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Isoflavone Extracts Enhance the Effect of Epidermal Growth Factor Receptor Inhibitors in NSCLC Cell Lines

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Abstract. *Aim: We investigated the effects of the pharmacological inhibition in vitro of epidermal growth factor receptor (EGFR) in combination with isoflavones. Materials and Methods: Four anticancer drugs (erlotinib, gefitinib, afatinib and AZD9291) were combined with soy and red clover isoflavone extracts and used in cellular proliferation assays. The antitumor activity of inhibitors alone and in combination with isoflavone extracts was compared on three non-small cell lung cancer (NSCLC) cell lines with affiant EGFR genotype: A549 (EGFR wt); H1795 (EGFR T790M); HCC827 (EGFR del E746-A750). Results: Combined treatment with extracts significantly enhanced the antiproliferative activity of all inhibitors against these cell lines. Bioactive compounds of extracts may synergize the antitumor efficacy of the inhibitors. Conclusion: To date, as far as we are aware, this is the first report of the combined effect of isoflavone extracts and EGFR inhibitors on human NCSLC cell growth. Sequential treatment with these drugs combined with isoflavones may represent the basis for a new therapeutic approach.*

Epidermal growth factor receptor (EGFR)-targeted therapies using small molecules, such as tyrosine kinase inhibitors (TKI) have recently been approved for non-small cell lung cancer (NSCLC). In particular, two EGFR tyrosine kinase inhibitors (TKI), gefitinib (ZD1839, Iressa) and erlotinib (OSI774, Tarceva), represent the first generation of molecular-targeted agents developed for the treatment of NSCLCs (1-3). Irreversible inhibitors such as afatinib and AZD9291 constitute second- and third-generation EGFR inhibitors. Progression-free survival (PFS) of patients with EGFR mutation receiving gefitinib, erlotinib or afatinib is improved when compared with standard chemotherapy, as demonstrated with randomized trials (4). Unfavorably, TKI therapies may become ineffective due to acquired resistance that may develop after a duration of response of ~10 months up to 1 year (5, 6). A secondary mutation in exon 20 of *EGFR* within the residue at position 790 inducing a threonine to methionine substitution (T790M) was determined as the first cause of resistance identified on a genetic basis. T790M causes resistance through changes in adenosine triphosphate (ATP) affinity because T790M mutation restores the ATP affinity of the kinase, re-establishing ATP as the favored substrate rather than the administered TKI (7). Second-generation EGFR TKIs, such afatinib, form irreversible covalent bonds with EGFR and are potentially effective *in vitro* against cells with EGFR T790M. Third-generation EGFR TKIs, such as AZD9291/are being developed to overcome resistance to first- and second-generation *EGFR* TKIs. In preclinical studies, AZD9291 was active both *in vitro* and *in vivo* against cell lines and murine models harboring the *EGFR* T790M mutation (8). Very promising results from an ongoing phase I study on AZD9291 have been reported (9).

Recently, natural dietary compounds have captured attention because of their synergistic effects with anticancer drugs against various types of cancer. Natural phytochemicals may accomplish their antitumoral effect through targeting diverse cancer cell signaling pathways, giving rise to cell-cycle block and apoptosis, or regulating antioxidant status and detoxification (10). Much evidence has shown that isoflavones exert their pleiotropic effects on cancer cells by interfering in cellular signaling pathways including nuclear factor kappa-light-chain-enhancer of activated B-cells, serine/threonine kinase, mitogen-activated protein kinase, and p53.

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Isoflavones are principally recovered in members of the Leguminosae family (11). Genistein is the most extensively analyzed flavonoid, it is involved in numerous biological actions with potential beneficial effects, rightly becoming a focus for research (12). Genistein is also a well-known TKI and induces apoptosis in several types of cancer, such as lymphoma, leukemia, breast, prostate, head and neck, pancreatic and lung cancer (13).

In the present study, the effect of gefitinib, erlotinib, afatinib and AZD9291 (selective EGFR TKIs) combined with isoflavones from soy and red clover was assayed on a panel of NSCLC cell lines by using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) and crystal violet assays. Our aim was to evaluate *in vitro* whether these isoflavone extracts synergize with TKIs to inhibit NSCLC cell growth.

Materials and Methods

Samples and extraction. Fresh faba beans (*Vicia faba*), peas (*Pisum sativum*), ordinary beans (*Phaseolus vulgare*) and soybean (*Glycine max*) seeds were purchased at a local market, while dried red clover (Trifolium pretense L.) was obtained at a herbalist's shop. Standards of genistin, glycitin, daidzin, biochanin A and formonotenin were purchased from Sigma-Aldrich (St. Louis, MO, USA). Genistein, glycitein and daidzein aglycones were obtained by acid hydrolysis (using 3.4 N HCl at 50˚C for 200 min) of the corresponding β-glucosides, according to Chiang *et al*. (14), with slight modifications.

A protocol for the extraction of phenolic compounds was adapted from Rostagno *et al*. (15). Briefly, 0.5 g of the dry sample was suspended in 5 ml of 50% ethanol (v/v) and ultrasonicated for 20 min at 60˚C. The suspension was centrifuged for 10 min at ~2500×g and the supernatant was recovered. The pellet was re-extracted for 12 hours with an additional 5 ml of solvent and centrifuged. The two supernatants were combined and stored in a freezer at −20˚C for subsequent analysis (16, 17).

Determination of total phenolic compounds. The total polyphenol content in the extracts was determined using the Folin-Ciocolteau method (18). Fifty microliters of dried bean, pea, red clover and soya extracts were combined to 3 ml with distilled water, 250 μl of Folin and 750 μl of 7% (w/v) Na_2CO_3. After 8 min, 950 ml of distilled water was added. Samples were left for 2 h at room temperature in the dark and the absorbance at 765 nm was then determined spectrophotometrically microplate reader, (Cary 50 MPR; Varian, LAB X, LA Jolla, CA, USA). Total polyphenol content was expressed as gallic acid equivalents (GAE, in mg) per gram of dry material.

Determination of total flavonoid compounds. Total flavonoid content was determined using the aluminium chloride colorimetric assay (19). Briefly, 50 μl of extracts or standard solution of catechin (6.25, 12.5, 25, 50, 100 μg/ml) in 80% ethanol was added to 10 μl of 10% (w/v) aluminium chloride solution, followed by the addition of 150 μl of 95% ethanol. Ten microliters of 1 M sodium acetate was added to each test sample in a 96-well plate. The absorbance was measured at 510 nm with a microplate reader (Biotek, San Jose, CA, USA). Total flavonoid content was expressed as mg catechin equivalents (CAE, in mg) per gram of dry material. All samples were analyzed in triplicates.

Determination of antioxidant activity. The 2,2-diphenyl-1-picrylhydrazyl (DPPH) free radical-scavenging activity assay was performed as described by Rostagno *et al*. (15). Briefly 20 μl of extract (1 mg/ml) in absolute ethanol was added to 180 μl of DPPH reagent in a 96-well plate. The absorbance was measured at 517 nm after 90 min, with a microplate reader (Biotek). Experiments were performed in triplicates. The results were expressed as percentage inhibition (I%), which was calculated using the following formula:

$$I\%=[(A_{DPPH}-A_{Sample})/A_{DPPH}]\times 100$$

where: A_{DPPH} is the absorbance of the control DPPH at time t=0' and
A_{Sample} is the absorbance of the sample after time t=90'.

High-performance liquid chromatography (HPLC) analysis of isoflavones. Isoflavone extracts were analyzed using a modular HPLC system (1100 Series; Agilent Technology, Palo Alto, CA, USA) equipped with a 250×2.0 mm, 4 μm particle diameter, Jupiter® C18 reversed-phase column (Phenomenex, Torrance, CA, USA) that was maintained at 37˚C in a thermostatic oven. Separations were carried out at a 0.2 ml/min constant flow rate, applying a 5-65% linear gradient of eluent B (acetonitrile/0.1% trifluoroacetic acid) in 60 min, following 5 min of isocratic elution at 5% B. Eluent A was 0.1% trifluoroacetic acid in HPLC-grade water. A diode array detector was used to record UV-Vis spectra every 2 s in the 190-650 nm range. The HPLC separations were monitored recording at the following wavelengths: λ=254, 280, 320 and 360 nm; and elaborated with the HPLC ChemStation software vers. A.07.01 (Agilent Technology) furnished with the chromatographer.

Cell culture. Three NSCLC cell lines with different *EGFR* mutation status and sensitivity to EGFR TKIs were used: HCC827 (del E746-A750), NCI-H1975 (T790M), and A549 (wild-type). Cells were obtained from the American Type Culture Collection (ATCC; Manassas, VA, USA) and maintained in ATCC-specified growth medium.

EGFR inhibitors. Erlotinib and gefitinib were purchased from Genentech (South San Francisco, CA, USA); AstraZeneca (London, UK) commercial suppliers, respectively. Afatinib was provided by Boehringher Ingelheim (Ingelheim Am Rhein, Rheinland-Pfalz, Germany), AZD9291 by AstraZeneca. Stock solutions of TKIs were prepared in dimethyl sulfoxide at a concentration of 10 mmol/l and maintained at −20˚C. Drugs were diluted to 1 mmol/l in 50% ethanol for a working solution.

Measure of cellular viability by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. The three NSCLC cell lines were seeded at a density of 3,000 cells/well in 96-well plates for 24 h. After 24 h, the cells were incubated with different concentrations of inhibitors (2.5 nM to 15 μM based on cell line sensitivity). The effects of each drug alone and the combination of drug and extracts for 48 h were assayed. Cell survival was determined by a standard MTT assay by the addition of 20 μl of

MTT (Promega CellTiter 96® AQueous One Solution Cell; Promega, Madison, WI, USA) for 2 h. The color intensity was measured by microplate reader (Cary 50 MPR; Varian) at 412 nm. Wells containing cells without any treatment were used as positive controls and the OD value was used to represent 100% cellular viability.

The half-maximal inhibitory concentration (IC_{50}) values were determined by ED50plus v1.0 online software, (Tlalpan, Mexico DF, Mexico). Results represent the median of three separate experiments each conducted in triplicate.

Crystal violet assay. Cells were seeded at 3,000 per well in 96-well plates. Twenty-four hours after seeding, cells were exposed to drugs with/without extracts as described above. Forty-eight hours after treatment, cells were fixed with formaldehyde and stained with 0.01% crystal violet for 15 min at room temperature. Stained cells were washed with phosphate-buffered saline. Viability was determined by measuring the absorbance at 595 nm (20).

Statistical analysis. ANOVA tests were used for statistical evaluation of results in three independent replications. Values of p lower than 0.05 were considered significant.

Results and Discussion

Isoflavone extracts. Isoflavones, a subclass of the flavonoids, are non-nutrient plant compounds, which occur predominantly as β-glucosides (genistin, daidzin, glycitin), or as acetyl-β-glucosides and malonyl-β-glucosides, and are therefore polar, water-soluble compounds (21, 22). Precursors of daidzein and genistein, and especially the 4'-methyl ethers formononetin and biochanin A, respectively, are mainly found in red clover (23).

Like soy, red clover contains the isoflavones genistein, daidzein, biochanin A, and formononetin; however, soy contains higher amounts of genistein and daidzein, while the dominant isoflavones in red clover are biochanin A and formononetin (24-27).

Solvent extraction is the most frequently used technique to obtain crude extracts from matrices of vegetable origin. The most common solvents employed are water, ethanol, acetone and acetic acid, used pure or in a mixture (16). It is known that the amount of extractable substances in the course of an extraction with solvents depends both on the peculiarities of the raw vegetable material and the solvent used (28). Clearly, for each specific plant matrix, there is a need to test and select the solvent and the most suitable conditions for maximizing the extraction yield and antioxidant activity of the extracts obtained (29-31). For soybean, extraction with acetone/acetic acid was more efficient, while for red clover, extraction with ethanol provided the best yield for the extract.

Total polyphenols, flavonoid content and antioxidant activities of the extracts from peas, fava beans, beans, soybean seeds and red clover were determined as reported in Table I. The content of phenolic compounds was found to range from 1.5 mg/g GAE for beans to 130 mg/g GAE for red clover, while total flavonoids ranged from 0.4 mg/g CAE for peas to 71 mg/g CAE for red clover.

Table I. *Total polyphenol and flavonoid content, and antioxidant activity of the extracts from peas, fava beans, beans, soybean seeds and red clover.*

Dried sample	Total phenols (mg GAE/g)	Total flavonoids (mg CAE/g)	Inhibition (%)*
Soybean	26.40	17.60	60
Fava bean	37.03	28.52	85
Pea	1.50	0.04	30
Bean	1.64	0.13	60
Red clover	130.00	71.00	88

GAE: Gallic acid equivalent; CAE: catechin acid equivalent. *By free radical-scavenging activity assay.

The radical-scavenging properties reported as % $DPPH^{\bullet}$ inhibition ranged from 30 to 88%, the highest being that for red clover extract. Thus, total phenolics, total flavonoids and antioxidant potential determined as radical-scavenging property were roughly correlated.

HPLC analysis of isoflavone extracts. The expected isoflavones both in the glycoside and aglycone forms were detected in the HPLC chromatogram of the ethanolic extracts of soybean flour (Figure 1A). Isoflavone components were assigned on the basis of the retention time (t_r) and UV spectral properties, also in comparison to those of the standards. Acetyl and malonyl derivatives were identified through the elution order, by comparison with literature data (32). Isoflavones were also monitored in the extracts from other legumes, including faba beans, peas and ordinary beans. Considering its well-established pseudo-estrogenic properties, red clover (Trifolium pratense) was monitored for the occurrence of isoflavones. The HPLC chromatogram of the ethanolic extracts from dried red clover is shown in Figure 1B. Along with minor peaks corresponding to the isoflavone components detected in soy, red clover exhibited dominant peaks at longer t_r. By comparison with literature data (33) and on the basis of the UV-Vis spectra, these components were identified as formonotenin (t_r 49.9 min; λ_{max} 260, 300 nm) and biochanin A (t_r 60.1 min; λ_{max} 248 nm). Analogously to soy, red clover isoflavones can occur also as acetyl- and malonyl-glucoside derivatives (34). These components are most likely the minor peaks with t_r in the 35-46 range, showing UV spectra characteristic of formonotenin and biochanin A. A number of additional metabolites structurally related to isoflavones have been described (33). However, a definitive characterization of these species would require the application of complementary analytical techniques. On the

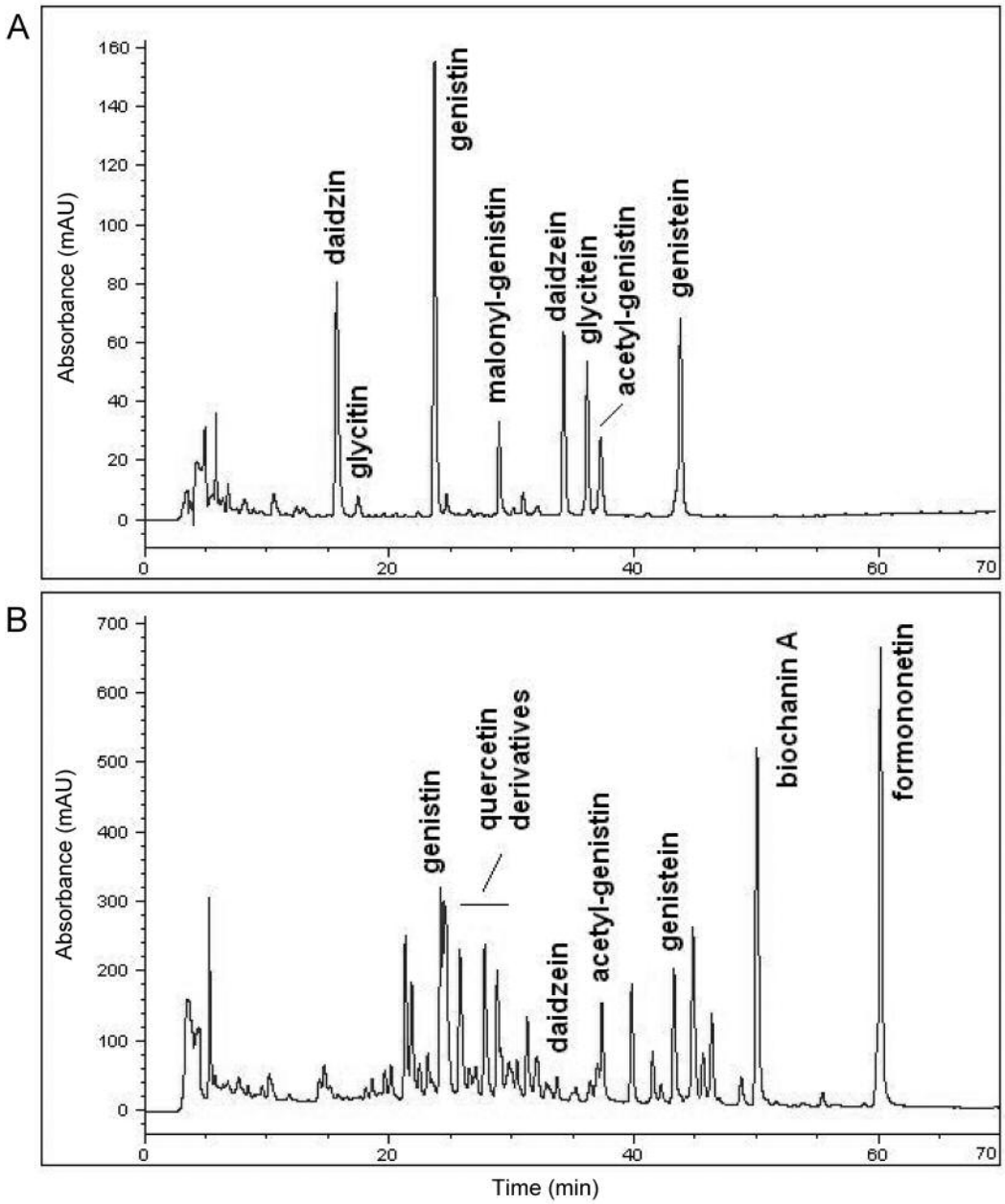

Figure 1. *Reversed-phase high-performance liquid chromatography chromatograms of isoflavones from soy (A) and red clover (B) acquired at λ=254 nm. The main isoflavone components were assigned by spectrophotometric analysis using a diode array detector and comparison with individual standards.*

other hand, the HPLC peaks at t_r 20-35 min were members of different metabolite classes and included flavonols such as quercetin glucoside and quercetin rutinoside (rutin) showing characteristic absorption bands with λ_{max}=355-360 nm.

Induction of growth inhibition by erlotinib, gefitinib, afatinib and AZD9291. The viability of A549, HCC827 and H1975 cell lines treated with gefitinib, erlotinib, afatinib and AZD9291 was determined by the MTT assay. Both erlotinib, gefitinib, afatinib and AZD9291 demonstrated growth inhibition of all three cell lines, but the sensitivity of the cell lines varied markedly. HCC827 cells with mutation in the EGFR gene, (del E746-A750) were highly sensitive to gefitinib and erlotinib at low nanomolar concentrations. A549 cells with wild-type *EGFR* were sensitive only at higher concentrations of EGFR TKIs (>9 μM), while H1975 cells

were sensitive only at maximum concentrations (>20 μM). Likewise for the irreversible inhibitors afatinib and AZD9291, we observed that HCC827 cells were the most responsive of the three cell lines, and H1975 was more responsive to AZD9291 than to afatinib (IC_{50}=4.2 μM *vs*. 12 μM, respectively). After 48 h of treatment, growth of cells was significantly inhibited in a dose-dependent manner and for each inhibitor, an IC_{50} value was calculated from the dose–response curves of the NSCLC cell lines. In Table II, IC_{50} values for TKI against the three cell lines are reported.

Soy and red clover isoflavones alone did not exhibit significant growth inhibition. Neither soy nor red clover extracts had any significant inhibitory effect against all three cell lines, although a trend indicating a reduction of vitality was found at increasing doses ($p>0.05$). An indicative, albeit weak, inhibitory effect was observed for HCC827 cells at the highest concentration, not significant in any case ($p>0.05$ data not shown).

Effects of gefitinib alone and in combination with extracts on A549, HCC827 and H1975 cell proliferation. To evaluate the effect of GEFITINIB alone and in combination with extracts on the cell viability of A549, HCC827 and H1975 cells *in vitro*, crystal violet assays were performed for 48 h. Concentrations above 200 μg/ml of isoflavones, resulted in a marked reduction in cell viability when used in combination with gefitinib.

It was found that the inhibitory rates with gefitinib alone, and in combination with extracts, were higher compared with the control group ($p<0.01$). There was no significance difference in vitality between cells treated with the extracts alone and control groups ($p>0.05$). However, the inhibitory rate in the group treated with gefitinib in combination with soy or red clover extract was higher compared with the agent alone ($p<0.05$; Table III). The induction of growth inhibition by gefitinib in combination with red clover extract was higher than that observed with soy extract, although not significantly.

The IC_{50} value (230 nM) calculated for gefitinib alone was reduced (135 nM) in the presence of soy extract and in combination with red clover extract (100 nM) for the HCC827 cell line.

The combination of isoflavone extracts and gefitinib showed the highest levels of cytotoxicity, with inhibitory concentration IC_{50} values at 13.47 μM and 12.9 μM for the A549 cell line, respectively, with soy and red clover extracts, in corresponding order, as compared with drug alone: IC_{50} value of 18.6 μM.

Ultimately, even for the H1975 cell line carrying the mutation which confers drug resistance, the IC_{50} value calculated for the drug alone 48.5 μM was reduced to 42 μM and 31 μM when the drug was given in combination with soy and red clover extract, respectively.

Table II. *Half-maximal inhibitory concentration (IC_{50}) values for tyrosine kinase inhibitors against three cell lines*.

	Median IC_{50}±SD			
Cell line	Gefitinib	Erlotinib	Afatinib	AZD9291
HCC827	230.0±1.9 nM	70.0±2.2 nM	132.0±0.4 nM	512.0±1.5 nM
A549	18.6±1.4 μM	9.2±0.1 μM	11.0±0.4 μM	8.4±0.2 μM
H1975	48.5±2.8 μM	24.8±1.1 μM	12.0±0.2 μM	4.2±0.7 μM

SD: Standard deviation.

Effects of erlotinib alone and in combination with extracts on NSCLC cell lines. In accordance with the results obtained with gefitinib, HCC827 cells were highly sensitive to erlotinib (IC_{50}=70 nM), whereas A549 cells were on average sensitive (IC_{50}=9.2 μM) and H1975 cells were scarcely responsive (IC_{50}=24.8 μM). Due to the presence of the T790M mutation, which confers resistance to treatment with TKI inhibitors, H1975 required a higher concentration of inhibitor compared to the HCC827 and A549 cell lines.

We observed a reduction of the IC_{50} values for all three cell lines when erlotinib was used in combined treatments with isoflavones of soy or red clover. Table III reports all the IC_{50} values.

Effects of afatinib alone or in combination with extracts on cellular viability. Dose–response curves for afatinib and extracts were generated for the sensitive EGFR mutant and wild-type NSCLC cell lines.

The IC_{50} values for afatinib combined with soy or red clover extracts were calculated. HCC827 cells were more responsive to afatinib compared with A549 cell line, while H1975 was the least responsive. For all the cell lines investigated, we observed an enhanced inhibitory effect when TKIs were used in combination with soy or red clover extracts (Table IV).

Inhibition by AZD9291 irreversible TKI alone and in the presence of isoflavones. Treatment with AZD9291, a third-generation irreversible EGFR TKI, as single agent for 48 h resulted in a dose-dependent inhibition of growth for HCC827, A549 and H1975 cell lines. The IC_{50} values for AZD9291 alone and in combination with soy or red clover extracts were compared for the three cell lines (Table IV). Soy and red clover extracts markedly synergized AZD9291-induced cell death, both in HCC827 and H1975, as well as in NSCLC cells with wild-type *EGFR* (A549).

Conclusion

Soy and red clover extracts enriched in isoflavones enhanced cell growth inhibition induced by erlotinib, gefitinib, afatinib, and AZD9291. Most likely related to its high

Table III. *Half-maximal inhibitory concentration (IC_{50}) values for gefitinib and erlotinib, alone and with soy or red clover extract, against three cell lines.*

	Median IC_{50}±SD					
	Gefitinib			Erlotinib		
Cell line	Alone	+ Soy extract	+ Red clover extract	Alone	+ Soy extract	+ Red clover extract
HCC827	230.0±1.9 nM	135.0±6.8 nM	100.0±5.1 nM	70.0±2.2 nM	38.0±3.4 nM	36.0±2.3 nM
A549	18.6±1.4 μM	13.5±0.7 μM	12.9±0.2 μM	9.2±0.1 μM	5.8±0.4 μM	5.3±0.4 μM
H1975	48.5±2.8 μM	42.0±2.0 μM	31.0±2.0 μM	24.8±1.1 μM	21.7±0.3 μM	20.2±0.9 μM

SD: Standard deviation.

Table IV. *Half-maximal inhibitory concentration (IC_{50}) values for afatinib and AZD9291, alone and combined with soy or red clover extract, against three cell lines.*

	Median IC_{50}±SD					
	Afatinib			AZD9291		
Cell line	Alone	+ Soy extract	+ Red clover extract	Alone	+ Soy extract	+ Red clover extract
HCC827	132.0±0.4 nM	87.0±0.8 nM	70.0±2.1 nM	512.0±1.5 nM	445.5±8.7 nM	400.0±3.7 nM
A549	11.0±0.4 μM	9.6±0.4 μM	6.9±0.6 μM	8.4± 0.2 μM	7.1±0.8 μM	5.2±0.3 μM
H1975	12.0±0.2 μM	10.6±0.7 μM	9.8±0.1 μM	4.2±0.7 μM	3.9±0.1 μM	3.5±0.5 μM

SD: Standard deviation.

isoflavone content, red clover extract was the most effective. Soy and red clover isoflavones in combination with TKI could represent a potential treatment to contribute to arresting tumor growth or to reducing the doses of the chemotherapeutics in the treatment of NSCLC.

As far as we are aware, this is the first report on the inhibitory effect of isoflavone extracts and AZD9291 on NSCLC cell growth.

Further studies are required to elucidate the mechanisms and to confirm the anticancer synergistic activity of TKI-isoflavones *in vivo*.

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