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Research Article

Synergistic Anticancer Effects of Gemcitabine and Resveratrol on A549 Non-Small Cell Lung Cancer Cell Line

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Abstract

Background: Non-small cell lung cancer (NSCLC) remains the leading cause of cancer-related mortality worldwide. Gemcitabine is an established effective agent in the treatment of NSCLC. However, resistance and dose-dependent toxicity limit the clinical efficacy of gemcitabine. Resveratrol, a polyphenolic compound, has been proposed as a chemosensitizing agent capable of modulating multiple survival pathways. ***Objective***: To assess whether resveratrol enhances the anticancer activity of gemcitabine in A549 NSCLC cells. ***Methods***: The viability of A549 cells was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) method. The synergistic interaction was analyzed using a constant molar ratio of 1:75 (gemcitabine: resveratrol). The combination index (CI) and dose reduction index (DRI) values were calculated across multiple effect levels. To see how the combination changed the ability of cells to divide and migrate, we used the clonogenic and wound-healing assays, respectively. ***Results***: The data showed that combining gemcitabine and resveratrol increased cytotoxicity compared to using either drug alone. This was shown by CI values that were consistently below 1.0 for all effect levels that were tested. The DRI values for both drugs were greater than 1.0 at all the effect levels, suggesting dose-sparing potential. In addition, the combination significantly decreased the colony formation and cell migration compared with individual drug treatments. ***Conclusion***: Resveratrol potentiated the anticancer effects of gemcitabine in A549 cells via synergistic cytotoxicity and enhanced suppression of the clonogenic survival and migratory potential.

Keywords: Gemcitabine; Non-small cell lung cancer; Resveratrol; Drug synergism.

التأثيرات التآزرية المُضادّة للسرطان للجيمسيتابين والريسفيراترول في خلايا سرطان الرئة ذو الخلايا غير الصغيرة A549

الخلاصة

الخلفية: يُعَدُّ سرطان الرئة ذو الخلايا غير الصغيرة السببَ الرئيسي لوفيات السرطان على مستوى العالم. ويُعد الجيمسيتابين عاملًا علاجيًا فعّالًا ومعتمدًا في معالجة سرطان الرئة ذو الخلايا غير الصغيرة، إلا أنّ كفاءته السريرية تُقيَّد بظهور المقاومة الدوائية والسمّية المعتمدة على الجرعة. الريسفيراترول، وهو مركب متعدد الفينولات، طُرح كعامل مُحَسِّس للعلاج الكيميائي قادر على تعديل العديد من مسارات البقاء الخلوي. **الهدف**: تقييم ما إذا كان الريسفيراترول يعزّز الفعالية المضادة للسرطان للجيمسيتابين في خلايا سرطان الرئة ذو الخلايا غير الصغيرة من نوع A549. **الطرائق**: تم تقييم حيوية خلايا A549 باستخدام اختبار بروميد 3-(4,5-ثنائي ميثيل ثيازول-2-يل)-2,5-ثنائي فينيل تترازوليوم وتم تحليل التداخل التآزري بين الدوائين باستخدام نسبة مولارية ثابتة قدرها 1:75 (جيمسيتابين:ريسفيراترول). جرى حساب قيم مؤشر الجمع ومؤشر تقليل الجرعة عبر مستويات متعددة من التأثير. تم تقييم تأثير المزيج العلاجي على إمكانية تكوين المستعمرات السرطانية وقدرة الخلايا على الهجرة باستخدام اختبار تكوين المستعمرات واختبار التئام الجروح على التوالي. **النتائج**: أشارت البيانات إلى أن الجمع بين الجيمسيتابين والريسفيراترول أحدث زيادة تآزرية في السُّمّية الخلوية مقارنةً باستخدام كلّ دواءٍ منفردًا، كما يتضح من قيم مؤشر الجمع التي بقيت أقل من 1 عبر جميع مستويات التأثير المدروسة. كانت قيم مؤشر تقليل الجرعة لكلّ من الدوائين أكبر من 1 عند جميع مستويات التأثير، مما يشير إلى إمكانية خفض الجرعة. بالإضافة إلى ذلك، أدى المزيج العلاجي إلى انخفاض معنوي في تكون المستعمرات وهجرة الخلايا مقارنةً بالعلاج بكل دواء على حدة. **الاستنتاج**: عزّز الريسفيراترول التأثيرات المضادة للسرطان للجيمسيتابين في خلايا A549، مُحْدِثًا سُمّية خلوية تآزرية مع تعزيز تثبيط تكون المستعمرات السرطانية والقدرة على الهجرة الخلوية. تدعم هذه النتائج اعتمادَ المعالجة المشتركة بالجيمسيتابين والريسفيراترول كاستراتيجية واعدة لتحسين مخرجات علاج سرطان الرئة ذو الخلايا غير الصغيرة.

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INTRODUCTION

Lung cancer is the most common cause of cancer-related deaths worldwide [1]. Non-small cell lung cancer (NSCLC) constitutes approximately 80% to 85% of all lung cancer cases [2]. Chemotherapy continues to be the standard treatment for NSCLC, despite the emergence of targeted therapies and immunotherapies [3]. While chemotherapeutic agents produce a good initial response and a modest increase in overall survival, patients will ultimately develop drug resistance [4]. Gemcitabine is a pyrimidine nucleoside antimetabolite active against several human malignancies, including NSCLC, pancreatic, bladder, breast, and ovarian cancer [5-8]. Nevertheless, drug resistance and side effects (such as

anemia and leukopenia) limit the therapeutic effectiveness of gemcitabine [9]. Given that different drugs work in different ways at the molecular level, combining several treatments in combination therapy is one way to possibly make the treatments more effective and create a stronger anticancer response. Additionally, it has been demonstrated that combination therapy can mitigate the detrimental side effects accompanying high-dose medications [10]. One of the promising strategies for both the treatment and prevention of cancer is the use of phytochemicals [11]. Resveratrol (trans-3, 4′, 5-trihydroxystilbene) is a polyphenolic compound found in various plants such as grapes, berries, and peanuts. Because of its safety and ability to target several signaling pathways involved in cancer cell survival, tumor development, angiogenesis, and metastasis, resveratrol may be considered an optimal chemical for cancer therapy [12,13]. Numerous studies have confirmed the anticancer properties of resveratrol in many malignancies, including NSCLC, breast, colorectal, pancreatic, and liver cancers [14-18]. The current work aimed to assess the capacity of resveratrol to augment the efficacy of gemcitabine in the treatment of NSCLC, utilizing the A549 cell line as an *in vitro* model. Establishing a synergistic interaction between gemcitabine and resveratrol could pave the way for more effective and safer chemotherapeutic approaches for NSCLC. This study aims to assess whether resveratrol enhances the anticancer activity of gemcitabine in A549 NSCLC cells.

METHODS

Cell lines and culture conditions

Human NSCLC cell line A549 was obtained from the American Type Culture Collection (ATCC). The cells were cultured in RPMI-1640 medium supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin. The cells were maintained at 37 °C in a humidified incubator with 5% CO_2.

Cell viability assay

The MTT assay method (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was employed to assess the cell viability after treatment with gemcitabine and resveratrol. To sum up, 4000 A549 cells were put into 96-well culture plates and left to grow overnight. The next day, they were treated with different amounts of gemcitabine and resveratrol (Hangzhou Hyper Chemicals Limited, China) for 72 hours. Control cells were treated with equivalent concentrations of vehicle (0.1% DMSO). After treatment, each well received 0.5 mg/ml of MTT solution, and the cells were incubated at 37 °C for 3 hours. The MTT solution-containing media was then carefully discarded. Afterward, 100 µl of dimethyl sulfoxide (DMSO) was introduced to each well to dissolve the formed formazan crystals, and the absorbance was measured at 570 nm with a 630 nm reference wavelength utilizing a microplate reader (Promega, USA). The cell viability percentage was determined using the following formula [19]:

Cell Viability (%) = × 100%

Assessment of synergistic drug interaction

After establishing the IC_{50} value of each drug, the drugs were tested in combination. Using the constant ratio combination design, the synergistic interaction between gemcitabine and resveratrol was analyzed by calculating the combination index (CI) and dose reduction index (DRI) based upon the Chou and Talalay median effect principle [20,21], using CompuSyn software (Version 1.0, CompuSyn, Inc., and Paramus, NJ, USA). A549 cells were divided into four groups: a control group, a gemcitabine-treated group, a resveratrol-treated group, and a combination (gemcitabine + resveratrol)-treated group. The two drugs were used at a constant ratio relative to their IC_{50} values. Thus, the concentration used corresponded to 0.125, 0.25, 0.5, 1, and 2 times the IC_{50} of each agent. The CI values reflect how two drugs interact with each other when given in combination. CI < 1 signifies a synergistic effect, CI = 1 signifies an additive effect, and CI > 1 signifies an antagonistic effect. The DRI quantifies the extent to which the dose of each drug in the combination can be lowered at a certain effect level, relative to the dose of each drug when administered alone. DRI >1 signifies that combinations could result in decreased drug doses relative to the doses of each drug given alone.

Clonogenic assay

A549 cells were seeded on 6-well plates at a population density of 300 cells per well. The next day cells were exposed to gemcitabine or resveratrol, alone or in combination for 72 h. The drugs were tested at concentrations corresponding to half their IC_{50} values for each drug. Subsequently, the drug-containing medium was substituted with a fresh, drug-free medium. After 7 days, the cells were fixed using ethanol for 5 minutes and subsequently stained with a solution of 0.5% crystal violet for 30 minutes. The cells were washed thrice with water and air-dried. The percent colony area was measured utilizing ImageJ software (Colony Area Plugin) [22].

Wound healing assay

A549 cells were inoculated in 24-well plates at a density of 1×10^4 cells per well and cultured until confluent. A scratch was then created in the cell monolayer utilizing a sterile 200 µL pipette tip. After that, the cells were washed thrice with sterile phosphate buffer saline (PBS). Then cells were immediately treated with gemcitabine and/or resveratrol at half their IC_{50} values for 72 h. The uncovered areas were captured and analyzed with ImageJ software. The original wound area (at zero time) and the residual wound areas (after 72 h) were measured, and the percentage of wound closure was calculated using the following formula [23]:

Wound closure (%) = × 100%

Statistical analysis

Statistical analysis was performed using GraphPad Prism 8.0 Software (GraphPad Software, Inc.). All values are presented as the mean ± SD of 3 independent experiments. Differences between groups were analyzed using one-way analysis of variance (ANOVA) followed by Tukey's post hoc test. Statistical significance was set at $p< 0.05$.

RESULTS

The IC_{50} value for each drug was first determined as a basic requirement for the constant ratio combination design. A549 cells were treated with gemcitabine (0.049-25 µM) or resveratrol (20-200 µM) for 72 hours. As shown in Figure 1, both gemcitabine and resveratrol inhibited cell growth in a concentration-dependent manner. The calculated IC_{50} value for gemcitabine was 0.92 µM, whereas that for resveratrol was 68.72 µM. Then, to determine whether the drugs synergistically inhibit cell growth, the cells were treated with gemcitabine and resveratrol in combination.

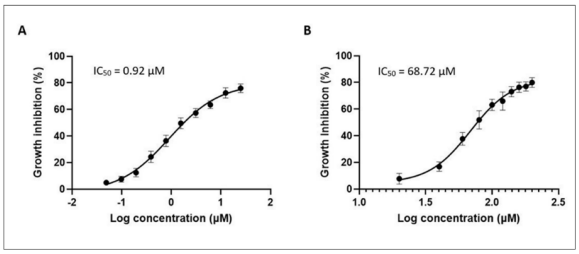

Figure 1: Cytotoxic effects of gemcitabine and resveratrol in A549 cells. Cells were treated with a range of concentrations of **A**) gemcitabine (0.049-25 µM, or **B**) resveratrol (20-200 µM, B) for 72 h. The cell viability was determined using the colorimetric MTT assay. Data are presented as mean ± SD (n = 3).

Guided by the IC_{50} values determined for the single drugs, the combination of gemcitabine and resveratrol was evaluated at a 1:75 constant ratio for 72 hours. Figure 2A shows the dose-response curves of the individual and combined drugs against A549 cells. The fraction of cells affected (Fa) following each treatment was utilized to perform synergy analysis with CompuSyn software.

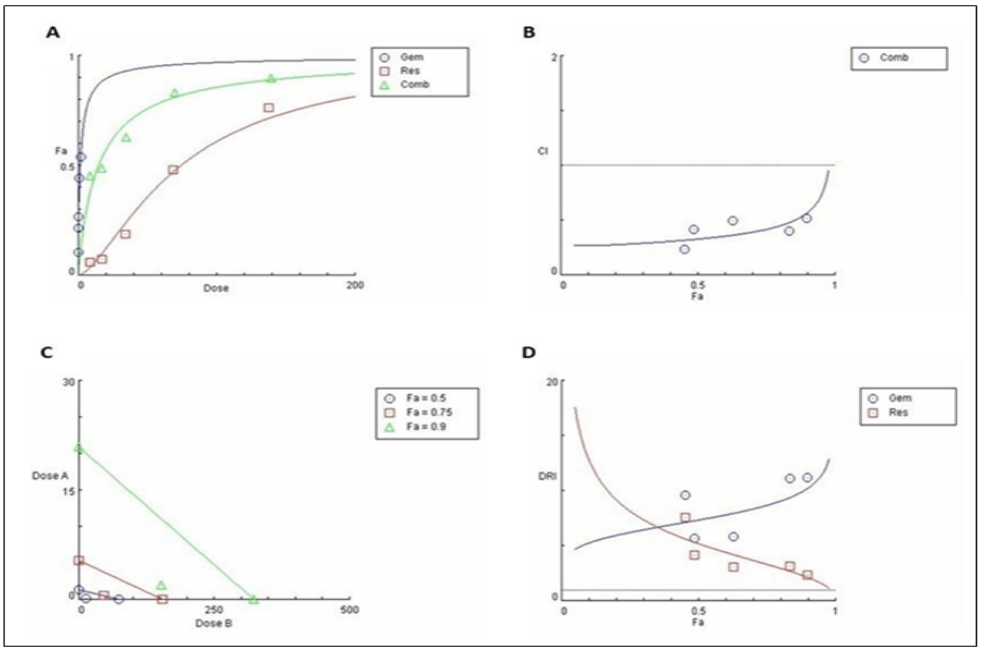

Figure 2: The graphic representations obtained from CompuSyn Report for cytotoxicity data of gemcitabine, resveratrol and their combinations in A549 cells at 72 h. **A**) Dose-effect curves of gemcitabine, resveratrol and their combinations. **B**) Combination index plot revealing 5 combination data points. CI <1 signifies synergism, CI =1 signifies additivity, and CI >1 signifies antagonism. **C**) Isobologram illustrating resveratrol (x-axis) and gemcitabine (y-axis) doses required for inhibition at 50% (Fa 0.5), 75% (Fa 0.75), and 90% (Fa 0.9) for each drug separately. Synergism is demonstrated by the dose pair plotted as a point (symbol) beneath its respective Fa isobole (line of additivity). **D**) DRI for gemcitabine and resveratrol are presented, DRI values greater than 1 indicate favorable drug interactions. Gem = gemcitabine, Res = resveratrol, Comb = combination.

The CI values were below 1.0 across the entire Fa range, indicating synergistic interaction between the two drugs. The degree of synergy varied with the effect level. Strong synergy was detected at low effect levels (CI= 0.28 at Fa= 0.25), whereas moderate synergy persisted even at high effect levels (CI= 0.73 at Fa= 0.95) (Figure 2B, Table 1). The isobologram analysis is a dose-oriented approach that offers a graphical presentation of the nature of interaction of two drugs. The isobologram was plotted at Fa values of 0.5, 0.75, and 0.90.

Table 1: Computerized quantitation of synergism (CI and DRI values) at selected effect levels (Fa = 0.25–0.95) for gemcitabine and resveratrol combinations in A549 cells after 72 h of treatment.

Affected fraction (Fa)	CI	DRI for gemcitabine	DRI for resveratrol
0.25	0.28371	6.21556	8.14192
0.50	0.32874	7.34347	5.19317
0.75	0.41716	8.67606	3.31236
0.9	0.57088	10.2505	2.11273
0.95	0.72976	11.4816	1.55602

CI<1.0 indicates synergism. CI: Combination Index, DRI: Dose Reduction Index, Fa: Fraction affected (e.g., Fa 0.5 = 50% cell death / IC50).

The data points were all below the additivity line (Figure 2C), further suggesting synergism at these effect levels. The DRI analysis demonstrated that the gemcitabine-resveratrol combination has the potential to reduce the doses of both gemcitabine and resveratrol. Across all the effect levels, both agents had DRI values larger than 1 (Figure 2D, Table 1). For example, the DRI at Fa= 0.50 was 7.34 for gemcitabine and 5.19 for resveratrol, indicating more than a sevenfold and fivefold reduction in the dose, respectively. For gemcitabine, the DRI values increased with higher effect levels, rising from 6.22 at Fa= 0.25 to 11.48 at Fa= 0.95. Collectively, the data suggests that the gemcitabine-resveratrol combination synergistically inhibited the growth of human NSCLC A549 cells. The colony formation assay was performed to investigate whether combinatorial treatment with gemcitabine and resveratrol could impair A549 cells colony forming ability more potently than individual compounds. As Figure 3 illustrates, individual treatment with gemcitabine and resveratrol reduced colony formation of A549 cells by 57.16% and 29.05%, respectively ($p< 0.0001$ and $p= 0.0067$).

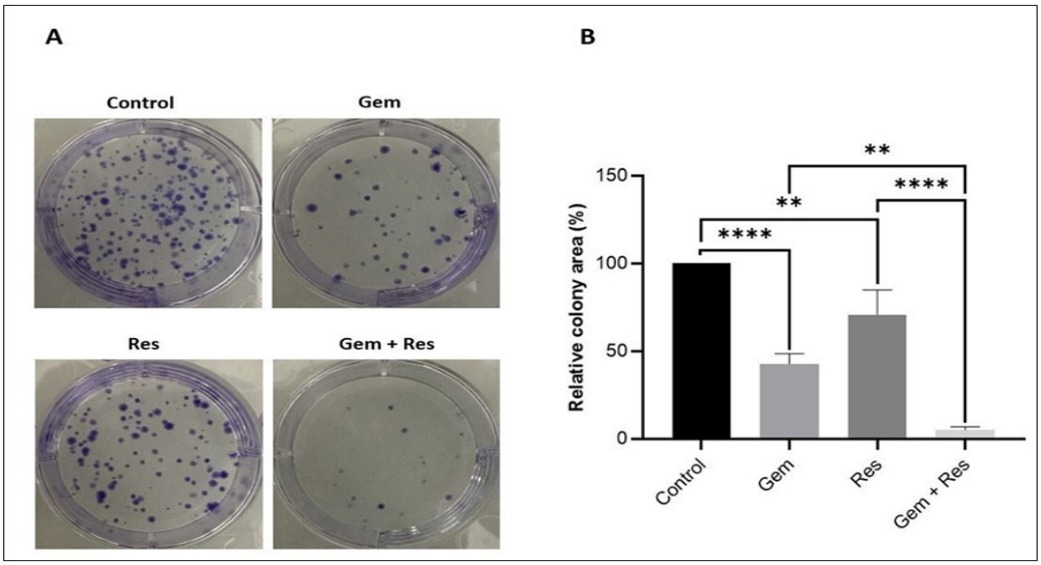

Figure 3: Inhibition of colony formation of A549 cells by gemcitabine in combination with resveratrol. Cells were treated with gemcitabine (0.46 µM) and/or resveratrol (34.36 µM) for 72 h. **A**) Representative images of colony formation from three independent experiments are presented. **B**) The colony area (%) in each treatment group was quantified and normalized to that of the control group. Data are shown as mean ± SD (n = 3). One-way ANOVA and Tukey's multiple comparison test were used in statistical analyses, $^{**}p< 0.01$, $^{****}p< 0.0001$. Gem = gemcitabine, Res = resveratrol.

The combination treatment resulted in a marked decrease of 94.86% ($p< 0.0001$), higher than the inhibition achieved with either gemcitabine or resveratrol alone ($p= 0.0013$, $p< 0.0001$, respectively), suggesting potentiation of the inhibitory effect on the clonogenic potential of A549 cells. The bi-directional wound healing assay was used to compare how well gemcitabine and resveratrol stopped A549 cells from migrating when used together versus when used alone. Each treatment group was normalized to its corresponding 0-hour values. As shown in Figure 4, the cells displayed slower migration after treatment with either gemcitabine or resveratrol ($p = 0.0166$, $p = 0.0009$, respectively). Importantly, the combination treatment repressed cell migration more potently than the individual treatment with either gemcitabine or resveratrol ($p= 0.0005$, and $p= 0.0075$, respectively).

DISCUSSION

Chemotherapy is the most common approach for managing NSCLC in clinical practice, with gemcitabine being one of the main chemotherapeutic agents in the treatment strategy [24,25]. However, resistance to gemcitabine is often recognized as an important limitation of lung cancer treatment [26]. The present study was conducted on the human NSCLC A549 cell line to explore the potential of resveratrol to potentiate the anticancer effects of gemcitabine. The constant ratio combination design was utilized to analyze the nature of gemcitabine-resveratrol interaction, as it allows computer simulation of CI and DRI values at all effect levels. Therefore, the IC_{50} values for single drugs were initially determined to establish this fixed ratio. The results indicated that the inhibitory effect of gemcitabine on A549 cells occurs in a dose-dependent manner, consistent with previous studies [27,28]. The IC_{50} value at 72 hours was 0.92 µM. Similarly, resveratrol demonstrated a dose-dependent cytotoxic effect, with an IC_{50} value of 68.72 µM at 72 hours, which is consistent with the inhibitory range reported in previous studies [29,30]. The IC_{50} value for gemcitabine was approximately 75-fold greater in potency than that of resveratrol. Therefore, a 1:75 constant molar ratio of gemcitabine to resveratrol was applied to test the combination. The study demonstrated that gemcitabine and resveratrol are synergistic against A549 cells, as evidenced by CI values consistently below 1 across the full range of effect levels and confirmed by isobologram analysis. Importantly, the DRI values for gemcitabine were higher at high effect levels. Combining chemopreventive or chemosensitizing compounds with chemotherapeutic drugs in lower doses may be a

good way to lower drug toxicity and make standard chemotherapy regimens work better, according to some evidence. This strategy may be especially useful for cancers that are hard to treat because of pharmacological resistance, which is promoted by different molecular pathways [31].

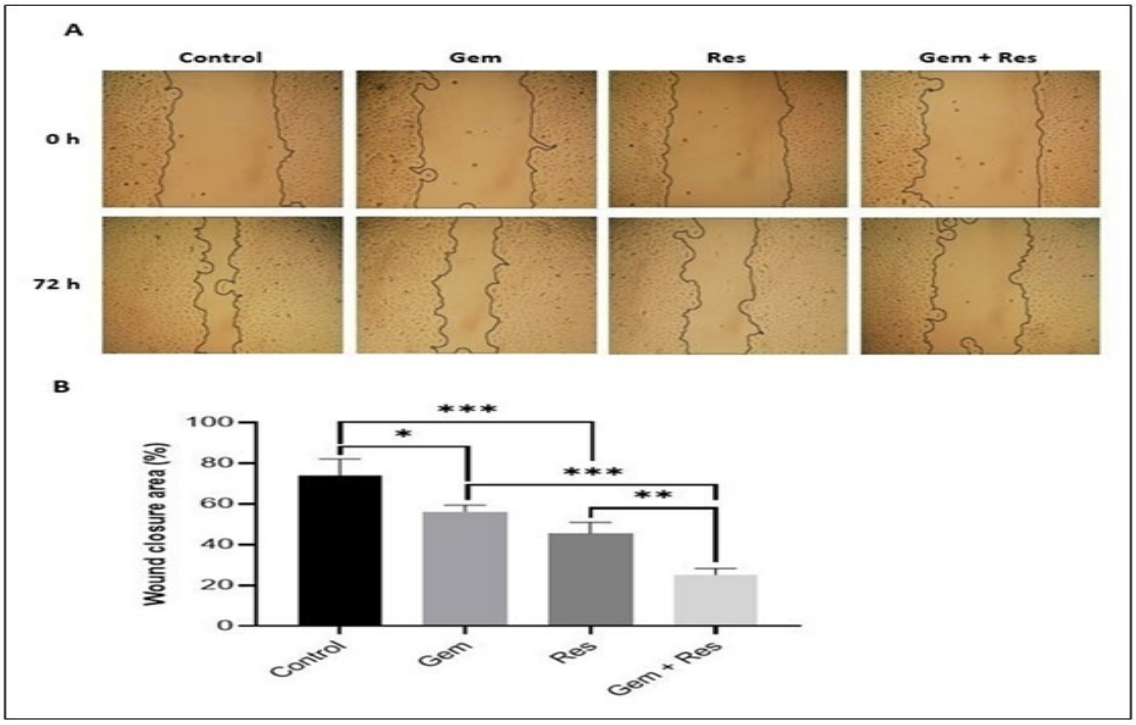

Figure 4: The individual and combined effect of gemcitabine and resveratrol on the migration of A549 cells. The cells were treated with gemcitabine (0.46 µM), resveratrol (34.36 µM) or combination of both for 72 h and cell migration was assessed by *in vitro* wound-healing assay. **A**) Representative images of the wound area for each group at 0 and 72 h. Scale bar = 100 µm. **B**) Quantification of the wound healing rate (closure area, %) after 72 h. Data are presented as mean ± SD (n = 3). Statistical significance was analyzed using one-way ANOVA and Tukey's multiple comparison test, *p< 0.05, **p< 0.01, ***p< 0.001. Gem = gemcitabine, Res = resveratrol

One of the characteristics of resveratrol is its ability to inhibit cell survival signaling, resulting in either direct activation of the apoptotic signaling cascade or suppression of the anti-apoptotic mechanisms in this context. Resveratrol can make cancer cells more sensitive to chemotherapeutic or cytotoxic drugs when the survival and anti-apoptotic pathways are turned off [32]. This makes the anticancer effects stronger when given with these drugs. The clonogenic and wound healing assays revealed that the combination therapy resulted in an enhanced reduction in the ability of A549 cells for unlimited proliferation and migratory potential. The clonogenic assay is a better way to measure long-term growth suppression than the MTT assay because it checks how well cancer cells can go through more than one round of mitosis over a longer period of time [33]. The clonogenic assay results suggest that combining gemcitabine with resveratrol might make it easier to stop cancer cells from multiplying over time. This could lower the chance that a cancer patient's tumor will come back. Cell migration is an intricate process vital for physiological development, regeneration, and tissue repair. Cell migration, however, promotes metastasis, the primary cause of mortality in cancer patients [34]. The wound healing assay results suggest that gemcitabine in conjunction with resveratrol may inhibit the metastatic potential of NSCLC cells. In line with this study, research has demonstrated that resveratrol may enhance the antitumor effects of gemcitabine, particularly in pancreatic cancer. For instance, resveratrol has been shown to boost the cancer-fighting effects of gemcitabine both in the lab and in a mouse model of human pancreatic cancer by changing markers of growth, invasion, angiogenesis, and metastasis [35]. Also, Jiang *et al*. (2016) found that resveratrol could make pancreatic cancer cells more sensitive to gemcitabine by decreasing the expression of YAP [36]. Wu *et al.* (2024) further reported that resveratrol enhances the sensitivity of Mia PaCa-2 and Panc-1 pancreatic cancer cells to gemcitabine through inhibition of the c-Met/PARP1 axis [37]. A recent study by Qin *et al.* (2020) using the HCC827 NSCLC model demonstrated that resveratrol promotes tumor microvessel growth, resulting in improved blood perfusion and drug delivery into the tumor, thereby enhancing the anticancer efficacy of gemcitabine. However, the study did not reveal significant *in vitro* synergy [38]. On the other hand, the results obtained from this study revealed a distinct synergism, suggesting that the interaction between gemcitabine and resveratrol may be cell line-specific and dependent on experimental settings. In A549 cells, numerous studies have shown that resveratrol might potentiate the sensitivity of cells to various chemotherapeutic drugs. For example, it has been demonstrated that resveratrol enhances the sensitivity of A549 cells to cisplatin via modulation of autophagy [39]. In addition, it has been suggested that resveratrol might improve the antitumor effects of paclitaxel in A549 cells by changing the expression of COX-2 [40]. Moreover, resveratrol has been shown to

synergistically augment the apoptotic effects of As_2O_3 in A549 cells through induction of oxidative stress [41]. Overall, these results support the idea of using resveratrol along with gemcitabine to treat NSCLC, which could lead to better treatment outcomes through synergistic interaction. However, more work is needed to understand the molecular mechanisms behind the observed synergy, particularly its impact on apoptosis and key oncogenic signaling pathways in NSCLC.

Conclusion

The results demonstrated that resveratrol enhances the inhibitory effects of gemcitabine on cell viability, proliferation, and migration of NSCLC A549 cells. These data suggest that combination treatment with gemcitabine and resveratrol might be a promising strategy to improve the outcome of clinical treatment for NSCLC. Further studies will seek to clarify the molecular mechanisms underlying the cooperative interactions between gemcitabine and resveratrol.

Conflict of interests

The authors declared no conflict of interest.

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Data sharing statement

Supplementary data can be shared with the corresponding author upon reasonable request.

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