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PAPER

Quenching the Firefly Bioluminescence by Various Ions

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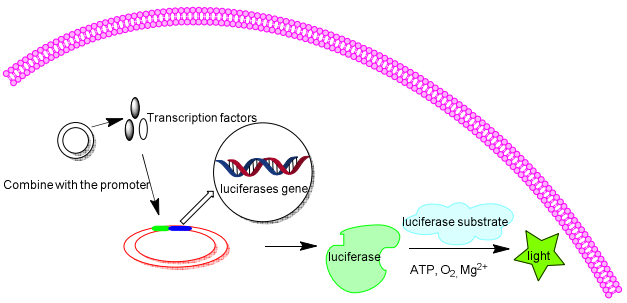
The luciferase reporter gene assay system is broadly applied in various biomedical aspects, including signaling pathway dissection, transcriptional activity analysis, genetic toxicity testing. It significantly improves the experimental accuracy and reduces the experimental error by the addition of an internal control. In the current research, we discovered some specific ions that could selectively inhibit firefly luciferase while having negligible effect on renilla luciferase in vitro in the dual-reporter gene assay. We showed that these ionic compounds had a high potential of being utilized as quench-and-activate reagents in the dual-reporter assay. Furthermore, results from kinetic studies on ion-mediated quenching effects indicated that different ions have distinct inhibition modes. Our study is anticipated to guide a more affordable design of quench-and-activate reagents in biomedicine and pharmaceutical analysis.

Introduction

Bioluminescence refers to the production and emission of visible light by an enzyme-catalyzed reaction within a living organism. It is widely found in various living organisms, such as bacteria, insects, and marine organisms.[1] Bioluminescence imaging (BLI) is a convenient yet reliable technique developed over the past decade that enables the sensitive and non-invasive study of real-time biological processes in living animal models. It should be noted that BLI can be carried out in absolute darkness while it does not require an excitation light source, thus avoiding the potential side effects derived from the excitation light.[2]

The benefit of bioluminescent enzymes (luciferases) has been widely recognized as genetic reporters for its sensitivity and efficiency (Scheme 1).[3] The two commonly used reporters, firefly and renilla luciferases, could oxidize different substrates to generate quantifiable bioluminescence. The firefly luciferin–luciferase reaction is the most well-studied bioluminescent system and is widely used in the biomedical field. Firefly luciferase (Fluc) gene encodes a monomeric 61 kDa protein, in which its activity does not depend on posttranslational modification.[3] Thus, once the translation is completed, it possesses genetic reporter gene function. Fluc can catalyze a two-step oxidation of D-Luciferin (LH2) into oxyluciferin in the presence of ATP, Mg^{2+} and oxygen, and then emit yellow to green light with an emission wavelength from 550 to 620 nm (Scheme 2). In fact, the emission colour is influenced by the substitution of the amino acid residue in luciferase，which can range from yellow-green to red.[4, 5] Bioluminescent intensity allows for the quantification of the luciferase activity. Major factors accounting for its altered shift in the emission wavelength include pH,[6] solvent polarity,[7] and modifications of luciferase.[5] Slight differently, renilla luciferase (Rluc) maintains its active form as a single polypeptide chain of 36 kDa and becomes inactivated once it self-associates to form higher molecular weight species.[8] It catalyzes coelenterazine oxidation to produce bioluminescence, coelenteramide, and CO_2 (Scheme 2).



Scheme 1. The fundamental principle of the luciferase reporter gene.

The differences in the enzyme structures and their substrates between Fluc and Rluc make it possible to measure the activity of the two luciferases by adjusting the reaction conditions, which have been widely applied in the dual-luciferase reporter gene assay. Such a dual-luciferase reporter gene assay has been adopted in various fields of research to improve the experimental accuracy and reduce the error. For example, Yong Zhong X *et al*. performed promoter deletion analysis by a

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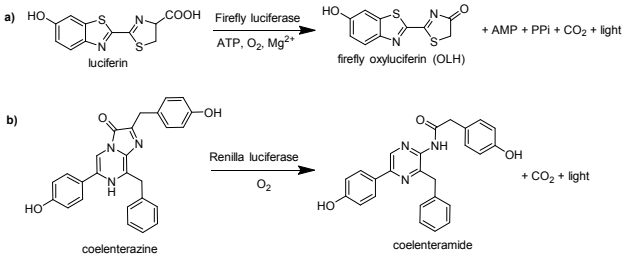
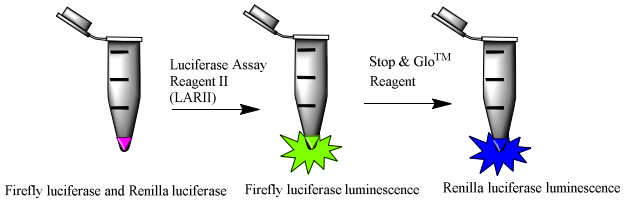
†Electronic Supplementary Information (ESI) available: The results of dual luciferase reporter gene simulated assay using other's ionic compounds *in vitro*.

dual-luciferase reporter system[9] to identify significant regulatory regions participated in the transcriptional control of gene expression. Amirali Afshari *et al*. reported a dual-reporter approach that enables quantitative monitoring of the activity of the NF-κB-mediated inflammatory pathway at real-time and to screen drugs in a high-throughput manner[10]. Because firefly luciferase bioluminescence spectra are pH-sensitive, Gabriele V. M. Gabriel *et al*. provided a new analytical strategy to monitor intracellular pH, and gene expression and/or ATP concentration using the firefly luciferase gene[11]. The dual luciferase reporter gene assay has the potential to characterize signaling pathways necessary for biology and disease processes. Moreover, this approach is expected to aid drug screens to yield new therapeutic candidates.



Scheme 2. Biochemical reactions catalyzed by Fluc (a) and Rluc (b) to produce bioluminescence.

In the dual reporter assay system, one luciferase is selected as an experimental reporter, and the other is considered as an internal control. First, one of the two enzyme-mediated luminescence reactions is initiated by addition of its corresponding substrate into the system. The luminescence signal generated by the first enzyme reaction is then measured. Next, the first enzymatic reaction is selectively and specifically quenched by adding a quench-and-activate, which quenches the first enzymatic reaction and triggers the second enzymatic reaction at the same time (Scheme 3). As a result, Fluc and Rluc activity can be sequentially detected in a single sample.



Scheme 3. The principle of dual-Luciferase reporter assay system. The Luciferase Assay Reagent II (LAR II) is added to the sample; the Fluc luminescence signal is then measured. Subsequently, the above reaction is terminated by adding Stop & Glo reagent to the same sample, and the Rluc luminescence is measured.

The key point of the entire assay system is to find an excellent quench-and-activate reagent. Although commercial detection reagents are available in the dual reporter assay system, commercial formulations have not been described because of the proprietary nature of the components.[12] In fact, several research groups noticed the Fluc inhibition[13] and found various Fluc inhibitors by high-throughput screening (HTS).[14] In 2004, Hugo Fraga *et al*. firstly reported to identify the luciferyl adenylate by HPLC under a nitrogen atmosphere.[15] After that, they found that coenzyme A induced stabilization of Fluc luminescence was because of a thiolytic reaction, which splited dehydroluciferyl-adenylate (L-AMP) and gave rise to dehydro-luciferyl-CoA (L-CoA), a much less powerful inhibitor.[16] Moreover, they indicated that hydrogen peroxide was generated in a side reaction, which D-LH2-AMP was oxidized into dehydroluciferyl adenylate (L-AMP).[17] In 2008, Rui Fontes *et al*. demonstrated that inorganic pyrophosphate and tripolyphosphate influenced Fluc luminescence because they reacted with L-AMP, which was also a LH2-AMP oxidation product in a side reaction, to obtain products that were less powerful inhibitors.[18] João M.M. Leitão *et al*. summarizes the major series of inhibitors of Fluc and the corresponding inhibition mechanism,[19] including substrate-related compounds, intermediates or products,[20,21,22,23,24] anesthetics,[25] fatty acids[26] and other inhibitors. Douglas S. Auld *et al*. discovered PTC124 (3-[5-(2-fluorophenyl)-1,2,4-oxadiazol-3-yl]benzoic acid) with an IC_{50} of 7 nM, but it was inactive against the renilla reniformis luciferase.[27,28] Besides, Pekka K. Poutiainen *et al*. conjugated the Fluc-specific inhibitor compounds with cell penetrating peptide as a versatile tool for analysis of cellular uptake of biomolecules.[29] In previous studies, we found a series of aryl triazoles[30] and 2-phenylnaphthalenes[31] as Fluc inhibitors. Meanwhile, some ions were evaluated the effects on Fluc. In 1970, McElroy WD. *et al*. observed that the change of ionic strength can have an influence on Fluc and identified a specific anion inhibition mechanism againstFluc.[32] The divalent ions can replace Mg^{2+} and different anions can change the total ionic strength, thereby affecting the bioluminescent reaction. For the divalent ions the Ni^{2+} and Co^{2+} and the alkaline earth are the strongest inhibitors of the luciferase bioluminescent reaction[33] and for different anions nitrate, thiocyanate, iodide and bromide are the most powerful inhibitors with Ki about 20 μM,[34,35] while only for chloride a noncompetitive inhibition mechanism is discussed.[35] In general, cells are required to lyse firstly in the dual luciferase assay.[36] This causes damage to the cells, and the process is complicated. Compared with organic compounds, inorganic salts have some significant advantages, such as low toxicity, easy accessibility, low cost and better water solubility. Therefore, some ions as quench reagents can inhibit rapidly Fluc activity without any changes of pH or killing the cells. Based on the properties of inorganic salts, we intend to explore a non-invasive detection method in the dual luciferase assay. In this article, we discovered that some specific anions could selectively inhibit Fluc while having little effect on Rluc *in vitro* and *in cellulo*, which may find broad application in improved dual luciferase reporter gene assay.

Biological evaluation

Inhibitory assay in vitro

In the preliminary screening, 22 ionic compounds were evaluated for their inhibitory activity on purified recombinant Firefly (*Photinus pyralis*) luciferase and recombinant *Renilla reniformis* luciferase. We simulated the standard protocol of dual luciferase reporter gene assay to evaluate their inhibitory activity. We increased concentrations of the salts to compare their inhibitory activities against Fluc and Rluc. As shown in Figure 1, we found several ionic compounds that can inhibit the Fluc activity above 500 μM, such as NaSCN, KI, $Na_2S_2O_3$, NaN_3, $Na_4P_2O_7$. *However, these* ionic compounds have little effect on Rluc activity below 50 mM. Thus, *these* ionic compounds have potential as quench-and-activate reagents and were applied in the dual-luciferase reporter assay in the presence of the enzyme. Moreover, we also discovered some metal ions (e.g., Fe^{3+}, Co^{2+}, Zn^{2+}, Cu^{2+}, Fe^{2+}), which had a serious impact on luciferase activities above 500 μM. A few of ions (e.g., F^-, NH_4^+) had little influence on luciferase activities at 500 mM. Some anions (e.g., $S_2O_4^{2-}$, NO_2^-) did not inhibit the luciferase activities until their concentrations in the buffer solution were above 5 mM (Figure S1 in the Supporting Information).

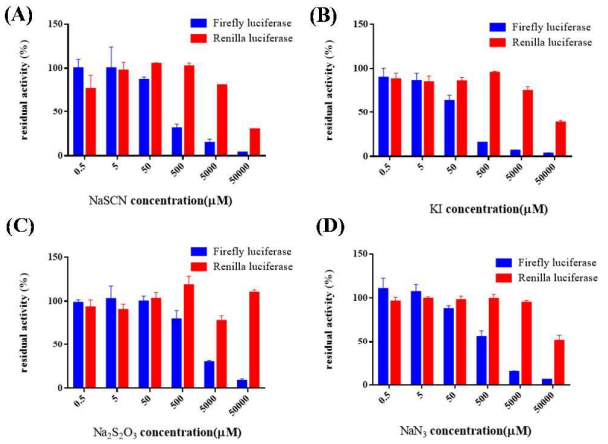


Fig. 1. Dual luciferase reporter gene simulated assay using NaSCN (A), $Na_2S_2O_3$ (B), KI (C) and NaN_3 (D).

Since the dual luciferase reporter gene assay is widely used in cellulo, we further evaluated their inhibitory activity in transfected ES-2-Fluc cells and ES-2-Rluc cells (a human ovarian cancer cell line transfected with Fluc and Rluc, respectively). We incubated increasing concentrations of NaSCN, KI, $Na_2S_2O_3$, NaN_3 with ES-2-Fluc cells and ES-2-Rluc cells for 1.5 h, and then added their corresponding enzyme substrates and tested their bioluminescence intensity immediately using an IVIS Kinetic (Caliper Life Sciences, USA) equipped with a cooled charge-coupled device (CCD) camera for bioluminescence imaging. As shown in Fig. 2, the impact on Fluc and Rluc activities in cellulo caused by these four ionic compounds is consistent with their effects in the presence of the enzyme. Their inhibitory potencies *in cellulo* are slightly weaker than in the presence of the enzyme. It indicates that these four ionic compounds can be used as quench-and-activate reagents in cellulo.

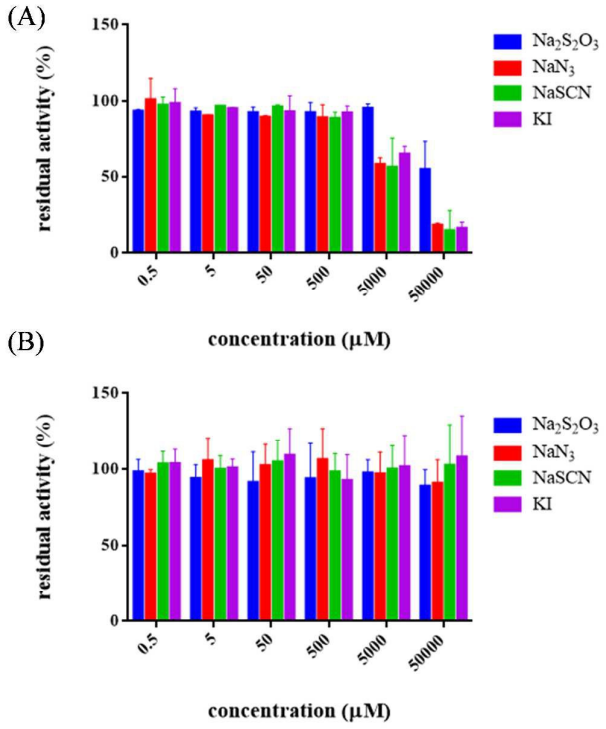


Fig. 2. Dual luciferase reporter gene simulated assay *in cellulo*. (A) ES-2-Fluc cell expressing Fluc bioluminescence inhibition assay. (B) ES-2-Rluc cell expressing Rluc bioluminescence inhibition assay.

Cell viability assay

To exclude the possibility that cell death might cause the decrease in bioluminescence, we used SRB assay to evaluate the cell viability caused by these ions. We tested the ES-2-Fluc cell viability after 1.5 h incubation with 250, 125, 62.5 and 31.25 mM of NaSCN, KI, $Na_2S_2O_3$ and NaN_3, which showed reasonable cell tolerability *in vitro*. Fig. 3 revealed that cells suffered little damage basically at the level up to 250 mM. Therefore, it is proved that our inhibitory assay in cellulo was valid.

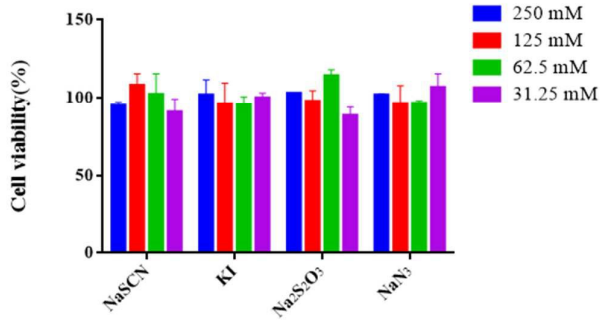


Fig. 3. SRB cell viability results of NaSCN, KI, $Na_2S_2O_3$ and NaN_3.

Enzyme inhibition kinetics assay

To assess their kinetic characteristics of inhibition, we further carry out their enzyme inhibition kinetics assays. We fixed the concentration of ATP at 100 μM, and measured the residual enzyme activity against increasing concentrations of amino-luciferin after incubation with four ionic compounds (NaSCN, KI, $Na_2S_2O_3$, NaN_3) solutions for 10 min. Then, we fixed the concentration of amino-luciferin at 2.5 μM, and measured the residual enzyme activity against increasing concentrations of ATP in the same way. Using the GraphPad Prism software, the Lineweaver-Burk plots are estimated, and the Michaelis constant V_{max} and maximum rate K_m were obtained. On the one hand, increasing concentrations of the four ionic compounds also caused an obvious decrease in V_{max} for amino-luciferin whereas the K_m remained essentially unchanged in a dose-dependent way (Tables 1-4). This demonstrates that the ionic compounds inhibition mode of luciferase is noncompetitive with respect to amino-luciferin. Therefore, we believe that the ionic compounds will not influence the binding of luciferase and amino-luciferin. On the other hand, change trends of K_m and V_{max} for ATP are the same with them for amino-luciferin with increasing concentrations of NaN_3 (Table 3), thus it showed that NaN_3 inhibition of luciferase is noncompetitive for ATP. With increasing concentrations of $Na_2S_2O_3$, the K_m for ATP clearly increased while the V_{max} for ATP basically maintained constant (Table 2), so it indicates that $Na_2S_2O_3$ inhibition of luciferase is competitive with respect to ATP. As the concentrations of KI and NaSCN increase, the V_{max} for ATP significantly decreased while the K_m increased (Tables 1 and 4). We suppose that their inhibition of luciferase belongs to mixed noncompetitive inhibition. In other words, KI and NaSCN not only could binds to the active site of luciferase to compete with ATP, but might bind the luciferase at allosteric sites, so that have an impact on luciferase activity. These results could be explained by the possible differences in the ionic strength and chemical properties of these ionic compounds.

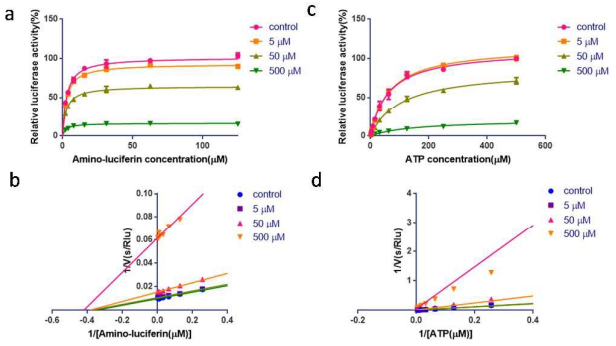


Fig. 4. Kinetics of inhibition of luciferase by KI. (a) Amino-luciferin saturation assay with increasing concentrations of KI (5, 50 and 500 μM). (b) Double-reciprocal plot of data in (a). (c) ATP saturation assay with increasing concentrations of KI (5, 50 and 500 μM). (d) Double-reciprocal plot of data in (c).

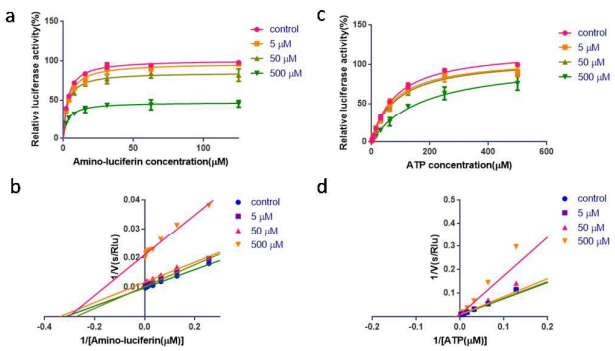


Fig. 5. Kinetics of inhibition of luciferase by $Na_2S_2O_3$.

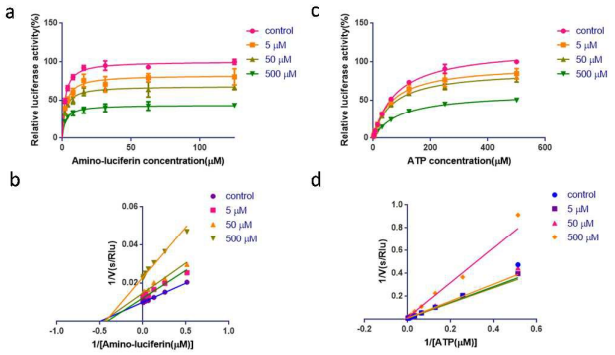


Fig. 6. Kinetics of inhibition of luciferase by NaN_3.

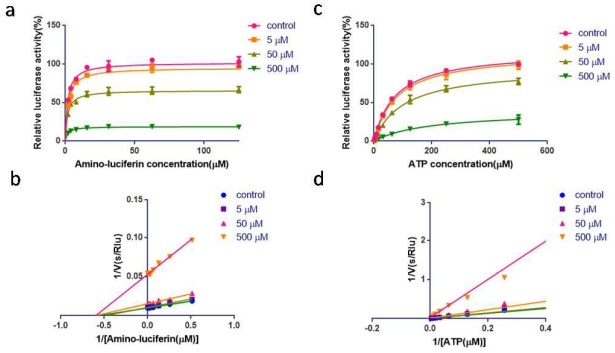


Fig. 7. Kinetics of inhibition of luciferase by NaSCN.

Table 1. Estimated K_m and V_{max} of amino-luciferin and ATP by KI. The values are shown by means±SD of three independent assays performed in triplicate

	Concentration (μM)	No inhibitor	5 μM	50 μM	500 μM
Aminoluciferin	Vmax (Rlu s-1)	101±1.16	93.1±0.99	64.7±0.94	16.2±0.34
	Km (μM)	2.83±0.19	2.65±0.17	2.58±0.23	2.38±0.31
ATP	Vmax (Rlu s-1)	111.3±2.82	115.9±2.21	85.78±1.873	20.79±1.05
	Km (μM)	60.99±4.86	65.22±3.83	102.8±6.12	148.9±18.30

Table 2. Estimated K_m and V_{max} of aminoluciferin and ATP by $Na_2S_2O_3$.

	Concentration (μM)	No inhibitor	5 μM	50 μM	500 μM
Aminoluciferin	V_{max} (Rlu s^{-1})	101±0.80	97.2±1.21	85.3±1.52	47.4±1.09
	K_m (μM)	3.13±0.14	3.60±0.25	2.92±0.31	3.28±0.43
ATP	V_{max} (Rlu s^{-1})	119.5±2.21	108.7±4.11	108.5±3.36	104.2±6.34
	K_m (μM)	82.26±4.42	76.40±8.56	83.05±7.44	171.5±24.39

Table 3. Estimated K_m and V_{max} of aminoluciferin and ATP by NaN_3.

	Concentration (μM)	No inhibitor	5 μM	50 μM	500 μM
Aminoluciferin	V_{max} (Rlu s^{-1})	101±1.13	82.6±2.48	68.2±1.32	43.5±0.94
	K_m (μM)	2.01±0.15	2.40±0.45	2.16±0.27	2.31±0.32
ATP	V_{max} (Rlu s^{-1})	118.6±2.04	95.95±2.79	88.77±2.12	59.88±1.01
	K_m (μM)	82.02±4.10	63.53±5.74	65.60±4.82	90.97±4.33

Table 4. Estimated K_m and V_{max} of amino-luciferin and ATP by NaSCN.

	Concentration (μM)	No inhibitor	5 μM	50 μM	500 μM
Aminoluciferin	V_{max} (Rlu s^{-1})	102±1.68	95.1±1.80	65.8±1.04	18.9±0.25
	K_m (μM)	1.78±0.20	2.05±0.26	1.67±0.19	1.67±0.16
ATP	V_{max} (Rlu s^{-1})	117.1±1.91	115.2±3.26	95.13±3.05	38.92±3.21
	K_m (μM)	74.39±3.62	79.47±6.59	103.4±9.03	191.1±35.58

Conclusions

In summary, a series of ionic compounds were evaluated for their Fluc and Rluc inhibition activity. On one hand, our experimental results indicated that some ions, such as metal ions and several special anions, could significantly inhibit the luciferase activity at relatively high concentrations. These interesting results indicate the caution to avoid introduction of these ions when we prepared the buffer solution for luciferase-mediated reactions. On the other hand, we found several ionic compounds can selectively inhibit Fluc while having little influence on Rluc. It implied that these ionic compounds of this selective characteristic could be used as quench-and-activate reagents in a dual luciferase reporter gene assay. We further evaluated their kinetic characteristics of inhibition. More interesting, we found that these ionic compounds inhibition modes for amino-luciferin are the same while inhibition modes for ATP are not exactly the same. Thus, our studies can be helpful for further research on the bioluminescent system and might contribute to expanding its application in biological and medical fields.

Acknowledgements

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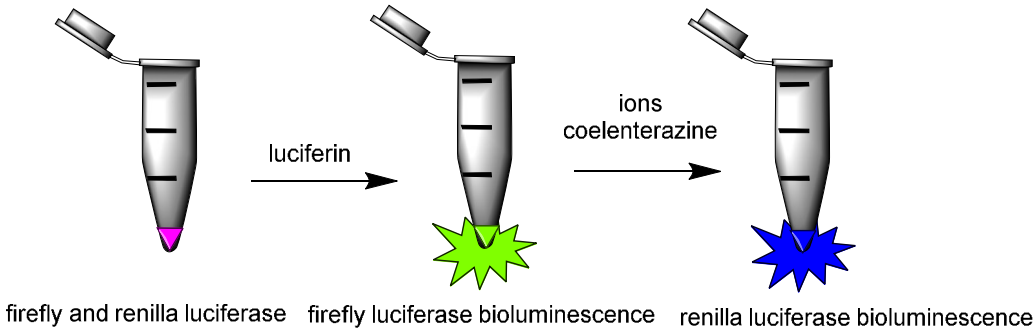

Some specific ions could selectively inhibit firefly luciferase while having negligible effect on renilla luciferase, which may be used in improved dual luciferase reporter gene assay.