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The Novel Capsazepine Analog, CIDD-99, Significantly Inhibits Oral Squamous Cell Carcinoma *In Vivo* Through a TRPV1-Independent Induction of ER Stress, Mitochondrial Dysfunction, and Apoptosis

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Abstract

Background: Oral squamous cell carcinoma (OSCC) is a deadly disease with a mere 40% five-year survival rate for patients with advanced disease. Previously we discovered that capsazepine (CPZ), a transient receptor potential channel, Vanilloid subtype 1 (TRPV1) antagonist, has significant anti-tumor effects against OSCC via a unique mechanism-of-action that is independent of TRPV1. Thus we developed novel CPZ analogs with more potent anti-proliferative effects (CIDD-24, -99, and -111).

Methods: Using OSCC xenograft models, we determined the efficacy of these analogs *in vivo*. TRPV1 interactions were evaluated using calcium imaging and a rat model of orofacial pain. Anti-cancer mechanism(s)-of-action were assessed by cell cycle analysis and mitochondrial depolarization assays.

Results: CIDD-99 was the most potent analog demonstrating significant anti-tumor effects *in vivo* ($p < 0.001$). CIDD-24 was equipotent to the parent compound CPZ, but less potent than CIDD-99. CIDD-111 was the least efficacious analog. Calcium imaging studies confirmed that

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Conflicts of Interest

Dr. Cara Gonzales and Dr. Stanton McHardy have a patent pending for this technology. All other authors have no conflict of interest.

CIDD-99 neither activates nor inhibits TRPV1 confirming that TRPV1 activity is not involved in its anti-cancer effects. All analogs induced an S-phase block, dose-dependent mitochondrial depolarization, and apoptosis. Histological analyses revealed increased apoptosis and reduced cell proliferation in tumors treated with these analogs. Importantly, CIDD-99 had the most dramatic anti-tumor effects with 85% of tumors resolving leaving only minute traces of viable tissue. Additionally, CIDD-99 was non-noxious and demonstrated no observable adverse reactions

Conclusion: This study describes a novel, highly efficacious, CPZ analog, CIDD-99, with dramatic anti-tumor effects against OSCC that may be efficacious as a lone therapy or in combination with standard therapies.

Keywords

oral cancer; capsazepine analog; mitochondria

Introduction

Head and neck squamous cell carcinoma (HNSCC) is an insidious disease resulting in over 50,000 new cases and 10,000 deaths in the United States annually (1, 2). Greater than 50% of all HNSCC patients will die within five years of their diagnosis (1). This is due to late-detection and consequent advanced disease, which is resistant to conventional treatments. Notably, 75% of new patients have advanced disease at the time of diagnosis (3–5). In addition, the majority of tumors (60%) are found in the oral cavity (OSCC); the site with the highest recurrence rates and a median survival of a mere 6 months (3–7). Hence there is a great need to develop new methods of treatment for OSCC.

Previously, we identified a novel anti-cancer function of Vanilloids in which both capsaicin (TRPV1 agonist) and its synthetic analog CPZ (TRPV1 antagonist) have anti-proliferative effects against multiple OSCC cell lines (8). We also demonstrated significant anti-tumor effects of CPZ *in vivo* using three OSCC xenograft models, which yielded no adverse effects on non-malignant tissues and no liver or kidney toxicities. Interestingly, we showed that the anti-cancer effects of CPZ are through a second mechanism-of-action that does not involve TRPV1; rather, induction of reactive oxygen species (ROS) and apoptosis (8). Based upon these findings, we generated potent CPZ analogs (30 total) with significantly greater anti-proliferative effects *in vitro* (9). Lead compounds CIDD-24 (compound 17) and CIDD-111 (compound 29) displayed significant anti-tumor effects *in vivo*, which were more potent than the parent compound CPZ. We also confirmed that these lead compounds are efficacious against multiple cancer types including OSCC. Here we describe the efficacy of CIDD-24, CIDD-111, and the novel, more potent analog, CIDD-99, against a panel of OSCC cell lines and validate their anti-tumor effects *in vivo*. Mechanisms-of-action are investigated using calcium imaging, behavior studies, and mitochondrial function testing.

Materials and Methods

Reagents

Working stocks of CPZ and analogs were made fresh by diluting each compound in 100% EtOH to a final stock concentration of 100mM.

Cell Lines

Cal-27, SCC-4, SCC-9, HeLa, H460, MDA-231, and PC-3 cells were obtained from ATCC (Manassas, VA, USA). HSC-3 cells were provided by Dr. Brian Schmidt at New York University College of Dentistry. Cell lines were authenticated by Genetica DNA Laboratories (Cincinnati, OH, USA) and maintained in DMEM supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin and incubated at 37°C in 5% CO₂. OKF6-TERT-2 cells were obtained from Harvard Medical School Cell Culture Core Collection, Cambridge, MA and cultured as previously reported (10). CHO cells overexpressing TRPV1 (CHO-TRPV1) were provided by Dr. Ardem Patapoutian at the Scripps Research Institute and cultured as described (11).

MTS Cell Viability Assays

Cell viability was determined using the Cell Titer 96 @ Aqueous Non-Radioactive Cell Proliferation Assay (Promega, Madison, WI, USA) as described (8, 12). Cells were plated and treated for 24h with serum free DMEM containing CPZ analogs at the indicated concentrations. Pre-treatments with N-acetyl-cysteine (NAC; 10mM) were performed for 30 min followed by treatment with NAC and CPZ analogs at the indicated times and concentrations. Final EtOH concentrations were maintained at 0.1%. Test groups were compared to vehicle-treated controls (n=4 per group).

Animal Studies

All animal studies followed the international guidelines on animal welfare in accordance with the National Institutes of Health guide for the care and use of laboratory animals and complied with the ARRIVE guidelines and the 2013 AVMA euthanasia guidelines. All studies were approved by the University of Texas Health San Antonio (UTHSA) Institutional Animal Care and Use Committee. Six week-old female athymic nude mice (Harlan, Indianapolis, IN, USA) were used in a laminar air-flow cabinet under pathogen-free conditions for anti-tumor studies. Six-week old male Sprague-Dawley rats, weighing approximately 300g (Envigo, Laboratories, Indianapolis, IN, USA) were used for behavioral studies. Mice and rats were provided with a 12h light/12h dark schedule at controlled temperature and humidity with food and water ad libitum and were acclimated for one week prior to study initiation.

Anti-tumor Studies

Mice were injected subcutaneously in the flank with 3×10^6 Cal-27 cells as previously described (8, 12). When tumors grew to 100mm³, mice were stratified into five experimental groups (n=5 per group) that received the following treatments via intra-tumor injection every other day for four weeks: vehicle control (100 µl of 0.25% EtOH diluted in sterile saline), 120µg of CPZ, CIDD-24, CIDD-99, or CIDD-111 diluted in 100µl sterile saline (final concentration of 0.25% EtOH). In a separate experiment, mice were inoculated as described, but treated with 240µg (12mg/kg) of CIDD-99 by intra-peritoneal (IP) injection or vehicle control every other day (n=10 per group) for three weeks. Mice were monitored daily for tumor growth (using digital calipers) and cachexia. Tumor volumes were calculated by the elliptical formula: $1/2(\text{length} \times \text{width}^2)$ (8). At experimental conclusion, mice were

euthanized and tumors fixed and processed for histological analysis. Hematoxylin and eosin (H&E) staining was performed by the UTHSA South Texas Reference Laboratory Histopathological Core facility.

Eye-Wipe Testing

Rats were placed in a temperature-controlled (22–25°C) behavioral laboratory in individual mirrored testing boxes (30×30×30 cm) in which they were allowed to acclimate for at least 1h. One drop (40μl) of a solution of 0.01% (w/v) capsaicin, CIDD-24, CIDD-99, or CIDD-111 in sterile saline, was instilled onto one eye of each freely moving animal (n=6 per group), as described (14,15). Time spent grooming or closing the affected eye was recorded for a total of 4 min, with observers blinded to the treatment allocation groups.

Calcium Imaging Studies

Calcium imaging was performed using FLIPR Calcium 6 Evaluation Kit (Molecular Devices, San Jose, CA, USA) according to the manufacturer's protocol and as previously described (9). CHO-TRPV1 cells (9×10^3) were seeded in a 384 well plate, loaded with Calcium 6 dye for 2h and the experiment run on Pherastar FS multimode plate reader (BMG Labtech, Cary, NC, USA) testing $1 \mu\text{M}$ of CIDD-24, CIDD-99, and CIDD-111 compared to 100nM capsaicin (positive control) and $1 \mu\text{M}$ CPZ (negative control; $n=3$ per group). Effects on calcium influx were measured by changes in relative fluorescent units (535nm). Additionally, cells were pre-treated for 10 min with $1 \mu\text{M}$ CPZ or CIDD-99 followed by 100nM capsaicin to determine potential TRPV1 inhibitory effects of CPZ analogs.

Cell Cycle Distribution

Cal-27 cells were cultured to 50% confluency, treated with 10μM CPZ, CIDD-24, CIDD-99, or CIDD-111 for 24h, harvested, and fixed in 70% EtOH followed by treatment with RNase A and propidium iodide staining. FACS analysis of DNA profiles of the percentage of cells in G1, S, and G2/M was performed.

JC-1 Assay

JC-1 assays were performed as previously described (13). Cells were treated for 2h with analogs or vehicle control (n=3 per group) at the indicated concentrations and stained with 1μM JC-1 dye (Thermo Fisher Scientific, Waltham, MA) for 15 min at 37°C. Cells were then washed and examined by FACS analysis.

Western Blot Analysis

Cal-27 cells treated for 24h or 48h with vehicle, CIDD-24, CIDD-99, and CIDD-111 at the indicated concentrations were harvested and lysed in Laemmli Lysis Buffer. Cell lysates (40μg) were separated using electrophoresis in 10% SDS-PAGE, separated proteins were transferred to PVDF membrane, and membrane blocked in 5% milk solution. Rabbit polyclonal antibodies (Cell Signaling, Danvers, MA, USA) against cPARP (#5625S; 1:1250), Bip (#3177S; 1:1000), and CHOP (#2895T; 1:1000) and rabbit monoclonal anti- α / β -tubulin antibody (Cell Signaling, #CS2148; 1:1,000) were diluted in 6ml diluent (1% milk in PBS-0.1% Tw-20) and incubated overnight at 4°C (12). Membranes were washed with

PBS-Tw-20, incubated with ECL Plus detection solution (GE Healthcare, South San Francisco, CA, USA) for 1 min, and signal was detected by radiographic exposure for 30 seconds.

Statistical Analyses

Statistical analyses were performed using GraphPad Prism4 (San Diego, CA, USA). Tumor growth was analyzed by two-way ANOVA with repeated measures and Bonferroni's post-hoc tests. Cell viability tests with and without NAC were evaluated by student's t-test. Calcium imaging, behavioral testing, and JC-1 results were analyzed by one-way ANOVA and Dunnett's multiple comparison post-tests. A p value less than 0.05 was considered statistically significant.

Results

Novel CPZ analogs CIDD-24, CIDD-99, and CIDD-111 have anti-proliferative effects against OSCC cell lines *in vitro*.

We previously generated two lead CPZ analogs, CIDD-24 and CIDD-111, which were significantly more potent than CPZ in HeLa-derived xenografts and efficacious against multiple cancer cell lines including H460 (non-small cell lung cancer; NSCLC), MDA-231 (breast cancer), PC-3 (prostate cancer), HeLa (cervical cancer) and HSC-3 (OSCC) (9). The present study confirms the anti-proliferative effects of an additional lead compound, CIDD-99, along with CIDD-24 and CIDD-111 against multiple OSCC cell lines including Cal-27, HSC-3, SCC-4, and SCC-9 cells (Figures 1A-D). The chemical structures of these compounds are depicted in Figure 1E-G. CIDD-99 was the most potent with a GC_{50s} ranging from 1–10 μ M depending on the cell line tested, followed by CIDD-111 whose GC_{50s} ranged from 2.5–40 μ M, and CIDD-24 whose GC_{50s} ranged from 20–30 μ M. Given that analogs CIDD-24 and CIDD-111 have anti-proliferative effects against numerous cancer types, we confirmed that CIDD-99 is cytotoxic against the same panel of cancer cell lines with GC_{50s} ranging from 1–7.5 μ M (Figure 1H).

Anti-tumor effects of lead analogs CIDD-24, CIDD-99, and CIDD-111 against Cal-27-derived mouse xenografts.

To determine the efficacy of these analogs against OSCC tumor growth *in vivo*, we generated Cal-27-xenografts that were treated every other day via intra-tumor injection. Similar to the effects seen in SCC-4 xenografts (8), CPZ induced a 50% reduction in tumor volume by day 28 compared to vehicle control ($p < 0.001$; Figure 2A). CIDD-99 was the most efficacious analog tested and significantly reduced tumor volumes by day 18 with an overall 75% reduction by day 28 ($p < 0.01$; Figure 2A). CIDD-24 was the next most effective analog displaying a significant reduction in tumor volumes by day 24; approximately 60% ($p < 0.05$; Figure 2A). CIDD-111 proved least efficacious with a slight, but significant reduction in tumor volumes on day 28 ($p < 0.01$; Figure 2A). At experimental conclusion, the average tumor volumes were 541 mm^3 (vehicle control), 269 mm^3 (CPZ), 211 mm^3 (CIDD-24), 134 mm^3 (CIDD-99), and 333 mm^3 (CIDD-111; Figure 2B). There were no observable adverse effects on adjacent non-malignant tissues (e.g. erythema, swelling, ulceration; Figure 2C). We also confirmed that our most efficacious compound, CIDD-99, had no

cytotoxic effects on non-malignant OKF6-TERT-2 keratinocytes at the Cal-27 GC₅₀ (5μM) *in vitro*, although higher concentrations did induce a slight, but insignificant reduction in cell viability (Figure 2D). Lastly, no changes in body weight and neurological function were noted with any of the compounds tested. On one occasion, CIDD-24 induced respiratory distress. Notably, the animal quickly recovered with no further incidences.

We validated efficacy of the lead compound CIDD-99 by systemic administration via IP injections. This method-of-delivery proved to have no observable adverse effects while maintaining excellent anti-tumor efficacy with some of the tumors completely resolving ($p<0.001$; Figure 3A). At experimental conclusion, the average tumor volumes were 480mm³ (vehicle control) and 65.6mm³ (CIDD-99; Figure 3B). Again, no changes in body weight, motor function, and respiration were noted.

Histological examination of tumor specimens treated by intra-tumor injection revealed that vehicle control and CPZ-treated specimens were large with central necrotic cores surrounded by wide bands of viable tumor cells (Figure 3C). Islands of cancer cells were also found throughout the necrotic cores (Figure 3C). Tumors treated with CIDD-24 and CIDD-99 were much smaller in size and maintained a lesser periphery of viable tumor cells with primarily necrotic cores (Figures 3D and 3E). These necrotic cores also contained large inflammatory infiltrates, not seen in vehicle control tumors. The most potent compound, CIDD-99, yielded the smallest tumor volumes and in some cases there were no tumor cells remaining (Figure 3E, left panel). Nearly all of the tumors from animals treated by IP injection of CIDD-99 either completely resolved or were too small to analyze histologically. Representative photographs of IP-treated control tumors are shown in Figure 3F (top panel) and of CIDD-99 IP-treated tumors (Figure 3F, bottom panel).

CIDD-24 is a TRPV1 agonist whereas CIDD-99 and CIDD-111 have no effect on TRPV1 mediated calcium influx *in vitro* and no effect on nocifensive behaviors in a rat model of orofacial pain.

In order to assess if these lead analogs mediate TRPV1 activity, we performed calcium imaging analysis of CHO-TRPV1 cells. These studies revealed a significant influx of calcium in response to CIDD-24 compared to capsaicin ($p<0.001$) whereas CIDD-99 and CIDD-111 failed to induce calcium influx (Figure 4A). Using a rat model for orofacial pain, we confirmed that, like capsaicin, CIDD-24 significantly stimulates nocifensive behaviors (Figure 4B; $p<0.01$) whereas CIDD-99 and CIDD-111 do not. These *in vivo* results recapitulate the TRPV1 activity seen *in vitro*. To determine if CIDD-99 inhibits TRPV1 activity, CHO-TRPV1 cells were pre-treated with CIDD-99 or CPZ (control) followed by capsaicin. We previously demonstrated that CIDD-111 does not inhibit TRPV1 (9). Likewise, CIDD-99 failed to inhibit capsaicin-induced calcium influx (Figure 4C) indicating that CIDD-99 does not inhibit TRPV1 activity while maintaining its significant anti-tumor efficacy.

CPZ analogs CIDD-24, CIDD-99, and CIDD-111 induce a pronounced S-phase block, mitochondrial dysfunction, ER stress, and apoptosis.

In an effort to determine the potential mechanisms-of-action, we performed cell cycle distribution analysis of Cal-27 cells treated with lead analogs compared to the parent compound CPZ. All analogs induce an S-phase block as does CPZ (Figure 2 5A-5B). Western blot analysis showed that these analogs induce a concentration-dependent increase in apoptosis as measured by cleaved PARP (c-PARP; Figure 5C). Furthermore, co-treatment of Cal-27 cells with the anti-oxidant NAC reversed the anti-proliferative effects of CIDD-24, CIDD-99, and CIDD-111 suggesting that ROS are mediating apoptosis (Figure 5D).

Lastly, we evaluated the effects of these analogs on mitochondrial function as measured by mitochondrial transmembrane potential using JC-1 staining. All lead compounds induced an apparent increase in the aggregate to monomer ratio at lower concentrations indicative of hyperpolarization of the mitochondria; however a dramatic depolarization of the mitochondrial transmembrane potential was seen at 50–100µM with all three analogs and the parent compound, CPZ (Figure 6A-6D). Endoplasmic reticulum (ER) stress is shown to disturb the accuracy of dyes used to analyze membrane polarization due to the large amount of Ca²⁺ released into the cytosol (14). This interference gives the impression that the membrane is hyperpolarized due to changes in mitochondrial pH when in fact ER released Ca²⁺ is causing this hyperpolarization. To confirm this, we performed western blot analysis of ER stress markers, BiP and CHOP. BiP is constitutively active, but upregulated under conditions of ER stress, whereas CHOP is only expressed under ER stress (15). Indeed, we confirmed BiP and CHOP expression are upregulated with these treatments (Figure 6E).

CIDD-99 sensitizes Cal-27 cells to cisplatin, gefitinib, and radiation in vitro.

In order to determine the potential efficacy of CIDD-99 as an adjuvant therapy, we conducted cell viability assays of combination treatments of CIDD-99 with cisplatin, gefitinib (EGFR inhibitor), and radiation *in vitro*. We determined that very low concentrations of CIDD-99 (500nM) caused significant synergistic anti-proliferative effects against Cal-27 cells when used in combination with 25µM cisplatin, 100nM gefitinib, and increasing doses of radiation; 0–10 Gray (Figures 6F and 6G).

Discussion

We previously reported the significant anti-tumor effects of CPZ using three OSCC xenograft models (8). We confirmed that the mechanism-of-action was independent of its TRPV1 interactions and involved induction of ROS and subsequent apoptosis. The present studies demonstrate significant anti-tumor efficacy of novel CPZ analogs CIDD-24, CIDD-99, and CIDD-111 against OSCC *in vitro* and *in vivo*. CIDD-99 proved to be the most potent of the three analogs *in vitro* and in OSCC xenograft models. We also demonstrate significant anti-tumor efficacy of CIDD-99 when administered systemically. Importantly, no observable adverse side effects were noted with CIDD-99. Maintaining the anti-cancer effects while eliminating the TRPV1 activity is critical to further develop CPZ analogs as cancer therapeutics. This was illustrated by CIDD-24, which induced respiratory distress on one occasion. We attribute this adverse effect to its ability to activate TRPV1,

which plays a role in regulating airway function under normal conditions and in diseases such as asthma and chronic cough (16). Behavioral studies confirmed that CIDD-24 induces nocifensive behaviors consistent with TRPV1 activation. In addition to mediating pain and respiration, TRPV1 plays a role in thermoregulation; thus compounds that activate TRPV1 also induce hypothermia whereas TRPV1 inhibitors induce hyperthermia (17). Consequently, early clinical trials evaluating TRPV1 inhibitors to treat pain were closed due to severe hyperthermia (17–20). CIDD-99 proved to be the most potent analog with no TRPV1 interactions and confirmed to be non-noxious in rat models of orofacial pain, making it an excellent candidate for further development. CIDD-111 was the least efficacious analog; most likely due to its poor solubility.

Numerous studies describe the anti-proliferative effects of Vanilloids leading to apoptosis via a TRPV1-independent mechanism (21–24). This is postulated to be due to Vanilloids' structural similarity to Coenzyme-Q (Co-Q), high lipophilicity, and poor redox potential, which together enable Vanilloids to freely cross the plasma membrane and enter the mitochondria where they compete with Co-Q and inhibit electron transport (22). This results in increasingly high levels of ROS and ensuing apoptosis (21, 22). Our previous studies of CPZ in multiple OSCC cell lines and xenograft models confirmed ROS induction and apoptosis *in vitro* and *in vivo*. In the present study, the antioxidant NAC reversed the cytotoxicity of CPZ analogs indicating that ROS are also key players in their anti-proliferative effects. Furthermore, this study confirms that both CPZ and its analogs induce mitochondrial dysfunction as measured by a dramatic depolarization of the mitochondrial transmembrane potential at higher concentrations. However, the seeming hyperpolarization of the mitochondrial transmembrane potential at lower concentrations implicates ER stress as an additional regulator of apoptosis (14). Indeed western blot analyses confirmed that lower concentrations of these analogs induce ER stress as measured by upregulation of CHOP and BiP. Therefore, we propose that the anti-cancer mechanisms-of-action of these analogs include disruption of electron transport and induction of ROS, which leads to ER stress and further ROS generation. This cycle of mitochondrial dysfunction, ER stress, and increasing ROS levels, ultimately results in apoptosis. Similar to our findings, numerous ROS-inducing natural compounds have been developed to target a variety of cancers, which are also shown to induce S-phase arrest due to oxidative stress and DNA damage from high levels of free radicals (25–27). These effects appear to be cancer-specific due to existing oxidative stress. Likewise, our study shows no adverse effects on non-malignant tissues surrounding OSCC xenografts treated by intra-tumor injection and no cytotoxic effects on OKF6-TERT-2 keratinocytes *in vitro*.

OSCC patients with advanced or recurrent disease have no effective therapies available to them and a mean overall survival with recurrence is only six months. Our studies indicate that CIDD-99 is efficacious as a single therapy; however we also demonstrate synergistic effects with standard treatments including cisplatin, gefitinib, and radiation. Therefore, CIDD-99 may prove useful as an adjuvant therapy as well. Taken together, we demonstrate that CIDD-99 is a novel, potent, anti-cancer agent, which may be efficacious for advanced or recurrent OSCC that warrants further study.

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References

1. Cancer Facts and Figures In. American Cancer Society, 2015.
2. Cancer Facts and Figures 2018 In., 2018.
3. BASELGA J, TRIGO JM, BOURHIS J et al. Phase II multicenter study of the antiepidermal growth factor receptor monoclonal antibody cetuximab in combination with platinum-based chemotherapy in patients with platinum-refractory metastatic and/or recurrent squamous cell carcinoma of the head and neck. *J Clin Oncol* 2005; 23: 5568–77. [PubMed: 16009950]
4. CHANG JH, WU CC, YUAN KS et al. Locoregionally recurrent head and neck squamous cell carcinoma: incidence, survival, prognostic factors, and treatment outcomes. *Oncotarget* 2017; 8: 55600–55612. [PubMed: 28903447]
5. LEON X, HITT R, CONSTENLA M et al. A retrospective analysis of the outcome of patients with recurrent and/or metastatic squamous cell carcinoma of the head and neck refractory to a platinum-based chemotherapy. *Clin Oncol (R Coll Radiol)* 2005; 17: 418–24. [PubMed: 16149284]
6. TAM S, ARASLANOVA R, LOW TH et al. Estimating Survival After Salvage Surgery for Recurrent Oral Cavity Cancer. *JAMA Otolaryngol Head Neck Surg* 2017.
7. VERMORKEN JB, STOHLMACHER-WILLIAMS J, DAVIDENKO I et al. Cisplatin and fluorouracil with or without panitumumab in patients with recurrent or metastatic squamous-cell carcinoma of the head and neck (SPECTRUM): an open-label phase 3 randomised trial. *Lancet Oncol* 2013; 14: 697–710. [PubMed: 23746666]
8. GONZALES CB, KIRMA NB, DE LA CHAPA JJ et al. Vanilloids induce oral cancer apoptosis independent of TRPV1. *Oral Oncol* 2014; 50: 437–47. [PubMed: 24434067]
9. DE LA CHAPA JJ, VALDEZ M, RUIZ F et al. Synthesis and SAR of novel capsazepine analogs with significant anti-cancer effects in multiple cancer types. *Bioorganic & Medicinal Chemistry* 2018.
10. RUPAREL S, BENDELE M, WALLACE A et al. Released lipids regulate transient receptor potential channel (TRP)-dependent oral cancer pain. *Mol Pain* 2015; 11: 30. [PubMed: 26007300]
11. ROONEY L, VIDAL A, D'SOUZA AM et al. Discovery, optimization, and biological evaluation of 5-(2-(trifluoromethyl)phenyl)indazoles as a novel class of transient receptor potential A1 (TRPA1) antagonists. *J Med Chem* 2014; 57: 5129–40. [PubMed: 24884675]
12. GONZALES CB, DE LA CHAPA JJ, SAIKUMAR P et al. Co-targeting ALK and EGFR parallel signaling in oral squamous cell carcinoma. *Oral Oncol* 2016; 59: 12–9. [PubMed: 27424178]
13. DE LA CHAPA JJ, SINGHA PK, LEE DR et al. Thymol inhibits oral squamous cell carcinoma growth via mitochondria-mediated apoptosis. *J Oral Pathol Med* 2018.
14. PERRY SW, NORMAN JP, BARBIERI J et al. Mitochondrial membrane potential probes and the proton gradient: a practical usage guide. *Biotechniques* 2011; 50: 98–115. [PubMed: 21486251]
15. ZEESHAN HM, LEE GH, KIM HR et al. Endoplasmic Reticulum Stress and Associated ROS. *Int J Mol Sci* 2016; 17: 327. [PubMed: 26950115]
16. JIA Y, LEE LY Role of TRPV receptors in respiratory diseases. *Biochim Biophys Acta* 2007; 1772: 915–27. [PubMed: 17346945]
17. SZOLCSANYI J Effect of capsaicin on thermoregulation: an update with new aspects. *Temperature (Austin)* 2015; 2: 277–96. [PubMed: 27227029]
18. STEINER AA, TUREK VF, ALMEIDA MC et al. Nonthermal activation of transient receptor potential vanilloid-1 channels in abdominal viscera tonically inhibits autonomic cold-defense effectors. *J Neurosci* 2007; 27: 7459–68. [PubMed: 17626206]

19. GAVVA NR Body-temperature maintenance as the predominant function of the vanilloid receptor TRPV1. *Trends Pharmacol Sci* 2008; 29: 550–7. [PubMed: 18805596]
20. MCGARAUGHTY S, SEGRETÍ JA, FRYER RM et al. Antagonism of TRPV1 receptors indirectly modulates activity of thermoregulatory neurons in the medial preoptic area of rats. *Brain Res* 2009; 1268: 58–67. [PubMed: 19236852]
21. HAIL N JR. Mechanisms of vanilloid-induced apoptosis. *Apoptosis* 2003; 8: 251–62. [PubMed: 12766485]
22. ZIGLIOLI F, FRATTINI A, MAESTRONI U et al. Vanilloid-mediated apoptosis in prostate cancer cells through a TRPV-1 dependent and a TRPV-1-independent mechanism. *Acta Biomed* 2009; 80: 13–20. [PubMed: 19705615]
23. ATHANASIOU A, SMITH PA, VAKILPOUR S et al. Vanilloid receptor agonists and antagonists are mitochondrial inhibitors: how vanilloids cause non-vanilloid receptor mediated cell death. *Biochem Biophys Res Commun* 2007; 354: 50–5. [PubMed: 17214968]
24. PECZE L, JOSVAY K, BLUM W et al. Activation of endogenous TRPV1 fails to induce overstimulation-based cytotoxicity in breast and prostate cancer cells but not in pain-sensing neurons. *Biochim Biophys Acta* 2016; 1863: 2054–64. [PubMed: 27180305]
25. YANG S, ZHANG Y, LUO Y et al. Hinokiflavone induces apoptosis in melanoma cells through the ROS-mitochondrial apoptotic pathway and impairs cell migration and invasion. *Biomed Pharmacother* 2018; 103: 101–110. [PubMed: 29635122]
26. PREYA UH, LEE KT, KIM NJ et al. The natural terthiophene alpha-terthienylmethanol induces S phase cell cycle arrest of human ovarian cancer cells via the generation of ROS stress. *Chem Biol Interact* 2017; 272: 72–79. [PubMed: 28506552]
27. XU Z, ZHANG F, BAI C et al. Sophoridine induces apoptosis and S phase arrest via ROS-dependent JNK and ERK activation in human pancreatic cancer cells. *J Exp Clin Cancer Res* 2017; 36: 124. [PubMed: 28893319]

Panel E-G: Chemical structures of CIDD-24, CIDD-99, and CIDD-111. **Panel H:** MTS cell viability assay using a panel of solid tumor types (HeLa, H460, MDA-231, and PC-3) treated with CIDD-99 for 24h (n=4 per group). Data is presented as mean $\pm$ standard deviation (SD).

Figure 2. Effects of intra-tumor injection of CPZ analogs on growth of Cal-27 xenografts and normal epithelium.

Panel A: Tumor volumes (mean $\pm$ SD) of Cal-27-derived xenografts treated by intra-tumor injection of 120 μ g of CPZ, CIDD-24, CIDD-99, and CIDD-111 every other day for 28 days. Significant reduction in tumor volumes is seen by day 18 for CIDD-99, day 24 for CIDD-24, day 26 for CPZ, and day 28 for CIDD-111 (n=5 per group; *p<0.05, **p<0.01, ***p<0.001). **Panel B:** Scatter plot of Cal-27-derived tumor volumes on day 28. Mean volume of vehicle control treated tumors is 541 mm³ whereas mean tumor volumes of CPZ, CIDD-24, CIDD-99, and CIDD-111 treated tumors are 269 mm³, 211 mm³, 134 mm³, and 333 mm³ respectively (n=5 per group). **Panel C:** Representative photograph of Cal-27-derived xenograft treated by intra-tumor injection with CIDD-99 (120 μ g) every other day for 28 days. Arrow indicates healthy adjacent non-malignant epithelium with no erythema, ulceration, or swelling. **Panel D:** Cell viability assay of OKF6-TERT-2 keratinocytes treated with CIDD-99 for 24h (n=4 per group).

Figure 3. Effects of systemic administration of CPZ analogs on growth of Cal-27 xenografts and histological changes.

Panel A: Tumor volumes (mean $\pm$ SD) of Cal-27-derived xenografts treated by IP injections of 12 mg/kg CIDD-99 every other day for 22 days. Significant reduction in tumor volumes is seen by day 6 and throughout the remainder of the study (n=10 per group; *p<0.05, **p<0.01, ***p<0.001). **Panel B:** Scatter plot of Cal-27-derived tumor volumes on day 22. Mean volume of vehicle control treated tumors is 480 mm³ and CIDD-99 treated tumors is 65.6 mm³ (n=10 per group). **Panels C-E:** Representative photomicrographs of H & E stained tumors treated by intra-tumor injection; top row, 4x magnification (scale bar = 500 μ M) and bottom row, 10x magnification of area outlined in 4x above (scale bar = 250 μ M). **Panel C:** Representative photomicrographs of H & E stained tumors treated with vehicle control (left panels) and CPZ (right panels). Wide bands of viable tumor cells line the periphery of the tumors with islands of tumor cells within the necrotic cores. Tumor core is indicated by asterisk (**). **Panel D:** Representative photomicrographs of H & E stained tumors treated

with CIDD-24 (left panels) and CIDD-111 (right panels). Narrower bands of tumor cells line the periphery of the tumors with large necrotic cores (indicated by asterisk; **) and inflammatory infiltrates. Panel E: Representative photomicrographs of H & E stained tumors treated with CIDD-99. CIDD-99-1 (left panels) is representative of four of the five tumors treated with CIDD-99 and demonstrates the extremely small size of the remaining tissue, which is devoid of viable tumor cells, has a large inflammatory infiltrate, and is necrosed. CIDD-99-2 (right panels) demonstrates the largest of the tumors treated with CIDD-99 via intra-tumor injection, which does have some viable tumor cells along the periphery, a large necrotic core (**), and a large inflammatory infiltrate. Panel F: Representative photograph of tumors treated by IP injection of vehicle control (top row) or 12mg/kg CIDD-99 (bottom row), which demonstrates the dramatic reduction in tumor volume following 22 days of treatment with CIDD-99.

Figure 4. Evaluation CPZ analogs' TRPV1 interactions *in vitro* and *in vivo*.

Panel A: Calcium imaging of CHO-TRPV1 cells treated with CIDD-24, CIDD-99, and CIDD-111 (1 μ M), or capsaicin positive control (100 nM). Calcium influx as measured by mean relative fluorescent units (RFU) $\pm$ SD is depicted. **Panel B:** Eye-wipe testing in response to 0.01% w/v of CIDD-24, CIDD-99, and CIDD-111 vs. capsaicin (n=6 per group). Nocifensive behaviors measured in seconds (mean $\pm$ SD) is depicted. **Panel C:** CHO-TRPV1 cells treated with CIDD-99 or CPZ (1 μ M) alone or pre-treated with CIDD-99 or CPZ (1 μ M), followed by capsaicin (100 nM). Calcium influx as measured by mean RFU $\pm$ SD is depicted; **p<0.01, ***p<0.001.

Figure 5. Effects of CPZ analogs on cell cycle distribution and apoptosis.

Panel A: Cell cycle distribution of Cal-27 cells treated with 10 μ M CIDD-24, CIDD-99, and CIDD-111 for 24h. **Panel B:** Quantification of cell cycle distribution (% cells) in G1, S, and G2 phase following treatment with vehicle control, CPZ (30 μ M), or 10 μ M CIDD-24, CIDD-99, and CIDD-111 for 24 h. **Panel C:** Western blot of c-PARP in Cal-27 cells treated with increasing concentrations of CIDD-24, CIDD-99, and CIDD-111 for 48h. **Panel D:** MTS assay of Cal-27 cells treated with CIDD-24, CIDD-99, and CIDD-111 (+/-) NAC (10mM) for 24 h; *p<0.05 and **p<0.01.

Figure 6. Effects of CPZ analogs on mitochondrial membrane potential and ER stress.

Panels A-D: Mitochondrial membrane potential analysis of Cal-27 cells treated for 2h with CIDD-24, CIDD-99, CIDD-111, and CPZ at the indicated concentrations. Quantification of JC-1 aggregates to monomers ratio (mean $\pm$ SD) is shown for CPZ (Panel A), CIDD-24 (Panel B), CIDD-99 (Panel C), and CIDD-111 (Panel D); $n=3$ per group. **Panel E:** Western blot analysis of BiP and CHOP expression in Cal-27 cells treated for 24h with 10, 25, and 50 μ M CIDD-24, CIDD-99, and CIDD-111 compared to CPZ. **Panel F:** Cell viability assay of Cal-27 cells treated for 24h with CIDD-99 (500 nM) and cisplatin (25 μ M) or gefitinib (100 nM). **Panel G:** Cell viability assay of Cal-27 cells treated for 24h with increasing concentrations of CIDD-99 following exposure to 2, 5, and 10 Gray (Gy) radiation; * $p<0.05$, ** $p<0.01$, *** $p<0.001$ vs control and ### $p<0.001$ between test groups.