Article

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Anal. Chem., **Just Accepted Manuscript** • Publication Date (Web): 11 May 2017

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ABSTRACT: Chalcone refers to an aromatic ketone and an enone that constitutes the central core for various important biological compounds in drug discovery. Moreover, the firefly luciferase (Fluc) as the bioluminescent reporter has been widely used in life science research and high-throughput screening (HTS). However, Fluc might suffer from direct inhibition by HTS compounds resulting in the occurrence of "false positives." In the current research, we discovered a series of chalcone compounds as Fluc inhibitors with favorable potency both in vitro and in vivo. Moreover, our compound 3i showed remarkable systemic inhibition in transgenic mice. Both enzymatic kinetics study and cocrystal structure demonstrated that compound 3i is competitive for substrate aminoluciferin, while noncompetitive for ATP. Besides, compound 3i exhibited excellent selectivity as a promising quenching agent in a simulated dual-luciferase reporter assay. We believed that our research would contribute to improving scientists' awareness of the Fluc inhibitors, pay attention to the bias results and even expand the utilization of bioluminescence in life science research.

INTRODUCTION

Chalcone is widely found in nature and existed in licorice, saffron, and other medicinal plants. Chalcone is not only the main component of natural products, but also a necessary precursor for synthetic manipulations. The core scaffold of chalcone is 1,3-diphenylpropenone (Fig. 1). Since the backbone structure of chalcones has greater flexibility and is capable of binding to different receptors, chalcones exhibit a broad range of therapeutic activity, such as anti-cancer, anti-parasitic, anti-HIV, antiviral, anti-fungal, antimalarial, anti-inflammatory and so on[1-3]. This fact has attracted the widespread concern of researchers, and they have found some promising compounds by acting at different targets. For example, chalcones can promote tumor cell apoptosis, inhibit angiogenesis and cell proliferation[4]. Zhang et al. synthesized xanthohumol (Xn), a natural product derived from hops, and its analogs, then discovered that an analog showed the highest cytotoxicity toward HeLa cells, with IC_{50} values of 1.4 μM[5]. Ram et al. synthesized oxygenated chalcone and bischalcone and exhibited excellent antimalarial activity against *Plasmodium berghei* in mice[6]. It is noteworthy that high-throughput screening (HTS), a relatively recent innovation, is increasingly being applied to drug discovery enterprise. HTS of compound libraries against pharmacological targets is a critical approach to drug discovery. Artese et al. performed an in silico HTS from commercially natural compounds and obtained a chalcone derivative that could significantly inhibit the telomerase activity[7]. More importantly, fully automated HTS systems based on luciferase enzyme as bioluminescent reporters have become widely utilized in chemical biology and drug discovery applications[8].

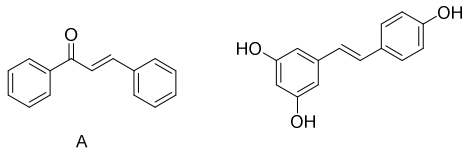


Figure 1. The structure of chalcone (A) and resveratrol (B)

Bioluminescence is a natural phenomenon through an enzyme-catalyzed reaction with the emission of visible light in a living organism. The firefly luciferin–luciferase system, the most widely applied bioluminescent system, has been well studied for more than 50 years. D-Luciferin (LH_2) can be oxidized into oxyluciferin under the participation of Fluc with an emission wavelength from 550 to 620 nm. Bioluminescence assay can be carried out in ab-

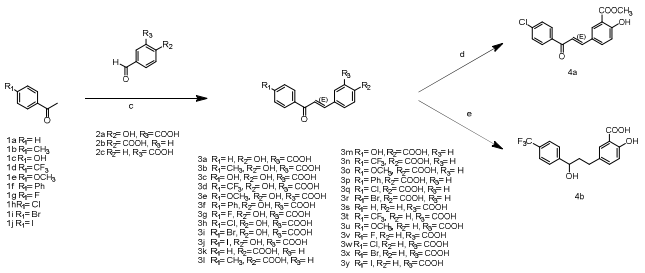
solute darkness without the demand of excitation light sources; thus, it can avoid the effect of the emission light and has a higher sensitivity compared with fluorescence methods[9]. FLuc-based bioluminescence assays are highly favored in HTS because of their excellent sensitivity[10]. However, the luciferase enzymes commonly used in the HTS may suffer from unexpected nonspecific activation or inhibition results. Consequently, it is essential to investigate the molecular mechanisms to achieve high accuracy and reliability[11].

In 1965, Ueda et al. firstly reported that general anesthetics, such as diethyl ether and halothane, inhibited the bioluminescence of firefly luciferase.[12] Fraga et al. found that coenzyme A induced stabilization of Fluc luminescence, because a thiolytic reaction splited dehydroluciferyl-adenylate(L-AMP) and generated dehydro-luciferyl-CoA(L-CoA), a much less powerful inhibitor[13]. In 2006, Bakhtiarova et al. suggested that resveratrol (Fig. 1B) could inhibit the firefly luciferase activity significantly with a K_i value of 2 μM, proposing that resveratrol might exist fundamentally flawed via luciferase reporter assays.[14] Auld et al. reported PTC124 (3-[5-(2-fluorophenyl)-1,2,4-oxadiazol-3-yl]benzoic acid) as a potent Fluc inhibitor with an IC_{50} of 7 nM[15,16]. Rui Fontes et al. indicated that the bioluminescence of luciferin could be inhibited by inorganic pyrophosphate and tripolyphosphate due to their reaction with L-AMP [17]. So far, many compounds containing different structures have been reported that could inhibit firefly luciferase, such as ions,[18] (E)-2-fluoro-4'-methoxystilbene[19], N-pyridin-2-ylbenzamides[20], 2-phenylnaphthalenes[21], aryltriazoles[22], 2-phenylnaphthalenes[23], 5-benzyl-3-phenyl-4,5-dihydroisoxazoles and 5-benzyl-3-phenyl-1,4,2-dioxazoles[11].

In the current study, we found that some chalcone derivatives could potently inhibit the firefly luciferase activity. Accordingly, it is of great significance to notify researchers of possible "false positives" when they use FLuc-based HTS to do some research on chalcones. We used resveratrol as the positive control to evaluate the activity of our chalcone compounds. In the current case, aminoluciferin was employed to study the kinetics profile of inhibition of firefly luciferase due to its higher affinity than the nature luciferin and longer emission wavelength (596 nm), which is beneficial to in vivo inhibition study[9]. We found (*E*)-5-[3-oxo-3-(4-bromophenyl)-1-propenyl]-2-hydroxy-benzoic acid (compound 3i) inhibited firefly luciferase with an IC_{50} value of 0.2 μM in vitro and (*E*)-5-[3-oxo-3-(4-phenylphenyl)-1-propenyl]-2-hydroxy-benzoic acid(compound 3f) inhibited firefly luciferase with an IC_{50} value of 2.49 μM in cellulo, which significantly stronger than resveratrol (2.33 μM in vitro and 35.94 μM in cellulo).We further evaluated the inhibitory activity of compound 3i in vivo and found that it exhibited 60.0% inhibition in xenografted balb/c-nu female mice by an intratumor injection and 18.8% inhibition in CAG-luc-eGFPL2G85 transgenic mice by a tail intravenous injection. Moreover, we investigated its kinetic mechanism of inhibition to obtain K_m and V_{max} values after incubation with different concentrations of compound 3i. In brief, compound 3i was competitive for aminoluciferin while noncompetitive for ATP. These results were consistent with the subsequent cocrystal structure of luciferase complex with compound 3i. The cocrystal result also demonstrated that compound 3i was bound in a pocket consisting of luciferase residues and occupied the catalytic active site of amino-luciferin. More interestingly, compound 3i could selectivity quench Fluc bioluminescence, which could be utilized as a promising quenching agent in a dual-luciferase reporter assay.

EXPERIMENTAL SECTION

Synthesis



Scheme 1. Synthesis of a series of chalcones by aldol condensation reaction. Reagents and conditions: (c) TsOH, AcOH, 85 °C, 12 h, 33-80%; (d) H_2SO_4, CH_3OH, 65 °C, 6 h, 88%; (e) $NaBH_4$, Pd/C, THF, H_2, rt, 4 h, 60%.

The synthetic route of chalcone compounds is outlined in Scheme 1. In brief, we started from the commercially available different substituted acetophenone or made some simple modification according to the method described by and Huang and Ley [24,25]. Then, the chalcone compounds were obtained by toluene sulfonic acid-catalyzed or sodium hydroxide-catalyzed aldol condensation reaction. In order to delve into their structure-activity relationship, we further performed the esterification of carboxylic acids and the reduction of α,β-unsaturated ketones. More synthetic details can be found in the supporting information.

Clone, protein expression and purification of firefly luciferase

Luciferase gene was amplified by PCR using Pgex-6p-2 plasmid contains the cDNA of Photinus pyralis luciferase. The synthetic primers used as follows (the restriction sites are underlined): forward primer, ATCggatccATGGAGGATGCGAAG, reverse primer, TCA ctcgagTTACAGTTTGCTTTTACC. The PCR product was cloned into the BamHI, and XhoI sites of pET-15b (Novagen) and the plasmid was overexpressed in E. coli strain BL21 (DE3). The E.coli cells were cultured in the LB medium containing 100 μg/mL ampicillin at 310 K to an absorbance (A600) reached 1.0 and were then induced with 1 mM IPTG (isopropyl β-D-1-thiogalactopyranoside) overnight at 288 K. The cells were harvested by centrifugation. Cells lysis was achieved by sonication method. After centrifugation at 28,000 × g for 45 min, the supernatant was applied to Ni-

NTA column (GE Healthcare) for affinity chromatography. The His-tagged luciferase was eluted with elution buffer (10 mM Tris–HCl pH 8.0, 500 mM NaCl, and 250mM imidazole). The purification process was then followed by size-exclusion chromatography with Superdex-200 column with 10 Mm Tris-HCl buffer pH 8.0 containing 500 mM NaCl and 0.3 mM dithiothreitol.

Bioluminescence assay materials and instruments

Ultrapure water purified in a Mill-Q filtration system was used to prepare all aqueous solutions. Measurements for bioluminescent assays were performed in 50 mM Tris buffer, pH 7.46 with 10 mM $MgSO_4$ at 37°C. Recombinant Renilla reniformis luciferase was purchased from RayBiotech. ATP and Tris base were purchased from Aladdin. Coelenterazine was purchased from Chemedir. Luminescence produced by the luciferase was measured with Omega microplate reader (POLARstar Omega, Germany). Bioluminescence imaging was performed using an IVIS Kinetic (Caliper Life Sciences, USA) equipped with a cooled charge-coupled device (CCD) camera. Circular regions of interest (ROI) were drawn and measured using Living Image software. The intensity of luminescence was reported as total photon flux within an ROI in photons per second.

Luciferase enzyme inhibition assay

Measurements for bioluminescent assays were performed in 50 mM Tris buffer, pH 7.60 with 10 mM $MgSO_4$ at 37 °C. All chalcone compounds were dissolved in dimethylsulfoxide at 50 mM, then they were further diluted to an increasing concentration ranging from 10 nM to 100 μM in Tris buffer prepared before. The recombinant firefly luciferase was purchased from Promega (E1702, USA). The luciferase was diluted to 20 μg/mL inTris buffer. Substrates solution is also prepared in the Tris buffer, containing 40 μM of aminoluciferin and 2 mM of ATP. To a 96-well plate (WHB, black) each well containing 50 μL of the luciferase solution, an amount of 50 μL of increasing concentrations of chalcone compounds was added as three replicates. After incubation at 25°C for 10 min, an amount of 100 μL of the substrates solution was added. An equal amount of DMSO in Tris buffer instead of compounds was set as a control group. Instead of compounds and luciferase, 100 μL Tris buffer was set as a blank group. Luminescence produced by the luciferase was measured with Omega microplate reader (POLARstar Omega, Germany). The log-(inhibitor) *versus* normalized response data were analyzed with the GraphPad Prism software(control was set as 100%).

ES-2-Fluc cell bioluminescence inhibition assay

ES-2 cells (human ovarian cancers cell line) were purchased from the Committee on Type Culture Collection of Chinese Academy of Sciences. ES-2 cells expressing firefly luciferase (ES-2-Fluc cells) were supplied by Cellcyto. The ES-2-Fluc cells were cultured in RPMI 1640 medium supplemented with 10% fetal bovine serum (FBS) at 37°C in a humidified atmosphere with 5% CO_2 incubator. In ES-2-FLuc cell bioluminescence inhibition assays, 100 μL cell suspension were added to a 96-well plate (Corning, 3603) to make the number of cells in each well at about 4×10^4. After incubated for 12 hours, 100 μL chalcone compounds of different concentrations (15.6 μM, 31.25 μM, 62.5 μM, 125 μM, 250 μM, 500μM, 1 mM) dissolved by RPMI 1640 medium without FBS were added as triplicates. Moreover, an equal amount of RPMI 1640 medium without compounds was added directly as a negative control. After further incubation for 12 hours, the medium was removed, and 100 μL amino-luciferin solution (100 μM, dissolved in Tris buffer of pH 7.60) was added. Also, the blank group (without cells) containing only 100 μL aminoluciferin solution were set. After the aminoluciferin solution had been added, the bioluminescence signal was measured immediately with IVIS Kinetic (Caliper Life Sciences, USA) instrument equipped with a cooled charge-coupled device (CCD) camera for bioluminescent imaging. The exposure time was set to one second. Circular regions of interest(ROI) were drawn and quantified using Living Image software.The data were reported as total photon flux within an ROI in photons per second and were analyzed using the GraphPad Prism software.

Mice model

Balb/c-nu female mice were purchased from Animal Center of China Academy of Medical Sciences when they were 8 weeks of age. To generate tumor xenografts in nude mice, ES-2-Fluc cells (1×10^7) were implanted subcutaneously under the right armpit region. Mice were kept in a standard 12:12 light–dark cycle either single- or group-housed with free access to food and water at 28 °C. Tumors were allowed to grow for two weeks before imaging. Transgenic mice harboring the CAG-luc-eGFP L2G85 transgene, constructed by the laboratory of Dr. Christopher H. Contag (Stanford University School of Medicine), were purchased from the Jackson Laboratory (Sacramento, California, USA). All animal studies were approved by the Ethics Committee of Qilu Health Science Center, Shandong University and complied with European guidelines for the care and use of laboratory animals.

Bioluminescence inhibition assay in mouse xenograft model

Day 1: Fifteen mice bearing ES-2-Fluc subcutaneous tumors were randomly divided into 3 groups (n=5, each): normal saline group, resveratrol group, inhibitor 3i group. The mice were anesthetized with isoflurane and then intraperitoneally injected with 100 μL aminoluciferin (0.5 mM, diluted by normal saline), followed immediately by bioluminescence imaging every 3 minutes for 30 to 40 minutes until the bioluminescence intensity reached a peak and got steady. Then, mice were fed with regular diet for 12 hours to metabolize away the aminoluciferin. After that, for inhibitor 3i group and resveratrol group, each mouse was injected with 50 μL resveratrol or 3i (200 μM) intratumorally, and for the normal saline group, 50

μL normal saline containing an equal amount of DMSO was used to instead of inhibitors.

Day 2: After feeding for another 12 hours (on the next day), all mice were intraperitoneally injected with 100 μL aminoluciferin (0.5mM, diluted by normal saline). Subsequently, bioluminescent imaging was performed every 3 minutes for 30 to 40 minutes. The relative activity for each mouse was calculated by dividing the peak total photon flux of day 1 by peak total flux of day 2. The residual activity of mice treated with inhibitors is calculated by the ratio of comparing relative activity of inhibition group with the saline group, setting the saline group as 100%.

In vivo inhibition assay in transgenic mice by tail intravenous injection

Pathogen-free luciferase-expressing transgenic mice (FVB-Tg(CAG-luc,-GFP)L2G85Chco/FathJ)17 were obtained from the Jackson Laboratory and housed in the Shandong University. Twelve transgenic mice were randomized into three groups: compound 3i group, resveratrol group, and normal saline group. For inhibition groups, 200 μL compound 3i or resveratrol (200 μM, dissolved in normal saline) were injected into the mice veins through tail intravenous injection. For the normal saline group, we used 200 μL normal saline instead. After 4 hours, the mice were anesthetized with isoflurane and injected with 100 μL amino-luciferin (0.5 mM, dissolved in normal saline) intraperitoneally. After injection with amino-luciferin, bioluminescent imaging was taken immediately every 3 minutes for 30 to 40 minutes until the bioluminescence intensity went through a peak and got steady. The inhibition rate was obtained by comparing the average bioluminescence flux of inhibition group with the normal saline group.

Dual-luciferase reporter gene assay simulation

Firstly, Fluc and Rluc dissolved in Tris-HCl buffer (20 μg/mL and 2.5 μg/mL, respectively) was mixed in a 96-well plate. Secondly, 50 μL Fluc substrates solution (20 μM aminoluciferin and 1 mM ATP in Tris-HCl buffer) were added to the mixture to initiate the firefly luminescence. Lastly, 100 μL mixture of compound 3i and coelenterazine (50 μM and 5 μM in Tris-HCl buffer, respectively) was added to quench the firefly luminescence and trigger renilla luminescence. Bioluminescence was measured immediately at wavelength 590 nm and 460 nm, emitted by Fluc and Rluc, respectively.

Crystallization of Fluc and Fluc-3i complex

Details of clone, expression and purification are described in the Supporting Information. Luciferase was concentrated to 8 mg/mL. Crystal of native luciferase was initially obtained by sitting-drop vapor diffusion at 293 K. After optimization, crystal was grown in hanging drops by mixing equal volumes of protein solution and reservoir solution (0.5 M Li_2SO_4, 15% PEG8000, 0.1 M Tris pH 8.0) at 293 K. To obtain crystal of Fluc-3i complex, native crystal was soaked in solution containing 1.2 mM inhibitor, 3.2 mM ATP and 12 mM $MgCl_2$ at 293 K for 1 min. The obtained crystal was flash-frozen in liquid nitrogen after soaking in a cryoprotectant solution consisting of the respective reservoir solution with 15-20% glycerol was used as a cryoprotectant, and all data were collected at 100 K in a nitrogen stream.

Data collection, processing and structure determination

X-ray diffraction data were collected at 100 K on beamline BL17U at SSRF, Shanghai, China equipped with a MAR Mosaic CCD 225 detector. The data were integrated and scaled using the HKL-200 program suite.[26] The crystals of Fluc-3i belong to space group P41212 with unit cell parameters: a=118.141 Å, b=118.141 Å, c=96.194 Å, α=β=γ=90° diffracted to 3.0 Å resolution. The structure of Fluc-3i was solved by molecular replacement using Phaser from the CCP4 suite of program [27,28] with firefly luciferase (PDB entry 1BA3) as the search model. The initial model of luciferase was refined using PHENIX[29] with additional rounds of manual rebuilding using the Coot molecular graphics program.[30] The compound 3i was added to the complex model by Coot based on the FO-FC density map of the ligand structure. The final R values were R_{work}=0.1954 and R_{free}=0.2698 based on a subset of 14148 of the reflections.

X-ray diffraction data collection and refinement statistics are presented in Table S1. The final model was checked and validated using PROCHECK[31], QMEAN[32] and ProQ[33], which indicated a good-quality model. The mean temperature factors for protein and solvent were calculated using BAVERAGE from the CCP4 program suite. Molecular graphics was illustrated with PyMOL.[34] The atomic coordinates and structure factors of Fluc-3i have been deposited in the Protein Data Bank with accession codes 5WYS.

Statistical analysis

Data values were expressed as means ± SD or SEM of at least two independent experiments and evaluated using Student's *t*-test for unpaired samples.

RESULTS AND DISCUSSION

In an initial screening, all chalcones were evaluated for their inhibitory activity on purified recombinant firefly (Photinus pyralis) luciferase. To determine their IC_{50} values and to generate the accurate concentration–response curves (CRCs) (Fig. 2A), we used the increasing concentrations of chalcones from 10 nM to 100 μM incubation with firefly luciferase for 10 min and measured the luminescence intensity with Omega microplate reader (POLARstar Omega, Germany) while resveratrol served as a positive control. As shown in Table S1, some chalcone compounds showed significantly enhanced inhibitory activity compared to the positive control resveratrol. Among them, compound 3i showed the most potent inhibitory activity with an IC_{50} value of 0.2 μM and exceeded 10-fold potency than the positive control resveratrol (IC_{50}=2.33 μM). Compared with other common inhibitors in the assay, the IC_{50} value of compound 3i also exceeded

L-luciferin (IC_{50}=115 μM), dehydroluciferin (IC_{50}=2.3 μM), and dehydroluciferyl coenzyme A (IC_{50}=5 μM), but was no better than L-AMP (IC_{50}=6 nM) and so on[35-37]. Because the luciferase-based assay was based on a readout of luminescence intensity at 590 nm, the inhibition quenching could cause the false-positive result. Therefore, we determined the absorbance spectra (250-700 nm) of compound 3i and FLuc emission spectrum in absence or presence of compound 3i. As shown in Fig. 3B, compound 3i could significantly decrease the luminescence intensity while having no impact on the maximum emission wavelength of Fluc. Moreover, compound 3i did not show any absorption at above 460 nm (Fig. 3). Therefore, compound 3i was unable to absorb the visible light produced by Fluc.

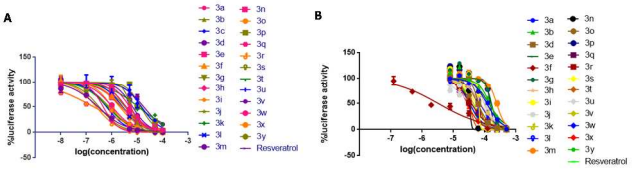


Figure 2. (A) Concentration–response curves for active chalcone compounds in a recombinant firefly luciferase inhibition assay; (B) CRCs for active chalcone compounds in cellulo. Representative graphs are selected from one experiment performed in triplicate.

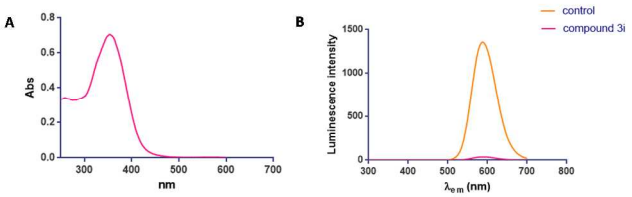


Figure 3. (A)Light absorbance spectrum for compound 3i and (B) bioluminescence emission spectrum with or without compound 3i

To investigate the effect of chalcones on firefly luciferase in cellulo, we performed a cell-based assay with ES-2-Fluc ovarian cancer cells that transfected with firefly luciferase. ES-2-Fluc cells were treated with various increased concentrations of inhibitors incubation for 12 h. After incubated for 12 h, the medium was removed, and the aminoluciferin solution was added. The corresponding CRCs are shown in Fig. 2B. As shown in Table S1, compound 3f exhibited potent inhibitory activity with an IC_{50} value of 2.49 μM, that is >10-fold stronger than resveratrol (IC_{50} =35.94 μM). Analysis of structure–activity relationship (SAR) reveals that the exposed carboxyl group on the right benzene ring is essential for maintaining high potency. The esterification of carboxylic acids will lead to the loss of activity. The activity of the carboxyl group at meta-position is a little better than the para-position. Hydroxy group at the R_2 position is not essential for the activity. Notably, the α, β-unsaturated ketone is also necessary for inhibitory activity. Besides, substituted group at the R_1 position can increase the efficacy of inhibition, in which halogen is more favorable than other substituted group. Among them, bromine-substituted and iodine-substituted chalcones have a higher potency than fluorine-substituted and chloro-substituted. This phenomenon might be explained by the size of the atomic radius.

Since the bioluminescence-based assay is widely applied in vivo, we further evaluated inhibitory activity of compound 3i in a well-established nude mice xenograft tumor model described previously in detail by us[21,38]. To construct the expression of firefly luciferase in nude mice xenografts, ES-2-Fluc cells were grafted subcutaneously into 8-week-old female mice under the right armpit region. After two weeks of tumor growth, bioluminescence signal of total flux was measured after intraperitoneal injection of amino-luciferin solution. The bioluminescence signal of the first day was set as the calibration value. After 12 h, we injected inhibitors (resveratrol and compound 3i, 200 μM) into the tumor and measured its bioluminescence intensity after waiting for another 12 h. The relative activity was obtained by calculating the ratio of the first and second day of bioluminescence intensity. As shown in Fig. 4A, the ratio of the blank normal saline group was increased to 135.3%, which may be due to tumor growth for 24 h. Therefore, we set the saline group as 100% and obtained residual total flux percentage by calculating the ratio of the relative activity of inhibition group (resveratrol and compound 3i) and the blank normal saline group. As shown in Fig. 4B, the residual activity of the bioluminescence was 40.0% after injection of compound 3i, while the residual activity of the positive control resveratrol group was 66.5%. Inhibition rate of inhibition group can be calculated by 100% -residual total flux (%). This indicated that compound 3i showed stronger inhibitory activity than resveratrol, and our compound 3i (200 μM) showed the inhibitory activity of 60.0%in the nude mouse xenograft tumor model.

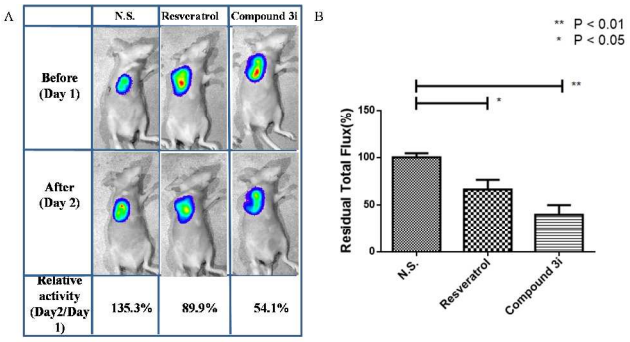


Figure 4. (A) Representative bioluminescence imaging in a nude mice xenograft tumor model. Relative activity was obtained by the total flux ratio of day 2 to day 1; (B) Quantification of residual activity, calculated by the ratio of relative activity of inhibition group to saline group. ** <0.01, * <0.05 (t-test, determined by GraphPad Prism software).

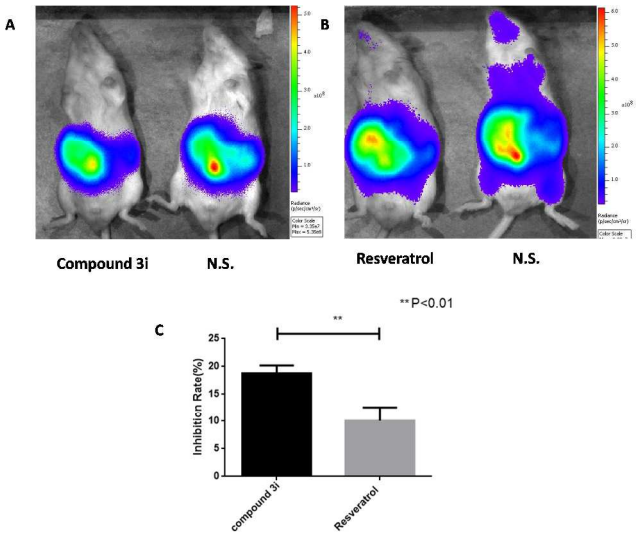


Figure 5. Representative bioluminescence imaging of inhibition caused by compound 3i (A) and resveratrol (B) compared with normal saline in transgenic mice; (C) quantification of inhibition rate caused by compound 3i and resveratrol. ** <0.01 (t-test, obtained by GraphPad Prism software).

To investigate whether our compound 3i can exhibit systemic inhibition effects in the body, we further evaluated its inhibition activity in CAG-luc-eGFP L2G85 transgenic mice by tail intravenous injection. The transgenic mice directed by the CAG promoter can show widespread expression of firefly luciferase in the whole body. For the inhibition group, the transgenic mice were tail vein injection of compound (200 μL of 200 μM solution in saline); for the saline group, transgenic mice received 200 μL of saline instead. After 4 h, the bioluminescence intensity was measured after intraperitoneal injection of aminoluciferin (100 μL, 0.5 mM, dissolved in saline). Inhibition rates of inhibitors can be calculated by 100% - residual total flux (%). As depicted in Fig. 5, compound 3i exhibited 18.8% inhibition in transgenic mice while the positive control resveratrol exhibited only 10.1% inhibition. This phenomenon also demonstrated that compound 3i showed more powerful inhibition than resveratrol in vivo.

To determine the inhibition mode, we continued to choose compound 3i for further concentration-dependent response in the presence of aminoluciferin and ATP. Maximum rate and Michaelis constant with or without compound 3i were determined with Michaelis–Menten kinetics, and the Lineweaver–Burk plots were estimated using GraphPad Prism software. For substrate aminoluciferin, the Michaelis constants (K_m) were significantly increased in the presence of the inhibitor, but the maximum rates (V_{max}) remained unaltered (Fig. S1 and Table S2). It indicated the inhibition mechanism for compound 3i was competitive with respect to the aminoluciferin. Therefore, we believe that compound 3i can be bound to the enzyme's active site where the substrate aminoluciferin also binds. For substrate ATP, compound 3i caused a significant decrease in V_{max}, while the K_m slightly decreased. We supposed that the inhibition mode belonged to noncompetitive inhibition. It revealed that compound 3i might combine with the luciferase outside the active site.

Structures of luciferase complexed with compound 3i were determined at 3.0 Å resolution by molecular replacement (see supporting information, Table S3) using the crystal structure of luciferase from *Photinus pyralis* (Protein Data Bank accession number: 1BA3) as a model. The final models of Fluc-compound 3i show that each asymmetric unit contains one monomer. All atoms of the Fluc-compound 3i model are well defined except for two amino-acid residues at the N-terminus, a loop from Ser198 to Ser201 and seven residues at C-terminus.

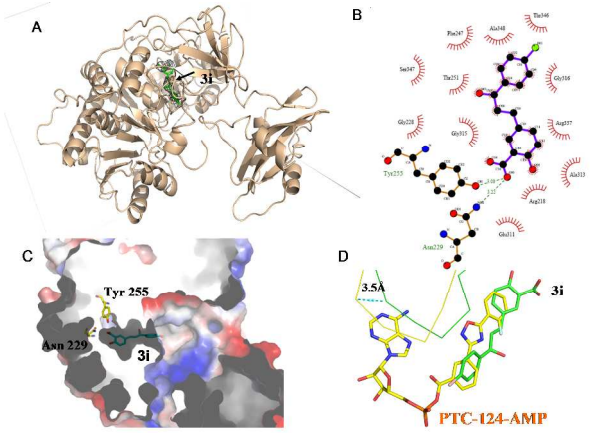


Figure 6. The overall structure of Fluc-compound 3i complex. (A): The whole Fluc-compound 3i complex structure is represented in wheat color cartoon model; compound 3i is drawn in green stick model and F_O-F_C OMIT map contoured at 1.5 σ shows electron density for compound 3i; (B): A schematic drawing of compound 3i binding site with Ligplot program; (C): The interaction of compound 3i with luciferase residues at the bottom of the pocket; (D): The located sites comparison of Fluc-compound 3i and Fluc-PTC124-AMP.

Apo-form crystals were soaked in the presence of compound 3i and the co-crystal structure electron density maps were examined for ligand binding (Fig. 6A). The compound 3i is bound in a pocket consisting of luciferase residues (Fig. 6B). Tyr255 and Asn229 forms hydrogen-bonded with 3i at the bottom of the pocket (Fig. 6C). The alignment of the Fluc-compound 3i structure to Fluc-PTC124-AMP structure (Protein Data Bank accession number: 3IES) indicated that the inhibitor 3i has the similar binding pattern with PTC-124. The hydrophobic pocket of Fluc is similar to PTC-124, except for a loop (Ala313-Leu319) of the luciferase has about 3.5 Å distance movement (Fig. 6D). These crystallographic results confirmed the competitive pattern of compound 3i for aminoluciferin by kinetics study. The atomic coordinates and structure factors of Fluc-3i have been deposited in the Protein Data Bank with accession codes 5WYS.

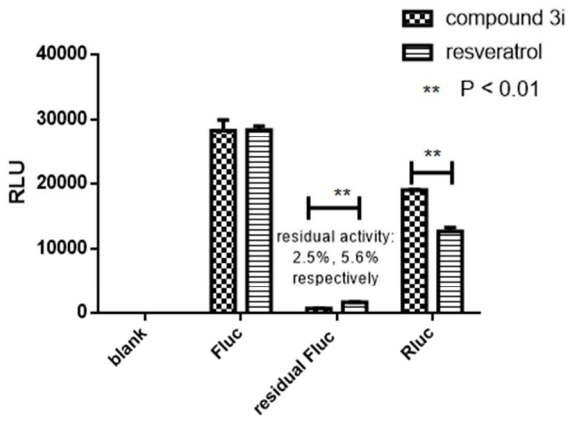


Figure 7. Dual-luciferase reporter assay simulation. Residual activity was obtained by RLU ratio of residual Fluc to Fluc. ** <0.01 (t-test, calculated by GraphPad Prism software).

The luciferase from the sea pansy *Renilla reniformis* (Rluc), another commonly used luciferase reporter, has been found some important applications in bioanalysis.[39] It can catalyze the coelenterazine oxidation to coelenteramide with the emission of blue light.[40] Rluc is commonly used together with Fluc in dual-luciferase reporter gene assay. Both reporters can be examined from the same sample by first measuring the Fluc activity and then quenching Fluc luminescence and measuring the RLuc activity following adding a quench-and-activate reagent.[8] In the process of enzymatic inhibition assays, we found that our chalcones inhibited firefly luciferase in an extremely rapid way. Therefore, we performed a dual-luciferase reporter assay simulation to evaluate the luciferase selectivity of compound 3i. First of all, Fluc substrate solution containing aminoluciferin (20 μM) and ATP (1 mM) were added into Fluc and Rluc mixture to generate the firefly bioluminescence. Then, a mixture of compound 3i (50 μM) and Rluc substrate coelenterazine (5 μM) was added to the same sample to quench firefly luminescence and trigger renilla luminescence. Bioluminescence generated by the luciferase-catalyzed reactions was measured immediately at wavelength 590 and 460 nm with an Omega microplate reader. As shown in Fig. 7, for the positive control, we found resveratrol quenched about 96% Fluc bioluminescence; however, it also eliminated 33.4% Rluc bioluminescence. Compound 3i eliminated nearly 98% of Fluc bioluminescence while having less effect on Rluc bioluminescence compared to the resveratrol. Therefore, compound 3i exhibited selectivity which quenched Fluc bioluminescence while having little impact on Rluc bioluminescence better than resveratrol. It indicated that compound 3i had a high potential of being applied in the dual-reporter assay as a promising Fluc quenching agent.

CONCLUSION

In summary, we discovered a series of chalcone compounds as potent firefly luciferase inhibitors. Compound 3i inhibited firefly luciferase with an IC_{50} value of 0.2 μM in vitro, approximately ten times greater potency than resveratrol. Compound 3f inhibited firefly luciferase with an IC_{50} value of 2.49 μM in cellulo, which also significantly stronger than resveratrol. In the animal experiment, compound 3i disclosed 60.0% inhibition in a well-established nude mouse xenograft tumor model and 18.8% inhibition in CAG-luc-eGFP L2G85 transgenic mice. In addition, kinetics of inhibition of luciferase by compound 3i and the cocrystal structure also indicated that compound 3i occupied the catalytic active site of substrate aminoluciferin. In other words, compound 3i was competitive for aminoluciferin while noncompetitive for ATP. Furthermore, compound 3i achieved its inhibition in an extremely rapid way when exposed to Fluc and had little effect on Rluc, which might be used as a promising quenching agent in a dual-luciferase reporter assay. Thus, our studies can be helpful for research about chalcones in drug discovery and might contribute to expanding its application in life science research.

ASSOCIATED CONTENT

Supporting Information

General methods, synthetic procedures, original spectra for structural characterization and additional figures and tables. This material is available free of charge via the Internet at http://pubs.acs.org.

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ACKNOWLEDGMENT

The present work was supported by grants from the National Program on Key Basic Research Project of China (Nos. 2013CB734000 and 2015CB150600), the National Natural Science Foundation of China (Nos. 81673393 and 31401626), the Taishan Scholar Program at Shandong Province, the Qilu Scholar Program at Shandong University, the Program for Changjiang Scholars and Innovative Research Team in University (No. IRT13028), the Major Project of Science and Technology of Shandong Province (No. 2015ZDJS04001), the Fundamental Research Funds of Shandong University (No. 2014JC008) and the National Institutes of Health (No. R01GM112003).

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