Non-pungent long chain capsaicin-analogs arvanil and olvanil display better anti-invasive activity than capsaicin in human small cell lung cancers

John D. Hurley, Austin T. Akers, Jamie R. Friedman, Nicholas A. Nolan, Kathleen C. Brown, and Piyali Dasgupta

Department of Pharmacology, Physiology, and Toxicology, Joan C. Edwards School of Medicine, Marshall University, Huntington, WV, USA

ABSTRACT
The nutritional compound capsaicin inhibits the invasion of many types of human cancers. The clinical development of capsaicin as an anti-cancer drug is limited due to its unfavorable side effects like burning sensation, stomach cramps, gut pain and nausea. This study compared the anti-invasive activity of capsaicin to non-pungent long chain capsaicin analogs, namely arvanil and olvanil, in human small cell lung cancer cells. Boyden chamber invasion assays revealed that arvanil and olvanil displayed improved anti-invasive activity relative to capsaicin in human SCLC cells. The results of the Boyden chamber assay were confirmed by the spherical invasion assay, and similar results were obtained. The anti-invasive activity of arvanil, olvanil and capsaicin were independent of TRPV and CB1 receptors. Furthermore, the anti-invasive activity of arvanil, olvanil and capsaicin was mediated by the AMPK pathway. Depletion of AMPK levels by siRNA methodology abrogated the anti-invasive activity of arvanil, olvanil and capsaicin. The non-pungent capsaicin analogs arvanil and olvanil display improved anti-invasive activity relative to capsaicin in human SCLC cells. These agents may represent the second generation of capsaicin-like compounds which are more potent than the parent molecule and have a better side effect profile.

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Introduction
Capsaicin is the spicy ingredient of chili peppers. Recent evidence has shown that capsaicin displays potent chemopreventive and anti-tumor activity in many types of human cancers. Apart from its growth-inhibitory activity, capsaicin has also been found to suppress invasion and migration of multiple human cancers like prostate cancer, melanoma, cholangiocarcinoma and fibrosarcoma. Small cell lung cancer (SCLC) is a highly invasive malignancy; limited stage SCLC patients show invasion of the tumor into the submucosa and peribronchial connective tissue at the time of presentation. Extended stage SCLC (which affects a majority of the patients) is characterized by the invasion of malignant cells beyond the hemithorax, in the pleural or pericardial effusion, blood and lymph (NCCC, UK). Agents like capsaicin which suppress the invasion of tumor cells may be of value in human SCLC therapy.

The clinical application of capsaicin is restricted by its unfavorable side-effect profile. Several convergent studies have shown that systemic administration of capsaicin in humans leads to intense gut pain, hyperalgesia, stomach cramps and nausea. These adverse side effects of capsaicin have led patients to abandon taking the drug. Such observations emphasize the need for novel capsaicin analogs, which retain the biological activity of capsaicin but do not produce the “heat-sensation” of capsaicin.

The analgesic activity of capsaicin has led to intense research focused on the design of novel capsaicin analogs. Structure activity relationship (SAR) studies have shown that long chain capsaicin compounds (Fig. 1A: with chain length R ≥ C16) are non-pungent, display better oral bioavailability and have lower “heat-sensation” than capsaicin. After examining SAR studies, we selected two capsaicin-like compounds, arvanil and olvanil (Fig. 1B). Arvanil and olvanil were selected because they are orally available, non-pungent, and have comparable pharmacological activity profile, relative to capsaicin.

The analgesic activity of capsaicin is mediated by the transient receptor potential vanilloid (TRPV) superfamily of ion channel receptors. Capsaicin functions as an agonist of the TRPV1 receptor. Arvanil and olvanil bind to TRPV1 with higher affinity than capsaicin.
A unique feature of arvanil and olvanil is that they are agonists of cannabinoid receptor (CB1). Although, the analgesic activity of capsaicin is mediated by TRPV1, its anti-tumor activities have been found to be independent of TRPV1.1 Similarly, the biological activity of arvanil and olvanil have been shown to be independent of TRPV1 or CB1 or both in various experimental systems. 17,18 For example, the apoptotic activity of arvanil in human leukemia cell lines was found to be independent of both TRPV1 and CB1,17,18 whereas its apoptotic effect on glioma cells required TRPV1 but not CB1.19

Previous studies show that capsaicin suppressed the invasion of human cancer cells in both cell culture and transgenic mouse models.2-5 The 5' AMP-activated protein kinase (AMPK) pathway has been shown to mediate the anti-invasive activity of capsaicin in cholangiocarcinoma cells in vitro.5 Furthermore, the AMP kinase pathway has been implicated in the pro-apoptotic, pro-autophagic and anti-inflammatory effects of capsaicin in several experimental models.20-22 Emerging data also show that dietary capsaicin stimulates glucose uptake in muscles and attenuates metabolic dysfunction in genetically obese mice via the AMPK pathway.23,24 Therefore the AMPK signaling network seems to an important converging point mediating the biochemical activities of capsaicin.

The effect of capsaicin on the invasion of human SCLC cells has not been studied. There are no published reports which have described the impact of olvanil and arvanil on invasion of human cancer cells. The main objective of the present studies was to compare the anti-invasive activity of arvanil, olvanil and capsaicin in human SCLC cells. We show that the non-pungent long chain capsaicin analogs arvanil and olvanil display better anti-invasive activity relative to capsaicin in human SCLC cell lines. We also wanted to analyze the role of TRPV1 and CB1 receptors in the anti-invasive activity of capsaicin olvanil and arvanil. We observed that the anti-invasive activity of arvanil, olvanil and capsaicin was independent of the TRPV1 and CB1 pathway. Most interestingly, arvanil, olvanil and capsaicin inhibited invasion of human SCLC cells by activation of the AMPK pathway. Our results suggest that arvanil and olvanil may represent the next-generation of capsaicin compounds with better anti-invasive activity and an improved side effect profile.

Materials and methods

Materials

Arvanil, olvanil, AM281 and dorsomorphine dichloride were obtained from Tocris Biosciences (Bristol, United Kingdom). Capsaicin and ruthenium red were purchased from Sigma-Aldrich (St. Louis, MO).

Cell lines and culture

The human SCLC cell lines DMS 53 and DMS 114 were obtained from American Type Culture Collection (Manassas, VA). DMS 114 was cultured in RPMI-1640 medium supplemented with 2 mM glutamine, 25 mM HEPES, 1 mM sodium pyruvate, 4.5 g/L glucose, 100 units/ml penicillin, 50 μg/ml streptomycin and 10% FBS.25 DMS 53 was cultured in RPMI-1640 containing with 2 mM glutamine, 1 mM sodium pyruvate, 1X non-essential amino acids, 1X insulin-transferrin-selenium (ITS; Invitrogen, ThermoFisher, Carlsbad, CA) supplement 100 units/ml penicillin, 50 μg/ml streptomycin and 5% FBS.26

Boyden chamber invasion assay

Boyden chamber assays were used to analyze the effect of arvanil, olvanil and capsaicin on the invasion of DMS 114 and DMS 53 cells.27 Corning® BioCoat™ Matrigel® invasion chambers containing 8.0 μm pore size transwell
filters (precoated with Matrigel) and companion 24 well plates were ordered from Corning (Corning, NY). The filters were rehydrated by incubating them in warm RPMI basal media according to manufacturer’s instructions. An aliquot of approximately $5 \times 10^5$ DMS 114 cells were seeded in the apical chamber in RPMI containing 0.1% FBS, in the presence or absence of the indicated compounds. RPMI media containing 20% FBS was used as the chemoattractant and added to the lower basolateral chamber. The plates were incubated at 37°C for 24 hours. After 24 hours, the invasion chambers were removed and non-invading cells on the upper surface of the filters were removed by wiping with cotton swabs. The cells that had invaded to the lower surface of the filters were quantified by staining with 0.5% crystal violet for 20 minutes. Cells which had invaded to the lower surface of the filters were extracted with DMSO. An aliquot of 100 μl of cell lysate was placed in a 96 well plate and the absorbance was read at 560 nm. The absorbance of control cells was assumed to be 1, and arvanil-, olvanil-, capsaicin-induced decreases in invasion were calculated as fold change from control.

The experiment was repeated using $5 \times 10^5$ DMS 53 cells using the protocol described above. Each sample was measured in duplicate and the entire assay was repeated two independent times.

**Spherical invasion assay**

The spherical invasion assay used was a modification of the assay used by Evenssen et al. DMS 114 cells were cultured to 70–80% confluence in RPMI containing 25 mM HEPES and 10% FBS. Subsequently, the cells were harvested and re-suspended in RPMI containing 25 mM HEPES and 20% FBS at a concentration of $5 \times 10^7$ cells/ml. The cell suspension was then treated with indicated doses of the test compounds and subsequently diluted 1:1 in Matrigel. A 6 μl aliquot of this mixture was pipetted as a drop onto the center of an 8-well chamber slide (Fig. 2A). These spots of cells mixed with Matrigel were allowed to polymerize at 37°C for 1 hour, after which they were bathed in 300 μl RPMI containing 25 mM HEPES and 10% FBS and incubated for 18 hours at 37°C. At this stage, DMS 114 cells grew within and up to the edge of the Matrigel spot. After 18 hours, the media was then gently aspirated. A second solution of phenol-red free Matrigel was prepared by diluting phenol-red free Matrigel with phenol-red free RPMI containing 25 mM HEPES and 20% FBS at a ratio of 1:1. Two hundred microliters of this diluted phenol-red free Matrigel solution was pipetted over the drops and incubated for 1 hour at 37°C (Fig. 2B). The cells were then bathed in 300 μl RPMI containing 25 mM HEPES and 10% FBS and incubated for 24 hours at 37°C. Subsequently, each spot was observed by phase contrast microscopy (Leica DMIL LED, Bannockburn, IL). Under the phase contrast microscope, the dark line demarcating the first pink Matrigel layer and the second white Matrigel layer was clearly visible (Fig. 2C). Three independent fields of these cultures were photographed by phase contrast microscopy (Leica DMIL LED). On the photographed image of these cultures, a dotted line was drawn to mark the interface between the primary and secondary Matrigel layers (marked ab, Fig. 2D). The NIH Image J software (version 1.47) was used to average the invading distance covered by the cells in the secondary Matrigel layer from the dotted line ab. In each photograph, seven distance measurements were recorded to recapitulate the variance in the invading distances of DMS 114 cells. This process was repeated in three independent photographic fields. The distance traveled by the invading cells (from the interface line ab) was counted in 21 (7 measurements X 3 fields) distinct areas, over three fields in a double blind fashion by three independent observers. The distance traveled by the control cells was assumed to be 1, and arvanil-, olvanil-, capsaicin-induced decreases in invasion were calculated as fold change from control. Each data point was performed in duplicate, and the whole experiment was repeated twice.

**MTT assay**

MTT assays were performed as described by Brown et al. DMS 114 and DMS 53 cells were plated in 96-well plates at a density of 5,000 cells/well. The plates were incubated overnight to allow complete reattachment of the cells. Subsequently, cells were treated with the indicated concentrations of arvanil, olvanil and capsaicin for 36 hours. After the indicated time points, 25 μl of sterile MTT solution (5 mg/ml in PBS) was added to each well, and the plates were incubated for 4 hours at 37°C. Then, the media was aspirated, and 150 μl of DMSO was added to each well to solubilize the formazan crystals. The absorbance of the plates was measured on an ELISA reader (Benchmark, Bio-Rad, Hercules, CA) at a wavelength of 540 nm. The absorbance of control cells was assumed to be 1, and arvanil-, olvanil-, capsaicin-induced decreases in cell viability were calculated as fold change from control. Each sample was performed in triplicate, and the entire experiment was repeated twice.

**Lysates and western blotting**

Cell lysates were made using the IGEPAL CA-630-based lysis protocol. DMS 114 cells were grown in 100 mm diameter tissue culture dishes to approximately 70–80%
confluence. Cells were harvested and washed three times with ice 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). Seventy microliters of lysis buffer was added for every 20 μl of packed cell volume. The lysate was rotated at 4°C for 40 minutes and subsequently spun at 15,000 g for 15 minutes at 4°C. The supernatant was collected and stored in −80°C for further analysis. The protein concentration of the lysate was measured using a Bradford Reagent (Bio-Rad). An aliquot of one hundred microgram of the protein was run on a 10% SDS-PAGE gel and transferred onto nitrocellulose membranes (Bio-Rad) using semi-dry transfer protocols.

Figure 2. Diagrammatic illustration of the spherical invasion assay. (A) The first layer is comprised of DMS 114 cells mixed in a 1:1 suspension with phenol-red containing Matrigel (light pink area). After 18 hours, DMS 114 cells grow and extend up to the boundary of this first layer. (B) After 24 hours, a second layer of 1:1 solution phenol-red free Matrigel, in phenol-red free RPMI (gray area) is added on top of the first Matrigel spot. The cells are incubated for 24 hours at 37°C. (C) After 24 hours, DMS 114 cells invade into the secondary Matrigel layer. The chamber slides are observed by phase contrast microscopy. (D) A representative photograph of untreated DMS 114 cells is shown. The black arrow indicates the vessels in second layer. The dotted line ab is taken as the interface between the two layers. The distance to which the cells have traveled (into the secondary Matrigel layer) is measured at seven sites (for each photograph) in a randomized double blind fashion by three independent observers, using NIH Image J Version 1.47. This process is repeated for three separate photographic fields per sample.

The equivalent loading of proteins on the SDS-PAGE gel was confirmed by staining of membranes with Ponceau S. Membranes were blocked for 1 hour in blocking buffer (1X PBS, 5% milk, 0.10% Tween-20) and placed in primary antibody (usually at a 1:500 dilution in 1X PBS, 0.10% Tween-20) overnight at 4°C. Membranes were washed three times in wash buffer (1X PBS, 0.10% Tween-20) for 5 minutes each. Primary antibody was detected using horseradish peroxidase–linked goat anti-mouse or goat anti-rabbit IgG or rabbit anti-goat antibodies (ThermoFisher, Waltham, MA; usually at a 1:500 dilution in 1X PBS, 5% milk, 0.10% Tween-20) overnight at 4°C. Membranes were washed thrice with wash buffer for ten minutes each. The proteins of interest were visualized with the enhanced chemiluminescent detection system ECL (SuperSignal™ West Dura Extended Duration Substrate,
ThermoFisher).\textsuperscript{31,32} Rabbit polyclonal phospho-AMPK-\(\alpha1/2\)- (dilution 1:1000 in 1X PBS, 0.10% Tween-20), goat polyclonal AMPK-\(\alpha1\) (dilution 1:500 in 1X PBS, 0.10% Tween-20) and goat polyclonal AMPK-\(\alpha2\) (dilution 1:500 in 1X PBS, 0.10% Tween-20; Santa Cruz Biotechnologies, Inc., Santa Cruz, CA) and GAPDH antibody (1:5000 in 1X PBS, 0.10% Tween-20; Trevigen Inc., Gaithersburg, MD) were used for the immünoblotting experiments. The results of the protein gel blotting assays were quantitated by densitometry by using NIH Image J Version 1.47.

\textbf{siRNA transfection and assays}

Chemically synthesized, double stranded AMPK-\(\alpha1\)-siRNA was purchased from Santa Cruz Biotechnologies, Inc. The transfection experiments were performed in DMS 114 cells. The transfection of 75 nM of AMPK-\(\alpha1\)-siRNA or control-siRNA in DMS 114 human SCLC cells was performed by using Oligofectamine reagent (Invitrogen, ThermoFisher, Carlsbad, CA), according to the manufacturers protocol. Twenty-four hours post-transfection, the cells were harvested, and a Boyden chamber invasion assay was performed.\textsuperscript{27} A non-targeting siRNA sequence (Santa Cruz Biotechnologies, Inc.) was used as a control-siRNA for the transfection experiments.\textsuperscript{31,32} Each transfection was performed in duplicate, and the entire assay was performed two independent times. The entire transfection experiment was repeated in a second human SCLC cell line, DMS 53.\textsuperscript{31,32}

Western blotting experiments were performed to assess the expression of proteins after siRNA transfection in DMS 114 and DMS 53 cells.\textsuperscript{31,32} The results of the western blotting assays were quantitated by densitometry using NIH Image J Version 1.47.

\textbf{Statistical analysis}

All data was plotted using GraphPad Prism Software, Inc. (La Jolla, CA), and was represented as the mean ± standard error of the mean (SEM). Results from the control and treated samples were compared using an analysis of variance followed by a Tukey’s post hoc test. All analyses were completed using a 95% confidence interval. Data was considered significant when \(P < 0.05\).

\textbf{Results}

\textbf{Capsaicin displays anti-invasive activity in human SCLC cells}

Boyden chamber invasion assays were used to measure the anti-invasive activity of capsaicin in DMS 114 human SCLC cells. We observed that capsaicin inhibited the invasion of DMS 114 cells in a concentration-dependent manner (Fig. 3A). The anti-invasive activity of capsaicin was significant from concentrations of 10 \(\mu M\) – 100 \(\mu M\) (*\(P<0.05\)). Next, we wanted to ascertain that the anti-invasive activity of capsaicin was not due to its growth-inhibitory activity. MTT assays show that capsaicin decreases the viability of DMS 114 human SCLC cells between 30 \(\mu M\) – 100 \(\mu M\) (Fig. 3B). Therefore, the anti-invasive activity of capsaicin displayed at 10 \(\mu M\) and 20 \(\mu M\) was independent of its growth-inhibitory activity. Since the magnitude of capsaicin-induced inhibition of DMS 114 cell invasion was greater at 20 \(\mu M\), we decided to use this concentration of capsaicin for all our subsequent experiments.

Subsequently, we analyzed the time-kinetics of the anti-invasive activity of capsaicin (Fig. 3C). We found that the treatment of DMS 114 cells with 20 \(\mu M\) capsaicin caused approximately a 29 ± 0.015 % decrease in the invasion of DMS 114 cells over 24 hours. The maximal anti-invasive activity of capsaicin was observed at 36 hours (Fig. 3C) and remained constant thereafter (approximately 53 ± 0.03% relative to controls). MTT assays revealed that 20 \(\mu M\) capsaicin did not display any growth inhibitory activity over 24 and 36 hours (Fig. 3D). Since our objective was to determine whether the anti-invasive of arvanil and olvanil was greater than capsaicin in human SCLCs, we chose the 24-hour time-point as a baseline for comparing the anti-invasive activity of all the three compounds.

\textbf{Arvanil and olvanil display greater anti-invasive activity than capsaicin in human SCLC cells in Boyden chamber invasion assays}

Figure 1 shows the chemical structures of arvanil, olvanil and capsaicin. The primary difference between these three compounds resides in the length of the aliphatic side chain R (Fig. 1A). Boyden chamber assays were performed to analyze the anti-invasive activity of arvanil and olvanil. Figure 4A and 4B show that both arvanil and olvanil display significant anti-invasive activity in DMS 114 human SCLC cells from 10 nM-100 \(\mu M\) (*\(P < 0.05\)). In contrast, capsaicin showed anti-invasive activity in DMS 114 from 10 \(\mu M\) – 100 \(\mu M\) (Fig. 3A). Such data suggests to us that the invasion-inhibitory ability of arvanil and olvanil may be greater than capsaicin. Next, we analyzed if the potent anti-invasive activity of arvanil and olvanil was due to their growth-inhibitory activity. MTT assays revealed that arvanil and olvanil decreased cell viability from 10 \(\mu M\) - 100 \(\mu M\) (Fig. 4C). Therefore, the anti-invasive activity of arvanil and olvanil were independent of their
Figure 3. Capsaicin suppresses invasion of DMS 114 human SCLC cells in a concentration-dependent and time-dependent manner. (A) Boyden chamber assays indicate that capsaicin inhibited the invasion of DMS 114 human SCLC cells in a concentration-dependent manner over 24 hours. (B) MTT assays show that capsaicin decreases the viability of DMS 114 cells from concentrations ranging from 30 μM-100 μM over 24 hours. (C) Time kinetics of the anti-invasive activity of 20 μM capsaicin from 24–48 hours. Values indicated by * are statistically significant relative to controls. The absorbance of control cells was assumed to be 1, and arvanil-, olvanil-, capsaicin-induced decreases in invasion were calculated as fold change from control. (D) MTT assays show that 20 μM capsaicin does not impact the viability of DMS 114 cells at 24 hours or 36 hours. However, the treatment of 20 μM capsaicin decreases cell viability in DMS 114 cells over 48 hours. The absorbance of control cells was assumed to be 1, and arvanil-, olvanil-, capsaicin-induced decreases in cell viability were calculated as fold change from control. Values indicated by * are statistically significant relative to controls. The figure represents the average of two independent experiments, and the data have been represented as mean ± SEM.
Figure 4. (For figure legend, see page 87)
growth-inhibitory activity at a concentration range of 10 nM-1 μM. Arvanil and olvanil showed the maximal anti-invasive activity at a concentration of 1 μM, and therefore, this concentration of arvanil and olvanil were chosen for subsequent experiments. The magnitude of the anti-invasive activity of arvanil (and olvanil) was similar to capsaicin; however, arvanil (and olvanil) showed the maximal anti-invasive activity at a 20-fold lower concentration relative to capsaicin. The results obtained in DMS 114 human SCLC cell line was verified in a second human SCLC cell line, namely DMS 53. Figure 4D demonstrates that 1 μM arvanil, 1 μM olvanil and 20 μM capsaicin display potent anti-invasive activity in human DMS 53 cells over 24 hours, as measured by the Boyden chamber assay. MTT cell viability experiments reveal that 1 μM arvanil, 1 μM olvanil and 20 μM capsaicin did not cause decrease in cell viability in DMS 53 human SCLC cells (Fig. 4E).

We also performed Boyden Chamber assays where the crystal-violet-stained cells were visualized by phase contrast brightfield microscopy. Untreated DMS114 cells showed robust invasion across the Matrigel-coated membrane in 24 hours (Supplementary Fig. 1, top left). When the cells were treated with 1 μM arvanil (Supplementary Fig. 1, top right) or 1 μM olvanil (Supplementary Fig. 1, bottom left), we observed a decrease in the number of invading cells. Similarly, the presence of 20 μM capsaicin (Supplementary Fig. 1, bottom right) suppressed the invasion of DMS 114 cells over 24 hours.

**Arvanil and olvanil show greater anti-invasive activity than capsaicin in human SCLC cells in spherical invasion assays**

The anti-invasive activity of arvanil, olvanil and capsaicin was confirmed by a second invasion assay, the spherical invasion assay (Fig. 2). In this assay, the invasive activity of cells was measured by their ability to invade into the secondary Matrigel layer.30 Untreated DMS 114 cells showed substantial invasive activity and migrated robustly across the interface (dotted line ab) into the secondary Matrigel layer (Fig. 5A; left column, top, middle and bottom picture). The invading cells are indicated by black arrows in Fig. 5A. The treatment of DMS 114 cells with 1 μM arvanil caused a substantial reduction in the distance to which these cells invaded the secondary Matrigel layer, across the interface line ab (Fig. 5A; right column, top picture). The treatment of DMS 114 cells with 1 μM olvanil yielded similar results (Fig. 5A; right column, middle picture). The anti-invasive activity of 20 μM capsaicin in DMS 114 cells is depicted in Fig. 5A (right column, bottom picture).

The amount of the invasion was calculated by measuring the distance invaded by the cells from the dotted interface line ab, as described in Methods. The results are graphically depicted in Figure 5B-C. We noticed that the trend observed in the Boyden chamber assays (Fig. 4A-B) was maintained in the spherical invasion assay. Arvanil and olvanil displayed greater anti-invasive activity than capsaicin in DMS 114 cells. The results of the spherical invasion assay were repeated in DMS 53 cells and similar results were obtained (Fig. 5C). The time point for the spherical invasion assay was 36 hours. MTT experiments showed that 1 μM arvanil, 1 μM olvanil and 20 μM capsaicin did not reduce the viability of DMS 53 cells over 36 hours (Fig. 5D).

**The anti-invasive activity of arvanil, olvanil and capsaicin were independent of the TRPV pathway**

The biological activity of capsaicin is mediated by the TRPV superfamily of transmembrane receptors.1 Specifically, capsaicin, arvanil and olvanil are high-affinity agonists for the TRPV1 receptor.12,15,16 The role of TRPV receptors in the anti-invasive activity of arvanil, olvanil and capsaicin was examined by using the generalized TRPV receptor antagonist ruthenium red.32 Boyden chamber assays showed that 10 μM ruthenium red did not reverse the anti-invasive activity of arvanil, olvanil or capsaicin (Fig. 6A), indicating the anti-invasive activity
Figure 5. (For figure legend, see page 89)
of these compounds were independent of the TRPV receptor pathway in DMS 114 human SCLC cells. We repeated these experiments in a second human SCLC cell line, namely DMS 53 (Supplemental Fig. 2A) and obtained similar results.

The results obtained from the Boyden chamber assay were repeated using the spherical invasion assay. Figure 7A shows that the invasion-inhibitory activity of arvanil, olvanil and capsaicin were not altered by 10 μM ruthenium red. These findings confirm that the ability of arvanil, olvanil and capsaicin to suppress the inhibition of human SCLC cells is not mediated by the TRPV signaling pathway.

The anti-invasive activity of arvanil, olvanil and capsaicin were independent of the CB1 pathway

Arvanil and olvanil are agonists for the cannabinoid receptor 1 (CB1). Therefore, the next series of experiments aimed to investigate if the CB1 receptors were mediating the anti-invasive activity of arvanil, olvanil and capsaicin. We used the CB1 receptor antagonist AM281 for our experiments. AM281 did not reverse the anti-invasive effects of arvanil, olvanil or capsaicin (Fig. 6B), in DMS 114 cells, as measured by Boyden chamber assays. These experiments were repeated in DMS 53 human SCLC cells and similar results were obtained (Supplemental Fig. 2B).

The results from the Boyden Chamber assay were repeated using the spherical invasion assay in DMS 114 cells and comparable results were obtained. The CB1 antagonist AM281 did not affect the anti-invasive activity of arvanil, olvanil and capsaicin (Fig. 7B) in human DMS 114 cells.

The anti-invasive activity of arvanil, olvanil and capsaicin were mediated by the AMPK pathway

Data from Lee et al. (2014) have shown that capsaicin suppresses the migration of human cholangiocarcinoma cells via the 5′ AMP-activated protein kinase-α (AMPK-α) kinase pathway. Similarly, cannabinoid agonists have been shown to exert anti-tumoral effects in pancreatic adenocarcinoma and hepatocellular carcinoma cells via the AMPK pathway. Since the AMPK pathway seemed to be involved in the bioactivity of capsaicin and cannabinoid ligands, we conjectured that the AMPK-α pathway may be responsible for the anti-invasive activity of arvanil, olvanil and capsaicin. Boydener chamber invasion assays revealed that the AMPK-α inhibitor dorsomorphine dichloride (also called compound C) abrogated the anti-invasive activity of arvanil, olvanil and capsaicin in DMS 114 and DMS 53 cells (Fig. 6C). The results obtained from the Boyden chamber experiments were verified by the spherical invasion assay in DMS 114 cells and similar results were obtained (Fig. 7C).

AMPK-α exists in two isoforms, AMPK-α1 and AMPK-α2. Immunoblotting experiments showed that DMS 114 and DMS 53 human SCLC cells expressed robust amounts of AMPK-α1 (Fig. 8A, topmost lane). However, AMPK-α2 was not detected in DMS 114 and DMS 53 (Fig. 8B). T47D human breast cancer cells were used as the positive controls for the immunoblotting experiments. Western blotting experiments were performed to test whether arvanil, olvanil and capsaicin induced activation of AMPK-α1 over 24 hours. Untreated DMS 114 cells had low amounts of phospho-AMPK-α (Thr172) (Fig. 8C and D, top row, leftmost lane). The treatment of DMS 114 cells with 1 μM arvanil (Fig. 8C, top row, second lane from the left) or 1 μM olvanil (Fig. 8D, top row, second lane from the left) caused robust activation and phosphorylation of AMPK-α1 kinase. Similarly, the treatment of DMS 114 with 20 μM capsaicin increased the levels of phosphorylated AMPK-α (Thr172) (Fig. 8E, top row, lanes 1 and 2 from the left). The results of these experiments were repeated in DMS 53 human SCLC cells and similar results were obtained (Fig. 8C-E, top panel, lanes 3 and 4 from the left).

The role of AMPK-α1 in the anti-invasive activity of arvanil, olvanil and capsaicin was confirmed by siRNA methodology. The transfection of AMPK-α1-siRNA in DMS 114 human SCLC cells (Fig. 9A, black bars) and
DMS 53 cells (Fig. 9A, white bars) reversed the anti-invasive activity of arvanil. The AMPK-siRNA experiment was repeated using olvanil (Fig. 9B) and capsaicin (Fig. 9C), and similar results were obtained. The transfection of a non-targeting control-siRNA did not have any effect on the invasion-inhibitory effects of arvanil, olvanil and capsaicin (Fig. 9A-C, third set of bars from the left). Finally, the transfection of AMPK-α1-siRNA did not influence the invasion of untreated DMS 114 (Fig. 9D; black bars) and DMS 53 cells (Fig. 9D; white bars). Western blotting experiments show that the transfection of AMPK-α1-siRNA suppressed the levels of AMPK-α1 in both DMS 114 (Fig. 9E) and DMS 53 cells (Fig. 9F).

The structures of arvanil and olvanil closely resemble capsaicin. However, these compounds are non-pungent and do not produce the unpleasant side effects of capsaicin. Therefore, it may be envisaged that these compounds represent the second-generation of capsaicin-like compounds with better anti-invasive activity and a better side effect profile. SCLC is a highly invasive tumor with a propensity for early metastasis. Long chain capsaicin compounds like arvanil and olvanil foster the hope of improved therapies in the treatment and management of SCLC.

Discussion

The invasion of tumor cells into the surrounding stroma, neighboring blood vessels and lymph nodes is an essential step for metastasis. It is well established that metastasis is the major cause of death in cancer patients. Among the many steps of metastasis, tumor invasion has been targeted by many therapeutic agents to suppress the distant spread of tumors. These agents include tyrosine kinase inhibitors, transmembrane receptor antagonist, small synthetic molecules against intracellular signaling agents and humanized antibodies. Recent evidence shows that nutritional compounds like capsaicin can suppress the invasion and migration of many types of tumor cells. The present study investigates the anti-invasive effect of capsaicin in human SCLC, which is a highly invasive and metastatic cancer. We find that capsaicin decreases the invasion of two human SCLC cell lines in both Boyden chamber assays and spherical
invasion assays. Our data agrees with that of other researchers who found that capsaicin suppresses the invasion and migration of prostate cancer, melanoma, fibrosarcoma and cholangiocarcinoma cells. Controversy exists on the effect of capsaicin on metastasis. Whereas data from Venier et al., (2015) show that capsaicin decreases invasion and metastasis burden in TRAMP model of prostate cancer,^2 other published reports have shown that capsaicin increases breast cancer and colon cancer metastasis.^39,41 Erin et al., (2004 and 2006) used a high concentration of capsaicin to deactivate sensory nerves and showed that such deactivation increased breast cancer metastasis.\(^{40,41}\) Similarly, Yang et al., (2013) have treated human colon cancer cell lines with high concentration of capsaicin (as high as 200 \(\mu\)M) to examine the pro-metastatic effect of capsaicin.\(^{39}\) However, it must be remembered that metastasis is a complex phenomenon and consists of many other steps apart from invasion. Our results show that capsaicin exerts anti-invasive activity at much lower concentrations (about 20 \(\mu\)M). At such low concentrations, capsaicin does not display any growth-inhibitory activity on human SCLC cell lines. Our published data show that the growth-inhibitory activity of capsaicin is displayed at about 50 \(\mu\)M.\(^{32}\) Therefore, the anti-invasive activity of capsaicin is independent of its growth inhibitory effects and occurs at lower concentrations.

A survey of literature shows that arvanil, olvanil and capsaicin display some similarities in their pharmacological profile. All the three compounds are high-affinity agonists of TRPV1 receptors. The affinity of arvanil and olvanil for TRPV1 is greater than that of capsaicin.\(^7\) Arvanil and olvanil upregulate the levels of intracellular calcium in a manner analogous to capsaicin.\(^{12}\) However, arvanil and olvanil are high affinity agonists of the cannabinoid receptor 1 (CB1), unlike capsaicin.\(^{15,16,42}\) Data from several laboratories also suggest interesting parallels in the growth-inhibitory activity of capsaicin, arvanil and olvanil. Although capsaicin is a TRPV1 ligand, its growth-inhibitory activity in most types of cancer cells has been shown to be independent of TRPV1.\(^{43}\) Similarly, published reports indicate that the growth-inhibitory activity of arvanil and olvanil (and related compounds) may be independent of TRPV1, CB1 or both.\(^{17-19}\)

Figure 7. Spherical invasion assays reveal that the anti-invasive activity of arvanil, olvanil and capsaicin did not involve the TRPV or CB1 pathway but were mediated by the AMPK pathway, in DMS 114 cells (A) Spherical invasion assays demonstrate that the anti-invasive activity of arvanil, olvanil or capsaicin were unaffected by the generalized TRPV antagonist ruthenium red in DMS 114 cells. (B) Spherical invasion assays reveal that invasion-inhibitory activity of arvanil, olvanil and capsaicin was not abrogated by the CB1 antagonist AM281 in DMS 114 cells. (C) The AMPK inhibitor dorsomorphine dichloride reverses the anti-invasive activity of arvanil, olvanil and capsaicin in DMS 114 cells, as measured by spherical invasion assays. The distance traveled by the control cells was assumed to be 1, and arvanil-, olvanil-, capsaicin-induced decreases in invasion were calculated as fold change from control. Values indicated by the same letter are not statistically significant. The figure represents the average of two independent experiments and the data have been represented as mean ± SEM.
Figure 8. Human SCLC cells express AMPK-α1 but not AMPK-α2. Arvanil, olvanil and capsaicin block activation of AMPK-α1 in human SCLC cells. (A) Immunoblotting experiments show that AMPK-α1 is robustly expressed in DMS 114 and DMS 53 cells. T47D human breast cancer cells were used as the positive controls for the experiment. GAPDH was used as the loading control for the protein gel blotting experiments, and the results were quantitated by densitometric analysis. (B) Western blotting experiments demonstrate that AMPK-α2 is not expressed by DMS 53 and DMS 114 human SCLC cells. T47D human breast cancer cells were used as the positive controls for the experiment. GAPDH was used as the loading control for the western blotting experiments, and the results were quantitated by densitometric analysis. (C) The treatment of 1 μM arvanil caused robust phosphorylation (and activation) of AMPK-α1 at threonine residue 172, in DMS 114 and DMS 53 cells over 24 hours (top panel). Total AMPK levels remained constant (middle panel). GAPDH was used as the loading control (bottom panel) for the protein gel blotting experiments, and the results were quantitated by densitometric analysis. The experiments were repeated using 1 μM olvanil (D) and 20 μM capsaicin (E) and similar results were obtained.
Figure 9. The AMPK-pathway mediated the anti-invasive activity of arvanil, olvanil and capsaicin in human SCLC cells. (A) Boyden chamber assays indicated that the transfection of AMPK-α1-siRNA significantly abrogated the anti-invasive activity of arvanil in DMS 114 cells (black bars). The experiment was repeated in human DMS 53 cells and comparable results were obtained (white bars). The transfection of a non-targeting control-siRNA did not have any effect on the anti-invasive activity of arvanil in DMS 114 and DMS 53 cells. (B) Depletion of AMPK-α1 levels by siRNA techniques ablated the anti-invasive activity of olvanil in DMS 114 (black bars) and DMS 53 cells (white bars). The anti-invasive activity of olvanil was not influenced by transfection of a control non-targeting siRNA in DMS 114 and DMS 53 cells. (C) AMPK-α1-siRNA reversed the anti-invasive activity of capsaicin in DMS 114 (black bars) and DMS 53 cells (white bars), whereas the control-siRNA have not effect on the anti-invasive activity of capsaicin in these two cell lines. The absorbance of control cells was assumed to be 1, and arvanil-, olvanil-, capsaicin-induced decreases in invasion were calculated as fold change from control. (D) The transfection of AMPK-α1-siRNA did not have any effect on the invasion of untreated DMS 114 (black bars) or DMS 53 cells (white bars). (E) Western blotting analysis showed that AMPK-α1 expression in DMS 114 cells and DMS 53 cells (F) was suppressed upon siRNA transfection. GAPDH was used as the loading control for the western blotting experiments, and the results were quantitated by densitometric analysis.
The growth-inhibitory activity of arvanil and olvanil has been studied in several cancers; however, there are no reports of their effect on the invasion of cancer cells. Our studies show for the first time that the non-pungent long chain capsaicin compounds olvanil and arvanil inhibit the invasion of human SCLC cells. The magnitude of the anti-invasive activity of olvanil and arvanil is similar to capsaicin but it is displayed at a 20-fold lower concentration relative to capsaicin. The anti-invasive activity of arvanil, capsaicin and olvanil was found to be independent of the TRPV and CB1 pathway. This is in agreement with data from several research laboratories which have shown that the growth-inhibitory effect of capsaicin in several human cancers is independent of TRPV receptors. Similarly, the apoptotic effects of olvanil and arvanil have been found to be autonomous of TRPV and CB1 pathways. Our data shows that the invasion-inhibitory activity of these compounds is yet another example of the TRPV- and CB1-independent effect of arvanil, olvanil and capsaicin.

The anti-invasive activity of arvanil, olvanil and capsaicin was mediated by activation of AMP kinase. Originally characterized as a regulator of fatty acid levels, cholesterol levels and lipid metabolism, the AMPK pathway has been recently recognized as a vital controller of cellular energy homeostasis. AMPK acts as a metabolic sensor which is activated by cellular stress conditions like ischemia, hypoxia and glucose deprivation. Several convergent studies show that AMPK controls intracellular energy pathways and maintains cell growth at normal levels via its downstream targets namely mTORC1, p53 and fatty acid synthase (FASN), and their associated metabolic processes. The tumor suppressor activity of AMPK is also due to its ability to regulate the classical tumor suppressor protein LKB1. Emerging evidence shows that the AMPK signaling network links metabolic syndrome to cancer. Clinical studies show that AMPK activation is decreased in lung cancer tumors isolated from patients who are active smokers compared to lung cancer tumors isolated from never smokers. This trend in also observed in SCLCs whose incidence is 90% correlated with smoking habits. The treatment of human SCLC cells with cisplatin or Titaonocene Y caused robust phosphorylation of AMPK-α1 at Thr172. Therefore, agents that cause the activation of AMPK-α1 may be useful for SCLC therapy. Capsaicin activates AMPK during cell migration, adipocyte differentiation, metabolic balance and glucose uptake processes. Similarly CB1 agonists regulate mitochondrial biogenesis, energy uptake and skeletal muscle metabolism via activation of the AMPK pathway. Although cannabinoid receptor ligands like R-methanandamide, arachidonoyl cyclopropamide (ACPA) or GW 405833 have been shown to induce activation of AMPK, our data is the first to report the activation of AMPK by arvanil and olvanil in human SCLC cells.

The α-subunit of AMPK is the catalytic subunit, and its phosphorylation at threonine residue 172 is essential for AMP activation. The α-subunit of AMPK exists in two isoforms, α1 and α2. We found that the AMPK-α1 subunit was robustly expressed in human SCLC cells whereas the AMPK-α2 subunit was not detected. Our data agrees with the results of other researchers who have not detected AMPK-α2 in the lung. The onset of extracellular stress causes the induction of AMPK-α2 specifically in the endothelial cells and smooth muscle cells of pulmonary vasculature. Our ongoing and future studies aim to explore the mechanisms by which olvanil, arvanil and capsaicin cause AMPK activation. However, the emphasis of this manuscript is to describe two novel non-pungent long chain capsaicin compounds which have better anti-invasive activity than capsaicin. Combination therapies involving capsaicin along with standard chemotherapeutic drugs and nutritional compounds have been investigated for several cancers. Recent studies have shown that capsaicin enhances the anti-inflammatory activity of the natural compound brassinin in human prostate cancer. Similarly, the combination of arvanil and temozolomide show better anti-tumor activity than either agent alone in mouse models of glioblastoma. This report describes two non-pungent long chain capsaicin compounds namely arvanil and olvanil which display anti-invasive activity comparable to capsaicin at lower concentrations. Arvanil and olvanil are structurally similar to capsaicin, recruit similar signaling pathways as capsaicin and display better pharmacological activity. Taken together, long chain capsaicin compounds like arvanil and olvanil may improve the efficacy and therapeutic index of capsaicin-based therapies in human small cell lung cancer.

### Abbreviations

| Acronym | Definition |
|---------|------------|
| AMPK    | 5′ AMP-activated protein kinase |
| CB1     | cannabinoid receptor1 |
| MTT     | [3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide] |
| RPMI    | Rosewell Park Memorial Institute |
| SAR     | Structure activity relationship |
| SCLC    | small cell lung cancer |
| siRNA   | small interfering RNA |
| TRPV    | Transient receptor potential vanilloid |

### Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.
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