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Research article

# DMU-212 against EGFR-mutant non-small cell lung cancer via AMPK/PI3K/Erk signaling pathway

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ABSTRACT

Although some important advances have been achieved in clinical and diagnosis in the past few years, the management of non-small cell lung cancer (NSCLC) is ultimately dissatisfactory due to the low overall cure and survival rates. Epidermal growth factor (EGFR) has been recognized as a carcinogenic driver and is a crucial pharmacological target for NSCLC. DMU-212, an analog of resveratrol, has been reported to have significant inhibitory effects on several types of cancer. However, the effect of DMU-212 on lung cancer remains unclear. Therefore, this study aims to determine the effects and underlying mechanism of DMU-212 on EGFR-mutant NSCLC cells. The data found that the cytotoxicity of DMU-212 on three EGFR-mutant NSCLC cell lines was significantly higher than that of normal lung epithelial cell. Further study showed that DMU-212 can regulate the expression of cell cycle-related proteins including p21 and cyclin B1 to induce G2/M phase arrest in both H1975 and PC9 cells. Moreover, treatment with DMU-212 significantly promoted the activation of AMPK and simultaneously down-regulated the expression of EGFR and the phosphorylation of PI3K, Akt and ERK. In conclusion, our study suggested that DMU-212 inhibited the growth of NSCLCs via targeting of AMPK and EGFR.

## 1. Introduction

Of all cancer types worldwide, lung cancer is one of the most prevalent and deadly, with very high incidence and mortality rates [1,2]. There are two well-known histological subtypes of lung cancer, NSCLC and small cell lung cancer (SCLC). NSCLCs represent 80–85% of lung cancers while SCLCs represent 15–20% [3,4]. Studies have been found in more than 50% of Asian patients with NSCLCs occur EGFR-mutation, thereby targeting EGFR is an effective therapeutic strategy for the treatment of NSCLC [5].

EGFR is a transmembrane protein and plays a critical role in regulating cell growth, survival, proliferation, and differentiation in mammalian cells via the activation of phosphatidylinositol 3-kinase (PI3K)-AKT pathways, extracellular-regulated kinase (ERK) pathways and so on [6,7]. However, Mutations of EGFR provide multiple advantages to tumors including promoting tumor progression, metastasis and angiogenesis of various of carcinoma cells [6,8].

Tyrosine kinase inhibitor (TKI) treatment is one of the therapeutic strategies for treating NSCLC with EGFR-mutation [9]. However, it is inevitable that most NSCLC patients developed acquiring resistance after receiving EGFR-TKI treatment [10]. Therefore, it is increasingly urgent to develop the new therapeutic strategies and anti-tumor drugs for NSCLCs with EGFR-mutations.

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Recent studies have found that activated AMPK pathway can inhibit the growth of gefitinib-resistant (G-R) NSCLCs partially through regulating the EGFR [11–13]. Therefore, targeting AMPK signaling pathway is a prospective therapeutic strategy to EGFR-mutation and resistant NSCLC.

Resveratrol (Fig. 1A), a phytoalexin present in natural foods such as grapes, blueberries and so on [14,15], has the inhibitory effects on the proliferation of a variety of tumor cells and the multidrug resistance, and can also against tumors by activating the immune system [16–18]. However, the low bioavailability and metabolic stability of resveratrol limits its application [19]. After structural modification [20], DMU-212 (Fig. 1B), as a derivative of resveratrol, has shown the promising metabolic stability *in vivo* and the excellent antiproliferative effects in breast, liver, melanoma, and ovarian cancer cells than resveratrol [21–23]. Our previous studies

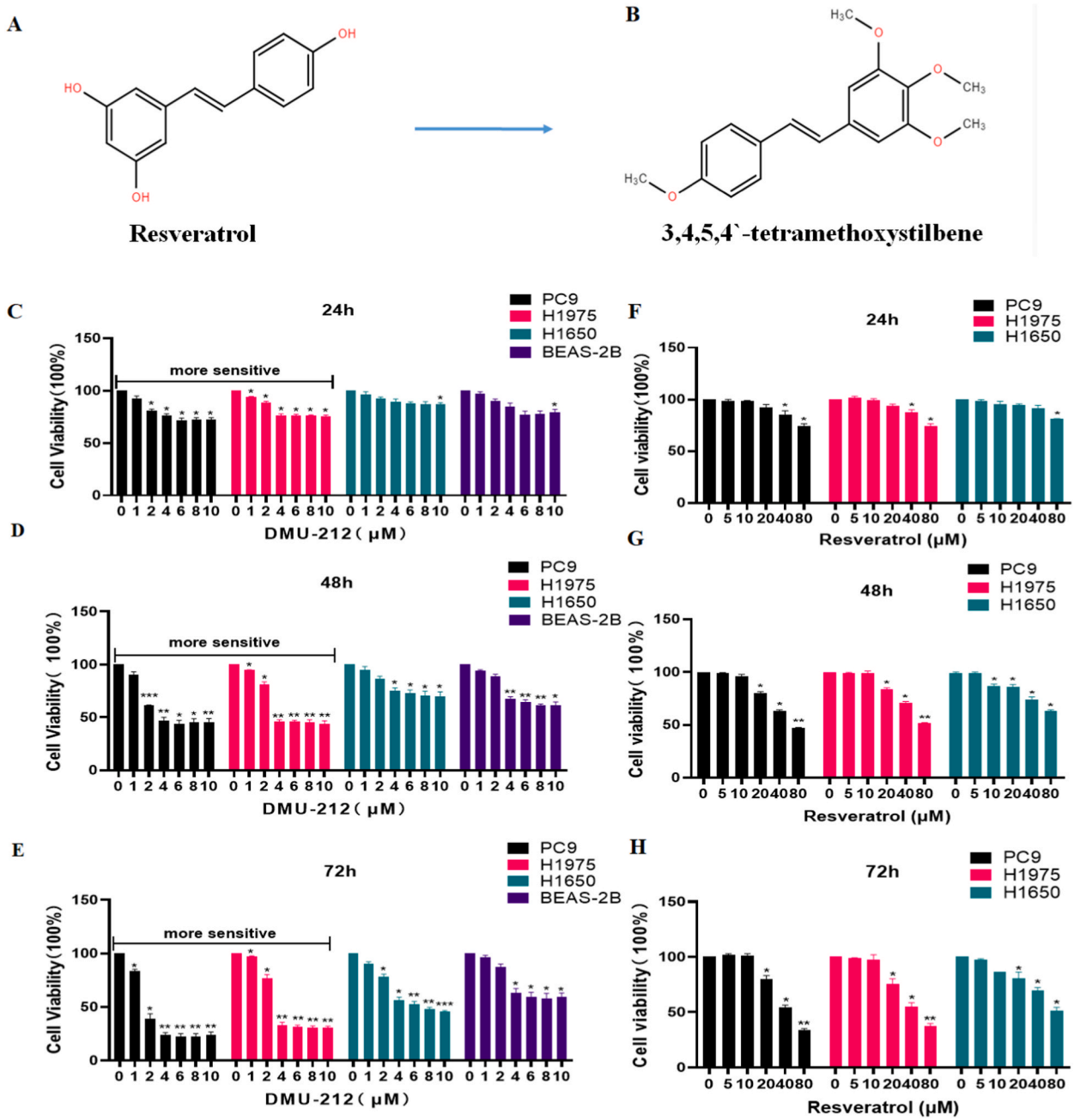


**Fig. 1.** The cytotoxic effects of DMU-212 and resveratrol on NSCLC cell lines. (A) The chemical structure of resveratrol. (B) The chemical structure of 3,4,5,4′-tetramethoxystilbene. (*C*–H) SRB assay showed the cell viability of three EGFR-mutant NSCLC cell lines (H1975, PC9, H1650) and one normal lung cell line (BEAS-2B) treated with DMU-212 or resveratrol for 24 h, 48 h and 72 h, all data presented as mean ± SD (n = 3), ***p < 0.001, **p < 0.01, *p < 0.05.

have found that DMU-212 exerted an inhibitory effect on G-R NSCLCs [24], while the effects and mechanisms of DMU-212 on other NSCLCs with EGFR genetic mutation remains unclear. Therefore, in present study, we explored the effects and underlying mechanisms of DMU-212 on NSCLC carrying EGFR mutation.

## 2. Materials and methods

### 2.1. Reagents

DMU-212 was obtained from Sigma (St Louis, MO, USA), and resveratrol was purchased from Herbest (ShanXi, China). Fetal bovine serum (FBS), antibiotics, RPMI-1640 medium and DMEM medium were purchased from Gibco (Carlsbad, CA, USA). Sulforhodamine B (SRB) was obtained from Meryer Chemical Technology (ShangHai, China) and Trichloroacetic acid (TCA) was purchased from aladdin (ShangHai, China). Tris-base was obtained from Sangon Biotech (Shanghai, China). The primary antibodies Cyclin B1, P21, *P*-PI3K, PI3K, *P*-AKT, AKT, *P*-EGFR (Tyr 1173), EGFR, *P*-Erk, *P*-STAT3 (Tyr705), STAT3, *P*-mTOR, mTOR, *P*-S6 and Erk were purchased from Cell Signaling Technology (Danvers, MA, USA), and other primary antibodies GAPDH, β-actin, S6, *P*-AMPK and AMPK were obtained from Santa Cruz (Dallas, TX, USA). The AffiniPure Goat Anti-Mouse secondary antibodies and the AffiniPure Goat Anti-Rabbit secondary antibodies were purchased from Jackson ImmunoResearch Labs (PA, USA). Annexin V/Propidium iodide (PI) staining kit was obtained from BD Biosciences (San Jose, CA, USA).

### 2.2. Cell lines and cell culture

H1975, PC9, H1650, and BEAS-2B cells were purchased from the American Type Culture Collection (ATCC) (Manassas, VA, USA). All lung cancer cell lines were cultured with RPMI-1640 medium, while BEAS-2B cells were cultured in DMEM. Culture medium was supplemented with 10% FBS and 1% antibiotics (penicillin–streptomycin). The cells were cultured in a 37 °C incubator with 5% $CO_2$ level.

### 2.3. SRB assay

Cells were seeded in 96-well culture plates at a density of 5000 cells/well and incubated for 24 h. The following day, cells were treated with various concentrations of DMU-212 for 24 h, 48 h and 72 h. Subsequently, Cells in 96-well plates were fixed by adding into 50 μL cold 50% TCA directly to medium supernatant and were incubated at 4 °C for 1 h. After washing the plates four times with tap water and air-drying at room temperature, cells were incubated with 100 μL of 4% SRB solution at room temperature for 1 h and rinsed four times with 200 μL 1% acetic acid. Finally, after air-drying plates at room temperature, the protein-bound dyes were solubilized by adding 100 μL 10 mM tris base solution and were measured at 515 nm in a microplate reader to analyze the cell viability.

### 2.4. Cell cycle analysis

H1975 or PC9 cells were seeded in 60 mm dish at $5 \times 10^5$ cells/well and incubated overnight. After treatment with different concentrations of DMU-212 (0, 0.75, 1.5, 3, 6 μM) for 24 h, cells were collected and rinsed with cold PBS and fixed with 70% ethanol for 2 h at 4 °C. After washing three times with cold PBS, the cell pellets were resuspended in 500 μL of 1 × PI staining solution and incubated for 30 min at 4 °C in the dark. Subsequently, the cell cycle was assessed using a BD Aria III Flow Cytometer (BD Biosciences, San Jose, California, USA), and each phase (sub-G1, G1, S, G2) was analyzed using FlowJo software.

### 2.5. Western blot

H1975 ($4 \times 10^5$ cells/well) or PC9 ($5 \times 10^5$ cells/well) were seeded in 6 cm dishes and incubated overnight. The cells were harvested after treatment with different concentrations (0, 0.75, 1.5, 3, 6 μM) of DMU-212 for 24 h and were lysed in 1 × RIPA lysis buffer containing proteinase inhibitors for 30 min on ice. After centrifugation, the supernatants were collected and quantified by BCA protein assay kit.

10 μg of protein sample were separated using 10%–12% SDS-polyacrylamide gel electrophoresis and transferred to a polyvinylidene difluoride (PVDF) membrane. The membranes were then blocked with 8% skim milk for 1–2 h and incubated with various primary antibodies (1:1000) overnight at 4 °C. Subsequently, the membranes were exposed to anti-rabbit or anti-mouse secondary antibodies (0.2 μg/ml) for 1 h at RT. The signal of bands on the PVDF membrane were visualized on an Amersham Imager 600 (AI600) scanner according to the instructions of the manufacturer and analyzed by Scion Image software.

### 2.6. Statistical analysis

All data were expressed as mean ± standard deviation (SD) of three replicate experiments. The statistical analysis was processed by GraphPad Prism 9.0 software. One-way analysis of variance (ANOVA) was chosen to analyze the differences between a number of groups (>2 groups) and Dunnett's test was conducted to compare among different groups. Student *t*-test was used to determine the difference between the two groups. The differences were defined as statistically significant at $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$, $^{****}p < 0.0001$.

## 3. Results

### 3.1. DMU-212 selectively inhibited the growth of EGFR-mutated NSCLC cells

Firstly, the cytotoxicity of DMU-212 and resveratrol was analyzed in a series of EGFR-mutant NSCLCs including PC9, H1975 and H1650 as well as normal lung epithelial cells BEAS-2B cells. The $IC_{50}$ values were shown in Table 1. As shown in Fig. 1C–E, the results found that the viability rates of these three NSCLC cells were about 70%–80% when incubated with DMU-212 (0–10 μM) at 24 h. However, after incubation with DMU-212 at 48 h or 72 h, the viability rates of PC9 and H1975 cells ranged from 25% to 50%. We also found that H1975 ($IC_{50} = 3.94 \pm 1.02$ μM, 72 h) and PC9 ($IC_{50} = 2.04 \pm 0.52$ μM, 72 h) cells were more sensitive to the treatment of DMU-212 than H1650 cells ($IC_{50} = 6.98 \pm 0.81$ μM, 72 h). Moreover, the cell viability of BEAS-2B cells was significantly higher than that of the other three NSCLCs at 24 h, 48 and 72 h, especially compared with PC9 cells and H1975 cells. Additionally, our results found that resveratrol treatment also significantly inhibited the growth of PC9 ($IC_{50} = 59.96 \pm 17.80$ μM, 72 h), H1650 ($IC_{50} = 85.04 \pm 13.97$ μM, 72 h) and H1975 ($IC_{50} = 58.15 \pm 14.69$ μM, 72 h) cells (Fig. 1F–H), while the effective concentration of resveratrol on these cells is 10–20 times that of DMU-212. The results showed that DMU-212 has a better effect on inhibiting the proliferation of EGFR-mutated NSCLCs than resveratrol. These results indicated that DMU-212 selectively inhibited the proliferation of EGFR mutant NSCLC cells in a dose and time dependent manner and exhibited lower cytotoxic activity in normal lung epithelial cells.

### 3.2. DMU-212 induced cell cycle arrest in EGFR-mutated NSCLC cells

To further determine the mechanism of DMU-212 in inhibiting proliferation of EGFR-mutated NSCLC cells, we examined the effect of DMU-212 on cell cycle by flow cytometry. As shown in Fig. 2A–C, treatment with DMU-212 for 24 h dose-dependently decreased the number of cells in G1 phase of cell cycle and significantly increased the number of cells in G2/M phase in H1975 and PC9 cells. In particular, the cell cycle of PC9 cells was completely blocked in G2/M phase after DMU-212 treatment.

Moreover, the results also showed that treatment with DMU-212 for 24 h significantly down-regulated the protein expression of cyclin B1 and up-regulated the protein expression of p21 (Fig. 2D–I). These data suggested that DMU-212 induced the G2/M phase arrest of cell cycle in both H1975 and PC9 cells.

### 3.3. DMU-212 inhibited the growth of EGFR-mutated NSCLC cells by activating AMPK

Studies have found that activation of AMPK can regulate the energy metabolism of tumor cells to induce cell cycle arrest, which is an important pathway to inhibit the growth and proliferation of tumor cell [25]. Therefore, we determined the effect of DMU-212 on the activation of AMPK and the changes of related downstream components in H1975 and PC9 cells.

As shown in Fig. 3A–D, DMU-212 significantly induced the phosphorylation of AMPK (Thr172) in both H1975 and PC9 cells, and simultaneously decreased the expression of *P*-mTOR and *P*–S6 (Fig. 3*E* and F), suggesting that DMU-212 may inhibit the growth of NSCLC cells with EGFR mutations via the activation of AMPK.

### 3.4. DMU-212 inhibited EGFR and its downstream pathway in EGFR-mutated NSCLC cells

EGFR mutations aberrantly activate its downstream proteins PI3K/AKT pathway and Erk pathway, playing an important role in the progression of NSCLC [26]. In addition, our previous studies have shown that targeting the crosstalk between the AMPK and EGFR pathways could be a major therapeutic strategy against NSCLCs with EGFR mutation [11,12]. Consequently, we examined the effect of DMU-212 on the activation of EGFR, Erk, PI3K and AKT in H1975 and PC9 cells. Our results showed that DMU-212 inhibited the phosphorylation of EGFR and Erk after treatment with DMU-212 (Fig. 4A and B). Moreover, as shown in Fig. 4C and D, the results also found that the phosphorylation of PI3K and AKT were significantly downregulated after treatment with DMU-212. These data altogether demonstrated that DMU-212 could prevent EGFR-induced activation of Erk and PI3K/Akt signaling pathways and thus suppressed the proliferation of EGFR-dependent NSCLC cells.

STAT3 is a key downstream protein of the EGFR pathway and highly activated in EGFR-TKI resistant NSCLC [27–30]. Dysregulated STAT3 activation involved in multiple processes including cell proliferation, migration, invasion, immune evasion and drug resistance

**Table 1**
The $IC_{50}$ value of DMU-212 and resveratrol in EGFR-mutant NSCLC cell lines or normal lung cells.

| Drugs | Cell line | Cell type | EGFR gene status | $IC_{50}$ Value of DMU-212 (24 h) | $IC_{50}$ Value of DMU-212 (48 h) | $IC_{50}$ Value of DMU-212 (72 h) |
|---|---|---|---|---|---|---|
| DMU-212 | PC9 | NSCLC | Exon19 deletion | >10 μM | 5.23 ± 1.17 μM | 2.04 ± 0.52 μM |
| Resveratrol | | | | >80 μM | 76.59 ± 11.47 μM | 59.96 ± 17.80 μM |
| DMU-212 | H1975 | NSCLC | L858R and | >10 μM | 6.15 ± 1.18 μM | 3.94 ± 1.02 μM |
| Resveratrol | | | T790 M | >80 μM | 98 ± 16.8 μM | 58.15 ± 14.69 μM |
| DMU-212 | H1650 | NSCLC | Exon19 deletion | >10 μM | >10 μM | 6.98 ± 0.81 μM |
| Resveratrol | | | | >80 μM | >80 μM | 85.04 ± 13.97 μM |
| DMU-212 | BEAS-2B | Human normal lung epithelial cells | Wild type | >20 μM | >10 μM | 10.88 ± 2.18 μM |

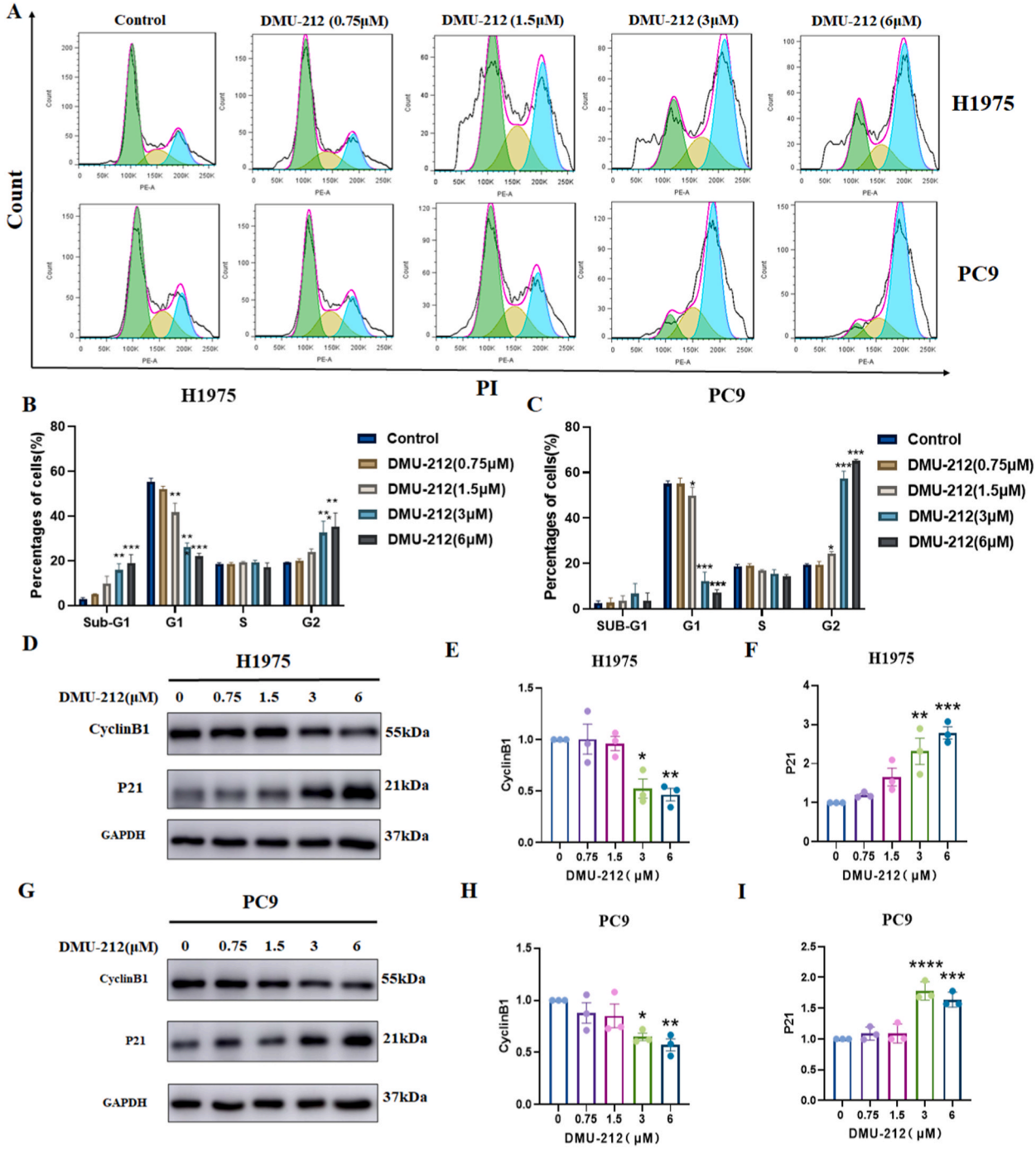


**Fig. 2.** DMU-212 induced cell cycle arrest in H1975 cells and PC9 cells. (A–C) Flow cytometric analysis of cell cycle in H1975 and PC9 cells treated with different concentrations of DMU-212. The mean value of each subgroup was obtained from three independent experiments, ***p < 0.001, **p < 0.01. (D and G) Western blot analysis to determine expression of cyclin B1 and p21 in H1975 and PC9 cells after 24 h treatment with increased concentrations of DMU-212. (E, F, H and I) Expression levels of each target protein/GAPDH were presented to show change in related proteins. Statistical analysis result of the protein level represented as mean ± SD (n = 3), ****p < 0.0001. ***p < 0.001, **p < 0.01, *p < 0.05.

in malignant tumors [31]. Therefore, we determined the effect of DMU-212 on activated STAT3 in gefitinib-resistant (G-R) NSCLC cells (H1975 cells). As shown in Fig. 4E and F, DMU-212 inhibited the phosphorylation of STAT3 in H1975 cells. This result demonstrated that DMU-212 can target EGFR/STAT3 pathway, and thus inhibit the proliferation of G-R NSCLC cells.

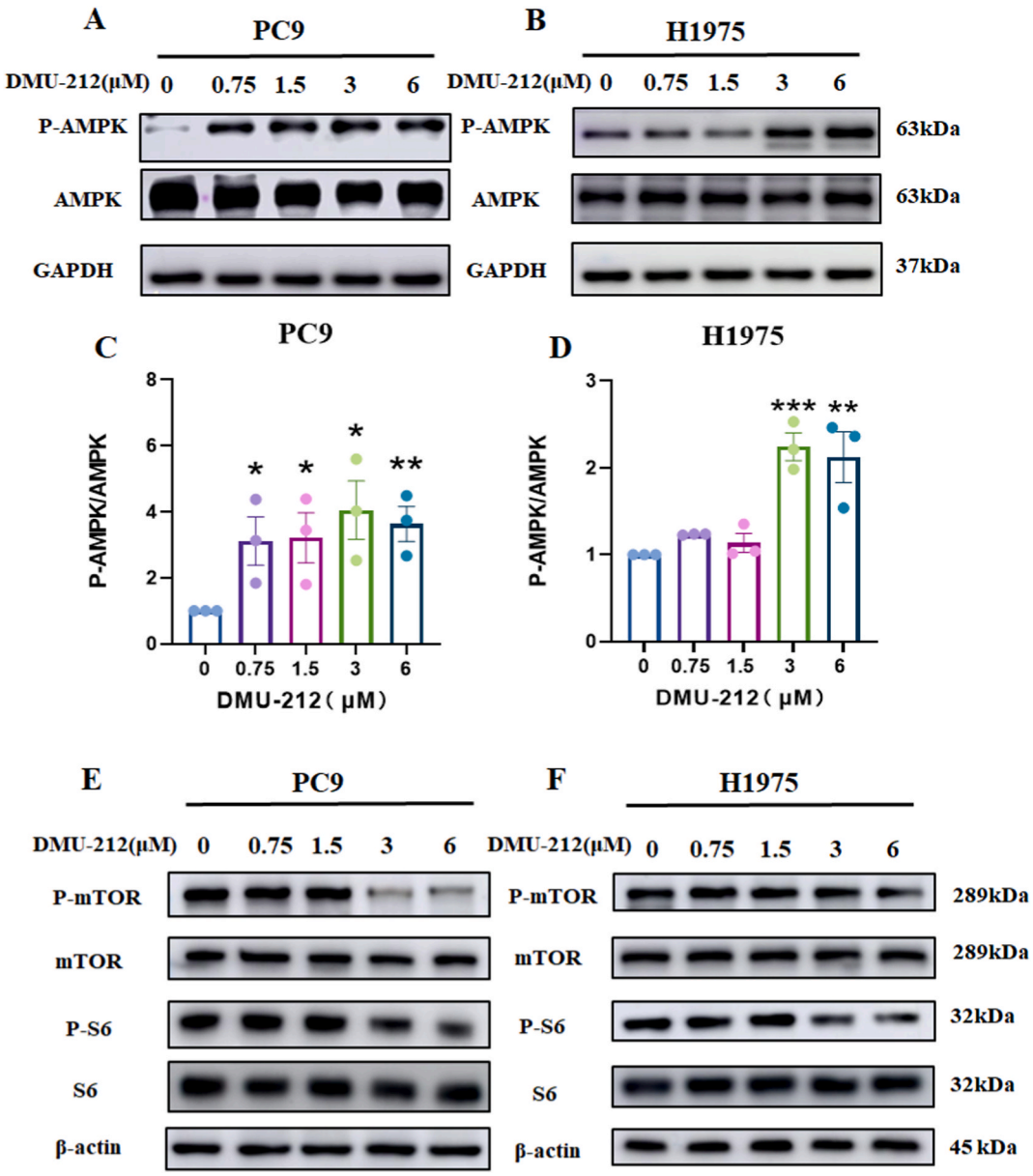


**Fig. 3.** DMU-212 significantly activated the AMPK pathway (A–B) The phosphorylation of AMPK was increased both in H1975 cells and PC9 cells, (*C*–D) The relative expression level of *P*-AMPK/AMPK was presented to show the changes of *P*-AMPK. (*E*–F) The phosphorylation of AMPK downstream target was down-regulated with DMU-212 treatment. Statistical analysis result of the protein level was represented as mean ± SD (n = 3), ***p < 0.001, **p < 0.01, *p < 0.05.

## 4. Discussion

EGFR-TKI has been widely established for NSCLC patients, but systemic disease progression will eventually occur in most patients who continue to receive EGFR-TKI [32], which is usually due to the emergence of acquired drug resistance. The EGFR-T790 M mutation accounts for nearly 50% of all subtypes of lung cancer and is the most common mechanism of acquiring resistance to EGFR-TKI in NSCLC [33,34]. Therefore, the development of new drugs targeting T790 M mutation is a major measure to overcome acquired drug resistance in clinical practice. Natural compounds have always been regarded as potential resources for novel agent development. DMU-212, an analog modified from natural chemo-preventive agent (resveratrol), has been found to be effective on TKI sensitive and resistant NSCLCs in our study.

We first discovered that DMU-212 significantly inhibited the proliferation of three EGFR mutant NSCLCs and was especially potent in PC9 and H1975 cells which obtained EGFR mutation. Then, we found the inhibition of cell proliferation was caused by the G2/M

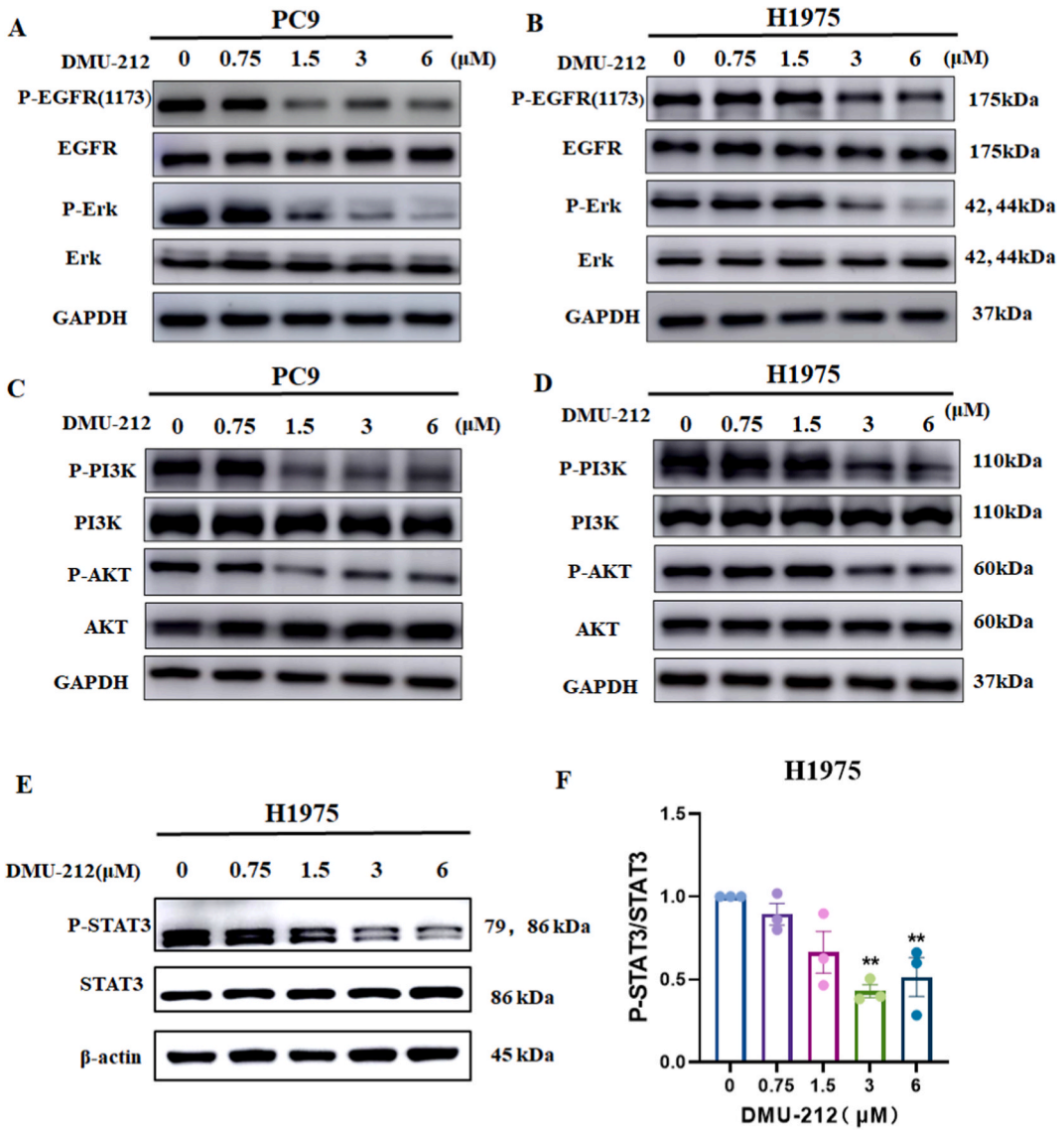


**Fig. 4.** The activation of EGFR was inhibited in EGFR mutant cells. (A–B) DMU-212 specifically decreased the phosphorylation of tyrosine residue 1173 on EGFR and affected the phosphorylation of downstream protein Erk in H1975 and PC9 cells. (*C*–D) EGFR downstream pathway PI3K/AKT was significantly inhibited after treatment with DMU-212 for 24 h. (*E*–F) The phosphorylation of STAT3 was significantly inhibited after DMU-212 treatment in H1975 cells. The relative expression level of *P*-STAT3/STAT3 was presented to show the changes of *P*-STAT3. Statistical analysis result of the protein level was represented as mean ± SD (n = 3), ***p < 0.001, **p < 0.01, *p < 0.05.

phase arrest of PC9 and H1975 cell cycle distribution induced by DMU-212. P21 (also known as $p21^{WAF1/CIP1}$), a well-known Cyclin-dependent kinase (CDK) inhibitor, is involved in the process of cell cycle progression from G1 to S to G2 and then to mitosis through the regulation of CDKs. In the CDK family of proteins the most important are CDK1 (Also called Cdc2), CDK2, CDK4 and CDK6 in throughout cell cycle progression, and cyclins are their regulatory subunits. CDKs combine with cyclins to form heterodimers to regulate the cell cycle via phosphorylation of downstream substrate Rb. In addition, cell cycle progression triggered by CDK-cyclin complex can be affected and inhibited by p21 [35]. The CDK1-cyclin B complex is an important marker of G2/M phase. Blocking synthesis of cyclin B1 and the inactivation of CDK1 results in the disruption of cell division and the termination of G2/M transformation [36]. Here, in our results, the expression of p21 was increased while the expression of cyclin B1 was decreased. This shows that DMU-212 induces cell cycle arrest at G2/M phase through up-regulated p21 expression promoting the degradation of cyclin B1. As a cell energy sensor enzyme, AMPK is involved in maintaining energy homeostasis and is considered a key molecule controlling cell growth in the occurrence and development of cancer [37]. It has been reported that the cell cycle arrest can be induced via the activation of AMPK in myeloma, ovarian and breast cancer cells with metformine treatment [38–40]. Moreover, metformin can promote apoptosis and inhibit angiogenesis by activating AMPK pathway, preventing the proliferation of cancer cells [41–43].

Resveratrol is a natural AMPK activator like metformin [44]. In this study, we found that its analog DMU-212 significantly induced the phosphorylation of AMPK in G-R/G sensitive NSCLC via with as shown Western blot analysis. The data suggests that the blockade of G2/M phase induced by DMU-212 may be partly realized through the activation of AMPK pathway. However, whether DMU-212 induces growth inhibition in AMPK dependent or AMPK independent manners needs to be further explored.

In anti-cancer therapy, multi-target treatment is usually more effective than single-target treatment. For example, mice with bladder cancer treated with metformin and gefitinib survived much longer than those treated with either drug alone [45]. The synergistic action may map the crosstalk between mechanisms. Since EGFR is expressed in more than 60% of NSCLCs, EGFR has become a popular target for the treatment of these cancers [6]. Activated EGFR can transduce downstream RAS/MAPK/ERK and PI3K/AKT signaling pathways, which are essential for a variety of cancer cell functions including survival, proliferation, autophagy and metabolism [46]. Studies have demonstrated that inhibition of PI3K/AKT signaling pathway promotes p21 induced G2/M phase arrest in the cell cycle [11,47]. A recent study also found that EGFR-ERK aixs is a key upstream signaling factor of Grainyhead-like 1 (GRHL1) which regulates G2/M phase and promotes cell cycle progression of lung cancer [48]. Moreover, several literatures have revealed that these two downstream pathways of EGFR are related to glycolysis. The blocking of GLUT1 endocytosis mediated by active Akt results in increased glucose uptake [49–51], and the activation of Erk also promoted aerobic glycolysis via the phosphorylation of Pyruvate kinase M2 (PKM2) [52]. In this study, DMU-212 significantly decreased the phosphorylation of EGFR and the downstream proteins (PI3K, AKT, Erk) in cells with EGFR-mutation, resulting in cell cycle arrest and inhibition of cell growth. Interestingly, Makinoshima et al. [49] reported that the glycolytic flux of lung adenocarcinoma cells carrying EGFR mutations was reduced through inhibition of the PI3K/AKT/mTOR pathway, which may indicate that the synergistic inhibition of EGFR and energy metabolism may be a more effective anti-cancer strategy for EGFR-expressing cancer cells. Therefore, further exploration is needed to describe in more detail the molecular mechanism by which EGFR stimulates downstream pathways to control GLUT1 expression and activity under the action of DMU-212.

Several studies have reported that EGFR-TKI resistance is associated with abnormal activation of STAT3 [53,54]. Thus, targeting STAT3 has been recognized as an attractive target for cancer therapy. Our results also initially indicated that the activation of STAT3 was suppressed by DMU-212 in G-R NSCLC cells, which may imply that DMU-212 can alleviate TKI-induced resistance.

Taken together, DMU-212, a derivative of natural cancer chemo-preventive agent, effectively inhibited EGFR-mutant NSCLC cells through the crosstalk between AMPK/EGFR pathways (Fig. 5). DMU-212 shows promising potential for patients with NSCLCs and other cancers expressing EGFR.

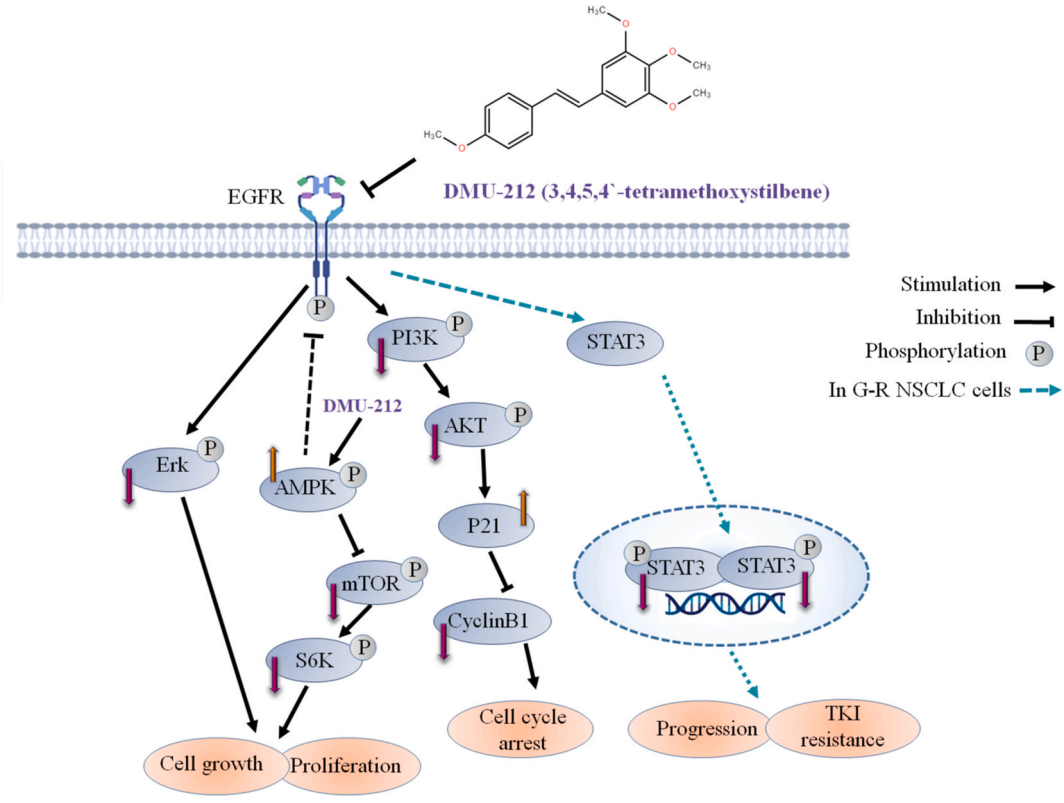


**Fig. 5.** The schematic diagram of underlying mechanism of DMU-212 action on EGFR-mutant NSCLC cells.

## Author contribution statement

Xiao-Ping Zhao: Conceived and designed the experiments; Performed the experiments; Analyzed and interpreted the data; Wrote the paper.

Xiao-Li Zheng; Min Huang; Ya-Jia Xie; Xiao-Wen Nie: Performed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Ali Adnan Nasim: Contributed reagents, materials, analysis tools or data; Wrote the paper.

Xiao-Jun Yao; Xing-Xing Fan: Conceived and designed the experiments; Contributed reagents, materials, analysis tools or data; Wrote the paper.

## Data availability statement

Data supporting the results of this study are available from the corresponding authors.

## Declaration of competing interest

The authors have no financial or commercial conflict of interest to disclose.

## Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2023.e15812.

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