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Capsaicin Synergizes with Camptothecin to Induce Increased Apoptosis in Human Small Cell Lung Cancers via the Calpain Pathway

Jamie R. Friedman^{1,§}, Haley E. Perry^{1,§}, Kathleen C. Brown¹, Yin Gao², Ju Lin², Cathryn D. Stevenson¹, John D. Hurley¹, Nicholas A. Nolan¹, Austin T. Akers¹, Yi Charlie Chen², Krista L. Denning³, Linda G. Brown³, and Piyali Dasgupta^{*,1}

¹Department of Biomedical Sciences, Joan C. Edwards School of Medicine, Marshall University, Huntington, WV, 25755

²Department of Biology, Alderson Broaddus University, Philippi WV, 26416

³Department of Pathology, Joan C. Edwards School of Medicine, Marshall University, Huntington, WV, 25755

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 anti-cancer 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 fosters hope for novel nutrition based combination therapies in SCLC.

Graphical abstract

* Correspondence to: Piyali Dasgupta, Department of Pharmacology, Physiology, and Toxicology, Joan C. Edwards School of Medicine, Marshall University, 1700 3rd Avenue, Huntington, WV, 25755. dasgupta@marshall.edu.

§ These authors contributed equally to the work presented here and should therefore be regarded as equivalent first authors.

The authors declare no conflict of interest

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1. 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 [1, 2]. 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 [3]. Another drawback with the cisplatin-etoposide regimen is its toxicity, which may render SCLC patients more susceptible to adverse symptoms upon subsequent treatments [4]. Patients with recurrent SCLC have very limited options, as the only standard chemotherapy with an FDA-approved drug, camptothecin (Fig. 1a), has an objective response rate of approximately 3% and little or no survival benefit [5]. Clinical trials have explored the possibility of camptothecin-based combination regimens for standard of care therapy for SCLC patients [4]. 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 [6, 7]. Our published data shows that capsaicin (the spicy compound of chili peppers; Fig. 1b) can induce robust apoptosis in human SCLC cells in cell culture and mouse models [8, 9]. 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.

A survey of literature shows that capsaicin increases the therapeutic index of several anti-cancer 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 [10]. Similarly, the treatment of stomach cancer cells with a combination of cisplatin and capsaicin caused greater than apoptosis than either of these agents given singly [11, 12]. A similar effect was also observed when capsaicin was given in combination with the doxorubicin analog pirarubicin [13]. The present manuscript investigates for the first time the anti-cancer 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 [14, 15].

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) [16]. 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.

2. Materials and Methods

2.1 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).

2.2 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 with 2 mM glutamine, 25 mM HEPES, 1mM sodium pyruvate, 4.5g/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).

2.3 Preparation of lysates

Cell lysates were made using detergent-based lysis protocol as described previously [17]. 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 [17]. The protein concentration of the lysate was measured using Bradford Reagent (Bio-Rad Laboratories, Hercules, CA, USA).

2.4 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.

2.5 Cell death ELISA assay

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 in Section 2.2), and the lysates were prepared as detailed above [17]. The protein concentration of the lysate was measured using Bradford Reagent (Bio-Rad Laboratories, Hercules, CA, USA). Twenty micrograms of lysate was 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.

2.6 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 [16]. H69 cells (1.5×10^6) were suspended in 100 μ l cold serum-free medium, mixed with 100 μ l cold BD Matrigel Matrix (BD Biosciences, San Jose, CA) and treated with 10 μ M capsaicin or 1 μ M camptothecin or a combination of 10 μ M capsaicin and 1 μ M camptothecin [18, 19]. 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.

2.7 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 [9, 18–20]. The caspase-3 activity assay was performed with four independent sets of tumor lysates prepared from control CAM H69 tumors, 10 μ M capsaicin-treated CAM H69 tumors, 1 μ M camptothecin-treated CAM H69 tumors, and H69 CAM tumors treated with a combination of 1 μ M camptothecin and 10 μ M 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.

2.8 Measurement of calpain activity

H69 human SCLC cells were treated with 10 μ M capsaicin or 1 μ M camptothecin or a combination of 10 μ M capsaicin and 1 μ M 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 μ l of calpain substrate for 60 minutes at 37°C [9, 21]. 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 wavelengths of 490 and 520 nm, respectively. Each sample was measured in duplicate and the whole experiment was repeated three times with independent sets of lysate. 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 (Section 2.6) [9, 18–20]. The tumor lysates were made as described above (Section 2.7). 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.

2.9 Statistical analysis

All data was plotted using GraphPad Prism 5 Software, Inc (La Jolla, CA, USA), and results were represented as the mean $\pm$ 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 was considered significant when $P < 0.05$.

All data involving combinatorial interactions between camptothecin and capsaicin were evaluated by the Chou-Talalay isobologram analysis using the method of non-constant ratios [14, 15]. The Chou-Talalay isobologram analysis (Calculusyn 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 [14, 15]. This method was used to examine whether camptothecin and capsaicin displayed a synergistic increase in apoptotic activity. The Chou-Talalay isobologram analysis yield 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.

3. Results

3.1 A concentration of 10 μ M capsaicin does not cause significant apoptosis ($P < 0.05$) 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 μ M and subsequently causes robust apoptosis at 50 and 100 μ M in DMS 114 human SCLC cells (Fig. 2a). The highest concentration at which capsaicin did not induce significant apoptosis ($P = 0.05$) was 10 μ M 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 (Fig. 2b and c). Our eventual goal was to test if a 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 μ M capsaicin for all our subsequent experiments.

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 μ M, and after that the levels of cell death rose significantly over 24 hours ($P = 0.05$) (Fig. 3a). The experiment was repeated in H69 and H82 cells and similar results were obtained (Fig. 3b and c). Based on the data of these two assays we selected 10 μ M capsaicin for our subsequent experiments.

3.2 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 (Fig. 4a). DMS 114 cells were treated with multiple concentrations of camptothecin (0.01 μ M -100 μ M) in the presence or absence of 10 μ M capsaicin. The 10 μ M capsaicin was added 45 minutes before the addition of camptothecin. Fig. 4a 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 (Fig 2 and 3). The Chou-Talalay analysis was used to determine if capsaicin synergized with camptothecin to induce enhanced apoptotic activity [14, 15]. 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 [14, 15]. Figure 4a shows that the maximal synergy was observed for the combination of 10 μ M capsaicin and 1 μ M camptothecin (CI=0.095). The combination of 10 μ M capsaicin and 10 μ M camptothecin was also synergistic (CI= 0.15); however, the magnitude of synergy was decreased. Fig. 4b represents the normalized isobologram of the capsaicin-camptothecin combination in DMS 114 cells [14, 15]. 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. Fig. 4b shows that treatment number 3 (10 μ M capsaicin and 1 μ M camptothecin) showed the maximal synergistic interaction.

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 μ M 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 (Fig. 4c) in DMS 114 cells over 24 hours. The values for the combination indices (CI) showed that 10 μ M capsaicin displayed the maximal synergy with 1 μ M camptothecin (CI=0.067) followed by 10 μ M capsaicin and 10 μ M camptothecin (CI=0.261) (Fig. 4c). Fig. 4d shows the corresponding normalized isobologram in DMS 114 cells. The pattern of CI in the isobologram corresponds to the Cell Death ELISA Kit (Fig. 4c). As can be observed, the combination of 10 μ M capsaicin (CPZ) and 1 μ M camptothecin (CPT) has the lowest CI out of all other combinations.

The results of these experiments were repeated in H82 human SCLC cells (Fig. 5a) and H69 human SCLC cells (Fig. 6a). Capase-3 Activity assays show that the synergistic interaction between 10 μ M capsaicin (CPZ) and 1 μ M camptothecin (CPT) is stronger in H82 than in DMS 114 and H69 cells. Fig. 5b 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 (Fig. 6b). These combinatorial apoptotic activities of camptothecin and capsaicin was proved in H82 cells (Fig. 5c) and H69 cells (Fig. 6c) 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 (Fig. 5d and 6d). Taken together, the combination of 10 μ M capsaicin (CPZ) and 1 μ M 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.

3.3 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 *in vivo*. For this purpose, we selected the chicken CAM model [16, 18, 19]. 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 [22, 23]. 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 camptothecin. An aliquot of 1.5×10^6 H69 cells were treated with 1 μ M camptothecin in the presence or absence of 10 μ M 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. Fig. 7a shows that the combination of 10 μ M capsaicin and 1 μ M camptothecin displayed significantly greater anti-tumor activity than either of these agents alone ($P = 0.05$). 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 μ M capsaicin and 1 μ M camptothecin showed significantly greater apoptosis than 10 μ M capsaicin alone or 1 μ M camptothecin alone ($P = 0.05$) (Fig. 7b). Each sample was measured in duplicate and the entire experiment was repeated three independent times.

3.5: 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 [24–27]. Similarly, our published data and that of others have also shown a role for calcium signaling pathway and calpain activation in the apoptotic effects of capsaicin [9, 28]. 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 (Fig. 8a) in H69 cells [9, 28]. The experiment was repeated in H82 cells and similar results were obtained (Fig. 8b). 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 (Fig. 8c). The experiment was repeated in a second human SCLC cell line H82, and similar results were obtained (Fig. 8d).

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 (Fig. 9a). These experiments were repeated in H82 human SCLC cells and similar results were obtained (Fig. 9b). Similarly, we observed that calpeptin suppressed the

synergistic apoptotic activity of capsaicin and camptothecin in H69 human SCLC cells (Fig. 9c), as measured by the Cell Death ELISA Kit. The assay was repeated in the human SCLC cell line H82, and similar results were obtained (Fig. 9d).

3.6 SCLC cells treated with 10 μ M capsaicin and 1 μ M 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 μ M capsaicin and 1 μ M camptothecin was confirmed by the measurement of calpain activity in H69 and H82 cells. Fig. 10a shows that the treatment of H69 human SCLC cells with 10 μ M capsaicin and 1 μ M 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 (Fig. 10a; white bars). The experiment was repeated in the variant human SCLC cell line and analogous results were obtained (Fig. 10b).

We also observed that the combination of 10 μ M capsaicin and 1 μ M 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 (Fig. 10c; white bars) [29]. The experiment was repeated in H82 cells and similar results were obtained (Fig. 10d).

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. Fig. 11 shows that the calpain activity in the H69 tumors treated with 10 μ M capsaicin and 1 μ M camptothecin is substantially higher than those treated with 10 μ M capsaicin alone and 1 μ M camptothecin alone. Our data suggests 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.

4. 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 [4, 30]. 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 [31].

Camptothecin and its related compounds are used for second line therapy for a variety of cancers including SCLC [5]. Patient-oriented studies show that camptothecin is active against brain metastases in SCLC [4, 30]. The clinic profile of camptothecin, its broad-spectrum anti-tumor activity, and its lack of cross resistance with other anti-cancer agents

has prompted clinical studies investigating the feasibility of camptothecin being used in a first-line setting for SCLC patients [4, 5, 31].

A unique feature of camptothecin is its ability to induce enhanced anti-cancer activity with multiple anti-neoplastic compounds [31, 32]. In many of these studies, the interactions between camptothecin and other cancer chemotherapeutic drugs was found to be potentially synergistic [4, 5, 31]. Although the combinatorial activity of other anti-cancer drugs (like cisplatin) have 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 [14–15]. Hormann et al. (2003) showed that the apoptotic efficacy of the camptothecin analog topotecan was increased in the presence of the flavonoid genestein [33]. However, they did not perform any statistical analysis to show whether the topotecan-genestein 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 [10–14].

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 chicken chorioallantoic membrane (CAM) constitute an established model to study tumor growth *in vivo* [34–36]. 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 [22, 23]. This is in contrast to athymic mouse models where dosages are translated to mg/kg bodyweight, 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* [37, 38]. Our previous publications have already shown that the administration of capsaicin does cause any gross discomfort, in mice [8, 9, 39]. Our published reports reveal that capsaicin displays significant bioavailability in the lungs of mice [40]. 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 superfamily of calcium-regulated intracellular cysteine proteases [41, 42] 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 [24–27]. Our published data and that of other research laboratories show that calpains are also vital regulators of capsaicin-induced apoptosis [9, 28]. 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 NF κ B [29, 43]. Several of these proteins have been shown to be downstream targets of both camptothecin- and capsaicin-induced apoptosis [44, 45]. 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.

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List of Abbreviations

SCLC	small cell lung cancer
CAM	chorioallantoic membrane
CI	combination index
STR	short tandem repeat
RPMI	Roswell Park Memorial Institute
EDTA	ethylene diamine tetraacetic acid
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
SPF	specific pathogen-free

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Fig. 1.
Structure of camptothecin (a) and capsaicin (b).

Fig. 2.

Concentration-dependent apoptotic activity of capsaicin in human SCLC cells over 24 hours, as measured by the Caspase-3 activity kit (Section 2.4). **(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 (described in Section 2.9) 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 ($P \geq 0.05$). Values represented by the same letter are not statistically significantly different from each other.

Fig. 3.

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 1.5–2.0-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 ($P > 0.05$). Values represented by the same letter are not statistically significantly different from each other.

Fig. 4.

Capsaicin (CPZ) sensitizes human SCLC cells to the apoptotic activity of camptothecin (CPT) over 24 hours. **(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 (as described in Section 2.4). The solid black line (with circular symbols) represents the concentrations of camptothecin (10 nM – 100 μM) along with 10 μM capsaicin. The data was 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 0.05). **(b)** The normalized isobologram generated from the Chou-Talalay analysis of Fig. 4a. 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 was 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 μ M camptothecin and 10 μ M capsaicin (CI=0.067) followed by 10 μ M camptothecin – 10 μ M capsaicin combination (CI=0.261) in 24 hours. **(d)** The normalized isobologram obtained after the Chou-Talalay analysis of Fig, 4c. The combination treatment 3 (1 μ M camptothecin and 10 μ M capsaicin) has the lowest CI followed by treatment #4 (10 μ M camptothecin and 10 μ M capsaicin).

Fig. 5.

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 ($P < 0.05$; depicted by solid black lines) in H82 cells. Chou-Talalay isobologram analysis demonstrate 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 ($P > 0.05$). **(b)** The normalized isobologram generated from the Chou-Talalay analysis of Fig. 5a. 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 matches the caspase-3 activity assay; the

utmost synergy was observed with a combination of 1 μ M camptothecin and 10 μ M capsaicin (CI=0.014) followed by blend of 10 μ M camptothecin – 10 μ M capsaicin (CI=0.058) in H82 cells over 24 hours. **(d)** The normalized isobologram obtained after the Chou-Talalay analysis of Fig. 5c. The combination regimen 3 (1 μ M camptothecin and 10 μ M capsaicin) has the lowest CI followed by regimen #4 (10 μ M camptothecin and 10 μ M capsaicin).

Fig. 6.

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 – 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 ($P > 0.05$). **(b)** The normalized isobologram generated from the Chou-Talalay analysis of Fig. 6a. 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 was 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 Fig. 6c. The combination treatment # 3 (1 μM

camptothecin and 10 μ M capsaicin) has the lowest CI followed by treatment condition #4 (10 μ M camptothecin and 10 μ M capsaicin).

(a)

(b)

Fig. 7.

The combination of 1 μ M camptothecin and 10 μ M 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 μ M capsaicin (as a single agent) did not significantly suppress the growth of H69 tumors xenotransplanted on chicken CAM (tumor weights = $92 \pm 18\%$ relative to control; P 0.05). The treatment of H69 tumors with 1 μ M camptothecin caused a decrease in tumor volumes (tumor volumes = $66 \pm 20.6\%$ relative to control). However, the combination of 1 μ M camptothecin and 10 μ M capsaicin decreased tumor volumes down to about $36 \pm 10\%$ 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 μ M camptothecin and 10 μ M capsaicin (13–16) displayed robust apoptotic activity which was significantly higher than any of the drugs as single agents (P 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 0.05).

Fig. 8.

The combinatorial apoptotic activity of 1 μ M camptothecin and 10 μ M capsaicin was mediated by intracellular calcium and the calpain pathway. **(a)** Caspase-3 activity assays indicate that the presence of 10 μ M 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 μ M calpeptin suppressed the synergistic apoptotic activity of 1 μ M camptothecin and 10 μ M 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 0.05).

Fig. 9.

H69 human SCLC cells were treated with 1 μ M camptothecin, 10 μ M capsaicin or a combination of 1 μ M camptothecin and 10 μ M capsaicin in the presence or absence of 10 μ M BAPTA-AM for 24 hours. 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 μ M camptothecin and 10 μ M 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 0.05).

Fig. 10.

The combination of 1 μ M camptothecin and 10 μ M capsaicin potently stimulates calpain activity in human SCLC cells. **(a)** H69 human SCLC cells treated with 1 μ M camptothecin or 10 μ M capsaicin or a combination of both for 24 hours. Cell lysates were made and calpain activity was measured. The combination of 1 μ M camptothecin and 10 μ M 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 μ M camptothecin and 10 μ M 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 μ M camptothecin and 10 μ M 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 ($P < 0.05$).

Fig. 11.

Elevation of calpain activity in H69 tumors treated with a combination of 1 μ M camptothecin and 10 μ M capsaicin. Four independent tumor lysates were used for the assay for each sample. Calpain activity assays show that 10 μ M 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 μ M camptothecin and 10 μ M capsaicin (13–16) displayed a greater magnitude of increase in calpain activity, relative of the drugs as single agents (P 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 0.05).