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Research paper

Aidi injection, a traditional Chinese medicine extract, reverses Gefitinib resistance in non-small cell lung cancer cells

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ABSTRACT

Introduction: Aidi injection is a traditional Chinese medicine containing multiple anti-tumour and immunomodulatory phytochemicals. While it synergistically enhances the efficacy of conventional chemotherapy in patients with non-small cell lung cancer (NSCLC), its effect on epidermal growth factor receptor (EGFR) tyrosine kinase inhibitor (TKI) sensitivity in NSCLC remains unclear. This study aimed to investigate the effect and mechanisms of Aidi injection on the sensitivity of human NSCLC cell lines to gefitinib.

Methods: Effect of Aidi injection on gefitinib sensitivity was assessed by MTT, colony formation and apoptosis assays in three NSCLC cell lines (A549, HCC827 and H1975). The association between the expression of genes and the overall survival was analysed by accessing TCGA lung adenocarcinoma datasets. The effect of Aidi injection on multidrug resistance-associated protein 2 (MRP2, encoded by ABCC2 gene) function and gefitinib sensitivity was compared between parental HEK293 cell and HEK293 overexpressing MRP2 cells (HEK/MRP2). The principle components of Aidi injection were determined by LC-MS/MS and the interaction of Aidi components with MRP2 protein explored using molecular docking.

Results: Aidi injection enhanced gefitinib sensitivity ($P < 0.05$) and increased gefitinib-induced apoptosis rate ($P < 0.05$) in three NSCLC cell lines. Analysis of TCGA lung adenocarcinoma dataset showed that patients with a high expression of *ABCC2* had significantly poorer survival ($P = 0.007546$). Aidi injection inhibited MRP2 activity in a concentration-dependant manner in HEK/MRP2 cells ($P < 0.05$). The combination of gefitinib with Aidi injection gave additive or weakly synergistic growth inhibition in HEK/MRP2 cells but exhibited antagonistic cytotoxicity in HEK293 cells. There were 11 main chemical components contained in Aidi injection, including astragalosides II and IV, cantharidin, etheutheroside E, ginsenosides Rb1, Rc, Rd, Re and Rg1, isofraxidin, and syringin. Docking studies showed strong affinity of Ginsenoside_Re towards MRP2.

Conclusions: Aidi injection may have the potential to be an adjuvant regimen to prevent and/or reverse common gefitinib resistance in NSCLC. The in silico and principle component analyses gives insight on ginsenoside_Re being a potential MRP2 inhibitor in Aidi injection.

1. Introduction

Worldwide lung cancer is one of the most leading cause of cancer death, of which non-small cell lung cancer (NSCLC) accounts for 80% by pathological type. NSCLC is also the most common type of lung cancer with a poor prognosis. The first-line treatment of NSCLC currently based on the presence or absence of driver gene alterations, such as epidermal growth factor receptor (EGFR) mutation, anaplastic lymphoma kinase (ALK), Proto-Oncogene Tyrosine-Protein Kinase reactive oxygen species (ROS-1) translocation and B-Raf proto-oncogene (BRAF) mutation [1,2]. EGFR mutation is the most frequent gene mutation and occurs in approximately 10% to 30% of unselected patients with NSCLC [1,3]. A series of well-designed randomised controlled clinical trials have shown beneficial therapeutic efficacy for EGFR tyrosine kinase inhibitor gefitinib relative to standard chemotherapy for NSCLC patients [4-6]. Although gefitinib has been widely adopted as the standard and preferred

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targeted regimen for treating EGFR mutation positive NSCLC, tumour resistance represents major clinical limitations. Acquired resistance to gefitinib or other first- and second-generation EGFR-TKIs inevitably develops within few months, and it is mainly mediated by the acquisition of the T790 M secondary mutation of EGFR [7]. Besides, the commonly characterised mechanism of anti-cancer drug resistance is altered cell membrane transport, which is typically due to ATP-binding Cassette (ABC) transporter-mediated drug efflux. MRP2 (an ABC transporter encoded by the ABCC2 gene) confers resistance to various anticancer drugs using energy derived from ATP hydrolysis. Downregulation of *ABCC2* in claudin-2 knockdown lung adenocarcinoma A549 cells enhanced sensitivity to gefitinib in vitro [8]. In addition, inhibition of *ABCC2* by a model inhibitor MK-571 reversed gefitinib resistance in A549 cells [8].

Aidi injection (Z52020236, China Food and Drug Administration), is an extract of ginseng, Astragalus membranaceus and Acanthopanax, has been approved for clinical use in China since 2002. It contains multiple anti-tumour and immunomodulatory phytochemicals, including ginsenoside, astragaloside, eleutheroside E, isofraxidin, syringin and coniferin [9]. Aidi injection has been widely used in China for the treatment of lung cancer, liver cancer, colorectal cancer, and gastric carcinoma [10-12]. Aidi injection has been suggested to synergistically enhance the efficacy of docetaxel-based or platinum-based chemotherapy in patients with NSCLC [10,12,13]. Aidi injection was also reported to significantly decrease the incidence of hepatotoxicity, gastrointestinal toxicity, and radiation-induced toxicity as well as renal toxicity [9]. The overall clinical effects of Aidi has been attributed to the induction of apoptosis, anti-proliferation and anti-angiogenesis, and the relief of chemotherapy-associated side effects. However, the effect of Aidi injection on EGFR-TKI sensitivity in NSCLC remains unclear.

With this background evidence, we carried out the study described here to provide the preclinical evidence of Aidi injection on the sensitivity of human NSCLC cells to gefitinib. First, we compared the effects of Aidi administration on gefitinib induced cytotoxicity and apoptosis in three NSCLC cell lines, which have different EGFR genotypes and ABCC2 expression levels. Next, we accessed the Cancer Genome Atlas (TCGA) lung adenocarcinoma datasets and demonstrated a significant association between ABCC2 gene expression and overall survival. Then, we experimentally verified the concentration-dependant inhibitory effects of Aidi injection on ABCC2 by using HEK293 parental and HEK293 overexpressing MRP2 cell models. The interaction of gefitinib and Aidi injection was determined by growth inhibition assay in isogenic HEK293 cells after treatment with gefitinib and Aidi injection at a fixed dose ratio. Further, in this study, the main components contained in Aidi injection was analysed by LC-MS/MS. Based on these, the interaction of each component from Aidi injection was evaluated using molecular docking method.

2. Methods

2.1. Reagents and antibodies

The cell culture media RPMI 1640, TrypLE express, penicillin-streptomycin, L-glutamine, PBS were from Life technologies (Auckland, NZ). Foetal bovine serum was from MediRay (NZ). The apoptosis kit, gefitinib, anti-EGFR rabbit monoclonal antibody and goat anti-rabbit IgG H&L (HRP) secondary antibody were procured from Invitrogen (Carlsbad, CA, US). Gefitinib was dissolved in dimethyl sulfoxide (DMSO) to make a stock concentration of 100 mM. Aidi injection extract was provided by Yibai Pharmaceuticals Ltd (Guizhou, China) with a ginsenoside Re (C48H82O18) concentration of 0.31 mg/mL. The drugs were diluted to the required concentration in a sterile environment with the cell culture media and were used in in vitro experiments. The final concentration of DMSO never exceeds 0.1% (v/v) in culture.

2.2. Cell culture

The human HEK293 cell line and NSCLC cell lines HCC827, H1975 and A549 were purchased from the American Type Culture Collection (ATCC, Manassas, VA, USA). ABCC2-overexpressing HEK293 cell line was a kind gift from Netherland Cancer Institute. All cell lines were culture and maintained in RPMI 1640 supplemented with 10% (v/v) foetal bovine serum, 2 mmol/L L-glutamine, 100 units of penicillin/streptomycin per mL, in a humidified atmosphere of 5% CO2 at 37ºC.

2.3. Cell proliferation assays and drug synergy studies

NSCLC cells were seeded at a density of 5000 cells per well in 96-well plates and grown overnight at 37 ˚C and 5% CO_2. After overnight incubation, cells were incubated with multiple concentrations of gefitinib and a combination of gefitinib and Aidi injection for 72 h followed by MTT [3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide] assay. Cell proliferation at different drug concentration was quantified by measuring photometric absorbence at 570 nm normalised to the mean absorbence of control untreated cells. Half-maximal inhibitory concentrations (IC_{50}) were determined from the images under the growth inhibition curves using Prism 8.0 software.

For combination gefitinib and Aidi injection therapy studies, HEK293 parental and HEK overexpressing MRP2 cells were treated with gefitinib and Aidi injection at a fixed-dose ratio. HEK293 and HEK293/MRP2 cells were seeded at a density of 3000 cells per well in 96-well plates and grown overnight at 37 ˚C and 5% CO_2. Cells were incubated with gefitinib, Aidi injection or a combination of both simultaneously for 72 h after which cytotoxicity was measured. Eight serial two-fold dilutions were examined at the following starting doses for HEK293 and HEK293/MRP2: Gefitinib and Aidi at 1.25, 2.5, 5, 10, 20, 40, 80 and 160 μM. Interactions between gefitinib and Aidi injection were assessed by calculating IC_{50} values and combination index (CI). Synergy (CI<1), additive effect (CI=1) and antagonism (CI>1) are determined using the below formula.(eq. (1)).

$$CI = \frac{D1}{Dx1} + \frac{D2}{Dx2} \quad (1)$$

where, D1 and D2 are concentrations of drug 1 and drug 2 that produce a given effect when given in combination, and Dx1 and Dx2 are concentrations of drug 1 and drug 2 that produce a given effect when given alone.

2.4. Colony formation

NSCLC cells were seeded in 96-well plates at a density of 1000 cells per well for H1975 and A549 cells and 2500 cells per well for HCC827 cell. After a day, cells were treated with gefitinib and a combination of gefitinib and Aidi for 72 h. The cells were allowed to incubate for 7 days until substantially good size colonies were formed in control plates. The medium was changed every 3 days, and at the end of the experiment, cells were fixed with ice-cold methanol for 15 mins and stained with 0.1% crystal violet for 15 mins at room temperature. The stained plates were thoroughly washed with water and air-dry for a few mins before analysed under the microscope.

2.5. Apoptosis assay

Annexin V-FITC apoptosis detection kit was used to assess the apoptotic effect of the combination of gefitinib and Aidi in NSCLC cell lines. The cells were double-stained with annexin-V fluorescein isothiocyanate (FITC) and propidium iodide (PI) in the dark for 15 mins at room temperature according to the manufacturer's instructions. Flow cytometric data were collected on a flow cytometry instrument (Beckman Coulter, MoFlo XDP) by measuring the fluorescence emission at 530 nm using

400 nm excitation and analysed with Kaluza software (Beckman Coulter, Brea, CA, USA).

2.6. Western blotting

Cells were harvested, washed with ice-cold PBS, and lysed with RIPA buffer supplemented with Roche Protease inhibitor. Protein concentration was measured by Bradford assay (Bio-rad, Richmond, CA). Equal amounts of protein were loaded in 10% polyacrylamide gel and electrophoresed. After electrophoresed, the proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane (Bio-rad, Richmond, CA). The PVDF membrane containing proteins were blocked with 2% non-fat milk for an hour at room temperature. Immunoblotting was performed using specific antibodies (primary EGFR in 1:1000 and secondary anti-rabbit antibody in 1:10,000 in 2% non-fat milk) and detected by Supersignal West Pico plus chemiluminescent reagent (Life Technologies, Auckland, NZ).

2.7. Association of gene expression with survival in patients with NSCLC

The association between the expression of ABCB1/ABCC1–3/ABCG2 and the overall survival was analysed by accessing TCGA lung adenocarcinoma datasets (http://xena.ucsc.edu/). The mRNA levels were shown as $\log_2(x+1)$ transformed RSEM normalised count [14]. The patients were divided into two groups based on the median values. The follow-up started at the date of diagnosis and ended at the date of death or 2000 days any individual was known to be alive. Basic clinical characteristics were retrieved and Kaplan–Meier curves generated for the Log-rank test.

2.8. MRP2 substrate accumulation

The parental HEK293 and MRP2-expressing HEK293 cells of density 500,000 cells/mL were incubated with the nonfluorescent precursor, 5-(and-6)-Carboxy-2′,7′-dichlorofluorescein diacetate (CDCFDA). CDCFDA is transported across the cell membrane by passive diffusion and is rapidly converted into a fluorescent MRP2 substrate CDCF. The accumulation of CDCF was performed by pre-treating cells with Aidi injection at two concentrations (equivalent to Ginsenoside Re 0.031 and 0.052 mg/mL) or vehicles at 37 ˚C for 15 min, followed by incubation with 5 μM of CDCFDA. After incubation for another 30 min, the cells were washed twice with ice-cold PBS and then lysed with 0.2 mL of 0.5% tween 20. The intracellular level of CDCF was analysed with a fluorescence plate reader (Spark, USA) with a standard laser for excitation at 488 nm and a filter emission at 530 nm.

2.9. LC-MS/MS analysis

Aidi injection was prepared as 1 in 25 dilution in 50% methanol. After ultrasonication for 30mins the cooled mixture was filtered through microporous filter membrane (0.2 μm). LC-MS/MS analysis was performed by using Agilent 6420 Triple quadrupole LC/MS system, and Phenomenex Kinetex Evo C18 (2.1 × 150 mm, 1.7 μm) column, the injection volume was 10μl, and temperature was 50 ˚C, gas flow rate was 10 L/min, and gradient elution was set as follows: 0 min, 5%B; 0.5 min, 5%B; 2 min, 15%B; 9 min, 20%B; 11 min, 50%B; 13 min, 50%B; 14 min, 80%B; 16 min, 80%B; 17 min, 5%B. Flow rate: 0.3 ml/min. (A:0.1% formic acid in Milli-Q water, B: 0.1% formic acid in acetonitrile.) The mass spectrometry analysis was performed in positive ion scanning mode, with a scanning range of 100–1200 m/z with 4000 V capillary voltage, and fragmentor voltage of 180 V. Multiple reaction monitoring (MRM) transitions for gensenoside Rb1, Rc, Rd and Re were 1131.6->365, 1101.7->335, 969.9->789.3 and 969.4->789.3 respectively and collision energy were 65, 65, 55 and 45 eV respectively.

2.10. In silico analysis

The main software and tools used for molecular docking studies were PubChem, MGL tools 1.5.7, AutoDock Vina 1.1.2 and PyMOL. The Aidi components which were detected in LC-MS/MS and gefitinib were used as ligands and downloaded from PubChem in SDF format. All the ligand structures were energy-minimised using MGL tools. MPR2 protein structure was obtained from ModBase database (Sequence database link: B2RMT8; UniProtKB - Q92887) [15]. The MRP2 protein in pdb file was converted to PDBQT format using MGL tools. The water molecules were deleted, missing residues were repaired, polar hydrogen atoms were added and charges were equally distributed to the MRP2 molecule prior to docking study. All the ligands were docked with receptor molecule using AutoDock Vina 1.1.2 [16]. The receptor and ligands were represented in PDBQT file format prior to docking. For docking, the MRP2 molecule was enclosed inside a grid box, with grid spacing of 1 Å, keeping the receptor rigid and the ligand as a flexible molecule. After defining the binding site and receptor-ligand preparation, docking runs were launched. The interaction energy between the ligand and receptor was calculated for the entire binding site and expressed as affinity (kcal/mol).

2.11. Statistical analysis

Statistical analyses were carried out using GraphPad Prism 8.0 software (GraphPad Software, San Diego, CA). Data were collected using a minimum of three experiments and expressed as mean values ± SD. The difference between the mean values for different experimental conditions was evaluated by Student's *t*-test or by one-way or two-way ANOVA followed by post-hoc tests, and p values were indicated where appropriate in the figures. The results were considered significant at p values less than 0.05.

3. Results

3.1. EGFR and ABCC2 expression in NSCLC cell lines

A representative NSCLC cell line of each group, including gefitinib insensitive A549 cells (wild type EGFR), gefitinib-resistant H1975 cells (L858R/T790M double EGFR mutations) and gefitinib-sensitive HCC827 cells (EGFR exon 19 deletion) was selected in this study (Table 1). Western Blotting method was used to detect the presence of EGFR protein in the NSCLC cell lines. EGFR protein is expressed in all A549, H1975 and HCC827 cells in the presence and absence of gefitinib (refer to Figure S1 in the Supplementary Material). The high expression of wild type/mutant EGFR in NSCLC cells is a major driver of cell proliferation. The ABCC2 mRNA expression in A549 (ranked 10/59), HCC827 (ranked 29/59) and H1975 (ranked 34/59) cells were compared to other lung cancer cell lines based on Barretina dataset [17] stored in ONCOMINE (https://www.oncomine.org).

3.2. Combination of gefitinib and Aidi inhibits cells proliferation in NSCLC in vitro

A pilot study was undertaken to determine which combinatory ratio of gefitinib and Aidi is effective in growth inhibition of NSCLC cell lines and IC_{50} values were calculated in NSCLC (H1975 and HCC827) cell lines (Table S1). Aidi exhibited concentration-dependant effects on gefitinib sensitivity in both H1975 and HCC827, as high-concentration Aidi (equivalent to ginsenoside Re concentration of 0.052 mg/mL) significantly enhanced the gefitinib sensitivity while Aidi at the lower concentrations (equivalent to ginsenoside Re concentration of 0.044 or 0.039 mg/mL) had no apparent effects (Table S1).

Based on our pilot studies, Aidi injection at a concentration equivalent to ginsenoside Re concentration of 0.052 mg/mL was selected to further explore its effect on antiproliferative activity of gefitinib

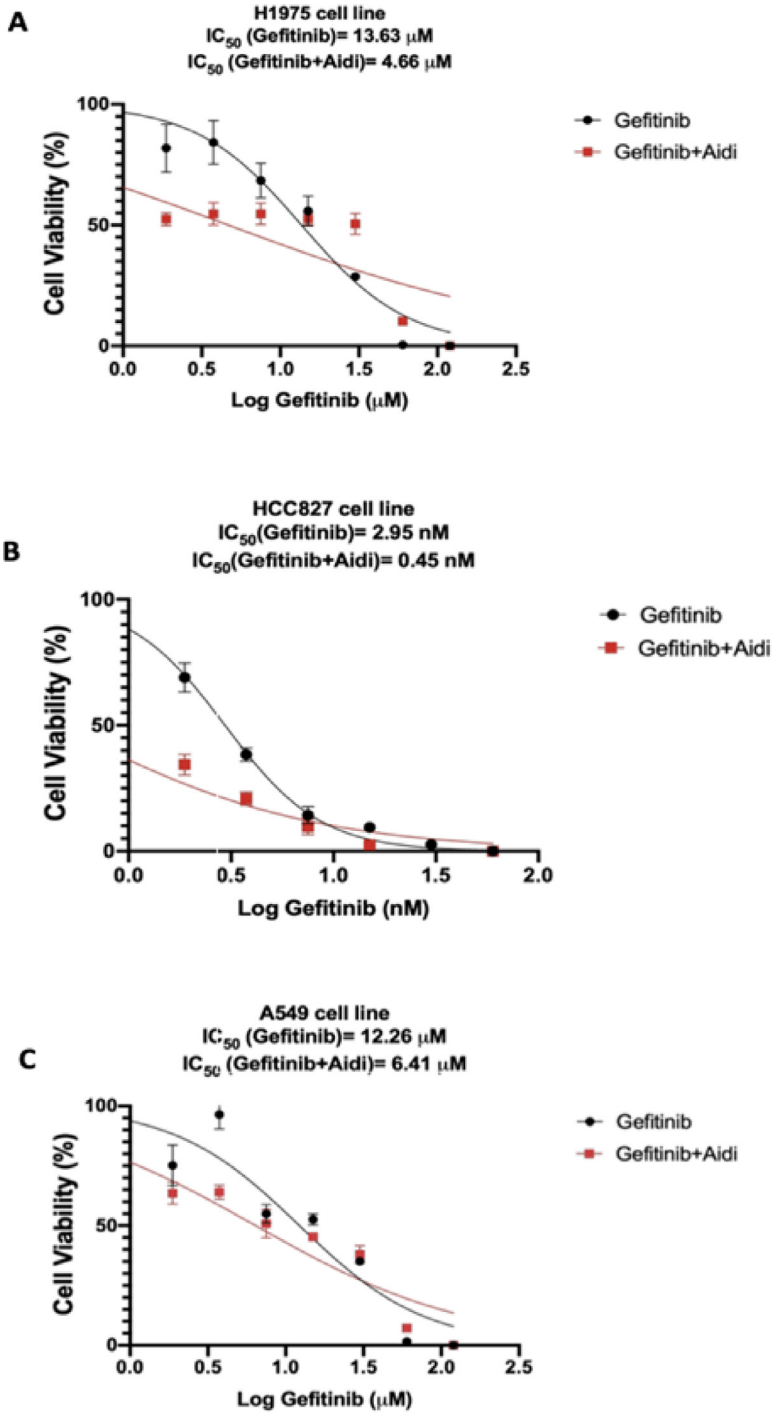

Fig. 1. Representative gefitinib-induced inhibition of growth of NSCLC cell lines. (A) H1975, (B) HCC827 and (C) A549 cells treated with different drug concentrations of gefitinib, either alone or in combination with Aidi. Symbols are means and standard errors of the mean [n = 4]. Solid lines are non-linear regression fits (Y = Bottom + (Top − Bottom)/ (1 + 10^ (LogIC50 − X)) to the data.

Table 1
EGFR mutation genotypes and ABCC2 mRNA level in HCC827, H1975 and A549 cell lines.

	EGFR L858R	EGFR T790M	EGFR exon 19 deletion	ABCC2 expression*
H1975	+	+	–	**−0.35737**
HCC827	–	–	+	**2.0226**
A549	–	–	–	**5.16488**

* ABCC2 mRNA levels were shown as log2 (median-centred intensity).

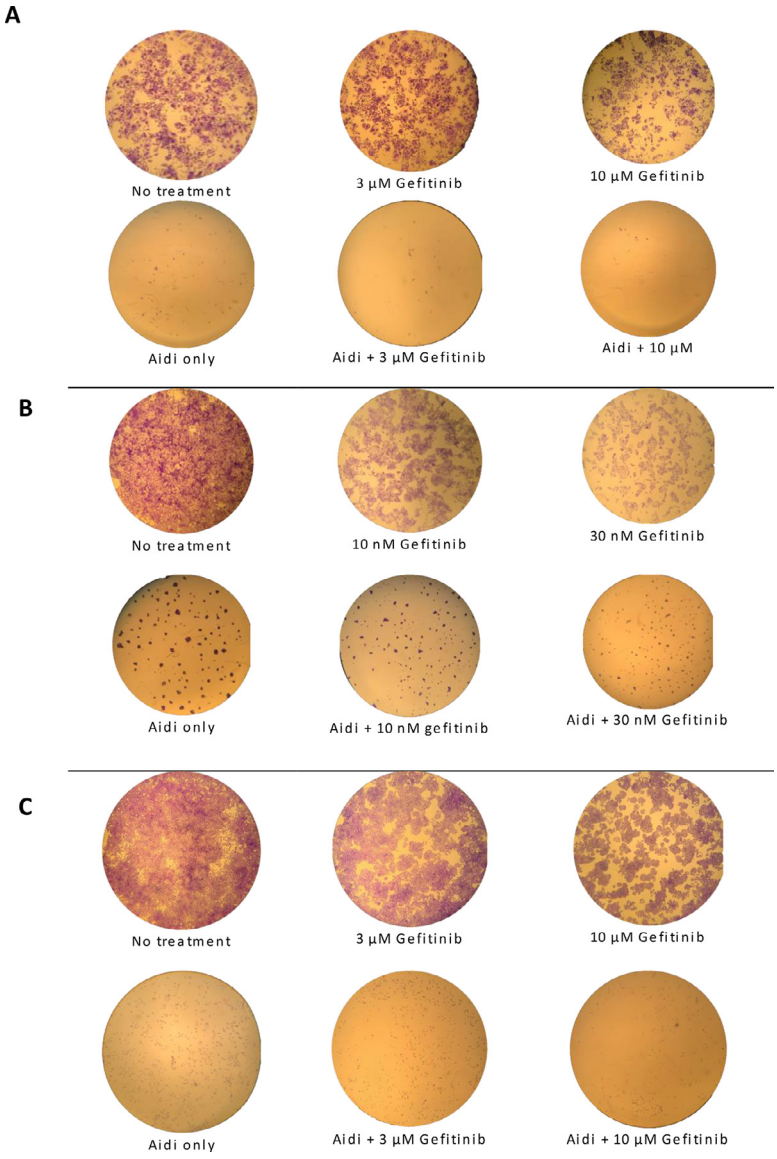

Fig. 2. NSCLC cell lines morphology after clonogenic assay in (A)H1975, (B) HCC827 and (C) A549. Cells were treated with 3 μM and 10 μM of gefitinib in H1975 and A549 cells, and 3 nM and 10 nM of gefitinib in HCC827 cells, either alone or in combination with Aidi for 10 days.

Table 2
Comparison of IC50 values of gefitinib in NSCLC cell lines after treatment with gefitinib alone and in combination with Aidi at a concentration (equivalent to ginsenoside Re concentration of 0.052 mg/mL).

Cell lines	IC_{50} (Mean ± SD) Gefitinib + NS	Gefitinib + Aidi	*p* values	Relative Resistance*
H1975	10.02 ± 4.37 μM	2.76 ± 2.20 μM	< 0.05	3.6
HCC827	6.00 ± 0.54 nM	0.98 ± 0.35 nM	< 0.001	6.2
A549	10.32 ± 2.32 μM	4.54 ± 3.34 μM	< 0.05	2.3

* Relative Resistance = the IC_{50} of gefitinib in NSCLC cell lines / the IC_{50} of gefitinib in combination with Aidi in NSCLC cell lines.

in a panel of NSCLC cell lines (i.e., A549 cells with wild type EGFR, gefitinib-resistant H1975 cells, and gefitinib-sensitive HCC827 cells). HCC827 cells were highly sensitive to gefitinib with the IC_{50} value of 6.0 ± 0.54 nM compared with H1975 and A549 cells with IC_{50} value of 10.02 ± 4.37 μM and 10.32 ± 2.32 μM, respectively. Combination treatment with gefitinib and Aidi injection significantly enhanced gefitinib induced antiproliferation effect in A549, H1975 and HCC827 cells (Fig. 1). Aidi injection significantly reduced gefitinib-induced growth inhibition IC_{50} values in three NSCLC cell lines examined (Table 2), resulting in a 2.3 – 6.2-fold increment in gefitinib sensitivity.

3.3. Colony formation results

The morphological characteristics of the A549, H1975 and HCC827 cell lines were further evaluated by colony formation assay after treatment with gefitinib alone and in combination with Aidi. The therapeutic potential of the gefitinib and Aidi combination in preventing the appearance of cell colonies were explored by treating cells with gefitinib and Aidi combination for 72 h. After 10 days of incubation, we observed more colony formation in all NSCLC cell plates treated with gefitinib only. With increased gefitinib concentration the colony formation de-

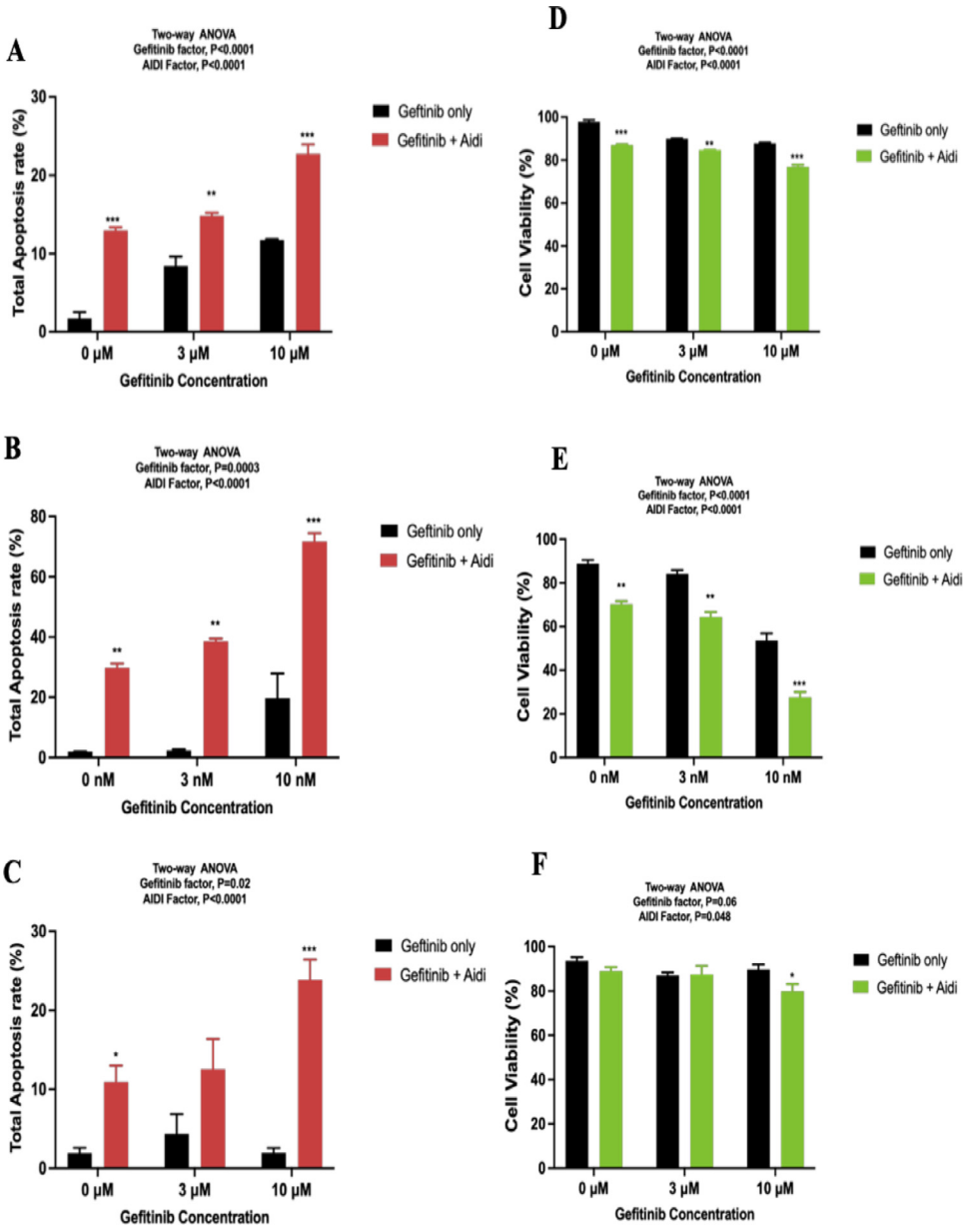

Fig. 3. Total Apoptosis rate in NSCLC after treatment with gefitinib and Aidi. NSCLC cell lines were treated with different concentration of gefitinib, either alone or in combination with Aidi for 72 h before stained with Annexin V-FITC/PI and the apoptosis rate were assessed by flow cytometry. The bar graphs represent the total apoptosis rate in (A) H1975, (B) HCC827, (C) A549 cells and cell viability in (D) H1975, (E) HCC827, (F) A549 cells after gefitinib and Aidi treatment. Data are presented as mean and SD of replicate samples. Asterisks are P values (*, $p<0.05$; **, $p<0.01$; ***, $p<0.001$) from Sidak post-tests that followed a two-way ANOVA.

creased in the NSCLC cell lines. We observed the intracellular space increased, cell numbers markedly reduced, and cell colonies prominently decreased in response to the combination of increased gefitinib concentration and Aidi treatments compared with cells treated with gefitinib alone. Moreover, treatment with Aidi only markedly reduced cell colonies in plates which speculate that Aidi alone may have anticancer effects in NSCLC cell lines (Fig. 2).

3.4. Combination of gefitinib and Aidi enhances apoptosis in NSCLC cell lines

We analysed the apoptotic effects of Aidi and its combination with gefitinib in NSCLC cell lines to increasing doses of gefitinib. The H1975 and HCC827 cell lines underwent apoptosis in a dose-dependant manner with gefitinib treatment only and A549 cells were less sensitive to gefitinib. The apoptosis rate in H1975 was 8.43 ± 1.2% and 11.74 ± 0.11% after treated with 3 μM and 10 μM of gefitinib respectively for 72 h. The apoptosis rate in HCC827 cells were 2.33 ± 0.32% and 19.70 ± 8.16% after treatment with 3 nM and 10 nM of gefitinib respectively for 72 h. The apoptosis rate in H1975 cells was 14.85 ± 0.36% and 22.75 ± 1.19% after treatment with 3 μM and 10 μM of gefitinib in combination with Aidi respectively for 72 h and in HCC827 were 38.57 ± 0.90% and 71.63 ± 2.82% after treatment with 3 nM and 10 nM of gefitinib in combination with Aidi respectively for 72 h. Furthermore, Aidi treatment only for 72 h induced apoptosis, with apoptosis rate 12.96 ± 0.41%, 29.78 ± 1.41% and 10.95 ± 2.06% in H1975, HCC827 and A549 cells respectively (Fig. 3). The gefitinib and Aidi combination, either in high dosage (10 μM gefitinib for H1975 and A549 cells; and 10 nM for HCC827 cells) or in low dosage (3 μM gefitinib for H1975 and A549 cells; and 3 nM for HCC827 cells), significantly increased the rate of apoptosis as compared to gefitinib treatment only (Fig. 4).

3.5. Association of ABCC2 expression with survival in patients with NSCLC

Oncogenomics data from 505 patients with lung adenocarcinoma from TCGA Lung Adenocarcinoma (LUAD) dataset showed that patients with a high expression of *ABCC2* (n = 254) had significantly poorer survival than patients with a low expression (n = 251) (Fig. 5A, P-value = 0.007564). However, differential expression of *ABCC2* had no

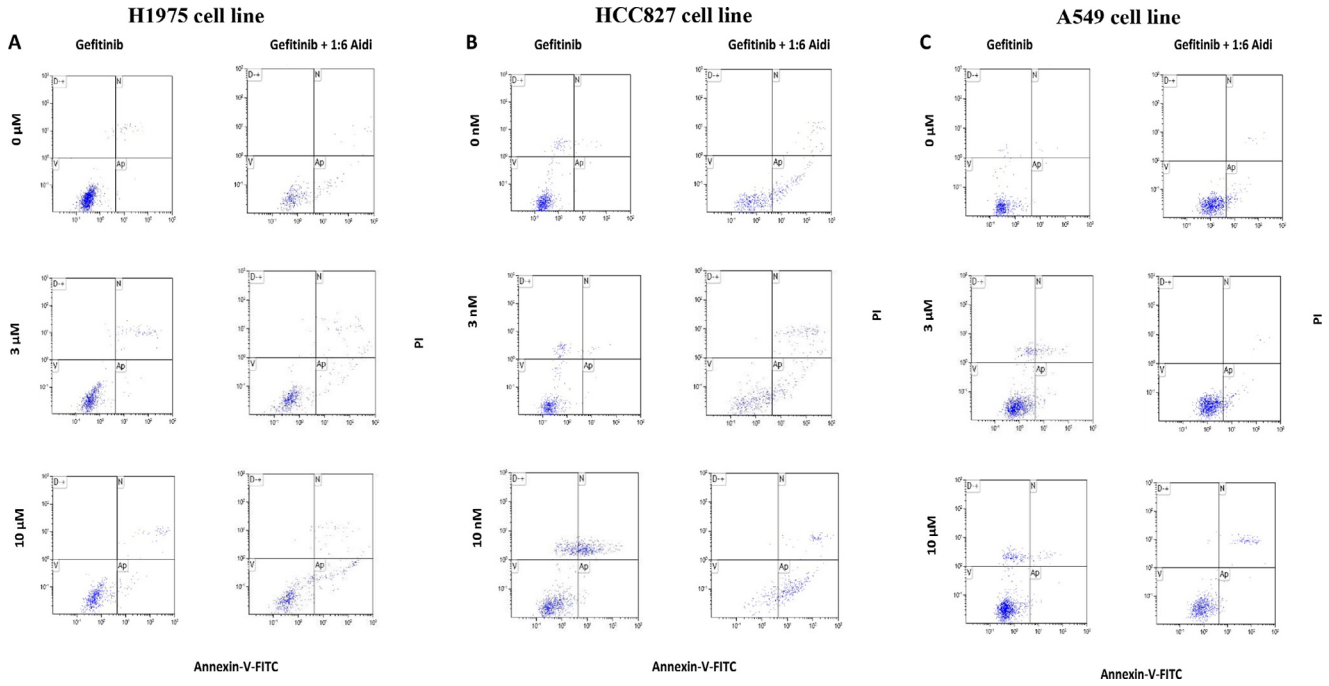

Fig. 4. Histogram of apoptotic changes in (A) H1975, (B) HCC827 and (C) A549 cell lines with gefitinib treatment alone or in combination with Aidi injection. After incubation of cells with different concentration of gefitinib and Aidi solution, the cells were subsequently stained with Annexin-V FITC and PI. The fluorescence intensity was measured by flow cytometry. Viable cells (V) are both Annexin-V and PI negative. At an early stage of Apoptosis (Ap), cells bind with only Annexin-V. At the late stage of apoptosis (N), the cells bind with both Annexin-V FITC and PI. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

apparent effects on overall survival in patients from TCGA Lung Squamous Cell Carcinoma (Fig. 5B, *P*-value = 0.8413).

Analysis of ABCB1, ABCC1 and ABCG2 gene expression in both TCGA lung cancer datasets were performed. TCGA Lung Adenocarcinoma (LUAD) dataset but not TCGA Lung Squamous Cell Carcinoma dataset showed that patients with a high expression of ABCB1 ($n = 252$) had significantly better survival than patients with a low expression ($n = 253$) (Figure S2, *P*-value = 0.01881). ABCC1 and ABCG2 gene expression had no apparent association with survival (Figure S2).

3.6. Inhibitory effect of Aidi injection on MRP2

The effect of Aidi injection on the efflux of the fluorescent substrate CDCF from MRP2-expressing cells was measured. In the absence of Aidi injection, CDCF accumulation in MRP2-expressing HEK293 cells was only 28% of that in parental HEK293 cells (Fig. 6A and 6B). In the presence of Aidi injection (equivalent to Ginsenoside Re 0.034 and 0.052 mg/mL), the cellular accumulation of CDCF significantly increased by 186% ($P < 0.05$) and 362% ($P < 0.01$, Fig. 6A), respectively; in MRP2-expressing HEK293 cells. Aidi injection had no effects on the cellular accumulation of CDCF in parental HEK293 cells (Fig. 6B).

3.7. Interaction of gefitinib and Aidi injection

Gefitinib has demonstrated efficacy in the treatment of NSCLC patients. However, some patients are refractory to gefitinib treatment, and strategies to improve its therapeutic efficacy are required. Thus, we evaluated the interactions between gefitinib and Aidi injection in HEK293 parental and HEK293 overexpressing MRP2 cell lines.

The cytotoxic effects of gefitinib in HEK293 (Fig. 7C) and HEK293/MRP2 cells (Fig. 7F), analysed showing that gefitinib inhibited cell proliferation in a dose-dependant manner. The IC_{50} values of gefitinib and Aidi after 72 h for HEK293 is 14.53 μM and 6.46 μM (Fig. 7B) respectively. Whereas, the IC_{50} values of gefitinib and Aidi after 72 h for HEK293/MRP2 is 9.30 μM and 23.45 μM (Fig. 7E) respectively. Interactions between gefitinib and Aidi injection were evaluated in both HEK293 and HEK293/MRP2 cell lines. The CI value of gefitinib and Aidi showed that the combination of these drugs is antagonistic (CI = 1.94) in HEK293 cells (Fig. 7A). In HEK293/MRP2 cells, the combination of gefitinib and Aidi injection had remarkably improved cytotoxicity over single-drug therapies. The CI of gefitinib and Aidi combination is additive or weakly synergistic (CI=0.97) in HEK293/MRP2. The data revealed that the combination therapy of gefitinib and Aidi injection were antagonistic in HEK293 cells and additive or weakly synergistic in HEK293 overexpressing MRP2.

3.8. Principle chemical composition

There were eleven principal components identified in Aidi injection by LC-MS/MS analysis, including astragalosides II and IV, cantharidin, eleutheroside E, ginsenosides Rb1, Rc, Re, Rd and Rg1, isofraxidin, and syringin.(Fig. 8). All principal components were further confirmed by using MRM analysis. The concentration of ginsenosides Rb1, Rc, Rd and Re in Aidi injection were 13.9, 8.6, 8.7 and 310.0 μg/mL, respectively. Fig. 9.

3.9. Docking studies using autodock Vina

In the present study, Aidi components were analysed through molecular docking study using AutoDock Vina and results were evaluated using PyMOL. Docking results against receptor molecule from PyMOL showed that all Aidi components at least formed one hydrogen-bond (H-bond) with MRP2. All ginsenosides Rb1, Rc, Rd, Re and Rg1 form 5 to 7 maximum H-bond. Relative binding affinities were scored for the Aidi components and gefitinib as ligands, represented as kcal/mol in Table 4. The docking scores for various ligands were represented in Table 4. The affinity values closed to −10 kcal/mol indicate efficient binding. Astragaloside II and IV, ginsenoside Rb1, Rc, Rd and

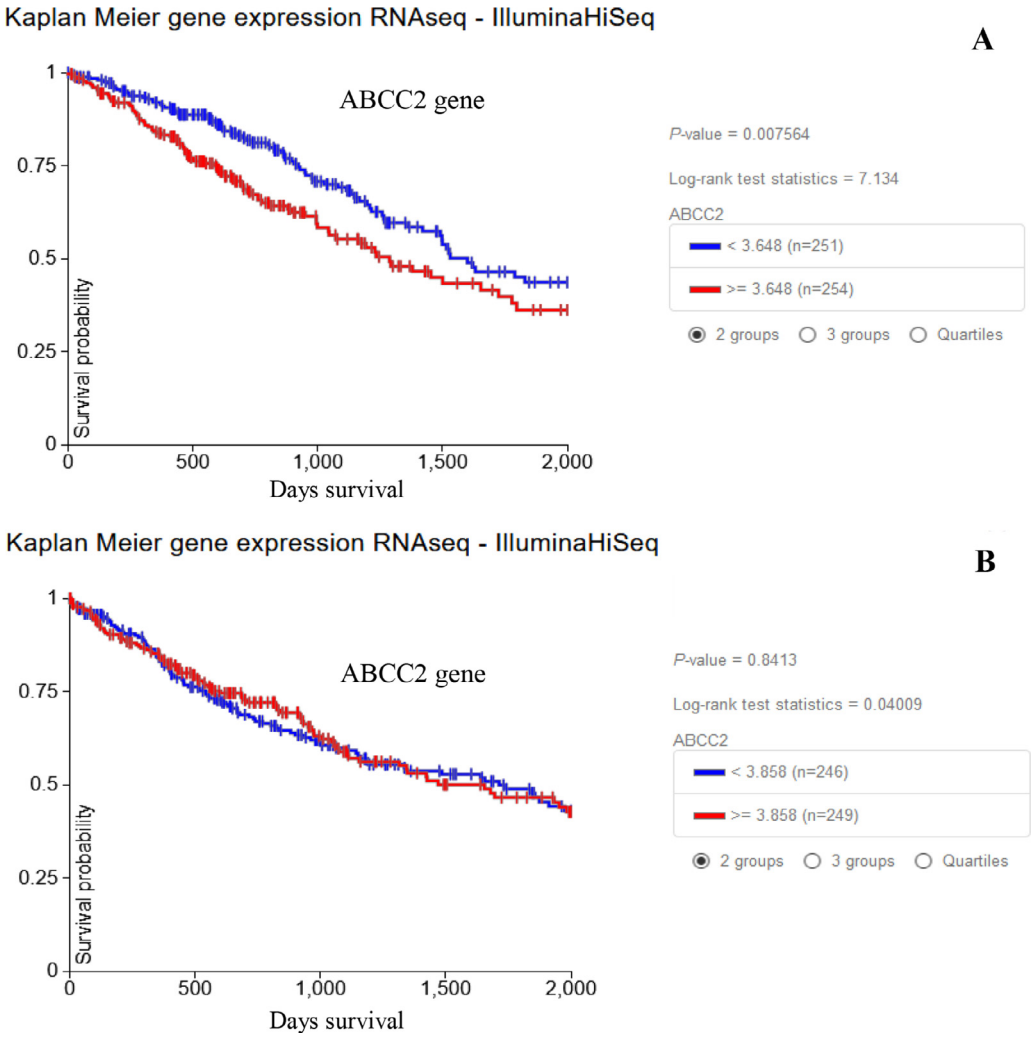

Fig. 5. ABCC2 gene expression in lung cancer patients. Overall survival curves stratified by expression of ABCC2 gene (encoding MRP2 protein) in patients with (A) lung adenocarcinoma and (B) lung squamous cell carcinoma. Log-rank test was used to compare the difference of survival curves. Statistical significance was set at P value ≤ 0.05.

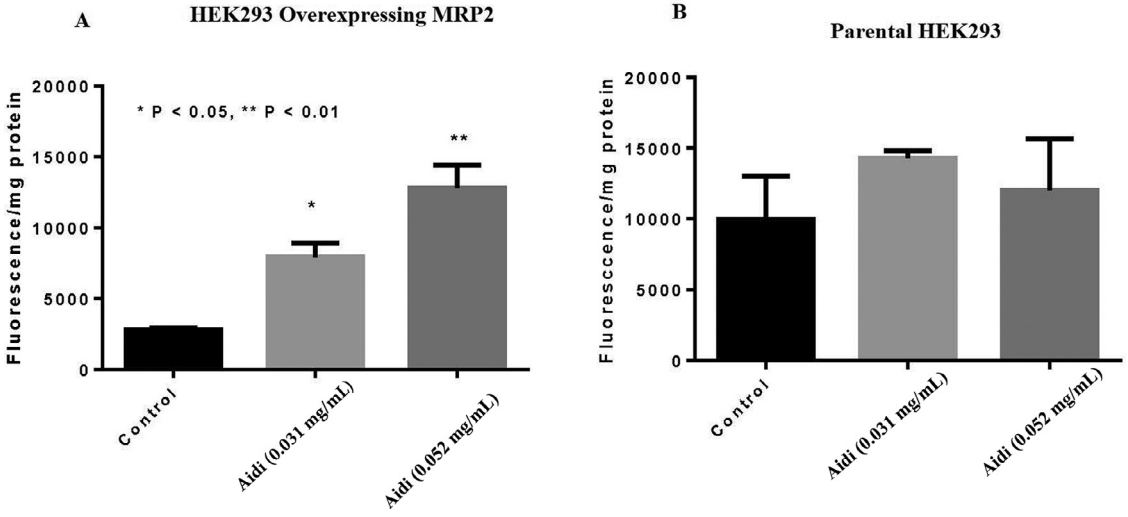

Fig. 6. Aidi injection increased the cellular accumulation of a MRP2 substrate CDCF in (A) HEK293 overexpressing MRP2 cells but not in (B) parental HEK293 cells. Cells pre-treated with Aidi injection equivalent to Ginsenoside Re 0.031 mg/mL; Cells pre-treated with Aidi injection equivalent to Ginsenoside Re 0.052 mg/mL. Data are Mean ± SD, Asterisks are P values (*, $p<0.05$; **, $p<0.01$) from Dunnet's post-tests that followed one-way ANOVA.

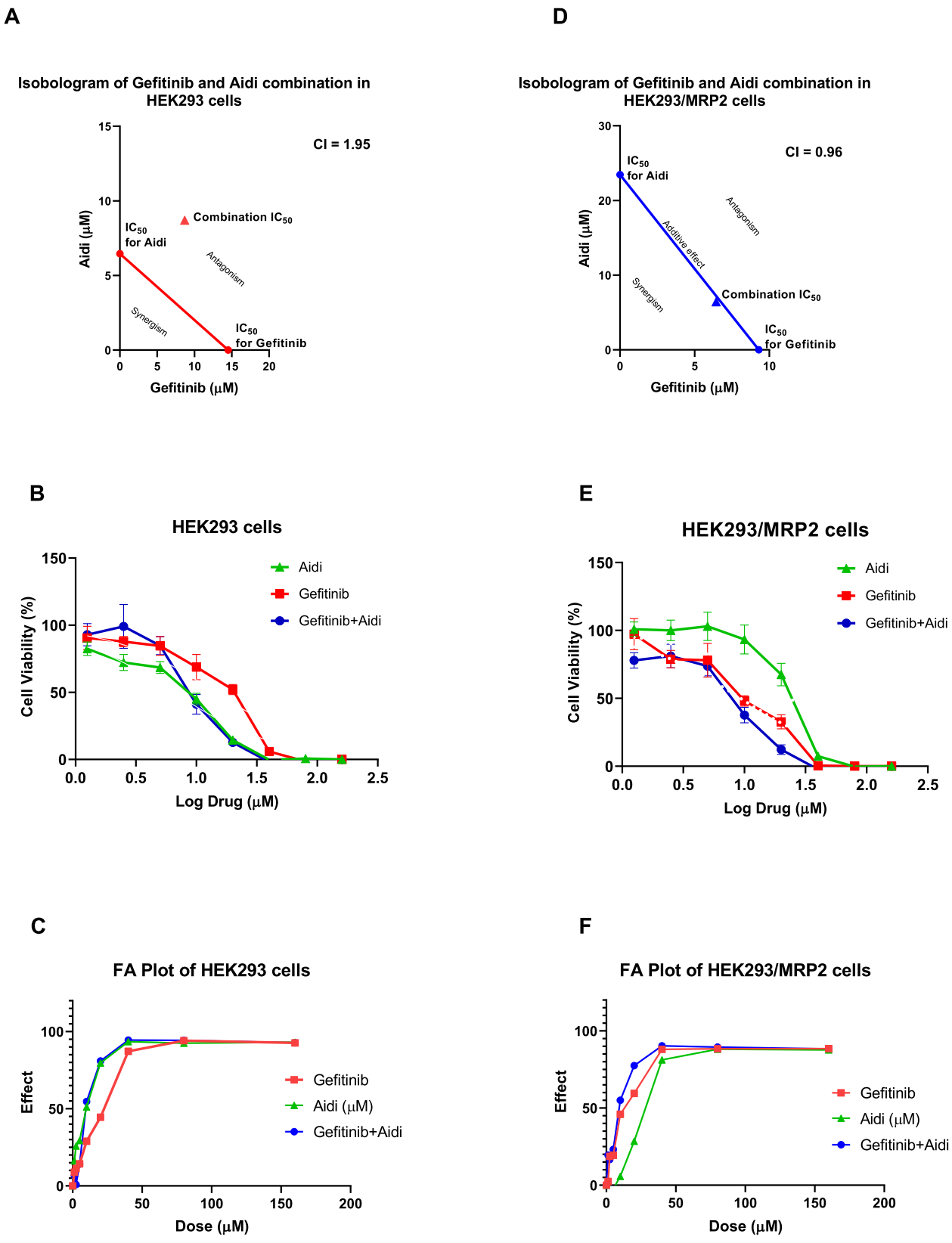

Fig. 7. Combination effect of gefitinib and Aidi injection in HEK293 and HEK293 overexpressing MRP2 (HEK293/MRP2) cells. (A) Isobologram in HEK293 cells, showing antagonism, as the observed datapoint are on the right side of curve. (B) Growth inhibition curve in HEK293 cells, showing the growth inhibition of gefitinib, Aidi and the combination of gefitinib and Aidi. (C) FA plot in HEK293 cells showing the dose vs effect. The effect is antagonistic. (D) Isobologram in HEK293/MRP2 cells showing additive, because the observed datapoint are almost on the left side of curve. (E) Growth inhibition curve in HEK293/MRP2 cells, showing the growth inhibition of gefitinib, Aidi and the combination of gefitinib and Aidi. (F) FA plot in HEK293/MRP2 cells showing the dose vs effect. The effect is additive.

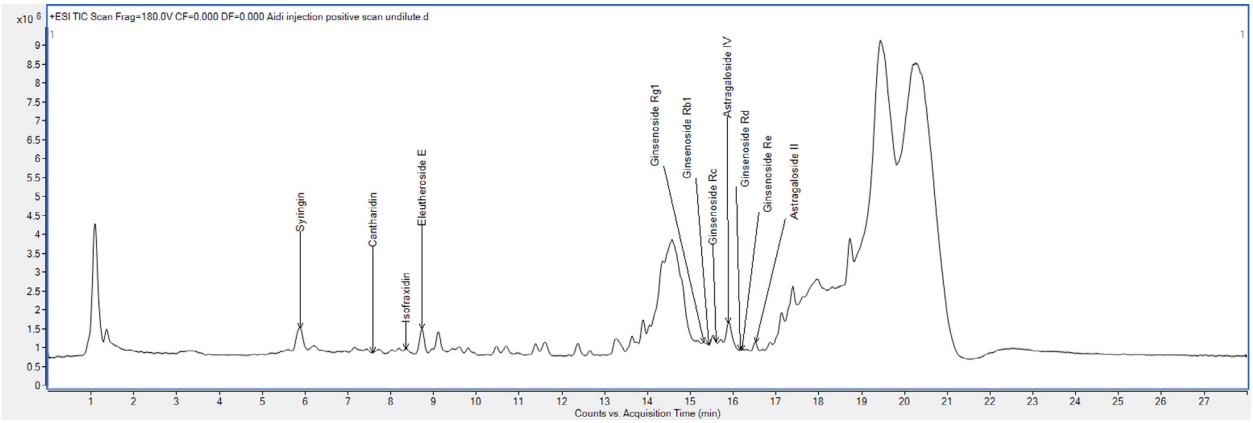

Fig. 8. Eleven principle components of Aidi injection were identified in a representative LC/MS total ion chromatograph.

Re has higher affinity score comparatively than other Aidi components which is mainly due to the structural difference between components and also number of active H-bond it is forming with MRP2 macromolecule. All ginsenoside components have common residue binding site Lys677 (Lysine) of MRP2 protein. Gefitinib and ginsenoside Re form H-bond with common residue Asp786 (Aspartic Acid) (Fig 9). We also observed binding affinity of some Aidi components, astragaloside (II, −10.2 kcal/mol; IV, −10.4 kcal/mol) and ginsenoside (Rb1, −10.0 kcal/mol; Rc, −10.2 kcal/mol; Rd, −10.1 kcal/mol; Re, −10.3 kcal/mol; Rg1, −9.4 kcal/mol) were higher than gefitinib (−7.4 kcal/mol).

4. Discussion

Combination therapy of EGFR-TKI gefitinib and other anticancer drugs has been shown promising results to overcome resistance to EGFR-TKIs in advanced NSCLC [18,19]. While some clinical trials of combination therapy failed to demonstrate a survival benefit due to the lack of patients' selection based on EGFR-mutational status [18]. This discovery gave a new viewpoint to this treatment strategy to better select patients who may most benefit from this combination therapy. In an individual patient based on tumour heterogeneity, multiple resistance mechanisms may be present simultaneously or sequentially. So, it is fundamental to perform a genetic examination before treatment to combine tailored treatments and proposed individualised therapy [20,21]. International standard oncology practice guidelines now recommend undertaking genetic testing before systemic treatment of patients with advanced lung cancer [22]. Genetic testing enables the identification of key genetic drivers of lung cancer in individual patients and the selection of the most appropriate targeted drug [23]. Genotype-directed targeted therapy of advanced lung cancer can achieve survival outcomes that are unprecedented compared to those achieved by standard treatments not selected by genotyping [24]. However, the best tumour response in some NSCLC patients is a progressive disease and in others, tumour resistance develops promptly. Intrinsic and acquired resistance to EGFR-targeting agents is an important issue in clinical practice. Acquired mutations in the EGFR tyrosine kinase domain, the activation of bypass signalling pathways, enhanced extrusion of intracellular active moieties, and phenotypic or histologic transformation have been identified as mechanisms of acquired resistance to first- and second-generation EGFR-TKIs [25]. The original EGFR mutation may remain detectable at the time of occurrence of new acquired resistance molecular changes. So continuing treatment beyond progression may be the best treatment option with the addition of other therapeutic agents. Importantly, gefitinib-based EGFR-TKI therapy may benefit the patient in combination therapy, given that the appropriate combination therapy target is identified. Therefore, there is an urgent need to identify alternative regimens for patients who will have such unoptimistic outcomes from gefitinib-based target therapy.

Aidi injection (Z52020236, China Food and Drug Administration) has been used as a safe and multitarget adjunct therapy in Chinese patients with NSCLC. Our in vitro experimental studies reported here provided the proof-of-principle evidence that Aidi injection enhanced gefitinib sensitivity in NSCLC cell lines with different mutation subtypes. The NSCLC patients with an exon 19 mutation in their EGFR gene respond to gefitinib, but after their tumours develop a T790M mutation, these tumours no longer respond to gefitinib. Our results demonstrated that the combination of both gefitinib and Aidi injection enhanced the antitumour activity of gefitinib in NSCLC cells carrying different EGFR mutations. Although enhanced expression of EGFR was previously shown in NSCLC [26], the antitumor activity of gefitinib in NSCLC correlates with EGFR mutations but not EGFR protein levels [27], which is consistent with our current results. The H1975 cell line carrying the L858R and T790M mutations was resistant to gefitinib whereas the HCC827 cell line with an exon 19 deletion mutation was less resistant. Given the fact that T790M mutations account for the major causes of acquired gefitinib resistance, our studies suggest Aidi injection might be used to reverse common gefitinib resistance in NSCLC.

It has been recently reported that downregulation or inhibition of MRP2 (encoded by ABCC2 gene) reversed gefitinib resistance and cisplatin (a model MRP2 substrate) resistance in A549 cells in vitro [8]. Given the pivot roles of gefitinib and platinum drugs in NSCLC treatment, we hypothesised that *ABCC2* gene may be a prognostic marker and thus performed gene association studies by accessing TCGA lung cancer datasets (http://xena.ucsc.edu/). Our results showed that lung adenocarcinoma patients with a high expression of *ABCC2* had a significantly poorer survival, suggesting a novel therapeutic target for lung adenocarcinoma. Thus we explored MRP2 inhibitory effects of Aidi injection by using isogenic HEK293 cell models. MRP2 is an ABC transporter and has been reported to confer resistance to various anticancer drugs such as gefitinib, gemcitabine, doxorubicin, cisplatin, methotrexate and oxaliplatin [8,28]. In the last two decades, more than 70% of the ABC transporter inhibitors reported were natural products or synthetic derivatives of these products [29]. Aidi injection is a traditional Chinese medicine containing multiple phytochemicals derived from ginseng, Astragalus membranaceus and Acanthopanax. Our results showed Aidi injection significantly increased the cellular accumulation of a model substrate CDCF in MRP2-overexpressing HEK293 cells in a concentration-dependant manner, but not in parental HEK293 cells, which indicates Aidi injection contains an efficient MRP2 inhibitor(s). Inhibition of MRP2 by Aidi injection alone increased the apoptosis rate in all three NSCLC cell lines examined (Table 3). We and others recently reported that silencing MRP2 alone increased the apoptosis rate in Caco-2 and HepG2 cells [30,31], suggesting MRP2 might also protect cells from apoptosis independently of cytotoxic drug efflux.

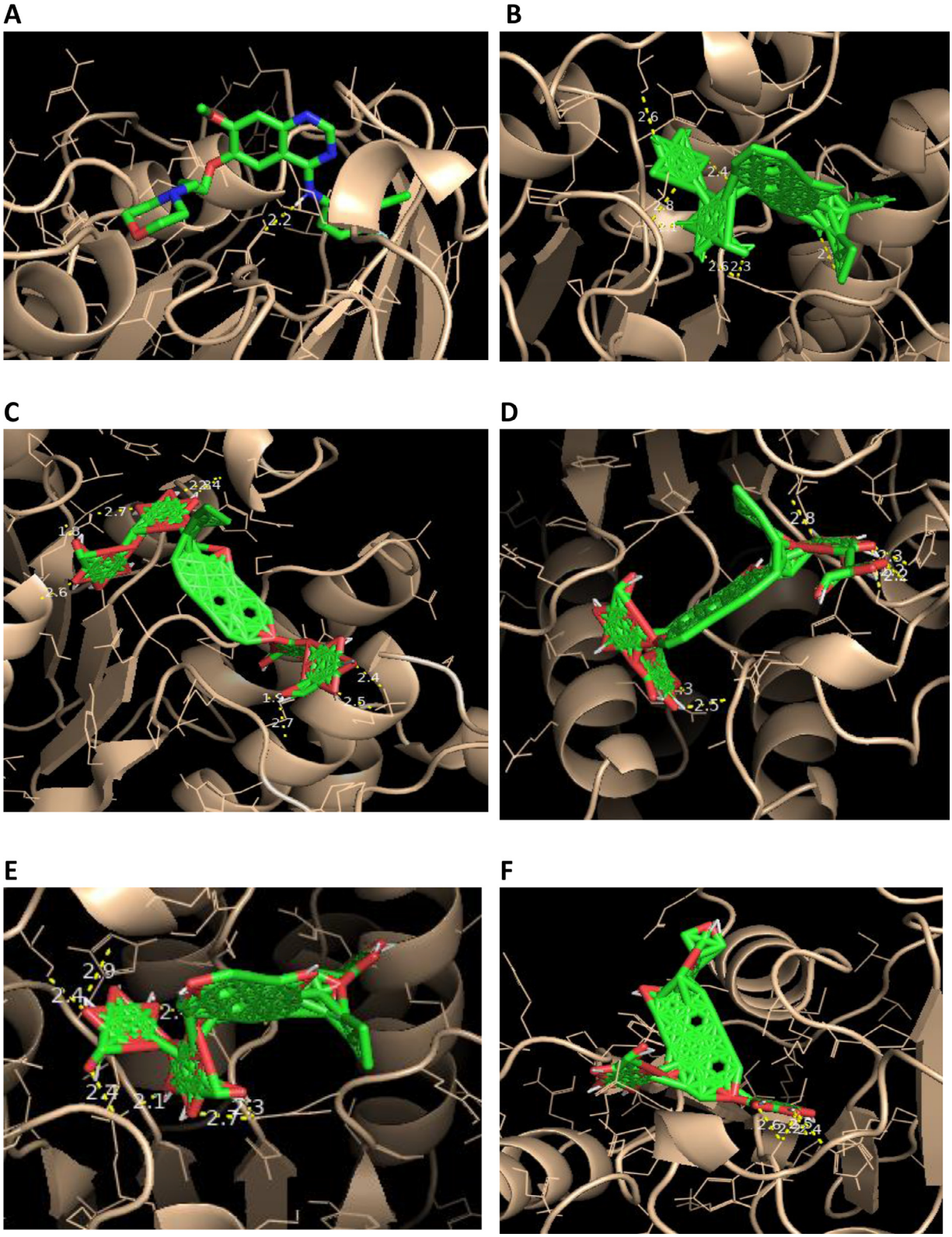

Fig. 9. Ligands docked on MRP2 macromolecule. Binding of (A) Gefitinib and (B-F) Aidi components with high binding affinity against MRP2, including (B) Ginsenoside Re, (C) Ginsenoside Rb1, (D) Ginsenoside Rc, (E) Ginsenoside Rd and (F) Astragaloside IV. Ligands are shown in green chemical structure and MRP2 are depicted in wheat colour in ribbon form. H-bond are shown in yellow dotted lines. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 3
Data analysis of apoptosis assay in NSCLC cell lines with gefitinib alone and in combination with Aidi (H1975 and A549 cells were treated with 3 μM and 10 μM gefitinib; HCC827 cells treated with 3 nM and 10 nM gefitinib).

Treatment	H1975 cells Total Apoptosis	*p*-values*	*p*-values‡	HCC827 cells Total Apoptosis	*p*-values*	*p*-values‡	A549 cells Total Apoptosis	*p*-values*	*p*-values‡
No treatment	1.7 ± 0.8			1.94 ± 0.07			1.91 ± 0.67		
Aidi only	12.96 ± 0.41			29.78 ± 1.41@@			10.95 ± 2.06		
Gefitinib (3 μM/3 nM)	8.43 ± 1.2			2.33 ± 0.32			4.34 ± 2.53		
Gefitinib(3 μM/3 nM)+Aidi	14.85 ± 0.36	<0.001	0.19	38.57 ± 0.90	0.001	0.15	12.54 ± 3.82	0.09	0.96
Gefitinib(10 μM/10 nM)	11.74 ± 0.11			19.70 ± 8.16@@			1.96 ± 0.61		
Gefitinib(10 μM/10 nM)+Aidi	22.75 ± 1.19	<0.001	<0.01	71.63 ± 2.82	<0.001	<0.01	23.88 ± 2.54	<0.01	0.03

* *p*-values compared with the apoptosis induced by gefitinib only.
‡ *p*-values compared with the apoptosis induced by Aidi only.

Table 4
Binding Affinity and hydrogen bond (H-bond) interaction of different ligands with MRP2 receptor macromolecule.

Ligands	Binding Affinity (kcal/mol)	Interacting residues	Number of H-bonds with MRP2	Distances of H-bond (from MRP2)
Astragaloside_II	−10.2	Thr701, Gln777, Trp709, Try703	6	2.0, 2.8, 1.9, 2.5, 2.3, 2.9
Astragaloside_IV	−10.4	Thr873, His875	4	2.6, 2.2, 2.5, 2.4
Canthridin	−5.8	Lys860, His865, Thr866	5	2.2, 2.3, 3.3, 2.4, 2.7
Eleutheroside	−8	Lys797, Asn801, His865, Thr866	5	2.4, 2.7, 2.6, 2.1, 2.5
Ginsenoside_Re	−10.3	Asp786, Thr873, Lys677, Gln706	7	2.5, 2.3, 2.6, 2.1, 2.8, 2.6, 2.4
Ginsenoside_Rb1	−10	Ser708, Lys766, Lys677, Gly763, Glu870, Ser678	6	2.5,2.4, 2.5,2.7, 1.8,2.6
Ginsenoside_Rc	−10.2	Lys677, Gln767, Thr873, His875, Ser789	6	2.8, 2.2, 2.2, 2.3, 2.3, 2.5
Ginsenoside_Rd	−10.1	Lys677, Gln706, Asp786, Glu870, Thr873	7	2.4, 2.3, 2.7, 2.1, 2.4, 2.9, 2.3
Ginsenoside_Rg1	−9.4	Lys677, Glu870, Glu871, Thr873, His875	5	2.5, 2.4, 2.2, 2.3, 2.4
Isofraxidin	−5.6	Ile661	1	1.9
Syringin	−6.3	His694, Thr696, Ile661	4	2.1, 2.2, 2.4, 2.5
Gefitinib	−7.4	Asp786	1	2.2

In the present study, we analysed and identified eleven main chemical components from Aidi injection, including astragalosides II and IV, cantharidin, eleutheroside E, ginsenosides Rb1, Rc, Re, Rd and Rg1, isofraxidin, and syringin. Out of all the components ginsenosides Rb1, Rc, Re and Rd have high binding affinity towards MRP2 forming maximum H-bond. Previously, it has been reported that ginsenoside can induce apoptosis of cancer cells and can inhibit the growth of tumour cells. Ginsenoside Rd can inhibit the growth of tumour cells, such as gastric, lung and colorectal cancers [32-34]. Ginsenoside Rb1, Rc, Re, Rg1 have certain anti-cancer effects [35-37]. In molecular docking we observed that ginsenoside form maximum H-bond with MRP2 with high binding affinity. The high H-bond between ligand-receptor complexes makes the complex more stable, so H-bonding could improve the affinity and as a result could improve the anti-cancer action. In vitro studies further support the in silico analysis showing Aidi injection alone has anti-cancer effect [38]. Our results indicated the high binding affinity of astragaloside II and IV, and ginsenoside Rb1, Rc, Re and Rd towards MRP2. Ginsenoside Re is the most abundant component in Aidi injection that also exhibited the best MRP2 binding affinity (with maximum H-bonds) and thus could be considered as a potential lead anti-cancer molecule in Aidi injection.

Interactions between gefitinib and Aidi injection were further explored by using parental HEK293 cells and MRP2-overexpressing HEK293 cells. The main reason for using drugs combination is to understand mutual enhancement of the therapeutic benefit effects, decreased side effects and prevention of drug resistance. The combination of gefitinib and Aidi gave additive or weakly synergistic (CI = 0.97) growth inhibition in MRP2-overexpressing HEK293 cells but exhibited antagonistic (CI = 1.94) cytotoxicity in parental HEK293 cells with low level of MRP2 expression. Our results also indicate that gefitinib may not be an MRP2 substrate as there is no significant differences in gefitinib sensitivity between parental HEK293 cells and MRP2-overexpressing HEK293 cells (Fig. 7C and 7E). Also, we observed from molecular docking results that gefitinib has less binding affinity with MRP2 protein. Based on our results it is reasonable to suggest that effect of Aidi injection on gefitinib sensitivity is MRP2 dependant although gefitinib may not be an MRP2 substrate. However, the mechanism is remaining to be elucidated and further studies are warranted. In addition, a combination of gefitinib and Aidi injection might reduce their toxicity in cells or tissues with low MRP2 expression, such as skin tissues (https://www.proteinatlas.org/ENSG00000023839-ABCC2/tissue). Skin rash is one of the major adverse effects of gefitinib but there is no current clinical evidence that Aidi injection diminishes such a side effect.

4.1. Limitations

The major limitations of the current study are the lack of separation and isolation of the specific MRP2 inhibitor(s) from Aidi injection. We and others recently reported that silencing MRP2 alone increased the apoptosis rate in Caco-2 and HepG2 cells [30,31], suggesting MRP2 might also protect cells from apoptosis independently of cytotoxic drug efflux. Inhibition of MRP2 by Aidi injection alone increased the apoptosis rate in all three NSCLC cell lines examined. However, the mechanism is remaining to be elucidated and further studies are warranted.

5. Conclusions

In conclusion, the combination of gefitinib and Aidi injection, a Chinese herbal medicine extract, is effective in reversing or preventing gefitinib resistance in NSCLC cells. ABCC2 gene (encoding MRP2) expression has been reversely associated with the survival rate in patients with lung adenocarcinoma. Aidi injection inhibited MRP2 functions and ginsenoside Re is one of the most abundant components in Aidi injection that also exhibited the highest MRP2 binding affinity. The combination of gefitinib with Aidi injection gave additive or weakly synergistic growth inhibition in MRP2 overexpressing HEK293 cells but exhibited antagonistic cytotoxicity in parental HEK293 cells. The in vitro findings provide strong evidence that a combination of gefitinib and Aidi injection might be a promising therapeutic strategy for the treatment of gefitinib-resistance NSCLC patients and should be further evaluated for clinical studies.

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Declaration of Competing Interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

CRediT authorship contribution statement

Riya Biswas: Data curation, Formal analysis, Methodology, Visualization, Writing – original draft. **Chun-mei Yang:** Data curation, Methodology, Visualization. **Wei Lu:** Data curation, Validation. **Ji He:** Methodology, Investigation. **Tony Chen:** Methodology, Validation. **Fang Tian:** Conceptualization, Writing – review & editing. **Yan Li:** Conceptualization, Methodology, Writing – review & editing, Supervision, Funding acquisition.

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Data availability

TCGA Lung Adenocarcinoma and Squamous Cell Carcinoma datasets can be accessed on UCSC Xena platform (https://tcga.xenahubs.net).

Supplementary materials

Supplementary material associated with this article can be found, in the online version, at doi:10.1016/j.eujim.2021.101368.

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