

The combination astemizole–gefitinib as a potential therapy for human lung cancer

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Abstract: Lung cancer is a major cause of cancer mortality. Thus, novel therapies are urgently needed. Repositioning of old drugs is gaining great interest in cancer treatment. Astemizole is an antihistamine proposed to be repositioned for cancer therapy. This drug targets several molecules involved in cancer including histamine receptors, ABC transporters and the potassium channels Eag1 and HERG. Astemizole inhibits the proliferation of different cancer cells including those from cervix, breast, leukemia and liver. Gefitinib is widely used to treat lung cancer; however, no response or drug resistance occurs in many cases. Here, we studied the combined effect of astemizole and gefitinib on the proliferation, survival, apoptosis and gene and protein expression of Eag1 channels in the human lung cancer cell lines A549 and NCI-H1975. Cell proliferation and survival were studied by the MTT method and the colony formation assay, respectively; apoptosis was investigated by flow cytometry. Gene expression was assessed by real-time polymerase chain reaction (RT-PCR), and protein expression was studied by Western blot analysis and immunocytochemistry. We obtained the inhibitory concentrations 20 and 50 (IC₂₀ and IC₅₀, respectively) values for each drug from the cell proliferation experiments. Drug combination at their IC₂₀ had a superior effect by reducing cell proliferation and survival in up to 80% and 100%, respectively. The drugs alone did not affect apoptosis of H1975 cells, but the drug combination at their IC₂₀ increased apoptosis roughly four times in comparison to the effect of the drugs alone. Eag1 mRNA levels and protein expression were decreased by the drug combination in A549 cells, and astemizole induced subcellular localization changes of the channel protein in these cells. Our in vitro studies strongly suggest that the combination astemizole–gefitinib may be a novel and promising therapy for lung cancer patients.

Keywords: astemizole, gefitinib, potassium channels, lung cancer

Introduction

Lung cancer is the major cause of cancer-related deaths worldwide.^{1,2} Most of the primary lung cancers are non-small-cell lung cancer (NSCLC).^{3,4} The most important oncogene drivers in NSCLC patients are mutations in the epidermal growth factor receptor (EGFR) gene; actually, activating mutations are a prime therapeutic target.^{5,6} The most common “sensitizing mutations” in EGFR (exon 19 deletions and exon 21 L858R mis-sense substitutions) result in constitutive activation of the receptor without ligand binding. In accordance, some of the EGFR tyrosine kinase inhibitors (TKIs) target mutant EGFRs.^{5,7–9} The first-generation EGFR inhibitors such as gefitinib were the first EGFR-targeted therapies to be registered and later approved by the US Food and Drug Administration (FDA) as a treatment for lung cancer.^{6,10} Unfortunately, although many patients initially respond to EGFR-targeted therapies, most of them eventually developed resistance and relapsed.¹¹ One of the mechanisms of drug resistance involves the extrusion of TKIs by ATP-binding cassette (ABC) multidrug efflux pumps.^{12,13} ABCG2 (breast cancer resistance protein) transports gefitinib which also interacts with

ABCB1 and ABCC1.¹⁴ Because drug resistance decreases the efficacy of the drug, it is necessary to find alternative therapeutic strategies for lung cancer patients.

Drug repositioning involves the identification of novel indications for already existing, well-characterized and well-tolerated drugs reducing costs and bypassing safety concerns; this reposition strategy has emerged as an attractive alternative for cancer treatment.^{15–18} Astemizole is an anti-histamine that has gained enormous interest to be repositioned for cancer treatment because it targets several molecules involved in cancer including H1 histamine receptors, P-glycoproteins and members of the potassium channel family *ether à-go-go*.^{19–28} Human ether à go-go-1 (Eag1, Kcnh1 and Kv10.1) is a voltage-gated channel that displays oncogenic properties and has gained great interest in cancer research.^{25,26,29,30} The distribution of Eag1 is very restricted in normal tissues. It is mainly expressed in the brain, but low amounts can be detected in placenta, testes and adrenal glands and transiently in myoblasts.^{29,31,32} Conversely, Eag1 is overexpressed in most human tumors, including liver, cervical, lung, breast, colon and prostate cancer.^{28,32–35} Astemizole decreases tumor cell proliferation in vitro in breast, liver, cervical and lung cancer cell lines as well as in in vivo breast and liver cancer models.^{16,24,28,36} In addition, astemizole potentiates the effect of other anticancer drugs in leukemia and breast cancer cells and downregulates Eag1 mRNA expression in breast cancer cells; in addition, astemizole binds to chromatin.^{36–39} Interestingly, the use of astemizole and loratadine was associated with reduced mortality from different cancers, and astemizole induced sensitization to chemotherapy and reversion of multidrug resistance in NSCLC cells.¹⁶

Therefore, because of the high mortality-to-incidence ratio of lung cancer and the potential use of anti-histamines in cancer treatment as well as due to the multitarget properties of astemizole and its synergism with anticancer drugs, here we investigated whether the combination astemizole–gefitinib may be a potential anticancer treatment for lung cancer cells.

Materials and methods

Cells lines and reagents

The human NSCLC cell lines A549 and NCI-H1975 were obtained from the American Type Culture Collection (Manassas, VA, USA) and cultured according to the manufacturer's instructions. Astemizole and DMSO were purchased from Sigma-Aldrich Co. (St Louis, MO, USA). Gefitinib (Iressa[®], ZD1839) was kindly provided by AstraZeneca plc (Cambridge, UK). The anti-Eag1 antibody was purchased from Novus International (Littleton, CO, USA) and the anti-actin antibody from Sigma-Aldrich Co.

Metabolic activity

Cell proliferation (assessed by metabolic activity) was assayed by a colorimetric method with 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) as described previously.²⁸ Briefly, 3×10^3 cells per well were seeded in 96-well plates and incubated for 72 hours in culture medium either alone or in the presence of astemizole, gefitinib or DMSO as vehicle. MTT (0.5 mg/mL) was added 4 hours before completing the whole incubation time. Absorbance data were obtained with a microplate photometer (Sunrise Touchscreen).

Colony formation assay

Cell survival was studied with the colony formation assay. Briefly, 2×10^2 A549 and 5×10^2 NCI-H1975 cells were cultured in 60 mm Petri dishes to allow the growth of colonies from single separated cells. Twenty-four hours after plating, the cells were incubated for 72 hours in culture medium alone or in the presence of DMSO or the drugs. Afterward, A549 and NCI-H1975 cells were left to grow for 6 and 11 days more, respectively, in the absence of the drugs. Then, cells were fixed in ethanol (absolute grade) for 15 minutes, stained with crystal violet (1%) for 15 minutes and then rinsed four times with water, observed with a microscope and counted.

Apoptosis

Apoptosis was studied by flow cytometry as described previously.²⁸ Briefly, 4×10^4 cells were seeded in culture plates and incubated during 72 hours in culture medium alone or in the presence of astemizole, gefitinib or vehicle (DMSO). Camptothecin (apoptosis inductor) and methanol (necrosis inductor) 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 by propidium iodide (PI). Experiments were performed by flow cytometry (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 Summit 4.3 software.

Immunocytochemistry

Cell lines were grown on charged glass slides and boiled for antigen retrieval, then blocked with endogenous peroxidase blocker (Bio SB, Santa Barbara, CA, USA) for 10 minutes and then incubated in the presence of 1:500 anti-Eag1 antibody overnight at 4°C. The slides were then incubated with secondary biotin antibody (Bio SB) for 15 minutes and then incubated with streptavidin polymer (Bio SB) for 15 minutes.

The specific staining reaction was completed by incubating the slides in the presence of diaminobenzidine in buffer reaction solution (Bio SB) and observed as a brown staining. Sections were counterstained with hematoxylin (Dako). The slides were observed in an Olympus IX51 microscope, Olympus DP70 camera (Tokyo, Japan).

Real-time polymerase chain reaction (RT-PCR)

Total RNA was extracted from cell cultures with TRIzol reagent. Five micrograms of total RNA was reverse transcribed using the Moloney Murine Leukemia Virus Reverse transcriptase (M-MuLV) (New England BioLabs Inc., Ipswich, MA, USA). RT-PCR was performed with 1 μ L of cDNA using the TaqMan™ detection system (Thermo Fisher Scientific) and the Universal PCR Master Mix reagents kit (Thermo Fisher Scientific). Probes previously developed from TaqMan were used to study *Eag1* (ID: Hs00924320_m1) and *Gusb* (ID: Hs00939627_m1, as a constitutive gene) expression. The PCR reaction protocol was 95°C for 15 seconds and 60°C for 1 minute (40 cycles). Data were analyzed with the $2^{-\Delta\Delta C_t}$ method.

Western blot

Cells were washed, scrapped and centrifuged, and the obtained pellet was resuspended in lysis buffer supplemented with protease inhibitors. Lysis was completed with the freezing–thawing process; the lysate was centrifuged and the supernatant collected. Forty micrograms of protein was

separated on sodium dodecyl sulfate polyacrylamide gel electrophoresis (10%), transferred to a nitrocellulose membrane and incubated with either the anti-*Eag1* antibody (1:750) or the anti-actin antibody (1:100,000). The relative protein quantification was performed with the ImageJ software (NIH, Bethesda, MD, USA).

Statistical analysis

Statistical analysis was performed with analysis of variance (ANOVA) followed by the Tukey–Kramer test using GraphPad Prism software version 5.0 (La Jolla, CA, USA). The differences between the drug combination groups and either the drug-alone groups at the corresponding concentration or the control or the vehicle are shown. The analysis of Western blot data was performed with the Student's *t*-test. *P*-values <0.05 were considered to be statistically significant.

Results

Concentration-dependent effect of astemizole and gefitinib on the proliferation of lung cancer cells

We first investigated the effect of astemizole and gefitinib alone on the proliferation of A549 and NCI-H1975 lung cancer cells, assessed by the metabolic activity. The effect of gefitinib is well known in both cell lines,^{40,41} and the effect of astemizole has been previously reported in A549 cells.¹⁶ As expected, both drugs decreased the metabolic activity in a concentration-dependent manner in both cell lines (Figure 1).

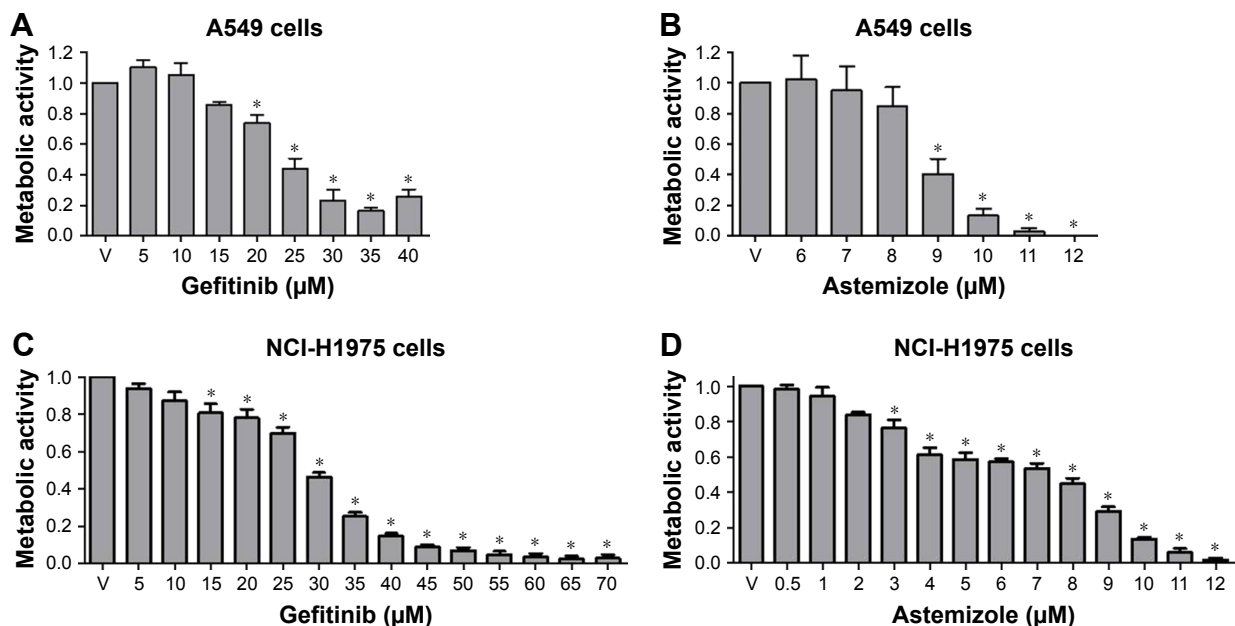


Figure 1 Effect of astemizole or gefitinib on the metabolic activity of lung cancer cells.

Notes: Astemizole or gefitinib decreased the proliferation of A549 (A and B) and NCI-H1975 (C and D) lung cancer cells in a concentration-dependent manner. Four different cell cultures were performed with six technical replicates each. Mean \pm SD, **p*<0.05 vs vehicle (V [DMSO]).

Table 1 Inhibitory concentrations (ICs) of astemizole or gefitinib in the proliferation of human lung cancer cells

Cell line	Astemizole (μM)		Gefitinib (μM)	
	IC ₂₀	IC ₅₀	IC ₂₀	IC ₅₀
A549	7.5	9.2	15	26.48
NCI-H1975	7.75	8.85	21.56	29.62

From these experiments, we obtained the IC₂₀ and IC₅₀ values for each drug in each cell line (Table 1).

Enhanced effect of the combination astemizole–gefitinib on the proliferation, survival and apoptosis of lung cancer cells

Cell proliferation experiments combining the drugs at their IC₂₀ and IC₅₀ concentrations were performed. The combination

of the drugs had superior anti-proliferative effects in both cell lines. Astemizole-IC₂₀ in combination with gefitinib-IC₂₀ decreased metabolic activity by 80%, whereas the combination of astemizole-IC₂₀ and gefitinib-IC₅₀ decreased metabolic activity by 95% in A549 cells (Figure 2A). In the NCI-H1975 cells, the combinations of astemizol-IC₂₀ plus gefitinib-IC₂₀ and astemizole-IC₂₀ plus gefitinib-IC₅₀ showed a decrease of metabolic activity by 65% and 90%, respectively (Figure 2A). Based on these results, for the rest of the experiments, we decided to focus on the drug combination at the IC₂₀ found in the cell proliferation assays (Table 1).

Cell survival (assessed by the colony formation assay) was decreased in a very pronounced manner by the drug combination in comparison with the drugs alone in both cell lines (Figure 2B). In this assay, the combination

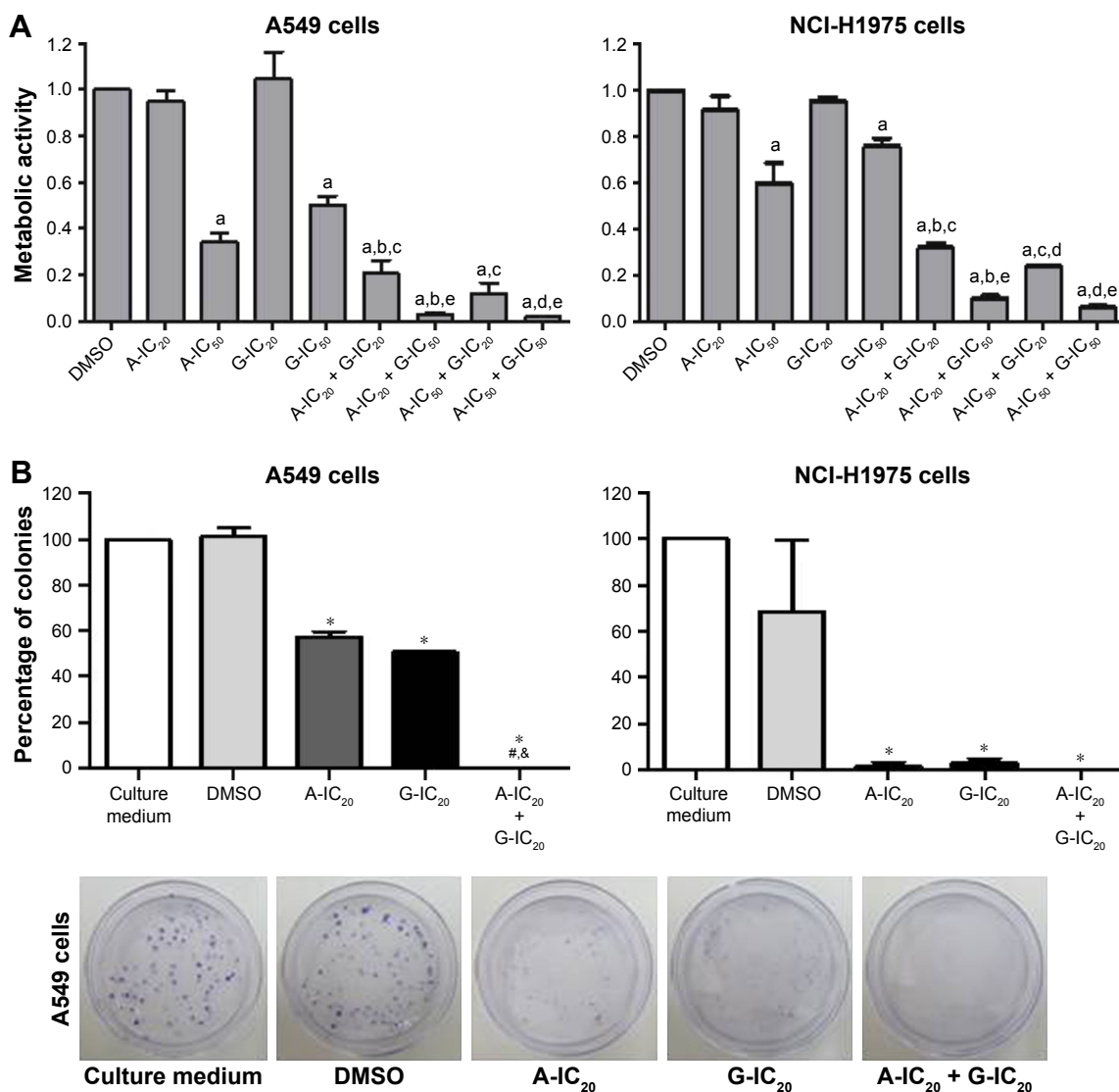


Figure 2 (Continued)

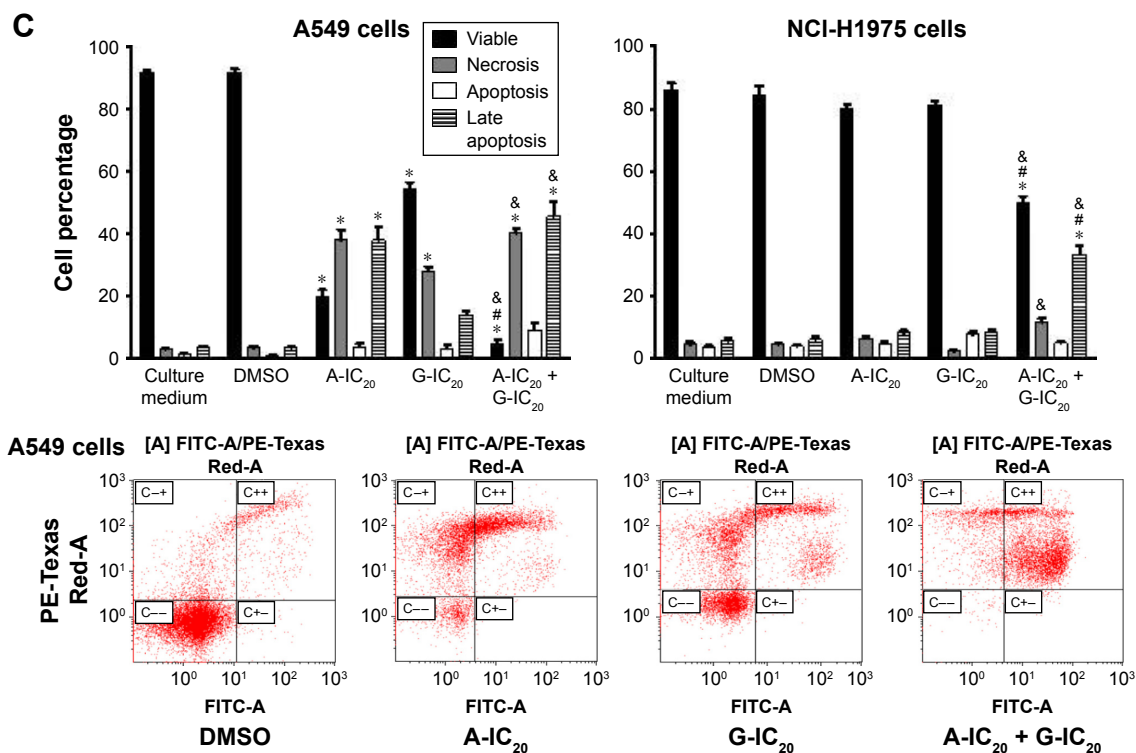


Figure 2 Enhanced effect of the combination astemizole–gefitinib on the metabolic activity, survival and apoptosis of lung cancer cells.

Notes: The combinations of the drugs at different ICs had outstanding effects on metabolic activity (A) in comparison with the effects of the drugs alone at their corresponding concentrations. Some of the most superior effects were observed when combining astemizole and gefitinib at their IC₂₀ (A-IC₂₀ and G-IC₂₀, respectively), and A-IC₂₀ with G-IC₅₀ in both cell lines. Four different cell cultures were performed with eight technical replicates each. Mean \pm SD, * p <0.05 vs vehicle (DMSO), [#] p <0.05 vs A-IC₂₀, [§] p <0.05 vs G-IC₂₀, [¶] p <0.05 vs A-IC₅₀ and [¶] p <0.05 vs G-IC₅₀. Colony formation assays (B) showed that both drugs decreased cell survival, but the effect was significantly stronger with the drug combination in both cell lines. Representative images of A549 colonies are shown in the bottom of the panel. Three different cell cultures were performed with three technical replicates each. Mean \pm SD, * p <0.05 vs medium and vehicle (DMSO), [#] p <0.05 vs A-IC₂₀ and [§] p <0.05 vs G-IC₂₀. Astemizole, gefitinib and the drug combination significantly increased apoptosis in comparison with controls in A549 cells (C), but the effect of the combination was not higher than that produced by astemizole alone. However, in NCI-H1975 cells, the drugs alone did not increase apoptosis in comparison with controls, but the drug combination was the only condition that clearly increased apoptosis in comparison with the rest of the groups. Representative plots from FACS experiments in A549 cells indicating the different quadrants separating the cell populations (bottom panels). Four different cell cultures were tested with three technical replicates each. Mean \pm SD, * p <0.05 vs vehicle (DMSO), [#] p <0.05 vs A-IC₂₀, [§] p <0.05 vs G-IC₂₀. **Abbreviations:** A, astemizole; G, gefitinib; FACS, fluorescence-activated cell sorting.

decreased cell survival completely in both cell lines. Some representative images are shown in Figure 2B. Then, we studied apoptosis by flow cytometry. The drug combination decreased the percentage of viable cells more than the drugs alone in A549 cells, but the effect of the combination on apoptosis was not higher than that produced by astemizole alone (Figure 2C). However, while the drugs alone did not increase apoptosis in comparison with control experiments (in the absence of the drugs) in NCI-H1975 cells, the drug combination increased apoptosis almost four times in comparison with the effect of any of the drugs alone (Figure 2C).

The combination astemizole–gefitinib regulates Eag1 channel expression

Because Eag1 channel is one of the potential targets of astemizole, and channel expression is regulated by this drug in breast cancer cells,³⁶ we wondered if the drug treatment might regulate Eag1 expression in both cell lines.

The drug treatment did not modify Eag1 mRNA levels or protein expression in NCI-H1975 cells (data not shown). However, while the drugs alone did not affect Eag1 mRNA expression in A549 cells, the drug combination decreased Eag1 mRNA levels by 75% in comparison with the expression in either control experiments or the presence of any drug alone (Figure 3A). Western blot analysis also revealed that the drug treatment significantly decreased Eag1 protein expression in A549 cells (Figure 3B and C). Immunocytochemistry studies showed that control cells treated with either the vehicle or astemizole displayed strong brown immunostaining (Figure 3D–E). Despite that immunostaining was not that strong in the gefitinib-treated cells (Figure 3F), the weakest signal was observed in the cells treated with the drug combination (Figure 3G). Interestingly, astemizole induced subcellular accumulation of the channel in some parts of the cell adjacent to the nucleus (Figure 3E). This subcellular rearrangement of Eag1 after treatment was exhibited only in A549 cells.

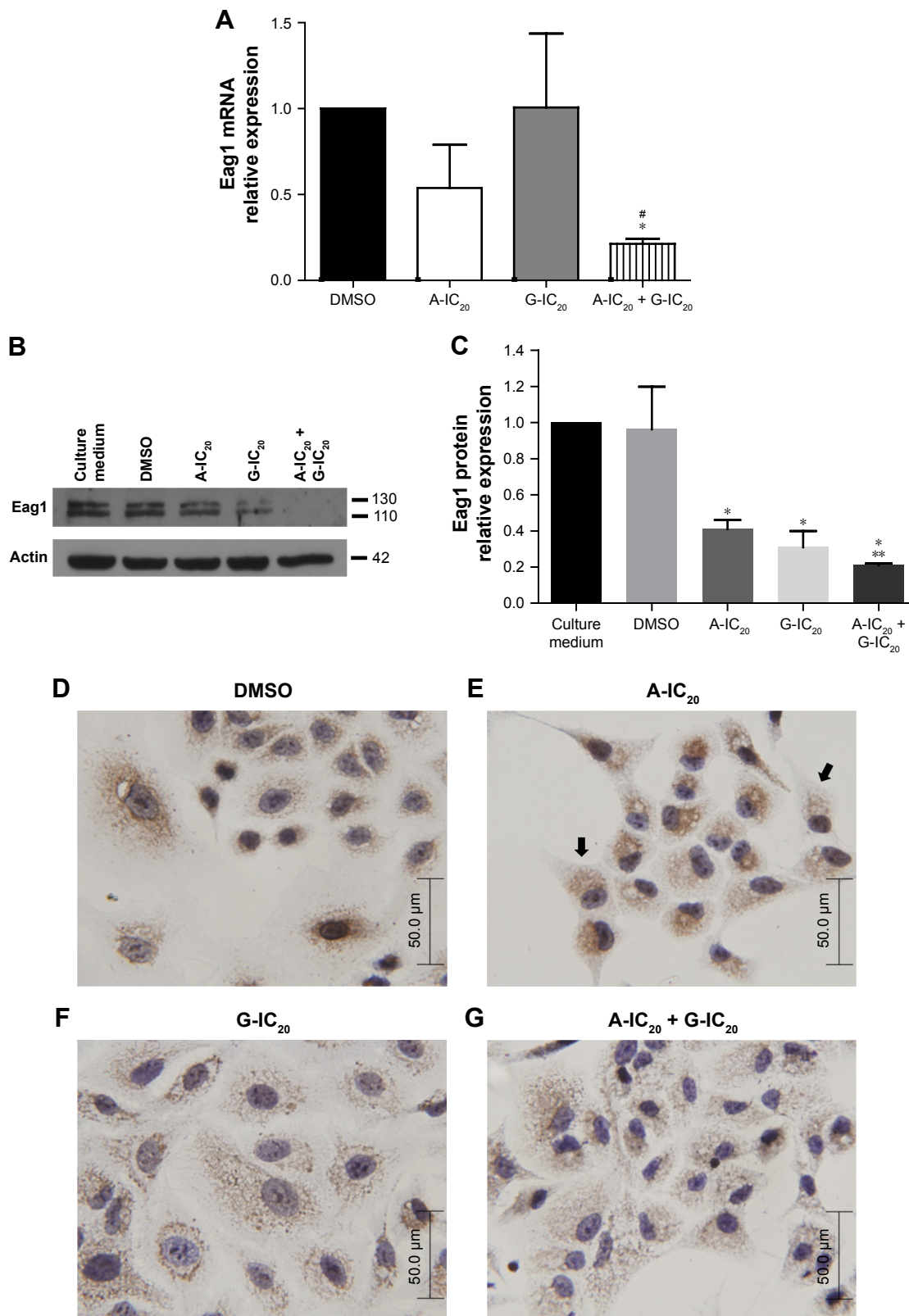


Figure 3 The combination astemizole–gefitinib downregulates Eag1 expression in A549 cells.

Notes: (A) Relative Eag1 mRNA expression was decreased only in the cells treated with the drug combination. Mean \pm SD, $^*p < 0.05$ vs vehicle (DMSO), $^{\#}p < 0.05$ vs G-IC₂₀. Western blot analysis revealed that Eag1 protein expression is clearly downregulated by the drug treatment in a significant manner (B and C). Mean \pm SD, $^*p < 0.05$ vs medium and vehicle (DMSO), $^{**}p < 0.05$ vs A-IC₂₀. The presence of Eag1 protein is revealed by immunocytochemistry as brown immunostaining (D–G). Vehicle-treated and astemizole-treated cells show strong Eag1 expression in the cytoplasm (D and E, respectively), but astemizole induced subcellular localization changes in some areas adjacent to the nucleus. Gefitinib-treated cells (F) showed less immunostaining, but the weakest signal was observed in the cells treated with the drug combination (G). Three different cell cultures were performed for all the experiments. Original magnification = 200 \times .

Abbreviations: A, astemizole; G, gefitinib.

Discussion

Lung cancer is the main cause of cancer-related deaths worldwide, and thus, identifying new therapeutic strategies is urgently needed.^{1,2} Unfortunately, most of the patients either do not respond or develop resistance to one of the most common treatments for lung cancer, namely, gefitinib.¹¹ Recently, the anti-histamine astemizole has gained great interest by its anticancer effects, either alone^{20,22,23,25,28,42} or in combination.^{16,20,36,37} In addition, epidemiological studies associated the use of astemizole and loratadine with reduced mortality from different cancers.¹⁶

The effect of gefitinib is well known in the A549 and NCI-H1975 lung cancer cells studied,^{40,41} whereas the effect of astemizole has been previously reported in A549 cells.¹⁶

Because astemizole has several targets involved in cancer,^{19–28,36,37,39} the possible mechanisms explaining the anti-proliferative effects here observed include blockage of oncogenic Eag1 potassium channels, decrease of Eag1 mRNA expression, inhibition of ABC multidrug transporters (which generate resistance to gefitinib)¹⁴ and antagonism of the histamine receptor H1. On the other hand, gefitinib may decrease cell proliferation by at least two possible mechanisms. A potential mechanism is the well-known EGFR-dependent pathway inhibition.^{6,10} The other is EGFR-independent by blocking H2-histamine and H4-histamine receptors, since gefitinib is able to antagonize these receptors and induce cytostasis and differentiation in leukemia cells.⁴³

Here, we also observed that the combination astemizole–gefitinib at low concentrations had superior effects on the metabolic activity, survival and apoptosis of human lung cancer cells. The combination of the drugs at their IC₂₀ decreased cell proliferation in up to 80%, whereas the combination of astemizole-IC₂₀ and gefitinib-IC₅₀ almost completely abolished proliferation in A549 cells. The drug combination also inhibited completely the survival of both cell lines. On the other hand, increased apoptosis was exclusively observed in NCI-H1975 cells only in the presence of the drug combination but not with the drugs alone. Some of the plausible mechanisms explaining the enhanced effects of the drug combination are the convergence on the histamine pathways (both can antagonize different histamine receptors), the increase in gefitinib concentration due to the blockage of ABC transporters by astemizole and/or the decrease in the expression of oncogenic Eag1 channels. Further studies are needed to elucidate the precise mechanism of the enhanced effects of the combination like testing the effect of other antihistamines, silencing or overexpressing the drug targets including histamine receptors, Eag1 potassium channels and ABC transporters, as well as testing the effect of other EGFR inhibitors.

In addition, we found that astemizole induced subcellular accumulation of Eag1 channels. More studies are needed to elucidate in which subcellular compartments this channel relocalization may be taking place and if this rearrangement may be associated with the anti-proliferative mechanism of astemizole. The cell lines displayed some differences in the drug responses. Despite that both cell lines were derived from biopsies of patients with NSCLC, the differences may reflect the heterogeneity of lung cancer cells observed in patients. However, the superior effect of the drug combination was maintained in both cell lines in several experimental approaches.

Astemizole is a non-sedating second-generation anti-histamine that does not cross the blood–brain barrier.⁴⁴ This molecule was withdrawn from the market in several countries especially because severe cardiac side effects including prolongation of the Q-T segment and *Torsade de Pointes* were observed in cases of overdose.^{20,45–47} Our results show very strong effects when low concentrations of astemizole and gefitinib were combined. Then, astemizole may be safely administered at proper dose, and especially in combination with gefitinib, the dose may be even lowered. Despite that several studies explaining the precise mechanism of the combination effect are needed, these results suggest the combination astemizole–gefitinib as a novel therapeutic strategy for lung cancer that may help to decrease mortality from this disease.

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Disclosure

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