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

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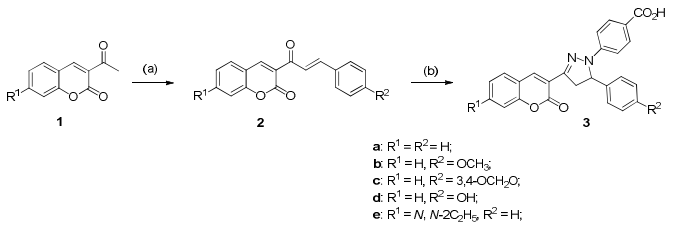
A series of novel fluorescent pyrazoline coumarin derivatives were synthesized. From them we screened a compound possessing strong inhibitory effects on lung cancer cells. By taking advantage of its fluorescence, LC-MS/MS and chemoinformatics, we identified successfully HSP90 as its target.

Drug target deconvolution is vital in new drug discovery.[1] It is not only helpful for elucidating the biological mechanism of disease, rational drug design and efficient structure-activity relationship (SAR) studies, but also beneficial for early discovery of drug side effects and toxicity.[2] Despite chemical proteomics facilitates drug target identification and verification, drug target deconvolution is still in trouble.[3] Fluorescent drugs based on synthetic small molecules are potent tools to monitor biological events in living system.[4, 5] Fluorescence emitted by drug can also give great aid to drug target identification.[6] But to date, due to limitations such as cell permeability, in vivo activity and toxicity, very few fluorescent anti-cancer drugs are developed.

Many pyrazoline derivatives are known to display a wide range of biological activities such as anti-inflammatory,[7] antimalarial,[8] antitumor,[8] antidepressants and anticonvulsants,[9] etc. Coumarin is a kind of old compounds which have recently drawn much attention due to its broad pharmacological activities. Many coumarins and their derivatives show antitumor, anti-inflammatory, anti-coagulant and anti-oxidant effects, as well as anti-microbial and enzyme inhibition properties.[10] In addition, pyrazoline and coumarin are also used as fluorescent chromophores.[11-15] Here, we synthesized a series of fluorescent pyrazoline coumarin derivatives and evaluated their anti-lung cancer proliferation activity. By taking advantage of fluorescence combined with LC-MS/MS and chemoinformatics technique, we identified the target of screened compound **3e**.

As a molecular chaperone, heat shock protein 90 (HSP90) is involved in folding and stabilization of some client proteins regulating the survival of cancer cells. Thus, HSP90 inhibitors are promising therapeutic agents for cancer treatment. Currently, some HSP90 inhibitors include geldanamycin and its derivatives (i.e., tanespimycin, alvespimycin, IPI-504), synthetic and small molecule inhibitors (i.e., AUY922, AT13387, STA9090, MPC3100), other inhibitors of HSP90 and its isoforms (i.e., shepherdin) have been developed.[16, 17] But limitations such as cytotoxicity and poor solubility demand the development of novel compounds targeting HSP90. Here we synthesized a novel fluorescent HSP90 inhibitor which showed strong growth inhibitory effects on lung cancer cells.

Compounds **3a-3e** were synthesized from substituted chromenone **1** according to reported methods (Scheme 1).[18, 19] The structures of compounds were characterized by ^{1}H NMR, IR, and MS. Moreover, representative crystal structure (**3e**) was determined by X-ray diffraction analysis (Fig. S1, ESI†).



(a) substituted-benzaldehyde, piperidine, EtOH, reflux 1-30 h; (b) 4-hydrazinylbenzoic acid, acetic acid, EtOH, reflux 4-7 h;

Scheme 1 Synthesis of Compound **3a-3e**

The morphology study (Fig. S2, ESI†) and viability assay (Fig. S3, ESI†) of the A549 lung cancer cells treated with pyrazoline coumarin derivatives **3a**-**3e** for 48 h were performed. Results showed compounds **3a**-**3e** could inhibit the growth of A549 cells. Among these pyrazoline coumarin derivatives, compound **3e** had the most powerful anti-proliferation effect (IC_{50} at 48 h = 7.9 μM, see Table. S1, ESI†). Further study indicated that compound **3e** also strongly inhibited H322 and H1299 lung cancer cells growth (Fig. S4 and Table. S1, ESI†), but it had no inhibitory effect on the growth of normal cells HUVECs (Fig. S4, ESI†). The study on the antiproliferation mechanism of these compounds showed that compound **3e** at 20 μM could induce apoptosis (Fig. S5, ESI†) rather than autophagy (Fig. S6, ESI†) and necrosis (Fig. S7, ESI†) in A549 cells, while other compounds at the test range of concentration did not cause apoptosis (Fig. S5, ESI†), autophagy (Fig. S6, ESI†) and necrosis (Fig. S7, ESI†) in A549 cells. Therefore, compound **3e** was a promising anti-cancer small molecule.

Fluorescent imaging of compounds **3a**-**3e** in A549 cells was detected by fluorescence microscopy. After incubation of A549 cells with 20 μM compound **3e** for 2 h, strong fluorescence could be seen in the cytoplasm of A549 cells offering a visual evidence of the compound entering into cells and pattern of the intracellular distribution (Fig. 1a). Moreover, at 36 h after treatment, fluorescence images of the cells revealed obvious morphological changes such as shrinkage or a disorder in cell shape (Fig. 1b), implying that the fluorescence distribution of compound **3e** could be useful for monitoring apoptosis processes. Other compounds exhibited no obvious fluorescence.

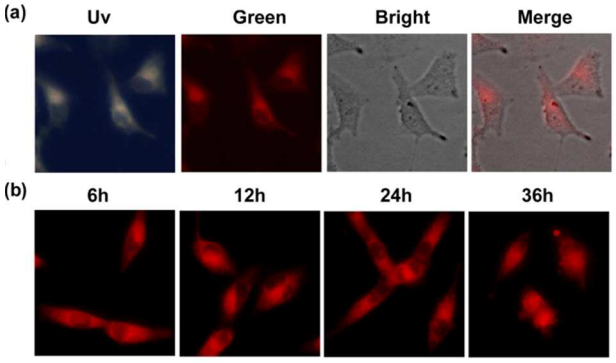


Fig. 1 Fluorescence microscope images of the intracellular compound **3e** distribution in A549 cells. (a) Cells were incubated with 20 μM compound **3e** for 2 h and photographed under different excitation light and bright light. Overlaid image was also presented. (b) Cells were incubated with 20 μM compound **3e** for 6, 12, 24 and 36 h respectively and photographed under green excitation light.

In order to identify the target of compounds, cell lysates from A549 cells treated with compounds **3a**-**3e** were analyzed by Native PAGE. Results showed that the lane of compound **3e** treatment proteins had a strong fluorescence strip. Yet other compounds did not exhibit strong fluorescence signal (Fig. 2). Then the strong fluorescence strip was cut and analyzed by LC-MS/MS. The results of LC-MS/MS were shown in Table S2. Small molecules with similar chemical structures often bind to similar targets.[20] Chemical similarities between drugs and ligand sets had successfully predicted and tested experimentally many unanticipated drug targets.[21] PubChem (http://pubchem.ncbi.nlm.nih.gov) hosted by the US National Institutes is a public database for biological properties of small molecules.[22] Information on drug-target interactions prediction is freely available for academic research in PubChem.[23] So protein targets prediction was performed using the database and the results were shown in Figure S8 and S9 (ESI†). Then overlapping of the PubChem prediction results and the LC-MS/MS results revealed that HSP90 was the unique shared protein target.

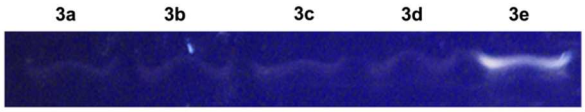


Fig. 2 Native PAGE analysis of total cell lysates from A549 cells treated with 20 μM compounds **3a**-**3e** respectively for 24h.

Previously we had noted that the binding of a small molecule to its protein target could interfere with the interaction between its protein target and corresponding antibody in cell lysates.[24] So we used the monoclonal antibody of HSP90 against full length recombinant HSP90 to immunoprecipitate HSP90 and examine whether compound **3e** could interfere with this process. The results showed that the compound blocked the binding of HSP90 with its antibody in a dose-dependent way suggesting that the compound might bind to HSP90 directly (Fig. S10, ESI†).

To clarify the potential interaction mode between compound **3e** and HSP90, the compound was docked into the crystal structure of HSP90. Results showed that the compound can accommodated into the socket of HSP90 catalytic site with ATPase activity easily (Fig. S11, ESI†).

HSP90 is an crucial molecular chaperone whose function is to assist nascent proteins and misfolded proteins to adopt correct conformations.[25] When HSP90 is exposed to its inhibitors, the amount of its client proteins will decrease due to the degradation by the ubiquitin-proteasome system. Client proteins usually are specific oncogenic proteins in different cancer types.[16] The levels of some HSP90 client proteins in A549 cells treated with compound **3e** were determined by western blot. The results showed after treatment with 20 μM compound **3e** for 24 h, there was a statistically significant decrease of p-AKT, AKT and NFκB (p65) which is a downstream protein of HSP90 client protein Iκβ (Fig. 3). Moreover, the inhibition of HSP90 is often accompanied by the rise of HSP90 itself or other heat shock proteins.[26, 27] But as newly discovered HSP90 inhibitor Oleocanthal,[28] compound **3e** did not increase the levels of HSP90 and HSP70, indicating compound **3e** and Oleocanthal had a different mechanism of action from common inhibitors (Fig. 3).

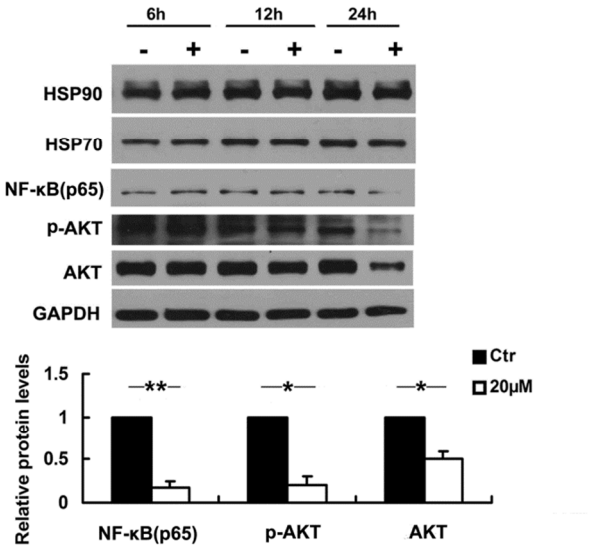


Fig. 3 Western blot analysis of HSP90, HSP70, NF-κB (P65), p-AKT and AKT proteins in A549 cells treated for different time with 20 μM compound **3e**. The graph represented the quantitation of NF-κB (p65), p-AKT and AKT protein levels in A549 cells treated for 24 h with 20 μM compound **3e**. Data are mean ± SEM. **p*<0.05, ***p*<0.01, n = 3. The inset is a representative from three independent experiments.

Conclusions

Due to its critical role in regulating the stability, activity and intracellular sorting of the client proteins involved in multiple oncogenic processes, HSP90 is an actively pursued protein target in anti-cancer drug development. Yet no HSP90 inhibitor has been FDA-approved to date. Problems such as cytotoxicity and poor solubility demand the development of novel compounds targeting HSP90.[16] Here we synthesized a novel HSP90 inhibitor with dual function of exhibiting fluorescence and strong growth inhibitory effects on lung cancer cells. So far, no fluorescent HSP90 inhibitor has been reported. The fluorescence emitted by drug molecule is very useful for monitoring the drug distribution, concentration and pathways travelled by drug in cells or even in living body which is still a challenge in pharmacokinetic studies.[29] So the new fluorescent HSP90 inhibitor synthesized by us would be of great value in pharmaceutical research and design of new therapeutics with improved properties and fewer side effects.

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Notes and references

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† Electronic Supplementary Information (ESI) available: Details of experimental procedures, data for growth inhibition, LC-MS/MS, chemoinformatics prediction, molecular docking, crystal structure, IR, ^{1}H NMR and ^{13}C spectra, HRMS. CCDC reference numbers 986432.

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