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*Original Article*

# Luteolin overcomes acquired resistance to osimertinib in non-small cell lung cancer cells by targeting the HGF-MET-Akt pathway

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**Abstract:** Osimertinib, a third-generation epidermal growth factor receptor tyrosine kinase inhibitor (EGFR-TKI), has overcome the acquired resistance of first- and second-generation EGFR-TKIs due to the EGFR T790M mutation in non-small cell lung cancer (NSCLC). However, acquired resistance to osimertinib remains a significant clinical challenge. Luteolin, a natural flavonoid from traditional Chinese medicine, has exerted antitumor effects in various tumors. In this study, we investigated whether the natural flavonoid luteolin can enhance the antitumor effects of osimertinib in NSCLC cells. We established an acquired osimertinib-resistant cell line, H1975/OR, and evaluated the effects of luteolin and osimertinib alone and in combination on proliferation, migration, invasion, and apoptosis of H1975/OR cells. The potential mechanisms by which the combination of luteolin and osimertinib exert their effects were investigated by PCR, western blot, gene silencing, molecular docking, SPR and kinase activity analysis. The combination of luteolin and osimertinib inhibited the proliferation, migration, and invasion of H1975/OR cells and promoted apoptosis. We identified mesenchymal-epithelial transition factor (MET) amplification and overactivation as important resistance mechanisms of H1975/OR cells. The combination downregulated the gene and protein expression of MET and inhibited its protein phosphorylation, thereby blocking the activation of the downstream Akt pathway. Additionally, the mediated effects of MET on the synergistic effect of luteolin and osimertinib were confirmed by silencing of MET. Luteolin strongly bound with nonphosphorylated MET by occupying the active pocket of MET and inhibiting its activation. Notably, the combination also downregulated the expression of autocrine hepatocyte growth factor (HGF), the sole ligand of MET. In conclusion, luteolin can synergize with osimertinib to overcome MET amplification and overactivation-induced acquired resistance to osimertinib by suppressing the HGF-MET-Akt pathway, suggesting the clinical potential of combining luteolin with osimertinib in NSCLC patients with acquired resistance.

**Keywords:** Non-small cell lung cancer, luteolin, osimertinib, mesenchymal-epithelial transition factor receptor, acquired resistance

## Introduction

According to global cancer statistics, lung cancer is the second most prevalent disease after breast cancer and the leading cause of cancer death worldwide [1]. Non-small cell lung cancer (NSCLC) is estimated to constitute around 85% of all lung cancers [2], and driver gene mutation is one of its main pathogenic mechanisms [3]. Epidermal growth factor receptor (EGFR) is the most common driver gene, and its mutation rate in clinical NSCLC patients is approximately 41.7-44.8% [4, 5]. The emergence of EGFR-tyrosine kinase inhibitors (TKIs) brings hope to

the clinical treatment of NSCLC patients. It specifically binds to the ATP-binding site in the EGFR kinase domain, which suppresses kinase activity, thereby preventing phosphorylation of EGFR protein and blocking the signaling pathways related to the growth, proliferation and migration of NSCLC cells [6]. The effective rate of osimertinib in treating patients with EGFR mutation lung adenocarcinoma is as high as 80%, and the progression-free survival (PFS) is 10-14 months [7-10]. However, the emergence of osimertinib resistance is becoming increasingly prevalent in clinical practice, and this needs to be addressed as soon as possible.

Mesenchymal-epithelial transition factor (MET) receptor is encoded by the proto-oncogene MET. Acquired resistance caused by MET gene amplification accounts for approximately 5-10% of all EGFR-TKI-resistant patients [11], and has an even higher rate (5-50%) in osimertinib-resistant patients [12]. The primary mechanism of MET amplification-induced resistance is to bypass EGFR by driving activation of the ErBb3/PI3K/Akt pathway [13]. Studies have found that MET amplification reduces the sensitivity of tumor cells to osimertinib [14]. Notably, the overproduction of hepatocyte growth factor (HGF), the unique ligand for MET, in tumor cells (autocrine) and mesenchymal cells (paracrine) leads to abnormal activation of the MET signaling pathway via the ligand-dependent mechanism [15, 16], suggesting that the cause of resistance is not limited to the target itself. Activation of the HGF/MET signaling pathway is also associated with increased tumor invasion and metastatic potential [17]. Furthermore, the MET gene amplification and T790M mutation can occur concurrently, in a reciprocal and complementary manner [17]. Post-progression survival (PPS) of patients with both the T790M mutation and MET amplification was 10.7 months, which was much lower than that of patients with T790M mutation alone (24.5 months) or MET amplification alone (14.1 months). The disease control rate of MET/T790M-positive patients treated with a combination of MET and T790M inhibitors would be significantly improved [17]. This suggests that the combination of these two targeted therapies is worthy of further exploration.

Luteolin is widely distributed in nature; it is initially isolated from the leaves, stems, and branches of Resedaodorata L; it is found in various traditional Chinese medicines such as Scutellaria barbata, Elsholtzia rugulosa, Dendranthema morifolium (Ramat.) Tzvel, and Lonicera japonica; and it has been demonstrated to possess anti-inflammatory, antitumor, antioxidant, intestinal flora regulation, and nerve protection effects [18-22]. Studies have revealed that luteolin can impede the progression of a variety of cancers including lung cancer, breast cancer, glioblastoma, prostate cancer, and colon cancer [23-27]; and its superior transdermal properties make it a treatment option for skin cancer [28]. The main anticancer mechanisms of luteolin include inducing cell apoptosis, arresting the cell cycle, and inhibiting cancer cell invasion and migration. Moreover, luteolin has been identified as an adjunct therapy due to its synergistic effect with PD-1 blockers [28]. Nevertheless, the synergistic effect of luteolin with osimertinib as well as its effects on osimertinib-induced resistance in NSCLC remains unexplored.

In this study, we evaluated the effect and mechanism of luteolin on resistance to osimertinib. Our findings demonstrate that luteolin and osimertinib synergically inhibit proliferation, migration, and invasion, as well as promote apoptosis in the H1975/OR cell line. The underlying mechanism is believed to involve the downregulation of MET and inhibiting its phosphorylation, thereby blocking the activation of the downstream Akt pathway. These results suggest that luteolin, a natural MET inhibitor, has great potential in overcoming the acquired resistance to osimertinib.

## Materials and methods

### *Chemicals and reagents*

Luteolin was purchased from Pufei De Biotech Co., Ltd. (JOT-10088; China). Osimertinib (AZD9291) was purchased from MedChemExpress (HY-15772; USA). Luteolin and osimertinib were dissolved in DMSO (Meilunbio, China) and were diluted to different concentrations with cell media immediately before use. Met and Akt antibodies were purchased from Boster Biological Technology (USA). p-Met (Y1234/1235) antibody was obtained from Cell Signaling Technology (USA). HGF and p-Akt (Ser473) antibodies were purchased from Affinity Biosciences (USA).

*Cell lines and cell culture*

Human normal lung epithelial Beas-2B cell line was obtained from meilunbio (China). The human NSCLC cell line H1975 with EGFR L858R and T790M mutations was a kind donation from the central laboratory of Qingdao Haici Hospital. We used gradient dosing culture and generational screening to construct an acquired osimertinib-resistant cell line named H1975/OR, which could stably grow and serially passage in cell media with 1.0 μg/mL osimertinib [13]. The H1975 cell line was cultured without osimertinib. All cell lines were authenticated by short tandem repeat (STR) analysis, and were cultured in DMEM medium (Meilunbio, China) supplemented with 15% fetal bovine serum (Gibco, USA) in a fully humidified incubator at 37°C under 5% $CO_2$. Photographs of cell morphology were taken with a Nikon Eclipse Ti2 inverted microscope (Nikon Corporation, Japan).

*Cell proliferation and combinational index assays*

Cell Counting Kit-8 (CCK-8) assays (Meilunbio, China) were used to evaluate cell proliferation. Following seeding in 96-well plates (3000 cells/well), the cells were treated with different drugs for 48 h. CCK-8 reagent was placed into 96-well plates (10 μl/well) before incubation for 2 h. The absorbance at 450 nm was measured by a Victor Nivo Multimode Microplate Reader (PerkinElmer, USA). CompuSyn software (ComboSyn, USA) was used to calculate the combinational index (CI) for drug interactions.

*Wound-healing assays*

Wound-healing assays were performed to evaluate cell migration. Pipettes were used to make scratches, and cells were exposed to different tested drugs for 0 h, 24 h, and 48 h. Photographs were taken using a Nikon Eclipse Ti2 inverted microscope. The wound area was measured using ImageJ software (National Institutes of Health, USA).

*Transwell assays*

Cell invasions were assessed using 8 μm pore size transwell insert chambers (Biofil, China), which were coated with matrigel (Corning, USA). Briefly, H1975/OR cells ($1.5 \times 10^5$/well) were seeded in the upper chamber with serum-free medium, while 20% FBS medium was added to the lower chamber. Both the upper and lower chambers were exposed to the different tested drugs for 24 h. Next, the non-invading cells in the upper chamber were carefully scraped off by cotton swabs. The invading cells were fixed with 4% paraformaldehyde (Biosharp, China) for 20 min, and were stained with 0.1% crystal violet (Solarbio, China) for 15 min. The stained cells were photographed on a Nikon Eclipse Ti2 inverted microscope.

*Apoptosis and cell cycle assays*

Cells were seeded in 6-well plates ($1 \times 10^6$/well), cultured overnight, and treated with different drugs for 24 h. We used Annexin V-FITC/PI Apoptosis Kit (Multi Sciences, China) to measure apoptosis and detect it by staining cells with annexin V and propidium iodide solution, followed by flow cytometry. Cell cycle experiments were performed using a Cell Cycle Staining Kit (Multi Sciences, USA). The percentages of the cells in the G1, S, or G2/M phases were analyzed with FlowJo software (BD Bioscience, USA).

*Real-time quantitative polymerase chain reaction (RT-qPCR)*

Genomic DNA was isolated by PreScript III RT ProMix (Enzy Valley, China) according to the manufacturer's instructions. 50 ng of genomic DNA was amplified using 2 × Universal qPCR SYBR Green Master Mix (Low Rox) (Meilun, China). The amplification protocol was 1 cycle at 95°C for 1 min, 45 cycles at 95°C for 10 s, and 60°C for 30 s, followed by a melt curve. Sample genomic DNA was assayed in triplicate for MET and GAPDH using the following primers: Met, forward 5'-TCAGGAGGTGTTTGGAAAG-3' and reverse 5'-GCAGTATGATTGTGGGGAA-3'; GAPDH, forward 5'-GGATTTGGTCGTATTGGG-3' and reverse 5'-GGAAGATGGTGATGGGATT-3'. GAPDH was used as a reference gene, and the relative Met copy number was determined by the 2^ (-TΔΔCt) method.

*Western blot analysis*

In brief, 25 μg of whole-cell protein lysates were separated by 10% denatured polyacrylamide slab gels (Boster, China), and subsequently transferred to a polyvinylidene fluoride mem-

brane (Millipore, USA) by electroblotting. Subsequently, the blots were incubated with the appropriate primary antibodies and then with secondary antibodies before being detected using an fg supersensitive ECL luminescence reagent (Meilun, China). The luminescence of the antibody was detected and captured by a ChemiDoc XRS+ imaging system (Bio-Rad, USA), and then relative quantitative analysis was performed using ImageJ software.

*MET small interfering RNA (siRNA) and transfection*

MET siRNA and control siRNA were chemically synthesized from GenePharma Biotechnology, and were transiently transfected using GP-transfect-Mate (GenePharma, China). After 48 h of transfection, the expression of MET in H1975/OR cells was verified by RT-qPCR.

*Molecular docking*

Molecular docking was performed using AutoDockTools and PyMOL. Nonphosphorylated MET (PDB ID: 8AN8) were obtained from the RCSB protein database (PDB bank). Using Glide Grid, a grid box with 20 A on all sides was generated and centered on the existing ligand. The luteolin 3D structure was downloaded from PubChem. MET was hydrogenated, and the docking binding energy of luteolin and MET was acquired by AutoDockTools. The top ranked docked pose was imported into PyMOL to overlay with the cocrystal structure of nonphosphorylated MET for a binding mode comparison.

*Surface plasmon resonance (SPR) analysis*

The activator for chip preparation was made by mixing 400 mM EDC and 100 mM NHS (GE) just before injection. The mixture was then injected into Fc1 and Fc2 sample channels for 800 s at a flow rate of 10 μL/min. Subsequently, 100 μg/mL of MET His Tag, Human in 10 mM NaAc (pH 4.5) was injected into the Fc2 sample channel for 300 s at a flow rate of 10 μL/min, resulting in an immobilization level of approximately 20556.7 RU. To deactivate the chip, 1 M Ethanolamine hydrochloride (GE) was injected into Fc1 and Fc2 sample channels at a flow rate of 10 μL/min for 800 s. In the running analyte by kinetics/affinity, luteolin was diluted with the Running Buffer (1*PBS-P, 5% DMSO, GE) to 8 concentrations (5.00, 2.50, 1.25, 0.625, 0.3125, 0.15625, 0.078125, 0.0390625, and 0 μM). The diluted luteolin was injected into Fc1-Fc2 of the channel at a flow rate of 30 μL/min for an association phase of 60 s, followed by a dissociation phase of 120 s. Both the association and dissociation processes were conducted in the Running Buffer.

*MET kinase activity assay*

MET kinase inhibition activity of the luteolin was evaluated using the ADP-Glo™ kinase assay (Promega, USA) according to the manufacturer's instructions. The luminescence was detected using a Victor Nivo Multimode Microplate Reader (PerkinElmer, USA).

*Statistical analysis*

We used GraphPad Prism 8 software to produce graphs, analyze data distribution, and perform statistical analyses. To investigate significant differences between the indicated groups, we employed analysis of variance (ANOVA) or t-test. All experiments were repeated three times, and the results are presented as means ± SEM.

## Results

*Construction of the H1975/OR cell line with acquired resistance to osimertinib*

The H1975 cell line harboring the EGFR L858R/T790M mutation was utilized to simulate the emergence of acquired resistance to osimertinib in vitro. This cell line has been demonstrated to be resistant to first- and second-generation EGFR-TKIs, such as gefitinib and erlotinib, yet highly sensitive to osimertinib (**Figure 1A**). This finding is consistent with patients who developed the T790M resistance mutation after the application of first- and second-generation EGFR-TKIs [29]. The osimertinib resistance of parental H1975 cells was induced by the gradient dosing culture. After 6 months of passage, the H1975/OR cell line, which could be stably passaged in cell medium containing 1.0 μg/mL osimertinib, was finally obtained through screening and elimination. The results showed that H1975/OR cells had significantly enhanced osimertinib resistance compared to the parental H1975 cells (**Figure 1A** and **1B**). The proliferation difference between the two

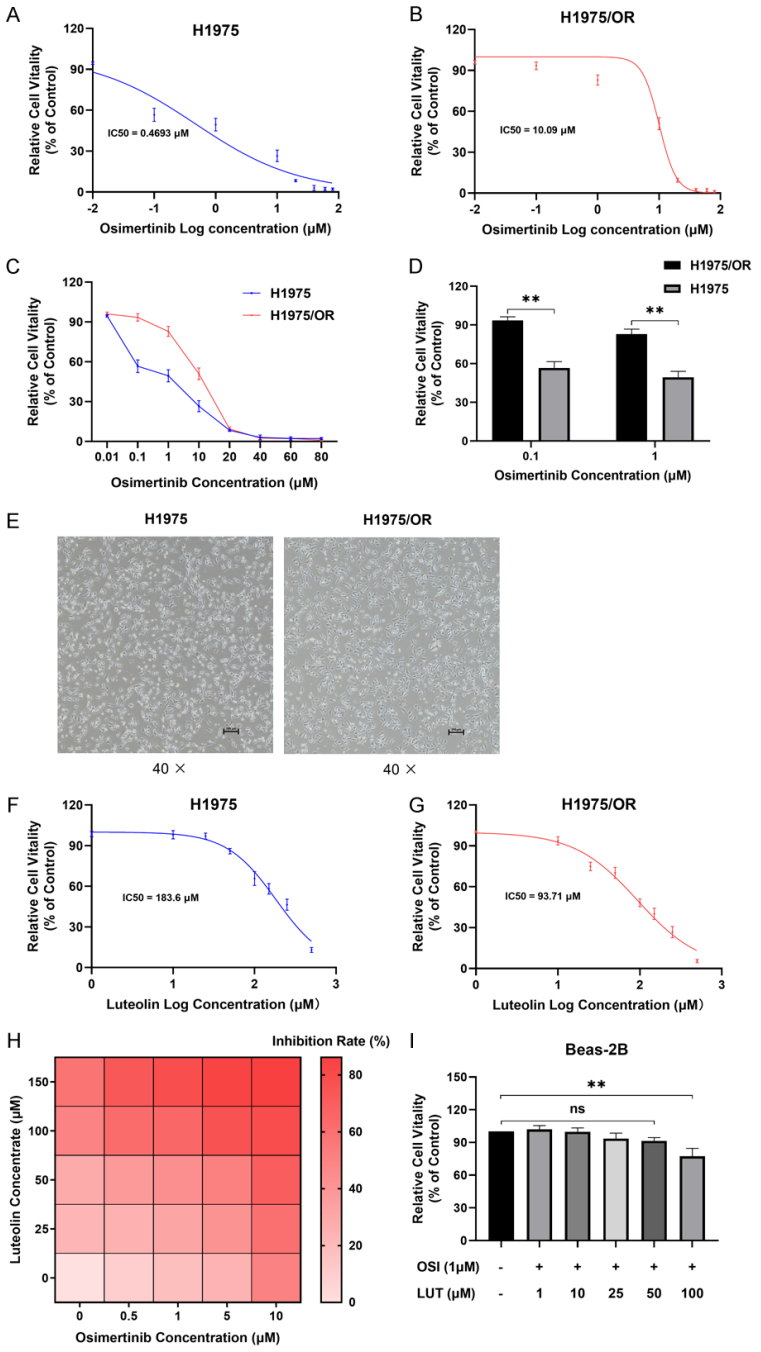


**Figure 1.** Synergistic effect of the combination of luteolin and osimertinib on the proliferation inhibition of osimertinib-acquired resistance cells, which are relatively more sensitive to luteolin. A, B. The inhibition of cell proliferation by osimertinib (0.01-80 μM) in H1975 and H1975/OR, and osimertinib inhibition fitting curve. IC50 has been indicated. C, D. Comparison of proliferation inhibition rates of H1975 and H1975/OR by osimertinib. The data was expressed as a percentage of the control. E. Cell morphology was observed and photographed with an inverted fluorescence microscope at 4 × magnification. F, G. The inhibition of cell proliferation by luteolin (1-250 μM) in H1975/OR and H1975 cells, and luteolin inhibition fitting curve. IC50 has been indicated. H. The combined inhibition of cell proliferation by luteolin (25-150 μM) with osimertinib (0.5-10 μM). The light to dark red color represents the gradually increasing inhibition rate, and the color card shows the inhibition rate corresponding to different depths of red. I. The inhibition of cell proliferation by the combination of different concentrations luteolin (LUT) and 1 μM osimertinib (OSI) in Beas-2B. All results are presented as means ± SD of three experiments. **, P < 0.01, ns, no significance.

cell lines was particularly significant at the concentrations of 0.1 μM and 1 μM osimertinib (**Figure 1C** and **1D**). The drug resistance index (RI = IC50 of resistant cells/IC50 of parental cells) was 21.50. It is generally believed that a drug-resistant cell line can be judged by an RI greater than 10. Therefore, the H1975/OR cell line with acquired resistance to osimertinib was successfully constructed. Furthermore, after the construction of the resistant cell line, an inverted microscope was used to observe the morphological changes between H1975 and H1975/OR at 40 × magnification (**Figure 1E**). It was noticed that after resistance to osimertinib, the differentiation of H1975/OR cell morphology was increased, the cell morphology became more slender, and closer contact between the cells could be observed. This cellular morphological alteration was similar to that of the resistant cell lines in other studies [30] and provided the cellular morphological basis for resistance of the H1975/OR cell line to high concentrations of osimertinib.

*Higher sensitivity of luteolin to the acquired osimertinib-resistant cell line versus parental*

To search for natural compounds with advantages in inhibiting the proliferation of H1975/OR cells, we performed

**Table 1.** Drug interactions of luteolin and osimertinib

| LUT (μM) | OSI (μM) | Combination index | Drug Interaction |
|---|---|---|---|
| 25 | 0.5 | 0.952 | Nearly Additive |
| 25 | 1 | 0.778 | Moderate Synergism |
| 25 | 5 | 0.917 | Nearly Additive |
| 25 | 10 | 0.505 | Synergism |
| 50 | 0.5 | 0.899 | Slight Synergism |
| 50 | 1 | 0.725 | Moderate Synergism |
| 50 | 5 | 0.759 | Moderate Synergism |
| 50 | 10 | 0.358 | Synergism |
| 100 | 0.5 | 0.534 | Synergism |
| 100 | 1 | 0.484 | Synergism |
| 100 | 5 | 0.307 | Synergism |
| 100 | 10 | 0.286 | Strong Synergism |
| 150 | 0.5 | 0.457 | Synergism |
| 150 | 1 | 0.332 | Synergism |
| 150 | 5 | 0.247 | Strong Synergism |
| 150 | 10 | 0.217 | Strong Synergism |

Combination Index (CI): 0.90~1.10, Nearly Additive; 0.85~0.90, Slight Synergism; 0.70~0.85, Moderate Synergism; 0.3~0.7, Synergism; 0.1~0.3, Strong Synergism.

a prescreening of several natural compounds known to be anti-lung cancer agents. The obtained results showed that luteolin had a better inhibitory effect on H1975/OR cells than on H1975 cells (IC50: 93.71 μM vs. 183.6 μM) (**Figure 1F** and **1G**). It can be hypothesized that the mechanism by which luteolin inhibited the proliferation of H1975/OR cells may be related to overcoming the resistance mechanism of osimertinib, suggesting that luteolin has the potential to overcome osimertinib resistance.

*Synergistic proliferation inhibition of the combination of luteolin and osimertinib in the acquired osimertinib-resistant cell line*

To explore whether luteolin and osimertinib have the value of combined application, we measured the combination index (CI) of different concentrations of luteolin combined with different concentrations of osimertinib. This result shows that the combination of luteolin and osimertinib has a concentration-dependent synergistic inhibitory effect on the proliferation of H1975/OR cells (**Figure 1H** and **Table 1**). In other words, the combination of luteolin and osimertinib inhibited the proliferation of H1975/OR cells more effectively than either drug alone. In addition, we evaluated the toxicity of the combination of different concentrations luteolin and 1 μM osimertinib on the Beas-2B cells. The results showed that the combination still had no significance toxic effects when luteolin was increased to 50 μM (**Figure 1I**), suggesting that the combination of luteolin and osimertinib has good potential in clinical application safety.

*Inhibition of the combination of luteolin and osimertinib on the migration and invasion of the acquired osimertinib-resistant cell line*

The abovementioned results showed that H1975/OR cells still showed resistance to osimertinib at a concentration of 1 μM. Therefore, we used different concentrations of luteolin combined with osimertinib at a concentration of 1 μM in subsequent experiments to explore the effect and mechanism of luteolin combined with osimertinib on the acquired osimertinib-resistant cell line. In the wound-healing assays, the results revealed no discernible migration difference between the osimertinib and the control group after 24 h of treatment (**Figure 2A** and **2B**). In comparison, the two groups treated with different concentrations of luteolin combined with osimertinib showed significantly inhibited migration of H1975/OR cells after both 24 and 48 h of treatment (**Figure 2A-C**). In addition, there was a positive correlation between luteolin concentration and migration inhibition rate (**Figure 2A-C**). According to these results, luteolin and osimertinib combined to inhibit H1975/OR cell migration significantly. Unexpectedly, treatment with 1 μM osimertinib alone was found to increase the migration of H1975/OR cells as compared to the control group. This difference was statistically significant after 48 h of osimertinib treatment (**Figure 2C**). This finding suggests that the increased migration of H1975/OR cells may be a stress response to osimertinib treatment. Additionally, the transwell assay showed that the combination of low concentrations of luteolin and osimertinib could significantly inhibit the invasion of H1975/OR cells (**Figure 2D**).

*Enhanced induction of apoptosis in the acquired osimertinib-resistant cell line by the combination of luteolin and osimertinib*

Luteolin has been shown to promote apoptosis and arrest the cell cycle in lung cancer and

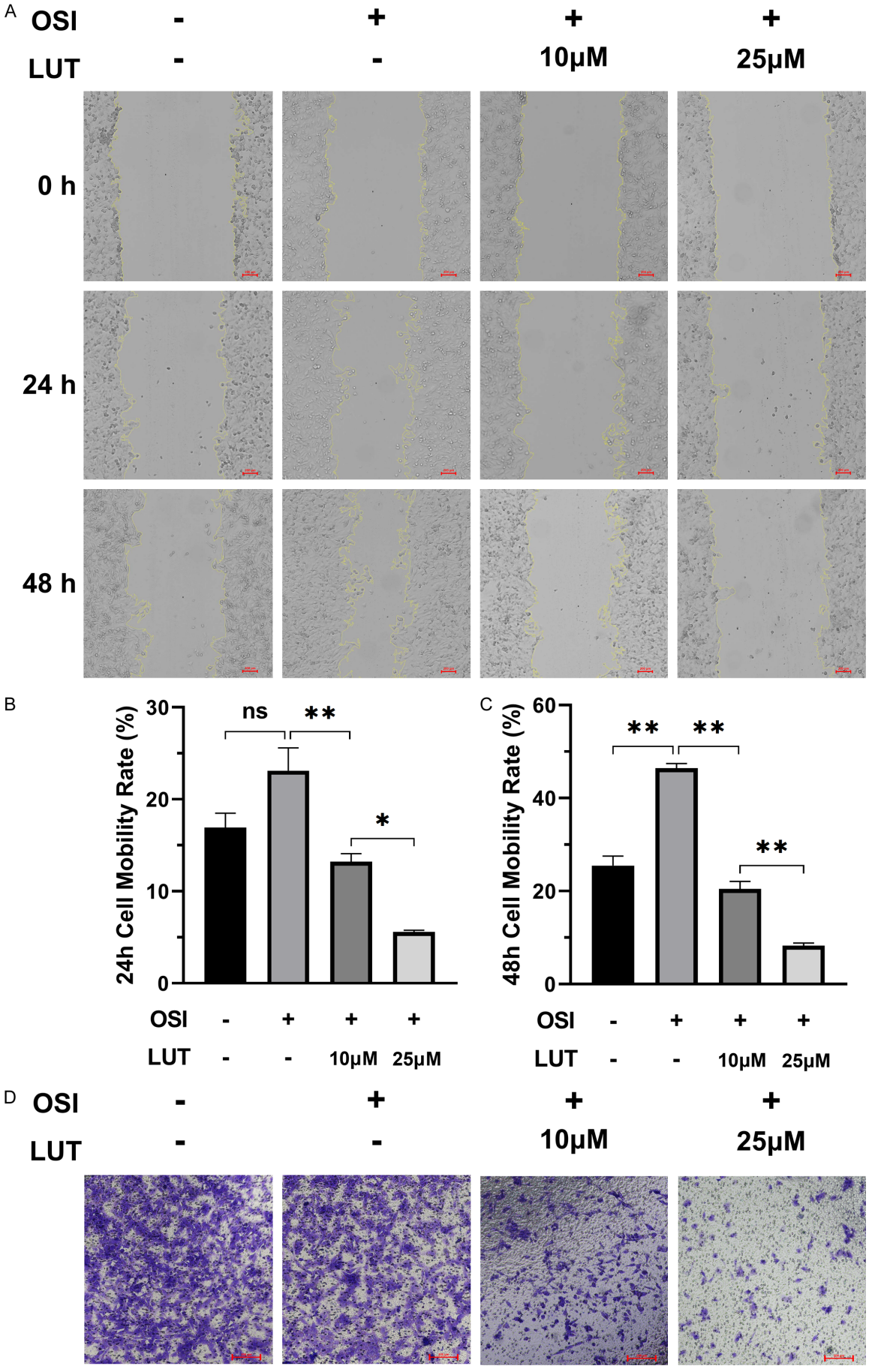
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**Figure 2.** The migration and invasion inhibition of the combination of luteolin and osimertinib on H1975/OR cells. A. The scratches were taken with an inverted microscope after exposure to different drugs for 0 h, 24 h, and 48 h, respectively. The scratch area was automatically identified by ImageJ, and the edges are connected with yellow lines. B and C. The 24 h and 48 h cell migration results were expressed as the increasing % of 0 h. D. The invading cells were stained and photographed on an inverted microscope after exposure to different tested drugs for 24 h. OSI "+" indicates osimertinib at 1 μM. The data are means ± SEM of three experiments. ns, no significance, *, $P < 0.05$, **, $P < 0.01$.

other tumors [19]. To further explore the effect of luteolin combined with osimertinib on apoptosis and the cell cycle of H1975/OR cells, flow cytometry was used. The obtained results showed that low concentrations of luteolin combined with osimertinib could significantly promote the apoptosis of the acquired resistant cells but had no effect on the cell cycle arrest at high concentrations (**Figure 3A** and **3B**). These results suggested that the combination of luteolin and osimertinib inhibited H1975/OR cells mainly by promoting apoptosis.

*Amplification and overactivation of MET were the acquired resistance mechanism of the H1975/OR cell line, and the combination of luteolin and osimertinib inhibited the activation of MET*

The abovementioned results demonstrated that the combination of luteolin and osimertinib synergistically affected the proliferation, migration, and apoptosis of H1975/OR cells. In subsequent experiments, it was crucial to clarify the acquired resistance mechanism of osimertinib-resistant H1975/OR cells to investigate the anti-resistance effect of luteolin. We first screened the common acquired resistance targets MET, HER2, VEGF, STAT3, BRAF, and CDH1 and used RT-qPCR to detect whether there were differences in the expression of related resistance genes between H1975/OR cells and parental H1975 cells. In addition, the effect of luteolin combined with osimertinib on the expression of osimertinib resistance-related genes was also explored. The obtained results showed that the copy number of the MET gene in H1975/OR cells was significantly higher than that in parental H1975 cells, and the expression level of the MET gene could not be downregulated by osimertinib alone (**Figure 4A**), indicating that MET gene amplification was the acquired resistance mechanism in the H1975/OR cell line. Different concentrations of luteolin combined with osimertinib decreased the expression level of the MET gene in a concentration-dependent manner (**Figure 4A**), indicating that luteolin could inhibit the expression of the MET gene related to osimertinib resistance.

MET is a tyrosine kinase receptor, the phosphorylation of which can activate the Akt pathway, thereby bypassing EGFR-TKIs targeting EGFR and inducing acquired resistance [13]. We found the expression level of MET, p-MET, and p-Akt in H1975/OR cells were significantly higher than those in parental H1975 cells and were not downregulated by osimertinib (**Figure 4B**). In addition, expression levels of MET, p-MET, and p-Akt in H1975/OR cells were decreased in a gradient manner following treatment with various concentrations of luteolin combined with osimertinib (**Figure 4B**). These results suggested that luteolin effectively inhibited the activation of MET and the expression of downstream Akt pathway, indicating that luteolin could inhibit the downstream cancer-promoting pathway by inhibiting MET overactivation, thereby playing a role in anti-osimertinib resistance.

*Confirmation of MET-mediated effects on the synergistic effect of luteolin and osimertinib through MET silencing*

To further confirm the role of MET in mediating the synergistic effect of luteolin and osimertinib, we investigated the effects of MET silencing on this combined treatment in H1975/OR cells. First, we successfully silenced MET expression in H1975/OR cells using MET siRNA and confirmed the silencing efficiency through RT-qPCR analysis. RT-qPCR results showed that both control siRNA + luteolin and osimertinib group and MET siRNA group effectively downregulated the expression of MET. However, there was no significant difference in MET expression between the MET siRNA + luteolin and osimertinib group and the MET siRNA group (**Figure 5A**).

In the subsequent cell proliferation, migration, invasion, and apoptosis experiments, we

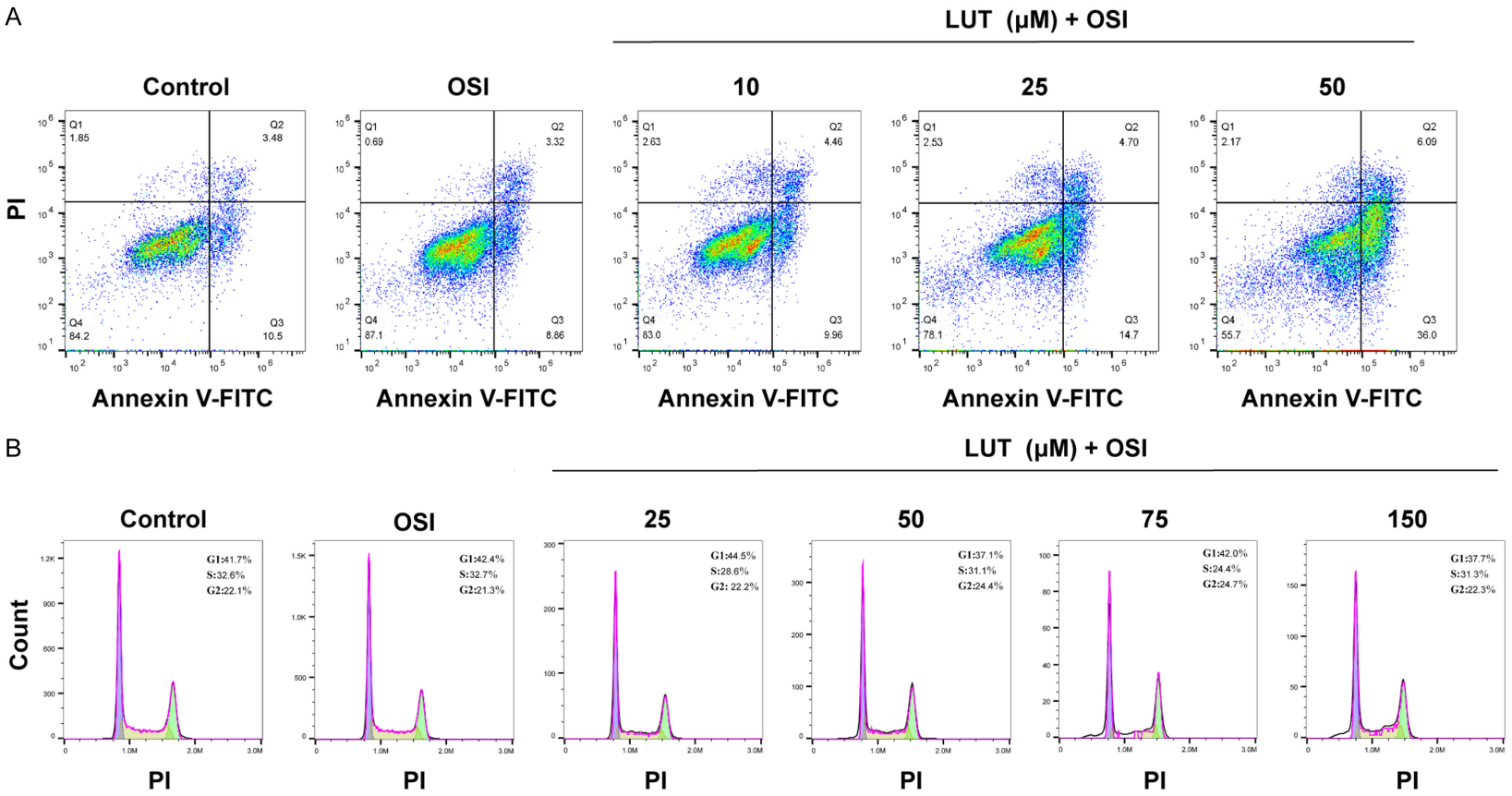



**Figure 3.** The enhanced apoptosis of the combination of luteolin and osimertinib but non-arrested the cell cycle in H1975/OR cells. The H1975/OR cells were treated with 1 μM osimertinib (OSI) alone or combined with 10-150 μM luteolin (LUT) for 24 h. Apoptosis (A) and the cell cycle (B) were detected with flow cytometry.

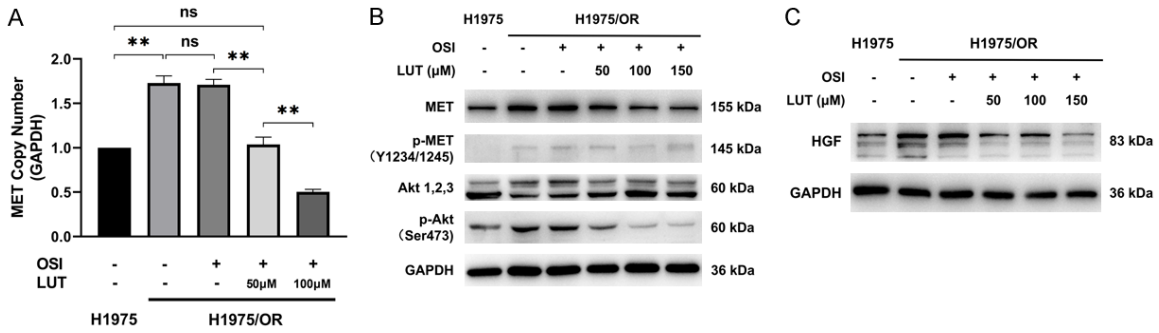


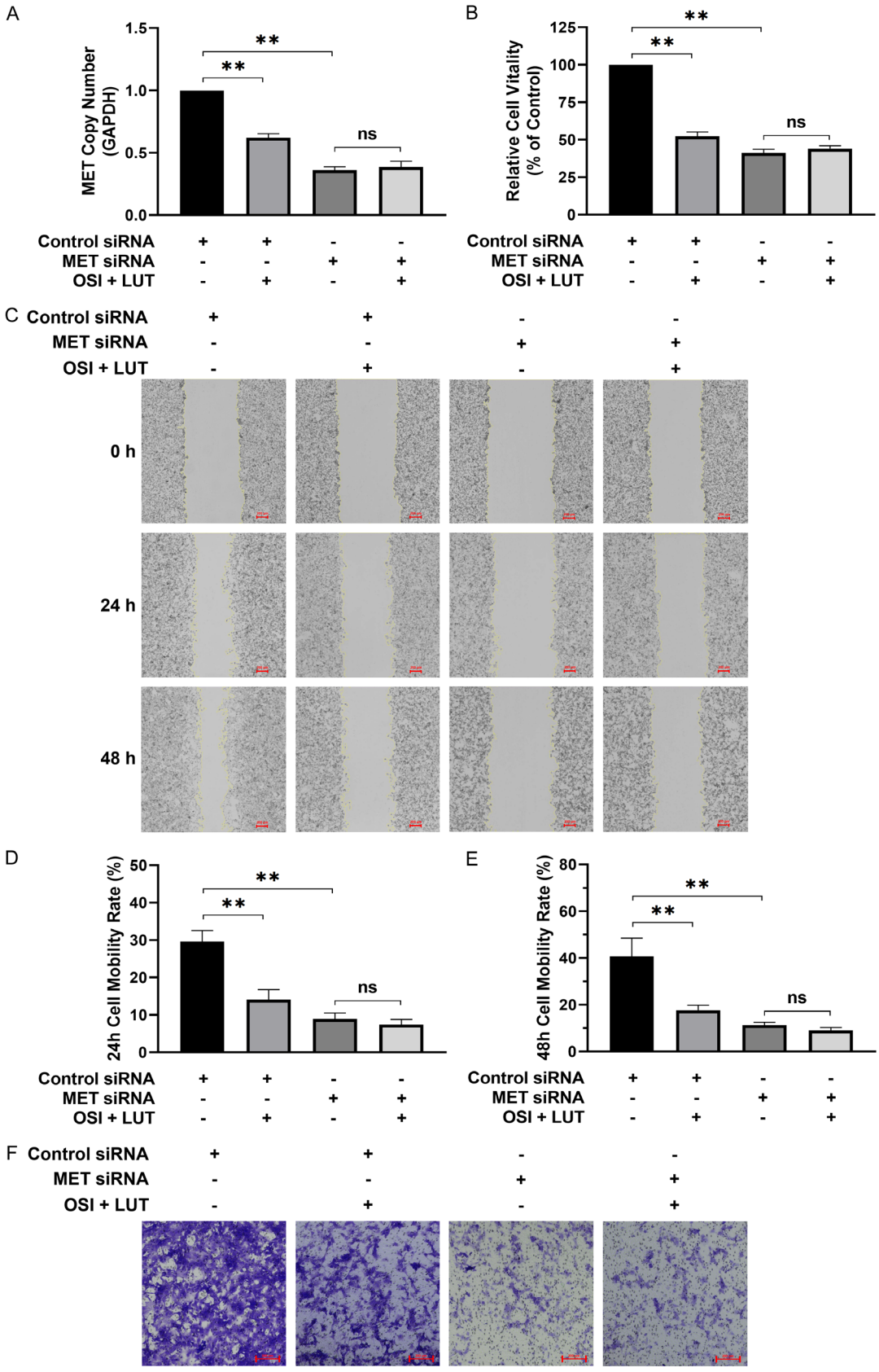

**Figure 4.** The inhibition of HGF-MET-Akt pathway in MET-amplified osimertinib-acquired resistant H1975/OR cells by the combination of luteolin and osimertinib. A. Relative MET gene copy numbers in H1975 and H1975/OR cell lines at different luteolin and osimertinib concentrations. B. MET, p-MET, Akt 1,2,3, and p-Akt protein expression in H1975 and H1975/OR cells at different luteolin and osimertinib concentrations. C. HGF protein expression in H1975 and H1975/OR cell lines at different luteolin and osimertinib concentrations.

obtained results that compared with the control siRNA group, both the control siRNA + luteolin and osimertinib group and the MET siRNA group significantly inhibited the proliferation, migration and invasion, and promote apoptosis of H1975/OR cells (**Figure 5B-G**). However, there was no significant difference between the MET siRNA + luteolin and osimertinib group and the MET siRNA group in these aspects (**Figure 5B-G**). These results showed that while MET siRNA and the combined treatment exhibit individual anti-cancer effects in osimertinib resistance cells with MET amplification and overactivation, their combination does not lead to further improvement in anti-cancer capabilities. These findings also strongly support the crucial role of identifying acquired resistance mechanisms to osimertinib in patients before subsequent treatment.

*Strong binding and inhibition of luteolin on MET in a stable conformation*

The chemical structure of luteolin shows that the B ring with conjugated double bonds is in the same plane as the C ring, which facilitates access to the enzyme substrate binding site. The two adjacent hydroxyl groups on the B ring also provide a basis for inhibiting kinase activity (**Figure 6A**). This structure suggests that luteolin has the potential to inhibit the activation and expression of MET, the target of resistance to osimertinib. To further explore whether luteolin exerted its inhibitory effect by directly binding to the MET protein while evaluating the binding strength of luteolin and MET in the spatial structure and to determine the best binding conformation of luteolin and the MET protein, we performed simulated molecular docking using luteolin (PubChem CID: 5280445) as a small molecule ligand and nonphosphorylated MET protein (PDB ID: 8AN8) as the receptor protein. It was found that the binding energies of the first nine conformations were all < -7 kcal/mol, indicating a strong binding ability of luteolin to the MET protein (**Table 2**). In the best binding conformation (**Figure 6B**), luteolin was deeply embedded in the active pocket of the MET protein and tightly bound to residues Tyr1159, Met1160, and Pro1158 in the ATP active site through four adjacent hydrogen bonds of its B ring (**Figure 6C** and **6D**). This binding acted as a key entering the lock hole to deadlock the protumor pathway of MET. We noted that the binding site and mode of luteolin to MET protein were similar to those of crizotinib, another MET/ALK/ROS multitargeted ATP-competitive protein kinase inhibitor in clinical application. However, compared with crizotinib, luteolin had more hydrogen bonding in the hinge region of MET (residues 1159-1162), which meant a more stable conformation.

To further verify the binding affinity between luteolin and MET, SPR binding analysis was performed. The RU values evaluating luteolin's binding to MET demonstrated a dose-dependent manner. And the determined equilibrium dissociation constant (KD) between luteolin and MET was about 0.5088 μM (KD = 5.088 × $10^{-7}$ M) (**Figure 6E**), indicated that luteolin had a strong affinity for MET. In addition to investigating the binding characteristics, we sought to elucidate the effect of luteolin treatment on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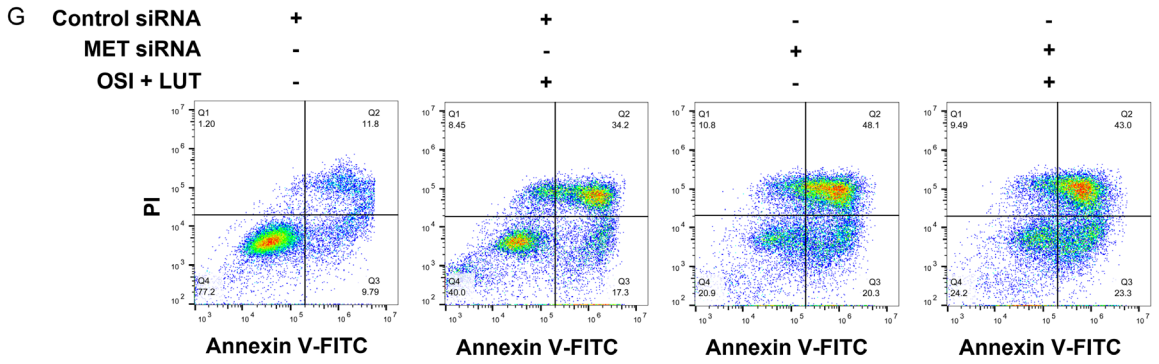


**Figure 5.** The effects of MET silencing on the synergistic effect of luteolin and osimertinib. A. Relative MET gene copy numbers of MET siRNA with or without osimertinib (1 μM) and luteolin (50 μM) in H1975/OR cells. B. The cell proliferation inhibition effect of MET siRNA with or without osimertinib (1 μM) and luteolin (50 μM) in H1975/OR cells. C. The cell migration inhibition effect of MET siRNA with or without osimertinib (1 μM) and luteolin (10 μM) in H1975/OR cells. D and E. The 24 h and 48 h cell migration results were expressed as the increasing % of 0 h. The data are means ± SD of three experiments. ns, no significance, *, $P < 0.05$, **, $P < 0.01$. F. The cell invasion inhibition effect of MET siRNA with or without osimertinib (1 μM) and luteolin (10 μM) in H1975/OR cells. G. The cell apoptosis effect of MET siRNA with or without osimertinib (1 μM) and luteolin (50 μM) in H1975/OR cells.

MET kinase activity. The results showed that luteolin significantly inhibited MET kinase activity with concentration gradient (IC50 = 9.103 μM) (**Figure 6F**). These compelling evidence suggests that luteolin inhibited MET kinase activity by binding within the ATP-binding pocket of MET.

*Reduced expression of HGF by the combination of luteolin and osimertinib*

The only known ligand of the MET protein is HGF. HGF can bind to tyrosine residues Tyr1234 and Tyr1235 of the MET protein, regulating kinase activity and inducing the autophosphorylation of MET, thus activating downstream pro-cancer pathways to induce osimertinib resistance [31]. Consequently, we investigated whether combination therapy with luteolin and osimertinib could affect autocrine HGF protein expression in acquired resistant cells. Surprisingly, we found that the expression of autocrine HGF was also upregulated in the H1975/OR cells and the combination therapy downregulated the expression of HGF (**Figure 4C**). Based on the aforementioned results, it can be deduced that the HGF-MET axis played a major role in the development of acquired resistance in the H1975/OR cell line. Moreover, this combination therapy of luteolin and osimertinib was found to suppress the activation of the HGF-MET axis and further inhibit its downstream Akt pathway.

## Discussion

Since Goodman and Gilman et al. reported the application of nitrogen mustard in treating non-Hodgkin's lymphoma in 1946 [32], humans have embarked on the journey to fight against malignant tumors using drugs. Through subsequent research, a series of drugs have been proven to have antitumor effects and are widely used in clinical treatment. The development of drug resistance has become an essential obstacle to drug therapy in cancer. The modern medical mechanism of drug resistance in tumor cells involves a variety of genes, signaling pathways, and environmental factors. The common ones mainly include drug target alteration, drug efflux, abnormal activation of signaling pathways, DNA damage repair, epithelial-mesenchymal transition, the influence of exosomes, and changes in the tumor microenvironment [33].

Compared with first- and second-generation EGFR-TKIs, osimertinib has improved efficacy and safety, with mPFS increasing from 10.2 months to 18.9 months [34], and can improve the median progression-free survival (mPFS) to 10 months in patients treated with first- and second-generation EGFR-TKIs [35]. Acquired resistance refers to the emergence of drug resistance in EGFR-TKI treatment, the further selection of existing gene mutations in NSCLC cells, and new mutations or abnormal gene expression under the treatment's selective pressure. Approximately 50% of the patients

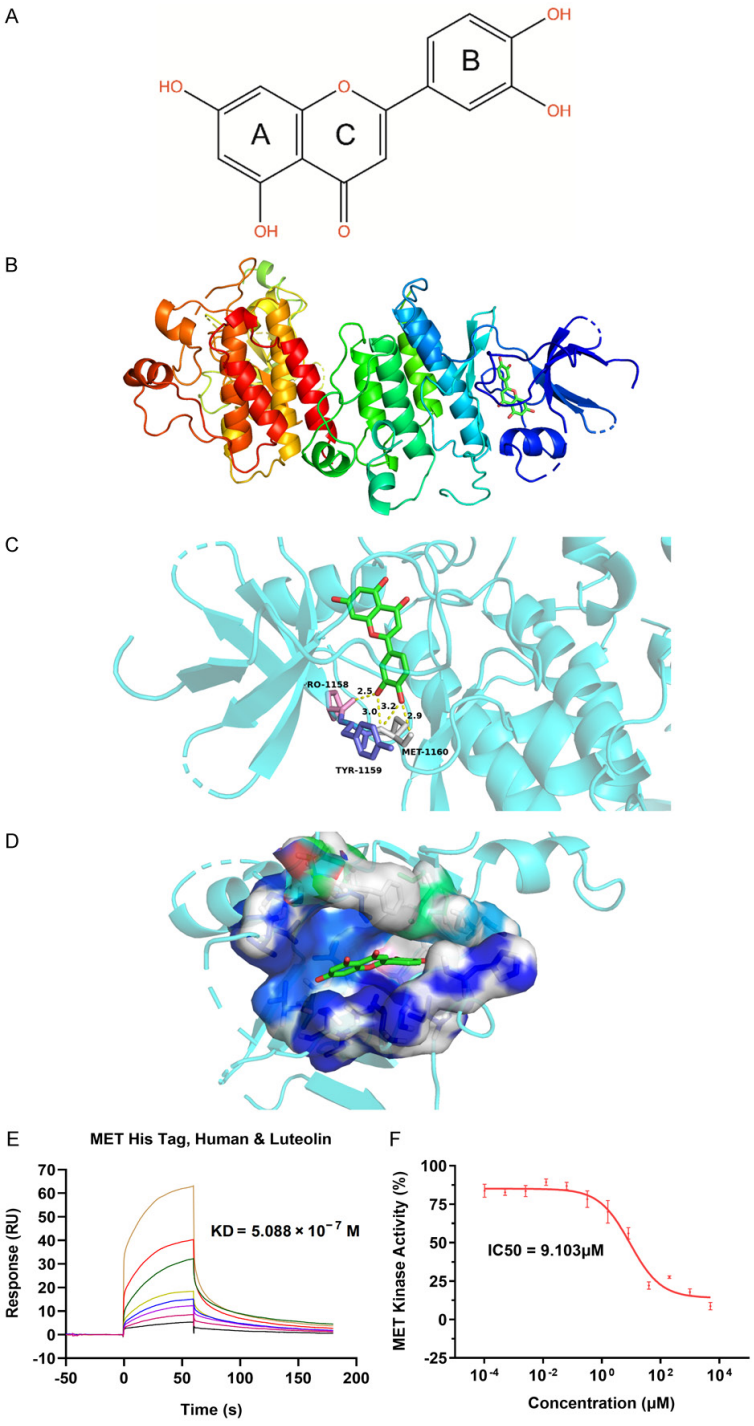


**Figure 6.** Stable binding of luteolin to MET by molecular docking analysis. A. The structural formula for luteolin. A, B, or C are the sequence number of the ring structure. B, C. The binding conformation of luteolin to the MET kinase domain. The colored structure shows the 3D conformation of the non-phosphorylated MET protein, and the red-green stick shows the 3D conformation of luteolin. Oxygen atoms: red stick. H-bond and length: yellow dotted line and attached number. PRO1158, TYR1159, and MET1160 active residues of MET binding to luteolin are represented by pink, purple, and grey sticks, respectively. D. Surface representation of luteolin docking in the binding pocket of MET kinase. E. The sensorgram of luteolin binding to MET-immobilized chip. The luteolin concentrations were 0.0390625, 0.078125, 0.15625, 0.3125, 0.625, 1.25, 2.50, and 5.00 μM (from bottom to top). F. The effect of luteolin treatment on MET kinase activity was measured by ADP-Glo™ kinase assay. The data are means ± SD of three experiments.

who acquired resistance to first- and second-generation EGFR-TKIs had the acquired T790M mutation [36]. The third-generation EGFR-TKI osimertinib (AZD9291) could irreversibly covalently bind to the amino acid C797 in intracellular EGFR, thereby inhibiting the phosphorylation of EGFR, and the activation of downstream signaling provided a way to overcome resistance caused by the T790M mutation. However, the reemergence of osimertinib resistance invariably develops.

Acquired resistance mechanisms to osimertinib can be broadly categorized into EGFR-dependent and EGFR-independent mechanisms. EGFR-dependent mechanisms of resistance to osimertinib involve the emergence of EGFR tertiary mutations or amplifications. These tertiary mutations include alterations in C797, G796, L792, L718, G719, and G724 [37-40]. On the other hand, EGFR-independent mechanisms of resistance encompass a wide range of factors such as MET amplification, HER2 amplification, activation of the RAS-MAPK pathway, activation of the PI3K pathway, overexpression of Mcl-1, overexpression of AXL, alterations in cell-cycle genes, oncogenic fusions, epithelial-to-mesenchymal transition (EMT), as well as histologic and pheno-

**Table 2.** Affinity Assessment of luteolin and MET

| Mode | Affinity (kcal/mol) | Distance From Best Mode (rmsd l.b.) | Affinity Assessment |
|---|---|---|---|
| 1 | -8.6 | 0.0 | Strong |
| 2 | -8.6 | 1.4 | Strong |
| 3 | -8.3 | 1.3 | Strong |
| 4 | -8.0 | 2.4 | Strong |
| 5 | -7.4 | 2.7 | Strong |
| 6 | -7.2 | 22.1 | Strong |
| 7 | -7.2 | 37.4 | Strong |
| 8 | -7.1 | 51.8 | Strong |
| 9 | -7.1 | 26.2 | Strong |
| 10 | -6.9 | 26.6 | Medium |
| 11 | -6.9 | 37.2 | Medium |
| 12 | -6.8 | 37.3 | Medium |
| 13 | -6.7 | 31.4 | Medium |
| 14 | -6.6 | 67.4 | Medium |
| 15 | -6.6 | 37.1 | Medium |
| 16 | -6.5 | 3.1 | Medium |
| 17 | -6.5 | 21.9 | Medium |
| 18 | -6.4 | 37.0 | Medium |
| 19 | -6.1 | 67.4 | Medium |
| 20 | -6.0 | 56.1 | Medium |

Affinity < -4, weak binding or no binding; -7~-4, medium binding; < -7, strong binding.

typic transformations [14, 41-47]. Among them, MET amplification is the most common EGFR-independent mechanisms [12]. Notability, despite considerable research, there are still many mechanisms of acquired resistance to osimertinib that have yet to be fully elucidated.

Unlike primary resistance, the mechanism and time of acquired resistance are difficult to predict. The implementation of liquid biopsy and integration with RNA sequencing data to monitor the response to osimertinib and detect the molecular alterations responsible for treatment failure is highly warranted. Moreover, the speed of new EGFR-TKI development is far less than the occurrence of clinical drug resistance, and the application of new drugs still cannot avoid the reemergence of acquired resistance through a general review of the current four generations of EGFR-TKIs [29, 48, 49]. The battle between new drugs and new resistance mechanisms will eventually be protracted, and it will be difficult to avoid the latter gaining the upper hand. Therefore, it may be more effective to increase the efficacy of existing EGFR-TKIs or to delay drug resistance by studying the combination of EGFR-TKIs and natural compounds while developing new drugs targeting resistance mutations. Therefore, in this study, we explored whether acquired resistance could be overcome by combining natural compounds and osimertinib and successfully found luteolin to play a role in anti-acquired resistance by acting on MET.

MET amplification and overactivation were acquired resistance mechanisms with a high incidence in clinical practice. In recent years, an increasing number of drugs targeting MET amplification and overactivation have been studied. However, there are few studies on the efficacy of traditional Chinese medicine monomers and compounds in overcoming resistance to osimertinib caused by MET amplification and overactivation.

Many studies have proven that the natural product luteolin has synergistic effects with antitumor drugs in targeted therapy, immunotherapy, and chemotherapy. Luteolin has been confirmed to enhance the proliferation inhibition and apoptosis of erlotinib in glioblastoma cell lines [50]. Another study found that luteolin could synergistically inhibit colorectal cancer by inhibiting AMPK and oxaliplatin [51]. The combination of a PD-1 blocker and luteolin or its derivative apigenin had a synergistic effect and could inhibit PD1 expression in KRAS mutant NSCLC [52]. Luteolin could also synergistically inhibit estrogen receptor-α and cyclin-dependent kinase 4/6 pathways in combination with indole-3-methanol to inhibit estrogen α-positive breast cancer [53]. In this study, we combined luteolin with osimertinib, and it was found that, similar to other studies mentioned above, luteolin had a synergistic effect with osimertinib, indicating a unique synergistic effect of luteolin as a natural product.

In this study, the effect of luteolin in overcoming resistance to osimertinib was demonstrated. However, the clinical application of luteolin and its derivatives has been hindered by their fragile chemical structure and poor solubility. Therefore, improving the stability and solubility of luteolin by nanoparticle assembly may be a new direction for luteolin. Finally, we hope this

study will inspire the emergence of more effective and diverse EGFR-TKI resistance studies.

## Conclusions

This study has investigated the effect and mechanism of luteolin combined with osimertinib on anti-acquired resistance. After constructing an acquired osimertinib-resistant cell line, we conducted assays of cell proliferation, migration, invasion, apoptosis, cell cycle, gene copy number, protein expression and molecular docking. According to the results, we have found that luteolin synergistically interacted with osimertinib to inhibit the proliferation, migration and invasion while promoting apoptosis of MET-amplified osimertinib-acquired resistant NSCLC cells, which have proved to be relatively more sensitive to luteolin Subsequently, we clarified that the amplification and overactivation of MET were the acquired resistance mechanism of the H1975/OR cell line, and the combination of luteolin and osimertinib inhibited the activation of HGF-MET-Akt pathway. Our findings provide evidence for further exploration into luteolin and its derivatives for overcoming acquired resistance to osimertinib and potentially other EGFR-TKIs.

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## Disclosure of conflict of interest

None.

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