30 | 12 | |
| OSU Kinghorn Lab | A06622 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06623 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06624 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06625 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06626 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06627 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06628 | 77 | X | 02/08/2011 |
| OSU Kinghorn Lab | A06629 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06630 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06631 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06632 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06633 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06634 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06635 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06636 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06637 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06638 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06639 | 94 | X | 02/08/2011 |
| OSU Kinghorn Lab | A06640 | 58 | X | 02/08/2011 |
| OSU Kinghorn Lab | A06641 | 86 | X | 02/08/2011 |
| OSU Kinghorn Lab | A06642 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06643 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06644 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06645 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06646 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06647 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06648 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06649 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06650 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06651 | 81 | X | 02/08/2011 |

APPENDIX II (Continued)

| | | | | |
|------------------|--------|-----|---|------------|
| OSU Kinghorn Lab | A06652 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06653 | 66 | X | 02/08/2011 |
| OSU Kinghorn Lab | A06654 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06655 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06656 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06657 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06658 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06659 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06660 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06661 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06662 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06663 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06664 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06665 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06666 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06667 | 94 | X | 02/08/2011 |
| OSU Kinghorn Lab | A06668 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06669 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06670 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06671 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06672 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06673 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06674 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06675 | 66 | X | 02/08/2011 |
| OSU Kinghorn Lab | A06676 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06677 | 77 | X | 02/08/2011 |
| OSU Kinghorn Lab | A06678 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06679 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06680 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06681 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06682 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06683 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06684 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06685 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06686 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06687 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06688 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06689 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06690 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06691 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06692 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06693 | N/A | X | 02/08/2011 |

APPENDIX II (Continued)

| | | | | |
|------------------|----------------|-----|---|------------|
| OSU Kinghorn Lab | A06694 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06695 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06696 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06697 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06698 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06699 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06700 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06701 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06702 | 46 | X | 02/08/2011 |
| OSU Kinghorn Lab | A06703 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06704 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06705 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06706 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06707 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06708 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06709 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06710 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06711 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06712 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06713 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06714 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06715 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06716 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06717 | N/A | X | 02/08/2011 |
| OSU Kinghorn Lab | A06281D003 | 40 | X | 02/22/2011 |
| OSU Kinghorn Lab | A06281D003F1 | N/A | X | 02/22/2011 |
| OSU Kinghorn Lab | A06281D003F2 | 53 | X | 02/22/2011 |
| OSU Kinghorn Lab | A06281D003F3 | N/A | X | 02/22/2011 |
| OSU Kinghorn Lab | A06281D003F4 | N/A | X | 02/22/2011 |
| OSU Kinghorn Lab | A06281D003F5 | N/A | X | 02/22/2011 |
| OSU Kinghorn Lab | A06333D003 | 98 | X | 02/22/2011 |
| OSU Kinghorn Lab | A06333D003F1 | N/A | X | 02/22/2011 |
| OSU Kinghorn Lab | A06333D003F2 | N/A | X | 02/22/2011 |
| OSU Kinghorn Lab | A06333D003F3 | N/A | X | 02/22/2011 |
| OSU Kinghorn Lab | A06333D003F4 | 99 | X | 02/22/2011 |
| OSU Kinghorn Lab | A06333D003F5 | 82 | X | 02/22/2011 |
| OSU Kinghorn Lab | A06319D26712K1 | N/A | X | 02/22/2011 |
| OSU Kinghorn Lab | AA6061D4-K1 | N/A | X | 02/22/2011 |
| OSU Kinghorn Lab | AA6061D4-K2 | N/A | X | 02/22/2011 |
| OSU Kinghorn Lab | A06723 | 19 | X | 06/06/2011 |
| OSU Kinghorn Lab | A06746 | 21 | X | 06/06/2011 |
| OSU Kinghorn Lab | A06747 | 27 | X | 06/06/2011 |

APPENDIX II (Continued)

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|------------------|--------|----|---|------------|
| OSU Kinghorn Lab | A06748 | 22 | X | 06/06/2011 |
| OSU Kinghorn Lab | A06752 | 49 | X | 06/06/2011 |
| OSU Kinghorn Lab | A06754 | 20 | X | 06/06/2011 |
| OSU Kinghorn Lab | A06768 | 23 | X | 06/06/2011 |
| OSU Kinghorn Lab | A06776 | 23 | X | 06/06/2011 |
| OSU Kinghorn Lab | A06799 | 28 | X | 06/06/2011 |
| OSU Kinghorn Lab | A06810 | 38 | X | 06/06/2011 |
| OSU Kinghorn Lab | A06811 | 51 | X | 06/06/2011 |
| OSU Kinghorn Lab | A06817 | 48 | X | 06/06/2011 |
| OSU Kinghorn Lab | A06819 | 47 | X | 06/06/2011 |
| OSU Kinghorn Lab | A06820 | 34 | X | 06/06/2011 |
| OSU Kinghorn Lab | A06821 | 21 | X | 06/06/2011 |
| OSU Kinghorn Lab | A06823 | 32 | X | 06/06/2011 |
| OSU Kinghorn Lab | A06824 | 26 | X | 06/06/2011 |
| OSU Kinghorn Lab | A06826 | 22 | X | 06/06/2011 |
| OSU Kinghorn Lab | A06827 | 27 | X | 06/06/2011 |
| OSU Kinghorn Lab | A06828 | 35 | X | 06/06/2011 |
| OSU Kinghorn Lab | A06831 | 34 | X | 06/06/2011 |
| OSU Kinghorn Lab | A06832 | 25 | X | 06/06/2011 |
| OSU Kinghorn Lab | A06834 | 36 | X | 06/06/2011 |
| OSU Kinghorn Lab | A06836 | 38 | X | 06/06/2011 |
| OSU Kinghorn Lab | A06837 | 86 | X | 06/06/2011 |
| OSU Kinghorn Lab | A06838 | 79 | X | 06/06/2011 |
| OSU Kinghorn Lab | A06840 | 38 | X | 06/06/2011 |
| OSU Kinghorn Lab | A06841 | 23 | X | 06/06/2011 |
| OSU Kinghorn Lab | A06842 | 20 | X | 06/06/2011 |
| OSU Kinghorn Lab | A06843 | 20 | X | 06/06/2011 |
| OSU Kinghorn Lab | A06844 | 30 | X | 06/06/2011 |
| OSU Kinghorn Lab | A06847 | 20 | X | 06/06/2011 |

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Experience

| | |
|---|-----------------------|
| University of Illinois at Chicago , Rockford, IL Research Specialist , Biopharmaceutical Sciences | Sept. 2012 – present |
| University of Illinois at Chicago , Chicago, IL Research Assistant , Medicinal Chemistry and Pharmacognosy | May 2009 – Aug. 2012 |
| University of Illinois at Chicago , Chicago, IL Research Student , Biological Sciences | Aug. 2008 – May 2009 |
| National Institute of Biological Sciences , Beijing, China Internship | Jul. 2007 – Jun. 2008 |
| China Agricultural University , Beijing, China Lab Trainee | Aug. 2006 – Jun. 2007 |

Education

| | |
|--|---------------------------------|
| University of Illinois at Chicago Master of Science / Dept. of Medicinal Chemistry and Pharmacognosy GPA: 3.6/4.0 Thesis: Natural products anticancer drug discovery and mechanistic studies of hapalosin and silvestrol | Sept. 2009 – May. 2013 expected |
| China Agricultural University Aug. 2004 – Jun. 2008 Bachelor of Science / Dept. of Biological Sciences GPA 3.7/4.0 Thesis: Construction and expression of artificial miRNA in <i>Arabidopsis</i> | |

Publication

-
- Nicholas Oberlies, Mario Figueroa, Tyler Graf, Audrey Adcock, David Kroll, **Jilai Yang**, Steven Swanson, Ulyana Munoz-Acuna, Esperanza Carcache de Blanco, Rajesh Agarwal, Mansukh Wani, Blaise Darveaux, and Cedric Pearce. Cytotoxic Epipolythiodioxopiperazine Alkaloids from Filamentous Fungi of the Bionectriaceae. *The Journal of Antibiotics*. 2012 Sept; 65: 559-564
 - Jilai Yang**, Qi Shen, David J. Newman, A. Douglas Kinghorn and Steven M. Swanson. Silvestrol-inhibits protein synthesis by down regulating the Akt/mTOR pathway. The 103rd annual meeting of American Association of Cancer Research
 - Daniel D. Lantvit, Qi Shen, **Jilai Yang**, Terry G. Unterman and Steven M. Swanson. Disruption of growth hormone signaling improves the efficacy of conventional chemotherapy in a rat model of mammary cancer. The 103rd annual meeting of American Association of Cancer Research.
 - Jilai Yang**, Qi Shen, Steven Swanson. Silvestrol induces apoptosis in cancer cells through AKT dependent pathway. The 52nd annual meeting of American Society of Pharmacognosy
 - Qi Shen, **Jilai Yang**, Jiachen Zi, Jimmy Orjala, and Steven M. Swanson. The novel depsipeptide Hapalosin B is a potent inhibitor of anticancer drug resistance associated with ABCB1 overexpression. The 52nd annual meeting of American Society of Pharmacognosy
 - Zhang H, Ohyama K, Boudet J, Chen Z, **Yang J**, Zhang M, Muranaka T, Maurel C, Zhu JK, Gong Z. Dolichol biosynthesis and its effects on the unfolded protein response and abiotic stress resistance in *Arabidopsis*. *Plant Cell*. 2008 Jul;20(7):1879-98