Supplementary Information

Discovery of a novel fluorescent HSP90 inhibitor and its anti-lung cancer effect

Su-Yun Bai, [#ac] Xi Dai, [#b] Bao-Xiang Zhao*[b] and Jun-Ying Miao*[a]

[a] Institute of Developmental Biology, School of Life Science, Shandong University, Jinan 250100, P.R. China.
[b] Institute of Organic Chemistry, School of Chemistry and Chemical Engineering, Shandong University, Jinan 250100, P.R. China.
[c] School of Basic Medical Sciences, Taishan Medical University, Taian 271000, P.R. China
[#] Equal contribution

Fax: +86 531 88565610
E-mail: miaojy@sdu.edu.cn, bxzhao@sdu.edu.cn

SUPPLEMENTARY RESULTS

S1. Crystal structure of compound 3e by X-ray

Crystal of compound **3e** suitable for X-ray diffraction was obtained by slow evaporation of a solution of the solid in ethyl alcohol at room temperature for 5 days. The crystal with approximate dimensions of 0.12 mm x 0.10mm x 0.08 mm for **3e** was mounted on a Bruker Smart Apex II CCD equipped with a graphite monochromated MoKα radiation (λ = 0.71073 Å) by using φ and ω scan modes and the data were collected at 298 K. The structure of the crystal was solved by direct methods and refined by full-matrix least-squares techniques implemented in the SHELXTL-97 crystallographic software. The non-hydrogen atoms were refined anisotropically. The hydrogen atoms bound to carbon were located by geometrical calculations, with their position and thermal parameters being fixed during the structure refinement. The final refinement converged at R1 = 0.0661, wR2 = 0.2163 for **3e**.

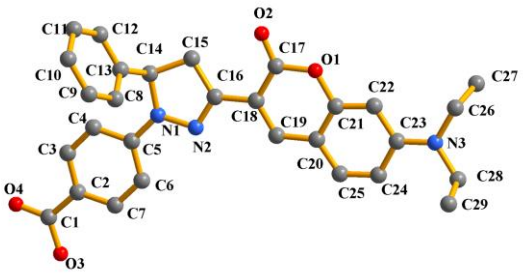

Fig. S1. Crystal structure of compound **3e**.

S2. Effects of the compounds on the morphology of A549 cells

The morphology of the cells treated with pyrazoline coumarin derivatives **3a**-**3e** was monitored under a phase contrast microscope. No morphological changes were found after treatment with these compounds except **3e** at 20 μM for 48 h, meanwhile the number of the cells treated with compounds **3a**-**3e** decreased. However, after treatment with compound **3e** at 20 μM for 48 h, most cells became globular and detached from the bottom of culture dish, indicating that apoptosis

might occur **(Figure S2)**.

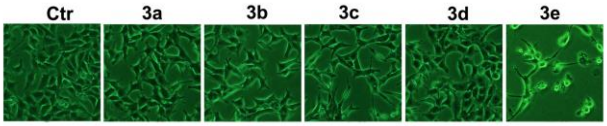

Fig. S2. Morphology image of A549 cells after treatment with compounds **3a-3e** at 20 μM for 48 h (200 ×).

S3. Effects of the compounds on the proliferation of A549, H322, H1299 lung cancer cells and normal cells HUVECs

SRB assay results showed that all the compounds had inhibitory effects on the growth of A549 cells **(Figure S3)**. Among them, compound **3e** displayed the most potent inhibitory effect. So it was chosen to further examine its effect on the viability of other lung cancer cells including H322 and H1299. Results indicated it also inhibited H322 and H1299 lung cancer cells' growth in dose-dependent manner **(Figure S4)**. More importantly, compound **3e** had no inhibitory effect on the growth of normal cells HUVECs **(Figure S4)**. Growth inhibitory properties IC_{50} (μM) for compound **3e** in three lung cancer cells at 48 h were shown in **Table S1**.

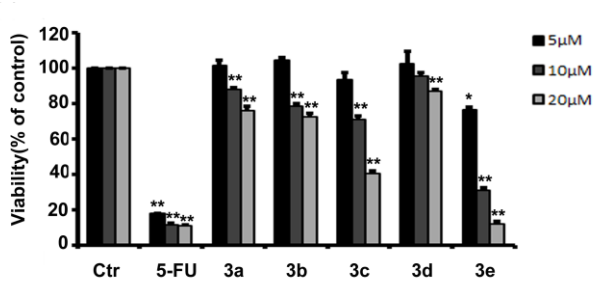

Fig. S3. Viability of A549 cells after treatment with 5-FU or compounds **3a**-**3e** for 48 h at the concentration of 5, 10 or 20 μM (*p<0.05 and **p<0.01 vs. control; n = 3).

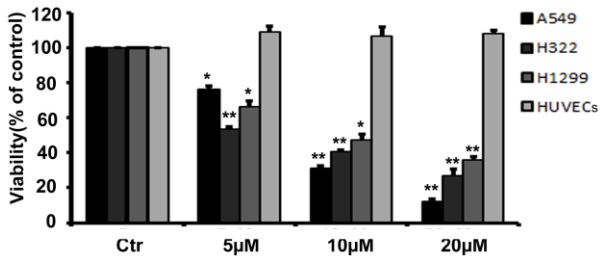

Fig. S4. Viability of H322, H1299 and HUVECs cells after treatment with 5, 10 or 20 μM compound **3e** for 48 h (*p<0.05 and **p<0.01 vs. control; n = 3).

Table S1. IC_{50} (μM) values for compound **3e** at 48 h in A549, H322 and H1299 lung cancer cell lines.

Cell lines	**A549**	**H322**	**H1299**
Compound **3e** (μM)	7.9±0.2	6.0±0.3	9.9±0.6

S4. Compound 3e induced apoptosis in A549 lung cancer cells

To detect the antiproliferation mechanism of the compounds, Hoechst 33258 staining, Western blotting, Acridine orange (AO) staining and LDH assays were performed. Cell shrinkage, membrane blebbing, DNA fragmentation and chromatin condensation are the typical characteristics of apoptotic cells. Hoechst 33258 staining assay showed that treatment with compound **3e** at 20 μM for 48 h could induce apoptosis in A549 cells (**Figure S5a**). The increase of Cleaved PARP expression also indicated the occurrence of apoptosis induced by compound **3e** (**Figure S5b**). Autophagy is another cell death mechanism. The increase of acidic vesicular organelles (AVO) is a well-known characteristic of autophagy.[1-3] Acridine orange (AO) staining results demonstrated 20 μM of compounds **3a-3e** for 48 h did not induce autophagy (**Figure S6**). Necrosis, as an unwanted side effect of anticancer agents, can be detected by LDH assay. The results showed there was no significant difference (p>0.05) in the LDH release level between the

control group and the compounds treatment group (**Figure S7**) implying that these compounds at the test range of concentration did not cause necrosis in A549 cells .

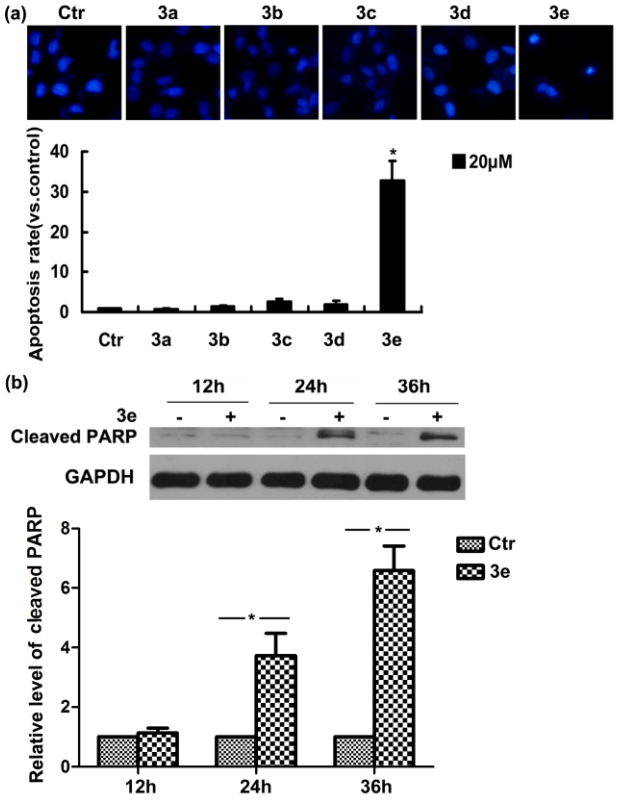

Fig. S5. Compound **3e** induced apoptosis in A549 cells. **(a)** After treatment with 20 μM compounds **3a**-**3e** or 0.1% DMSO (control) for 48 h, cells were stained with Hoechst 33258 and the apoptosis rate was calculated (**p*<0.05 vs. control). **(b)** Cleaved PARP expression was examined using Western blotting (**p* < 0.05 vs. control). The inset is a representative from three independent experiments.

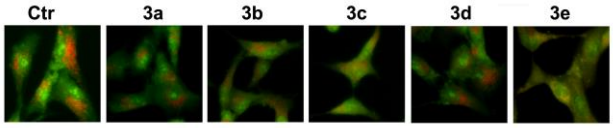

Fig. S6. Compounds **3a**-**3e** at 20 μM did not increase the acidic vesicle level in A549 cells at 48 h.

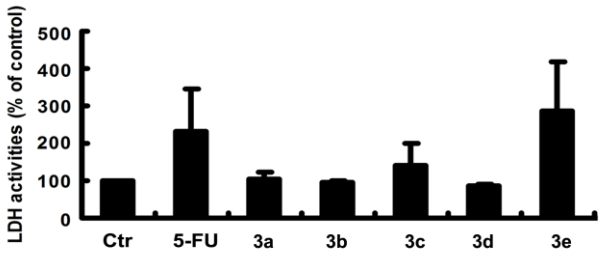

Fig. S7. Effects of 5-FU and compounds **3a**-**3e** (20 μM) on the release of LDH from A549 cells at 48 h (p>0.05 vs control; n = 3).

S5. Identification of compound 3e interactor by LC-MS/MS

The LC-MS/MS results were shown in **Table S2**.

Table S2. Proteins identified by LC-MS/MS.

NO.	NCBInr gi	Proteins	Score	Matches
1	306890	chaperonin (HSP60)	788	16(13)
2	35505	pyruvate kinase	756	17(14)
3	31645	glyceraldehyde-3-phosphate dehydrogenase	606	15(12)
4	4757756	annexin A2 isoform 2	476	13(9)
5	10716563	calnexin precursor	414	11(6)
6	30795231	brain acid soluble protein 1	380	8(7)
7	4758012	clathrin heavy chain 1	319	10(7)
8	4505763	phosphoglycerate kinase 1	278	11(6)
9	825671	B23 nucleophosmin (280 AA)	249	7(4)
10	2662291	cytochrome b5	226	3(3)
11	6807647	hypothetical protein	206	7(5)
12	**83699649**	**heat shock 90kDa protein 1, alpha**	**204**	**7(5)**
13	9507215	tubulin alpha-8 chain isoform 1	200	7(3)
14	4507677	endoplasmin precursor	199	7(4)
15	28336	mutant beta-actin (beta'-actin)	197	10(5)
16	4557032	L-lactate dehydrogenase B chain	187	5(4)
17	5174735	tubulin beta-4B chain	183	8(3)
18	47079	2-phosphopyruvate-hydratase alpha-enolase	182	6(3)
19	5031857	L-lactate dehydrogenase A chain isoform 1	179	9(4)
20	5031635	cofilin-1	164	4(2)
21	4204880	heat shock protein	162	4(4)
22	63055057	beta-actin-like protein 2	143	5(3)
23	177207	4F2 antigen heavy chain	142	3(2)

24	4557305	fructose-bisphosphate aldolase A isoform 1	138	8(4)
25	31170	unnamed protein product	135	2(2)
26	5107666	Chain A, Structure Of Importin Beta Bound To The Ibb Domain Of Importin Alpha	127	3(2)
27	763431	albumin-like	99	2(1)
28	1633054	Chain A, Cyclophilin A Complexed With Dipeptide Gly-Pro	94	3(2)
29	4505415	NAD(P)H dehydrogenase [quinone] 1 isoform a	89	5(2)
30	4506667	60S acidic ribosomal protein P0	85	2(2)
31	5821140	ASY	78	3(1)
32	5174447	guanine nucleotide-binding protein subunit beta-2-like 1	77	2(1)
33	37433	unnamed protein product	74	2(2)
34	4503483	elongation factor 2	72	3(1)
35	927056	eukaryotic translation elongation factor 1 alpha 1-like 14	72	2(2)
36	31092	unnamed protein product	71	3(2)
37	386758	GRP78 precursor	69	2(1)
38	4139784	Chain A, Canine Gdp-Ran Q69l Mutant	67	3(2)
39	340219	vimentin	66	2(1)
40	9836652	BSCv	62	1(1)
41	62420916	actin-like protein	59	5(1)
42	12002034	brain my043 protein	54	3(0)
43	119620305	hCG21219	53	2(1)
44	119569640	hCG2041308	53	1(1)
45	1082886	tumor necrosis factor type 1 receptor associated protein TRAP-1	52	1(1)
46	1710248	protein disulfide isomerase-related protein 5	51	1(1)
47	4758988	ras-related protein Rab-1A isoform 1	48	1(1)
48	5852834	bridging integrator-2	47	2(0)
49	1015321	alanyl-tRNA synthetase	43	1(1)
50	36146	ribosomal protein S12	37	1(0)
51	385321	neutrophil cytosolic factor 3; NCF-3	30	1(0)
52	4929687	CGI-109 protein	30	1(0)
53	1931584	eIF-3 p110 subunit	28	1(0)
54	36796	t-complex polypeptide 1	25	2(0)
55	4507115	fascin	25	1(0)
56	671527	gamma subunit of CCT chaperonin	25	2(0)
57	3095186	cargo selection protein TIP47	23	1(0)
58	22209028	Thioredoxin-related transmembrane protein 1	22	1(0)
59	567112	OMM protein (Ig gamma3) heavy chain	17	1(0)
60	121944798	immunoglobulin A heavy chain variable region	13	1(0)

S6. Searching the possible targets of compound 3e in the PubChem database

Protein targets search was performed in the PubChem database. Similar Compounds search was

used to predict the protein target of compound **3e**. Similar Compounds search type allows science researchers to locate records that are similar to a chemical structure query using pre-specified similarity thresholds. Similarity is measured using the Tanimoto equation and the PubChem dictionary-based binary fingerprint. This fingerprint consists of series of chemical substructure "keys". Each key denotes the presence or absence of a particular substructure in a molecule. The fingerprint does not consider variation in stereochemical or isotopic information. Collectively, these binary keys provide a "fingerprint" of a particular chemical structure valence-bond form. The degree of similarity is dictated by the Threshold parameter. A threshold of "100%" effectively acts as an "exact match" to the provided chemical structure query (ignoring stereo or isotopic information), while a threshold of "0%" would return all chemical structures in the PubChem Compound database. Various predefined thresholds between 99-60% are allowed. Here the threshold was set 80%. After the structure of compound **3e** was searched in PubChem Compound, the target proteins of active compounds with similar structure that were tested in bioassays were collected in PubChem (**Fig. S8**). Then the data obtained were collected as the basis of the next step study. Active chemicals targeting HSP90 were listed (**Fig. S9**).

Protein Targets	BioAssays	Compounds		
		Active	Activity Concentration	
			<=1μM	<=1nM
TPA: Tim23p [Saccharomyces cerevisiae S288c] (gi: 285814664)	3	2		
NPC1 gene product [Homo sapiens] (gi: 255652944)	1	2	2	
Chain A, Crystal Structure Of The Human 2-Oxoglutarate Oxygenase Loc390245 (gi: 221046486)	1	2		
Chain A, Jmjd2a Tandem Tudor Domains In Complex With A Trimethylated Histone H4-K20 Peptide (gi: 162330054)	1	2		
IDE gene product [Homo sapiens] (gi: 155969707)	5	2		
POLI gene product [Homo sapiens] (gi: 154350220)	2	2	1	
ATAD5 protein [Homo sapiens] (gi: 116283940)	2	2	1	
Microtubule-associated protein tau [Homo sapiens] (gi: 92096784)	2	2	1	
huntingtin [Homo sapiens] (gi: 90903231)	2	2		
HSP90AA1 protein [Homo sapiens] (gi: 83318444)	2	2		
TRPV1 gene product [Homo sapiens] (gi: 74315350)	2	2		
GLS protein [Homo sapiens] (gi: 71051501)	1	2		
Chain A, Crystal Structure Of The Apo Form Of Vibrio Harveyi Luxp Complexed With The Periplasmic Domain Of Luxq (gi: 67463988)	1	2		
vitamin D3 receptor isoform VDRA [Homo sapiens] (gi: 63054845)	1	2		
caspase 7, apoptosis-related cysteine peptidase [Homo sapiens] (gi: 55960760)	2	2		
IDH1 [Homo sapiens] (gi: 49168486)	1	2		
envelope glycoprotein [Human immunodeficiency virus 1] (gi: 45357394)	3	2		
CHRM1 gene product [Homo sapiens] (gi: 37622910)	3	2		
aldehyde dehydrogenase 1 family, member A1 [Homo sapiens] (gi: 30582681)	1	2		
mitogen-activated protein kinase kinase kinase kinase 2 [Homo sapiens] (gi: 22035600)	4	2		

Fig. S8. Some protein targets predicted in PubChem database.

#	Structure	Substance	Compound	Activity Outcome	Type	Value [μM]	BioAssay	Target	Links
1		MLS001172710 [SID49676442]	T0508-1612 [CID4611621]				Luminescence-based primary biochemical high throughput screening assay to identify inhibitors of the Heat Shock Protein 90 (HSP90) [AID1789, Type: screening]	HSP90AA1 protein [Homo sapiens] [gi:83318444]	View
2		MLS000779248 [SID26660325]	MLS000779248 [CID9675345]				Luminescence-based primary biochemical high throughput screening assay to identify inhibitors of the Heat Shock Protein 90 (HSP90) [AID1789, Type: screening]	HSP90AA1 protein [Homo sapiens] [gi:83318444]	View
3		MLS000779248 [SID26660325]	MLS000779248 [CID9675345]				Luminescence-based confirmation biochemical high throughput screening assay for inhibitors of the Heat Shock Protein 90 (HSP90) [AID1846, Type: screening]	HSP90AA1 protein [Homo sapiens] [gi:83318444]	View
4		MLS001172710 [SID49676442]	T0508-1612 [CID4611621]				Luminescence-based confirmation biochemical high throughput screening assay for inhibitors of the Heat Shock Protein 90 (HSP90) [AID1846, Type: screening]	HSP90AA1 protein [Homo sapiens] [gi:83318444]	View

Fig. S9. Active chemicals targeting HSP90 which have similar structure as compound **3e**.

S7. Compound 3e directly binds to HSP90

Immunoprecipitations (IPs) was used to detect the interaction between compound **3e** and HSP90. The results showed that the compound blocked the binding of HSP90 with its monoclonal antibody in a dose-dependent way suggesting that the compound might bind to HSP90 directly (**Fig. S10**).

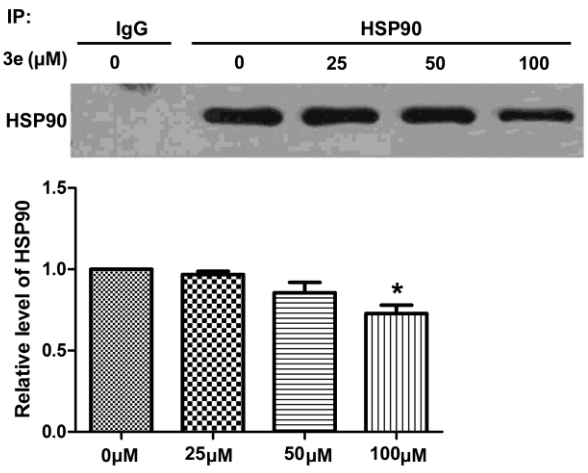

Fig. S10. Equal amounts of A549 cell lysates were incubated with compound **3e** at different

concentrations for 2 h and then immunoprecipitated using anti-HSP90. Western blots of IPs with HSP90 showed the interference of compound **3e** with the interaction between HSP90 and its antibody (*p<0.05 vs control; n = 3).

S8. Molecular docking of compound 3e to HSP90

To clarify the potential interaction mode between compound **3e** and HSP90, the compound was docked into the crystal structure of HSP90. Molecular docking with compound **3e** was performed with AutoDock 4.2 to confirm the suitability of HSP90 for docking. The compound accommodated into the socket of HSP90 catalytic site with ATPase activity easily with an inhibition constant of 145.70 μ M (**Fig. S11**).

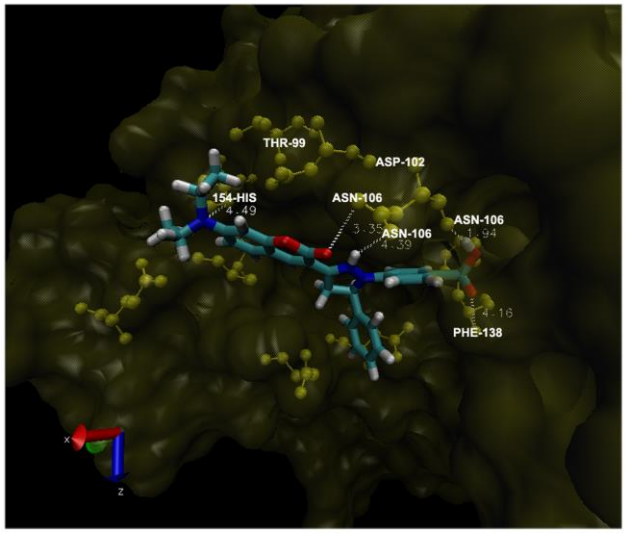

Fig. S11. Putative binding mode of compound **3e** docked into HSP90 catalytic site. Docking predicted the formation of HSP90-compound **3e** complex involves a hydrogen bond between the compound and the amino acid residue Asn106. Four other possible hydrogen bonds may also form between the compound and the amino acid residues Asn106, His154 and Phe138.

EXPERIMENTAL SECTION

Cell culture

A549, H322 and H1299 lung cancer cell lines were grown in RPMI 1640 medium (Gibco, 31800-089) supplemented with 10% (v/v) bovine calf serum (Hyclone) at 37 ℃ with 5% CO_2 atmosphere. The cells were seeded in 96 well plates or other appropriate dishes containing the medium at the density of 6250/cm^2, 24 h later cells were treated with different compounds supplemented with RPMI 1640 medium containing 1% (v/v) bovine calf serum for different time. Human umbilical vein endothelial cells (HUVECs) were obtained in our laboratory as described by Jaffe et al.[4] HUVECs were cultured in M199 medium (Gibco, 31100-035) supplemented with 15% heat-inactivated fetal bovine serum and 2 ng/ml FGF-2 in a humidified incubator at 37 ℃ with 5% CO_2. Passage number of HUVECs used in experiments was not greater than 10.

Cell viability assay

Cells were seeded onto 96-well plates and treated with small molecules synthesized at different concentration for 48 h, respectively. SRB assay was used to determine the proliferation/viability percentage with cells treated only by the solvent 0.1% (v/v) DMSO as a control. The results shown were the average of triplicate assays.

Hoechst 33258 staining

Cells were plated in 24-well plates before treatment with DMSO or 20 μM compounds. Forty-eight hours after treatment, the cells were stained with 10 μg/ml Hoechst 33258 (sigma, 861405) for 10 min at 37 ℃, then gently washed once with PBS and photographed by an Olympus (Japan) BH-2 fluorescence microscope. A minimum of 500 cells was counted, and each experiment was conducted in triplicate.

Acridine Orange (AO) staining to detect autophagy

One minute after stained with 100 μg/mL acridine orange in the medium at room temperature, cells were gently washed once with PBS, and then observed under an Olympus (Japan) BH-2 fluorescence microscope.

LDH assay

Lactate dehydrogenase (LDH) assay was performed on A549 cells treated with DMSO or 20 μM compounds for 48 h using a LDH kit (Nanjing Jiancheng, China) according to the manufacturer's instructions.

Identification of compound 3e interactor by LC-MS/MS

After washed with ice-cold PBS A549 cells were lysed in western and IP lysis buffer (Beyotime, P0013) containing 20 mM Tris–HCl (pH 7.5), 1% Triton X-100, 150 mM NaCl, and proteinase

inhibitor mix. The cellular debris was removed by centrifugation for 10 min at 12000 x g at 4 ℃. Total protein extracts from A549 cells were resolved on a 9% native PAGE. The luminous gel band from compound **3e** treated cells was cut under UV light. After fixed by fixing solution containing 40% ethyl alcohol, 10% glacial acetic acid and 50% ultrapure water, the gel band was washed with ultrapure water and analyzed by LC-MS/MS (Academy of Military Medical Sciences).

Molecular docking of compound 3e to HSP90

X-ray crystal structure of human HSP90 containing ATPase activity domain (pdb ID: 1YET) was downloaded from the Protein Data Bank. Molecular docking of compound **3e** to HSP90 was performed using Autodock 4.2. After water and other ligands were removed from the ligand and receptor Autodock pdbqt files and polar hydrogen atoms were added, compound **3e** was docked to HSP90 with all the parameters set as previously reported.[5] VMD was used to the output of complexes, the study of modeling, images and the related calculation such as the length of hydrogen bonds and the distances between atoms.

Western blot analysis

After seeding 24 h, cells were treated with small molecule or DMSO (control group) for 24 h and then whole-cell extracts were obtained. Following separated by 12% SDS-PAGE and transferred onto a PVDF membrane (Millipore, IPVH00010), the membrane was incubated overnight at 4 ℃ with one of the following primary antibodies: anti-HSP90 (Sc-69703, Santa Cruz Biotechnology), anti-HSP70 (Sc-1060-R, Santa Cruz Biotechnology), anti-NFκB p65 (Sc-109, Santa Cruz Biotechnology), anti-pAKT (cod.4060, Cell Signaling), anti-AKT (cod.4691, Cell Signaling), anti-PARP (cod.9542, Cell Signaling) and anti-GAPDH (Sc-47724, Santa Cruz Biotechnology).

Then the membrane was incubated with peroxidase-conjugated secondary antibody (Santa Cruz Biotechnology) for 1 h at room temperature, and detected by an enhanced chemiluminesence detection kit (Thermo Fisher, 34080). Quantity one software was used to analyze the relative quantity of proteins.

Immunoprecipitation

Immunoprecipitation was performed as described by Li et al.[6] Simply speaking, after A549 cells were lysed in western and IP buffer (Beyotime, P0013) and centrifuged at 4 ℃, the remaining supernatant was precleared with protein A/G agarose beads (Beyotime, P2012) for 1 h at 4 ℃. Then the supernatant was collected by centrifugation and incubated with anti-HSP90 or normal mouse IgG (as control) and Protein A/G beads overnight at 4 ℃. Finally the beads were washed three times with IP buffer followed by elution with 2 × SDS loading buffer and detection by western blot assay.

Statistical analyses

Data was analyzed by SPSS 13.0 (Statistical Package for the Social Sciences) and expressed as mean ± SEM. Results from at least 3 independent experiments were analyzed by one-way ANOVA. p-value of <0.05 was considered statistically significant.

Synthesis of compounds

Compound **1** was synthesized from substituted-salicylaldehyde and ethyl acetoacetate according to the reported method. [7]

Compound **2** was synthesized from compound (**1**) and substituted-benzaldehyde according to reported method.[8]

Compound **2** (1 mmol) and 4-hydrazinylbenzoic acid (1.2 mmol) were dissolved in dry EtOH (50 ml), and 0.5 mL acetic acid was added. The mixture was reflux for 4 h. After cooling to room temperature, the mixture poured into ice water. The ensuing solid was filtered and washed with EtOH. The crude product recrystallized from ethanol to give **3**. [9]

3-cinnamoyl-2H-chromen-2-one (2a). Yield 67%; yellow solid; mp 175-176 ^{o}C. IR (KBr, cm^{-1}) ν: 3022 (ArH), 1726 (C=O), 1610 (C=C). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 7.34-7.69 (9H, m, ArH), 7.88 (1H, d, *J* = 15.8 Hz, -CO-CH=), 7.96 (1H, d, *J* = 15.8 Hz, =CH-Ar), 8.5980 (1H, s, 4-H of coumarin). HRMS: m/z $[M+H]^+$ calcd for $C_{18}H_{13}O_3$: 277.0865, found 277.0858.

3-(3-(4-methoxyphenyl)acryloyl)-2H-chromen-2-one (2b). Yield 69%; yellow solid; mp 168-170 ^{o}C. IR (KBr, cm^{-1}) ν: 3062 (ArH), 2975 (CH_3), 1717 (C=O), 1603 (C=C). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 3.86 (3H, s, OCH_3), 6.93 (2H, d, *J* = 6.96 Hz, ArH), 7.33-7.41 (2H, m, ArH), 7.63-7.65 (2H, d, *J* = 6.96 Hz, ArH), 7.66-7.68 (2H, m, ArH), 7.82 (1H, d, *J* = 15.6 Hz, -CO-CH=), 7.87 (1H, d, *J* = 15.6 Hz, =CH-Ar), 8.58 (1H, s, 4-H of coumarin). HRMS: m/z $[M+H]^+$ calcd for $C_{19}H_{15}O_4$: 307.0970, found 307.0972.

3-(3-(benzo[1,3]dioxol-5-yl)acryloyl)-2H-chromen-2-one (2c). Yield 67%; yellow solid; mp 211-212 ^{o}C. IR (KBr, cm^{-1}) ν: 3028 (ArH), 2917 (CH_2), 1720 (C=O), 1577 (C=C). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 6.03 (2H, s, OCH_2O), 6.83-7.68 (7H, m, ArH), 6.84 (1H, d, *J* = 8.0, =CH), 7.40 (1H, d, *J* = 8.0, =CH), 8.58 (1H, s, 4-H of coumarin). HRMS: m/z $[M+H]^+$ calcd for $C_{19}H_{13}O_5$: 321.0763, found 321.0752.

3-(3-(4-hydroxyphenyl)acryloyl)-2H-chromen-2-one (2d). Yield 66%; yellow solid; mp 248-256 ^{o}C. IR (KBr, cm^{-1}) ν: 3298 (OH), 3018 (ArH), 1701 (C=O), 1571 (C=C). ^{1}H NMR (DMSO, 400 MHz): δ = 6.84 (2H, d, *J* = 8.6 Hz, ArH), 7.41-7.50 (2H, m, ArH), 7.46 (1H, d, *J* = 15.9 Hz, =CH), 7.62 (2H, d, *J* = 8.6 Hz, ArH), 7.70 (1H, d, *J* = 15.9 Hz, =CH), 7.73-7.77 (1H, m, ArH), 7.94 (1H, dd, *J* = 7.8 and 1.5 Hz, ArH), 8.62 (1H, s, 4-H of coumarin), 10.16 (1H, s, OH). HRMS: m/z $[M+H]^{+}$ calcd for $C_{18}H_{13}O_4$: 293.0814, found 293.0805.

3-cinnamoyl-7-(diethylamino)-2H-chromen-2-one (2e). Yield 42%; yellow solid; mp 149 ^{o}C. IR (KBr, cm^{-1}) ν: 3082 (ArH), 1710 (C=O), 1593 (C=C). ^{1}H NMR ($CDCl_3$, 400 MHz): δ = 1.26 (6H, t, 7.1 Hz, CH_3), 3.48 (4H, q, 7.1 Hz), 6.56-7.70 (8H, m, ArH), 7.84 (1H, d, *J* = 15.7 Hz, -CO-CH=), 8.14 (1H, d, *J* = 15.7 Hz, =CH-Ar), 8.56 (1H, s, 4-H of coumarin). HRMS: m/z $[M+H]^{+}$ calcd for $C_{22}H_{22}NO_3$: 348.1600, found 348.1606.

4-(3-(2-oxo-2H-chromen-3-yl)-5-phenyl-4,5-dihydro-1H-pyrazol-1-yl)benzoic acid (3a). Yield 48%; yellow solid. IR (KBr, cm^{-1}) ν: 3447 (COOH), 3042 (ArH), 1733 (C=O), 1599 (C=C). ^{1}H NMR (DMSO-d_6, 400 MHz): δ = 3.28 (1H, dd, *J* = 18.2 and 5.2 Hz, pyrazole, 4-H*a*), 4.04 (1H, dd, *J* = 12.4 and 18.2 Hz, pyrazole, 4-H*b*), 5.62 (1H, dd, *J* = 5.2 and 12.4 Hz, pyrazole, 5-H), 7.01-7.87 (13H, m, ArH), 8.58 (1H, s, C4-H of coumarin), 12.35 (1H, s, COOH). ^{13}C NMR (100 MHz, DMSO-d_6): 45.16, 62.92, 112.86 (2C), 116.39, 119.53, 119.99, 121.17, 125.25, 126.11 (2C), 128.11, 129.42, 129.57 (2C), 131.25 (2C), 132.69, 140.09, 142.06, 146.23, 146.96, 153.58, 158.50, 167.60. HRMS: m/z $[M+H]^{+}$ calcd for $C_{25}H_{19}N_2O_4$: 411.1339, found 411.1429.

4-(5-(4-methoxyphenyl)-3-(2-oxo-2H-chromen-3-yl)-4,5-dihydro-1H-pyrazol-1-yl)benzoic acid (3b). Yield 34%; yellow solid. IR (KBr, cm^{-1}) ν: 3420 (COOH), 3070 (ArH), 2977 (CH_3), 1730 (C=O), 1599 (C=C). ^{1}H NMR (DMSO-d_6, 400 MHz): δ = 3.26 (1H, dd, *J* = 18.2 and 5.1 Hz, pyrazole, 4-H*a*), 3.71 (3H, s, CH_3), 4.00 (1H, dd, *J* = 12.2 and 18.2 Hz, pyrazole, 4-H*b*), 5.61 (1H, dd, *J* = 5.1 and 12.2 Hz, pyrazole, 5-H), 6.89-7.87 (12H, m, ArH), 8.57 (1H, s, C4-H of coumarin), 12.33 (1H, s, COOH). ^{13}C NMR (100 MHz, DMSO-d_6): 45.06, 55.71, 62.41, 112.90 (2C), 114.91 (2C), 120.05, 121.03, 121.60, 125.26, 126.54, 127.40 (2C), 129.26, 129.51, 131.22 (2C), 140.02, 146.95, 149.31, 153.56, 154.10, 159.11, 160.47, 167.64. HRMS: m/z $[M+H]^+$ calcd for $C_{26}H_{21}N_2O_5$: 441.1450, found 441.1437.

4-(5-(benzo[d][1,3]dioxol-5-yl)-3-(2-oxo-2H-chromen-3-yl)-4,5-dihydro-1H-pyrazol-1-yl)benzoic acid (3c). Yield 21%; yellow solid. IR (KBr, cm^{-1}) ν: 3427 (COOH), 3072 (ArH), 2964 (CH_2), 1728 (C=O), 1598 (C=C). ^{1}H NMR (DMSO-d_6, 400 MHz): δ = 3.26 (1H, dd, *J* = 18.2 and 5.0 Hz, pyrazole, 4-H*a*), 4.99 (1H, dd, *J* = 12.2 and 18.2 Hz, pyrazole, 4-H*b*), 5.58 (1H, dd, *J* = 5.0 and 12.2 Hz, pyrazole, 5-H), 5.98 (2H, s, OCH_2O), 6.75-7.87 (11H, m, ArH), 8.57 (1H, s, C4-H of coumarin), 12.31 (1H, s, COOH). ^{13}C NMR (100 MHz, DMSO-d_6): 45.10, 62.68, 101.60, 106.36, 109.16, 112.89 (2C), 116.65, 119.48, 119.54, 120.04, 121.12, 125.37, 129.43, 131.25 (2C), 132.69, 135.90, 140.08, 146.31, 146.91, 147.15, 148.30, 153.57, 158.54, 167.63. HRMS: m/z $[M-H]^-$ calcd for $C_{26}H_{17}N_2O_6$: 453.1087, found 453.1099.

4-(5-(4-hydroxyphenyl)-3-(2-oxo-2H-chromen-3-yl)-4,5-dihydro-1H-pyrazol-1-yl)benzoic acid (3d). Yield 21%; yellow solid. IR (KBr, cm^{-1}) ν: 3336 (COOH), 1717 (C=O), 1596 (C=C).

^{1}H NMR (DMSO-d_6, 400 MHz): δ = 3.24 (1H, dd, *J* = 18.2 and 5.2 Hz, pyrazole, 4-H*a*), 3.98 (1H, dd, *J* = 12.2 and 18.2 Hz, pyrazole, 4-H*b*), 5.53 (1H, dd, *J* = 5.2 and 12.2 Hz, pyrazole, 5-H), 6.71-7.86 (12H, m, ArH), 8.55 (1H, s, C4-H of coumarin), 9.40 (1H, s, OH), 12.27 (1H, s, COOH). ^{13}C NMR (100 MHz, DMSO-d_6): 45.18, 62.57, 112.88 (2C), 116.22 (2C), 116.40, 119.57, 120.11, 121.00, 125.27, 127.37 (2C), 129.42, 131.18 (2C), 132.28, 132.67, 139.97, 146.20, 147.00, 153.55, 157.29, 158.51, 167.67. HRMS: m/z $[M-H]^-$ calcd for $C_{25}H_{17}N_2O_5$: 425.1137, found 425.1147.

4-(3-(7-(diethylamino)-2-oxo-2H-chromen-3-yl)-5-phenyl-4,5-dihydro-1H-pyrazol-1-yl)benzoic acid (3e). Yield 32%; red solid. IR (KBr, cm^{-1}) ν: 3403 (COOH), 3040 (ArH), 2973 (CH_2), 1713 (C=O), 1593 (C=C). ^{1}H NMR (DMSO-d_6, 400 MHz): δ = 1.34 (6H, t, *J* = 6.9, CH_3), 3.24 (1H, dd, *J* = 18.2 and 5.0 Hz, pyrazole, 4-H*a*), 3.46 (4H, q, *J* = 6.8, CH_2), 3.99 (1H, dd, *J* = 12.2 and 18.2 Hz, pyrazole, 4-H*b*), 5.57 (1H, dd, *J* = 5.0 and 12.2 Hz, pyrazole, 5-H), 6.56-7.74 (12H, m, ArH), 8.41 (1H, s, C4-H of coumarin), 12.27 (1H, s, COOH). ^{13}C NMR (100 MHz, DMSO-d_6): 12.83 (2C), 44.66 (2C), 45.60, 62.53, 96.61, 108.56, 110.06, 111.43, 112.37 (2C), 120.26, 126.05 (2C), 127.99, 129.53 (2C), 130.70, 131.24 (2C), 141.20, 142.33, 147.27, 147.47, 151.59, 156.79, 159.56, 167.69. HRMS: m/z $[M-H]^-$ calcd for $C_{29}H_{26}N_3O_4$: 480.1923, found 480.1934.

Reference List

1. Y. Chen, E. McMillan-Ward, J. Kong, S. J. Israels and S. B. Gibson, *Journal of cell science*, 2007, **120**, 4155-4166.
2. Y. Chen, E. McMillan-Ward, J. Kong, S. J. Israels and S. B. Gibson, *Cell death and differentiation*, 2008, **15**, 171-182.
3. Y. Chen, M. B. Azad and S. B. Gibson, *Cell death and differentiation*, 2009, **16**, 1040-1052.
4. E. A. Jaffe, R. L. Nachman, C. G. Becker and C. R. Minick, *The Journal of clinical*

investigation, 1973, **52**, 2745-2756.

5. L. Margarucci, M. C. Monti, C. Cassiano, M. Mozzicafreddo, M. Angeletti, R. Riccio, A. Tosco and A. Casapullo, *Chemical communications*, 2013, **49**, 5844-5846.
6. H. Li, N. Liu, S. Wang, L. Wang, J. Zhao, L. Su, Y. Zhang, S. Zhang, Z. Xu, B. Zhao and J. Miao, *Biochimica et biophysica acta*, 2013, **1833**, 2092-2099.
7. H. S. Jung, T. Pradhan, J. H. Han, K. J. Heo, J. H. Lee, C. Kang and J. S. Kim, *Biomaterials*, 2012, **33**, 8495-8502.
8. Q. Zou, Y. Fang, Y. Zhao, H. Zhao, Y. Wang, Y. Gu and F. Wu, *Journal of medicinal chemistry*, 2013, **56**, 5288-5294.
9. S. Khode, V. Maddi, P. Aragade, M. Palkar, P. K. Ronad, S. Mamledesai, A. H. Thippeswamy and D. Satyanarayana, *European journal of medicinal chemistry*, 2009, **44**, 1682-1688.