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RESEARCH

Empagliflozin promotes autophagy and apoptosis in A549 lung cancer cells

Empagliflozin A549 akciğer kanseri hücrelerinde otofaji ve apoptozu artırır

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Abstract

Purpose: The objective of the present study is to investigate antiproliferative effects of empagliflozin (EMPA) and to determine whether it induces autophagic cell death in lung cancer cells.

Materials and Methods: A549 lung cancer cells were treated with increasing concentrations of EMPA, and cell viability was assessed by MTT assay. Autophagy and apoptosis related markers (LC3B, caspase-3, caspase-9) were examined by western blotting. Clonogenic assays were performed following treatment with EMPA alone or in combination with everolimus (RAD001) or hydroxychloroquine (HCQ).

Results: EMPA significantly reduced A549 cell viability in a dose-dependent manner. The IC50 values were 54.11 μM at 24 h and 109.6 μM at 48 h. EMPA also markedly decreased clonogenic capacity by 66% and induced morphological alterations. In addition, the expression levels of LC3B and cleaved caspase-3 were increased. Our findings demonstrate that EMPA activates autophagy and triggers caspase-3 dependent apoptotic cell death.

Conclusion: Empagliflozin exerts significant antiproliferative activity in lung cancer cells through the induction of autophagy and caspase-3 mediated apoptosis. Its ability to modulate autophagic and apoptotic pathways suggests that EMPA may represent a potential candidate for future combination based therapeutic strategies in lung cancer. Further mechanistic investigations and in vivo studies are warranted to validate these findings.

Keywords: Empagliflozin, SGLT2, lung cancer, autophagy, apoptosis

Öz

Amaç: Bu çalışmanın amacı, empagliflozin'in (EMPA) antiproliferatif etkilerini araştırmak ve akciğer kanseri hücrelerinde otofajik hücre ölümünü tetikleyip tetiklemediğini belirlemektir.

Gereç ve Yöntem: A549 hücreleri EMPA' nın artan konsantrasyonları ile muamele edildi ve hücre canlılığı MTT testi ile değerlendirilmiştir. Otofaji ve apoptoz ile ilişkili belirteçler (LC3B, kaspaz-3, kaspaz-9) western blotlama ile incelenmiştir Klonojenik analizler, hücrelerin EMPA tek başına veya everolimus (RAD001) ya da hidroksiklorokin (HCQ) ile kombinasyon halinde uygulanmasının ardından gerçekleştirilmiştir.

Bulgular: EMPA, A549 hücre canlılığını doza bağımlı olarak anlamlı şekilde azaltmıştır. IC50 değerleri 24 saat için 54.11 μM, 48 saat için ise 109.6 μM olarak belirlenmiştir. EMPA ayrıca klonojenik kapasiteyi %66 oranında belirgin biçimde azaltmış ve hücresel morfolojide değişikliklere yol açmıştır. Bununla birlikte, LC3B ve kesilmiş kaspaz-3 ekspresyon düzeylerinde artış gözlenmiştir. Bulgularımız, EMPA'nın otofajiyi aktive ettiğini ve kaspaz-3 bağımlı apoptotik hücre ölümünü tetiklediğini göstermektedir.

Sonuç: Empagliflozin, akciğer kanseri hücrelerinde otofaji ve kaspaz-3 aracılı apoptozun indüksiyonu yoluyla belirgin antiproliferatif etki göstermektedir. Otofajik ve apoptotik yolakları modüle edebilme yeteneği, EMPA'nın akciğer kanserinde gelecekteki kombinasyon temelli tedavi stratejileri açısından potansiyel bir aday olabileceğini düşündürmektedir. Ancak bu bulguların doğrulanması için ileri mekanistik çalışmalar ve in vivo araştırmalar gereklidir.

Anahtar kelimeler: Empagliflozin, SGLT2, akciğer kanseri, otofaji, apoptoz

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INTRODUCTION

Lung cancer is one of the most prevalent and lethal malignancies worldwide and continues to represent a major public health challenge despite advances in screening, molecular diagnostics, and targeted therapy[1]. According to global cancer statistics, lung cancer remains one of the most commonly diagnosed cancers and is the leading cause of cancer related mortality in both men and women. Its aggressive biological behavior, coupled with the tendency for late-stage diagnosis, contributes substantially to its poor prognosis[1,2].

Lung cancer is broadly classified into two major histological categories based on morphological, clinical, and molecular features. Non Small Cell Lung Cancer (NSCLC) accounts for approximately 80–85% of all cases and comprises three primary subtypes: adenocarcinoma, squamous cell carcinoma and large cell carcinoma[3]. Small Cell Lung Cancer (SCLC) represents approximately 15–20% of lung cancer cases and is distinguished by rapid doubling time, early metastatic dissemination, and nearly universal TP53 and RB1 alterations. Lung cancer remains the leading cause of cancer-related death, responsible for more deaths annually than colorectal, breast, and prostate cancers combined. Five-year survival rates remain low generally below 20% across all stages primarily because most cases are diagnosed at advanced or metastatic stages[4]. Survival outcomes vary between subtypes: NSCLC shows a moderately better prognosis with the advent of targeted therapies and immunotherapy, whereas SCLC continues to exhibit a markedly poor prognosis despite its initial responsiveness to therapy. Therefore, novel treatment options are still urgetly needed for lung cancer.

Empagliflozin is a highly selective sodium glucose cotransporter-2 (SGLT2) inhibitor widely used in the management of type 2 diabetes mellitus (T2DM)[5]. As one of the leading agents in this class, empagliflozin, empagliflozin (EMPA) reduces renal glucose reabsorption, thereby promoting urinary glucose excretion[6]. In recent years, the expression of SGLT2 has been shown to be elevated in several tumors types. It has been suggested that SGLT2 inhibitors suppress tumor proliferation by lowering the glucose level. Therefore, there has been growing interest in both the potential cancer related risks and the antitumor effects of SGLT2 inhibitors such as canagliflozin, dapagliflozin and empagliflozin.

Autophagy is an evolutionarily conserved cellular process and catabolic pathway that enables the degradation of long-lived proteins, dysfunctional organelles, and intracellular pathogens through lysosome mediated mechanisms[7]. In normal cells, autophagy contributes to cellular homeostasis and exerts tumor suppressive effects; however, in tumor cells and components of the tumor microenvironment, it may shift toward a cytoprotective, pro-survival function. Autophagy is commonly triggered by metabolic and cellular stressors, including nutrient deprivation, starvation, hypoxia, genomic damage, and exposure to chemotherapeutic or radiotherapeutic agents[7-9].

It has been demonstrated that canagliflozin and dapagliflozin suppress cancer cell proliferation and promote apoptosis by inhibiting the mTOR/p70S6K signaling pathway and activating AMPK in both in vitro and in vivo models[5, 10]. Although the body of research on empagliflozin is more limited than that on these agents, existing studies likewise suggest that empagliflozin reduces cell proliferation and enhances apoptotic responses. However, the regulatory role of empagliflozin in the mTOR–autophagy axis remains to be elucidated. Thus, a critical gap exists regarding the molecular mechanisms underlying empagliflozin-induced growth inhibition and its potential to trigger autophagy-associated cell death in lung cancer.

This study provides a novel contribution to the literature by specifically evaluating the relationship between empagliflozin treatment, autophagy, and apoptosis in lung cancer cells, an area that remains insufficiently explored. Unlike previous reports that mainly describe general antiproliferative effects, the present work focuses on molecular markers of autophagy and programmed cell death, particularly LC3, caspase-3 and caspase-9. Therefore, the objective of the present study is to investigate the antiproliferative effects of empagliflozin and to determine whether it triggers autophagic cell death in lung cancer cells.

MATERIALS AND METHODS

Drug preparation

Empagliflozin (EMPA), hydroxychloroquine (HCQ) and everolimus (RAD001) were purchased from Sigma Aldrich (Sigma-Aldrich Corporation, St. Louis, MO). EMPA (stock solution: 10 mM) and RAD001 (stock solution: 5 mM) were solved by DMSO. Stock solution was aliquoted and stored -20 ^{0}C until use. HCQ was dissolved in double-distilled water (stock solution: 2 mM) and freshly prepared before use. All serial dilutions were prepared with DMEM.

Cell culture

The human lung cancer cell lines A549 were purchased from the American Type Culture Collection (Manassas, VA). Cells were cultured in Dulbecco's modified Eagle medium (DMEM, Gibco, Gaithersburg, USA) supplemented with 10% Fetal Bovine Serum (FBS, Gibco, Gaithersburg, USA) and a 100 U/ml penicillin-streptomycin solution (Gibco, Gaithersburg, USA). All cells were incubated with 5% CO_2 at 37°C.

This study was conducted exclusively using in vitro cell culture models and did not involve human participants or experimental animals; therefore, ethics committee approval was not required.

All experimental procedures were performed in the Cell Culture Laboratory of the Faculty of Medicine at Bilecik Seyh Edebali University. The laboratory operates in accordance with institutional quality assurance standards, which ensure traceability, reproducibility, and data integrity through systematic documentation and regular equipment calibration. All experiments, treatment applications, and molecular analyses were carried out by the corresponding author.

Cell viability assays

Cells were seeded in 96-well culture plates at a density of 5 × 10^3 cells per well and treated with varying concentrations of empagliflozin (25, 50, 100, 250, and 500 μM) for 24 or 48 hours. Following treatment, cell viability was assessed using the MTT assay. Briefly, cells were exposed to 1 mg/mL MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) (Sigma-Aldrich, USA) for 1 hour at 37°C. After removing the MTT reagent, the resulting formazan crystals were dissolved in 200 μL of DMSO. Absorbance was subsequently recorded at 570 nm to quantify cell viability.

Western blot analysis

A549 cells were plated into T25 flasks. After 24 hours adhesion, EMPA IC50 concentration was applied, following pretreatment with HQ 25 μM, RAD001 20 μM for 2 h. The cells were harvested, washed twice with ice-cold PBS, and lysed in lysis buffer at 4 °C after empagliflozin treatment. Total protein levels were quantified using a BCA protein assay kit (Thermo Fisher Scientific, MS, USA). Equal amounts of total proteins were were separated by 10% and 15% SDS-PAGE gels and subsequently transferred onto membranes. The membranes were blocked for 1 hour in blocking solution containing 0.1% Triton X-100 and % non-fat dry milk prepared in TBS-Tween 20.

Then they were incubated with primary antibodies against LC3B (Affinity Biosciences, Cincinnati, USA), Caspase-9 and Caspase-3 (Santa Cruz Biotechnology, Santa Cruz, CA). After washing with TBS-T, the membranes were incubated with horseradish peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology, Santa Cruz, CA). β-actin primary and anti-mouse secondary antibodies (Santa Cruz Biotechnology, Santa Cruz, CA) were used to detect β-actin as a loading control. Bound antibodies on the membranes were detected by ECL detection reagents using an imager (Elabscience, TX, USA). All experiments were independently repeated at least triplicate.

Clonogenic assay

Cells were seeded into 6 well-plates (2 x 10^3). 24 hours later was pretreated with HQ 25 uM, RAD001 20uM for 2 h. Then, the cells were treated with EMPA IC50 concentrations. Colonies were grown for 2-week and then stained with 10% crystal violet. Experiments were repeated three independent experiments.

Statistical analysis

Statistical analysis was performed using the one-way ANOVA and the statistical differences were determined by Graphpad Prism9 software. The data were presented as mean ± standard deviation (SD) of

three independent experiments. The half-maximal inhibitory concentration (IC50) calculation were calculated using a non-linear regression algorithms based on cell viability by GraphPad Prism. P values equal or less than ≤ 0.05 were considered as statistically significant. All Experiments were performed in triplicate and independently repeated three times.

RESULTS

We first evaluated the antiproliferative effects of EMPA on lung cancer cell lines using the MTT assay. Cells were cultured in 96-well plates and treated with EMPA at concentrations of 25, 50, 100, 250, and 500 μM for 24 and 48 h. EMPA significantly decreased cell proliferation in A549 cells in a dose-dependent manner (Fig. 1A). The half maximal inhibitory concentration (IC50) was also calculated by dose-response curves and were 54.11 μM and 109.6 μM at 24 h and 48 h, respectively (Fig. 1B). The IC50 value at 24 hour was lower and more effective compared to a 48-hour exposure. Based on these results, the 24 hour IC50 concentration (54.11 μM) was applied for subsequent clonogenic and western blot experiments.

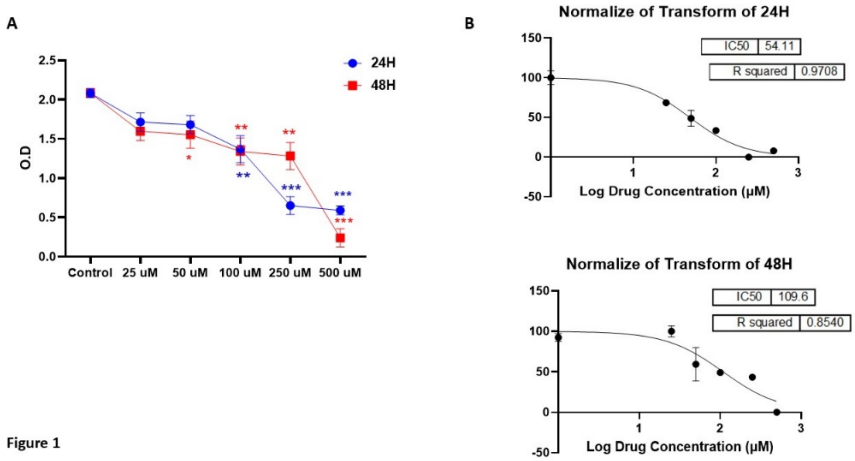

Figure 1

Figure 1. EMPA leads to inhibition of cell proliferation. (A, B) A549 cells were treated with EMPA and cell proliferation was determined by MTT assay after 24 and 48 hours, (*p< 0.05, **p <0.01, ***p < 0.001). All experiments were independently performed three times.

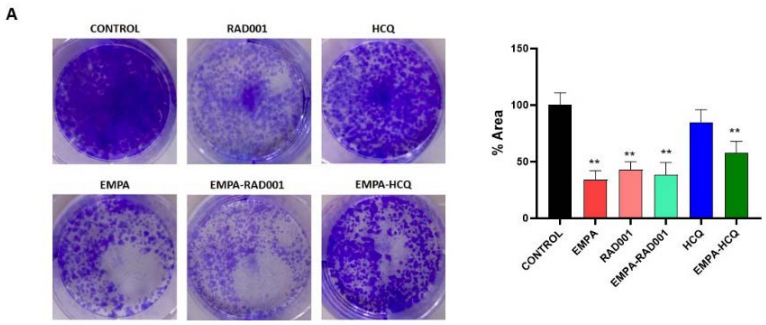

Figure 2. EMPA inhibits colony formation. (A) A549 cells were treated with EMPA and RAD001 or HCQ. The cell viability was performed by colony formation assay. One-way ANOVA was used for significance analysis. The results of colony formation assay are represented as the mean ± standard deviation (SD) values obtained from at least three different experiments (n=3). The percent of colonies area was quantified by ImageJ program and normalized against controls (*p< 0.05, **p <0.01, ***p < 0.001).

RAD001 is an mTOR inhibitor while HCQ inhibits autophagic flux. We further assessed the effects of EMPA, alone or in combination with RAD001 or HCQ on the clonogenic survival of A549 cells. Colonies were grown for 2–3 weeks after treatment, stained with 10% crystal violet, and colony area was calculated and compared with the control group. As shown in Figure 2A, EMPA markedly decreased colony formation of A549 by 66%. The combination of EMPA with RAD001 resulted in 62% diminish in colony formation whereas RAD001 alone decreased it by 57%. HCQ alone reduced clonogenic activity by 15%, whereas combination treatment resulted in a 42% reduction. These findings indicate that EMPA suppresses cell proliferation and clonogenic capacity in lung cancer cells in vitro.

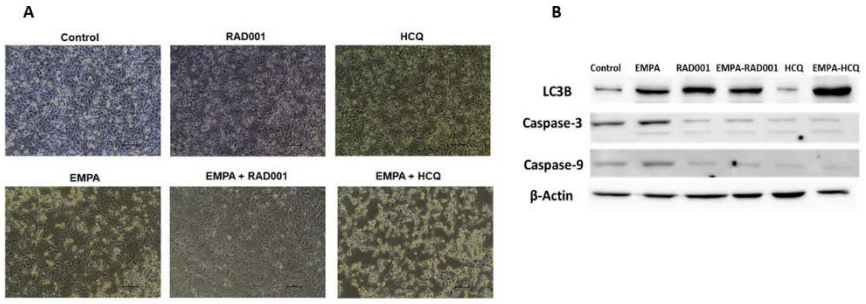

Figure 3. EMPA induces autophagy and apoptosis. (A) Morphology images following EMPA and combined with drugs. (B) A549 cells were exposed to EMPA either RAD001 or HCQ and 24 hours later cells were collected and analyzed by western blot for the expression of LC3B, caspase-3 and caspase-9. B-actin were used as loading control. n=3 biological replicates.

Furthermore, the results in Figure 3A showed that EMPA alone or in combination treatments remarkably altered the morphology of A549 cells.

Apoptosis and autophagy are referred to as programmed cell death I and II respectively, and are also represent crucial mechanisms in cancer therapy. To examine whether EMPA causes cell death, we evaluated the expression of LC3, caspase-3, and caspase-9 by western blot assay. EMPA markedly increased LC3 levels compared to control, indicating activation of autophagy. Consistent with this finding, Figure 3B demonstrates that EMPA induces cleavage of caspase-3. However, caspase-9 protein levels remained unchanged in A549 cells. Our results demonstrate that EMPA activates both autophagy and apoptosis related pathways.

DISCUSSION

Lung cancer constitutes a major global public health burden worldwide in terms of both incidence and mortality. Its development and progression result from dysregulation of various signal pathways depending on oncogene and suppressor genes or epigenetic alterations. EGFR, ALK, and KRAS mutations are frequently found in lung cancer. The PI3K/AKT/mTOR cascade which is downstream of EGFR signal transduction plays a crucial role in proliferation, survival and apoptosis[3]. Secondary mutations in EGFR mutated tumors, resistance to specific targeted therapies and chemotherapy, and the risk of early relapse remain significant challenges in the treatment of lung cancer[11]. Drug combinations and simultaneous targeting of multiple signaling pathways may enhance treatment efficacy[11, 12].

EMPA, SGLT2 inhibitors, were initially developed for type 2 diabetes treatment, but it has become the subject of intense research in recent years due to their unexpected effects on cancer biology. Numerous studies have reported that these molecules disrupt cellular energy balance, leading to AMPK activation and subsequent suppression of the mTOR pathway, resulting in cell cycle arrest, increased apoptosis and decreased tumor growth[13, 14].

Here, we investigated the effects of EMPA on lung cancer cell proliferation and examined whether it induces autophagic cell death. Our results suggest

that EMPA reduces cell viability and induces both autophagic and apoptotic cell death in A549 cells. Emerging evidence also supports the anticancer activity of EMPA. Xie et al. showed that EMPA suppresses tumor growth in cervical cancer nude mice by activating AMPK[15]. Abdelhamid further reported that EMPA decreases cell viability in hepatocellular carcinoma[16]. It has been shown that Empa decreases cell proliferation in MCF-7, 4T1 and A549 cells[17-19]. Application of 100 μg/mL EMPA reduced cell viability by 51.49% in A549. Furthermore, the IC50 value of EMPA was found to be 110 μg/mL at 40 h. Consistent with these studies, our results revealed that treatment with 100 μM EMPA resulted in an approximately 64% decrease in cell viability. Moreover, the IC50 value in our study was 54.11 μM at 24 h. The observation that EMPA exhibited an IC50 values of 54.11 μM at 24 h and 109.6 μM at 48 h suggests that cellular responses to the treatment may vary in a time-dependent manner. Although the mechanisms underlying this difference have not been fully elucidated, the reduced efficacy observed at prolonged exposure may be associated with pharmacodynamic processes. In particular, prolonged exposure may trigger adaptive cellular responses, including the activation of cytoprotective mechanisms such as autophagy and metabolic reprogramming, which could contribute to a gradual decrease in drug sensitivity over time.

Faradi et al. demonstrated that EMPA interacts with caspase-3 in silico analysis. In addition, it has been reported that EMPA induces apoptosis by increasing the levels of cleaved caspase-3 and PARP in cervical cancer cells[20]. In line with these findings, our data demonstrated a significant increase in cleaved caspase-3 expression, whereas caspase-9 expression remained unchanged. The lack of significant changes in caspase-9 expression, may suggest the involvement of a caspase-9 independent apoptotic mechanism. Taken together, these findings suggest a potential crosstalk between autophagy and apoptosis, through which EMPA may engage parallel or interconnected cell death pathways to mediate its anticancer activity.

Autophagy is known to function as a cell protective mechanism under normal physiological conditions. In contrast, it plays a dual role, acting either as a tumor suppressor or as an oncogenic process depending on the cellular context in cancer. It has been shown that EMPA increases autophagy through AMPK/mTOR pathway in non alcoholic fatty liver disease[21]. Abdelhamid et al. also found that EMPA increases autophagy in hepatocellular carcinoma[16]. On the contrary, EMPA suppressed LC3B expression in a DOX-induced cardiotoxicity mouse models[16]. Our findings indicate that EMPA increases autophagic activity. Drug resistance decreases the effectiveness of cancer treatment. Numerous studies have shown that the use of mTOR inhibitors like everolimus (RAD001) or autophagy inhibitors such as HCQ with chemotherapy drugs raises treatment response[22-24]. Similarly, inhibition of autophagy enhances chemosensitivity to anticancer drugs[24]. Dox combined with EMPA has been shown to increase apoptosis and to be effective at lower doses[25]. Karzoon et al. showed that EMPA in combination with tamoxifen was more effective in MCF7 cells[26].

Consistent with these results, EMPA alone reduced the clonogenic area by 66%, indicating a significant suppression of long term proliferative capacity. Notably, co-treatment with RAD001 further decreased clonogenic survival by 62%, demonstrating that the combination was more effective than RAD001 alone (57%) This enhanced inhibitory effect likely reflects the complementary actions of the two agents. Mechanistically, RAD001-mediated inhibition of mTORC1 suppresses cellular growth and protein synthesis, while EMPA induces metabolic stress and disrupts energy homeostasis. The simultaneous targeting of these pathways may synergistically impair survival signaling. Furthermore, RAD001-induced autophagy, which can be cytoprotective when activated alone, may shift toward a pro-death mechanism in the presence of EMPA-associated metabolic and oxidative stress, thereby further limiting clonogenic survival.

At the molecular level, the observed upregulation of LC3B suggests that EMPA activates autophagic flux. While HCQ alone exerted minimal cytotoxic effects, its combination with EMPA markedly enhanced antiproliferative activity, indicating that autophagy may serve as a cytoprotective mechanism in response to EMPA induced cellular stress. Moreover, these findings imply that the biological outcome of EMPA–RAD001 cotreatment is highly context dependent, highlighting the need for optimized dosing regimens and carefully designed sequential treatment strategies in future studies.

Altogether, these results highlight the antitumor potential of EMPA in A549 cells and suggest that its combination with RAD001 or HCQ may enhance therapeutic outcomes by effectively overcoming cellular resistance mechanisms. Our data further

indicate that EMPA exerts its cytotoxic effects through the simultaneous induction of autophagy and apoptosis-related cellular responses, pointing to a dual cell death mechanism that may enhance therapeutic efficacy and reduce the likelihood of resistance development in A549 cells.

This study has several limitations. First, the experiments were conducted exclusively in the A549 lung cancer cell line, which may not fully capture the molecular and phenotypic heterogeneity of lung cancer. Second, although our findings suggest an interplay between EMPA induced autophagy and apoptotic signaling, the precise mechanistic relationships between these pathways remains to be fully elucidated. In particular, a more comprehensive assessment of autophagic flux, including the evaluation of additional markers such as p62/SQSTM1 alongside LC3 processing, is warranted to better characterize autophagy induction. Finally, in vivo studies will be essential to validate the biological relevance of these observations and to determine their potential translational implications.

In conclusion, this study demonstrates that EMPA exerts antiproliferative effects in A549 lung cancer cells through the concurrent modulation of autophagy and apoptosis-related pathways. Although EMPA has shown anticancer activity and multiple potential molecular targets across different cancer types, its clinical applicability in oncology remains to be fully established. The present findings suggest that combination treatment strategies may help overcome resistance mechanisms by simultaneously targeting distinct signaling pathways, thereby enhancing therapeutic efficacy. In this context, EMPA-based combination approaches may represent a promising avenue for future clinical investigation, warranting further preclinical and translational studies to clarify their potential role in lung cancer therapy.

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Peer-review: Externally peer-reviewed.
Conflict of Interest: The authors declare that they have no conflict of interest.
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