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Potential Applications of Capsaicinoids in Small Cell Lung Cancer Therapy

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POTENTIAL APPLICATIONS OF CAPSAICINOIDS IN SMALL CELL LUNG CANCER THERAPY

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APPROVAL OF THESIS

We, the faculty supervising the work of Jamie Rae Friedman, affirm that the dissertation, *Potential Applications of Capsaicinoids in Small Cell Lung Cancer* meets the high academic standards for original scholarship and creative work established by the Biomedical Sciences Program and the Joan C. Edwards School of Medicine. This work also conforms to the editorial standards of our discipline and the Graduate College of Marshall University. With our signatures, we approve the manuscript for publication.

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DEDICATION

I would like to dedicate this work to my family (both human and canine) who have supported me throughout all of my studies. To my parents, Steve and Kathy, always told me I could achieve anything I wanted and stayed by my side through the ups and downs. To my brother, Scott, who was always a phone call away to make me laugh and watch sports together even though we were 8 hours away. My parents and brother let me talk about my project often, even though they usually didn't have a clue what I was talking about. To my Pop-pop and Mom-mom, Stan and Alma, who always were interested in my research, facetiming me almost daily to check in. My aunts and uncles always sent words of encouragement to me, reminding me that eventually I would actually finish. I also can't forget to mention the Friedman Dogs, Jake, Sosa, and Josie. Josie has worked hard by my side (usually napping) so that she could earn her puppy PhD with me. Lastly, I'd like to dedicate this to my grandparents who were diagnosed with lung cancer and inspired my interest in cancer related research, Pete and Rae. And to my Grammy, Shelia, who I know has been with me through this whole journey. I love you all.

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ABSTRACT

Lung cancer continues to be the leading cause of cancer related mortality worldwide. Lung cancer is not a single disease but an umbrella that encompasses two major classifications, non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). SCLC represents about 15-20% of all lung cancer cases and is almost exclusively diagnosed in smokers. Typically, patients will respond very well to first line treatment, but face inevitable relapse. The fact that SCLC still carries a grim 5-year survival rate of less than 5% highlights the lack of advancement in treatment options to effectively improve patient response and survival. Capsaicinoids, in particular Capsaicin (the spicy compound in chili peppers), have previously been reported to be an advantageous adjunct treatment with traditional chemotherapeutic options in several cancer types. One challenge to the use of capsaicin is the variety of side effects, such as gastrointestinal pain, sweating and ulcers that are frequently reported with clinical administration. Synthetic non-pungent capsaicinoids, which show many of the same bioactive properties as capsaicin may, however, be a promising alternative. The studies in this dissertation investigated the use of capsaicin and several non-pungent analogs as chemotoxic or adjuvant therapy for SCLC. Utilizing various *in vitro* and *in vivo* models we investigated the synergistic effects of capsaicin and camptothecin. We provide new evidence that capsaicin synergistically sensitizes SCLC to the effects of camptothecin, inducing a rise in intracellular calcium levels and activating the calpain pathway to induce apoptosis. Analysis of the antineoplastic capacity of various capsaicinoid analogs found arvanil to be the most potent capsaicinoid at inducing apoptosis in SCLC cell lines. Similar to capsaicin, arvanil also induced apoptosis in SCLC cell lines by raising intracellular calcium levels leading to increased calpain activity. The chemotoxic potency and non-pungent character of arvanil supports the future investigation of the adjuvant use of

arvanil with camptothecin or other chemotherapeutic agents to find a combination therapy that provides the same synergistic effects as capsaicin, while lacking the adverse side effect profile. Taken together, these studies demonstrate that capsaicin and arvanil have the potential to successfully treat SCLC in combination with conventional chemotherapeutics, as well as possibly treating other cancer types.

CHAPTER 1: INTRODUCTION

LUNG CANCER AND CAPSAICIN

Lung cancer

In 2018 cancer was the second leading cause of death worldwide. The incidence of cancer in men has gone down slightly from 2008 to 2014, while the incidence of cancer in women has stayed consistent since 1999 ("Cancer," 2018; Cronin et al., 2018). The mortality associated with cancer, however, has decreased in both sexes (Cronin et al., 2018). Lung cancer is the second most common form of cancer in both men and women, second to prostate and breast cancer respectively. Lung cancer is the leading cause of cancer related death regardless of sex. The mortality rate for lung cancer is about 53 per 100,000 for men; approximately 2.8 times higher than for prostate cancer (Cronin et al., 2018). For women, the mortality rate is about 35 per 100,000, which is about 1.8 times higher than for breast cancer. Smoking has been identified as a leading risk factor for lung cancer. It is estimated that tobacco use causes between 80 and 90% of all lung cancers (Latimer & Mott, 2015). While higher smoking rates among men likely contribute to the apparent gender difference in lung cancer mortality rates, other risk factors for lung cancer including first- or second-hand smoke, as well as environmental exposures, such as asbestos and radon affect both genders equally.

Lung cancer is comprised of two major groups based on tissue histology, non-small cell lung cancer (NSCLC) and small cell lung cancer (SCLC). NSCLC, which accounts for approximately 80-85% of lung cancers, is further divided into subtypes; lung adenocarcinoma (LAC), squamous cell carcinoma (SCC), large cell carcinoma (LCC) and neuroendocrine carcinoid tumors (Friedman et al., 2019). Classification of each subtype of NSCLC is based on the tissue of origin of the tumor. LAC, which is the most common form of lung cancer,

originates from the mucus-secreting glands in the lungs and is typically found in the outer regions of the lung. SCC develops in the central air passages of a patient's lungs and is the most common type of NSCLC found in smokers. LCC is found anywhere in the lung and is characterized by rapid growth and spread. Each subtype is then further characterized by defined stages of progression (Latimer & Mott, 2015). NSCLC has a staging system ranging from stage 0 to stage 4, being the most critical. Treatment for NSCLC includes surgical removal for early stage disease. Later stages of NSCLC are treated with chemotherapy, radiation, and targeted immunotherapies. Generally speaking, NSCLC has a better survival rate, especially for those diagnosed in early stages (Latimer & Mott, 2015). In recent years, new treatment options have become available for NSCLC and have led to improved therapeutic outcomes and survival rates.

SCLC differs in a variety of ways from NSCLC. SCLC represents about 15-20% of all lung cancer cases. While NSCLC can occur in patients who are never-smokers, SCLC is found almost exclusively in smokers. SCLC arises from neuroendocrine cells of the lung (Alvarado-Luna & Morales-Espinosa, 2016). SCLC also generally spreads much quicker and more aggressively than NSCLC. There are no subtypes of SCLC, it is simply divided into two stages; limited where the cancer is confined to one lung or extensive when the cancer has spread to secondary locations outside of the single hemithorax region. About two-thirds of those diagnosed with SCLC are already in the extensive stage. Initial treatment consists of a platinum-based chemotherapy (cisplatin or carboplatin) combined with etoposide (Pietanza, Byers, Minna, & Rudin, 2015). Despite high initial response rates of 60-80%, SCLC commonly develops chemotherapeutic resistance, which leaves patients with no viable treatment options (Alvarado-Luna & Morales-Espinosa, 2016; Bunn et al., 2016; Pietanza et al., 2015). Irinotecan is used as a second-line agent in the treatment of refractory SCLC, however, it also induces frequent tumor

resistance in patients within 14-26 weeks (S. L. Wood, Pernemalm, Crosbie, & Whetton, 2015). Radiotherapy is utilized in patients whose cancer is confined to the chest, and prophylactic cranial irradiation is also used in some cases due to the common feature of secondary brain metastases in SCLC (Pietanza et al., 2015). When diagnosed in the limited stage, the 5-year survival rate for SCLC is about 25%. Extensive stage SCLC has a 5-year survival rate of almost zero (Latimer & Mott, 2015). Despite the urgent need for more effective therapy, treatment options for SCLC have not seen any significant progress in decades (Pietanza et al., 2015; Qiu et al., 2017). Current therapeutic modalities only extend patient survival by 4 to 8 months, compared to non-treatment. SCLC has been described as a "graveyard" for drug development research. Given the grim outlook for those diagnosed with SCLC, identifying effective, novel adjuvant or secondary treatment options is imperative.

Capsaicin

Natural compounds have been associated with medicinal benefits for hundreds of years (Basith, Cui, Hong, & Choi, 2016). Capsaicin is the main pungent ingredient isolated from chili peppers. It has commonly been utilized for its analgesic activity, and ability to treat pain and inflammation associated with a variety of diseases (Chapa-Oliver & Mejia-Teniente, 2016; X. F. Huang, Xue, Jiang, & Zhu, 2013). Capsaicin is considered to be the prototypical agonist of the transient receptor potential vanilloid (TRPV) 1 receptor (Elokely et al., 2016; Hazan, Kumar, Matzner, & Priel, 2015). The TRPV receptors are a family of cation channels and are responsible for our sense of temperature. The analgesic activity of capsaicin is believed to involve TRPV1 signaling (Jara-Oseguera, Simon, & Rosenbaum, 2008). Diabetic neuropathy, arthritis, and skin disorders are just a few of the maladies in which capsaicin has been shown to have potent therapeutic pain-relieving properties (Janusz et al., 1993; Luo, Peng, & Li, 2011; Rollyson et al.,

2014). Capsaicin has also been shown to have potent antineoplastic activity against several cancer types, including prostate, lung, and bladder (Basith et al., 2016; Chapa-Oliver & Mejia-Teniente, 2016; Pramanik, Boreddy, & Srivastava, 2011; Srinivasan, 2016). There has also been evidence that capsaicin has the ability to enhance the cytotoxic effect of FDA approved chemotherapeutic agents, suggesting a potential role for capsaicin as an effective adjuvant therapy to improve chemotherapeutic response- and survival-rates in SCLC (Dai et al., 2018; Vendrely et al., 2017; N. Wang, Chaoran, Zhang, Zhai, & Lu, 2018; Zheng et al., 2016).

A potential hindrance to the use of capsaicin as a therapeutic agent, particularly following oral administration, is the spectrum of unpleasant side effects associated with its pungent nature. These side effects, which often include gastric pain, sweating, ulcers, and tearing of the eyes, frequently cause patients to discontinue use, rendering any clinical trials useless or inconclusive (Fuhrer, Vogelsang, & Hammer, 2011; Hammer, 2006; Hammer, Fuhrer, Pipal, & Matiasek, 2008; Hammer & Vogelsang, 2007). One potential way to circumvent this issue is to explore the use of natural or synthetic derivatives of capsaicin (capsaicinoids), which may lack many of these adverse effects. Various capsaicinoids have been extensively evaluated in order to determine if they are able to maintain their anti-cancer activity, while minimizing their side effect profile. Many of these compounds show much promise as viable treatment options for lung cancer, as well as, other types of cancer (Luo et al., 2011; Macho et al., 2003).

Statement of hypothesis

There continues to be a lack of treatment options for those diagnosed with SCLC. The studies in this dissertation focus on evaluating two major aspects of the use of capsaicin as an adjuvant antineoplastic agent against SCLC to improve therapeutic response. The combinatorial effects of capsaicin with a current chemotherapeutic agent is assessed first based on the

hypothesis that capsaicin is able to sensitize human SCLC cells to the chemotherapeutic effects of camptothecin. These studies evaluate the possible synergy that occurs when these agents are used concurrently both *in vitro* and *in vivo.* Signaling mechanisms controlling these effects are also examined. Second, a study of a panel of synthetic capsaicin analogs was conducted to identify potentially potent anti-cancer agents with equal or greater antineoplastic activity than capsaicin, but without the adverse side effect profile. The signaling pathways activated by these analogs are also examined and compared to capsaicin. Taken together, these studies have laid the foundation for the use of capsaicin and capsaicinoids as potential adjuvant SCLC treatment options. The long-term implications of this study will hopefully lead a way to increasing the dismal survival rate of SCLC patients, as well as potentially utilizing the knowledge found to treat additional types of cancers.

CHAPTER 2: CAPSAICINOIDS ENHANCE CHEMOSENSITIVITY TO CHEMOTHERAPEUTIC DRUGS

A manuscript submitted to *Advances in Cancer Research.*

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ABSTRACT

Cytotoxic chemotherapy is the mainstay of cancer treatment. Conventional chemotherapeutic agents do not distinguish between normal and neoplastic cells. This leads to severe toxic side effects, which may necessitate the discontinuation of treatment in some patients. Recent research has identified key molecular events in the initiation and progression of cancer, promoting the design of targeted therapies to selectively kill tumor cells while sparing normal cells. Although the side effects of such drugs are typically milder than conventional chemotherapies, some off-target effects still occur. Another serious challenge with all chemotherapies is the acquisition of chemoresistance upon prolonged exposure to the drug. Therefore, identifying supplementary agents that sensitize tumor cells to chemotherapy-induced apoptosis and help minimize drug resistance would be valuable for improving patient tolerance and response to chemotherapy. The use of effective supplementary agents provides a two-fold advantage in combination with standard chemotherapy. Firstly, by augmenting the activity of the chemotherapeutic drug it can lower the dose needed to kill tumor cells and decrease the incidence and severity of treatment-limiting side effects. Secondly, adjuvant therapies that lower the effective dose of chemotherapy may delay/prevent the development of chemoresistance in tumors. Capsaicinoids, a major class of phytochemical compounds isolated from chili peppers, have been shown to improve the efficacy of several anti-cancer drugs in cell culture and animal models. The present chapter summarizes the current knowledge about the chemosensistizing activity of capsaicinoids with conventional and targeted chemotherapeutic drugs, highlighting the potential use of capsaicinoids in novel combination therapies to improve the therapeutic indices of conventional and targeted chemotherapeutic drugs in human cancers.

INTRODUCTION

The term capsaicinoid refers to the class of compounds found in the capsicum family (also known as chili peppers) (Luo et al., 2011). The most common capsaicinoid is capsaicin, responsible for the spicy characteristics of chili peppers. Other natural capsaicinoids isolated from peppers include: capsiate, found in Japanese CH-19 sweet peppers; capsiconiate, found in *Capsicum baccatum L.*; and resiniferatoxin (RTX), isolated from the cactus plant *Euphorbia resinifera* and the Nigerian plant *Euphorbia poissonii* (Friedman et al., 2018; Luo et al., 2011). All capsaicinoids display potent analgesic activity (Basith et al., 2016; Chapa-Oliver & Mejia-Teniente, 2016; Evangelista, 2015; Srinivasan, 2016). Capsaicin is a common ingredient in over-the-counter pain-relieving lotions and creams (Basith et al., 2016; Evangelista, 2015). The analgesic activity of capsaicinoids is mediated by the transient receptor potential vanilloid (TRPV) family of receptors, which is comprised of six members (TRPV1-TRPV6) (Satheesh et al., 2016). All capsaicinoids are high affinity agonists of the TRPV1 receptor (Satheesh et al., 2016). However, several lines of evidence have shown that capsaicinoids exert biological functions that are independent of TRPV1 receptor activation or are mediated by other TRPV receptors (Chapa-Oliver & Mejia-Teniente, 2016; Chow, Norng, Zhang, & Chai, 2007; Clark & Lee, 2016; Diaz-Laviada & Rodriguez-Henche, 2014; Friedman et al., 2018; Lau et al., 2014; Shintaku et al., 2012).

Early studies found that capsaicinoids exert potent chemopreventive activities in a variety of human cancers including lung, prostate, pancreatic, cholangiocarcinoma and skin cancer. Subsequent research demonstrated that capsaicinoids display anti-neoplastic activity in human breast, lung, prostate, gastric, renal, oral and hepatocellular carcinoma (Basith et al., 2016; Chapa-Oliver & Mejia-Teniente, 2016; Srinivasan, 2016). However, conflicting evidence also

exists on the anti-cancer activity of capsaicin. The long-term dietary administration of chili peppers was found to produce neoplastic changes in the liver and cecum (Hoch-Ligeti, 1951). More recent published reports indicated that capsaicin promoted the survival and growth of bladder, colon and skin cancers (Bode & Dong, 2011; Hoch-Ligeti, 1951; Toth & Gannett, 1992). Similarly, studies by Erin et al., (2004 and 2006) showed that the administration of capsaicin at high doses (125 mg capsaicin/kg body weight) increased breast cancer aggressiveness and promoted mammary tumor metastasis to the lung and heart (Erin, Boyer, Bonneau, Clawson, & Welch, 2004; Erin, Zhao, Bylander, Chase, & Clawson, 2006). The aim of their studies was to demonstrate that capsaicin caused denervation of sensory neurons in breast carcinomas and such denervation promoted breast cancer metastasis (Erin et al., 2004; Erin et al., 2006). Apart from these few published reports, the majority of studies have confirmed that low doses of capsaicin suppress the growth and progression of human cancers (Figure 1).

While capsaicinoids are accepted to be high affinity agonists of the TRPV1 receptor, the anti-tumor activity of capsaicinoids appears to be predominantly independent of TRPV1 and involves multiple molecular mechanisms, including activation of cell death mechanisms, inhibition of mitogenic pathways and blockage of mitochondrial respiration, tumor angiogenesis and metastasis (reviewed in (Chapa-Oliver & Mejia-Teniente, 2016; Clark & Lee, 2016; Diaz-Laviada & Rodriguez-Henche, 2014; Friedman et al., 2018; Srinivasan, 2016). Recent studies have indicated that capsaicinoids sensitize human cancer cells to the apoptotic effects of anti-cancer drugs and compounds. These include phytochemicals, synthetic small molecules, conventional chemotherapeutic drugs, as well as novel targeted signal-transduction inhibitors. The present chapter will focus on the chemosensitization activity of capsaicinoids on classifications of FDA-approved chemotherapeutic drugs that are used clinically for the

treatment of cancer patients. We will discuss the signaling pathways underlying the combinatorial growth-inhibitory activity of capsaicinoids and the anti-cancer drugs *in vitro* and *in vivo*. Finally, we will discuss the pharmacokinetic nature of the interaction between capsaicinoids and the chemotherapeutic drug, whether it is additive, synergistic or antagonistic and the statistical methods used to determine such drug-drug interactions.

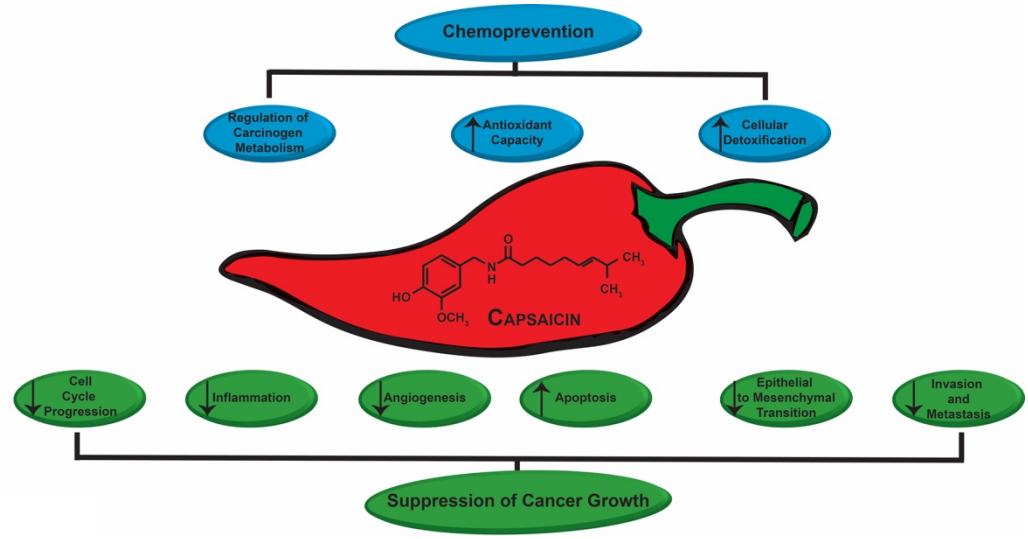

Figure 1. A schematic of the various anticancer and chemopreventive mechanisms triggered by capsaicin in experimental models of carcinogenesis and metastasis.

ANTIMETABOLITES

Antimetabolites are a class of chemotherapeutic drugs which function by mimicking endogenous molecules required for cell cycle progression (Chabner et al., 2011; Peters, 2014). By mimicking these compounds, antimetabolites become incorporated into the DNA or RNA of replicating cells, causing errors in these essential molecules. These errors lead to DNA/RNA damage and subsequently cause cell death (Longley, Harkin, & Johnston, 2003; Longley & Johnston, 2005). Knowledge of nucleic acid biosynthetic processes allowed for the development of various antimetabolites, the earliest with clinical utility included methotrexate, 6-mercaptopurine (6-MP) and 5-fluorouracil (5-FU; Figure 2A) (Burchenal et al., 1953; Farber &

Diamond, 1948; Heidelberger et al., 1957; Jolivet, Cowan, Curt, Clendeninn, & Chabner, 1983; Rutman, Cantarow, & Paschkis, 1954). Two shortcomings of antimetabolites are the dose-dependent toxicities and development of drug resistance (Longley & Johnston, 2005; N. Zhang, Yin, Xu, & Chen, 2008). One strategy to overcome these problems is to combine these potent antimetabolite drugs with a bioactive nutritional compound that will act synergistically to reduce the IC_{50} of the main chemotherapeutic treatment (Cheung-Ong, Giaever, & Nislow, 2013). The exploration of capsaicin in combination with antimetabolite agents is ongoing and has provided some promising results.

Several studies have examined the combinatorial anti-cancer activity of 5-FU and capsaicin. 5-FU is an integral part of the treatment regimens for a variety of malignancies, including skin, colorectal, breast, pancreatic and gastrointestinal cancer (Sorrentino, Kim, Foderaro, & Truesdell, 2012). Side effects of 5-FU include cardiotoxicity, along with diarrhea, mucositis, myelosuppression, and thrombophlebitis (Sorrentino et al., 2012). Combination chemotherapy involving 5-FU is the cornerstone of gastrointestinal tract adenocarcinomas (Chabner et al., 2011; Tang, Feng, Liang, & Cai, 2016). The combination of capsaicin and 5-FU was investigated in HGC-27 metastatic gastric cancer cells. Meral et al., (2014) showed that various concentration combinations of 5-FU and capsaicin effectively inhibited the growth of HGC-27 cells. Treatment with 50 μM 5-FU along with 12, 25, 50 or 100 μM capsaicin showed significant decrease in cell viability when compared to the control at 24 and 48 hours (Meral et al., 2014). The authors inferred that capsaicin at high concentrations ranging from 25-100 μM sensitized HGC-27 human gastric cancer cells to 5-FU-induced apoptosis at 48 hours (Meral et al., 2014). This group also assessed cell injury and found that 25-200 μM of capsaicin significantly altered both LDH and glucose concentrations, while low concentrations of capsaicin

(12 μM) caused no elevation of LDH or glucose levels. Such observations suggest that the combinatorial growth-inhibitory activity of 5-FU and capsaicin is maximal at 50 μM 5-FU and 12 μM capsaicin, while capsaicin (as a single agent) displays marginal growth-inhibitory activity and no cell injury (Meral et al., 2014). Although the authors claimed the interaction between 5-FU and capsaicin to be synergistic, statistical analysis such as the Chou-Talalay isobologram method to confirm synergy between the two drugs was not performed. The isobologram analysis yields a factor known as combination index (CI). A value of CI lower than 1 indicates synergy between the drugs. The CI equals 1 for additive drug interactions. If the CI is greater than 1, the drugs are antagonistic to one another (Chou, 2008, 2010).

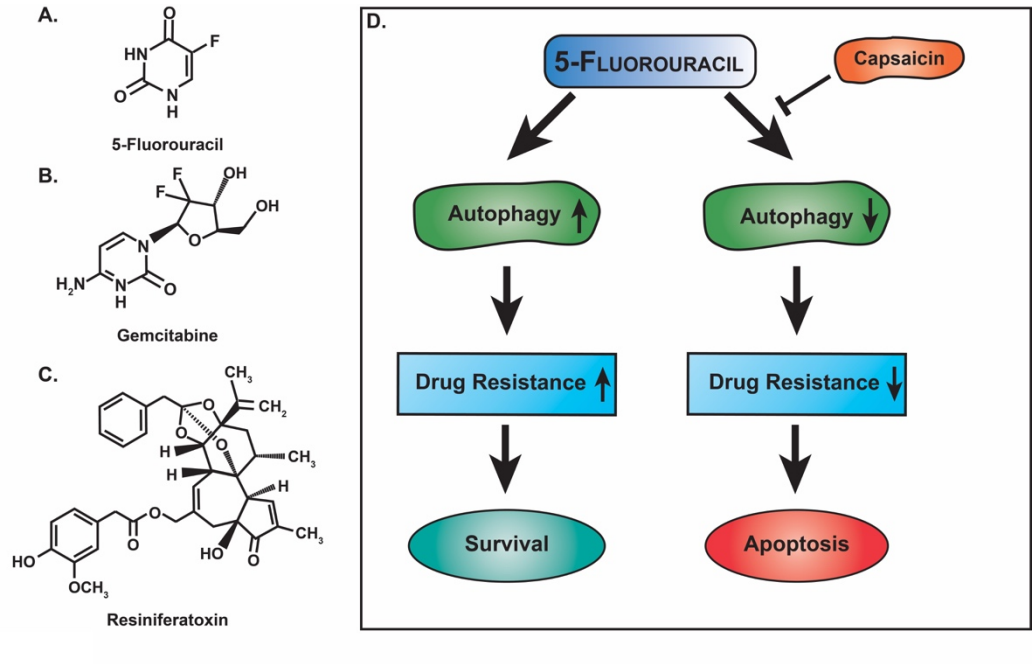

Figure 2. Antimetabolites
(A) 5-Fluorouracil. (5-FU). (B) Gemcitabine. (C) Resiniferatoxin (RTX). (D) Signaling mechanisms underlying 5-FU induced drug resistance in human CCA cells. The presence of capsaicin along with 5-FU downregulates autophagy and triggers downstream apoptosis in human CCA cells.

Human cholangiocarcinoma (CCA) is a diverse group of hepatobiliary cancers, which originate from the biliary tree (Banales et al., 2016). CCAs are classified into intrahepatic (iCCA), perihilar (pCCA) and distal (dCCA) based on their anatomical location. The main challenge of CCA therapy is its aggressive clinical course and chemotherapy-refractory nature (Banales et al., 2016). Hong et al., (2015) analyzed the cytotoxicity of 5-FU in a panel of human CCA cell lines, namely QBC939, MZ-ChA-1 and SK-ChA-1 (Z. F. Hong et al., 2015). They observed that all three cell lines were relatively resistant to 5-FU and displayed growth-inhibitory activity only at high concentrations above 100 µM. A similar trend was observed with 25-150 µM capsaicin at 48 hours, with IC_{50} values approximately 100 µM in all the three cell lines. Subsequently, the authors used varying concentrations of capsaicin (0, 20, 40 and 80 µM)

in the presence or absence of varying 5-FU concentrations (0, 20, 40 and 80 μM). Capsaicin sensitized QBC939 human CCA cells to 5-FU-induced apoptosis at multiple concentrations (Z. F. Hong et al., 2015). Chou-Talalay isobologram analysis was performed and the interaction between 5-FU and capsaicin was found to be synergistic at two combinations. The presence of 40 μM capsaicin along with 40 μM 5-FU lowered the IC_{50} of 5-FU from 126 μM to 35 μM (CI=0.69). Maximal synergy was observed at 40 μM capsaicin and 80 μM 5-FU (CI=0.48) in QBC939 cells. When stained for Annexin-V the combination of 40 μM capsaicin along with 40 μM 5-FU showed significantly more Annexin-V-positive cells than that of either drug alone (Z. F. Hong et al., 2015). The authors also showed capsaicin sensitized human CCA to the apoptotic activity of 5-FU in athymic mouse models, lending strength to the study. The CCA-tumor-bearing mice were administered 60 mg 5-FU/kg body weight/day, 150 mg capsaicin/kg body weight/day or a combination of both. Notably, 5-FU and capsaicin alone did not have any impact on tumor volumes, while the combination of the two drugs showed significantly greater anti-tumor activity (Z. F. Hong et al., 2015). Terminal deoxynucleotidyl transferase dUTP nick end labeling (TUNEL) staining on the CCA tumors (isolated from athymic mice) showed significant increase in apoptosis in tumor-bearing mice treated with the combination of 5-FU and capsaicin than either drug administered as single agents (Z. F. Hong et al., 2015).

Autophagy is one of the mechanisms underlying 5-FU-induced resistance of gastrointestinal cancers (Tang et al., 2016). Established tumors need autophagy to protect themselves from hostile microenvironments like hypoxia, nutrient deprivation and chemotherapeutic drugs (Levy, Towers, & Thorburn, 2017; White, Mehnert, & Chan, 2015). The synergistic anti-cancer activity of 5-FU and capsaicin was due to the ability of capsaicin to inhibit 5-FU-induced autophagy of QBC939 human CCA cells. The treatment of QBC939 with

40 μM 5-FU upregulated the expression of autophagic genes beclin1, atg5 and the autophagic biomarker protein LC3II. The presence of 40 μM capsaicin along with 5-FU decreased the levels of beclin1, atg and LC3II comparable to untreated cells. When the autophagy inhibitor 3-methyladenine (3-MA) was incorporated along with 5-FU the above-mentioned 5-FU induced alterations in beclin1, atg5 and LC3II were also reversed supporting that capsaicin has the ability to chemosensitize resistant cells by inhibiting drug-induced autophagy (Z. F. Hong et al., 2015). The mechanistic target of rapamycin (mTOR) pathway is a vital controller of cellular autophagy (Levy et al., 2017; Paquette, El-Houjeiri, & Pause, 2018). mTOR is a serine/threonine protein kinase comprised of two distinct complexes called mTORC1 and mTORC2. The mTORC1 complex plays a crucial role in autophagic signaling processes. The activity of mTORC1 is regulated by a diverse range of upstream signals such as growth and stress signals, as well as cytoplasmic kinases like extracellular related kinase (ERK), phosphoinositol-3 kinase (PI-3K)/Akt and ribosomal S6 kinase (RSK) (Paquette et al., 2018). Hong et al., (2015) observed that 5-FU inhibited the phosphorylation of Akt (Ser473) and pS6 (Ser235/236), while the presence of capsaicin reversed 5-FU inhibition of Akt and pS6 phosphorylation (Z. F. Hong et al., 2015). These findings suggest that capsaicin reduces chemoresistance to 5-FU by inhibiting 5-FU-induced autophagy via the Akt/mTOR pathway to induce cell death (Figure 2D).

Gemcitabine (Figure 2B), an antimetabolite pyrimidine analog, is the first-line monotherapy treatment for pancreatic cancer (Chabner et al., 2011). Vendrely et al., (2017) examined the effect of three nutritional compounds namely, capsaicin, resveratrol and sulforaphane on the anti-tumor activity of gemcitabine. Chou-Talalay isobologram analysis showed that capsaicin and gemcitabine displayed synergistic growth-inhibitory activity in CAPAN-2 human pancreatic cancer cells (CI=0.5). The maximal synergistic activity was

observed with a combination of resveratrol, capsaicin and gemcitabine (CI=0.05). Subsequently, the authors tested the anti-tumor activity of these drug combinations in athymic mice (Vendrely et al., 2017). CAPAN-2 human pancreatic tumor-bearing mice were treated with 12.5 mg gemcitabine/kg body weight three times a week by intraperitoneal (i.p.) injection. Resveratrol (50 mg/kg body weight) and capsaicin (5 mg/kg body weight) were administered by oral gavage three times a week. Treatment with resveratrol and capsaicin did not sensitize CAPAN-2 tumors to the growth-suppressive effects of gemcitabine at the standard dose. Interestingly, when the dose of gemcitabine was reduced by 33% (8.3 mg/kg body weight) such that gemcitabine alone did not have any impact on the growth rate of CAPAN-2 tumors in athymic mice, the combination with capsaicin and resveratrol to the low-dose gemcitabine fully restored the anti-tumor response to gemcitabine equivalent to the full dose (12.5 mg/kg body weight). Histological examination of the tumors showed increased therapeutic response in tumors treated with low dose gemcitabine (8.3 mg/kg body weight), resveratrol and capsaicin (hereafter referred as C+R+G_L) compared to the other treatment groups. The anti-tumor activity of C+R+G_L correlated with increased Bax/Bcl2 ratio and decreased phospho-Akt/total Akt ratio, suggesting that C+R+G_L selectively stimulated pro-apoptotic pathways with concomitant downregulation of cell survival mechanisms (Vendrely et al., 2017).

Resiniferatoxin (RTX; Figure 2C) is a naturally occurring capsaicinoid isolated from the latex of the cactus *Euphorbia resinifera* (Friedman et al., 2018). Hartel et al., (2006) examined the combinatorial apoptotic activity of RTX with gemcitabine or 5-FU. Although RTX alone displayed robust apoptotic activity in MIA-PaCa-2 and CAPAN-1 human pancreatic cancer cells, it showed no synergistic interaction with gemcitabine or 5-FU in these studies. Statistical

analysis could not confirm whether the interaction between RTX and 5-FU (or gemcitabine) was additive (Hartel et al., 2006).

PLATINUM-BASED DRUGS

Platinum-based drugs are the standard of care for the treatment of many solid tumors and are a vital component in both curative-intent and palliative combination chemotherapy regimens (Chabner et al., 2011; Dasari & Tchounwou, 2014; Dilruba & Kalayda, 2016; Fuertes, Alonso, & Perez, 2003). Combination chemotherapy involving cisplatin has been used for cancers of the lung, ovaries, testes, solid head and neck tumors, and sarcomas (Chabner et al., 2011). The first platinum drug, Cisplatin (cis-diamminedichloroplatinum; Figure 3A), was originally described in the late 1900s for its ability to block binary fission in bacteria (Oun, Moussa, & Wheate, 2018). Cisplatin directly binds to DNA causing intra/interstrand crosslinking, resulting in DNA damage and subsequent apoptosis (Chabner et al., 2011).

A major drawback to the clinical use of platinum-based drugs is the dose-limiting toxicities, including nephrotoxicity, which can lead to acute, and often-irreversible kidney injury (Bai et al., 2017; Dilruba & Kalayda, 2016; Manohar & Leung, 2018; Oun et al., 2018). Ototoxicity resulting in damage to cochlear hair follicles can cause permanent hearing loss (Dilruba & Kalayda, 2016; Paken, Govender, Pillay, & Sewram, 2016). Neurotoxicity leading to pain, weakness or numbness in the extremities, especially the hands and feet, has been reported in patients. Hepatotoxicity, evidenced by increased hepatic enzymes in the serum, as well as gastrointestinal and hematological toxicities have also been reported (Dasari & Tchounwou, 2014; Manohar & Leung, 2018; Oun et al., 2018). With toxicities affecting almost every major organ system, one can see why dose monitoring and using the lowest dose possible is crucial when treating patients. Newer drugs of this class, including carboplatin and oxaliplatin, have

similar mechanisms of action as cisplatin, but display divergent dose-limiting toxicity profiles (Kilari, Guancial, & Kim, 2016). The administration of carboplatin is associated with a lower incidence of nausea and vomiting (relative to cisplatin) and a lower risk of nephrotoxicity and ototoxicity in cancer patients (Dilruba & Kalayda, 2016; Oun et al., 2018). However, carboplatin possesses greater myelosuppressive activity, while oxaliplatin has the lowest risk of nephrotoxicity and ototoxicity amongst these drugs (Oun et al., 2018).

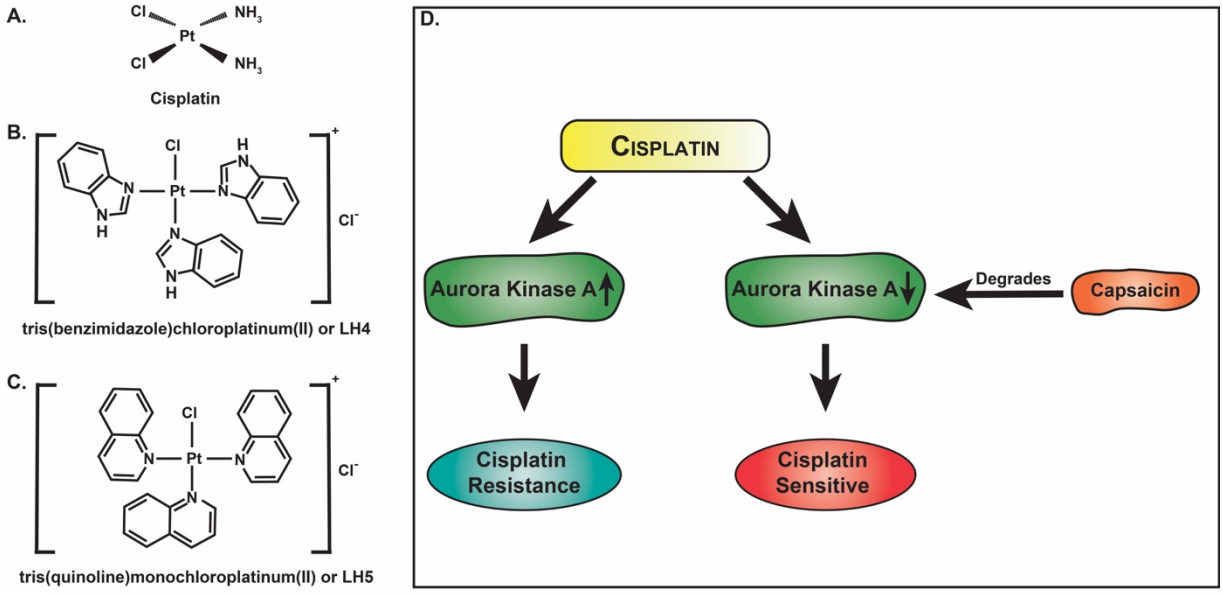

Figure 3. Platinum-based drugs
(A) Cisplatin. (B) LH4. (C) LH5. (D) Stomach cancer cells acquire resistance to cisplatin by increased expression of Aurora Kinase A. When capsaicin is added it stimulates the degradation of Aurora Kinase thereby circumventing cisplatin induced drug resistance.

The clinical efficacy of platinum-based drugs is further limited by the development of chemoresistance in tumor cells (Dasari & Tchounwou, 2014; Kilari et al., 2016). Almost all solid tumors eventually develop resistance to cisplatin and its related compounds. Cisplatin-based combination therapy frequently gives excellent initial outcomes in lung cancer patients. However, the disease often relapses, and the tumor is unresponsive to cisplatin (Fennell et al., 2016). The mechanisms underlying cisplatin resistance involve multiple pathways, including the

elevation and conjugation by glutathione (GSH) and binding by metallothionein, which minimize DNA binding by active forms of platinum-based drugs (Amable, 2016; Galluzzi et al., 2012; Oun et al., 2018). Cisplatin resistance also involves the activation of DNA repair pathways, dysregulation of p53 tumor suppressor gene function, amplification of the pro-survival Ras/MAPK signaling pathway, and upregulation of heat shock proteins (HSP) in neoplastic cells (Amable, 2016; Dasari & Tchounwou, 2014). Furthermore, the acquisition of cisplatin resistance also involves down-regulation of drug uptake transporters and upregulation of the functional activity of efflux transporters (Kilari et al., 2016). Despite these limitations, cisplatin-based chemotherapy remains a standard of care for a variety of solid tumors (Dasari & Tchounwou, 2014; Dilruba & Kalayda, 2016). All of these observations have led to intense research to identify potential compounds that can improve the therapeutic index of cisplatin, improve its toxicity profile, circumvent drug resistance mechanisms, and augment its cytotoxic activity in human cancers.

Recent interest has increased focus on natural fruit- and vegetable-derived compounds as potential agents to reduce cisplatin nephrotoxicity and increase tumor sensitivity to chemotherapy (Athira, Madhana, & Lahkar, 2016; Gomez-Sierra, Eugenio-Perez, Sanchez-Chinchillas, & Pedraza-Chaverri, 2018). Garufi et al., (2016) investigated the growth-inhibitory activity of cisplatin with capsaicin in human p53 mutant glioblastoma cells (Garufi, Pistritto, Cirone, & D'Orazi, 2016). The authors treated U373 human glioblastoma cells with 100 μM capsaicin, 2.5 μg/ml cisplatin or a combination of both drugs over 24 hours and observed that the combination of capsaicin with cisplatin induced 3-fold higher cell death relative to either capsaicin or cisplatin alone (Garufi et al., 2016). This study further showed that capsaicin reactivated p53 function in U373 cells, using over-expression and siRNA techniques to confirm

the role of the p53 wild type pathway in the growth-suppressive activity of capsaicin as a single agent. The authors speculated that capsaicin was sensitizing U373 cells to cisplatin by restoration of p53 wild type activity. The caveat of the study is the authors did not present direct experimental evidence (by p53-siRNA and p53 plasmid vectors) showing that the enhanced growth-inhibitory activity of cisplatin-capsaicin combination was mediated by the p53 pathway. Notably, cell authentication experiments have shown that the U373 MG cell line (available at ATCC and EACC) is genetically distinct from the original U373 MG cell line isolated by Westmark et al., (1973) in Uppsala, Sweden (Ishii et al., 1999; "Misidentified Cell Lines," ; Westermark, 1973). The U373 MG cell line obtained by ATCC and EACC was identical to another human glioma cell line U251. Subsequently, both ATCC and EACC have discontinued this cell line from their inventory.

Huh et al., (2011) analyzed the cooperative growth-suppressive activity of cisplatin and capsaicin in human stomach cancers using a panel of Korean human stomach cancer cell lines (Huh, Lee, Lee, Park, & Han, 2011). They treated SNU01, SNU-5, SNU-16, SNU-601, SNU-638 and SNU-668 cells with 100-500 μM cisplatin for 6 hours. The authors observed that out of all the cell lines tested, SNU-668 was the most resistant to cisplatin, with an IC_{50} value of over 500 μM (Huh et al., 2011). Subsequently, they tested whether 300 μM capsaicin could sensitize SNU-668 cells to cisplatin-induced apoptosis. The combination of 20 μM cisplatin with 300 μM capsaicin decreased viability of SNU-668 cells approximately 7-fold, relative to vehicle treated or cisplatin only treated cells (Huh et al., 2011). Similarly, the combination of cisplatin and capsaicin displayed 5-fold lower cell viability, compared to SNU-668 cells treated with capsaicin alone. By using flow cytometry, Huh and colleagues were able to detect that the treatment of SNU-668 cells with both capsaicin (300 μM) and cisplatin (20 μM) resulted in an accumulation

of G1- and S-phase cells. When SNU688 cells were treated with cisplatin alone, the cells accumulated in the G2/M-phase, which led to the conclusion that capsaicin overcame cisplatin-induced G2/M-phase arrest in SNU-688 cells and caused accumulation in G1 (Huh et al., 2011). TUNEL apoptosis assays revealed the treatment with 20 μM cisplatin alone caused apoptosis in 5.16% of SNU-668 cells; 300 μM capsaicin alone in 22.4% of cells; however, the combination induced apoptotic cell death in 48.5% of cells. Capsaicin induced a greater magnitude of apoptosis in SNU-668 cells than cisplatin probably due to the fact that SNU-688 cells are cisplatin resistant and therefore have a low apoptotic response towards cisplatin. The combination of cisplatin and capsaicin abolished the expression of both Bcl-2 and Bcl-xL. Notably, cisplatin increased the expression of Bcl-2 in SNU-688, implying that Bcl-2 may be one of the mechanisms underlying a cisplatin resistant phenotype.

Several lines of evidence show that overexpression of Aurora kinase A (Aurora A) leads to tumor-acquired chemotherapeutic resistance, especially to cisplatin (Figure 3D**)** (Kuang et al., 2017; Polacchini et al., 2016; L. Wang, Arras, et al., 2017; Xu et al., 2014). Aurora A is a serine threonine kinase involved in several steps of mitosis, such as centrosome function, spindle assembly, chromosome alignment and mitotic entry (Borisa & Bhatt, 2017). Treatment with cisplatin was found to increase the expression of Aurora A levels in SNU-668 cells (Huh et al., 2011). In contrast, the treatment of SNU-668 with 300 μM capsaicin almost abolished the levels of Aurora A in these cells. The combination of cisplatin and capsaicin also decreased expression of Aurora A below detectable levels, effectively inhibiting cisplatin-induced increase in Aurora A. Experiments using the proteasome inhibitor MG132 revealed that capsaicin suppressed the levels of cellular Aurora A via enhancement of proteasomal degradation (Huh et al., 2011). Such capsaicin-induced degradation of Aurora A prevents cisplatin-induced nuclear factor kappa-B

(NF-κB) activation and survival of drug resistant SNU-338 cells (Huh et al., 2011). Therefore, the inclusion of capsaicin along with cisplatin may provide a valuable strategy for overcoming cisplatin resistance in human stomach cancer cells.

In other studies, the combination of cisplatin and capsaicin robustly decreased viability of the human neuroblastoma cell line KELLY (Altun, Altun, Olgun, Pamukoglu, & Olgun, 2016). KELLY cells were treated with 5 μM capsaicin, 100 μM cisplatin or a combination of both for 24 hours. WST-1 assays revealed that 5 μM capsaicin had no effect on the viability of these cells. In contrast, 100 μM cisplatin decreased the viability by approximately 25% (Altun et al., 2016). When 5 μM capsaicin was combined with 100 μM cisplatin there was a dramatic decrease in the viability (by about 88%). Annexin-V/PI flow cytometric analysis revealed that the combination of cisplatin and capsaicin induced greater magnitudes of apoptosis than either drug alone (Altun et al., 2016).

Arzuman et al., (2014) synthesized a new platinum-based compound tris(benzimidazole)chloroplatinum(II) or LH4 (Figure 3B) (Arzuman, Beale, Chan, Yu, & Huq, 2014) and examined the combinatorial activity of LH4 with various phytochemicals including capsaicin. MTT assays were used to determine the concentration-dependent growth-inhibitory activity of LH4 and cisplatin at concentrations ranging from 0.16-20 μM in three variants of the human ovarian cancer cell line A2780 at 72 hours. These three variant ovarian cancer cell lines are characterized by their chemoresistant behavior; A2780 is cisplatin sensitive, $A2780^{cisR}$ is cisplatin resistant, and $A2780^{ZDO473R}$ is picoplatin resistant (Arzuman et al., 2014). They observed that capsaicin decreased the viability of all three lines. Using Chou-Talalay isobologram, the authors found that the combination of LH4 and capsaicin was synergistic in A2780 (CI=0.41), $A2780^{cisR}$ (CI=0.48) and $A2780^{ZDO473R}$ (CI=0.36) cells (Arzuman et al., 2014).

They also investigated whether pretreatment with one of the drugs could enhance synergistic growth suppressive activity. They pretreated all the cell lines with LH4 and then treated the cells with capsaicin four hours later and revealed that the interaction remained synergistic in A2780 (CI=0.52), A2780^{cisR} (CI=0.94) and A2780^{ZDO473R} (CI=0.84) cells. A noteworthy observation is the magnitude of synergy between LH4 and capsaicin was maximal when both drugs were added simultaneously to the cells, especially in the platinum resistant A2780 cell lines (Arzuman et al., 2014). The combination of capsaicin and LH4 added together to A2780 and A2780^{cisR} showed higher intracellular platinum levels than when LH4 was given alone (Arzuman et al., 2014). The combination of LH4 and capsaicin induced a higher magnitude of intracellular platinum accumulation in A2780^{cisR} cells (6-7 fold higher than LH4 alone) than in the parent line A2780 (1.5 fold higher than LH4 alone). The combination of LH4 and capsaicin showed no changes in platinum-DNA binding in A2780 and A2780^{cisR} cells, relative to LH4 alone. However, when the cells were treated with capsaicin followed by LH4, there was a robust increase in the amounts of platinum-DNA complexes in A2780^{cisR} human ovarian cancer cells (Arzuman et al., 2014).

A monofunctional platinum (II) drug tris(quinoline)monocloroplatinum(II) or LH5 (Figure 3C) (Arzuman, Beale, Yu, & Huq, 2016) was used to evaluate the combinatorial growth-suppressive effects of LH5 and capsaicin in human ovarian cancer cells. The authors observed that the greatest synergistic effect was seen when both LH5 and capsaicin were used to treat the cells at the same time (the 0/0 protocol) in ED_{50}, ED_{75}, and ED_{90} measured across A2780, A2780^{cisR} and A2780^{ZDO473R} human ovarian cancer cells (Arzuman et al., 2016). The picoplatin resistant cell line A2780^{ZDO473R} showed the lowest degree of synergy, when compared to the other two cell lines. Although the authors have not performed any experiments to investigate the signaling pathways underlying the synergistic growth-suppressive activity of LH5 and capsaicin,

they speculate that capsaicin induces an accumulation of cells in the G1-phase, while LH5 causes cell cycle arrest in the G2-phase and subsequent apoptosis via the p53 pathway. Experiments involving pretreatment of LH5 followed by capsaicin 4 hours after (0/4 protocol) showed greater magnitude of synergy and lower CI values than the 4/0 protocol, when LH5 was added 4 hours after capsaicin (Arzuman et al., 2016). Further studies are warranted to investigate why the 0/0 protocol showed a greater synergistic interaction than pretreatment of either drug.

Wang et al., (2018) have demonstrated that capsaicin synergizes with cisplatin in osteosarcoma cells xenografted in athymic mice (Y. Wang et al., 2018). As a proof of concept, they investigated the combinatorial activity of cisplatin and capsaicin in three human osteosarcoma cell lines, namely MG63, 143B and HOS. The combination of 50 μM or 100 μM capsaicin synergistically decreased cell viability of MG63 and 143B cells across a range of cisplatin concentrations (16.7-66.7 μM). Furthermore, the combination of 100 μM capsaicin with 16.7 μM cisplatin induced a greater magnitude of apoptosis and cell cycle arrest in all osteosarcoma cell lines than either drug administered alone (Y. Wang et al., 2018). This correlated with enhanced upregulation of pro-apoptotic and cell cycle inhibitory proteins (Bax, caspase-3, cytochrome C, p21, p18) and decreased expression of pro-survival biomarkers (Bcl-2, survivin, cyclins D1, D3 and cdk4/6). Most interestingly, the presence of 100 μM capsaicin increased the anti-invasive activity of cisplatin (Y. Wang et al., 2018). The functional activity of matrix metalloproteinase-2 and -9 (MMP2 and MMP9) was suppressed to a greater extent when cisplatin and capsaicin were added together than either drug alone. Finally, the administration of 20 mg capsaicin/kg body weight along with 4 mg cisplatin/kg body weight decreased the growth rate of 143B human osteosarcoma tumors xenotransplanted in athymic mice more potently than either drug administered alone (Y. Wang et al., 2018).

Innovative studies have also examined the ability of capsaicin to diminish/minimize the toxic side effects of cisplatin. A major toxic side effect of cisplatin is ototoxicity, which is defined as hearing loss due to temporary or permanent damage to the sensory hair cells in the cochlea (Oun et al., 2018; Paken et al., 2016). Altun et al., (2016) evaluated the possibility of using capsaicin as a preventative agent to combat cisplatin-induced ototoxicity in murine mouse ear organ corti cells (HEI-OC1) (Altun et al., 2016). The treatment of HEI-OC1 with 100 μM cisplatin caused a 64% decrease in cell viability. Notably, 5 μM capsaicin did not impact the viability of HEI-OC1 cells (Altun et al., 2016). The combination of capsaicin and cisplatin displayed lower growth-inhibitory activity (20% decrease in cell viability) relative to cisplatin alone (64% decrease in cell viability). Gene expression analysis revealed that the combination of cisplatin and capsaicin upregulated mitosis-related cell cycle genes (cdc25c), DNA repair genes (Fancg, Mif, M1H3), and downregulated apoptosis-related genes (Bax, PARP2, p53), leading to survival of HEI-OC1 ear organ corti cells (Altun et al., 2016).

Capsaicin was also shown to protect against cisplatin-induced nephrotoxicity. Shimeda et al., (2005) induced nephrotoxicity in Sprague-Dawley rats by i.p. injection of 5 mg cisplatin/kg body weight followed by 10 mg capsaicin/kg body weight by oral gavage twice daily for six days after cisplatin administration. Cisplatin-induced nephrotoxicity was characterized by decreased body weight, kidney GSH content and kidney superoxide dismutase (SOD) activity, an increase in kidney weight, kidney malondialdehyde levels, serum creatinine, and blood urea nitrogen (BUN) levels. The administration of capsaicin along with cisplatin significantly decreased BUN, serum creatinine and kidney malondialdehyde levels and increased kidney SOD activity (Shimeda et al., 2005). Capsaicin administration also reversed the cisplatin-induced increase in kidney weight, returning kidneys to normal weights. The authors went on to show that capsaicin

mitigated cisplatin-induced nephrotoxicity by scavenging free radicals (generated by cisplatin-treatment) via the SOD pathway.

Jung et al., (2014) observed that capsaicin abrogated the growth-suppressive effect of cisplatin in HK2 human renal proximal tubule cells. Subsequently, they extended their studies to a mouse model to confirm the protective activity of capsaicin in cisplatin-induced nephrotoxicity in C57BL6 mice (Jung et al., 2014; Shimeda et al., 2005). Their study design was slightly different from Shimeda et al., (2005). The differences in cisplatin treatment regimens was most likely due to rats being more susceptible to cisplatin nephrotoxicity than mice (Katayama et al., 2011; Perse & Veceric-Haler, 2018). They orally administered three doses of capsaicin (2.5, 5, or 10 mg/kg body weight) once a day for five consecutive days. A single dose of cisplatin (5 mg/kg body weight) was i.p. injected on day 4 (twelve hours after the administration of capsaicin). The administration of cisplatin induced an 8-fold increase in serum creatinine and a 4-fold increase in BUN. The pretreatment of mice with capsaicin at doses of 5 mg/kg body weight or 10 mg/kg body weight decreased cisplatin-induced increases in serum creatinine and BUN levels. Notably, the dose of 10 mg/kg body weight capsaicin, completely normalized creatinine and BUN levels in mice. The kidneys of mice treated with cisplatin showed tubular and glomerular injury, namely tubular dilation, vacuole formation and necrosis. However, when the mice were pre-administered capsaicin, their kidney reverted to near normal morphology, with only slight changes in glomeruli and minor edema of the tubular cells (Jung et al., 2014).

Several convergent studies have shown that oxidative stress, inflammation, proinflammatory cytokines and toll-like receptors (TLR) contribute to tubular toxicity and vascular injury observed in cisplatin-induced renal injury (Cenedeze et al., 2007; Kuhad, Pilkhwal, Sharma, Tirkey, & Chopra, 2007; Mitazaki et al., 2013; Mukhopadhyay et al., 2010;

Ramesh & Reeves, 2002; Sahu, Kuncha, Sindhura, & Sistla, 2013; Valles, Lorenzo, Bocanegra, & Valles, 2014). Jung et al., (2014) observed that cisplatin induced the upregulation of proinflammatory cytokines (TNF-α, IL-1β and IL-6), TLR4 and its ligands (high mobility group box 1, HMB1; advanced glycation end product, AGE) in the kidney. The pre-administration of capsaicin (10 mg/kg body weight) abolished cisplatin-induced alterations in TNF-α, IL-1β, IL-6, TLR, HMB1 and AGE in the kidneys of mice (Jung et al., 2014). Next, the authors examined the effect of capsaicin on cisplatin-induced production of ROS in the kidney. The enzymes of the NADPH oxidase family (NOX) play a vital role in ROS generation by catalyzing the transfer of electrons from NADPH to molecular oxygen (Bedard & Krause, 2007). Out of seven NOX isoforms, NOX4 (and to a lesser extent NOX2) have been implicated in cisplatin-induced nephrotoxicity (Sedeek, Nasrallah, Touyz, & Hebert, 2013). Jung et al., (2014) measured ROS levels in HK2 immortalized human proximal tubule cells (Jung et al., 2014). They observed that the treatment of HK2 cells with 30 μM cisplatin induced robust ROS activity. The presence of 100 μM capsaicin along with cisplatin decreased the ROS levels close to those observed in vehicle-only treated cells. Similarly, the combination of 30 μM cisplatin and 100 μM capsaicin reversed cisplatin-induced NOX4 expression in HK2 cells over 24 hours. IHC of kidney tissues from cisplatin-treated mice showed vigorous expression of NOX-4 and 4-hydroxynonenal (4-HNE; a biomarker for lipid peroxidation), which was reduced by pre-treatment with 10 mg capsaicin/kg body weight (Jung et al., 2014). These findings suggest that capsaicin reduced oxidative stress and ROS formation mediated by cisplatin treatment.

A previous study showed that increased expression of heme oxygenase-1 (HO-1) via overexpression or pharmacological induction confers protection from cisplatin-induced ototoxicity in both cell culture and *in vivo* mouse models (So et al., 2008). Traditionally, the

principal function of HO-1 is to catalyze the breakdown of heme into biliverdin, carbon monoxide and iron (Loboda, Damulewicz, Pyza, Jozkowicz, & Dulak, 2016). Several congruent studies have indicated that the induction of HO-1 functions as a defense mechanism, shielding cells from the damaging effects of oxidative stress (Bolisetty, Zarjou, & Agarwal, 2017). The disruption of HO-1 activity in rats or deletion of the HO-1 gene in mice results in exacerbation of cisplatin-induced renal injury, emphasizing the protective role of this enzyme in cisplatin-induced nephrotoxicity (Bolisetty et al., 2017). Jung et al., (2014) found that HO-1 was substantially decreased in the kidneys of cisplatin-treated mice. When the mice were pretreated with 2.5, 5.0 and 10 mg capsaicin/kg body weight (before the administration of cisplatin), there was an induction of HO-1 protein levels in kidney homogenates (Jung et al., 2014). The protective effect of capsaicin on the growth-suppressive activity of cisplatin (in HK2 cells) was reversed in the presence of pharmacological inhibitors of the HO-1 pathway (namely ZnPP 1X) or HO-1 siRNA. Notably, the treatment of HK2 cells with 100 μM capsaicin caused robust upregulation of HO-1 levels starting at 6 to 24 hours. Taken together, the data of Jung et al., (2014) indicate a pivotal role of the HO-1 pathway in the cytoprotective activity of capsaicin against cisplatin-induced nephrotoxicity (Jung et al., 2014). We believe that the protective activity of capsaicin against cisplatin-induced nephrotoxicity is extremely clinically relevant. Such observations may facilitate the design and application of novel capsaicin-cisplatin based combination therapies that will improve the therapeutic index of cisplatin in cancer patients. Furthermore, the synergistic cytotoxic activity of cisplatin and capsaicin suggests that lower doses of cisplatin (along with capsaicin) may be required to achieve optimal therapeutic response in patients. The ability to lower the dose of cisplatin administered to a patient, while maintaining

or increasing potential cancer cell kill would be predicted to minimize the adverse effects and toxicity profile of cisplatin, while improving health outcomes for cancer patients.

ANTHRACYCLINES

Anthracyclines are a class of chemotherapeutic drugs originally isolated from *Streptomyces peucetius* bacteria (Arcamone et al., 1969; Chabner et al., 2011). Daunorubicin (Figure 4A) is considered to be the prototypical anthracycline, being the first anthracycline shown to have anti-cancer properties (Davis & Davis, 1979). A derivative of daunorubicin, doxorubicin (Figure 4B), was the second anthracycline to be discovered and evaluated for its antineoplastic activity (Baboota et al., 2014; Tacar, Sriamornsak, & Dass, 2013). Additional derivatives and metabolites have since joined the anthracycline chemotherapy family, including epirubucin, idarubicin and pirarubicin (Cersosimo & Hong, 1986; Hollingshead & Faulds, 1991; Minotti, Menna, Salvatorelli, Cairo, & Gianni, 2004). Anthracycline drugs have the ability to treat a variety of cancers, including both solid tumors and hematological malignancies (Chabner et al., 2011). Doxorubicin intercalates into the DNA and RNA and inhibits topoisomerase II function, preventing DNA and RNA replication, and inducing cell death in both normal and cancerous cells (Baboota et al., 2014; Tacar et al., 2013) .

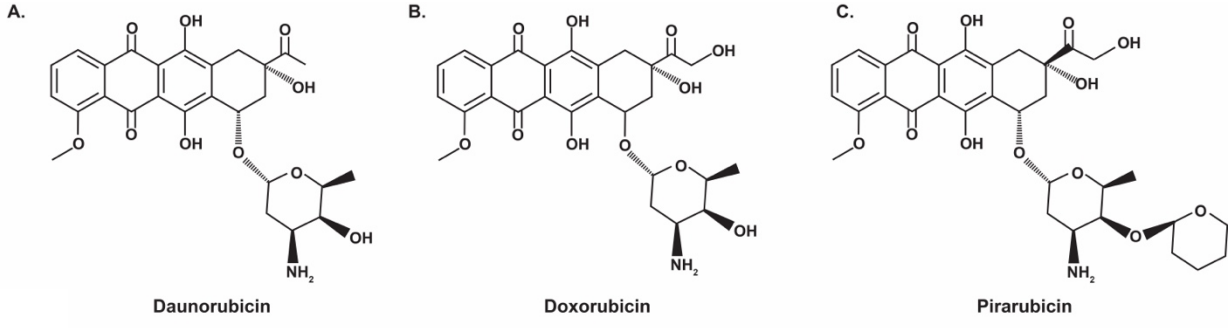

Figure 4. Anthracyclines
(A) Daunorubicin. (B). Doxorubicin. (C). Pirarubicin.

Similar to other nonspecific chemotherapeutic agents, doxorubicin has a variety of toxic side effects associated with its use. Apart from nausea, gastrointestinal problems and neurological disturbances, doxorubicin has been documented to cause toxicity in the liver, brain and kidneys (Tacar et al., 2013). The dose-limiting toxicity of doxorubicin is acute and chronic cardiotoxicity, characterized by myofibrillar loss, cytoplasmic vacuolization, apoptosis, interstitial edema, and fibroplasia (Chatterjee, Zhang, Honbo, & Karliner, 2010; Y. Shi, Moon, Dawood, McManus, & Liu, 2011). Due to the highly documented cardiotoxicity associated with doxorubicin, different types of clinical restrictions have been put in place for use in patients. Different types of drugs, such as beta-blockers or angiotensin II inhibitors, are now administered with doxorubicin in hopes of lowering the chance of the patient experiencing cardiotoxicity (Chatterjee et al., 2010). Due to the variety and severity of toxicities, the ability to use a lower dose of doxorubicin while maintaining its antineoplastic qualities would be a valuable therapeutic strategy to improve both the short-term and long-term safety of patients.

The second generation doxorubicin analog, pirarubicin (also called THP-Adriamycin or THP-doxorubicin; Figure 4C), displays a wide spectrum of anti-tumor activity amongst solid tumors of the urinogenital system like bladder cancer (Arakawa et al., 2011; Crijnen & De Reijke, 2018). Almost 80% of all bladder cancers are superficial and do not invade surrounding muscles (Crijnen & De Reijke, 2018). Intravesical chemotherapy involving pirarubicin is the standard of care for non-invasive bladder cancer after transurethral resection of bladder tumors (TURBT) (Arakawa et al., 2011; Crijnen & De Reijke, 2018). A drawback of this therapeutic regimen is that the five-year recurrence rate amongst bladder cancer patients is relatively high (greater than 30%) (Crijnen & De Reijke, 2018). The capsaicin-receptor TRPV1 functions as a tumor suppressor in bladder cancer (Mistretta et al., 2014; Santoni et al., 2012). Capsaicin has

been shown to induce robust apoptosis and prevent metastasis of human bladder cancers via a TRPV1-dependent mechanism (Amantini et al., 2009; Santoni et al., 2012). Based on these findings, Zheng et al., (2016) investigated the anti-tumor activity of the combination of the TRPV1 agonist capsaicin in addition to the current standard of care, pirarubicin, in human bladder cancer cell lines (Zheng et al., 2016).

The authors chose two human bladder transitional cell carcinoma cell lines (5637 and T24) for their studies. The 5637 cells expressed robust amounts of TRPV1 (at both mRNA and protein levels), whereas T24 cells are null for TRPV1 (Zheng et al., 2016). Their studies revealed that capsaicin displayed growth-suppressive activity only in TPRV1 expressing 5637 cells (IC_{50} =150 μM) but not in the TRPV1 null T24 cells. Subsequently, they treated 5637 cells with a range of concentrations of pirarubicin (0-800 μM) in the presence or absence of 150 μM capsaicin for 12 hours and measured cell viability. They observed that the presence of capsaicin along with pirarubicin decreased the IC_{50} value of pirarubicin from 566 nM to 335 nM (Zheng et al., 2016).

The predominant mechanism by which anthracycline compounds like pirarubicin suppress cell growth is intercalation between DNA strands, causing S- or G2/M-phase cell cycle arrest (Figure 5). Flow cytometry analysis revealed that the treatment of 5637 cells with 150 μM capsaicin caused G0/G1 arrest at 12 hours. When pirarubicin was combined with capsaicin, there was an elevation in the number of cells arrested in G2/M- and S-phase and a concomitant decrease in the fraction of cells in G0/G1-phase (Zheng et al., 2016). The presence of TRPV1 antagonist capsazepine partially reversed the observed elevation of G2/M- and S-phase populations observed in the pirarubicin plus capsaicin treated 5637 cells. These findings suggest

that the TRPV1 pathway is at least in part responsible for capsaicin-induced sensitization of 5637 cells to the anti-proliferative activity of pirarubicin.

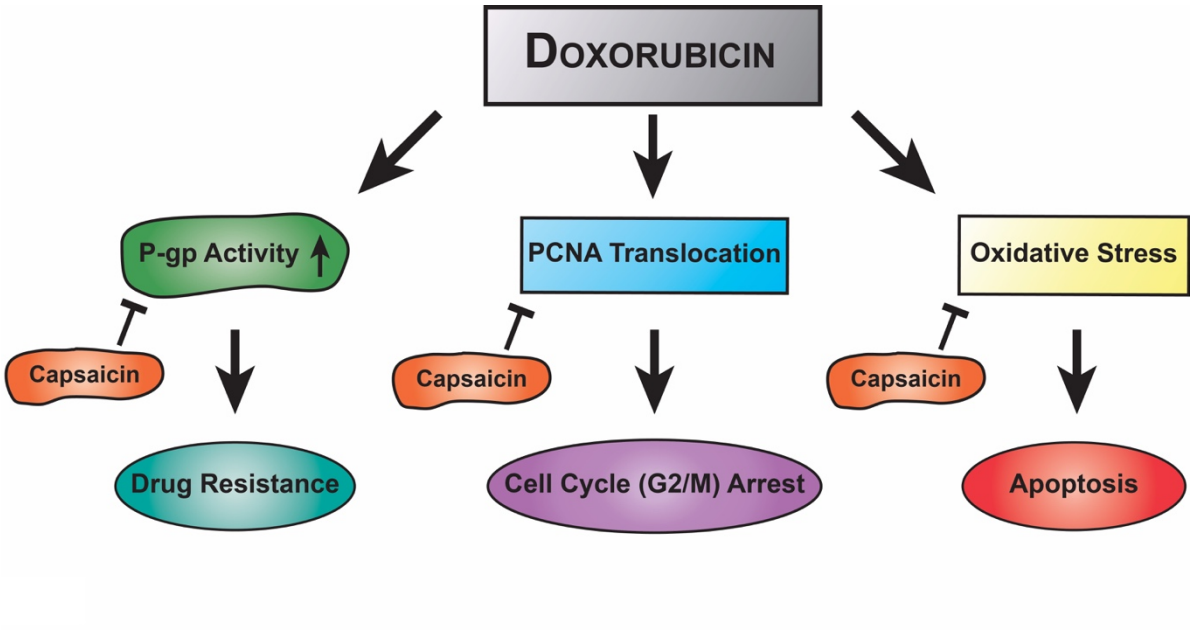

Figure 5. A simplified schematic of the signaling mechanisms of the synergistic anticancer activity of doxorubicin and capsaicin in human cancer cells.

One of the important proteins involved in the entry of cells into S-phase is proliferating cell nuclear antigen (PCNA), which becomes elevated as cells progress from G1- to S-phase. Translocation of PCNA from the cytosol to the nucleus then facilitates cell cycle progression from S- to G2/M-phase (Zheng et al., 2016). The combination of capsaicin and pirarubicin did not induce significant elevation of PCNA relative to each drug alone; however, it potently blocked nuclear translocation of PCNA, thereby arresting the 5637 cells in S- and G2/M-phase (Zheng et al., 2016).

A major factor that limits the efficacy of chemotherapy is the acquisition of multi-drug resistance in tumors during prolonged treatment. The molecular basis of such drug resistance (intrinsic or acquired) is multifactorial; however, drug efflux and drug metabolism mechanisms

play a major role in this process (Chabner et al., 2011). Drug efflux is mediated by membrane-bound transporters that pump many of these anticancer drugs back out of the cell. This process decreases the bioavailability of the anti-cancer drug, and ablates their ability to kill cancer cells (Miller, 2003). Out of the seven subfamilies of ATP-binding cassette (ABC) transporters, the most extensively studied efflux transporter protein is the P-glycoprotein (P-gp), whose functional activity is elevated in a diverse array of human cancers (Z. Chen et al., 2016). Li et al., (2018) investigated the effect of capsaicin on the functional activity of P-gp in doxorubicin-resistant Caco-2 human colon carcinoma cells (H. Li, Krstin, Wang, & Wink, 2018). The efflux of the fluorescent dye Rho123 was used as a measure of cellular P-gp activity. The authors observed that the treatment of Caco-2 cells with capsaicin resulted in a concentration-dependent cellular retention of Rho123, implying that capsaicin blocked the activity of P-gp in Caco-2 cells. Furthermore, they confirmed the inhibitory effect of capsaicin on P-gp activity in a second resistant human leukemic cell line CEM/ADR5000 and obtained similar results (H. Li et al., 2018). This is in contrast with the observations of Sadzuka et al., (2008) who did not see any effect of capsaicin on doxorubicin influx or efflux in Ehlrich ascitis carcinoma (EAC) cells and M5076 ovarian carcinoma cells (Sadzuka, Hatakeyama, Daimon, & Sonobe, 2008). Such differences may be explained by alterations in experimental design and in the nature of the cancer cell lines, such as differences in expression or function of P-gp, used in the two experiments. Sadzuka et al., (2008) measured total doxorubicin influx and efflux in neoplastic cells, whereas Li et al., (2018) specifically measured P-gp activity in human colon cancer and leukemia cell lines (H. Li et al., 2018; Sadzuka et al., 2008). There were also species differences of the cell lines used in the two research papers. The experiments by Sadzuka et al., (2008) were performed in M5076 murine ovarian sarcoma and mouse ascites carcinomas whereas the studies

by Li et al., (2018) used doxorubicin resistant human colon cancer and leukemia cell lines for their investigations (H. Li et al., 2018; Sadzuka et al., 2008). Whereas capsaicin blocks human P-gp activity, it may have minimal impact on the functional activity of the abovementioned mouse ABC transporters. If capsaicin does not regulate the functional activity of murine ABC transporters, then doxorubicin transport in M5076 and EAC cells may not be sensitive to capsaicin.

Subsequently, Li et al., (2018) explored the synergistic growth-inhibitory activity of doxorubicin and capsaicin in Caco-2 cells (H. Li et al., 2018). They combined the serial dilution doses of doxorubicin with fixed, non-toxic doses of capsaicin (IC_{10}, IC_{20} or IC_{30}) and determined the effect of these combinations on the viability of Caco-2 cells (H. Li et al., 2018). All three combinations of doxorubicin and capsaicin displayed synergistic growth inhibitory activity ($CI<1$) and significantly reduced the IC_{50} value of doxorubicin in Caco-2 cells. The magnitude of the CIs was very similar across all three groups. They repeated the experiments in CEM/ADR5000 cells and obtained analogous results. The magnitude of CI was higher (indicating less synergy) in CEM/ADR5000 cells relative to Caco-2 cells (H. Li et al., 2018).

Studies in mouse models have indicated that capsaicin minimizes the acute cardiotoxic side effects of doxorubicin administration. Patel & Mehta, (2017) administered capsaicin at two doses (1 mg/kg body weight and 2 mg/kg body weight) to mice via intraperitoneal injections daily for ten days (Patel & Mehta, 2017). On day eight they injected the mice with 20 mg doxorubicin/kg body weight via i.p. Mice that were administered vehicle only or doxorubicin alone served as one of the control groups of the study. The authors observed that the acute administration of doxorubicin caused a reduction in the body and heart weights of the mice. However, doxorubicin-induced decreases of body weight and heart weights were abrogated when

the mice were pretreated with 1 mg capsaicin/kg body weight before doxorubicin administration (Patel & Mehta, 2017). The authors observed that the acute administration of doxorubicin caused cardiotoxicity as evidenced by elevation in creatinine kinase-muscle/brain (CK-MB), LDH and tissue malonaldehyde (MDA) levels. Similarly, the amounts of antioxidant enzymes like SOD, myocardial catalase (CAT) and GSH were reduced. The pre-administration of 1 mg capsaicin/kg body weight significantly abrogated doxorubicin-induced elevation of CK-MB, LDH and MDA levels (Patel & Mehta, 2017). Furthermore, capsaicin elevated the levels of SOD, CAT and GSH (which were lowered by the administration of doxorubicin). Histological analysis of heart sections from doxorubicin treated mice showed inflammation and cardiac necrosis as evidenced by vascular dilatation and loss of myofibrils network. In contrast, the heart sections of mice pretreated with 1 mg capsaicin/kg body weight before administration of doxorubicin were morphologically and histologically normal and comparable to vehicle-treated mice (Patel & Mehta, 2017).

CAMPTOTHECIN ANALOGS

Camptothecins are natural topoisomerase-1 inhibitors isolated from the Chinese tree *Camptotheca accuminata* (Chabner et al., 2011). Camptothecin (Figure 6A) and its related analogs form a tertiary complex with single-stranded DNA (during S-phase of the cell cycle) and topoisomerase 1, leading to DNA damage and cell death (Chabner et al., 2011). Two synthetic analogs of camptothecin, topotecan and irinotecan (Figure 6B and 6C), are used clinically for the treatment of ovarian, SCLC and colorectal cancer (Chabner et al., 2011). Irinotecan is a prodrug, which is cleaved by carboxylesterases in the tumor, liver or red blood cells to generate the active drug SN-38, which displays 10-100 fold more potent anti-tumor activity than irinotecan (Chabner et al., 2011).

The dose-limiting toxicities of topotecan are mainly hematological in nature, causing neutropenia, with or without thrombocytopenia in cells (Chabner et al., 2011). The dose-limiting toxicity of irinotecan is severe diarrhea (experienced by about 35% of patients) with or without neutropenia. Another challenge with irinotecan is poor bioavailability in the plasma and tissues (Chabner et al., 2011). Although the active metabolite SN-38 can be measured in the plasma (shortly after intravenous infusion), the amount of SN-38 is only 4% of the amount of irinotecan injected, suggesting that only a small portion of the pro-drug is converted to the active form of the drug. In contrast, intravenous topotecan is detected at about 25-35% in the plasma (Chabner et al., 2011).

Studies in our laboratory focus on the cell biology of small cell lung cancer (SCLC). SCLC is characterized by rapid doubling time, aggressive clinical course and a dismal survival rate (Gazdar, Bunn, & Minna, 2017; Ujhazy & Lindwasser, 2018). Cisplatin-based chemotherapy is the cornerstone for SCLC therapy. Initially, SCLC patients respond very well to therapy, with 80-100% of patients showing remission; however, the tumors typically relapse within a year and frequently do not respond to chemotherapy or radiation (Ujhazy & Lindwasser, 2018). A significant drawback with the cisplatin-etoposide regimen is its toxicity, which frequently renders SCLC patients more susceptible to adverse symptoms upon subsequent treatments. Patients with recurrent SCLC have very limited treatment options. The standard second-line chemotherapy for recurrent SCLC, camptothecin (topotecan, irinotecan), has an objective response rate of approximately 3% and little or no survival benefit (Ardizzoni, 2004; Horita et al., 2015). A subset of patients also presents with refractory platinum-resistant SCLC, which does not respond to cisplatin-based combination therapy from the beginning (Lara et al., 2015).

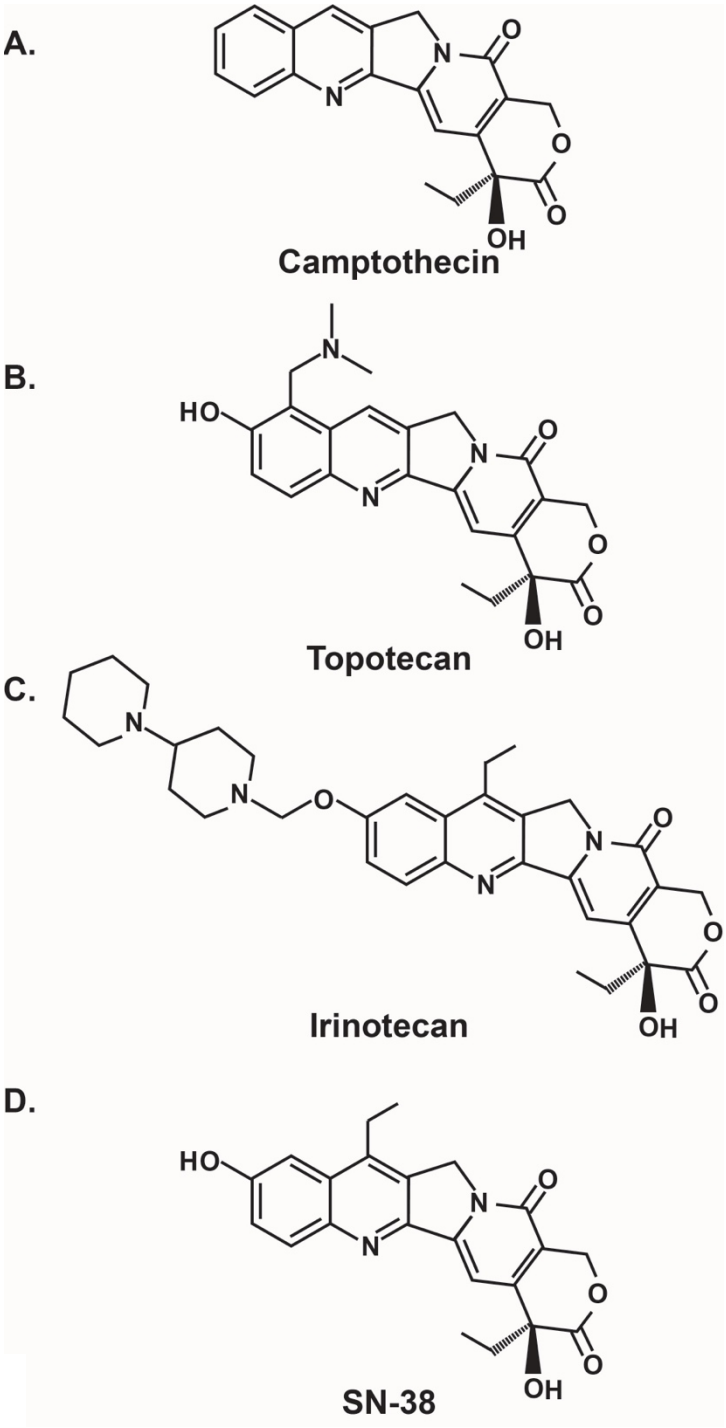

Figure 6. Camptothecin analogs
(A) Camptothecin. (B) Topotecan. (C) Irinotecan. (D) SN-38.

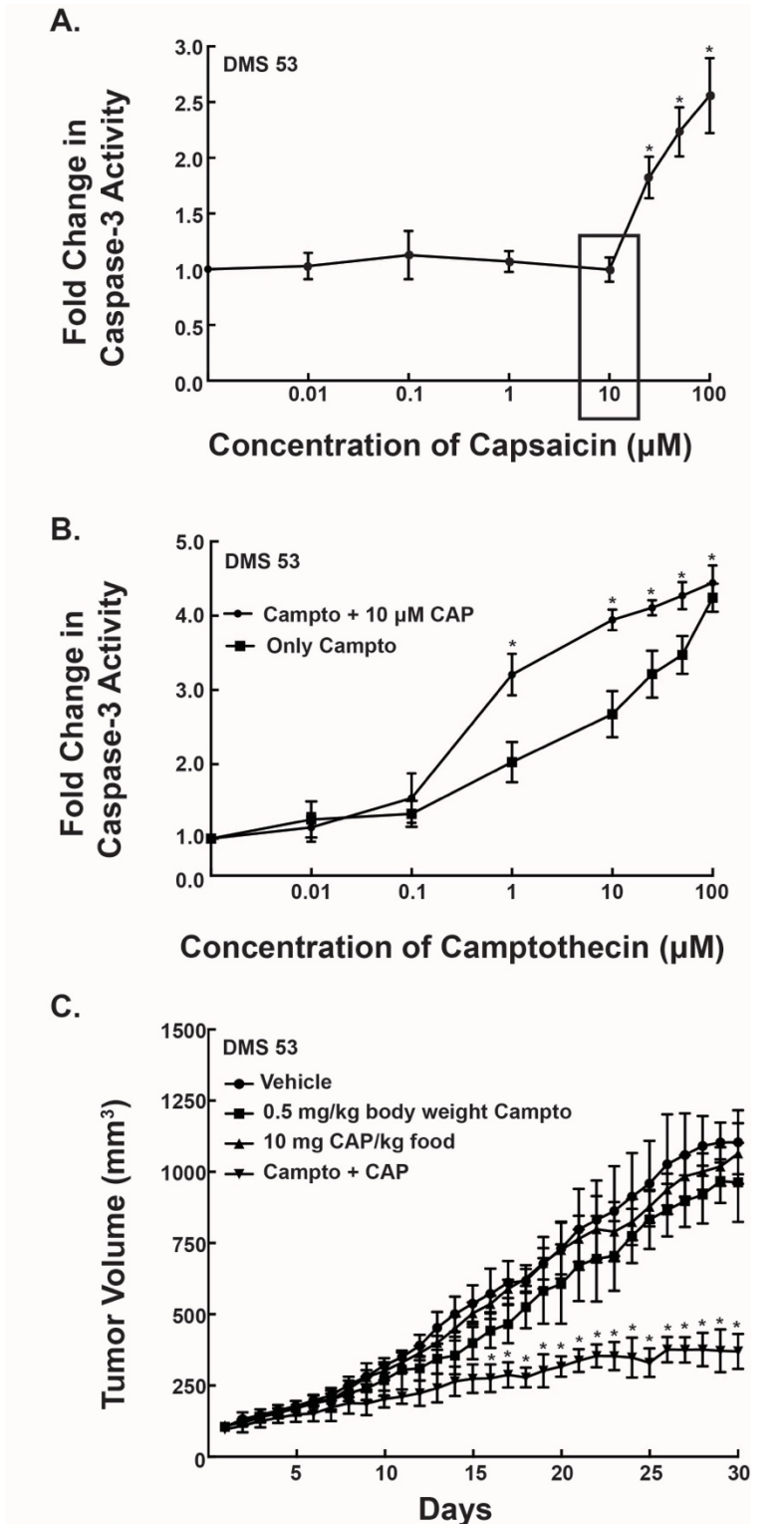

Figure 7. Combinatorial effects of capsaicin and camptothecin in small cell lung cancer (A) Caspase-3 activity of DMS53 human small cell lung cancer cells when treated with various concentrations of capsaicin (CAP). Data points denoted with a * are statistically significant relative to control ($p \leq 0.05$). (B) Caspase-3 activity of DMS53 human small cell lung cancer cells when treated with various concentrations of camptothecin (campto) alone or in combination with

10 μM capsaicin. Data points denoted with a * are statistically significant relative to campto alone ($p \leq 0.05$). (C) Tumor volumes of DMS53 xenograft mice treated with vehicle, 10 mg capsaicin/kg food, 0.5 camptothecin/kg body weight (administered twice weekly), capsaicin in combination with camptothecin as above-mentioned doses. Doses denoted with * are statistically significant relative to the vehicle, capsaicin alone and camptothecin alone ($p \leq 0.05$).

Studies in our laboratory have explored the ability of capsaicin to increase the anti-neoplastic activity of camptothecin in human SCLC cell lines. Using a panel of three human SCLC cell lines, namely DMS 114, NCI-H69 and NCI-H82, we performed apoptosis assays using multiple concentrations of capsaicin (0-100 μM) and observed that 10 μM was the highest concentration of capsaicin that did not trigger cellular apoptosis (Friedman et al., 2017). These assays were repeated in DMS 53 human SCLC cells with similar results (Figure 7A). Apoptosis was then evaluated following treatment with a concentration range of camptothecin (10 nM-100 μM) in the presence or absence of 10 μM capsaicin (Figure 7B). Isobologram analysis demonstrated that capsaicin and camptothecin synergistically induce apoptosis (CI<1) in DMS 53 SCLC cells (Table 1**)**. Signal transduction studies revealed that the combinatorial apoptotic activity of capsaicin and camptothecin was mediated by increased intracellular calcium and subsequent activation of the calpain family of calcium-sensitive proteases (Friedman et al., 2017). This chemosensitizing mechanism of capsaicin on camptothecin-induced apoptosis was further verified in H69 chicken chorioallantoic membrane (CAM) tumor models (Friedman et al., 2017). Using a DMS 53 human SCLC xenograft mouse model, we found that the dietary administration of capsaicin (10 mg/kg food in AIN-76A diet) along with 0.5 mg/kg camptothecin (i.p. injection, twice/week) displayed greater anti-tumor activity relative to either drug administered as a single agent (Figure 7C). Future studies in our laboratory aim to examine the combinatorial activity of capsaicin with irinotecan, given its clinical use in the treatment of lung cancer patients.

Concentration of Camptothecin	Concentration of Capsaicin	Combination Index (CI)
100 nM	10 μM	0.031
10 μM	10 μM	0.03
25 μM	10 μM	0.045
50 μM	10 μM	0.055
100 μM	10 μM	0.066

Table 1. Combination index of the growth inhibitory activity of camptothecin along with capsaicin in DMS53 human small cell lung cancer cells
CI<1: Synergy CI=1: Additive CI>1: Antagonistic

The pharmacokinetics of irinotecan is complex and involves several transporters and drug metabolizing enzymes (Mathijssen et al., 2001). Briefly, carboxylesterase enzymes transform irinotecan to SN-38 (Figure 6D), which is subsequently converted by UGT1A1 to form SN-38G (SN-38 Glucuronide), or by CYP3A4 to form APC (7-ethyl-10-[4-N-(5-aminopentanoic acid)-1-piperidino]-carbonyloxycamptothecin (Mathijssen et al., 2001). The OAT1B1 transporter predominantly mediates the hepatic uptake of SN-38 (Iusuf et al., 2014). Several studies have indicated that capsaicin regulates bioavailability of drugs metabolized by CYP3A4 and inhibits the activity of OAT1B1 (F. Chen, Zhai, Zhu, & Lu, 2015; Duan et al., 2013; X. J. Zhai, Shi, Chen, & Lu, 2013).

Wang et al., (2018) explored if the presence of capsaicin could increase the bioavailability of irinotecan and SN-38 in both cell culture and rat models (N. Wang et al., 2018). They pretreated Sprague-Dawley rats daily with 3 mg capsaicin/kg body weight (by oral gavage) for seven days. On the seventh day, 20 mg irinotecan/kg body weight was injected intravenously, 30 minutes after capsaicin administration. They observed that the plasma concentration of active SN-38 was about 1.5-fold higher in the capsaicin-pretreated mice relative to mice treated with irinotecan only. The presence of capsaicin prevented SN-38 from binding to

plasma protein and increased the unbound fraction of SN-38 by about 2.0-2.5-fold. Similarly, the liver/plasma ratio (L/P ratio) for SN-38 was substantially lower in capsaicin-pretreated mice compared to mice injected with irinotecan only. Taken together, these findings suggest that the presence of capsaicin increases the bioavailability of SN-38 in the plasma so that it can display greater anti-cancer activity (N. Wang et al., 2018). An innovative study by L. Wang et al., (2017) involved the synthesis of an oral self-emulsifying hydrophobic drug delivery system in which both SN-38 and capsaicin were encapsulated (referred to as 1-SEEDS) (L. Wang, Chen, et al., 2017). Subsequently, the authors characterized the drug release profiles, morphology, and droplet size of these polymeric encapsulated drug systems. They measured the effect of 1-SEEDS on the viability of a panel of human cancer cell lines, namely HCT-116, SW680 (colon carcinoma), MCF-7, MDA-MB231 (breast carcinoma) and H1299 (lung carcinoma). The growth-inhibitory activity of 1-SEEDS in colon and breast cancer cell lines was 1.5-3.0-fold higher than SN-38 alone. Furthermore, 1-SEEDS induced almost 2-fold higher apoptosis than SN-38 alone and 4-fold higher apoptosis than capsaicin alone in HCT-116 cells (L. Wang, Chen, et al., 2017). Studies in an athymic mice bearing HCT-116 human colon cancer tumors showed that 30 mg 1-SEEDS/kg body weight suppressed the growth of HCT-116 tumors to a greater extent than SN-38 or capsaicin-alone. The administration of 1-SEEDS did not produce any toxic side effects, like hemolysis or alteration in the body weights of mice (L. Wang, Chen, et al., 2017). Future research in the development of targeted drug delivery systems may make delivery of both an anti-cancer drug and a chemosensitizing agent to the site of the tumor, in a selective efficacious manner, a clinical reality. This would greatly improve the anti-tumor activity of these compounds in human cancers.

TARGETED SIGNAL TRANSDUCTION INHIBITORS

The proteasome inhibitor bortezomib (also called Velcade; Figure 8A) is used for the treatment of patients suffering from multiple myeloma (Kouroukis et al., 2014). Bhutani et al., (2007) analyzed the combinatorial apoptotic activity of capsaicin and bortezomib in U266 human multiple myeloma cells *in vitro* (Bhutani et al., 2007). The treatment of U266 cells with 25 μM capsaicin along with 20 nM bortezomib increased the fraction of apoptotic cells by about 4-fold compared to 20 nM bortezomib alone and by about 8-fold relative to 25 μM capsaicin alone. The combinatorial apoptotic effects of capsaicin and bortezomib were mediated by disruption of STAT3 activation and phosphorylation (Bhutani et al., 2007). Notably, a recent "Editor Note" alerted readers about certain ambiguities in the figures of this published report, involving the apoptosis assays performed with bortezomib and capsaicin (Bhutani et al., 2018). Figure 6A of the paper by Bhutani et al., (2007) describes the combinatorial apoptotic activity of thalidomide and capsaicin in U266 cells. Figure 6B shows that capsaicin sensitizes U266 cells to bortezomib-induced apoptosis. The images used to represent controls and capsaicin-treated cells are identical between Figure 6A and B. The editorial note mentions that the authors were unable to provide the original data for these images at the time of institutional review (Bhutani et al., 2018).

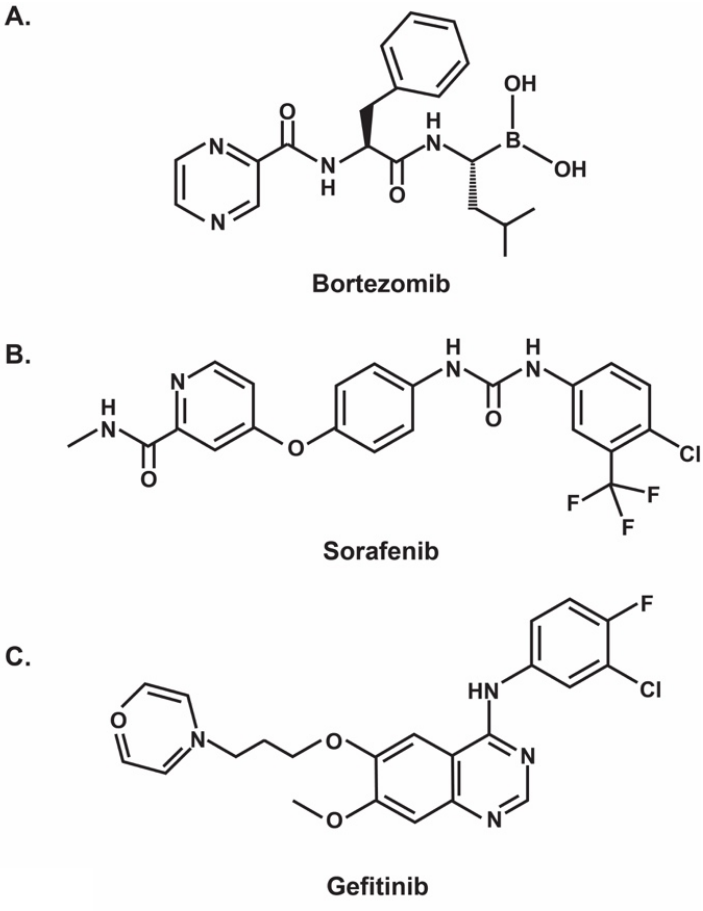

Figure 8. Targeted signal transduction inhibitors
(A) Bortezomib. (B) Sorafenib. (C). Gefitinib.

The anti-cancer drug sorafenib (Figure 8B) is an inhibitor of multiple receptor and cytoplasmic kinases including vascular endothelial growth factor receptor 1 and 2 (VEGFR-1, VEGFR-2), and platelet-derived growth factor-β (PDGF-β) -tyrosine kinase (Wilhelm et al., 2008). Sorafenib is the only systemic treatment for patients with unresectable hepatocellular carcinoma (HCC). Clinical studies indicate that it prolongs overall survival of HCC patients by approximately 2.8 months (Daher, Massarwa, Benson, & Khoury, 2018). A challenge with sorafenib therapy is the acquisition of drug resistance upon long-term treatment (Niu et al., 2017). Cell culture data suggest that sorafenib resistance may be partially abrogated by the use of PI3K/Akt inhibitors (Matter, Decaens, Andersen, & Thorgeirsson, 2014; B. Zhai et al., 2014; H.

Zhang, Wang, Liu, & Cao, 2018). Capsaicin triggers apoptosis and autophagy in many types of human cancers by blocking the PI3K/Akt/mTOR pathway (Clark & Lee, 2016; Diaz-Laviada & Rodriguez-Henche, 2014; Srinivasan, 2016). With this finding in mind, Dai et al., (2018) investigated the combinatorial growth-suppressive activity of sorafenib and capsaicin in LM3, Hep3B and HuH7 human HCC cell lines (Dai et al., 2018). The combined treatment of capsaicin and sorafenib decreased the viability of all HCC cell lines to a greater magnitude than either drug alone. The authors confirmed these findings using colony formation assays in LM3 cells, showing that multiple concentrations of capsaicin (80, 100 and 120 μM) and sorafenib (2, 3 and 4 μM) showed synergistic inhibition (CI<1) of colony formation in all possible combinations (Dai et al., 2018). The synergistic induction of apoptosis (by capsaicin and sorafenib) was accompanied by an increase in pro-apoptotic proteins like Bax, cleaved caspase-3, cleaved PARP and decrease of pro-survival proteins like Bcl-2 (Dai et al., 2018). Similarly, the combination of capsaicin and sorafenib displayed synergistic autophagic activity upregulating autophagic biomarkers Beclin-1 and LC3A/B-II and decreasing the levels of autophagy-specific substrate P62. Furthermore, Boyden chamber and wound healing assays revealed that the treatment with capsaicin (80 μM) along with sorafenib (4 μM) synergistically inhibited (CI<1) the migration and invasion of LM3 cells (Dai et al., 2018). A notable point here is that it is unclear whether this observed anti-migratory and anti-invasive activity is due to cell death or due to inhibition of cell motility pathways. The combination of capsaicin and sorafenib decreased the expression of pro-invasive proteins like vimentin, N-cadherin, MMP2 and MMP9, and increased the expression of the epithelial biomarker E-cadherin. The authors further confirmed the synergistic anti-tumor activity of sorafenib and capsaicin in LM3 human HCC tumors xenografted in athymic mice. The administration of 2.5 mg capsaicin/kg body weight together with 50 mg sorafenib/kg body

weight showed greater decrease in tumor volumes than when the drugs were administered as single agents (Dai et al., 2018). The combinatorial anti-neoplastic activity of sorafenib and capsaicin correlated with inhibition of EGFR, PI3K/Akt and activation of their downstream substrates like mTOR and p70S6 kinase.

A similar study exploring the synergistic growth inhibitory activity of sorafenib and capsaicin was performed by Zhang et al., (2018), using PLC/PRF/5, HuH7 and HepG2 human HCC cells (S. S. Zhang et al., 2018). Chou-Talalay isobologram analysis revealed that 100 μM capsaicin showed synergistic growth-suppressive activity with 3, 10 and 30 μM sorafenib. The interaction between capsaicin and lower concentrations of sorafenib (0.3 or 1 μM) showed a moderate-to-slightly antagonistic interaction. No synergy between capsaicin and sorafenib was observed in normal liver cells. The combination of 200 μM capsaicin and 50 mg sorafenib/kg body weight synergistically inhibited the growth of PLC/PRF/5 tumors in athymic mouse model (S. S. Zhang et al., 2018). Analogous to the results of Dai et al., (2018), the combination of 5 or 10 μM sorafenib with 100 μM capsaicin showed robust apoptosis with inhibition of ERK and STAT3 activation (Dai et al., 2018; S. S. Zhang et al., 2018).

Parashar et al., (2019) synthesized folic acid functionalized nanoparticles, enabling the co-administration of the epidermal growth factor receptor (EGFR) inhibitor gefitinib (Figure 8C) along with capsaicin (Parashar et al., 2019). Overexpression and activating mutations in EGFR have been reported in 10-15% of caucasian non-small cell lung cancer (NSCLC) and in about 50% of Asian NSCLC patients. The EGFR inhibitors, namely gefitinib and erlotinib, are first line therapies for such NSCLC patients (Hirsh, 2018). The authors characterized these gefitinib-capsaicin-folic acid based nanoparticles (referred hereafter as Gnb-CAP-NP) by transmission electron microscopy (TEM), measuring the particle size, drug loading, efficiency, drug

entrapment efficiency and release kinetics (Parashar et al., 2019). Cell viability and cell proliferation assays showed that Gnb-CAP-NP displayed greater growth-suppressive activity relative to capsaicin-loaded folic acid nanoparticles (CAP-NP) or gefitinib-loaded nanoparticles (Gnb-FA-NP) in A549 human NSCLC cells (Parashar et al., 2019). The encapsulated formulations of gefitinib and capsaicin decreased the viability of A549 cells to a greater magnitude than the unmodified compounds. The interaction between gefitinib and capsaicin was synergistic (CI<1).

The enhanced growth-inhibitory effect of Gnb-CAP-NP was observed to be due to both apoptosis and cell cycle arrest (Parashar et al., 2019). Cell cycle analysis revealed that Gnb-CAP-NP induced G0/G1-phase arrest in A549 cells. Gnb-CAP-NP decreased the expression of the pro-invasive protein MMP9, and the cell cycle regulatory protein p16, as well as an increase of pro-apoptotic proteins (caspase-3 and caspase-9) in A549 cells. The intravenous administration of Gnb-CAP-NP (releasing 20 mg/kg gefitinib and 10 mg/kg capsaicin simultaneously) robustly suppressed urethane-induced lung carcinogenesis in both male and female albino Wistar rats (Parashar et al., 2019). Drug delivery systems capable of co-administration of the chemotherapeutic drug as well as the chemosensitizer may represent a promising breakthrough for the treatment and management of human cancers.

RADIATION THERAPY

Radiation therapy is a modality of cancer therapy that involves administration of high doses of radiation to kill cancer cells and shrink tumors. Radiotherapy induces the death of cancer cells by causing DNA damage (Baskar, Dai, Wenlong, Yeo, & Yeoh, 2014; Eriksson & Stigbrand, 2010). Apoptosis is the predominant mechanism of cell death in response to radiation therapy, especially in hematopoietic cells and their malignant counterparts. However, radiation

therapy can also initiate cell death by other mechanisms including cell cycle arrest, senescence, and mitotic catastrophe (Eriksson & Stigbrand, 2010). As with chemotoxic therapeutics, the predominant challenges of radiation therapy are also disease recurrence and tumor resistance. The main reason for innate or acquired resistance to radiation therapy is still unknown, but current evidence suggests that multiple pathways like hypoxia and DNA-repair enzymes contribute to radiation resistance of neoplastic cells. Therefore, radiosensitizers like dietary compounds, gold nanoparticles, and HSP inhibitors have potential to improve the efficacy of radiation therapy for cancer.

Combined modality therapy refers to the combination of radiotherapy with drugs which enhance the lethal effect of radiation on cancer cells (Tannock, 1989). The radiosensitizing ability of capsaicin has been studied in human prostate cancer. Venier et al., (2013, 2015) evaluated the effects of capsaicin and radiation therapy using three distinct human prostate cell lines, namely LNCAP (androgen-receptor positive, wild type p53), DU145 (androgen-receptor negative, mutant p53 P223L, and V274F) and PC3 (androgen-receptor negative, p53 null; (Venier, Colquhoun, Klotz, Fleshner, & Venkateswaran, 2013; Venier et al., 2015). The authors wanted to compare the radiosensitizing ability of capsaicin between prostate cancer cells and normal prostate epithelial cells. They selected RWPE-1 as the normal prostate epithelial cell line (undetectable androgen receptor expression, p53 null, Rb null). The authors measured the radiosensitizing ability of capsaicin using colony formation assays. They treated LNCAP, PC-3 and RWPE-1 cells with radiation in the presence or absence of capsaicin for 5 days (Venier et al., 2015). The capsaicin was added one hour prior to irradiation. The sensitizer enhancement ratio (SER) was calculated by dividing the area under the curve (AUC) for vehicle (DMSO) and the AUC for capsaicin-treated cells. The authors observed that the radiosensitizing activity of

capsaicin was not concentration-dependent. LNCAP showed robust radiosensitizing activity of capsaicin at all three concentrations (0.01, 1 and 10 μM; SER greater than 1). However, RWPE-1 only showed synergistic radiosensitization with capsaicin at the 10 μM dose (Venier et al., 2015). It was found that the treatment of LNCAP and PC3 cells with radiation and capsaicin increased the expression of the DNA damage marker phospho-H2AX and decreased the number of cells in S-phase. The combinatorial growth-inhibitory activity of capsaicin and radiation was determined to be independent of TRPV1 and correlated with decreased levels of phospho-NF-κB (Venier et al., 2013; Venier et al., 2015). Previous studies by the same research group showed that the radiosensitization of capsaicin coincided with upregulation of Bax, Bad, p21and p27 and decrease in the levels of androgen receptor in a panel of human prostate cancer cell lines (Klotz et al., 2011; Venier et al., 2012). Subsequently, the authors confirmed the radiosensitizing ability of capsaicin in athymic mice xenografted with LNCAP tumors (Venier et al., 2015). Mice were given a dose of 6 Gray (Gy) units of ionizing radiation on day 16 in the presence or absence of capsaicin. HPLC analysis confirmed the presence of capsaicin and its metabolites in the serum of mice. The combination of capsaicin and radiation significantly decreased the growth rate of LNCAP tumors compared to vehicle-treated mice or mice administered each drug alone (Venier et al., 2015). IHC analysis of the tumors revealed that radiation upregulated the expression of NF-κB, which was decreased when capsaicin was included in the treatment regimen. Notably, they did not observe any alteration of total NF-κB expression when they treated LNCAP cells *in vitro* with radiation alone, capsaicin alone or in combination. However, they did observe that capsaicin suppressed radiation-induced phospho-NF-κB levels in both LNCAP cells (in cell culture) and in LNCAP tumors excised from athymic mice. The combination of radiation and capsaicin resulted in sustained DNA damage in LNCAP tumors as evidenced by increased

expression of the DNA damage marker γ-H2AX in the sections prepared from LNCAP tumors xenografted in athymic mice (Venier et al., 2015). Moreover, LNCAP tumor bearing mice administered radiation in the presence of capsaicin showed significantly lower Ki-67 index (a marker for cell proliferation) and androgen receptor expression. Conflicting data exists on the radiosensitizing activity of capsaicin. Nishino et al., (2016) did not find any combinatorial growth-inhibitory activity of radiation and capsaicin in A549 human lung adenocarcinoma cells (Nishino et al., 2016). However, such divergent results can be explained by differences in experimental design in the two studies. Whereas the studies described by Venier et al., (2015) pretreated the cells with 10 μM capsaicin one hour prior to irradiation, Nishino et al., (2016) treated cells with 10 μM capsaicin 5 minutes after irradiation (Nishino et al., 2016; Venier et al., 2015). The data obtained from drug-combination studies are often dependent on the treatment protocol (pretreatment versus adding both drugs simultaneously versus post-treatment). Also, Nishino et al., (2016) used A549 human lung cancer cells for their studies. Other studies have reported that capsaicin decreased the viability of A549 cells at high concentrations (above 200 μM) via inflammatory signaling and genotoxic stress mechanisms rather than apoptosis (Halme et al., 2016; Lewinska, Jarosz, et al., 2015; Reilly et al., 2003). Such observations seem to suggest that capsaicin may have divergent bioactivity depending on the nature of the cell line, the concentration of capsaicin being used, or the time sequence of the experiments. The studies by Klotz et al., (2011) and Venier et al., (2012, 2013 and 2015) used prostate cancer cells, whereas Nishino et al., (2016) investigated the radiosensitizing activity of capsaicin in lung adenocarcinoma and melanoma cells (Klotz et al., 2011; Nishino et al., 2016; Venier et al., 2013; Venier et al., 2012; Venier et al., 2015). A strength of the research report by Venier et al., (2015)

is that they validated their findings both in cell culture and athymic mouse models of prostate cancer (Venier et al., 2015).

CONCLUSIONS AND FUTURE DIRECTIONS

The capsaicinoid family of nutritional compounds, isolated from chili peppers, can sensitize multiple types of human cancers to the apoptotic effects of conventional, as well as targeted anti-cancer drugs. However, only a handful of targeted therapies, namely sorafenib, gefitinib and bortezomib, have been combined with capsaicin to investigate their combinatorial growth inhibitory activity. It is hoped that several classes of targeted therapies like anti-angiogenic agents, immunotherapies, hormonal agents and signal transduction inhibitors will display synergistic anti-cancer activity upon combination with capsaicinoids. Recent research has characterized novel drug delivery systems like liposomes and nanoparticles which simultaneously release the chemosensitizer (capsaicin) and the anti-cancer drug namely SN-38 or gefitinib.

The clinical application of capsaicin is limited by its unpleasant side effects including gut pain, hyperalgesia, stomach cramps and nausea (Drewes et al., 2003; Hammer, 2006; O'Neill et al., 2012). This drawback could be circumvented by natural non-pungent capsaicinoids, like the capsiates, which retain the anti-tumor activity of capsaicin but do not produce the “heat-sensation” of capsaicin and RTX (Friedman et al., 2018). Apart from natural non-pungent capsaicinoids, structure-activity relationship studies have generated synthetic non-pungent capsaicin analogs (Friedman et al., 2018). Future research involving the chemosensitization activity of these non-pungent capsaicin analogs will facilitate the development of clinically relevant combination therapies for the treatment and management of cancer.

CHAPTER 3: CAPSAICIN SYNERGIZES WITH CAMPTOTHECIN TO INDUCE INCREASED APOPTOSIS IN HUMAN SMALL CELL LUNG CANCERS VIA THE CALPAIN PATHWAY

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ABSTRACT

Small cell lung cancer (SCLC) is characterized by excellent initial response to chemotherapy and radiation therapy with a majority of the patients showing tumor shrinkage and even remission. However, the challenge with SCLC therapy is that patients inevitably relapse and subsequently do not respond to the first line treatment. Recent clinical studies have investigated the possibility of camptothecin-based combination therapy as first line treatment for SCLC patients. Conventionally, camptothecin is used for recurrent SCLC and has poor survival outcomes. Therefore, drugs which can improve the therapeutic index of camptothecin should be valuable for SCLC therapy. Extensive evidence shows that nutritional compounds like capsaicin (the spicy compound of chili peppers) can improve the anticancer activity of chemotherapeutic drugs in both cell lines and animal models. Statistical analysis shows that capsaicin synergizes with camptothecin to enhance apoptosis of human SCLC cells. The synergistic activity of camptothecin and capsaicin is observed in both classical and variant SCLC cell lines and *in vivo*, in human SCLC tumors xenotransplanted on chicken chorioallantoic membrane (CAM) models. The synergistic activity of capsaicin and camptothecin are mediated by elevation of intracellular calcium and the calpain pathway. Our data foster hope for novel nutrition-based combination therapies in SCLC.

INTRODUCTION

Small cell lung cancer (SCLC) accounts for about 15–20% of all lung cancer cases and is the most aggressive type of lung cancers (Kahnert, Kauffmann-Guerrero, & Huber, 2016; Koinis, Kotsakis, & Georgoulias, 2016). Cisplatin or carboplatin in combination with etoposide is the standard of care for SCLC patients. Although this regimen initially works very well in SCLC patients with a response rate of greater than 80%, the disease inevitably relapses within a year, at

which point the tumor is non-responsive to cisplatin-based combination therapies (Alvarado-Luna & Morales-Espinosa, 2016). Another drawback with the cisplatin- etoposide regimen is its toxicity, which may render SCLC patients more susceptible to adverse symptoms upon subsequent treatments (Stewart, 2004). Patients with recurrent SCLC have very limited options, as the only standard chemotherapy with an FDA-approved drug, camptothecin (Figure 9A), has an objective response rate of approximately 3% and little or no survival benefit (Asai, Ohkuni, Kaneko, Yamaguchi, & Kubo, 2014). Clinical trials have explored the possibility of camptothecin-based combination regimens for standard of care therapy for SCLC patients (Stewart, 2004). Therefore, agents which can increase the therapeutic efficacy of camptothecin may improve the outcomes of SCLC therapy. Several convergent studies have shown that dietary compounds can sensitize neoplastic cells to the apoptotic effects of chemotherapeutic drugs (Ho & Cheung, 2014; Mohan, Narayanan, Sethuraman, & Krishnan, 2013). Our published data show that capsaicin (the spicy compound of chili peppers; Figure 9B) can induce robust apoptosis in human SCLC cells in cell culture and mouse models (Lau, Brown, Dom, & Dasgupta, 2012; Lau et al., 2014). Therefore, we conjectured that low doses of capsaicin (where it does not cause cell death) may sensitize human SCLC cells to the apoptotic activity of camptothecin and its derivatives.

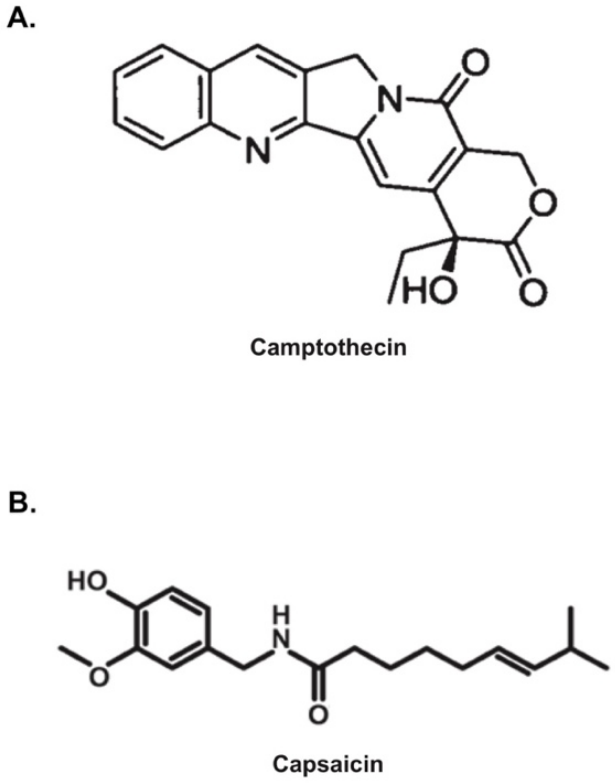

Figure 9. Structure of (A) camptothecin and (B) capsaicin

A survey of literature shows that capsaicin increases the therapeutic index of several anticancer treatments. The administration of capsaicin increased the therapeutic efficacy of radiation in prostate cancer. Monofunctional platinum-based drugs, like LH5, showed increased apoptotic activity in combination with capsaicin (Arzuman et al., 2016). Similarly, the treatment of stomach cancer cells with a combination of cisplatin and capsaicin caused greater apoptosis than either of these agents given singly (Huh et al., 2011; Wiwanitkit, 2012). A similar effect was also observed when capsaicin was given in combination with the doxorubicin analog pirarubicin (Zheng et al., 2016). The present manuscript investigates for the first time the anticancer activity of the combination of capsaicin and camptothecin. We show that low doses of capsaicin (where it does not cause any apoptosis) synergizes with camptothecin to induce high levels of cellular apoptosis in human SCLCs. We confirmed the synergistic apoptotic activity of

capsaicin and camptothecin in classical human SCLC cell lines (NCI-H69 and DMS 114), as well as the variant human SCLC cell line NCI-H82. Another innovative feature about our study is that we have analyzed the synergistic interaction between these two drugs by the Chou-Talalay isobologram method (Chou, 2008, 2010).

The apoptotic activity of capsaicin-camptothecin combination was confirmed using two independent apoptosis assays. Subsequently, we show that the combination of capsaicin and camptothecin enhances apoptosis (compared to these agents given alone) *in vivo*, in human SCLC tumors xenotransplanted on chicken chorioallantoic membranes (CAM) (Nowak-Sliwinska, Segura, & Iruela-Arispe, 2014). We also examined the signaling pathways underlying the combinatorial synergistic apoptotic activity of capsaicin and camptothecin. We found that the synergistic apoptotic activity capsaicin and camptothecin was mediated by elevation of intracellular calcium and activation of the calpain pathway both in cell culture and in chicken CAM models. The results of our studies may lead to improved treatment regimens for SCLC.

MATERIALS AND METHODS

Reagents

Camptothecin, capsaicin, BAPTA-AM (1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis(acetoxymethyl ester) and calpeptin were purchased from Sigma-Aldrich (St. Louis, MO, USA). All cell culture reagents, including RPMI-1640, FBS, Trypsin-EDTA, and HEPES, were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Sodium pyruvate, glucose, and penicillin-streptomycin solutions were obtained from Corning (NY, USA).

Cell culture

The human SCLC cell lines NCI-H82, NCI-H69 (hereafter referred to as H82 and H69) and DMS 114 were purchased from ATCC (Manassas, VA). The ATCC used Short Tandem Repeat (STR) profiling for authentication of these cells. H69 and H82 were cultured in RPMI-1640 supplemented with 2 mM glutamine, 4.5 g/L glucose, 100 units/ml penicillin, 100 units/ml streptomycin and 10% fetal bovine serum (FBS). DMS 114 was cultured in RPMI-1640 containing 2 mM glutamine, 25 mM HEPES, 1 mM sodium pyruvate, 4.5 g/L glucose, 100 units/ml penicillin, 100 units/ml streptomycin and 10% FBS. All cell lines were maintained in a 37°C humidified incubator with 5% carbon dioxide (NuAire Laboratory Equipment, Plymouth MN).

Preparation of lysates

Cell lysates were made using detergent-based lysis protocol as described previously (Hurley et al., 2017). Cells were harvested and washed three times with cold PBS. Cells were then lysed with M2 lysis buffer (20 mM Tris, pH 7.6, 0.5% IGEPAL-CA-630, 250 mM NaCl, 3 mM EGTA, 3 mM EDTA, 4 µM DTT, 5 mM PMSF, 1 mM sodium fluoride, 1 mM sodium orthovanadate, 25 µg/ml leupeptin, 5 µg/ml pepstatin, 5 µg/ml aprotinin, and 25 µg/ml trypsin-chymotrypsin inhibitor) and the lysates were prepared as detailed elsewhere (Hurley et al., 2017). The protein concentration of the lysate was measured using Bradford Reagent (Bio-Rad Laboratories, Hercules, CA, USA).

Measurement of caspase-3 activity

DMS 114 human SCLC cells were cultured to 80% confluence as described above. On the day of the experiment, the medium of the cells was changed to RPMI medium containing 1% FBS. Subsequently, cells were treated with the indicated concentrations of the relevant drugs for

24 hours at 37°C. A few of the drug treatments involved treating the human DMS 114 cells with both camptothecin and capsaicin. In these cases, capsaicin was added 45 minutes before camptothecin and then the cells were incubated for 24 hours at 37°C.

Cell lysates were made using the Caspase-3 Activity Kit (EMD Millipore Corporation, Billerica, MA, USA). The protein concentration of the lysate was measured using Bradford Reagent (Bio-Rad Laboratories, Hercules, CA, USA). An aliquot of the cell lysate containing one hundred micrograms of protein was used for the measurement of caspase-3 activity, according to the manufacturer's protocol.

Each sample was measured in triplicate and the whole experiment was repeated three times using independent sets of cell lysates. Caspase-3 Activity in untreated lysates was considered to be equal to 1, and the activity observed in treated lysates was calculated as fold increase relative to the untreated control sample. The experimental procedure was identical in H69 and H82 cells.

Cell death ELISA

DMS 114 human SCLC cells were cultured to 80% confluence in T-75 tissue culture flasks (Nunc, Roskilde, Denmark). On the day of the experiment, the medium of the cells was changed to RPMI medium containing 1% FBS. The DMS 114 human SCLC cells were treated with the indicated concentration of the appropriate drug for 24 hours at 37°C. A few experiments involved treating the human DMS 114 SCLC cells with both camptothecin and capsaicin. In these cases, capsaicin was added 45 minutes before camptothecin, and then the cells were incubated for 24 hours at 37°C.

Cells were then lysed with M2 lysis buffer (described above), and the lysates were prepared as detailed above (Hurley et al., 2017). The protein concentration of the lysate was

measured using Bradford Reagent (Bio-Rad Laboratories, Hercules, CA, USA). Twenty micrograms of lysate were used for each sample. Cellular apoptosis was measured by the Cell Death ELISA Kit (Roche Life Sciences, Indianapolis, IN, USA), according to manufacturer's protocol. The absorbance value of control untreated cells was taken as 1, and the absorbance of drug-treated cells were graphically represented as fold-increase relative to the control. The protocol was identical for H69 and H82 human SCLC cells. Each sample was measured in duplicate and the entire experiment was repeated three times with independent sets of lysates.

Chicken chorioallantoic membrane (CAM) assay

Specific pathogen-free (SPF) fertile chicken eggs (Charles River Laboratories, North Franklin, CT) were incubated at 37.5°C with 75% relative humidity and continuously rotated slowly by an automatic egg turner (G.Q.F. Manufacturing Company, Savannah, GA). At Day 9, eggs were candled and windows opened on the shell to expose the CAM (Nowak-Sliwinska et al., 2014). H69 cells (1.5×10^6) were suspended in 100 μL cold serum-free medium, mixed with 100 μL cold BD Matrigel Matrix (BD Biosciences, San Jose, CA) and treated with 10 μM capsaicin or 1 μM camptothecin or a combination of 10 μM capsaicin and 1 μM camptothecin (K. C. Brown et al., 2013; Lau et al., 2013). These cells were applied to the CAM of each chicken embryo. Eggs were incubated at 37°C for seven days before tumor implants were removed, photographed and weighed. A total of eight eggs were assayed for each group.

Preparation of tumor lysates from CAM

Chicken CAM experiments were performed as described above. After the H69 human SCLC tumors were excised, they were snap frozen in liquid nitrogen. An aliquot of 30 mg of the tumor was weighed and used to make tumor lysates. Tumor lysates were prepared using T-Per lysis buffer (Pierce Biotechnology, Rockford, IL, USA), according to manufacturer's protocol

(K. C. Brown et al., 2013; Dasgupta et al., 2011; Lau et al., 2014; Lau et al., 2013). The caspase-3 activity assay was performed with four independent sets of tumor lysates prepared from control CAM H69 tumors, 10 μM capsaicin-treated CAM H69 tumors, 1 μM camptothecin-treated CAM H69 tumors, and H69 CAM tumors treated with a combination of 1 μM camptothecin and 10 μM capsaicin. The cellular apoptosis in these lysates was measured by using the Caspase-3 Activity Kit (Chemicon, Temecula, CA, USA). Each sample was measured in triplicate and the entire experiment was repeated four times with independent sets of lysates.

Measurement of calpain activity

H69 human SCLC cells were treated with 10 μM capsaicin or 1 μM camptothecin or a combination of 10 μM capsaicin and 1 μM camptothecin (for 24 hours) in RPMI medium containing 1% FBS. Subsequently, cells were harvested and washed twice with PBS. Cell lysates were prepared using the assay buffer provided in the Sensolyte 520 Calpain Activity Assay Kit (Anaspec, Freemont, CA, USA). An aliquot of the cell lysate containing two hundred micrograms of protein was used for each replicate sample. The samples were incubated with 50 μl of calpain substrate for 60 minutes at 37°C (Guha et al., 2010; Lau et al., 2014). The rest of the assay was performed according to manufacturer's instructions. The fluorescence intensity was measured using a Biotek Synergy2 spectrofluorometer (Biotek Instruments, Winooski, VT, USA) at excitation and emission wave- lengths of 490 and 520 nm, respectively. Each sample was measured in duplicate and the whole experiment was repeated three times with independent sets of lysates. Calpain activity in untreated lysates was considered to be equal to 1, and the activity observed in treated lysates was calculated as fold increase relative to the untreated control sample.

The calpain enzyme activity assay was also performed using the tumor lysates from H69 tumors xenografted on chicken CAM (K. C. Brown et al., 2013; Dasgupta et al., 2011; Lau et al., 2014; Lau et al., 2013). The tumor lysates were made as described above. An aliquot of two hundred micrograms of the protein was used in the calpain assay. The methodology and data representation of the assay was similar to the one described for H82 and H69 cells.

Statistical analysis

All data were plotted using GraphPad Prism 5 Software, Inc (La Jolla, CA, USA), and results were represented as the mean ± standard deviation (SD). Results from the control and treated samples were compared using an analysis of variance (ANOVA) followed by a Tukey posthoc multiple comparison test. All analyses were completed using a 95% confidence interval. Data were considered significant when $p \leq 0.05$.

All data involving combinatorial interactions between camptothecin and capsaicin were evaluated by the Chou-Talalay isobologram analysis using the method of non-constant ratios (Chou, 2008, 2010). The Chou-Talalay isobologram analysis (Calcusyn Graphing Software Version 2.11, Biosoft Inc., Ferguson, MO, USA) is an established method to determine if two drugs exhibit synergistic, additive or antagonistic interactions (Chou, 2008, 2010). This method was used to examine whether camptothecin and capsaicin displayed a synergistic increase in apoptotic activity. The Chou-Talalay isobologram analysis yields a parameter called the combination index (CI). A CI below 1 is taken to be an indicator of synergism. The lower the value of the CI, the stronger the synergy between the drugs.

RESULTS

A concentration of 10 μM capsaicin does not cause significant apoptosis ($p \leq 0.05$) in human small cell lung cancer (SCLC) cell lines.

The first series of experiments analyzed the concentration dependent apoptotic activity of capsaicin in human SCLC cell lines over 24 hours, using the caspase-3 activity assay. We observed that the capsaicin displays little apoptotic activity until 10 μM and subsequently causes robust apoptosis at 50 and 100 μM in DMS 114 human SCLC cells (Figure 10A). The highest concentration at which capsaicin did not induce significant apoptosis ($p \leq 0.05$) was 10 μM in DMS 114 cells. The caspase-3 activity assay was repeated in two additional human SCLC cell lines, H69 and H82, and similar results were obtained (Figure 10B and C). Our eventual goal was to test if low doses of capsaicin (where it does not display apoptotic activity) could sensitize human SCLC cells to camptothecin-induced apoptosis. Therefore, we selected the concentration of 10 μM capsaicin for all our subsequent experiments.

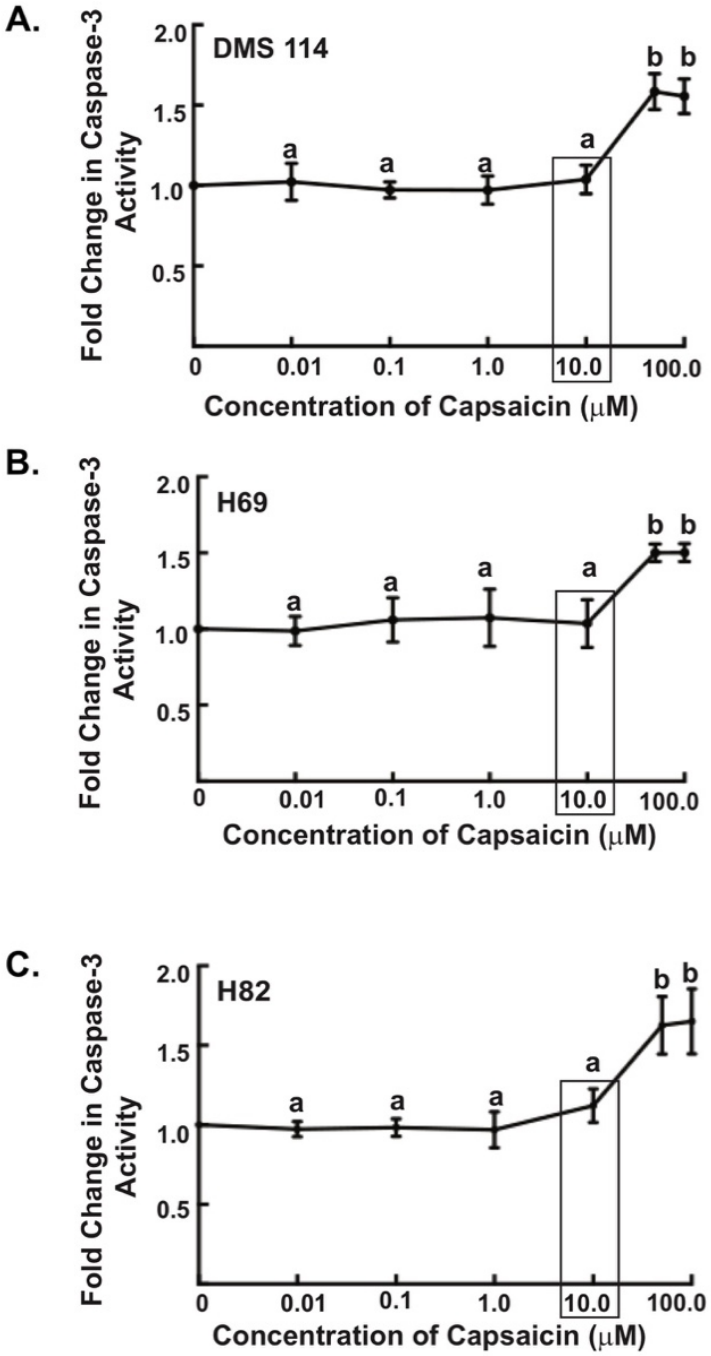

Figure 10. Concentration dependent apoptotic activity of capsaicin in human SCLC cells over 24 hours, as measured by the caspase-3 activity kit

(A) Capsaicin displayed very little apoptotic activity until 10 μM, after which it induced robust apoptosis at 50 μM and 100 μM in DMS 114 cells. (B) The apoptotic activity of capsaicin was confirmed in H69 as well as H82 human SCLC cells (C) Each sample was measured in triplicate and the whole experiment was repeated three times using independent sets of cell lysates. Caspase-3 activity in untreated lysates was considered to be equal to 1, and the activity observed

in treated lysates was calculated as fold increase relative to the untreated control sample. Statistical analysis showed that 10 μM capsaicin (indicated by the box on the graph) was the highest concentration at which capsaicin did not display significant apoptotic activity ($p \leq 0.05$). Values represented by the same letter are not statistically significantly different from each other.

The results obtained from the caspase-3 activity assay were verified using a second apoptosis assay, the Cell Death ELISA Kit (Roche Life Science). We obtained similar results as the caspase-3 activity assay. The treatment of DMS 114 cells with varying doses of capsaicin caused little cell death until 10 μM, and after that the levels of cell death rose significantly over 24 hours ($p \leq 0.05$) (Figure11A). The experiment was repeated in H69 and H82 cells and similar results were obtained (Figure 11B and C). Based on the data of these two assays we selected 10 μM capsaicin for our subsequent experiments.

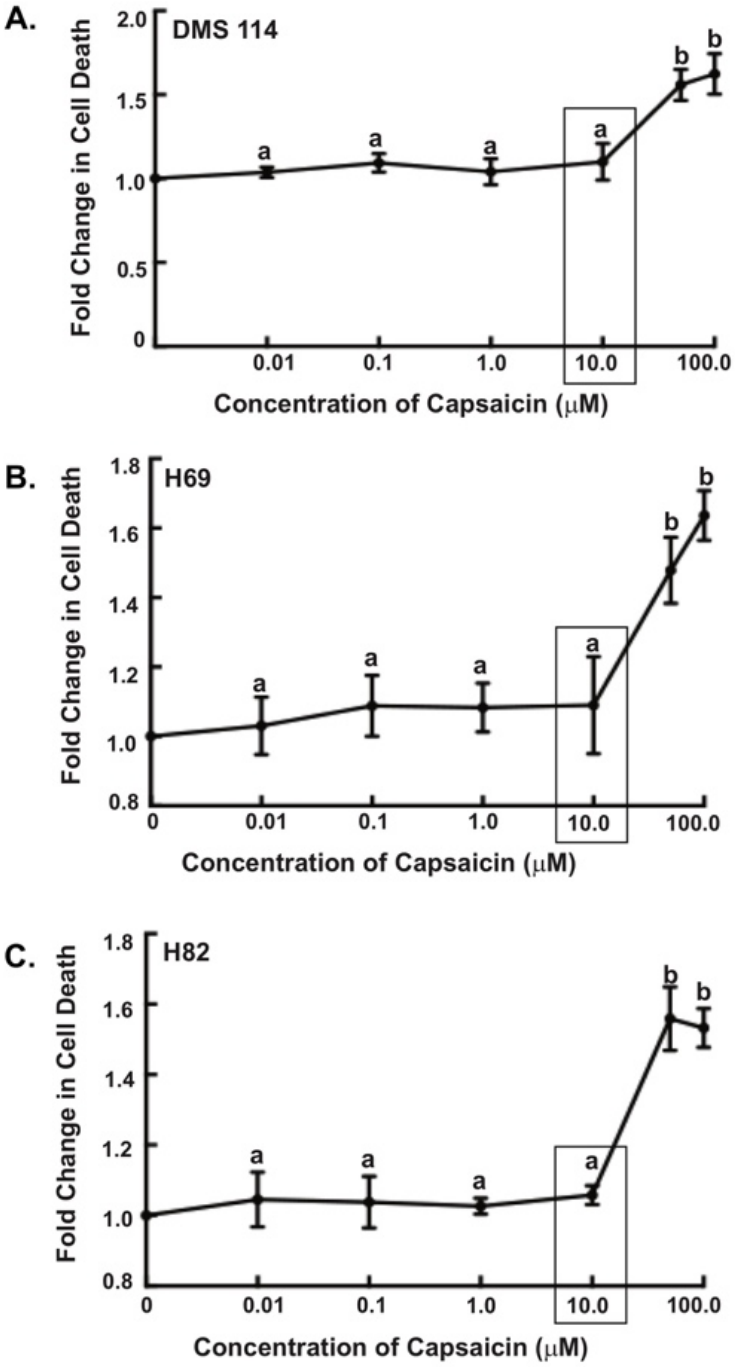

Figure 11. Cell Death ELISA assays were used to confirm the apoptotic activity of capsaicin over 24 hours

(A) The apoptotic activity of capsaicin was minimal in DMS 114 human SCLC cells until a concentration of 10 μM, after which it caused between 1.5 and 2.0-fold apoptosis at 50 μM and 100 μM over 24 hours. (B) The pro-apoptotic activity of capsaicin was verified in a classical human SCLC cell line H69 and as well as a variant human SCLC cell line H82 (C) and analogous results were obtained. Each sample was measured in triplicate and the whole

experiment was repeated three times using independent sets of cell lysates. The absorbance obtained in untreated lysates was considered to be equal to 1, and the activity observed in treated lysates was calculated as fold increase relative to the untreated control sample. The highest concentration at which capsaicin did not display significant apoptotic activity was 10 μM ($p \leq 0.05$). Values represented by the same letter are not statistically significantly different from each other.

The combinatorial apoptotic activity of camptothecin and capsaicin is greater than these drugs treated alone in human SCLC cells

Caspase-3 activity assays were performed to test the combinatorial apoptotic activity of capsaicin (referred as CPZ) and camptothecin (referred as CPT) in DMS 114 human SCLC cells (Figure 12A). DMS 114 cells were treated with multiple concentrations of camptothecin (0.01–100 μM) in the presence or absence of 10 μM capsaicin. The 10 μM capsaicin was added 45 min before the addition of camptothecin. Figure 12A shows that the combination of camptothecin and capsaicin (indicated by solid black line with the black round dots) possessed greater apoptotic activity than corresponding concentrations of camptothecin alone (dotted line with black square dots) or capsaicin alone (Figure 10 and 11). The Chou-Talalay analysis was used to determine if capsaicin synergized with camptothecin to induce enhanced apoptotic activity (Chou, 2008, 2010). As mentioned in Materials and Methods, a combination index (CI) below 1 is an indicator of synergism; the lower the magnitude of CI (below 1) the greater the synergy (Chou, 2008, 2010). Figure 12A shows that the maximal synergy was observed for the combination of 10 μM capsaicin and 1 μM camptothecin (CI = 0.095). The combination of 10 μM capsaicin and 10 μM camptothecin was also synergistic (CI = 0.15); however, the magnitude of synergy was decreased. Figure 12B represents the normalized isobologram of the capsaicin-camptothecin combination in DMS 114 cells (Chou, 2008, 2010). The symbols on the isobologram indicate the CI of the two drugs namely camptothecin (CPT) and capsaicin (CPZ). The numbers 1–6 next to the symbols on the isobologram represent the different combination regimens (described in the

legends). The closer the CI is to the zero value the stronger is the synergy between the two drugs. Figure 12B shows that treatment number 3 (10 μM capsaicin and 1 μM camptothecin) showed the maximal synergistic interaction.

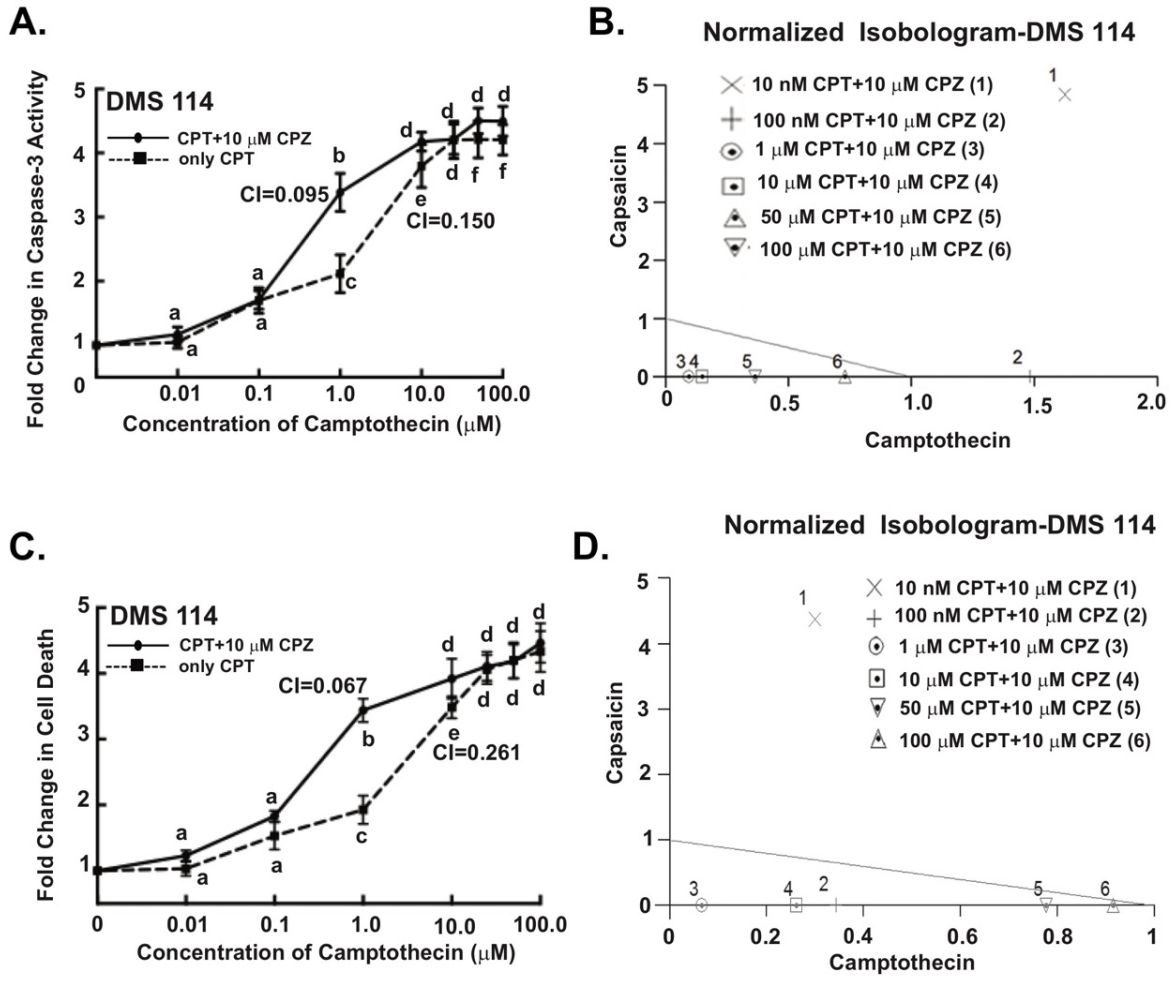

Figure 12. Capsaicin (CPZ) sensitizes human SCLC cells to the apoptotic activity of camptothecin (CPT)

(A) Concentration dependent apoptosis observed in DMS 114 cells human SCLC cells in response to camptothecin (10 nM–100 μM; depicted by the dotted lines with square symbols). Apoptosis was measured by Caspase-3 Activity assays. The solid black line (with circular symbols) represents the concentrations of camptothecin (10 nM–100 μM) along with 10 μM capsaicin. The data were evaluated by the Chou-Talalay isobologram and the combination indices (CI) were determined. A CI value below 1 indicates synergy, the lower the CI value the stronger is the synergy. The maximal synergy was observed for the combination of 1 μM camptothecin and 10 μM capsaicin (CI = 0.095). The combination of 10 μM camptothecin and 10 μM capsaicin (CI = 0.15) showed lower synergy than the 1 μM camptothecin–10 μM capsaicin combination. Each sample was measured in triplicate and the experiment was performed three independent times. Values indicated by the same letters are not statistically significant ($p \leq 0.05$). (B) The normalized isobologram generated from the Chou-Talalay analysis of Figure 12A. The CI of the various combination treatments are numbered 1–6 on the graph (the details of the treatments are described in the legends). We observed that treatment # 3 (corresponding to 1 μM camptothecin and 10 μM capsaicin) has the lowest CI. (C) The results

obtained from the caspase-3 activity assay were verified using the Cell Death ELISA assay in DMS 114 cells and similar results were obtained. The apoptotic activities of varying concentrations of camptothecin (CPT) are represented by the dotted lines with square symbols over 24 hours. When 10 μM capsaicin (CPZ) was added to each of these treatments, the magnitude of cell death was substantially increased (solid black lines with circular symbols). The maximal synergy was obtained with a combination of 1 μM camptothecin and 10 μM capsaicin (CI = 0.067) followed by 10 μM camptothecin–10 μM capsaicin combination (CI = 0.261) in 24 hours. (D) The normalized isobologram obtained after the Chou-Talalay analysis of Figure 12C. The combination treatment #3 (1 μM camptothecin and 10 μM capsaicin) has the lowest CI followed by treatment #4 (10 μM camptothecin and 10 μM capsaicin).

The synergistic apoptotic activity of capsaicin and camptothecin in DMS 114 cells was verified by a second apoptosis assay, the Cell Death ELISA Kit (Roche BioSciences). We observed similar results as the caspase-3 activity assay. The combination of 10 μM capsaicin (CPZ) with varying concentrations of camptothecin (CPT) produced increased apoptosis (indicated by solid black line with the black round dots) compared to camptothecin alone (dotted line with black square dots) or capsaicin alone (Figure 12C) in DMS 114 cells over 24 hours. The values for the combination indices (CI) showed that 10 μM capsaicin displayed the maximal synergy with 1 μM camptothecin (CI = 0.067) followed by 10 μM capsaicin and 10 μM camptothecin (CI = 0.261; Figure 12C). Figure 12D shows the corresponding normalized isobologram in DMS 114 cells. The pattern of CI in the isobologram corresponds to the Cell Death ELISA Kit (Figure 12C). As can be observed, the combination of 10 μM capsaicin (CPZ) and 1 μM camptothecin (CPT) has the lowest CI out of all other combinations. The results of these experiments were repeated in H82 human SCLC cells (Figure 13A) and H69 human SCLC cells (Figure 14A). Capase-3 Activity assays show that the synergistic interaction between 10 μM capsaicin (CPZ) and 1 μM camptothecin (CPT) is stronger in H82 than in DMS 114 and H69 cells. Figure 13B represents the normalized isobologram of the capsaicin-camptothecin combination in H82 as measured by the caspase-3 activity assay. A similar isobologram was also obtained for H69 cells (Figure 14B). These combinatorial apoptotic activities of camptothecin

and capsaicin were proved in H82 cells (Figure 13C) and H69 cells (Figure 14C) using the Cell Death ELISA Kit. The pattern of synergy observed in the isobologram in both cell lines closely parallels the results obtained by the caspase-3 activity assay (Figure 13D and 14D). Taken together, the combination of 10 μM capsaicin (CPZ) and 1 μM camptothecin (CPT) displayed the maximum synergy in all three human SCLC cell lines, and this combination was used for the signal transduction experiments outlined later in the manuscript.

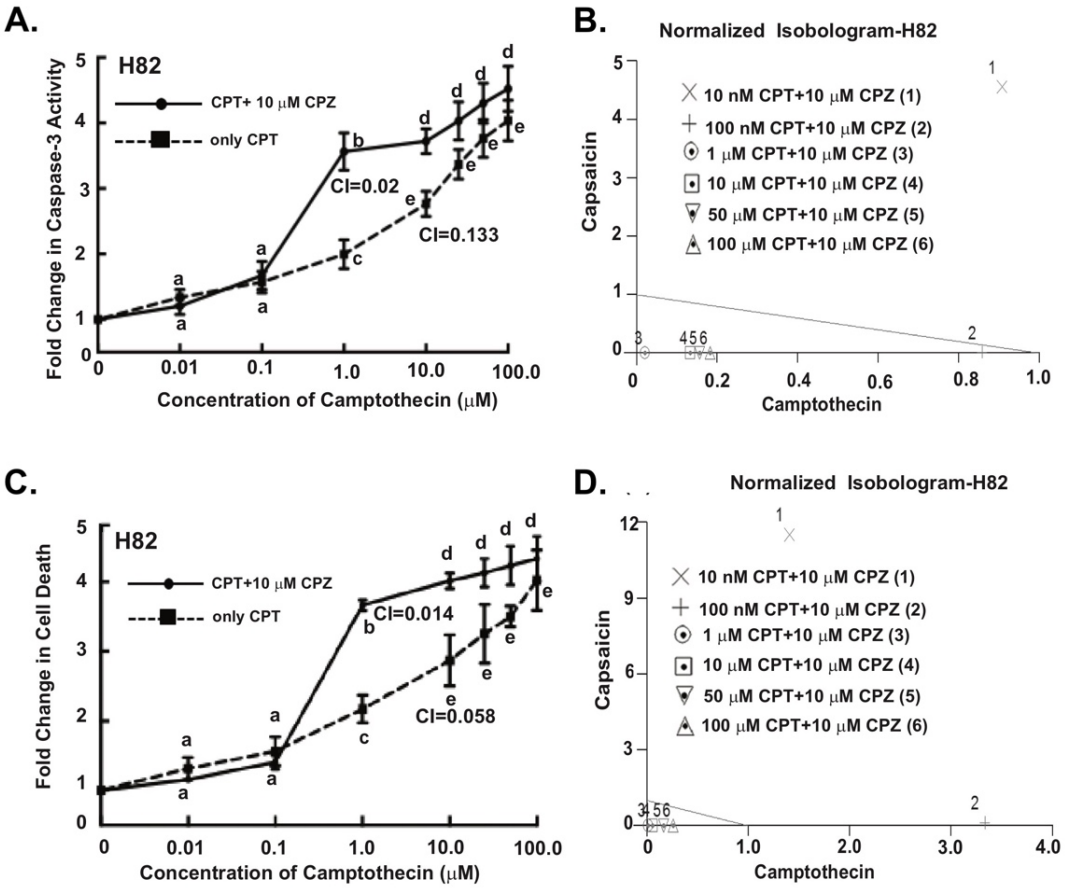

Figure 13. The synergistic apoptotic activity of capsaicin (CPZ) and camptothecin (CPT) is observed in multiple SCLC cell lines

(A) Caspase-3 activity assays were used to measure the pro-apoptotic activity of a range of camptothecin concentrations (10 nM–100 μM; dotted lines with square symbols) in H82 human variant SCLC cells over 24 hours. When 10 μM capsaicin was added along with camptothecin there was a significant increase in cellular apoptosis ($p \leq 0.05$; depicted by solid black lines) in H82 cells. Chou-Talalay isobologram analysis demonstrates that the greatest synergy was observed for the combination of 1 μM camptothecin and 10 μM capsaicin (CI = 0.02). Furthermore, the combination of 10 μM camptothecin and 10 μM capsaicin (CI = 0.133) exhibited poorer synergy than the 1 μM camptothecin–10 μM capsaicin combination in H82 cells. Each sample was measured in triplicate and the experiment was performed three independent times. Values indicated by the same letters are not statistically significant ($p \leq 0.05$). (B) The normalized isobologram generated from the Chou-Talalay analysis of Figure 13A. The CI of the various combination treatments represented as points within the isobologram (numbered 1–6 on the graph; the details of the treatments are described in the legends). (C) The data obtained in the Cell Death ELISA assay (with H82 cells), closely match the caspase-3 activity assay; the utmost synergy was observed with a combination of 1 μM camptothecin and10 μM capsaicin (CI = 0.014) followed by blend of 10 μM camptothecin–10 μM capsaicin (CI = 0.058) in H82 cells over 24 hours. (D) The normalized isobologram obtained after the

Chou-Talalay analysis of Figure 13C. The combination regimen 3 (1 μM camptothecin and 10 μM capsaicin) has the lowest CI followed by regimen #4 (10 μM camptothecin and 10 μM capsaicin).

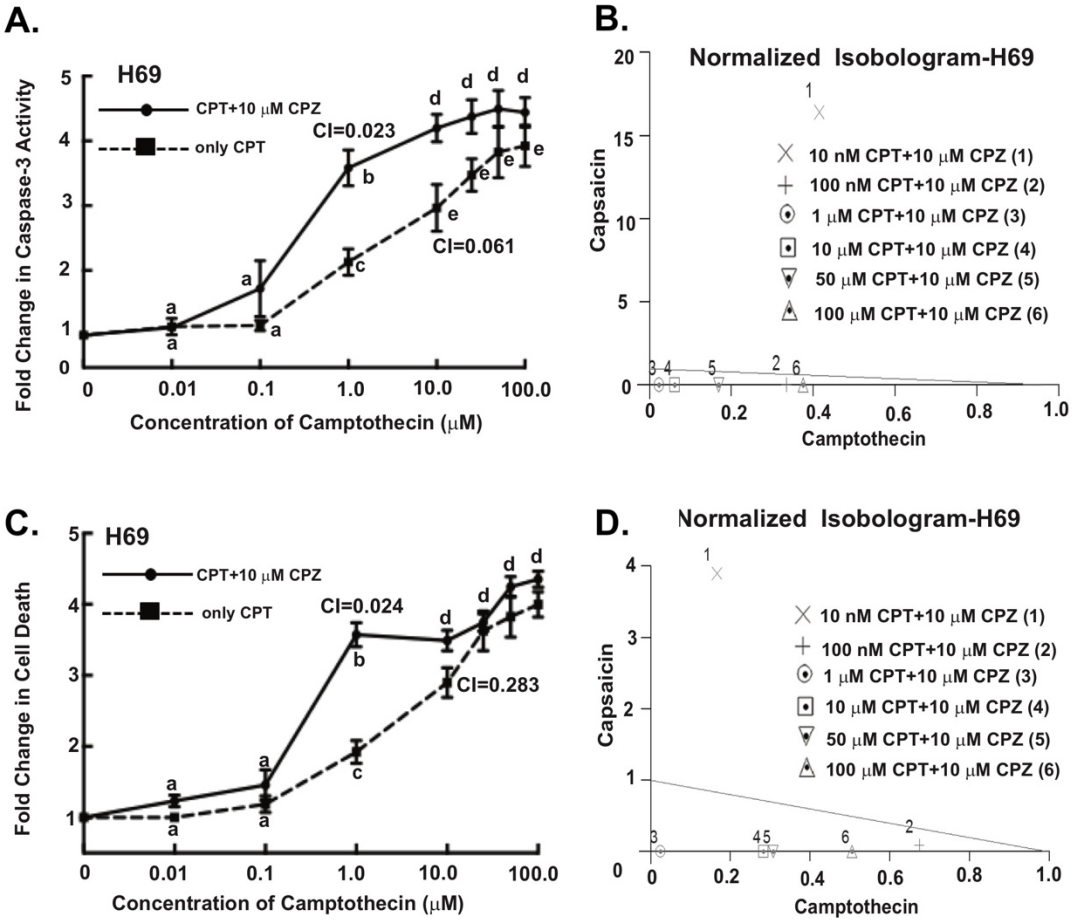

Figure 14. Camptothecin (CPT) and capsaicin (CPZ) induce synergistic cell death in the classical human SCLC cell line H69

(A) Caspase-3 activity assays demonstrate that camptothecin (at concentrations ranging from 10 nM to 100 μM; dotted lines with square symbols) induce 4–5-fold apoptosis in H69 cells over 24 hours. When 10 μM capsaicin was added along with each of these concentrations of camptothecin there was upregulation of apoptotic cell death (shown by solid black lines) in H69 cells. The highest synergy was observed 1 μM camptothecin and 10 μM capsaicin (CI = 0.023), as measured by the Chou-Talalay analysis. We also noted that the amalgamation of 10 μM camptothecin and 10 μM capsaicin (CI = 0.061) exhibited decreased synergy compared to the 1 μM camptothecin–10 μM capsaicin combination in H69 cells. Each sample was measured in triplicate and the experiment was performed three independent times. Values indicated by the same letters are not statistically significant ($p \leq 0.05$). (B) The normalized isobologram generated from the Chou-Talalay analysis of Figure 14A. The CI of the various combination modalities are represented as points within the isobologram (these points are numbered 1–6 on the graph; the details of the treatments are labelled in the legends) in H69 cells. (C) The outcomes obtained from the caspase-3 activity assay were confirmed using the Cell Death ELISA assay in H69 cells

and parallel results were obtained. (D) The normalized isobologram obtained after the Chou-Talalay analysis of Figure 14C. The combination treatment # 3 (1 μM camptothecin and 10 μM capsaicin) has the lowest CI followed by treatment condition #4 (10 μM camptothecin and 10 μM capsaicin).

Capsaicin synergizes with camptothecin to display increased apoptotic activity *in vivo* in chicken chorioallantoic membrane (CAM) assay

We wanted to study whether capsaicin and camptothecin display synergistic apoptotic activity i*n vivo*. For this purpose, we selected the chicken CAM model (K. C. Brown et al., 2013; Lau et al., 2013; Nowak-Sliwinska et al., 2014). The advantage of the chicken CAM model is that the concentrations used in cell culture can be directly translated in the chicken CAM experiments. In contrast, the doses in mice experiments are usually expressed in mg/kg body weight, and it is difficult to correlate these doses to the concentration of the drugs used in cell culture models (Kain et al., 2014; Lokman, Elder, Ricciardelli, & Oehler, 2012). Keeping these considerations in mind we opted for the chicken CAM model to determine whether capsaicin could sensitize H69 human SCLC cells to the apoptotic activity of capsaicin. An aliquot of $1.5X10^6$ H69 cells were treated with 1 μM camptothecin in the presence or absence of 10 μM capsaicin and then implanted on the chorioallantoic membrane of a fertilized chicken egg. The H69 cells formed tumors on the chicken CAM. The chicken CAM was incubated at 37°C for seven days, and then the tumors were excised and weighed. Figure 15A shows that the combination of 10 μM capsaicin and 1 μM camptothecin displayed significantly greater anti-tumor activity than either of these agents alone ($p \leq 0.05$). An aliquot of these tumors was snap frozen in liquid nitrogen and lysates were made. Four independent sets of lysates were made for every treatment. Caspase-3 activity apoptosis assays showed that the tumors treated with 10 μM capsaicin and 1 μM camptothecin showed significantly greater apoptosis than 10 μM capsaicin

alone or 1 μM camptothecin alone ($p \leq 0.05$; Figure 15B). Each sample was measured in duplicate and the entire experiment was repeated three independent times.

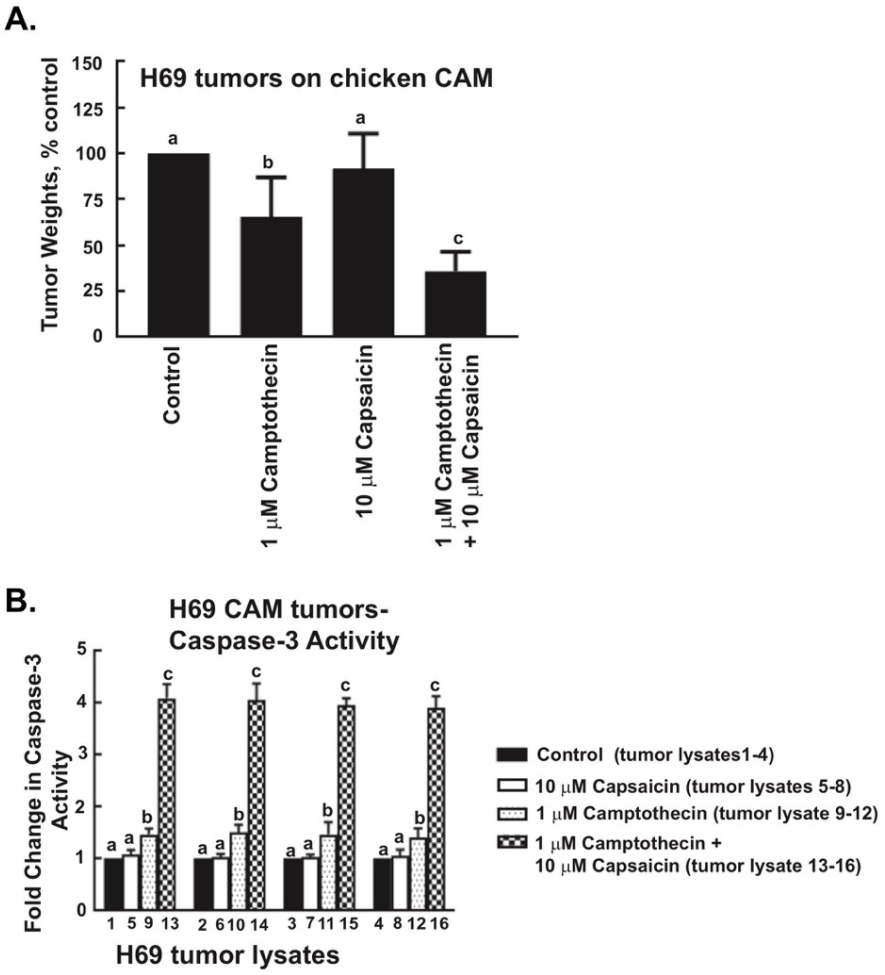

Figure 15. The combination of 1 μM camptothecin and 10 μM capsaicin inhibited the growth of human SCLC tumors *in vivo* in chicken chorioallantoic membrane (CAM) model
The tumor weights in the control group were taken as 100, and the tumor volumes in the rest of the samples were calculated as percentage of control. (A) Chicken CAM assays showed that the treatment of 10 μM capsaicin (as a single agent) did not significantly suppress the growth of H69 tumors xenotransplanted on chicken CAM (tumor weights = 92 ± 18% relative to control; $p \leq 0.05$). The treatment of H69 tumors with 1 μM camptothecin caused a decrease in tumor volumes (tumor volumes = 66 ± 20.6% relative to control). However, the combination of 1 μM camptothecin and 10 μM capsaicin decreased tumor volumes down to about 36 ± 10% relative to control. Each group was comprised of eight chicken CAMs. (B) After seven days, the tumors were excised and snap frozen in liquid nitrogen. Four independent tumor lysates were made for each sample. Caspase-3 apoptosis assays reveal that the control tumors (1–4) and capsaicin-treated tumors (5–8) displayed very little apoptotic activity. Camptothecin-treated H69 tumors (9–12) induced about 1.5-fold increase in caspase-3 activity. However, the H69 tumors treated

with both 1 μM camptothecin and 10 μM capsaicin (13–16) displayed robust apoptotic activity which was significantly higher than any of the drugs as single agents ($p \leq 0.05$). Each sample was measured in triplicate and the experiment was performed four independent times. Values indicated by the same letters are not statistically significantly different ($p \leq 0.05$).

The synergistic activity of capsaicin and camptothecin was dependent on intracellular calcium and the calpain pathway

The signal transduction pathways underlying the combinatorial activity of capsaicin and camptothecin was probed by using specific chemical inhibitors. Multiple convergent studies have shown that the elevation of intracellular calcium and subsequent activation of the calpain pathway is important in mediating camptothecin-induced apoptosis in several experimental systems (Cao, Deng, & May, 2003; Mandic et al., 2002; D. E. Wood & Newcomb, 1999; D. E. Wood et al., 1998). Similarly, our published data and those of others have also shown a role for calcium signaling pathway and calpain activation in the apoptotic effects of capsaicin (Lau et al., 2014; Oh & Lim, 2009). We conjectured that perhaps these two drugs were converging on the calcium-calpain pathway and the amplification of this signaling network was responsible for the synergistic apoptotic activity of capsaicin and camptothecin.

Caspase-3 Activity apoptotic assays were used to determine the role of intracellular calcium in the apoptotic effects of capsaicin and camptothecin over 24 hours. The calcium chelator BAPTA-AM potently abrogated the apoptotic activity of camptothecin-capsaicin combination (Figure 16A) in H69 cells (Lau et al., 2014; Oh & Lim, 2009). The experiment was repeated in H82 cells and similar results were obtained (Figure 16B). The role of the calpain pathway in the synergistic apoptotic activity of capsaicin and camptothecin was analyzed by the calpain inhibitor calpeptin. The presence of calpeptin ablated the synergistic apoptotic activity of capsaicin and camptothecin (in H69 human SCLC cells), as measured by the caspase-3 activity

(Figure 16C). The experiment was repeated in a second human SCLC cell line H82, and similar results were obtained (Figure 16D).

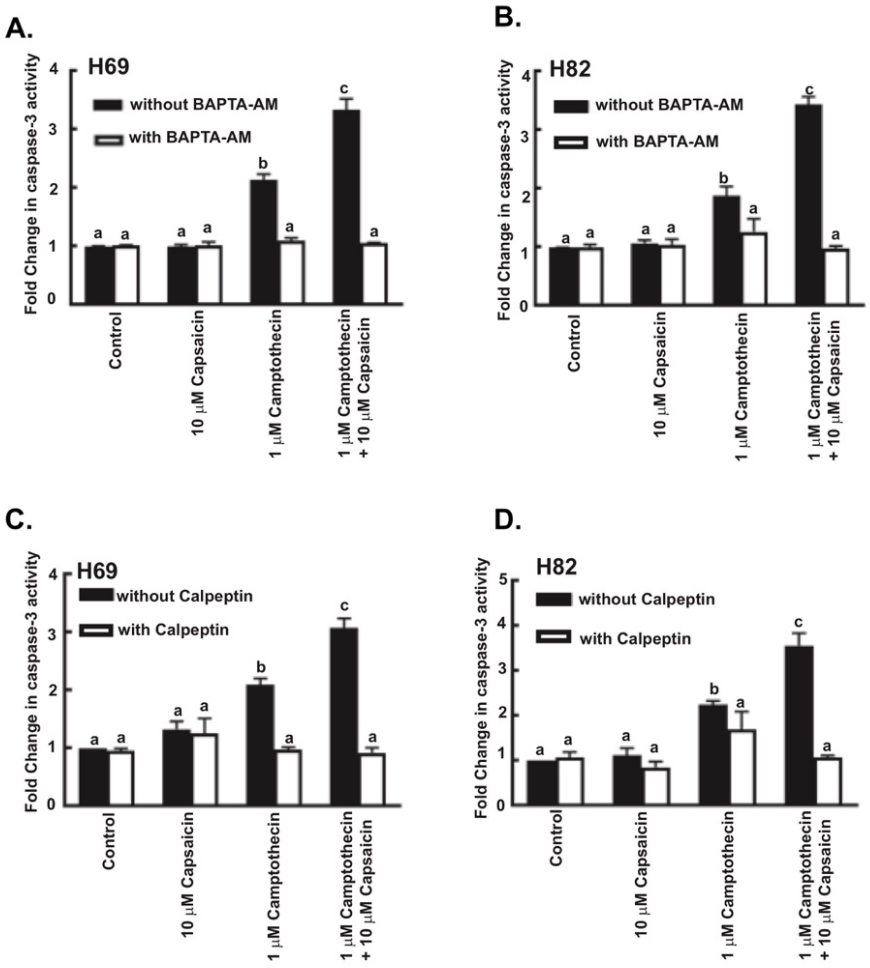

Figure 16. The combinatorial apoptotic activity of 1 μM camptothecin and 10 μM capsaicin was mediated by intracellular calcium and the calpain pathway
(A) Caspase-3 activity assays indicate that the presence of 10 μM BAPTA-AM abrogated the combinatorial apoptotic activity of capsaicin and camptothecin in both H69 and H82 (B) human SCLC cells over 24 hours. (C) Similarly, the treatment of H69 human SCLC cells with 10 μM calpeptin suppressed the synergistic apoptotic activity of 1 μM camptothecin and 10 μM capsaicin (as measured by caspase-3 activity assays) in H69 cells. (D) The experiment was repeated in H82 cells and similar results were obtained in 24 hours. Each sample was measured in triplicate and the experiment was performed three independent times. Values indicated by the same letters are not statistically significant ($p \leq 0.05$).

The results obtained with BAPTA-AM and calpeptin were verified by using a second apoptosis assay, the Cell Death ELISA Kit. The presence of BAPTA-AM reversed the

combinatorial apoptotic activity of capsaicin and camptothecin in H69 human SCLC cells in 24 hours (Figure 17A). These experiments were repeated in H82 human SCLC cells and similar results were obtained (Figure 17B). Similarly, we observed that calpeptin suppressed the synergistic apoptotic activity of capsaicin and camptothecin in H69 human SCLC cells (Figure 17C), as measured by the Cell Death ELISA Kit. The assay was repeated in the human SCLC cell line H82, and similar results were obtained (Figure 17D).

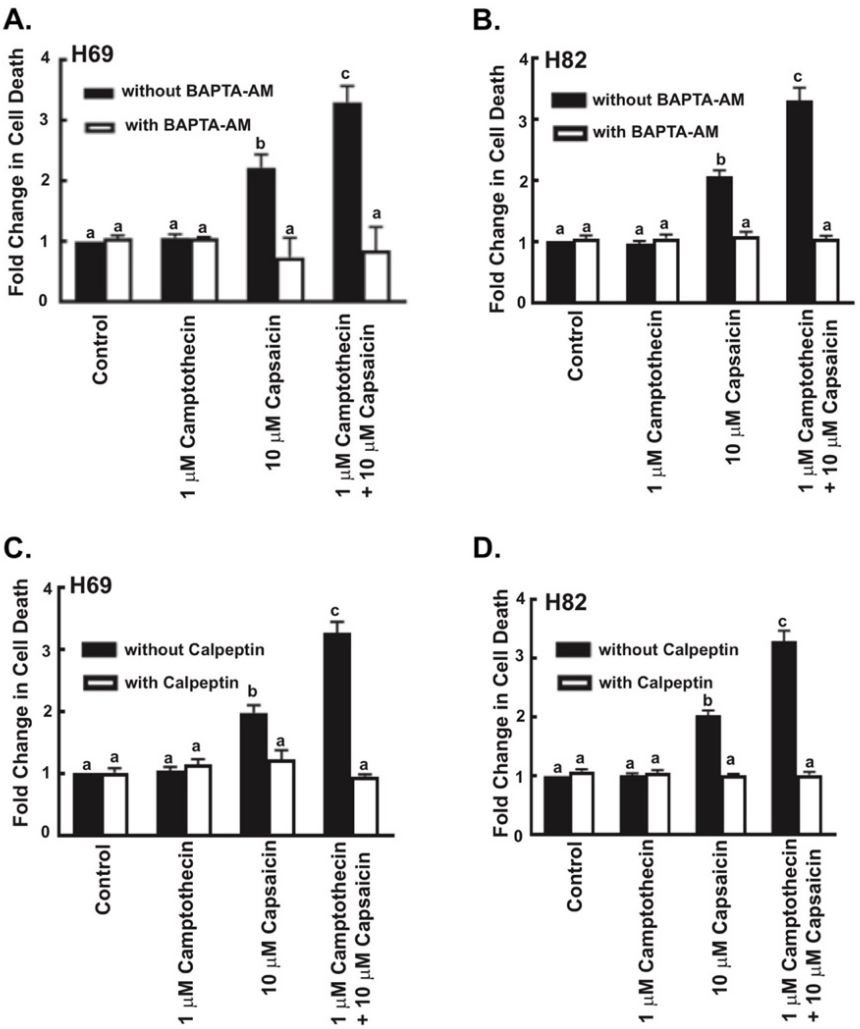

Figure 17. H69 human SCLC cells were treated with 1 μM camptothecin, 10 μM capsaicin or a combination of 1 μM camptothecin and 10 μM capsaicin in the presence or absence of 10 μM BAPTA-AM for 24 hours

(A) Cell death ELISA assays show that BAPTA-AM reversed the apoptotic activity of the capsaicin-camptothecin combination in H69 human SCLC cells. (B) The experiment was repeated in H82 human SCLC cells and analogous results were obtained. (C) The calpain inhibitor calpeptin suppressed cell death induced by a combination of 1 μM camptothecin and 10 μM capsaicin in H69 cells over 24 hours. (D) The entire experiment was repeated in the H82 variant human SCLC cells and similar results were obtained. Each sample was measured in duplicate and the experiment was performed three independent times. Values indicated by the same letters are not statistically significant ($p \leq 0.05$).

SCLC cells treated with 10 μM capsaicin and 1 μM camptothecin show increased calpain activity relative to each of the drugs alone

The role of the calpain pathway in the synergistic apoptotic activity of 10 μM capsaicin and 1 μM camptothecin was confirmed by the measurement of calpain activity in H69 and H82 cells. Figure 18A shows that the treatment of H69 human SCLC cells with 10 μM capsaicin and 1 μM camptothecin produces a potent increase in calpain activity (over 24 hours), which is significantly greater than each of these drugs as single agents. The calpain activity (induced by capsaicin-camptothecin combination) was abrogated by the intracellular calcium chelator BAPTA-AM (Figure 18A; white bars). The experiment was repeated in the variant human SCLC cell line and analogous results were obtained (Figure 18B).

We also observed that the combination of 10 μM capsaicin and 1 μM camptothecin caused a 4–5-fold increase in calpain activity in H69 human SCLC cells in 24 hours, which was suppressed by the calpain pathway inhibitor calpeptin (Figure 18C; white bars) (Lopatniuk & Witkowski, 2011). The experiment was repeated in H82 cells and similar results were obtained (Figure 18D).

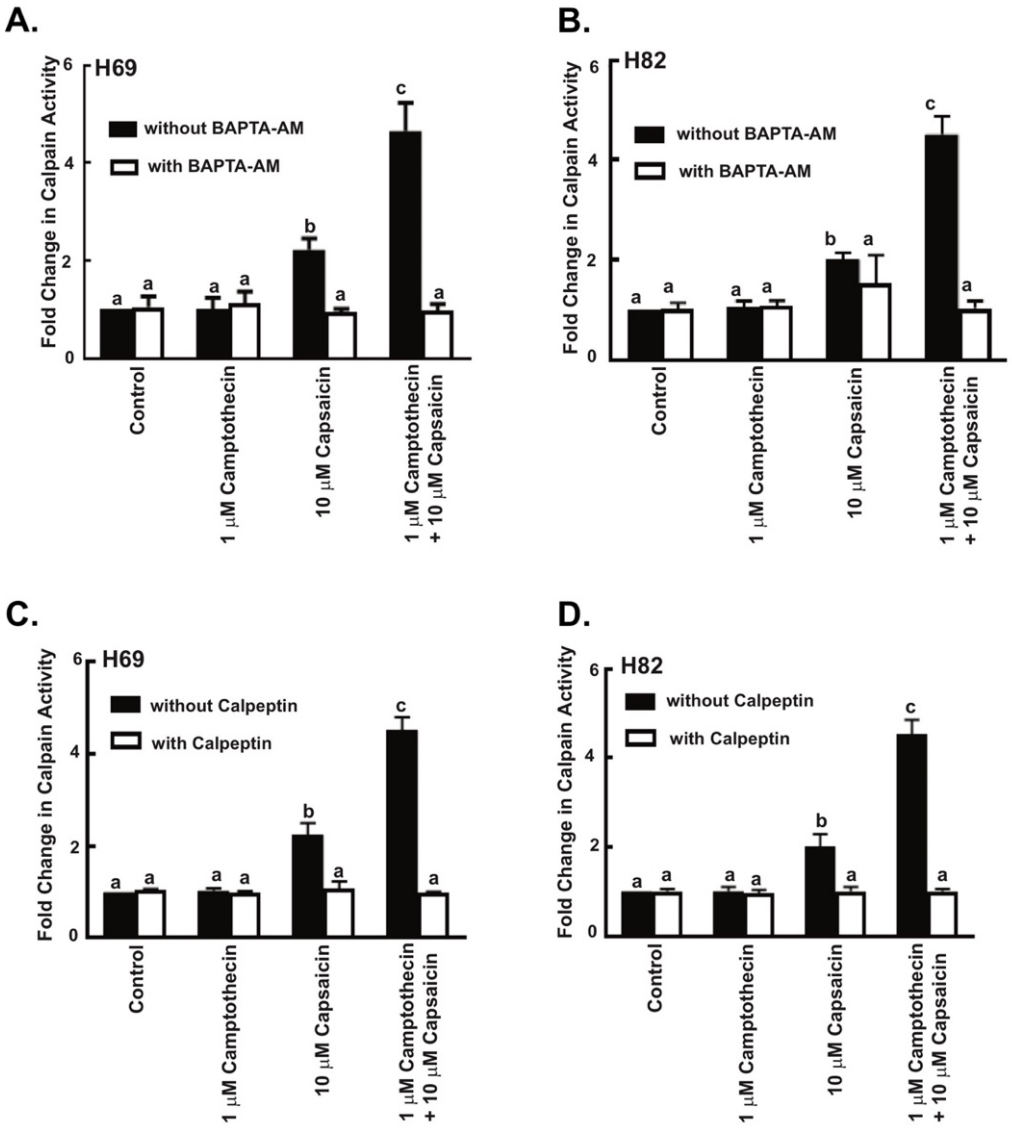

Figure 18. The combination of 1 μM camptothecin and 10 μM capsaicin potently stimulates calpain activity in human SCLC cells

(A) H69 human SCLC cells treated with 1 μM camptothecin or 10 μM capsaicin or a combination of both for 24 hours. Cell lysates were made and calpain activity was measured. The combination of 1 μM camptothecin and 10 μM capsaicin induced greater than 4-fold increase in calpain activity, which was greater than either drugs used as single agents. The elevation of calpain activity (in response to the combination of 1 μM camptothecin and 10 μM capsaicin) was dependent on the calcium pathway, as demonstrated by its abrogation by BAPTA-AM. (B) The results of these experiments were confirmed using H82 human SCLC cells. (C) The combination of 1 μM camptothecin and 10 μM capsaicin elevated specifically calpain activity in H69 cells, and such elevation of calpain activity was blocked by the calpain-specific inhibitor calpeptin over 24 hours. (D) The calpain enzyme assay was repeated in a

second human SCLC cell line H82 and comparable results were obtained. Each sample was measured in duplicate and the experiment was performed three independent times ($p \leq 0.05$).

Finally, we tested whether calpain activity was upregulated in the H69 tumors implanted on chicken CAM which had been treated with a combination of camptothecin and capsaicin. Four tumor lysates were analyzed per treatment regimen. Figure 19 shows that the calpain activity in the H69 tumors treated with 10 μM capsaicin and 1 μM camptothecin is substantially higher than those treated with 10 μM capsaicin alone and 1 μM camptothecin alone. Our data suggest that the synergistic apoptotic activity of capsaicin and camptothecin involves elevation of intracellular calcium which in turn induces enhanced activation of calpain pathway, leading to cellular apoptosis.

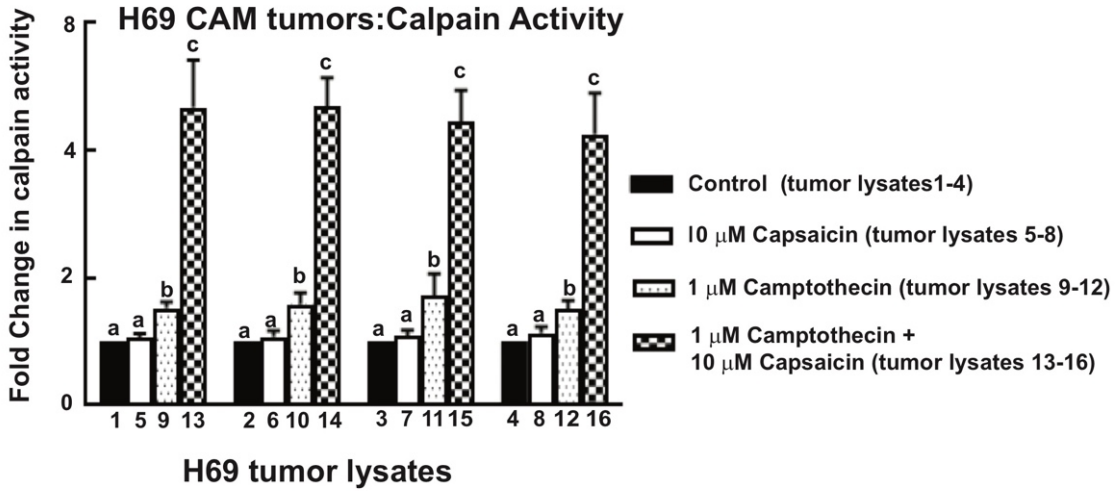

Figure 19. Elevation of calpain activity in H69 tumors treated with a combination of 1 μM camptothecin and 10 μM capsaicin
Four independent tumor lysates were used for the assay for each sample. Calpain activity assays show that 10 μM capsaicin-treated tumors (5–8) displayed very little increase of calpain activity relative to control tumors (1– 4). Camptothecin-treated H69 tumors (9–12) induced modest elevation increase in calpain activity. However, the H69 tumors treated with both 1 μM camptothecin and 10 μM capsaicin (13–16) displayed a greater magnitude of increase in calpain activity, relative of the drugs as single agents ($p \leq 0.05$). Each sample was measured in duplicate and the experiment was performed four independent times. Values indicated by the same letters are not statistically significant ($p \leq 0.05$).

DISCUSSION

Camptothecin is primarily an inhibitor of topoisomerase 1. Topoisomerase 1 is an enzyme which relaxes supercoiled DNA during DNA replication. During DNA replication and repair, the enzyme topoisomerase 1 creates single strand breaks in the DNA. Camptothecin forms a tertiary complex with topoisomerase 1 and the cleaved DNA, thereby blocking the annealing of DNA sister strands (Nagourney, Sommers, Harper, Radecki, & Evans, 2003; Stewart, 2004). This camptothecin-DNA-Topoisomerase 1 complex causes DNA damage and eventually leads to cellular apoptosis. Other mechanisms of camptothecin-induced cell death include cell cycle arrest at the G1 or G2/M phase (depending on the dose of drug used), generation of reactive oxygen species, causing activation of apoptotic proteases of the calpain family, and direct induction of cytosolic calcium which triggers apoptotic proteins of the Bcl-2 family, leading to cell death (Gokduman, 2016).

Camptothecin and its related compounds are used for second line therapy for a variety of cancers including SCLC and is well tolerated (Asai et al., 2014). Patient-oriented studies show that camptothecin is active against brain metastases in SCLC (Nagourney et al., 2003; Stewart, 2004). The clinic profile of camptothecin, its broad-spectrum anti-tumor activity, and its lack of cross resistance with other anticancer agents has prompted clinical studies investigating the feasibility of camptothecin being used in a first-line setting for SCLC patients (Asai et al., 2014; Gokduman, 2016; Stewart, 2004).

A unique feature of camptothecin is its ability to induce enhanced anticancer activity with multiple anti-neoplastic compounds (Gokduman, 2016; O'Brien, Eckardt, & Ramlau, 2007). In many of these studies, the interactions between camptothecin and other cancer chemotherapeutic drugs was found to be potentially synergistic (Asai et al., 2014; Gokduman, 2016; Stewart,

2004). Although the combinatorial activity of other anticancer drugs (like cisplatin) has been investigated with multiple dietary compounds, there are very few such studies involving camptothecin. This study investigates for the first time the potential combinatorial apoptotic activity of camptothecin and capsaicin in SCLC. We selected a concentration of capsaicin which did not induce any cell death in SCLC, and when we combined it with varying concentrations of camptothecin we found that the two agents synergistically enhance apoptosis within a range of concentrations. A rare feature of our studies is that we used the Chou-Talalay isobologram analysis to determine whether the interactions between capsaicin and camptothecin were truly synergistic (Chou, 2008, 2010). Hormann et al., (2012) showed that the apoptotic efficacy of the camptothecin analog topotecan was increased in the presence of the flavonoid genistein (Hormann, Kumi-Diaka, Durity, & Rathinavelu, 2012). However, they did not perform any statistical analysis to show whether the topotecan-genistein combination was additive or synergistic. Similarly, several studies have shown that capsaicin increased the therapeutic efficacy of cisplatin or radiation in stomach and prostate cancer, but rigorous statistical analyses of the nature of the interaction between the two therapies were absent (Arzuman et al., 2016; Chou, 2008; Huh et al., 2011; Wiwanitkit, 2012; Zheng et al., 2016).

The present manuscript also shows that the combination of capsaicin and camptothecin showed increased anti-tumor activity *in vivo* (compared to the agents administered singly) in chicken CAM models. Previous studies have shown that human cancer cells implanted on CAM constitute an established model to study tumor growth *in vivo* (Canela et al., 2017; Dehelean et al., 2013; Michaelis et al., 2015). The advantage of the chicken CAM model is that we can take the optimal concentrations, found in cell culture models, and directly apply them in the *in vivo* setting (Kain et al., 2014; Lokman et al., 2012). This is in contrast to athymic mouse models

where dosages are translated to mg/kg body weight, and it is difficult to correlate whether the concentration of the drug *in vitro* is similar to the dose of the drug in the tumor microenvironment *in vivo* (de Jong, Essers, & van Weerden, 2014; M. Liu et al., 2013). Our previous publications have already shown that the administration of capsaicin does not cause any gross discomfort in mice (K. C. Brown et al., 2010; Lau et al., 2012; Lau et al., 2014). Our published reports reveal that capsaicin displays significant bioavailability in the lungs of mice (Rollyson et al., 2014). Taken together, our data suggest that the combination of camptothecin and capsaicin has the potential for being a feasible strategy for therapy and management of human SCLCs.

Several convergent studies have shown that the calpain super-family of calcium-regulated intracellular cysteine proteases (Ono, Saido, & Sorimachi, 2016; Potz, Abid, & Sellke, 2016) are involved in the biological activities of camptothecin. Calpains have been shown to mediate camptothecin-induced apoptosis and play a role in camptothecin-induced DNA damage and drug resistance (Cao et al., 2003; Mandic et al., 2002; D. E. Wood & Newcomb, 1999; D. E. Wood et al., 1998). Our published data and those of other research laboratories show that calpains are also vital regulators of capsaicin-induced apoptosis (Lau et al., 2014; Oh et al., 2008). Therefore, we conjectured that perhaps an intracellular calcium and calpain pathway was the converging point for the two drugs. We show that the combination of camptothecin and capsaicin amplifies cellular calpain activity leading to a large increase in cellular apoptosis.

Although, the results presented in this manuscript are unique and innovative, our study has a few limitations. One of the limitations of the study is that the synergistic apoptotic activity of capsaicin and camptothecin has not been investigated in athymic mouse models. It is well established that several pro-apoptotic and pro-survival proteins are substrates of the calpain

pathway. These include p53, Bcl-2, Bcl-xl, Bid, Bax, caspase-3, caspase-7, - 8, and -9, caspase-12, and NFjB (Lopatniuk & Witkowski, 2011; Moretti, Del Bello, Allavena, & Maellaro, 2014). Several of these proteins have been shown to be downstream targets of both camptothecin- and capsaicin-induced apoptosis (Clark & Lee, 2016; Legarza & Yang, 2006). However, we do not know the precise calpain substrates that are key players in the combinatorial activity of capsaicin and camptothecin. Finally, we have yet to investigate whether capsaicin and camptothecin display synergistic apoptotic activity in cisplatin- resistant human SCLC cells. These studies are currently underway in the laboratory and will form the basis of a future publication.

Conflict of interest

The authors declare no conflict of interest

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CHAPTER 4: ANTICANCER ACTIVITY OF NATURAL AND SYNTHETIC CAPSAICIN ANALOGS

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ABSTRACT

The nutritional compound capsaicin is the major spicy ingredient of chili peppers. Although traditionally associated with analgesic activity, recent studies have shown that capsaicin has profound antineoplastic effects in several types of human cancers. However, the applications of capsaicin as a clinically viable drug are limited by its unpleasant side effects, such as gastric irritation, stomach cramps, and burning sensation. This has led to extensive research focused on the identification and rational design of second-generation capsaicin analogs, which possess greater bioactivity than capsaicin. A majority of these natural capsaicinoids and synthetic capsaicin analogs have been studied for their pain-relieving activity. Only a few of these capsaicin analogs have been investigated for their anticancer activity in cell culture and animal models. The present review summarizes the current knowledge of the growth-

inhibitory activity of natural capsaicinoids and synthetic capsaicin analogs. Future studies that examine the anticancer activity of a greater number of capsaicin analogs represent novel strategies in the treatment of human cancers.

INTRODUCTION: CAPSAICIN

Capsaicin (trans-8-methyl-N-vanillyl-6-noneamide; Figure 20A) is the principal, pungent ingredient of chili peppers in the plant genus *Capsicum*. The compound can be found predominantly within the white pith and membrane of both cayenne and chili peppers (Chapa-Oliver & Mejia-Teniente, 2016). It is a potent analgesic and is used topically to treat pain and inflammation associated with a variety of diseases (Basith et al., 2016; O'Neill et al., 2012). The analgesic activity of capsaicin is mediated by transient receptor potential subfamily vanilloid member 1 receptor (TRPV1), which belongs to the transient receptor potential superfamily of cation-channel receptors (J. Chen et al., 2014). The transient receptor potential vanilloid receptor family is comprised of six members (TRPV1-6). Capsaicin functions as the classic agonist of the TRPV1 receptor (Caterina et al., 1997). The binding of TRPV1 to capsaicin trigger a plethora of molecular events ultimately inducing to depletion of substance P, and desensitization of sensory neurons leading to its analgesic activity. This paved the way for the isolation, design, and synthesis of capsaicin-like compounds (which were TRPV1 agonists) that displayed more potent analgesic activity than capsaicin.

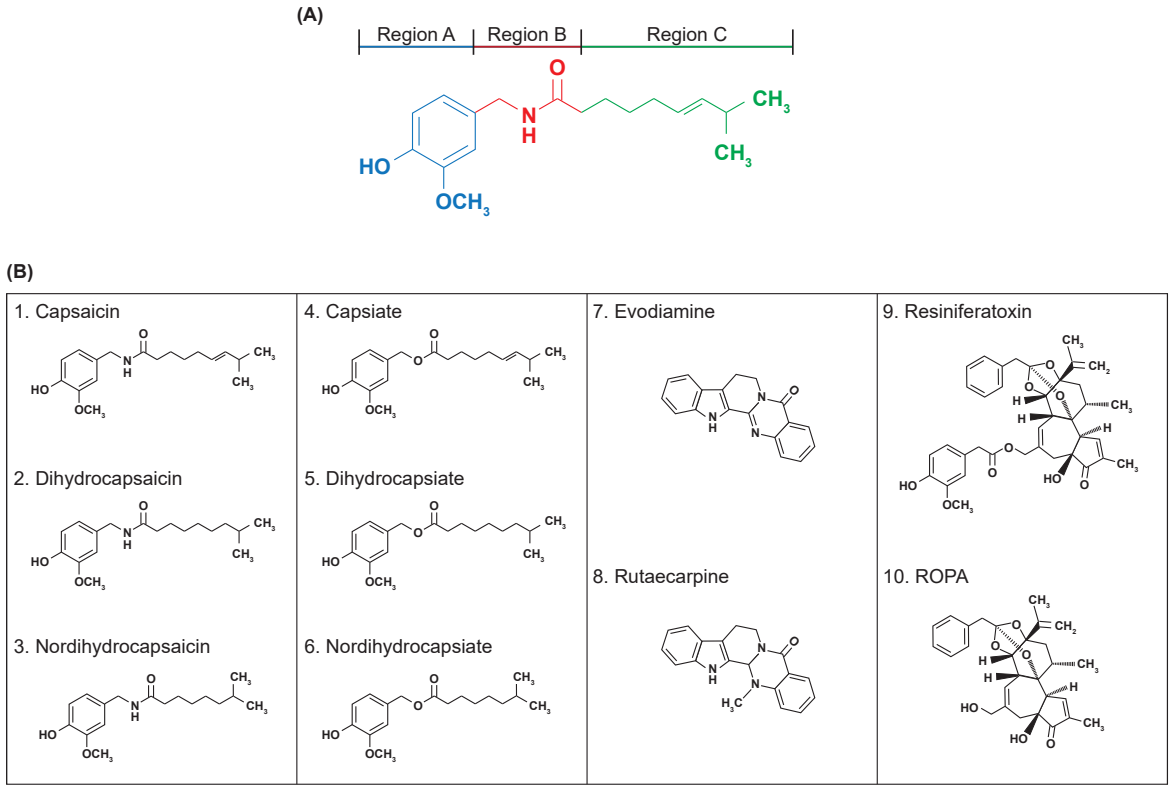

Figure 20. Structures of natural capsaicinoids
(A) Pharmacophore of capsaicin. The blue structural moiety represents Region A; the red portion of the structure represents Region B; the green alkyl side chain represents Region C. (B) Structures of natural capsaicinoids, which have been investigated for their growth-inhibitory activity in cell culture or animal models.

Emerging evidence shows that capsaicin displays anticancer activity in several human cancers, both in cell culture and mouse models (for excellent reviews please refer to (Basith et al., 2016; Chapa-Oliver & Mejia-Teniente, 2016; Clark & Lee, 2016; Diaz-Laviada & Rodriguez-Henche, 2014; Srinivasan, 2016)). This led researchers to conjecture that natural and synthetic TRPV1 agonists would display growth-inhibitory effects analogous to capsaicin. Because a large number of TRPV1 agonists (which had been tested for analgesic activity) had already been described in literature, they were initially investigated for their anticancer activity. However, a majority of research studies have shown that the anticancer activity of capsaicin and

capsaicin analogs is completely independent of TRPV1 receptor. This is true of both natural capsaicinoids and synthetic capsaicin mimetics (the reader is referred to excellent reviews and papers (Basith et al., 2016; Chapa-Oliver & Mejia-Teniente, 2016; Clark & Lee, 2016; Ziglioli et al., 2009). Although these natural synthetic capsaicin mimetics are TRPV1 ligands, their anticancer activity does not involve the TRPV1 receptor (Lau et al., 2012). The anticancer activity is mediated through the direct interaction of these compounds with key signaling molecules of the cytoplasmic, mitochondrial, and metabolic survival pathways (Basith et al., 2016; Chapa-Oliver & Mejia-Teniente, 2016; Clark & Lee, 2016; Diaz-Laviada & Rodriguez-Henche, 2014; Srinivasan, 2016). The cellular pathways underlying the anticancer activity of capsaicin are not fully understood; however, multiple mechanisms such as increase of intracellular calcium, induction of calpain activity, reactive oxygen species (ROS) generation, inhibition of coenzyme Q, suppression of mitochondrial respiration, and inhibition of transcription factors like p53, signal transducer and activator of transcription (STAT) 3, and nuclear factor κB have been involved (for excellent reviews, see (Bode & Dong, 2011; Cho, Lee, & Choi, 2017; Clark & Lee, 2016; Fernandes, Cerqueira, Soares, & Costa, 2016; Lau et al., 2012)). In addition to suppressing the growth of human cancer cells, capsaicin promotes the apoptotic activity of cancer chemotherapy agents by multiple mechanisms (Arzuman et al., 2016; Clark & Lee, 2016; Friedman et al., 2017; Huh et al., 2011; Vendrely et al., 2017). For example, capsaicin has been reported to inhibit p-glycoprotein efflux transporters in KB-C2 human endocervical adenocarcinoma cells. The presence of capsaicin in vinblastine-treated KB-C2 cells increases the concentration of vinblastine in the cellular microenvironment and thereby sensitizes these cells to undergo apoptosis (Khan, Maryam, Mehmood, Zhang, & Ma, 2015). The p-

glycoprotein is a well-characterized transmembrane ATP-binding cassette, multidrug resistance 1 transporter involved in efflux of numerous drugs and other xenobiotics (Silva et al., 2015).

The development of capsaicin as a clinically useful drug for pain relief or cancer therapy is hindered by its adverse side effects. The topical or oral administration of capsaicin in humans causes skin redness, hyperalgesia nausea, intense tearing in the eyes, conjunctivitis, blepharospasm (sustained, forced, involuntary closing of the eyelids), vomiting, abdominal pain, stomach cramps, bronchospasm, and burning diarrhea (Drewes et al., 2003; Evangelista, 2015; Hammer, 2006). Clinical trials exploring the pain-relieving activity of capsaicin have shown that such side effects have results in patients discontinuing use of capsaicin due to its strong pungency and nociceptive effect (Drewes et al., 2003; Evangelista, 2015; Hammer, 2006). Such observations have led to research focused on the discovery and design of capsaicin-like compounds, which display greater anticancer activity than capsaicin with a gentler side effect profile. Another incentive for the design of capsaicin-based drug candidates is to obtain compounds endowed with improved pharmacological activity, bioavailability, biologic half-life, selectivity, specificity, and therapeutic index relative to capsaicin (Lau et al., 2012). The anticancer activity of capsaicin is covered in several review articles (Chapa-Oliver & Mejia-Teniente, 2016; Clark & Lee, 2016; Khan, Bi, Qazi, Fan, & Gao, 2015). However, the anticancer activity of these natural and synthetic capsaicin-like compounds has yet to be summarized. The present review fills this void of knowledge and discusses the growth-suppressive activity of natural and synthetic capsaicin-like compounds in human cancers. Specifically, the growth-inhibitory activity of these in both tissue culture and animal models will be discussed. We believe that this detailed discussion of the anticancer activity of capsaicin analogs is both timely and relevant, for the potential applications of such compounds in cancer therapy.

STRUCTURE ACTIVITY RELATIONSHIP OF CAPSAICIN

The potential clinical application of capsaicin is restricted by its unfavorable side effect profile. Clinical studies investigating the analgesic activity of capsaicin have shown that oral capsaicin administration in humans leads to intense abdominal pain, hyperalgesia, stomach cramps and nausea (Basith et al., 2016; O'Neill et al., 2012). These adverse side effects have caused patients to abandon taking capsaicin. This has led to intense research involving capsaicin structure activity relationship studies to isolate or develop new, less irritating analogs (Drewes et al., 2003; Evangelista, 2015; Hammer, 2006). A second driving force behind the identification and synthesis of capsaicin analogs is that of novel drug discovery that aims to generate new capsaicin mimetics with better pharmacological and therapeutic profile than the parent molecule. The structure of capsaicin can be broken down into three major areas, which are depicted in Figure 20A. The three major regions are as follows: aromatic (Region A), amide (Region B), and the hydrophobic (Region C) (Basith et al., 2016; Chapa-Oliver & Mejia-Teniente, 2016; Clark & Lee, 2016; Diaz-Laviada & Rodriguez-Henche, 2014; X. F. Huang et al., 2013; Srinivasan, 2016).

ANTINEOPLASTIC ACTIVITY OF NATURAL CAPSAICIN ANALOGS

Capsiates

Data from several independent research laboratories have led to the discovery of natural capsaicin-like compounds that resemble the structure of capsaicin but contain variations in Regions A (aromatic), B (amide), or C (hydrophobic). There are few published reports about natural capsaicin-like compounds (capsaicinoids) that have alterations in Region A (Gavaraskar, Dhulap, & Hirwani, 2015; Ogasawara, Matsunaga, Takahashi, Saiki, & Suzuki, 2002). However, several capsaicinoids having variations in Region B have been reported to suppress the growth of

human cancer cells in cell culture. The non-pungent capsaicinoid, capsiate (Figure 20B3), is isolated from a strain of peppers called CH-19 Sweet. Apart from capsiate, CH-19 Sweet is also the source for two additional capsiate-like compounds, namely dihydrocapsiate and nordihydrocapsiate (Figure 20B4 and B5) (Macho et al., 2003; Watanabe, Ohnuki, & Kobata, 2011). These three compounds differ from capsaicin in Region B; dihydrocapsiate and nordihydrocapsiate contain an ester bond instead of an amide bond between the vanillyl motif and the fatty acid side chain. Dihydrocapsiate and nordihydrocapsiate also differ in Region C relative to capsaicin. Dihydrocapsiate and nordihydrocapsiate have only saturated bonds in the alkyl chain of Region C instead of a single double bond observed in capsaicin. Macho et al., (2003) studied the antiapoptotic activity of capsiate, dihydrocapsiate, and nordihydrocapsiate in Jurkat human acute T-cell leukemia cells. They observed that all three compounds induced apoptosis in a concentration-dependent manner when incubated with Jurkat cells.

Several convergent studies have indicated that capsaicin may also function as a tumor promotor in skin cancer, breast cancer, and colon cancer (Bode & Dong, 2011). In contrast, all capsaicin-like compounds (natural capsaicinoids or synthetic capsaicin mimetics) have shown only growth-inhibitory activity toward numerous cell lines (Basith et al., 2016). Nordihydrocapsiate further showed potent chemopreventive activity in an *in vivo* two-stage model of mouse skin carcinogenesis. These findings would suggest that, in this experimental model and with application of a promotor, nordihydrocapsiate may provide protection against skin cancer (Macho et al., 2003). The mechanism of action of these capsiates was similar to capsaicin and was mediated by inhibition of transcription factor nuclear factor kB, elevation of reactive oxygen species, and loss of mitochondrial membrane potential (Figure 21A) (Macho et al., 2003; Watanabe et al., 2011). Most interestingly, nordihydrocapsiate showed better

proapoptotic activity than capsaicin in Jurkat cells, as reflected by the IC_{50} values (nordihydrocapsiate, IC_{50}=75 mM; capsaicin, IC_{50}=125 mM) (Macho et al., 2003). Both capsiate and dihydrocapsiate displayed antiangiogenic activity in cell culture and mouse models (Figure 21A). These compounds suppressed vascular endothelial growth factor–induced angiogenesis in human umbilical cord endothelial cells via direct suppression of Src kinase activity and phosphorylation of its downstream substrates, such as $p125^{FAK}$ and vascular endothelial cadherin. Most interestingly, capsiate and nordihydrocapsiate do not affect autophosphorylation of the vascular endothelial growth factor receptor kinase insert domain/fetal liver kinase (Min et al., 2004; Pyun et al., 2008). The antiangiogenic activities of the two compounds were comparable to each other and to capsaicin. Such non-pungent capsaicinoids (capsiate and its related compounds) may be more applicable in cancer therapy than capsaicin.

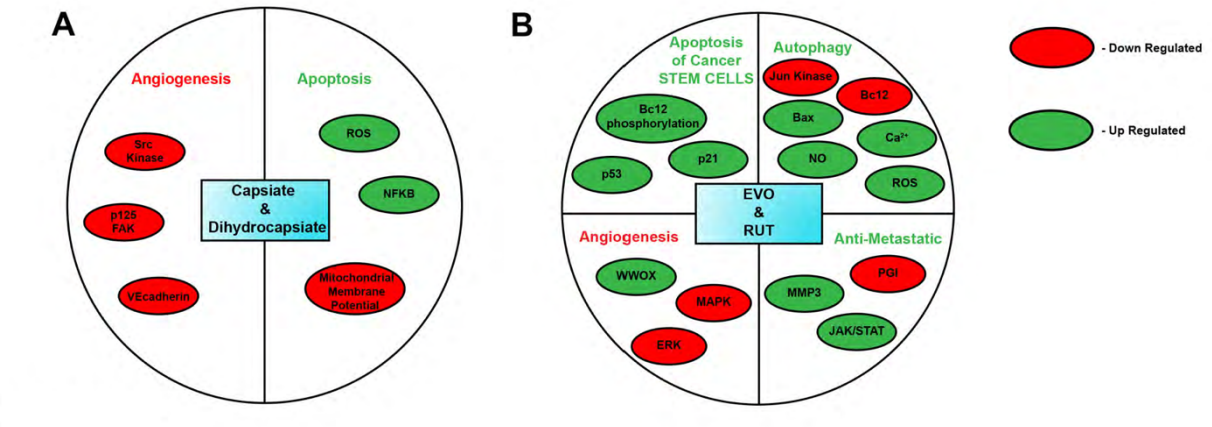

Figure 21. Signaling pathways underlying the anticancer activity of natural capsaicinoids
(A) Capsiate and dihydrocapsiate. (B) Evodiamine and Rutaecarpine.

Evodiamine and Rutaecarpine

Evodiamine (EVO; Figure 20B6) and rutaecarpine (RUT; Figure 20B7) are alkaloids isolated from the fruit of the Chinese medical plant *Evodia rutaecapra*, otherwise known as Evodia fruit (Wu, Chien, Chen, & Chiu, 2016; Wu, Chien, Liu, Chen, & Chiu, 2017; Yu, Jin,

Gong, Wang, & Liang, 2013). Capsaicin and EVO share pharmacophore elements, but their lipophilic moiety (Region C) is different, encompassing a saturated isononenyl unsaturated group in capsaicin, and two phenyl rings in evodiamine (De Petrocellis et al., 2014; Pearce et al., 2004; S. Wang et al., 2015; T. Wang, Wang, & Yamashita, 2009). Wang et al., (2012, 2015) have performed docking and molecular modeling on the pharmacophore of EVO and capsaicin and observed a remarkable similarity between the pharmacophore of the two compounds (S. Wang et al., 2015; Z. Wang et al., 2012). EVO has been characterized as a potent, selective agonist of the TRPV1 receptor, similar to capsaicin (Ivanova & Spiteller, 2014; S. Wang et al., 2016). Cell culture studies show that EVO displays growth-inhibitory activity in human breast cancer, prostate cancer, leukemia, urothelial cell carcinoma, gastric cancer, osteosarcoma, oral cancer, non-small lung cancer, colon cancer, glioma, glioblastoma, thyroid cancer, melanoma, and cervical cancer cells (M. C. Chen et al., 2010; Du et al., 2013; Fang et al., 2014; Gavaraskar et al., 2015; Hu et al., 2016; J. Huang et al., 2015; Kan et al., 2007; Khan, Bi, et al., 2015; T. J. Lee et al., 2006; Sachita, Kim, Yu, Cho, & Lee, 2015; Shen et al., 2015; C. S. Shi et al., 2017; Wu et al., 2017; F. Yang et al., 2017). However, EVO has been shown to be an antagonist of the aryl hydrocarbon receptor as well (Yu et al., 2010). The growth-suppressive activity of EVO is mediated by cell cycle arrest, apoptosis, and autophagy, which involve a symphony of mechanisms (Figure 21B), including downregulation of survivin, Akt, STAT3, Mcl-1, B-cell lymphoma-2 (Bcl-2) and cdc-p15, and upregulation of caspase-3, phosphatase and tensin homolog, Bcl-2 associated killer, Bax, Fas ligand, microRNA-429, matrix metalloproteinase-9, Jun kinase, cyclin B1, cdc25c, and cdc2-p161 (T. C. Chen, Chien, Wu, & Chen, 2016; Fan et al., 2017; Fang et al., 2014; Han et al., 2016; J. Huang et al., 2015; Khan, Bi, et al., 2015; T. J. Lee et al., 2006; Y. L. Li et al., 2016; Liu, Huang, Wu, & Wen, 2016; Meng et al., 2015; Peng et al.,

2015; L. Wei, Jin, Cao, & Li, 2016; Wu et al., 2017; F. Yang et al., 2017; Zhu et al., 2011; Zou et al., 2015). EVO-induced autophagy in human glioblastoma cells is mediated by Jun kinase, Bcl-2, and elevation of Bax, intracellular calcium, and induction of ROS/nitric oxide (A. J. Liu, S. H. Wang, K. C. Chen, et al., 2013; A. J. Liu, S. H. Wang, S. Y. Hou, et al., 2013). The antitumor activity of EVO has been explored in athymic mouse models of human hepatocellular carcinoma, colon cancer, and renal carcinoma (Wu et al., 2016; J. Yang et al., 2013; C. Zhang et al., 2010). The anticancer activity of EVO in hepatocellular cancer (Figure 21B) may be attributed to its ability to suppress β-catenin–mediated angiogenesis (L. Shi et al., 2016). In contrast, EVO suppressed the growth of human renal carcinoma cells *in vivo* by inducing phosphorylation of Bcl-2 (Wu et al., 2016). In addition, EVO targeted breast cancer stem-like cells by activating p53 and p21 expression (Han et al., 2016). In gastric cancer stem cells, EVO inhibited proliferation via inhibition of the Wingless/β-catenin pathway (Wen et al., 2015).

EVO has been shown to induce apoptosis in drug-resistant human cancer cells. EVO displays antiproliferative activity in camptothecin-resistant human leukemia cells (Pan et al., 2012).The mechanism of EVO-induced G2/M arrest involves the inhibition of topoisomerase 1 and 2 (Y. C. Lee et al., 2015). Similarly, EVO induces cell cycle arrest in Taxol-resistant ovarian cancer cells and in Adriamycin-resistant human breast cancer cells (Liao et al., 2005; Zhong, Tan, Wang, Qiang, & Wang, 2015). EVO triggers apoptosis in human colon cancer cells resistant to oxaliplatin and cisplatin (Ogasawara, Matsubara, & Suzuki, 2001; Wen et al., 2015). EVO sensitizes human cancer cells to the apoptotic effects of chemotherapeutic agents. EVO synergizes with doxorubicin and gemcitabine to produce increased apoptosis in breast cancer and pancreatic cancer cells, respectively (S. Wang et al., 2014; W. T. Wei et al., 2012). Likewise, EVO enhances the efficacy of erlotinib in human lung cancer and in human ovarian cancers (Y.

L. Li et al., 2016). Moreover, EVO sensitizes U87MG human glioblastoma cells to the proapoptotic effects of tumor necrosis factor–related apoptosis-inducing ligand. Hu et al., (2016) observed that EVO sensitizes human gastric cancer cells to the growth-suppressive effects of radiotherapy *in vitro* and *in vivo* (Hu et al., 2016). In addition to promoting apoptosis in various cancer cells, EVO alters the ATP-binding cassette subfamily G member 2 breast cancer–resistant protein transporter to increase chemosensitivity of colorectal cancer cells. EVO was not a substrate inhibitor of ABCG2, as EVO diminished ABCG2 protein expression in HCT-116/L-OHP cells, which increased cancer chemosensitivity to cisplatin (Sui et al., 2016). Additional studies are needed to explore whether EVO can modify ABCG2 protein expression in other cancer cells.

EVO displays antimigratory, anti-invasive, and anti-metastatic activity in human lung cancer, breast cancer, and nasopharyngeal cancer cells *in vitro* and in mouse models. EVO exerts anti-metastatic activity by multiple mechanisms, such as regulation of matrix metalloproteinase-3 activity, p38 kinase activity, extracellular signal-regulated kinase activity, and Janus kinase/STAT pathway, and downregulation of phosphoglucose isomerase (Du et al., 2013; Peng et al., 2015; Zhao et al., 2015).

RUT is the second major alkaloid isolated from *E. rutaecapra*. It is a potent agonist of TRPV1 (Ivanova & Spiteller, 2014). RUT displayed antiproliferative activity in three-dimensional spheroid models of human breast cancer cells (Guo et al., 2016). The antineoplastic activity of EVO and RUT has led to intense research involving design and synthesis of second-generation EVO-like or RUT-like analogs with improved anticancer activity (Figure 21B). The reader is referred to some excellent reviews on this subject (Y. H. Hong et al., 2010; Li-Weber,

2013; Song et al., 2013; Yu et al., 2013). Further studies are needed to investigate whether TRPV1 signaling pathway plays a role in the anticancer activity of EVO and Rut.

Resiniferatoxin

The capsaicin analog resiniferatoxin (RTX; Figure 20B8) is a tricyclic diterpene isolated from the latex of the cactus plant *Euphorbia resinifera* (Iadarola & Gonnella, 2013). RTX is one of the most potent TRPV1 agonists ever described in literature (D. C. Brown, 2016). As can be seen in the figure above, the structure of capsaicin and RTX closely resembles each other, except that Region C is a diterpene moiety of the daphnane class (Carnevale & Rohacs, 2016). Furthermore, pharmacophore clustering and docking studies reveal a close similarity between the two compounds (Athanasiou et al., 2007; Carnevale & Rohacs, 2016; Elokely et al., 2016; Hartel et al., 2006; Y. H. Lee, Im, Kim, & Lee, 2016). Based on previous studies, four sites represent the pharmacophore of RTX, as follows: 1) 4-hydroxy-3-methoxyphenyl, 2) C20 ester, 3) C3-keto, and 4) orthophenyl groups (X. F. Huang et al., 2013). The growth-inhibitory activity of RTX has been investigated in multiple human cancer cells. Of these, RTX caused robust apoptosis in human bladder cancer cell lines (T24, 5637) and in athymic mouse models xenografted with T24 bladder cancer cells (Farfariello et al., 2014). However, it did not trigger cell death in normal human urothelial cells. This observation is interesting because RTX selectively targeted human bladder cancer cells, but not the normal urothelial cells.

RTX mimics capsaicin-producing selective apoptosis for human cancer cells while sparing the normal cells (Lau et al., 2014). However, RTX differs by inducing prolonged cell cycle arrest (within G0 phase) in IEC-18 rat ileal epithelial cells. Such differences can be explained by the fact that the IEC-18 is an immature epithelial cell line derived from rat intestinal crypt, and therefore its growth characteristics cannot be compared with normal primary

adult epithelial cells (Frey et al., 2004). Additionally, species-specific differences between rat and human cell lines may explain the varying response of RTX between IEC-18 and normal urothelial cells. In agreement with other studies, the growth-suppressive effects of RTX were found to be independent of TRPV1 receptor and involved a decrease of cyclin D1 at mRNA and protein levels (Frey et al., 2004). The compound resiniferanol-9, 13, 14 ortho-phenylacetate (ROPA) is a hydrolysis product of RTX (Figure 20B9). Frey et al., (2004) investigated the growth-inhibitory activity of ROPA on IEC-18 cells (Frey et al., 2004). ROPA was found to induce a transient protein kinase C–dependent cell cycle arrest in G_1 phase. The cell cycle–inhibitory effects of ROPA were accompanied by a decrease in cyclin D1 levels and simultaneous upregulation of p21 expression (Figure 22A) (Frey et al., 2004). In contrast, RTX did not have any effect on p21 levels in IEC-18 cells. A remarkable observation was that the growth- inhibitory activity of ROPA as well was found to be independent of the TRPV1 receptor family (Frey et al., 2004). The apoptotic activity of RTX was mediated by diverse mechanisms (Figure 22A) such as mitochondrial depolarization, generation of reactive oxygen species, suppression of mitochondrial respiration, blockage of protein kinase C, inhibition of cyclin D1, and induction of $p21^{waf1/Cip1}$ (Athanasiou et al., 2007; Farfariello et al., 2014; Hartel et al., 2006; Vercelli et al., 2014; Ziglioli et al., 2009).

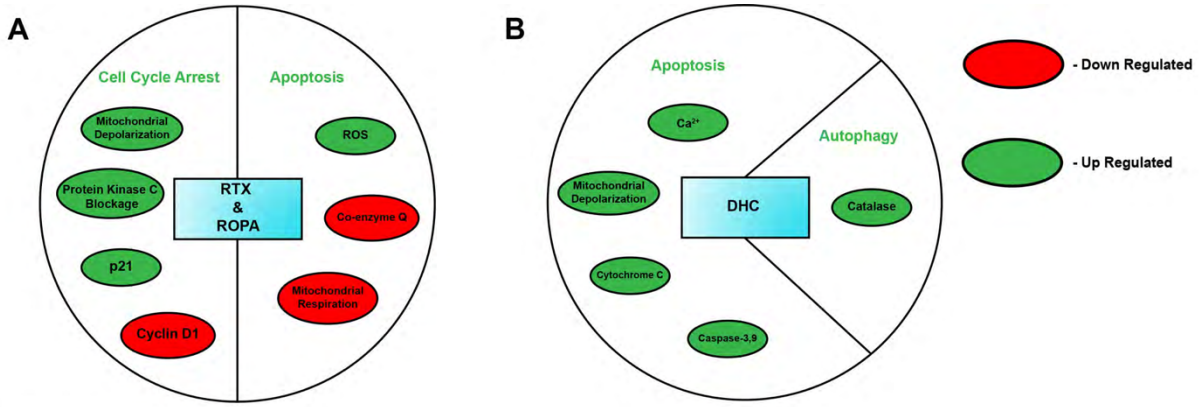

Figure 22. Signal transduction pathways mediating the antitumor activity of natural capsaicinoids
(A) RTX and ROPA. (B) DHC.

Dihydrocapsaicin

The capsaicin analog dihydrocapsaicin (DHC) differs from capsaicin in the hydrophobic Region C. It contains a saturated bond between C6 and C7 carbon atoms of Region C (Figure 20B2). DHC is less pungent than capsaicin based on the Scoville heat unites. The anticancer activity of DHC has been observed in several human cancer cell lines, including human breast cancer cells, colon cancer cells, and gliomas (Oh et al., 2008; Oh & Lim, 2009). A majority of these studies have been done in cell culture. An intriguing observation was that DHC showed greater growth-inhibitory activity than capsaicin in these cell lines. The growth-inhibitory effects of DHC (Figure 22B) were mediated via cell cycle arrest, apoptosis, and autophagy inhibition of cellular metabolism (Halme et al., 2016; Oh et al., 2008). The antitumor activity of DHC was observed in athymic mouse models of human gliomas as well (Xie et al., 2016). However, the drawback with DHC is that it has pungent and irritant properties like capsaicin (Schneider, Seuß-Baum, & Schlich, 2014).

ANTINEOPLASTIC ACTIVITY OF SYNTHETIC CAPSAICIN ANALOGS

N-Acylvanillamides

Among all synthetic analogs of capsaicin, the N-acylvanillamides (N-AVAMs) are of the most extensively researched for their analgesic activity (X. F. Huang et al., 2013; Kobata et al., 2010; Melck et al., 1999). There are numerous studies that have investigated their anticancer activities in diverse human cancer cell lines (Sanchez-Sanchez et al., 2015; Sancho et al., 2003; Stock et al., 2012). This class of compounds is modified in the hydrophobic Region C of capsaicin (Figure 23). Early studies experimented with substituting the acyl side chain with saturated long-chain lipophilic groups. However, these compounds were inactive (Melck et al., 1999). The introduction of long-chain unsaturated fatty acids fully restored the analgesic activity of these compounds. The N-AVAMs are non-pungent and do not have the unfavorable side effects of capsaicin. Structure activity studies experimented with the magnitude of unsaturation in these side chain and the length of the side chain to yield capsaicin analogs with improved analgesic activity and binding profile to TRPV1 (X. F. Huang et al., 2013). Recent studies examined the growth-inhibitory activity of these unsaturated N-AVAMs (UN-AVAMs). Tuoya et al., (2006) demonstrated that the UN-AVAM dohevanil (Figure 23A) induced a greater magnitude apoptosis in MCF-7 human breast cancer cells than capsaicin *in vitro* (Tuoya et al., 2006). Appendino et al., (2005) synthesized a panel of UN-AVAM compounds with varying affinity for human TRPV1 receptor (Appendino et al., 2005; Appendino, Minassi, Morello, De Petrocellis, & Di Marzo, 2002). Of these compounds, several studies have investigated the growth-inhibitory activity of olvanil (Figure 23B), rinvanil (Figure 23C), and phenylacetylrinvanil (PhAR; Figure 23D). The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays showed that olvanil decreased the viability of C6 rat

glioma cells and EFM-19 breast cancer cells (Figure 24) (X. F. Huang et al., 2013; Melck et al., 1999). Apart from being a potent TRPV1 agonist, these compounds displayed weak binding and activation of cannabinoid receptor 1 (CB1) and competitively inhibited the anandamide membrane transporter. Luviano et al., (2014) studied the growth-inhibitory activity of rinvanil and PhAR in J774, P388, and WEHI-3 mouse leukemic cell lines (Figure 24). PhAR showed improved growth-inhibitory activity relative to rinvanil and capsaicin in all of the cell lines studied (Luviano et al., 2014). Additionally, PhAR displayed some selectivity for leukemic cell lines relative to normal mouse bone marrow cells (Luviano et al., 2014). However, contradictory findings were found later by Sánchez-Sánchez et al., (2015), who analyzed the antiproliferative and cytotoxic activity in a panel of human cervical cancer cell lines, namely HeLa, CaSki, and ViBo. The growth-inhibitory activity of these compounds varied from cell line to cell line and did not correlate with their binding profile to human TRPV1 receptor (Sanchez-Sanchez et al., 2015). The researchers also observed that rinvanil showed selective growth-inhibitory effects on the cervical cancer cells relative to normal lymphocytes, whereas PhAR showed no selectivity between normal and tumor cells (Sanchez-Sanchez et al., 2015). Such variance in results may be attributed to the nature of the cancer, species-specific differences (human cell lines versus mouse cell lines) and the disparity in the methodology used in the two studies. Whereas the studies performed by Luviano et al., (2014) studied the growth- inhibitory effects of PhAR and rinvanil by the Sulforhodamine B assay, Sánchez-Sánchez et al., (2015) used the lactate dehydrogenase assay to evaluate the effect of PhAR and rinvanil on normal lymphocytes (Luviano et al., 2014; Sanchez-Sanchez et al., 2015).

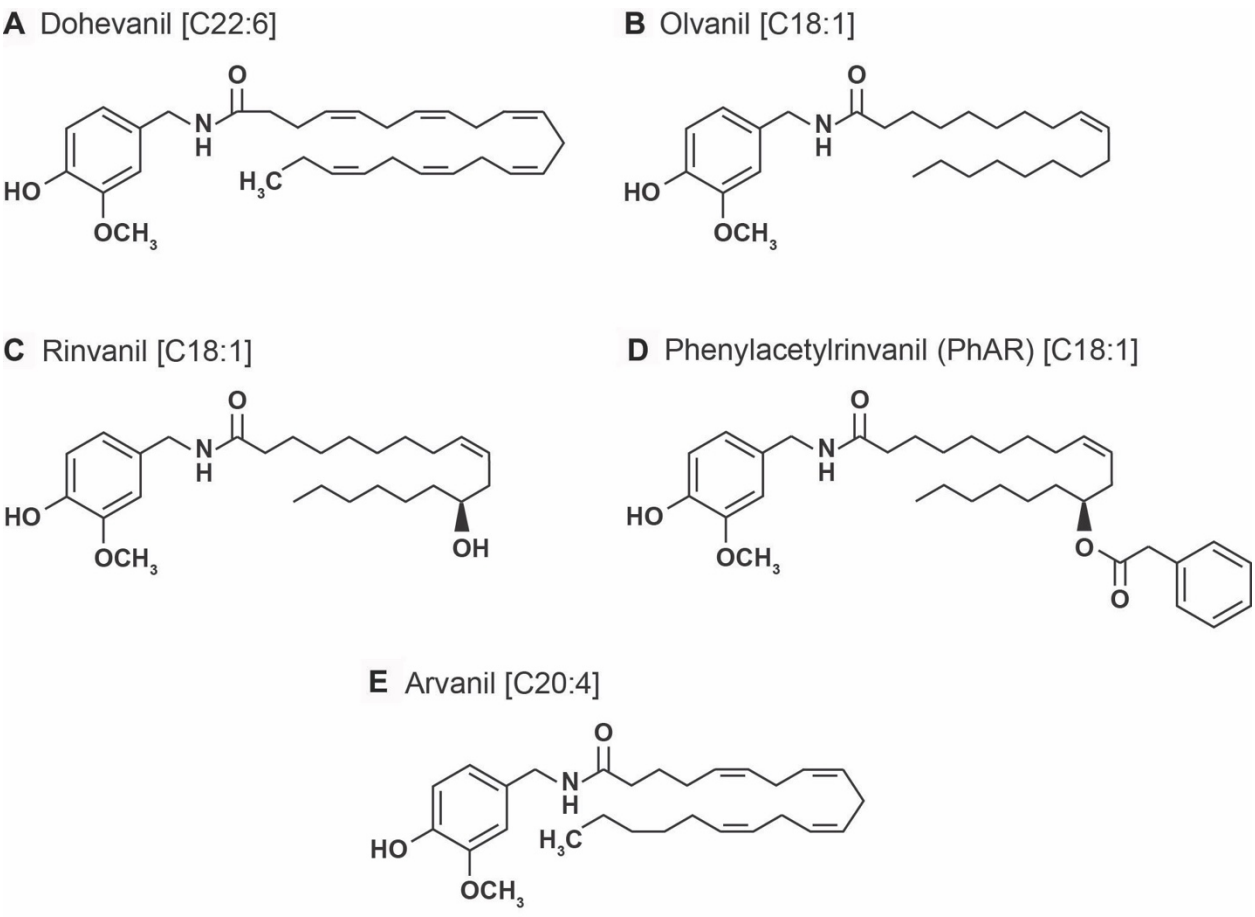

Figure 23. A panel of UN-AVAMs that have been investigated for their growth-suppressive activity in cell culture or mice models
(A) Dohevanil. The first number in the rectangular brackets indicates the chain length and the number after the colon indicates the number of double bonds. For example: [C22:6] written next to dohevanil means dohevanil has a chain length of 22 carbon atoms and 6 double bonds in this alkyl side chain. (B) Olvanil [C18:1]. (C) Rinvanil [C18:1]. (D) Phenylacetylrinvanil [C18:1]. (E) Arvanil [C20:4].

Di Marzo et al., (2002) developed arvanil, an extremely powerful TRPV1 agonist (Figure 23E). Arvanil is a very potent agonist of the TRPV1 and CB1 receptor (Di Marzo et al., 2002). It also induces robust inhibition of anandamide membrane transporter and fatty acid amide hydroxylase (De Petrocellis, Bisogno, Davis, Pertwee, & Di Marzo, 2000; Di Marzo et al., 2002; Glaser et al., 2003; Melck et al., 1999). Experiments in cell culture systems showed that arvanil suppressed the growth of C6 mouse glioma cells, Jurkat human T-cell leukemia cells, human

breast cancer cells (MCF-7, T-47D, and EFM-19 cell lines), and prostate cancer cells (DU145, PPC-1, and TSU cell lines; Figure 24) (De Lago et al., 2006; Di Marzo, Melck, De Petrocellis, & Bisogno, 2000; W. Li & MooreII, 2014; Melck et al., 1999; Sancho et al., 2003). A majority of these studies showed that the growth-suppressive activity of arvanil was independent of TRPV1 and CB1 receptor (Melck et al., 1999). Stock et al., (2012) investigated the antineoplastic activity of arvanil in HG-astrocytoma cells organotypically grown in mouse brain slices (Figure 24). Arvanil suppressed the growth of HG-astrocytoma at a relatively low concentration of 50 nM (Stock et al., 2012). Subsequently, Stock et al., (2012) confirmed the antineoplastic activity of arvanil in HG-astrocytoma tumors implanted in immunocompromised severe combined immunodeficiency mice. They observed that arvanil suppressed the tumor growth rate of HG astrocytomas better than temozolomide (the standard of care for astrocytoma patients). The survival time of mice administered with arvanil was greater than vehicle-treated mice (Stock et al., 2012). This study administered a combination of arvanil and temozolomide, which showed an increase in survival times compared with either agent administered alone or mice administered with vehicle only (Stock et al., 2012). Stock et al., (2012) observed that the anticancer activity of arvanil in human astrocytomas was dependent on the TRPV1 receptor only (Stock et al., 2012). These results are divergent from those found in human breast and prostate cancer cells (Melck et al., 1999). Such different observations may be due to differences in the cell biology of neuronal and non-neuronal human cancer cells. Small cell lung cancer is a neuroendocrine tumor characterized by rapid doubling time, aggressive clinical course, and a dismal 5-year survival rate. The N-AVAMs arvanil and olvanil suppressed the invasion of human small cell lung cancer cell lines via the 5’ AMP-activated protein kinase pathway (Hurley et al., 2017).

RPF, Epoxide-Based Analogs

de-Sa-Junior et al., (2013) synthesized a capsaicin mimetic called RPF101 (Figure 25A). The structure of RPF101 differs from capsaicin, primarily in Region B, where the amide group has been replaced by a bioisosteric sulfonamide (de-Sa-Junior et al., 2013). The alkyl side chain in Region C was replaced with a benzene moiety. The antiproliferative and apoptotic activity of RPF101 in MCF-7 human breast cancer was greater than capsaicin. RPF101 caused cell shrinkage and pyknosis (Figure 24) in three-dimensional spheroid cultures of MCF-7 cells (de-Sa-Junior et al., 2013). RPF101 caused a disruption of mitochondrial membrane potential, dysregulation of microtubule formation, and mitotic catastrophe to induce cell cycle arrest and apoptosis in human breast cancer cells (Figure 24) (de-Sa-Junior et al., 2013). The research group further modified RPF101 to produce an analog RPF151 (Figure 25B) with better stability and aqueous solubility properties (Ferreira et al., 2015). In addition, RPF151 displayed lower hyperalgesia relative to capsaicin. MTT assays showed that RPF151 decreased cell viability better than capsaicin in MDA-MB-231 human breast cancer cells. However, RPF151 did not differentiate between MCF-10A normal human breast epithelial cells and breast cancer cells (Ferreira et al., 2015). The mechanism of action of RPF151 was divergent from RPF101. RPF151 induced cell cycle arrest at S-phase with concomitant decrease in cyclin A, D1, and D3 (Figure 24). RPF151 also induced apoptosis in MDA-MB-231 cells via downregulation of p21, reduction of mitochondrial membrane potential, and activation of the tumor necrosis factor–related apoptosis-inducing ligand pathway (Ferreira et al., 2015). The antineoplastic activity of RPF151 was analyzed by nude mice model of human breast cancer, where it showed higher antitumor activity than capsaicin. Most remarkably, the growth-suppressive activity of RPF151 is independent of the TRPV1 receptor.

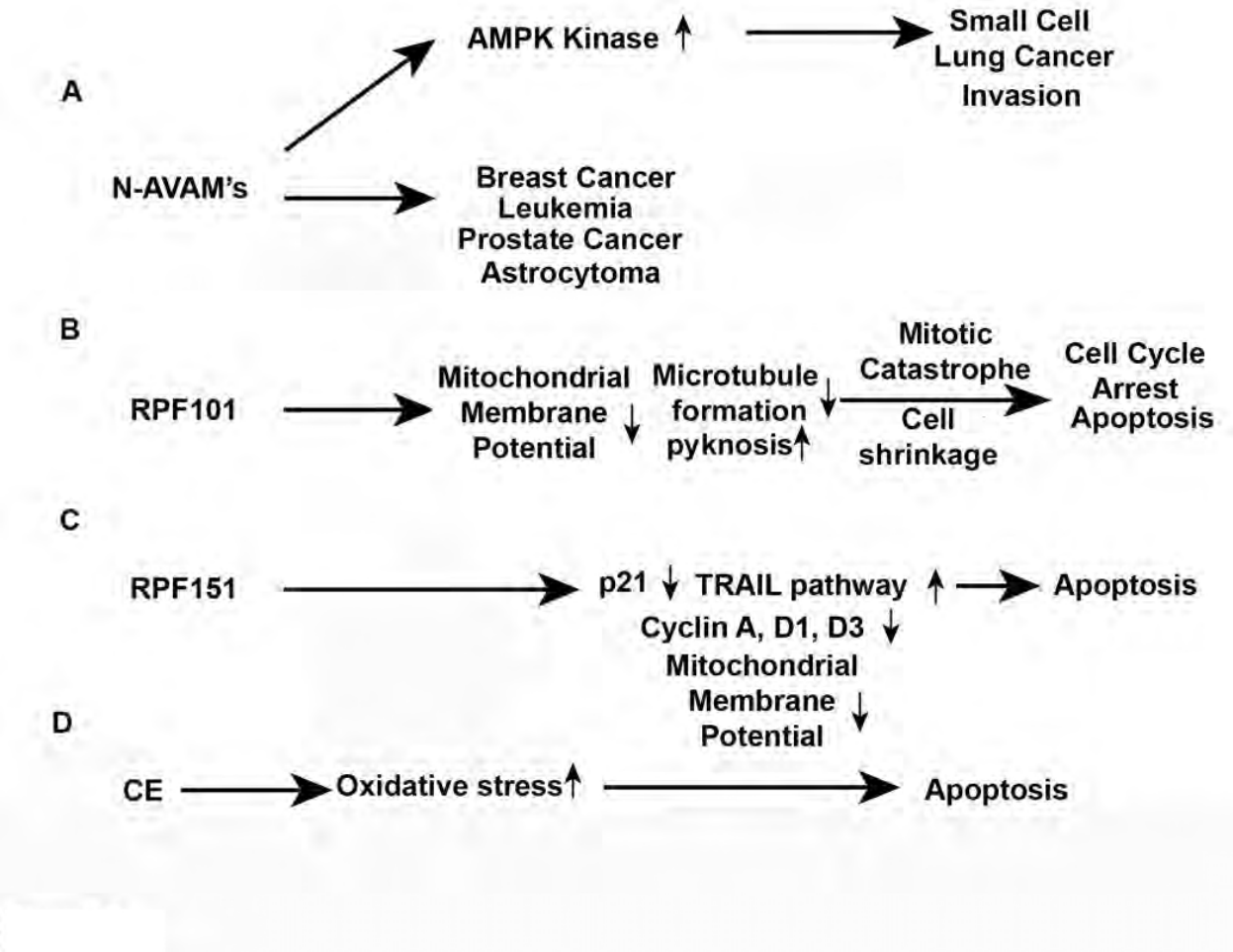

Figure 24. Signaling pathways underlying the antiproliferative and proapoptotic activity of synthetic capsaicin analogs

Lewinska et al., (2015) synthesized a constrained capsaicin analog that contained an epoxide motif in Region C of the capsaicin (Figure 25C). The growth-suppressive activity of capsaicin epoxide (CE) compared with capsaicin was studied in a diverse array of cell lines using the MTT assay (Lewinska, Chochrek, Smolag, Rawska, & Wnuk, 2015). Both capsaicin and CE did not reduce the viability of human dermal fibroblasts. However, CE decreased the viability of NIH/3T3 murine embryonic fibroblasts better than capsaicin (Lewinska, Chochrek, et al., 2015). The varying results in this study could be due to species and lineage differences between human

dermal fibroblasts and the NIH/3T3 cells. Similarly, the growth-inhibitory activity of CE was found to be better than capsaicin in prostate cancer, breast cancer, cervical cancer, and renal cancer cell lines. The human breast cancer cell line MCF-7 was found to be most responsive to CE-induced cell death (Lewinska, Chochrek, et al., 2015). CE was shown to trigger robust apoptosis in these cell lines by inducing oxidative stress (Figure 24).

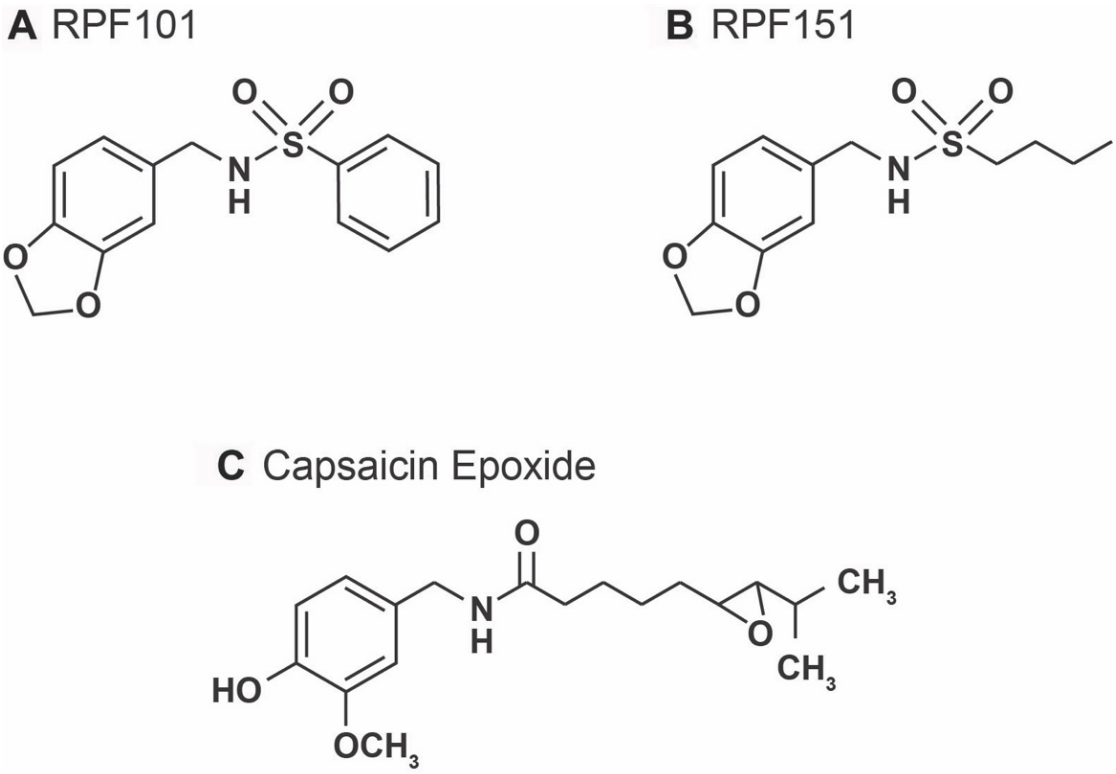

Figure 25. Capsaicin analogs containing a sulfonamide and epoxide motif in their structure trigger apoptosis in human cancer cells
(A) RPF101. (B) RPF151. (C) Capsaicin epoxide.

Miscellaneous Capsaicin Analogs.

The TRPV1 antagonist capsazepine (Figure 26A) displayed potent antitumor activity in human prostate cancer and osteosarcoma cells (J. K. Huang et al., 2006; J. H. Lee et al., 2017; Teng et al., 2004). Gonzales et al., (2014) showed that capsazepine suppressed the growth of human oral squamous cell carcinoma in cell culture and xenograft models in athymic mice

(Gonzales et al., 2014). The apoptotic activity of capsaicin was found to be independent of TRPV1 (J. K. Huang et al., 2006). The apoptotic activity of capsazepine was induced by endoplasmic reticulum stress, increase of ROS, followed by increase of intracellular calcium in a phospholipase C–independent pathway (Figure 27). Capsazepine was also found to be an inhibitor of Janus kinase/STAT3 signaling in prostate cancer cells (J. K. Huang et al., 2006). Capsazepine also sensitized A549 lung cancer cells to radiation therapy (Nishino et al., 2016). Thomas et al., (2007, 2011, 2012) synthesized the capsaicin analog nonivamide (Figure 26B), which decreased the viability of the immortalized human lung epithelial cell line BEAS-2B overexpressing TRPV1 (referred in this work as TRPV1-OE cells) (Thomas et al., 2011; Thomas et al., 2012; Thomas et al., 2007). Nonivamide and its analog N-(3-4 dihydroxybenzyl)nonivamide (Figure 26C) displayed potent growth-suppressive activity in TRPV1-OE cells, and this process was mediated by the ROS oxidative stress pathway (Thomas et al., 2007). Damiao et al., (2014) synthesized a variety of capsaicin analogs (Figure 26 C–E) and tested for their cytotoxicity in B16F10 (mouse melanoma), SK-MEL-28 (human melanoma), NCI-H1299, NCI-H460 (human lung cancer), SK-BR-3, and MDA-MB-231 (human breast cancer) cell lines (Damiao et al., 2014). The capsaicin analog N-(benzo[d] [1,3]dioxol-5-ylmethyl)-4-methoxybenzamide (Figure 26D) decreased the viability of human NCI-H1299 cells and mouse melanoma cells, comparable to capsaicin. Benzo[d][1,3]dioxol-5-ylmethyl hexanonate (Figure 26E) showed greater growth-inhibitory activity in SK-MEL-28 cells than capsaicin (Damiao et al., 2014), whereas its growth-suppressive effects are similar to capsaicin in mouse melanoma cells. Furthermore, the authors performed exploratory data analysis and molecular modeling on these both, N-(benzo[d][1,3]dioxol-5-ylmethyl)-4-methoxybenzamide and benzo[d][1,3]dioxol-5-ylmethyl hexanonate. These in silico experiments suggested that aryl

amides, esters, and alkyl esters may be promising scaffolds to develop capsaicin mimetics with improved anticancer activity (Damiao et al., 2014). The compound MRS1477 (Figure 26F), a positive allosteric modulator of TRPV1, was found to be very robust in inducing apoptosis in human breast cancer cells *in vitro* and in athymic mouse model (Naziroglu et al., 2017). The growth-inhibitory effects of MRS1477 were observed at five-fold lower concentration relative to capsaicin. The proapoptotic activity of MRS1477 was mediated by the TRPV1 receptor (Naziroglu et al., 2017).

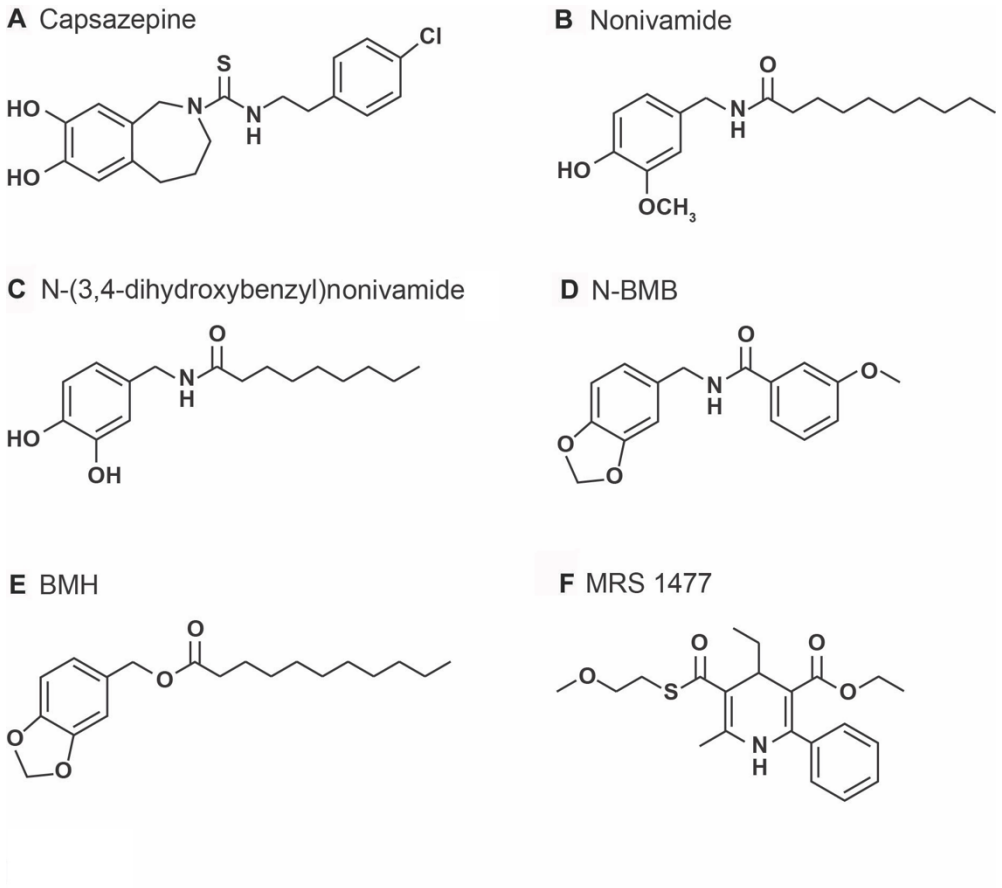

Figure 26. Miscellaneous capsaicin analogs that display growth-inhibitory activity in human and mouse cancer cell lines
(A) Capsazepine, a TRPV1 antagonist. (B) Nonivamide. (C) N-(3-4 dihydroxybenzyl)nonivamide. (D) N-(Benzo[d][1,3]dioxol-5-ylmethyl)-4-methoxybenzamide (N-BMB). (E) Benzo[d][1,3]dioxol-5yl-methyl hexanonate (BMH).

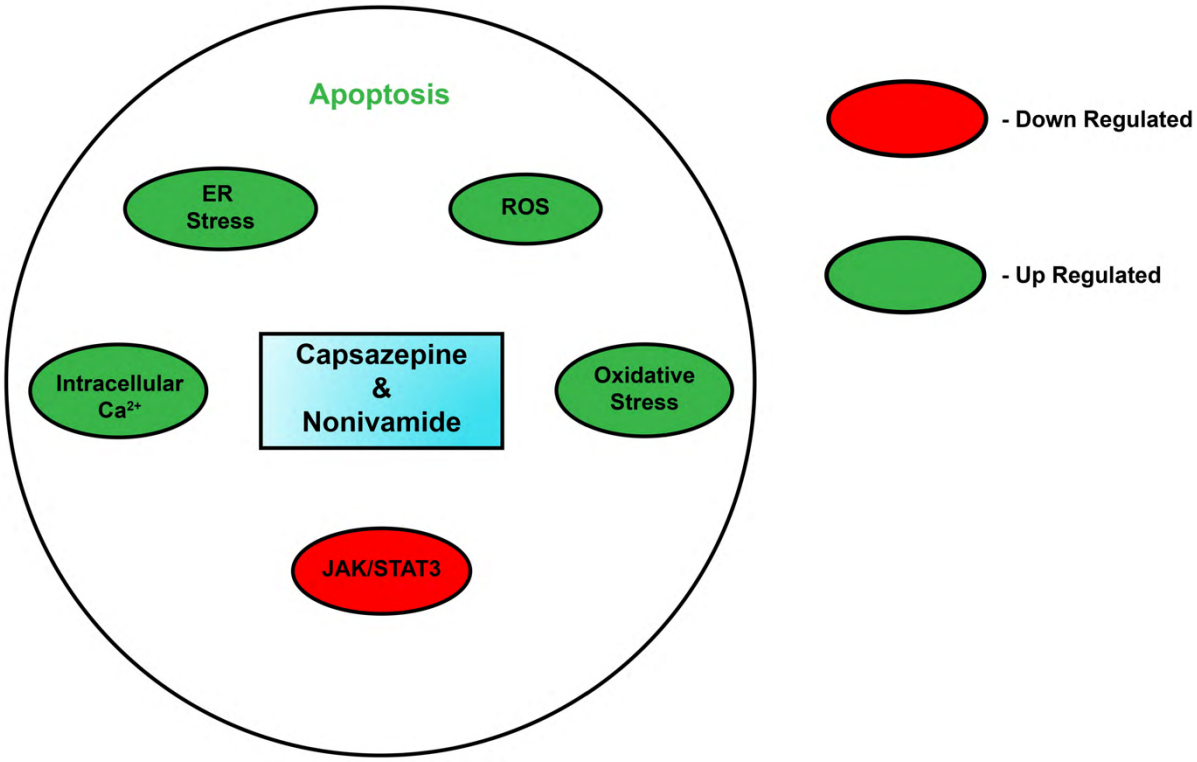

Figure 27. Molecular mechanisms underlying the apoptotic activity of capsazepine and nonivamide

CONCLUSIONS AND FUTURE DIRECTIONS

The nutritional compound capsaicin has shown potent anticancer activity in multiple human cancers. However, the therapeutic potential of capsaicin has been limited by its unpleasant side effects. This has led to intense research focused on the discovery and design of natural and synthetic capsaicin-like compounds. A variety of natural capsaicinoids has been isolated from peppers and other natural sources. Similarly, synthetic capsaicin analogs have been designed by manipulating the pharmacophore of capsaicin. Another aim of the rational design of capsaicin analogs has been to find compounds that will display better bioactivity and greater therapeutic index. A promising class of synthetic non-pungent capsaicin mimetics are long-chain unsaturated N-AVAMs. An exciting development in the field of capsaicin analogs has been the synthesis of allosteric TRPV1 modulators for cancer therapy. However, a majority of these

capsaicin mimetics have been tested for their analgesic activity and not their anticancer activity. The growth-inhibitory activity of some capsaicin analogs has been predominantly analyzed in cell culture and not in animal models. Such data underline the importance of examining the antineoplastic of different types of synthetic capsaicin mimetics in athymic mouse and patient-derived xenograft models. Capsaicin, capsiate, and EVO have been shown to display potent antiangiogenic activity in both cell culture and mouse models. In contrast, there are no reports of the antiangiogenic activity of other natural and synthetic capsaicin analogs. Another promising area of research is the combinatorial anticancer activity of these capsaicin analogs with conventional chemotherapy or radiation. The development of non-pungent second-generation capsaicin mimetics with anticancer and antiangiogenic activity will pave the way for novel treatment regimens in human cancers.

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Authorship Contributions

Participated in research design: Rimoldi, Dasgupta.

Contributed new reagents or analytic tools: Friedman, Brown, Nolan.

Wrote or contributed to the writing of the manuscript: Miles, Akers, Colclough, Rimoldi, Friedman, Brown, Nolan, Valentovic, Seidler, Dasgupta.

CHAPTER 5: ANTI-CANCER ACTIVITY OF SYNTHETIC CAPSAICINOIDS IN SMALL CELL LUNG CANCER

INTRODUCTION

Small cell lung cancer (SCLC) represents 15-20% of all lung cancer cases. SCLC is characterized as being aggressive showing rapid growth and quick dissemination (Bunn et al., 2016; Herbst, Heymach, & Lippman, 2008; Kalemkerian et al., 2013). Frequently, patients are already in the extensive stage of disease at diagnosis, with the five-year survival rate being almost non-existent. One of the major obstacles with SCLC treatment is its pervasiveness for developing resistance to first line platinum-based chemotherapeutic options (Alvarado-Luna & Morales-Espinosa, 2016; S. L. Wood et al., 2015). After resistance has developed, patients have limited options for the next step of their treatment. Chemotherapeutic approaches typically only extend a patient's life by 4-8 months, emphasizing both the severity of the disease and the continued lack of efficacious therapeutic modalities (Bunn et al., 2016; Latimer & Mott, 2015). The lack of successful treatment options has plagued patients diagnosed with SCLC for decades. The search for novel compounds or drugs to treat or augment chemotherapy currently used to treat SCLC is imperative to improving response to therapy and reducing the mortality rates associated with this disease (Alvarado-Luna & Morales-Espinosa, 2016; Bunn et al., 2016; Koinis et al., 2016; Polley et al., 2016).

Capsaicin, the major pungent compound in chili peppers, has been studied extensively for its analgesic properties. Along with being a potent analgesic, it has also been shown to have anti-cancerous properties in a handful of malignancies such as lung cancer, prostate cancer and melanoma (Basith et al., 2016; K. C. Brown et al., 2010; Lau et al., 2014; Meral et al., 2014; Patowary, Pathak, Zaman, Raju, & Chattopadhyay, 2017). Capsaicin is an agonist of the

transient receptor potential vanilloid (TRPV) family of receptors which can sense heat. This receptor is why a chili pepper elicits a noxious heat sensation (Chapa-Oliver & Mejia-Teniente, 2016; Chow et al., 2007; Clark & Lee, 2016; Diaz-Laviada & Rodriguez-Henche, 2014; Friedman et al., 2018; Lau et al., 2014; Shintaku et al., 2012). The clinical application of capsaicin is hindered by its unpleasant side effect profile related to the heat sensation of capsaicin (Basith et al., 2016; Patowary et al., 2017). Despite being a proven analgesic, gastrointestinal burning, diarrhea, and abdominal discomfort are just a few of the many adverse reactions reported by patients when being treated in clinical trials (Fuhrer et al., 2011; Hammer, 2006). The frequency of patient discontinuation of capsaicin therapy renders many trial outcomes useless, making the proposal and implementation of capsaicin as a medicinal agent challenging. One potential solution to this issue is to investigate the use of natural and synthetic analogs of capsaicin (here after called capsaicinoids), both of which have also been shown to be potent analgesics with similar anti-cancer effects of capsaicin (Appendino et al., 2002; Di Marzo et al., 2002; X. F. Huang et al., 2013; Janusz et al., 1993). Due to the structural similarity and the comparable analgesic activity of many of these capsaicinoids, it is plausible that these compounds may also maintain similar, if not better, anti-cancer properties (potentially due, in part, to therapeutic tolerability). In fact, previous studies have shown that non-pungent long-chain capsaicinoids prevented invasion of lung cancer cells more effectively than capsaicin, suggesting that further investigation to evaluate their potential clinical use is warranted (Hurley et al., 2017).

The studies presented in this chapter evaluate the anti-cancer capabilities and mechanism of capsaicinoids in SCLC. In order to compare the antineoplastic abilities of capsaicinoids to that of capsaicin, a variety of assays were performed. We evaluated the cytotoxic potency of a panel

of synthetic capsaicinoids to induce cell death in three SCLC cell lines first by MTT assay and then by measuring caspase-3 activity, as well as a cell death ELISA. Following the evaluation of the anti-cancer properties of multiple capsaicinoids, the most potent capsaicinoid was chosen to further investigate the anti-cancer mechanisms of action and to compare it to that of capsaicin, which had been established in previous studies by our laboratory (Lau et al., 2014). The selected synthetic capsaicinoids evaluated in these studies are considered to be non-pungent, which means they lack the adverse clinical side effects that are reported for capsaicin. The overall aim of these studies was to determine if the synthetic capsaicinoids provide an equal, if not better anti-cancer agent than capsaicin, and to evaluate and compare their mechanisms of action to that of capsaicin. Identifying non-pungent capsaicinoid compounds with equivalent or greater potency than capsaicin, in conjunction with understanding their mechanism of cytotoxicity, will greatly benefit the search for adjuvant therapy options with potential to augment current standard-of-care chemotherapeutic regimens and improve patient response and survival rates.

METHODS

Reagents

Capsaicin, arvanil, olvanil, BAPTA-AM (1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis(acetoxymethyl ester), Ruthenium Red (RR), AM-281, and calpeptin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Livanil and Linvanil were synthesized by our collaborator Dr. John Rimaldi at the University of Mississippi. All cell culture reagents, including RPMI-1640, FBS, Trypsin-EDTA, and HEPES, were purchased from American Type Culture Collection (ATCC; Manassas, VA, USA). Sodium pyruvate, glucose, and penicillin-streptomycin solutions were obtained from Corning (NY, USA). Alveolar Epithelial Cell Medium was purchased from ScienCell Research Laboratory.

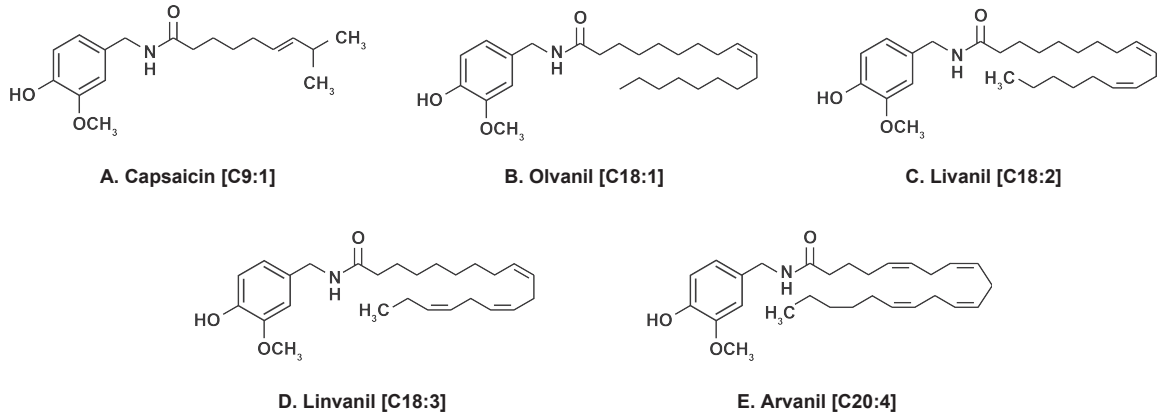

Figure 28. The molecular structures of capsaicin, olvanil, livanil, linvanil, and arvanil
The first number following the compound names in the brackets denotes the chain length. The number following the colon represents the number of double bonds within the chain. For example: Capsaicin has a 9-carbon chain with one double bond and is designated [C9:1].

Cell culture

Three human SCLC cell lines were utilized: NCI-H69 (H69), NCI-H82 (H82), and DMS 114. H69 is considered a classical representative SCLC cell line, isolated from a 55-year-old male. H82 is a variant SCLC cell line, isolated from a 40-year-old. Both cell lines were isolated from men via pleural effusion (Broers et al., 1988; Carney et al., 1985; Gazdar, Carney, Nau, & Minna, 1985). DMS 114 is a human SCLC cell line isolated from a 68-year-old male and characterized by Pettengill et al., (1980). Both H69 and H82 are suspension cell lines which grow in aggregates. DMS 114 is an adherent cell line (Pettengill et al., 1980). All three cell lines were grown and maintained in RPMI-1650 with 2 mM glutamine, 25 mM HEPES, 1 mM sodium pyruvate, 4.5 g/L glucose, 100 units/mL penicillin, 100 units/mL streptomycin and 10% fetal bovine serum (Friedman et al., 2017; Lau et al., 2014). All cell lines were purchased from ATCC and grown in accordance with their suggestions. They were maintained in an incubator at 37°C and 5% CO_2. These cell lines have been widely utilized in SCLC studies and possess characteristics similar to human patients (Broers et al., 1988; Carney et al., 1985; Pettengill et al.,

1980). Human pulmonary alveolar epithelial (HPAEpiC) cells were purchased from ScienCell Research Laboratory. HPAEpiCs were used to represent "normal" lung cells. They were maintained in Alveolar Epithelial Cell Medium (basal medium) supplemented with 10% of fetal bovine serum, 1% epithelial cell growth supplement, and 1% penicillin/streptomycin solution in accordance with the supplier's suggestions. They were maintained in an incubator at 37°C and 5% CO_2.

Treatment of cultured cells

Cells were grown to 70-80% confluency. Cells were then treated with each compound at varying concentrations (10 nM, 100 nM, 1 μM, 2.5 μM, 5 μM, 10 μM, 20 μM) for 24 hours with RPMI-1640 + 1% FBS. Vehicle controls were treated with 0.2% DMSO. When cells were treated with BAPTA-AM (10 μM), RR (1 μM), or AM-281 (10 μM) the inhibitors were added 45 minutes before the capsaicinoid.

Preparation of lysates

Cell lysates were prepared according to a previously established protocol (Brown et al., 2010). Cells were harvested, washed 3 times with cold PBS and added to M2 Lysis Buffer (20 mM Tris, pH 7.6, 0.5% IGEPAL CA-630, 250 mM NaCl, 3 mM EGTA, 3 mM EDTA, 4 μM DTT, 5 mM PMSF, 1 mM sodium fluoride, 1 mM sodium orthovanadate, 25 μg/ml leupeptin, 5 μg/ml pepstatin, 5 μg/ml aprotinin and 25 μg/ml trypsin-chymotrypsin inhibitor). The lysates were incubated on an orbital rocker at 4°C for 40 minutes, and cleared by centrifugation at 15,000 g for 15 minutes at 4°C. The supernatants were collected and stored at -80°C. Protein concentrations were determined by Bradford assay (Bio-Rad Laboratories, Hercules, CA, USA).

Cell viability assay

Cells were seeded in 96-well plates at a density of 1.0 x 10^4 cells per well and allowed to incubate for 24 hours to reduce cell stress. The cells were treated in RPMI + 1% FBS in triplicate with each compound and concentration as indicated. After 24-hour incubation the cells were analyzed using an MTT cell viability assay. MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was added to the cells and allowed to incubate for 2-4 hours. During this time, viable cells reduced the MTT to purple formazan. The media was removed and DMSO was added to solubilize the formazan. Absorbance was measured at 540 nm in a microplate reader. Each cell line was performed independently with each compound in triplicate on the plate. Each plate was performed three times. The cell viability of the treatment groups were compared to the vehicle controls.

Caspase-3 activity assay

The colorimetric Caspase-3 Activity Assay was purchased from EMD Millipore (Cambridge, MA) and run in accordance with the manufacturer's specifications, using 100-150 μg protein aliquot in a 96-well plate in duplicate. Lysates were normalized using the Bradford protein quantification method. The Caspase-3 Activity Assay detects the chromophore p-nitroaniline (pNA). Following addition of the DEVD-pNA substrate to the cell lysate, pNA is released following cleavage of the substrate by active Caspase-3. Optical density of pNA was detected at 405 nm using a microplate reader. This assay was performed twice on three independent sets of cell lysates (n=6). The procedure was repeated for all cell lines.

Cell death ELISA

The Roche Cell Death Detection sandwich ELISA was purchased from Roche. The supplied 96-wells are precoated with anti-histone antibodies. Nucleosomes associated with

apoptosis bind to the anti-histone antibodies if they are present in the sample. A secondary antibody (anti-DNA-POD) with a reporter peroxidase was then added. Color change following addition of the ABTS solubilizing solution was then analyzed by measuring absorbance at 405 nm (reference wavelength approximately 490 nm) with a microplate reader to provide a quantitative reading. Due to the specificity of the antibodies to apoptotic-associated nucleosomes, this assay shows apoptosis but not necrosis-related cell death. The manufacturer's protocol was followed using the previously described cell lysates normalized by Bradford protein quantification method. The absorbance value of untreated control cells was set as 1 and the absorbance of the compound treated cells were reported as a fold increase in cell death relative to the control. The protocol was identical for all cell lines.

Calpain activity assay

SCLC cells were treated with either calpeptin or BAPTA-AM (10 μM) 45 minutes prior to adding 20 μM arvanil in RPMI media containing 1% FBS, as described above, for 24 hours. Cells were harvested and washed twice with PBS. Lysates were made using the provided buffer in the Sensolyte 520 Calpain Activity Assay Kit (Anaspec, Freemon, CA, USA). Two hundred micrograms of lysates were used and incubated with 50 μL of calpain substrate for 60 minutes at 37°C. The assay was performed according to manufacturer's specifications. Fluorescence was measured in a microplate reader with an excitation wavelength of 490 nm and an emission wavelength of 520 nm.

Statistical analysis

Data were analyzed and plotted using GraphPad Prism 5 Software, Inc (La Jolla, CA, USA), and represented as the mean ± the standard deviation. The results were compared by one-

way analysis of variance (ANOVA) followed by a Tukey post-hoc multiple comparison test. Data were considered significant at $p \leq 0.05$.

RESULTS

Arvanil, linvanil, and livanil reduce cell viability in SCLC cell lines

Initial experiments were conducted to evaluate the effects of four capsaicinoid compounds on cell viability in three SCLC cell lines. Previously published studies from our laboratory found that 10 μM capsaicin has no effect on the cell viability of SCLC cell lines; thus 10 μM capsaicin was selected as the reference concentration for comparing the potency of the capsaicinoid compounds (Hurley et al., 2017). To evaluate and compare the cytotoxic potency of the capsaicinoid compounds relative to capsaicin, cell viability was measured by MTT assay following a 24-hour treatment of each cell line with 10 μM capsaicinoid (arvanil, linvanil, livanil, olvanil; Figure 28) or 10 μM capsaicin. In H69 cells there was a significant decrease in cell viability following treatment with 10 μM of arvanil, linvanil, and livanil but not olvanil or capsaicin (Figure 29A). Livanil decreased cell viability by about 20%, while linvanil decreased cell viability by about 30%. These findings were consistent in H82 (Figure 29B) and DMS 114 (Figure 29C) cells as well. Arvanil demonstrated the most potent effect on cell viability in all three cell lines, decreasing cell viability by about 50% (Figure 29 A-C). Therefore, arvanil was selected as the representative capsaisinoid in subsequent experiments.

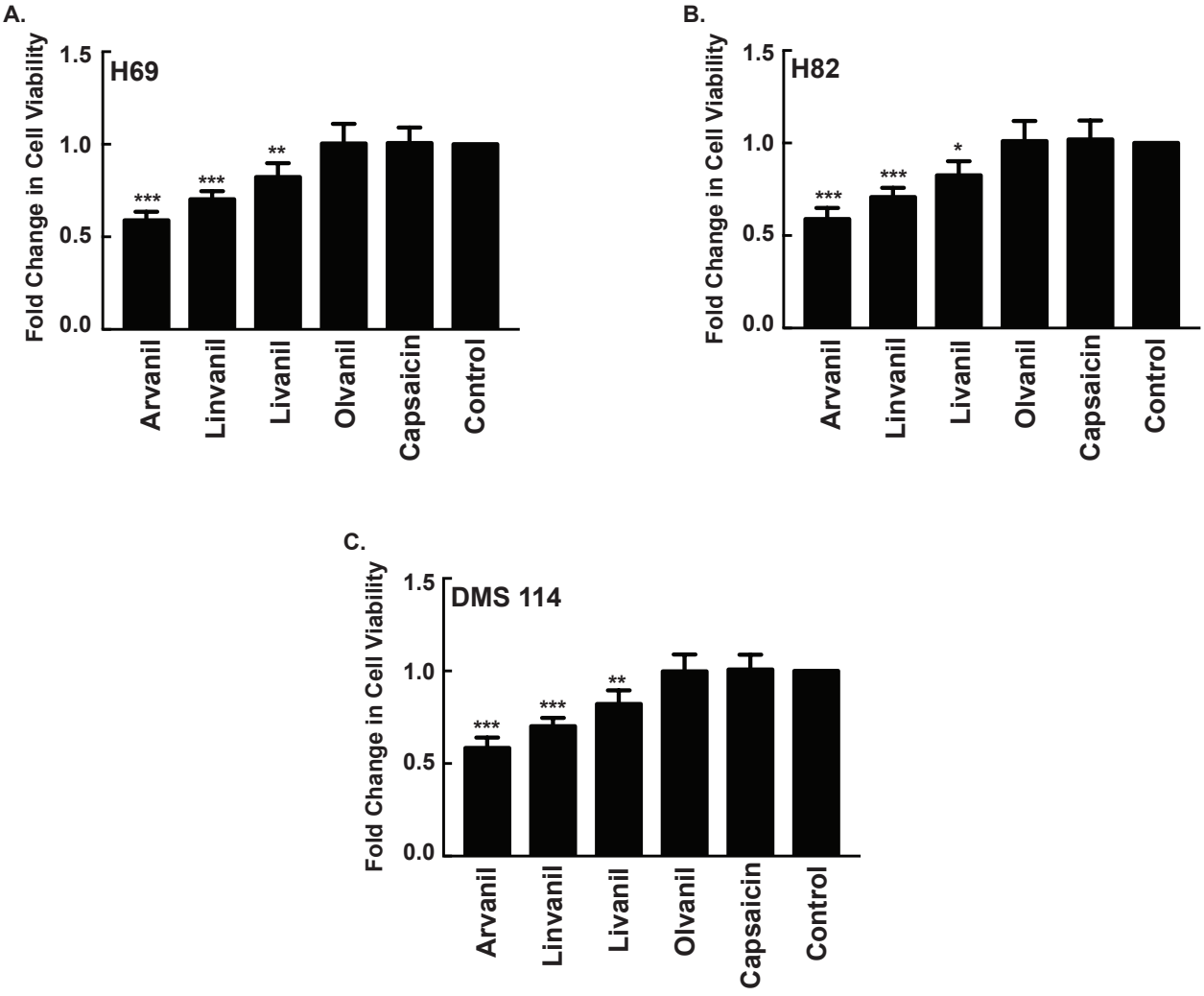

Figure 29. Effect of capsaicinoids on SCLC viability
The effect of capsaicin, olvanil, livanil, linvanil, and arvanil (10μM) on cell viability over 24 hours was compared in three SCLC cell lines by MTT assay; (A) H69, (B) H82 and (C) DMS 114. * denotes statistical difference from control. Data is represented as the mean ± SD. * $p \leq 0.05$, ** $p \leq 0.01$, *** $p \leq 0.001$.

Arvanil induces apoptotic cell death in SCLC cells but not normal lung cells

To elucidate the mechanism of cytotoxicity, each cell line was treated for 24 hours with a concentration range of arvanil or capsaicin (10 nM, 100 nM, 1 μM, 2.5 μM, 5 μM, 10 μM, 20 μM) and caspase-3 activity was measured. Arvanil significantly increased caspase-3 activity at lower concentrations than the capsaicin treated cells. Capsaicin treatment failed to induce

caspase-3 activity at all concentrations, in all cell lines. In H69 cells, arvanil significantly induced caspase-3 activity starting at a concentration of 2.5 μM (Figure 30A). In H82 cells, arvanil significantly induced caspase-3 activity at a concentration of 5 μM (Figure 30B). DMS 114 cells were found to be the most susceptible to arvanil, showing induced caspase-3 activity at 1 μM (Figure 30C). This showed that arvanil is more potent than capsaicin at inducing the apoptotic pathway in human SCLC cells.

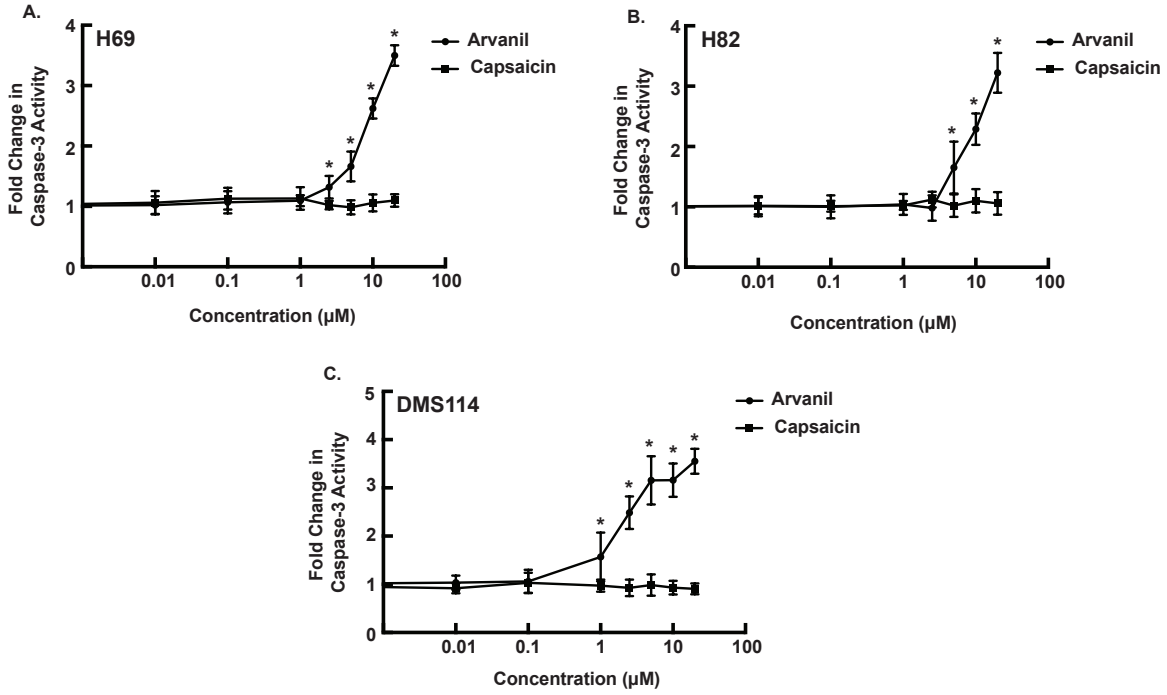

Figure 30. Comparison of caspase-3 activity induced by arvanil and capsaicin in SCLC cell lines
Caspase-3 Activity Assay was used to compare the ability of arvanil and capsaicin (at identical concentrations; 10 nM, 100 nM, 1 μM, 2.5 μM, 5 μM, 10 μM, 20 μM) to induce caspase-3 activity at 24 hours in (A) H69, (B) H82 and (C) DMS 114 cells. Data is represented as the mean ± SD. * denotes statistically different from control; $p \leq 0.05$.

Next, we wanted to evaluate whether arvanil-induced cell death was selective for cancer cells by analyzing arvanil-induced cell death in normal lung cells. In order to evaluate the effects on normal cells, normal human pulmonary alveolar epithelial cells (HPAEpiCs) were treated for

24 hours with either 20 μM arvanil or capsaicin. Twenty micromolar was selected due to the fact that it was statistically significant in all three SCLC cell lines. Previous data from our laboratory also suggests that 20 μM capsaicin is ineffective at inducing significant cell death in various lung cancer cell lines. Neither compound caused a significant increase in caspase-3 activity in HPAEpiC cells (Figure 31A). This was further examined using the cell death ELISA to verify the caspase-3 activity assay. Again, treatment with 20 μM arvanil or capsaicin failed to cause a significant increase in cell death activity in HPAEpiCs (Figure 31B). This data suggests that arvanil may be capable of selectively killing cancer cells while leaving normal lung cells unharmed.

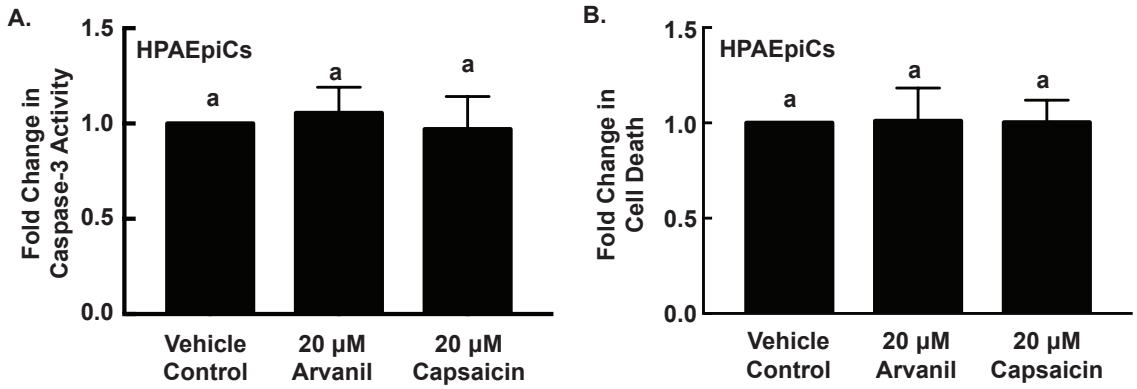

Figure 31. Effect of arvanil and capsaicin on caspase-3 activity and cell death in normal human pulmonary alveolar epithelial cells
Caspase-3 activity and cell death were evaluated in HPAEpiC cells following 24 hour treatment with 20 μM arvanil or capsaicin. Results of the (A) caspase-3 activity assay and (B) cell death ELISA showed that neither arvanil nor capsaicin caused a significant increase in caspase-3 activity or cell death in HPAEpiCs. Data is represented as the mean ± SD. Statistical significance was considered at $p \leq 0.05$.

Arvanil does not induce cell death via the TRPV receptor in SCLC

Next, we wanted to evaluate the signaling pathways through which arvanil may be initiating its anti-cancerous properties. Capsaicin is considered the prototypical TRPV agonist, and previous research in our laboratory has shown that capsaicin-induced cell death is TRPV6 receptor-dependent in SCLC (Lau et al., 2014). To evaluate whether arvanil-induced cell death is

also TRPV receptor-dependent, we used Ruthenium Red (RR), a potent generalized TRPV receptor antagonist, to evaluate its ability to abrogate the effects of arvanil in SCLC. If arvanil-induced cell death is TRPV receptor-dependent, RR should be able to block the effects of arvanil. Figure 32 shows that when SCLC were pretreated with 10 μM of RR, arvanil still induced a significant increase in caspase-3 activity in the three SCLC cell lines.

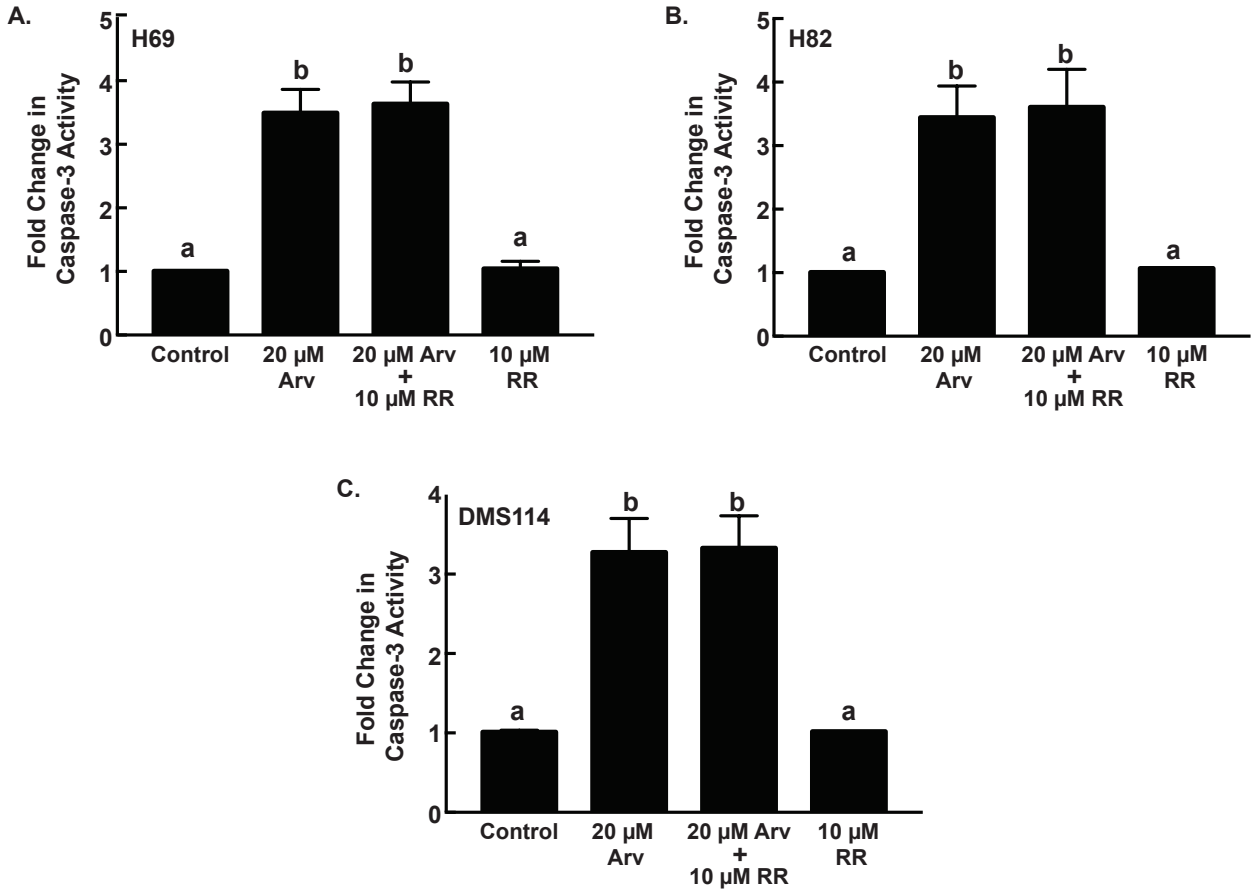

Figure 32. Effect of TRPV receptor inhibition on arvanil-induced caspase-3 activity in SCLC

To evaluate the role of TRPV receptor activation in arvanil-induced apoptosis, caspase-3 activity was measured in SCLC cell lines in response to10 μm arvanil (Arv) following pretreatment with 10 μM ruthenium red (RR). (A) In H69 cells arvanil still induced a significant increase in caspase-3 activity in the presence of RR. Similar results were found in (B) H82 and (C) DMS

114 cells. Data is represented as the mean ± SD. Values represented by the same letter indicate no statistical difference from each other at $p \leq 0.05$.

To validate the caspase-3 activity assay, the lysates treated with RR were then used to perform a cell death ELISA. As seen with caspase-3 activity, pretreatment with RR was unable to inhibit arvanil-induced cell death in all SCLC cell lines, H69, H82, and DMS 114 (Figure 33). The inability of RR to prevent arvanil from inducing caspase-3 activity or cell death suggests that the anti-cancer effects of arvanil are TRPV receptor independent.

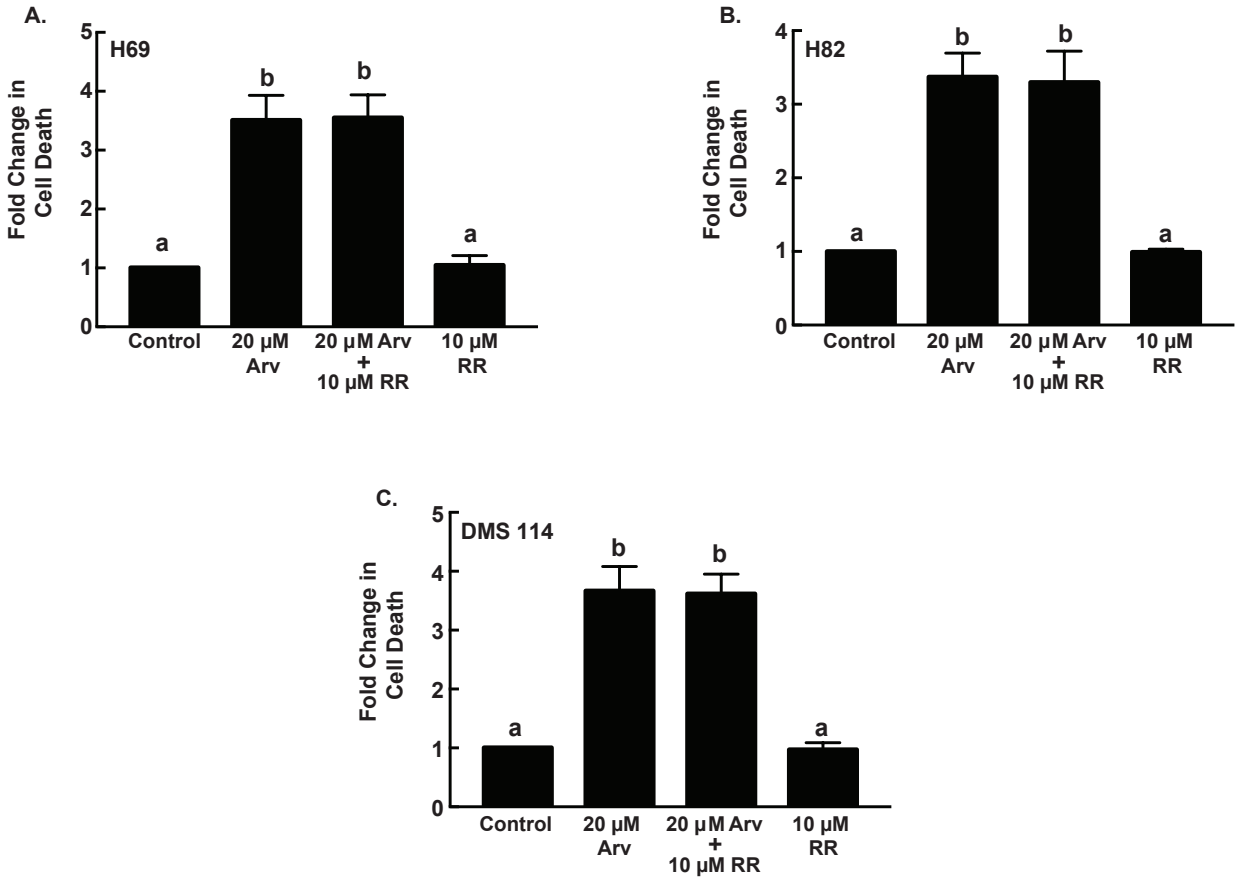

Figure 33. Effect of TRPV receptor inhibition on arvanil-induced cell death in SCLC
Fold change of cell death was evaluated when arvanil (Arv) was treated with 10 µM ruthenium red (RR) to see if it could reverse the anti-cancer activity. (A) In H69 cells arvanil was able to induce significant increases in cell death, as well as in (B) H82 cells and (C) DMS 114 cells.

Data is represented as the mean ± SD. Values represented by the same letter indicate no statistical difference from each other at $p \leq 0.05$.

Arvanil does not induce cell death of SCLC via the cannabinoid 1 receptor

After finding that TRPV receptor inhibition had no effect on the anti-cancer properties of arvanil, a literature review showed that arvanil is a cannabinoid 1 receptor (CB1) agonist (Di Marzo et al., 2002). AM 281 is a selective CB1 antagonist. To evaluate whether arvanil-induced cytotoxicity is dependent on CB1 interaction, cells were treated with 1 μM AM 281 45 minutes before being treated with 20 μM arvanil. Blocking the CB1 receptor had no effect on arvanil-induced caspase-3 activity in all three SCLC cell lines (Figure 34). Similar results were found when these treatment groups were assessed by cell death ELISA. AM 281 had no effect on arvanil-induced cell death in SCLC cells (Figure 35). The inability of AM281 to prevent arvanil from inducing caspase-3 activity or cell death suggests that the anti-cancer effects of arvanil are CB1 receptor independent.

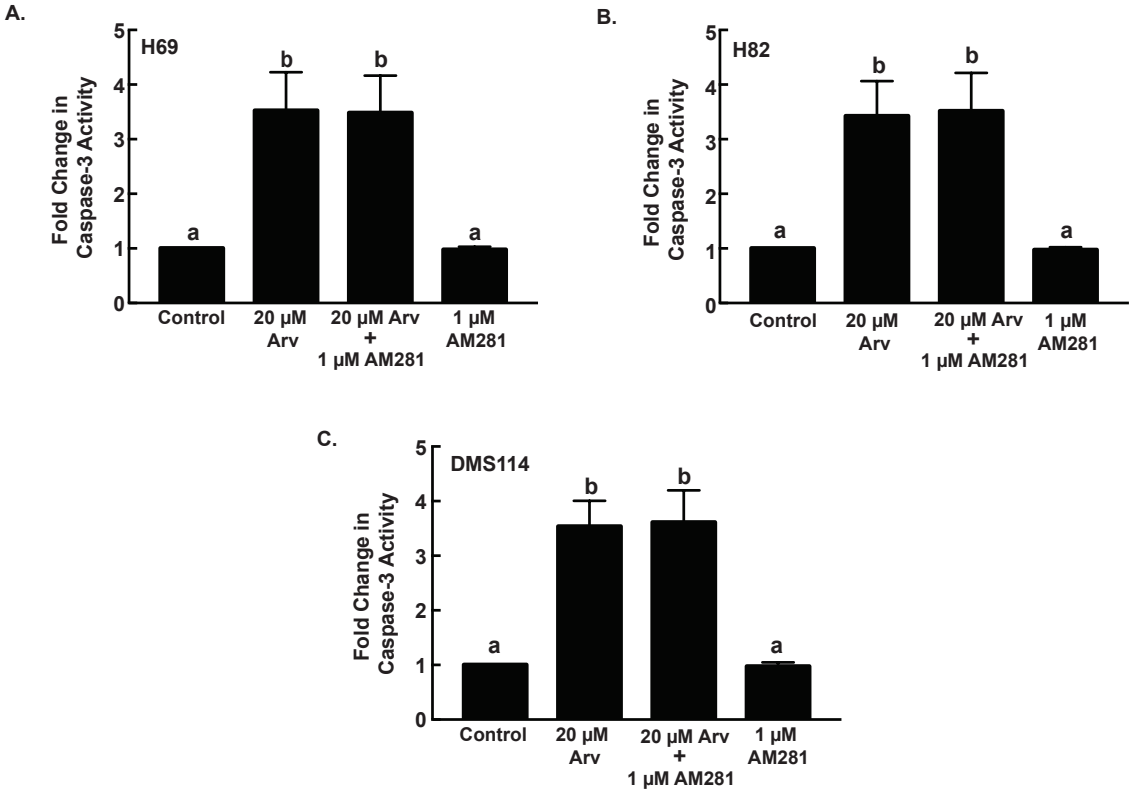

Figure 34. Effect of cannabinoid 1 receptor inhibition on arvanil-induced caspase-3 activity in SCLC

To evaluate the role of the cannabinoid 1 receptor (CB1) in the anticancer activity of arvanil in SCLC, caspase-3 activity was evaluated in response to arvanil (Arv) following pretreatment with 1 µM AM-281. Following treatment with AM-281 to inhibit the CB1 receptor, arvanil was still capable of inducing a significant increase in caspase-3 activity in all three cell lines; (A) H69, (B) H82 and (C) DMS 114. Data is represented as the mean ± SD. Values represented by the same letter indicate no statistical difference from each other at $p \leq 0.05$.

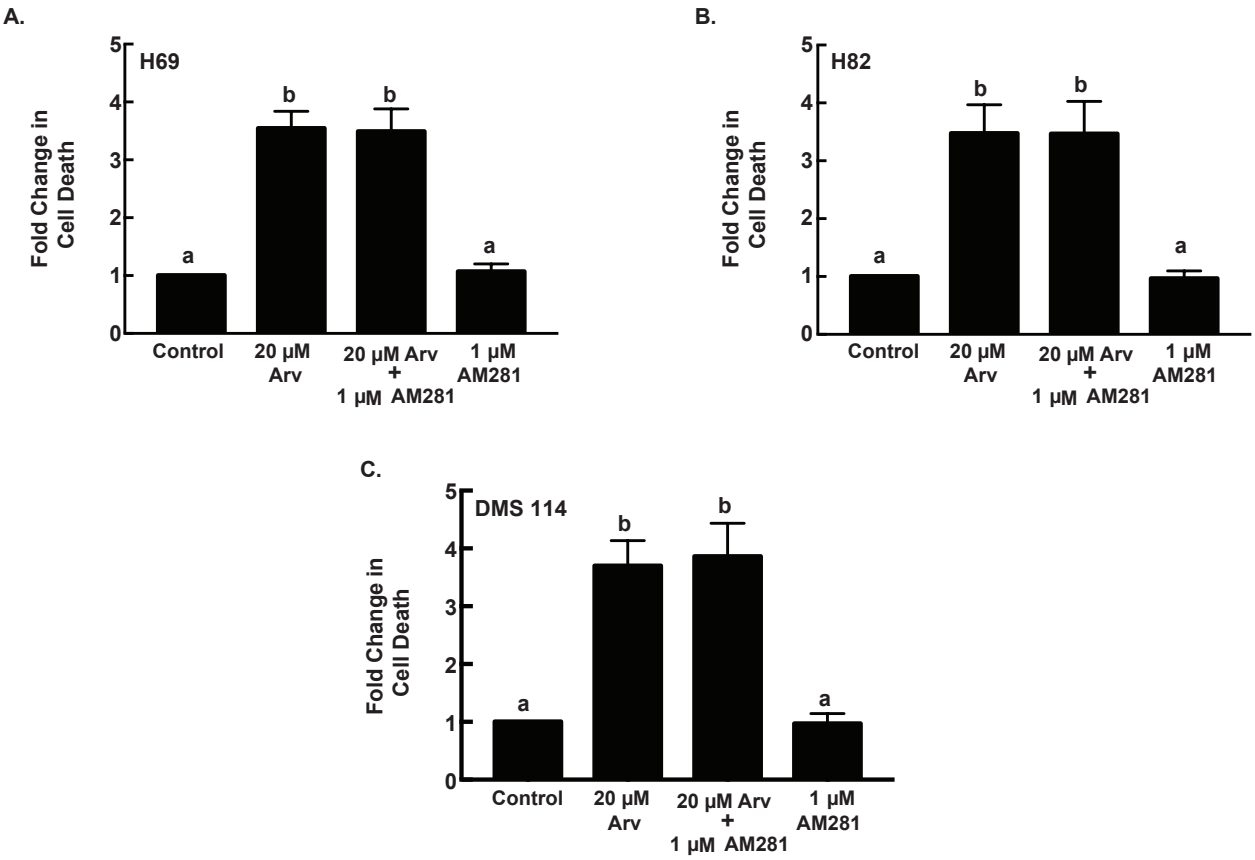

Figure 35. Effect of cannabinoid 1 receptor inhibition on arvanil-induced cell death in SCLC

To evaluate the effect of CB1 receptor inhibition on arvanil-induced cell death, SCLC cells were treated with arvanil following pretreatment with 1 µM AM-281. CB1 inhibition had no effect on inhibiting arvanil-induced cell death in SCLC. Arvanil was able to induce significant cell death in the presence of AM-281 in all three SCLC cell lines; (A) H69, (B) H82 and (C) DMS 114. Data is represented as the mean ± SD. Values represented by the same letter indicate no statistical difference from each other at $p \leq 0.05$.

Arvanil induces apoptosis via intracellular calcium and the calpain pathway in SCLC

Since arvanil did not appear to induce its activity via the same receptors utilized by capsaicin, we wanted to evaluate intracellular mechanisms that may play a role in producing the anti-cancer properties of arvanil in SCLC. Previous studies in the laboratory have demonstrated a relationship between capsaicin and intracellular calcium signaling, which activates the calpain pathway and leads to apoptosis (Friedman et al., 2017; Lau et al., 2014). To determine whether

intracellular calcium also plays a role in the apoptotic signaling of arvanil, our investigations used the chemical calcium chelator BAPTA-AM to evaluate the effect of calcium modulation on arvanil-induced apoptosis in SCLC. When H69, H82 and DMS 114 SCLC cells were pretreated with 10 μM BAPTA-AM, 20 μM arvanil failed to induce caspase-3 activity, which remained at control levels (Figure 36). The results of this experiment were further verified using the cell death ELISA. In line with the caspase-3 activity assay, BAPTA-AM was able to ablate the induction of cell death by arvanil in all three SCLC cell lines (Figure 37).

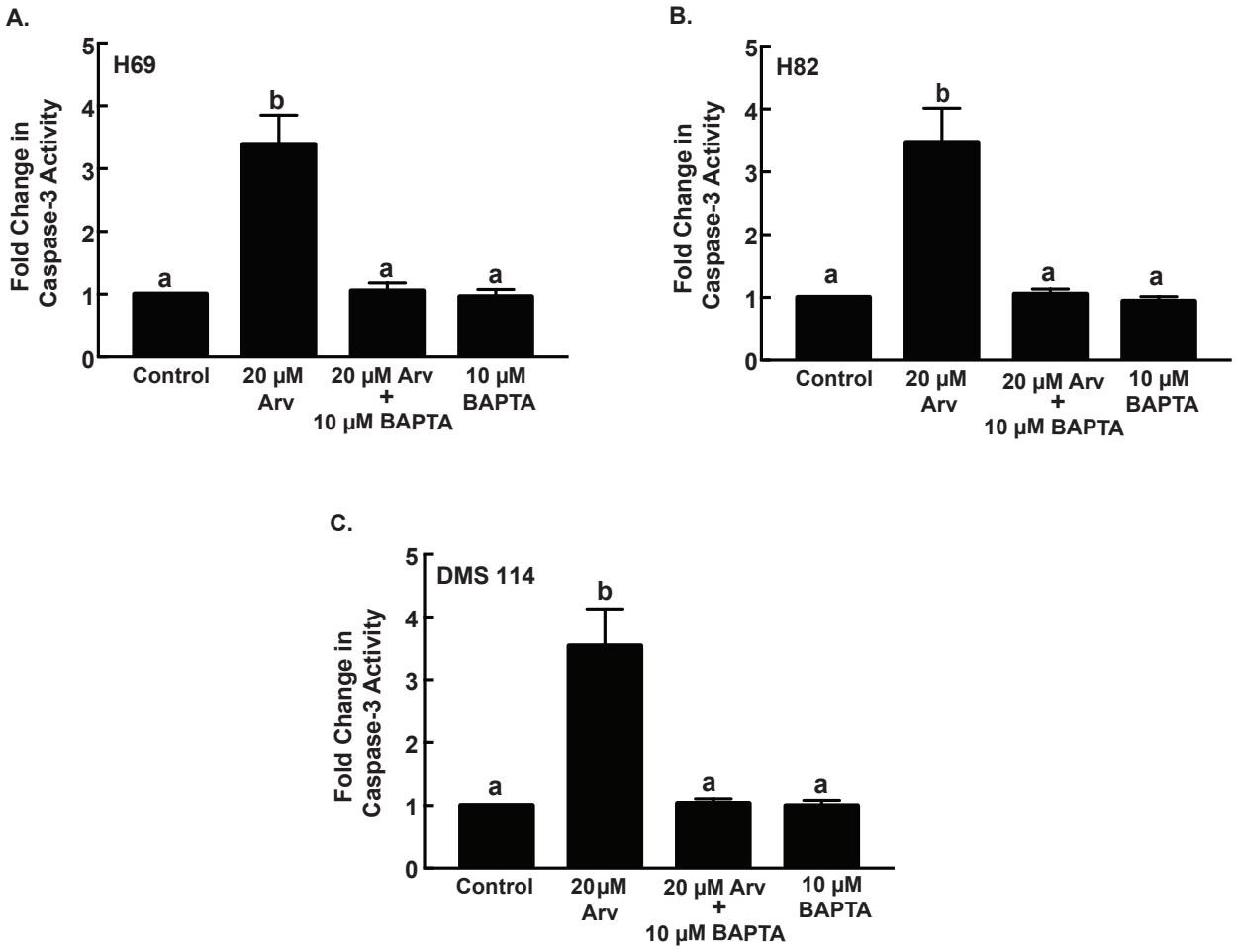

Figure 36. Effect of intracellular calcium chelator on arvanil-induced caspase-3 activity in SCLC

To evaluate the role of intracellular calcium in the apoptotic activity of arvanil in SCLC, caspase-3 activity was evaluated following treatment of three SCLC cell lines with arvanil in the presence of 10 µM BAPTA-AM, a potent calcium chelator. Arvanil was unable to induce caspase-3 activity in the presence of BAPTA-AM in (A) H69, (B) H82 and (C) DMS 114 cells. Data is represented as the mean ± SD. Values represented by the same letter indicate no statistical difference from each other at $p \leq 0.05$.

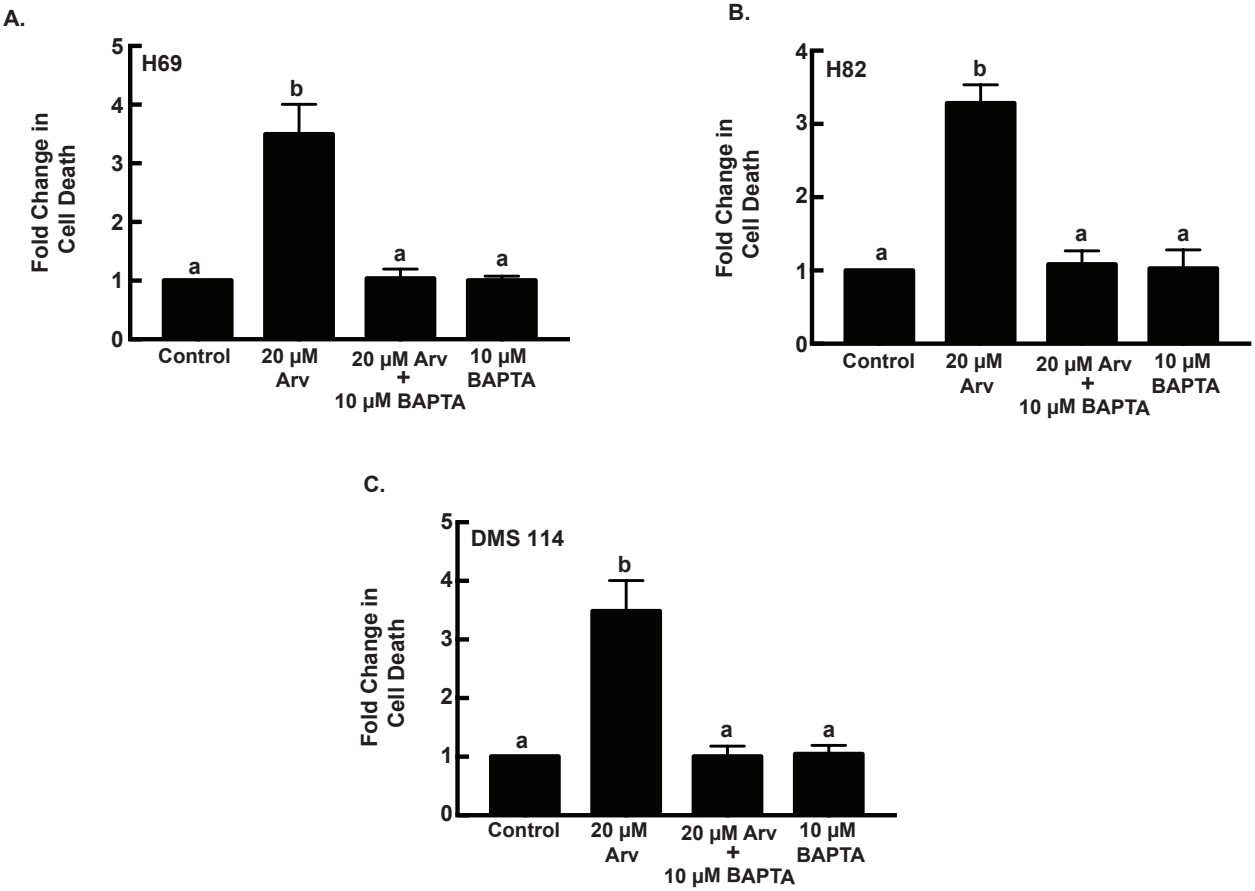

Figure 37. Effect of intracellular calcium chelation on arvanil-induced cell death in SCLC To evaluate the role of intracellular calcium in the anti-cancer activity of arvanil in SCLC, cell death was evaluated following treatment of three SCLC cell lines with arvanil in the presence of 10 µM BAPTA-AM, a potent calcium chelator. Arvanil was unable to induce cell death in the presence of BAPTA-AM in (A) H69, (B) H82 and (C) DMS 114 cells. Data is represented as the mean ± SD. Values represented by the same letter indicate no statistical difference from each other at $p \leq 0.05$.

Given that intracellular calcium appears to be involved in the apoptotic mechanism of action of arvanil, we wanted to evaluate whether arvanil-induced modulation of intracellular calcium also induced the calpain pathway similar to capsaicin. To evaluate the role of the calpain pathway, SCLC cell lines were treated with arvanil in the presence of calpeptin, a calpain pathway inhibitor. Treatment with calpeptin significantly inhibited the activity of arvanil as

measured by caspase-3 activity assay in H69, H82, and DMS 114 SCLC cell lines (Figure 38), which was further verified using the cell death ELISA (Figure 39).

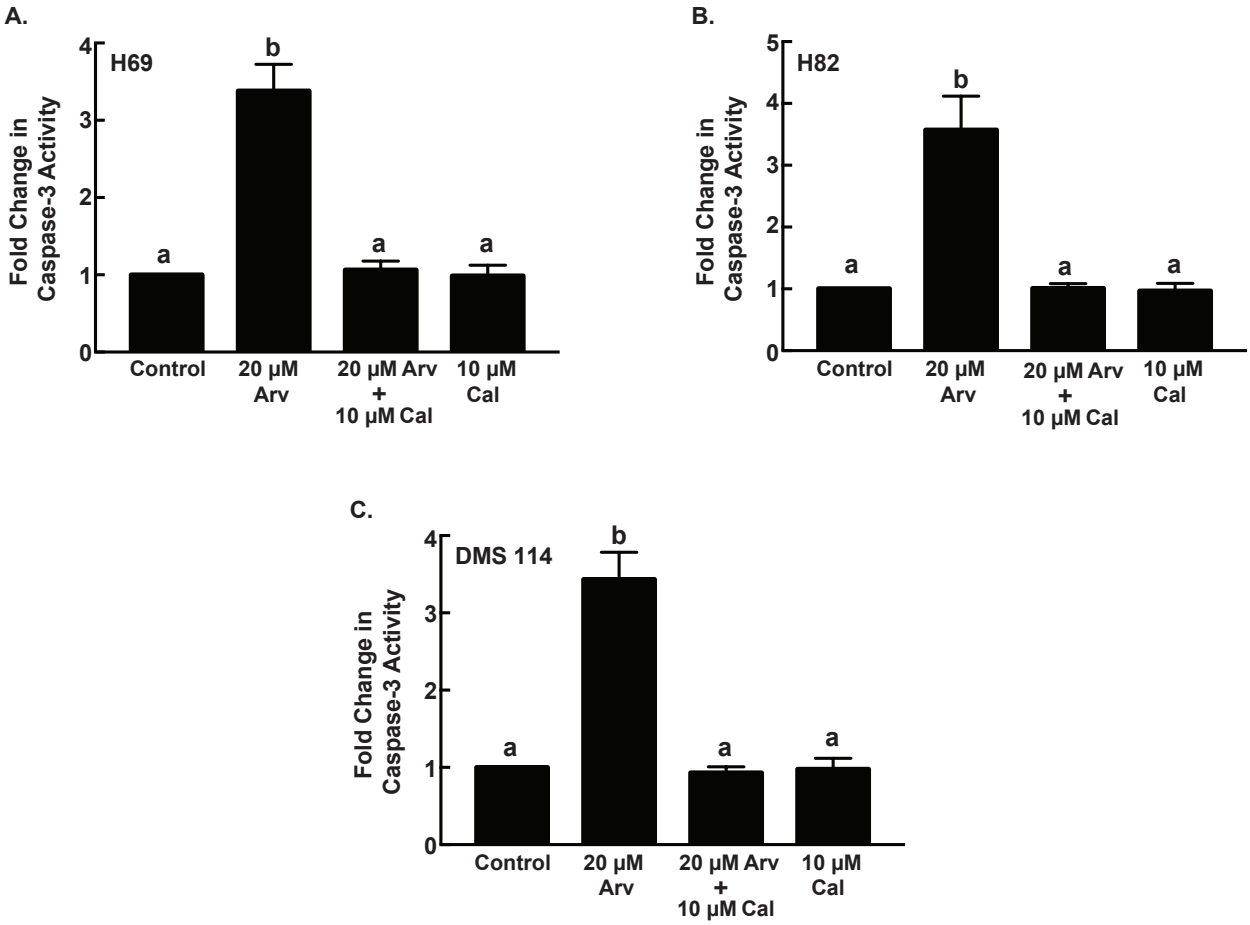

Figure 38. Effect of calpeptin on arvanil-induced caspase-3 activity in SCLC
To evaluate the role of the calpain pathway in arvanil-induced caspase-3 activity in SCLC, caspase-3 activity was evaluated following treatment of three SCLC cell lines with 20 µM arvanil in the presence of 10 µM calpeptin (Cal), a calpain pathway inhibitor. Calpeptin effectively abrogated the anti-cancer activity of arvanil, as arvanil was unable to induce apoptosis in the presence of calpeptin in all three cell lines; (A) H69, (B) H82 and (C) DMS 114 cells. Data is represented as the mean ± SD. Values represented by the same letter indicate no statistical difference from each other at $p \leq 0.05$.

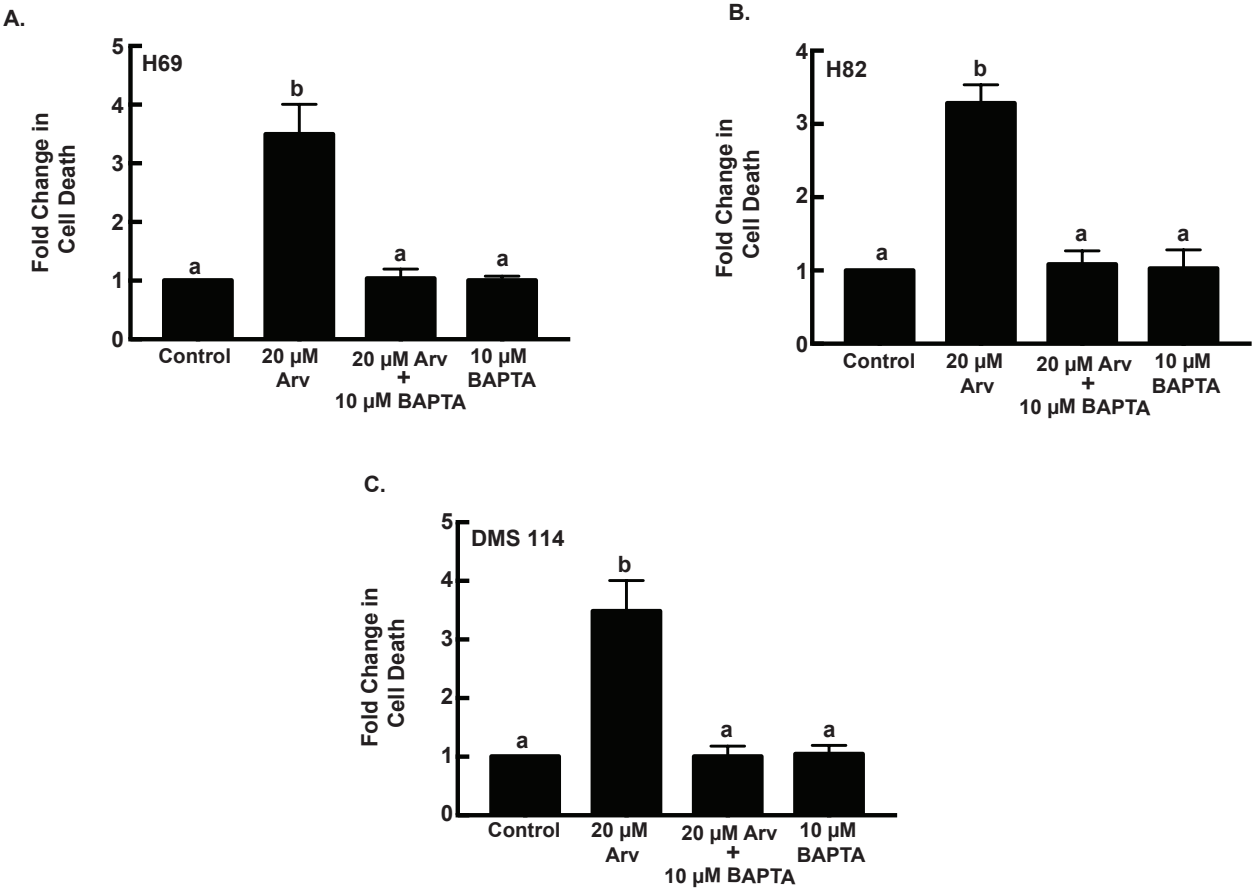

Figure 39. Effect of calpeptin on arvanil-induced cell death in SCLC
To evaluate the role of the calpain pathway in the anti-cancer activity of arvanil, cell death was evaluated in all three SCLC cell lines following treatment with 20 µM arvanil in the presence of 10 µM calpeptin (Cal), a calpain pathway inhibitor. Calpeptin effectively abrogated the anti-cancer activity of arvanil, as arvanil was unable to induce cell death in the presence of calpeptin in all three cell lines; (A) H69, (B) H82 and (C) DMS 114. Data is represented as the mean ± SD. Values represented by the same letter indicate no statistical difference from each other at $p \leq 0.05$.

Arvanil causes increased calpain pathway activity in SCLC

To further verify that arvanil was inducing apoptosis in the SCLC cell lines via induction of the calpain pathway, a calpain activity assay was performed using two of the cell lines, H69 and H82. The calpain activity assay measured the amount of active calpain proteases present in cell lysates from cells that had been treated with arvanil, with or without pretreatment with BAPTA-AM. In both H69 (Figure 40A) and H82 (Figure 40B), calcium chelation effectively

prevented the induction of calpain activity in response to arvanil. This suggests that calcium plays a role in the arvanil-induced calpain pathway activation in SCLC cells. To further verify that activation of the calpain pathway plays a role in arvanil-induced cytotoxicity, a calpain activity assay was performed again on cells treated with arvanil in the presence of calpeptin, a calpain pathway inhibitor. Similar to the effects of BAPTA-AM, the presence of calpeptin brought the arvanil-induced calpain activity to control levels in both SCLC cell lines (Figure 40C and D).

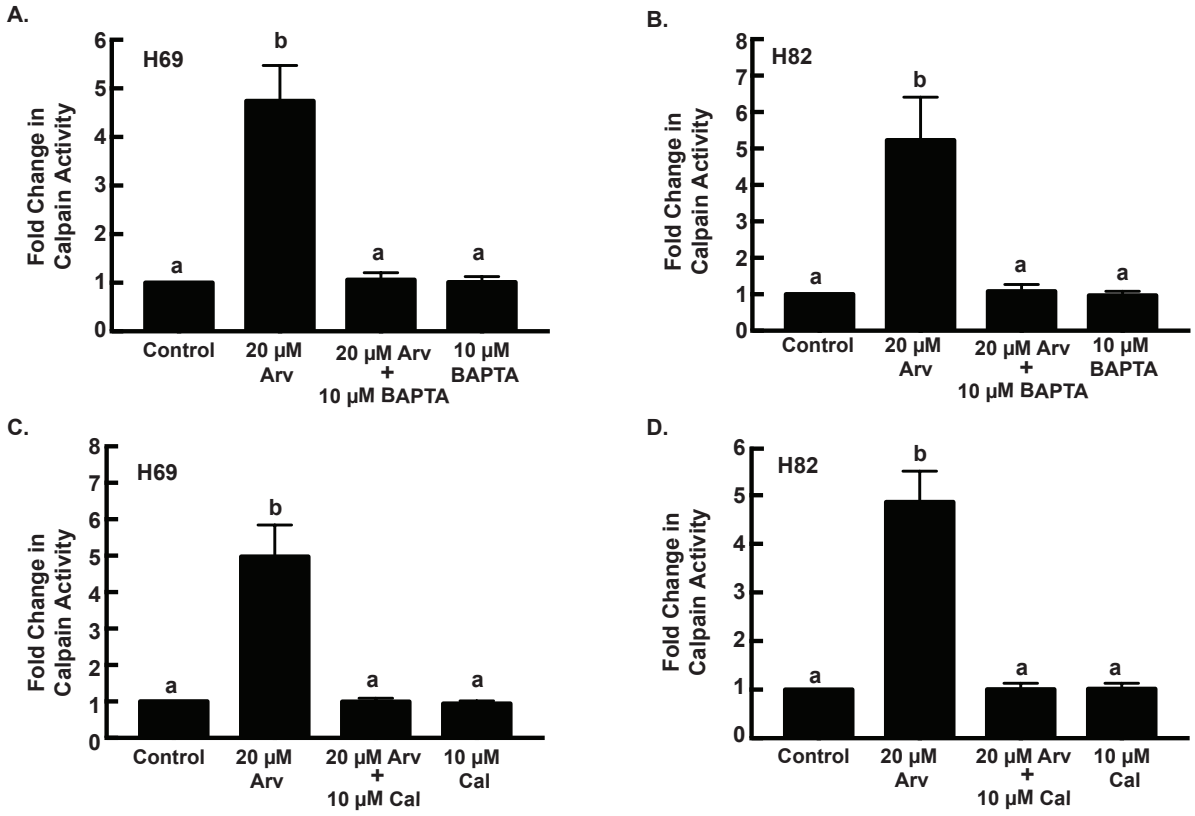

Figure 40. Arvanil-induced calpain activity in the presence of calcium chelator (BAPTA-AM) and calpain pathway inhibitor (calpeptin)
To evaluate the role of the calpain pathway in the anti-cancer activity of arvanil, a calpain activity assay was used following treatment of SCLC cell lines with 20 μM arvanil in the presence or absence of 10 μM BAPTA-AM (calcium chelator) or 10 μM calpeptin (Cal; calpain pathway inhibitor). Pretreatment with BAPTA-AM abrogated arvanil-induced calpain activity in (A) H69 and (B) H82 SCLC cell lines. Similarly, calpeptin effectively abrogated arvanil-induced calpain activity in (C) H69 and (D) H82 SCLC cell lines. Data is represented as the mean ± SD. Values represented by the same letter indicate no statistical difference from each other at $p \leq 0.05$.

CONCLUSIONS AND DISCUSSION

Small cell lung cancer remains a malady in which there are few therapeutic options that significantly improve patient survival (Alvarado-Luna & Morales-Espinosa, 2016). Initial responses to the first-line platinum-based combination treatments, such as cisplatin, plus etoposide may cause initial hope in patients, but the inevitability of relapse is rarely avoided (Pietanza et al., 2015; S. L. Wood et al., 2015). The studies in this chapter provide evidence that

synthetic capsaicinoids may provide unique viable options to improve clinical response in SCLC treatment. One of the main problems with current chemotherapy options is the large incidence of side effects and the lack of cell selectivity, given the fact that standard chemotherapy often kills normal cells along with the cancer cells (McGowan et al., 2017; Oun et al., 2018; Willers, Azzoli, Santivasi, & Xia, 2013). In these studies, we found arvanil to be efficacious in its ability to kill SCLC cells while having no apparent negative effect on normal human pulmonary alveolar epithelial cells. This suggests that arvanil may have the unique capacity to selectively target cancerous cells while preserving normal cells in the surrounding tissue. Previous work from our laboratory shows that in SCLC capsaicin induces apoptosis via the TRPV6 receptor leading to increased activity of the calpain pathway (Lau et al., 2014). Despite its structural similarity to capsaicin, arvanil's anti-cancerous properties appear to be independent of both the TRPV and CB1 receptors, as shown in Figures 33-36. While it remains unclear which receptors are responsible for eliciting arvanil's anti-cancer properties in SCLC, the fact that arvanil does not act via the TRPV receptor suggests that arvanil should not induce a heat sensation similar to capsaicin, and supports other studies which indicate that arvanil is non-pungent. Lacking the heat sensation would lead to a more tolerable side effect profile, promoting patient compliance with therapy. One similarity capsaicin and arvanil have are the induction of the calpain pathway, with the calcium chelator BAPTA-AM and calpain inhibitor calpeptin being able to reverse the apoptotic activity seen in SCLC.

These studies provide evidence that arvanil induces elevated intracellular calcium levels, which leads to calpain pathway activation in SCLC cells. Calpains are a family of calcium-activated proteases. The source of intracellular calcium that activates calpain activity is thought to be the mitochondria, the endoplasmic reticulum, or an influx of extracellular calcium

(Harwood, Yaqoob, & Allen, 2005; Laszlo Kovacs, 2014; M. J. Lee, Kee, Suh, Lim, & Oh, 2009; Moretti et al., 2014; Ono et al., 2016; D. E. Wood & Newcomb, 1999). It is generally accepted that calpains play a role in cellular apoptotic signaling, as well as necrotic signaling. Due to the fact that arvanil treatment led to increased calpain activity as well as caspase-3 activity, it is logical to postulate that the calpain activity is responsible for triggering arvanil-induced apoptosis in this model. Further studies would be necessary to determine the specific mechanism by which the calpain pathway is activating apoptosis since calpains are involved in so many different biological pathways. Calpains have a growing list of substrates, such as apoptosis inducing factor (AIF), p53, Bax, Bid, PARP, and cytosolic proteins/enzymes (Lopatniuk & Witkowski, 2011; Ozaki, Yamashita, & Ishiguro, 2009; Potz et al., 2016; D. E. Wood et al., 1998). Future studies to evaluate the role of caspase and calpain activation in capsaicinoid-induced cytotoxicity will need to be performed in order to understand how the two pathways may work together or independently. These studies will further our understanding of capsaicinoid-induced apoptosis and how they can potentially be used in conjunction with other chemotherapeutic agents to bolster their activity, reduce side effects, and improve clinical outcomes in SCLC.

Further studies are also needed to evaluate livanil and linvanil, which also demonstrated favorable anti-cancer activity in SCLC. If these analogs act in a similar way, they could prove useful in other cancer types, with survival rates comparable to SCLC. Identifying the mechanisms of action could also lead to additional modification of the capsaicin molecule to allow it to target specific receptors/proteins. Long term ramifications of such studies could lead to further modifications based on capsaicin as the parent compound, to be used concurrently with current chemotherapeutic treatments in SCLC, as well as other cancer types. The possibility of

additional interventions for cancer that could augment or deviate from the commonly used standard-of-care treatments leads to hope for patients who have run out of options.

CHAPTER 6: CONCLUSIONS AND DISCUSSION

Over the past several decades, researchers have demonstrated that capsaicinoids display a wide variety of biological and physiological activities. Many of these compounds, capsaicin in particular, demonstrate analgesic, positive cardiovascular, anti-obesity, anti-inflammatory, and anti-tumor effects. The studies presented in this dissertation focused heavily on the anti-tumor effects of capsaicinoids in SCLC. Our goal was to substantiate and promote the use of capsaicinoids as a relevant chemopreventive or adjuvant chemotherapeutic option that may lead to the development of new and advanced treatment strategies for SCLC.

Despite a decrease in smoking rates, SCLC rates remain consistent (Bunn et al., 2016; Cronin et al., 2018; Jamal et al., 2016). Efforts to develop effective therapeutic treatments for SCLC have been hindered by a variety of obstacles (Alvarado-Luna & Morales-Espinosa, 2016; Bunn et al., 2016; Pietanza et al., 2015). Of considerable challenge is the fact that the majority of SCLC patients are frequently diagnosed in later stages of the disease, after the cancer has metastasized to distant parts of the body. SCLC treatment is further complicated by the frequency of patients with refractory disease (relapse less than 45 days post treatment), resistant disease (relapse 45-90 days post treatment), or the rapid acquirement of chemoresistance to first line treatments in patients presenting with therapy sensitive disease (relapse more than 90 days post treatment) (Pietanza et al., 2015). Regardless of the initial classification, for those with refractory SCLC, or those who acquire chemoresistance, second line treatments rarely work, with the median survival in patients with relapsed disease ranging from two to six months (Foy et al., 2017; Gazdar et al., 2017; Pietanza et al., 2015). Though some advances have been made in NSCLC treatment with the use of targeted therapies like gefitinib or erlotinib, the primary treatment for patients with SCLC has not changed in several decades and remains limited to

platinum-based combination therapy in conjunction with radiotherapy (Latimer & Mott, 2015; S. L. Wood et al., 2015). The complexity of SCLC and its classification as a recalcitrant cancer underline the need to identify new novel treatment options that will improve patient response to chemotherapy, reduce side effects, and offer improved overall outcomes (Zeman, Brzezniak, & Carter, 2017).

In Chapter 2, we reviewed and discussed the potential of capsaicin to be utilized as an adjunctive treatment option in combination with several clinically relevant and widely used chemotherapeutics. Numerous studies demonstrated that not only is capsaicin an effective monotherapy for potentially treating various cancer types, but it also demonstrates the capacity to effectively improve the response to chemotherapeutic agents when used in combination (Basith et al., 2016; Chapa-Oliver & Mejia-Teniente, 2016; Friedman et al., 2018; Srinivasan, 2016). Our published data presented in Chapter 3 further supported such findings, successfully demonstrating that capsaicin enhanced the anti-cancer activity of camptothecin, as evidenced by a greater induction of apoptosis at lower concentrations than camptothecin alone in three separate human SCLC cell lines. The presence of using a quantitative method to assess drug synergy is also a somewhat rare feature of our study. A mathematical calculation method allows for one to see if a drug combination is synergistic quantitatively, not just by the subjective opinion of a researcher (Chou, 2010). Using the Chou-Talaly method to analyze the data, we were able to demonstrate that the interaction of capsaicin and camptothecin was in fact synergistic in the SCLC cell lines and not simply additive or antagonistic. Another point verifying the synergistic effects was the use of 10 μM capsaicin to sensitize the SCLC cell lines when we have shown that at that concentration, capsaicin does not cause any apoptotic activity in these cells at 24 hours. In the CAM model, H69 xenografted tumors showed a significant

increase in caspase-3 activity, as well as a significant decrease in tumor weight. Similar to the previous studies in our laboratory, both the *in vitro* and *in vivo* models showed evidence of a rise in intracellular calcium levels leading to increased calpain activity. The origin of the rise in intracellular calcium is most likely due to an influx of calcium via the TRPV6 cation channel, as documented in other studies (Lau et al., 2014). TRPV6 was shown to be expressed in the SCLC cell lines utilized in our studies, and its role in the anti-cancer activity of capsaicin was recognized by using siRNA knock down of TRPV6 to reverse the effects of capsaicin. Future studies investigating capsaicin and camptothecin could utilize TRPV6 siRNA to evaluate its role in the ability of capsaicin to augment the effect of camptothecin in SCLC cells. It also may be advantageous to investigate a capsaicin/camptothecin treatment option in cancers which have already been shown to have higher levels of TRPV6 levels, for example prostate, colon, breast, thyroid, and ovarian carcinomas (Lehen'kyi, Raphael, & Prevarskaya, 2012).

Despite its potential to sensitize human SCLC to the antineoplastic activity of camptothecin, capsaicin's potent and pungent side effects are a challenge to patient compliance, compromising its efficacy. For this reason, additional studies in our laboratory were conducted to investigate the use of non-pungent synthetic capsaicinoids which lack the characteristic heat sensation associated with capsaicin administration. These analogs would lack the common gastrointestinal discomfort or burning sensation side effects which typically cause discontinuation of treatment. In Chapter 4 we discussed in detail multiple aspects of both natural and synthetically derived capsaicin analogs. Some of the most intriguing studies were the derivatization of unsaturated N-acylvanillamides or UN-AVAMs. These are compounds that maintain the main pharmacophore or "head" region of the capsaicin molecule but vary in the length and saturation of the hydrophobic fatty acid chain (Figure 23). These compounds are non-

pungent and lack the negative side effects of capsaicin (Appendino et al., 2005; X. F. Huang et al., 2013; Tuoya et al., 2006). While arvanil, our UN-AVAM of interest, was originally reported to be both a CB1 and TRPV1 agonist, several anti-cancer studies have shown that arvanil induces growth suppression independently of both CB1 and TRPV1 (Di Marzo et al., 2002; Sancho et al., 2003). It was therefore of great interest to investigate the potential anti-cancer activity of these capsaicinoids in SCLC.

Through our investigation discussed in Chapter 5, we found that arvanil was in fact able to induce apoptosis in several SCLC cell lines, similar to capsaicin. To compare the efficacy and potency of these capsaicin analogs, we chose to compare the effects of each compound to the effects found for capsaicin at 10 μM, a concentration at which capsaicin was unable to induce apoptosis in SCLC cell lines. Arvanil proved to be the most effective analog, inducing apoptosis in one SCLC cell line at as low as 1 μM (Lau et al., 2014). Importantly, we also noted that arvanil concentrations as high as 20 μM, which induced significant apoptosis and cell death in all SCLC cell lines, had no effect on normal human lung cells in culture. If arvanil were to be utilized in the clinic, not only would it lack capsaicin's adverse side effect profile, but it appears to have the highly beneficial advantage of targeting cancerous cells while leaving normal cells intact. This finding is important given the commonly associated off target effects of conventional chemotherapeutics (Chabner et al., 2011; Cheung-Ong et al., 2013; Dasari & Tchounwou, 2014; McGowan et al., 2017; Oun et al., 2018; Tannock, 1989). The addition of an adjuvant therapy such as arvanil could lower the required dose of a toxic chemotherapy and would help reduce the side effects associated with normal tissue damage. As described in Chapter 2, the side effect profiles of commonly used chemotherapeutic drugs are extensive and often lead to death of

healthy tissue along with the cancerous tissue. If arvanil has little to no cytotoxic effects on normal cells, its addition would not contribute further off targets effects.

Unlike our previous studies with capsaicin, which indicated a role for TRPV6 in the mechanism of action, the anti-cancer activity of arvanil proved to be independent of the TRPV receptor family, maintaining its antineoplastic activity in the presence of a general TRPV receptor antagonist. Studies also showed that CB1 had no role in arvanil's antineoplastic activity. Despite these upstream differences, the anti-cancer mechanisms of arvanil did demonstrate some similarities to capsaicin. When treating SCLC cell lines with arvanil, the membrane permeable calcium chelator, BAPTA-AM, was able to reverse the effects, indicating a role for increased intracellular calcium in arvanil's anti-cancer effects. The origin of this calcium, however, is still unclear. The rise in intracellular calcium level caused by capsaicin in SCLC cells was found to be dependent on TRPV6 receptor activity, indicating that capsaicin most likely induced an influx of calcium from the extracellular space via the 7TRPV6 receptor. To evaluate if increased intracellular calcium is caused in part by a TRPV independent influx of extracellular calcium in arvanil treated cells, further studies would be necessary, possibly by using a membrane impermeable calcium chelator. Also similar to capsaicin, arvanil caused an increase in calpain pathway activity. The mechanistic role of the calpain pathway in cancer is somewhat unclear; however, calpains are known to play an important role in cell cycle regulation and in some instances, have been shown to promote cell progression in cancer. Despite claims of calpains being important in the development of cancer, it is also known that calpain activity is required for a handful of chemotherapeutic agents, such as cisplatin (Leloup & Wells, 2011). If calpain activity were inhibited, such chemotherapeutic drugs could be rendered ineffective at promoting cell death by the inability to cleave vital signaling proteins like caspase-3. So, despite the

knowledge of calpains' interaction in the development of cancer, it is also a vital signaling pathway involved in the induction of cell death when trying to treat cancer (Mandic et al., 2002; Moretti et al., 2014). In this dissertation, increased calpain activity was implicated in causing cell death with capsaicin and camptothecin treatments, as well as treatments with the capsaicinoid arvanil alone. This may suggest that in SCLC, increasing calpain activity with capsaicinoids may offer a means of intervention.

There is still much to learn about how arvanil may function as an anticancer agent. Future studies from this research will investigate the downstream pathways involved in arvanil's signaling mechanism. Calpain related substrates involve many that are required for the induction of apoptosis, like p53, PARP, BCL-2, BAX, Bid, AIF, and caspases (Harwood et al., 2005; Leloup & Wells, 2011; Moretti et al., 2014). Identifying which substrates are cleaved/activated in addition to caspase-3, would potentially identify additional targetable mechanisms for capsaicinoids in SCLC. *In vivo* models of SCLC are also still lacking and will contribute greatly to elucidating the effects of arvanil in SCLC. Currently, studies are being planned to utilize a patient derived xenograft (PDX) mouse model of SCLC. These models more effectively mimic the tumor microenvironment than other orthotopic or syngeneic mouse models. Furthermore, the results from such studies are critical to supporting the development of much needed human trials and applications.

I believe that the most promising research to come from continuing these studies will ultimately be the development of clinical combination therapies with capsaicinoids such as arvanil and chemotherapeutic agents. The ability of arvanil to show apoptotic activity equivalent to capsaicin but at lower concentrations suggests it too might work at sensitizing human SCLC to chemotherapeutic agents such as camptothecin. It would also be important to combine capsaicin

and arvanil with additional chemotherapeutics, such as irinotecan, etoposide, or cisplatin, to see if they are able to sensitize SCLC lines to a variety of agents. Irinotecan is commonly the second line of treatment for SCLC. If combination with capsaicin or arvanil improves patient response it would be extremely clinically relevant to patients with refractory or recurrent disease. Future studies evaluating the efficacy of combination treatments with capsaicin or arvanil and chemotherapy in chemoresistant cell lines will also greatly further the field and help identify new treatment modalities for therapy resistant patients. Using cisplatin resistant cell lines to mimic the common occurrence of SCLC relapse, would have very relevant translational implications. Xenografts of the resistant cell lines or PDX models from patients whose tumor was resistant to cisplatin would mimic a human patient and provide an insight to see if combination therapies with capsaicin or arvanil would work in a clinic. If these compounds were proven effective against resistant tumors, it would further support the development of new therapy modalities to treat patients with resistant disease.

Unfortunately, treatment failure, harsh side effects, and chemotherapy resistance aren't issues limited to SCLC patients, as patients with nearly every type of cancer suffer many of the same fates. An adjunctive treatment, such as capsaicin or arvanil, could prove helpful in treating a multitude of cancer types. I believe that the use of capsaicin or arvanil in conjunction with an FDA approved chemotherapeutic agent is the most viable and advantageous application of the knowledge obtained in these studies. Arvanil has even greater possibilities due to its non-pungent nature. The use of combination therapy with an already FDA approved drug allows for a fast track into clinical application, which means patients could begin to use capsaicin and arvanil in the near future. The universal use of naturally derived products as a means of medicinal intervention supports the use of compounds like capsaicinoids in cancer intervention, and more

importantly, analogs of natural compounds such as arvanil, may hold even more promise than their natural parent compounds. The potential therapeutic uses of capsaicinoids and their synthetic analogs as single drugs or combination therapies are just starting to become realized, and is exciting for medical doctors, researchers, and patients alike.

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APPENDIX A: IRB LETTER

MARSHALL UNIVERSITY
www.marshall.edu

Office of Research Integrity

February 28, 2019

Jamie Friedman
Department of Biomedical Sciences
Byrd Biotechnology Science Building, Room 416
Joan C. Edwards School of Medicine
Marshall University

Dear Jamie:

This letter is in response to the submitted dissertation abstract entitled *"Potential Applications of Capsaicinoids in Small Cell Lung Cancer."* After assessing the abstract it has been deemed not to be human subject research and therefore exempt from oversight of the Marshall University Institutional Review Board (IRB). The Code of Federal Regulations (45CFR46) has set forth the criteria utilized in making this determination. Since the study does not involve human subjects as defined in DHHS regulation 45 CFR §46.102(f) it is not considered human subject research. If there are any changes to the abstract you provided then you would need to resubmit that information to the Office of Research Integrity for review and determination.

I appreciate your willingness to submit the abstract for determination. Please feel free to contact the Office of Research Integrity if you have any questions regarding future protocols that may require IRB review.

Sincerely,

Bruce F. Day, ThD, CIP
Director

APPENDIX B: JPET PERMISSIONS LETTER

January 30, 2019

Jamie Friedman
Marshall University
Biomedical Sciences
1700 Third Ave.
Huntington, WV 25701

Email: friedman4@marshall.edu

Dear Jamie Friedman:

This is to grant you permission to include the following article in your dissertation entitled "Potential applications of capsaicinoids in small cell lung cancer therapy" for Marshall University:

> JR Friedman, NA Nolan, KC Brown, SL Miles, AT Akers, KW Colclough, JM Seidler, JM Rimoldi, MA Valentovic, and P Dasgupta (2018) Anticancer Activity of Natural and Synthetic Capsaicin Analogs, *J Pharmacol Exp Ther*, 364(3):462-473; DOI: https://doi.org/10.1124/jpet.117.243691

On the first page of each copy of this article, please add the following:

> Reprinted with permission of the American Society for Pharmacology and Experimental Therapeutics. All rights reserved.

In addition, the original copyright line published with the paper must be shown on the copies included with your dissertation.

Sincerely yours,

Richard Dodenhoff
Journals Director

APPENDIX C: ABBREVIATIONS

3-MA…3-methyladenine

4-HNE…4-Hydroxynonenal

5-FU…5-fluorouracil

6-MP…6-mercaptopurine

ABC…ATP-binding cassette

AGE…advanced glycation end product

AIF…apoptosis inducing factor

ANOVA…analysis of variance

Arv…arvanil

AUC…area under the curve

Aurora A…Aurora kinase A

BAPTA-AM…(1,2-Bis(2-aminophenoxy)ethane-N,N,N',N'-tetraacetic acid tetrakis (acetoxymethyl ester)

Bcl-2…B-cell lymphoma 2

BMH…Benzo[d][1,3]dioxol-5yl-methyl hexanonate

BUN…blood urea nitrogen

Cal…calpeptin

CAM…chicken chorioallantoic membrane

CAP-NP…capsaicin-loaded folic acid nanoparticles

CAT… catalase

CB1…cannabinoid receptor 1

CCA…Human cholangiocarcinoma

CCK-8…Cell Counting Kit-8

CDK1…cyclin B-bound cdc2

CE…capsaicin epoxide

CI…combination index

CK-MB…creatinine kinase-muscle/brain

CPT…camptothecin

CPT…Camptothecin

CPZ…capsaicin

dCCA…distal cholangiocarcinoma

DHC…dihydrocapsaicin

EAC…Ehlrich ascitis carcinoma

ED_{50}…50% cytotoxic activity

ED_{75}…75% cytotoxic activity

ED_{90}… 90% cytotoxic activity

EGFR…epidermal growth factor receptor

ERK…extracellular related kinase

EVO…Evodiamine

FBS…fetal bovine serum

Gnb-CAP-NP…gefitinib-capsaicin-folic acid-based nanoparticles

Gnb-FA-NP…gefitinib-loaded nanoparticles

GSH…glutathione

H&E…hematoxylin and eosin staining

H2-DCFDA…2’ 7’ dichlorodihydrofluorescien diacetate

HCC…hepatocellular carcinoma

HEI-OC1…mouse ear organ corti cells

HMB1…high mobility group box 1

HO-1…heme oxygenase-1

HPAEpiC…Human pulmonary alveolar epithelial cells

HSP…heat shock proteins

i.p…intraperitoneal

iCCA…intrahepatic cholangiocarcinoma

IHC…immunohistochemistry

L/P ratio…liver/plasma ratio

LAC…lung adenocarcinoma

LCC…large cell carcinoma

MDA…malonaldehyde

MMP…matrix metalloproteinase

mTOR…mechanistic target of rapamycin

MTT…3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

N-AVAM…N-acylvanillamides

N-BMB…N-(benzo[d][1,3]dioxol-5-ylmethyl)-4-methoxybenzamide

NF-κB…nuclear factor kappa-B

NOX…NADPH oxidase family

NSCLC…non-small cell lung cancer

P-gp…p-glycoprotein

pCCA…perihilar cholangiocarcinoma

PCNA…proliferating cell nuclear antigen

PDGF-β…platelet-derived growth factor-β

PDX…patient derived xenograft

PhAR…phenylacetylrinvanil

PI-3K…phosphoinositol-3 kinase

pNA…p-nitroaniline

Rho123…rhodamine 123

ROPA…resiniferanol-9, 13, 14 ortho-phenylacetate

ROS…reactive oxygen species

RR…Ruthenium Red

RSK…ribosomal S6 kinase

RTX…resiniferatoxin

RUT…rutaecarpine

SCC…squamous cell carcinoma

SCLC…small cell lung cancer

SD…standard deviation

SER…sensitizer enhancement ratio

SN-38G…SN-38 Glucuronide

SOD…superoxide dismutase

SPF…Specific pathogen-free

STAT…signal transducer and activator of transcription

STR…short tandem repeat

TEM…transmission electron microscopy

TLR…toll-like receptors

TRPV… transient receptor potential vanilloid

TUNEL…Terminal deoxynucleotidyl transferase dUTP nick end labeling

TURBT…transurethral resection of bladder tumors

UN-AVAMs…unsaturated N-acylvanillamides

VEGFR…vascular endothelial growth factor receptor

APPENDIX D: VITA

Jamie Rae Friedman

(302) 893-3846 • Friedman4@marshall.edu • Jamie.Friedman29@gmail.com

EDUCATION

- **Marshall University, Huntington WV**
  - Doctor of Philosophy in Biomedical Sciences
  - Area of emphasis in Pharmacology and Toxicology
  - Expected Graduation May 2019, GPA: 3.15

- **Bethany College, Bethany WV**
  - Bachelor of Sciences in Biology, with an emphasis in Biochemistry
  - Graduated May 2015, GPA 3.4 *Cum Laude*
  - Presidents List, Deans List

Professional and Research Experience

- **Scientist I**
  - BioAgilytix Labs, LLC, Research Triangle Park, NC
  - April 2019-Present

- **Graduate Assistant**
  - Laboratory of Dr. Piyali Dasgupta, Department of Biomedical Sciences
  - Joan C. Edwards School of Medicine, Marshall University
  - *Growth inhibitory effects of synthetic capsaicin analogs on human small cell lung cancer in vitro and in vivo*

- **2014 NASA Undergraduate Research Fellowship**
  - Supervisor Dr. Jennifer Franko, WVU
  - *The effects of estrogen exposure on cell proliferation in UV exposed Epithelial Tissues.*
  - Presented at Bethany College Research Day April 2015

- **Biology Lab Proctor**
  - 2013-2015 Teaching Assistant to Dr. William Hicks and Dr. Jennifer Franko

- **Peer Reviewing**
  - Asked to peer review for *Journal of Pharmacology and Experimental Therapeutics*

PUBLICATIONS

- **Friedman, J.R.,** Richbart, S.D., Merrit, J.C., Brown, K.C., … Dasgupta, P. (2018). Acetylcholine signaling system in progression of lung cancers, *Pharmacology & Therapeutics*, ISSN 0163-7258, accepted in press.

- **Friedman, J.R.**, Brown, K.C., Miles, S.L., Nolan, N.A., …Valentovic, M.A., and Dasgupta, P. (2017). Anti-cancer Activity of Natural and Synthetic Capsaicin Analogs. *Journal of Pharmacology and Experimental Therapeutics,* 64(3):462-473.

- **Friedman, J. R.**, Perry, H.E., Brown, K.C., Gao, Y., Lin, J., Stevenson, C.D., . . . Dasgupta, P. (2017). Capsaicin synergizes with Camptothecin to induce increased apoptosis in human small cell lung cancers via the calpain pathway. *Biochem Pharmacol.* 129, 54-66.

- Hurley, J.D., Akers, A.T., **Friedman, J.R.,** Nolan, N.A., Brown, K.C. and Dasgupta, P. (2016). Non-pungent long chain capsaicin-analogs arvanil and olvanil display better anti-invasive activity than capsaicin in human small cell lung cancers**,** *Cell Adhesion and Migration,* 11(1), 80-97.

Under Review

- **Friedman, J. R.,** Richbart, S. D., Merrit, J. C., Perry, H. E.,…Valentovic, M.A., and Dasgupta, P. (2018) Capsaicinoids enhance chemosensitivity to chemotherapeutic drugs. *Advances in Cancer Research.*

PRESENTATIONS

Oral Presentations

- **Friedman, J.R.** Anti-cancer activity of non-pungent capsaicin analogs: A structure-activity study, Oncology Grand Rounds, Marshall University, Joan C. Edwards School of Medicine, May 9th, 2018.

- **Friedman, J.R.**, Gadapalli, R.S., Akers, A.T., Nolan, N.A.,…Dasgupta, P. Anti-cancer activity of non-pungent capsaicin analogs: A Structure-Activity Study, Symposium – Carcinogenesis: Models, Mechanisms, and Modulators, Experimental Biology 2018, San Diego, California, April 24th 2018.

- **Friedman, J.R.**, Nolan, N.A., Akers, A.T., Richbart, S.D.,…Dasgupta, P. Anti-metastatic activity of capsaicin in human lung adenocarcinoma, Marshall University Research Day 2018, Joan C. Edwards School of Medicine, Huntington, WV, March 30th 2018.

- **Friedman, J.R.** Non-pungent long chain capsaicin analogs, arvanil and olvanil display better anti-invasive activity than capsaicin in human small cell lung cancers, Oncology Grand Rounds, Marshall University Joan C. Edwards School of Medicine, May 11th, 2016.

Selected for Oral Presentation

- **Friedman, J. R.,** Richbart, S. D., Merrit, J. C., Brown, K. C.,…Dasgupta, P. Capsaicin and natural capsaicin-like compounds suppress metastasis in lung adenocarcinoma. Minisymposium - Mechanisms of Carcinogenesis, Experimental Biology 2019, Orlando, Florida, April 9th 2019.

Poster Presentations

- **Friedman, J.R.,** Perry, H.E., Brown, K.C., Akers, A.T., Nolan, N.A.,…Dasgupta, P. Capsaicin synergizes with camptothecin to show enhanced apoptotic activity in small cell lung cancer, Experimental Biology 2017, Chicago, Illinois, April 22-26 2017.

- **Friedman, J.R.,** Perry, H.E., Brown, K.C., Akers, A.T., Nolan, N.A.,…Dasgupta, P. Capsaicin sensitizes human small cell lung cancer cells to the pro-apoptotic activity of camptothecin, Pathobiology for Investigators, Students and Academicians (PISA), Pittsburgh, Pennsylvania, September 25-27 2017.

- **Friedman, J.R.,** Akers, A.T., Hurley, J.D., Brown, K.C., Nolan, N.A., and Dasgupta, P. Non-pungent long chain capsaicin analogs, arvanil and olvanil display better anti-invasive activity than capsaicin in human small cell lung cancers, Center for Clinical and Translational Science 2016 Spring Conference, University of Kentucky.

Selected for Poster Presentation

- **Friedman, J. R.,** Richbart, S. D., Merrit, J. C., Brown, K. C.,…Dasgupta, P. Capsaicin and natural capsaicin-like compounds suppress metastasis in lung adenocarcinoma. EB Scientific Highlights Poster Session, Experimental Biology 2019, Orlando, Florida, April 6th 2019.

HONORS

- **Best Research Performance in this Academic Year 2016-2017**
    - Graduate Student Award with a $2,000 stipend to attend a National Conference

GRANTS

- **MU-ADVANCE Path Forward 2016**
    - $500 Travel Award for female STEM students

- **Gans Fund Research Grant 2014**
    - STEM Grant of Bethany College

LEADERSHIP EXPERIENCE

- **Biomedical Sciences Graduate Student Organization at Marshall University**
    - *Historian 2016-Present*