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CONCISE ARTICLE

Synthesis and biological evaluation of a series of aryl triazoles as firefly luciferase inhibitors

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Haixiu Bai,[a] Peng Zhu,[a] Wenxiao Wu,[a] Jing Li,[a] Zhao Ma,[a] Wei Zhang,[a] Yanna Cheng,[b] Lupei Du[a] and Minyong Li[a*]

[a] *Department of Medicinal Chemistry, Key Laboratory of Chemical Biology (MOE), School of Pharmacy, Shandong University, Jinan, Shandong 250012, China.*
[b] *Department of Pharmacology, School of Pharmacy, Shandong University, Jinan, Shandong 250012, China.*

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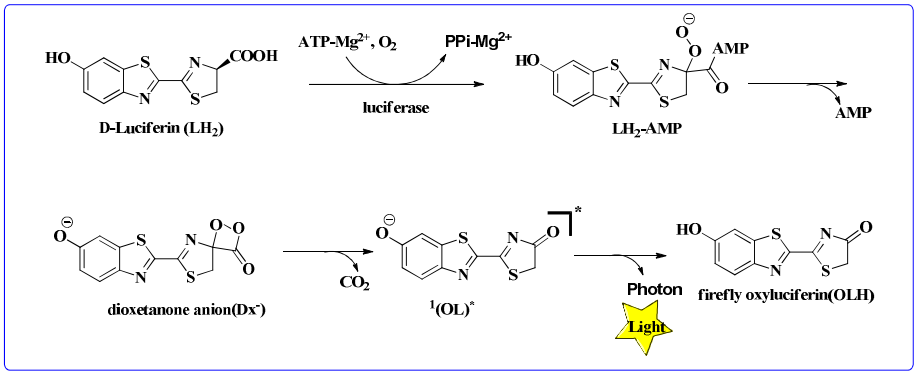
As the most studied bioluminescent system, firefly luciferase is widely applied in many aspects, such as developing small molecule probes, bioluminescent imaging, high-throughput screening, dual luciferase reporter, etc. Considering that a false positive phenomenon often emerges while researchers conduct high-throughput screening based on firefly luciferase, and that the triazole core is a "privileged" scaffold in drug design and development, we herein report a series of triazoles with potent inhibitory activity in vitro and in vivo, comparable to that of the well-known inhibitor resveratrol. More interesting, a kinetics study disclosed that these triazoles exhibited a brand new inhibition mode, mixed noncompetitive for substrate aminoluciferin while noncompetitive for ATP. Henceforth, these compounds can notify researchers for possible "false positives". Moreover, they will shed light on luciferase structure–function mechanistic exploration and help expand its application in various areas.

Introduction

Bioluminescence is the production and emission of visible light by a chemical reaction within a living organism. It occurs widely in marine vertebrates and invertebrates, as well as in some insects, fungi, microorganisms and terrestrial invertebrates. Firefly luciferase, as the most studied bioluminescent enzyme, can catalyze the oxidation of luciferin and emit yellow to green lights upon a two-step reaction. In such a firefly luciferin–luciferase system, oxygen, ATP and magnesium ions are necessary as co-factors. In the first step, firefly luciferase catalyzes the reaction between luciferin and ATP to produce a luciferin–adenylate conjugate, and then the conjugate undergoes oxygenation and cyclization to form a 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, as well as oxyluciferin (OLH), CO_2 and AMP (**Scheme 1**).[1] The wavelength of the bioluminescent light can range from 530nm to 640 nm, depending on multiple intermolecular interactions (mostly hydrogen-bonding, **π–π** stacking and electrostatic interaction), polarity of the solvent, pH and the microenvironment of the enzyme etc. The unique characteristics of the firefly bioluminescence system can ensure its extensive application in many areas, such as bioluminescent imaging, quantitative high-throughput screening (qHTS), luciferase reporter gene assay, detection of ATP, etc.

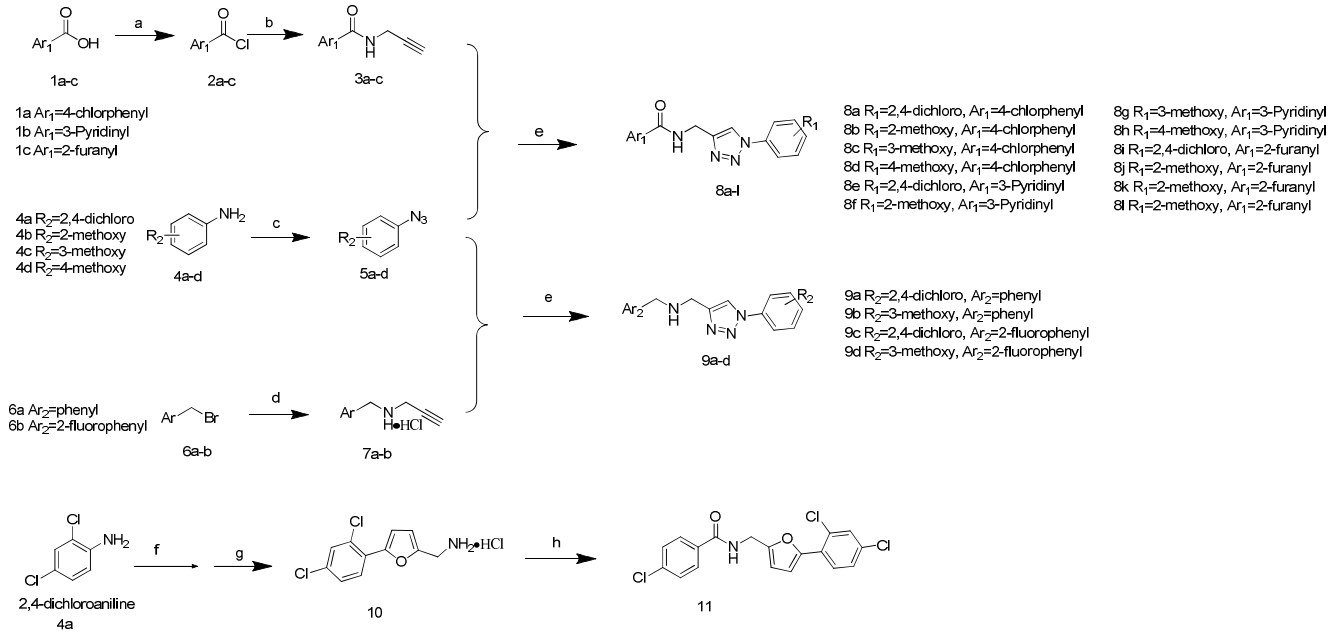


Scheme 1. Mechanism of firefly bioluminescence.

Despite the prevalent applications, there are still a few drawbacks and limitations in luciferase-luciferin system. One case is quantitative high-throughput screening (qHTS) assay based on luciferase bioluminescence, which has become widely used in chemical biology and drug discovery applications. In 2006, D.M. Kemp claimed that resveratrol can potently inhibit firefly luciferase activity with a K_i value of 2 μM, cautioning researchers that previous study about resveratrol showing therapeutic value in various field might be fundamentally flawed since they all utilized the firefly luciferase assay.[2] These interesting results somewhat explain the "false positives" that stand out as "promising compounds" through the bioluminescence-based qHTS but turn out to be false alert in further bioassay. Ever since then, several research groups reported their "false positives" with potent firefly luciferase inhibitory activity, in which most of the inhibitors are small molecules with rigid structures, commonly containing a heterocycle moiety, such as thiazole, imidazole, oxadiazole, or pyridine ring. [3-9]

In the current article, we report a series of novel aryl triazole derivatives (Table 1) with firefly luciferase inhibitory activity both in vitro and in vivo. It's well known that triazole core has been generally considered as a "privileged" scaffold for a variety of drug candidates, including antivirals, antifungal agents, H_1/H_2 histamine receptor blockers, cholinesterase active agents, etc., and as a result, it has been widely used in many therapeutic agents, such as fluconazole and ribavirin. In a high throughput screening that relies on firefly luciferase reporter-gene assay, small molecular inhibitors of firefly luciferase, as these triazoles, are extremely likely to pop out as "hits". Nevertheless, these "hits" could turn out to be "false positives" when further evaluated with other bioassays. According to our preliminary structure-activity relationship analysis, the triazole structure is a valid inhibition core for firefly luciferase. Therefore, identification of these firefly luciferase inhibitors may help us to avoid futile effort in the initial screening.

Moreover, considering that some of our triazole compounds demonstrated good inhibitory potency in vitro, in cellulo and in vivo, they may be applied as quenching reagents in firefly luciferase-based assays, such as dual luciferase reporter assays. These compounds' inhibition modes through kinetics assay were well examined as well. In brief, these triazoles exhibited a novel inhibition mode compared to those inhibitors reported before. The inhibition is mixed noncompetitive for substrate luciferin while noncompetitive for ATP. This indicated that besides competing with luciferin for the active site, these triazoles might have yet another way to inhibit the bioluminescence. Also, since many triazoles are reported as free radical scavengers,[10, 11] and the intermediate in bioluminescent luciferin-luciferase undergoes dioxetanone anion radical form, we examined if our triazole inhibitors could quench the bioluminescence through the DPPH free radical scavenging assay. Hopefully, our efforts on biological activity and mechanism search will help for further investigation onto the luciferase-catalyzed systems and further expand its application in many areas.



Scheme 2. Synthesis of triazoles **8a-l** and **9a-d**. Reagents and conditions: (a) $SOCl_2$, toluene, 90 °C; (b) 3-aminopropyne, CH_2Cl_2, 0 °C; (c) HCl/$NaNO_2$, H_2O; NaN_3; (d) 3-aminopropyne, 0 °C; HCl/EtOH; (e) **5a-d**; $CuSO_4 \cdot 5H_2O$, sodium L-ascorbate, EtOH, *t*-BuOH, H_2O. Synthesis of compound **11**. Reagents and conditions: (f) HCl/$NaNO_2$, 0 °C; (g) $CuCl_2$, furfurylamine, room temperature; (h) 4-chlorobenzoyl chloride **4a**, Et_3N, 0 °C

Results and discussions:

Chemistry:

The synthesis of triazoles **8a-l**, **9a-d** and aryl furan compound **11** was outlined in **Scheme 2**. In brief, triazole compounds **8a-l** and **9a-d** were synthesized through Cu(I)-catalyzed azide-alkyne click chemistry in a one-pot reaction from appropriate azidobenzenes **5a-d** and substituted N-benzyl-2-propynyl amines **3a-c** and **5a-b** in mixture solvent of *tert*-butyl alcohol and water in the absence of light.[12] Azidobenzenes **5a-d** were prepared from corresponding anilines, first forming diazonium salt upon reaction with $NaNO_2$ in acid condition, and then followed by substitution by NaN_3. Substituted N-benzyl-2-propynyl amines **3a-c** were easily prepared from aromatic acids **1a-c** through chloridization and amidation in an ice-salt bath. Intermediates **7a-b** were conveniently prepared from benzyl bromides **6a-b** through nucleophilic substitution by 2-propynylamine in an ice-salt bath.

Compound **11** was synthesized starting from 2,4-dichloroaniline in three steps. First of all, 2,4-dichloroaniline turned into diazonium salt upon reaction with $NaNO_2$ in an acid condition, then the diazonium salts reacted with furfurylamine through catalyst $CuCl_2$ by forming (5-(2,4-dichlorophenyl)furan-2-yl) methanamine **10**. Finally, compound **11** was produced by compound **10** reacting with 4-chlorobenzoyl chloride using triethylamine as the base in tetrahydrofuran in an ice bath.

Biological evaluation

Inhibitory assay in vitro

In the preliminary screening, all compounds were evaluated for their inhibitory activity on purified recombinant firefly (*Photinus pyralis*) luciferase. We tested the inhibitory activity of increasing concentrations of the compounds to get concentration−response curves (CRCs) to determine their IC_{50} values. Figure **1a** shows the concentration−response curves (CRCs) of compounds with IC_{50} < 100 μM. As shown in Table 1, most of the triazoles showed moderate to good inhibitory activity. Among them, compound **8a** showed the best potency with an IC_{50} value of 3.28 μM, comparable to that of the positive control resveratrol. Compound **11** with a furan ring instead of triazole ring was inactive.

Since bioluminescence-based assay is widely used in cellulo and in vivo, we further evaluated their inhibitory activity in ES-2-Luc cells (human ovarian cancer cell line transfected with firefly luciferase). We incubated increasing concentrations of inhibitors with ES-2-Luc cells for 12 hr, and then tested their bioluminescence intensity using an IVIS Kinetic (Caliper Life Sciences, USA) equipped with a cooled charge coupled device (CCD) camera for bioluminescent imaging. As we can see in fig 1b, for compounds 8c, 9c, 9d and 9b, their inhibitory activity remained in cellular. However, some of the compounds with fine inhibitory potency in enzyme level, including the most potent compound 8a, only showed negligible to none potency in cellular (8i, 8e, 8k, 8g, 9a). Irrational lipo-hydro partition coefficient logP might be the reason for loss of potency. We used Chembiodraw ultra 12.0 software to predict clogP of these compounds. As we can see in Table 1, for compounds that retained potency in cellulo, their clogP range from 3.06 to 3.38, with only one exception 9c. Nevertheless, for compounds that lost potency, their clogP turned out to be either larger than 4.0 (8a and 9a) or lower than 2.8 (8i, 8e, 8k, 8g). Thus, we can ascribe the loss of potency to poor permeability in cell membrane caused by inappropriate lipo-hydro partition coefficient.

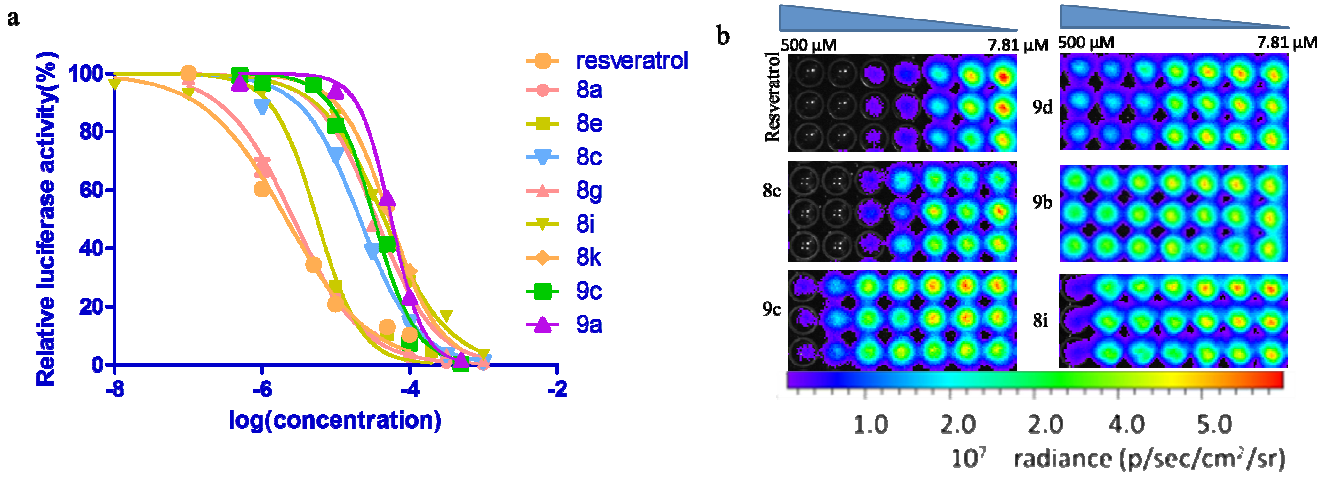
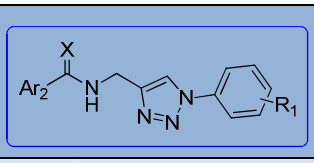
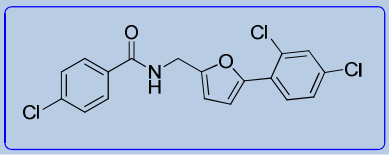
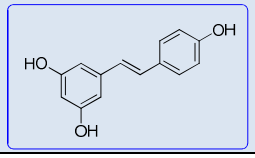


Fig 1. a) Concentration−response curves for active compounds in recombinant firefly luciferase inhibition assay; b) Bioluminescence imaging of ES-2-Luc cells incubated with increasing concentrations of inhibitors. For each concentration, three wells in vertical are treated the same as parallel group. Representative graphs are chosen from one experiment performed in triplicate.

Table 1 Firefly luciferase enzymatic and cellular inhibition activity of novel triazoles [a]

General Structure: 

compounds	R_1	Ar_2	X	Enzymatic IC_{50} (μM)[a]	Cellular IC_{50} (μM)[a]	clogP
8a	2,4-dichloro	4-chlorphenyl	O	3.28±0.43	>200	4.61
8b	2-methoxy	4-chlorphenyl	O	>100	>200	3.37
8c	3-methoxy	4-chlorphenyl	O	22.3±1.10	55.6±4.02	3.37
8d	4-methoxy	4-chlorphenyl	O	>100	>200	3.37
8e	2,4-dichloro	3-pyridinyl	O	6.65±0.78	>200	2.72
8f	2-methoxy	3-pyridinyl	O	>100	>200	1.47
8g	3-methoxy	3-pyridinyl	O	33.2±2.52	>200	1.47
8h	4-methoxy	3-pyridinyl	O	>100	>200	1.47
8i	2,4-dichloro	2-furanyl	O	47.9±2.26	>200	2.67
8j	2-methoxy	2-furanyl	O	>100	>200	1.43
8k	3-methoxy	2-furanyl	O	56.6±2.44	>200	1.43
8l	4-methoxy	2-furanyl	O	>100	>200	1.43
9a	2,4-dichloro	phenyl	H,H	53.3±3.25	>200	4.62
9b	3-methoxy	phenyl	H,H	>100	190±3.90	3.38
9c	2,4-dichloro	2-fluorophenyl	H,H	44.1±4.72	102±3.74	4.78
9d	3-methoxy	2-fluorophenyl	H,H	>100	102±3.74	3.53
11				>100	>200	4.93
Resveratrol				2.27±0.12	30.1±3.21	3.06

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

Preliminary structure-activity relationship can be summarized as following: (a) compound 11 with a furan ring instead of triazole core completely lost potency. This indicates that the triazole core is essential for inhibitory potency, changing it to furan ring will lead to thoroughly loss of potency; (b) the amide linker between the aryl ring Ar_2 and triazole core is crucial for inhibitory activity, probably by restricting the flexibility of the molecular, which is in accordance with the previous reported findings. In this case, our compound 9a, 9c, 9b and 9d proved to be much less potent than 8a, 8e, 8c and 8g; (c) introduction of electron withdrawing group at the phenyl ring is favourable. Compounds with electron withdrawing group like 2,4-dichloro (8a, 8e) or 3-methoxy (8c, 8g) proved to be much more potent than corresponding compounds with electron donating group 2-methoxy (8b, 8f) or 4-methoxy (8d, 8h). (d) Replace the aromatic ring Ar_2 with a more electron rich ring, e.g. a foran ring (8i, 8j, 8k, 8l) will lead to tremendously loss of potency. Correspondingly, introduce of electron-withdrawing group in Ar_2 (9c) will help to improve potency compared to 9a.

Cell viability Assay

To rule out the possibility that the decrease in bioluminescence might also be caused by cell death through incubation with our compounds, we tested ES-2-Luc cell viability of all compounds after 12-hour incubation with 500, 250, 125 and 62.5 μM of compounds 8c, 9c, 9d and 9b, which showed good activity in cellulo. The cell viability was

evaluated by using MTT assay according to standard protocol. Figure 2 revealed that cells suffered only slight damage at 500 μM. As a result, our compounds did not cause any significant damage in cell viability at < 250 μM concentrations.

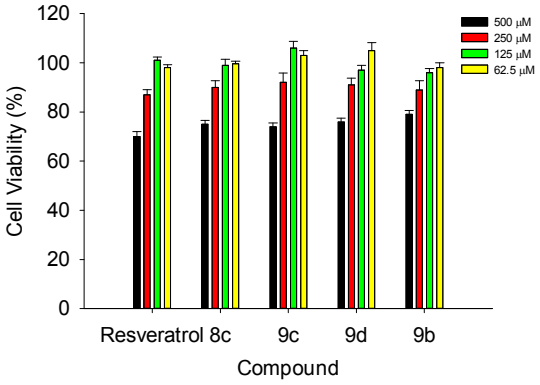


Fig 2. Viability of ES-2-Luc cells after incubation with various concentrations of compounds.

In vivo inhibition assays

Subsequently, compound 8c, most potent both in enzyme level and in cellular were chosen for inhibition assay in xenografted balb/c-nu male mice. To avoid the individual variation of mouse, we tested the bioluminescence as baseline by injecting 100μL of luciferin (0.5 mM) intraperitoneally into the mouse in the first day, and then give the mouse 12 hours to metabolize away the luciferin. After that, we injected 25 μL of inhibitor 8c or resveratrol (5 mM) into the tumor and waited for another 12 hours, then we injected luciferin intraperitoneally into the mouse to measure its bioluminescence again.[13] For normal saline group, we injected an equivalent amount of sterile saline instead of inhibitors as blank group. The sterile saline was prepared containing 15% DMSO, as inhibitors group, to eliminate influence caused by DMSO. Due to the 24-hr growth of the tumor, we can see that the blank normal saline group suffered an increase of 146.13% in total bioluminescence. Therefore, we calculated inhibition rate by comparing with the saline group. As shown in Fig 3, compound 8c displayed good inhibitory activity with 38.31% inhibition compared to that of positive control resveratrol 57.66% inhibition. These interesting results suggest that our compound 8c presented reasonable bioluminescence quenching behavior in vivo. To avoid the artificial influence, for the same mouse before and after injection with inhibitors, we calculated its bioluminescence intensity using ROIs of the same size. For some of the mice that developed two tumors in close proximity, we chose the bigger one for intratumor injection. As turned out in Figs 3b-c, the bioluminescence signal of the bigger tumor attenuated, while the small one changed little. We can assume that the 25 μL inhibitors intratumorly injected targeting the bigger tumor did not reach the small one. Therefore, we set the ROIs as circles that surrounded only the bigger tumor instead of the whole one. By comparison, the inhibitory effect is quite obvious.

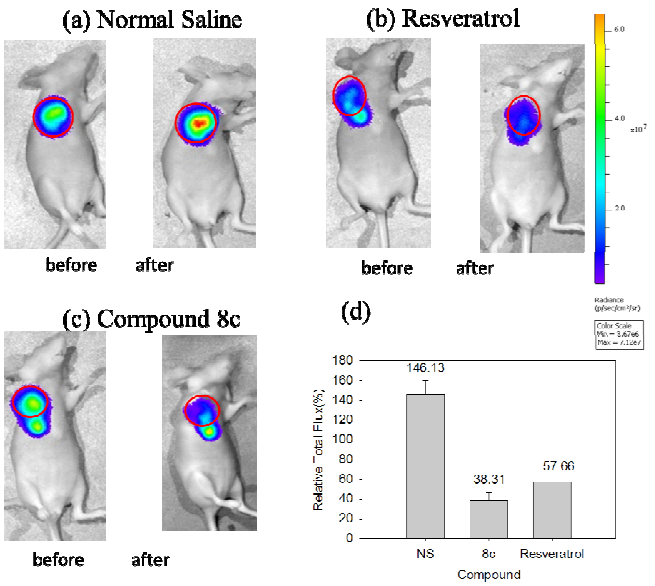


Fig 3. Bioluminescence imaging of inhibitory activity in xenografted tumors in nude mice. (a) Bioluminescence imaging of normal saline as blank group. (b) Bioluminescence imaging of resveratrol inhibition activity. (c) Bioluminescence imaging of compound 8c inhibition activity. (d) Quantification of relative total flux. Representative graphs are chosen from one experiment performed in triplicate. ROIs were drawn over individual wells. Error bars are SD for triplicated measurements

Enzyme inhibition kinetics assay.

We chose the most potent inhibitor 8a to investigate its kinetic characteristics of inhibition. First of all, we fixed the concentration of ATP constant at 1 mM, and tested the enzyme activity against increasing concentrations of luciferin after inhibition by compound 8a (Fig 4a). Then, we fixed the concentration of luciferin at 500 μM, and measured the same way for ATP (Fig 4c). By Lineweaver–Burk plot (Figures 4b and 4d), we get Michaelis–Menten parameters K_m and V_{max} to evaluate the inhibition mode for luciferin (table 2). The inhibition of enzyme by 8a caused luciferin a significant increase in K_m and a decrease in V_{max} in a dose-dependent way. This indicates a typical mixed noncompetitive inhibition, suggesting the inhibitor not only binds into the active site of luciferase to compete with aminoluciferin, but also might have some other way to quench the bioluminescence, possibily by interfering with the luciferase-luciferin complex or aminoluciferin–adenylate conjugate. Another possibility is that the inhibitor can also bind to an allosteric site in luciferase distorting the active site to a nonoptimal conformation for aminoluciferin, thus resulting in a decrease in V_{max}. Since some triazoles are reported to be free radical scavengers, in the case of ATP, the V_{max} obviously decreased while the K_m remained almost unaltered in a dose-depent manner. This indicates that the inhibition mode for ATP is noncompetitive. Thus, we are quite sure that the inhibitor won't interfere with the aminoluciferin–adenylate conjugate. For now, most of the reported inhibitors are competitive or noncompetitive with

respect to luciferin. The triazole inhibitors in our study exhibited a new inhibition mode.

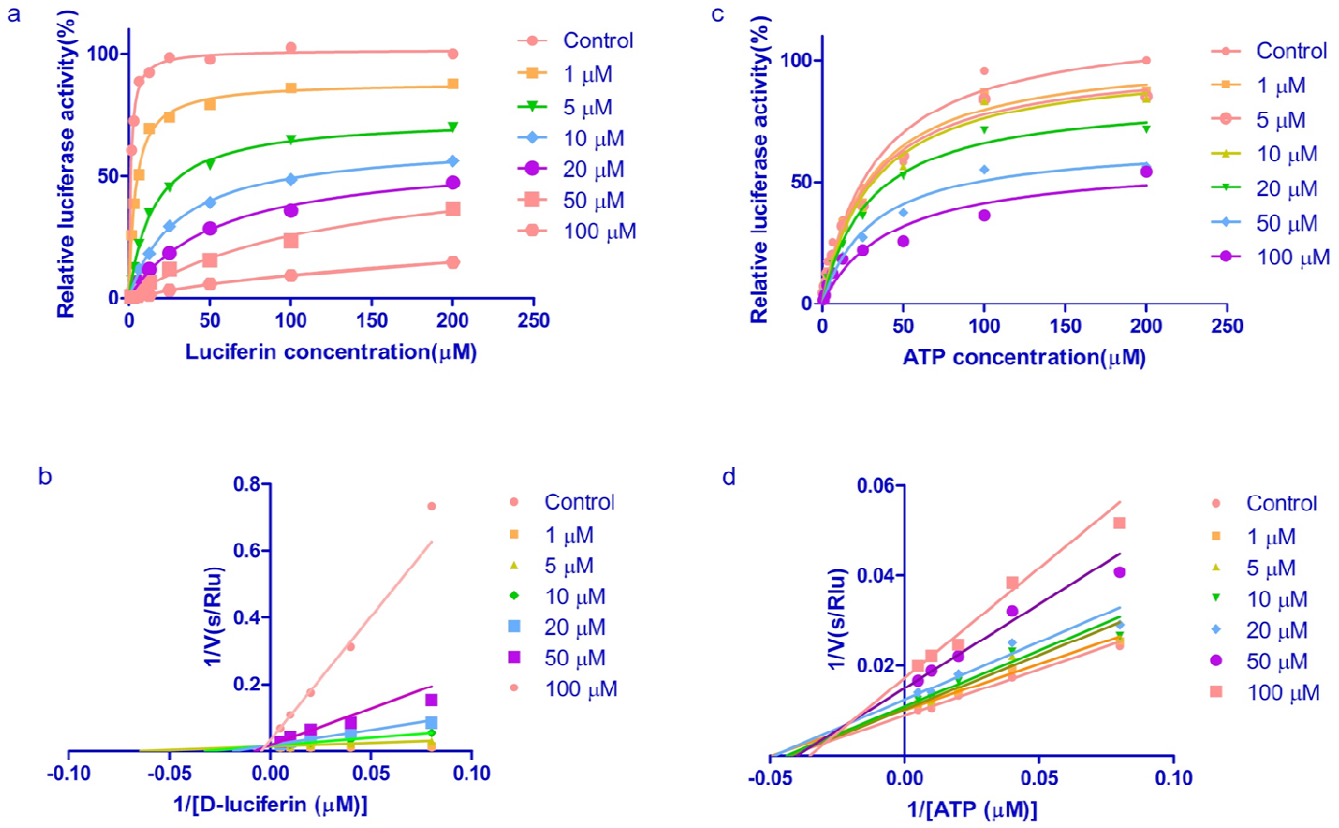


Fig 4. Kinetics of inhibition of luciferase by **8a**. (a) Luciferin saturation assay with increasing concentrations of **8a** (1, 5, 10, 20, 50 and 100 μM). (b) A Lineweaver–Burk plot of data in (a). (c) ATP saturation assay with increasing concentrations of **8a** (1, 5, 10, 20, 50 and 100 μM). (d) A Lineweaver–Burk plot of data in (c). The lines of (a) and (c) are fitted to Michaelis-Menten assay using GraphPad Prism software. The Lineweaver-Burk plots are estimated using GraphPad Prism software.

Table 2. Estimated V_{max} and K_m of luciferin and ATP

	Concentration (μM)	no inhibitor	1 μM	5 μM	10 μM	20 μM	50 μM	100 μM
Luciferin[a]	V_{max} (Rlu/s)[c]	97.9±2.78	86.7±1.21	78.2±5.43	73.5±10.13	65.5±11.23	63.5±5.91	43.5±16.2
	K_m (μM)[c]	0.98±0.09	3.75±0.33	12.8±1.74	26.8±4.79	47.2±8.80	111±21.5	222±21.3
ATP[b]	V_{max} (Rlu/s)[c]	120±2.81	104±0.85	102±1.16	97.7±1.53	88.5±2.28	74.5±5.86	59.1±0.45
	K_m (μM)[c]	37.6±3.52	31.1±1.08	30.3±0.84	31.0±0.38	32.4±2.38	34.7±3.36	37.3±3.89

[a] Dependence of luciferase activity on concentration of luciferin at 1 mM ATP was determined in absence (no inhibitor) or presence of 1 μM, 5 μM, 10 μM, 20 μM, 50 μM and 100 μM of compound 8a.
[b] Dependence of luciferase activity on concentration of ATP at 500 μM luciferin was determined in absence (no inhibitor) or presence of 1 μM, 5 μM, 10 μM, 20 μM, 50 μM and 100 μM of compound 8a.

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

Conclusion

In summary, a series of triazoles were synthesized through Cu(I)-catalyzed azide-alkyne click chemistry in a one-pot reaction then evaluated for their firefly luciferase inhibition activity. Some triazoles presented reasonable inhibition activity both in vitro and in vivo. The most potent compound **8a** demonstrated suitable efficiency with IC_{50} of 3.28 μM in firefly luciferase inhibition assay. Compound **8c** exposed inhibition activity with IC_{50} of 22.3 μM in the enzymatic inhibition assay and 55.6 μM in the cellular level, comparable

to the positive control resveratrol. Our follow-up in vivo experimental results indicated that compound **8c** has reasonable potency in mouse, in which it can effectively inhibit the bioluminescence in Balb/c-nu male mice bearing ES-2-luc subcutaneous tumors even more potently than control resveratrol. Moreover, a preliminary SAR exploration implies that the triazole core is a valid firefly luciferase inhibitor core. Kinetics study indicated a mixed noncompetitive mechanism for substrate luciferin and noncompetitive for ATP. As the triazole core is a "privileged" scaffold for a variety of drug candidates, this article may notify researchers to pay attention to "false positives" that might emerge in qHTS. Moreover, the brand new inhibition mode of these triazoles can be helpful for further investigation into the amazing luciferin-luciferase system and might help to expand its application in various areas since our compounds exhibited considerable potency in cellulo and in vivo.

Acknowledgements
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† Electronic Supplementary Information (ESI) available: [experimental procedures, ^{1}H-NMR and HR-MS spectra]. See DOI: 10.1039/b000000x/

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Synthesis and biological evaluation of a series of aryl triazoles as firefly luciferase inhibitors

Haixiu Bai,[a] Peng Zhu,[a] Wenxiao Wu,[a] *Jing* Li,[a] Zhao Ma,[a] Wei Zhang,[a] Yanna Cheng,[b] Lupei Du[a] and Minyong Li[a*]

[a] Department of Medicinal Chemistry, Key Laboratory of Chemical Biology (MOE), School of Pharmacy, Shandong University, Jinan, Shandong 250012, China.

[b] Department of Pharmacology, School of Pharmacy, Shandong University, Jinan, Shandong 250012, China.

Graphical abstract:

A series of novel aryl triazoles was synthesized as firefly luciferase inhibitors in vitro and in vivo. More interestingly, these compounds are mixed noncompetitive for luciferin and noncompetitive for ATP.