Synergistic Effects of 2-Deoxy-D-Glucose and Cinnamic Acid with Erlotinib on NSCLC Cell Line #

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Abstract

Non-small cell lung cancer (NSCLC) is a deadly solid tumor with poor prognosis due to its metastasis and resistance to current treatments. Tyrosine kinase inhibitors (TKIs), such as erlotinib, are efficient in treating NSCLC, but the emergence of chemical resistance and side effects severely limits their single use. Objective: in this study, the combination treatments of either 2-deoxy-D-glucose (2DG) or cinnamic acid (CINN) with erlotinib (ERL) were tested for their possible synergistic effect on the proliferation and migration capacity of NSCLC cells. Methods: In this study, the NSCLC model A549 cell line was used to study the effects of single compounds and their combinations on the cell growth inhibition, clonogenicity potential, and migration capacity. To determine the effectiveness of this combination, the present study also aims to quantify the Combination -Index and the Dose-Reduction-Index to find out the nature of drug’s combination by utilizing CompuSyn software. Results: Our data showed that single use of 2 DG, CINN or erlotinib for 48 h elicit a dose-dependent inhibition of proliferation of NSCLC cells. 2DG or CINN markedly increase the sensitivity of NSCLC cells to erlotinib in all the tested doses. Combination of either 2DG or CINN with erlotinib also reduced the clonogenicity of NSCLC cells up to 67% and 85%, respectively, as compared to erlotinib single treatment. Additionally, CINN and erlotinib combination therapy decreased the migratory capacity of A549 cells by 3 times and further induced much more apoptotic cell death phenotypes. Conclusion: treatment with the combination of either 2DG or cinnamic acid with erlotinib exhibited a better inhibitory influence on NSCLC cells than individual-drug therapy. These compounds can be a prospective adjunct to erlotinib usage, and therefore, can increase the efficiency of NSCLC treatment.

Keywords: Non-small cell lung cancer (NSCLC); Cinnamic acid (CINN); 2-deoxy-D-glucose (2DG); Erlotinib (ERL).

Introduction

Non-small cell lung cancer is one of the most common malignant tumors in men and women and a leading cause of mortality worldwide (1). Erlotinib and gefitinib are the first-generation TKIs used to treat NSCLC with EGFR mutations. Erlotinib is an epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI) in NSCLC cells (2). However, the relatively rapid emergence of resistance to erlotinib monotherapy substantially limits overall therapeutic benefit (3, 4).
In this sense, combined therapies against NSCLC cancer are indicated. Therefore, there is an urgent need for novel treatment options to enhance chemosensitivity and overcome resistance to NSCLC. To obtain stronger biocompatibility, less toxicity, and better therapeutic potential, which may simultaneously target many of the differential weaknesses of malignancy, the research for combinatorial therapeutics has expanded dramatically in recent years.\(^{(5)}\)

Cinnamic acid (CINN) is a phenolic compound present in plant cell walls either in free or bound form.\(^{(6)}\) CINN has been studied for a wide range of biological purposes, including anti-inflammatory, antimicrobial, and platelet aggregation.\(^{(7-8)}\) Its anticancer action is the most notable among them, both in vitro as well as in vivo.\(^{(9,10,11)}\) A cutting-edge method of drug development is the pharmacological targeting of cancer metabolism.\(^{(12)}\) The metabolic activity differs greatly from that of normal cells.\(^{(14)}\) Among the most commonly recognized use of the glucose analog 2-Deoxy-D-glucose (2DG) is as a glucose metabolism inhibitor.\(^{(14)}\) Because 2DG acts as a competitive inhibitor of glycolysis in cancer cells and because many malignancies have increased levels of hexokinase and glucose absorption, it has been proposed as a molecular cancer therapy.\(^{(16)}\)

The purpose of this study was to investigate the anti-proliferative and anti-migratory effects of erlotinib plus either 2DG or CINN in NSCLC, as well as to further analyses any possible mechanisms at play.

## Material and Methods

### Chemicals

Cinnamic acid was obtained from (Sigma-Aldrich Chemical Company Inc.). 2-DG was purchased from (Elabscience, China), while erlotinib was supplied by Shanghai Roche Pharmaceuticals Ltd, China. Both cinnamic acid and erlotinib were dissolved in dimethylsulfoxide (DMSO) (ZHENGZHOU MEIYA CHEMICAL PRODUCTS CO., LTD.), while 2-DG was reconstituted in distilled water. All compounds were filter sterilized using 0.22 μM Millipore syringe filter. Antibiotics (100 U/mL penicillin and 100 g/mL streptomycin), RPMI-1640 medium and fetal bovine serum (FBS) were purchased from Euroclone, Italy’s Euroclone.

### Cell cultures

Human lung non-small cell carcinoma cell line A549 was obtained from the American Type Culture Collection (ATCC). Cells were cultured in RPMI 1640 supplemented with 10% fetal bovine serum (FBS) at 37 °C in a humidified atmosphere of 5% CO2. Cells were grown as monolayer, media was replaced every 3 days and passed when cells confluency extended ~70%.

In vitro cell proliferation-inhibition assays

The MTT assay was used to determine the cytotoxic effects of the analyzed compounds.\(^{(13)}\) A549 cells were seeded at a density of 5 × 10^3 in 96-well plates. Following overnight culture, cells were then treated with CINN, 2DG, or erlotinib. In 96-well plates, cells were exposed to each medication for 48 hours before 10 μL of MTT reagent (Sigma Aldrich) was added to each well. The medium was aspirated after 4 hours of incubation (37 °C, 5% CO2) to generate formazan crystals, which were then dissolved in 100 μl of DMSO solution. The optical density of each well was measured at 560 and 600 nm with a GloMax® Microplate Reader (Promega, USA). Each individual experiment was repeated at least three times.

### Clonogenic assay

Following the procedure described by Franken et al., 300 A549 cells were seeded in a 6-well culture plate for analysis of their clonogenic potential.\(^{(18)}\) The media were changed with the appropriate treatments every three days. On a 40X inverted phase contrast microscope, colonies with 50–200 cells were counted. Each treatment was carried out in triplicate and twice, and the entire surface area of the plate was scored for colonies.

### Morphological changes study

In this assay, A549 cells were cultured in a 24-wells culture-plate. When the cells reach 70% confluency, they were then treated for 48 hours with the indicated compounds at their IC50. After 48 hours, the cell culture medium was aspirated and the cells were mounted by adding 500 μL of 1% formaldehyde in PBS for 5 minutes at 25°C and stained by using 0.5 percentage crystal violet for half an hour. The wells then washed gently with phosphate buffer saline.\(^{(12)}\) The morphological modifications of the cells were inspected under an inverted microscope (OPTIKA, IM3) at 400× total magnifications and imaged by a digital camera (OPTIKA, Italy).

### Analysis of Combined Drug Effects

Using Composyn software (Biosoft, Cambridge, UK), we examined the effects of the medicine combination. We used the combination index (CI), which is derived from Chou and Talalay's median effect principle, to determine if the outcome of therapy with the two drugs was additive or synergistic. The following formula was used to produce CI: \( CI = D1/(Dx)1+D2/(Dx)2 \), where D1 and D2 are the compound concentrations 1 and 2 used together to achieve x% of the total medication effect, and (Dx)1 and (Dx)2 are the concentrations of the separate agents to achieve the same efficacy. An additive action between the two chemicals is indicated by a CI of 1, antagonism is indicated by a CI > 1, and synergism is shown by a CI < 1.\(^{(19)}\)
Study of cancer cell migration

Concisely, trans-well chambers with transparent PET membrane with a 8.0-μm pore size (SPLInsertTM) was used (12). A549 cells (1 × 10^5) were cultured in 300μl serum-free RPMI medium and placed in the upper trans-well chamber in the company of tested compounds (CINN alone, ERL alone, CINN+ERL and control). Complete medium was added to the bottom chamber as a chemoattractant culture. The transwell was kept for 24 h at 37 °C. Immigrated cells on the top surface of the filter were mechanically removed using a cotton swab and 4% paraformaldehyde was used to fix the migrating cells on the bottom surface for 30 minutes. Samples were cleaned three times with PBS, stained for 30 minutes with crystal violet (0.5 mM) and then examined and captured on camera under a microscope (Optika, IM-3, Italy). The average of cancer cell counts in 10 randomly chosen fields for each replications. The cell images were analyzed using ImageJ software (ImageJ Software, NIH, USA).

Statistical analysis

All data are given as mean±SD of triplicates. The Student's t test was utilized to compare between two groups, while the one-way analysis of variance (ANOVA) was applied for comparing three or more groups. GraphPad Prism 7.2 was utilized to estimate the IC50 for each compound (GraphPad 7 Software Inc., USA). By the CompuSyn tool, combination index, Fa-CI, and DRI-Fa parameters were calculated (CompuSyn Inc, USA). Changes that had a p-value < 0.5 were reflected significant differences.

<table>
<thead>
<tr>
<th>Drugs</th>
<th>2DG (mM)</th>
<th>Cinnamic acid (µM)</th>
<th>Erlotinib (µM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>IC50</td>
<td>48.85</td>
<td>30.92</td>
<td>14.7</td>
</tr>
</tbody>
</table>

Results

Determination of dose-response curves and half-maximal inhibitory concentration (IC50) values of 2DG, CINN or erlotinib in NSCLC cells

Before evaluating the effect of combination treatment of either 2DG or CINN with erlotinib on the growth of A549 cells, we first investigated cell viability and the half-maximal inhibitory concentration (IC50) values of each single compound in NSCLC A549 cells by using MTT assay for 48 h. As shown in Figure 1, the treatments with either 2DG, CINN, or erlotinib reduced the growth and proliferation of A549 cells in a dose-dependent manner. Although, 2DG significantly reduced A549 cells viability by 45% (P<0.01) and 55% (P<0.01) at 50 and 100 mM concentrations, respectively (Fig 1A). However, CINN markedly caused a much more pronounced effects, it cause 65% (P<0.001) and 74% (P<0.0001) decrease in cells viability at 50 and 100 µM concentrations, respectively (Fig 1B). Moreover, erlotinib applied a significant reduction of cell viability in A549 cells line at nearly all the tested concentrations. The percentage reduction in the number of viable cells treated with only erlotinib was ranging from 25–96% (Fig 1D). The dose response curves of these compounds on A549 cells were also determine, with 2DG IC50 values of 48.85 mM, whereas the IC50 for each of CINN and erlotinib were 30.92 µM and 14.7, respectively (Table 1). Therefore we chose 50 mM of 2DG, 30 µM of cinnamic acid and 15 µM of erlotinib for the subsequent experiments.

Table 1. Half-maximal inhibitory concentration of 2DG, cinnamic acid and erlotinib on A549 cells for 48 hours.

![Graph A](image1.png)

![Graph B](image2.png)
The Combined effects of 2DG or CINN with Erlotinib on lung cancer

Figure 1. The efficacy of 2DG, CINN and ERL on the NSCLC cells viability. A549 cells treated with a range of concentrations of 2DG (A), CINN (B) and ERL. (C) were counted at 48 h using MTT assay. (D) The data are represented as means of triplicate samples for each group. 2DG, 2-Deoxy D glucose; CINN, cinnamic acid; ERL, erlotinib. P<0.0001 (****), P<0.001 (**), P<0.01 (*).

The combination of erlotinib with cinnamic acid or 2DG synergistically repressed the survival of cultured NSCLC cells

Erlotinib is well known for having an anticancer effect on a variety of malignancies, thus it is desirable to increase its therapeutic potential at lower doses. In this study, combining non-toxic concentrations (½ IC50 and IC50) of either 2DG or CINN with 15 µM erlotinib in A549 cells for 48 h was performed to assess their synergy. In A549 cells, the combination of 30 µM CINN with 15 µM erlotinib caused 67% decrease in cell viability of A549 cells at 48 h, compared to erlotinib alone (p<0.001). At the same time point, the combination of 2DG (50 mM) with erlotinib exerted a decrease of only 58% compared to erlotinib only treatment (p<0.001).

Figure 2. Synergistic effects of 2DG or cinnamic acid with erlotinib on the viability of NSCLC cells. A549 cells were treated with (½ IC50 and IC50) of either 2DG or CINN alone, or combined with 15 µM erlotinib in A549 cells for 48 h, as indicated. The cells were then processed for MTT assay. Data are represented as means of triplicate samples for each treatment. p < 0.05 (#) versus control; p < 0.001 (**), p < 0.05 (*) versus the group treated with erlotinib only.

Cinnamic acid-erlotinib combination suppresses the clonogenic potential of NSCLC cells

The clonogenicity of A549 cells was investigated after treatment with erlotinib together with either 2DG or cinnamic acid. This study demonstrated that, compared to control, 2DG and cinnamic acid repressed the clonogenic potential of A549 by 10% and 20%, respectively. Although the erlotinib only treatment resulted in 52% decrease in colony formation, as compared to control. However, the combination treatment of 2DG+ ERL or CINN+ ERL exerted a higher decrease in colony number (about 84% and 93%, respectively as compared to the control group (p<0.001)). Interestingly, the combined treatment of erlotinib with either 2DG or CINN also significantly reduced clonogenicity of A549 by more than 67% and 85% cells, respectively, as compared to erlotinib only treatment (Figure 3).

Furthermore, CINN+ ERL treatment significantly reduced the number of cells in each colony compared to the erlotinib-only group (p=0.0068). Furthermore, CINN+ERL dramatically reduced the number of cells in each colony compared to the control group (p= 0.0001). Similarly, the quantitative study also showed that the addition of 2DG to erlotinib treatment significantly reduced the number of cells in colonies when compared to the control group (p = 0.0002). (Figure 3 B, D). The average number of colonies and the number of cells of a single clone were decreased dramatically when CINN was added to erlotinib.
The Combined effects of 2DG or CINN with Erlotinib on lung cancer

Changes in apoptotic morphology of lung cancer cells after combination of erlotinib with 2DG or CINN.

Cellular morphology changes were also studied by contrast phase inverted microscope as a preliminary investigation of the factors that led to cell death that was brought on by the combination of erlotinib with either 2DG or CINN in NSCLC cells. A shown in Figure 4, A549 cells displays endothelial-like morphology with characteristic shape. Treatment with Erlotinib 15 μM resulted in cell death with little to no variation in A549 cell morphology after 48 h compared to the untreated control. However, the combined treatment induced dramatic changes in cellular morphology including membrane blebbing, cells rounding, shrinking, and loss of contact with neighboring cells (Figure 4). As a result, it was proposed that the mode of cell death brought on by combination therapy probably due to apoptosis.

Cinnamic acid chemosensitize A549 cells to erlotinib treatment

Cells were co-treated with either erlotinib and/or CINN at different concentrations range (0.195–100 μM) for 48 h. MTT assay was performed to access cell viability. The preliminary results showed that CINN significantly decreased the IC50 of erlotinib and therefore chemosensitized lung cancer cells to

Figure 3. Effect of the combination of 2DG or cinnamic acid with erlotinib on the clonogenicity of NSCLC cells. (A) Photos (magnification 1x) show demonstrative wells in which A549 cells were treated with concentrations of 2DG, CINN and ERL for 1 week. After that cells were stained with cv dye. (C) Colony mass between 5 ±200 cells was calculated and plotted. (B) Pictures (4x magnification) depict a single colony that the cells formed as a representative example, as well as (D) the mean analysis of the results. The number of colonies was normalized to a control value of 100. Data are means with standard deviations of triplicates separate experiments. p < 0.05 (#) versus control; p < 0.01 (**), p < 0.05 (*) versus the group treated with erlotinib only.

Figure 4. Effects of 2DG and CINN alone or in combination with erlotinib on the morphology of lung cancer cells. The representative photomicrograph displays morphological alterations in A549 cancer cells after they were exposed to various combinations for 48 hours at their predicted IC50 (total magnification 400x). Arrows show membrane blebbing in red, loss of cell-cell contact in orange, and broken nuclei into two or more apoptotic entities in black.
erlotinib. The IC50 parameter for ERL was 14.91 µM, while the administration of ERL together with CINN resulted in a decrease in the IC50 parameter of erlotinib to 3.61 µM (Figure 5A). The Combination Index (CI) calculated using the CompuSyn analyses confirmed this result (Table 2, Figure 5B). As shown Table 2, CI score was reduced, when the concentration of CINN + erlotinib amplified. The CI score of all the ERL+ CINN combinations was less than 1 which indicates a high synergistic effect at all tested concentrations. Additionally, after CINN with ERL, the Dose-Reduction Index (DRI) was also determined by using CompuSyn, which evaluates how many-times a single drug dose can be declined as administered in combine with another drug. As illustrated in Figure 5C and Table 2, the DRI revealed that the CINN+ ERL group at all concentrations exhibit a favorable dose-reduction index >1. The combination of CINN with ERL resulted in a dose reduction for erlotinib by 5 folds to inhibit cell growth by 75% (Fa= 0.75). While at Fa = 0.5 (50 % growth inhibition), the DRI for erlotinib was 8 folds (Table 2, Figure 5C).

![Figure 5](https://example.com/figure5.png)

**Figure 5. Dose-effect relationship of Cinnamic acid and erlotinib combination in lung cancer cell lines.** (A) dose-response plots were obtained from MTT cytotoxicity test at 48 hours treatment (analyzed by Graph pad prism). (B) The CI for CINN with ERL. CI<1, CI=1 and CI>1 indicate synergism, additive effects and antagonism, respectively. (C) Dose reduction index (DRI) of CINN and ERL combinations were showed, the DRI value >1 signified a good drug’s combinations while DRI less than 1 indicating negative combinations. Data obtained via CompuSyn analysis (D) Isobolograms showing the doses necessary for inhibition at 25% (Fa =0.25), 50% (Fa =0.5) and 75% (Fa =0.75) aimed at each drug separately. Isobolograms indicate the nature of the drug interaction at constant ratios in lung cancer cell line. Particular drug combination at diverse effect levels, data points under the line = synergistic, on the line = additive and above the line = antagonistic effects. The degree of synergism in this drug combination is reflected by the distance of the data point from its respective line (same color). Experiments were accomplished in triplicate. Data are represented as mean ± SD. Fa, fraction affected; CI, combination index; p < 0.001 (****), p < 0.001 (**), p < 0.01 (**), p < 0.05 (*) versus the group treated with erlotinib only.

**Table 2. The combination index and the dose reduction index are analyzed to calculate the degree of drug combination.** This table illustrated the affected fractions (Fa), and corresponding CI and DRI

<table>
<thead>
<tr>
<th>Affected Fraction (Fa)</th>
<th>ERL (µM)</th>
<th>CINN (µM)</th>
<th>CI Value</th>
<th>DRI for ERL</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>2.73</td>
<td>13.42</td>
<td>0.095</td>
<td>12.6</td>
</tr>
<tr>
<td>0.5</td>
<td>9.42</td>
<td>38.21</td>
<td>0.159</td>
<td>7.84</td>
</tr>
<tr>
<td>0.75</td>
<td>32.48</td>
<td>108.8</td>
<td>0.266</td>
<td>4.88</td>
</tr>
<tr>
<td>0.97</td>
<td>473.02</td>
<td>1046.95</td>
<td>0.829</td>
<td>1.75</td>
</tr>
</tbody>
</table>
The 2DG or Cinnamic acid -erlotinib combination reduces cell migration capabilities

Transwell® migration tests were used to assess how 2DG and CINN affected A549 cells. As shown in Figure 6A and B, the number of migrated cells in the individually-treated erlotinib group was significantly reduced compared with the control group (p< 0.0069). A Transwell assay suggested that the migratory ability of A549 cells was significantly decreased after combination treatment of erlotinib with 2DG or CINN, and the inhibitory effect of the combined treatment was more pronounced compared to the control group (p= 0.0192 and 0.0003) respectively.

properties through the EMT process (14). Therefore, in the treatment of NSCLC, inhibition of migration ability along with the induction of cancer cell death can be an important therapeutic strategy for suppressing cancer cell metastasis (15). To achieve even better results in NSCLC containing EGFR mutations, we therefore sought to assess in this trial whether we can supplement erlotinib treatment with the combination of CINN or 2 DG. The study of the effects of the combination of chemotherapeutic drugs with particular cancer inhibitors is becoming increasingly important due to the positive finding that combination treatment is preferable to single-medication treatment. Although several pathways can be addressed, combining various medicines can be more effective (additive or synergistic) (16).

The 2DG is a synthetic glucose analog in which the 2-hydroxyl group is replaced by hydrogen (17). Acting as a D-glucose mimic, 2DG blocks the first step of glycolysis (18). Many cancers have an increase in glucose absorption and hexokinase levels and so 2DG has been proposed as a molecular cancer treatment based on its activities as a competitive glycolysis inhibitor in cancer cells (17). On the other hand, CINN is a well-known natural aromatic carboxylic acid that has been used in traditional Chinese and Indian medicine for over centuries for its antimicrobial, anti-inflammatory, and anticancer activities (6, 8, 11, 19).

From the present results, it has shown that ERL with 2DG showed less proliferative, clonogenic and metastasis potential as compared to individual treatment. Interestingly, the combination of ERL with CINN exerted much more effect. It is demonstrated that CINN with erlotinib at non-toxic concentration, augmented its growth inhibitory and anti-migratory effect on A549 cells more powerfully than 2DG+ERL. The CI plot revealed CI ≤ 1 for all combines of CINN + ERL utilized, which reveals synergistic effect between ERL and CINN at much lower concentrations than single erlotinib treatment. This augmentation potential may mediate at molecular level by induction of apoptotic cell deaths, CINN has been shown to have anticancer activity because it increases the expression of proapoptotic genes for example FAS, BAX, caspase3, PARP, and reduces the expression of anti-apoptotic protein BCL2 (10, 20, 21). Many previous studies have shown that CINN demonstrated antitumor potential against different types of cancers cell lines but has no effect on normal skin fibroblast cells (9, 10, 22, 23, 28). In 2022, Yang et al. demonstrated
that novel chloropyrame-cinnamic acid hybrids had cytotoxic effects on breast cancer cells \(^{(25)}\). Morphological analysis in the current study further indicated that the anti-tumor activity of CINN plus ERL may be due in part to the induction of apoptosis. Their combination can forcefully inhibit cell propagation and migration.

In conclusion, co-administration of cinnamic acid with erlotinib increases the inhibitory effects of erlotinib on tumorigenicity and migration of NSCLC cells. These outcomes are much more than the effects observed after the 2DG + ERL combination. Taken together, these results imply that cinnamic could be a promising erlotinib combo drug to increase erlotinib's therapeutic potential in NSCLC cells at lower doses.

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**Conflict of Interests**

All authors declare that there is no conflict of interests.

**Ethics Statements**

This article was approved by the ethical committee of the Pharmacology and Toxicology Department/ College of Pharmacy/ Mustansiriyah University.

**Author contributions:**

Wood S, Al-Khafajy performed the investigation, methodology and writing –original draft. Munaf H, Zalzala performed data analysis, writing-editing and publication. Zakariya Al-Mashhadani performed writing-review and publication.

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