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Synthesis and evaluation of novel isoxazolyl chalcones as potential anticancer agents

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ABSTRACT

A series of novel isoxazolyl chalcones were synthesized and evaluated for their activities *in vitro* against four types of human non-small cell lung cancer cells, including H1792, H157, A549 and Calu-1 cells. The preliminary biological screening showed that compounds **5d** and **5f–i** exhibited significant cytotoxicity, particularly, compounds **5f** and **5h** were identified as the most potent anticancer agents with IC_{50} values 1.35–2.07 μM and 7.27–11.07 μM against H175, A549 and Calu-1 cell lines, respectively. Compounds **5f–i** could induce apoptosis in A549 cells by death receptor 5 (DR5) mediated extrinsic pathways. The preliminary structure–activity relationship study showed that compounds bearing electron withdrawing groups (EWG) at the 2-position of the phenyl ring in Ar group were more effective than those with EWG at 4-position. These results further demonstrated that the scaffolds designed in this work might lead to the discovery of novel anti-lung cancer agents.

1. Introduction

Lung cancer is a deadly disease in the world today and causes more deaths than any other type of cancer [1]. Despite great advances in developing anticancer drugs in the past decades, the discovery of novel anti-lung cancer agents with new mechanism of action, high selectivity and excellent therapeutic efficacy is still a major work to chemists.

Isoxazole derivatives as a special class of compounds exhibited many interesting biological activities, for example, antituberculosis activity [2], antioxidant activity [3], antidepressant [4], antifungal activity [5], herbicidal activity [6], insecticidal activity [7,8], DNA-intercalating agent [9] and anticancer activities [10–13]. Chalcones, which bear a three-carbon α, β-unsaturated carbonyl system between two aromatic rings, also showed a broad spectrum of biological activities including anti-inflammatory [14], antimalarial [15], anti-invasive [16], antibacterial [17], and anticancer [18–20]. In addition, Chalcones are capable of inducing apoptosis [21,22]. As a result, these compounds are regarded as promising anticancer agents (Fig. 1) [23–26]. Owing to interaction with the amino groups of nucleic acids, a number of clinically useful anticancer drugs have genotoxic effects. However, chalcones have not been found to show such undesired side effects [27]. In order to improving chalcone compound bioavailability, different substituents chalcones on aryl or heteroaryl rings have been synthesized [28].

In the design of new drugs, the combination of different pharmacological monomers may lead to the finding of novel candidates with interesting biological activity [29,30]. In this study, we aimed to design and synthesize novel compounds with both isoxazole and chalcone entities in one molecule and investigate their cytotoxicity to human non-small cell lung cancer cells.

2. Results and discussion

2.1. Chemistry

The synthesis of isoxazolyl chalcones **5a–i** has been achieved by the reaction between 1-(5-methyl-3-(4-nitrophenyl)isoxazol-4-yl)ethanone **4** and aromatic aldehydes in the presence of ethanol and sodium hydroxide at room temperature for 3–6 h as shown in Scheme 1. The intermediate **4** was prepared by 1,3-dipolar cycloaddition reaction at room temperature in dichloromethane.

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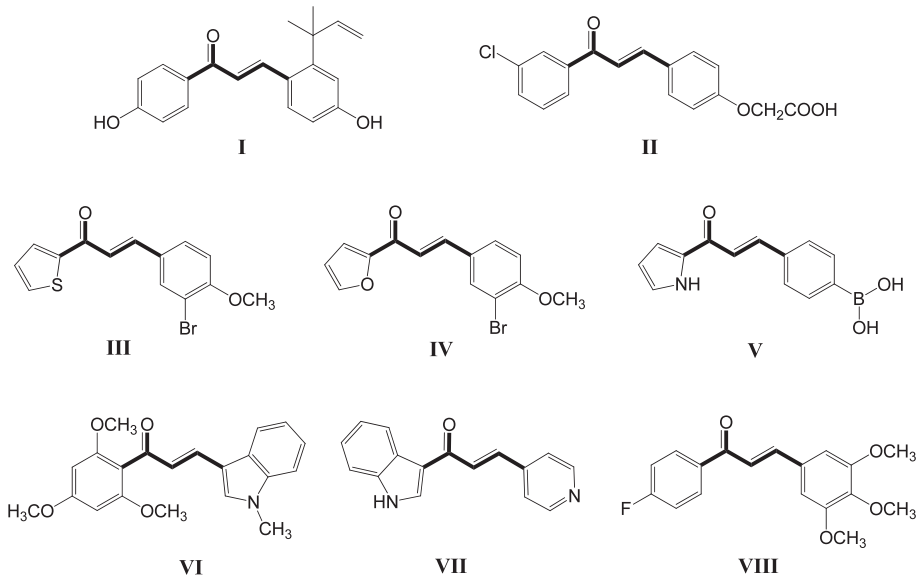
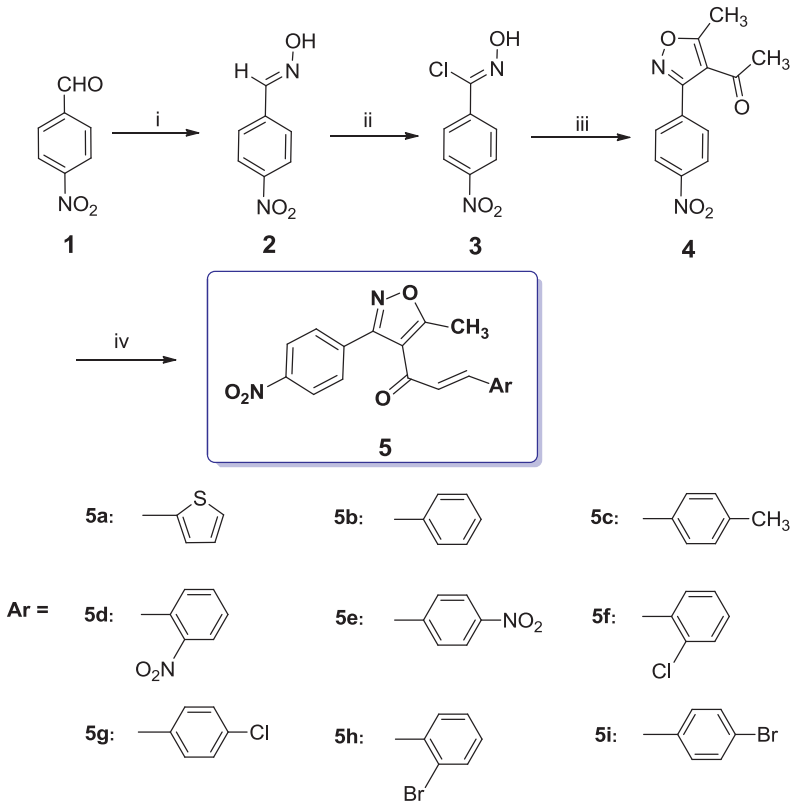


Fig. 1. Examples of anticancer agents based on chalcones, already reported in literature.



Scheme 1. Synthesis of compounds **5a–i**. (i) $NH_2OH.HCl$, Na_2CO_3, EtOH, reflux, 6 h, 93%; (ii) DMF, NCS, 0 °C, then rt, 12 h; (iii) CH_2Cl_2, Et_3N, 30 min; acetylacetone, rt, 12 h, 80%; (iv) aromatic aldehydes, EtOH, NaOH (aq), rt, 3–6 h, 49–92%.

The crucial intermediate **3** was prepared from 4-nitrobenzaldehyde oxime **2** in the presence of N-chlorosuccinimide (NCS).

The structures of compounds **5a–i** were made on the basis of their spectral data and elemental analysis. The IR spectrum of compound **5f** showed the absorption band at 1666 cm^{-1} assigned to C=O stretching mode. The presence of N=C had been identified by the characteristic vibration at 1603 cm^{-1}. The presence of NO_2 and C—Cl had been identified at 1523, 1352 and 753 cm^{-1}, respectively. The ^1H NMR spectra (400 MHz) of compound **5f** indicated four aromatic proton signals in *p*-NO_2 phenyl moiety appeared at the range of $\delta = 8.32$ and $\delta = 8.07$ ppm as doublet peaks ($J = 8.44$ Hz. Four aromatic proton signals in *o*-Cl phenyl moiety appeared at the range of $\delta = 7.20–7.84$ ppm as multiplet peaks ($J = 8.44$ Hz). Two proton signals in double bond appeared at $\delta = 7.87$ and $\delta = 7.09$ ppm as doublet peaks ($J = 16.00$ Hz). Three protons of methyl moiety appeared at the range of $\delta = 2.75$ ppm as single peak. The ^{13}C NMR spectrum of compound **5f** showed characteristic signals at 13.13 (CH_3), 116.43, 123.34, 124.53,

127.96, 128.18, 131.57, 134.20 (C—Cl), 139.07, 148.37, 160.18 (C=N), 174.30 and 184.74 (C=O) accounting for the presence of different kinds of carbon atoms in the molecule.

2.2. Effects of compounds **5a–i** on the Viability of Human Non-small Cell Lung Cancer Cells

The synthesized compounds were evaluated for their *in vitro* anticancer activity using Sulforhodamine B (SRB) assays. The growth-inhibitory experiments were undertaken in four human non-small cell lung cancer cell lines, H1792, H157, A549 and Calu-1 cells. The obtained data showed most compounds had inhibitory effects on the growth of four cells in a dosage dependent manner. Compounds **5a**, **5b**, **5c** and **5e** could mild inhibit the cell growth at 40 μM after 48 h of the treatment. At 80 μM concentration, all compounds showed significantly inhibitory effects (Fig. 2). Exposure of H1792, H157, A549 and Calu-1 cells to compounds **5d**, **5f**, **5g** and **5h** at 20 μM for 48 h resulted in cell viabilities decreasing from 100% to 79.9–51.5%, 58.1–20.5%, 60.9–20.9% and 20.5–9.0%. Exposure of H1792, H157, A549 and Calu-1 cells to compounds **5d**, **5f**, **5g** and **5h** at 40 μM for 48 h, the cell viabilities reduced more significantly from 100% to 45.2–11.9%, 27.9–7.4%, 32.5–14.1% and 11.7–1.4%, respectively. When the concentration of compounds **5f**, **5g** and **5h** increased to 80 μM, the cell viabilities of H1792, H157, A549 and Calu-1 cells reduced to 28.4–7.9%, 5.9–5.2%, 10.3–3.9% and 5.3–0.5%.

In addition, we observed that the structure of the Ar group in compounds **5a–i** played an important role in their anticancer activity. When Ar was 4-nitrophenyl, 4-chlorophenyl and 4-bromophenyl, the growth inhibitory property of these compounds was inferior to that of compounds with 2-nitrophenyl, 2-chlorophenyl and 2-bromophenyl, respectively. When Ar was 2-thienyl, phenyl and 4-methylphenyl, the toxicity of these compounds was poor. These results indicated that compounds bearing EWG at the 2-position of the phenyl ring were more effective. Growth inhibitory properties (IC_{50}) for compounds **5a–i** are listed in Table 1. IC_{50} values were determined from log plots of percent of control vs concentration.

2.3. Compounds **5d** and **5f–i** induce apoptosis in A549 cells by cell cycle analysis

According to the result of SRB assay, we found that **5d**, **5f**, **5g**, **5h** and **5i** were the effective compounds against cancer cells, so we investigated the five compounds thoroughly. By flow cytometric analysis of cell cycle, we found that **5f**, **5g**, **5h** and **5i** increased the percentage of sub-G_0 phase and decreased the percentage of G_0/G_1 phase, while **5d** increased the percentage of sub-G_0 phase weakly in A549 cells. These results suggested that **5f**, **5g**, **5h** and **5i** induced apoptosis in A549 cells. In addition, we found that **5f**, **5g** and **5h** induced apoptosis in a dose dependent manner (Fig. 3).

2.4. Compounds **5f–i** induce apoptosis in A549 cells

Then we determined whether **5f–i** could induce apoptosis in A549 cells by western blot. A549 cells were treated with concentrations (0, 20 and 40 μM) of **5f–i** respectively for 48 h, then the cells were harvested and prepared for western blot analysis. We found that **5f–i** could induce the up-regulation of DR5 in a dose-dependent manner (Fig. 4), suggesting that **5f–i** activated the extrinsic pathways of apoptosis. Next, we found that **5f–i** induced the cleaved bands of caspase 8, caspase 9, caspase 3 and PARP, indicating that **5f–i** induced apoptosis.

3. Conclusion

In summary, we designed and synthesized a series of isoxazolyl chalcones **5a–i**. Most of the compounds showed significantly cytotoxic effect on human non-small cell lung cancer cells. Preliminary structure–activity relationship study showed that compounds **5d**,

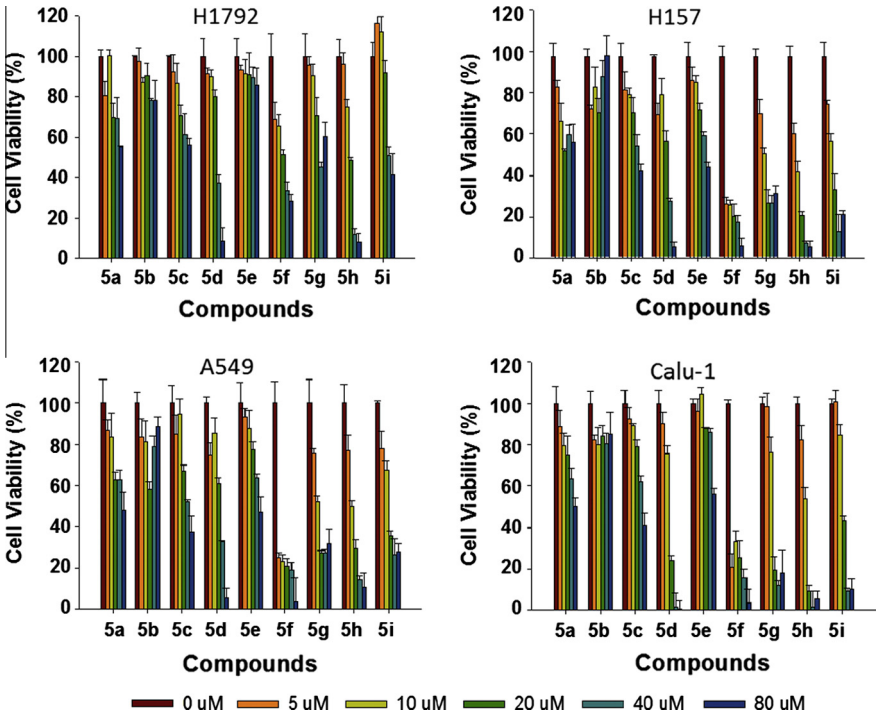


Fig. 2. Cytotoxic effect of nine synthesized isoxazolyl chalcones on human non-small cell lung cancer cells. Cancer cells were treated with various concentrations (0, 5, 10, 20, 40, 80 μM) of compounds **5a–i** for 48 h. A viability assay was carried out. Experiments were performed in triplicate; data are expressed as means of the triplicate determinations of a representative experiment in% cell viability of untreated cells (100%).

Table 1
Growth inhibitory properties IC_{50} (μM) for compounds **5a–i** at 48 h.

Compound	Lung cancer cell lines			
	H1792	H157	A549	Calu-1
5a	ND[b]	90.22	70.14	83.57
5b	Inactive[a]	ND[b]	Inactive[a]	Inactive[a]
5c	80.55	60.38	50.67	62.26
5d	28.20	17.13	19.32	12.14
5e	Inactive[a]	68.05	72.33	ND[b]
5f	19.30	1.35	1.48	2.07
5g	63.38	ND[b]	12.87	20.40
5h	19.63	7.27	11.07	8.98
5i	ND[b]	12.77	17.71	ND[b]

IC_{50}: concentration of compound that inhibits cell proliferation by 50%.
[a] Inactive: IC_{50} > 100 μM.
[b] ND: not determined.

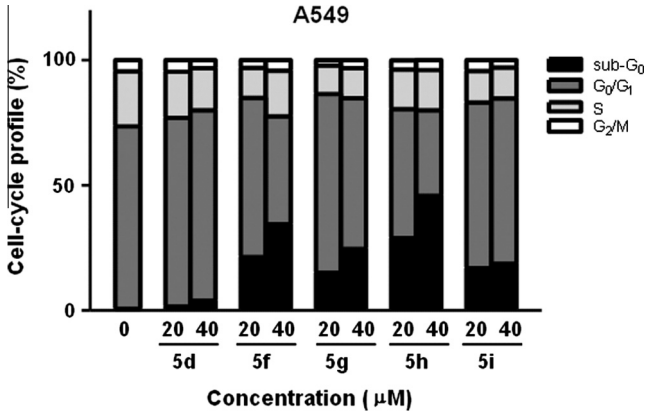


Fig. 3. A549 cell was treated with concentrations (0, 20 and 40 μM) of **5d** and **5f–i** respectively for 48 h, then the cells were harvested and stained with propidium iodide for detection of cell cycle by flow cytometry.

5f and **5h** bearing EWG at the 2-position of the phenyl ring in Ar group were more effective than compounds **5e**, **5g** and **5i** with EWG at 4-position, respecitively. In A549 cells, compounds **5f–i** could induce apoptosis by death receptor 5 (DR5) mediated extrinsic pathways. These results suggested isoxazolyl chalcones bearing EWG at the 2-position of the phenyl ring could be used as lead compounds to develop novel potent anti-lung cancer agents.

4. Experimental section

Melting points were determined in open glass capillaries in anelectrical melting point apparatus which was uncorrected. The infrared (IR) spectra were recorded on Perkin–Elmer IR Spectrophotometer using the KBr pellet technique. ^{1}H NMR spectra was recorded in DMSO-d_6 on a Bruker NMR spectrometer at 400 MHz using tetramethylsilane (TMS) as internal standard. Chemical shifts are expressed in δ, ppm. ^{13}C NMR spectra were recorded in a Bruker Avance 400 (100 MHz, DMSO-d_6 as solvent). The reactions were monitored by thin layer chromatography (TLC) on silica gel plates using a mixture of petroleum ether and ethyl acetate. Chemicals were obtained from commercial supplies and used without purification. DR5 antibody was purchased from ProSci (Poway, CA). Caspase 3 antibody was purchased from Imgenex (San Diego, CA). Caspase 8, caspase 9 and PARP antibodies were purchased from Cell Signaling Technology (Danvers, MA).

4.1. Synthesis

4.1.1. Synthesis of 4-nitrobenzaldehyde oxime (**2**)

4-Nitrobenzaldehyde (0.2 mol, 30.2 g) and hydroxylamine hydrochloride (0.2 mol, 13.9 g) were added to an ethanolic solution (250 mL) of anhydrous sodium carbonate (0.1 mol, 10.6 g) and the reaction mixture was heated and refluxed for 6 h, and the reaction was completed (TLC monitoring known). The ethanol was evaporated, water was added and the mixture was extracted with dichloromethane. The organic layer was dried over sodium sulfate and after evaporation of the solvent the 4-nitrobenzaldehyde oxime was obtained as a yellow solid (30.9 g, 93%), M.p. 122–124 °C and it was used without further purification.

4.1.2. Synthesis of 1-(5-methyl-3-(4-nitrophenyl)isoxazol-4-yl)ethanone (**4**)

1-Chloropyrrolidine-2,5-dione (NCS) (0.15 mol, 20 g) was added in the solution of nitrobenzaldehyde oxime (0.15 mol, 25 g) and 100 mL N,N-dimethyl formamide (DMF) in ice bath. After the addition the reaction mixture was stirred for 12 h at room temperature

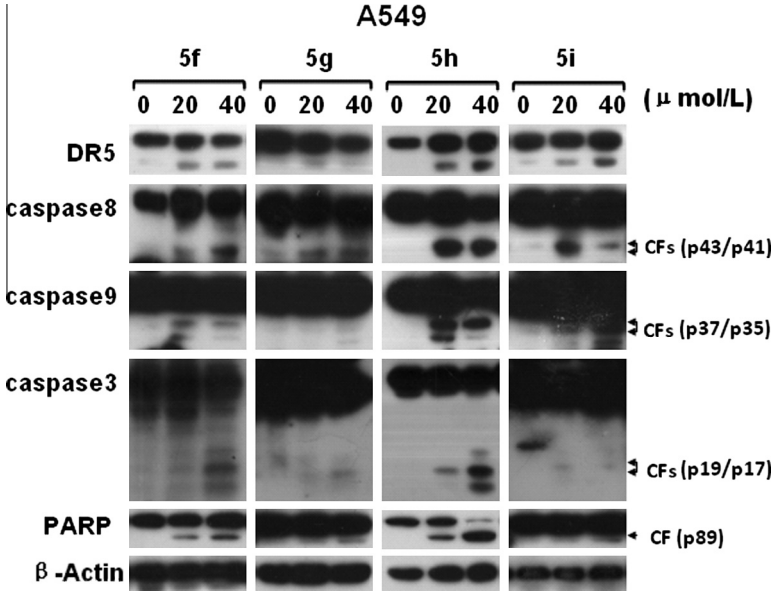


Fig. 4. A549 cell was treated with concentrations (0, 20 and 40 μM) of **5f–i** respectively for 48 h, then the cells were harvested and prepared for western blot analysis. β-Actin expression were used as loading controls. CFs, cleaved forms.

and the reaction was completed (TLC monitoring known). After that saturated NaCl solution (150 mL) and ethyl acetate (150 mL) was added and stirred. The organic phase was separated and washed with water (150 mL). The organic phase was dried over sodium sulfate and concentrated under reduced pressure. Dichloromethane (150 mL) and triethylamine (0.24 mol, 24 g) were added to the residues and stirred at room temperature for 30 min. Then acetyl acetone (0.2 mol, 20 g) was added and stirred for 12 h. Water was added, the organic phase was separated, dried with sodium sulfate and concentrated under reduced pressure to give a yellow powder (29.5 g, 80%), M.p. 134–136 °C. IR (KBr, cm^{-1}) ν: 3443, 1677, 1609, 1529, 1361; ^1H NMR (400 MHz, DMSO-d_6, δ): 2.34 (s, 3H, CH_3), 2.79 (s, 3H, $COCH_3$), 7.84 (d, 2H, PhH, J = 8.60 Hz), 8.33 (d, 2H, PhH, J = 8.60 Hz); ^{13}C NMR: (100 MHz, DMSO-d_6): 13.63 (CH_3), 30.46 (CH_3), 116.72, 123.33, 130.72, 135.16, 148.28, 160.50 (C═N), 174.71, 191.82 (C═O).

4.1.3. General procedure for the synthesis of compounds ***5a–i***

5-Methyl-3-(4-nitrophenyl)-4-acetyl-iso-oxazole (8 mmol, 2 g) and aromatic aldehydes (8 mmol) were added to the solution of ethanol (150 mL) and 10% aq NaOH (3 mL). The reaction mixture was stirred for 3–6 h at room temperature and the reaction was completed (TLC monitoring known). The precipitate was filtered and washed with water to obtain the compounds **5a–i**. The pure products were crystallizeed in ethanol.

*4.1.3.1. 1-(5-Methyl-3-(4-nitrophenyl)isoxazol-4-yl)-3-(thiophen-2-yl)prop-2-en-1-one (**5a**).* Yield: 65%; M.p. 163–164 °C; Yellow solid; IR (KBr, cm^{-1}) ν: 3442, 1659, 1591, 1524, 1353; ^1H NMR (400 MHz, DMSO-d_6, δ): 2.69 (s, 3H, CH_3), 6.58 (d, 1H, COCH═, J = 15.60 Hz), 7.14–7.81 (m, 3H, Thiophene ring), 7.74 (d, 1H, ═CH, J = 15.60 Hz), 7.86 (d, 2H, PhH, J = 8.40 Hz), 8.34 (d, 2H, PhH, J = 8.40 Hz); ^{13}C NMR: (100 MHz, DMSO-d_6): 12.93 (CH_3), 116.35, 123.64, 126.81, 128.82, 130.50, 131.08, 134.72, 137.63, 139.00, 148.36, 159.99 (C═N), 173.71, 184.43 (C═O); Anal. Calcd for $C_{17}H_{12}N_2O_4S$: C, 59.99; H, 3.55; N, 8.23. Found: C, 59.93; H, 3.72; N, 8.15.

*4.1.3.2. 1-(5-Methyl-3-(4-nitrophenyl)isoxazol-4-yl)-3-phenylprop-2-en-1-one (**5b**).* Yield: 81%; M.p. 180–183 °C; White solid; IR (KBr, cm^{-1}) ν: 3422, 1657, 1592, 1524, 1350; ^1H NMR (400 MHz, DMSO-d_6, δ): 2.70 (s, 3H, CH_3), 7.03 (d, 1H, COCH═, J = 16.00 Hz), 7.28–7.63 (m, 5H, PhH), 7.61 (d, 1H, ═CH, J = 16.00 Hz), 7.84 (d, 2H, PhH, J = 8.40 Hz), 8.46 (d, 2H, PhH, J = 8.40 Hz); ^{13}C NMR: (100 MHz, DMSO-d_6): 12.99 (CH_3), 116.45, 123.51, 125.67, 128.75, 130.50, 131.18, 130.61, 130.61, 133.97, 134.66, 145.14, 148.32, 160.15 (C═N), 173.58, 185.46 (C═O); Anal. Calcd for $C_{19}H_{14}N_2O_4$: C, 68.26; H, 4.22; N, 8.38. Found: C, 68.09; H, 4.74; N, 8.52.

*4.1.3.3. 1-(5-Methyl-3-(4-nitrophenyl)isoxazol-4-yl)-3-p-tolylprop-2-en-1-one (**5c**).* Yield: 49%; M.p. 203–206 °C; White solid; IR (KBr, cm^{-1}) ν: 3422, 1657, 1592, 1524, 1350; ^1H NMR (400 MHz, DMSO-d_6, δ): 2.32 (s, 3H, CH_3), 2.69 (s, 3H, CH_3), 6.98 (d, 1H, COCH═, J = 16.00 Hz), 7.21 (d, 2H, PhH, J = 7.60 Hz), 7.50 (d, 2H, PhH, J = 7.60 Hz), 7.63 (d, 1H, ═CH, J = 16.00 Hz), 7.85 (d, 2H, PhH, J = 8.40 Hz), 8.32 (d, 2H, PhH, J = 8.40 Hz); ^{13}C NMR: (100 MHz, DMSO-d_6): 12.95 (CH_3), 21.02 (CH_3), 116.48, 123.52, 124.68, 128.68, 129.67, 131.25, 134.69, 141.24, 145.27, 148.31, 160.11 (C═N), 173.43, 185.42 (C═O); Anal. Calcd for $C_{20}H_{16}N_2O_4$: C, 68.96; H, 4.63; N, 8.04. Found: C, 68.34; H, 4.96; N, 7.89.

*4.1.3.4. 1-(5-methyl-3-(4-nitrophenyl)isoxazol-4-yl)-3-(2-nitrophenyl)prop-2-en-1-one (**5d**).* Yield: 77%; M.p. 188–190 °C; Gray solid; IR (KBr, cm^{-1}) ν: 3442, 1660, 1605, 1521, 1350; ^1H NMR (400 MHz, DMSO-d_6, δ): 2.76 (s, 3H, CH_3), 7.02 (d, 1H, COCH═, J = 16.00 Hz), 7.68–7.90 (m, 4H, PhH), 7.88 (d, 1H, ═CH, J = 16.00 Hz), 8.06 (d, 2H, PhH, J = 8.40 Hz), 8.35 (d, 2H, PhH, J = 8.40 Hz); ^{13}C NMR: (100 MHz, DMSO-d_6): 13.04 (CH_3), 116.24, 122.86, 123.69, 124.79, 128.61, 129.56, 130.31, 131.59, 134.48, 139.57, 148.45 (C—N), 160.16 (C═N), 174.18, 185.11 (C═O); Anal. Calcd for $C_{19}H_{13}N_2O_6$: C, 60.16; H, 3.45; N, 11.08. Found: C, 60.52; H, 3.63; N, 10.93.

*4.1.3.5. 1-(5-Methyl-3-(4-nitrophenyl)isoxazol-4-yl)-3-(4-nitrophenyl)prop-2-en-1-one (**5e**).* Yield: 79%; M.p. 181–182 °C; Gray solid; IR (KBr, cm^{-1}) ν: 3443, 1677, 1608, 1528, 1350; ^1H NMR (400 MHz, DMSO-d_6, δ): 2.77 (s, 3H, CH_3), 7.08 (d, 1H, COCH═, J = 16.00 Hz), 8.06 (d, 2H, PhH, J = 8.44 Hz), 7.72 (d, 2H, PhH, J = 8.44 Hz), 7.90 (d, 1H, ═CH, J = 16.00 Hz), 8.07 (d, 2H, PhH, J = 8.40 Hz), 8.36 (d, 2H, PhH, J = 8.40 Hz); ^{13}C NMR: (100 MHz, DMSO-d_6): 13.58 (CH_3), 116.69, 122.82, 123.32, 126.87, 129.19, 130.25, 134.83, 140.34, 148.24 (C—N), 151.56, 160.51 (C═N), 174.26, 185.32 (C═O); Anal. Calcd for $C_{19}H_{13}N_2O_6$: C, 60.16; H, 3.45; N, 11.08. Found: C, 60.74; H, 3.42; N, 11.21.

*4.1.3.6. 3-(2-Chlorophenyl)-1-(5-methyl-3-(4-nitrophenyl)isoxazol-4-yl)prop-2-en-1-one (**5f**).* Yield: 66%; M.p. 162–164 °C; White solid; IR (KBr, cm^{-1}) ν: 3443, 1666, 1603, 1523, 1352, 753; ^1H NMR (400 MHz, DMSO-d_6, δ): 2.75 (s, 3H, CH_3), 7.09 (d, 1H, COCH═, J = 16.00 Hz), 7.20–7.84 (m, 4H, PhH), 7.87 (d, 1H, ═CH, J = 16.00 Hz), 8.07 (d, 2H, PhH, J = 8.44 Hz), 8.32 (d, 2H, PhH, J = 8.44 Hz); ^{13}C NMR: (100 MHz, DMSO-d_6): 13.13 (CH_3), 116.43, 123.34, 124.53, 127.96, 128.18, 131.57, 134.20 (C—Cl), 139.07, 148.37, 160.18 (C═N), 174.30, 184.74 (C═O); Anal. Calcd for $C_{19}H_{13}ClN_2O_4$: C, 61.88; H, 3.55; N, 7.60. Found: C, 61.34; H, 3.81; N, 8.08.

*4.1.3.7. 3-(4-Chlorophenyl)-1-(5-methyl-3-(4-nitrophenyl)isoxazol-4-yl)prop-2-en-1-one (**5g**).* Yield: 87%; M.p. 217–220 °C; White solid; IR (KBr, cm^{-1}) ν: 3426, 1658, 1600, 1529, 1352, 784; ^1H NMR (400 MHz, DMSO-d_6, δ): 2.71 (s, 3H, CH_3), 7.06 (d, 1H, COCH═, J = 16.00 Hz), 7.48 (d, 2H, PhH, J = 8.44 Hz), 7.61 (d, 2H, PhH, J = 8.44 Hz), 7.67 (d, 1H, ═CH, J = 16.00 Hz), 7.86 (d, 2H, PhH, J = 8.40 Hz), 8.33(d, 2H, PhH, J = 8.40 Hz); ^{13}C NMR: (100 MHz, DMSO-d_6): 13.02 (CH_3), 116.40, 123.67, 124.64, 126.31, 128.60, 129.61, 130.44, 134.59, 135.51 (C—Cl), 149.40, 160.18 (C═N), 174.50, 185.40 (C═O); Anal. Calcd for $C_{19}H_{13}ClN_2O_4$: C, 61.88; H, 3.55; N, 7.60. Found: C, 61.29; H, 3.67; N, 8.34.

*4.1.3.8. 3-(2-Bromophenyl)-1-(5-methyl-3-(4-nitrophenyl)isoxazol-4-yl)prop-2-en-1-one (**5h**).* Yield: 56%; M.p. 135–137 °C; White solid; IR (KBr, cm^{-1}) ν: 3416, 1666, 1602, 1523, 1353, 760; ^1H NMR (400 MHz, DMSO-d_6, δ): 2.49 (s, 3H, CH_3), 6.70 (d, 1H, COCH═, J = 16.00 Hz), 6.89–7.87 (m, 4H, PhH), 7.69 (d, 1H, ═CH, J = 16.00 Hz), 8.06 (d, 2H, PhH, J = 8.44 Hz), 8.32 (d, 2H, PhH, J = 8.44 Hz); ^{13}C NMR: (100 MHz, DMSO-d_6): 13.41 (CH_3), 116.20, 123.29 (C-Br), 124.59, 127.99, 128.27, 132.74, 134.53, 142.79, 148.09, 160.18 (C═N), 174.25, 184.73 (C═O); Anal. Calcd for $C_{19}H_{13}BrN_2O_4$: C, 55.23; H, 3.17; N, 6.78. Found: C, 55.31; H, 3.40; N, 6.72.

*4.1.3.9. 3-(4-Bromophenyl)-1-(5-methyl-3-(4-nitrophenyl)isoxazol-4-yl)prop-2-en-1-one (**5i**).* Yield: 92%; M.p. 198–200 °C; White solid; IR (KBr, cm^{-1}) ν: 3443, 1660, 1600, 1529, 1353, 779; ^1H NMR (400 MHz, DMSO-d_6, δ): 2.71 (s, 3H, CH_3), 7.08 (d, 1H, COCH═, J = 16.00 Hz), 7.60 (d, 2H, PhH, J = 8.40 Hz), 7.62 (d, 2H, PhH, J = 8.40 Hz), 7.67 (d, 1H, ═CH, J = 16.00 Hz), 7.87 (d, 2H, PhH, J = 8.40 Hz), 8.33(d, 2H, PhH, J = 8.40 Hz); ^{13}C NMR: (100 MHz, DMSO-d_6): 13.63 (CH_3), 116.40, 123.67 (C—Br), 124.63, 126.34, 128.44, 130.45, 132.27, 134.74, 143.75, 148.32, 160.16 (C═N),

173.66, 185.39 (C=O); Anal. Calcd for $C_{19}H_{13}BrN_2O_4$: C, 55.23; H, 3.17; N, 6.78. Found: C, 55.23; H, 3.21; N, 7014.

4.2. Cell culture

Human non-small cell lung cancer (NSCLC) cell lines H1792, A549, H157 and Calu-1 were purchased from the American Type Culture Collection and maintained in monolayer culture in RPMI-1640 medium with 5% fetal bovine serum in a 5% CO_2, humidified incubator at 37 °C.

4.3. Cell survival assay

H1792, A549, H157 and Calu-1 cells were seeded in 96-well cell culture plates with 100 μL RPMI-1640 medium with 5% fetal bovine serum, and treated with the indicated agents on the second days. At the end of the treatment, sulforhodamine B (SRB) assay was used to measure the viable cell numbers as follows: discarded the old medium and added 100 μL 10% ice-cold TCA to each well, incubated at 4 °C for 1 h; removed the TCA and washed the plates five times with distilled water and dried in the air; then added 50 μL SRB solution to each well of the dried 96-well plates and shaked for 5 min on titer plate shaker, washed the plates with 1% v/v acetic acid five times to remove the unbound dye and dried the washed plates in the air; then solubilised the bound SRB by adding 100 μL 10 mM buffered Tris Base (pH = 10.5) to each well and shaked for 5 min; finally, read the plates on microplate reader with the working wavelength 540 nm. The optical density (OD) of SRB in each well was directly proportional to the cell number, so the OD values can be plotted against concentration.

4.4. Cell cycle analysis

A549 cells were seeded in 6-well plates, on the second day cells were treated with the indicated chemicals for the indicated time. Cells were harvested and fixed with 70% ice-cold ethanol at −20 °C overnight. Then cells were centrifuged, washed with PBS and resuspended with 1 mL staining solution (50 μg/mL RNase A and 50 μg/mL propidium iodide) for 45 min in the dark, followed by analysis with FACSCalibur cytometer (Becton Dickinson, San Jose, CA).

4.5. Western blot analysis

Cells were seeded and treated with the indicated agents on the second days. At the end of the treatment, cells were harvested with PBS. Used the lysis buffer to resuspend the cell pellet, and incubated on ice for 30 min, then centrifuged at 12,000 rpm at 4 °C for 15 min. The concentrations of the protein in the supernatant was measured using Bradford measurement (Bio-Rad), then prepared the protein samples for the electroblotting. Whole-cell protein lysates (40 μg) were separated through 12% SDS–PAGE and transferred to an Immuno-Blot polyvinylidene difluoride membrane by electroblotting; then the blots were blocked with 5% non-fat milk in PBS containing 0.1% Tween-20 for 1 h, probed or reprobed with the indicated primary antibodies at 4 °C overnight. After that, the blots were incubated with the indicated HRP-conjugated secondary antibody at room temperature for 1 h; finally, the antibody binding was detected by the ECL reagents following the manufacturer's protocol.

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