JRVM
Journal of Research in Veterinary Medicine

RESEARCH ARTICLE

J Res Vet Med. 2025: 44 (1) 35-41
DOI:10.30782/jrvm.1671365

Synergistic Antiproliferative Impact of Capsaicin and Leflunomide in Murine Lung Cancer Cells

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Received 07-04-2025 Accepted 13-05-2025

Abstract

The emergence of drug resistance and limited efficacy of conventional chemotherapeutics have prompted the search for alternative anticancer strategies. This study aimed to investigate the antiproliferative effects of Capsaicin (CAP) and Leflunomide (LEF), alone and in combination, in murine Lewis lung carcinoma (LLC-1) cells. Following IC_{50} determination via the Sulforhodamine B (SRB) assay, combination experiments were conducted using sub-IC_{50} concentrations, and synergistic interactions were assessed through SynergyFinder software and CellTiter-Glo validation. Capsaicin and Leflunomide exhibited dose-dependent cytotoxicity, with IC_{50} values of 177.68 µM and 41.53 µM, respectively. Although limited cytotoxicity was observed with monotherapies at low doses, their combination produced a statistically significant synergistic effect (Combination Index = 0.13), resulting in enhanced growth inhibition and reduced colony formation capacity. Mechanistically, the combination is proposed to induce cancer cell death through dual targeting of intracellular calcium/oxidative stress (via TRPV1 activation by Capsaicin) and pyrimidine biosynthesis (via DHODH inhibition by Leflunomide). These findings highlight a promising multi-targeted strategy that suppresses cancer cell proliferation while potentially minimizing systemic toxicity. The Capsaicin–Leflunomide combination may offer a novel therapeutic approach in the treatment of aggressive and drug-resistant lung tumors, warranting further investigation in preclinical models.

Keywords: Lung cancer, Capsaicin, Leflunomide, Synergistic effect, Combination therapy.

Introduction

Cancer is one of the most serious health problems on a global scale and is among the leading causes of death (1). Lung cancer, in particular, attracts attention with its high mortality rates, and its treatment becomes difficult because current chemotherapeutic agents often cause side effects and resistance development in cancer cells (2). Therefore, the research of new molecules and combinations in different types of cancer, including lung cancer, is becoming increasingly important (3,4).

In recent years, the use of natural compounds in cancer treatment has increased interest. Capsaicin (CAP), the main active ingredient of hot pepper, stands out with its anti-inflammatory and anticancer properties and acts through various cellular-molecular pathways (5,6). One of the most well-known mechanisms of Capsaicin is that it increases intracellular calcium ion levels by activating TRPV1 receptors on the cell membrane (7). This increase results in the triggering of oxidative stress and activation of apoptotic pathways (8,9). It also shows suppressive effects on cancer cell proliferation by inducing the phosphorylation of stress-related kinases such as p38 MAPK and JNK (10). Its low potential for side effects makes Capsaicin attractive as a complementary agent to chemotherapy (11).

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On the other hand, Leflunomide (LEF) is an immunosuppressant drug generally used in the treatment of autoimmune diseases, and its active metabolite teriflunomide is reported to suppress proliferation in cancer cells by inhibiting the Dihydroorotate Dehydrogenase (DHODH) enzyme (12,13). DHODH plays an important role in pyrimidine biosynthesis; inhibiting this enzyme contributes to cell cycle arrest in rapidly proliferating tumor cells by creating nucleotide deficiency. Leflunomide has also inhibited cellular growth via AMPK activation and mTOR signal suppression (14,15).

Combining natural compounds with pharmacological agents can increase treatment efficacy while reducing side effects (16). In this context, the combination of Capsaicin and Leflunomide is thought to act through multiple complementary mechanisms, one by triggering oxidative stress and regulating calcium signaling, the other by blocking the cell cycle by inhibiting pyrimidine synthesis. Such an approach may suppress the growth of cancer cells more effectively and reduce the likelihood of developing resistance to treatment.

This study aimed to investigate the anti-cancer effects of the combined use of Capsaicin and Leflunomide in mouse lung cancer cell lines. The two agents could create synergistic effects through different biochemical pathways. The findings are anticipated to shed light on the development of new combined treatment strategies and contribute to creating more effective and reliable protocols for various cancer types, especially lung cancer.

Material and Methods

Cell Culture

This study used the mouse lung cancer cell line LLC-1 (Lewis Lung Carcinoma), available in our unit. The murine Lewis lung carcinoma (LLC1) cell line was preferred due to its extensive use in syngeneic in vivo mouse models, which allows for future translational studies. Cells were grown in DMEM (Dulbecco's Modified Eagle's Medium) (ATCC; Cat. No. 30-2002) containing 10% FBS (Fetal Bovine Serum) (ATCC; Cat. No. 30-2020) and 0.1% penicillin-streptomycin (100 IU/ml, 100 µg/ml) (Gibco™; Cat. No. 15140-122) in a humidified incubator at 37 °C and 5% CO 2. In each passage, when the cells reached a specific density (e.g., 80-90% confluency), they were collected with trypsin (0.25% trypsin-EDTA) and re-seeded.

Compounds Used in the Experiment

• Leflunomide (Lef) (MedChemExpress; Cat. No. HY-B0083) was prepared as a stock solution (187.5 mM concentration in DMSO) and stored at -20°C throughout the study.

• Capsaicin (Cap) (MedChemExpress; Cat. No.: HY-10448) was prepared as a stock solution (50 mM concentration in DMSO) and stored at -20°C throughout the study.

Before the experimental applications, the stock solutions were diluted to the desired working concentrations with the culture medium (or serum medium) and added to the cells. Control groups containing the same amount of DMSO but no active substance were prepared for the solvent control.

Determination of IC_{50} Values of Inhibitors

IC_{50} (half maximum inhibitory concentration) values were determined separately for Leflunomide and Capsaicin after cell proliferation procedures. For this purpose, the cell viability test Sulforhodamine B (SRB) method was applied as described in the literature (17). The SRB assay was selected due to its high reproducibility, sensitivity, and cost-effectiveness for adherent cell lines. This method is widely used to assess drug cytotoxicity in various cancer cell lines. In brief, LLC-1 cells were seeded in 96-well plates at 5×10^4 cells/well, and the cells were incubated for 24 hours. The next day, media containing different concentrations of Leflunomide (0, 3.1, 6.25, 12.5, 25, 50 µM) and Capsaicin (0, 31.25, 62.5, 125.5, 250, 500 µM) were added to the medium and incubated for 72 hours. At the end of the incubation period, the absorbance values of the plates were measured at 510 nm in a microplate reader by following the SRB staining protocol. The percentage of live cells in each concentration was evaluated as a percentage compared to the control group. IC_{50} values were calculated using a 4-parameter non-linear dose-response curve in the GraphPad Prism Program (18). Initial drug concentrations were determined based on previous studies using Capsaicin and Leflunomide in cancer models (19, 20). A wide dose range was tested to determine IC_{50} values for each drug, which were then used to establish the dose matrix for combination analysis.

Combination Experiment and Matrix Creation

Combination experiments were performed after determining the IC_{50} values of Leflunomide and Capsaicin. The aim was to demonstrate the synergy or antagonism status by simultaneously applying each compound at concentrations below their IC_{50} values (for example, with variations such as $IC_{50/2}$, $IC_{50/4}$, $IC_{50\times2}$). For this purpose, LLC-1 cells were re-seeded in 96-well plates and incubated for 24 hours. This time, inhibitors (Leflunomide 6.25 µM and Capsaicin 62.5 µM) were prepared in various combinations at the de-

termined concentrations in the same cell plates and added to the cells. A combination matrix was created as a control group (only vehicle/DMSO), single agent, and combined groups. After the cells were incubated for 72 hours again, SRB was applied for a cell viability test. The obtained absorbances were converted to percentage values compared to the control group and analyzed using SynergyFinder (https://synergyfinder.org/) software. Combination Index (CI) synergy parameters were evaluated. In addition, a different and more sensitive method, the Cell-Titer-Glo (Promega, Cat. No. G9241) protocol, was used to confirm cell viability tests. This time, cells were seeded in triplicate, and the luminescence was measured in a luminosity plate reader (GloMax Discover-Promega) after the inhibitor application process described above.

Synergy Analysis

The obtained absorbance/viability values were uploaded to the SynergyFinder platform and analyzed. This software calculates CI (Combination Index) or Synergy Scores using models such as Bliss Independence or Loewe Additivity (20). CI < 1 indicates synergy, CI = 1 indicates additive effect, and CI > 1 indicates antagonism. In this study, combination doses that showed synergistic effects were selected and used for further experiments.

Colony Formation Assay (CFA)

The effects of Leflunomide and Capsaicin alone and in combination, in accordance with the determined synergistic dose, on the colony formation capacity of cells, which is an *in vitro* indicator of their anti-tumorigenic potential, were tested. Colony formation assays were performed as described in the literature (21). In Brief, LLC-1 cells were seeded in 24-well plates at low density (500 cells/well). After an initial incubation period of 24 h, the cells were treated with a medium containing Leflunomide and Capsaicin at selected doses, separately and in combination. The medium was renewed regularly, and the cells were incubated for approximately 7–10 days (or until colony formation). At the end of incubation, the wells were gently washed with PBS and fixed with methanol. Then, they were stained with crystal violet. The colonies formed were photographed, and the number of colonies belonging to each group was calculated. The inhibitory effect on clonal proliferation after drug application was evaluated by normalizing the colony number to the control group (22).

Statistical Analysis

All experiments were performed with at least three independent replicates (n=3). The values obtained were expressed as mean ± standard deviation (SD). One-way ANOVA or Student's t-test was used when appropriate for comparisons between groups. Statistical significance was accepted as $p < 0.05$. Synergy and antagonism were evaluated according to the SynergyFinder report in combination studies.

Results

The effects of Capsaicin and Leflunomide on cell viability were investigated at different concentrations. In the analyses, the IC_{50} value for Capsaicin was determined as 177.68 µM, and the IC_{50} value for Leflunomide was determined as 41.53 µM. While the reducing effect of Capsaicin on cell viability became evident at higher concentrations, the effect of Leflunomide was observed at lower doses. The IC_{50} values for both compounds show that they suppress cell viability dose-dependently (Figure 1).

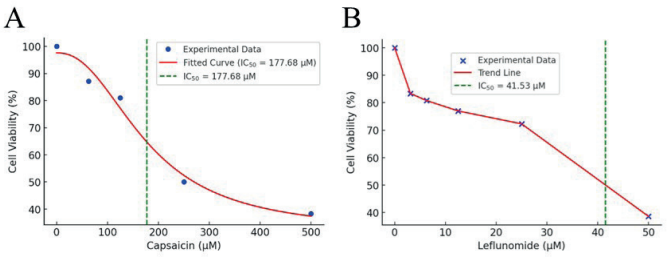

Figure 1: Determination Of The Effective Concentration Range Of Capsaicin And Leflunomide İn LLC-1 Cells.

Cell viability was determined by the SRB method. The dose-response curve was plotted in GraphPad Prism, and IC_{50} values were determined using the 4-parameter nonlinear regression method. (A) The effect of Capsaicin on cell viability at different concentrations is shown. The red curve represents the sigmoidal dose-response curve fitted to the experimental data. IC_{50} value was calculated as 177.68 µM and is shown as a green dashed line. (B) The effect of Leflunomide on cell viability is shown. The red line represents the trend line. IC_{50} value was calculated as 41.53 µM and is shown as a green dashed line.

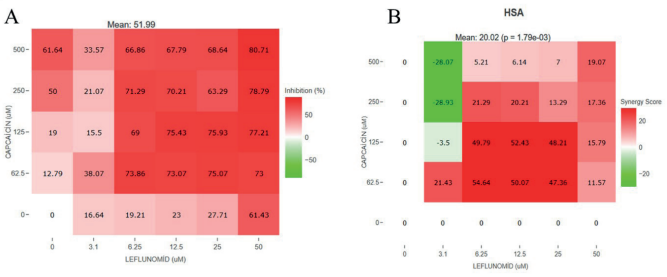

Figure 2: (A) Cell Inhibition Rates (%) of Capsaicin and Leflunomide, Alone and in Combination (A). Synergy Score Analysis of Capsaicin and Leflunomide (B).

Shows the cell inhibition percentages of the combination of Capsaicin and Leflunomide at different doses. Each cell represents the inhibition percentage for a given combina-

tion concentration. Shades of red indicate higher inhibition rates, while lighter areas indicate lower inhibition levels. The mean inhibition value is 51.99. (B) Synergy scores calculated using the HSA (Highest Single Agent) model are shown. Green colors represent antagonistic effects (negative synergy), and red colors represent synergistic effects (positive synergy). The mean synergy score was 20.02, with a p-value of 0.001, indicating a statistically significant synergistic interaction.

Table 1: Combination Index Table

Drug1	Drug2	Conc1	Conc2	CI	Response
Leflunomide	Capcaicin	6,25	62,5	**0,13**	73,86
Leflunomide	Capcaicin	12,5	62,5	0,18	73,07
Leflunomide	Capcaicin	25	62,5	0,24	75,07

CI < 1: Synergistic effect, CI = 1: Additive effect, CI > 1: Antagonistic effect. In the synergy score table specified in the literature (according to the Chou-Talalay method), the dose with the lowest CI (0.13) value was selected, and experiments were continued with these doses. The table was prepared based on Synergy Fynder's results.

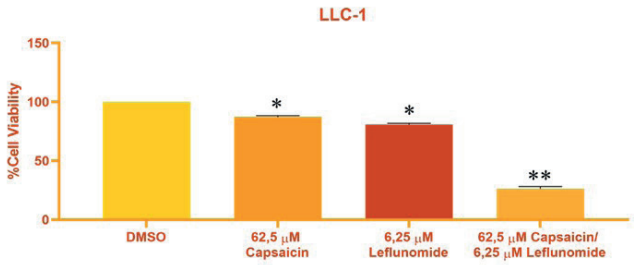

Figure 3: Cell proliferation obtained with 62.5 µM of Capcaicin and 6.25 µM Leflunomide for LLC-1 cells using CellTiterGlo assay.

Values represent the mean and standard deviation of a representative experiment performed in duplicate. *p<0.01, **p<0.001 compared to DMSO control.

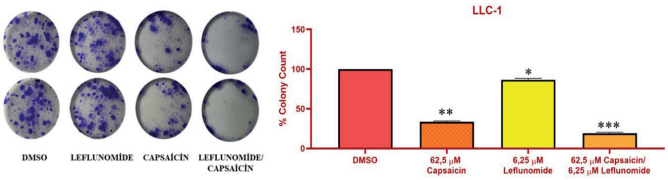

Figure 4: Photographic pictures of the colonies (A). Simultaneous Inhibition of Capcaicin (32.5 µM) and Leflunomide (3.25 µM) Diminishes the Colony Formation Capacity of LLC-1 cells (B).

Colony numbers were counted by Image J (Schneider, Rasband & Eliceiri, 2012), and values were normalized to DMSO, which was set to 100%. *p<0.05, **p<0.01, *** p<0.001 "Effects of different groups on cell viability (OD value). Data are presented as mean ± standard deviation (n=2). Statistical differences between the groups were evaluated using the Student's t-test. p < 0.05 was considered significant.

Discussion

In this study, it was observed that Leflunomide and Capsaicin did not show significant cytotoxic effects when applied alone at sublethal concentrations in a mouse lung cancer cell line but provided synergistic cell death induction when applied in combination. This synergy supports the idea that simultaneous suppression or overstimulation of multiple molecular pathways targeted in cancer therapy may suppress tumor cell proliferation more effectively (16,23). One of the mechanisms of the potent antiproliferative effect of combination therapy is that Leflunomide inhibits the enzyme Dihydroorotate Dehydrogenase (DHODH) and disrupts pyrimidine biosynthesis, thus driving cancer cells into nucleotide starvation (12,24). DHODH inhibition can disrupt DNA and RNA synthesis, particularly in rapidly dividing cancer cells, and thus arrest the cell cycle in the G1/S phase (25). However, pyrimidine deficiency can also disrupt the cellular energy balance, triggering AMPK activation and making cells more susceptible to metabolic stress (15).

On the other hand, Capsaicin triggers intracellular calcium signaling via TRPV1 receptors, which leads to increased levels of reactive oxygen species (ROS) (7,26). Increased oxidative stress can lead to the disruption of mitochondrial membrane integrity, cytosolic cytochrome c release, and the activation of caspase pathways, which can initiate apoptotic cell death (10). Moreover, TRPV1 activation can trigger the intracellular unfolded protein response (UPR) by increasing Endoplasmic Reticulum (ER) stress, which can further enhance apoptotic signals (27).

Another important point is that Leflunomide can affect p53-dependent and independent cell death pathways via DHODH inhibition. Previous studies have reported that p53 mutant cells may be more sensitive to DHODH inhibition (28). In combination therapy, combining Leflunomide and Capsaicin may also be effective via p53-independent mechanisms, providing advantages in drug resistance development.

The metabolic stress caused by Leflunomide and the increase in oxidative stress caused by Capsaicin lead to overcoming the defense mechanisms of cancer cells. Cancer cells generally try to survive under stressful conditions by gaining metabolic flexibility; however, the disruption of pyrimidine synthesis and increased oxidative load may limit their ability to adapt (29). This dual suppression may render the antioxidant defense systems in cancer cells inadequate. For example, it has been reported that Capsaicin may reduce the resistance of cancer cells to oxidative stress

by inhibiting the Nrf2 pathway (one of the main pathways regulating the cellular antioxidant response) (30). This suggests that the combination therapy may suppress cell growth and weaken the cancer cells' defense mechanisms. Previous studies have shown that Capsaicin alone can exhibit anticancer activity but is often insufficient at low doses (31). Similarly, although Leflunomide has been reported to inhibit proliferation in some cancer cell lines, it is known that high doses can lead to toxic side effects (12). The synergistic effect of these agents used at low doses in our study demonstrates the potential of multi-target treatment approaches that can provide higher efficacy with lower toxicity. Although the IC_{50} value of Capsaicin determined in this study appears relatively high compared to values reported in some other cancer cell lines, this observation may reflect intrinsic resistance mechanisms specific to the LLC1 murine lung carcinoma cells. Furthermore, Capsaicin is a highly lipophilic molecule with poor aqueous solubility and limited cellular uptake under standard *in vitro* conditions. These physicochemical properties often necessitate the use of higher concentrations to achieve effective intracellular accumulation in 2D monolayer cultures. It should also be noted that *in vitro* cytotoxicity data do not always directly translate to in vivo efficacy or toxicity, as pharmacokinetics, metabolism, and tissue distribution can substantially alter drug behavior.

In addition, it has been reported in the literature that using natural compounds together with chemotherapeutic agents can delay or prevent the development of drug resistance (16). New experimental and clinical studies on the combination of Capsaicin and Leflunomide can be evaluated as an effective alternative treatment strategy, especially in resistant lung cancer cases.

Another important aspect of our findings is that both agents can affect different cellular and molecular targets, creating a "dual inhibition strategy" (32). While Leflunomide limits the production of pyrimidines required for DNA and RNA synthesis, Capsaicin triggers mitochondrial stress and apoptotic mechanisms via TRPV1. This combined attack can severely limit the adaptability of cancer cells and overload cellular stress responses, generating an irreversible death signal (33,34,35).

However, our study has some limitations. Firstly, all findings were obtained *in vitro*, and it is not yet clear how the synergistic effect of Leflunomide and Capsaicin will be shaped in vivo models. Therefore, tumor reduction rates, effects on metastatic spread, and drug resistance mechanisms need to be investigated in more detail in mouse models. In addition, detailed preclinical and clinical studies are needed on the pharmacokinetics, tissue targeting, toxicity profile, and long-term efficacy of the combination therapy. In addition, determining different dosing protocols and investigating the potential for use with other chemotherapeutic drugs are among the possible strategies to increase treatment success. Second, the combination's cellular signaling pathways have not yet been clearly elucidated. More comprehensive mechanistic studies should be conducted using proteomic, genomic, or metabolomic approaches. In particular, it is important to investigate the possible synergistic interactions between TRPV1 activation and DHODH inhibition in detail. In addition, conducting analyses on more cell lines to understand how Leflunomide and Capsaicin act on different types of cancer may provide more data for clinical applications. One of the another limitations of the current publication is the prevention of cytotoxic effects of the drug on healty lung epithelial cells. Future studies will include normal cell models for better assessment of therapeutic selectivity and possible side effects. On the other hand, it should be noted that this study is based only on *in vitro* data and should be supported by in vivo studies.

Conclusion

The data obtained in this study show that Leflunomide and Capsaicin, even at sublethal doses, have a strong anticancer effect in combination with mouse lung cancer cells. This effect is thought to occur through the combined induction of intracellular metabolic and oxidative stress. In this respect, the study makes an important contribution to multicomponent treatment approaches. Further preclinical and clinical studies may verify the safety and efficacy of this combination and allow it to be evaluated as an alternative or complementary treatment to chemotherapy.

In terms of clinical application potential, a targeted treatment strategy at low doses may carry a lower risk of toxicity compared to traditional chemotherapeutic agents while providing similar or higher efficacy. Especially in aggressive tumors such as lung cancer and with a high potential for developing resistance to treatment, the combination of Leflunomide and Capsaicin is promising in terms of both preventing resistance mechanisms and improving patient tolerability.

In conclusion, this study provides important findings that the combined use of Leflunomide and Capsaicin may have potent anticancer effects on lung cancer cells. Despite various limitations, it is anticipated that the data obtained will shed light on the development of new combined treatment strategies in malignancies with similar treatment difficulties, especially lung cancer.

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