

The Effects of Nebivolol-Gefitinib-Loratadine Against Lung Cancer Cell Lines

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Abstract. Background/Aim: Non-small-cell lung cancer (NSCLC) is the most frequently diagnosed malignancy and the first cause of cancer-related death. Thus, finding alternative therapeutic options is crucial. Drug repurposing offers therapeutic options in a simplified and affordable manner, especially to cancer patients in developing countries. Several drugs including antihistamines and beta-adrenergic receptor blockers (beta-blockers) display antiproliferative properties on cancer cells. Interestingly, NSCLC patients who had used either antihistamines or beta-blockers showed improved response to chemotherapy or reduced mortality in comparison to non-users of any of these drugs. However, combination therapy is gaining substantial interest in many cancers including non-EGFR mutated NSCLC. Here, we investigated the antineoplastic effect of the combination of the antihistamine loratadine, the beta-blocker nebivolol, and the tyrosine-kinase inhibitor gefitinib on NSCLC cell lines. Materials and Methods: A-549 and NCI-H1975 cell lines were used. The effect of nebivolol, gefitinib,

and loratadine on the metabolic activity was studied using the MTT assay. The inhibitory concentrations (IC_{20} and IC_{50}) were calculated and used in the drug-combination experiments. Apoptosis was investigated using flow cytometry; and cell survival using the colony formation assay. Results: The combination nebivolol-loratadine-gefitinib produced a significant synergistic effect on inhibiting the metabolic activity and colony formation, as well as on promoting apoptosis in both cell lines. Noteworthy, the effect on the cell line carrying the EGFR mutation (NCI-H1975) was very similar to the cell line that does not exhibit such mutation (A-549 cells). Conclusion: The nebivolol-gefitinib-loratadine combination may be a promising alternative for lung cancer treatment.

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Lung cancer is the leading cause of cancer-related deaths worldwide (1). Non-small cell lung cancer (NSCLC), accounts for approximately 80% of all lung cancer cases and is the subtype in which oncogenic mutations are most relevant (2, 3). The clinical treatment for NSCLC patients includes monoclonal antibodies, chemotherapeutic agents, and molecular targeted therapy (3-10). However, these therapies usually become ineffective after a relatively short period of time, in most cases due to the development of resistance to treatment (11, 12). Therefore, finding better alternative therapeutic options is imperative. To this end, drug combination and repurposing strategies have been used. These therapeutic options possess several advantages including reduced toxicity, low probability of resistance, alternative therapeutic targets, relatively short approval time, and in some cases lower costs (13-18). Antihistamines and beta-adrenergic receptor blockers (beta-blockers) have gained interest in the repurposing of drugs for cancer treatment, including lung cancer. Recent works have demonstrated that the use of antihistamines like loratadine in patients with NSCLC was

associated with a significant reduction in mortality (19-21). In addition, nebivolol is a beta-blocker that has demonstrated antiproliferative effects on NSCLC cells (22). Moreover, some studies found that the beta-blocker use was related to longer overall survival and better progression-free survival in patients with NSCLC (23-26). Actually, our group has demonstrated that the combination of antihistamines with other drugs is a potential therapeutic alternative for liver and lung cancer (27, 28). In this work, we evaluated the potential antineoplastic effect of the loratadine, nebivolol and gefitinib combination on NSCLC human cell lines.

Materials and Methods

Cell lines and reagents. The human lung cancer cell lines A549 and NCI-H1975 were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA), and cultured according to the suppliers instructions. The A549 cell line does not have *EGFR* mutations but has a *RAS* mutation. The NCI-H1975 cell line exhibits the *EGFR* T790M mutation that confers resistance to first-generation tyrosine kinase inhibitors, such as erlotinib and gefitinib. The cells were seeded and cultured for 24 h and then exposed to the different drugs. Gefitinib, loratadine and nebivolol, as well as DMSO were purchased from Sigma Aldrich Co. (St Louis, MO, USA).

Metabolic activity. Metabolic activity was assayed using a colorimetric method with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as previously described (27). Briefly, 3,000 A549 and 3,500 NCI-H1975 cells per well were seeded in 96-well plates and incubated for 72 h in culture medium alone or in the presence of either gefitinib, loratadine, nebivolol or their combinations or DMSO as vehicle. MTT (0.5 mg/ml) was added 4 h before completion of the incubation time. Absorbance data were obtained with a Thermo Fisher MultiskanSkyHigh microplate spectrophotometer (Waltham, MA, USA) using two filters (595 nm and 690 nm). The inhibitory concentrations (IC_{20} and IC_{50}) were obtained by analyzing the concentration-response curves of each drug from the MTT assays.

Apoptosis. Apoptosis was studied using flow cytometry as previously described (27). Briefly, cells (80,000 A549 and 120,000 NCI-H1975) were seeded in 60 mm Petri dishes and incubated for 72 h in culture medium alone or in the presence of the individual drugs or selected combinations, or DMSO. Camptothecin (apoptosis inducer) and methanol (necrosis inducer) were used as controls. Apoptosis was determined with the Annexin V-FITC kit (Thermo Fisher Scientific, Waltham, MA, USA) binding to phosphatidylserine and DNA staining with propidium iodide (PI). Experiments were performed using a flow cytometer (CYAN ADP; Dako, Glostrup, Denmark). Percentages of viable (FITC-negative and PI-negative), apoptotic (FITC-positive and PI-negative) and late apoptotic (FITC-positive and PI-positive) cells were obtained by quadrant analysis using the Kaluza 2.1 software (Beckman Coulter, Inc. Brea, CA, USA).

Colony formation assay. Cell survival was studied using the colony formation assay. Briefly, 250 A549 and 2,500 NCI-H1975 cells were cultured in 60 mm Petri dishes to allow the growth of colonies from single separated cells. 24 h after plating, the cells were incubated for 72 h in culture medium alone or in the presence of the individual

Table I. Inhibitory concentrations [IC (μM)] obtained for each drug and cell line.

	A549			NCI-H1975	
	IC_{10}	IC_{20}	IC_{50}	IC_{20}	IC_{50}
Gefitinib	10.44	13.71	21.84	5.6	13.1
Loratadine	16.30	21.17	33.13	13.92	39.37
Nebivolol	12.21	14.17	18.29	11.74	19.10

drugs or selected combinations, or DMSO. Afterwards, cells were left to grow for 10 and 14 days more, respectively, in the absence of the drugs. Then, cells were fixed in ethanol (absolute grade) for 15 min, stained with crystal violet (1%) for 15 min, rinsed four times with water, observed and counted.

Statistical analysis. Data statistical analysis was performed using the GraphPad Prism software 8.1 (La Jolla, CA, USA). One-way ANOVA analysis, followed by Tukey-Kramer test was performed. p -Values <0.05 were considered as statistically significant.

Results

Calculation of the IC s of gefitinib, loratadine, and nebivolol on the NSCLC cell lines. Concentration-response curves of the drugs were obtained using the MTT assay. We used different concentrations of each drug and compared their effect against that of DMSO (data not shown). From these curves, the IC_{20} and IC_{50} were calculated for both cell lines and used in the next experiments; in the case of the A549 cells the IC_{10} was also studied (Table I).

Enhanced effect of the combinations on the metabolic activity in both cell lines. First, we determined the effect of the drugs alone on the metabolic activity of A549 and NCI-H1975 cells at the corresponding IC s (Figure 1A). Then, the metabolic activity experiments with drug combinations were performed at different IC s. We observed a strong metabolic activity inhibition with the loratadine-nebivolol combination in A549 cells, thus we decided to test combinations with lower concentrations (IC_{10}) of these drugs in this cell line. Even at low concentrations (IC_{10}) some of the two-drug combinations resulted in significantly higher inhibition of the metabolic activity (Figure 1B). Interestingly, the loratadine-nebivolol combination was more effective than the combination of any of these drugs with gefitinib. The strong effect of the two-drug combinations was also observed at the other IC s tested in both cell lines (Figure 1C). In accordance, all the three-drug combinations inhibited almost completely the metabolic activity in both cell lines (Figure 1B and D).

Apoptosis was increased in A549 and NCI-H1975 cells especially by the drug combinations. With the results

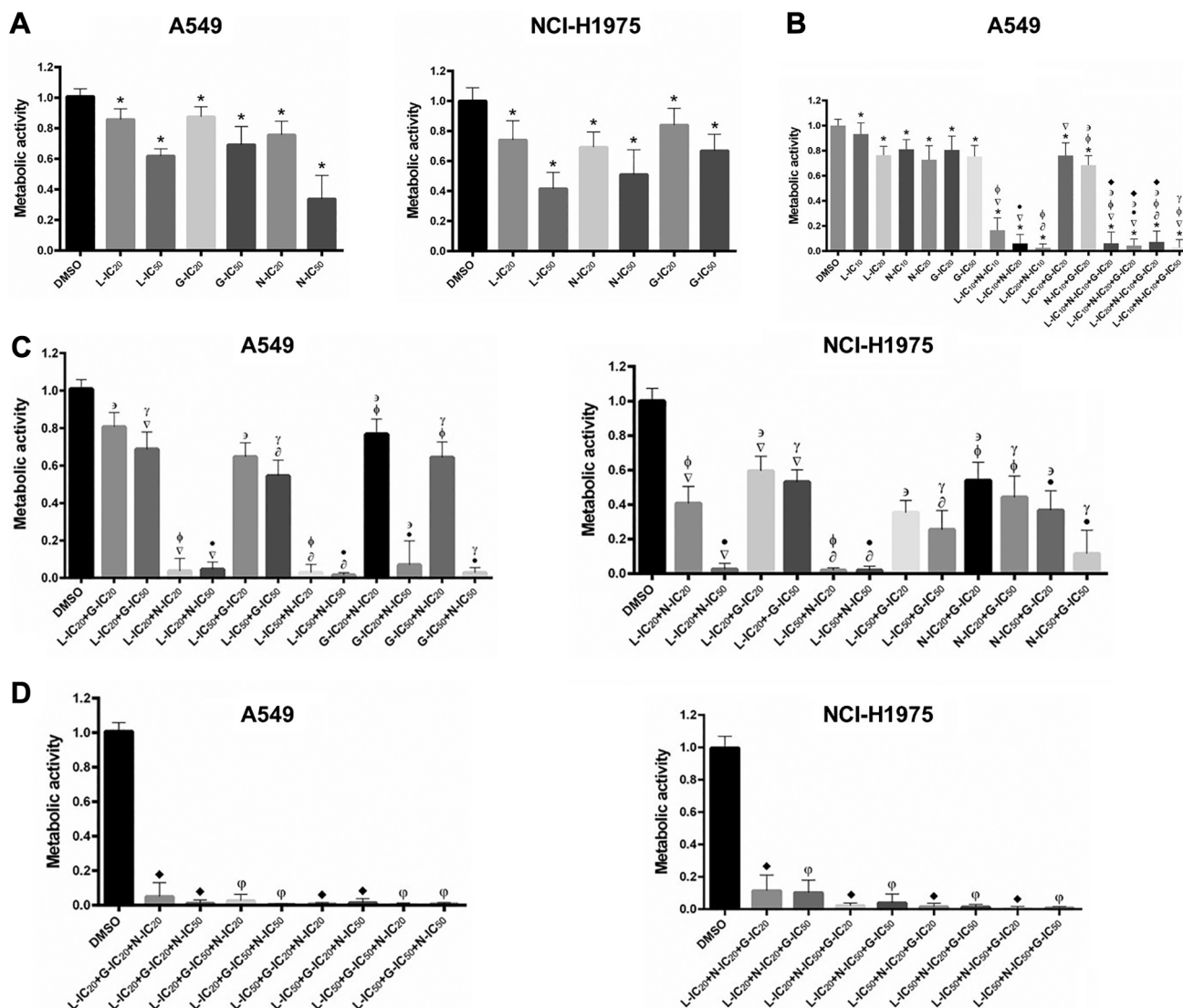


Figure 1. The combinations of gefitinib (G), loratadine (L) and nebivolol (N) abolish the metabolic activity in A549 and NCI-H1975 cell lines. A) Drug-alone effect; B) L-IC₁₀ and N-IC₁₀ and their two- and three-drug combination effects on the A549 cell line; C) two-drug combinations effects at different ICs on both cell lines; D) three-drug combination effects. The combination of two or three drugs significantly inhibited metabolic activity in both cell lines compared to the effect of individual drugs. Cells were cultured in the presence of the drug combinations for 72 h and results are shown as the mean \pm S.D. of eight replicates for each group and from three different experiments. Statistically significant differences vs. the DMSO group (*), vs. L-IC₂₀ (∇) (or L-IC₁₀ in panel B), vs. L-IC₅₀ (\emptyset) (or L-IC₂₀ in panel B), vs. N-IC₅₀ (\bullet) (or N-IC₂₀ in panel B), vs. G-IC₂₀ (\oplus), vs. G-IC₅₀ (γ), vs. groups using G-IC₂₀ (\blacklozenge) in the two-drug combination assay and vs. groups using G-IC₅₀ (ϕ) also in the two-drug combination assay. $p < 0.05$ in all cases.

obtained in the metabolic activity experiments, the effect of the drug-alone and selected drug combinations on apoptosis was studied. In A549 cells, loratadine and gefitinib alone showed no effect on apoptosis when compared to the control. However, nebivolol alone and its combinations increased apoptosis (Figure 2A). Interestingly, the drugs alone did not increase apoptosis in NCI-H1975 cells, but the combinations did (Figure 2B).

Drug combination effect on cell survival. Cell survival (assessed by the colony formation assay) was abolished completely in most cases by the drug combinations in comparison with the drugs alone in A549 cells. Surprisingly, the particular triple combination L-IC₁₀+N-IC₁₀+G-IC₅₀ showed no effect on the cell survival of these cells (Figure 3A). Interestingly, single-drug treatment significantly decreased cell survival in some cases in NCI-H1975 cells,

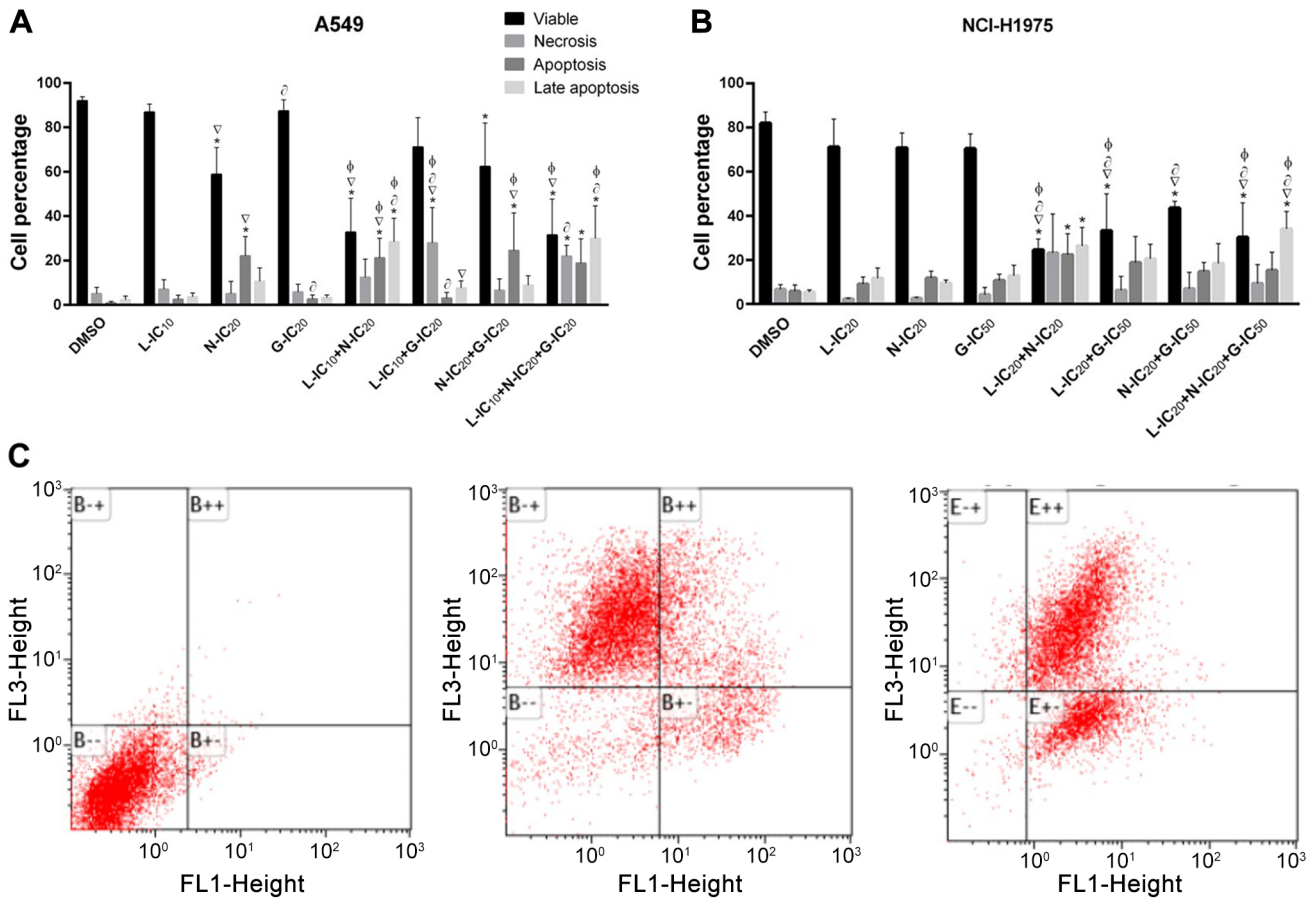


Figure 2. Apoptosis induction by gefitinib (G), loratadine (L), nebivolol (N), and their combinations in lung cancer cell lines. A) Nebivolol and drug combination increased apoptosis in comparison with the DMSO group in A549 cells. B) Drug combination increased apoptosis vs DMSO and drug-alone in NCI-H1975 cells. Cells were cultured in the presence of the combinations or individual drugs for 72 h. Results are shown as the mean±S.D. of three different experiments. C) Representative plots from the flow-cytometry data indicating the different quadrants separating the cell populations: viable cells (left panel), necrosis cells (center panel), apoptosis and late apoptosis cells (right panel). Statistically significant difference vs. the DMSO group (*), vs. L-IC₁₀ (∇) (or L-IC₂₀ in panel B), vs. N-IC₂₀ (∂), vs. G-IC₂₀ (ϕ) (or G-IC₅₀ in panel B). $p < 0.05$ in all cases.

while all the drug combinations tested completely abolished cell survival in these cells (Figure 3B).

Discussion

The current panorama in the management of lung cancer worldwide is almost hopeless. To date, lung cancer continues to be the deadliest neoplasm and represents the sixth cause of death worldwide (1, 29). Unfortunately, despite the existence of several therapeutic strategies offered to these patients, they either do not respond to the treatment or develop resistance in a short period of time, as in the case of gefitinib-treated patients (3, 4, 5-9, 10, 11, 12). Gefitinib is a tyrosine kinase inhibitor (TKI) of the epidermal growth factor receptor (EGFR), which is over-expressed in 15 to 50% of the patients with NSCLC (30-32). In many countries, gefitinib is the first-choice treatment for patients

with some common EGFR mutations; however, due to multiple resistance mechanisms, its effectiveness is limited to a few months (33, 34). For this reason, new TKIs such as afatinib and osimertinib have been developed; however, these have shown only a modest improvement compared to gefitinib (35, 36).

In this work, we demonstrate that the combinations of two or three drugs (gefitinib, loratadine and nebivolol) have a greater effect on the lung cancer cell lines A549 and NCI-H1975 compared to the effect of each drug alone. In addition, we clearly show that both cell lines are sensitive to the three drugs studied, but with different sensitivity. The inhibitory concentrations found were very similar to those reported (where available) in several studies including our own work (19, 20, 32, 37). In the drug combination experiments, we observed a synergistic effect in both cell lines when combining nebivolol with loratadine. Interestingly, we observed a greater

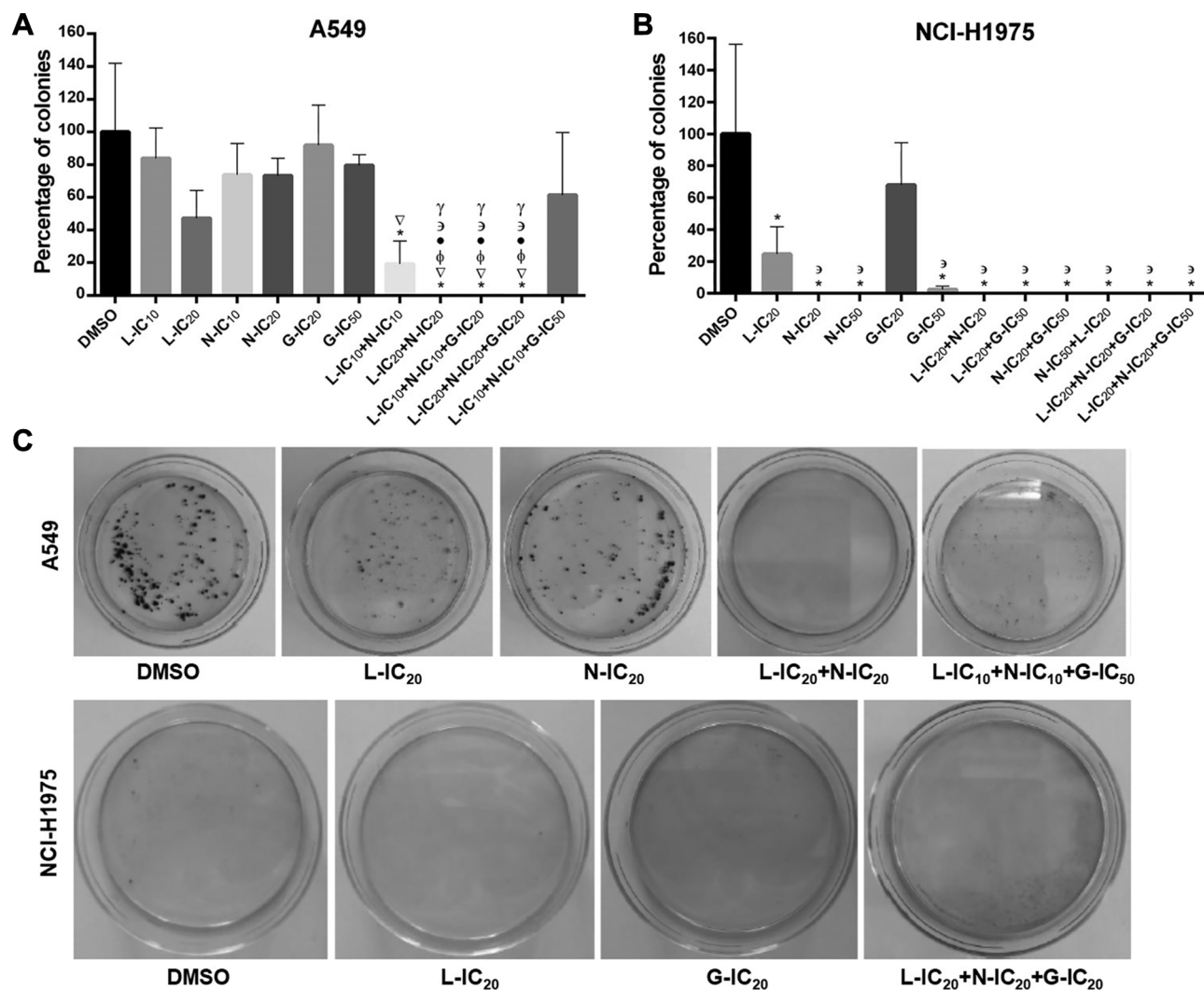


Figure 3. Drug combinations of gefitinib (G), loratadine (L), and nebivolol (N) suppress cell survival in A549 and NCI-H1975 cells. The combinations tested were able to completely inhibit the formation of colonies. Percentage of colonies formed under each condition in A) A549 and B) NCI-H1975 cell lines. Cells were cultured in the presence of the individual drugs or their combinations or DMSO for 72 h. Results are shown as the mean±S.D. from three different assays. C) Representative examples of colony formation in Petri dishes, cells were stained with crystal violet (1%). Statistically significant differences vs. the DMSO group (*), vs. L-IC₁₀ (▽), vs. N-IC₁₀ (φ), vs. N-IC₂₀ (●), vs. G-IC₂₀ (≡), vs. G-IC₅₀ (γ). $p < 0.05$ in all cases.

sensitivity in the A549 cell line, therefore we also decided to use the IC₁₀ of both drugs in these cells. Noteworthy, even at these low ICs used, we still observed a significant synergistic effect. In a few cases, antagonistic effects were observed when combining gefitinib with any of the other drugs; however, this was avoided when using higher ICs of the drugs.

Remarkably, all the three-drug combinations studied abolished almost completely the metabolic activity and colony formation even when using the IC₁₀ of loratadine and nebivolol. The two-drug combination of loratadine and nebivolol displayed notable effects on apoptosis, as well as in combination with gefitinib.

The potential molecular mechanisms explaining the observed anticancer effects are depicted in Figure 4. Loratadine accumulation in the lysosomes causes the leak of proteolytic enzymes and H⁺ and Ca⁺⁺ ions into the cytoplasm inducing the apoptotic process (20). Nebivolol could trigger several processes such as cell arrest, mitochondrial respiration inhibition (38), and proteasome-mediated EGFR degradation (22). In addition, the activation of the beta-adrenergic receptor leads to increased levels of PKA, which in turn enhances cell proliferation *via* the RAS-RAF-MEK- ERK signaling pathways. As a consequence, beta-blockers like nebivolol decrease cell proliferation. Besides, nebivolol is also capable of inhibiting

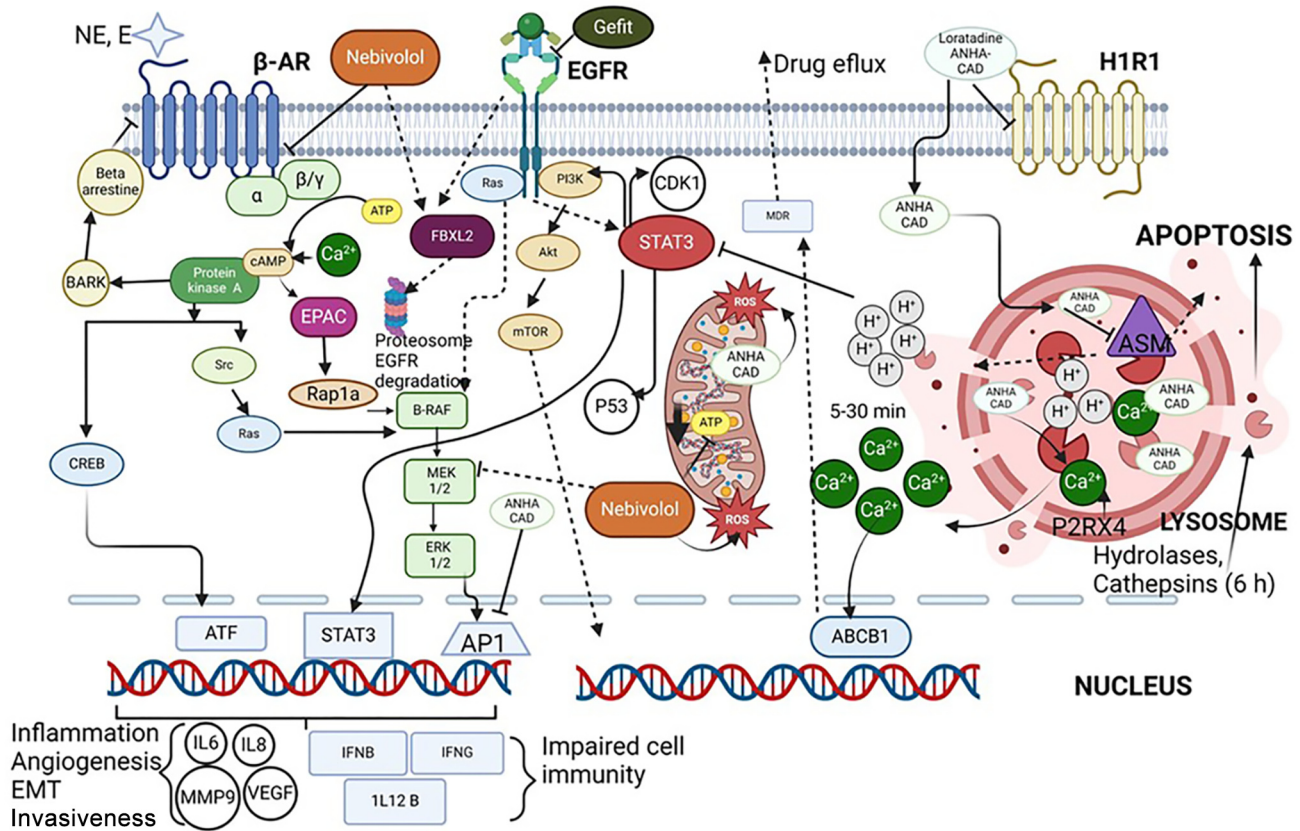


Figure 4. Potential molecular mechanisms involved in the anticancer effects of the combination gefitinib-loratadine-nebulivol on lung cancer cells. The β-AR and EGFR signaling pathways are proliferative in nature; their overstimulation (β-AR) or oncogenic mutations (EGFR) lead to uncontrolled proliferation of neoplastic cells. However, antagonism of these receptors by nebulivol and gefitinib, respectively, inhibits these pathways. Additionally, nebulivol promotes apoptosis by producing oxidative stress at the mitochondrial level while simultaneously inhibiting mitochondrial respiration and facilitating the degradation of the EGFR receptor in proteasomes through the activation of FBXL2. It is also theorized that it can directly inhibit MEK, in the MAPK proliferative pathway. Furthermore, the lysosomal accumulation of loratadine, an amphiphilic cationic antihistamine (ANHA-CAD) (19) stimulates the release of Ca²⁺ and H⁺, producing inhibition of multidrug-resistant (MDR) proteins by Ca²⁺ and acidification of the cytosol by H⁺, which dephosphorylates and inactivates STAT3, thus inhibiting the STAT3-PI3K-Akt-mTOR signaling. The accumulation of loratadine inside lysosomes stimulates P2RX4, which is responsible for expelling Ca²⁺ into the cytosol, and inhibits ASM (acid sphingomyelinase), which causes an accumulation of ceramides and destabilization of the lysosomal membrane, producing its fragmentation and consequently the release of hydrolases and cathepsins into the cytosol, which favors the initiation of apoptosis and pyroptosis.

MEK kinase, which has been associated with resistance to treatments with TKIs. Furthermore, nebulivol has recently been shown to enhance the sensitivity of breast cancer cells to the fibroblast growth factor receptor (FGFR) inhibitor erdafitinib by reduction in AKT activation (39). However, nebulivol can bind to the FBXL2 chaperone, which becomes more efficient in degrading the EGFR receptor regardless of the resistance mutations, which could be favoring the disappearance of cellular resistance to gefitinib (22, 40). Therefore, the signaling pathways targeted by loratadine and/or nebulivol may create a suitable environment leading to tumor cell eradication.

We demonstrate that combining two or three-drugs-gefitinib, loratadine, and nebulivol- is a potential approach for lung cancer treatment. Although several studies explaining the precise mechanism of the combination effect are needed, these

results suggest that the triple combination gefitinib-loratadine-nebulivol could serve as a novel therapeutic strategy for lung cancer. The potential anticancer effect of this combination on lung cancer cells displaying other mutations deserves further investigation. We propose this combination as a worthy translational approach (for instance in Phase II clinical trials), that may help to decrease the mortality from this disease.

Conflicts of Interest

The Authors declare no conflicts of interest in relation to this study.

Authors' Contributions

JLM-L and EH-G performed the experiments; JLM-L, EH-G, MdGC-L and JC analyzed the data; JLM-L, MdGC-L and JC wrote

the paper; JLM-L, MDGC-L, RV-V and JC made substantial contributions to the manuscript; all Authors contributed to the design of the experiments, reviewed, and approved the manuscript.

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