Identification and Characterization of Potential New Anticancer Drugs from Natural Sources

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THESIS

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TO MY GRANDMOTHER

for her support and love

and for always believing in me.

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WC

CONTRIBUTIONS OF AUTHORS

Chapter I is a literature review that provides background information about the field and emphasizes the importance of my research. Chapter II summarizes the research methods and materials used to execute the experiments. Portions of this chapter have been published previously.[1-13] Chapter III is a published manuscript for which I was the primary author.[1] Chapter IV represents a series of experiments that are currently unpublished. Dr. Jinhong Ren generated figure 15. Dr. Christian Erxleben generated figure 25. Chapter V contains a discussion of the results obtained from these experiments and future directions for the field to explore. Appendix A includes several published manuscripts for which I was one of the contributing author.[2-13] Dr. Jimmy Orjala, Dr. Nick Oberlies, Dr. Chun-Tao Che and Dr. Brian Murphy contributed to the compounds isolation.

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LIST OF ABBREVIATIONS

3MA	3-methyladenine
5-AZA	5-azacytidine
ACD	Autophagic cell death
ACT	Adoptive cell transfer
ADC	Antibody-drug conjugate
AIF	Apoptosis-inducing factor
ALCL	Anaplastic large cell lymphoma
AO	Acridine orange
AR	Androgen receptor
ATG	Autophagy-related gene
BGCs	Biosynthetic gene clusters
CAR	Chimeric antigen receptor
Cas9	CRISPR-associated protein 9
CDC	Centers for Disease Control and Prevention
CID	Chemical inducer of dimerization
CML	Chronic myeloid leukemia
CRISPR	Clustered regularly interspaced short palindromic repeats
DAPI	4',6-diamidino-2-phenylindole
DAQA	Diazaquinomycin A
DHA	Docosahexaenoic acid
DISC	Death-initiated signaling complex
DNMT	DNA methyltransferase

LIST OF ABBREVIATIONS (continued)

DSB	double-strand break
ECL	Enhanced chemiluminescence
EGFR	Epidermal growth factor receptor
eIF	Eukaryotic initiation factor
EPA	Eicosapentaenoic acid
ERα	Estrogen receptor alpha
ERK	Extracellular signal–regulated kinase
ETP	Epipolythiodioxopiperazine
FADD	Fas-Associated protein with Death Domain
FBS	Fetal bovine serum
FDA	U.S. Food and drug administration
FUSION	Functional signature ontology
G418	Geneticin
HDAC	Histone deacetylase
hERG	Human *either-a-go-go* related gene
HGSC	High-grade serous ovarian cancer
HHT	Homoharringtonine
HIF-1α	Hypoxia-inducible factor 1 alpha
HMTase	Histone methyltransferase
Hsp	Heat-shock protein
HTS	High-throughput screening
i.p.	Intraperitoneal

LIST OF ABBREVIATIONS (continued)

K63	Lysine 63
LC3	Light chain 3
LMP	Lysosomal membrane permeabilization
MAPK	Mitogen-activated protein kinase
MAPKKK	MAPK kinase kinase
MDC	Monodansylcadaverine
MEF	Mouse embryonic fibroblast
MOE	Murine oviductal epithelium
MOMP	Mitochondrial outer membrane permeabilization
NCI	National Cancer Institute
NCI-60	National Cancer Institute 60
NExT	National Cancer Institute Experimental Therapeutics
NF-κB	Nuclear factor-κB
NP	Natural product
PARP	Poly (ADP-ribose) polymerase
PCD	Programmed cell death
PDX	Patient-derived xenograft
PE	Phosphatidylethanolamine
PI	Propidium iodide
RAL	Resorcylic acid lactone
ROS	Reactive oxygen species
RTK	Receptor tyrosine kinase

LIST OF ABBREVIATIONS (continued)

SAHA	Suberoylanilide hydroxamic acid
SAR	Structure-activity relationship
SEER	Surveillance, Epidemiology and End-Results
sgRNA	single guide RNA
STS	Soft tissue sarcoma
SV40	Simian virus 40
TAK1	Transforming growth factor (TGF)-β-activating kinase 1
TGF	Transforming growth factor
TNF	Tumor necrosis factor
TNFR	TNF receptor
Topo II	DNA-topoisomerase II
TRADD	Tumor necrosis factor receptor type 1-associated DEATH domain protein
TRAIL	TNF-related apoptosis-inducing ligand
WHO	World health organization

SUMMARY

In 2014, the World Health Organization (WHO) reported that cancer was the leading cause of death worldwide, accounting for 8.2 million deaths in 2012. According to the National Cancer Institute (NCI) and the Centers for Disease Control and Prevention (CDC), cancer ranks second among all causes of death in the United States. The American Cancer Society estimates that there will be 1,685,210 new cases of cancer in 2016, and 595,690 cancer related deaths.[14] Cancer chemotherapy is an established adjuvant to surgery and radiation to treat successfully solid tumors, lymphomas, and leukemias. Although many anticancer drugs are approved and clinically available, the problems of resistance to and toxicity of existing agents necessitates the discovery of new drug leads.

For decades, secondary metabolites from plants, fungi and bacteria have been found to exhibit potent anticancer activity. Natural products are selected by evolution to reach their molecular target within cells and to interact with biologically active protein folds. Secondary metabolites may serve their producing organisms by improving their competitive strength. Natural products have been essential components of drug discovery and are direct sources of small molecule therapies and scaffolds for semisynthesis. Driven by the need for new anticancer targets, this thesis focuses on exploring new molecules for the novel regulation in cell death to find compounds with good specificity and potency against cancer cells without altering normal cells.

Silvestrol is a cyclopenta[b]benzofuran that was isolated from the fruits and twigs of *Aglaia foveolata*, which is indigenous to the island states of Southeast Asia. Previous testing of silvestrol revealed that it is a potent inhibitor of protein synthesis and has

cytotoxic activity similar to or more potent than many Food and Drug Administration (FDA) approved anticancer agents. Silvestrol treatment of cultured human cell lines derived from melanoma (MDA-MB-435) or colon cancer (HT-29) induced cell cycle arrest at the G2 stage. Silvestrol treatment also induced caspase-3 activation and apoptotic cell death in a time- and concentration-dependent manner. Treatment of ATG7-null mouse embryonic fibroblast (MEF) cells with silvestrol resulted in impaired transformation of LC3-I to LC3-II. This suggests that silvestrol caused a conventional autophagy response that ultimately progressed to apoptosis.

Strebloside was isolated from the stem bark of *Streblus asper* in Southeast Asia, and its chemical structure shares a common cardiac glycoside structural motif. Strebloside was studied in a hollow fiber assay to determine if it had any potency in vivo. Strebloside was able to inhibit the growth of ovarian cancer cell model OVCAR3 and also triple negative breast cancer cell model MDA-MB-231. From these data, ovarian cancer models were chosen due to its high sensitivity to strebloside. Strebloside was active in OVSAHO, OVCAR4 and OVCAR5, but was most potent in OVCAR3. Furthermore, strebloside blocked cell cycle progression at the G2-phase in OVCAR3 cells after 72 h treatment at 100 nM. Strebloside treatment also induced PARP cleavage indicating apoptosis activation in OVCAR3 after 72 h at 100 nM. Strebloside potently inhibited cell growth and induced cell death in human ovarian cancer cells through induction of caspase-mediated apoptosis. Strebloside also behaved in a manner consistent with being a cardiac glycoside, which might limit its clinical use.

Natural products have been essential components of anticancer drug discovery; they serve both as a direct source of small molecule therapies and as an inspiration for the semisynthesis of novel, biologically active derivatives. Bioassay-guided fractionation and isolation provide the screening platform to identify unique chemical structures. Extracts and fractions from collected plants, fungi, cyanobacteria and actinomycetes were screened in a panel of cancer cell lines derived from melanoma, colon cancer, breast cancer and ovarian cancer. Several potential drug leads have been isolated and their structures elucidated. Among these potential drug leads, phyllanthusmin and verticillin A showed potent anticancer activity. However, their mechanisms of action require further investigation.

The mechanism by which silvestrol and strebloside block cell proliferation and induce apoptosis deserves further study. The targets of both silvestol and strebloside are different from conventional chemotherapeutic agents. The possibility of use new targets for anticancer therapies can decrease drug resistance, providing better healthcare to human beings. Based on the cytotoxic potential of these drug leads, they may provide a novel and promising strategy to improve cancer therapy. Structurally complex natural products are being isolated from diverse biological organisms living in aquatic and terrestrial ecosystem all over the world. Overall, anticancer drug discovery from natural sources remains an important approach for novel drug development.

I. INTRODUCTION

A. Cancer Statistics

Cancer, or malignant tumors and neoplasms, is a generic term for a large group of diseases that can affect any part of the body. The defining feature of cancer is the rapid growth of abnormal cells that invade adjacent tissues ultimately metastasizing to other organs. Metastases are the major cause of death from cancer. In 2014, WHO reported that cancer is the leading cause of death worldwide, accounting for 8.2 million deaths in 2012.[15] According to the National Cancer Institute (NCI) and Centers for Disease Control and Prevention (CDC), cancer ranks second among all causes of death in the United States.[16] The American Cancer Society estimates that there will be 1,685,210 new cases of cancer in 2016, and 595,690 cancer related deaths.[14] There are six major histologic categories of cancer: carcinoma, sarcoma, myeloma, leukemia, lymphoma and mixed types. The most common cancers are projected to be breast cancer, lung and bronchus cancer, and prostate cancer.[14]

B. Hallmarks of Cancer

Although each cancer exhibits a unique set of phenotypes, cancers do share a group of common characteristics. The hallmarks of cancer proposed by Hanahan and Weinberg include genomic instability and mutation, tumor promoting inflammation, sustaining proliferative signaling, evading growth suppressors, resisting cell death, replicative immortality, inducing angiogenesis, invasion and metastasis, deregulating cellular energetics and avoiding immune destruction[17]. The ten hallmarks represent a

group of molecular, biochemical, and cellular traits shared by most cancers. This model provides a valuable framework into the development treatments against cancers.

C. Cancer Therapy

The main types of cancer treatment include: surgery, radiation therapy, chemotherapy, targeted therapy and immunotherapy.[18] Surgery is used to prevent, diagnosis, stage, and treat cancer.

Cancer chemotherapy is an established adjuvant to surgery and radiation for the successful treatment of solid tumors, lymphomas, and leukemias. Chemotherapeutic agents target rapidly dividing cells. In addition to cancer cells, certain normal cells, such as bone marrow, the digestive tract and hair follicles are also affected, leading to adverse side effects. Based on their mechanism of action, chemotherapeutic agents can be categorized as alkylating agents, antimetabolites, antitumor antibiotics, topoisomerase inhibitors and mitotic inhibitors.[19] Although many anticancer drugs are approved and clinically available, problems of resistance and toxicity necessitate the discovery of new drug leads.[17,20,21]

Targeted therapies, including monoclonal antibodies and small molecule inhibitors,[22] block the proliferation of cancer cells by interfering with specific molecules required for tumor development and growth. With their distinct mechanism of action, these agents provide individual cancer treatment. Targeted therapies do have some limitations. One is that cancer cells can become resistant to them through protein mutation so the targeted therapy no longer interacts with it. Also, the tumor can finds new pathways to achieve tumor growth bypassing the target.[17,20,21] Another limitation is

some identified proteins are difficult to develop as targets because of the protein's structure.

Immunotherapy is the treatment that uses the patient's immune system to combat their disease. The main types of immunotherapy now being used to treat cancer include cytokines, monoclonal antibodies, and vaccines. Progress is also being made with adoptive cell transfer (ACT), i.e, chimeric antigen receptor (CAR) T-cell therapy.[23] The major challenge of immunotherapy is that not all patients' cancers express the required antigen that the monoclonal antibody or vaccine recognizes. In general, the response rates to immunotherapy are around 30 percent. The disadvantage is the risk of inducing an autoimmune response.

Despite the successes that cancer research has yielded including novel biomarkers, targets, and molecular pathways for cancer diagnosis and therapy, the complexity of cancer limit the clinical efficacy of cancer therapy. Therefore, no methods available presently for cancer are fully satisfactory. Traditional cancer treatments usually have severe side effects in part due to their lack of specificity. In contrast, targeted therapies and immunotherapies block specific molecules required in tumorigenesis. However, resistance often occurs regardless of the drug target.[20,21,24] Not surprisingly, cancer cells typically contain multiple genetic and epigenetic abnormalities. As resistance remains a major challenge, the current rationale is to effectively utilize existing drugs in combination. In addition, combination therapies usually are the most promising and effective in the treatment of human disease. One major benefit of combination therapy is that they reduce drug resistance. Another advantage is when drugs with different effects are combined, each drug can be used at

its optimal dose, with tolerable side effect. Tailoring cancer treatment raises new questions. The financial cost of targeted therapy and immunotherapy may become an important issue in health care economics.[22] With this in mind, agents with different mechanisms of action are needed. More potent and less toxic drugs continue to be pursued.

D. Cell Death in Anticancer Therapy

Cell death can be classified based on morphological appearance, enzymological criteria, immunological characteristics, or functional aspects. Apoptosis involves chromatin condensation and nuclear fragmentation, finally forming small round bodies that contain intact cytoplasmic organelles or fragments of the nucleus.[25] Apoptotic bodies eventually are engulfed by resident phagocytic cells. While apoptosis involves the rapid demolition of all cellular structures and organelles, autophagy is a slow phenomenon. Aurophagy starts with parts of the cytoplasm are sequestered within double-membraned vacuoles and finally digested by lysosomal hydrolases. Massive autophagic vacuolization is observed in some instances of cell death, which has been named autophagic cell death. Necrosis is usually defined in a negative fashion because of the rupture of the plasma membrane. The main features of necrosis include a gain in cell volume that finally culminates in rupture of the plasma membrane. Necrosis is considered to be lethal because of the often association with pathological cell loss and because the ability of necrotic cells to promote local inflammation that may support tumor growth.[26]

Targeted therapies are often cytostatic, whereas chemotherapeutic agents are cytotoxic by damaging or stressing cells, which lead to cell death. Accumulated data suggest that various chemotherapeutic agents can kill tumor cells through the induction of apoptosis.[27,28] This calls for better understanding of the regulatory mechanisms that control cell survival and death pathways in human cancers.

E. Apoptosis and Cancer

One of the cell death signaling pathways is apoptosis. Apoptosis or programmed cell death is a key regulator of physiological control and tissue homeostasis. Two major pathways to caspase-dependent apoptosis have been identified: the extrinsic pathway and the intrinsic pathway. The extrinsic pathway involves stimulation of members of the tumor necrosis factor (TNF) receptor–nerve growth factor receptor superfamily such as TNF receptor (TNFR), CD95 (Fas) receptor or TNF-related apoptosis-inducing ligand (TRAIL) receptor (death receptors), while the intrinsic pathway, in which mitochondrial outer membrane permeabilization (MOMP) occurs and results in assembly of a caspase-activating complex between caspase 9 and Apaf-1.[29-31]

In the extrinsic pathway, death receptor stimulation typically results in the recruitment and activation of caspase 8 by the adaptor proteins Fas-Associated protein with Death Domain (FADD) and Tumor necrosis factor receptor type 1-associated DEATH domain protein (TRADD). This leads to the formation of a death-initiated signaling complex (DISC), which can propagate death signals in two ways: proteolysis of the BH3-only protein Bid, which will translocate to mitochondria and MOMP, and by direct proteolysis of downstream caspases, which results in their activation. During

intrinsic pathway, some BH3-only proteins act as sentinels for cell stress, damage or infection and can be mobilized to initiate MOMP through post-translational modification. This process triggers oligomerization of Bax and/or Bak in the mitochondrial outer membrane, forming channels that permit the escape of multiple proteins from the mitochondrial intermembrane space.[32-34]

Some of the mitochondrial proteins [apoptosis-inducing factor (AIF)] released as a result of MOMP can promote caspase-independent apoptosis.[35] Additionally, caspase-independent death can also result from stimuli that cause lysosomal membrane permeabilization (LMP) with the release of cathepsin proteases.[36]

Tissue homeostasis is the result of a delicate balance of proliferation on one side and cell death on the other side. Apoptosis is a cellular death program that is inherent to all mammalian cells. It plays an important role in the regulation of various physiological and pathological conditions. Dysregulation of apoptotic pathways can not only promote tumorigenesis but also render cancer cells resistant to chemotherapy.[37,38] Furthermore, apoptosis of circulating tumor cells can have an impact on the metastatic process.[39,40] Development of novel molecules that activate apoptosis by targeting different pathways will lead to effective cancer therapies, paving the way to bypass treatment resistance in various human cancers.[41]

F. Natural Products as Sources of New Drugs

Primary metabolites, such as nucleotides, amino acids and organic acids, perform metabolic roles in growth and development. In contrast, secondary metabolites, traditionally referred to as natural products, are a vast and diverse group of organic

compounds that have, for the most part, unknown cellular functions. However, natural products are selected by evolution to reach their molecular target within cells. They are thought to serve their producing organisms by improving their competitive strength. These organic substances have relatively small molecular weight (< 3,000 daltons) and have diverse structures.[42] Natural products and their derivatives have historically been invaluable therapeutic agents. Since ancient times, biologically active secondary metabolites have been used in traditional medicine. Natural products, including plants, animals and minerals, have been the basis of treatment of human diseases.[43-45] They have been essential components of drug discovery and serve as both direct source of small molecule therapies and synthetic derivatives.

Plants

Plants have formed the basis for traditional medicine used for centuries in countries such as China and India. Higher plants continue to afford many new drugs to treat disease. Modern scientists continue to take advantage of historical ethnopharmacological information to inform modern drug discovery efforts. Morphine and codeine isolated from *Papaver somniferum* are still in clinical use. Ethnomedical usage of medicinal plants has been introduced into modern therapy, namely, quinine and artemisinin, two of the major anti-malaria drugs. Other significant drugs developed from traditional medicinal plants include: atropine (anticholinergic), colchicine (antigout), ephedrine (bronchodilator), and physostigmine (cholinesterase inhibitor). Current drug discovery has mainly relied on bioactivity-guided isolation, leading to the discoveries of

the important anticancer agents, paclitaxel from *Taxus brevifolia* and camptothecin from *Camptotheca acuminate*.[46,47]

Microorganisms

Microorganisms were identified as sources of valuable natural products. In 1928, penicillin from *Penicillium rubens* was first discovered by Alexander Fleming. Unique microorganisms are abundant on land, in freshwater and at sea. Terrestrial microorganisms are a plentiful source of structurally diverse bioactive substances including antibiotics, immunosuppressive agents, hypolipidemic agents.[47] However, unlike the long-standing medical use of terrestrial microbes, marine organisms have shorter utilization history in the treatment of human disease. Therefore, much attention has been given to marine organisms due to their considerable biodiversity and the fact that the oceans cover over 70% of the world's surface.

i. Fungi

Fungi are one of the more diverse kingdoms of life. However, less than 100,000 have been described in the literature among the 1.5 to 5.1 million estimated species of fungi.[48-50] The fungal sources of new metabolites have been broadened from terrestrial strains to marine habitats and living plants with their endophytes. Fungal secondary metabolites yield commercial importance for the pharmaceutical or agricultural industries. β-lactam antibiotics, mainly penicillins and cephalosporins, are among the world's blockbuster drugs. Other important drugs include immunosuppressive agents (e.g., cyclosporin) and antilipidemics (e.g., lovastatin and mevastatin).[51]

ii. Cyanobacteria

Cyanobacteria constitute a unique group of phytosynthetic bacteria and populate diverse habitats through the world. Cyanobacteria are a rich source of novel natural products with diverse chemical scaffolds and a variety of biological activities.[52] Further, cyanobacterial metabolites show biological activities such as antifungal, antibiotic, and anticancer activities.[53] Brentuximab vedotin targets CD30 and in 2011 became the first FDA approved drug based on a cyanobacterial metabolite. Brentuximab vedotin is an antibody-drug conjugate[54] for the treatment of Hodgkin's lymphoma and systemic anaplastic large cell lymphoma (ALCL).

iii. Actinomycetes

Actinomycetes are gram-positive bacteria found in both terrestrial and aquatic environments that contain a high percentage of GC nucleotides. To date, approximately 23,000 bioactive secondary metabolites have been discovered from microorganisms, with 10,000 of them being produced by actinomycetes.[55,56] *Streptomyces* is the largest genus of actinobacteria. They produce over two-thirds of the clinically useful antibiotics including macrolides (e.g., erythromycin), aminoglycosides (e.g., streptomycin), tetracyclin, chloramphenicol and vancomycin. In addition, a number of the secondary metabolites produced by *Streptomyces* like actinomycin D, bleomycin, doxorubicin have been used as chemotherapy drugs, along with *Streptomyces* derived antifungals nystatin and amphotericin B.[46]

Animals

Teprotide, which was isolated from the venom of Brazilian viper (*Bothrops jararaca*), led to the design of inhibitor of angiotensin-converting enzyme, captopril and enalapril, used in the treatment of cardiovascular disease. The isolation of exendin-4 from the venom of the Gila monster, *Heloderma suspectum,* led to the development of extenatide polypeptide, Byetta®, used to control blood sugar in adults with type 2 diabetes. Bivalirudin is a leech anti-platelet protein that is genetically engineered from hirudin, the substance in the saliva of the leech (*Haementeria officinalis*). Bivalirudin is an inhibitor of collagen-induced platelet aggregation and stops blood clotting.[46,47]

Marine organisms

To date, there are seven therapeutic agents that derived from the marine environment.[57] The first marine derived product to gain FDA approval was ziconotide, isolated from the venom of the cone snail *Conus magus*. Ziconotide is currently marketed as Prialt® for the treatment of severe chronic pain. The complex alkaloid ecteinascidin-743 isolated from colonial tunicate *Ecteinascidia turbinata* was granted approval in Europe and the USA for the treatment of soft tissue sarcomas (STS) under the trade name Yondelis®.[47] Cytarabine, or Ara-C, is a semisynthetic derivative of a *C*-nucleoside isolated from the Caribbean sponge, *Cryptotheca crypta* and is currently indicated for the treatment of several types of leukemia. Eribulin mesylate was isolated from the Japanese sponge *Halichondria okadai*[58] and is approved for the treatment of breast cancer and liposarcoma. Lovaza is the only FDA- approved Omega-3 prescription drug approved for reducing serum triglycerides. The product is composed

of eicosapentaenoic acid (EPA) and docosahexaenoic acid (DHA)[59] obtained from fish. In addition, a further 13 agents are in phase I, II, or III clinical trials.[57]

For decades, secondary metabolites from plants, fungi and bacteria have been found to have powerful anticancer activity.[47,60,61] Among all anticancer drugs in the market during the 1940s to date, more than 60% of anticancer drugs are of natural origin, or resulting from modifications of natural products, including natural product (NP) as its original structure (13%), semisyntheticly modified NP (28%), synthetic NP mimic (9%), synthetic compounds with NP pharmacophore (10%) and synthetic NP mimic with NP pharmacophore (4%).[62] Since natural sources have been largely unexplored for medicinal purposes, secondary metabolites from nature undoubtedly will be a promising field for drug discovery.

G. New Drug Targets

It is well established that many FDA approved anticancer drugs kill cancer cells by inducing apoptosis. However, traditional anticancer chemotherapeutics targeting DNA replication or cell division have severe side effects. DNA damage and chromosomal instability caused by these agents can significantly increase a patient's risk of developing secondary malignancies. Drugs targeting signaling oncoproteins were developed subsequently to increase specificity and thus reduce side effect, but have limitations such as the development of resistance.[21,24] Therefore, there is an urgent need to identify new molecular targets to treat first line cancer and relapsed cancer.

Protein synthesis

Protein synthesis is a complex biochemical reactions. A large number of factors are involved in the interaction that catalyzes the assembly of ribosomes, mRNA templates, and aminoacylated tRNAs. Translation initiation is regulated by eukaryotic initiation factors (eIF4F). eIF4F is composed of three subunits: eIF4E, eIF4A and eIF4G. At the level of the ribosome recruitment, first, eIF4E binds to the cap structure present at the 5' end of mRNAs. Second, eIF4A, a DEAD-box RNA helicase that unwinds 5' mRNA structure implicated in preparing a ribosome landing pad for 43S pre-initiation complexes (40S ribosomal subunit and associated factors). Last, eIF4G, a large scaffolding protein involved in recruiting the 43S pre-initiation complex via its interaction with 40S-associated eIF3. [63]. Elevated eIF4E expression and increased eIF4F activity has been repeatedly implicated in malignancy. Studies showed that the altered translation of pro-survival and pro-growth signals that contribute to oncogenesis,[64] angiogenesis, and chemoresistance.[65] Therefore, inhibition of the eIF4F checkpoint suppresses tumor growth and offers a rational way for developing therapeutics.[66] In October 2012, homoharringtonine (HHT), a natural plant alkaloid derived from *Cephalotoxus fortune*, was approved by the FDA as the first protein translation inhibitor for the treatment of chronic myeloid leukemia (CML). Undoubtedly, this success in drug discovery will launch a new platform for scientists and the pharmaceutical industry to explore biological pathways and uncover new targets.

Autophagy

Autophagy is an essential homeostatic process. It involvs the lysosomal degradation of cytoplasmic organelles or cytosolic components. Multiple forms of cellular stress, including nutrient or growth factor deprivation, hypoxia, reactive oxygen species, DNA damage, protein aggregates, damaged organelles, or intracellular pathogens can stimulate autophagy. Different phases of autophagy could be activated by both stimulus-dependent and stimulus-independent signaling pathways.[67] However, excessive levels of cellular autophagy can result in autophagic cell death.

The three main types of autophagy are macroautophagy, microautophagy and chaperone-mediated autophagy. The term “autophagy” usually indicates macroautophagy. In particular, the main difference between microautophagy and macroautophagy is that microautophagy sequesters cytoplasm directly at the lysosomal surface of the lysosomal membrane, whereas the sequestering membrane in macroautophagy is derived from the plasma membrane. Chaperone-mediated autophagy involves the direct translocation of cytosolic proteins. The processes contain a particular pentapeptide motif across the lysosomal membrane and requires the action of cytosolic and lysosomal chaperones to unfold substrates. In organisms from yeast to mammals, macroautophagy can buffer against the effects of starvation by mobilizing nutrients that result from macromolecular degradation.[68] Contrast to the ubiquitin–proteasome system, which specifically recognizes only ubiquitinated proteins for proteasomal degradation, autophagy is generally thought to be a nonselective degradation system. Several processes involved in autophagy have been characterized. First, cytosolic material is sequestered by an expanding membrane sac, the

phagophore, resulting in the formation of autophagosome.[69] Second, autophagosome subsequently fuses with a lysosome. Finally, the cargo-containing autophagolysosome is then lysed, and the contents are degraded and reused.[70]

Control of autophagy relies on proteins encoded by a set of autophagy-related genes (Atg). Beclin-1 (Atg6) is one of the critical proteins regulating autophagy induction and can stimulate autophagy when overexpressed in mammalian cells.[71,72] Along with Beclin-1, a component of a class III PI3-kinase- containing complex, Atg1 is essential for the formation of a preautophagosomal structure. After formation of the preautophagosomal structure, dual ubiquitin-like conjugation pathways are required for the growth of autophagosomal membranes and autophagosome formation. These pathways, which require the E1-like enzyme Atg7, catalyze the conjugation of Atg12 to Atg5 and Atg8 to phosphatidylethanolamine (PE). Consistent with this function, Atg7 is required for the formation of autophagosomes. Autophagy is a dynamic process even under basal conditions. Therefore the autophagy biomarker, MAP1LC3B (Atg8) is turned over constantly, indicating that the increase in LC3B-II and accumulation of LC3B-positive puncta do not necessarily reflect an induction of autophagy, but can also result from impaired LC3B-II degradation.[73] SQSTM1/p62 is another widely used autophagy marker. It binds directly to both LC3B and ubiquitin.[74,75] The level of p62 is believed to reflect autophagosome turnover because of its degradation. Since akin to LC3B, p62 is itself sequestered by the autophagosome during this process and degraded in the autolysosome.

Recently, two distinct forms of autophagy in cancer have been elucidated: canonical and non-canonical autophagy. In contrast to classical or canonical autophagy,

non-canonical autophagy is a process that does not require the entire set of autophagy-related proteins, in particular Beclin 1, to form the autophagosome.[76,77]

Whereas apoptosis is a well-known form of programmed cell death (PCD), the detailed events of autophagy require further study. If nutrient deprivation persists, progressive autophagy can ultimately lead to autophagic cell death (ACD). The interplay between apoptosis and autophagy is an under-studied area. The potential use of autophagy inducers or inhibitors as a treatment could mark a major step forward in how we treat cancer.

Cancer is a result of dysregulation of cell differentiation, proliferation, and survival. Since autophagy contributes both to cell death and cell survival, its role in carcinogenesis is complex. Thus, whether autophagy plays a protective, inhibitive, or a death- promoting role remains to be fully elucidated. And while apoptosis related proteins are clearly understood, autophagy related cell regulation still needs to be explored further. Appropriate modulation of autophagy, inhibition its cytoprotective effect or promotion of autophagic cell death, might be a new anticancer target.[68,78] More importantly, when and how to use autophagy inhibitors or inducers as a treatment is a question of utmost importance.

Na^+/K^+-ATPase

Na^+/K^+-ATPase (Sodium pump) is an integral membrane protein found in all animal cells. It consists two subunits: a large catalytic α-subunit (110 kDa) and a glycosylated β-subunit (55 kDa). This enzyme pumps sodium out of cells while pumping potassium into cells by utilizing the energy from ATP hydrolysis. Na^+/K^+ -ATPase helps

maintain a variety of physiological functions such as cell excitability, contractility, and secondary active transport.[79] Even though the ion transport function of Na^+/K^+-ATPase is well documented, it has been implicated that Na^+/K^+-ATPase serves multiple roles in cells. Recent evidence points to Na^+/K^+-ATPase acts in various signal transduction pathways involving Src, Akt, MAPK and EGFR.[80-83] Activation of these pathways typically results in increased proliferation, while inhibiting these signaling pathways reduce tumor growth and proliferation, considered as cancer therapies.

Histone methyltransferase (HMTase)

Two major mechanisms of epigenetic regulation of gene expression are covalent modification of DNA and histones, the two core components of eukaryotic chromatin. The methylation of lysine residues in histones, especially in the N-terminal tails of histone H3 and H4, play an important role in gene regulation. HMTases catalyze the methylation of histones, thereby influencing chromatin structure and changing the pattern of gene expression. Aberrant HMTase activity has a fundamental role in driving human cancer.[84,85] Genes such as BRCA, cyclin, p19 are regulated by histone methylation, which affects DNA repair and the cell cycle. DNA methyltransferase (DNMT) and histone deacetylase (HDAC) inhibitors have been under extensive development for human cancer therapy.[59] In contrast, development of HMTase inhibitors as therapeutic agents are still at early stage.[86] Thus, use of HMTase inhibitors to re-induce the expression of epigenetically silenced tumor suppressor gene can lead to suppression of cancer growth or sensitization of cancer cells to therapeutic agents.

HDAC

Chromatin structure is regulated by epigenetic regulators for the control of vital cellular processes including gene transcription, DNA replication, and DNA repair.[87] Recently, it has become apparent either the aberrant recruitment of epigenetic regulators, or mutations in genes encoding epigenetic regulators, can be important in several human disease, including cancer.[88,89] HDACs are key enzymes of epigenetic regulation. Genetic knockdown of HDAC1, -2, -3, and -6 in a variety of tumor types such as breast, colon, and lung induced cell cycle arrest and apoptosis.[90] Acetylation of nonhistone proteins has been shown to modulate protein functions by altering protein-protein interaction, stability and cellular localization. Many nonhistone proteins were well-characterized such as E2F, p53, c-Myc, nuclear factor-κB (NF-κB), hypoxia-inducible factor 1 alpha (HIF-1α), estrogen receptor alpha (ER α), androgen receptor (AR), MyoD, heat-shock protein (Hsp)-90, Stat3, Smad7, α-tubulin, β-catenin, and many others.[91-93] Thus, HDACs are considered to be new and promising anticancer targets. Several HDAC inhibitors have been approved for clinical use.[94]

The proteasome

The ubiquitin proteasome pathway represents the major pathway for protein degradation. Scientists have discovered that more than 80 % of cellular proteins are degraded through this pathway.[95] The 26S proteasome is found in the nucleus and cytoplasm of all eukaryotic cells. It consists of a barrel-shaped 20S proteolytic core, composed of 2 identical α-subunit ring and 2 β-subunit rings, plus capped with two 19S regulatory complexes. The defects within this pathway are associated with a number of

diseases, including cancer. It has been reported that proteasome inhibition contributes to the inhibition of NF-κB, inhibition of angiogenesis, impaired DNA repair, altered cell cycle, increase of pro-apoptotic proteins and endoplasmic reticulum stress. Proteins destined for degradation are first polyubiquitinated by E1, E2 and E3 ubiquitinating enzymes. Upon recognition by 19S, ubiquitinated proteins are directed to the 20S core where proteolytic cleavage is mediated by subunit β1 (caspase-like activity), β2 (trypsin-like activity), and β5 (chymotrypsin-like activity).[96] Cancer cells generally have higher levels of proteasome activity than normal cells, making the proteasome a rational therapeutic target in oncology.

Transforming growth factor (TGF)-β-activating kinase 1 (TAK1)

TAK1 is a serine/threonine kinase and a MAPK kinase kinase (MAPKKK) that can be activated by different cytokines, such as TGF-β, IL-1β, TNFα and toll-like receptor ligands.[97] TAK1 may play diverse roles in various cancers. For instance, ablation of TAK1 can cause carcinogenesis in liver.[98] However, in skin cancer, pancreatic cancer, colon cancer and ovarian cancer, inhibition of TAK1 can induce cell death, reverse chemoresistance, apoptosis, sensitizes cells to cisplatin-induced cytotoxicity.[99-102] Therefore, targeting TAK1 is a promising therapeutic approach.

H. New Potential Antitumor Agents of Natural Origin

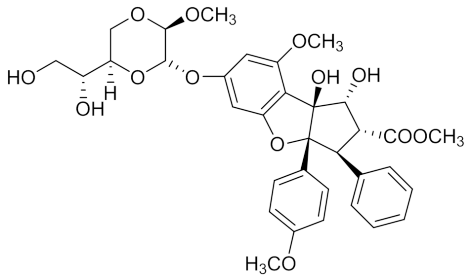

Figure 1. Structure of silvestrol.

Silvestrol

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, which is unique to members of the genus Aglaia. Silvestrol was isolated from extracts of the fruits and twigs of *Aglaia foveolata* (M. Roemer) Merrill (syn. A. pyramidata Hance).[103] It is a translation initiation inhibitor that acts as a chemical inducer of dimerization (CID) by facilitating the engagement between eIF4A and RNA.[104,105] Furthermore, silvestrol was found to possess potent anticancer activities in both the hollow fiber assay and the P-388 lymphocytic leukemia system in mice.[106] Reports have shown that tight control of protein synthesis is essential for normal cellular function and unrestrained protein synthesis can promote tumorigenesis. There is significant interest in treating cancers by blocking protein synthesis. Due to its ability to block protein synthesis and induce G2/M growth arrest in cultured human cancer cell lines, silvestrol is one of the most exciting

candidate anticancer compounds. The potent ability of silvestrol to inhibit protean synthesis and induce cancer cell death makes this unique rocaglate derivative an intriguing molecule for further development as a candidate cancer drug.

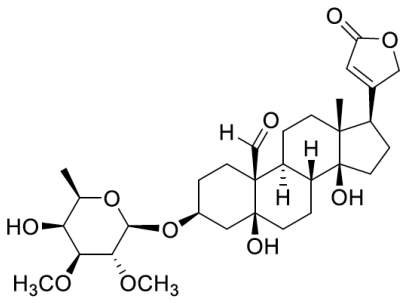

Figure 2. Structure of strebloside.

Strebloside

Streblus asper (Moraceas) is a small tree in India, Sri Lanka, Vietnam and Thailand. This tree has been used in Ayurvedic medicine for the treatment of cardiac disorders, epilepsy and edema.[107] Strebloside was first isolated from *S. asper* stem bark and its chemical structure shares a common cardiac glycoside structural motif. Strebloside has potent cytotoxicity against HT-29 cells. Na^+/K^+-ATPase is a ubiquitous membrane protein and relays signals through protein-protein interactions mediating cell proliferation, differentiation and apoptosis.[80] Cardiac glycosides are a group of natural compounds that inhibit Na^+/K^+-ATPase. Members in this family have been used in the clinic for years. Due to its ability to block ion pump might induce cell cycle arrest and apoptosis, cardiac glycoside such as strebloside could serve as good anticancer drugs.

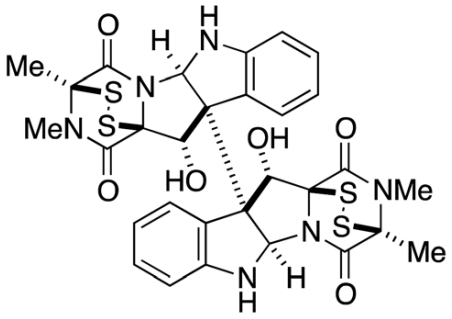

Figure 3. Structure of verticillin A.

Verticillin A

Epipolythiodioxopiperazine (ETP) alkaloids are bioactive secondary metabolites isolated largely from terrestrial and marine fungi. It has been reported that ETPs show potent cytotoxic, antibacterial activities.[54] Modifications of histones are major mechanisms of epigenetic regulation of gene expression. The methylation of lysine residues in histones is catalyzed by HMTases affecting cellular differentiation. Aberrant HMTase activity has a fundamental role in human cancers are established.[84,85] In comparison with normal cells, human cancer cells exhibit DNA hypomethylation, which leads to tumorigenesis, and hypermethylation of tumor-suppressor genes, which mediates gene silencing.[108] Verticillin A, a selective HMTase inhibitor, was derived from a mushroom extract. It selectively inhibits HMTases such as SUV39H1, SUV39H2 and G9a.[109] Therefore, it has the potential to suppress malignancy.

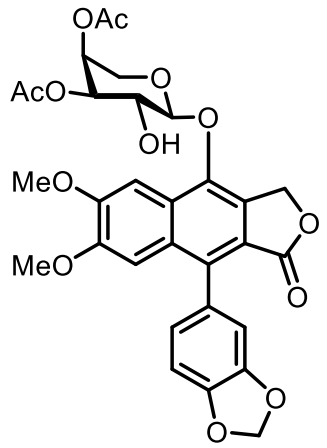

Figure 4. Structure of phyllanthusmin D.

Phyllanthusmin D

Many species of plants in the Phyllanthus genus have been utilized in Asian traditional medicine. Phyllanthusmins were isolated from *Phyllanthus poilanei*. Their structures are similar to aryltetralin lignin lactones, including the natural product podophyllotoxin and the semi-synthetic antitumor agent etoposide (VP-16). Etoposide is known to stabilize the DNA-topoisomerase II (topo II) complex. Several other arylnatphtalene lignin lactones have been isolated with some reported to have activity against topo IIa.[110,111] Phyllanthusmin D showed promising activity in an *in vivo* hollow fiber assay with no sign of gross cytotoxicity. However, the cytotoxic activities of phyllanthusmin C and D were not due to their ability to inhibit topo II.[112] Explore the structure-activity relationship (SAR) and probe the mechanism of action of phyllanthusmin would help to clarify and development this potential natural product.

I. Program Overview

The search of anticancer compounds from natural sources leads to the collaborative multidisplinary research project. The program project entitled “Discovery of Anticancer Agents of Diverse Natural Origin” (P01 CA125066) has been funded by the U.S. National Cancer Institute, NIH. Projects 1-3 focus on the isolation of tropical plants, aquatic cyanobacteria, and filamentous fungi, respectively. Cores 1-3 offer support in *in vitro* and *in vivo* testing, synthesis/analogue development and pharmacokinetics, and biostatistics, respectively. An organizational scheme of this program project is shown in Figure 5. The team consists of three outstanding natural products groups, The Ohio State University (OSU), the University of Illinois at Chicago (UIC), and the University of North Carolina at Greensboro (UNCG). The principal investigator of this program project is Dr. A. Douglas Kinghorn, OSU.

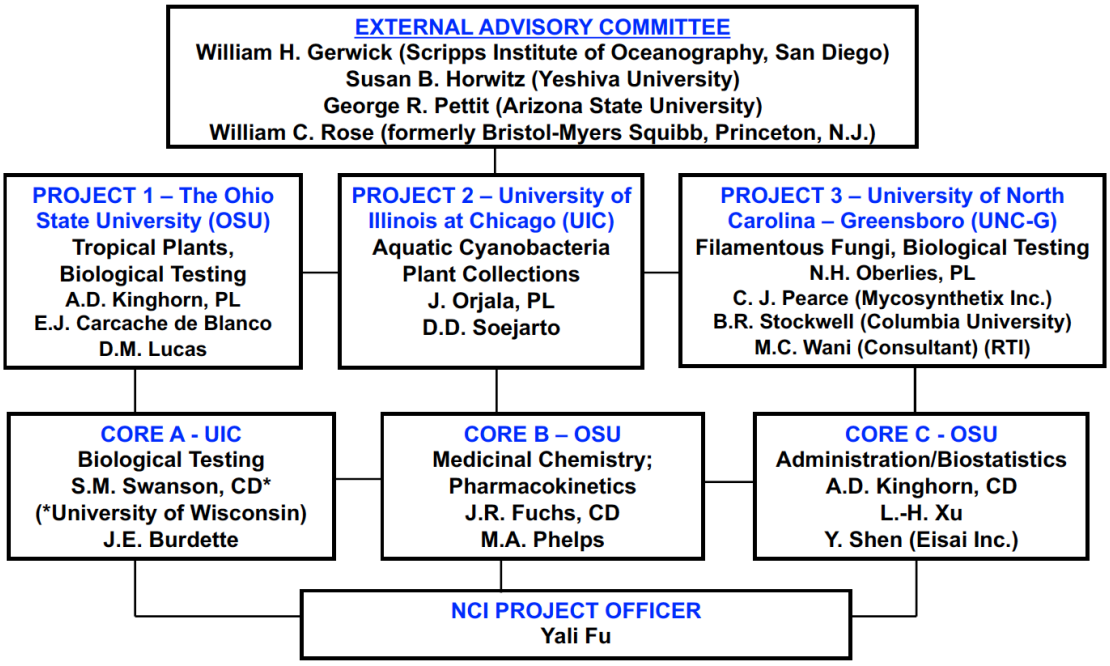

Figure 5. Organizational scheme of the program project.

This research project directs the purification, structural characterization, chemical modification, and biological evaluation. Driven by the need of new anticancer targets, we expect to explore for novel regulation in cell death in prospect of finding novel compounds with good specificity and potency against cancer cells without altering normal cells. Samples were collected from plants (Dr. Kinghorn in OSU and Dr. Soejarto in UIC), fungi (Dr. Oberlies in UNCG) and cyanobacteria (Dr. Orjala). In the processes of bioassay-guided fractionation and isolation, our collaborators further extract, fractionate and isolate pure compounds based on our bioassay results. Several lead compounds from this project was isolated and will be described in this thesis including silvestrol, strebloside, phyllanthusmin, icacinolactone G, homoisoflavonoids, verticillin A, merocyclophanes and diazaquinomycin A. In UIC, we focus on anticancer drug screening and study its mechanism of action. Therefore, potential drug leads were prioritized for further investigation and discussed in this thesis.

Aims of the study

Drug discover and drug repurposing are two main strategies in drug development. Drug discovery focuses on looking for new compounds or new targets while drug repurposing repositions known drugs or known targets but new applications. Silvestrol will serve as an example in drug discovery and strebloside will be the example in drug repurposing. In chapter III, we characterized the mechanism of action of silvestrol using human melanoma cells. It is plausible that silvestrol induces autophagy by inhibiting translation. Studies have shown that there is probable cause linking apoptosis and autophagy, by analyzing the two of them together that we can not only

clarify ACD but also explain the critical transition from survival to cell death in response to cellular stress. In chapter IV, we identified the signaling networks of strebloside in ovarian cancer cells. It has been shown in literature that cardiac glycosides inhibit Na^+/K^+-ATPase and further block cancer cell growth. The goal of this study is to evaluate if strebloside share common cardiac glycoside activity and whether it exhibits better anticancer effect. Strebloside was assessed with digitoxin, a clinical used cardiac glycoside, for its activity.

II. MATERIALS AND METHODS

A. Reagents and Antibodies

The isolation of silvestrol, {6-*O*-demethyl-6-[6-(1,2-dihydroxyethyl)-3-methoxy-1,4-dioxan-2-yl]-aglafolin}, has been described previously,[103] and this compound was provided for present study in >99% purity. Suberanilohydroxamic acid (SAHA; vorinostat), vinblastine, 3-methyladenine (3-MA), bafilomycin A1, acridine orange and monodansylcadaverine were obtained from Sigma-Aldrich Corp. (St. Louis, MO). Homoharringtonine was purchased from Santa Cruz Biotechnology (Santa Cruz, CA). Primary and secondary antibodies were from commercial sources and used according to the recommendations of the supplier. Secondary anti- rabbit antibodies coupled to horseradish peroxidase were from Cell Signaling Technology, Inc.

B. Cell Culture

Human melanoma cancer cells MDA-MB-435, human breast cancer cells MDA-MB-231, human colon cancer cells HT-29, and human ovarian cancer cells OVCAR3 were purchased from the American Type Culture Collection (Manassas, VA). Cells were propagated in RPMI 1640 medium supplemented with fetal bovine serum (10%), penicillin (100 units/ml), and streptomycin (100 µg/ml). OVSAHO, and Kuramochi cells were grown in RPMI 1640 with 10% fetal bovine serum, and 1X penicillin-streptomycin. OVCAR4 cells were grown in RPMI 1640 with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin-streptomycin. OVCA 432 and OVCAR5 cells were maintained in Minimum Essential Media (MEM) supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine, 1% non-essential amino acids, 1% sodium pyruvate, and 1%

penicillin/streptomycin. OVCAR8 cells were grown in DMEM with 10% fetal bovine serum, and 1% penicillin-streptomycin. Normal immortalized human ovarian surface epithelial cells (IOSE 80) were a gift from Dr. Nelly Auersperg at the University of Vancouver and were maintained in 50% v/v Medium 199 and 50% v/v MCDB (Sigma-Aldrich, St. Louis, MO), 15% FBS, 1% L-glutamine, 1% penicillin/streptomycin, and 0.055% epithelial growth factor (EGF) (PeproTech Inc, Rocky Hill, NJ). Wild type and ATG7-deficient mouse embryonic fibroblast cells (MEF and MEF-atg7$^{-/-}$) were kindly provided by Dr. Masaaki Komatsu (Tokyo Metropolitan Institute of Medical Science). MEFs were maintained in Dulbecco's Modified Eagle's Medium, supplemented with 10 % fetal bovine serum, and penicillin (100 units/ml), and streptomycin (100 µg/ml). Cultured cells were maintained at 37°C in 5% CO_2.

For the evaluation of LC3 puncta, the plasmid EGFP-LC3 was transfected into MDA-MB-435 cells with Lipofetamine 2000 Reagent from Invitrogen (Carlsbad, CA). Stable cell lines were selected using antibiotic resistant plasmids containing the gene of interest. The EGFP-LC3 plasmid was kindly provided by Dr. Wei-Pang Huang (Department of Life Science, National Taiwan University, Taipei, Taiwan). MDA-MB-435 cells stably expressing EGFP-LC3 were selected using 400 µg/mL G418 (Gemini Bio-products, West Sacramento, CA) and maintained in RPMI 1640 media containing 200 µg/mL G418.

Primary murine oviductal epithelial (MOE) cells (also called murine tubal epithelial cells or MTEC) were pooled from multiple oviducts to establish one cell line from 8-week-old female CD1 mice. Once the primary isolated MOE cells became established in culture, the MOE^{LOW} cells (passages 8–25) were continuously passaged

to generate the experimentally aged, MOE^{HIGH} cells (passages 85–120). Both MOE^{low} and MOE^{high} cells were maintained in complete medium containing α-MEM modified Eagle's medium (CellGro, Manassas, VA, USA) supplemented with ribonucleosides, deoxynucleosides, and L-glutamine and supplemented with 10% fetal bovine serum, 2 mM L-glutamine (Life Technologies, Grand Island, NY, USA), 2 μg/ml epithelial growth factor, 5 μg/ml insulin, 5 μg/ml transferrin, 5 ng/ml sodium selenite (Roche; Indianapolis, IN, USA), 1 mg/ml gentamycin (CellGro; Manassas, VA, USA), and 18.2 μg/ml β-estradiol (Sigma Aldrich; St Louis, MO, USA). MOE SCR cells were maintained in MOE medium containing 0.5 μg/ml puromycin and MOE PTEN/KRAS cells were maintained in MOE medium containing 0.5 μg/ml puromycin and 200 μg/ml hygromycin. MOE cells were maintained in a humidified atmosphere containing 5% CO_2 at 37°C.

C. Cell Viability Assay

Cells in log phase growth were harvested by trypsinization followed by two PBS washings to remove all traces of enzyme. Cells were seeded at 5,000 cells per well in 96-well clear, flat-bottom plates and incubated overnight. Silvestrol, SAHA, HHT, bortezomib, strebloside or digitoxin dissolved in DMSO, vinblastine dissolved in water was then diluted and added to the appropriate wells. The cells were incubated for 72 h and evaluated for viability with a commercial absorbance assay (CellTiter 96® AQueous One Solution Cell Proliferation Assay, Promega, Madison, Wisconsin) that measured viable cells. IC_{50} values were expressed in nM relative to the solvent (DMSO) control.

D. Cell Cycle Assay

In Chapter III, MDA-MB-435 cells were plated in 6-well plates and treated with vehicle, or silvestrol for 24 h. In Chapter IV, OVCAR3 cells were plated in 6-well plates and treated with vehicle, strebloside or digitoxin for 72 h. After treatment, the cells were collected by trypsinization, fixed in 70% ethanol, washed in PBS, resuspended in 1 mL of PBS containing 1 mg/mL RNase and 50 µg/mL propidium iodide, incubated in the dark for 30 min at room temperature, and analyzed using EPICS flow cytometer (Beckman-Coulter; Brea, CA). The data were analyzed using Multicycle software (Phoenix Flow Systems; San Diego, CA).

E. Confocal Microscopy

For detection of acidic vesicular organelles with monodansylcadaverine staining, MDA-MB-435 cells were plated 1 day before their treatment and incubated overnight. After a 24-hour treatment, cells were incubated for 10 minutes with monodansylcadaverine (50 mM) and subsequently observed by confocal microscopy (Zeiss LSM 710; Jena, Germany). For live cell imaging detection with acridine orange, MDA-MB-435 cells were grown on MatTek 35 mm glass-bottomed culture dishes (MatTek Corp., Ashland, MA) in complete medium. Cells were exposed to silvestrol for the indicated time, then incubated with acridine orange (1 µg /mL) for 15 min and observed under a confocal microscopy. For LC3 puncta imaging, MDA-MB-435 cells stably transfected with the EGFP-LC3 plasmid were grown on MatTek 35mm glass-bottomed culture dishes, followed by silvestrol treatment for 24 h. The subcellular distribution of EGFP-LC3 was observed by confocal microscopy.

F. Caspase Activity Assay

MDA-MB-435 cells in log-phase growth were seeded in white-walled, clear-bottomed 96-well microtiter plates and incubated overnight. The next morning, DMSO (vehicle), vinblastine or silvestrol were added to the cells to a final volume of 100 µl. After 24-48 hour incubation, caspase 3/7 activity was assessed using a commercial luminescence kit according to the manufacturer's instructions (Caspase-Glo® 3/7 Assay, Promega Corp.). The Caspase-Glo reagent, which also serves to lyse the cells, was added to each well (100 µL) and the contents were mixed gently and incubated at room temperature for 90 min. The resulting luminescence was measured using a Synergy microplate reader (BioTek Instruments, Winooski, VT).

G. Morphological Analysis of Apoptotic Cells

MDA-MB-435 cells were incubated with DMSO, 25 nM silvestrol, or 1nM vinblastine for 24 hours. The cells were then washed with PBS, fixed with 3.7% paraformaldehyde and permeabilized with 0.1% Triton X-100. The cells were incubated for 5 min in the dark with DAPI (250 ng/ml). The stained cells were viewed by fluorescence microscopy. The percentage of apoptotic cells was calculated as the ratio of apoptotic cells to total cells counted, and at least 3 fields in each well were counted.

H. Flow Cytometry

For the apoptosis assay, MDA-MB-435 cells were grown to confluence, and incubated with DMSO or 25 nM silvestrol for the indicated time intervals. The cells were trypsinized, washed in PBS, and centrifuged at 200x g for 5 min. Then, the pellet was resuspended in 100 µL binding buffer and added 2 µL Annexin V-FITC and 2 µL propidium iodide (PI). After 15 min incubation at room temperature, FITC and PI fluorescence was detected using a FACScalibur flow cytometer (Becton Dickinson, San Diego, CA) and subsequently analyzed by CellQuest software (BD Biosciences; Franklin Lakes, NJ).

I. qPCR Analysis

Total RNA was isolated using the RNeasy kit (Qiagen, Valencia, CA) and reverse transcribed into cDNA with the high-capacity cDNA Reverse transcription kit (Applied Biosystems, Carlsbad, CA). cDNAs were plated in triplicate in 96-well plates and followed by adding TaqMan Master Mix (Applied Biosystems) and primer probe for a final volume of 25 uL. The plates were sealed, centrifuged for 1 minute, and then ran by using FAM and VIC as the detector probes for each assay.

J. Immunoblot Analysis

In Chapter III, whole cell lysates were prepared from MDA-MB-435 cells treated with silvestrol at different time intervals or with inhibitors. In Chapter IV, whole cell lysates were prepared from OVCAR3, OVSAHO or OVCA432 cells treated with strebloside or digitoxin. After treatment, cells were harvested, washed twice with PBS

and re-suspended in RIPA Lysis and Extraction Buffer and incubated on ice for 15 min. After centrifugation (4°C, 15 min and 14,000 g) supernatants containing cellular proteins were collected. Protein concentration was determined by the BCA assay (Pierce, Rockford, IL). Cell lysates were adjusted for protein content and equal amounts (25 µg) separated by SDS-PAGE. Proteins were immobilized onto PVDF membranes. After saturating with 5% (w/v) non-fat milk in TBST for 1 h at room temperature, the membranes were incubated with primary antibody overnight at 4°C. The next day, membranes were washed in TBST (5×5 min) then further incubated with horseradish peroxidase-conjugated IgG secondary antibody at room temperature for 1 hour followed by extensive washing with TBST (5×5 min). Finally, the proteins were visualized using an enhanced chemiluminescence (ECL) reagent. (Antibody dilutions are listed in Table I)

TABLE I. ANTIBODY CONCENTRATIONS FOR IMMUNOFLUORESCENCE

Antibody	Concentration	Species Raised	Company
Actin	1:1000	Rabbit	Sigma-Aldrich
Cyclin B	1:300	Rabbit	Cell Signaling
Cyclin D	1:300	Rabbit	Cell Signaling
p62	1:300	Rabbit	Cell Signaling
LC3	1:300	Rabbit	Cell Signaling
Caspase 3	1:300	Rabbit	Cell Signaling
PARP	1:1000	Rabbit	Cell Signaling
p53	1:1000	Rabbit	Cell Signaling
p21	1:500	Rabbit	Cell Signaling
pErk	1:1000	Rabbit	Cell Signaling
Erk	1:1000	Rabbit	Cell Signaling
Mcl-1	1:1000	Rabbit	Cell Signaling
Bcl-xL	1:1000	Rabbit	Cell Signaling

K. Animals

Seven-week-old immunodeficient NCr *nu*/*nu* mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA) and housed in cages at room temperature with a relative humidity of 50–60% under 12:12 h light–dark cycle. All animal work was approved by University of Illinois at Chicago Animal Care and Use Committee (protocol number 13-057), and the mice were treated in accordance with the institutional guidelines for animal care.

L. *In Vivo* Hollow Fiber Assay

Seven-week-old immunodeficient NCr *nu*/*nu* mice were divided into seven groups, including vehicle negative control group (12 mice), paclitaxel positive control group (12 mice), and strebloside treatment groups with a dose of 1, 5, 10, 15, and 30 mg/kg, respectively (6 mice for each 5-treatment group). Strebloside was dissolved initially in ethanol and subsequently diluted with Tween 20. The mixture was diluted with distilled water to 5% ethanol and 5% Tween 20. Hollow fibers were implanted into the abdominal cavity of mice on day 0. Then the mice were injected i.p. once daily for four days (from day 3 to day 6) with strebloside (different dose as indicated), the vehicle negative and paclitaxel (5 mg/kg) positive controls, respectively. On day 7, all the remaining mice were sacrificed. The fibers were retrieved, and viable cell mass was evaluated by a modified MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] assay. The percentage of net growth for the cells in each treatment group was calculated by subtracting the day 0 absorbance from the day 7 absorbance and dividing

this difference by the net growth in the vehicle control (minus value between the day 7 and the day 0). Each mouse was weighed daily during the study.

M. Na^+/K^+-ATPase Activity Assay

Na^+/K^+-ATPase activity was assessed using a luminescent ADP detection assay (ADP-Glo Max Assay; Promega, Madison, Wisconsin) that measures enzymatic activity by quantitating the ADP produced during the enzymatic first half-reaction. Specifically, 10 µL of assay buffer containing Adenosine 5'-triphosphatase from Porcine Cerebral Cortex (Sigma) were added to the wells of a 96-well plate followed by 10 µL of DMSO or DMSO containing strebloside or digitoxin. Then each well received 5.0 µL of ATP to initiate the reaction. Final assay concentrations contained different concentration of strebloside or digitoxin. After 15 min incubation at 37°C, 25 µL ADP-Glo Reagent was added to terminate the reaction, and plates were incubated at room temperature for 40 min to deplete remaining ATP. Then 50 µL of Kinase Detection Reagent was added, and plates were incubated for 60 min at room temperature to convert ADP to ATP. ATP was measured via a luciferin/luciferase reaction using a Synergy Mx (BioTek, Winooski, VT) to assess luminescence.

N. Molecular Modeling

The crystal structure of the Na^+/K^+-ATPase in complex with digoxin (PDB code 4ret) was optimized using the Protein Preparation Wizard. [Schrödinger Release 2016-1: Schrödinger Suite 2016-1 Protein Preparation Wizard; Epik version 3.5, Schrödinger, LLC, New York, NY, 2016; Impact version 7.0, Schrödinger, LLC, New York, NY, 2016;

Prime version 4.3, Schrödinger, LLC, New York, NY, 2016.] Restrained minimization was performed on hydrogens in OPLS3 force field. The 3D structures of Digitoxin and Strebloside were prepared using LigPrep [Schrödinger Release 2016-1: ligprep, version 3.7, Schrödinger, LLC, New York, NY, 2016.] and OPLS3 force field was used for ligands geometric optimization with neutralize ionization and the chiralities were assigned based on their natural structures. Default values were used for other parameters during protein and ligand preparations. Following molecular dockings were performed using the GOLD v5.2.2[113] with the above prepared protein and ligands. The active site for the enzyme was defined within 10 Å around the catalytic site of (-28.6, -19.8, 70.9) in the crystal structure. Scaffold constraints with tetracyclic steroid rings were used for the docking pose prediction. The best scoring pose for each compound was extracted for further analysis.

O. Electrophysiological Recordings and Analysis

Potassium currents were recorded under voltage-clamp with an EPC10 patch clamp amplifier interface (HEKA Electronics, Lambrecht_Pfalz, Germany) For data acquisition, pulse generation, and analysis, a computer (Dell, Round Rock, TX) running PULSE software (HEKA Electronics) was used. All experiments were conducted at room temperature (20–24°C). Data are presented as mean ± SE of 3 experiments. The patch pipettes were made from Corning type 7052 glass (Garner Glass, Claremont, CA). Whole-cell currents were obtained conventionally from dialyzed cells voltage-clamped through ruptured membrane patches. The solution bathing the cells contained (mM): 140NaCl, 10HEPES, 1$CaCl_2$, 1$MgCl_2$, 5KCl, 10glucose pH adjusted to 7.4 saline was supersfused at 1ml/min. Pipette solution: 140KCl, 2$MgCl_2$, 1$CaCl_2$, 10HEPES,

2.5EGTA (~50nM free Ca^{2+}) pH adjusted to 7.2 The pulse protocol was run a minimum of 10 times before the perfusion with strebloside to ensure that the currents were stable.

P. Clonogenic Assay

OVCAR3 and OVSAHO cells were plated (200 cells/60 mm-dish) and incubated for 21 days. After incubation, cells were washed, fixed with 4% (*w/v*) paraformaldehyde, and stained with 0.04% (*w/v*) crystal violet for 30 min at room temperature. Plates were washed with deionized H_2O, dried, and photographed using the FluorChem E documentation system (Protein Simple, Santa Clara, CA, USA). Numbers of foci were analyzed using Image J software (NIH) from three biological replicates.

Q. Soft Agar Colony Formation Assay

OVCAR3 and OVSAHO cells were suspended (1.5×10^4) in 0.35% (*w:v*) agar on top of a 0.5% (*w:v*) base agar layer. Cells were incubated for 21 days with media changed every 3 days. Final colonies were imaged on a Nikon Eclipse TS100 microscope and colonies were counted using Image J software from three biological replicates.

R. Luciferase Assay

Cells were plated at a density of 25,000 per well into 24-well plates and incubated overnight. Cells were transfected with 0.05 µg/well of an expression construct containing the NF-κB binding element promoter upstream of the luciferase gene using

Mirus TransIT LT1 (Mirus Bio LLC, Madison, WI) according to the manufacturer's instructions. Cells were transfected for 24 hr in serum-supplemented media. Cells were then treated with TNFα at 10 ng/mL (Sigma Aldrich), strebloside (100 nM) or digitoxin (100 nM) for 4 hr. Normal cell luciferase activity was measured using a Synergy Mx (BioTek, Winooski, VT).

S. Statistical Analysis

The data were analyzed by one-way analysis of variance followed by a paired student t-test comparing untreated controls and treatment groups. P-values of 0.05 or less were considered statistically significant.

III. SILVESTROL INDUCES EARLY AUTOPHAGY AND APOPTOSIS IN HUMAN MELANOMA CELL

(Reprinted with permission from *BMC cancer* **16**, 17, doi:10.1186/s12885-015-1988-0 (2016).)

A. Introduction

Skin cancer is the most commonly diagnosed cancer. Melanoma accounts for less than two percent of skin cancers, but approximately 75 percent of skin cancer deaths are a result of melanoma.[114] Melanoma is often considered one of the most aggressive and treatment-resistant human cancers. Discoveries have shown that melanoma frequently harbors mutations that attenuate tumor suppressor genes such as p53 and PTEN, leading to cell cycle dysregulation. Melanoma also frequently exhibits enhanced activation of receptor tyrosine kinases like epidermal growth factor receptor (EGFR) and MET, as well as BRAF and small G proteins such as Ras.[115] Together, these aberrant signaling networks render melanoma resistant to conventional chemotherapeutic drugs.

For decades, secondary metabolites from plants, fungi and bacteria have been found to contain powerful anticancer activity.[42,47,61,116] The genus *Aglaia* of the plant family 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, as well as other southeast Asian countries. Previous phytochemical studies on *Aglaia* species have shown that among all the isolates, cyclopenta[*b*]benzofurans, also known as rocaglate or rocaglamide derivatives, deserve further study due to their unusual carbon skeletons[117,118] and potent biological activities. Silvestrol is a rocaglate derivative

containing a dioxanyl ring and was isolated from the tropical tree *Aglaia foveolata.* Silvestrol is toxic against human cancer cell lines propagated *in vitro* or *in vivo* with a potency similar to that of paclitaxel or camptothecin.[103] It is a translation initiation inhibitor that prevents ribosome loading onto a mRNA template by targeting the eukaryotic initiation factor, eIF-4A[104,105]. Silvestrol was found to possess potent anticancer activities in both the *in vivo* hollow fiber assay and the P-388 lymphocytic leukemia mouse model[103]. The compound has been found to show promising *in vitro* and *in vivo* activities against certain B-cell malignancies,[119] and is currently under pre-clinical development in the National Cancer Institute Experimental Therapeutics (NExT) program. However, the mechanism of action of silvestrol responsible for inducing cellular death is still unclear. Tight control of protein synthesis is essential for normal cellular function and survival, but unrestrained protein synthesis can promote tumorigenesis. Therefore, silvestrol's ability to block protein synthesis is of significant interest in treating cancers.

Autophagy is an essential, homeostatic process involving the lysosomal degradation of cytoplasmic organelles or cytosolic components. Autophagy is a physiological process involved in the routine turnover of proteins or intracellular organelles.[120] The process of autophagy starts by sequestering cytosolic proteins or organelles into autophagosomes that then fuse with lysosomes to form autolysosomes for the degradation of sequestered contents by lysosomal hydrolases.[121] Control of autophagy relies on proteins encoded by a set of autophagy-related genes.[122] First, autophagosome nucleation is mediated by Beclin 1 (Atg6), a class III phosphatidylinositol 3-kinase complex.[71,72] Later, the Atg12-Atg5 complex and

microtubule-associated protein 1 light chain 3 (LC3, Atg8) are required for the elongation of autophagosomes. During autophagy, LC3-II is increased from the conversion of LC3-I, which is considered an autophagosomal marker.[123] Autophagy may protect against cancer by promoting autophagic cell death or contribute to cancer cell survival. Importantly, autophagy and apoptosis often occur in the same cell, mostly in a sequence in which autophagy precedes apoptosis. Loss or gain of either autophagy or apoptosis influences numerous pathological processes.[124,125] Proteins involved in pathways that modify autophagy might provide novel anticancer targets.[68,78]

Tight regulation of protein synthesis is critical for cell survival during nutrient and growth factor deprivation. In the presence of adequate nutrients, protein synthesis is stimulated and autophagy is inhibited.[126,127] Tumor growth requires new protein synthesis. Therefore, use of silvestrol that inhibits translation could be a useful therapeutic strategy.[128] Oncogenic effects arising from the ectopic expression of the eukaryotic initiation factor eIF-4E has been reported.[128] Moreover, down-regulation of eIF-4E, which is the rate-limiting factor for translation, has been shown to have an anti-tumor effect.[66] Considerable attention has therefore been focused on targeting other components of the protein translation machinery. As a member of translation inhibitors with a unique structure, silvestrol previously showed histological selectivity for several cancer cell types, perhaps through the depletion of short half-life pro-growth or pro-survival proteins, including cyclin D and Mcl-1. Given its ability to modulate tumor cell growth, the current study evaluates whether silvestrol induces both apoptosis and autophagy to induce cell death, and further defines the mechanism of this agent.

B. Results

Cytotoxic effect of silvestrol on human melanoma cells

Many anticancer strategies currently used in clinical oncology such as γ-irradiation, suicide gene therapy or immunotherapy, have been linked to activation of the intrinsic and/or extrinsic pathway of apoptosis in cancer cells. Silvestrol displayed an unprecedented profile in the National Cancer Institute 60 (NCI-60) human cancer cell line panel, exhibiting sub-nanomolar potency as an anticancer compound in many of the cell lines tested. Thus, silvestrol was investigated for its ability to kill cancer cells by inducing apoptosis. To explore the molecular mechanism of silvestrol, the MDA-MB-435 human melanoma cancer cell line was treated. The IC_{50} values of silvestrol were determined using the MTS cell viability assay. As shown in Table II, silvestrol-induced cytotoxicity was concentration-dependent when tested between the range of 100 μM and 0.01 nM. The IC_{50} value (half maximal inhibitory concentration) of silvestrol was 1.6 nM in MDA-MB-435 cells and its response to standard chemotherapy drugs is shown in Table II. Its cytotoxicity against other cell lines was listed in Table III. Surprisingly, silvestrol appeared to be more potent than another translation inhibitor, homoharringtonine (HHT), which is approved for the treatment of chronic myeloid leukemia.[129]

	IC_{50} (nM)
Vinblastine	1.3
Silvestrol	1.6
Bortezomib	5.7
Homoharringtonine	20
SAHA	475

TABLE II. CYTOTOXICITY OF SILVESTROL ON HUMAN MELANOMA CELLS
Concentration-dependent response for silvestrol-induced cytotoxicity in MDA-MB-435 cells. Melanoma cells were treated with vinblastine, silvestrol, bortezomib, homoharringtonine, and SAHA for 3 days. Cytotoxicity was determined by the MTS assay with the viability of control cells defined as 100 %. Dose-response data represent mean viability ± SE (n = 3 wells per treatment)

TABLE III. IC_{50} OF SILVESTROL IN HUMAN CANCER CELL LINES

	Silvestrol
MDA-MB-435	1.60 nM
MDA-MB-231	3.18 nM
OVCAR3	0.47 nM
HT-29	7.09 nM

Silvestrol decreases proliferation

To examine whether silvestrol-induced toxicity is associated with cell cycle arrest, cells were treated with 25 nM silvestrol for 1, 2 or 3 days followed by a MTS assay. As shown in Figure 6A, silvestrol reduced the proliferation rate of cells in a time-dependent manner. Similar results were found in the HT-29 human colon cancer cell model (Figure 7). By 24 h, decreases in cyclin B1 and cyclin D1 expression were observed in silvestrol-treated cells relative to controls (Figure 6B). Lastly, to evaluate cell cycle distribution, cells were treated with silvestrol for 24 h. The data suggested that silvestrol blocks progression through the cell cycle at the G_2-phase (Figure 6C and 6D). Silvestrol induced cell cycle arrest was also observed in HT-29 human colon cancer cells (Figure 8). Taken together, these results indicate that silvestrol blocks the cell cycle at least in part by inhibiting cyclin expression.

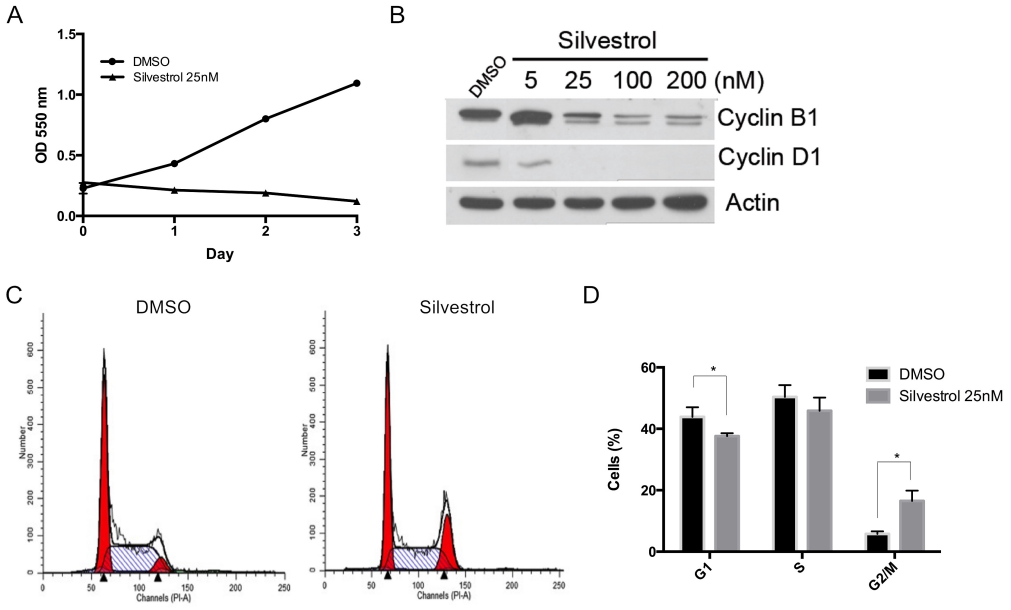

Figure 6. Silvestrol inhibits cell proliferation in MDA-MB-435 human melanoma cells.

(A) Cells were exposed to silvestrol at 25 nM for the indicated times and evaluated for survival by the MTS assay. **(B)** Immunoblot analysis of cyclin B1 and cyclin D1. Cells treated with DMSO and different doses of silvestrol for 24 h and harvested. Cell lysates were resolved in SDS-PAGE and probed with specific antibodies against cyclin B1, cyclin D1 and β-actin. **(C)** Silvestrol inhibits cell proliferation by inducing G2-phase accumulation. Cells were treated with DMSO or silvestrol for 24 h prior to analysis by flow cytometry. **(D)** Graph displaying differences in cell cycle phases. Data represented as means ± SEM, * $p \leq 0.05$

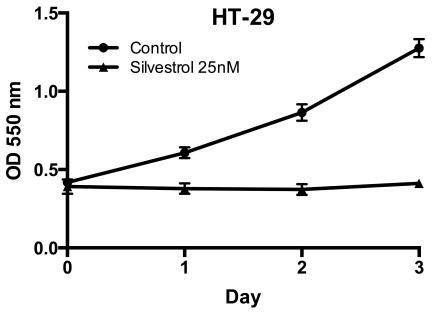

Figure 7. Silvestrol inhibits cell proliferation in HT-29 human colon cancer cells
Cells were exposed to silvestrol at 25 nM for the indicated times and evaluated for survival by the MTS assay.

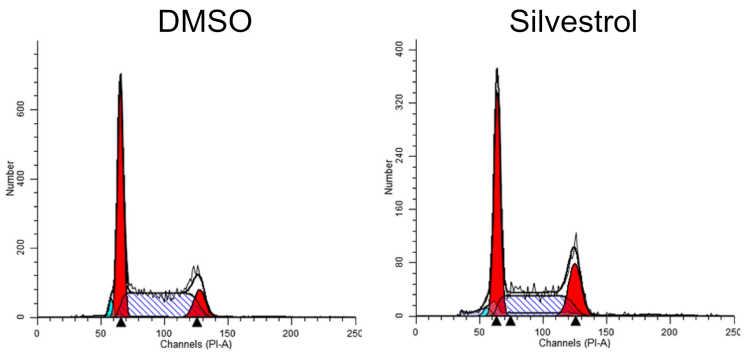

Figure 8. Silvestrol inhibits cell proliferation by inducing G2-phase accumulation in HT-29 cells.
Cells were treated with DMSO or silvestrol for 24 h prior to analysis by flow cytometry.

Silvestrol induces activation of caspase-3/7 and apoptosis

To provide some insight into the potential mechanism of silvestrol-induced cell death, the ability of silvestrol to activate apoptosis was tested. First, apoptotic cells were identified by chromatin morphology using DAPI (6,4'-diamidino-2-phenylindole) staining. Silvestrol induced chromatin condensation in MDA-MB-435 cells compared to the negative control and the positive control, vinblastine (Figure 9A). Next, flow cytometry was conducted using annexin V (AnnV) staining and propidium iodide (PI) staining to label MDA-MB-435 cells undergoing apoptosis from treatment with or without silvestrol. In the presence of silvestrol, $AnnV^{+}PI^{+}$ (late-stage apoptosis) cells significantly increased (Figure 9B).

Caspase activation is a hallmark of the early stages of apoptotic cellular death. Within the identified major caspases, the effector, or executioner caspases are caspase-3, -6, and -7.[130] Additionally, caspase-3/7 activation can be detected by the cleavage of a luminogenic substrate containing the sequence DEVD. Caspase-3/7 activation was detected in MDA-MB-435 cells in response to silvestrol treatment at 24 and 48 hours (Figure 9C). Similarly, the increase in caspase 3/7 activity was also detected in cells treated with vinblastine. Western blots further confirmed silvestrol induced an increase in the abundance of cleaved poly ADP ribose polymerase (PARP) and cleaved caspase 3 after 48 h treatment (Figure 9D). An increase in cleaved caspase 3 and cleaved PARP expression was observed after 48 h and comparable to that induced by vinblastine. Next, HHT served as translation inhibitor control. Notably, cells treated HHT exhibited no increase in cleaved caspase 3 or PARP. Silvestrol is a translation inhibitor. Thus at the protein level, the westerns show a lower expression but

a higher activity. Taken together, these findings indicate that silvestrol can induce apoptosis via activation of a caspase-3-dependent pathway in MDA-MB-435 cells. Furthermore, different translation inhibitors, such as silvestrol and HHT, do not equally induce apoptosis.

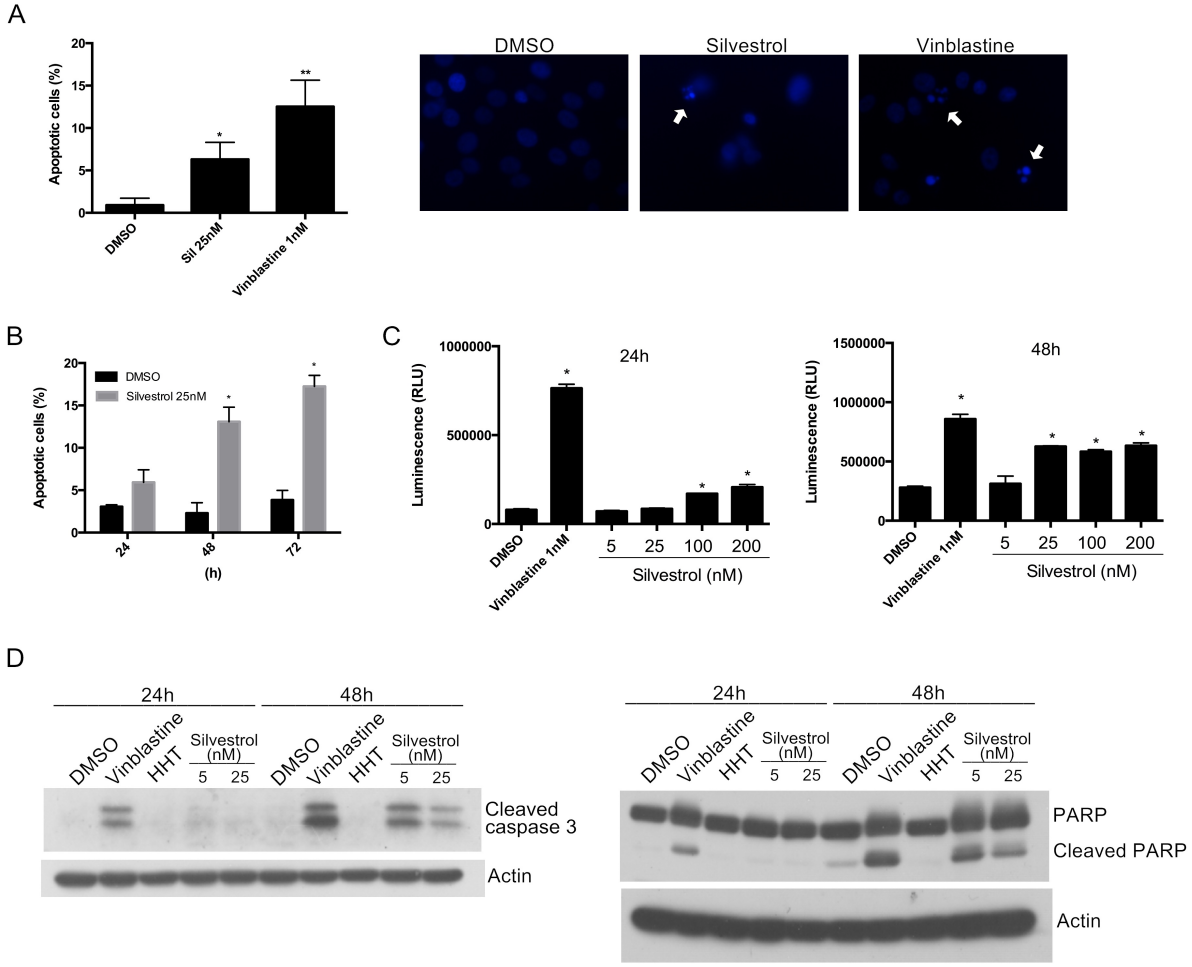

Figure 9. Silvestrol induces apoptosis in MDA-MB-435 cells.
(A) Quantification of apoptosis was performed using DAPI staining. Apoptotic cells were identified by condensation and fragmentation of the nuclei. **(B)** Silvestrol induced apoptosis is time-dependent. MDA-MB-435 cells were treated with DMSO or 25 nM silvestrol for 24 to 72 h, and the Annexin V-FITC/PI double-staining analysis was performed. The early apoptosis (Annexin V-FITC positive, PI negative) and necrotic/late apoptotic (Annexin V-FITC positive, PI Positive) stages were quantified as apoptotic cells. **(C)** Cells in logarithmic growth were treated with DMSO, silvestrol, or vinblastine for 24 h or 48 h. Caspase 3/7 activity was assessed as production of a luminescent product. **(D)** Immunoblot analysis of caspase 3 and PARP. The cleavages of caspase 3 and PARP were detected in cells treated with DMSO, 1 nM vinblastine, 30 nM homoharringtonine (HHT) or silvestrol for the indicated times and harvested for protein analysis. Data presented as means ± SEM, * $p \leq 0.05$

Silvestrol induces morphological features of autophagy

In order to determine additional novel mechanisms of silvestrol-mediated toxicity, a transcriptional array was performed for cancer signal transduction pathways. Preliminary data suggested that silvestrol could induce autophagy in human colon cancer cells (Figure 10). Autophagy involves sequestering cytoplasmic proteins into lytic components and is characterized by the formation and promotion of acidic vesicular organelles. Therefore, to investigate silvestrol-induced toxicity further, the potential of this compound to induce autophagy was studied using biochemical and morphological criteria. In MDA-MB-435 cells, the transcriptional response to silvestrol was analyzed by using qPCR analyses. Autophagic mRNA LC3B and p62 were upregulated in the presence of silvestrol (Figure 11A). Silvestrol's ability to induce LC3 and p62 was blocked when combined with the autophagy inhibitor, 3-methyladenine (3MA) (Figure 10A). In HT-29 colon cancer cells, silvestrol-induced autophagy related gene expression was also inhibited by 3MA (Figure 12). Consequently, these results were consistent with the hypothesis that silvestrol exposure can induce gene transcription associated with autophagy. Next, western blots were performed under the same conditions to confirm changes in protein expression consistent with autophagy. The expression of endogenous LC3 was analyzed in lysates derived from MDA-MB-435 cells that had been treated with silvestrol for up to 24 h. Silvestrol treatment induced early autophagy in the MDA-MB-435 cells, which was characterized by cleavage of LC3 and a decline in the p62 levels relative to control (Figure 11B). These results imply silvestrol is inducing the early stages of autophagy in MDA-MB-435 cells.

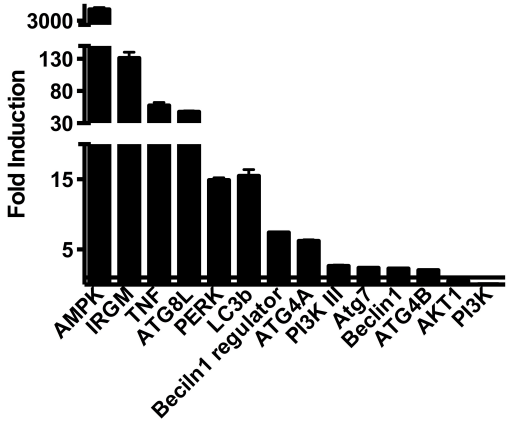

Figure 10. Expression of autophagy related genes induced by silvestrol treatment.

HT-29 cells were treated with 25 nM silvestrol for 16 h and mRNA of cells were assessed. Equivalent mRNA loading was assessed by internal control.

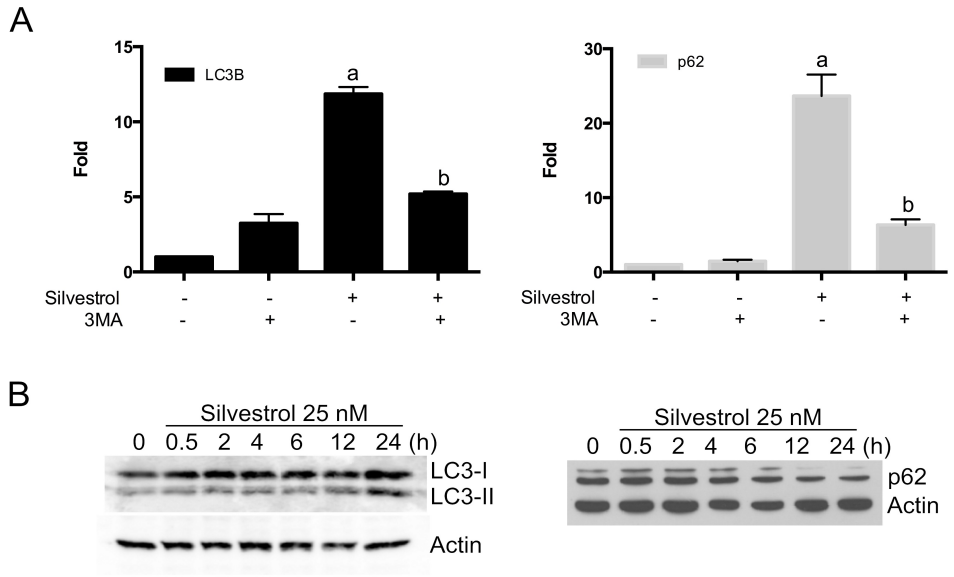

Figure 11. Silvestrol induces transcriptional and translational changes in MDA-MB-435 cells

(A) Cells were incubated with 3MA (10 mM), silvestrol (25 nM), or both for 24 h. mRNA expression was assessed by qPCR. **(B)** Silvestrol induced p62 degradation and LC3-II accumulation. MDA-MB-435 cells were treated with 25 nM silvestrol from 0-24 h and harvested for protein analysis. The data are represented as means ± SEM, * $p \leq 0.05$

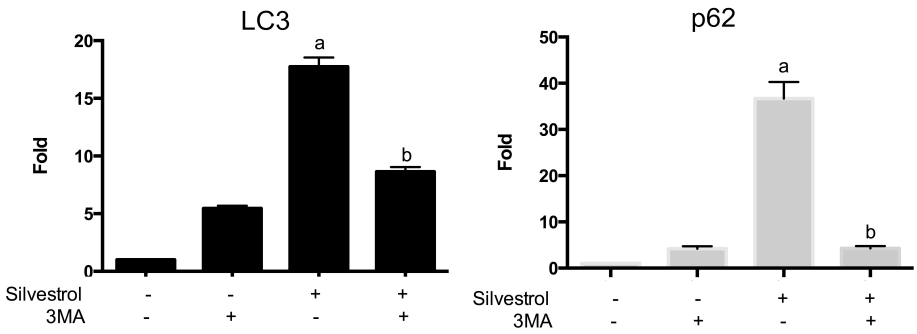

Figure 12. Silvestrol induces transcriptional changes in HT-29 cells.
Cells were incubated with 3MA (10 mM), silvestrol (25 nM), or both for 24 h. mRNA expression was assessed by qPCR. The data are represented as means ± SEM, * $p \leq 0.05$

Silvestrol induces autophagosome accumulation

In order to quantify autophagolysosome-containing cells, treated cells were stained with monodansylcadaverine (MDC). Vital staining of silvestrol-treated MDA-MB-435 cells with MDC, a specific autophagolysosome marker, revealed increased accumulation of the dye relative to control cells (Figure 13A). The increase in autophagolysosome was comparable to that observed with HHT and SAHA, which is known to induce autophagy.[131] To quantify the development of the acidic vesicular organelles, silvestrol-treated cells were stained with acridine orange. Representative photomicrographs of control, SAHA-treated, HHT-treated, and silvestrol-treated cells are shown. Both silvestrol and SAHA enhanced autophagosome formation in MDA-MB-435 cells (Figure 13A).

To better understand the role of silvestrol in autophagosome formation, LC3 protein location was evaluated. LC3, a homolog of yeast Atg8, was used as an autophagy marker. LC3 is specifically localized to autophagic structures, including the autophagosome and its precursor structures, the phagophore and the autolysosome. Under normal nutritional conditions, LC3 protein is distributed diffusely in the cytoplasm. Upon induction of autophagy (e.g., by starvation), autophagosomes are formed, which is an important early step of autophagy induction, while LC3 gets redistributed to a vacuolar pattern. The EGFP-LC3 fusion protein has a similar distribution pattern to endogenous LC3 and can be used as an alternative marker of autophagy induction since it appears as cytoplasmic puncta under fluorescence microscopy. Therefore, the appearance of multiple LC3-positive puncta suggests the induction of autophagy. MDA-MB-435 EGFP-LC3 cells were used to study the cell response to silvestrol. When

comparing with control cells, cells treated with silvestrol or SAHA displayed multiple EGFP-LC3 puncta, representing autophagic vacuoles that were formed in the cytoplasm (Figure 13B).

An increased number of autophagosomes could result from either increased formation or decreased degradation. 3MA, a PI3K inhibitor, blocks autophagy induction. Bafilomycin A1 is a potent inhibitor for vacuolar-type H^+-ATPase that is required for fusion of the autophagosome with the lysosome. Therefore, 3MA and bafilomycin A1 were employed to assess LC3-II accumulation as a marker of autophagosome formation in MDA-MB-435 cells. As illustrated in Figure 13C, silvestrol-induced LC3-II accumulation was attenuated by 3MA. In contrast, silvestrol could still induce LC3-II accumulation in the presence of bafilomycin A1, suggesting that the increase of LC3-II was not due to the blockage of autophagic degradation.

In mammalian cells, Atg7 is essential for the autophagy conjugation system, the formation of autophagosomes, and degradation of proteins and organelles. To evaluate the relationship between autophagy and silvestrol-induced cell death, silvestrol-induced cytotoxicity in wild-type and Atg7-null mouse embryonic fibroblasts (MEFs) were compared. The cytotoxicity induced by silvestrol was more sensitive in wild-type compared to $Atg7^{-/-}$ MEF cells (Figure 13D), suggesting autophagy was involved in silvestrol-induced cell death. Taken together, these findings suggest that silvestrol upregulates gene transcription and triggers the protein translation of the early autophagy pathway components as well as caspase mediated apoptosis.

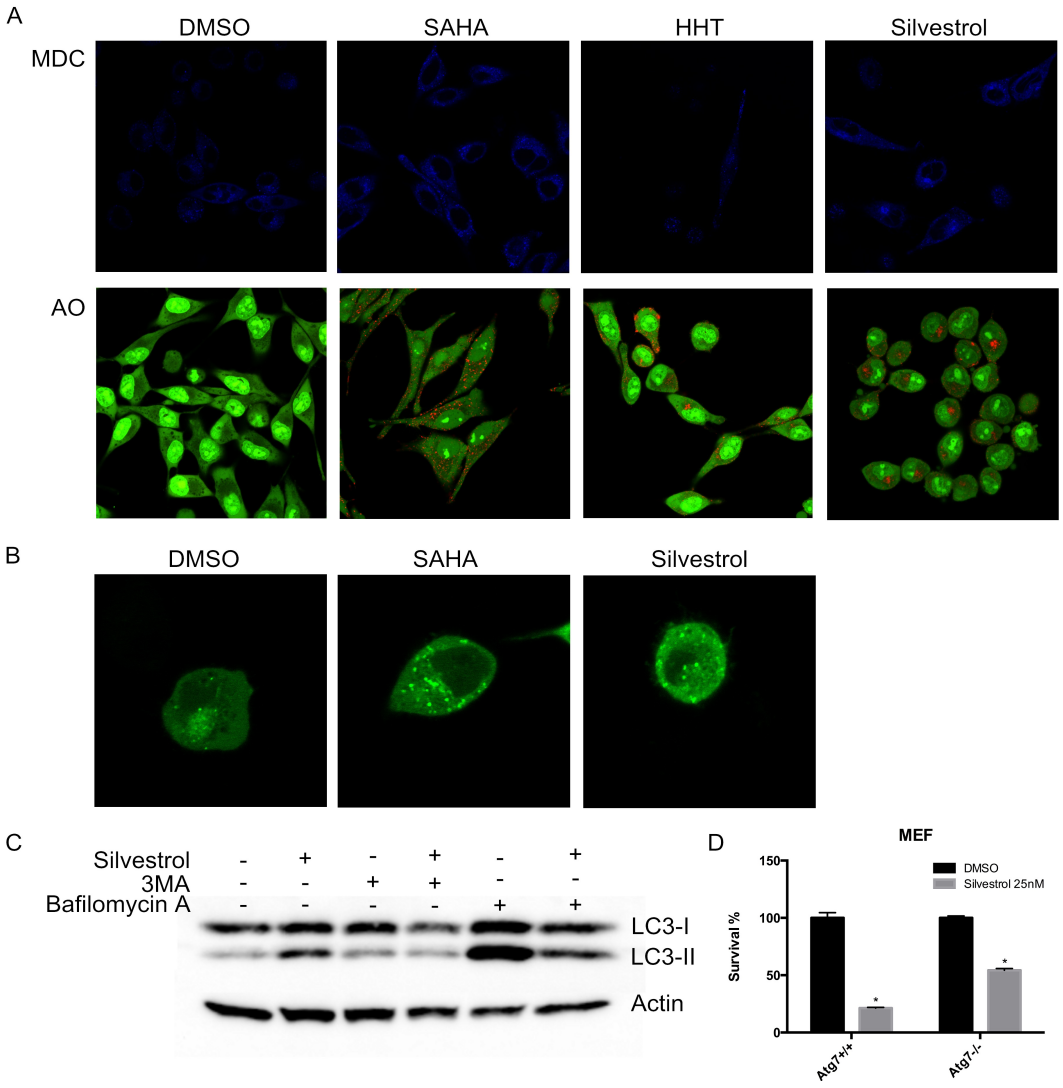

Figure 13. Silvestrol treatment induces autophagy in MDA-MB-435 cells.
(A) Cells were treated with either DMSO, 5 μM SAHA, 30 nM homoharringtonine (HHT), or 25 nM silvestrol for 24 h and subsequently stained with monodansylcadaverine (MDC) or acridine orange (AO). Both silvestrol and SAHA promoted vacuole formation (indicated by blue staining in MDC and red staining in AO). Images are representative of a pattern of staining observed in at least three independent experiments. **(B)** Silvestrol induces autophagy by mediating EGFP-LC3 translocation. Representative pictures of cells treated with DMSO, 5 μM SAHA or 25 nM silvestrol for 24 h. **(C)** Cells were treated with 25 nM silvestrol in the presence or absence of 3MA (10 mM), bafilomycin A1 (50 nM) and then harvested for protein analysis. Cell lysates were resolved in SDS-PAGE and probed with specific antibodies against LC3 and Actin. **(D)** Wild-type (WT) or Atg7-/- MEFs were treated with 25 nM silvestrol for 72 h. Cytotoxicity was determined by MTS assay with the viability of control cells defined as 100 %. The data are represented as means ± SEM, * $p \leq 0.05$

C. Discussion

Silvestrol is a plant-derived natural product that represents a promising lead structure in anti-cancer drug discovery. In this study, the cytotoxic potential of silvestrol against human melanoma cells was investigated. Silvestrol dramatically reduced the viability of MDA-MB-435 cells. Additionally, decreases in cyclin B1 and cyclin D1 expression were observed in silvestrol-treated cells, with blockage of the cell cycle at the G_2-phase. Silvestrol also induced apoptotic features such as nuclear chromatin condensation and caspase-3 activation. Silvestrol included early autophagosome accumulation, such as LC3-II accumulation and time-dependent p62 degradation. The upstream inhibitor, 3MA, but not the downstream inhibitor, bafilomycin A, blocked autophagy processes induced by silvestrol. Taken together, these studies have provided insight into the potential of silvestrol to induce cell death in melanoma.

Previous data have demonstrated that silvestrol can induce cell cycle arrest and apoptosis.[132,133] However, little is known about the molecular mechanism(s) mediating these effects. Cell death can occur through multiple pathways, and the induction of multiple mechanisms of cell death might be useful in cancer therapy. Inhibition of protein synthesis leading to autophagy and apoptosis is a promising new strategy for anticancer therapy. For instance, the histone deacetylase (HDAC) inhibitor, SAHA, is a strong autophagy inducer and also initiates caspase-dependent apoptosis.[134,135] In contrast, sanguilutine is another natural product that has been documented to induce autophagy, but it does not induce caspase-dependent cell death in human A375 melanoma.[136] These studies support that silvestrol induces both early autophagy and caspase-mediated apoptosis in human melanoma cells. These activities are different

when compared to homoharringtonine, another protein synthesis inhibitor, highlighting the different mode of translation inhibition. Driven by the need for new anticancer targets, the exploration of small molecules that regulate cell death through several mechanisms may provide valuable cancer chemotherapeutic agents.

There is accumulating evidence that modulation of protein translation with depletion of short half-life survival factors can enhance therapeutic responses.[133] The effect of silvestrol on cyclin B and cyclin D expression is consistent with these reports. Further analysis of selectively translated mRNAs modulated by silvestrol may be useful to understand specific pathways involved in cancer progression.

There are strong correlations between defects in autophagy regulation or execution and cancer development.[75] This may be due to the fact that autophagy deficiency results in increased DNA damage and gene amplification, decreased cellular differentiation and protein catabolism, especially during stress. In addition, evidence is accruing in the literature that suggests that chemotherapeutic agents can induce autophagic cell death in apoptosis deficient cancer cells while autophagy might potentiate some anticancer drugs against cancer.[131,134,137-139] In contrast, other studies suggest that genetic or pharmacological inhibition of autophagy enhances efficacy of cancer chemotherapeutic agents.[140,141] Suppression of early stage of autophagy by Atg7 knockout reduced silvestrol-induced cytotoxicity indicating that autophagy assists in some of the silvestrol-induced cell death. Combination therapies with silvestrol and other chemotherapeutic agents are under active investigation for melanoma and other cell lines. Although silvestrol and HHT are both as translation inhibitors, HHT can induce autophagy but has a limited ability to drive the apoptotic pathway suggesting that the

mechanism of translational inhibition impacts autophagy and its relationship to apoptosis. Cycloheximide, another translational inhibitor demonstrated autophagy inhibition.[142] Subtle differences in the binding site of the specific translational inhibitor may underlie changes in autophagy.

The functional relationship between the two self-destructive processes, autophagy and apoptosis, is complex and under-studied. In general, it appears that similar stimuli can induce either apoptosis or autophagy in a mutually exclusive manner.[125] Generally, autophagy represents a stress adaptation that avoids cell death, but in several scenarios, autophagy can also lead to autophagic cell death.[36,143-145] For example, in previous reports inhibition of the early steps of autophagy reduced the activation of caspase 8-mediated apoptosis, while inhibition of the late steps of autophagy increased caspase-dependent cell death.[146] A recent study indicates that p62 might act as a key factor that influences autophagy to induce cell death or survival.[74,75] It has been shown p62 silencing induced autophagy activation and caused cell death. In fact, the protein p62 can interact with TRAF6, which is a lysine 63 (K63) E3 ubiquitin ligase that promotes TRAF6 oligomerization, activation of NFκB, and cell survival during tumorigenesis.[75,147] Thus, the elimination of p62 suppresses tumorigenesis. Based on the current study, silvestrol similarly lead to the degradation of p62 and cancer cell death.

In summary, silvestrol is an unusual rocaglate derivative with a dioxanyl ring that potently inhibits cell growth and induces cell death in human melanoma cells through induction of early autophagy and caspase-mediated apoptosis. These findings provide insight into the mechanisms of cell death and signaling from silvestrol in melanoma.

Understanding the interplay between autophagy and apoptosis could potentially inform the development of future chemotherapy agents and improve combination therapies that stimulate these pathways. Future studies on silvestrol could focus on structure/ligand based drug design in concert with structural biology, synthetic chemistry, biochemical analysis and pharmacokinetics.[148] Based on the cytotoxic potential of silvestrol, it will provide a novel and promising strategy to improve the anticancer treatment.

IV. Strebloside-induced cytotoxicity in ovarian cancer cells is mediated through cardiac glycoside signaling networks

A. Introduction

Drug repurposing is a strategy that offers promising opportunities for increasing the number of small molecules that can be used to treat disease. Extensive searches for potent anticancer drug leads revealed that some drugs can be repurposed for cancer. Cardiac glycosides, such as digoxin, are prescribed to treat cardiovascular disease, and they also displayed anticancer activity. Lower mortality rates have been reported in women with breast cancer treated with cardiac glycoside as compared to women not consuming cardiac glycosides.[149] Epidemiologic studies have demonstrated a reduced incidence of leukemia, lymphoma, renal, prostate and lung cancer with regular cardiac glycoside intake.[150,151] Other reports have demonstrated that cardiac glycoside have synergistic anticancer effect when used in combination with chemotherapeutic agents. Specifically, the combination of oleandrin and cisplatin resulted in greater cytotoxicity in prostate, breast, lung, pancreatic, colorectal and melanoma cell lines than either agent alone.[152] A synergistic cytotoxicity was observed in colorectal cancer patients when digoxin was combined with oxaliplatin.[153] Several cardiac glycoside were studied in clinical trials as anticancer treatment.[154] In contrast to some of the positive studies, in the Surveillance, Epidemiology and End-Results (SEER)-Medicare database analysis, digoxin use during chemotherapy was not associated with improved overall survival in patients with epithelial ovarian cancer treated with surgery and platinum chemotherapy.[155] Recent reports revealed that bufalin induces cell cycle arrest and apoptosis in endometrial and ovarian cancer.[156] However,

little has been studied on cardiac glycosides in high-grade serous ovarian cancer (HGSC).

Cardiac glycosides are a class of chemical compounds used clinically for arrhythmia and heart failure that specially inhibit Na^+/K^+-ATPase. Apart from its function on the ion pump, Na^+/K^+-ATPase interacts with different signaling proteins and many of these have been studied for their role in reducing cancer cell viability. Inhibition of Na^+/K^+-ATPase induced Ca^{2+} accumulation and increased reactive oxygen species (ROS), followed by growth arrest and cell death. Na^+/K^+-ATPase inhibition also activated signal transduction pathways including Src, EGFR and MAPK and reduced p53 synthesis.[80-83] A study on hepatocellular carcinoma using quantitative proteomics and bioinformatics showed proteins involved in mitotic cell cycle (e.g., Cyclin D1 and CDK) and chromosome segregation (e.g., AURKA and SMC2) were primarily decreased from treatment with cardiac glycosides.[157] Due to the involvement of the Na^+/K^+-ATPase in numerous cellular functions, changing the chemical structure of the cardiac glycosides to alter the downstream signaling might be one strategy to repurpose this class of molecules for cancer.

To date, several cardiac glycosides have been reported to exhibit cytotoxicity toward human cancer cells.[158] *Streblus asper* is a small tree found in tropical countries such as India, Sri Lanka, Malaysia, Philippines and Thailand. Various parts of this plant have been used in Ayurveda and other folk medicines for the treatment of leprosy, toothache, diarrhea, epilepsy and cancer.[107] Strebloside was isolated and identified from *S. asper*. Among the numerous cardiac glycoside obtained, strebloside showed potent cytotoxicity against HT-29 and KB cells.[159]

In order to determine if the cardiac glycoside, strebloside, can exert unique signaling to induce cytotoxicity, it was compared to digitoxin in several cancer cell lines. To investigate the anticancer effect of strebloside, a variety of tumor cells were tested and strebloside showed potency against OVCAR3 and MDA-MB-231 *in vivo* in a hollow fiber assay. In the current study, high-grade serous ovarian cancer cell lines were used. The results showed strebloside binds to Na^+/K^+-ATPase and further affects cell cycle, cell proliferation and induces apoptosis in ovarian cancer cells. Moreover, strebloside displayed similar signal transduction pathways as digitoxin. Activation of cell death by strebloside appears to be mediated through the Na^+/K^+-ATPase and its downstream signaling. Its anticancer activity mimicked that of other cardiac glycosides.

B. Results

In vivo evaluation of strebloside

Strebloside was tested in an *in vivo* hollow fiber assay to determine its possible antitumor efficacy. Immunodeficient NCr *nu*/*nu* mice were implanted with MDA-MB-231 human breast cancer cells, OVCAR3 human ovarian cancer cells, and MDA-MB-435 human melanoma cells placed in hollow fibers. The mice were injected ip daily for four days with strebloside at doses of 1.0, 5.0, 10.0, 15.0 or 30.0 mg/kg. Control animals were injected with either vehicle or paclitaxel (5 mg/kg). The relative MDA-MB-231, OVCAR3 and MDA-MB-435 cell growth values from all mice were calculated. The results showed that the values in MDA-MB-231 and OVCAR3 from the treatment of strebloside at 5.0 to 30.0 mg/kg (i.p.) were all statistically significantly different compared to vehicle control (i.p.), but not MDA-MB-435, implying some cell specific effect *in vivo* (Figure 14). No gross toxicity was observed in the mice treated at the doses employed. These findings suggest that strebloside may posess anticancer potential.

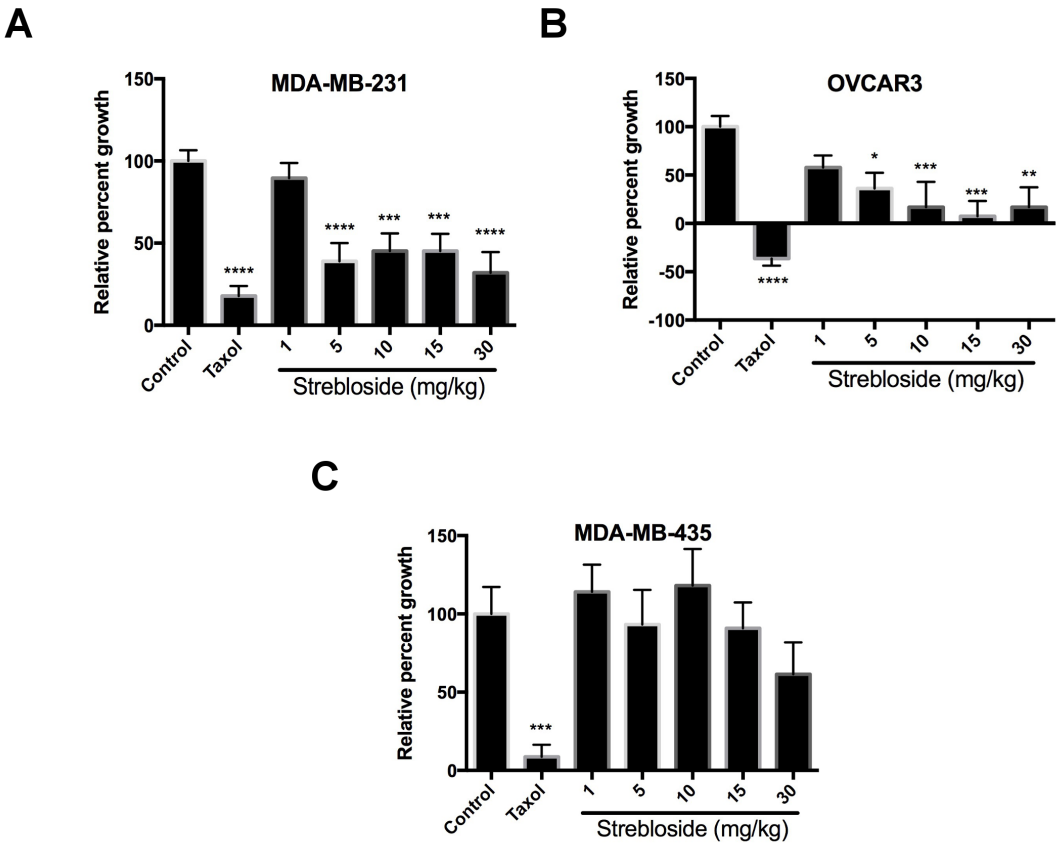

Figure 14. *In vivo* biological evaluation of strebloside.
Effect of strebloside on the growth of human breast MDA-MB-231 **(A)** and ovarian OVCAR3 **(B)** and melanoma MDA-MB-435 **(C)** cancer cells implanted in NCr *nu*/*nu* mice tested in an *in vivo* hollow fiber assay. The results are shown as the average percentage cell growth relative to control. Columns, mean in each group (n = 12 for the control group and n = 6 for the treatment group); bars, SE; *$p \leq 0.1$, **$p \leq 0.05$, ***$p \leq 0.01$, and ****$p \leq 0.001$ for significant differences from the control treatment.

Na^+/K^+-ATPase inhibition by strebloside

Strebloside's chemical structure is highly similar to other cardiac glycosides, such as digoxin, digitoxin and ouabain. Therefore, strebloside was tested for the ability to inhibit Na^+/K^+-ATPase activity. The cellular enzyme adenosine 5′-triphosphatase from porcine cerebral cortex was incubated with various concentrations of strebloside, and digitoxin was used as positive control. Strebloside inhibited Na^+/K^+-ATPase activity in a concentration-dependent manner (Figure 15). Strebloside not only resembles a cardiac glycoside based on its chemical structure, but it also blocks the Na^+/K^+-ATPase pump based on its ability.

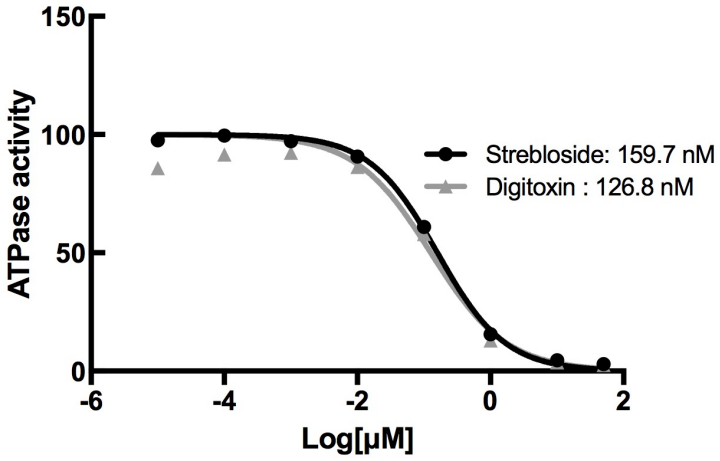

Figure 15. ATPase Assay of Na^+/K^+-ATPase treated with strebloside and digitoxin. Na^+/K^+-ATPase activity was determined and displayed as the percentage of activity relative to the untreated sample.

Molecular modeling of strebloside, digitoxin in Na^+/K^+-ATPase

A computer simulated docking analysis was performed to further compare the structure of digitoxin and strebloside in the binding pocket of the Na^+/K^+-ATPase. The binding mode for digitoxin and strebloside are similar from the overlapping of the two docking complexes (Figure 16), with 14-OH forming a H-bond with T797. In addition, the 3'-OH in the glycosyl portion of digitoxin forms a H-bond with the sidechain of R880 and the 19-OH in strebloside forms a H-bond with Q111. Van der Waals force interactions are also detected among F786, L793 and the steroid portion of these two compounds as well as for the L125, A323, T797 interactions with the lactone part. [Pictures are made by Chimera[160]]

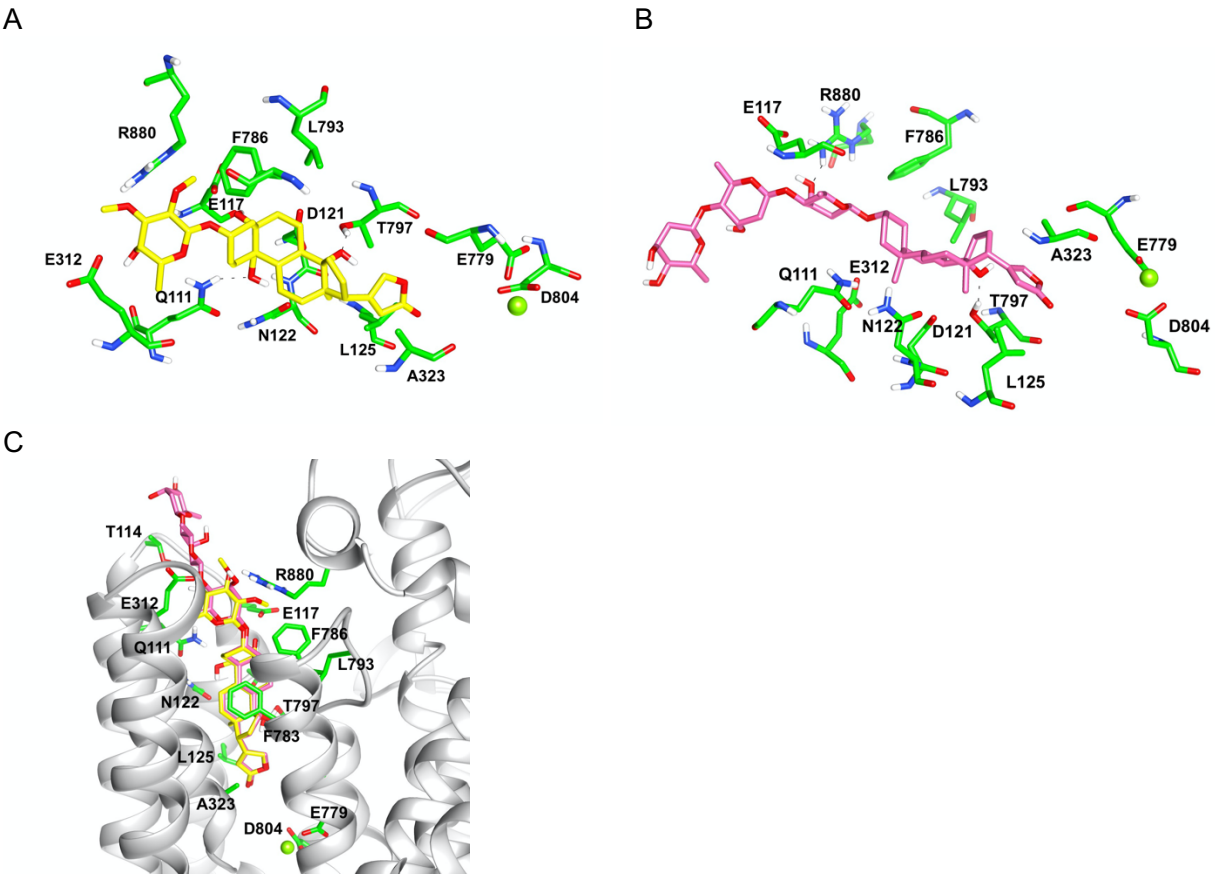

Figure 16. Strebloside (yellow) and digitoxin (pink) bound to Na^+/K^+-ATPase.
(A) The predicted strebloside-binding mode. Strebloside and the amino acids in the active site are depicted by yellow and green sticks. The 14-OH and 19-OH of strebloside form H-bonds with the sidechains of T797 and Q111, respectively. **(B)** The predicted digitoxin-binding mode. Digitoxin and the amino acids in the binding cavity are depicted in pink and green sticks. The 14-OH of digitoxin forms a H-bond with sidechain of T797. And 3'-OH in the glycosyl part forms H-bond with sidechain of R880. The Mg^{2+} ion occupying cation site II is represented by a green sphere in panels A and B. **(C)** Overlapping the docking poses of strebloside and digitoxin. Digitoxin, strebloside and the amino acids in the binding cavity are depicted in pink, yellow and green sticks, respectively. And the Na^+/K^+-ATPase is shown in gray ribbon. (Data and figure generated by Dr. Jinhong Ren in Dr. Michael Johnson's laboratory at UIC)

Strebloside inhibits cell proliferation

Cardiac glycosides that inhibit the Na^+/K^+-ATPase can also reduce cell viability. Previously, strebloside demonstrated *in vivo* activity against OVCAR3 and MDA-MB-231. Strebloside also demonstrated significant cytotoxic activity in human melanoma (MDA-MB-345) and colon cancer cells (HT-29). IC_{50} values are displayed in Table 1. To further analyze the use of strebloside in high-grade serous ovarian cancer cells, several additional lines were tested. including OVCAR3, OVSAHO, Kuramochi, OVCAR4, OVCAR5 and OVCAR8. Strebloside was active in all lines tested (Table IV). To determine whether this toxic action was specific to cancer cells, the ovarian surface epithelial cell line IOSE-80, which expresses SV40 large T antigen, was treated with strebloside and digitoxin. The cardiac glycosides were more potent in the normal immortalized cell line as compared to the tumor cell lines consistent with previous reports in breast epithelial cells and cancer cells[161] (Table V). One reason that cardiac glycosides have continued to be pursued for anti-cancer activity is due to their efficacy in xenograft models. However, this has been suggested to be due to the ability of these compounds to selectively kill human cells as compared to murine cells due to differential expression in Na^+/K^+-ATPase. In support of these previous results, after 3 days of strebloside and digitoxin treatment, growth of human cancer cells was inhibited, while murine oviductal epithelial cells (MOE) was not growth inhibited (Table VI). The Cancer Genome Atlas Network notes that several key pathways are altered in HGSC. For example, PTEN is lost and KRAS is amplified in tumors. However, MOE with silenced PTEN and activated KRAS, which was sufficient to drive tumorigenesis,[162] but was not affected by either cardiac glycoside (Table VI). Thus, murine cells are more resistant

than human cells to the anti-proliferative effects of these cardiac glycosides. Despite being active in human cancer cells, the antitumor activity of cardiac glycosides is probably due to selective toxicity in human cells versus rodent cells.

TABLE IV. IC_{50} OF STREBLOSIDE IN HUMAN CANCER CELL LINES

	Strebloside (nM)	Digitoxin (nM)
MDA-MB-435	78.7	43.3
MDA-MB-231	643.0	482.0
HT-29	101.0	67.6

TABLE V. IC_{50} OF STREBLOSIDE IN HUMAN HIGH-GRADE SEROUS OVARIAN CANCER CELL LINES

	Strebloside (nM)	Digitoxin (nM)
OVCAR3	134.1	117.1
OVSAHO	560.6	250.2
Kuramochi	3437.0	5911.0
OVCAR4	457.3	425.6
OVCAR5	541.2	321.0
OVCAR8	91.1	71.5

TABLE VI. IC_{50} OF STREBLOSIDE IN HUMAN AND MURINE NORMAL CELLS

	Strebloside (nM)	Digitoxin (nM)
IOSE	50.9	48.4
MOE Low	$> 5\times10^4$	$> 5\times10^4$
MOE High	$> 5\times10^4$	$> 5\times10^4$
MOE Scr	$> 5\times10^4$	$> 5\times10^4$
MOE PTEN/KRAS	$> 5\times10^4$	$> 5\times10^4$

To further assess the anti-proliferative phenotype of strebloside, OVCAR3 and OVSAHO cells were tested for *in vitro* anti-cancer activity. A clonogenic assay was employed to monitor the ability of strebloside treated cells to form colonies as compared to control. The cells were treated with drug or solvent control every 3 days for the entire assay. The negative control group formed a significant number of colonies after 21 days, while neither OVCAR3 nor OVSAHO cells formed colonies when exposed to strebloside (Figure 17A and 17B). Next, the cells were grown in soft agar to test whether strebloside inhibited anchorage independent growth. Strebloside treated cells formed significantly less soft agar colonies than DMSO control in OVCAR3, while no difference was detected in OVSAHO (Figure 17C and 17D).

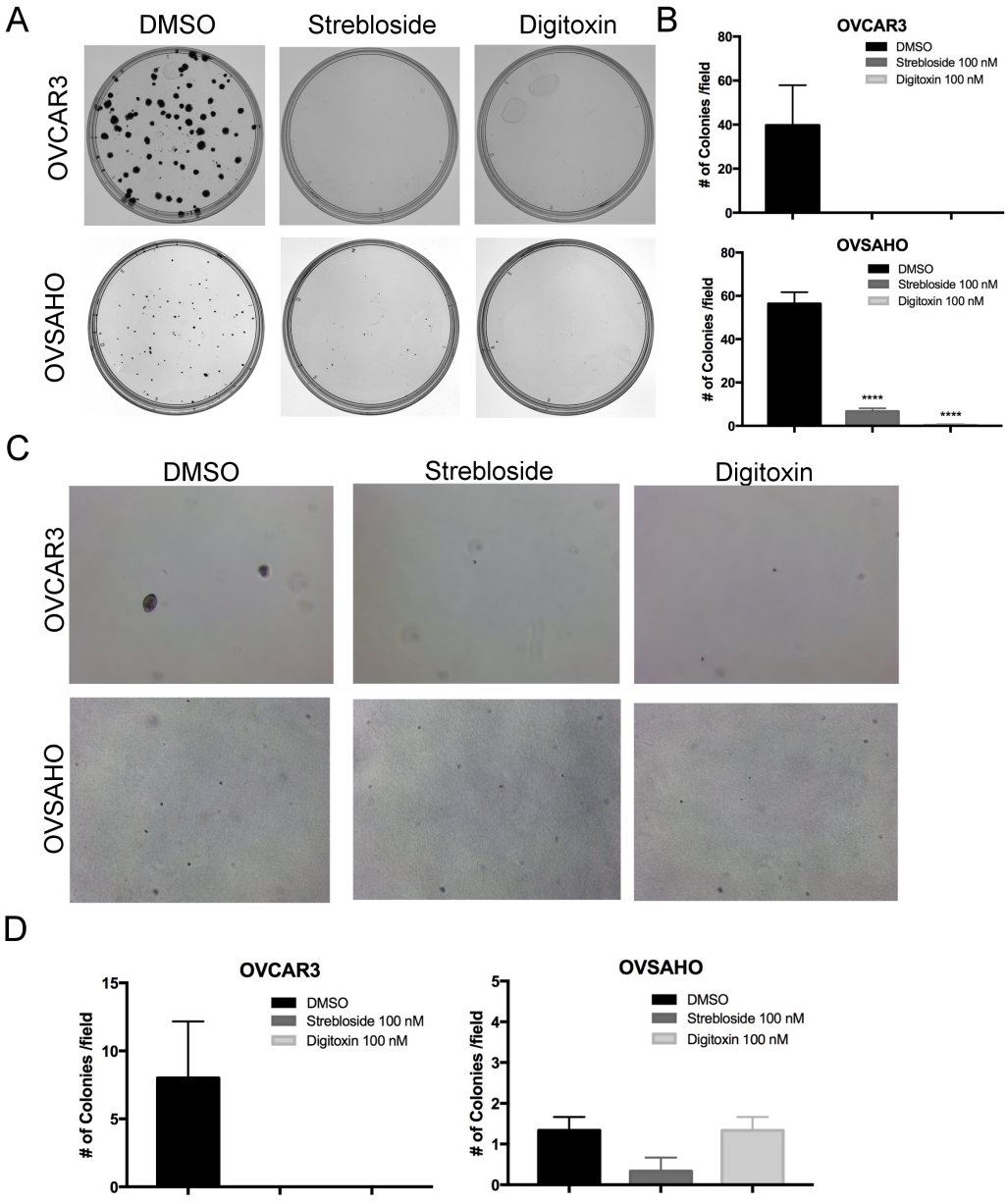

Figure 17. Strebloside inhibited 2D colony formation and 3D soft agar colony growth.

(A and B) OVCAR3 and OVSAHO cells were treated with strebloside and digitoxin then tested for 2D colony formation after 21 days using a clonogenic assay. **(C and D)** OVCAR3 and OVSAHO cells were treated with strebloside and digitoxin then analyzed for soft agar colony formation after 21 days. **** $p \leq 0.0001$

Strebloside blocks cell cycle

To investigate if cells undergo cell cycle arrest as a consequence of strebloside treatment, strebloside treated cells were collected and subjected to flow cytometry after propidium iodide staining. Figure 18A shows strebloside induced an accumulation of cells in the G2/M phase of the cell cycle in OVCAR3, similar to cells treated with digitoxin (Figure 18B). Next, the cyclin dependent kinase inhibitor p21 was measured using western blot as marker of cell cycle arrest. Quantitative densitometry confirmed that strebloside and digitoxin treated cells expressed more p21 protein than control (Figure 18C and 18D). Similar results were observed in OVSAHO (Figure 19). OVCAR3 and OVSAHO cells treated with either strebloside or digitoxin both demonstrated a significant increase in p21 expression, suggesting p21 enhances the blockage of cell cycle.

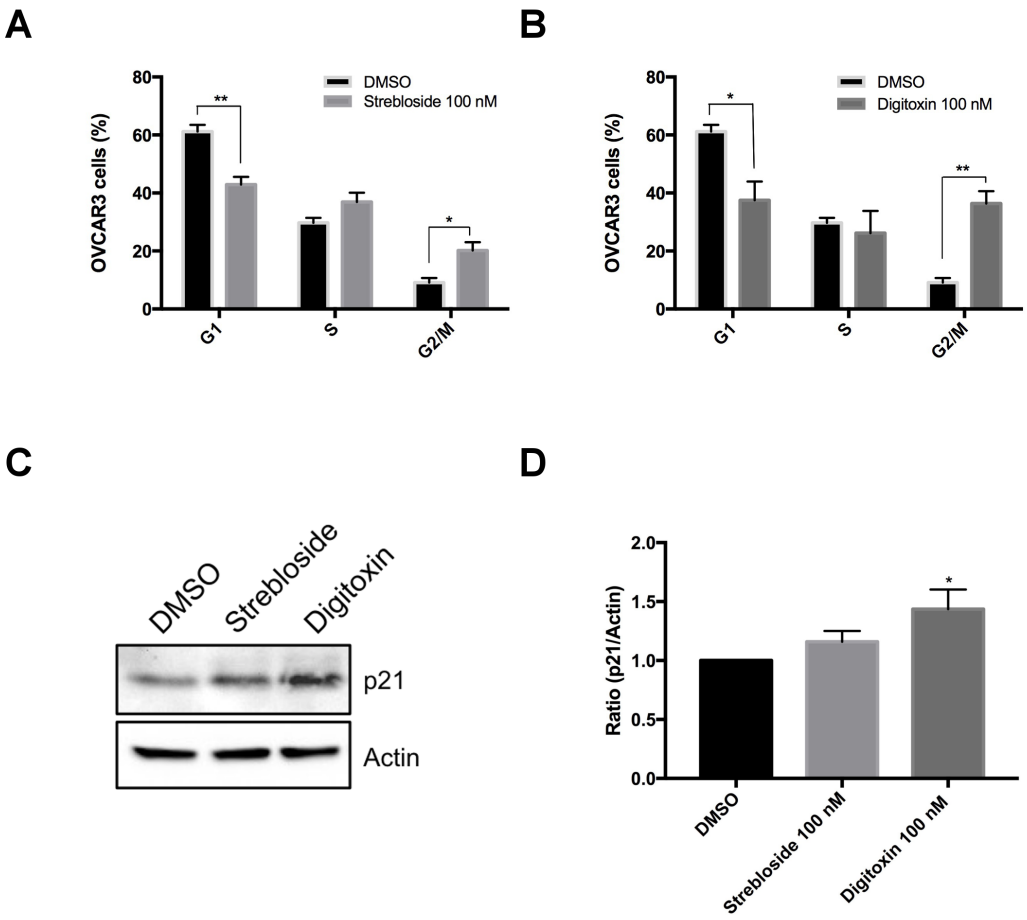

Figure 18. Strebloside inhibits cell proliferation in OVCAR3 human ovarian cancer cells.

(A and B) Cells were treated with DMSO, strebloside or digitoxin for 72 h prior to analysis by flow cytometry. Graph displaying differences in cell cycle phases. **(C)** Western blot of OVCAR3 cell lysates treated with either strebloside or digitoxin were probed for p21 and actin. (**D)** Quantitative densitometry of western blots indicate increase expression of p21 in OVCAR3 cells (*n* = 3). Data represented as means ± SEM, * $p \leq 0.05$, ** $p \leq 0.01$

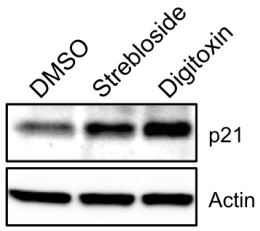

Figure 19. Strebloside inhibits p21 expression in OVSAHO human ovarian cancer cells.
Western blotting of OVSAHO cell lysates treated with either strebloside or digitoxin were probed for p21 and actin.

Strebloside induces apoptosis

To determine the mechanism for cardiac glycoside induced cytotoxicity, western blot analyses were performed. As shown in figure 20A, treatment of OVCAR3 cells with either strebloside or digitoxin led to a significant increase in cleaved caspase 3 as well as its downstream target cleaved PARP. Quantitative densitometry was performed on OVCAR3 cells. Cells exposed to either Strebloside or digitoxin expressed significantly higher levels of cleaved PARP and cleaved caspase 3 when compared to control cells (Figure 20B and 20C). There was also a decrease in the anti-apoptotic proteins Mcl-1 and Bcl-xL after either strebloside or digitoxin treatment (Figure 20D and 20E). Quantitative densitometry confirmed that streblosdie or digitoxin treated cells expressed less Mcl-1 and Bcl-xL protein than control cells (Figure 20F and 20G). Next, western blotting and quantitative densitometry were performed on OVSAHO cells exposed to either strebloside or digitoxin. Cleaved PARP expression levels in strebloside or digitoxin treated cells were higher than the levels in control cells (Figure 21A and 21B). These results suggest that the apoptotic effect of strebloside in OVCAR3 cells is mediated by the increase of cleaved caspase 3, cleaved PARP and suppression of pro-survival proteins Mcl-1 and Bcl-xL.

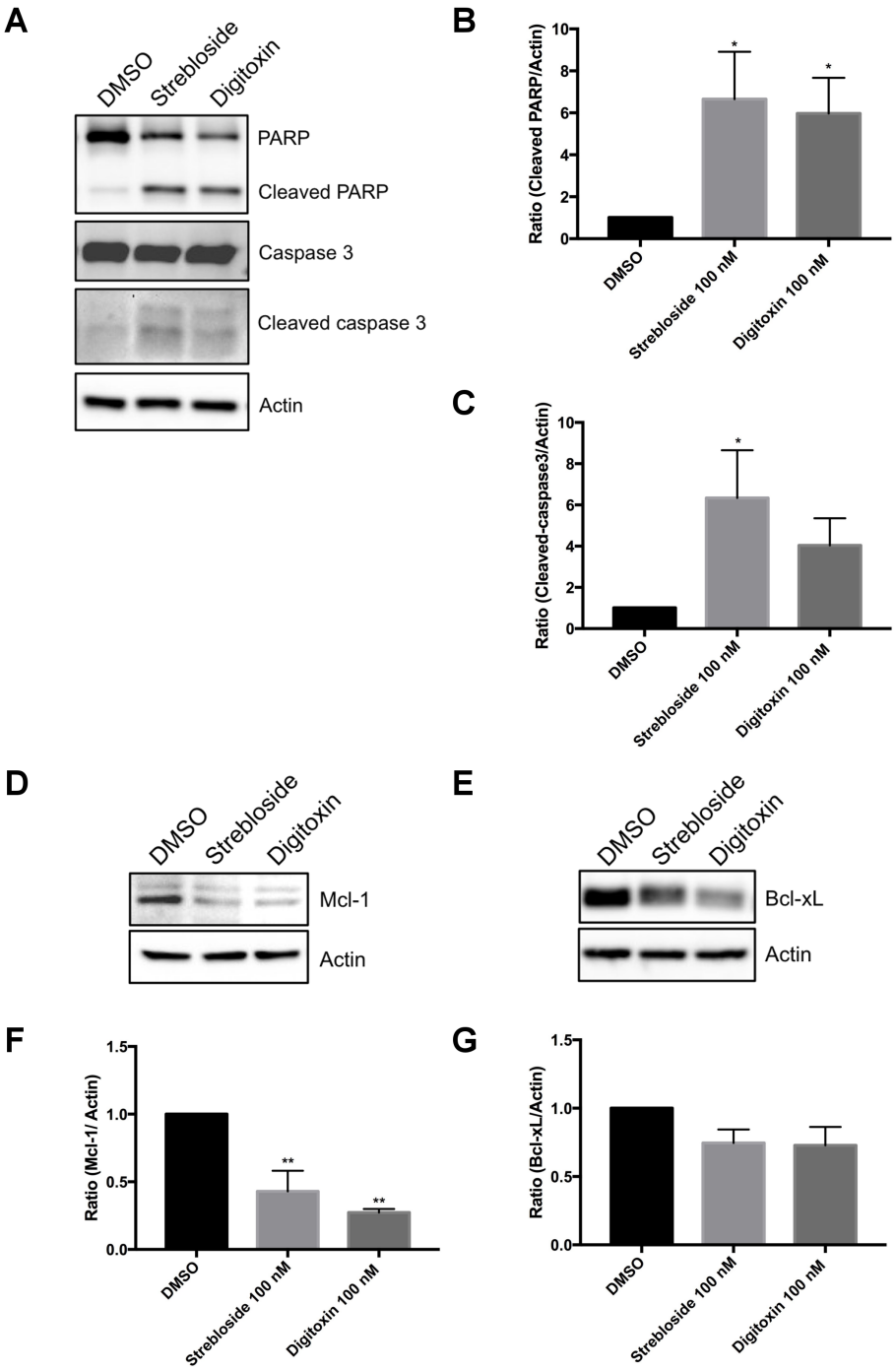

Figure 20. Strebloside induces apoptosis in OVCAR3 cells.
(A) Western blot of OVCAR3 cell lysates treated with strebloside and digitoxin were probed for PARP, caspase 3, cleaved caspase3, and actin. **(B and C)** Quantitative densitometry of western blots indicate increase expression of cleaved PARP and cleaved caspase 3 in OVCAR3 cells (n = 3). **(D and E)** Western blotting of OVCAR3 cell lysates treated with strebloside or digitoxin were probed for Mcl-1, Bcl-xL and actin. **(F and G)** Quantitative densitometry of western blots indicate decreased expression of Mcl-1 and Bcl-xL in OVCAR3 cells (n = 3). * $p \leq 0.05$, ** $p \leq 0.01$

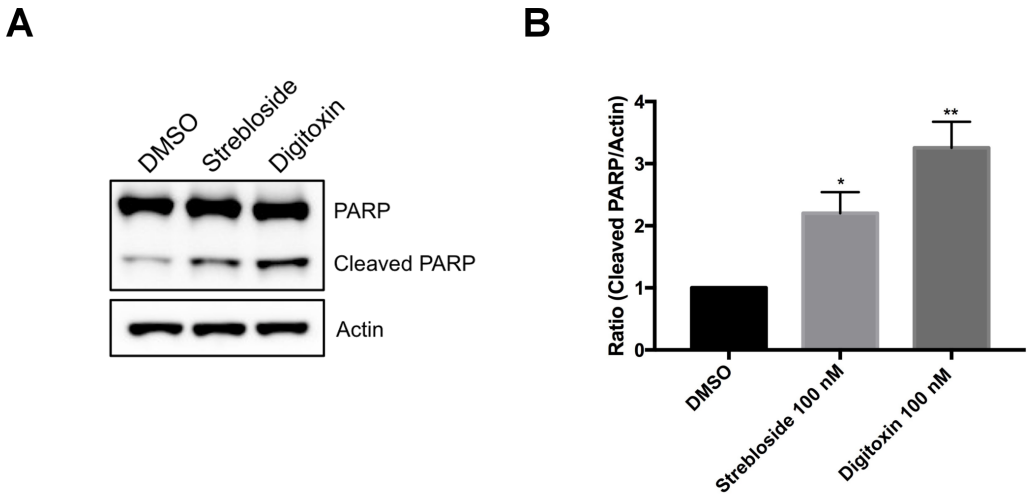

Figure 21. Strebloside increases cleaved PARP in OVSAHO human ovarian cancer cells.
(A) OVSAHO cells were treated with strebloside or digitoxin at 100 nM for 72 h and subjected to western blotting. **(B)** Quantitative densitometry of western blots indicate increase expression of cleaved PARP in OVSAHO cells significantly higher than control (n = 3). * $p \leq 0.05$, ** $p \leq 0.01$

Strebloside decrease of mutant p53 expression is ERK pathway dependent

In order to reveal some of the pathways that might be modified by strebloside, western blots of OVCAR3 and OVSAHO lysates were performed and the results were compared to digitoxin as a positive control. Given the high percentage of p53 mutation in HGSC, p53 has been a central target for mechanism-driven cancer drug discovery. Furthermore, it has been reported that the cardiac glycosides digoxin and ouabain, reduce p53 protein levels.[163] Since most of these studies were done with cells that express either wild-type or mutant p53, and high grade serous ovarian cancer cells should all express mutant p53, the ability for strebloside to reduce p53 protein levels was examined. Treatment of strebloside and digitoxin resulted in the disappearance of p53 (Figure 22A and 22B). Since p53 expression was not detectable in OVSAHO cells, OVCA432 were treated with strebloside or digitoxin, which resulted in decreased expression of p53 (Figure 23A and 23B). In addition, inhibition of the Na^+/K^+-ATPase can transduce intracellular signals via multiple pathways, including mitogen-activated protein kinases (MAPK). Previous reports demonstrate that a MAPK inhibitor was able to block cardiac glycoside induced repression of p53.[163] Extracellular signal-regulated kinases (ERKs) are members of MAPK. Phosphorylated ERK and total ERK were probed in response to strebloside and digitoxin treatment. Both cell lines showed induced activation of ERK after treatment with strebloside (Figure 22C and 22D) or digitoxin (Figure 22E and 22F). These data are consistent with previous reports where cardiac glycoside-induced cell death was mediated by activation of ERK, followed by the decreases in p53. Overall, these findings suggest the MAPK pathway is

downstream of Na^+/K^+ ATPase and contributes to decreased mutant p53 expression in HGSC cells.

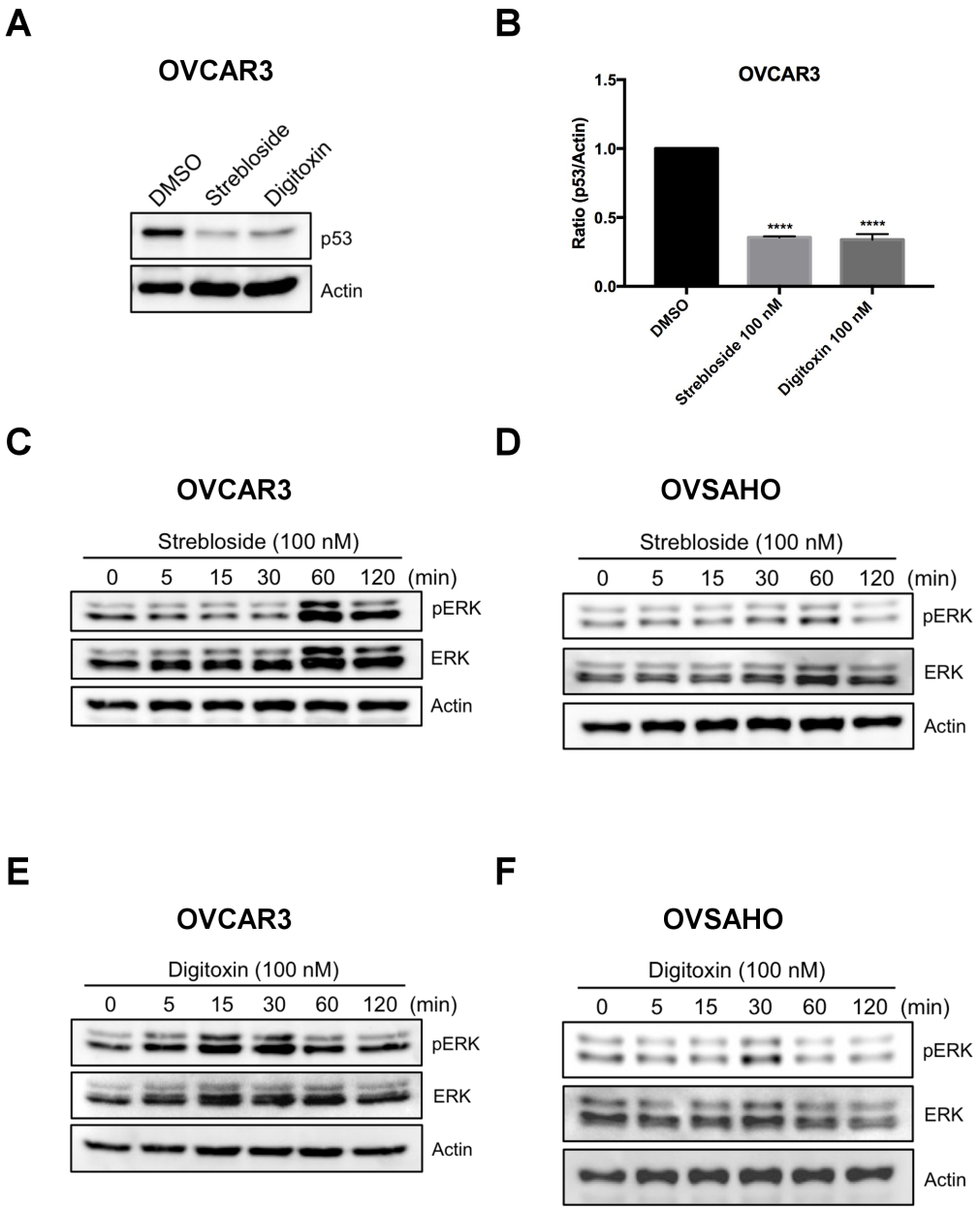

Figure 22. Strebloside decreases p53 expression and activates ERK pathway. **(A)** OVCAR3 cells were treated with strebloside or digitoxin at 100 nM for 72 h and subjected to western blot analysis. **(B)** Quantitative densitometry of western blots indicate significantly decreased expression of p53 in OVCAR3 cells relative to control (*n* = 3). **(C and E)** OVCAR3 cells were exposed to strebloside or digoxin at 100 nM for indicated periods of time, and analyzed by western blotting. **(D and F)** OVSAHO cells were exposed to strebloside or digoxin at 100 nM for indicated periods of time, and analyzed by western blot. **** $p \leq 0.0001$

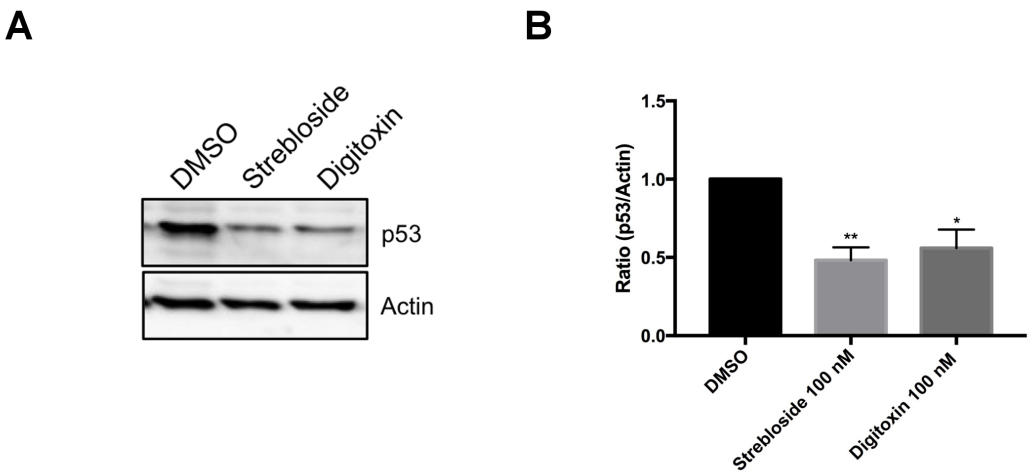

Figure 23. Strebloside decreases p53 expression in OVCA432 human ovarian cancer cells.
(A) OVCA432 cells were treated with strebloside or digitoxin at 100 nM for 72 h and subjected to western blotting. **(B)** Quantitative densitometry of western blots indicate that p53 expression is significantly decreased in OVCA432 cells relative to control (n = 3). * $p \leq 0.01$, ** $p \leq 0.001$

Strebloside inhibits NF-κB transcription activity

Cardiac glycosides have also been shown to inhibit cell survival by inhibiting NF-κB signaling. To further characterize how strebloside modulates signal transduction pathways that may contribute to their ability to hinder tumor growth, a luciferase assay was employed that quantifies regulation of the NF-κB promoter. OVCAR3 cells were transfected with a reporter gene of NF-κB and exposed to strebloside or digitoxin. When cells were incubated with TNF-α, activation in NF-κB reporter activity was observed. By contrast, cotreated TNF-α with strebloside or digitoxin decreased NF-κB signaling 29% and 50%, respectively (Figure 24A and 24B). These findings taken together suggest strebloside shares common features of cardiac glycosides, which are reported in the literature to inhibit NF-κB signaling.[164]

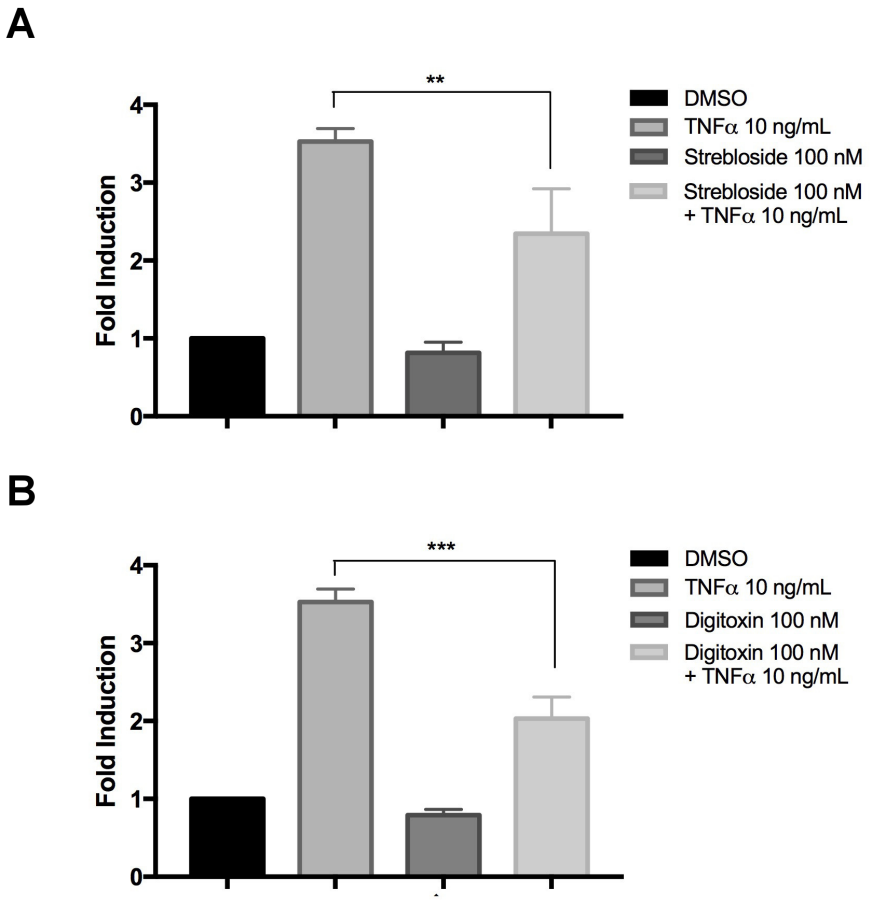

Figure 24. Strebloside inhibits NF-κB activation.
(A) OVCAR3 cells were transfected with plasmids pNF-κB-luc overnight, then cotreated with 100 nM strebloside with or without 10 ng/ml TNF-α for 4 h. Cell extracts were collected and analyzed. **(B)** OVCAR3 cells were transfected with plasmids pNF-κB-luc overnight, then cotreated with 100 nM digitoxin with or without 10 ng/ml TNF-α for 4 h. Cell extracts were collected and analyzed. ANOVA was performed for fold change to analyze significance compared to untreated. Data represented as mean ± SEM, ** $p \leq 0.01$, *** $p \leq 0.001$

Electrophysiological characterization of strebloside

Many drugs cause side effects that can upset the balance between benefit and risk of a specific therapy. One of the most important undesired effect of drugs involves prolongation of the cardiac action potential (acquired long QT syndrome; aLQT2) which is mostly caused by inhibition of the human *either-a-go-go* related gene (hERG) potassium channels and can be associated with ventricular fibrillation and sudden death. Several drugs have been removed from the market or have received restrictive labeling because of their association with aLQT2. Our electrophysiological study revealed that strebloside did not exert any change on any of the hERG1 biophysical parameters that were measured including peak current amplitude, current-voltage relationship, voltage-dependence of current deactivation and activation (Figure 25). These data suggest that strebloside is neither a hERG1 blocker nor activator and that strebloside should not cause aLQT2 in clinical practice.

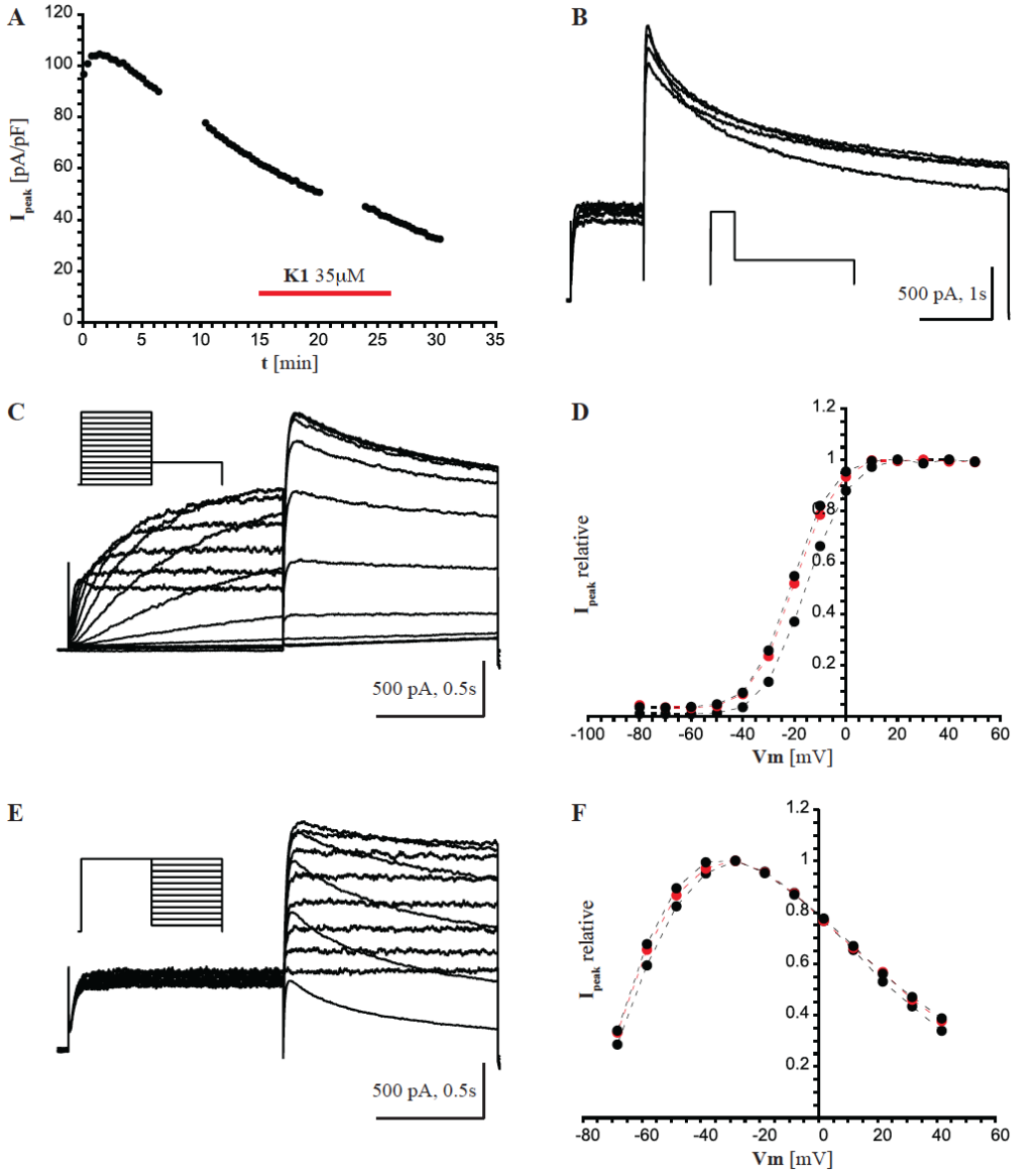

Figure 25. Strebloside does not affect hERG1.
(A) hERG1 current whole-cell recording over time with example traces and voltage protocol. **(B)** Example traces and voltage protocol. **(C)** Current-voltage relationships. **(D)** Activation curves, **(E)** Voltage-dependence (IV curves were determined during the 'gaps' in A). **(F)** deactivation curves before, during [165] and after strebloside (35mM). (Data and figure generated by Dr. Christian Erxleben in Dr. Saverio Gentile's laboratory at LUC)

C. Discussion

Cardiac glycosides are clinically used for the treatment of heart failure and atrial arrhythmia. Strebloside was isolated from *Streblus asper* and possessed a common cardiac glycoside structure, yet few studies have focused on its potential cardiac glycoside activity. In this study, we asked if strebloside also exhibited anticancer action in ovarian cancer cells and compared this action to digitoxin, one of the most commonly prescribed cardiac glycosides. *In vivo* hollow fiber assay results showed that strebloside inhibited MDA-MB-231 and OVCAR3 cell growth. ATPase assay and molecular modeling also displayed that strebloside bind Na^+/K^+-ATPase. Strebloside increased p21, a marker of cell cycle progression, and increased the percentage of cells in the G2/M phase of the cell cycle. In addition, strebloside-treated cells showed apoptosis features such as increased cleaved caspase 3 and cleaved PARP protein levels and decreased Mcl-1 and Bcl-xL. Consistent with other cardiac glycosides, strebloside activated ERK and decreased mutant p53. Furthermore, strebloside inhibited NF-κB transcription activity. Thus, strebloside could be a potential anticancer drugs candidate; however, strebloside did not exhibit better anticancer affects than digitoxin.

Cardiac glycosides possess multiple anticancer activities. There are four isoforms of the α subunit of Na^+/K^+-ATPase, namely α1, α2, α3 and α4. The expression level of α1/3 subunits of Na^+/K^+-ATPase correlates with the susceptibility of cancer cells toward cardiac glycosides. Cancer cells with higher ratio of α3 to α1 isoform are more sensitive to cardiac glycoside treatment. Relative lack of α3 in some human tumor cells and rodent cells may explain their unresponsiveness to cardiac glycosides.[166] In fact, the differences of the isoforms of Na^+/K^+-ATPase location between normal and

malignant cells determine its binding capability.[167] The data in the present study demonstrate that murine cells are more resistant to cardiac glycosides than human cells regardless of whether they were considered normal or malignant. Notably, strebloside and digitoxin show different cytotoxicity in HGSC and normal cells, with normal cells being more susceptible. Similarly, MCF10A are more susceptible than MCF7 cells to digitoxin.[161]

The tumor suppressor protein p53 is a transcription factor that controls many cellular functions such as DNA repair, cell cycle and apoptosis. It is the most commonly mutated gene in all human cancers.[168] Approaches are currently under investigation to target p53 and its regulators, including gene therapy to restore p53 function, inhibition of MDM2, refolding of mutant p53 to wild-type p53, eliminating mutant p53, and targeting p53 family proteins like p63 and p73.[169,170] Some of these p53-targeted therapies have entered clinical trials.[169,171] A recent study suggests that digoxin and ouabain reduced mutant p53 level through MAPK signaling pathways, initiated on the inhibition of Na^+/K^+-ATPase.[163] Remarkably, strebloside, as well as digitoxin, induces ERK phosphorylation and down-regulation of mutant p53 in ovarian cancer cells, indicating the common effect of cardiac glycosides. Since cardiac glycosides seem to also harm normal cells and despite structural differences they all similarly modify the Na^+/K^+-ATPase, the ability to use them for targeting p53 is unlikely to be able to avoid these alternative deleterious problems.

The altered expression of anti-apoptotic Bcl-2 protein family is a common pattern in cancer.[172] Most cancer cell models overexpress one or more of the three major proteins: Bcl-2, Bcl-xL and Mcl-1. Mcl-1 repression occurs in different cancer cell

models after treatment with cardiac glycosides. Cardenolides (digoxin, digitoxin, ouabain) or bufadienolides (cinobyfagin and proscillaridin) all have been reported to downregulate Mcl-1.[173] Strebloside also resulted in Mcl-1 downregulation to activate apoptosis. Furthermore, the NF-κB transcription factors are expressed in many tissue types in processes related to growth, differentiation, and inflammation. Constitutive NF-κB signaling also has been identified in tumors of epithelial origin including breast, colon, lung and ovarian carcinomas.[174] Recent work suggested NF-κB conveyed poor outcomes in ovarian cancer.[175] Cardiac glycosides potently induce mitotic arrest, through downregulation of the nuclear transcription factor, NF-κB.[176] Consistent with this finding, strebloside induced G2/M phase arrest and inhibition of NF-κB activity. These findings suggest cardiac glycosides may also be useful in targeting the pro-inflammatory component responsible for cancers. Despite the structural differences between strebloside and digitoxin, similar downstream signals appear to arise from both compounds in both normal and cancer cells.

In conclusion, both strebloside and digitoxin share in common their Na^+/K^+-ATPase binding mode based on molecular docking and inhibition. Similar to digitoxin, strebloside downregulated antiapoptotic proteins such as Mcl-1 and Bcl-xL and induced apoptosis, by initiating caspase signaling and PARP cleavage. The inhibition of Na^+/K^+-ATPase by strebloside was found to affect the mitotic cell cycle, proliferation, repression of p53, and NF-κB. However, treatment with strebloside does not appear vastly different from digitoxin, which has displayed mixed results for breast and ovarian cancers based on epidemiological studies of women consuming these compounds long-term for treatment of cardiovascular disease. Antibody-drug conjugates (ADCs) that enhance the

selective targeting of cancer cells will maximize the cardiac glycoside efficacy, while reducing their system side effect by preventing nonspecific uptake by normal cells. This study demonstrates that the key signaling events from strebloside are almost identical to other more common cardiac glycosides, and therefore will likely suffer from the same side effects.

V. DISCUSSION AND FUTURE DIRECTIONS

Drug discovery and drug repurposing are two main strategies in drug development. Drug discovery focuses on searching new compounds or new targets while drug repurposing emphasis on known drugs or targets but new applications. The purpose of this project was to screen new anticancer drug leads from natural sources and investigate their mechanisms of action. This work showed that silvestrol and strebloside have potent anticancer activity. Our data illustrate that silvestrol induces autophagy and apoptosis. In addition, cardiac glycosides are documented to exhibit cytotoxicity against several human cancer models. Data from the current project suggest that strebloside induces apoptosis through blocking Na^+/K^+-ATPase. Our findings illuminate the importance on natural product drug discovery. First, screening compounds from nature sources remain promising field for drug development. Second, efforts on the mechanism of action of silvestrol provide new insight in understanding of apoptosis and autophagy, which are critical in drug discovery. Lastly, the influence of strebloside focusing on specific signal transduction pathways offers the chance on drug repurposing. However, this chapter will also focus on some of the issues with natural product, such as target identification, compound solubility and selectivity, and supply problems of natural products. For each of the compounds investigated in this thesis, these issues generated hurdles that in both instances halted further work on the compounds.

A. Phenotypic versus Target-based Screening

Two types of screens have been applied on early stage drug development—phenotypic screens and target-based screens. The former measures the effects, or phenotypes, that compounds induce either *in vitro* or *in vivo* such as in cells, tissues or animals, whereas the latter the looks at the effect of compounds on a purified target protein via *in vitro* assays. Screening for drug leads in phenotypic screens provides an opportunity to realize the value of natural products. In this project, phenotypic screens help to recognize cytotoxic compounds. According to the cytotoxicity results, we can prioritize potential drug leads for further mechanism of action study. This approach successfully identifies active molecules such as silvestrol and strebloside; however, emerging data from research studies imply silvestrol is toxic in dog model, whereas strebloside selectively kill human cancer cells is due to its species specific cytotoxicity. While animal models seldom exactly replicate human disease, phenotypic screening is unable to adequately address the concern whether compounds deserve further evaluated for clinical toxicological test. Different model organisms and target assay may help to assess the priority of potential drug leads. For example, sample testing in cell lines from murine can rule out the chance that it has different effect between mouse and human. Spontaneous or mutagen induced tumor models in rodents can provide the environment that the host and the tumor are from the same species.

Eukaryotic-specific inhibitors possess capacity to alter cell proliferation and/or protein synthesis. Cycloheximide has been shown to block the translation elongation in eukaryotic cells. It binds the ribosome and inhibits eEF2-mediated translocation and induces apoptosis. Inhibition of eukaryotic translation elongation by lactimidomycin was

proposed through binding the ribosomal E-site and prevents translocation of the P-site tRNA into the E-site. Silvestrol was proposed as a translation inhibitor by interrupting eIF4A related translation initiation and induced both autophagy and apoptosis. Interestingly, another translation inhibitor HHT blocked mRNA translation at ribosome A-site to prevent translation elongation but only showed autophagy induction. Whether these binding site differences affect downstream signal transduction pathways, is yet to be experimentally validated. Other binding targets might be involved in silvestrol induced apoptosis and autophagy. To address this question, different strategies could be performed in the future. Development of cells with eIF4A overexpression or knockdown might improve our understanding if other proteins are involved in silvestrol-induced cell death. In addition, the attachment of biotin to a small molecule provides a powerful tool in target validation. As biotinylated compounds, they bind to and can precipitate interacting biomolecules in live biological systems. This system may help to pull down proteins besides eIF4A that might interact with silvestrol. Together, different approaches are needed for drug development and utilized to prioritize potential drug leads. Additionally, the key to unravel the need of new anticancer drug is to study these mechanism of action. These specific targets can eventually serve as cancer therapeutic targets.

B. Microfluidics as A New Tool for Drug Discovery

The development of *in vivo* animal models constitute translational investigation in anticancer therapies that have shown promise in *in vitro* models. Rodent models are used to study gene and protein function. Despite the conveniences, the major limitation

of xenograft models is that mice used have compromised immune systems, so do not represent the behavior, failing to recapitulate the complex tumor-host interaction.[177-179]

Although strebloside, like other cardiac glycosides, was highly efficient in inhibiting the growth of human cancer cells in xenograft or hollow fiber assay, without significant toxicity to the host. In this case, it is species differences between murine and human cells that complicate the analysis. Rodent cells are more resistant than human cells to the cardiac glycoside cytotoxicity. Indeed, rodent cells were 1000 times more resistant than human cells. Data also showed the selectivity toward the human cancer cells versus the human non-malignant cells were not high. Dereplication should play essential role in natural product anticancer drug discovery. For example, strebloside is structurally similar to cardiac glycoside and presumably possesses similar biological effect, ruling itself out though it exhibits potent anticancer activity. Target based study such as hERG activity assay help to predict potential side effect before clinical trial. This means strebloside is not a good anticancer drug. Applications of strebloside against other diseases deserve further investigation. Microfluidic devices build minimal function units that recapitulate tissue- and organ- level functions. Organ-on-chips are microengineered biomimetic systems that represent key functional units of living human organs because they use primary human tissues and/or cells.[180] In the future, microfluidics experiment will compare strebloside activity among different human cells, tissues or organs, evaluating its safety profile. Furthermore, evidence demonstrates that silvestrol exhibits nephrotoxicity in dogs, indicating species difference or off target effect. Future experiments will aim at performing microfluidics experiment to improve preclinical screening prediction and validate potential drug leads.

C. Targeted Delivery for Cancer Therapy

The lack of effective prevention and the frequent chemoresistance of tumors have contributed to the difficulty of treating cancer. Additionally, quite often chemotherapeutic drugs are frequently associated with severe side effects due to nonspecific biodistribution to healthy tissues. Therefore, it is highly desirable to design effective therapies that selectively target tumor cells while the normal cells are not affected. This can greatly reduce the systemic side effects and improve prognosis.

Nanocarriers

Many existing chemotherapeutic drugs, repurposed drugs and newly developed small-molecule anticancer compounds have high lipophilicity and low water-solubility. Nanotechnologies that can be applied to cancer therapy such as dendrimers, liposomes and micelles have been widely investigated for targeted drug delivery to tumors. In such compounds, nanotechnology could serve as an alternative for solubilization approach instead of using surfactants and co-solvents, avoiding adverse side effects. Silvestrol induces apoptosis and autophagy was measured. In addition, both silvestrol and its analogue episilvestrol exhibit potent anticancer activity. However, silvestrol has poor solubility. Previous *in vivo* experiments by Lucas and colleagues determined silvestrol have a relatively low (~10%) systemic availability when administered i.p. using a saline/DMSO vehicle. Although silvestrol metabolite silvestric acid displays better solubility, unfortunately, the activity is lost. Micelles such as dendron can maintain the efficacy of silvestrol *in vitro*. Advances in nanocarriers have made this an emerging

platform for cancer therapy. Some nanocarrier-based drugs have reached the FDA approval.[247,248] Continued research is needed to determine whether the nanocarrier can effectively encapsulate chemotherapeutic drugs and specifically target the cancer cells while sparing the healthy cells. Further studies are required to evaluate if cancer cells become resistant to nanocarrier as they do to other cancer treatment that are currently being used in the clinic. Future studies will be focused on embedding silvestrol in micelles to find if the activity of silvestrol maintained *in vivo*.

Antibody Drug Conjugates

Targeted drug delivery by drug conjugates is a new emerging class of anticancer therapy. This strategy aims to target tumor cells specifically while sparing normal cells, thus resulting in high efficacy and low toxicity. Drug conjugation systems, namely ADC, immunotoxins and immunoliposomes, combine the specific targeting moiety of an antibody or similar construct with the efficacy of a toxic drug.[183] Over 40 distinct ADCs, targeting an array of antigens and utilizing a variety drugs and linkers, are under clinical evaluation. Two ADCs, Kadcyla® (trastuzumab emtansine) and Adcetris® (brentuzimab vedotin), have been FDA approved and widespread use in the oncology clinic.[184] Noticeably, natural products were used as the cytotoxic drug payload. The expanding ADCs under clinical investigation certainly attest to the interests and hopes. Strebloside shares common cardiac glycoside structure and signaling transduction pathways. It inhibits cell growth, induces apoptosis, it also deceases mutant p53 and inhibits NF-κB activation. However, strebloside shows species specific cytotoxicity against human cells compared to murine cells, but not tumorigenic specific. This might explain the narrow

therapeutic index possibility of cardiac glycosides. Since strebloside shows similar activity as digitoxin, if this strategy was to be employed it would likely proceed with the already FDA approved compound, digitoxin. An interesting approach to circumvent the issue about the cell selectivity would be to exploit an ADC system that delivers cardiac glycosides such as digoxin or digitoxin to selectively kill cells overexpressing a specific receptor, such as the FSHR. FSHR is overexpressed in ovarian cancer cells. The ADC will be designed such that it could be used as a therapeutic agent, irrespective of the cell of origin.

D. Microbial Natural Products as Preferred Sources of New Drugs and Drug Leads

Aside from the number of existing microbial species is estimated at 10^5 to 10^6, less than 1 percent of microbial community can be cultivated, implying the vast biodiversity of microbial natural products remains underappreciated.[185] In light of the properties of microorganisms, microbial natural products favor themselves in drug discovery and development. Due to the complexity of structure, natural products from plants are usually hard to be synthesized. The yield of natural products from plants is also low. Only 0.008% (w/w) of silvestrol was obtained from dried twigs of *A. folveolata*. Unlike plants, microbial natural products can be produced by large-scale fermentation and can be engineered at metabolic pathways. This greatly bypasses the supply issue of natural products.

Collecting plants from foreign countries also requires agreement that covers the rights and the expectations of countries where the project operated, making difficulties on sample collection. Microorganisms are abundant on land, in freshwater and at sea,

thus collection of microorganism sample is relatively easy. Thereby microbes hold great promise for the discovery of novel natural products. Whole genome sequencing has revealed the biosynthetic potential for natural products in microorganisms. Genome analysis has led to identification of gene clusters in *Streptomyces*, which known to make a few compounds, each one has the potential to produce up to 30 natural products on average.[186] Currently, the key focus areas of the research community includes polyketide biosynthesis and engineering, microbial genome mining, and synthetic biology. In this project, diazaquinomycin A will be prioritized and its BGC will be further exploded. Future studies will be focused on manipulating microorganism gene cluster. These new secondary metabolites can eventually serve as cancer therapeutics. Long-term goal is to apply bioinformatics on actinomycetes BGCs and to utilize the predictions to focus other potential strains, ending in new compound discovery. The strategies provide easier and faster ways for new drug discovery. The use of genome mining is of great value in narrowing down the potential gene clusters from data obtained by broad prediction softwares such as antiSMASH, significantly reduced the costs and time to develop new drug.

Tremendous efforts are focused on improving diagnosis, reducing remission and increasing overall survival. It is clear there is a large unmet need to discover new anticancer drugs. Our findings contribute the knowledge of natural product drug discovery.

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APPENDICES

APPENDIX A: Identification of novel therapeutics from natural sources

APPENDIX B: Copyright permission to reprint material from published manuscripts

APPENDIX A

IDENTIFICATION OF NOVEL THERAPEUTICS FROM NATURAL SOURCES

A. Introduction

According to the most recent United States mortality data, cancer is the second leading mortality in the United States, after heart disease. Lung and bronchus cancer is responsible for the greatest number of death, followed by prostate cancer in men or breast cancer in women, and colorectal cancer. Due to adopting healthier lifestyle, earlier detection of certain cancers, as well as improvements in treatment, cancer death rates have decreased.[14] However, development of resistance to the existing chemotherapeutic drugs occurs in most cases. As a result, treating recurring cases of cancer is challenging and new drugs are desperately needed.

For over 40 years, small molecules derived from plants and microbes have provided a number of useful therapeutic drugs.[187] Natural products have made their major impact as direct treatments or drug templates in anti-neoplastics and anti-infectives. An estimated 40% of the pharmacophores found in natural product compounds are absent in synthetic compounds.[188] Moreover, secondary metabolites are produced by organisms, and therefore are intrinsically bioactive and complementary for binding and interacting with their target macromolecules.[189] The search for naturally occurring lead compounds remains of interest with novel biochemical mechanisms of action.

Among all natural sources, microorganisms produce a wealth of structurally diverse metabolites with remarkable range of biological activities applied in medicine and agriculture.[190] Genomics has revealed many metabolite biosynthetic gene clusters

(BGCs) are not expressed in many microorganisms.[190,191] Strategies that activate silent BGCs in bacteria and fungi have been developed.[190,192,193] The application of epigenetic modifiers, such as histone deacetylase inhibitors and DNA methyltransferase inhibitors, has been shown to upregulate suppressed genes and led to the production of new molecules.[191,194] HDAC inhibitor, SAHA, treated *A.niger* resulted in the isolation of a new metabolite, nygerone A.[195] Treatment of *P. citreonigrum* with the DNMT inhibitor, 5-azacytidine (5-AZA), produced two new compounds, including in the total ten additional secondary metabolites.[196] Recent data have demonstrated these nuclear enzymes are controlled by ubiquitin-proteasome processing, implicating proteasomes as a crucial player in degrading enzymes that repress gene transcription.[197]

The National Cancer Institute (NCI) 60 Human Tumor Cell Lines Screen has been developed and adopted in global research for more than 20 years. The screen utilizes 60 different human tumor cell lines including tumors originating from colon, lung, ovarian, prostate, breast, renal, CNS, melanoma and leukemia. It helps to identify and characterize novel compounds with growth inhibition or killing tumor cells. To date, the majority of FDA approved anticancer drugs have been screened multiple times in the NCI-60 panel.[198]

Identifying potential novel therapeutics by performing bioassay-guided fractionation and isolation of plants, fungi and bacterial secondary metabolites remains important. Multiple different human cell lines were used in this thesis to determine cytotoxicity, including human colon cancer cells HT-29, human melanoma cancer cells MDA-MB-435, human breast cancer cells MDA-MB-231 and human ovarian cancer

cells OVCAR3 which have been in use by NCI-60 since 1980s. By screening extracts, fractions or pure compounds, mechanistic analysis will be further performed for study pure compounds' mechanism of action.

B. Results

Phyllanthusmins

Phyllanthus is a large plant genus with many species used in Asian traditional medicine systems.[112] Using a bioassay guided fractionation approach, phyllanthusmin D and phyllanthusmin E were isolated.[112] The phyllanthusmins are members of the arylnaphthalene subclass of lignin lactones. Many naturally occurring arylnaphthalene lignan lactones have been reported to possess potent cytotoxicity toward small panels of human cancer cell lines.[199,200] Synthesized phyllanthusmin analogues were evaluated *in vitro* utilizing four cancer cell lines. These compounds were isolated and synthesized by the Fuchs lab.

TABLE VII. IC_{50} OF PHYLLANTHUSMIN D

	IC_{50}(μM)	
Compound	**MDA-MB-435**	**HT-29**
Phyllanthusmin D	0.25	0.13

APPENDIX A (continued)

TABLE VIII. IC_{50} OF PHYLLANTHUSMINS

	IC_{50}(µg/mL)			
Compounds	**MDA-MB-435**	**MDA-MB-231**	**OVCAR3**	**HT-29**
PHY 1	0.03	1.64	0.62	0.29
PHY 3	0.98	3.95	2.95	8.98
PHY 4	0.09	0.64	1.29	0.04
PHY 7	0.08	0.36	1.45	0.09
PHY 9	> 25	24.63	22.49	> 25
PHY 11	0.15	3.23	0.62	0.16
PHY 13	0.03	0.23	0.82	0.30
PHY 14	0.09	1.72	1.46	0.13
PHY 15	0.29	0.82	1.30	0.11
PHY 16	12.73	> 25	18.07	9.70
PHY 17	1.21	20.37	11.74	0.73
PHY 18	0.38	3.15	2.73	0.24
PHY 19	0.94	3.59	4.82	0.71
PHY 20	0.32	1.38	1.79	0.77

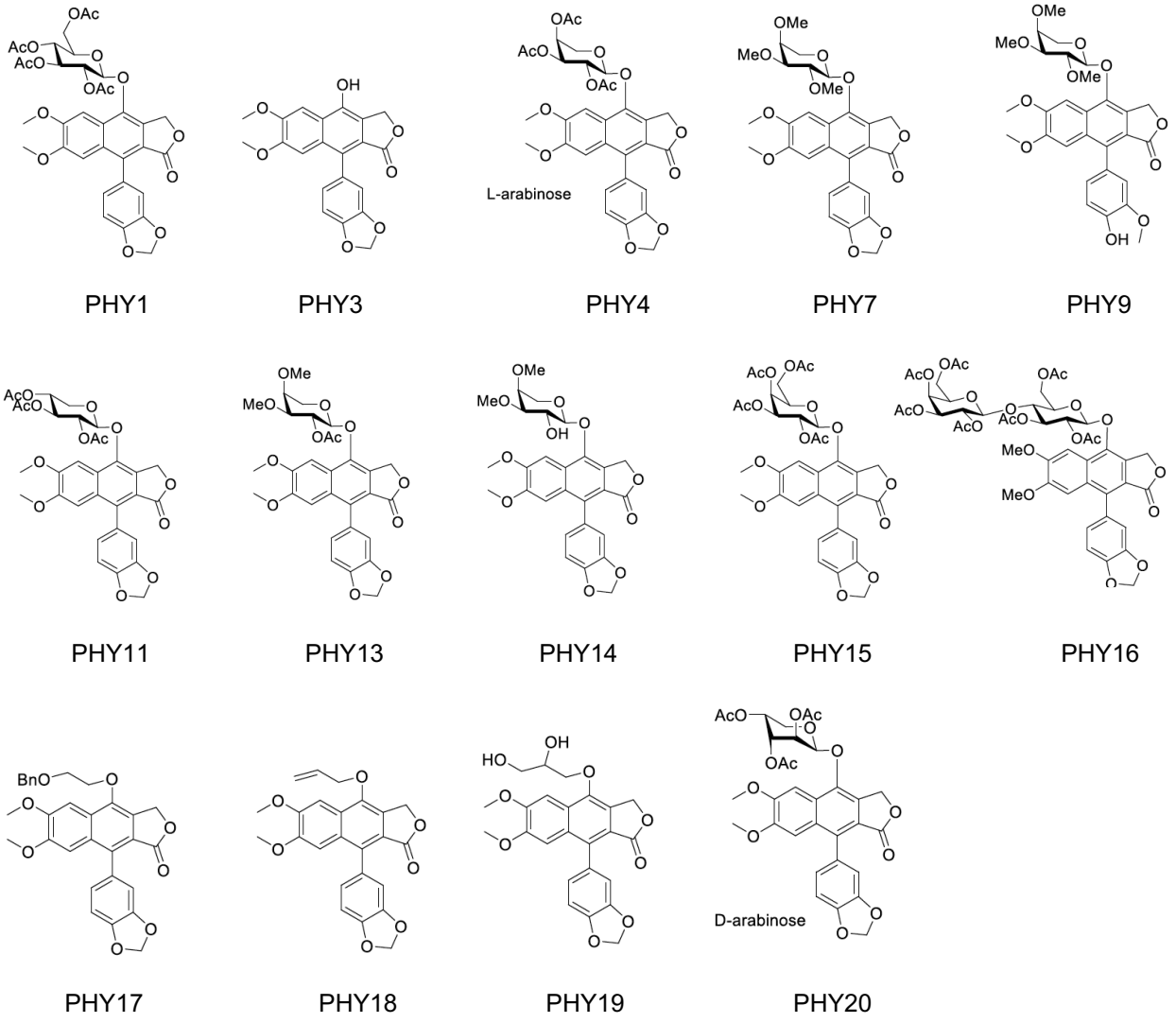


Figure 26. Structures of phyllanthusmins.

APPENDIX A (continued)

(9*β*H)-pimarane, (9*β*H)-17-norpimarane and 17-norpimarane diterpenoids

Icacina trichantha Oliv. (Icacinaceae) is a traditional herbal medicine used in Nigeria and other regions of West Africa.[201] Its tuber is used to treat food poisoning, constipation, and malaria. It was also found to show hypoglycemic, sedative, analgesic, and bacterial-inhibitory effects.[201,202] 17-hydroxyicacinol, icacinol, humirianthol, humirianthenolide C, and icacenone were obtained from the tubers of this plant.[4] These compounds were isolated by the Che lab.

TABLE IX. CYTOTOXIC ACTIVITIES OF (9*β*H)-PIMARANE AND DERIVATIVES

	IC_{50}(μM)	
Compounds	**MDA-MB-435**	**HT-29**
17-hydroxyicacinol	5.61	18.27
Icacinol	1.25	4.23
Humirianthol	1.65	4.94
Humirianthenolide C	0.66	3.00
Icacenone	6.44	12.35

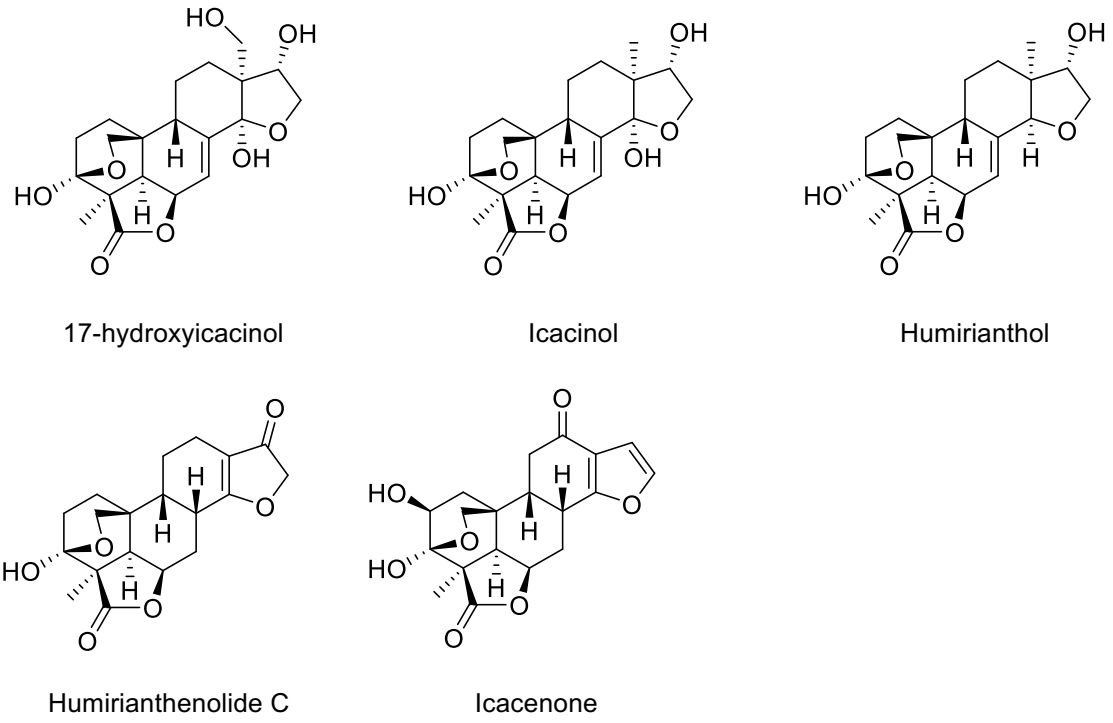


Figure 27. Structures of (9*β*H)-pimarane and derivatives.[4]

APPENDIX A (continued)

Further investigation led to the discovery of seven new 17-norpimarane and (9*β*H)-17-norpimarane diterpenoids, icacinlactones A-G, which were isolated from the tuber of *Icacina trichantha*.[12] These compounds were isolated by the Che lab.

TABLE X. CYTOTOXIC ACTIVITIES OF ICACINLACTONES A-G

	IC_{50}(μM)		
Compound	**MDA-MB-435**	**MDA-MB-231**	**OVCAR3**
Icacinlactone A	> 20	> 20	17.76
Icacinlactone B	> 20	> 20	> 20
Icacinlactone C	> 20	> 20	> 20
Icacinlactone D	> 20	> 20	> 20
Icacinlactone E	> 20	> 20	> 20
Icacinlactone F	6.16	8.94	10.50
Icacinlactone G	> 20	> 20	> 20

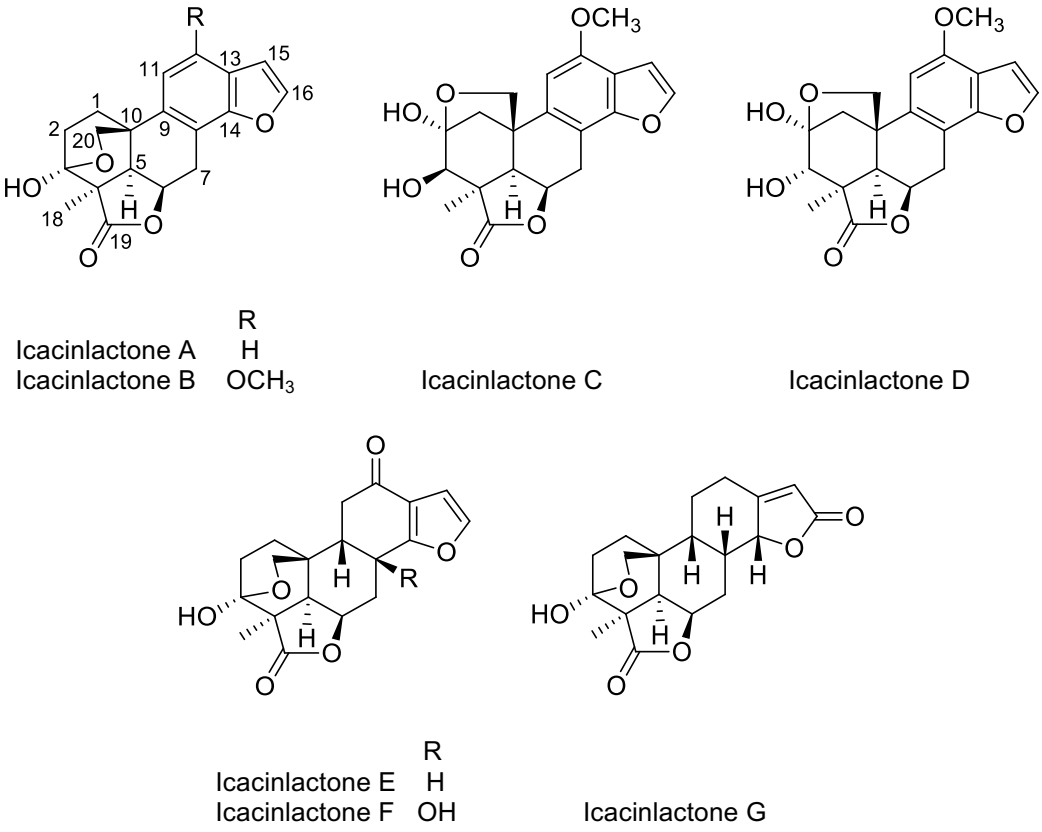


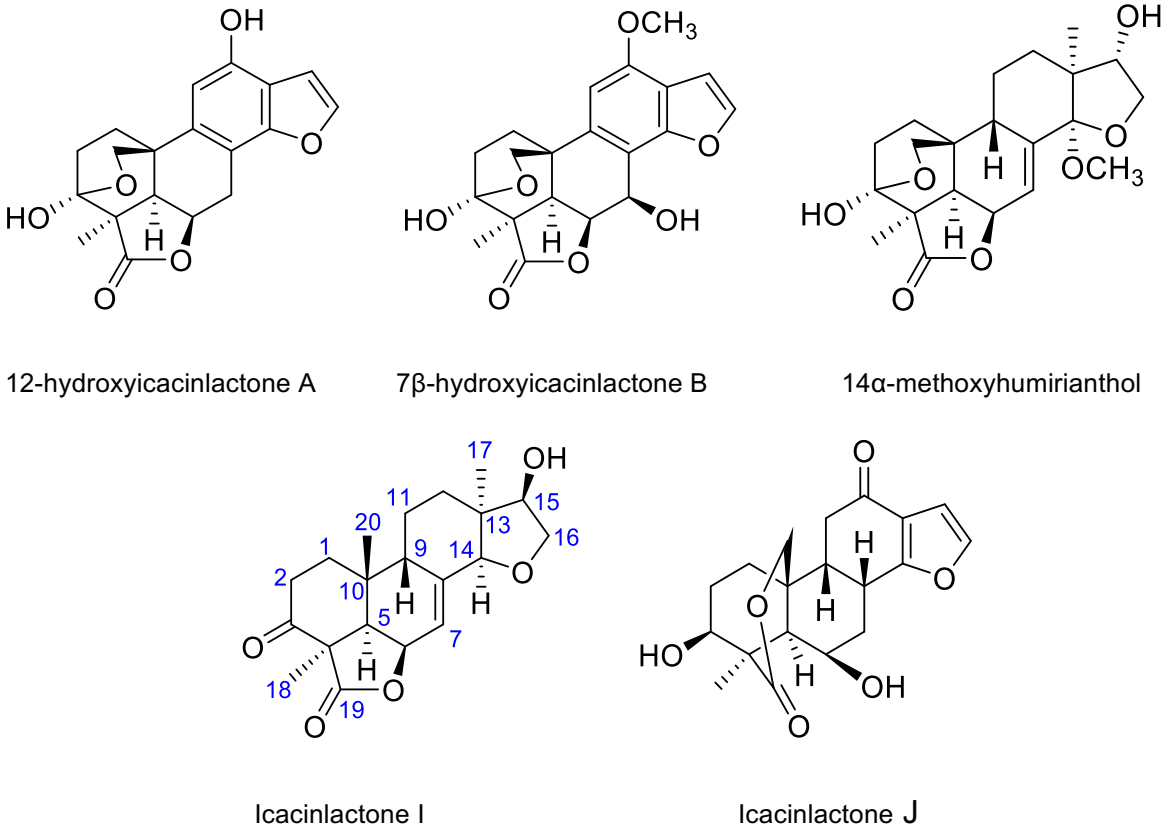
Figure 28. Structures of icacinlactones A-G.[12]

APPENDIX A (continued)

Recently, five new diterpenoid lactones, 12-hydroxyicacinlactone A, 7β-hydroxyicacinlactone B, 14α-methoxyhumirianthol, icacinlactone I, and icacinlactone J were obtained.[11] These compounds were isolated by the Che lab.

TABLE XI. CYTOTOXIC ACTIVITIES OF DITERPENOID LACTONES

Compound	IC_{50}(μM)		
	MDA-MB-435	MDA-MB-231	OVCAR3
12-hydroxyicacinlactone A	> 20	> 20	> 20
7β-hydroxyicacinlactone B	> 20	> 20	> 20
14α-methoxyhumirianthol	7.04	> 20	> 20
Icacinlactone I	> 20	> 20	> 20
Icacinlactone J	> 20	> 20	> 20



12-hydroxyicacinlactone A | 7β-hydroxyicacinlactone B | 14α-methoxyhumirianthol

Icacinlactone I | Icacinlactone J

Figure 29. Structures of diterpenoid lactones.[11]

APPENDIX A (continued)

Next, the tuber of *I. trichantha* was extracted with 80% aqueous MeOH by percolation, followed by solvent partition into petroleum ether-soluble, EtOAc-soluble, and n-BuOH-soluble fractions, successively. Six new pimarane derivatives, icacinlactone K, icacintrichanone, icacinlactone L, 7α-hydroxyicacinlactone B, 7α-hydroxyicacenone, and 2β-hydroxyhumirianthenolide C, were purified from the EtOAc- and n-BuOH-soluble fractions. Compounds were tested against three cancer cell lines, MDA-MB-435, MDA-MB-231 and OVCAR3.[13] These compounds were isolated by the Che lab.

TABLE XII. CYTOTOXIC ACTIVITIES OF PIMARANE DERIVATIVES

	IC_{50}(μM)		
Compound	**MDA-MB-435**	**MDA-MB-231**	**OVCAR3**
Icacinlactone K	> 20	> 20	> 20
Icacintrichanone	> 20	> 20	> 20
Icacinlactone L	> 20	> 20	> 20
7α-hydroxyicacinlactone B	> 20	> 20	> 20
7α-hydroxyicacenone	2.91	7.60	7.53
2β-hydroxyhumirianthenolide C	1.48	2.85	3.23

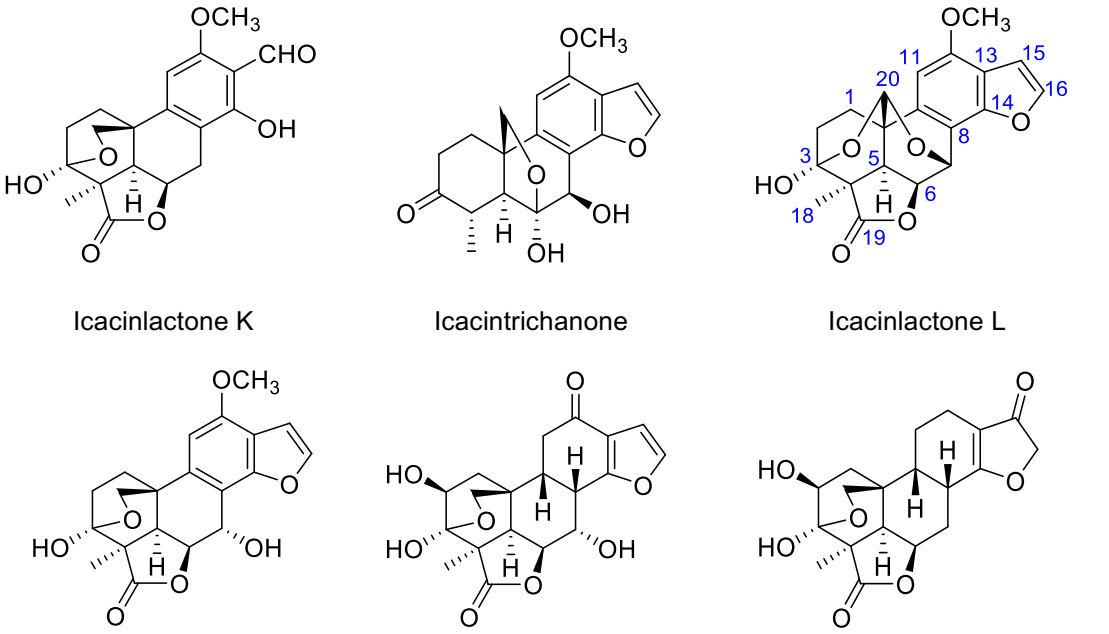


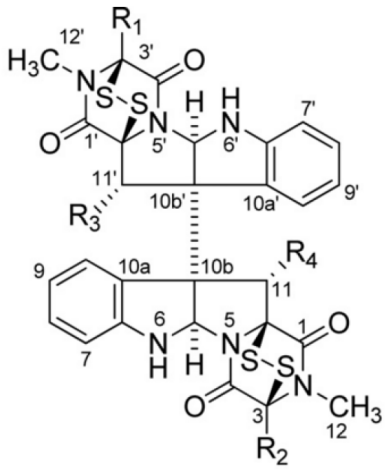
Figure 30. Structures of pimarane derivatives.[13]

Verticillin A

Fungi play important roles in the environment. They have the ability to occupy almost all niches, both natural and human-made. Not surprisingly, their properties are of interest and studied for use in medicine and food.[203] Bioactivity-directed fractionation of two filamentous fungi of the Bionectriaceae, strains MSX 64546 and MSX 59553 from the Mycosynthetix library, led to the isolation of a new dimeric epipolythiodioxopiperazine alkaloid. Verticillin H, along with five related analogs, Sch 52900, verticillin A, gliocladicillin C, Sch 52901, 11′-deoxyverticillin A were isolated.[54] All compounds were evaluated for cytotoxic activity against MDA-MB-435, MDA-MB-231 and OVCAR3. These compounds were isolated by the Oberlies lab.

TABLE XIII. CYTOTOXIC ACTIVITIES OF DIMERIC EPIPOLYTHIODIOXOPIPERAZINE

	IC_{50}(nM)		
Compound	**MDA-MB-435**	**MDA-MB-231**	**OVCAR3**
Verticillin A	1.15	10.75	5.05
Verticillin H	4.67	28.58	22.42
Sch52901	0.81	8.91	2.77
Sch52900	3644	20610	6754
Gliocladicillin C	1.61	32.99	10.11
11-Deoxyverticillin A	0.96	10.64	9.78



	R_1	R_2	R_3	R_4
Verticillin A	CH_3	CH_3	OH	OH
Verticillin H	CH_2CH_3	CH_2CH_3	OH	OH
Sch52901	CH_2CH_3	CH_3	OH	OH
Sch52900	$CH(OH)CH_3$	CH_3	OH	OH
Gliocladicillin C	CH_2CH_3	$CH(OH)CH_3$	OH	OH
11-Deoxyverticillin A	CH_3	CH_3	H	OH

Figure 31. Structures of dimeric epipolythiodioxopiperazine.

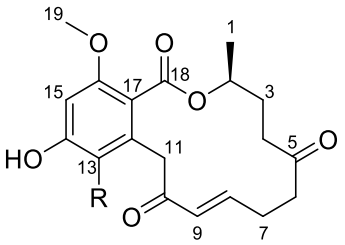
APPENDIX A (continued)

Greensporones

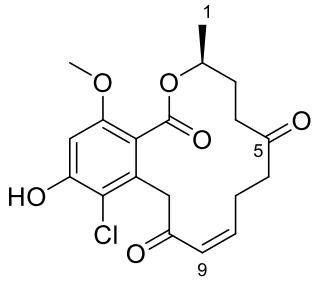
Freshwater fungi represent an understudied source of bioactive secondary metabolites, as approximately 125 compounds have been described, or less than 1% of the over 14,000 compounds that have been characterized from fungi.[204] Hence, studies on freshwater fungi have been initiated in pursuit of new chemical diversity.[205] An aquatic fungus, *Halenospora* sp., was sampled from a submerged wood substrate in a stream. Promising bioactivity of the organ extract led to the isolation of 14 new, but structurally related, resorcylic acid lactones (RALs) [greensporone A, greensporone B, 8,9-dihydrogreensporone A, dechlorogreensporone A, greensporone C, *O*-desmethylgreensporone C, 8,9-dihydrogreensporone C, greensporone D, greensporone E, dechlorogreensporone D, 8,9-dihydrogreensporone D, greensporone F, dechlorogreensporone F, and greensporone G]. The cytotoxicities of these compounds were tested against MDA-MB-435 melanoma and HT-29 colon cancer cell lines. Greensporone C was the most potent, with IC_{50} values of 2.9 μM and 7.5 μM, respectively.[5] These compounds were isolated by the Oberlies lab.

TABLE XIV. ACTIVITIES OF GREENSPORONES AGAINST TWO HUMAN TUMOR CELL LINES

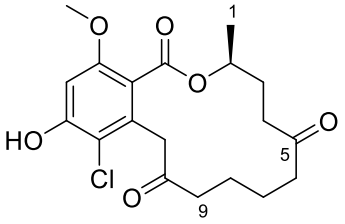
Compound	IC_{50}(μM)	
	MDA-MB-435	HT-29
Greensporone A	14.1	14.1
Greensporone B	> 20	> 20
8,9-dihydrogreensporone A	> 20	> 20
Dechlorogreensporone A	14.1	14.1
Greensporone C	2.9	2.9
O-desmethylgreensporone C	14.5	14.5
8,9-dihydrogreensporone C	> 20	> 20
Greensporone D	-	-
Greensporone E	> 20	> 20
Dechlorogreensporone D	11.2	11.2
8,9-dihydrogreensporone D	> 20	> 20
Greensporone F	-	-
Dechlorogreensporone F	> 20	> 20
Greensporone G	> 20	> 20



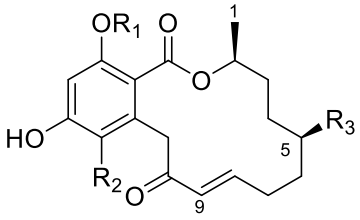
	R
Greensporone A	Cl
Dechlorogreensporone A	H



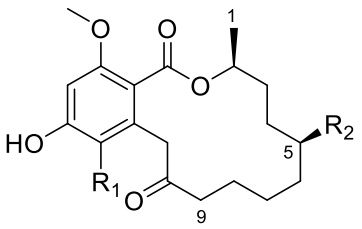
Greensporone B



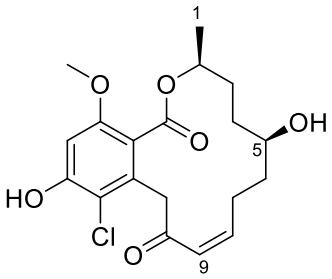
8,9-dihydrogreensporone A



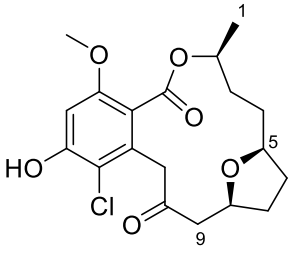
	R_1	R_2	R_3
Greensporone C	CH_3	H	H
O-desmethylgreensporone C	H	H	H
Greensporone D	CH_3	Cl	OH
Dechlorogreensporone D	CH_3	H	OH



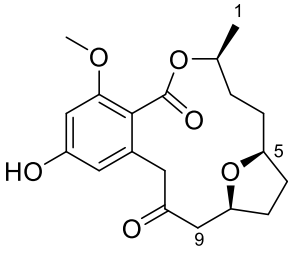
	R_1	R_2
8,9-dihydrogreensporone C	H	H
8,9-dihydrogreensporone D	Cl	OH



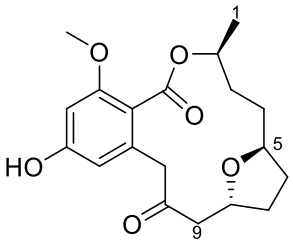
Greensporone E



Greensporone F



Dechlorogreensporone F



Greensporone G

Figure 32. Structures of greensporones.[5]

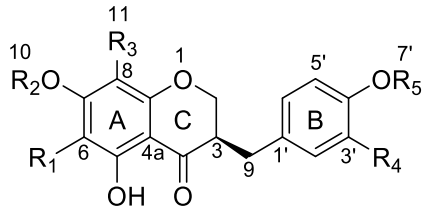
APPENDIX A (continued)

Homoisoflavones

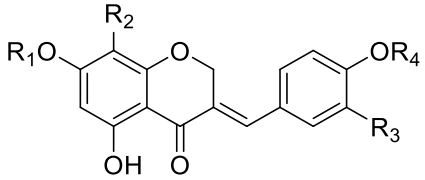
Homoisoflavonoids are a rare class of natural compounds. More than 240 natural homoisoflavonoids have been reported with a wide range of biological activities, including antimicrobial, antimutagenic, antioxidant, and anti-inflammatory effects.[206] Eight new and ten known compounds were isolated from the bulbs of *Bellevalia eigii*, native plants of Jordan. Of these, the serious of 16 homoisoflavonoids comprise seven new analogues 7-*O*-methyl-3′ -hydroxy-3,9-dihydropunctatin, 6-hydroxy-7-*O*-methyl-3,9-dihydropunctatin, 7,4′-di-*O*-methyl-3′-hydroxy-3,9-dihydropunctatin, 7-*O*-methylpunctatin, 7-*O*-methyl-3′-hydroxypunctatin, 5-hydroxy-7,8-dimethoxychroman-4-one, and 7-*O*-methyl-8-demethoxy-3-hydroxy-3,9-dihydropunctatin and nine known analogues 7-*O*-methyl-3,9-dihydropunctatin, 8-*O*-demethyl-7-*O*-methyl-3,9-dihydropunctatin12−14, 5,7-dihydroxy-3-(3′-hydroxy-4′-methoxybenzyl)-4-chromanone, 5,6-dihydroxy-3-(4-hydroxybenzyl)-7-methoxy-4-chromanone, 4′,5,7 trihydroxyhomoisofl avanone, 3′,4′,5,7-tetrahydroxyhomoisofl avanone, demethyleucomin, punctatin and isomuscomosin. In addition, the known moupinamide and a new compound, bellegimycin, were also isolated. Cytotoxic activities of all compounds were measured against human melanoma cell MDA-MB-435 and human colon cancer cell HT-29. 5,7-dihydroxy-3-(3′-hydroxy-4′-methoxybenzyl)-4-chromanone and 7,4′-di-*O*-methyl-3′-hydroxy-3,9-dihydropunctatin were the most potent on the MDA-MB-435 cell line, with IC_{50} value of 1.0 and 1.1 μM, respectively.[10] These compounds were isolated by the Oberlies lab.

TABLE XV. CYTOTOXIC ACTIVITIES OF HOMOISOFLAVONOIDS

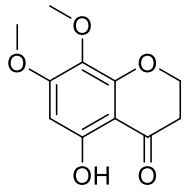
Compound	IC_{50}(μM)	
	MDA-MB-435	HT-29
7-*O*-methyl-3,9-dihydropunctatin	> 20	> 20
8-*O*-demethyl-7-*O*-methyl-3,9-dihydropunctatin	> 20	> 20
7-*O*-methyl-3′-hydroxy-3,9-dihydropunctatin	> 20	> 20
5,7-dihydroxy-3-(3′-hydroxy-4′-methoxybenzyl)-4-chromanone	1.0	> 20
5,6-dihydroxy-3-(4-hydroxybenzyl)-7-methoxy-4-chromanone	> 20	> 20
6-hydroxy-7-*O*-methyl-3,9-dihydropunctatin	> 20	> 20
4′,5,7-trihydroxyhomoisoflavanone	> 20	> 20
3′,4′,5,7-tetrahydroxyhomoisoflavanone	> 20	> 20
7,4′-di-*O*-methyl-3′-hydroxy-3,9-dihydropunctatin	1.1	17.3
7-*O*-methylpunctatin	13.2	> 20
Demethyleucomin	> 20	> 20
Punctatin	> 20	> 20
7-*O*-methyl-3′-hydroxypunctatin	4.6	> 20
5-hydroxy-7,8-dimethoxychroman-4-one	> 20	> 20
7-*O*-methyl-8-demethoxy-3-hydroxy-3,9-dihydropunctatin	> 20	> 20
Isomuscomosin	> 20	> 20
Moupinamide	> 20	> 20
Bellegimycin	> 20	> 20



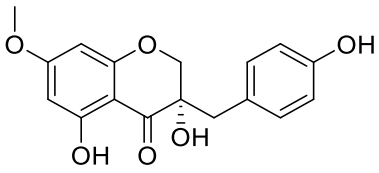
	R_1	R_2	R_3	R_4	R_5
7-*O*-methyl-3,9-dihydropunctatin	H	CH_3	OCH_3	H	H
8-*O*-demethyl-7-*O*-methyl-3,9-dihydropunctatin	H	CH_3	OH	H	H
7-*O*-methyl-3′-hydroxy-3,9-dihydropunctatin	H	CH_3	OCH_3	OH	H
5,7-dihydroxy-3-(3′-hydroxy-4′-methoxybenzyl)-4-chromanone	H	H	H	OH	CH_3
5,6-dihydroxy-3-(4-hydroxybenzyl)-7-methoxy-4-chromanone	OH	CH_3	H	H	H
6-hydroxy-7-*O*-methyl-3,9-dihydropunctatin	OH	CH_3	OCH_3	H	H
4′,5,7-trihydroxyhomoisoflavanone	H	H	H	H	H
3′,4′,5,7-tetrahydroxyhomoisoflavanone	H	H	H	OH	H
7,4′-di-*O*-methyl-3′-hydroxy-3,9-dihydropunctatin	H	CH_3	OCH_3	OH	CH_3



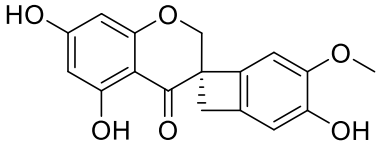
	R_1	R_2	R_3	R_4
7-*O*-methylpunctatin	CH_3	OCH_3	H	H
Demethyleucomin	H	H	H	H
Punctatin	H	OCH_3	H	H
7-*O*-methyl-3′-hydroxypunctatin	CH_3	OCH_3	OH	H



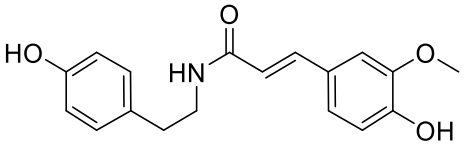
5-hydroxy-7,8-dimethoxychroman-4-one



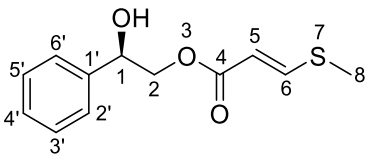
7-*O*-methyl-8-demethoxy-3-hydroxy-3,9-dihydropunctatin



Isomuscomosin



Moupinamide



Bellegimycin

Figure 33. Structures of homoisoflavones.[10]

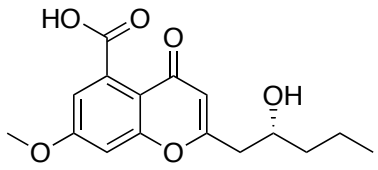
APPENDIX A (continued)

(*R*)-2-(2-hydroxypentyl)-5-carboxy-7-methoxychromone and (*E*)-2-(pent-1-en-1-yl)-5-carboxy-7-methoxychromone

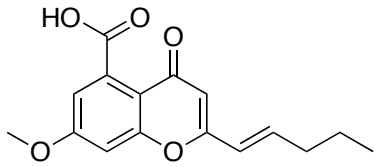
Proteasomes are responsible for proteolysis, including transcriptional factors. This study examined the capability of bortezomib, a proteasome inhibitor, to induce the production of secondary metabolites in a fungus. MSX 63935, order Pleosporales, had been shown previously to biosynthesize a series of resorcylic acid lactones of polyketide origin.[207] Mycosynthetix fungal strain 63935 (MSX 63935) was growing in the presence of proteasome inhibitor (bortezomib) and harvested. (*R*)-2-(2-hydroxypentyl)-5-carboxy-7-methoxychromone was isolated from bortezomib-dosed growths of MSX 63935 and compound (*E*)-2-(pent-1-en-1-yl)-5-carboxy-7-methoxychromone was isolated from a degraded solution of the former.[6] These compounds were isolated by the Oberlies lab.

TABLE XVI. CYTOTOXIC ACTIVITIES OF (*R*)-2-(2-HYDROXYPENTYL)-5-CARBOXY-7-METHOXYCHROMONE AND (*E*)-2-(PENT-1-EN-1-YL)-5-CARBOXY-7-METHOXYCHROMONE

	IC_{50}(μM)	
Compound	**MDA-MB-435**	**HT-29**
(*R*)-2-(2-hydroxypentyl)-5-carboxy-7-methoxychromone	> 20	> 20
(*E*)-2-(pent-1-en-1-yl)-5-carboxy-7-methoxychromone	> 20	> 20



(R)-2-(2-hydroxypentyl)-5-carboxy-7-methoxychromone



(E)-2-(pent-1-en-1-yl)-5-carboxy-7-methoxychromone

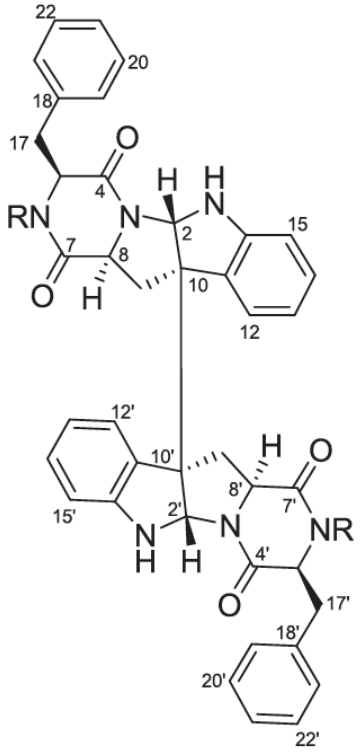
Figure 34. Structures of *(R)*-2-(2-hydroxypentyl)-5-carboxy-7-methoxychromone and *(E)*-2-(pent-1-en-1-yl)-5-carboxy-7-methoxychromone.[6]

Diketopiperazine dimer

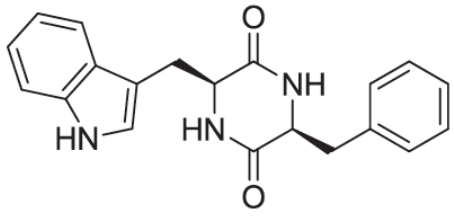
Prior investigations of filamentous fungi have resulted in the isolation of several new and biologically active natural products.[208-210] Metabolites from *Aspergillus sydowii* (MSX19583) were obtained from spruce litter collected in 1984 in Colorado, USA. Chemical separation of the extract afforded a new diketopiperazine dimer along with three known compounds including cyclo-(*L*-phenylalaninyl-*L*-tryptophanyl), *S*-sydonic acid, and *S*-sydonol. All compounds were found to be inactive, displaying IC_{50} values > 20 μM.[9] These compounds were isolated by the Oberlies lab.

TABLE XVII. CYTOTOXIC ACTIVITIES OF DIKETOPIPERAZINE DIMER

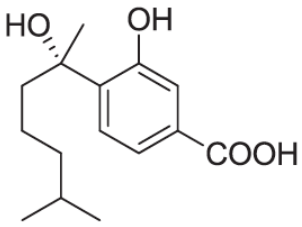
	IC_{50}(μM)	
Compound	**MDA-MB-435**	**HT-29**
Diketopiperazine dimer	> 20	> 20
cyclo-(*L*-phenylalaninyl-*L*-tryptophanyl)	> 20	> 20
S-sydonic acid	> 20	> 20
S-sydonol	> 20	> 20



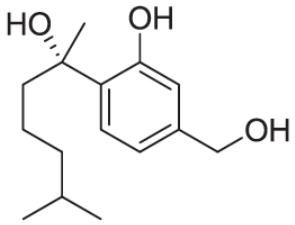
R=H
Diketopiperazine dimer



cyclo-(*L*-phenylalaninyl-*L*-tryptophanyl)



S-sydonic acid



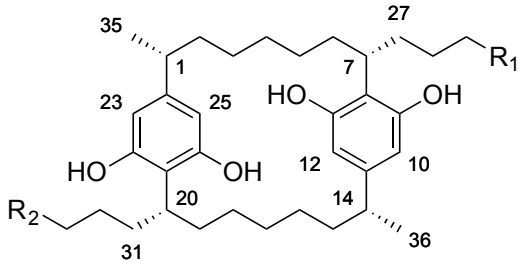
S-sydono

Figure 35. Structures of diketopiperazine dimer.[9]

APPENDIX A (continued)

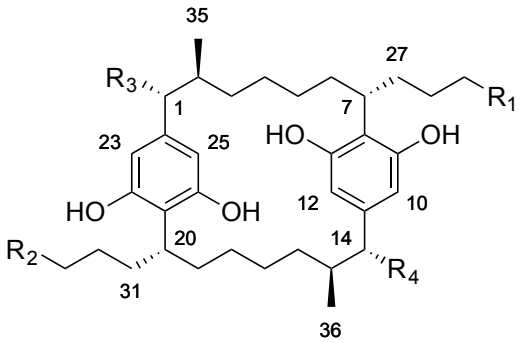
[7,7] Paracyclophanes

Cyanobacteria are prolific producers of structurally diverse and bioactive natural products.[211] Several metabolites containing a [7.7]paracyclophane skeleton have been discovered from cyanobacteria.[212-214] [7,7] paracyclophanes are cytotoxic secondary metabolites produced by freshwater and terrestrial cyanobacteria. Merocyclophane A, C, and D were isolated from UIC 10110 *Nostoc sp*.,[215] carbamidocyclophanes A, B, C, F, and G were isolated from UIC 10274 *Nostoc sp*.,[216] and ribocyclophanes A, B, and C were isolated from UIC 10279 *Nostoc sp* (structures not shown). All compounds were found to be cytotoxic.



	R_1	R_2
Merocyclophane A	CH_3	CH_3
Merocyclophane C	OH	CH_3
Merocyclophane D	OH	OH

Figure 36. Structures of merocyclophanes.



	R_1	R_2	R_3	R_4
Carbamidocyclophane A	$CHCl_2$	$CHCl_2$	$OCONH_2$	$OCONH_2$
Carbamidocyclophane B	$CHCl_2$	CH_2Cl	$OCONH_2$	$OCONH_2$
Carbamidocyclophane C	$CHCl_2$	CH_3	$OCONH_2$	$OCONH_2$
Carbamidocyclophane F	$CHCl_2$	$CHCl_2$	OH	$OCONH_2$
Carbamidocyclophane G	$CHCl_2$	$CHCl_2$	$OCOCH_3$	$OCONH_2$

Figure 37. Structures of carbamidocyclophanes.[2]

APPENDIX A (continued)

Carbamidocyclophanes F and G

Two carbamidocyclophanes were isolated from freshwater cyanobacterium *Nostoc* sp. UIC 10274. Evaluation of cytotoxic activities against human melanoma cell MDA-MB-435 and human colon cancer cell HT-29 revealed that carbamidocyclophane F exhibited anti-proliferative activity with an IC_{50} of 0.7 μM in both cell lines, while carbamidocyclophane G displayed IC_{50} of 0.5 μM in both cell lines.[2] These compounds were isolated by the Orjala lab.

TABLE XVIII. CYTOTOXIC ACTIVITIES OF CARBAMIDOCYCLOPHANES F AND G

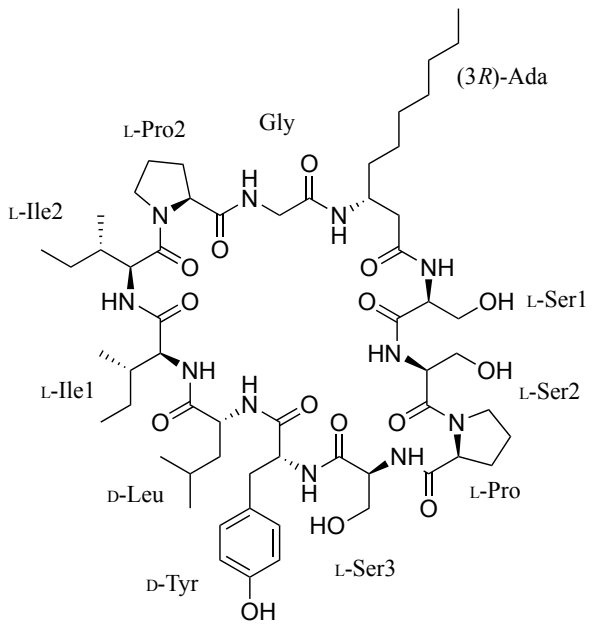
	IC_{50}(μM)	
Compound	**MDA-MB-435**	**HT-29**
Carbamidocyclophane F	0.7	0.7
Carbamidocyclophane G	0.5	0.5

Trichormamides A and B

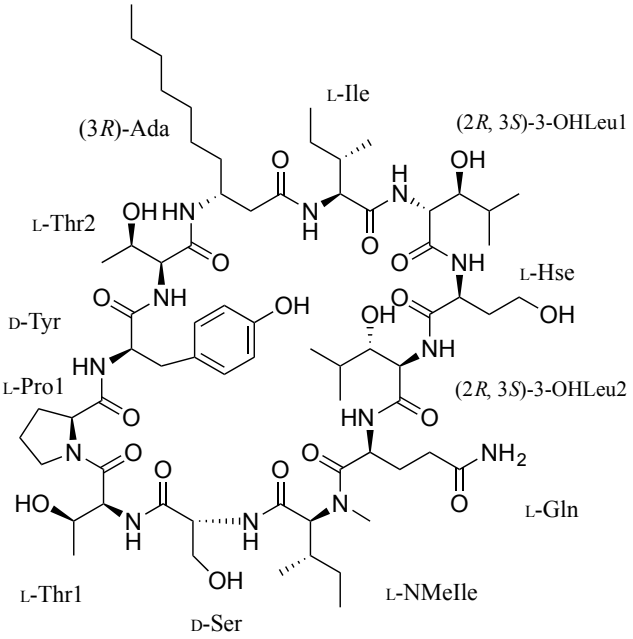
Cyanobacteria are prolific producers of cyclic lipopeptides. Many structurally diverse cyclic lipopeptides have been reported and studied for antimicrobial, antiproliferative, antitumor, 20S proteasome inhibitory, and allelopathic activities.[217-221] Bioassay-guided fractionation led to the isolation of two cyclic lipopeptides, named trichormamide A and B, from fresh water cyanobacterium *Trichormus* sp. UIC 10339. Trichormamide A is a cyclic undecapeptide while B is a cyclic dodecapeptide. Trichormamide B is cytotoxic against human colon cancer cell HT-29 and human melanoma cell MDA-MB-435 with the IC_{50} values of 0.8 μM and 1.5 μM, respectively.[3] These compounds were isolated by the Orjala lab.

TABLE XIX. CYTOTOXIC ACTIVITIES OF TRICHORMAMIDES A AND B

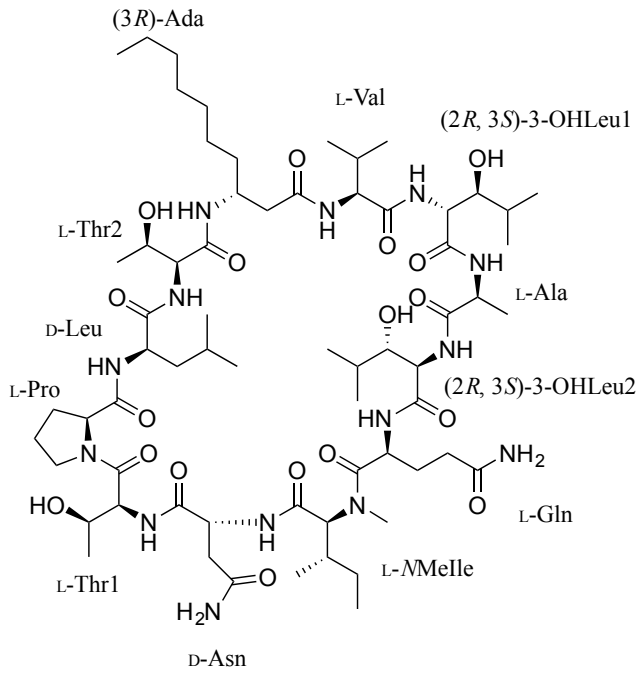
	IC_{50}(μM)	
Compound	**MDA-MB-435**	**HT-29**
Trichormamide A	9.9	16.9
Trichormamide B	0.8	1.5



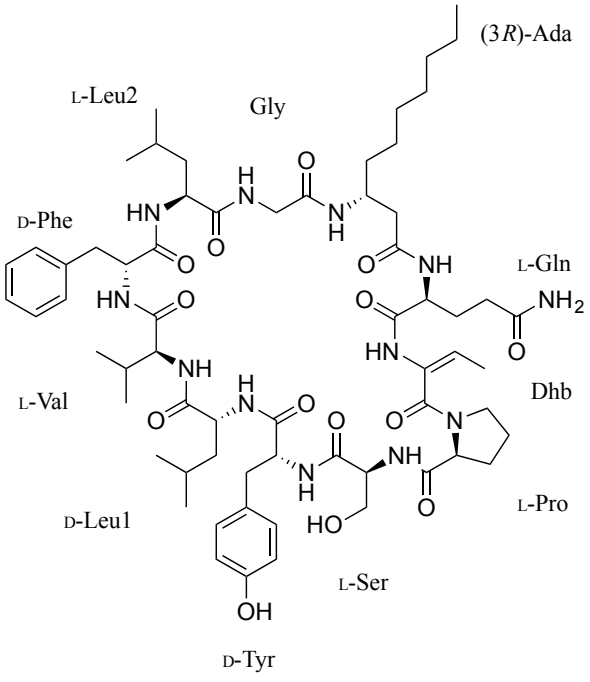
Trichormamides A



Trichormamides B



Trichormamides C



Trichormamides D

Figure 38. Structures of trichormamides A, B, C and D.[3,8]

Trichormamides C and D

Two cyclic lipopeptides, trichormamide C and D, were isolated from freshwater cyanobacterium *cf. Oscillatoria* sp. UIC 10045. Trichormamide C is a cyclic undecapeptide and D is a cyclic dodecapeptide. Both trichormamide C and D display antiproliferative activities against human colon cancer cell HT-29 and human melanoma cell MDA-MB-435. The IC_{50} values of trichormamide C against these two cell lines were 1.7 μM and 1.0 μM, respectively. As for trichormamide D, the IC_{50} values were 11.5 μM and 11.7 μM, respectively.[8] These compounds were isolated by the Orjala lab.

TABLE XX. CYTOTOXIC ACTIVITIES OF TRICHORMAMIDES C AND D

	IC_{50}(μM)	
Compound	**MDA-MB-435**	**HT-29**
Trichormamide C	1.7	1.0
Trichormamide D	11.5	11.7

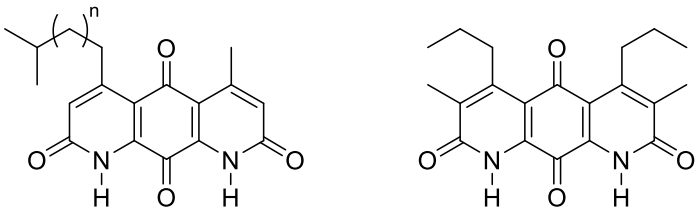
APPENDIX A (continued)

Diazaquinomycin A, H and J

Actinomycete bacteria have provided us with an abundance of bioactive compounds, including many clinically useful antibiotics and anticancer drugs.[62] Screening of aquatic-derived actinomycete secondary metabolite library led to the isolation of diaza-anthracene antibiotic diazaquinomycin A, H and J. Although diazaquinomycin A (DAQA) is known, DAQA exhibited a range of IC_{50} values when screened against a panel of human cancerous cell lines [MDA-MB-435 (0.1 μM), MDA-MB-231 (3.6 μM), HT-29 (5.7 μM), OVCAR3 (0.5 μM)].[7] These compounds were isolated by the Murphy lab.

TABLE XXI. CYTOTOXIC ACTIVITIES OF DAQA

	IC_{50}(μM)			
Compound	**MDA-MB-435**	**MDA-MB-231**	**OVCAR3**	**HT-29**
DAQA	0.1	3.6	0.5	5.7



DAQH n=5
DAQJ n=6

DAQA

Figure 39. Structures of diazaquinomycins H, J and A.[7]

C. Discussion

Among all these pure compounds, verticillin A shows potent anticancer activity. Verticillin A is also known to inhibit histone methyltransferase which no known drug target this enzyme. Unlike oncogenic mutations are fixed in the cancer genome, epigenetic alterations are potentially reversible, offering a unique therapeutic opportunity.[84,222,223] Therefore, verticillin A is a promising lead. However, due to its solubility, future direction will focus on nanocarrier platform selectively targeting cancer cells, while improving system biodistribution. Another potential drug lead is phyllanthusmin, although its mechanism of action is still unclear, it shows potent anticancer activity against several different cancer cell lines. Follow up study will use RNAseq and functional signature ontology (FUSION) maps to identify

Natural products represent the richest sources of novel molecular scaffolds. The discovery of new natural products promises significant advances, not only as drugs, but also drug leads. In spite of the success of natural products in drug discovery, natural products are significantly underrepresented.[47] It is estimated that only 5-15% of the approximately 300,000 species of higher plants have been systematically investigated, pharmacologically, and phytochemically.[47,224,225] In addition, it has been estimated that less than 1% of microorganisms seen microscopically have been cultivated.[47,185] Several challenges in natural product drug discovery include (1) low yields, (2) limited supply, (3) complex structures posing for structural modifications, and (4) complex structures precluding practical synthesis.[226] With the emerging field of synthetic biology, microorganisms can be engineered to overproduce the desired natural products or

engineered its metabolic pathways to produce natural product analogues. Therefore, microbial natural products may be preferred sources of new drugs and drug leads.

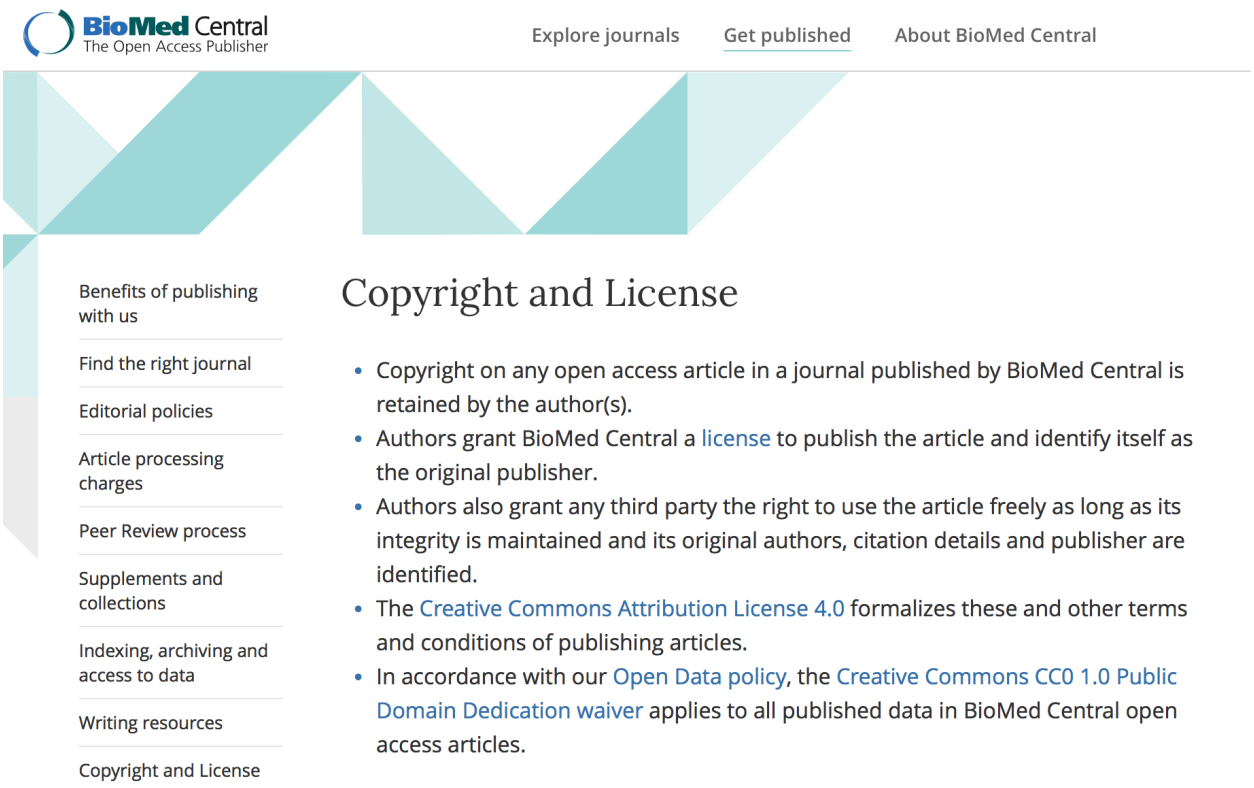
While terrestrial actinomycetes have been mined for their anti-bacterial and anti-cancer activity for generations, rediscovery of known agents continues to be an issue. Recently, the diversity of actinomycetes obtained from aquatic sources has become an intense area of investigation.[227] Marine and freshwater actinomycetes are capable of producing new classes of chemicals, same as their terrestrial counterpart. Additionally, previous studies on cyanobacterial secondary metabolites have been largely focusing on strains from marine environments. Freshwater cyanobacteria has been underexplored.[228] To this end, further study of these diverse organisms in chemistry and biological activity may find new anticancer compounds.

The rise in resistance to antibiotics and cancer chemotherapeutics is a major threat to modern health care. As a consequence, there is a need for new compounds. However, traditional bioactivity-based screening of extracts from the cultures of microorganisms, such as actinobacteria and filamentous fungi, has yielded known metabolites.[229] Owing to the microbial genome mining, pleiotropic and pathway-specific approaches are able to activate silent BGCs. Activate silent gene clusters leading to the discovery of new bioactive compounds.[190,192] This strategy helps potential strains in the strain library to generate structural diversity and chemical novelty compounds.

Biologically diverse organisms from land and sea continue to provide numerous chemically diverse secondary metabolites. Potent lead candidates discovered through bioassay-guided fractionation and isolation could serve as scaffolds for synthetic

modification. To satisfy the constant need for new drugs against the continuous emerging of drug resistance, it would be advantageous seeking potential drug leads from nature. Natural products remain of much interest as compounds that with novel biochemical mechanisms of action that might lead to better treatment for cancer.

APPENDIX B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2008 - 2011	**Pharmacy Manager/Pharmacist** *Chia-An Community Pharmacy,* Taipei, Taiwan

PUBLICATIONS

1. **Chen WL**, Ren Y, Swanson SM, Kinghorn AD, Burdette JE. "Strebloside blocks cell growth and induces apoptosis in human ovarian cancer cells" (In preparation)

2. Ren Y, **Chen WL**, Lantvit DD, Sass EJ, Shriwas P, Ninh TN, Chai HB, Zhang X, Soejarto DD, Chen X, Lucas DM, Swanson SM, Burdette JE, Kinghorn AD. "Cardiac Glycoside Constituents of *Streblus asper* with Potential Antineoplastic Activity" (Under review)

3. Guo B, Onakpa MM, Huang XJ, Santarsiero BD, **Chen WL**, Zhao M, Zhang XQ, Swanson SM, Burdette JE, Che CT. "Di-nor- and 17-nor-pimaranes from Icacina trichantha" *J Nat Prod.* **2016** 79(7):1815-1821.

4. **Chen WL**, Pan L, Kinghorn AD, Swanson SM, Burdette JE. "Silvestrol induces early autophagy and apoptosis in human melanoma cells" *BMC Cancer.* **2016** 16(1):17.

5. Zhao M, Onakpa MM, Santarsiero BD, **Chen WL**, Szymulanska-Ramamurthy KM, Swanson SM, Burdette JE, Che CT. "(9βH)-Pimaranes and Derivatives from the Tuber of Icacina trichantha" *J Nat Prod*. **2015** 78(11):2731-2737.

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Oral Presentations

2016 Center for Pharmaceutical Biotechnology seminar series, UIC, Chicago, IL

Poster Presentations

2016 Midwest Ovarian Cancer Coalition Workshop, Notre Dame, IN

2016 Yao Yuan Biotech-Pharma Symposium, Chicago, IL

2016 Women Health Research Day, Chicago, IL

2016 University of Illinois at Chicago Student Research Forum, Chicago, IL

2016 College of Pharmacy Research Days, Chicago, IL

2015 Cancer Research Forum Chicago, IL

2015 Illinois Symposium on Reproductive Sciences, Champagne, IL

2015 The American Society of Pharmacognosy, Copper Mountain, CO

2015 MIKI Medicinal Chemistry Meeting, Lawrence, KS

2015 College of Pharmacy Research Days, Chicago, IL

2014 Cancer Research Forum, Chicago, IL

2014 Illinois Symposium on Reproductive Sciences, Chicago, IL

2014 The American Society of Pharmacognosy, Oxford, MS

2014 MIKI Medicinal Chemistry Meeting, Chicago, IL

2014 College of Pharmacy Research Days, Chicago, IL

AWARDS

2015 University of Illinois at Chicago GSC Travel Award

2015 University of Illinois at Chicago Graduate College Student Travel Presenter's Award

2015 Scientific Poster Award-AAPS Students' Choice Award

2014 University of Illinois at Chicago GSC Travel Award

2014 Rush University Medical Center Scholarship

2014 NTUSPAA-NA Travel Award

2014 University of Illinois at Chicago Chancellor's Student Service and Leadership Award

2014 W.E. van Doren Scholar

2013 College of Pharmacy Image Competition Honorable Mention

LEADERSHIP

2015 - present **Student representative of Department of Medicinal Chemistry and Pharmacognosy**
UIC Graduate Student council, Chicago, IL

2014 - present **Student representative of College of Pharmacy**
UIC College of Pharmacy Graduate Student Committee, Chicago, IL

2015 - present **Chair**
American Association for Pharmaceutical Scientists -UIC Student Chapter

2014 - 2015 **Chair elect**
American Association for Pharmaceutical Scientists -UIC Student Chapter

2013 - 2014 **Vice-chair**
American Association for Pharmaceutical Scientists -UIC Student Chapter

PROFESSIONAL MEMBERSHIP

2015 - present **The American Association for the Advancement of Science**

2014 - present **The American Chemical Society, Division of Medicinal Chemistry**

2014 - present **The American Society of Pharmacognosy**
Associate Membership

2015 - present **Controlled Release Society**
Student Chapter

2013 - Present **American Association for Pharmaceutical Scientists**
Student Chapter