

# Peruvoside is a new Src inhibitor that suppresses NSCLC cell growth and motility by downregulating multiple Src-EGFR-related pathways

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## Research

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# Abstract

## Background

The tyrosine kinase Src plays an essential role in the progression of many cancers and is involved in several signalling pathways regulated by EGFR. To improve the efficacy of lung cancer treatments, this study aimed to identify novel compounds that can disrupt the Src-EGFR interaction to inhibit lung cancer progression and are less dependent on the EGFR mutation status than other compounds.

## Methods

We used Src pY419 ELISA as the drug-screening platform to screen a compound library of more than 400 plant active ingredients and identified peruvoside as a candidate Src-EGFR crosstalk inhibitor. Human Non-small cell lung cancer cell lines (A549, PC9, PC9/gef, H3255 and H1975) with different EGFR statuses were used to perform cell cytotoxicity and proliferation assays after peruvoside or dasatinib treatment. Src and Src-related protein expression was evaluated by western blotting in peruvoside-treated A549, H3255 and H1975 cells. The effects of peruvoside on cancer cell function were assessed in A549 cells. The synergistic effects of gefitinib and peruvoside were assessed by CI-isobologram analysis in gefitinib-resistant cell lines. The efficacy of peruvoside *in vivo* was determined using nude mice subjected to compound or vehicle treatments.

## Results

Peruvoside significantly suppressed the phosphorylation of Src, EGFR, and STAT3 in a dose- and time-dependent manner and somewhat suppressed their protein expression. Cell function assays revealed that peruvoside also inhibited the proliferation, invasion, migration, and colony formation of lung cancer cells *in vitro* and tumour growth *in vivo*. Furthermore, peruvoside sensitised gefitinib-resistant tumour cells (A549, PC9/gef and H1975) to gefitinib treatment, indicating that it may exert synergistic effects when used in combination with established therapeutic agents. Our data also demonstrated that the inhibitory effects of peruvoside on lung cancer progression might be attributed to its ability to regulate multiple proteins, including Src, PI3K, JNK, Paxillin, p130cas, and EGFR.

## Conclusions

Our findings suggest that peruvoside suppresses Non-small cell lung cancer malignancy by downregulating multiple Src-related pathways and may help develop new anticancer drugs and therapeutic strategies for lung cancer in the future.

## Introduction

Non-small cell lung cancer (NSCLC) is one of the leading causes of cancer-related mortality worldwide. The five-year survival rate of lung cancer patients after diagnosis has plateaued at only 12%-15% [1]. Mutations in oncogenic driver genes are usually the cause of dysfunction of normal cells. Most of these mutations occur in kinases related to signal transduction, such as HER2, KRAS, AKT1, MEK, and EGFR, causing them to be constitutively activated and overexpressed, thereby inducing abnormal cancer cell growth and metastasis, which leads to poor prognoses and poor treatment responses [2, 3]. Therefore, targeting these molecules to inhibit tumour growth is essential to improve the prognosis of NSCLC patients and increase the treatment efficiency for this disease [4].

In recent years, targeted therapies have been developed to treat NSCLC patients with certain driver mutations. EGFR tyrosine kinase inhibitors (TKIs), such as gefitinib and erlotinib, function by competing for the ATP-binding site and have been used in clinical treatment [5]. The sensitivity of these drugs is related to the position of the mutation on the tyrosine kinase domain of EGFR. For example, the lack of exon 19 and L858R mutation in exon 21 can increase sensitivity to the drug [6]. Conversely, the EGFR T790M mutation can lead to TKI resistance and is positively associated with lung cancer recurrence [7]. Drug resistance is a crucial issue in cancer treatment. Therefore, the development of new therapeutic strategies or targeted drugs, including the second-generation TKI afatinib [8] and third-generation TKIs osimertinib [9] and olmutinib [6], has become the main objective of current research focusing on cancer treatment. Nevertheless, approximately 10% of NSCLC patients show primary TKI resistance, and the underlying mechanism remains unclear.

The expression of the proto-oncogene Src is related to tumour development and a poor prognosis because Src regulates signalling pathways that affect its downstream protein expression [10]. Src is a member of Src family kinases (SFKs), which are non-receptor kinases. Under normal conditions, approximately 90%-95% of Src is phosphorylated at the Tyr530 position, which indicates a closed and inactive structure. In its activated form, Src is dephosphorylated at Tyr530 and auto-phosphorylated at Tyr416 in its kinase domain [11]. Mutations in EGFR and EGFR family-related proteins enhance Src expression and activate Src downstream signals through the ERK, Akt and STAT pathways [12]. Src and EGFR show synergistic effects through mutual phosphorylation and activation [13], and their co-expression induces cell transformation [14].

Because of the crosstalk between Src and EGFR, inhibiting Src may improve NSCLC treatment [15]. Several known Src inhibitors, including dasatinib (BMS-354825), saracatinib (AZD0530), and ponatinib (AP24534), have been developed as therapeutic agents, and their effectiveness against solid tumours has been evaluated in clinical trials [16]. Src inhibitors induce apoptosis in various NSCLC cells and inhibit cell survival and oncogenic malignant transformation regulated by EGFR [13]. Thus, inhibition of the Src pathway alone can induce cell apoptosis, and combinations with gefitinib can further enhance the effects of Src inhibitors on EGFR and HER2.

To improve the efficacy of lung cancer treatments, we aimed to identify compounds that disrupt the Src-EGFR interaction and are less dependent on the EGFR mutation status than other compounds. In this

study, using Src pY419 enzyme-linked immunosorbent assay (ELISA) as the drug-screening platform to screen a compound library of more than 400 plant active ingredients, we identified peruvoside (PubChem CID: 12314120) as a candidate Src-EGFR crosstalk inhibitor. We also investigated the functional mechanism underlying the ability of peruvoside to suppress lung cancer cell progression using *in vitro* and *in vivo* approaches. Our findings may promote the development of new anticancer drugs and therapeutic strategies useful to treat lung cancer in the future.

## Methods

### Cell culture

The human bronchial epithelial cell line BEAS2B (ATCC CRL-9609) and human lung adenocarcinoma cell lines A549 (ATCC CCL-185) and H1975 (ATCC CRL-5908) were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA). The human lung adenocarcinoma cell lines PC9, PC9/gef and H3255 were a kind gift from Dr. Chih-Hsin Yang (National Taiwan University Hospital, Taiwan). The cell lines were grown in RPMI (Gibco, Breda, The Netherlands) supplemented with 10% foetal bovine serum (FBS; Gibco) and 1% penicillin/streptomycin at 37 °C in a humidified atmosphere of 5% CO<sub>2</sub>.

### Drug treatment and herbal compound library

The herbal compound library, representing a collection of 415 pure products and their derivatives, was purchased from Sigma-Aldrich (St. Louis, MO) and ChromaDex (Irvine, CA) and contained a range of alkaloids, diterpenes, pentacyclic triterpenes, sterols, and many other diverse representatives. Peruvoside was purchased from Sigma-Aldrich, and a stock solution of peruvoside was prepared in dimethyl sulfoxide (DMSO) and stored at -20 °C. The compound was diluted in fresh medium before each experiment, and the final DMSO concentration was lower than 0.1%. To determine whether peruvoside promotes protein degradation through the ubiquitination pathway, A549 cells were treated with 10 μM MG132, a proteasome inhibitor (Sigma-Aldrich) for 2 h and then with peruvoside for an additional 24 h. The cell lysates were extracted and then subjected to western blot analysis of the specific proteins.

### Enzyme-linked immunosorbent assay (ELISA)

To accelerate the screening of the compound library, ELISA (Human Phospho-Src (Y419) DuoSet IC ELISA; R&D Systems, Minneapolis, MN, USA) was performed in the early stage. The details of the procedures, including plate preparation and signal detection, are described in the manufacturer's instructions. Briefly, A549 cells were seeded at  $1.5 \times 10^5$  cells per well and then were treated with various compounds for 24 h. After extraction and quantification, the cell lysates or standards were added to 96-well microplates coated with the diluted capture antibody for 2 h. Next, the detection antibody and streptavidin-HRP (1:2000) were consecutively added to each well. After incubating the samples with the substrate solution and stop solution, the absorbance was measured at 450 nm (570 nm as the reference) using a multilabel plate reader (Vector3; Perkin-Elmer, USA). The absorbance at 570 nm was subtracted from the absorbance at 450 nm to calculate the amount of Src phosphorylation.

# Western blot analysis

Western blotting was used to examine the protein activity and expression levels of Src and related proteins after peruvoside treatment. The detailed procedures were described previously [17]. Monoclonal mouse anti-glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (1:5000; Upstate Biotechnology, Lake Placid, NY, USA) was used as a loading control. All the experiments were performed at least three times in duplicate.

## Cell viability and proliferation assay

The PrestoBlue (Invitrogen) cell viability reagent was used to evaluate the cytotoxic and proliferative effects of peruvoside used for various durations as described previously [17]. Briefly, the tested cell lines were seeded in 96-well plates at a density of  $5 \times 10^3$  cells/well and incubated for 24 h. Next, the cells were treated with different concentrations of peruvoside for 24, 48, 72 and 96 h. Subsequently, PrestoBlue solution was added to the culture medium in the wells. After a further 1.5 h of incubation, the colour intensity was measured at 570 nm (600 nm as the reference) using a multi-label plate reader (Vector3; Perkin-Elmer, USA).

## Colony formation assay

For the anchorage-dependent growth assay, 500 cells were seeded in six-well plates and treated with peruvoside. After one week, the cells were washed with PBS and fixed with 4% paraformaldehyde. The fixed cells were stained with 0.05% crystal violet. For the anchorage-independent growth assay, CL1-5 cells were seeded at  $1 \times 10^3$  cells per well in soft agar. After solidification, the cells were treated with different concentrations of peruvoside. The cells were incubated for 2 weeks and then stained with 0.5 mg/ml of *p*-iodonitrotetrazolium violet. Colonies with a diameter greater than 1 mm were counted under an inverted microscope. The assay was performed as described previously [18].

## Cell migration and invasion assays

The motility of cancer cells was measured using a Transwell device, according to our previous study [19]. Briefly, Transwell membranes (8- $\mu$ m pore size, 6.5-mm diameter; Corning CoStar Corporation) coated with or without Matrigel (2.5 mg/ml; BD Biosciences Discovery Labware) were used for the invasion and migration assays, respectively. Medium with 10% FBS was added to the lower wells of the chambers, and the upper wells were filled with serum-free medium containing  $1 \times 10^4$  or  $2 \times 10^4$  A549 cells per well. The medium contained different concentrations of peruvoside (10 and 50 nM), and 0  $\mu$ M represented 0.1% DMSO as the solvent control. After 12 (migration) or 24 h (invasion) of incubation, the cells on the upper wells were removed, and the cells that migrated onto the lower surface of the membranes were fixed with methanol and stained with 20% Giemsa solution (Sigma-Aldrich). The cells were counted under a light microscope. The experiments were performed in triplicate.

## In vivo animal studies

The mouse experiments were approved by the Institutional Animal Care and Use Committee of the National Chung Hsing University, and tumorigenesis experiments were performed in the mice according to previously described protocols [17]. Six-week-old nude mice (obtained from the National Laboratory Animal Center, Taipei, Taiwan) were housed at six mice per cage. Briefly,  $1 \times 10^6$  A549 cells in 100  $\mu$ l of PBS were injected subcutaneously into the nude mice. When the tumour size reached 50–100 mm<sup>3</sup>, the mice were randomly grouped into peruvoside-treated or untreated groups. Peruvoside was suspended in DMSO and dosed i.p. once daily at 0.1 mg/kg for 4 weeks of treatment. After intraperitoneal injection, the mice were measured every 7 days for tumour appearance. After 4 weeks, the mice were killed, and their tumour sizes were analysed. The tumour volumes were estimated from the calliper-measured lengths (*a*) and widths (*b*) using the formula  $V = 0.4 \times ab^2$  [20].

## Real-time reverse transcription–PCR (RT-PCR) and immunohistochemical staining

The expression levels of Src and related genes were detected by real-time RT-PCR using an ABI prism 7300 sequence detection system (Applied Biosystems, Foster, CA). TATA-box-binding protein (TBP) was used as the internal control (GenBank X54993). The detailed procedures and calculations have been described previously [18]. Immunohistochemical staining was used to investigate the phosphorylation level of Src in tumour tissue from nude mice. Briefly, the paraffin-embedded tumour tissue sections were reacted with the rabbit anti-human Src pY418 polyclonal primary antibody (Invitrogen), and then the DAKO EnVision System, containing the horseradish peroxidase-conjugated anti-rabbit secondary antibody and enzyme substrate 3,3'-diaminobenzidine, was used to detect Src activity.

## Drug synergy analysis

To elucidate the synergistic effects of peruvoside and gefitinib on gefitinib-resistant lung cancer cells *in vitro*, A549, PC9/gef and H1975 cells were subjected to combination treatment and then cell viability assays. The above data were further analysed using CalcuSyn software (Biosoft, Cambridge, UK) and the combination index (CI)-isobologram equation, as described previously [21]. CI<1, CI=1 and CI>1 represent the synergistic, additive and antagonistic effects of the indicated compounds, respectively.

## Statistical analysis

All the *in vitro* experiments were performed at least in triplicate, and the results are presented as the mean  $\pm$  standard deviation where appropriate. Statistical analyses were performed using two-tailed Student's *t* test, and significant differences were defined as *P* values of less than 0.05.

## Results

### Drug bank screening via the Src pY419 ELISA platform

ELISA was performed to determine the inhibitory effect of the 415 pure compounds in the library on Src activity. The screening criteria and procedures of the drug bank are illustrated in Additional file 1: Fig. S1.

After two runs of ELISA screening, only 20 compounds showed a significant inhibitory effect compared with the control (Additional file 1: Fig. S2). Among these compounds, five had more than 50% inhibitory activity on Src phosphorylation and were identified as candidates. To focus our research, peruvoside (P7897), a cardiac glycoside with inotropic and chronotropic effects, was selected for further *in vitro* and *in vivo* studies.

## Effect of peruvoside treatment on cell viability and Src-related protein phosphorylation

Lung cancer A549 cells were treated with various concentrations of peruvoside and then were subjected to the cell cytotoxic assay at 24, 48, 72, and 96 h. The results are presented as the percentage relative to the DMSO-treated control. The corresponding  $IC_{50}$  values of each time point are shown in Table S1. The cytotoxicity results at 24 h indicated that cell viability was less influenced by peruvoside at 10 nM, a dose that is approximately equivalent to  $IC_{20}$ . Therefore, to determine the effects on cellular functions in subsequent experiments, we used concentrations no higher than 50 nM ( $IC_{50}$ ), which are more appropriate concentrations for the following investigations (Fig. 1a).

To determine the inhibitory effects of peruvoside on Src, EGFR and STAT3 phosphorylation, western blotting was performed. Peruvoside inhibited the activation and expression of Src, EGFR and STAT3 in A549 cells at 24 h in a dose- and time-dependent manner (Fig. 1b). Similar results were obtained in two other lung cancer cell lines, H3255 (Fig. 1c, left panel) and H1975 (Fig. 1c, right panel).

### *Inhibition of cell viability and proliferation ability in EGFR mutant lung cancer cell lines by peruvoside*

To identify the cytotoxic effect of peruvoside on EGFR mutant cell lines, PC9 and PC9/gef cells with exon 19 deletion, H3255 cells harbouring the L858R mutation and H1975 cells harbouring the L858R and T790M mutations were used. The viabilities of both TKI-sensitive or TKI-resistant cell lines were significantly inhibited by 50 nM peruvoside at all tested time points (Fig. 2a-d). Furthermore, the immortalised BEAS-2B cell line was used to evaluate the cytotoxicity of peruvoside in relatively normal cells. Peruvoside showed lower cytotoxicity in normal cells than in cancer cell lines (Additional file 1: Fig. S3). Additionally, the results from the cytotoxicity assays were analysed using CalcuSyn software (Biosoft, Cambridge, UK) to determine the  $IC_{50}$  in the five NSCLC cell lines and BEAS-2B cells. For example, the 24-h  $IC_{50}$  values in A549, PC9, PC9/gef, H3255, H1975 and BEAS-2B cells were 48 nM, 74 nM, 67 nM, 143 nM, 277 nM and 428 nM, respectively (Additional file 1: Table S1).

Dasatinib is a well-known Src inhibitor whose effective dose is usually 100 nM. The same concentration of peruvoside and dasatinib was used to treat the lung cancer cell lines with different EGFR mutation statuses and to further compare the inhibitory effect on proliferation ability. Both peruvoside and dasatinib suppressed A549, PC9, PC9/gef, H3255 and H1975 cell proliferation and peruvoside; in particular, had a marked inhibitory effect. Therefore, whether the cell line was EGFR wild-type or mutant, peruvoside effectively inhibited cell proliferation—even better than dasatinib (Fig. 3a-e).

# Anticancer effect of peruvoside on cancer cell functions

The colony formation, migration and invasion assays were performed to investigate the anticancer effects of peruvoside. We demonstrated that peruvoside inhibited the anchorage-dependent growth of A549 cells (Fig. 4a) and anchorage-independent growth of CL1-5 cells (Fig. 4b) in a concentration-dependent manner. Notably, peruvoside at a concentration as low as 5 nM, inhibited colony formation. To investigate the effects of peruvoside on cell motility, A549 cells were pre-treated with peruvoside for 24 h and then were subjected to Transwell migration and invasion assays for 12 h and 24 h, respectively. Peruvoside significantly inhibited the cell migratory and invasive abilities, even at the low concentration of 10 nM (Fig. 4c and d).

## Suppression of *in vivo* tumour growth by peruvoside

To further examine the effect of peruvoside *in vivo*, A549 cells were injected subcutaneously into nude mice. The mice were then treated with or without peruvoside (i.p., 0.1 mg/kg/day) and were observed every 7 days for tumour growth. Twenty-eight days after treatment, the peruvoside-treated group had an average tumour size of 129 mm<sup>3</sup> (95% CI = 86–178 mm<sup>3</sup>; P = 0.0063), which was significantly reduced compared with the average tumour size of 348 mm<sup>3</sup> (95% CI = 195–501 mm<sup>3</sup>) in the control group (Fig. 5a). After 4 weeks, the mice were killed, and their tumour sizes were analysed. The tumour weights were also significantly decreased from 0.55 g to 0.26 g (P = 0.0126; Fig. 5b). To determine the change in Src activity in tumours, the tissues were sectioned and immunostained with human anti-phospho-Src antibody. The immunohistochemistry data indicated that peruvoside significantly decreased Src Y418 phosphorylation in tumour tissues compared with control tissues (Fig. 5c). Additionally, the lung adenocarcinoma cell lines A549, PC9/gef, and H1975 were treated with different combinations of peruvoside and gefitinib for 72 h, and then the combined effects of the two compounds on the cells were evaluated. The results of the CI-isobologram analysis indicated that peruvoside and gefitinib had synergistic effects on the A549 (CI: 0.012 ~ 0.768), PC9/gef (CI: 0.15 ~ 0.915), and H1975 cell lines (CI: 0.353 ~ 0.628) (Additional file 1: Tables S2-S4). Specifically, the combination of 0.005, 0.075, or 0.01 μM peruvoside and low-dose gefitinib (0.01 or 0.05 μM) had synergistic effects on A549 cells (Fig. 5d, upper panels). Furthermore, the combination of 0.025 or 0.05 μM peruvoside and gefitinib increased the sensitivity of the PC9/gef and H1975 cell lines to gefitinib, even when peruvoside was administered at concentrations as low as 0.01 μM (Fig. 5d, middle and lower panels). In summary, 0.025 μM and 0.05 μM peruvoside could render A549, PC9/gef, and H1975 gefitinib-resistant lung cancer cells sensitive to lower gefitinib concentrations.

## Effect of peruvoside on Src downstream pathways

To determine the mechanisms by which peruvoside inhibits cancer cell functions, western blotting of previously reported Src-related signalling pathways was performed. These proteins included PI3K and AKT for the survival pathway; MEK and ERK for the proliferation pathway; and FAK, paxillin, p130 CAS and JNK for the migration and invasion pathways [22]. A549 cells were treated with the designated

concentrations of peruvoside for 24 h, and peruvoside inhibited PI3K, AKT, FAK, SAPK/JNK, Paxillin and p130 CAS phosphorylation in these cells. However, the phosphorylation of MEK and ERK was not reduced by peruvoside treatment (Fig. 6a). In addition to using the EGFR wild-type A549 cell line, we also used the EGFR mutant H3255 and H1975 cell lines to evaluate whether peruvoside can effectively inhibit the expression of these proteins in NSCLC cell lines with TKI-sensitive or TKI-resistant EGFR mutations. These two cell lines were treated with sublethal doses of peruvoside (50 nM and 100 nM) for 24 h and then subjected to western blotting. In the H3255 cell line, the proteins and pathways affected by peruvoside were similar to those in the A549 cell line (Fig. 6b), except for p-MEK, p-ERK and Paxillin. Furthermore, in the H1975 cell line, peruvoside suppressed the phosphorylation of PI3K, AKT, MEK, ERK FAK, SAPK/JNK, Paxillin and p130 CAS (Fig. 6c). Overall, the results in the three cell lines also showed that the expression of Src and some Src-related proteins, such as EGFR, STAT3 and FAK, was downregulated by peruvoside (Fig. 1 and Fig. 6).

## Reduced mRNA expression of Src and related pathways by peruvoside

The expression levels of Src and some of its related proteins were decreased compared with those in the control (Fig. 1 and Fig. 6). The reduced protein levels could be explained by transcription and protein stability alterations. Therefore, real-time PCR was performed to investigate the effect of peruvoside on the transcriptional regulation of the genes tested in this study. The mRNA expression levels of EGFR, Src, STAT3 and FAK were significantly downregulated by peruvoside, even when the drug was administered at a relatively low concentration (25 nM) (Fig. 7a). However, to explore whether the ubiquitin-proteasome system plays a role in peruvoside-induced protein reduction, the proteasome inhibitor MG132 was used in cell culture. Peruvoside decreased the expression of the tested proteins as described previously in this study, but co-treatment with MG132 could not restore their expression compared with that in the vehicle control (Fig. 7b). Therefore, we speculate that the protein reduction caused by peruvoside might occur via the regulation of RNA levels. However, further investigations are needed to clarify the detailed regulatory mechanisms.

## Discussion

The proto-oncogene c-Src is a non-receptor tyrosine kinase that plays a key role in multiple signalling pathways to regulate cell growth and metastasis in several cancer types [23]. The deregulation of its activity or expression is also related to drug resistance in cancer patients and is associated with poor prognoses [24]. ELISA is widely used as a diagnostic and analytical tool in clinical practice and basic research to detect and quantify specific antigens or antibodies in a given sample [25]. Moreover, ELISA has several advantages over other screening techniques because of its simplicity, selectivity, and sensitivity. In this study, using Src pY419 ELISA as the drug-screening platform to screen a compound library with more than 400 plant active ingredients, we found that peruvoside is a potential Src inhibitor

that significantly inhibits NSCLC cell functions *in vitro* and tumorigenesis *in vivo*. Moreover, we determined that peruvoside has a synergistic effect when used in combination with gefitinib.

Peruvoside, a cardiac glycoside (CG), is a natural ingredient extracted from oleander seeds. CGs are a class of organic compounds comprising a sugar (glycoside) and an aglycone (steroid) moiety. They are used to treat heart ailments, such as congestive heart failure, ischaemia and cardiac arrhythmia. Interestingly, in recent years, several studies have revealed that some CGs possess potent anticancer effects in various cancers [26]. Peruvoside has also been shown to have anti-proliferative and anticancer effects by regulating the expression of various key proteins involved in cell cycle arrest, caspase activation and autophagic cell death in several cancers, including myeloid leukaemia, breast cancer and lung cancer cells [27–29]. Furthermore, previous studies have indicated that peruvoside inhibits AKT phosphorylation and  $\beta$ -catenin expression in H460 lung cancer cells (EGFR wild-type) [29], as well as induces autophagy and apoptosis through MAPK, Wnt/ $\beta$ -catenin and mTOR signalling in A549 lung cancer cells (EGFR wild-type) [30]. However, these studies did not investigate the effect of peruvoside on NSCLC with activating and acquired resistance EGFR mutations, the Src-EGFR-related signalling pathways involved, or its effect on tumorigenesis *in vivo*. Moreover, the functional role of peruvoside in cancer and mechanism underlying its antitumour activity are still largely unknown. Here, we elucidated the multi-faceted role of peruvoside in NSCLC and signalling pathways in which it may be involved. To our knowledge, this is the first study to indicate that peruvoside can inhibit NSCLC progression by regulating multiple Src-related signalling pathways.

EGFR is overexpressed in approximately 40%-80% of NSCLC tumours; therefore, EGFR activity and mutations that can trigger downstream signalling pathways are important factors in lung cancer treatment that must be considered by clinicians attempting to manage this disease [31]. Similar to EGFR, c-Src is also overexpressed in many types of cancer and is co-overexpressed with EGFR in several types of tumours, including carcinomas of the colon, breast, and lung [14, 32]. A previous study showed that Src inhibitors not only suppress Src activity but also inhibit EGFR tyrosine kinase activation and downstream signalling pathways. Moreover, depending on the EGFR/Ras mutational profile, different Src inhibitors may exhibit divergent anticancer effects in NSCLCs [33]. Therefore, Src can serve as a therapeutic target to improve NSCLC treatment [13]. The Src inhibitor dasatinib has been approved for clinical use in patients with chronic myeloid leukaemia (CML) [34] and can improve the efficacy of cetuximab and cisplatin in tumour-negative breast cancer (TNBC) when used in combination [35]. Moreover, dasatinib was recently shown to be a multi-kinase inhibitor that affects the STAT5, c-kit, and PDGFR pathways [36]. However, similar to gefitinib, dasatinib cannot inhibit the growth of NSCLC cells with wild-type EGFR (A549) or a T790M mutation (H1975) [37]. Compared to dasatinib, peruvoside had a cytotoxic effect on all NSCLC cell lines tested, namely A549, PC9, PC9/gef, H3255 and H1975 cells, regardless of their EGFR mutation status. Furthermore, peruvoside was relatively less toxic to BEAS-2B cells than to cancer cells in this study.

c-Src has been reported to bind to EGFR and phosphorylate tyrosine residues on Y845, resulting in the activation of various downstream pathways [38]. Therefore, c-Src and activated EGFR cooperate to

induce cell transformation, and cancer development is critical for EGFR-mediated oncogenesis [39]. Because of the crosstalk between Src and EGFR, inhibiting the activity of both proteins may facilitate the successful treatment of NSCLC patients without EGFR-activating mutations or with acquired resistance mutations. Previous studies have shown that the inhibition of c-Src kinase activity sensitises EGFR-TKI-resistant cells and significantly decreases AKT activation, cell survival and migration, indicating that Src inhibitors might overcome resistance to EGFR inhibitors in lung cancer cells [40]. Thus, the effects of combination therapy with dasatinib and EGFR TKIs (erlotinib, gefitinib and afatinib) on NSCLC were investigated in several clinical trials in recent years [41, 42]. However, these phase I/II clinical trials did not obtain ideal results because of few or no clinical responses in NSCLC patients with acquired EGFR-TKI-resistant mutations or with wild-type EGFR [41, 43]. Our data indicated that peruvoside significantly sensitised gefitinib-resistant lung adenocarcinoma cells (A549, PC9/gef, and H1975) to gefitinib treatment *in vitro*, suggesting that this compound may reduce the gefitinib dose, enhance gefitinib efficacy, and decrease targeted therapy costs and patient loads. These findings indicate that peruvoside may be a new candidate compound that can be used instead of dasatinib in combination therapy regimens comprising one of the two kinase inhibitors.

Src has been identified as an important oncogenic driver in many signalling pathways to enhance cancer cell motility, tumorigenesis, angiogenesis, and metastasis [22]. Among these pathways, some pivotal pathways have been demonstrated to modulate cancer progression, including the PI3K/AKT, STAT3, MEK/ERK, JNK, FAK, Paxillin, and p130cas pathways [23]. The PI3K/AKT pathway can be activated by EGFR and Src, leading to aberrant cell survival and cell cycle progression [44]. Our data showed that peruvoside inhibits the activity of Src and EGFR as well as the phosphorylation and expression of PI3K in EGFR mutant (H3255 and H1975) and wild-type (A549) cells. FAK-Src signalling through Paxillin, ERK, and p130cas regulates actin cytoskeletal reorganisation to promote cell migration [45]. Furthermore, JNK is the transcriptional regulator of matrix metalloproteinase (MMP)-2 and MMP-9; thus, JNK activation can lead to proteolysis and increased cell invasion [46]. Our data revealed that peruvoside significantly represses FAK, JNK, Paxillin, and p130cas phosphorylation or protein expression in H3255, H1975 and A549 cells, leading to the inhibition of cancer cell invasion and migration. A previous study showed that MEK and ERK might be involved in the Src-related signalling pathway, resulting in increased cell proliferation [47]. In this study, peruvoside decreased MEK and/or ERK phosphorylation levels in the indicated cell lines but promoted their phosphorylation in A549 cells. Similar results were reported in a previous study [18] and suggested that peruvoside may also affect other signalling pathways, growth factors or protein kinases to inhibit cell growth in a certain cell line. Additionally, we found that the reductions in certain proteins caused by peruvoside may be due to its effect on transcriptional regulation rather than increased ubiquitination, which is one of the effects that is often observed with antitumour drugs such as palbociclib (a CDK inhibitor) and fludarabine (premature transcription chain terminator) [48].

In summary, our findings indicate that peruvoside may directly or indirectly affect the expression of Src and downstream or related proteins, thereby inhibiting cancer progression. However, we cannot rule out the possibility that peruvoside may affect multiple targets. Recently, based on the multiple target effects

of drugs inducing different anticancer responses, the concept of polypharmacology has been developed [49]. Multi-target drugs can treat diseases more effectively than single-target drugs, regardless of whether these multi-target drugs are used alone or in combination with other agents, and multi-target agents are expected to provide more efficacious and safer therapeutic solutions that are less prone to drug resistance phenomena [50]. For example, sorafenib, a VEGFR, PDGFR, KIT, FLT3, and RAF inhibitor, was recently confirmed in clinical trials for its effectiveness in advanced gastrointestinal stromal tumour patients [51]. Moreover, the safety and efficacy of anlotinib, a novel multi-target TKI that inhibits VEGFR2/3, FGFR1-4, PDGFR  $\alpha/\beta$ , c-Kit, and Ret [52], in patients with refractory advanced NSCLC have been verified in a randomised phase II trial.

## Conclusions

We found that peruvoside may have multi-target inhibitory effects on NSCLC and may have a synergistic effect when combined with gefitinib. These results indicate that peruvoside has potential as a treatment method for cancer. Therefore, whether it is used alone or in combination with other drugs, peruvoside may be the basis for future therapies and should be evaluated in future drug development studies.

## Abbreviations

CI: combination index

CML: chronic myeloid leukaemia

EGFR: epidermal growth factor receptor

FAK: *focal adhesion kinase*

FGFR: fibroblast growth factor receptors

FLT3: fms-like tyrosine kinase 3

JNK: jun amino-terminal kinases

mTOR: *mammalian target of rapamycin*

NSCLC: non-small cell lung cancer

PDGFR: *platelet-derived growth factor receptors*

PI3K: phosphoinositide 3-kinases

SAPK: stress-activated protein kinases

SFKs: src family kinases

STAT3: signal transducer and activator of transcription 3

TKIs: tyrosine kinase inhibitors

TNBC: tumour-negative breast cancer

## Declarations

### Availability of data and materials

All experimental data generated or analyzed during this study are included in this published article and its supplementary files.

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Not applicable.

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### Ethics declarations

### Ethics approval and consent to participate

The mouse experiments were performed with the approval of the institutional animal care and use committee of National Chung Hsing University (IACUC No.: 105-129).

### Consent for publication

All authors agree on publication of the results of the present manuscript.

### Competing interests

The authors declare that they have no competing interests.

### Additional information

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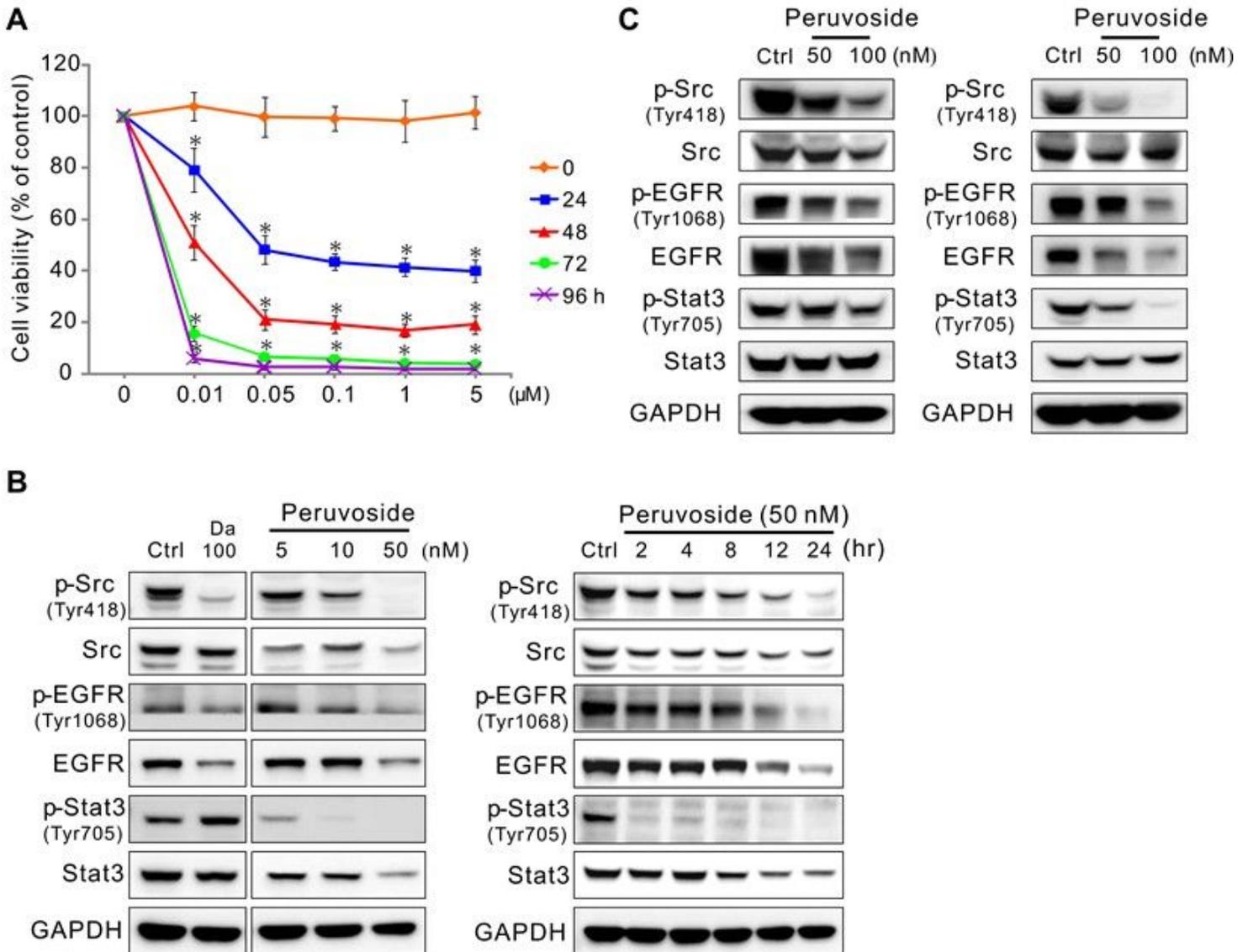
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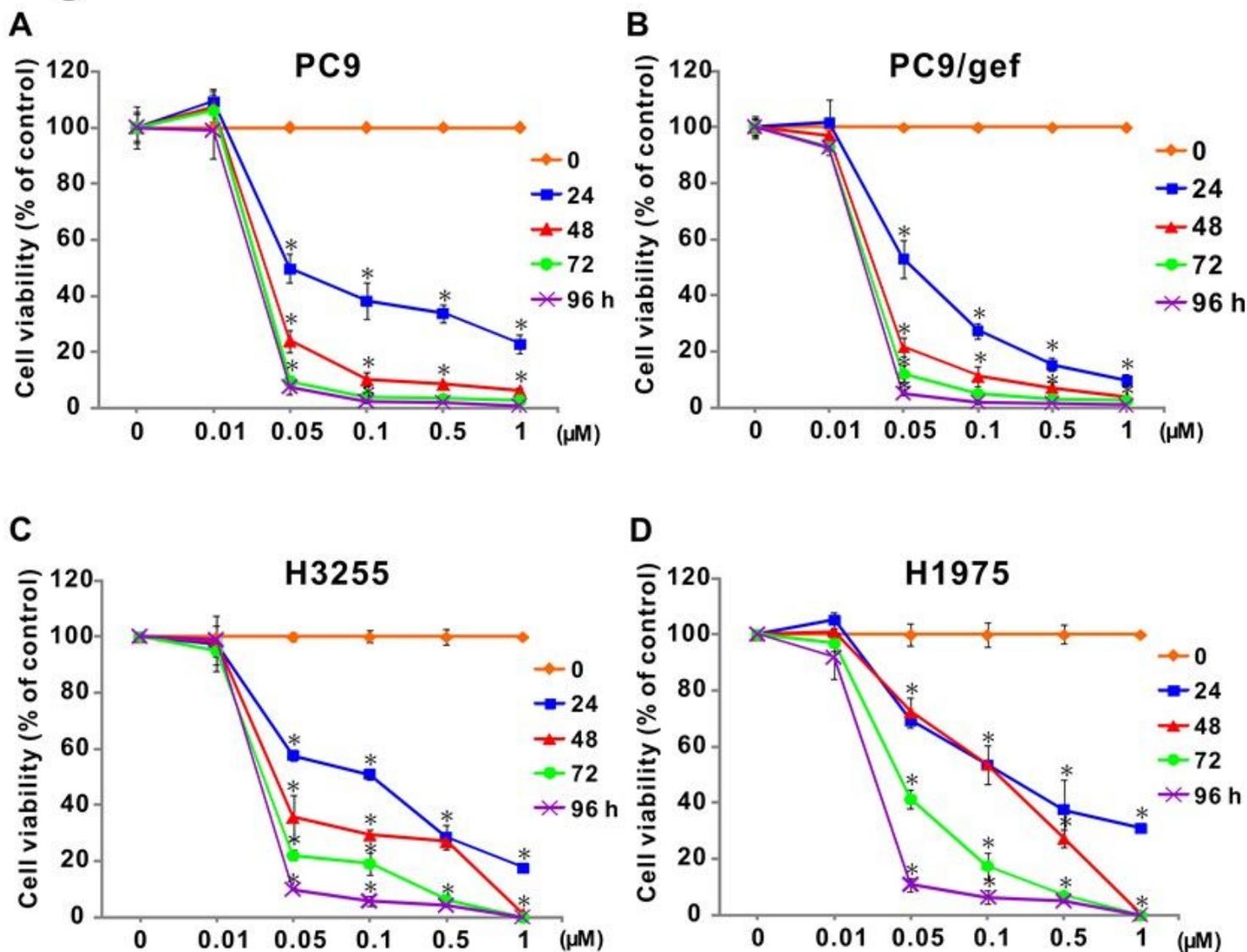
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## Figures

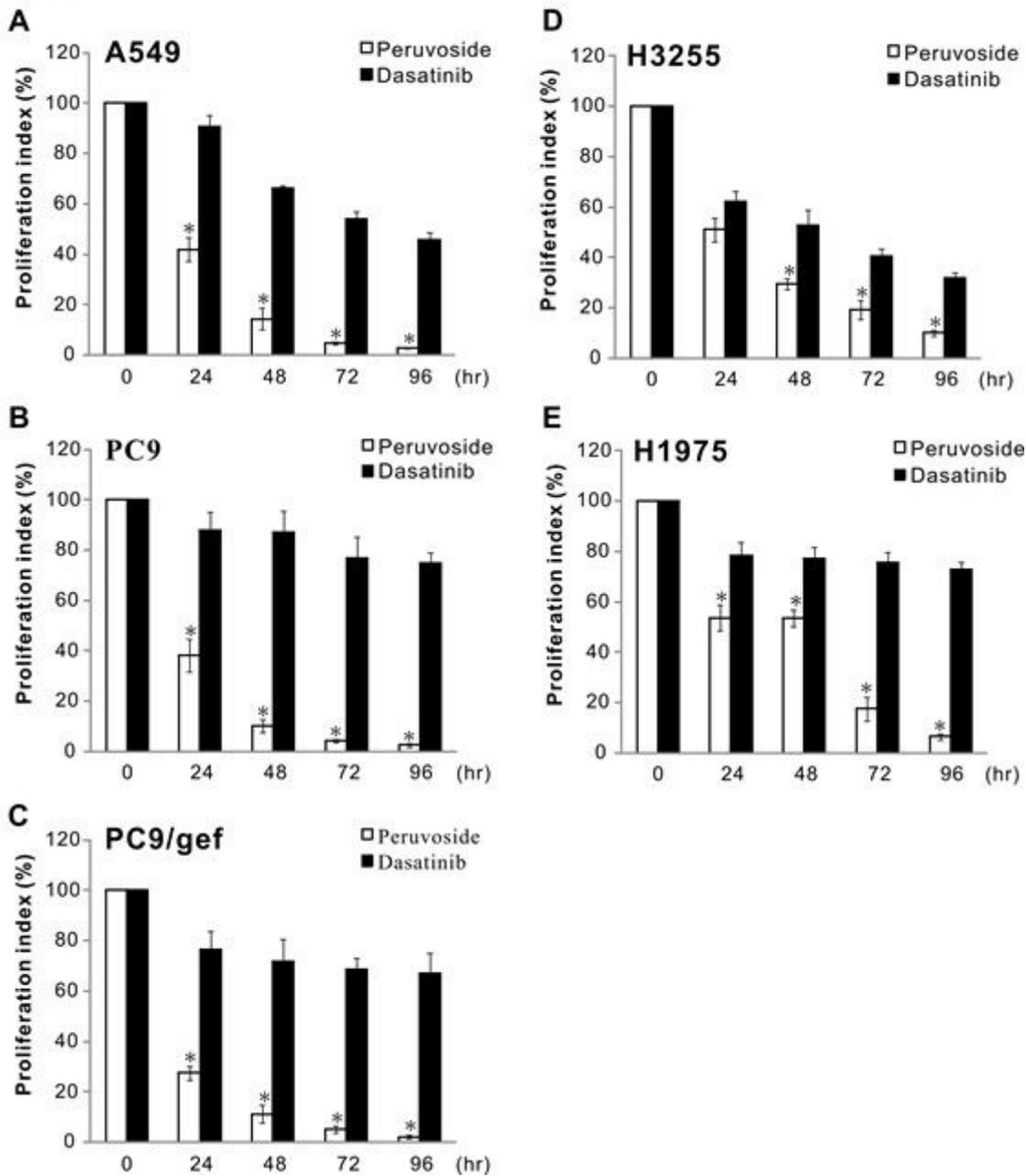
**Fig. 1****Figure 1**

Effects of peruvoside on Src, EGFR and STAT3 expression and the viability of different cell lines. (A) Inhibition of A549 cell viability by peruvoside, as determined by PrestoBlue reagent at different concentrations and time points. The results are presented as percentages of the vehicle control (0  $\mu$ M, 0.1% DMSO). The IC<sub>50</sub> values of the designated time points are shown in Table S1. Each experiment was performed independently and in triplicate. \*P<0.05 compared with the vehicle control. (B) Inhibition of the protein expression and phosphorylation of Src, EGFR, and STAT3 by peruvoside in A549 cells. Left panel: cells were treated with the indicated concentrations of peruvoside for 24 h and then were subjected to western blotting. Right panel: cells were treated with 50 nM peruvoside for the specified time and then were subjected to western blotting. (C) Inhibition of Src, EGFR, and STAT3 expression and phosphorylation by peruvoside in H3255 (left panel) and H1975 (right panel) cells. The cells were treated with the indicated concentrations of peruvoside for 24 h and then were analysed by western blotting. Ctrl: 0.1% DMSO; Da100: 100 nM dasatinib as a positive control. GAPDH served as a loading control.

**Fig. 2****Figure 2**

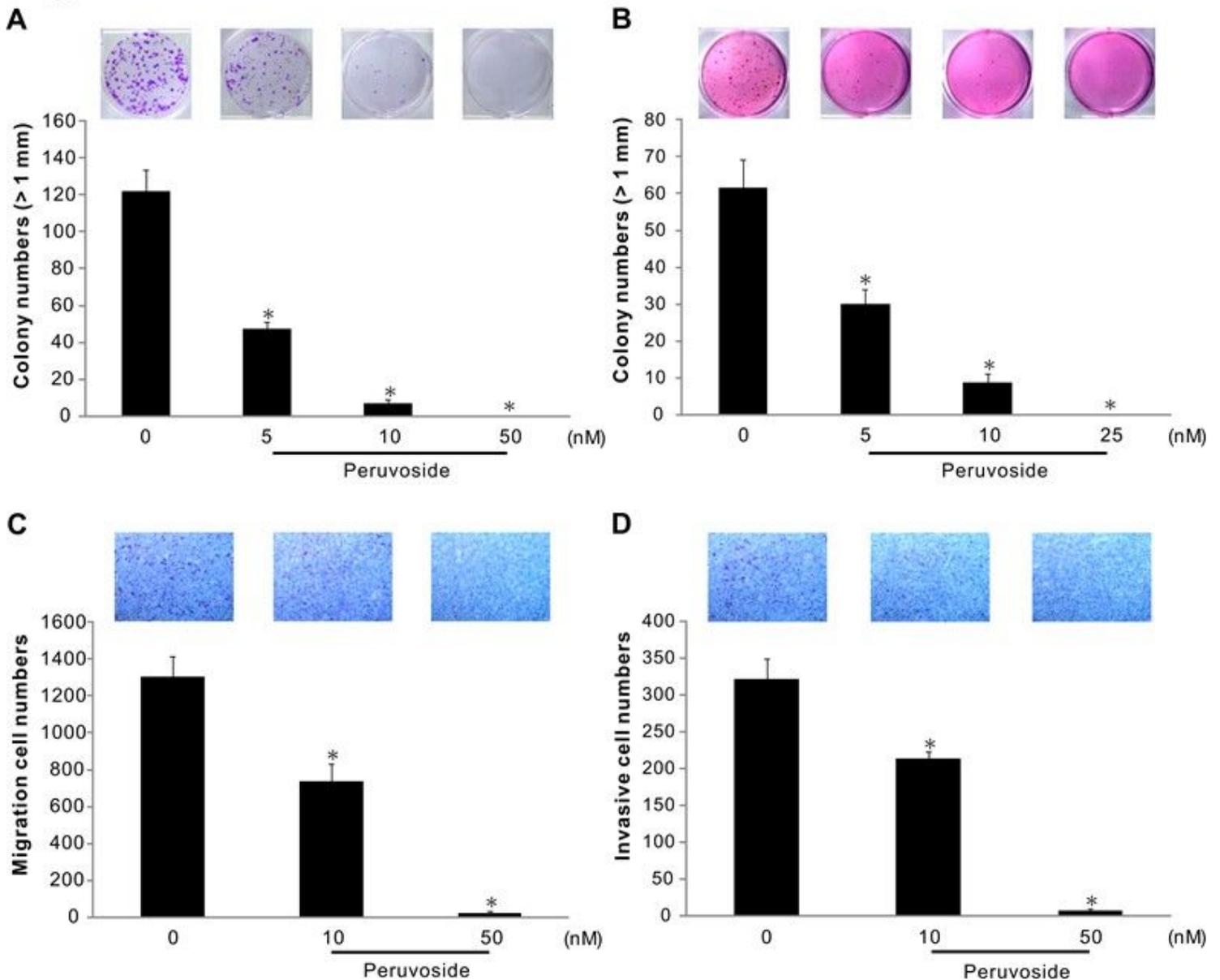
Cytotoxicity of peruvoside in NSCLC cell lines with different EGFR mutation statuses. PC9 (A), PC9/gef (B), H3255 (C) and H1975 (D) cells were treated with various concentrations of peruvoside for 24, 48, 72, 96 h and then were subjected to cell viability assays. The results are presented as percentages of the vehicle control (0 μM, 0.1% DMSO). The IC<sub>50</sub> values of the designated time points are shown in Table S1. Each experiment was performed independently and in triplicate. \*P<0.05 compared with the vehicle control.

**Fig. 3**

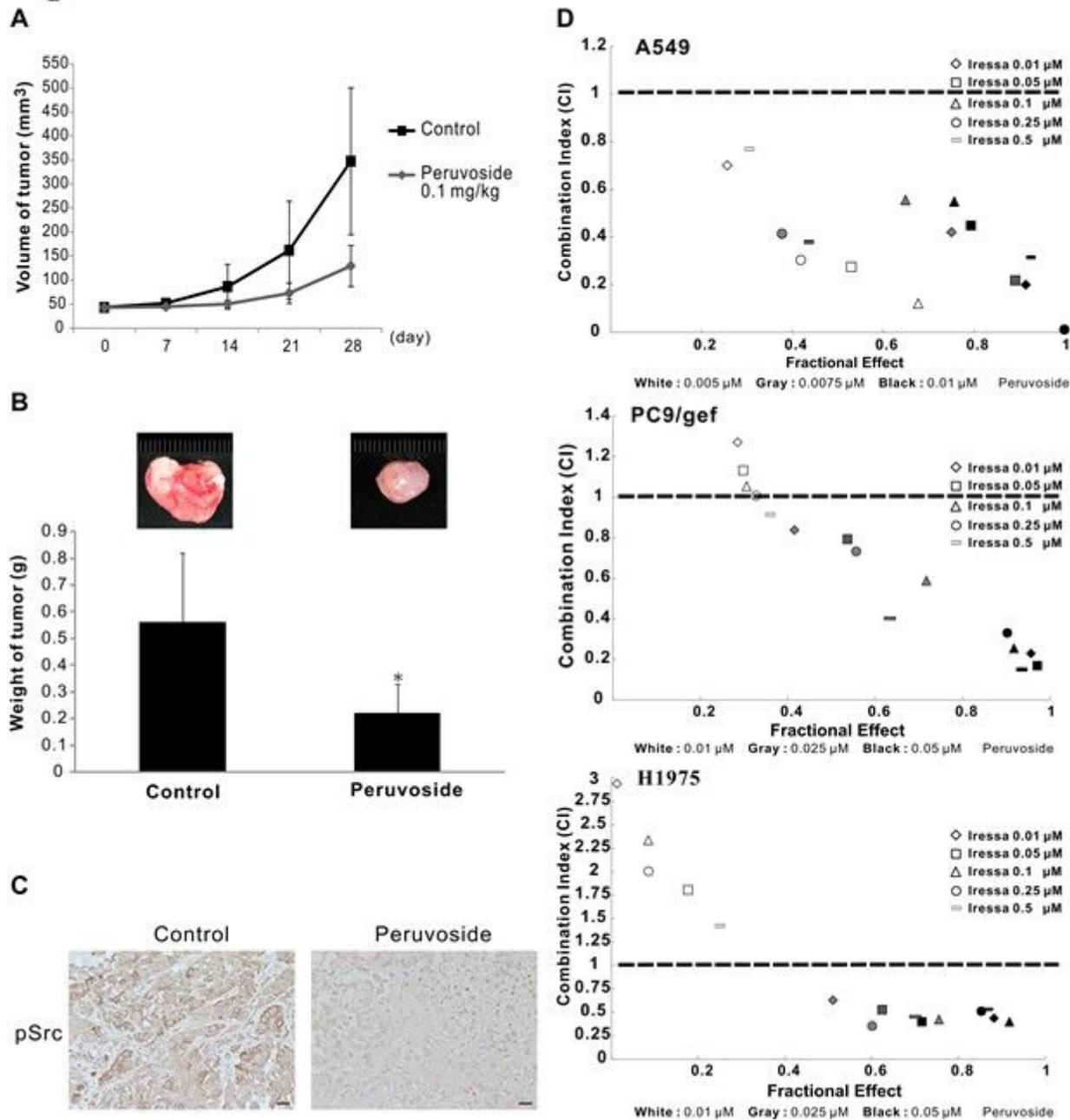


**Figure 3**

Comparison of the efficacy between peruvoside and dasatinib in NSCLC cell lines with different EGFR mutation statuses. The lung cancer cell lines A549 (A), PC9 (B), PC9/gef (C), H3255 (D) and H1975 (E) were treated with the same concentration of peruvoside and dasatinib to compare the inhibitory effect on the proliferation ability at the indicated time points, as determined by the PrestoBlue cell viability assay. The results are presented as percentages of the vehicle control (0  $\mu$ M, 0.1% DMSO). Each experiment was performed independently and in triplicate. \*P<0.05 compared with the vehicle control.

**Fig 4****Figure 4**

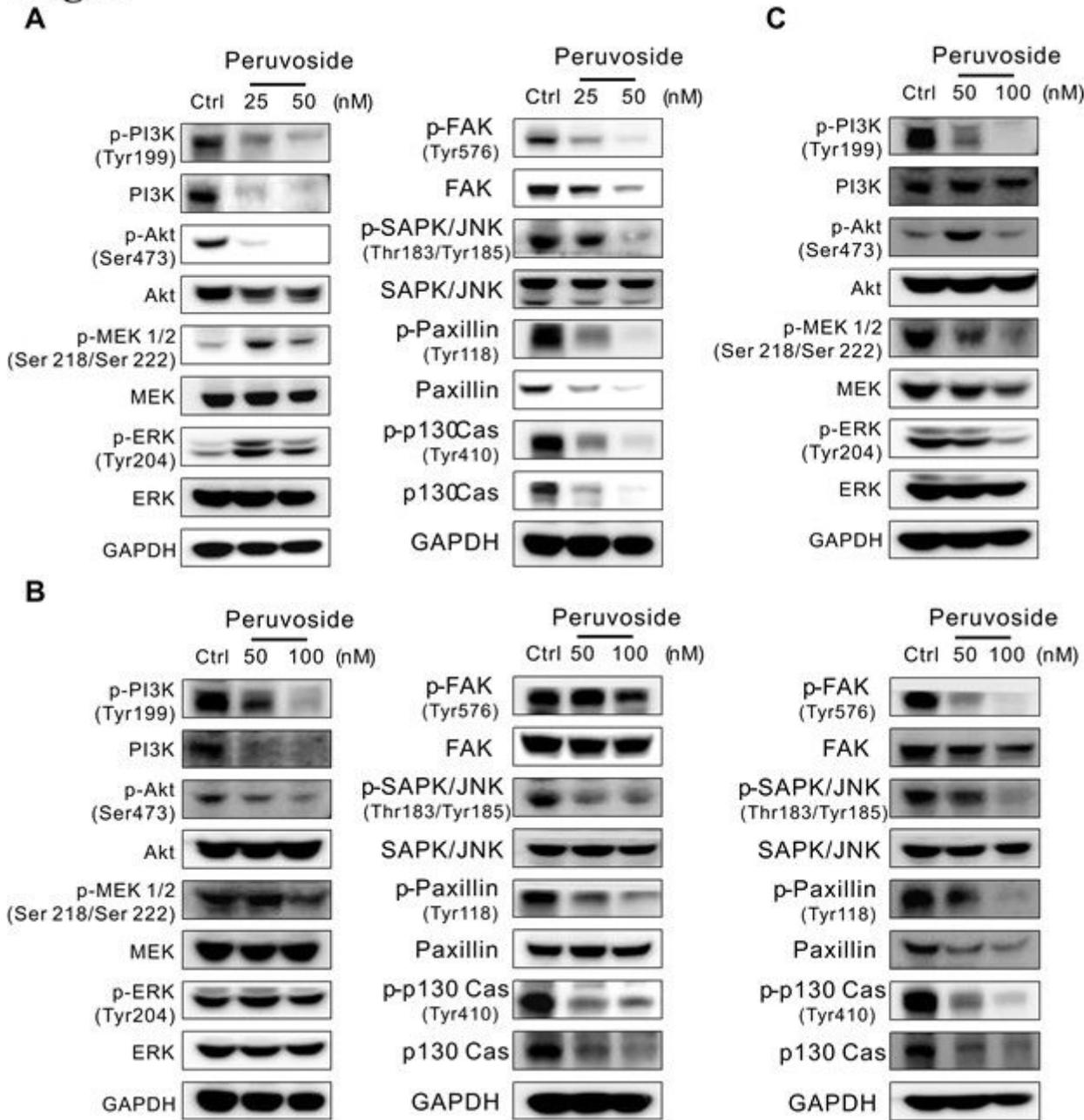
Suppressive effects of peruvoside on anchorage-dependent/independent cell growth and cell motility. The colony formation assay was used to determine the inhibitory effect of peruvoside on clonogenicity. (A) Anchorage-dependent cell growth in the A549 cell line. Colonies with diameters  $\geq 0.3$  mm were counted. (B) Anchorage-independent cell growth in the CL1-5 cell line. Colonies with diameters  $\geq 0.5$  mm were counted. Each experiment was performed independently and in triplicate. 0 nM: 0.1% DMSO. (C) Inhibitory effect of peruvoside on cancer cell migration, as determined by the Transwell assay without Matrigel. (D) Inhibitory effect of peruvoside on cancer cell invasion, as measured by the Transwell assay with Matrigel. Each experiment was performed independently and in triplicate. \* $P < 0.05$  compared with the vehicle control.

**Fig. 5****Figure 5**

Effects of peruvoside on tumour growth and drug synergism. The indicated number of live A549 cells was subcutaneously injected into mice divided into vehicle-treated (n=6) and drug-treated groups (n=6). (A) The tumour volumes were measured every 3-4 days. (B) Peruvoside decreased the tumour weight. The data are presented as means  $\pm$  standard deviation. (C) The p-Src expression level and distribution in murine tumour tissues were determined by immunohistochemical staining and observed using a light microscope (400 $\times$  magnification). The control represents 0.1% DMSO, and peruvoside represents 0.1 mg/kg of the compound. The scale bars represent 20  $\mu$ m. \*P<0.05 compared with the vehicle control (0.1% DMSO). (D) Synergistic effects of peruvoside and gefitinib on lung cancer cell lines, as determined by the cell viability assay. The indicated combinations of peruvoside and gefitinib were used to treat

gefitinib-resistant lung adenocarcinoma A549, PC9/gef and H1975 cells for 72 h. The CI was calculated using CalcuSyn software. Each experiment was performed independently and was repeated three times.

## Fig. 6

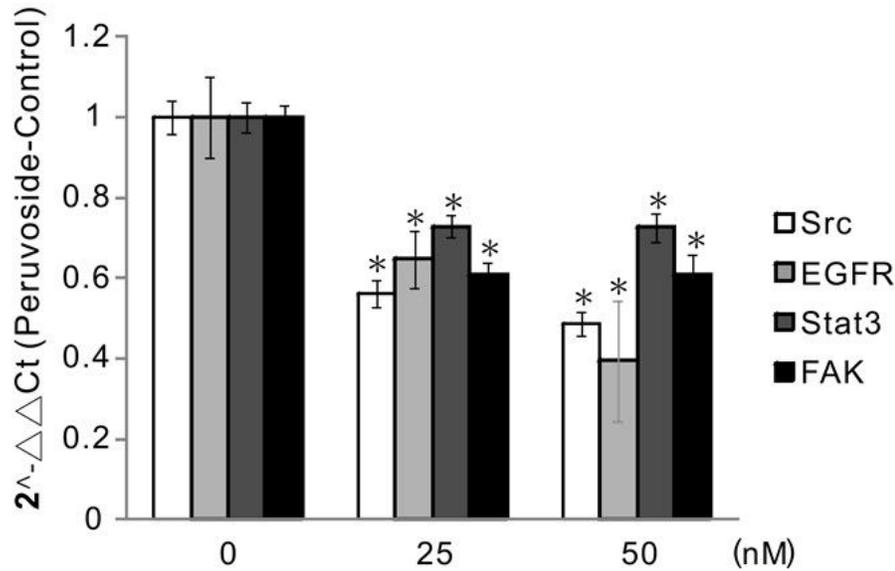


**Figure 6**

Effects of peruvoside on the expression and phosphorylation of Src and related proteins. Lung cancer A549 (A), H3255 (B), and H1975 (C) cells were treated with peruvoside at the indicated concentrations for 24 h. The expression and phosphorylation levels of PI3K, Akt, MEK, ERK, FAK, JNK, Paxillin, and p130cas were measured by immunoblot analysis using the corresponding antibodies. GAPDH was used as an internal control. Ctrl: 0.1% DMSO. Each experiment was performed independently and in triplicate.

# Fig. 7

A



B

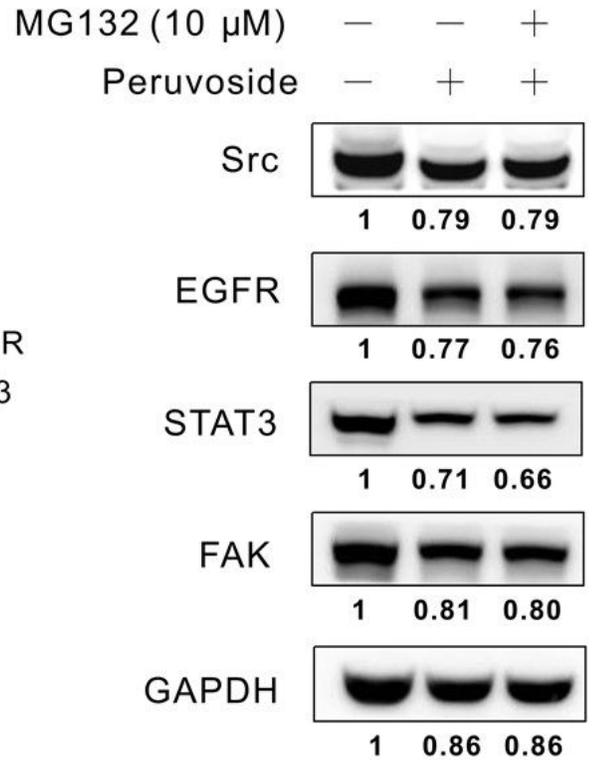


Figure 7

Peruvoside inhibits Src and Src-related gene transcription. (A) Repressive effects of peruvoside on Src, EGFR, STAT3, and FAK transcription in A549 cells, as determined by real-time RT-PCR. The relative gene expression levels were calculated using the comparative CT method ( $2^{-\Delta\Delta CT}$ ). TBP: internal control. Each experiment was performed independently and in triplicate. \* $P < 0.05$  compared with the control (0 nM: 0.1% DMSO). (B) Role of the ubiquitin-proteasome system in the protein decrease caused by peruvoside. MG132 and/or peruvoside was administered to A549 cells for 24 h, followed by western blotting of the designated proteins. GAPDH served as a loading control. Protein expression was quantified by ImageJ software (NIH), and the results are shown directly below the gel graph.

## Supplementary Files

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