ABSTRACT

Effective Targeting of Epithelial Mesenchymal Transition-driven Breast Cancer Stem Cells Using Ophiobolin A, a Natural Product

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Triple negative breast cancer (TNBC) is characterized by the lack of progesterone and estrogen hormone receptors and lack of HER2 overexpression, resulting in limited treatment options. Not only do TNBC patients face limited treatment options, but a TNBC diagnosis also associates with poor diagnostic outcomes due to high rates of metastasis and recurrence, processes ascribed to a subpopulation of cells called cancer stem cells (CSCs). CSCs act as highly-adaptable tumor-initiating cells, which colonize tumor growth in secondary metastatic sites. Cancer stem cells may arise naturally from dedifferentiated adult cells, yet, in tumors, the epithelial-to-mesenchymal transition (EMT) has been shown to yield CSCs. EMT is a normal biological process that becomes dysregulated in tumor formation, whereby stationary epithelial tumor cells gain a migratory mesenchymal phenotype. These mesenchymal cells are adapted to survive the process of metastasis and chemotherapy treatment, and they also display increased stem cell markers and properties. It may be through this de-differentiation process that cancer cells gain their stemness, making EMT an attractive process to target in the attempts to eliminate CSCs.

Many conventional therapies fail to successfully target CSCs due to the cells' reliance on inducing apoptotic cell death, upregulation of drug transporters, and slowed cell cycle. Compounds with cytotoxic activity directed towards CSCs are gaining interest in cancer biology, in particular natural products. Several natural products demonstrate promising effects against cancer hallmarks, contrasted by targeted therapies which fall victim to genetic redundancy, mutation, or epigenetic modification. Ophiobolin A (OpA) is a molecule that shows promise in targeting the elusive CSC population in TNBC. We demonstrate that CSC-derived EMT cells are differentially sensitive to OpA, and that treatment reduces EMT and CSC phenotypes such as migration, mammosphere formation, and chemoresistance. Moreover, OpA induces cellular changes consistent with non-apoptotic cell death, alters the morphology and function of mitochondria, and acts in a mitochondria-dependent manner. Finally, OpA is effective in reducing primary tumor volume in mice. In summary, we show that OpA, a natural product, demonstrates directed cytotoxicity against CSCs and may be useful for clinical application for TNBC patients.

Effective Targeting of Epithelial Mesenchymal Transition-driven Breast Cancer Stem Cells Using Ophiobolin A, a Natural Product

by

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A Dissertation

Approved by the Department of Biology

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Submitted to the Graduate Faculty of
Baylor University in Partial Fulfillment of the
Requirements for the Degree
of

Doctor of Philosophy

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August 2021

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ACKNOWLEDGMENTS

I am deeply appreciative to those who provided ample support throughout the duration of the projects cumulating in this dissertation, and to those who lifted me up everywhere else.

I would firstly like to thank my advisor, Dr. Joseph Taube, whose expertise and guidance was invaluable in formulating research questions and methodology. Your unwavering support both academically and in my adjacent pursuits truly helped me develop as a researcher and as a scientist. I am genuinely so lucky to have worked with you and to have benefitted from your advisement.

Many thanks to the members of the Taube Lab for support in experimentation advisement and execution, as well as for rigorous conversation and critique. Special words of thanks are extended to Sheridan Mikhail for her work with tissue staining, as well as Alec Ingros and Saawan Patel for not only their willingness and eagerness to help out with experiments, but especially for their humor. Thank you to Dr. Bernd Zechmann for assistance with TEM microscopy; conversations with you made the process that much more fun. I am also grateful to Dr. Erica Bruce and members of her lab for lending last-minute supplies and innumerable cell culture plates. I am also very thankful to the members of collaborations and of my committee: Drs. Leigh Greathouse, Bessie Kebaara, Daniel Romo, and Cheolho Sim.

My career trajectory would not be where it has landed without support of all my wild ideas and side hustles. I am truly thankful to those who saw my potential and

encouraged it. To Dr. Beth Allison Barr and Dr. Christopher Rios for their support with Present Your PhD, as well as all those who joined along the way. To Dr. Kate Carter and Dr. Michelle Valkanas for their support throughout the NCSE fellowship and helping me believe in myself as a science communicator. To those who nurtured my skills as an educator: Drs. Erika Abel and Marcie Moehnke, and Ms. Amanda Bracey. I am deeply thankful for your mentorship and guidance. I also thank those who work tirelessly to ensure the broad reach of science. To my teachers growing up who instilled in me, and surely many others, an early love of science that was my internal source of strength: Mr. Mark Nagy (7^{th} grade), Mrs. Pamela Gilmore (11^{th} grade, AP Biology). Also, to the network of science communicators that have shown, invited me into, and helped me grow within this incredible community.

This acknowledgement would not be complete without gratitude for those who helped me see the light at the end of the tunnel and the path forward. This dissertation is majorly a result of your enduring love and support. To my best friends, Megan Ed, Rachel Neerdaels (Hickson), Felicia Osburn. To Jason Morrison and Tyler Olson. And, most of all, to my parents, Ric and Michelle Reisenauer.

DEDICATION

To those who are my family, both by birth and by choice; y'all kept me fed, kept me focused, and filled my soul with love.

ATTRIBUTIONS

Chapter Three

Experiments performed by K.N.R. with contributions from S.S., P.D., P.S., S.P., H.S., S.M., and A.I. Isolation and characterization of ophiobolins by M.M., A.B., A.E., and A.K. Novel chemical syntheses by Y.T. and D.R. Study design, manuscript drafting by K.N.R. and J.H.T., with editing by D.R. and A.K.

Chapter Four

Viability, microscopy, and protein expression experiments by K.N.R. Cybrid and electron transport chain experiments by B.K. and lab. Study design and analysis by K.N.R. and J.H.T.

Chapter Five

STS Analogs Via C–H Borylation: K.M.G.: Design and synthesis of analogs, method development, author of manuscript. K.K: Design and synthesis of analogs, method development. J.L.W.: Project lead. K.N.R.: Design and execution of biological assays. J.T.: Project design, biological studies.

Total Synthesis and Anticancer Activity of (+)-Hypercalin C and Congeners: Synthesis and characterization of (+)-Hypercalin C and congeners by Y.T. Biological

assays by K.R. Study design, manuscript drafting by Y.T. and K.N.R., with editing by D.R. and J.H.T.

Pharmacophore-Directed Retrosynthesis Applied to Ophiobolin A: Simplified Bicyclic Derivatives Displaying Anticancer Activity: Retrosynthesis and pharmacophore chemistry by Y.T and M.M. Biological assays by K.R. Study design, manuscript drafting by Y.T. and K.N.R., with editing by A.E., D.R. and J.H.T.

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CHAPTER ONE

Introduction

Triple-Negative Breast Cancer (TNBC) Patients Suffer from a Lack of Treatment Options

Breast cancer is a heterogeneous collection of distinct diseases with unique etiologies and optimal treatments. Patients with tumors positive for estrogen receptor, progesterone receptor, or human epidermal growth factor receptor 2 (HER2) have benefited from improvements in targeted treatments. However, patients diagnosed with triple negative breast cancer (TNBC) still face poor prognoses due in part to the lack of effective targeted treatments. [1,2] Currently, anthracyclines (i.e., doxorubicin), taxanes (i.e., paclitaxel), and anti-metabolites (i.e., gemcitabine) are the main FDA-approved treatment options for TNBC patients. [3–5] Anthracyclines inhibit DNA synthesis, taxanes function by inhibiting cell division via stabilization of microtubules, and anti-metabolites target cell division by inducing DNA damage. [6,7] Single-agent chemotherapy in adjuvant or neo-adjuvant application is highly ineffective, yet remains the primary backbone of TNBC therapies.[8] Recent studies focus on combination treatments, yet these trials have yielded variable results.[9] These trial results promote decidedly controversial efficacy of these types of chemo-compounds, especially when considering that only half of TNBC patients treated with standard neo-adjuvant chemotherapy will respond to treatment.[9–12] Those who do not achieve a pathological complete response to chemotherapy treatment are six times as likely develop primary tumor recurrence and/or distant metastasis within three years of diagnosis. [10,13–22] Recurrence and metastasis increase the risk of mortality four-fold, despite continued

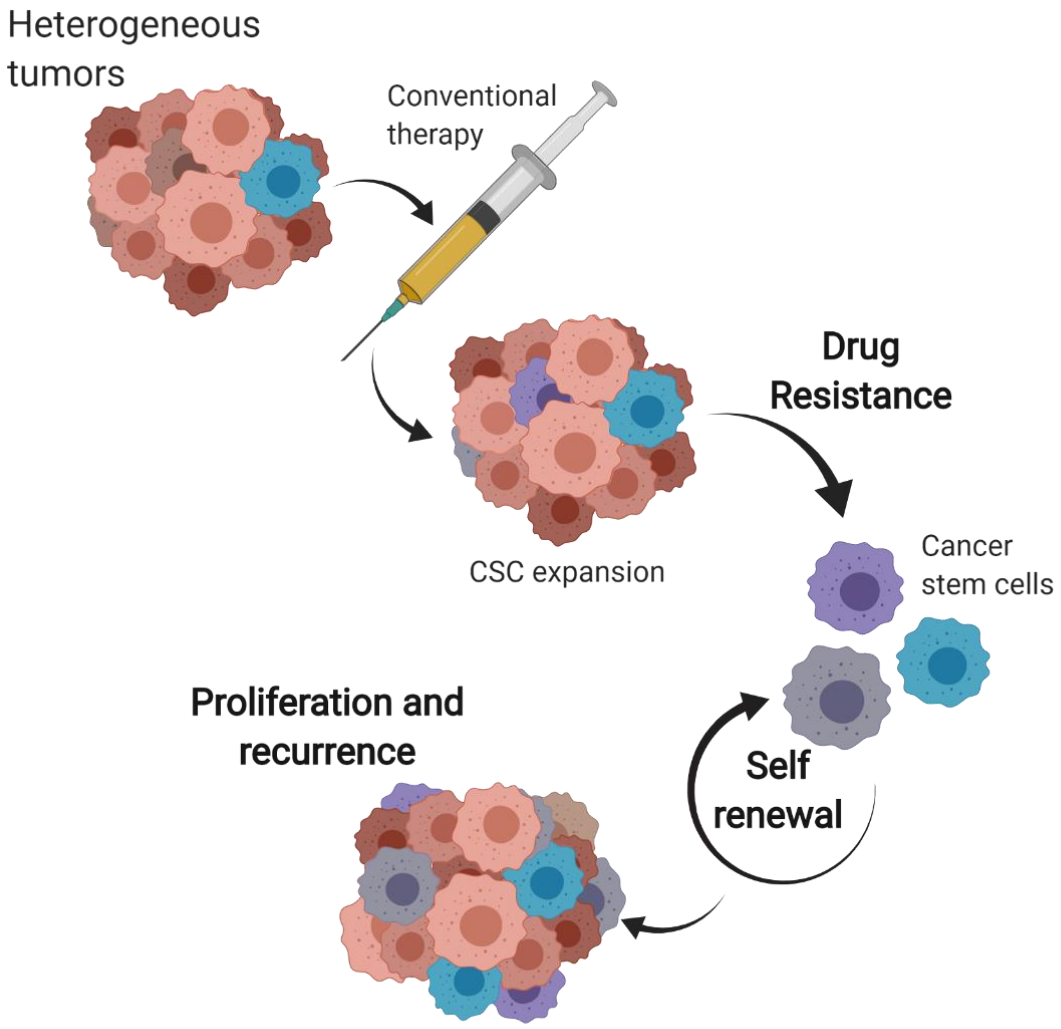
treatment or surgery.[10,23] These reasons make it imperative to develop treatments against aggressive TNBCs.

Cancer Stem Cells (CSCs) Comprise an Enriched Sub-Population in TNBC Tumors

Breast cancer tumors are able to be sub-divided into molecular subtypes, and TNBC are typically classified as claudin-low or basal-like tumors.[2,11] Refining these categories can prove difficult due to diverse tumor heterogeneity, which can be attributed to tumor evolution via the expansion of mutation-driven subpopulations and to variability of these subpopulations with tumorigenic (CSC) and non-tumorigenic (non-CSCs) properties.[24] Such subpopulations within the tumor exhibit diverse degrees of tumor initiating potential and resistance to chemotherapy. CSCs are characterized by their tumorigenic, metastatic, and treatment-resistant properties, and, while all tumors are comprised of a mixture of CSCs and non-CSCs, molecular-subtypes claudin-low and basal-like are highly enriched for the tumorigenic CSC subpopulation.[2] CSC populations can be measured by sorting them using their cell surface markers, $CD24^{lo}$ and $CD44^{hi}$, as well as increased ALDH1 activity, and ganglioside GD2 expression.[25–27]

CSCs Promote Tumor Outgrowth and Recurrence, and Are Resistant to Conventional Therapies

Because TNBC is predominantly comprised of claudin-low and basal-like tumors[14], which are particularly enriched for the tumorigenic CSC subpopulation, there is an increased likelihood of tumor and disease recurrence for patients with TNBC diagnoses. In fact, regardless of primary breast tumor subtype, all post-chemotherapy recurrent tumors



Scheme 1.1 Cancer stem cells promote proliferation and recurrence through drug resistance. Heterogeneous epithelial tumors are comprised of a mixture of cells, one key subtype being cancer stem cells (CSCs). When tumors are treated with conventional chemotherapies, the sensitive population of cells die and the CSC population, which is resistant, will expand. These cells are able to self-renew and, thusly, promote the expansion of the tumor. CSCs are able to initiate this expansion immediately following treatment, or decades later, prompting a recurrence of disease that is no longer sensitive to prior treatments.

are highly enriched for CSCs.[28] This makes targeting CSCs relevant not only to TNBC tumors, but also to tumor recurrence and the emergence of therapy resistance across all breast cancer subtypes and other carcinomas. CSCs' innate resistance to chemotherapy leads to rapid disease progression, which is another major contributing factor to TNBC

mortality.[13] This resistance stems from their relative quiescence, resistance to apoptosis, high expression of multidrug transporters, and increased capacity for DNA repair.[29] Currently, there are few plausible treatments for targeting CSCs due to our incomplete understanding of their biology and the fact that CSC populations themselves are heterogeneous.[30] Pursuing multi-therapy approaches to independently target CSCs and differentiated cells thusly becomes imperative to successfully targeting this lethal subpopulation in order to improve patient outcome.

Epithelial-Mesenchymal Transition (EMT) Promotes the Acquisition of CSCs

The epithelial-mesenchymal transition occurs during development and wound healing, but its aberrant activation in cancer tumors has been implicated in inducing dedifferentiation of cancer non-stem cells into CSCs.[31,32] In fact, the acquisition of EMT-driven CSC properties in a tumor has been linked to the induction of tumor recurrence, chemoresistance, and TNBC metastatic propensity.[31,33–36] In response to signals promoting EMT, carcinoma cells acquire a mesenchymal morphology, marked by profound gene expression decreases in E-cadherin (*CDH1*) and increases in N-cadherin (*CDH2*), fibronectin (*FN1*), and vimentin (*VIM*). EMT-inducing transcription factors such as Snail (*SNA1*), Twist (*TWIST1*), ZEB1 and ZEB2 also promote these changes at a transcriptional level.[37–39]

EMT and Metastasis

While a relationship between EMT and stem-like properties has been well characterized [31,32], there is still considerable debate surrounding the contribution of EMT

to metastasis.[40–42] Cells that promote metastasis must be capable of disseminating from the primary tumor, and must survive the journey to the secondary location to initiate growth of a new tumor mass. In order to successfully achieve this growth in the latter part of the metastatic cascade, it is accepted that EMT must be reversed to re-initiate cell cycle progression, and to enable re-epithelialization, proliferation, colonization and expansion of micro-metastases into macro-metastases with a histopathology similar to that of the primary tumor.[39,43] Work in the mouse mammary tumor virus (MMTV)-driven expression of the polyoma middle T (PyMT) oncogene mouse model has demonstrated a loss of E-cadherin expression in disseminating cancer cells, which is reversed within early rounds of cell division at the metastatic site.[44–46] In fact, reports that question the requirement of EMT in promoting metastasis of untreated tumors still demonstrate that metastasis of chemotherapy-treated tumors is accompanied by EMT.[41] Given that standard-of-care for many breast cancers involves chemotherapy[15,18], the role of EMT in chemotherapy-associated metastasis demonstrated by these studies signifies that EMT is likely to be highly clinically relevant for understanding and effectively targeting metastasis.

CSC-Specific Agents Are Distinct from Non-CSC Targeting Drugs

Solitary disseminated CSCs often remain in a quiescent state, rendering them resistant to conventional therapies that rely on targeting cell cycle processes responsible for driving increased rates of cell division, typical of proliferating tumor cells.[47] These disseminated cells contribute to the failure of current therapeutic methods and are responsible, at least in part, for cancer recurrence.[48] By targeting CSCs through

mechanisms independent of cellular proliferation, patients at risk for primary or metastatic recurrence could experience improved outcomes.

Experimental Approaches to Improve CSC Therapeutic Understanding

Improving TNBC-targeted therapies relies on improving understanding of CSCs and their role in promoting residual and recurring disease. Rigorous investigations into novel compounds impacting the biology of tumor re-growth are essential. It is important to note that CSCs comprise a small sub-population of the overall tumor, and they are difficult to grow and propagate outside the tumor microenvironment without risking loss of essential stemness features.[49–52] This makes acquiring the large quantities of these cells required to withstand drug screening and testing infeasible; instead, studies that utilize a surrogate system that allows for stable expansion and manipulation of the EMT/CSC phenotype show more promise. The studies using *in vitro* models are numerous and are successful in both observation and manipulation of EMT and CSC phenotypes both using established cancer cell lines from patients, as well as induced models. Advances in technology expand understanding of how these cells behave through 3D models and computational analysis.[53–57]

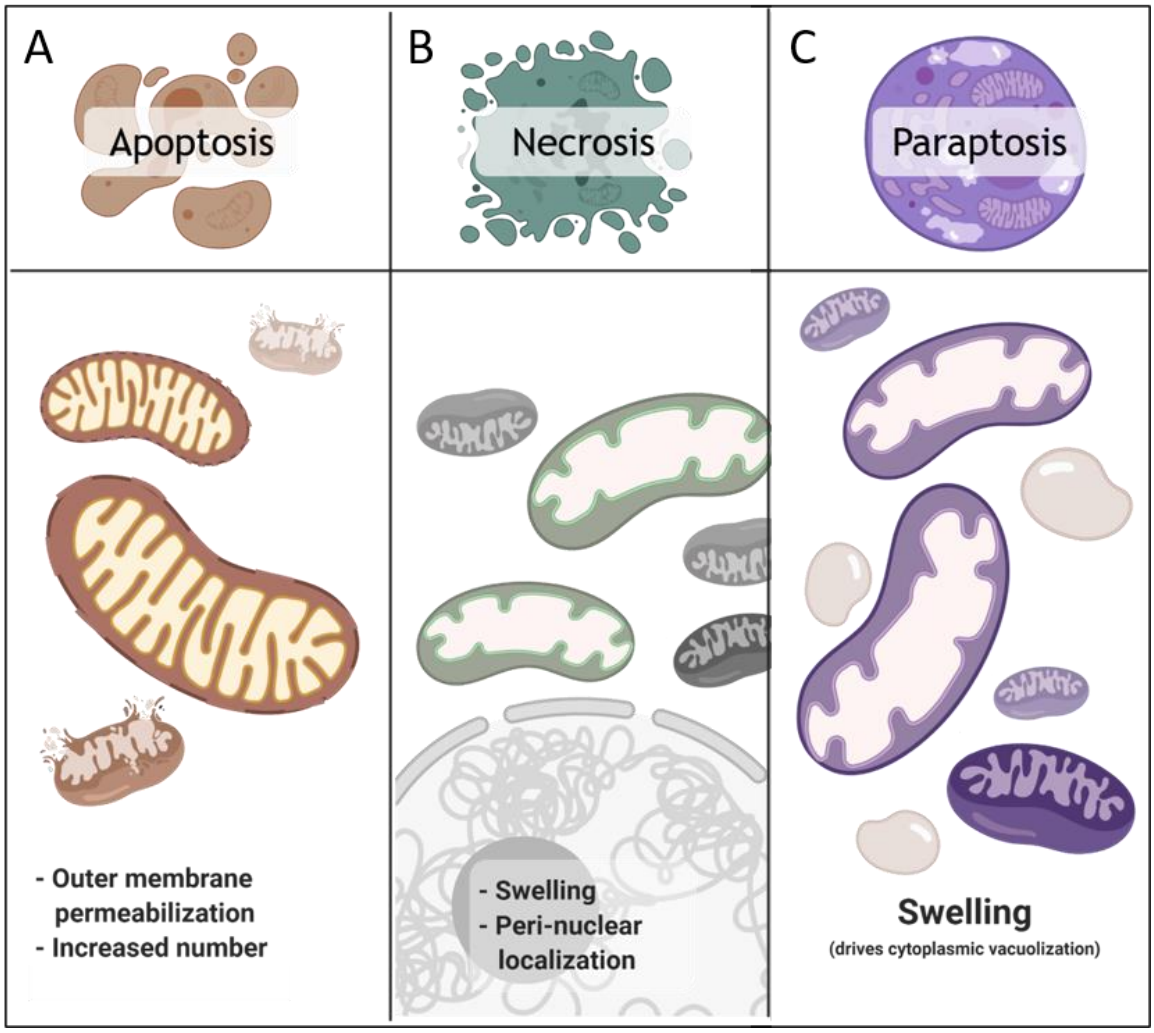
Natural Products Target EMT-Driven CSCs and Reduce Chemoresistance by Overcoming Apoptosis Resistance

The population of chemoresistant patients will only increase as treatments relying on growth factor receptor-targeting are easily evaded by tumor cells via mutation or activation of parallel pathways.[58] Natural products that induce non-apoptotic cell death have been demonstrated to be effectively disruptive at low concentrations and may elude

drug resistance mechanisms adopted by CSCs, providing alternatives or replacements to current targeted therapies.[59,60]

One such compound is Ophiobolin A (OpA), a fungal secondary metabolite part of a family of ophiobolins with a common sesterterpenoid (25 carbon) framework.[61,62] OpA exhibits nanomolar activity against specific cancer cell lines [e.g. 120 nM IC_{50} for A549 lung cancer cells[63] and 20 nM for Hs683 glioma cells[64]] and a wide therapeutic index [e.g. 21 μM IC_{50} for human peripheral blood mononuclear cells[65]]. Furthermore, OpA is selective for the CSC-enriched population of breast cancer cells, indicated by a selective reduction of the $CD44^{hi}/CD24^{lo}$ subpopulation of TNBC MDA-MB-231 cells and a decrease in mammosphere formation.[66] While the mechanism of action for OpA is yet to be elucidated, several studies report changes in mitochondrial properties such as calcium signaling and the calmodulin pathway[67,68], membrane polarity and permeability[65,69,70], and regulation of ion transporters.[62] OpA is also implicated in a variety of cell death pathways in cancer, such as apoptosis, autophagy, and paraptosis, depending on the type of cancer.[62,70–72]

In the following chapter, other natural products that play a role in evading CSC apoptosis resistance are described. Many are in clinical trials or have informed the synthesis of derivatives that improve challenges such as bioavailability and solubility. The field of natural product-based therapies in cancer is highly promising and offers novel solutions to a complex disease.



Scheme 1.2 Different cell death pathways uniquely affect mitochondria. (A) Apoptosis activation results in the outer membrane of mitochondria permeabilizing, releasing the inner contents into the cytoplasm. Further, the number of mitochondria increase (Márquez-Jurado et al., 2018). (B) Mitochondria during necrosis activation begin to swell and localize next to the nucleus. (C) Paraptosis, the most newly described mechanism of cell death in this scheme, is the least well characterized. However, it is understood that mitochondrial swelling plays a central role in promoting the characteristic cytoplasmic vacuolization of the mechanism of cell death.

CHAPTER TWO

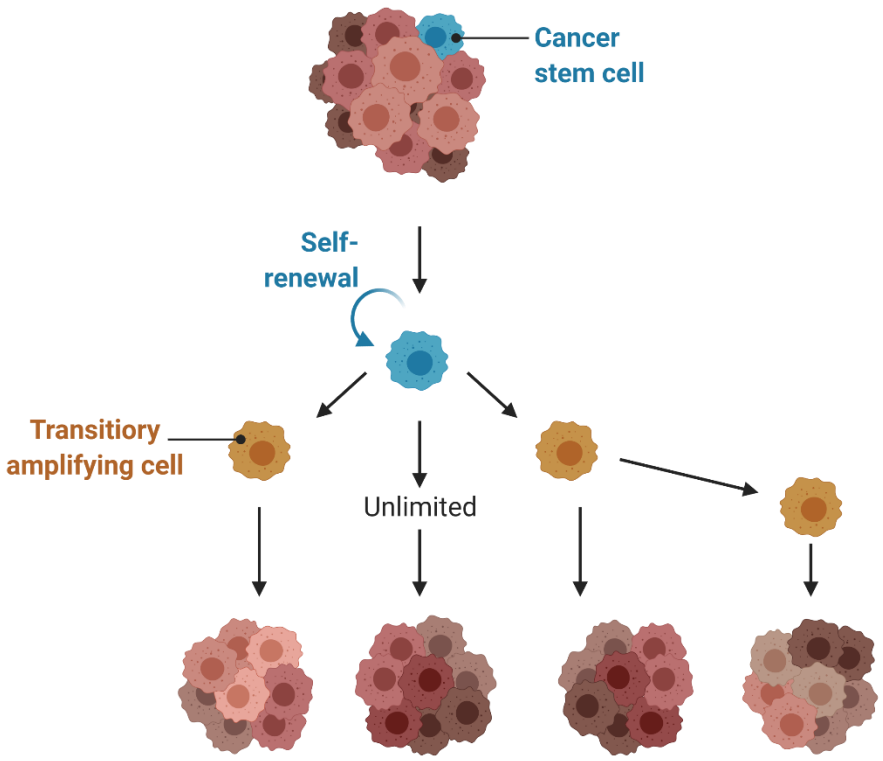
Targeting Epithelial Cancer Stem Cells Via Natural Products

Introduction

Conventional cancer therapies often fail to completely eradicate the disease, leaving intact a subpopulation of cancer stem cells (CSCs) capable of driving disease recurrence. Defined in the early 1990s in leukemia [73–75] CSCs also function in a range of solid cancers including breast [25], colorectal [74,76,77], and brain cancer.[78] Like normal stem cells, CSCs are further defined by their functional properties, such as their role in tumor renewal and growth, and their multipotent differentiation capacity.[79] However, CSCs are also linked to particular clinical observations such as tumor recurrence and chemoresistance.[80–82] This population exhibits an elevated ability to seed new tumors upon experimental implantation in appropriate animal hosts, compared to other cells in the tumor.[79] CSCs also divide slowly, facilitating resistance to chemotherapies and radiation that target fast-dividing cells. A better understanding of the biology CSCs has inspired treatment strategies no longer aimed at shrinking tumor bulk, but, rather, eliminating the CSC population that sustains long term growth.[80] Treatments that are able to address cues from the internal signaling, epigenetics, and environmental cues that result in CSC generation and renewal are required to succeed in mitigating disease. In particular, the use of naturally-evolved bioactive molecules which possess anti-CSC activities is illuminating novel therapeutic strategies likely to result in improved cancer treatments.

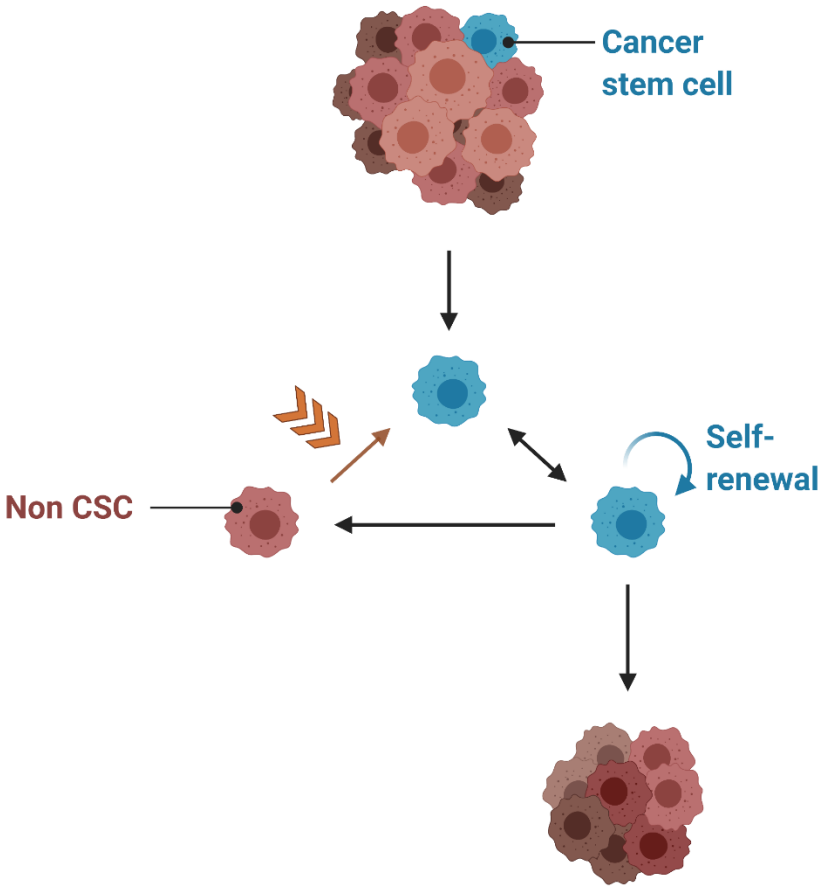
Phenotypic Heterogeneity: Cancer Stem Cells

Intratumoral heterogeneity can manifest through clonal evolution of genetically dissimilar cells or through phenotypic variation in which genetically identical cells exhibit divergent behaviors. Indeed, most, if not all, tumor types possess a subpopulation of cells that match common descriptions of CSCs, however, how they arise is more highly debated. Early evidence indicated a hierarchical model where CSC properties were restricted to cells with the appropriate intrinsic signaling and epigenetic states.[80–82] This model is founded in a theory that tumor growth is fueled by small numbers of CSCs that generate larger number of transitory-amplifying cells that then differentiate into non-dividing bulk tumor cells observed in examples of post-dormancy tumor recurrence, and metastasis (Scheme 2.1). A cornerstone of this model is that CSCs are able to initiate tumors in xenograft assays, compared to differentiated tumor cells (non-CSCs), which dictates that there is limited plasticity in the tumor hierarchy, i.e., differentiated tumor cells cannot convert into CSCs. However, technical and conceptual limitations result in the xenotransplantation strategy falling short.[24,80,83] In particular, transplantation assays involve dissociation of the tumor mass, thus, introducing external pressures such as loss of cell-to-cell contacts, attachment to the extracellular matrix, and microenvironmental signals. These variables do not rule out the potential for these cells to adapt to experimental conditions, thus disrupting a foundational concept of the unidirectional hierarchical model.[80]



Scheme 2.1 Hierarchical cancer stem cell model relies on unidirectional cell division. Tumors contain a small number of cancer stem cells that asymmetrically divide into transitory-amplifying cells, which then promote differentiation into distinct tumor clones and increase Intratumoral heterogenicity.

Recent studies that allow for *in situ* assessment and utilize more advanced techniques like genetic lineage-tracing and cell-ablation strategies, however, illuminate the inconsistencies in the fixed-hierarchy model and, instead, posit that CSCs are able to arise via phenotypic transitions when certain stimuli are applied, such as would be found in specific microenvironments or niches (Scheme 2.2).[80,84] For example, genetic lineage-tracing allows for the stable labeling and identification of unique cells within a tumor while avoiding mechanical disruption of the tumor mass, such as in models that use a *LacZ* reporter system.[85] A model such as this more accurately evaluates persistence, size, and composition of cell clones over time to determine stemness potential. Additionally, this expanded model allows for the complex interaction of CSCs with the tumor and the



Scheme 2.2 Dynamic cancer stem cell model incorporates multidirectional CSC formation. Cancer stem cells are able to be formed both via cell division, but also from de-differentiation of non-CSCs. Extra-cellular stimuli (orange bolt), often from the tumor microenvironment or nearby CSC niches, are required to induce this de-differentiation process in non-CSCs. Only CSCs are able to initiate tumor formation and renewal, however.

microenvironment, and the idea that CSCs do not have to be rare and/or quiescent.[86] It is the pressures of the CSC niches that direct the results of CSC cell divisions: two, one, or no additional stem-like progenitor cells. The signals from the niche create a competition for space within the local microenvironment and produce cells that differentiate and leave the niche. Interestingly, however, this process is not unidirectional, and daughter cells along a spectrum of differentiation are able to revert to a stem-like phenotype and replace diminishing CSCs in the niche.

Through the process of mutation accumulation, genetic changes may render CSCs progressively independent of the niche signals. One keynote study illuminates how lineage tracing helped not only debunk linear models of CSC proliferation, but also identifies niche independency. The hierarchical model would suggest that proliferative basal epidermal cells adhere to strict vertical niche proliferation, however cells instead spawn several lineages of differentiated cells depending on the influence of particular niches.[87] Similarly, in the normal developmental context, niche-independent effects are observed in intestinal crypts. When the foundational stem cell is lost, it is replaced with a neighboring non-stem cell which acquires pluripotency, thus negating the theory of crypts derived from a singular clones.[88] Additional studies have also utilized lineage tracing to describe advancements upon the fixed-hierarchy CSC model such as in the stomach[89], testis[90], and blood.[91]

These examples further shed light on the complications researchers have faced in identifying and eradiating this problematic subpopulation of cells. Modern technologies illuminated novel findings into CSC plasticity, quiescence, renewal, and therapeutic response, which have opened the door for the first successes of CSC-targeted therapies.

Molecular Hallmarks of Cancer Stem Cells

Since the first description in leukemia in the early 1990s[75], the role and phenotypes of CSCs has now been demonstrated in many cancers including breast [25], colorectal [74,76,77], and brain cancer.[78] These works, and others, demonstrate that in solid tumors only a fraction of cancer cells present the capacity to reform secondary tumors following their transplantation into immunodeficient mice. Increasing understanding of what promotes this tumorigenicity at a molecular level comes from experimental evidence focused on

identifying CSCs through their unique cell surface markers, changes in signaling pathways, and nuclear environment. Together, these characteristics illuminate the reliance of CSCs on various pathways and mechanisms that can be therapeutically targeted.

Cell Surface Markers

Surface Proteins: Common markers for stemness have become a way to distinguish CSCs within heterogeneous populations. Cell surface markers allow for quick sorting, using flow cytometry, or to identify localization via immunofluorescence. Key cell surface marker proteins corresponding to CSCs include CD24, CD29, CD44, and CD133, though not all are expressed in each cancer type and CSCs can exhibit high (hi), low (lo) or an absence (-) of these markers. The respective (low/-)/(hi) pattern of cell surface markers CD24 and CD44 is indicative of a CSC-enriched population in a wide range of cancers including breast, lung, pancreas, prostate, colorectal, renal, and ovarian.[92–94] Other key cell surface markers include CD133[78,94,95], found in liver, lung, breast, prostate, colon, and pancreas cancers and glioblastoma[78,96–98]; CD29, prominent in squamous cell carcinoma[99] and colorectal cancers[74,100]; and broadly EpCAM[74].

Efflux Proteins: Another characteristic distinguishing CSCs from the main tumor cell population is their increased expression of multidrug efflux proteins.[101–103] Typically, these proteins pump out toxins from the gastrointestinal tract, eliminate bile in hepatocytes, maintain homeostasis across the blood-brain and placental barriers, and excrete drugs from the body.[104] In CSCs, they confer multidrug resistance, which impairs the ability of commonly used chemotherapies to work effectively. The main overexpressed efflux proteins in CSCs are part of the ATP-binding cassette (ABC) transporter family: P-

glycoprotein (also known as multidrug resistance protein 1 (MDR1) or ABCB1), multidrug resistance associated proteins 1 and 2 (MRP1/ABCC1 and MRP2/ABCC2), and breast cancer resistance protein (BCRP/ABCG2).[105–107] These proteins are also overexpressed in glioblastoma, lung cancer, osteosarcoma, prostate and ovarian cancer, and nasopharyngeal carcinomas.[108–113] Therapeutically, four types of ABC transporter blockers are established for clinical use, with the most recent under development derived from natural compounds such as chloroquine, quercetin, genistein, curcumin, and others.[114] However, inconsistent results highlight the need for further understanding of the particular circumstances in which drug efflux is a critical liability constraining success of chemotherapies.[114–117]

Considering CSC models and the experiments used to define this subpopulation, cell surface markers play a key role in understanding how these cells work and in establishing the models used to describe CSCs. Xenotransplantation experiments assess the functional properties of CSCs in solid tumors, characterize the implicated cells based on cell surface markers, and can be used to derive cancer-specific estimates for frequency and abundance of CSCs within the tumor mass. The frequency of CSCs seems to be generally low, ranging from 1 per 100,000 to 1 per 1,000 cells.[118] Similar experimental methods of dissociating the tumor into single cells, passing them through fluorescence-activated cell sorting (FACS) to isolate subpopulations of interest, and then re-injecting these cells into new hosts have been used to generate these estimates. However, as previously discussed, this approach comes with limitations and introduces variation to the cell environment, which could alter the phenotype of the isolated cells. Nevertheless, as FACS sorting relies on the expression of specific membrane-bound proteins, this approach has provided strong links between cell surface markers and CSC activity.

Activated Signaling Pathways

Some current treatment approaches for CSCs target cellular signaling pathways implicated in stem cell physiology, in particular, the WNT, Notch, and Hedgehog (HH) pathways.[79,119] These pathways are involved in an intricate overlapping network that orchestrates self-renewal and proliferation in several types of normal stem cell settings but can promote neoplasia when deregulated.[120] Multiple mechanisms to target these pathways have already been identified and are in clinical application, however they still face the challenges of choosing the most appropriate inhibitor for each patient, identifying pharmacodynamic biomarkers, and selecting mechanism-based combination regimen and patient stratification according to recognized efficacy biomarkers.[119]

Wnt Signaling: The Wnt pathway is highly conserved and composed of three major sub-pathways: canonical, which acts through β-catenin activation and is implicated in tumorigenesis, and two types of non-canonical, one which drives cell polarity and regulates the cytoskeleton, and the other which regulates intracellular calcium levels. The Wnt signaling pathways can be activated by Wnt ligands which bind to receptor molecules (e.g., Frizzled) on the surface of target cells. Following this receptor-ligand interaction, transduction proteins including β-catenin activate target genes like MYC or CCND1 (cyclin D1). Not only is Wnt associated with tumorigenesis broadly, it also maintains the CSC subpopulation and induces epithelial-mesenchymal transition (EMT; described below) phenotypes.[32,121–126] Current therapies aim to prevent β-catenin translocation or degradation, neutralize Wnt ligands, or inhibit Wnt receptors.[119] Broadly there are three types of compounds in clinical or preclinical studies designed to target Wnt signaling: synthetic small molecules, natural products, and multiclonal antibodies (mAbs).[127] There

is preclinical expansion in all small molecule targets, unnamed outside of their alphanumerical codes. Clinically, studies involving molecules that target β-catenin are in Phase I/II in colorectal cancer and leukemia.[128–130] Studies involving molecules that target porcupine (PORCN), an acetyltransferase that promotes Wnt molecules to transport from the Golgi apparatus to the cell surface in order to bind to Frizzled (FZD) receptor, are primarily in Phase I and focus on metastatic colorectal cancer and other advanced solid tumors.[131–134] Small molecules that target Dishevelled (DSH), which relays Wnt signals to downstream effectors, but these are still in early phases.[135] Natural products such as derricin, carnosic acid, quercetin, and resveratrol are broadly undefined in their targets, though some preclinical findings suggest BCL9 and β-catenin as possible targets.[136–139] For cancer specifically, resveratrol is the only natural product in clinical trial, focused on colorectal cancer, cancer with metastases, and multiple myeloma.[137] mAbs target both Wnt proteins broadly as well as Frizzled receptor.[140–142] Phase I studies are underway in breast, pancreatic, liver, ovarian, non-small cell lung cancer, and several other solid tumors.

Notch Signaling: Like Wnt, Notch is another conserved pathway with multifactorial roles in proliferation, stemness, cell fate, differentiation, and angiogenesis. Notch works by signaling via transmembrane ligands and receptors between contiguous cells. Key modulators include DLL1/3-4, JAG1/2, and Notch1-4. Notch is one of the most commonly activated pathways in cancer cells, especially in cases of metastasis likely due to its role in angiogenesis promotion. From a therapeutic perspective, Notch is challenging to target because evidence shows that inhibition enhances chemoresistance[143], while upregulation of the pathway promotes CSC stemness.[144,145]

Complete pathway inhibition is not essential for efficacy, however. Instead, two major classes of inhibitors, which interfere with receptor cleavage or ligand-receptor interactions, are successful when combined with standard chemotherapies.[146,147] Inhibition of γ-secretase, a large protein complex that executes early stages of Notch activation, is an effective means to suppress Notch's proto-oncogenic function and is used in clinical applications.[147–149] By preventing the proteolytic cleavage that releases the active intracellular fragment, preclinical studies in gastrointestinal cancer[143] and glioblastoma[150] show decreases in stem cell markers and tumor burden following γ-secretase inhibition (GSI). Co-treatment with gemcitabine and GSIs demonstrate clinical antitumor activity and more than four months stable disease in pancreatic, tracheal and primary breast cancer.[151] In breast cancer models, GSIs not only induce apoptosis, but also suppress tumor self-renewal and metastasis.[152] Of the more than 100 GSIs that have entered clinical trials [146,147], many successfully suppress disease progression and CSC phenotypes, indicating that Notch disruption via γ-secretase inhibition is a promising approach for targeting CSCs.

mAbs directed against Notch receptors or ligands "lock" Notch receptors in inactive conformations and are in preclinical development.[147,149] Counter to GSIs, mAbs offer specific targeting, such as ligands like DLL-4 or receptors like Notch1-3. DLL-4 is an effective target for mAbs because it promotes growth and stem cell renewal, and mAb therapies that block DLL-4 promote necrosis and impaired tumor growth.[153] Humanized mAb tarextumab blocks both Notch2 and Notch3, which arrests growth in xenograft breast, small-cell lung, ovarian, and pancreatic cancers.[146] Moreover, tarextumab reduces CSC frequency, delays tumor recurrence after termination of chemotherapy, and synergizes with gemcitabine co-treatment to reduce tumor burden.[154]

GSIs and mAbs are currently the only approaches to Notch inhibition that have reached preclinical studies and beyond. However, research elucidating transcriptional, translational, and post-translational regulation of components in Notch signaling identify targets for future work, including NF-κB-promoted Jagged-1 expression and AP-1-driven Notch4 signaling.[147]

Hedgehog Signaling: The hedgehog (HH) pathway functions during embryonic development and in repair of normal tissues to ensure proper tissue patterning, and is also activated during EMT. There are three main ligands: Sonic hedgehog (SHH), Indian hedgehog (IHH), or Desert hedgehog (DHH). Ligand binding relieves the inhibitory effect of their Patched (PTCH) transmembrane receptors on Smoothened (SMO), which is also located in the cell membrane; SMO will then activate GLI transcription factors via enhanced nuclear localization, which will in turn activate HH target genes. Like Wnt, there are canonical and noncanonical pathways, which are SMO dependent and independent, respectively, with the latter being more readily attributed to tumor-associated signals.[119] A number of intracellular signaling pathways stimulate HH ligand expression and secretion, including crosstalk with TGF-β, KRAS–MAPK/ERK, PI3K–AKT, and IGF. SMO is the leading target for HH-focused therapies, but various resistance mechanisms have been described. SMO inhibitors are not effective when the receptor is activated downstream by other signaling pathways.[155] Moreover, mutations in SMO disrupt the inhibitor binding site and render therapies ineffective.[156]

Nuclear Environment (Transcription/Epigenetics)

As previously mentioned, the CSC phenotype is initiated and maintained by a network of overlapping pathways and signals, which alter gene expression via transcriptional and epigenetic factors. By definition, for a CSC to maintain identity yet also possess pluripotency, it requires reversible but semi-stable changes, which inherently calls for epigenetic regulation. Epigenetics refers to the mechanisms that control the reversible regulation of gene expression by modifying DNA or histone proteins, without altering the DNA sequence. The effector molecules can receive direction from a host of signaling pathways, from development, to proliferation, to survival.

Epithelial-Mesenchymal Transition: One of the molecular programs that sets CSCs in carcinomas, cancers of epithelial origin, apart from other cancers is the epithelial-mesenchymal transition (EMT). EMT occurs in normal physiology, e.g. epithelial wound healing, and development, e.g. formation of the neural crest.[121,157] EMT is defined as the process by which epithelial cells undergo phenotypic changes to a mesenchymal state, through changes in gene expression including activation of Snail, Slug, Twist, and ZEB1, and deactivation of E-cadherin, claudin-1, and ESRP1/2.[158] These changes cause cells to lose apical-basal polarity and anoikis sensitivity, and begin to appear longer and spindle-like, compared to boxy and cobblestoned. Enrichment for an EMT gene signature in primary tumors has been linked to tumor invasion, metastasis, and poor prognosis.[159] The reverse process, mesenchymal-epithelial transition (MET), is also important in disease progression, returning cells to an epithelial and highly-proliferative state. This EMT-MET plasticity can be conferred and maintained by epigenetic mechanisms. For example, TGF-β-induced EMT results in downregulation of miR-200c and miR-183, as well as

concomitant elevation of the stemness-related genes Bmi1 and Klf4, which are rescued by overexpression of miR-200c or p53. [160] Further, some histone markings contribute to epigenetic regulation of EMT-MET plasticity, allowing for flexible determination of gene expression.[160]

Importantly, cells that undergo EMT are also shown to be more stem-like, acquiring the ability to seed new tumors, at both primary and metastatic sites, and a resistance to therapies designed to target rapidly dividing cells[161–163]. For example, differentiated breast cancer cells activated with ectopic expression of EMT-TFs Twist or Snail acquire CSC properties.[31] Further, in xenograft breast cancer models, cells that independently undergo EMT and activate the Ras-MAPK pathway also acquire stemness characteristics.[32] This pattern is also evident in prostate[164,165], pancreatic[33,165], head and neck[166], liver [167], colorectal[168], and skin[31,32] cancers. Interestingly, in niches with strong cytokine and growth factor production, EMT can be activated via, for example, tumor-associated macrophages that secrete VEGF and IL6, and T cells that secrete IL17 and TGF-β, promote CSCs[169,170]

CSCs require the activity of histone modifiers such as histone deacetylases (HDACs), DNA methyltransferases (DNMTs), histone lysine methyltransferases and demethylases (KMTs/KDMs), and protein arginine methyltransferase (PRMTs). The amino acid residues located on the N- and C-terminal tails of histones can be modified to influence gene expression, including by acetylation, methylation and ubiquitylation. When occurring on promoters or enhancer regions, these modifications confer chromatin states that affect gene expression by altering the ability of protein complexes that bind specific post-translational modifications. Each of these enzymes prove valuable and targetable in

CSC-specific therapies, either alone or in combination with other epigenetic inhibitors or conventional therapies.

DNA Methyltransferases: DNA methyltransferases (DMNTs) catalyze the transfer of a methyl group from the methyl donor S-adenosylmethionine (SAM) to the fifth carbon atom of cytosine base in the CpG dinucleotides of gene promoter. This epigenetic mark inhibits transcription by recruiting proteins with methyl-CpG-binding domains to remodel the histone or chromatin, or by preventing recruitment of DNA binding proteins.[171–174] Generally, CpG methylation is required for differentiation whereas demethylation is essential for induction of the pluripotent state, especially in the cases of CSC generation and tumor initiation.[175–177]

DNMTs consist of the maintenance methyltransferase DNMT1, and de novo methyltransferases DNMT3A and DNMT3B. Broadly, DMNTs play vital physiological roles in mammalian development, genome stability and cell fate determination, where DNMT3A and DNMT3B contribute by establishing methylation at CpG dinucleotides[178,179]. These DNA methylation patterns are maintained by DNMT1 during cellular proliferation.[180] Dysregulation of DMNT1 is implicated in tumorigenesis; for example, DMNT1 promotes the triple negative breast cancer (TNBC), phenotype by hypermethylating and repressing estrogen receptor expression. DMNT1 also promotes EMT, autophagy, and CSC proliferation and stemness through hypermethylation of promoter regions of estrogen receptor, tumor suppressor genes, microRNAs, and epithelial markers. Inhibitors of DMNT1 like azacitidine, decitabine, and guadecitabine show promise of sensitizing TNBC patients to immune checkpoint blockade therapy through their hypomethylation activity.[181] The importance of both hypomethylation and DNMT1 in

CSCs suggests that specific localization is likely to be a more important determinant of cellular identity than the overall global patterning. In particular, hypomethylation at pluripotency loci and hypermethylation at tumor suppressor genes or those involved in differentiation is important in the generation of CSCs.[171]

Histone Lysine Methyltransferases and Demethylases: Histone lysine methyltransferases (KMTs) and demethylases (KDMs) post-translationally modify histone methylation marks and the resulting effects of these modifications have implications in regulation of gene expression, differentiation, DNA damage repair as well as in tumorigenesis.[182,183]

KMTs demonstrate large structural diversity of its active sites, allowing these enzymes to have high substrate specificity.[184,185] For example, KMT4 is the only enzyme that methylates at the H3K79 position.[186] Similarly, methylation of H3K27 is only driven by the catalytic subunit EZH2 (KMT6) of PRC2 complex.[184] Due to the structural differences between different KMTs, a number of small molecules have been successful in interrupting activity.[182,183,187,188]

KDMs can be grouped into two families: the lysine-specific demethylase (LSD) family and Jumonji C domain-containing (JmjC) family. KDM6A (UTX), is one of the most robustly studied lysine demethylases, and is responsible for demethylating H3K27. The loss of its activity plays a role in in multiple human malignancies, including multiple myeloma, esophageal squamous cell carcinoma and renal carcinoma.[186] However, no inhibitors of JmjC enzymes have advanced beyond biochemical studies.[184]

Histone Deacetylases: Encoded by 18 individual genes and clustered into 4 distinct classes, histone deacetylases (HDACs) are commonly expressed and provide ample

opportunity for therapeutic intervention. In fact, the potential of HDAC inhibitors (iHDACs) for the treatment of cancer was first proposed nearly 50 years ago when their capacity to induce differentiation of erythroleukemia cells was discovered.[189] One major challenge to HDAC-specific therapy is their reliance on operating in complexes with other proteins, which implicates HDACs in the disruption of multiple pathways, thus reducing specificity.[190] In fact, HDACs act both as tumor suppressors and as tumor promoters, depending on the cellular context, the protein complexes present, and their general expression levels.[191]

Therapeutically, there are several molecules under investigation. SAHA (suberoylanilide hydroxamic acid), one of the most commonly used histone deacetylase iHDACs, induces differentiation of various cancer cell types including those of breast and endometrial carcinomas[79], however results have been mixed and, in some cases, met with notable toxicity.[192,193] Valproic acid, another iHDAC, increases CSCs in breast cancer by inducing dedifferentiation through Wnt activation.[194] However, HDAC-specific therapies in clinical use face limitations like side- and off-target effects, which require further research to overcome.

An alternative approach to targeting histone acetylation is through bromodomain inhibitors. Bromodomains (BRDs) confer binding affinity to histone acetylation, are found in several proteins, and target chromatin-modifying enzymes to specific genomic sites to regulate transcription.[171] Combination therapy results are also encouraging. Pathania et al. reported that combining azacitidine (DNMT inhibitor) and butyrate (HDAC inhibitor) was capable of significantly decreasing breast cancer CSC population.[195] Further, the combination of azacitidine and HDAC inhibitor entinostat at low doses in a phase I/II

clinical trial showed sustained and favorable responses in treatment-resistant non-small cell lung cancer (NSCLC) patients.[196] Early results of phase III trials for HR+HER2– advanced-stage breast cancer patients also show promise wherein aromatase inhibitor therapy in conjunction with HDAC inhibitor tucidinostat increased median progression-free survival.[197] A broader summary of HDAC inhibitors in clinical trial was conducted by Eckschlager et al.[198]

Protein Arginine Methyltransferases: Protein arginine methyltransferases (PRMTs), just like DMNTs and KMTs, utilize SAM to confer post-translational modifications. With nine different known PRMTs, they mono- and di-methylated arginine.[199,200] Arginine methylation regulates binding of methylarginine to protein modules that read this mark, such as plant homeodomain (PHD) zinc fingers, Tudor domains, and SH3 domains [201]

PRMTs maintain stem cell traits in normal stem cells, such as pluripotent state maintenance, upregulation of Oct4, Sox2, and Nanog, suppression of anti-stem microRNAs, and somatic cell reprogramming.[202] PRMTs interplay with histone transcriptional marks to drive HOX gene activation, implicated in EMT-driven tumor growth and metastasis.[203–206] EMT can also be driven by PRMTs via *ZEB1* promoter activation and FOXP1-driven stem cell self-renewal and proliferation in breast cancer[207,208], and by PRMT activation of TGF-β.[209] PRMTs also are known to play an active role in enhancing CSCs in colon[210] and breast[207] cancers.

Although targeting PRMTs is not yet tested in clinical trials, promising preclinical work establishes a rationale for translating some of the PRMT findings in oncology into clinical trials. So far, inhibitors targeting PRMT5 and type I PRMTs have entered clinical

development for patients with hematological malignancies or advanced solid tumours.[199,211] Further, like other epigenetic regulators, PRMT-specific therapies show promise when combined with DMNT inhibitors and even with inhibitors for different PRMT classes.[212–214]

Overall, the CSC phenotype is not the result of activation or errors in a single pathway, rather it is the outcome of cross-talk between multiple signals. Ultimately, targeting one single pathway is unlikely to be sufficient. The development of CSC-inhibitors requires a working understanding of key nodes in the stem cell signaling network and how these nodes interplay. This knowledge is propelling the design of mechanism-based combination regimens that use standard chemotherapies with novel approaches to cover broad ground in eliminating cancer.

The Role of Natural Compounds in Cancer Stem Cell Targeting

Often metabolites, natural products modulate a variety of pathways, are useful in the treatment of a wide range of afflictions, and have been applied as medicines throughout human history, with the earliest use dating back to 2600 BC in Mesopotamia.[215] Clinical implementation of these natural products as drugs must overcome the challenges of limited availability, difficult identification of bioactive compounds, time-consuming collection of wild specimens, and incompatibility with high throughput screening.[216] To be relevant clinically, the mechanism of action needs to be clearly defined, which is challenging when natural products often have pleiotropic effects.

Natural phytochemicals are growing in interest for drug discovery and research in cancer fields due to promising effects against cancer hallmarks. Moreover, because cancer

develops different approaches to avoid drug effects, it can be challenging to identify a molecule that will not be rendered ineffective following events of genetic redundancy, mutation, or epigenetic modification. These phytochemicals are more robust against cancer's evasion mechanisms due to their broad effects, spurring research into targeting CSCs. Recent studies indicate that particular natural products target programmed cell death mechanisms or embryonic developmental pathways that are cornerstones to CSC survival. Therefore, identification of natural compounds which selectively inhibit the initiation, progression, metastasis of carcinogenesis and elimination of the CSCs at the same time, without cytotoxic effects in normal cells, has become important. In this review, we highlight the data illustrating six natural products that have been successful in targeting CSC populations and phenotypes.

Genistein

Investigation into the role of isoflavones present in soy was prompted by observations that populations with diets rich in soy experienced lower incidence of hormone-driven cancers such as breast and prostate.[217–219] 4,5,7-trihydroxyisoflavone, otherwise known as genistein, is a natural isoflavone phytoestrogen found in soybeans. Genistein has a similar molecular structure to estrogens, and, because it demonstrates weak estrogenic activity, is labeled as a phytoestrogen.[217] Genistein, like other flavonoid glycosides, is relatively hydrophobic and can enter cells without being cleaved, further, it does not have to be hydrolyzed to exert its biological effects. This means that the molecule doesn't have to pass through the acidic environment in the stomach or be processed by

bacterial galactosidase in the gastrointestinal tract, making it an interesting compound for direct-delivery anti-cancer studies.[220,221]

Genistein possesses a wide range of anti-cancer activities.[217] Its role in cell cycle regulation and apoptosis induction are linked to the molecule's ability to inhibit topoisomerase I and II[222], 5α-reductase[223] and protein histidine kinase.[224] Genistein is more effective in cancers carrying *TP53* mutations, those expressing a higher level of Plk1, a serine/threonine mitotic protein kinase, and against models of paclitaxel-resistant prostate cancer vs non-resistant cancer.[225,226] Together, this demonstrates that genistein acts through a mechanism of mitotic disturbance. Genistein also inhibits signaling pathways via disruption of NFκB and Akt[227] and inhibition of protein-tyrosine kinases, specifically epidermal growth factor (EGF) and Src.[228,229] Treatment with genistein, compared to other isoflavones, inhibits growth of gallbladder carcinoma cells and induces arrest at G2/M.[230] Genistein also inhibits the phosphorylation of human epidermal growth factor receptor 2 (HER2) protein, promoting the delay of tumor onset in HER2-overexpressing transgenic mice.[231] Genistein also antagonizes estrogen- and androgen-mediated signaling pathways[232,233], and prevents angiogenesis and metastasis via antioxidant properties[234,235]. Broadly, genistein has been studied in several cancers including leukemia[236], lymphoma[237], ovarian[238], melanoma[239], gastric[240], pancreatic[241,242], breast[232,243], head and neck[244–246], and prostate[233,247].

Similarly to other investigations into non-traditional compounds for cancer therapies, genistein is often studied in conjunction with standard chemotherapeutic agents for sensitivity or reactivity.[217] *In vitro*, genistein co-treatments with cisplatin, docetaxel, doxorubicin, or gemcitabine in prostate, breast, pancreas, and lung cancers[248–250] or CHOP

(cyclophosphamide, doxorubicin, vincristine, prednisone) in lymphoma[251] induce apoptosis. These results are further supported *in vivo* for pancreatic and prostate cancers with gemcitabine and docetaxel co-treatments.[241,252]

Promising outcomes with gemcitabine co-treatment drive investigation into its role in suppressing CSC-signaling pathways. Broadly, gemcitabine targets CSCs in breast[253], prostate[254], lung[255], head and neck[256], gastric[257,258], nasopharyngeal[259], bladder[260], renal[261], liver[262], and ovarian[238] epithelial cancers, and evidence in these studies overwhelmingly points to genistein suppression of the Hedgehog-Gli axis and chemoresistance reduction through suppressed ABCG2 expression and ERK1/2 activity.

Genistein decreases CSCs in MCF7, an epithelial-receptor-positive breast cancer cell line, though interestingly not one with a notably high proportion of CSCs.[253] Treatment decreases growth and proliferation, promotes apoptosis, and downregulates Hedgehog-Gli1 signaling. CSC-directed effects include decreased sphere formation and loss of the CD44+ population. In nude mice, genistein-treated MCF7 xenografts grow less and demonstrate decreased ALDH activity, compared to control treatment.

Prostate CSCs exhibit cell morphologies and gene expression consistent with an EMT phenotype, but, when treated with genistein, EMT features are suppressed. In vitro, genistein decreases tumorsphere-formation and CD44 expression, as well as tumor formation in vivo. Indeed, no tumor development was observed in both the genistein-alone and the combination (docetaxel/genistein) treatment groups. Mice treated with a combination of genistein and docetaxel survived longer than mice treated with genistein alone, indicating that genistein synergizes with docetaxel in this model. Finally, genistein was sufficient to suppress the growth-enhancing effect of Shh on proto-spheres as well as

Gli1 expression and activity. Together, these results indicate that the Hedgehog-Gli pathway contributes to pancreatic CSC maintenance, which can be suppressed using genistein in vitro and in vivo.[254]

Nasopharyngeal carcinoma stem like cells are also sensitive to genistein-regulation of Shh signaling-induced apoptosis.[259] In cell lines established through tumorsphere-forming assay, genistein inhibits sphere formation, decreases the number of EpCAM+ cells, downregulates the expression of nasopharyngeal CSCs markers (CD44, ALDH1, OCT4, Nanog), suppresses cell proliferation (identified via protein-level suppression of cyclin D1, c-MYC, and PCNA), and induces apoptosis (identified via flow cytometry and western blot of Bax and caspases 3,8,9). Further cementing the ability of genistein to target the Shh pathway, expression of SHH, SMO, and GLI1 is suppressed. These effects were mitigated when cells were given external activation of the Shh pathway via Smoothened activator purmorphamine, solidifying genistein's cytotoxic role in nasopharyngeal carcinoma stem like cells via Shh disruption.

In gastric cancer, genistein promotes cytotoxicity in CSCs via interruption of signaling cascades and efflux transporters.[257,258] Yu et al. sorted cells from MKN45, a human gastric cancer cell line, according to CD44 expression.[258] Shh signaling is upregulated in these cells, as are stemness-related proteins: CD44, Oct4, Bmi, Nestin, and ABCG2. When treated with genistein, these molecules are suppressed with downstream effects on cellular migration and sphere-formation. Genistein downregulates Gli1 significantly at the mRNA level. When stemlike cells are treated with shGli or genistein the effects on the CD44+ population of MKN45 cells are similar, indicating that genistein is functioning in gastric cancer by targeting Gli expression. Huang et. al. evaluated changes

in stemness-related phenotypes by focusing on CD44/54/24/90, OCT4, Sox2, and Nanog.[257,263–267] In gastric CSCs, genistein reduces the expression of all these markers. Inhibition of colony formation and tumor sphere formation and reduction in 5-FU and cisplatin resistance are also observed following genistein treatment of two heterogenous gastric cancer cell lines. Genistein-promoted chemosensitivity also drives repression of ABCC1, ABCC5 and ABCG2 expression and ERK 1/2 activity, indicating these as potential effectors of genistein cytotoxicity. When analyzed in mouse xenografts, genistein reduces tumor mass compared to control treatment[256].

Data from renal CSCs also support the ability of genistein to function through Shh inhibition. CSCs, isolated from 786-O and ACHN cells in a tumor sphere formation assay, were subjected to genistein treatment and observed for changes in CSC properties.[261] Genistein suppresses tumor sphere formation, decreases renal CSC markers (CD133, CD44, ALDH1, Oct4, Nanog), inhibits proliferation, and induces apoptosis. When renal CSCs are treated with a SMO inhibitor that suppresses Shh activation, CSC markers are also suppressed, phenocopying genistein. Treatment with genistein attenuates the activation of Shh pathway-associated proteins, unless countered with the SMO activator purmorphamine. In fact, the activator is able to fully counter genistein-induced renal CSC marker repression, as well as proliferation and apoptosis inhibition.

Other cancers provide further insight into the mechanisms by which genistein diminishes CSC proliferation and self-renewal including pathways that involve miRNAs, interleukins, FOXM1, and STAT3. Manganese superoxide dismutase (MnSOD) promotes invasion and migration in lung cancer, primarily through the interaction with FOXM1, which is elevated in lung cancer stem like cells.[255,268,269] When treated with sub-cytotoxic

concentrations of genistein (20 and 40 μM), sphere formation activity and protein expression levels of CD133, CD44, Bmi1 and Nanog decrease, indicating that genistein affects lung CSCs. Identifying whether genistein affects the MnSOD/FOXM1 axis, MnSOD and FOXM1 overexpression antagonizes the effects of 40 μM genistein; however, when knocked down, the inhibitory effects of only 20 μM genistein are enhanced.[255] FOXM1 also plays a role in driving genistein cytotoxicity against liver cancer stem cells.[262] Liver cancer stemlike cells promote favorable microenvironments for and regulate the initiation of hepatic carcinomas, and, by which, produce cytokines that alter the microenvironment. MHCC97H-derived spheres are enriched for CSCs, high in CD133, CD44, and EpCAM proteins, which readily form colonies and promote tumor formation. Genistein treatment reduces FOXM1 expression, abrogates sphere and colony formation, and decreases CD133, CD44, and EpCAM proteins. However, when FOXM1 is overexpressed, genistein loses its efficacy, indicating the role of FOXM1 in genistein's anti-CSC effects.

In models of head and neck cancer (HNC), the miR-34a/RTCB axis promotes sensitivity to genistein in CSC subpopulations. HNC tumor-initiating cells (HNC-TICs) promote self-renewal of the cancer, and decreased patient survival.[256,270] When treated with genistein, ALDH+ and CD44+ patient-derived HNC-TICs lose proliferative and self-renewal ability, coinciding with decreased cell survival. Treatment also suppresses classic EMT phenotypes like migration, invasion, and colony-formation, as well as EMT-associated proteins like Snail, ZEB1, Slug, vimentin, and upregulates E-cadherin. Co-treatment with doxorubicin, cisplatin, and 5-fluorouracil (5-FU) increases cell death. Mechanistically, genistein activity is linked to increased miR-34a expression, which

promotes ROS-mediated apoptosis, and decreases in self-renewal, migration, invasion, and ALDH1 activity (which are correlated to EMT properties). RTCB, a 3'-phosphate RNA ligase that has not been well-characterized and works by catalyzing unconventional RNA splicing during unfolded protein response,[271] is a target of miR-34a, and its miR-34a-driven suppression is a major driver of genistein-related cytotoxicity. Further, the above results are validated in vivo via subcutaneous HNC-TICs xenografts in nude mice.[256]

Bladder and ovarian cancers illustrate the role of interleukins in genistein-driven cytotoxicity. Arsenic induces bladder cancer by driving EMT and increasing CSC levels, as measured by CD44 expression.[272–274] This phenotype is driven by IL-8 overexpression, which is stimulated by HER2 phosphorylation.[260] Treatment with genistein inhibits the phosphorylation of HER2, which results in the downregulation of the downstream signaling pathways, and subsequently the progression of EMT, similarly to prostate and head and neck cancer, and the CD44+ CSC population. CSCs in ovarian cancer are influenced by macrophages in the tumor microenvironment.[238] Macrophages co-cultured with ovarian cancer stem-like cells induce SKOV3 cell stemness via IL-8/STAT3 signaling. Treatment with genistein downregulates ovarian stemness marker CD163 and macrophage-promoted phosphorylation of STAT3. Further, genistein decreases IL-10, increases IL-12 and nitric oxide, and reduces the macrophage- or stemlike cell-driven clonogenic capacity of the stemlike population of CD133/CD44 SKOV3 cells. Interestingly, the effects by genistein treatment are reversed by IL-8 addition or STAT3 overexpression and all these results are recapitulated in vivo.

Overall, the CSC-directed activity of genistein is robustly demonstrated for a host of cancers through its disruption of the cell cycle, signaling and hormone pathways, and

cell death cascades. It targets CSC differentiation and growth pathways including Shh and STAT3, re-activates cell death in resistant populations, and modulates the EMT phenotype. Based on these data, in-progress clinical trials in prostate,[275] colorecal,[276] and breast[277] cancers, identify genistein's ability to regulate metastasis and proliferation. It is likely that genistein will continue to be explored as an anti-cancer therapy and continue to provide promising results.

Curcumin

Known for its role in subcontinental cooking, turmeric (*Curcuma longer*) has been a diet staple for centuries. Turmeric has a long history of being used as medicine in an ancient Indian healing system that dates back over 5,000 years.[278] More than providing a rich, yellow color to foods or healing ailments, turmeric also is the source of the polyphenol curcumin. Chemically it is known as diferuloylmethane ($C_{21}H_{20}O_6$), and has been identified to play a broad role against various oncogenic pathway targets.[279]

However, biologically, this ancient medicine is now investigated for numerous anti-infectious[280], anti-oxidant[281], anti-inflammatory[282,283], liver-protective[284], heart-protective[285], blood clot suppressive[286], anti-arthritic[287], chemopreventive, and anti-carcinogenic[288–290] conditions. Studies in breast and lung cancers explore curcumin's role in multidrug resistance[291], and pancreatic and breast cancers unveil that curcumin mitigates the pro-migratory effects of EMT.[292–294] Numerous pathways are identified as curcumin targets,[295–297] and tumors treated with curcumin regain sensitivity to therapy.[278,298,299] Broadly, curcumin prevents metastasis and progression in multiple cancer types, including pancreatic cancer, breast cancer and chronic myeloid leukemia.[279]

Tamoxifen is one of the leading standard-of-care therapies for breast cancer, however, patients can develop tamoxifen-resistance, often accompanied by EMT-associated disease progression to metastasis. Interestingly, curcumin inhibits proliferation, migration, invasion in tamoxifen-resistant breast cancer cells. These phenotypes are accompanied by a decrease in E-cadherin, increase in N-cadherin, and decrease in long noncoding RNA (lncRNA) H19, which plays a role in activating EMT.[292,293]. Pancreatic cancer studies further the significance of the EMT-curcumin axis.[294] Via curcumin's role in blocking the hedgehog signaling pathway, the effects of EMT reverse. In hepatoma cells, curcumin inhibits TGF-β1-induced EMT by suppressing Smad2 signaling pathway activation,[295] and in another pancreatic cancer study, curcumin inhibits superoxide dismutase-induced migration, invasion and EMT-related gene expression.[300]

Identifying curcumin's role in inducing cell death is a challenging feat that suggests cancer-type specificity. In breast cancer, curcumin is both shown to induce apoptosis as well as paraptosis, a mechanism of cell death described in 2000 and is broadly categorized by its dissimilarity to other cell death phenotypes.[301,302] When applied to breast cancer cells, curcumin inhibits the phosphorylation of proteins downstream of AKT/mTOR signaling, inducing autophagy through activated lysosomal recycling, which leads to apoptosis-induced cell cycle arrest.[297] In an alternate study in breast cancer, curcumin was thoroughly identified to induce neither apoptosis, nor autophagy, but instead paraptosis.[303] When cells are treated with Z-VAD, a pan-caspase inhibitor that blocks apoptosis, along with curcumin or TRAIL, an inducer of apoptosis, only TRAIL signaling is mitigated by the co-treatment, as measured by western blot of caspase-3 and PARP. Overexpressing flag-tagged Bcl-2, Bcl-xL, survivin, and XIAP and observing a block of TRAIL, but not curcumin, via

western blot, further rule out curcumin-induced apoptosis. To understand if curcumin functions via autophagy, selenite, an inducer of autophagy in glioma, was compared to curcumin for effects on LC3. Only selenite increases LC3 fluorescence. When autophagy inhibitors 3-MA (3-methyladenine) or bafilomycin A are added, again, only selenite is affected. One of the key characteristics of paraptosis-induced cell death is vacuolization, which curcumin treatment robustly induces over a time course, observed via transmission electron microscopy. Both mitochondria and the endoplasmic reticulum swell during paraptosis, functioning as the source of vacuoles. Functionally, paraptosis requires protein synthesis and is inhibited with upregulation of AIP-1/Alix. Curcumin effects are abrogated by protein synthesis inhibitor cycloheximide, and by decreased AIP-1/Alix protein expression. Forced overexpression of these proteins attenuate curcumin's effects.

Mitochondrial superoxide drives cell death via curcumin in breast cancer and lung adenocarcinoma. Mitochondrial superoxide is known to induce tumor progression and cisplatin resistance, and curcumin is capable of overcoming this resistance, thus meditating disease progression. [268] Curcumin's drug resistant reversing effect in lung cancer cells is also evident via HIF-1α inhibition and activating caspase-3.[304] In colon cancer, multidrug resistance to vincristine, cisplatin, fluorouracil, and hydroxycamptothecin reduces following curcumin treatment in vitro and in vivo, likely due to suppression of multidrug resistance gene expression and P-glycoprotein.[305] In retinoblastoma, curcumin abrogates the effects of multidrug resistance associated protein 1 via its substrate binding site, thusly inhibiting the transport of the protein and increasing the accumulation of its substrate.[306] Key studies in breast, colon, glioma, esophageal, oral, and lung cancers illuminate a wide array of curcumin-driven anti-CSC effects.

Early studies on curcumin's effect on breast CSCs indicate that the compound could sensitize estrogen-receptor-positive MCF7 cells and TNBC MDA-MB-231 cells to the chemotherapeutic, mitomycin C.[307] Not only did treatment with curcumin increase sensitivity to mitomycin C, but also to paclitaxel, cisplatin, and doxorubicin, even more so in the CSC subpopulation of these cells than in a mixed population. Co-treatment with curcumin and mitomycin C ameliorates breast CSC sphere forming ability by reducing CD44(+)/CD24(-/low) population. Researchers posited that the effect was due to curcumin's function in reducing ABC transporters ABCG2/C1. Application of an ABC-transporter inhibitory molecule, which functions similarly to curcumin, also inhibits CSC properties both in vitro and in xenograft nude mice transplanted with MDA-MB-231 CSCs. Either mitomycin C or curcumin treatment partially reduces tumor burden, but combined therapy reduces tumor burden more than 90% in 30 days. Later studies built on evidence that curcumin improves therapeutic efficacy in breast cancer by introducing novel delivery mechanisms. A specific multi-target drug delivery nanoparticle with CD44-targeting delivers curcumin and salinomycin to breast CSCs as a means to impair EMT.[308]

Both curcumin and salinomycin are hydrophobic, and salinomycin also carries an unfavorable pharmacokinetic profile and cytotoxicity during systemic drug administration.[309] However, curcumin and salinomycin have complementary primary functions of limiting therapeutic resistance and eliciting cellular death, respectively, making co-delivery an attractive pursuit. When delivered together, compared to particles loaded with only one compound, cellular uptake into breast CSCs, drug release, and therapeutic efficacy improves. Co-treatment induces G1 cell cycle arrest and limits EMT, as measured by wound-healing assay, reduces adhesion, downregulates vimentin, and

upregulates E-cadherin. It should be noted that results were particularly improved when the nanoparticles were conjugated with hyaluronic acid, which specifically interacts with the CD44 receptor. Another study also sought to improve delivery and overcome curcumin's hydrophobic nature by increasing controlled release and long-term storage stability. Both breast cancer cells and breast CSCs respond to nanostructure-delivered curcumin defined via a reduction in mammosphere size and number, and curcumin is more readily internalized with this method.[310] Lastly, curcumin overcomes hypoxia-induced chemotherapy resistance, tumor progression, and metastasis.[311] MCF7 breast cancer cells were sorted based on CD44(+)/CD24(-), and were treated with curcumin under hypoxic and normoxic conditions. Under hypoxia, curcumin induces cell cycle arrest in CSCs equally to the normal population; however, in normoxic conditions, curcumin induces a later stage cell cycle arrest in CSCs.[310]

Studies focused on colon cancer CSCs address reactivation of cell death, targeting LGR5+ and CD44+ stem cells, and re-sensitization to chemotherapies. Early studies focused on targeting the CSC-promoting factors in colon cancer targeted the survival-promoting DCLK1+ CSCs[312]. When the broad CSC population was treated with curcumin, expression levels of stem cell markers (DCLK1/ CD44/ ALDHA1/ Lgr5/ Nanog) overall decrease in three-dimensional spheroid cultures and tumor xenografts. However, curcumin unexpectedly induces proliferation and autophagic survival of a subset of DCLK1+ CSCs. Spheres from colon cancer cells are majorly dissociated with curcumin therapy, but remaining DCLK1+ cells regrow within 30-40 days. These cells activate autophagy, suggesting a survival benefit that permits long-term persistence of colorectal cancer. To understand the role of DCLK1 in promoting colorectal cancer, especially after curcumin

treatment, DCLK1 was knocked down via siRNA. With this knockdown, CSCs both in vitro and in vivo initiate apoptotic death, eliminating this population of cells. When the siRNA and curcumin were applied, the co-treatment reduces the CSC phenotype, resulting in decreased sphere and tumor xenograft growth, even more so than curcumin alone. Another well-characterized population of colorectal CSCs is the LGR5+ population, which supports sphere formation and viability.[313] Curcumin-treated LGR5+ tumorsphere formation is inhibited and show decreases in cell viability in a dose-dependent manner, renewed apoptosis and induced autophagy.[313] When autophagy inhibitor hydroxychloroquine is added, curcumin, acting through the autophagy pathway, loses its proliferation inhibition. Other effects include inhibition of the extracellular matrix (ECM)-receptor interaction pathway and downregulation of transcription factor AP-2 alpha (TFAP2A). Interestingly, another article the year prior identifies curcumin as a potential adjuvant treatment drug for colon cancer by targeting CD44.[314] In addition to identifying that curcumin inhibits CSC phenotypes, as supported by multiple studies, real-time PCR and western blot identify the curcumin-induced downregulation of CD44. Observed via flow cytometry, the CD44+ population decreases following curcumin exposure, likely due to the increase in apoptosis in this subpopulation of cells. Interestingly, curcumin's effects are reduced with the addition of siRNA knockdown of CD44, supporting the importance of the CD44 protein as a means for curcumin to target CSCs. This effect is likely unique to the CD44(+) cells due to their increased cellular uptake of curcumin, compared to both a control and CD44(-) population. One of the major challenges to eliminating CSCs is their resistance to standard chemotherapies. 5-FU or 5-FU plus oxaliplatin (FOLFOX) is the backbone of colorectal cancer chemotherapeutics, however it is met with limited success.

In vitro, FOLFOX alone inhibits colon cancer cell growth, but enriches for CSCs.[299] When the remaining population of cells receive curcumin or a combination treatment with FOLFOX, the CSC population is markedly reduced. These cells show decreased expression of CD44, CD166, and epidermal growth factor receptor (EGFR), anchorage independent growth, and sphere formation. In contrast, the surviving population of FOLFOX-treated cells show increases in EGFR and DNA methyltransferase I; however, the opposite occurs when treated with curcumin and/or FOLFOX. Another study also addressed FOLFOX resistance in colon cancer by applying combination dasatinib, a Src inhibitor, and curcumin, but in vivo.[315] Previous work from this group identified that combination therapy in vivo induces 90% regression in spontaneous intestinal adenomas.[316] When treated with either dasatinib and/or curcumin, remnant adenomas show decreases in CSC markers, most markedly in combination therapy, although individual treatments are also significant. When using a FOLFOX-resistant cell line, CSC markers increase, consistent with the similar resistant line from the Yu et al. study. This CSC-marker increase leads to increased invasion and sphere-forming abilities, compared to the parent (not resistant) line. When these cells are treated with combination therapy, interestingly, they act synergistically to inhibit cellular growth, invasion, and sphere formation. This is likely due to the reduction in CSC population. Challenging colorectal CSCs with curcumin is clearly effective, though one group used a curcumin analog to evaluate the role of STAT3 activity in promoting disease progression.[317,318] STAT3 phosphorylation activity in ALDH+/CD133+ colon cancer cells is upregulated; treatment with the analog and curcumin inhibit this phosphorylation, as well as viability and sphere formation . The analog uniquely reduces

STAT3 downstream target expression, induces apoptosis, and suppresses tumor growth in two different xenograft models.

In glioma, defects in autophagy promote CSCs to block differentiation and induce radioresistance.[319] When these CSCs are treated in vitro with curcumin, they differentiate and fail to grow, due to curcumin-promoted autophagy activation.[320] Further, curcumin induces glioma-initiating cell differentiation and self-renewal from resected glioblastoma samples, as evidenced by increases in differentiation markers and decreases in neural stem/progenitor markers. When the ex vivo cells are dissociated and plated post-treatment, they demonstrate loss of sphere forming ability. These effects are reliant on curcumin's ability to activate autophagy. When challenged with 3-MA (which inhibits type III PI3K, an initiator of autophagy), curcumin's effects are abrogated; when given E64d (a lysosomal protease inhibitor which inhibits autophagic flux), curcumin's effects are enhanced. Further, xenografts increase autophagosome formation with curcumin. Intracranial tumors are small and confined with curcumin treatment, compared to no treatment, and curcumin increases survival, decreases toxicity, and increases differentiation markers in the xenografts.

A study in esophageal squamous carcinoma evaluated multiple cell lines to understand how curcumin functions against different backgrounds.[321] Treatment reveals a wide variety of sensitivities, and any cells that survive curcumin treatment show a significant loss in the ALDH1A1+ and CD44+ stem cell populations. In oral carcinoma, EMT is induced by hepatocyte growth factor (HGF) signaling via the HGF receptor c-Met and downstream activation of the pro-survival ERK pathway.[322] Treatment with curcumin inhibits HGF-induced EMT and cell motility in oral carcinoma CSCs via c-Met

blockade.[323] Further, curcumin inhibits the HGF-induced increases of vimentin by downregulating the expression of phosphorylated c-Met, an extracellular receptor tyrosine kinase.

Non-small cell lung cancer (NSCLC) studies unveil curcumin's role in targeting CSCs via the Hippo pathway and sensitization to chemotherapy. Zheng et al. treated co-cultured NSCLC and normal pulmonary epithelial cells with curcumin.[324] The treated CSC population is no longer able to form spheres, and curcumin selectively has no effect on the NSCLC and normal pulmonary epithelial cells. ALDH and stemness markers (CD133, EpCAM, and Oct1) decrease following treatment, and gene set enrichment analysis (GSEA) identifies curcumin's effect on the Hippo pathway. When effectors of this pathway are challenged along with curcumin treatment, the only change was in TAZ. TAZ overexpression abrogates curcumin's effects on stemness, indicating that curcumin affects Hippo specifically through TAZ. These results are different from previous studies showing that the Wnt, JAK2 and Hedgehog pathways are involved in curcumin's effects on lung cancer cell stemness,[325,326] implying that curcumin might have several targets or that high concentrations of curcumin might have off-target effects. In another study conducted by Abdul Satar et al., polyphenols like curcumin were analyzed for their ability to sensitize CSCs to cytotoxic agents such as cisplatin.[327] Sensitivity to cisplatin was determined by evaluating stemness characteristics like the expression of SOX2, NANOG, KLF4, ALDH, and chemoresistance proteins, proliferation, colony formation, and spheroid formation of cells. When treated with curcumin alone, cisplatin alone, or the combination of both at 24, 48, and 72 hours the aforementioned effects all decrease in lung CSCs.

While these studies are promising and led to many clinical trials, the therapeutic potential of curcumin is limited by its rapid metabolization, poor solubility, and inefficient absorption.[328] To overcome this, researchers are turning to curcumin hybrids and derivatives to increase potency, bioavailability, and half-life.[297] Allylated monocarbonyl analogues and enone analogues of curcumin promote mitotic arrest and apoptosis by reactive oxygen species-mediated stress[329,330]. Novel curcumin analogues exhibit high potency in hormone-resistant prostate cancer[331] and nasopharyngeal carcinoma.[332] In breast cancer, novel analogs are especially promising.[333–335] In one study, TNBC cells were treated with a molecule built with pharmacophores from both curcumin and chromone, a controller drug normally used as therapy as an alternative to glucocorticoids and here used to increase bioavailability.[335] This novel molecule decreases cell survival and colony formation, increases mitochondrial reactive oxygen species, and decreases EMT. When co-treated with paclitaxel, the treatment synergistically induces anti-proliferative action in TNBC cells.

Another synthetic analog of curcumin, called C1, is delivered via lipid nanoparticles.[336] This delivery aids in sustained release and is more effective than external treatment. Effects include depolymerized microtubules, generation of ROS, DNA damage, apoptosis, and 3D sphere-inhibition better than unencapsulated drug. In vivo, C1-containing nanoparticles suppress MCF7 mammosphere growth. Dimethoxycurcumin (DMC), a methylated and more stable analog of curcumin, is significantly more potent than curcumin in inducing cell death and reducing the clonogenicity of malignant breast cancer cells.[337] DMC reduces the tumor growth of xenografted breast MDA-MB 435S cells more strongly than curcumin and also induces paraptotic cell death. DMC-induced paraptosis

increases dilation of mitochondria and the endoplasmic reticulum and inhibits proteasomal activity, at a lower concentration than curcumin natural product.

The potential of curcumin as a therapy against cancer, inflammation, and bacterial infections is now well established. Modified versions of the molecule have overcome therapeutic delivery and efficacy challenges and have been used to treat a plethora of human diseases.[338] Overall, experimental data suggests curcumin efficacy and capability to eradicate CSCs in cancers, but rising concern for the safety and associated adverse effects of direct delivery has presented another setback for curcumin clinical use. Additional pre-clinical, animal model-based studies will enhance comprehension of the safe clinical use of curcumin. Likewise, studies to reduce the toxicity of nanoparticles should be encouraged. Further, the potential of different curcumin derivatives can also be tested for their influence on the viability of CSCs.

Ophiobolin A

Ophiobolins are a wide category of natural molecules, unified by their 25-carbon structures and phytotoxic activity. These secondary metabolites are broadly produced by fungi in the genera *Aspergillus, Bipolaris, Cephalosporium, Cochliobolus,* and *Drechslera*.[339] There are nearly 60 identified biogenic analogs of ophiobolins and studies predominantly focus on their bactericidal, fungicidal, nematocidal, and anti-calmodulin effects.[61,340–344]

In 1965, an ophiobolin was first characterized as a 25-carbon, five-membered ring compound, containing a hydroxyl, an α/β-unsaturated carbonyl ring, and a ketone.[345–347] Named ophiobolin A (OpA), it was another near-twenty years until the effects of this

specific molecule were described. In the interim, research focused on the chemistry of OpA and its sister molecules.

Initial publications on OpA followed its history of phytotoxicity, due in part to two significant plant disease epidemics.[348,349] It was found that OpA reduces potassium permeability through the interruption of transmembrane electrical potential and proton extrusion in maize.[69] Further, calmodulin, a calcium-binding messenger in eukaryotic cells, is an irreversible target of OpA, explaining *in situ* ion leakage and a decrease in glucose uptake in both bovine brain cells and maize cells.[350] Interestingly, there was one study amongst these early pursuits that evaluated OpA in animal cells following the finding that OpA impacts glucose uptake.[351] Ultimately, researchers found that OpA does not affect basal rate of glucose uptake in this model, but does work with insulin to depress stimulation of the system. Numerous studies identify the broad effects of OpA when applied to plants, though the exact mechanisms were left undescribed for decades. Alongside these plant studies were attempts to understand OpA in animals, finding that OpA is also toxic in animal models.[352] This work, along with others, established a protocol for OpA in animal models, and, combined with the molecule's diverse effects in plants, opened the door for research into OpA's anti-cancer properties.

OpA is found to function in a wide array of phenotypes from mobility inhibition in porcine sperm[65], to enhanced responses in mammalian cells compared to plant cells[353], to continuation of OpA as a phytotoxin.[354,355] The earliest mention of OpA in anti-cancer studies identified highly acute cytotoxicity against cell lines from human lung and colon carcinomas and melanoma, as well as in murine lymphoma.[356] The National Cancer Institute investigated OpA against the 60-cell line panel resulting in 50% growth inhibition

ranging from 16 to 400nM.[339] Glioblastoma became an early focus for OpA, since it faces severe therapeutic challenges due to difficulty of resection and high rates of chemoresistance, especially to apoptosis-inducing therapies.[357] In glioblastoma, OpA induces paraptosis, by inhibition of the calcium-activated potassium channel, BKCa (big conductance Ca2+-activated K+ channel).[62] The BKCa channels are found in the membranes of the mitochondria and endoplasmic reticulum, two organelles highly associated with paraptosis[301,302]. Further, OpA treatment alters the morphology of glioblastoma cells, which inhibits migration and is independent of necrosis and apoptosis-related mechanisms, as identified by Annexin V/PI staining and protein analysis of PARP cleavage. The authors describe a proposed mechanism by which OpA suppression of BKCa drives paraptosis: the closing of the channel obstructs the movement of K+ ions, which drives water to enter the cell in an attempt to maintain homeostasis, which then promotes vacuolization.

A few years later, these data were followed up in an *in vivo* model of glioblastoma.[64] OpA was delivered intraperitoneally at 10 mg/kg three times per week for a total of 21 days. Not only do the mice receiving OpA survive longer, but their tumor growth slows. One of the main questions this study sought to answer was if OpA was able to cross the blood-brain barrier, one of the most significant challenges to establishing effective glioblastoma therapies. When OpA was injected intravenously into the lateral tail vein of a tumor bearing mouse, it is detected 15 minutes post-administration in a brain tumor extract. The most recent glioblastoma study proposes OpA's mechanism of action through endoplasmic reticulum stress via disruption of thiol proteostasis.[68] Like prior sturdies, paraptosis is observed through dilation of the endoplasmic reticulum, vacuolization, and

non-apoptotic cell death. When the mechanism of cell death is further investigated, OpA's activity is independent of reactive oxygen species, but a high dose is effectively blocked by thiol antioxidants.

OpA is also studied in renal, breast, and cervical epithelial cancers. In renal cancer, cells were challenged with different calmodulin inhibitors such as, W-13 (40 μM), calmidazolium (20 μM), and ophiobolin A (5 μM) to block FOXO1a export, which is a downstream effector of the PTEN signal transduction pathway.[358] This pathway, along with the PI3K and Akt axes, controls aberrant cell growth and promotes several types of cancer, and is also modulated through calmodulin regulation of FOXO1a cellular localization.[359–363] When calmodulin is inhibited with OpA, FOXO1a re-localizes to the nucleus.[358] Huang and Chen also evaluated OpA in renal cancer, investigating its interaction with the ROS-scavenger, NAC (N-acetylsysteine).[364] OpA induces apoptosis, inhibits growth, and promotes ROS accumulation; further, treatment increases the expression of cytochrome c and cleaved caspase 3, and decreases Bcl-2 expression. When co-treated with NAC ROS are eliminated, OpA loses both its anti-proliferative activity and apoptosis-inducing effects. In 2016, a study focused on oncogenic signaling pathways in breast cancer identified OpA as an inhibitor of phosphorylation in PI3K/mTOR, Ras/Raf/ERK and CDK/RB pathways.[63] OpA induces apoptosis and cell cycle arrest, which decrease in parallel with ribosomal proteins, extracellular signal-regulated kinases (ERK) and retinoblastoma protein phosphorylation. Two significant studies more deeply evaluate OpA against a host of cancer cell lines. The findings demonstrate that OpA is more effective in mammal (IC_{50} <1 μM) cells than plant (IC_{50} ≥10 μM), following testing on eight cancer cell lines with a variety of multidrug resistance phenotypes.[353] Interestingly, OpA displays similar

inhibition concentration values on the parental lines as on the non-multidrug resistant cell lines, highlighting its potential usefulness against CSC-enriched cancers. A more recent panel of cells were also tested including cancer cell lines from breast (MCF7 and MDA-MB-231), glioma (U-87 MG), cervix (HeLa), and HeLa-derived epidermoid (KB 3-1).[71] There is little difference in the size of the cells after 24 hour-treatment at the lower concentration; however, treatment with 10 μM OpA shows a highly statistically significant change in size for several lines. Breast cancer cells decreased in size, and cervical cells decreased. This finding prompted the evaluation of necrotic and apoptotic cell death, as assayed by AV/PI staining. Of the epithelial cancers, only the TNBC MDA-MB-231 cells show evidence of early apoptosis. Other factors studied include mitochondrial area per cell, mitochondrial length, mitochondrial bifurcations, mitochondrial membrane depolarization, ROS generation, ER stress response, and intercellular calcium concentration. The mechanism, concentration, and duration of treatment on each cell line impact the type of cell death activated by OpA. Overall, as with other OpA studies, many of the aforementioned findings are supported by similar results in non-epithelial cancers.[70,72,365,366]

OpA's broad record of research has identified several potential target proteins, especially as means to treat cancer. As early as 1984 and 1985, Leung et. al. defined 9 μM OpA (4 μM at longer incubation) as an effective inhibitor of the calmodulin-dependent cyclic nucleotide phosphodiesterase, both on bovine brain calmodulin and in plants, such as maize. This effect is irreversible, time-dependent and calcium-dependent.[350,367] Supporting these data, studies find that OpA inhibits calmodulin by interacting with its lysine residues, resulting in poor activation of phosphodiesterase.[67,344] Multiple studies

have since utilized OpA for its inhibition of calmodulin, though in vitro growth inhibitory effects of OpA do not correlate with calmodulin mRNA expression level in the NCI 60-cell line panel.[71] Masi et. al. offers an explanation for these cellular effects as being related to the two different mechanisms of chemical reactivity to calmodulin.[339] To more effectively study small-molecule inhibitors, Manoharan et. al. establishes a cell lysate-based FRET-assay to detect residue-specific, covalent calmodulin inhibitors, such as OpA.[368] This assay confirms OpA reactivity with lysines 75, 77 and 148 on calmodulin, which also corroborates findings that K-Ras-directed activity of OpA depends on calmodulin.[66,71] Reports also identify KCNMA1, the gene that encodes the α-subunit of the large conductance, voltage and Ca2+-activated potassium (BKCa) channel as a target of OpA[62]. Due to the influence on cell cycle progression and cell migration when BKCa is defective, this channel is relevant to cancer research.[369,370]

One of the most challenging phenotypes of CSCs is their resistance to standard therapies. Ivermectin, which exhibits anti-cancer activities, was originally used to modulate invertebrate-specific glutamate-gated chloride channels.[343,371] Interestingly, ophiobolin M was co-treated with ivermectin for its nematocidal activity.[343] It was twenty years later when OpA was studied in CSCs. K-ras signaling, targeted through OpA inactivation of calmodulin, is important in breast cancer cells[66]. A novel nanoclustering-FRET assay differentially screens chemical libraries with K- and H-ras-derived FRET biosensors to identify new candidate CSC inhibitors, including OpA. OpA treatment on multiple cell lines decreases the proportion of CD44(+)/CD24(-) breast cancer CSCs at 200 nM as well as mammosphere formation. Our work has also identified OpA as selective for breast CSCs, specifically through its activity towards cells that have undergone EMT.[372]

We show that sub-cytotoxic doses of OpA (30 and 100nM) reduce mammosphere formation of MDA-MB-231 cells, and 100nM OpA reduces the prevalence of CD44(+)/CD24(-) breast cancer CSCs. Further, we demonstrate that OpA increases sensitivity to paclitaxel and doxorubicin, standard chemotherapies for breast cancer. Clearly, though, there is much work that can be done to further elucidate the role of OpA in mitigating disease burden from CSCs.

One of the great challenges to success thus far has been that OpA suffers from rapid clearance in vivo, requiring high rates of application, and limited duration toxicity, due to slow action or difficulty reaching the target. In a plant model, OpA delivered in nanoparticles increases stability, modulates release kinetics, and improves targeted application.[355] The research in plant systems reveal OpA's effects on membrane permeability and potential, ion flux, and even cell mobility. In the cancer field, OpA is thoroughly studied in glioblastoma, compared to other cancers, but recent studies focused on breast cancer stem cells are likely to drive promising results into further research. While there is little consensus on the mechanism of cell death OpA induces, if only to agree that it is highly variable, further work into understanding how and why different mechanisms are activated in different models, under different treatment schemes, or even within different sub-populations of cancer cells will help uncover the complexity of this emerging natural product.

Salinomycin

Discovered in 1973 from *Streptomyces albus*, monocarboxylic polyether ionophore salinomycin, was originally used as an anticoccidial drug for poultry. More recently, the

natural compound is linked to reduction of CSC populations in a variety of cancers. A 16,000-compound screen identified salinomycin as being able to inhibit growth of human breast cancer and decrease metastasis.[373–375] Namely, salinomycin reduces the number of cancer stem cells in breast cancer models, especially when compared to common chemotherapeutics, such as paclitaxel.[373,374] These early studies opened the door for further exploration into the compound's activity against CSC phenotypes such as tumor initiation and chemo-driven cell death resistance.

The presence of CSCs is linked to tumor formation and metastasis, which can be modeled in vitro using tumorosphere growth in non-adherent conditions and migration assays. Salinomycin abrogates the ability of breast, colorectal, gastric, lung, osteosarcoma, pancreatic, prostate, and squamous cell CSCs via the reduction in capability to form spheres.[309,376–380] These results could be related to increased sensitivity to anoikis following salinomycin treatment and reinstating a reliance on anchorage to the extracellular matrix.[381] Matrix metallopeptidases (MMPs) are associated with degradation of most extracellular matrix proteins during organogenesis, growth and normal tissue turnover, but are downregulated following treatment with salinomycin, decreasing the ability of cells to escape the primary tumor.[381] Further, treated cells express reduced oncogenes, induce oxidative stress, and/or a reduction in CSC fractions.[376,379] The change in these phenotypes is driven by salinomycin-induced cell death activation, which is especially helpful against cell-death resistant CSCs. The use of salinomycin triggers an increase in pro-apoptotic genes in cisplatin-resistant colorectal cancer cells[382], apoptosis activation in human hepatocellular carcinoma cells[383], and autophagy response in prostate and breast cancer cells. [384,385] The stimulation of programmed cell death by salinomycin is driven by

increased activity of caspases and PARP cleavage, and inhibition of STAT3 cleavage.[381] Together, these data demonstrate the role of salinomycin in limiting CSC-promoted disease progression.

The exact mechanism driving salinomycin cytotoxicity is unknown; however, pathways associated with conferring stemness- and CSC-related phenotypes are demonstrated to correlate with treatment. In colorectal cancer cells, salinomycin decreases the activity of the Wnt signaling cascade[386], Akt signaling pathway, Wnt/β-catenin, Hedgehog, and Notch pathways.[384,387] Salinomycin also inhibits lung and prostate Oct4-, Nanog-, and Sox2-expressing CSCs .[384,387,388]

More so than just targeting CSC-related pathways, improvements to salinomycin delivery have also yielded promising results. Polymeric micelles contain a hydrophobic core that can carry hydrophobic compounds, like salinomycin, and their hydrophilic exteriors more readily cross cell membranes, increase the micelle's stability, and protect the structure from rapid clearance.[389] This mechanism of delivery circumvents the stress of in vivo delivery with ethanol, which was the only way to overcome the drug's poor aqueous solubility.[373] Breast cancer CSCs treated with salinomycin-loaded micelles are more sensitive to treatment than heterogenous breast cancer cells.[390] Further, salinomycin-containing micelles selectively eliminate CSCs from the heterogeneous population of breast cancer cells, thus decreasing proliferation overall, and increase sensitivity to therapy with paclitaxel, both free and loaded into micelles. The purpose of micelle use is to improve in vivo use and decrease toxicity associated with required stabilizing loading solutions, which treatment of xenografts demonstrate. In vivo treatment with salinomycin-loaded micelles is only improved when delivered in combination with paclitaxel, documented via

decreased tumor size and weight after nearly three weeks post-cell injection. Potentially most interestingly, this delivery mechanism also reduced the percentage of CD44(+)/CD24(-) CSCs from the bulk tumor population better than free salinomycin.

Salinomycin was one of the first molecules to gain traction in the conversation about natural molecules targeting CSCs. It has been well characterized with effects ranging from reduction of CSC-related properties like tumor growth and chemosensitivity, to induction of cell death in CSC populations, to targeting CSC-reliant stemness pathways. These understandings are helpful in understanding why salinomycin is efficacious against CSCs and expands foundational understanding for CSC-targeted approaches, especially in other natural products.

Ivermectin

Research into ivermectin follows closely to that of salinomycin in that both are repurposed drugs, evaluated for their antitumor effects. Isolated from *Streptomyces avermitilis*, ivermectin is a polycyclic lactone member of the avermectin family[391].

Ivermectin, approved by the FDA for use in human medicine against onchocerciasis, known as river blindness[392,393], is well-tolerated and stable, with a similar chemistry to salinomycin. Studies in melanoma were the first to explore ivermectin as an anti-cancer therapy.[394,395]. To date, ivermectin has been further studied in breast[396,397], ovarian[398], neuroblastoma[399,400], and renal cell carcinoma cell lines,[401] and in immune deficient mice harboring human acute myeloblastic leukemia[402], glioblastoma[403], breast[404], glioma[405], and colon[406] carcinomas.

Functionally, ivermectin has broad activity including apoptosis in leukemia via the induction of mitochondrial dysfunction and oxidative stress[402,403], inactivation of p21-activated autophagy[404] increased sensitivity to tumor metastasis-promoting P2X4/P2X7 in breast cancer[407], and interactions with multidrug resistance protein[408], the Akt/mTOR and WNT-TCF pathways[126,403,406], the purinergic receptors[407,409], PAK-1 protein[398,404], certain cancer-related epigenetic deregulators such as SIN3A and SIN3B[397], RNA helicase[405], chloride channel receptors.[402] Clearly, ivermectin is well understood in general cancer settings, and it recently has become of interest in anti-CSC studies, with the initial few studies showing promising results.

In 2018, Dominguez-Gomez et al. analyzed ivermectin against breast CSCs after a screen identified the molecule as similar to salinomycin, a molecule which had been well-studied in cancer stem cells at this point.[373,396] Researchers used breast cancer cell line MDA-MB-231, which has a rich subpopulation of $CD44^{+}/CD24^{-}$ CSCs able to form spheres in culture. Treatment with ivermectin demonstrates differential sensitivity to the stem cell-enriched populations compared to total population. Further, ivermectin treatment decreases the capacity of these cells to form spheres. Molecularly, researchers identify decreased expression of Nanog, Sox-2, and Oct-4 at the mRNA and protein levels, indicative of decreases in stemness overall. Similar studies evaluate ivermectin's role against breast CSCs via inhibition of the SIN3-PAH2 interaction domain with MAD[397], and in PAK1/Stat3 complex regulation of *IL-6* transcription.[410]

Ivermectin also exhibits anti-CSC activity in high-grade gliomas, which are commonly resistant to chemo- and radiotherapies and experience high relapse rates and tumor recurrence, consistent with CSC phenotypes.[411,412] Its role in mitochondrial

disruption may be the key factor driving activity against glioma CSCs, which are notably reliant on oxidative phosphorylation, especially compared to glycolysis, which many therapies target.[411,413,414] Further, ivermectin, while indirectly through p-glycoprotein efflux transporters, penetrates the blood-brain barrier, making it attractive for glioma-targeted therapies.[408,415–417] In colon cancer, ivermectin downregulated the expression of intestinal stem cell genes *ASCL2* and *LGR5*[418–420] and antagonizes primary sphere formation in 2D colon cancer cell lines.[406]

Multiple studies identify ivermectin as a co-treatment to improve conventional chemotherapies. Ivermectin improves treatment with cisplatin, paclitaxel, daunorubicin and cytarabine, dasatinib, and dapafenibA.[402,421–424] Because ivermectin is able to interact with so many different drugs, each with unique mechanisms of action, and in multiple different cancers, it carries potential for synergistic effects, though these have not been robustly identified. These promising co-treatments prompted a phase I pharmacokinetic study, which demonstrated that ivermectin was tolerable up to 2mg/kg in humans, but no other clinical studies have been done to progress ivermectin as an anti-cancer therapy.[425] Ivermectin is known to have multiple targets and interact with many different therapies, suggesting it may be difficult to produce resistance and, thusly, a valuable molecule to pursue in the effort to target CSC-rich, chemotherapeutically-resistant cancers.

Chloroquine

Outside the cancer field, chloroquine is best known as an antimalarial agent. It works by inhibiting lysosomal acidification, which prevents autophagy via repression of autophagosome fusion and degradation. Typically, autophagy is activated during times of

stress associated with starvation, growth factor deprivation, or need for increased energy. In cancer, this programmed cell death response is thought to act as a survival pathway, leading to increased interest in determining mechanisms that inhibit autophagy in cancer.[426] Now, chloroquine is one of the most widely used drugs to inhibit autophagy *in vitro*, and is the only accepted mechanism of autophagy inhibition for clinical use, resulting in more than twenty clinical trials currently underway.[427,428]

Autophagy occurs via the formation of the autophagosome, a double-membrane-bound structure that delivers cytoplasmic contents to the lysosomes. Lysosomes then bind to the outer membrane of the autophagosome and digest the internal structures, releasing amino acids, sugars, fatty acids, nucleotides, and nucleosides. These then enter the cytosol and can re-enter cellular circuits. Chloroquine inhibits this process during the final stage by preventing the autophagosome binding with the lysosome.

Following the emerging trend of drug reuse/repurposing, chloroquine is well studied as a cancer therapy. In highly tumorigenic and metastatic breast cancer cell lines, chloroquine initiates cell death independently of autophagy, which is a characteristic repeated in several types of cancer.[427] When autophagy is inhibited via shRNA against autophagy-related proteins, chloroquine retains efficacy.[427] Chloroquine is also readily studied as a co-treatment and demonstrates the most cytotoxicity when used with compounds that inhibit PI3K or mTOR, compared to standard DNA-damaging therapies.[427] In TNBC, results describe the role of the Jak2-STAT3 signaling pathway and DNMT1 expression in chloroquine-activated cell death.[429] As another example in breast cancer, chloroquine treatment inhibits potassium currents through KV10.1, a voltage-gated potassium channel, which ultimately decreases migration.[430] In hepatoblastoma,

chloroquine treatment decreases proliferative cells and overall viability in both 2D and 3D culture, induces apoptosis, decreases NAD+ and aspartate, increases NAD+ oxidation shifts to NADH, and downregulates PARP1/2.[431]

Pancreatic ductal adenocarcinoma CSCs decrease *in vitro* following chloroquine treatment, but not due to chloroquine's inhibition of autophagy.[432] Chloroquine-treated pancreatic ductal adenocarcinoma tumors *in vivo* similarly lose tumorigenicity and invasiveness, especially with gemcitabine co-treatment where established tumors are eliminated entirely. Key stemness players in this cancer type are inhibited by chloroquine. Hedgehog decreases in CSCs only, resulting in migration differences between the dedifferentiated and differentiated populations. Pancreatic adenocarcinomas are highly reliant on CXCR4 (C-X-C chemokine receptor 4) signaling, which drives the metastatic spread of CSCs. CXCR4 also only responds to stimulation from CXCL12 (stromal-derived factor-1), and both are inhibited following chloroquine treatment, further deriving ERK and STAT3 phosphorylation[432]. Esophageal squamous cell carcinoma CSCs are also reliant on CXCR4, and, like in pancreatic ductal adenocarcinoma, chloroquine targets this signaling via the STAT3 pathways. This effect is also observed to be independent of autophagy.[433]

Chloroquine, in conjunction with standard therapy, has been tested against glioblastoma multiforme in multiple randomized controlled trials.[434,435]. Only in cases where patients were under the age of 60 did survival improve with the addition of chloroquine to therapeutic regimens.[434] To understand the mechanism of chloroquine in brain cancer, another research team evaluated combination treatment with chloroquine and paclitaxel with nanoparticle delivery to cross the blood-brain barrier.[436] In this case,

autophagy plays a significant role in affecting stemness-associated genes (SOX2, POU5F1 and NANOG) because cells treated with paclitaxel alone increase expression of these genes, but this affect is abrogated with the addition of chloroquine. Further, apoptosis is induced in combination therapy more than with paclitaxel alone, suggesting that chloroquine may play different roles in activating cell death depending on the target or co-treatment.[436]

Chloroquine treatment in breast cancers successfully decreases stemness, though the mechanism still seems to be loosely defined. One frequently-cited study in TNBC implies that autophagy is the main driver, though researchers were not able to detect even basal autophagy and relied on relatively minimal autophagosome presence in TEM imaging, LC3B immunofluorescence, and marginal increases in p62.[429] When the authors specifically evaluated the CD44(+)/ CD24(−/low) CSC population in breast cancer cells, chloroquine sensitized cells to paclitaxel through inhibition of autophagy in both preclinical and clinical settings. This is supported by a reduction in ALDH activity and in primary and secondary mammosphere formation.[429,437] Further, combination treatment with paclitaxel reduces CD44(+)/ CD24(−/low) CSCs in patients with drug-resistant recurrent tumors. The proposed mechanism implicates Jak2 and DNA methyltransferase 1, which are both critical for maintenance of breast CSCs.[438] However, these data could be muddied by paclitaxel playing a strong role in phosphorylating STAT3 and the similarity in expression in both the heterogeneous population and the CSC-only population.[429] Another study, published around the same time, evaluated JIMT-1 epithelial breast cancer cells bearing a population of CD44(+)/ CD24(−/low) CSCs.[437] Here, experimental suppression of autophagy decreases the CSC population, enhances CD24 transcription, and

decreases vimentin. These findings are repeated in MDA-MB-231 cells, a cell line with notably higher CSC proportions. However, it is important to recognize that these data follow the logic of chloroquine-driven autophagy, but researchers do not directly use chloroquine treatment, instead relying on its published downstream effects to draw similarities to chloroquine treatment and induced autophagy inhibition.[437]

Autophagy has also been described to inhibit stem cell phenotypes in colon cancer[312,439], urinary bladder cancer[440–442], and prostate cancer[443]. Further in-depth information about the role of autophagy and how natural products are used to target cancer stem cells using this specific mechanism of cell death can be found in this review[444].

Current thinking posits that chloroquine's activity is so highly specific and effective at eliminating CSCs, that the larger, differentiated population of cells can remain in the tumor and still be therapeutically problematic. Interestingly, in the aforementioned CSC-focused work, the role of chloroquine in mediating autophagy is highly varied. In some cases, autophagy is found to not be the driver of the observed effects, with chloroquine being able to maintain efficacy in the presence of autophagy blockers. In line with the challenge of determining the role of autophagy in CSCs overall, chloroquine's role in promoting CSCs remains obtuse. Further work is needed to understand the mechanisms and scenarios in which autophagy inhibition or activation becomes beneficial in anti-CSC therapies.

CHAPTER THREE

Epithelial-Mesenchymal Transition Sensitizes Breast Cancer Cells to Cell Death Via the Fungus-Derived Sesterterpenoid Ophiobolin A

This chapter published as: Reisenauer, K. N., Tao, Y., Das, P., Song, S., Svatek, H., Patel, S. D., Mikhail, S., Ingros, A., Sheesley, P., Masi, M., Boari, A., Evidente, A., Kornienko, A., Romo, D., & Taube, J. (2021). Epithelial-mesenchymal transition sensitizes breast cancer cells to cell death via the fungus-derived sesterterpenoid ophiobolin A. Scientific Reports, 11(1), 10652. https://doi.org/10.1038/s41598-021-89923-9

Abstract

The epithelial-mesenchymal transition (EMT) imparts properties of cancer stem-like cells, including resistance to frequently used chemotherapy, necessitating the identification of molecules that induce cell death specifically in stem-like cells with EMT properties. Herein, we demonstrate that breast cancer cells enriched for EMT features are more sensitive to cytotoxicity induced by ophiobolin A (OpA), a sesterterpenoid natural product. Using a model of experimentally induced EMT in human mammary epithelial (HMLE) cells, we show that EMT is both necessary and sufficient for OpA sensitivity. Moreover, prolonged, sub-cytotoxic exposure to OpA is sufficient to suppress EMT-imparted CSC features including sphere formation and resistance to doxorubicin. Growth of an orthotopically grown, CSC-rich mammary cell tumor, is suppressed by OpA treatment. These data identify a driver of EMT-driven cytotoxicity with significant potential for use either in combination with standard chemotherapy or for tumors enriched for EMT features.

Introduction

Breast cancer patients who have triple-negative breast cancer (TNBC) face poor prognoses driven by high rates of metastasis and early recurrence [1-6]. TNBC is characterized as histologically negative for estrogen receptor (ER), progesterone receptor (PR), and amplified human epidermal growth factor receptor-2 (HER2), preventing the use of hormone- or HER2-targeted therapies. Instead, treatment with anthracycline (doxorubicin) and/or taxanes is capable of providing 5-year survival in about half of TNBC patients [7-10].

TNBC is comprised of mostly basal-like and claudin-low intrinsic subtypes, both of which have been characterized as enriched with cancer stem-like cells [11-13]. Cancer stem-like cells (CSCs) are defined by their ability to re-initiate tumor growth upon transplantation and are hypothesized to fuel metastasis and primary tumor recurrence, resulting in an overall decrease in survival [14-17]. To improve TNBC patient outcomes, novel and specific approaches targeted at CSCs are needed.

One proposed mechanism driving the emergence of CSC-like cells is the epithelial-mesenchymal transition (EMT) [18,19]. EMT is a trans-differentiation process characterized by acquisition of a spindle-like morphology, loss of apical-basal polarity, increased motility, and a tolerance to anoikis. These phenotypic shifts are driven by gene expression changes mediated by transcription factors SNAIL (SNAI1), TWIST (TWIST1), and ZEB1, effects of which include upregulation of vimentin and downregulation of epithelial markers E-cadherin (CDH1) and miR-200c [20-26].

Cells that have undergone an EMT typically acquire CSC properties including decreased sensitivity to conventional chemotherapies used to treat TNBC. This chemoresistance is driven by drug efflux pumps, enhanced DNA repair capacity, and

epigenetic changes [16,27-32]. There are currently no approved therapies that specifically target CSCs. A leading pre-clinical compound is salinomycin, reported to decrease the sub-population of CSCs, tumor initiating capability, and chemoresistance, with negligible side effects [33]. Other naturally occurring compounds such as curcumin, and quercetin have been reported to reduce the effects of EMT by inhibiting key proteins associated with migration (SNAIL, MMP-2/9), anoikis tolerance (BCL2), cell-to-cell adhesion (N-cadherin), and signaling cascades (JAK/STAT, ERK) [34-37]. This breadth illustrates the potential for applying natural products to persistent issues in oncology.

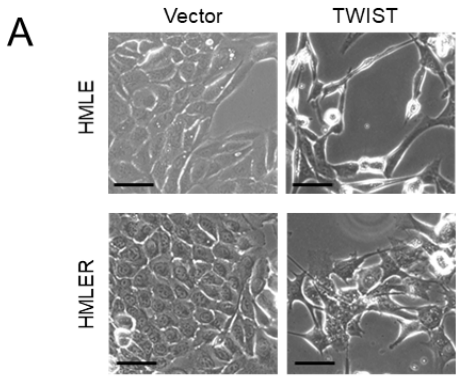
Ophiobolin A (OpA) is a natural product produced from fungi in the genera Aspergillus, Bipolaris, Cephalosporium, Cochliobolus, and Drechslera [38]. This sesterterpenoid (25-carbons) is a secondary metabolite that has long been studied for its phytotoxic effects in a variety of plants and has begun to be evaluated as a cytotoxic compound [38]. Published cell culture-based experiments describe a role for OpA in motility inhibition [39], membrane depolarization [40-43], roles in inflammation 44, and reduction in stemness [45]. In vivo data demonstrate that OpA is tolerated in mice and is effective against an orthotopic model of glioblastoma [40,46,47]. Herein, we investigated the applicability of OpA on EMT-enriched breast cancer and found that experimentally induced EMT enhances the susceptibility of mammary epithelial cells to OpA-induced cell death. Furthermore, breast cancer cell lines treated with OpA experience loss of EMT-associated stemness attributes, demonstrating that OpA induces selective cytotoxicity in cells that have undergone EMT. Additionally, OpA is effective in reducing tumor burden in mice with orthotopic, EMT-positive, mammary tumors, highlighting the potential of EMT-targeted cancer treatment.

Results

Mammary Epithelial Cells That Have Undergone EMT Are More Sensitive to OpA

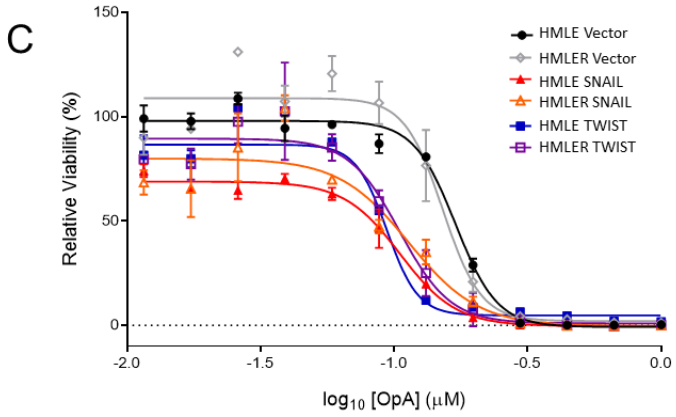
Given the previously published link between OpA and CSC-targeted activity [45], we investigated a potential link between OpA and EMT using an experimental model of EMT induction. Immortalized human mammary epithelial (HMLE) cells have an epithelial morphology (Fig. 3.1A) and express E-cadherin (Fig. 3.2A). We used HMLE cells, as well as HMLE cells transformed with the Ras oncoprotein (HMLER) that are induced to undergo EMT through lentiviral transduction of viruses driving expression of the EMT-inducing transcription factor (TF) TWIST, [26,48] resulting in the acquisition of a mesenchymal morphology (Fig. 3.1A) and protein expression (Fig. 3.2A). TWIST expression also induces stemness properties including a greater prevalence of cells expressing high levels of CD44 and low levels of CD24 ($CD44^{hi}/CD24^{lo}$) (Fig. 3.2B) and an increased sphere formation efficiency (Fig. 3.2C) [16,26]. To identify EMT-selective, highly active molecules, we measured the level of TWIST-induced sensitivity to molecules shown to inhibit CSC properties including salinomycin [49], ophiobolin A [45], curcumin [50], genistein [51], and disulfiram [52]. Only two such molecules demonstrated selectivity towards EMT-positive cells, salinomycin and OpA, and only OpA also demonstrated consistently sub-micromolar cytotoxic activity (Fig. 3.1B). Furthermore, induction of EMT through expression of TWIST or through another EMT-TF, SNAIL [53], in either HMLE or HMLER cells increased sensitivity to OpA-driven cytotoxicity (Fig. 3.1C). Indeed, EMT decreased the IC_{50} value from a mean of 137-147 nM for epithelial cells to a mean of 85-91 nM for mesenchymal cells (Fig. 3.1D). These results stand in stark contrast to EMT-driven

resistance to many commonly used chemotherapeutic drugs including doxorubicin and STS (Fig. 3.2D).



B

IC50 (μM)	HMLER				Selectivity Index
	Vector		TWIST		
	Mean	S.D. (+/-)	Mean	S.D. (+/-)	
Salinomycin	2.76	1.19	0.709	0.22	3.9
Ophiobolin A	0.138	0.01	0.091	0.005	1.5
Curcumin	0.075	0.009	0.099	0.02	0.8
Genistein	0.011	0.003	0.018	0.003	0.6
Disulfiram	>10.0	n.d.	>10.0	n.d.	n.a.



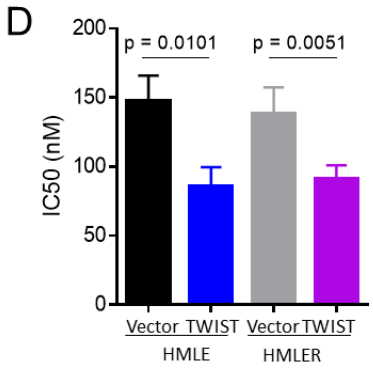


Figure 3.1 Sensitivity to OpA is enhanced by EMT. (A) Representative morphology of non-transformed, immortalized, mammary epithelial cells expressing TWIST or control vector. Scale bar represents 20 μm. (B) Cytotoxic activity of the indicated compounds was measured, in triplicate, by MTS assay. Mean and standard deviation of IC_{50} values are reported. Selectivity index is calculated as (HMLE Vector IC_{50}) / (HMLE TWIST IC_{50}). (C) Representative data indicating cytotoxicity for the indicated cell lines. Error bars represent standard deviation. (D) Mean and standard deviation of IC_{50} values for OpA, n = 3 or 4, two-tailed student's unpaired t-test used to test significance. n.d. = not determined, n.a. = not applicable

miR-200c Suppression Is Necessary for Sensitivity to OpA

Because we observed that OpA selectively impacts cells that have undergone EMT, we next evaluated whether reversing the EMT status of these cells would be sufficient to undermine OpA sensitivity. To do this, we introduced an epithelial-specific microRNA into HMLE-TWIST cells. miR-200c expression has been shown to be sufficient to reverse

EMT and associated CSC features [54]. First, we verified over-expression of miR-200c in HMLE Twist cells (Fig. 3.3A) and confirmed the expected effects on the prevalence of $CD44^{hi}/CD24^{lo}$ cells (Fig. 3.4A) and sphere formation (Fig. 3.4B). We next measured sensitivity to OpA and found that induction of miR-200c partially compromised sensitivity to OpA (Fig. 3.3B). This indicates that miR-200c-driven suppression of the CSC state impacts sensitivity to OpA.

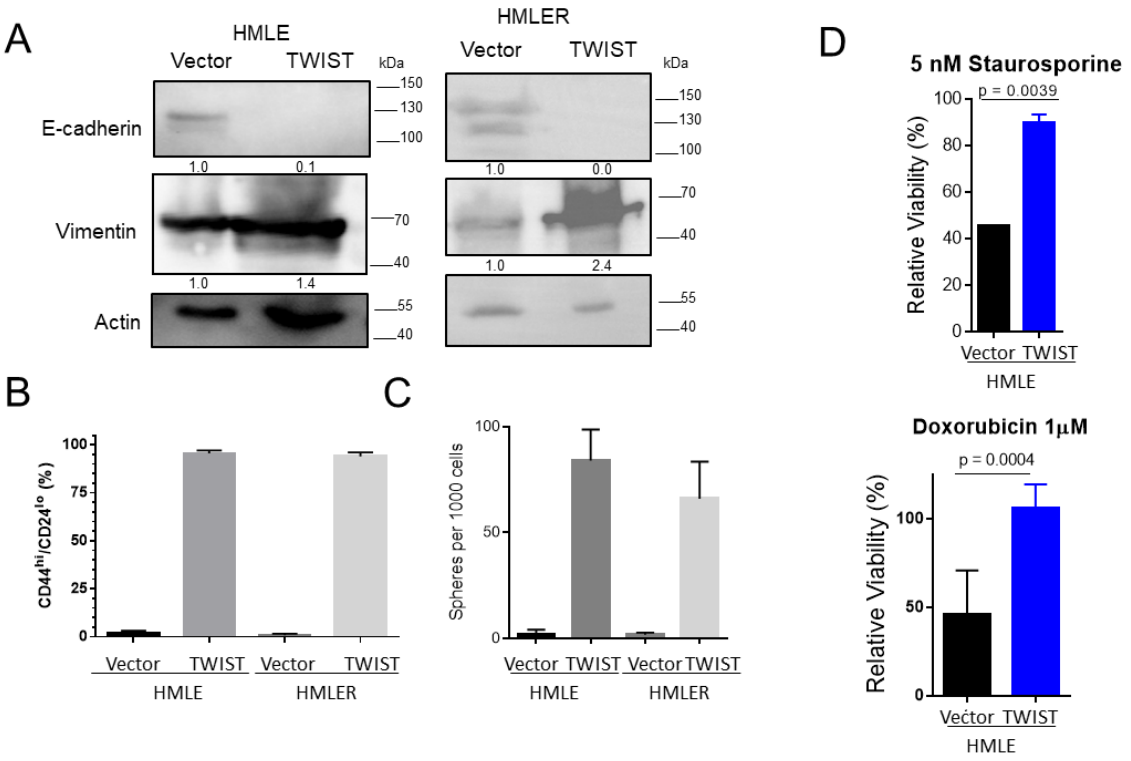


Figure 3.2 EMT and CSC features of cells overexpressing Twist. (A) Representative western blot showing E-cadherin and vimentin protein expression. Images cropped to show relevant bands. Band intensity is normalized to actin and provided as a quantification relative to vector cells. Full-length blots available. (B) Flow cytometry was performed to measure expression of CD44 and CD24. The percentage of cells with both high CD44 and low CD24 is shown. Mean and standard deviation are shown, n = 3. (C) Mammosphere formation assay was performed on the indicated cells. Mean and standard deviation are shown, n = 4. (D) HMLE cells with the indicated vectors were exposed to the indicated dose of the indicated compounds for 72 hours before viability was assessed using an MTS assay. Data are normalized to vehicle treatment. Mean and standard deviation are shown, n = 4. Students t-test used to generate p-values.

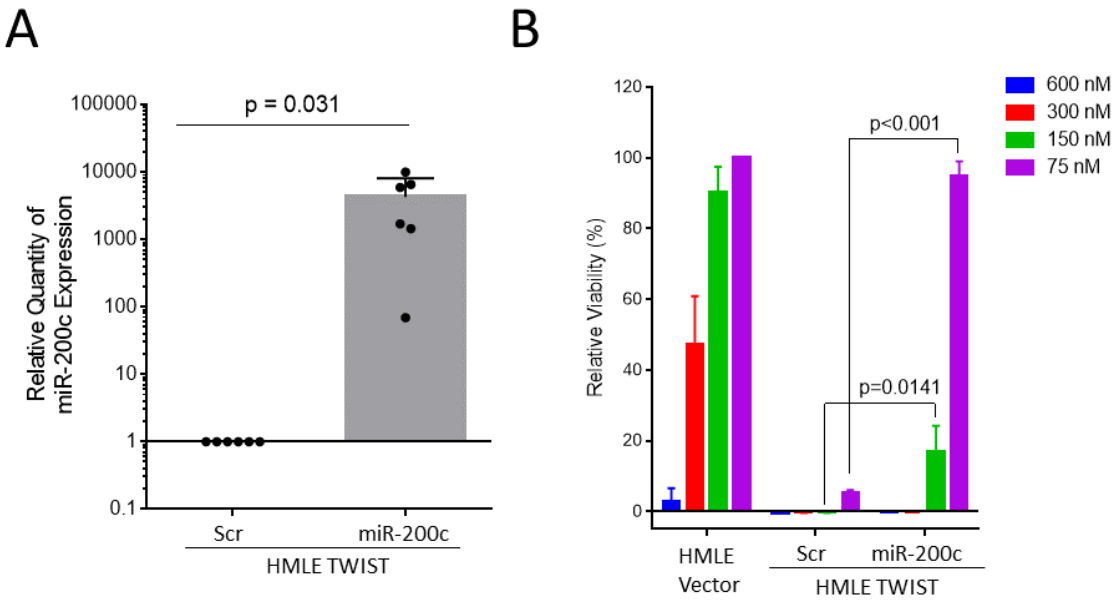


Figure 3.3 miR-200c enhances sensitivity to Ophiobolin. (A) Mean and standard deviation of miR-200c expression in HMLE TWIST cells expressing ectopic miR-200c or a control vector. n=6 (B) Mean and standard deviation of relative viability for indicated doses of OpA in HMLE and HMLE TWIST cells expressing ectopic miR-200c or a control vector. n=3, two-tailed student's unpaired t-test used to test significance.

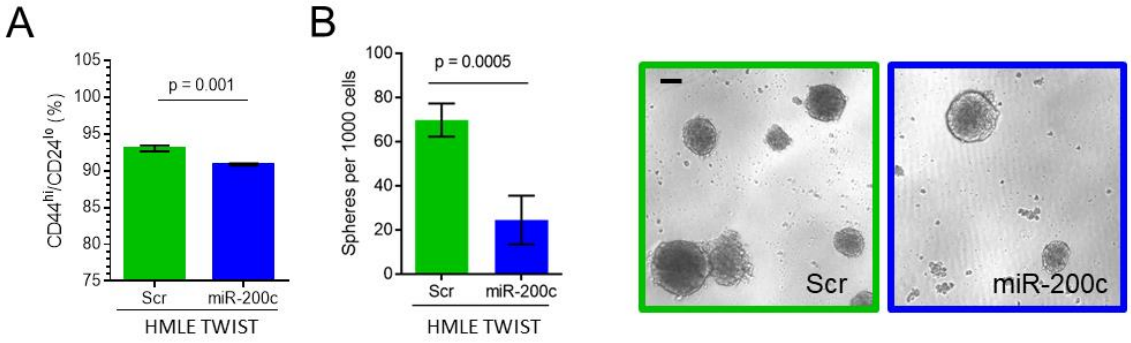


Figure 3.4 Suppression of CSC features of cells overexpressing miR-200c. (A) Flow cytometry was performed to measure expression of CD44 and CD24. The percentage of cells with both high CD44 and low CD24 is shown. Mean and standard deviation are shown, n = 3. (B) Mammosphere formation assay was performed on the indicated cells. Representative images are included, scale bar = 100 μm. Mean and standard deviation are shown, n = 4. Students t-test used to generate p-values.

Persistent Treatment with OpA Alters Cellular Phenotypes

Triple-negative breast cancer cell lines of the basal-like or claudin-low subtype typically exhibit greater EMT and CSC features [55]. To examine the effect of OpA on breast cancer cells, we measured the cytotoxic activity on the ER-positive, CSC-poor, epithelial-like MCF7 and triple-negative, CSC-rich, mesenchymal-like MDA-MB-231 cell lines. While both cell lines were highly responsive to an elevated dose of OpA (400 nM), the MDA-MB-231 cells displayed significantly greater cell death at an 80 nM dose (Fig. 3.5A). We next considered whether exposure to OpA, in addition to exerting a cytotoxic effect, might also abrogate EMT and CSC-associated cell phenotypes. To evaluate the impact of sub-cytotoxic doses of OpA on EMT and CSC phenotypes, we performed experiments on CSC-rich MDA-MB-231 cells using continuous, multi-day treatment of 30 nM OpA, 100 nM OpA, or vehicle (Fig. 3.5B- blue and purple arrows, respectively). Continuous treatment with a sub-cytotoxic doses of OpA triggered modest changes in cell morphology toward a more compact and cobblestone-like appearance, characteristic of epithelial cells (Fig. 3.5C). To evaluate the effect on EMT, we measured markers of EMT following exposure to OpA. Cells treated with 100 nM OpA, but not 30 nM, showed increased expression of CDH1 (E-cadherin) and decreased expression of CDH2 (N-cadherin), indicative of a partial EMT reversion (Fig. 3.5D, E). As EMT is necessary for the migratory capacity of MDA-MB-231 cells, we ascertained whether OpA could inhibit migration using a wound healing assay. Consistent with an effect on EMT properties, cells pre-treated with sub-cytotoxic doses of OpA failed to migrate in response to a scratch wound (Fig. 3.5F). We next measured the effect of OpA on anchorage-independent growth using a mammosphere assay and on the prevalence of CSC-associated $CD44^{hi}/CD24^{lo}$ cells.

Consistent with an effect on CSC properties, we observed that pre-treatment of MDA-MB-231 cells with OpA reduced sphere formation (Fig 3.5G). While pre-treatment at 30 nM had no effect on the prevalence of $CD44^{hi}/CD24^{lo}$ cells, pre-treatment at 100 nM had a minor, though statistically significant effect (Fig. 3.5H). In summary, persistent treatment of a CSC-rich breast cancer cell line with OpA diminishes sphere formation and migratory properties associates with CSC and EMT.

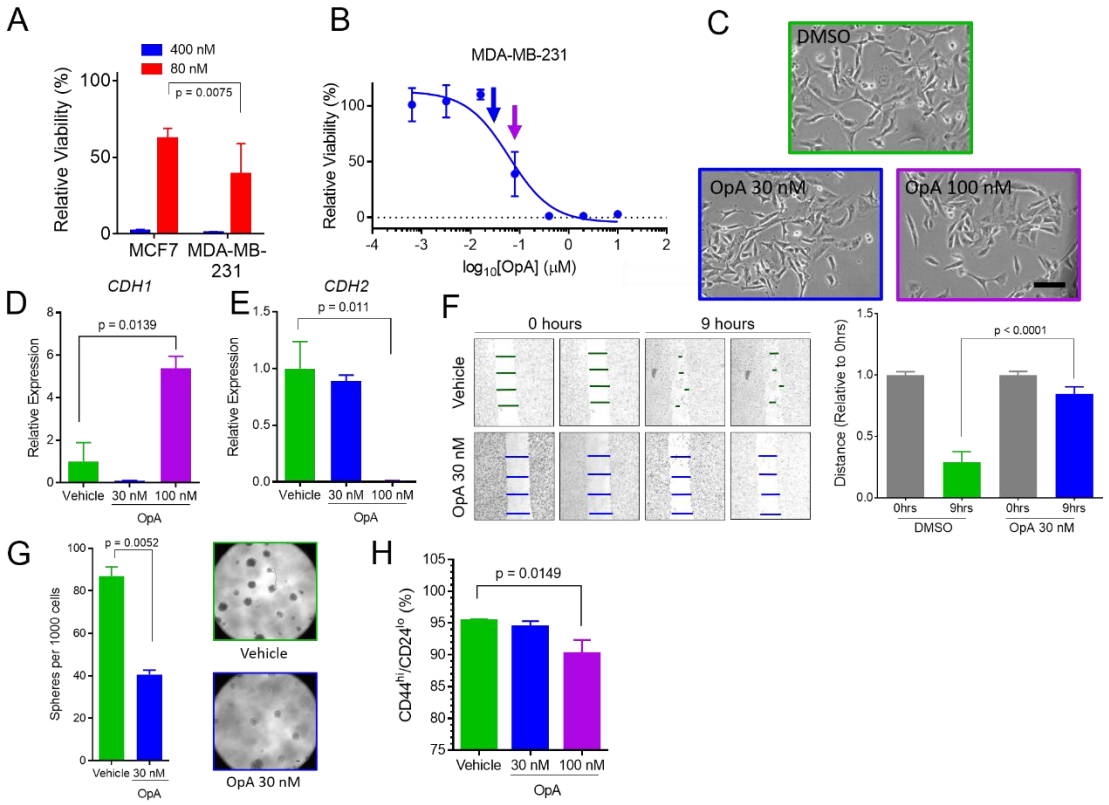


Figure 3.5 Treatment with OpA suppresses EMT-driven cell behavior. (A) Mean and standard deviation of relative viability for indicated doses of OpA in MCF7 and MDA-MB-231 cells, n = 8, two-tailed student's unpaired t-test used to test significance. (B) Representative data indicating cytotoxicity of OpA to MDA-MB-231 at the indicated doses. Blue and purple arrows indicate doses used for sub-cytotoxic pre-treatment in C-H. (C) Representative morphology of MDA-MB-231 cells treated with OpA at 30nM or 100 nM for 4 days followed by 24 hours of culture in clean media. Scale bar = 100 μm. (D/E) qRT-PCR for CDH1 (D) and CDH2 (E) from cells treated as in (C), n = 3, mean and standard deviation are shown, two-tailed student's unpaired t-test used to test significance. (F/G) MDA-MB-231 cells, treated as in (C) were subjected to a (F) sphere-forming assay n=8, unpaired t-test, scale bar = 100 μm or a (G) flow cytometry assay for CD44 and CD24. The percentage of cells with both high CD44 and low CD24 is shown. Mean and standard deviation are shown; n = 3, two-tailed student's

unpaired t-test used to test significance. (H) MDA-MB-231 cells, treated with OpA at 30nM for 4 days, were cultured in clean media for 12 hours then subjected to a wound healing assay. Representative images are shown from 0 hours post-scratch and 9 hours post-scratch.

OpA Treatment Increases Sensitivity to Chemotherapy

EMT-promoted stemness drives resistance to commonly used chemotherapies. One approach to overcoming this problem is to consider dual-treatment therapies that combine CSC-targeting compounds with conventional drugs. To examine the relationship between EMT and the combinatorial impact of OpA treatment, we co-treated cells with a dilution series of OpA and either doxorubicin or paclitaxel. Co-treatment of MDA-MB-231 cells with as little as 12.5 nM OpA enhanced the cytotoxic response from doxorubicin (Fig. 3.6A), while 50 nM OpA enhanced the cytotoxic response from paclitaxel (Fig. 3.7A). Notably, addition of 50 nM OpA was sufficient to maintain cytotoxic activity despite a 25-fold reduction in the dose of doxorubicin (Fig. 3.6A-orange bar) and a 5-fold reduction in the dose of paclitaxel (Fig. 3.7A-orange bar).When analyzed using Combenefit [56], these dose combinations tended toward synergistic effects (Fig. 3.6B, Fig. 3.7B). Combination treatment using the synthetic derivative, 3-deoxy-OpA (Fig. 3.7C) did not result in altered activity (Fig. 3.7D-F). To test whether the EMT state or degree of CSC enrichment was relevant for synergistic activity of OpA and doxorubicin, we performed combination treatment analysis on MCF7, HMLE vector and HMLE TWIST cells. MCF7 cells displayed less synergy and more antagonism than MDA-MB-231 cells (Fig. 3.6C). HMLE vector cells treated with OpA and doxorubicin also exhibited strong antagonism which was diminished in the HMLE TWIST cells (Fig 3.6D,E). The capacity of OpA to act in concert with clinically useful chemotherapeutic agents indicates that co-treatment may be useful to more effectively treat breast cancer.

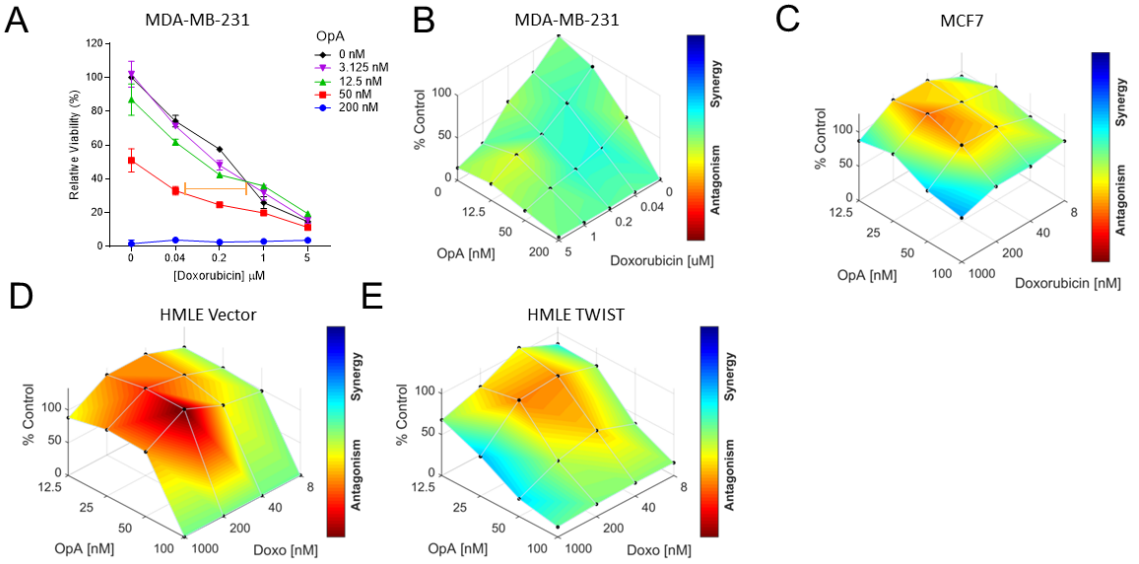


Figure 3.6 Combinatorial activity for OpA with doxorubicin and paclitaxel. (A/B) Representative data indicating cytotoxicity to a range of doses of OpA and doxorubicin for MDA-MB-231 (A), n = 4. (B) Data from (A) are represented using Combenefit. Blue-shaded areas represent dose combinations with synergistic effects. (C-E) Representative data indicating interactions between OpA and doxorubicin for MCF7 (C), HMLE Vector (D) and HMLE TWIST (E) cells.

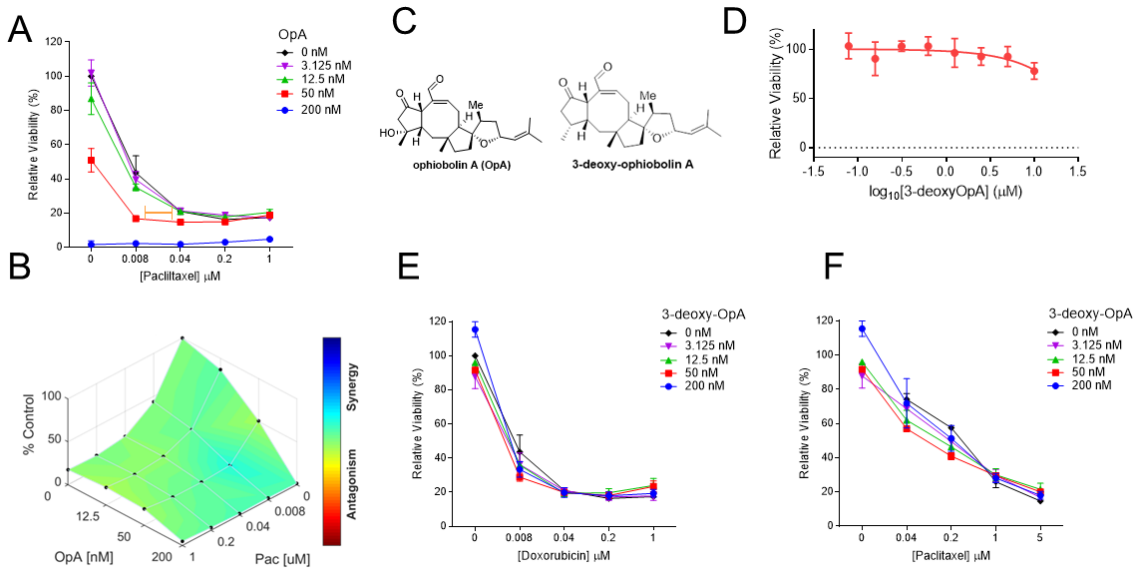


Figure 3.7 OpA synergizes with paclitaxel while deoxy-OpA is inactive towards MDA-MB-231 cells. (A) Representative data indicating cytotoxicity to a range of doses of OpA and paclitaxel for MDA-MB-231. (B) Data from (A) are represented using Combenefit. Blue-shaded areas represent dose combinations with synergistic effects. (C) Molecular structures of OpA and deoxy-OpA. (D) Representative data indicating cytotoxicity of

deoxy-OpA to MDA-MB-231 at the indicated doses. (E/F) Representative data indicating cytotoxicity of MDA-MB-231 to a range of doses for 3-deoxy-OpA and doxorubicin (E) or 3-deoxy-OpA and paclitaxel (F).

OpA Is Tolerated In Vivo and Suppresses Growth of Mammary Cell Tumors with Exogenous TWIST-Expression

We next assessed whether OpA treatment alone is sufficient to reduce growth of a mammary cell tumor, which is composed exclusively of EMT-positive cells, in mice. As such, immunocompromised mice were orthotopically injected with Ras-transformed HMLE cells constitutively expressing the TWIST transcription factor to induce EMT (HMLER-TWIST). Following the emergence of palpable tumors, mice were randomly assigned to either the control (DMSO diluted into saline) or OpA treatment groups. Thrice weekly injections for 3 weeks consisting of 10 mg/kg of OpA were not well tolerated as mice exhibited weight loss greater than 20% of initial body weight and two adverse outcomes were recorded prior to the final dose (Fig. 3.8A). However, a dose of 5 mg/kg was better tolerated with weight loss less than 15% and one adverse outcome, while a dose of 2.5 mg/kg had no statistically significant impact on body weight (Fig. 3.8A). A dose of 5 mg/kg of OpA was sufficient to significantly suppress the growth of HMLER-TWIST tumors (Fig. 3.8B) and to reduce the endpoint tumor volume of HMLER-TWIST tumors (Fig. 3.8C). We sought to ascertain whether OpA treatment contributes to increased cell death within treated tumor tissue by staining for cleaved caspase-3, a marker of apoptosis. Unexpectedly, staining for cleaved caspase-3 in the primary tumors revealed no significant difference between untreated and OpA-treated mice (Fig. 3.9). However, OpA has been shown to induce non-apoptotic cell death in other models [40,57]. Because HMLER-TWIST tumors metastasize to the lung and other organs [26], we analyzed lungs from OpA-treated mice to determine if metastatic burden was reduced. Despite the observed effects on

migration in vitro, there was no significant reduction in lung metastatic burden associated with OpA treatment (Fig. 3.9).

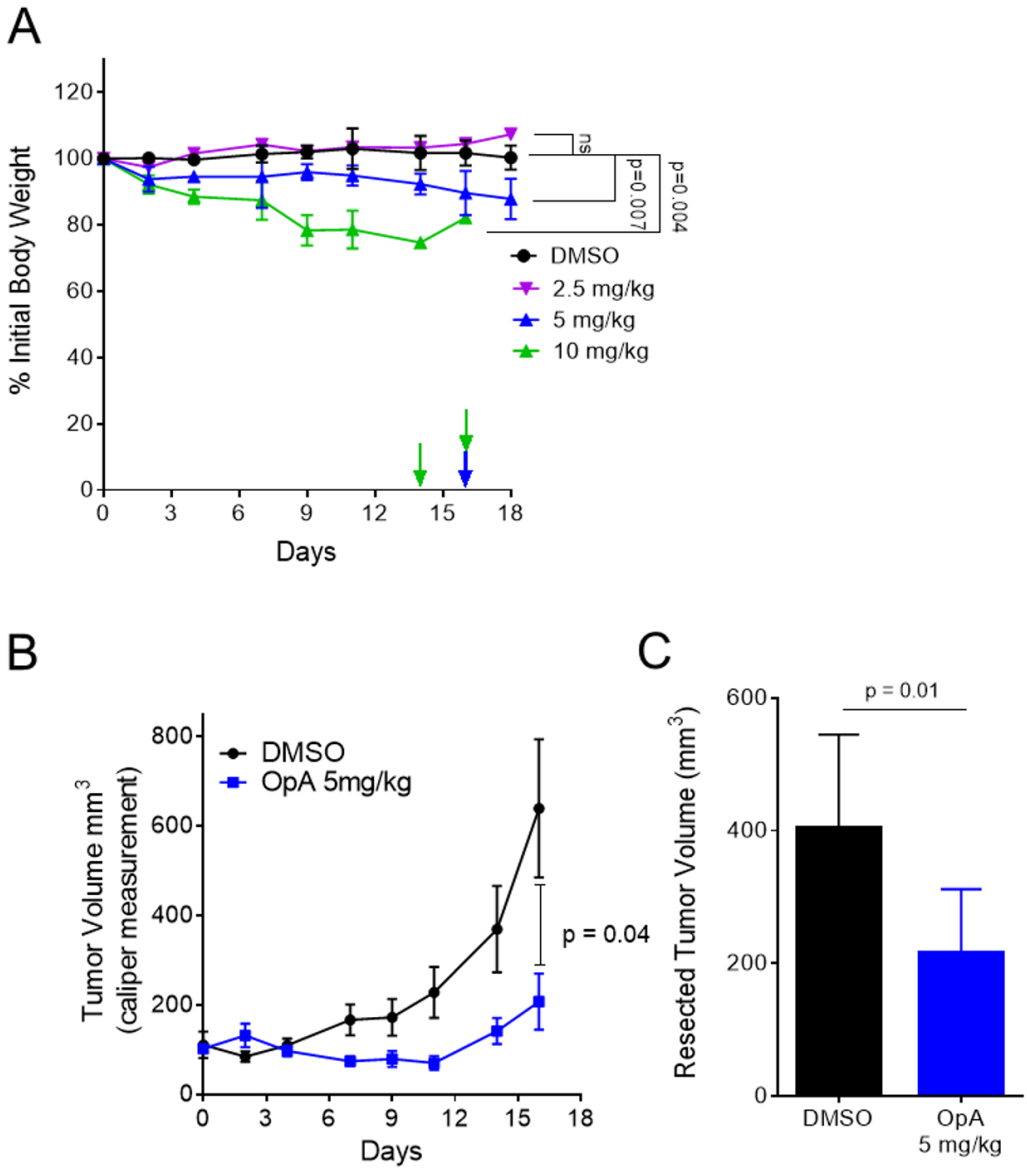


Figure 3.8 OpA is tolerated in vivo and suppresses tumor growth from cells over-expressing TWIST. (A/B) Mice, bearing tumors composed of HMLER-TWIST cells, were injected with 10 mg/kg (n=2), 5 mg/kg (n=5), 2.5 mg/kg (n=3) of OpA, or vehicle control (n=8), thrice weekly for three weeks. (A) Body weight was tracked. Arrows indicate endpoint criterion met for an individual animal. Statistical significance measured using the Holm-Sidak method with an alpha of 5%. (B) Tumor volume was measured at the indicated timepoints by caliper and is given as length x width divided by 2. n = 5. Statistical significance of the difference between volumes at 17 days is indicated as determined by two-tailed student's unpaired t-test. (C) End-point tumor volume was compared by two-tailed student's unpaired t-test, n=4.

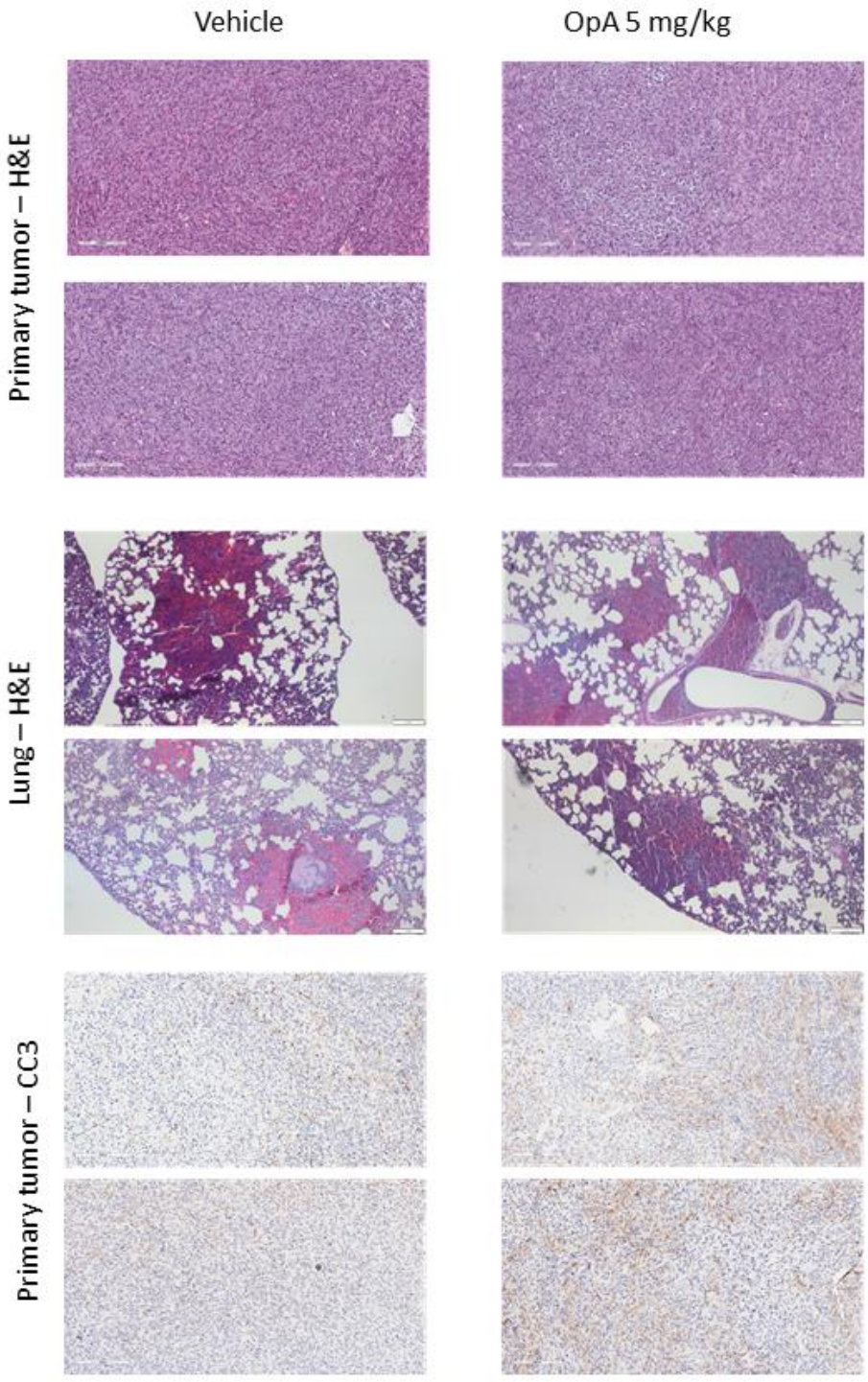


Figure 3.9 Histology of primary tumors and lungs of mice xenografted with HMLER Twist cells and treated with OpA or vehicle.

Discussion

Currently, conventional chemotherapeutic drugs are able to elicit high response rates in about half of TNBC patients; however, the remaining patients eventually develop progressive disease[2], with some even experiencing more aggressive and CSC-rich tumors after therapy [14,58]. Identification of molecules with specificity for CSC-rich cell populations will facilitate the development of novel therapies and may improve responses to currently available therapies.

While several other natural products have been linked to CSC-targeting [34,35,49,59-64], our work highlights a natural product that selectively kills breast CSCs exhibiting EMT features. Further, we show a reduction of EMT phenotypes such as migration, as well as reduction in sphere-forming capacity and changes to CSC-rich subpopulations in a TNBC cell model. Extending OpA's efficacy in reducing CSC-related properties, our data suggest increased sensitivity to conventional chemotherapeutics doxorubicin and paclitaxel when co-treated with OpA. Finally, we evaluate the efficacy of OpA in vivo and show suppressed growth of an EMT-positive, primary tumor.

Evolution-driven selection of natural products imparts biological activities useful for disease treatment and which may not be mimicked by selective kinase inhibitors. Other successful natural products that have driven cancer therapies include taxol, vinblastin, anthracyclines, daunomycin and doxorubicin [65]. Several studies [40,42,43,47,66-69] have evaluated one such natural product, OpA, in cancer settings, predominantly using in vitro models, and, similar to our present study, these studies report IC_{50} values in the low nanomolar range. Our work is one of the first to evaluate OpA *in vivo* and is the first to describe the impact of EMT on OpA sensitivity. By focusing on the effects on EMT and

stemness phenotypes, this work opens the door for the discovery of essential molecular pathways and for the investigation of OpA derivatives as a future cancer treatment.

Materials and Methods

Cell Lines

MCF7, and MDA-MB-231, were received from ATCC; HMLE, HMLER, HMLE Snail, HMLER Snail, HMLE TWIST, and HMLER TWIST were kindly gifted from Dr. Sendurai Mani (MD Anderson Cancer Center). Breast cancer cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Corning Inc., Kennebuck, ME, USA) supplemented with 10% fetal bovine serum (FBS) (Equitech-Bio Inc., Kerrville, Texas, USA) and 1X antibiotics (Penicillin/Streptomycin, Lonza, Basel, Switzerland). Immortalized human mammary epithelial cells (HMLE) and derivatives were maintained as in Elenbaas et. al [70]. Cell lines were tested monthly for mycoplasma and validated via STR testing. Incubation occurred at 37˚C with 5% CO2. miR-200c overexpression was generated using lentiviral transduction of pCMV-MIR (Origene Rockville, MD). Transduced cells were selected using puromycin.

Reagents

Curcumin, genestein, doxorubicin, and paclitaxel were obtained from Selleckchem (Houston, TX, USA), salinomycin from Cayman Chemicals (Ann Arbor, MI USA), and disulfiram from (Tocris Bioscience, Bristol, UK). OpA was produced by fermentation of the fungus Drechslera gigantea. It was extracted from the fungal culture filtrates, purified,

crystallized and identified by 1H NMR and ESI MS as previously reported [71]. The purity of OpA was >98% as ascertained by 1H NMR and HPLC analyses.

3-Deoxy OpA was synthesized from ophiobolin I [72,73] which was also obtained through fermentation as previously reported [71]. A two-step synthetic sequence involving conjugate reduction of the enone which proceeded with high diastereoselectivity (>19:1 by 600 MHz 1H NMR) followed by a Ru(IV)-mediated oxidation of the primary alcohol to the aldehyde delivered 3-deoxy OpA. It should be noted that the methyl group at C3 is epimeric with respect to the C3-methyl group in OpA. However, the importance of the C3-hydroxy group and/or the stereochemistry of this methyl group was verified through studies described below and 3-deoxy OpA served as a negative control.

Viability

Cells were plated with 2,000 cells per well in a 96-well plate and allowed to adhere overnight. Compounds, suspended in DMSO and diluted into PBS, or vehicle were added to the culture medium and incubated for 72 hours at 37˚C, 5% CO2. Following manufacturer suggested protocol, 20µL CellTiter 96® AQ_{ueous} One Solution Cell Proliferation Assay (MTS; Promega, Madison, WI, USA) was added and incubated 1-4 hours at 37˚C, 5% CO2. Absorbance was measured at 490 nm using a 96-well plate reader (Fisher Scientific, Hampton, NH, USA).

RNA Extraction and Detection

Cells were lysed in the presence of Trizol® Reagent (Thermo Scientific, Waltham, MA, USA) and total RNA extracted following manufacturer protocol recommendations.

Relative quantification of the mRNA levels was performed using the comparative Ct method with the formula $2^{-\Delta\Delta Ct}$. For microRNA analysis small nucleolar RNA U6 was used for normalization while for mRNA analysis GAPDH was used for normalization, Taqman and SYBR PCR Master Mixes were obtained from Applied Biosystems (Foster City, CA, USA; Thermo Scientific). All quantitative reverse transcription-PCR (RT-PCR) experiments were run in technical quadruplicates and biological triplicates and a mean value was used for the determination of mRNA levels.

Western Blotting and Antibodies

Cells were lysed in the presence of 100 microliters radio-immunoprecipitation (RIPA) buffer containing protease inhibitors (Alfa Aesar, Stoughton, MA, USA) on ice. Protein was quantified using the Bradford Assay (BioRad, Hercules, CA, USA). Twenty micrograms of total protein from each sample was resolved on a 4%–12% SDS-PAGE gel and transferred to PVDF membranes. Sister blots were then probed with antibodies including anti-E-cadherin (Cell Signaling, Danvers, MA, USA), anti-vimentin (Protein Technologies, Tucson, AZ, USA), or anti-β-actin (BD Biosciences, San Jose, CA) antibody. Chemiluminescent signals were detected with ECL™ prime (Thermo Fisher Scientific) using the Biorad ChemiDoc system. If necessary, blots were stripped with ECL Stripping Buffer (Li-Cor, Lincoln, NB, USA) following manufacturer protocol. Bands were quantified using ImageJ.

Mammosphere Assay

Cells were harvested and suspended in serum-free mammary epithelial growth medium (MEGM) supplemented with 1% methyl cellulose, 20 ng/mL FGF, 10 ng/mL EGF, and 4 μg/mL heparin. Cells were plated in 4 replicates in a flat-bottom ultra-low attachment 96-well plate (Corning) and allowed to grow at 37˚C, 5% CO2 for 10-14 days and were monitored microscopically to ensure that they did not become confluent during the experiment. 100 μL low-attachment media was added every 3-4 days. Wells were imaged using 4x magnification on a computer-assisted phase contrast microscope (Nikon, Tokyo, Japan). Spheres larger than 100 micrometers were counted.

Flow Cytometry

For flow cytometry, cells were harvested, counted and 10^5 cells were incubated with 5 μl of either CD44 (BV421 Mouse Anti-Human CD44 # 562890; BD Biosciences, San Jose, CA, USA) and/or CD24 (PE-Mouse Anti-human CD24 #555428; BD Biosciences) in PBS with 1% serum for 1 hour on ice, minimizing light exposure. Cells were then pelleted at low-speed and washed with PBS with 1% serum twice before measurement of fluorescence using BD FACS Melody (BD Biosciences).

Migration

For migration assay, cells were serum-starved overnight and scratch wounds were created using a sterile pipette tip on the cell monolayer or by plating cells in 2-well culture inserts (Ibidi, Madison, WI). Cell migration rates were determined by measuring the distance between cell fronts after the indicated number of days in culture. The distance

between the two edges at multiple points was quantified using ImageJ at the indicated timepoints.

Co-Treatment and Interaction

Cells were treated with compound or matched-percentage DMSO or other vehicle in serial dilutions and incubated for 72 hours before measuring viability using MTS (Promega, Madison, WI, USA). Interactions were quantified using the Combenefit program with the Loewe model and dose-response surface mapping [56].

Tumor Growth

Female Scid/bg (CB17.Cg-PrkdcscidLystbg-J/Crl) mice (5–8 weeks old) were obtained from Charles River Laboratories (Wilmington, MA, USA). Animals were maintained under clean room conditions in sterile filter top cages with autoclaved bedding and housed on high efficiency particulate air–filtered ventilated racks. Animals received sterile rodent chow and acidified water *ad libitum*. All of the procedures were conducted in accordance with the Institute for Laboratory Animal Research Guide for the Care and Use of Laboratory Animals and with Baylor University Animal Care and Use Committee guidelines. HMLER-TWIST cells were harvested, pelleted by centrifugation at 2000xg for 2 minutes, and resuspended in sterile serum-free medium supplemented with 30% to 50% Matrigel (BD Biosciences, San Jose, CA, USA). Cells ($2x10^6$ in 100 µl aliquots) were implanted into the left fourth mammary fat of each mouse and allowed to grow until measurable by caliper. Then, OpA or vehicle was administered by intraperitoneal injection three times weekly for three weeks at 2.5 mg/kg, 5 mg/kg or 10 mg/kg. Tumor volume and

body weight were recorded concurrently with injection protocol [74]. At designated times, mice were humanely euthanized, and tumors and lungs were collected. Experiments were approved by Baylor University IACUC (#1441130). This study was carried out in compliance with ARRIVE guidelines (http://www.nc3rs.org.uk/page.asp?id=1357),

Tissue Staining

Immunohistochemistry was performed on formalin-fixed, paraffin-embedded tissue. The Leica Bond Max automated platform was used to perform the immunohistochemistry. The antibodies used were as follows:(Ki67 Lot#GR94281-1, Caspase 3 Lot#GR3265151-4 (Abcam, Cambridge, MA). Antibodies were diluted at 1:200 and 1:1000, respectively.

Statistical Analysis

Unless otherwise stated, statistical differences were determined using a student's t-test. The GraphPad PRISM software v6 was used to perform these analyses. Statistical significance levels are annotated as n.s. = non-significant, *$p < 0.05$, **$p < 0.01$, ***$p < 0.001$, ****$p < 0.0001$.

Acknowledgements

We acknowledge the entire Taube Lab for invaluable discussion and advice. Also, we appreciate the assistance of Dr. Igor Bado (Baylor College of Medicine, Houston, TX, USA) for advice regarding Combenefit. We would like to acknowledge Dr. Gerard Nuovo (Discovery Life Sciences, Powell, OH, USA) for assistance with immunohistochemistry.

The authors also thank Dr. Maurizio Vurro (Institute of Sciences and Food Production, CNR, Bari, Italy) for the supply of Drechslera gigantea culture filtrates. This work was supported by the Cancer Prevention and Research Institute of Texas, grant #RP180771 to J.H.T. and D.R.

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CHAPTER FOUR

OpA Acts Through a Non-Apoptotic Mitochondria-Specific Mechanism

Introduction

Leading approaches to eliminating breast cancer rely on hormone therapy, chemotherapy, or novel targeted therapies. Because triple negative breast cancer (TNBC) patients have not benefitted from clinically successful targeted therapies, often due to increased CSC abundance compared to other cancers, chemotherapy remains the leading therapeutic approach, and even then only 50% of patients will respond.[9–11,445] The chemotherapy options for the management of TNBC include compounds that target DNA repair complexes (platinum compounds and taxanes), p53 (taxanes), or cell proliferation (anthracycline containing regimens).[446] As mentioned, challenges with these therapies are abundant. Failure comes from mechanisms like cancer stem cell-driven resistance due to slow-diving cells and drug efflux, or from mutation. Successes in overcoming these hurdles come from approaches that are able to target the cancer stem cell (CSC) sub-population, enhancing therapeutic effectiveness in non-responding TNBC patients.[447]

A Brief Review of Cell Death Pathways

Apoptosis: Apoptosis is one of the most well-studied mechanisms of programmed cell death. Activation of the pathway occurs following either intrinsic stimulation (growth factor deprivation, stress, PARP-signaling of DNA single-strand breaks, viruses) or

extrinsic stimulation (cell surface receptors such as Fas or TNFR1). Ultimately, both pathways promote the cleavage of caspase-3 and result in phenotypic changes characterized as blebbing, whereby the cytoskeleton dissociates from the membrane and the cytoplasm bulges outwards. The intrinsic pathway acts through BCL-2 inactivation, which frees BAX and BAK to promote cytochrome c release and mitochondrial fission. The extrinsic pathway bypasses mitochondrial activation of caspase-3 by instead using activated caspase-8.[448] The mitochondria can also be stimulated independently of caspases via poly-ADP-ribose (PAR), which is generated via PARP (PAR polymerase) cleavage following single strand breaks.[449] PARP cleavage renders the protein inactive, which acts as a substrate for caspase-3.

Necrosis and Necroptosis: Necrosis is a form of unprogrammed cell death resulting from cellular damage or pathogenic infiltration. Necroptosis is described as the programmed form of necrosis, independent of caspases and reliant on TNF-mediated RIPK signaling. RIPK signaling induces the formation of the necrosome which phosphorylates MLKL and prompts organelle degradation by MLKL-disruption of their membranes.[450] Furthermore, this drives inflammation to which mitochondria respond by opening the mitochondrial permeability transition pore (mPTP). mPTP opening destabilizes and depolarizes the mitochondria, leading to its eventual rupture.[451,452] Interestingly, apoptosis and necroptosis are intertwined, both able to regulate the other, namely at the intersection of caspase-8.[453]

Paraptosis: Emerging data indicate paraptosis as a mechanism of cytotoxicity distinct from apoptosis, characterized phenotypically by extensive vacuolization and swelling of the endoplasmic reticulum and/or mitochondria, and genetically by a reliance

on protein synthesis. [301,302,454–456] Due to variability in the literature in describing paraptosis-like cell death, there are not yet typified assays for paraptosis,[454] however, transmission-electron microscopy and fluorescence labeling of mitochondria readily characterize vacuolization and organelle swelling, and reversed cytotoxicity following inhibition of protein synthesis demonstrate evidence of pathways in the paraptosis program.[301] Assays also rely on demonstrating a lack of apoptosis- or necrosis-based cytotoxicity. Inducers of paraptosis include ligands that stimulate growth factor receptor families[457,458] or through the action of many natural products such as curcumin,[303] manumycin,[459] celastrol,[460] and paclitaxel.[461] While the mechanisms underlying paraptosis are not yet well understood,[454] several kinases have been implicated including the mitogen-activated protein kinase (MAPK) pathway, MEK-2, and Jun N-terminal kinase (JNK).[301] Each of these pathways is also implicated in EMT activation, suggesting that there may be a potential for a generalized increased sensitivity to paraptosis following EMT induction.[462–467]

Cancer Stem Cell Resilience is Mediated Through Apoptosis Resistance

CSCs contribute to cancer robustness through a variety of mechanisms; current innovative approaches are evaluating treatments that function broadly enough to overcome this resistance. One challenge to finding successful therapies is that CSCs possess evolved resistance to apoptosis.[29,74,79] This resistance is accomplished either through overexpression of antiapoptotic proteins or reduction of proapoptotic proteins. Through the intrinsic apoptotic pathway, mitochondria mediate this cell death program through the permeabilization of their membrane and the release of cytochrome c. Compounds like

daunorubicin and menadione that target mitochondrial membrane potential are shown overcome CSC apoptosis resistance and to promote mitochondria-induced apoptosis in CSCs.[468–472] This links a second challenge to CSC-targeted therapies; CSCs exhibit changes in mitochondrial function. CSCs are slow diving cells, which means that their metabolic requirements are less than that of highly proliferative cancer cells. Because of this, mitochondria look and behave differently in CSCs and changes in mitochondrial biogenesis and mtDNA copy number are both shown to increase resistance in CSCs.[468,473] Ultimately, therapies that can act through a mitochondrial-specific, non-apoptotic manner promise successful elimination of CSCs.

Role of Mitochondria and Mitochondrial Activity in Cell Death and EMT

Mitochondria undergo distinct dysfunctional changes that have been associated with progression to a metastatic and drug-resistant phenotypes.[474] The mechanistic link between metastasis and mitochondrial dysfunction is gradually emerging and recent reports connect these phenotypes to the activation of the EMT gene signature.[475–477] There is a cyclical interplay between alterations in mitochondrial metabolism driving EMT and EMT affecting the expression of metabolic genes.[478] Downregulation of oxidative phosphorylation (OXPHOS) is one of the altered metabolic processes associated with an EMT gene signature.[479] In 60% of patients with poor prognoses, OXPHOS, namely complex I and IV subunits of the electron transport chain (ETC), is downregulated and EMT is one of the most upregulated programs.[478,479] Further, the effects of ETC/OXPHOS inhibition are paralleled following mtDNA depletion, which induces the mitochondrial dysfunction of increased Ca^{2+} cytosolic release. This Ca^{2+} release drives retrograde

signaling, i.e., signals from the mitochondria that induce gene expression in the nucleus, that result in apoptosis resistance, multidrug resistance, invasion, and EMT.[478,480,481] EMT is triggered by transcriptional activation of SLUG, SNAIL, and TWIST, and the mesenchymal markers fibronectin, vimentin, and N-cadherin, with a corresponding decrease in the epithelial marker E-cadherin.

Herein, we demonstrate that OpA, a natural product that we previously demonstrated is highly effective against EMT-derived CSCs[372], mediates non-apoptotic cell death via a mitochondrial-dependent pathway. The EMT status is critical within the mitochondria to sustain OpA-mediated sensitivity and we observe functional changes to mitochondrial function and copy number following treatment with OpA. Phenotypic changes to mitochondria, including swelling, are also consistent with OpA treatment, only in mesenchymal cells. We do not observe increases in apoptotic markers after OpA treatment, but toxicity is lost with RIPK activation. Together, our data begin to elucidate the role of OpA in conferring toxicity to cells with EMT and CSC properties.

Results

Mesenchymal-cell Derived Mitochondria Play a Key Role in Conferring OpA Sensitivity

We have established that EMT is required for sensitivity to OpA,[372] and evidence points to the role of mitochondria in the activation of EMT and to the effect of OpA on mitochondria.[62,69,70,475] So, to further understand the role of OpA during EMT, we leveraged the ability of so-called mitochondrial cybrid models, developed by our collaborator, Dr. Benny Kaipparettu at Baylor College of Medicine. It was previously

demonstrated that cross-talk between mitochondria and the nucleus can influence tumor properties, even initiating metastasis.[482–484] When mitochondria from non-cancerous cells are introduced to highly aggressive cancer cells without mitochondria, the signals from the benign mitochondria repress oncogenic pathways.[482] We tested the cytotoxic activity of OpA in two cybrid models that involve epithelial HMLE and A1N1 cells, and mesenchymal MDA-MB-231 and SUM159 cells (Figure 4.1A). Cybrids were created by stripping epithelial HMLE cells of mitochondria, leaving a nuclear background and then introducing mitochondria from mesenchymal MDA-MB-231 cells. Additionally, cybrids were created by stripping mesenchymal SUM159 of mitochondria, leaving a nuclear background and then introducing mitochondria from epithelial A1N1 cells (Figure 4.1B). When treated with 0.5μM OpA for as little as 24 hours, cybrids containing mesenchymal mitochondria are more sensitive than epithelial cells, both with the same epithelial nuclear background (Figure 4.1C). However, in the same mesenchymal nuclear background, sensitivity is abrogated when epithelial mitochondria are introduced (Figure 4.1D). These results are also consistent at 48-hour and 72-hour exposure durations. These data indicate that the mitochondria present in EMT-positive cells play a significant role in OpA-mediated cell death. This is consistent with our previous findings that mesenchymal cells are more sensitive than epithelial cells to OpA.

OpA Affects Mitochondrial Biology and Phenotype

To evaluate the biological effect of OpA on mitochondria, we evaluated the activity of components of the electron transport chain (ETC) as well as mitochondrial copy number.

These experiments were also conducted in a collaboration with the Kaipparettu lab at Baylor College of Medicine. Following treatment with both 0.25µM and 0.5µM OpA, we observed changes in ETC activity of complex III (Figure 4.2). When we evaluated correlating pulldown experiment results, the top hits did not contain any subunits of complex III. COX6B1, however, was identified as a potential target of OpA, but it is part of complex IV (data via personal correspondence). Further, western blot analysis did not identify changes in any complexes' protein expression (data not shown), indicating that OpA may inhibit the activity of the complexes, but does not inhibit protein expression.

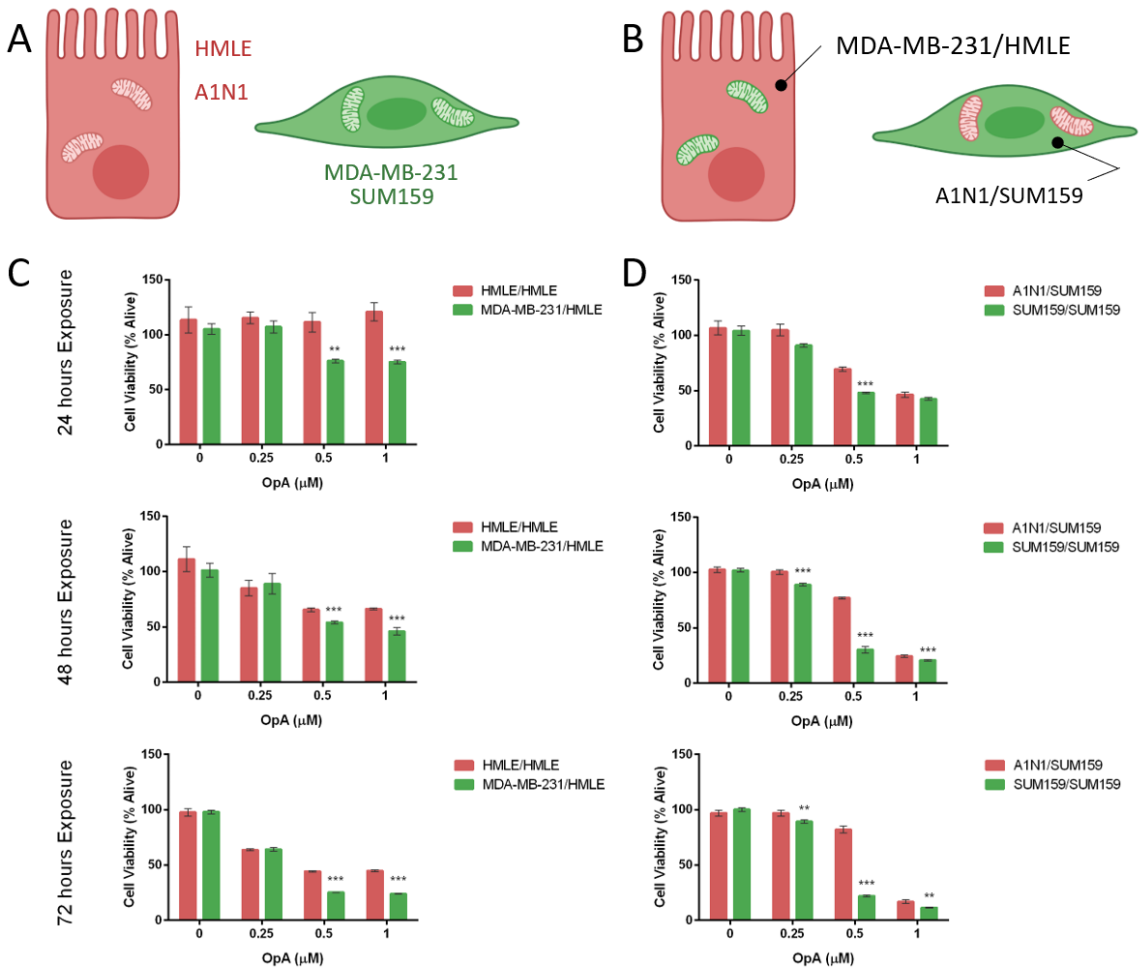

Figure 4.1 Mesenchymal mitochondria are necessary and sufficient to confer sensitivity to OpA. (A) Schematic of non-cybrid models, showing consistent epithelial (pink; HMLE and A1N1) and mesenchymal (green; MDA-MB-231 and SUM159) nuclear and mitochondrial backgrounds. (B) Schematic of cybrid models, showing the mesenchymal MDA-MB-231 mitochondria introduced to epithelial HMLE nuclear background and the epithelial A1N1 mitochondria introduced to mesenchymal SUM159 nuclear background. (C/D) Indicated cells or cybrids treated with OpA for 24, 48, or 72 hours before measuring viability.

Decreases in mitochondrial copy number are linked to activation of apoptosis, but is not characteristically detected in other mechanisms of programmed cell death.[485] When we observed mtDNA changes following OpA treatment in MDA-MB-231 cells, treatment with 0.5μM decreased mtDNA copy number, suggesting that mitochondria following OpA treatment do not correlate with the decrease in mitochondrial copy number typical of apoptosis.

Cell death pathways induce characteristic sub-cellular phenotypic changes like vacuolization, cytoplasmic degradation, and organelle swelling. To understand the cellular phenotypic changes induced by OpA and to correlate our findings to EMT-mediated sensitivity, we evaluated epithelial HMLE cells transformed with oncogenic Ras (HMLER), as well as either EMT-inducing transcription factors, Twist (HRTw) or Snail (HRSn). We chose to use two sub-IC_{50} doses of OpA (0.1μM and 0.3μM) in these cell lines. We recognize that not every cell in a tumor microenvironment will experience equal doses of a treatment, so we wanted to treat cells with a relevant dose that they would experience if they were located further from vasculature and the point of delivery. While this is well studied in clinic, it is also able to be assessed *in vitro*.[486–489] A similar concept was studied in a single-dish gradient experiment, demonstrating the rationale for using sub-IC_{50} doses.[490]

We observed intracellular vacuolization at both doses only in HRTw and HRSn cells, not in HMLER cells (Figure 4.3). Vacuolization did not increase with increased OpA exposure. Vacuolization is common in both necrosis and paraptosis, so we further examined cells using transmission electron microscopy (TEM) to evaluate the state of their organelles. Vacuolization, white arrows, is evident in both vehicle- and OpA-treated cells, but it is more quantified in OpA-treated HRSn cells, compared to HMLER + OpA cells, consistent with phase contrast data (Figure 4.4A,B). Endoplasmic reticulum, green arrows, were more readily visible in HRSn + OpA-treated cells, compared to HMLER, (Figure 4.4B), though statistical analysis did not reveal a difference in OpA-treated cells (data not shown).

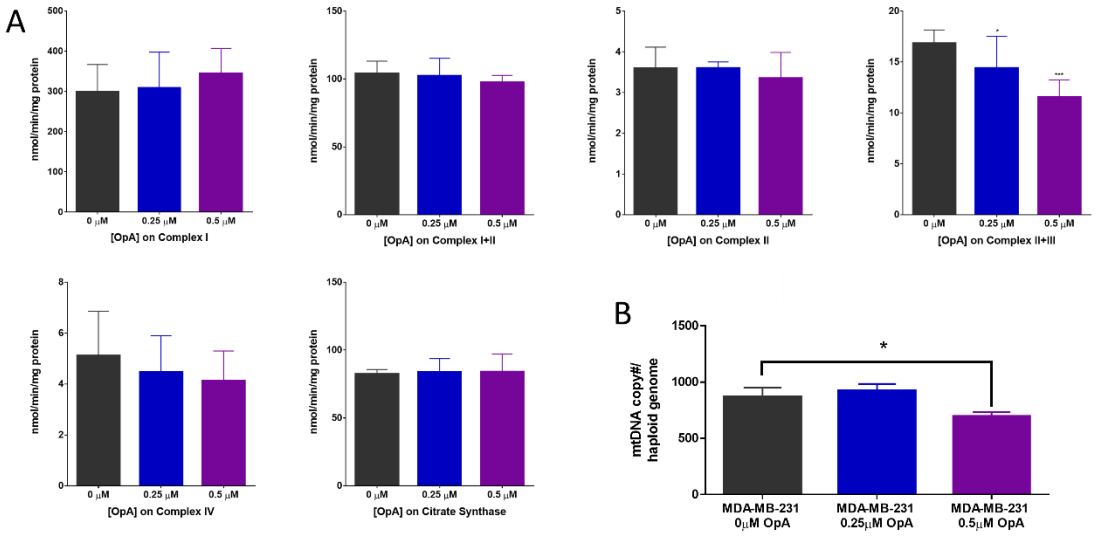

Figure 4.2 OpA treatment impacts electron-transport chain activity. (A) MDA-MB-231 cells were treated with indicated doses of OpA for 24 hours. Average of 3 experiments. Students t-test, compared to no treatment. (B) MDA-MB-231 cells treated with 0.25μM or 0.5μM OpA for 24 hours. *<0.05 *** <0.0001

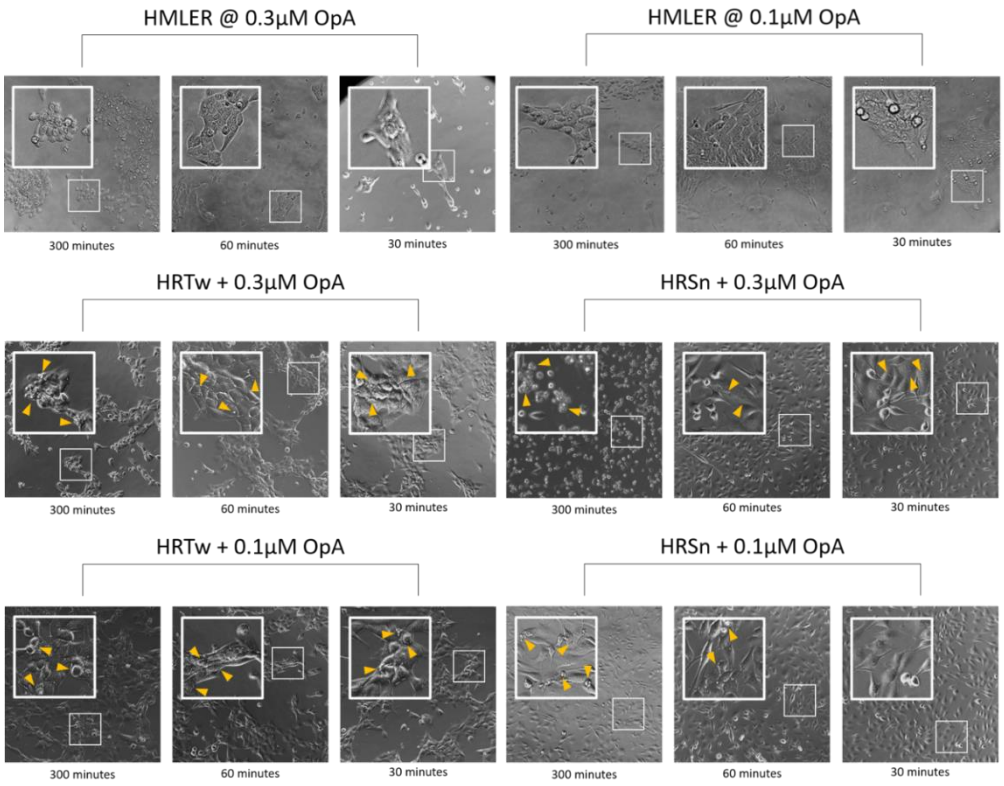

Figure 4.3 OpA induces vacuolization in EMT cells. HMLE cells transfected with Ras, as well as Snail or Twist were treated with 0.1μM or 0.3μm OpA for 30 minutes, 1 hour, or 5 hours before imaging in bright field at 20x. Yellow arrows indicate vacuoles. Images representative of three replicates.

Interestingly, there is an increase in baseline endoplasmic reticulum width in cells that have undergone EMT, compared to those that have not, and OpA treatment does not affect this difference in endoplasmic reticulum widths between EMT and non-EMT cells. Further, there is no difference endoplasmic reticulum morphology between OpA, doxorubicin, and DMSO treatment of HRSn cells. However, we observed distinct differences in mitochondrial phenotypes between treated and untreated HRSn cells. To evaluate further, we analyzed the areas of each mitochondrion using ImageJ and observed that mitochondria in treated cells were significantly larger than untreated cells (Figure 4.4C). Mitochondria treated with OpA appeared more vacuolized than those treated with

doxorubicin or vehicle, as evidenced by large open spaces within the membranes (Figure 4.4D). Together, these data indicate that OpA affects mitochondrial activity, number, and size, and induces vacuolization in the cytoplasm.

Effects of OpA Treatment are Consistent with a Non-apoptotic Cell Death Mechanism

Vacuolization and organelle swelling are indicative of several types of cell death, so, to more acutely determine the mechanism of cell death activated by OpA treatment, we examined changes in PARP protein expression, associated with apoptosis, following treatment with OpA, dimethoxycurcumin (DMC), an inducer of paraptosis[337], or doxorubicin, an inducer of apoptosis.[491–494] Because there are studies that suggest temporal importance of different types of cell death activation in OpA and other molecules, [71,455,456,495] we exposed cells across three time points to capture any variance in cell death initiation. Treatment with doxorubicin caused PARP cleavage in MDA-MB-231 cells across all three exposure times, but OpA did not cause PARP cleavage (Figure 4.5).

In another approach to understand the mechanism of cell death associated with OpA, we used an inhibitor of necroptotic cell death activation, nectostatin-1. Necroptosis is mainly mediated by RIPK1 (receptor-interacting protein [RIP] kinase 1), RIPK3, and MLKL (mixed lineage kinase domain-like pseudokinase). Necroptosis can be inhibited by necrostatin-1 (Nec-1), which is the first well-defined necroptosis inhibitor that exclusively inhibits RIPK1 activity.[496–498] 1.25 μM Nec-1 was needed to abrogate the negative effect of staurosporine on cell viability. However, only 0.24 μM Nec-1 was needed to block the effect of OpA on cell viability, indicating that OpA is more sensitive to inhibition by Nec-1 than staurosporine.

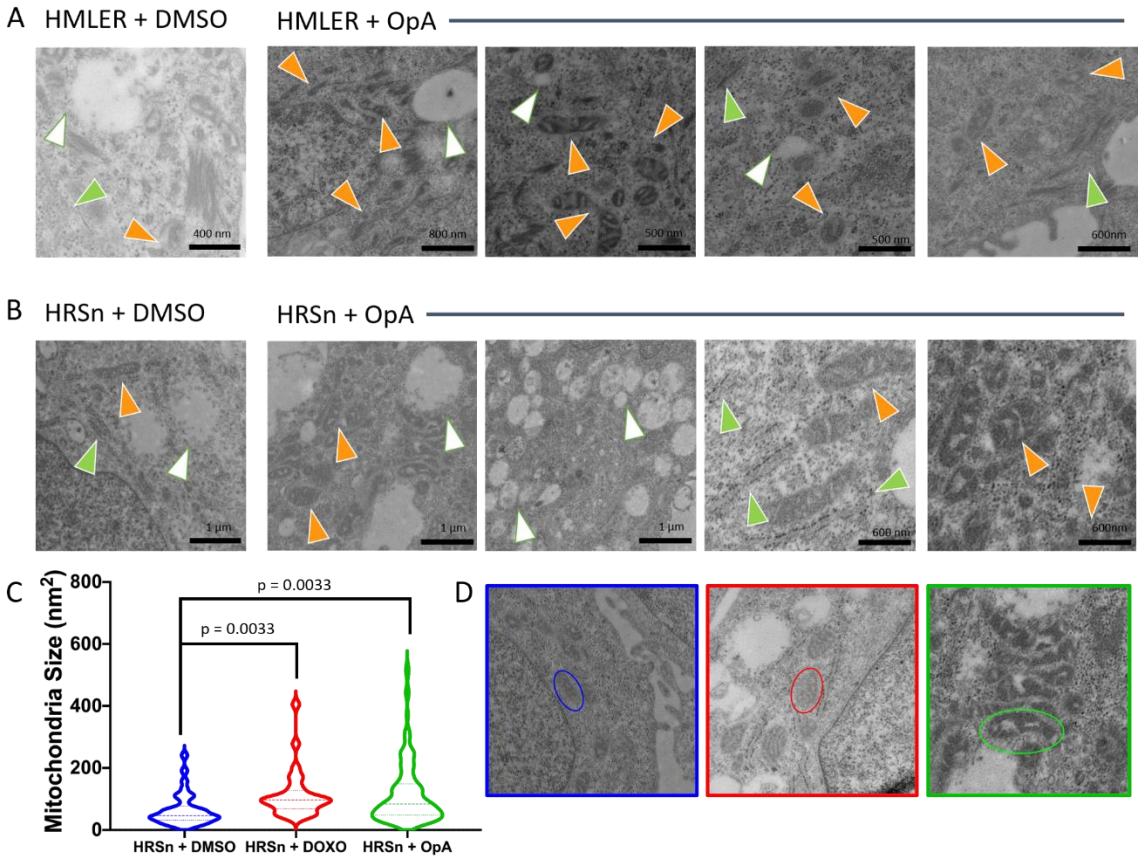

Figure 4.4 OpA increases mitochondrial size compared to control. HMLER ± Snail cells treated with 0.3µM OpA, 2µM doxorubicin (Doxo), or DMSO, 60 minute exposure before harvest. (A/B) Representative images of (A) HMLER and (B) HMLER-Snail cells from TEM imaging, scale bar as shown. White arrows indicate vacuoles; orange, mitochondria; green, endoplasmic reticulum. (C) Area of all mitochondria observed in collected images, normalized based on image scale. Students t-test, p values provided. (D) Representative images of mitochondria from each indicated treatment of HRSn cells. Blue is DMSO; Red, Doxo; Green, OpA.

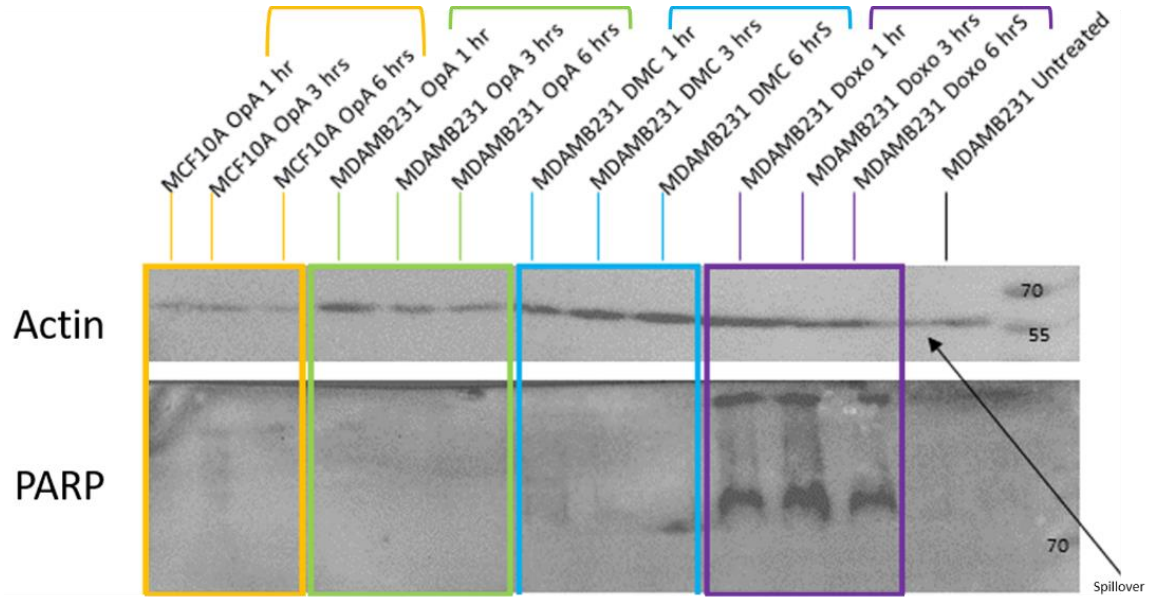

Figure 4.5 OpA does not induce apoptosis in breast cells. Representative western blot showing PARP protein expression and cleavage protein expression. Image cropped to show relevant bands. Full-length blots available.

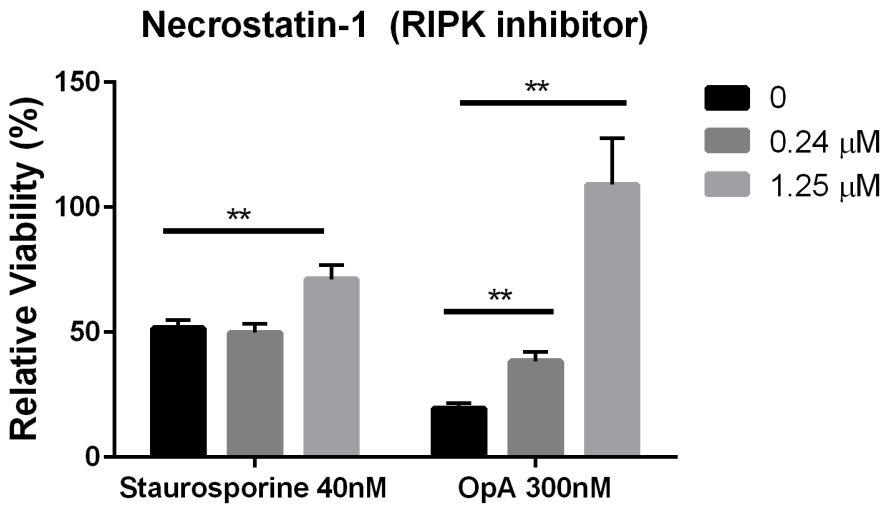

Figure 4.6 Ophiobolin A-driven cytotoxicity is sensitive to necroptosis inhibition. MDA-MB-231 cells treated for 72 hours with indicated doses. Students t-test, ** $p < 0.01$

Discussion

We previously described differential sensitivity to OpA in cells that had undergone EMT and gained CSC phenotypes.[372] Targeting the EMT and CSC-rich subpopulations of breast cancer promises improved therapeutic outcomes, reduced recurrence of disease, and improved survival in patients. We investigated the mechanisms by which OpA induces toxicity in mesenchymal cells by treating mitochondrial cybrids in which epithelial and mesenchymal mitochondria had been introduced into the opposite nuclear backgrounds. Prior studies have indicated the importance of mitochondrial crosstalk with the nucleus to alter phenotypes like energy usage and metabolism, aging, and metastasis.[482,499] Specifically in EMT settings, mitochondria are demonstrated to drive the program's activation. For example, phosphorylation of mitochondrial STAT3 drives p53/RAS signaling in ovarian cancer cells, which then activates EMT through Slug.[500] In breast and lung cancer cell lines, mutations in mtDNA genes ND4, ND5, and ND6 increase experimental metastasis.[501,502] We find that OpA sensitivity is abrogated in mesenchymal cells when epithelial mitochondria are introduced, and sensitivity is gained in epithelial cells when mesenchymal mitochondria are introduced. This indicates that OpA's toxicity is enacted through the mitochondria, either directly through impact to the organelle or through interruption of crucial mitochondrial-nuclear crosstalk. If OpA is impacting the function of mitochondria, one of the means to assess changes is through evaluation of the ETC. We observed changes in only one complex of the ETC, complex III, supporting the hypothesis that the mechanism-of-action for OpA occurs within the mitochondria.

We further sought to understand the impact of OpA on the mitochondria, as well as other organelles, through phenotypic changes, evaluated through microscopy. We were

able to identify cytoplasmic vacuolization using both brightfield and transmission electron microscopy. In both cases, vacuolization in EMT cells was more prevalent than in cells in which EMT had not be induced. We also noted changes in the widths of endoplasmic reticulum (ER) between EMT and non-EMT cells, though this difference was consistent in treated and non-treated cells, leading us to conclude that, while EMT may impact ER swelling, OpA likely does not play a significant role in this phenotype. In fact, our findings are consistent with studies linking EMT with activation of the unfolded protein response as a means to adapt to ER stress. [503,504] Other studies demonstrate that ER stress decreases epithelial markers and increases mesenchymal-like morphology.[505,506] Further, EMT is also implicated in stress to the mitochondria,[474,483] which is consistent with our data showing mitochondrial swelling in OpA-treated cells. We noticed an increase in intra-organelle vacuolization, which could be the driving factor to the increased size, rather than just larger organelles overall, though this would need to be confirmed with further experimentation.

These observed phenotypic changes prompted investigation into the mechanism of cell death activated following OpA treatment .We demonstrate that OpA does not activate apoptotic cell death. This is especially promising since many conventional chemotherapies that induce apoptosis fail to achieve success. We demonstrate that treatment with OpA does not induce cleavage of apoptosis-related PARP. Furthermore, we describe that OpA cytotoxicity is mitigated by co-treatment with RIPK inhibitor, necrostatin-1. These data drive interest in further elucidating necrotic or necroptotic mechanisms of cell death as necessary for OpA cytotoxicity. Nec-1 is widely used to target RIP kinase activity, and it works through either the tumor necrosis factor-alpha receptor-1 (TNFR1) signaling pathway or by the IDO-kynurenine (IDO-K) pathway, both associated with an

inflammatory response.[507–514] RIPK is associated with EMT, whereby its silencing inhibits Wnt/β-catenin-promoted EMT.[515] The Wnt/β-catenin pathway is also associated with CSC progression and cancer metastasis.[516] Furthermore, RIPK expression is higher in CSCs than in normal cancer cells.[517] When RIPK is downregulated via siRIPK in bladder CSCs proliferation is inhibited in vitro and xenograft tumor formation decreases. Together, these findings implicate RIPK in promoting both EMT and CSCs, and as a potential effector of OpA cytotoxicity.

Overall, our data point to the role of mitochondria and a mesenchymal phenotype in promoting sensitivity to OpA. We suggest that OpA works through a non-apoptotic pathway, and shows potential reliance on RIPK-induced necroptosis. Future work to understand the intra-mitochondrial role of OpA and further uncovering clues about the activated programmed cell death pathway could promote improved therapeutic options to sensitize and eliminate EMT-driven CSCs.

Methods

Cell Lines

MCF10A and MDA-MB-231 cells were ordered from ATCC; HMLE, HMLER, HMLER-Snail, and HMLER-Twist cells were received as a generous gift from Dr. Sendurai Mani. Mitochondrial cybrids and associated non-cybrid cells were created and maintained by Dr. Benny Kaipparettu and his lab.

Breast cancer cells were cultured in Dulbecco's Modified Eagle's Medium (DMEM) (Corning Inc.; Kennebuck, ME, USA) supplemented with 10% fetal bovine

serum (FBS) (Equitech-Bio Inc.; Kerrville, Texas, USA) and 1X antibiotics (Penicillin/Streptomycin, Lonza; Basel, Switzerland). Cell lines maintained as in Chapter 3[372]. Cell lines were tested monthly for mycoplasma and validated via STR testing. Culture conditions were 37 ˚C, 5% CO2.

Reagents

Doxorubicin was obtained from Selleckchem (Houston, TX, USA), salinomycin from Cayman Chemicals (Ann Arbor, MI USA), dimethoxycurcumin from Cayman Chemicals, and necrostatin-1 from Thermo Fisher Scientific (Waltham, MA, USA). OpA was obtained as mentioned in Chapter 3.[372]

Viability

Cells were plated at 2,000 cells per well in 96-well plates and allowed to adhere overnight. Compounds, suspended in DMSO and diluted into PBS, or vehicle were added to the culture medium and incubated for 24, 48 (in the case of cybrid viability studies), or 72 (cybrid studies and necrosis inhibitor studies) hours at 37˚C, 5% CO2. Cell viability was monitored after different drug treatment using the standard 3-(4, 5-Dimethyl-2-thiazolyl)-2, 5-diphenyl-2H-tetrazolium bromide (MTT) assay and validated using the sulforhodamine B (SRB) based colorimetric assay to rule out artifacts from mitochondrial alterations in MTT activity. The optical density of solubilized formazan salts was assessed at 570 nm in a Tecan Infinite M200 microplate reader (Männedorf, Switzerland).

Cybrids

Cybrid creation and maintenance were performed by the Kaipparettu lab as described in Kaipparettu, et al., 2013.

Microscopy

Brightfield microscopy was used to image vacuolization. Cells were plated in triplicate in a 96-well plate and allowed to reach confluency (>80% surface coverage). Wells were gently washed with PBS to clear any floating/dead cells, as to not confound observations following treatment application. OpA, suspended in DMSO and diluted into appropriate culture medium, or vehicle were added to clean culture medium and allowed to incubate for the indicated time. Cells were imaged using Nikon Eclipse Ts2R with Nikon DS Qi2 camera. Analysis conducted on Nikon NIS Elements imaging software (Nikon Instruments Inc.; Melville, NY, USA).

Transmission electron microscopy (TEM) was used to image vacuolization, mitochondria, and endoplasmic reticulum. Cells were harvested according to standard protocol and fixed for 45 minutes using 2.5% glutaraldehyde and 45 minutes using 1% osmium, with 3 x 10-minute PBS washes between. Samples were then dehydrated with increasing concentrations of ethanol and allowed to polymerize following infiltration with increasing ratios of resin to ethanol. Samples were imaged using Transmission Electron Microscope JEM-1010 (JEOL Inc.; Peabody, MA, USA) with XR16 (16 Megapixel) digital camera (AMT; Woburn, MA, USA)

Image analysis

TEM images were quantified using ImageJ whereby the area of the cell in each image was outlined, eliminating any blank space captured in the micrograph, then normalized based on scale bar. The same was repeated for each identified mitochondria in each sample image. Normalization followed the equation: area (686 pixels / 1 inch) (1 nm / scale bar pixel value). Protocol based on Lam et. al., 2021.[518]

Western blotting and antibodies

Cells were lysed in the presence of 100 microliters radio-immunoprecipitation (RIPA) buffer containing protease inhibitors (Alfa Aesar; Stoughton, MA, USA) on ice. Protein was quantified using the Bradford Assay (BioRad; Hercules, CA, USA). Twenty micrograms of total protein from each sample was resolved on a 4%–12% SDS-PAGE gel and transferred to PVDF membranes. Sister blots were then probed with antibodies anti-PARP (BD Biosciences; San Jose, CA,, USA) and anti-β-actin (BD Biosciences) antibody. Chemiluminescent signals were detected with ECL™ prime (Thermo Fisher Scientific) using the Biorad ChemiDoc system. If necessary, blots were stripped with ECL Stripping Buffer (Li-Cor; Lincoln, NB, USA) following manufacturer protocol. Bands were quantified using ImageJ.

Statistical analysis

Unless otherwise stated, statistical differences were determined using a student's t-test. The GraphPad PRISM software v6 was used to perform these analyses. Statistical

significance levels are annotated as n.s. = non-significant, *p < 0.05, **p < 0.01, ***p < 0.001, ****p < 0.0001.

Acknowledgments

TEM samples were prepared and imaged under the guidance of Dr. Bernd Zechmann. We thank collaborators the Kaipparettu lab, especially Dr. Junhyoung Park, for work on the mitochondrial cybrids, mtDNA copy number, and ETC experiments. Thank you as well to Dr. Ben Cravatt and Dr. Stephan Hacker for their work on OpA ligand interaction. Thanks also to Drs. Kornienko and Evidente for supplying purified OpA.

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CHAPTER FIVE

Anti-Cancer Activity of Natural Products, Congeners, and Derivatives

This chapter contains material from publications:

Gayler, K. M., Kong, K., Reisenauer, K., Taube, J. H. & Wood, J. L. STS Analogs Via C–H Borylation. *ACS Med. Chem. Lett.* **11**, 2441–2445 (2020). Reproduced with permission.

Tao, Y., Reisenauer, K., Taube, J. H. & Romo, D. Total Synthesis and Anticancer Activity of (+)-Hypercalin C and Congeners. *Angewandte Chemie* **131**, 2760–2764 (2019). Copyright Wiley-VCH GmbH. Reproduced with permission.

Tao, Y. *et al.* Pharmacophore-Directed Retrosynthesis Applied to Ophiobolin A: Simplified Bicyclic Derivatives Displaying Anticancer Activity. *Org. Lett.* **22**, 8307–8312 (2020). Reproduced with permission.

Introduction

Understanding the role of natural products as cancer therapies requires work across multiple fields of research. Contained within this chapter are the biological characterizations for three published studies focused on the natural products staurosporine, hypercalin C, Ophiobolin A (OpA), as well as natural congeners and synthetic derivatives.

Staurosporine

Staurosporine (STS), isolated by Ōmura and co-workers in 1977 from *Streptomyces staurosporeus*, exhibits potent inhibitory properties against a vast majority of the human kinome.[1,2] In cancers overexpressing human epidermal growth factor receptor, STS sensitivity is positively correlated to *EGFR* (otherwise known as *HER*-1) overexpression.[3] The activation of HER proteins promotes kinase-driven signal transduction pathways such

as MAPK/ERK and PI3K/AKT[4], which correlates with the identified kinase-inhibition activities of STS. STS is also associated with activation of apoptosis in lung carcinomas through the release of cytochrome c from the mitochondria.[5] Further, STS also induces mitochondrial dysfunction before nuclear apoptosis is activated, which is especially important in circumventing resistance in non-small cell lung carcinoma, where caspase-dependent cell death pathways are less efficient.[6–8] STS also induces apoptotic activity in pancreatic cells through p27, encoded by *CDKN1B* and responsible for cellular control of growth and differentiation, but not with G9R point mutation in p27.[9]

Since its isolation, numerous drug discovery efforts have attempted to capitalize on the potency of STS and several other naturally occurring indolocarbazole-containing natural products (ICZs) through the preparation of semisynthetic analogs. The ICZ moiety of STS has been demonstrated to play a key role in binding to the adenosine triphosphate (ATP) pocket of kinases, which acts by preventing the transfer of phosphate from DNA to the activated tyrosine site, directly inhibiting the activity of topoisomerase II.[10] As a pan-kinase inhibitor, STS has a wide variety if targets.[11] Related to its role in apoptosis induction, STS complexes with cAMP-dependent protein kinase and CDK2.[12] Studies focused on overcoming STS's promiscuous binding found success using methods like clickable cell-permeable probe to identify cellular targets. One study found STS binds to 18 protein kinases, 4 tyrosine kinases (c-Src, YES, CSK and BTK), 10 serine/threonine kinases, and several non-protein kinases (ex: lipid kinase, phosphofructokinase, pyruvate kinases).[13] Following confirmational pull-down and immunoblotting experiments, STS was unequivocally bound to ribosomal protein S6 kinase alpha-2 (RPS6KA),

phosphofructokinase (PFKM), creatine kinase B (CKB), phosphoribosyl pyrophosphate synthetase 2 (PRPS2), and adenosine kinase (ADK) in hepatic cells.

Although the homologous nature of the ATP binding sites in the human kinome has made improving selectivity and potency quite challenging,[14–16] several compounds have moved into clinical trials (e.g., CEP-1347) and at least one (e.g., Midostaurin) has been approved for use in cancer treatment.[17] Modifying the ICZ core in STS has been a central part of an effort to fill the void in structure-activity relation (SAR) studies, so we began investigating C–H activation borylation as an orthogonal approach. Through this methodology, we accessed several novel STS analogs that we investigated for biological activity to define the selectivity and potency of these molecules and to uncover the relevancy of borylation as a means to prepare biologically relevant STS analogs that would otherwise be inaccessible.

Hypercalin

The hypercalins and chinensins are members of the dearomatized, polyprenylated polycyclic acylphloroglucinol (PPAP) family of natural products isolated by Hostettmann from *Hypericum calycinum L.*[18] Several PPAPs undergo further oxidative cyclizations leading to polycyclic members that have attracted considerable synthetic interest;[19] however, the hypercalins and chinensins have not yet been synthesized. These natural products display minor structural differences, namely, substitution at the quaternary carbon center (C4, R^1) and the acyl group (C27, R^2). Members of this family possess a range of biological activities including antibacterial, antiviral, and cytotoxic.[20] For example,

hypercalins A–C showed growth-inhibitory activity against the Co-115 human colon carcinoma cell line with ED50 values ranging from 0.60–0.83 μM.[18]

In terms of anticancer activity, hypercalin C is one of the most potent members of this family.[18] We therefore targeted this congener and designed analogues for synthesis toward the goal of interrogating its proposed anticancer activity.[21]

OpA

The ophiobolins are fungal-derived sesterterpenoids consisting of over 50 members,[22,23] with ophiobolin A (OpA) being the first isolated and also one of the most bioactive congeners.[24–26] This family of natural products is drawing increasing interest[23,27,28] not only because of their high potency against various cancer cell lines[28,29] (e.g., chronic lymphocytic leukemia (CLL), IC_{50} = 1 nM;[29] P335, IC_{50} = 63 nM;[27] glioblastoma Hs683, IC_{50} = 620 nM[30,31]) for OpA but also for their unique cellular effects and selectivity observed. For example, both OpA and B[24] show selectivity toward cancer stem cells (CSCs) by significantly reducing the CD44(hi)/CD24(-/low) population in an MDA-MB-231 cell line.[32] Moreover, the ability of certain ophiobolins to induce paraptosis [28,30,33] or autophagy[34] makes these natural products valuable leads toward apoptosis-resistant cancer cells. Our interest in the ophiobolins stems from their complex structures, unique bioactivities and unknown cellular targets, and was further enhanced by our finding that OpA, but not 3-deoxy OpA, specifically induces cell death in stem-like cells with epithelial-mesenchymal transition (EMT) properties.[35] Several structure–activity relationship (SAR) studies of the anticancer effects of ophiobolins, derived mainly from derivatization of OpA or congeners, led us to apply our recently described pharmacophore-

directed retrosynthesis (PDR) strategy[36,37] to elaborate on SAR information with respect to effects on stem-like cancer cell. Pharmacophores are small portions of a molecule that are sufficient to induce recognition of a ligand by that molecule. From a biological perspective, pharmacophores that share similar toxicities as the "parent" molecule are likely to be sufficiently able to induce similar change and also inform the portion of the molecule that is most biologically relevant. We investigated several OpA pharmacophores to uncover the biologically active portion of the compound.

Results and Discussion

Anticancer Activity of Staurosporine Analogs

In our studies, we evaluated the cytotoxicity of STS and novel phenol analogs **13** and **14** against breast cell lines including tumor-derived MDA-MB-231 cells and nontransformed, immortalized, HMLE cells (Figure 5.1). The activities of **13** and **14** against the MDA-MB-231 cell line were found to be equipotent to STS. Interestingly, these studies also revealed that inclusion of a phenol at either the C9 or C10 position selectively diminishes activity against HMLE with 9-OH- and 10-OH-STS (**13** and **14**) being 5.6-fold and 12.0-fold less potent, respectively. Thus, activity toward the nontransformed mammary cell line, HMLE, was compromised indicating the possibility of an increased therapeutic index. (Figure 5.1)

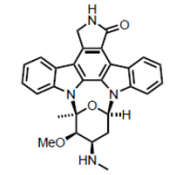
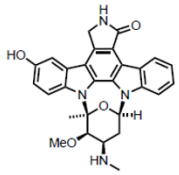
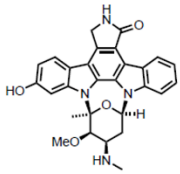
		MDA-MB-231		HMLE	
	Compound	**IC_{50} (nM)**	**SD**	**IC_{50} (nM)**	**SD**
	Staurosporine (**1**)	2.5	±0.2	4.9	±0.4
	**13**	2.0	n.d.	27.5	±10.8
	**14**	3.4	±1.3	58.7	±20.1

Figure 5.1 Cytotoxic Activity of Staurosporine (1), 13, and 14. MDA-MB-231 = human breast adenocarcinoma cell line, HMLE = immortalized human mammary epithelial cells, IC_{50} = half maximal inhibitory concentration, SD = standard deviation.

Anticancer Activity of (+)-Hypercalin C and Congeners

We measured the cytotoxicity of synthetic hypercalin C and its derivatives against HCT-116 colon cancer and MDA-MB-231 breast cancer cell lines (Figure 5.2). The bare cyclopentyl moiety bearing an additional alcohol **8** or aromatic moieties in place of the cyclohexanetrione, namely arene derivatives **15** and **13**, were not cytotoxic up to 100 μM. These results suggest that the cyclopentyl moiety alone, though rich in stereochemical information, does not significantly contribute to the observed cytotoxicity. This is further supported by derivative **21**[38] which retains some of the original cytotoxicity (6.5–7.7X decrease) while only possessing a simple isopentyl chain in place of the complex cyclopentane found in hypercalin C. Interestingly, the presence of oxygen in the arene ring, see derivative **13**, but a lack of acidic protons led to the initially observed cytotoxicity (at

less than 100 μM). The cytotoxicity of synthetic hypercalin C was found to be in the 3–4 μm range, consistent with previous reports;[18] however, dimethyl hypercalin C (**2**) showed a measurable decrease in cytotoxicity (approximately 5–7X). While this lends some credence to the proposed requirement of acidic enols for cytotoxicity, the difference is not significant enough to exclude alternative hypotheses regarding the mode of action of the hypercalins. On the other hand, the hydrogenated derivative **22** showed similar activity to the natural product, indicating that the unsaturation of the prenyl side chains has minimal effect on cytotoxicity.

Ophiobolin A Pharmacophore Anticancer Activity

We then assayed the cytotoxicity of the racemic, simplified OpA derivatives synthesized toward a breast cancer cell line (MDA-MB-231) and a noncancerous cell line (MCF-10A), to probe selectivity, if any, in comparison to OpA and a few naturally occurring OpA congeners (Figure 5.3). While significantly less potent than OpA, simplified bicyclic OpA derivatives (±)-**10** and (±)-**38** bearing the proposed pharmacophore displayed cytotoxicity (~7 and 11 μM, respectively) against MDA-MB-231 cells. The activity of the highly simplified and unstable ketoaldehyde (±)-**11** was unsurprisingly less active (~44 μM). The selectivity observed with OpA between a cancer vs noncancer cell line (~69 vs 200 nM, ~3-fold difference) is erased with the structurally simplified bicyclic derivatives (±)-**10** and (±)-**38**. The similar potency of these simplified bicyclic derivatives is noteworthy and suggests that the C3-stereochemistry as either a secondary or tertiary alcohol has little effect on bioactivity. Overall, the described SAR

data with racemic bicyclic derivatives of OpA suggest that cytotoxicity and selectivity increase in parallel with increasing complexity as expected.

A few closely related congeners of OpA were also assayed. Direct comparison of OpA and (+)-6-epi-OpA suggests the importance of the C6 relative stereochemistry for

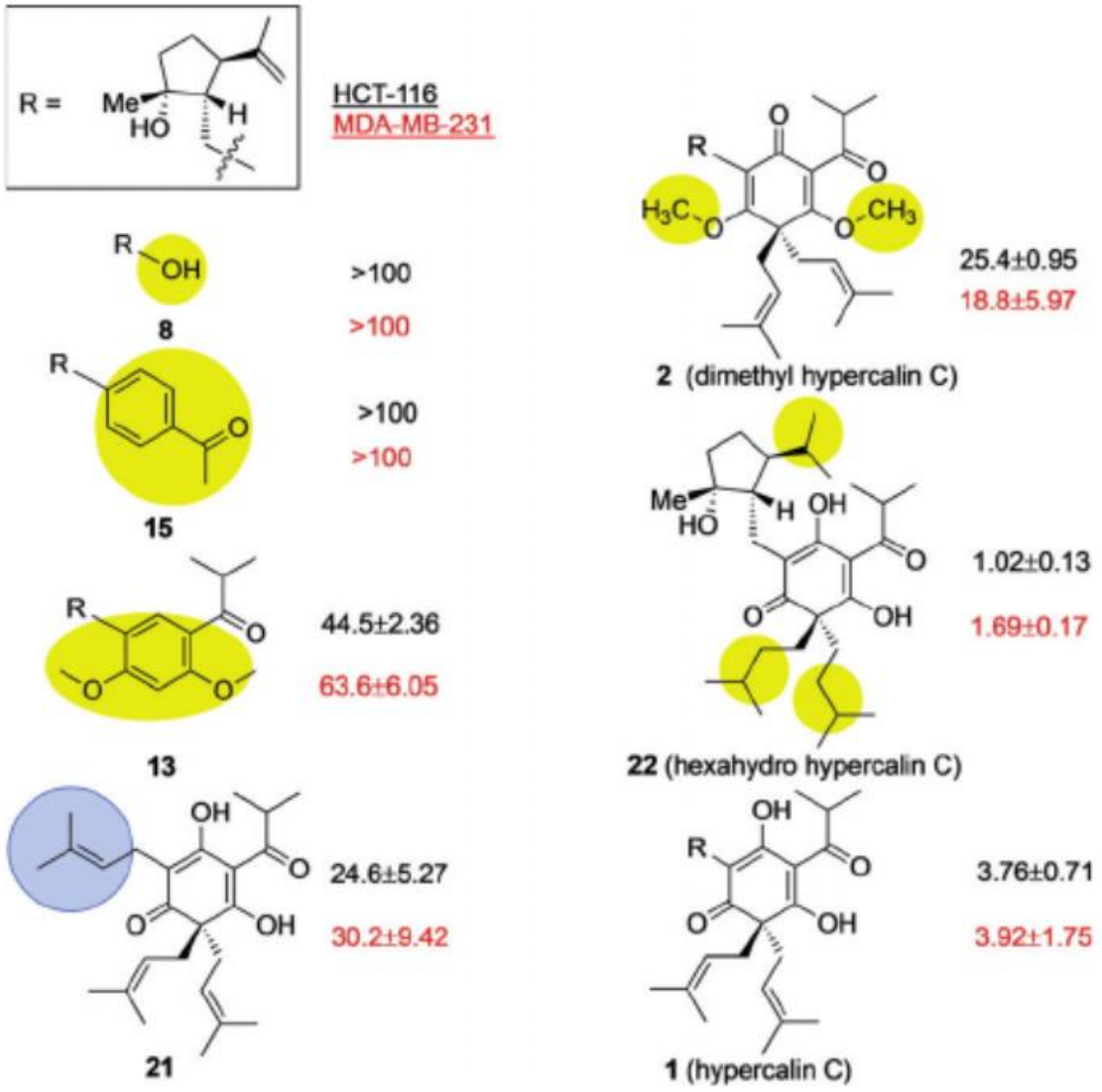
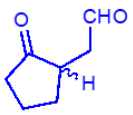
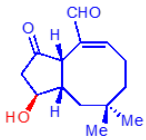
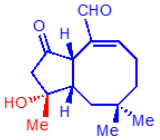
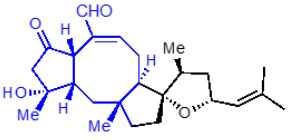


Figure 5.2 Cytotoxicity of hypercalin C and derivatives against HCT-116 and MDA-MB-231 cell lines IC50. IC_{50} (μM) values were determined for the indicated compounds with MDA-MB-231 (red) and HCT-116 (black) cells in vitro. Standard error of the mean represents the variation of three biological replicates, each with at least three technical replicates

cytotoxicity toward this breast cancer cell line leading to an ~12-fold drop in activity (~69 nM vs ~0.8 μM, Figure 5.3). Other structural changes also impact cytotoxicity including loss of the C3-hydroxyl through β-elimination in conjunction with epimerization at C6 leading to an 8-fold drop in activity (cf. (+)-3-anhydro-6-epi-OpA). Retaining the C6-stereochemistry but dehydration in combination with reduction of the aldehyde to the primary alcohol as found in (+)-OpI leads to a significant loss in cytotoxicity (~10-fold drop vs OpA) with insignificant selectivity between the cancer vs noncancer cell line, ~4.9 vs 4.2 μM, respectively.

OpA & Derivatives / Cell lines (IC_{50} μM)	 (±)-11	 (±)-38	 (±)-10	 (+)-ophiobolin A
MCF10A	197 ± 14.4†	13.4 ± 3.10	6.36 ± 1.99	0.198 ± 0.076
MDA-MB-231	44.2 ± 16.1†	11.0 ± 0.935	7.09 ± 2.20	0.069 ± 0.024

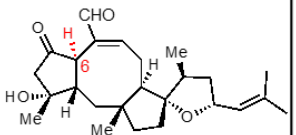
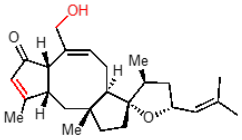
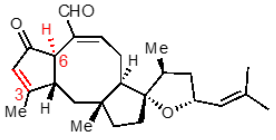
OpA & Derivatives / Cell lines (IC_{50} μM)	 (+)-6-epi-ophiobolin A	 (+)-ophiobolin I	 (+)-3-anhydro-6-epi-ophiobolin A
MCF10A	1.74 ± 0.614	4.16 4 ± 0.763	4.16 ± 0.763
MDA-MB-231	0.80 ± 0.28	0.552 ± 0.124	4.94 ± 0.921

Figure 5.3 Cell Viability Data of Synthesized, Simplified Analogs of Ophiobolin A and Natural Congeners. IC50 values are denoted in mean ± standard deviation of at least 3 independent replicates. (†) Ketoaldehyde (±)-11 was unstable in DMSO, and thus a freshly prepared sample was required to preclude inconsistencies observed when using stored DMSO stock solutions.

Conclusion

We analyzed three natural products, congeners, and derivatives in non-transformed mammary cells and in breast and colon cancer cell lines to understand their toxicity following novel synthesis approaches and in comparison with analog models. Viability was assayed across all compounds in MDA-MB-231 triple negative breast cancer cells. STS was 50% effective at 2.5 ± 0.2nM, hypercalin C at 3.92 ± 1.75μM, and OpA at 69 ± 24nM. Interestingly, one derivative of hypercalin C and one derivative of STS were more toxic to MDA-MD-231 cells than the unmodified counterparts, namely STS compound **13** (2.0nM), hexahydro-hypercalin C (1.69μM). No modified versions of OpA were more toxic than the unmodified version. Understanding the biological effects of natural products, their derivatives, and deciphering the smallest portion of the molecule that retains activity can further inform pharmacological and clinical applications of these molecules in the future.

Materials and Methods

Cellular Viability

Solid compounds were re-suspended in DMSO to yield a final concentration of 5 mM STS, 100 mM Hypercalin C and congeners, and 25 mM OpA and pharmacophores. Aliquots were stored at -80 ºC. Cells were plated on 96-well plates with 2,000 cells per well in appropriate media with 10% FBS. After 24 h, compounds were added to media and allowed to incubate for 72 h at 37 ºC, 5% CO2. Relative cell metabolic activity was

assessed by incubation with MTS assay reagent for 3 h (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega) according to the manufacturer's protocol.

Background absorbance (media only) was subtracted from all other wells and absorbance was then normalized to DMSO treatment at matching concentrations. The normalized relative viability values were graphed against the drug dosage and IC_{50} (drug concentration eliciting 50% of the maximum inhibition) values were calculated for each tested cell line using the "log(inhibitor) vs. response -- Variable slope" function in Prism6 (Graphpad). The experiments were repeated again at an additional two time points.

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CHAPTER SIX

Conclusion

Research Objectives

Successfully eliminating or sensitizing cancer cells to existing therapies requires innovative approaches. The goal of this work was to pursue natural products capable of targeting of cancer stem cells (CSCs) with a focus on Ophiobolin A (OpA) and how it preferentially kills cells that have undergone epithelial-mesenchymal transition (EMT). Our work evaluates toxicity of CSC-rich triple negative breast cancer cells to natural product OpA.

Our initial interest in OpA followed unusual results in which cells that had undergone EMT were more sensitive to the compound. The mesenchymal phenotype resulting from EMT not only promotes progression through the metastatic cascade, but also confers stemness properties through the initiation of CSCs.[31,32,544,545] In our in vitro models of EMT as well as in breast cancer cell lines, cells with increased EMT were more sensitive to OpA.[372] This research clearly illustrates the EMT requirement *in vitro*, but it also raises the question of whether OpA is able to impair EMT and CSC related progression of disease *in vivo*. While well-tolerated, we did not observe changes in metastatic burden using our model and further research is required to identify such changes.

EMT and CSC signatures both confer resistance to mechanisms of cell death, namely apoptosis, and experience metabolic changes related to their increased tumorigenicity.[474,481] OpA was originally investigated as a phytotoxin, and, interestingly,

early studies describe metabolic changes following treatment, mediated by OpA-induced changes to calcium transport.[69,350,351] This prompted us to investigate mitochondrial effects of OpA. One of the best means we had to study the crossover between mitochondria and EMT in OpA toxicity was by initiating a collaboration with the Kaipparettu lab to use their mitochondrial cybrid models.[482] We found that OpA sensitivity increased in the presence of mesenchymal mitochondria, even when against a nuclear background, and that sensitivity was lost in models where epithelial mitochondria were introduced to mesenchymal cells. These data were striking and solidified that OpA cytotoxicity works through mitochondria in cells that have a mesenchymal phenotype.

Since our objective was to investigate how OpA promotes cell death, we investigated the mitochondria during cell death activation. Previous research offers limited insight into cell death activation and the means by which OpA promotes cytotoxicity,[62,70,71] especially in breast cancer models. We observed notable differences in mitochondrial appearance and function following OpA treatment, which supported our hypothesis that OpA acts in a mitochondria-dependent manner. Further, we accumulated evidence for non-apoptotic cell death, but recommend further investigation into these findings, as discussed below.

Through extensive collaboration, we explored the biological effects of natural product pharmacophores[546] and derivatives[547,548]. Staurosporine, a pan-kinase inhibitor that has moved into clinical trials[535], is the most extensively studied, but is the least toxic to TNBC cells. Hypercalin C also demonstrated equally low-toxicity to cancer cells, but the work conducted in this study is helpful to inform future chemistry and synthesis experiments. It was promising to see that OpA maintained its selectivity against TNBC

cells and that the compound in its entirety is the most effective chemical structure. Toxicity measurements support the proposed pharmacophore and further suggest that cytotoxicity and selectivity increase in parallel with increasing complexity. The pharmacological studies conducted with structural sub-components provided valuable insight into future studies that may seek to establish a synthetic version of OpA.

Recommendations and Contributions

OpA is not the only natural product to suppress EMT and CSC phenotypes. Genistein suppresses both EMT and CSCs in prostate, head and neck, bladder, and ovarian cancers. Pancreatic, oral and breast cancer CSCs and EMT are suppressed following curcumin treatment. This shows promise for OpA, as genistein and curcumin have successfully entered clinical trials. Further, work with genistein and curcumin have informed synthesis of derivative forms that enhance in vivo bioactivity.

The work conducted with genistein and curcumin, while helpful in understanding the role of natural products in disrupting EMT and CSCs, tend to investigate these processes retroactively. Reduction in the EMT gene signature or CSC proportions are shown as helpful side-effects following treatment Our work offers an approach that centralizes EMT and CSCs, describing a reliance on the CSC and mesenchymal phenotypes. Instead observing changes in EMT-activated cells and CSCs *ex post facto,* our work shows the importance of these phenotypes in sensitizing cells to OpA toxicity. . This research with OpA demonstrates that the EMT/CSC axis is not only an essential part of OpA toxicity, but that the unique changes induced by EMT and present in CSCs, such as metabolic shifts and changes in cell death, aid in promoting OpA's effects. This unique

approach, rather than retroactive analysis, indicates potential therapeutic targets and "weaknesses" in these cells that OpA is taking advantage of to confer cell death.

As previously discussed in chapter 4, mitochondrial dysfunction is consistent with both EMT and CSC phenotypes.[474] We provide initial findings that support this, such as changes in mtDNA copy number and changes in the electron transport chain activity. However, further research that more deeply explores the intersection of metabolic changes in EMT-derived CSCs could provide valuable insight into OpA cytotoxicity. Focusing on changes in ion transport would be especially helpful because existing literature points to these changes following OpA treatment[62,68,69] as well as in promoting the retrograde signals that promote EMT.[478,480,481]

Our study offers suggestive evidence for mitochondrial-dependent mechanism of action, which we observed following transmission electron microscopy. In this study, we uncovered two key findings that should promote further investigation. The first being that mitochondria size increases with OpA treatment. Swelling is consistent with both necroptotic and paraptotic cell death, so further studies are needed to provide further evidence for one or the other. Secondly, we noticed that the endoplasmic reticulum (ER) did not undergo significant changes following OpA treatment, but we did notice that there was a difference between EMT and non-EMT cells. As discussed, ER stress is common with EMT induction.[503–506] Because cells that have undergone EMT are more sensitive to OpA, it could be that any OpA-driven effects on the ER are redundant or obscured by an already-stressed ER environment in EMT cells. Existing research links OpA and ER stress,[68,549] which would support the hypothesis that OpA still does mediate ER stress but no studies have been done in an EMT background to tease apart these mechanisms.

Uncovering the mechanism of programmed cell death responsible for conferring OpA toxicity to mesenchymal breast cancer cells is yet to be concretely elucidated. Existing literature describes a variety of mechanisms induced following OpA treatment including apoptosis, autophagy, and paraptosis.[62,68,71,72] Our findings indicate that apoptosis is not activated and that the mechanism of cell death may be reliant on RIPK, associated with necroptosis. To better understand the implications of these results, future studies could address additional markers of cell death pathways such as caspases 3 and 8, both of which play a role, either directly or indirectly, in apoptosis and necroptosis. Paraptosis has not been as well described as the aforementioned cell death pathways, yet evidence points to both reliance on protein synthesis and potential inhibition of AIP-1/Alix proteins.[302,550,551] Further experiments that interrupt protein synthesis, perhaps through cycloheximide treatment, and promote AIP-1/Alix expression, perhaps through constitutive overexpression, in conjunction with OpA treatment would provide further insight into the effects of OpA-driven cytotoxicity.

Final Remarks

The initial scope of this research was to use natural products against EMT and CSCs in TNBC. Selectivity in EMT cells against OpA showed promise and sparked interest in the extent to which the compound could reduce in vitro effects like migration, sphere formation, and resistance to chemotherapies. Successes here expanded work into more complex biological systems and showed early promise by reducing primary tumor burden. As our work continued to unveil the activities of OpA, we sought to go beyond our initial scope and further explore the mechanisms through which OpA enacted its toxicity.

Through collaboration with experts in biochemistry, microscopy, and mitochondria biology, we have begun to unpack the complex activity of this natural product.

It is clear that there are many overlapping themes that will contribute to continuing future work studying OpA. We clearly demonstrate the dependency on mitochondria in OpA-driven cell death, as well as activation of the EMT program. Early studies in OpA as a phytotoxin in plant models have supported our findings and may continue to provide clues into OpA's mechanism of action. Continued success in this line of research will undoubtedly provide evidence to support clinical application and improved outcomes for TNBC patients.

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