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Journal Name

ARTICLE

Discovery of a series of 2-phenylnaphthalenes as firefly luciferase inhibitors

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As the most convenient and efficient bioluminescence system, the firefly luciferase/luciferin complex has been widely used in life science research and high-throughput screening (HTS). Nonetheless, the interpretation of firefly luciferase-based assay data is often complicated by the occurrence of "false positives," in part because firefly luciferase (Fluc) is subjected to direct inhibition by HTS compounds that might inadvertently act as inhibitors of its catalytic site. Here we report a series of 2-phenylnaphthalenes as Fluc inhibitors with suitable potency both *in vitro* and *in vivo*. Besides, our compound 5 showed significant systemic inhibition in transgenic mice. Enzymatic kinetics study reveals that compound 5 is competitive for substrate aminoluciferin and noncompetitive for the second substrate ATP. Furthermore, compound 5 exhibited a good performance as a quenching agent in a dual-luciferase reporter assay. We anticipate that these Fluc inhibitors will contribute to the broader utilization of bioluminescence in life science research while circumventing or at least reducing the futile efforts on "false positives".

INTRODUCTION

Bioluminescence is the production and emission of light by a living organism, through natural enzyme-catalytic reactions. It occurs extensively in marine vertebrates and invertebrates, as well as in some fungi and microorganisms, including a number of bioluminescent bacteria and terrestrial invertebrates such as fireflies. Compared to chemiluminescence, the unique enzyme catalytic mechanism of bioluminescence makes it more convenient and efficient for detection, quantification, and application. Given that bioluminescence displays strong specificity, high sensitivity and has no background interference in bioassays, it has been widely used in biomedical, pharmaceutical, bioanalytical and bioimaging applications. Fluc is the most widely-used bioluminescence system up till now by catalyzing the oxidation of luciferin and emitting yellow to green lights with oxygen, ATP and magnesium ion acting as indispensable cofactors (Scheme 1).[1] In the first step, firefly luciferase catalyzes the reaction between luciferin and ATP, leading to formation of luciferin–adenylate conjugate, and

then the conjugate undergoes oxygenation, cyclization and forms dioxetanone anion (Dx⁻). Subsequently, the excited singlet state of OL [1(OL)*], a light emitter intermediate is generated. Upon the excited state 1(OL)* decay to the ground state oxyluciferin (OLH), a yellow to green bioluminescent light is produced. Firefly oxyluciferin (OLH), CO_2 and AMP are released at the same time. The glowing process can be inhibited by the two major products of the reaction, OLH and dehydroluciferyl-adenylate (L-AMP), which lead to the flash profile of firefly bioluminescence. Joaquim C. G. Esteves da Silva et al. demonstrated that OLH is a competitive inhibitor of luciferase (K_i = 0.50 ± 0.03 µM) while L-AMP act as a tight-binding competitive inhibitor (K_i = 3.8 ± 0.7 nM).[2] Besides, CoA can stabilize the light emission through thiolytic reaction between CoA and L-AMP, which gives rise to dehydroluciferyl-CoA (L-CoA), a much less powerful inhibitor.[3, 4] Firefly luciferase can also catalyze the synthesis of H_2O_2 from the same substrates when D-LH$_2$-AMP is oxidized into dehydroluciferyl adenylate (L-AMP).[5] The emission wavelength can vary from 530 to 640 nm, depending on parameters such as multiple intermolecular interactions (mostly hydrogen bonding, π–π stacking and electrostatic interaction), pH, solvent polarity, and the microenvironment of the enzyme. Luciferin, as the natural substrate of Fluc, can emit normal yellow to green light with a peak wavelength of 562 nm at neutral or alkaline pH, and red light peaked around 614 nm at acidic pH with low intensity and quantum yield. In 1966, White and McElroy claimed a modified substrate aminoluciferin (Scheme. 1) with red-shifted bioluminescence (596 nm) and a higher affinity than natural luciferin.[6] Up to now, luciferin and aminoluciferin are still the only two commonly used substrates

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in firefly luciferase research. The long wavelength is the advantage of Fluc application in bioluminescent imaging since it can penetrate into tissues up to 3 - 4 cm. This unique characteristic of the Fluc system enables its exceptionally functional applications both *in vitro* and *in vivo*, such as bioluminescent imaging, quantitative high throughput screening, luciferase reporter gene assay, diagnostic applications, and environmental monitoring.
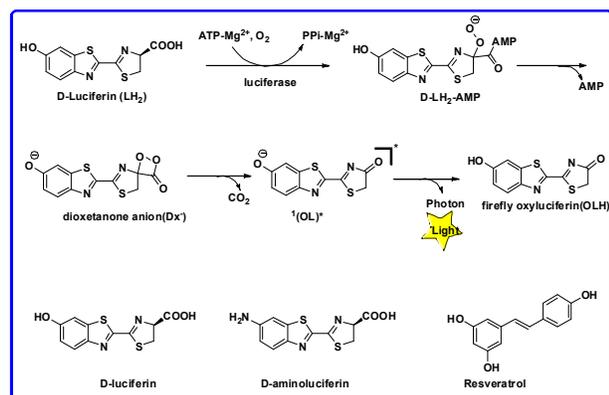
Scheme 1. Mechanism of firefly bioluminescence and structure of D-luciferin, D-aminoluciferin, and Resveratrol.

Despite its enormous contribution to molecular biology and pharmaceutical research, Fluc occasionally misleads our research directions to "false positives," especially in high-throughput screenings that rely on luciferase reporter assays. In 1965, Ueda *et al.* reported that general anesthetics inhibited the bioluminescence of purified firefly luciferase.[7] This phenomenon raised extensive research on external factors that might influence the luminescence process, since that might reduce the accuracy of bioassays. In 1998, Issaku Ueda et al. proved that fatty acids are specific inhibitors of firefly luciferase in competition with luciferin in micromolar ranges.[8, 9] In 2003, Neil D Perkins et al. observed a strong inhibition of reporter plasmids containing the firefly luciferase gene while investigating the effects of pifithrin-α on the transcriptional activity of NF-κB, pifithrin-α was proven to be inhibitor of firefly luciferase both *in vivo* and *in vitro*.[10] In 2006, Daniel M. Kemp et al. claimed that resveratrol (Structure shown in Scheme 1) can potently inhibit firefly luciferase activity with an IC$_{50}$ value of 2 μM, cautioning that some studies on resveratrol might be fundamentally flawed if based on luciferase reporter assays.[11] Ever since then, several more research groups suffered Fluc inhibitors oriented "false positives" in luciferase reporter gene-based assays. These Fluc inhibitors varied in structures, including the resveratrol structurally related NF-κB inhibitor (E)-2-fluoro-4'-methoxystilbene,[12] the drug candidate Ataluren,[13] N-(quinolin-2-yl) benzamides,[14] pyrrolo[2,3-d]pyrimidine analogues,[15] 5-benzyl-3-phenyl-4,5-dihydroisoxazoles and 5-benzyl-3-phenyl-1,4,2-dioxazoles,[16] natural compound with quinazolin-4(3H)-one core.[17] Some more Fluc inhibitors (*e.g.,* 2-phenylbenzothiazole,[18] pyrrolo [2,3-b] quinoxalines,[19] and aryl triazoles[20]) were also reported, along with their modes of action, mechanisms and

characteristics, In addition, some general anesthetics and alkanes are also reported to be Fluc inhibitors.[21-23] Fluc inhibitors and relative inhibition characteristics have been target for "false positives" discussion.[24, 25] As bioluminescence based reporter assays being more and more frequently used, Fluc inhibition and the modes of inhibition (MOI) raised extensive interests. Auld et al. reported novel MOI of Ataluren through the formation of multisubstrate adduct inhibitor (MAI), and further investigated the vital effect of its m-carboxylate on inhibitory potency.[13] They also demonstrated that the inhibitory activity can be relieved by free coenzyme A through promoting the thiolysis and dissociation of the MAI. Thorne et al. conducted a screening for Fluc inhibitors in NIH Molecular Libraries Small Molecule Repository among the 360,864 compounds, and found that more than 10% showed inhibitory potency against Fluc.[26, 27] Their structure-activity relationship analysis revealed that most of the inhibitors share similar scaffold, mostly being small, linear, and planar, commonly containing heterocyclic rings, e.g. thiazole, imidazole, oxadiazole, or pyridine ring. Albeit the inhibitors caused "false positives" trouble storm, they can be utilized in a dual-luciferase reporter assay kit (DLR, Promega Corporation, Wisconsin, USA). In the DLR Assay, the activities of firefly and Renilla luciferases are measured sequentially in a single sample. First, firefly luciferase reporter is measured by adding substrate luciferin to generate a luminescent signal. After quantifying the firefly luminescence, the bioluminescence is quenched, and the Renilla luciferase luminescence is initiated simultaneously by adding Stop & Glo Reagent to the same sample. Thus, Fluc inhibitors with good potency and high efficiency can be utilized as the quenching agents. Besides, Pekka K. Poutiainen *et al.* evaluated Fluc inhibitors as a versatile tool for real-time monitoring cellular uptake and trafficking of biomolecules.[28]

2-Phenylnaphthalenes are the scaffold of a marketed acne treatment medicine adapalene (differin),[29] and can be frequently found in pharmaceuticals, natural products, and agrochemicals.[30-33] This scaffold was also reported as a promising candidate for the treatment of cancer by targeting topoisomerase.[34-37] Moreover, 2-phenylnaphthalenes were reported to enhance estrogen receptor selectivity, thus may be therapeutically useful in treating certain chronic inflammatory diseases.[38] Hence, here in this paper we report a series of 2-phenylnaphthalenes as small molecule inhibitors of firefly luciferase to alert researchers of possible "false positives". We used resveratrol, a well-known potent firefly luciferase inhibitor, as the positive control to evaluate the activity of our compounds. The most potent compound 2-(4-(6-methoxynaphthalen-2-yl)phenyl)acetic acid (compound 5) inhibited firefly luciferase with IC$_{50}$ value of 0.13 μM *in vitro* and 10.8 μM *in cellulo*, which exceeded resveratrol in similar assays (2.38 μM *in vitro* and 29.6 μM *in cellulo*). Further *in vivo* assay of compound 5 (200 μM) showed 54.1% inhibition in xenografted balb/c-nu male mice by intratumor injection and 23.8% inhibition in CAG-luc-eGFP L2G85 transgenic mice by tail intravenous injection. To examine its kinetic features of inhibition, we conducted an enzyme kinetics assay to obtain K$_m$ and V$_{max}$ values under varying concentrations of inhibitor compound 5. Compared to the two major products of the

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reaction, OLH and L-AMP, which lead to the flash profile of firefly bioluminescence by competitive inhibition (K_i = 0.50 ± 0.03 µM) and tight-binding competitive inhibitor (K_i = 3.8 ± 0.7 nM) respectively,[2] our compound 5 was competitive for substrate aminoluciferin while noncompetitive for ATP. Compound 5 inhibited aminoluciferin with a K_i value of 0.12 µM, more potency than OLH. In addition, compound 5 could be utilized as a quenching agent in a dual-luciferase reporter assay and surpassed the respective component in the DLR commercial kit.

Results and Discussion

Chemistry

The synthesis route of 2- phenylnaphthalenes 4-14 is outlined in Scheme 2 using similar methods and experimental conditions published previously.[35] In brief, we started from the commercially available 2-bromo-6-methoxynaphthalene (1), and got 2-(6-methoxynaphthalen-2-yl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (2) in a very high yield through a palladium(0) catalyzed Suzuki coupling reaction with bis(pinacolato)diboron according to the method described by Takagi.[39] Through Suzuki cross-coupling reaction of 2-(6-methoxynaphthalen-2-yl)-4,4,5,5-tetramethyl-1,3,2-dioxaborolane (2) with the corresponding bromobenzene derivatives (3a-g) and hydrolysis of the ester bond, we got compounds 4-13. Ether cleavage was performed using boron tribromide to afford corresponding compounds 14. More synthetic details can be found in the Supporting Information.
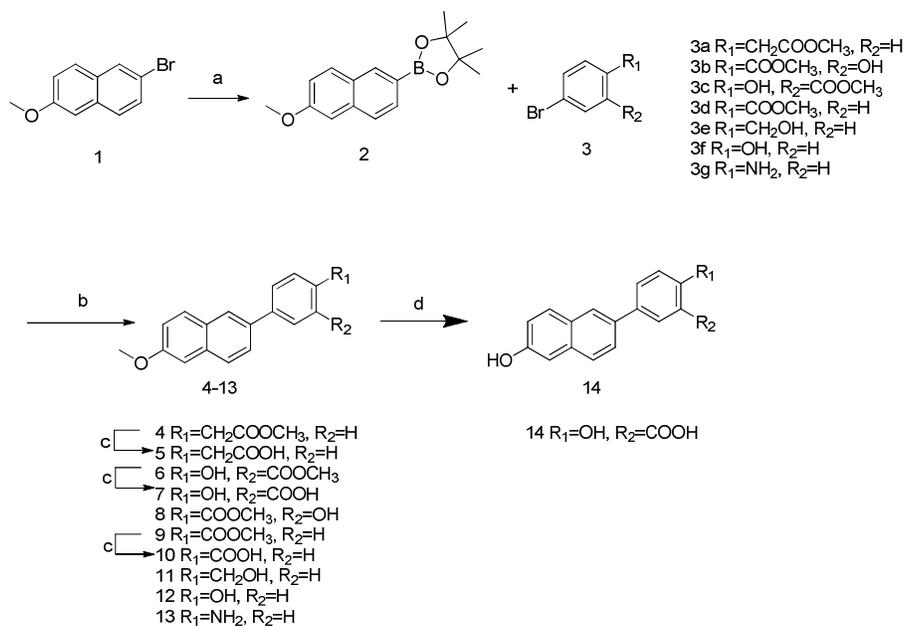
Scheme 2. Synthesis of 2-phenylnaphthalenes 4-14. Reagents and conditions: (a) bis(pinacolato)diboron, potassium acetate, PdCl₂(dp.pf), dioxane, 60 °C, 6 h. (b) potassium fluoride PdCl₂(dppf), dioxane, 90 °C, 12h. (c) NaOH, EtOH, reflux then HCl. (d) Boron tribromide, DCM, -78 °C, 12 h.

In vitro inhibition assays

We conducted a preliminary screening of 11 compounds. All compounds were evaluated for their inhibitory activity on QuantiLum recombinant firefly luciferase using the initial concentrations of 10 and 100 µM. Among them, five compounds showed more than 50% bioluminescence inhibition under the concentration of 10 µM. The inhibitory results are shown in Fig 1A. To confirm their inhibitory potency, we further used increasing concentrations of the compounds from 1 nM to 100 µM to obtain the accurate concentration–response curves (CRCs) to determine their IC_{50} values. Fig. 1B shows the concentration–response curves (CRCs) of compounds with IC_{50} < 10 µM. Results are summarized in Table 1: all of five compounds showed significantly enhanced inhibitory potency when compared to the positive control resveratrol. Among them, compound 5 is the most potent with

an IC_{50} value of 0.13 µM, approximately 20 fold more potent than the positive control resveratrol (IC_{50} = 2.38 µM). Next, we evaluated their inhibitory activity in cellulo using ES-2-Fluc cells (a human ovarian cancer cell line transfected with firefly luciferase expressing gene). We incubated increasing concentrations of inhibitors with ES-2-Fluc cells for 12 h, and then tested their bioluminescence intensity using a Caliper IVIS Kinetic in vivo optical imaging system (Caliper Life Sciences, now PerkinElmer, USA) equipped with a cooled charge-coupled device (CCD) camera for bioluminescence imaging. The corresponding CRCs are shown in Fig 1C. As shown in Table 1, compound 5 exhibited high potency with an IC_{50} value of 10.8 µM, about 3-fold more potent than resveratrol (IC_{50} = 29.6 µM). For compounds 7 and 10, their cellular inhibitory activities are almost comparable to resveratrol. For compounds 6 and 14, their inhibitory activities are less potent,

possibly due to poor membrane penetration ability. Structural-activity relationship (SAR) analysis reveals that the carboxyl acid on the benzene ring is essential for maintaining a strong inhibitory activity. Substitution of the benzene with hydroxyl group (*e.g.,* compounds 11 and 12) or amino group (*e.g.*, compound 13) abrogated its inhibitory activity. Protection of the carboxyl acid with a methyl ester (*e.g.,* compounds 4, 6, 8 and 9) also led to substantially reduced efficacy. The hydroxyl group on the naphthalene ring is not favorable, while introducing a methyl group to protect the hydroxyl group (e.g., compound 7 *vs* compound 14) can enhance the potency both in enzymatic level and in cellular assay.
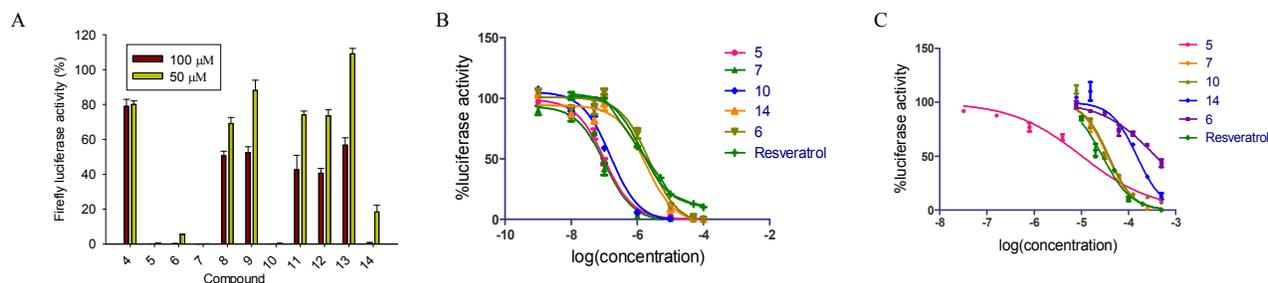

Fig 1. (A) Recombinant firefly luciferase inhibition assay initial screening. Inhibition potencies of all the compounds at concentrations of 10 and 100 µM were evaluated in the initial screening. (B) Concentration–response curves for active compounds in recombinant firefly luciferase inhibition assay; (C) CRCs for active compounds *in cellulo*; Representative graphs are chosen from one experiment performed in triplicate.

Table 1. Firefly luciferase enzymatic and cellular inhibition activity of 2- phenylnaphthalenes[a].

General structure:

Compounds	R_1	R_2	R_3	Enzymatic IC$_{50}$ (µM)[a]	Cellular IC$_{50}$ (µM)[a]
5	-CH$_3$	-CH$_2$COOH	-H	0.13±0.05	10.8±2.49
7	-CH$_3$	-OH	-COOH	0.14±0.05	28.2±6.84
10	-CH$_3$	-COOH	-H	0.22±0.08	47.4±8.41
14	-H	-OH	-COOH	0.87±0.62	146±17.5
6	-CH$_3$	-OH	-COOCH$_3$	2.26±0.04	331±54.3
Resveratrol				2.38±0.08	29.6±2.62

[a]Assays were performed in triplicate (n≥3); values are shown as mean±SD.

To rule out the possibility that the observed inhibitory potency *in cellulo* might be due to cytotoxicity, we conducted an MTT cell viability assay to evaluate the cytotoxicity of our compounds. Briefly, ES-2-Fluc cells were incubated with different concentrations of compounds (500 µM, 250 µM, 125 µM, 62.5 µM) for 12 hr before the cell viability measured by MTT method. Figure 2 displayed that the compounds 5, 10 and 14 were nontoxic to ES-2-Fluc cells when concentrations were below 250 µM. Compounds 6 and 7 presented low toxicity at 250 µM. Besides, the compounds only absorbed light below 320 nm (absorbance spectra of compound 5 shown in supporting information), thus making it less likely to interfere with the visible light of firefly luciferase bioluminescence.
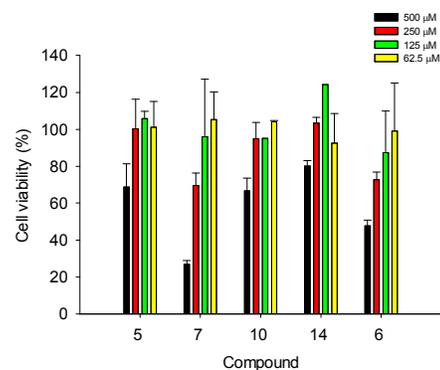

Fig 2. The viability of ES-2-Fluc cells after incubation with various concentrations of compounds.

In vivo inhibition assay by intratumor injection

Since the bioluminescence-based assay is widely used both *in vitro* and *in vivo*, we further evaluated inhibitory activity of compound 5 in well-established mouse xenograft tumor bioluminescence imaging models.[40-42] To avoid the individual variation of mouse, we tested the bioluminescence signal of total flux (photons/s/cm^2/steradian) by injecting aminoluciferin intraperitoneally into the mouse on the first day, and set it as the calibration value. Afterward, 12 hr were left for the mouse to metabolize away the aminoluciferin. After that, we injected inhibitors (200 µM in sterile normal saline) into the tumor and waited for another 12 hours before measuring its bioluminescence signal of the total flux (photons/s/cm^2/steradian) again. Bioluminescence imaging of the mice before and after compound inhibition is shown in Fig.

3A. The relative activity was calculated by bioluminescence total flux ratio of day 2 to day 1. For the normal saline group, we injected an equivalent amount of sterile saline as a blank group. The firefly luciferase residual activity was displayed in Fig 3B. Due to the 24 hr growth of the tumor, we can see that the blank normal saline group suffered an increase of 135.2% in total flux. Therefore, we calculated residual total flux percentage by the ratio of comparing the relative activity of inhibition group with the saline group, setting the saline group as 100%. As shown in Fig 3B, only 54.1% bioluminescence remained in the mouse injected with compound 5, while 67.0% left in the mouse injected with resveratrol. Notably, our compound 5 (200 µM) showed the inhibitory activity of 45.9% *in vivo*.
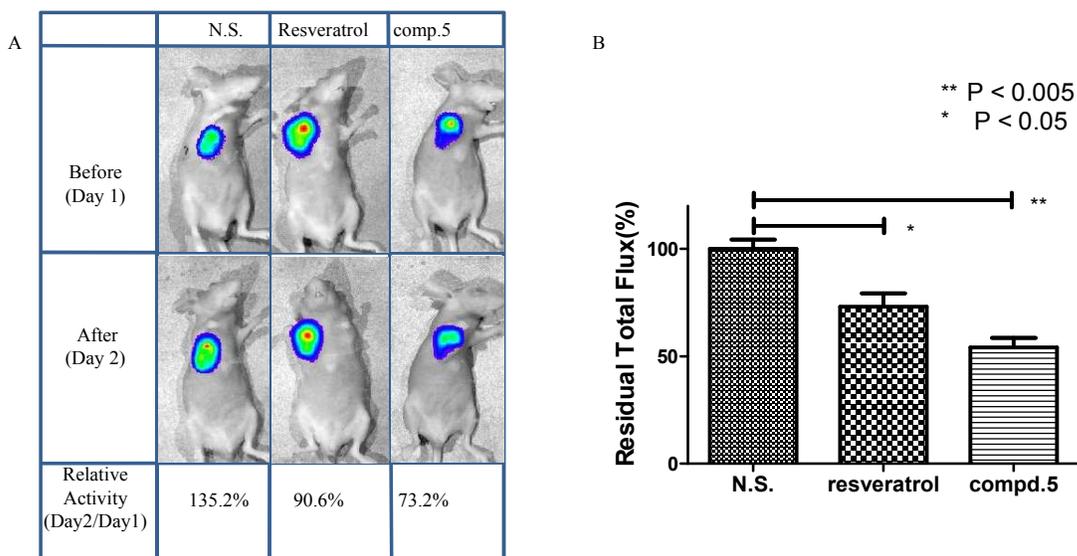

Fig 3. (A) Representative bioluminescence imaging of inhibition in xenografted tumors in nude mice. Relative activity was calculated by ratio of total flux of day 2 to day 1. (B) Quantification of residual total flux percentage, calculated by ratio of relative activity of inhibitors group to normal saline group. Inhibition rate of resveratrol and compound 5 can be calculated by 100%-residual total flux (%), which means 45.9% and 23.0% inhibition, respectively. ** < 0.005, * < 0.05 (t- test, calculated by GraphPad Prism software).

In vivo inhibition assay in transgenic mice by tail intravenous injection

Although compound 5 showed 45.9% inhibition in xenograft tumor in mouse, it is still not sure whether compound 5 can exert systemic inhibition effects in the body. Therefore, we further evaluated its potency in CAG-luc-eGFP L2G85 transgenic mice by tail intravenous injection. The transgenic

mice harboring the CAG-luc-eGFP L2G85 transgene exhibit widespread expression of firefly luciferase directed by the CAG promoter. As shown in Fig 4, our compound 5 demonstrated 23.8% systemic inhibition while resveratrol exhibited only 13.3% inhibition. This evidence indicates that compound 5 displayed better inhibition behavior than resveratrol *in vivo*.
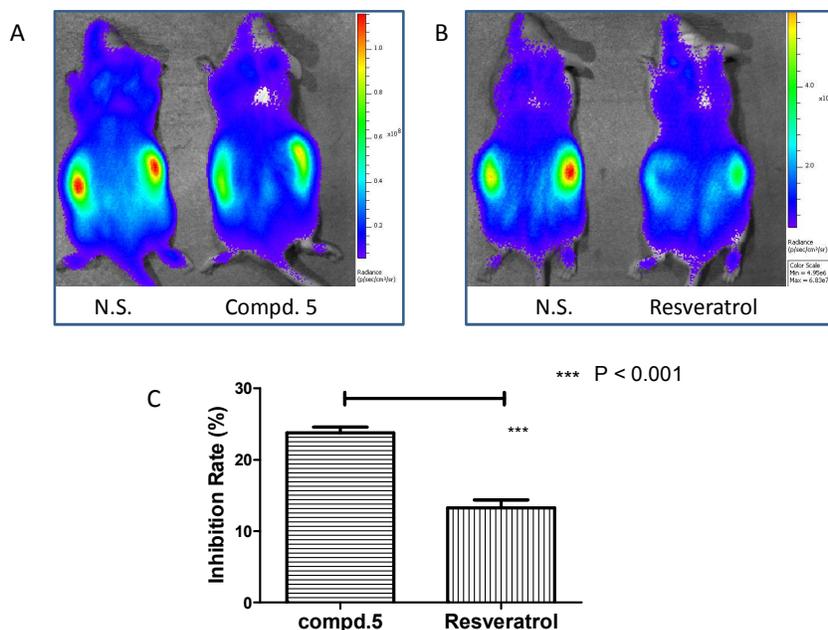
Fig 4. (A) Representative bioluminescence imaging of inhibition by compound 5 compared to normal saline in transgenic mice; (B) Representative bioluminescence imaging of inhibition compared to normal saline by resveratrol in transgenic mice; (C) Quantification of inhibition rate by compound 5 and resveratrol. *** < 0.001 (t- test, calculated by GraphPad Prism software).

Kinetics assay

We chose the most potent compound 5 for further analysis on kinetic parameters. The Michaelis-Menten kinetics parameters K_m and V_{max} of aminoluciferin and ATP in the absence and presence of increasing concentrations of compound 5 were examined (Fig. 5). First of all, we fixed the concentration of ATP at its K_m, and measured the enzyme activity against increasing concentrations of aminoluciferin after inhibition by compound 5 (Fig. 5A). Then, we fixed the concentration of aminoluciferin at its K_m, and measured the same way for ATP (Fig. 5C). By Lineweaver–Burk plot (Fig. 5B and 5D), we get Michaelis–Menten parameter K_m and V_{max} to analyze the inhibition mode for aminoluciferin (Table 2). For substrate aminoluciferin, compound 5 caused a significant increase in K_m in a dose-dependent way, while the V_{max} remains unaltered (Table 2), which usually arises from the inhibitor having an affinity for the active site of an enzyme where the substrate also binds. The substrate and inhibitor compete for access to the enzyme's active site. For the second substrate ATP, the K_m value remained almost unaltered while V_{max} obviously decreased in a dose-dependent manner. This phenomenon reveals a noncompetitive inhibition mode for ATP. The K_i value can be obtained from the IC_{50} values and kinetics data using the Cheng-Prusoff equation. Compound 5 potently inhibits firefly luciferase with a K_i value of 0.12 μM. Kinetics data in low

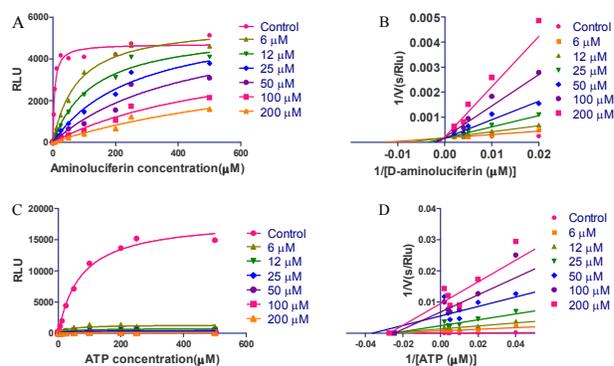inhibition concentration was shown in supporting information (Fig. S1 and Table S1).

Fig 5. Kinetics of inhibition of luciferase by compound 5. (A) aminoluciferin saturation assay with increasing concentrations (6, 12, 25, 50, 100 and 200 μM); (B) a Lineweaver–Burk plot of data in (A); (C) ATP saturation assay with increasing concentrations (6, 12, 25, 50, 100 and 200 μM); (D) a Lineweaver–Burk plot of data in (C). The lines of (A) and (C) are fitted to Michaelis–Menten assay using GraphPad Prism 5 software. The Lineweaver–Burk plots are estimated using GraphPad Prism software.

Table 2. Kinetic paramaters V_{max} and K_m of substrate, aminoluciferin and ATP.

	Concentration (μM)	no inhibitor	6 μM	12 μM	25 μM	50 μM	100 μM	200 μM
Amino-luciferin	V_{max} (Rlu/s)[a]	4611±100	5352±359	5459±12	6366±320	5691±416	4706±238	4824±148
	K_m (μM)[a]	5.06±0.33	71.6±6.80	126±5.60	313±39.4	392±57.8	557±61.6	919±20.4

		N.D.[c]	0.12	0.12	0.12	0.12	0.11	0.11
	K_i^b	N.D.[c]	0.12	0.12	0.12	0.12	0.11	0.11
ATP	V_{max} (Rlu/s)[a]	13379±55	1374±34	793±59.4	426±8.35	221±40.0	160±13.6	20±14.6
	Km (μM)[a]	48.7±5.44	33.8±1.86	42.1±6.98	33.0±6.41	29.9±6.41	38.7±1.92	39.4±2.66

[a] Michaelis constant V_{max} and maximum rate K_m were estimated with Michaelis–Menten kinetics equation using GraphPad Prism software. Values are showed by means ± SD of three independent assays performed in duplicate.

[b] K_i values were calculated by the Cheng-Prusoff equation.[43]

[c] N.D.: Not Determined since K_i value is a constant for inhibitors.

Dual-Luciferase reporter gene assay simulation

During the course of enzymatic inhibition assays, we found that our compounds inhibited firefly luciferase in an extraordinarily rapid way. The compounds reached its uppermost potency upon exposed to firefly luciferase within 30 seconds. Therefore, we designed a dual-luciferase reporter assay simulation, and used resveratrol and our compound 5 as a quenching agent. The assay requires a mixture of firefly luciferase (Fluc) and renilla luciferase (Rluc). First of all, firefly luciferase substrates aminoluciferin and ATP were added into luciferase mixture to initiate the firefly bioluminescence. Then, a mixture of inhibitor (50 μM) and Rluc substrate coelenterazine (5 μM) was added to quench firefly luminescence and initiate renilla bioluminescence. Bioluminescence of both was measured immediately at wavelength 590 nm and 460 nm, emitted by Fluc and Rluc, respectively. As shown in Fig 6, compound 5 eliminated about 97% of the firefly luciferase bioluminescence without significant influence on Rluc bioluminescence, while being compatible with Rluc substrate coelenterazine. For resveratrol, 5.64% Fluc bioluminescence remained. Besides, resveratrol also eliminated 33.4% Rluc bioluminescence. Thus, compound 5 showed potential as promising Fluc quenching agent in the combined application of bioluminescence systems.
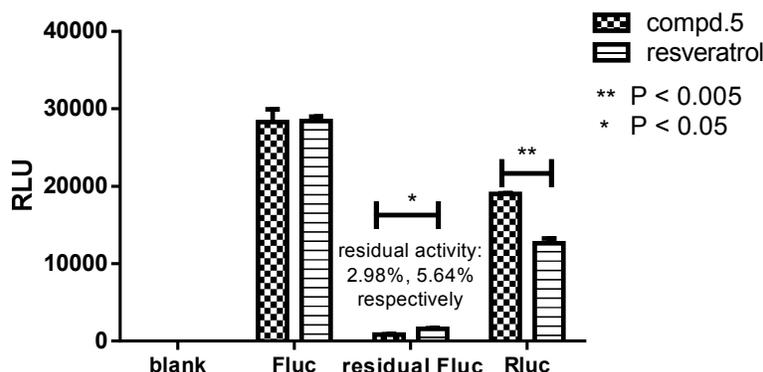

Fig 6. Dual-luciferase reporter assay simulation. Residual activity was calculated by RLU ratio of residual Fluc to Fluc. ** < 0.005, * < 0.05 (t- test, calculated by GraphPad Prism software).

Conclusion

In summary, here we disclosed a series of 2-phenylnaphthalenes compounds as firefly luciferase inhibitors. Compound 5 inhibited firefly luciferase with an IC$_{50}$ value of 0.13 μM *in vitro* and 10.8 μM *in cellulo*. Besides, kinetic assay indicates our compound 5 is competitive inhibitor with K_i value of 0.12 μM, more potent than OLH (K_i = 0.50 μM). In addition, compound 5 (200 μM) showed 45.9% inhibition *in vivo* on well-established mouse xenograft tumor models by intratumor injection. Besides, compound 5 showed 23.8% inhibition in transgenic mice by tail intravenous injection, indicating it can exert systemic inhibition. Surprisingly, the compound could exert its inhibition in an extremely rapid way upon exposure to firefly luciferase, or even to the glowing firefly luciferase-luciferin mixture in a dual-reporter assay. Notably, small molecules with the similar framework are ubiquitous in various compounds libraries designed for high throughput screening.

These results raise cautions in "false positives" for those researchers employing firefly luciferase-based quantitative high throughput screenings. Additionally, the ultra-fast and potent inhibition of our designed compounds can contribute to the crossed or combined application of the firefly luciferase system with other bioluminescence systems, *e.g.,* renilla luciferase.

Acknowledgements

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