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UNEXPECTED EMERGENCE OF LUCIFERASE-INHIBITORY ACTIVITY DURING STRUCTURAL DEVELOPMENT STUDY OF PHENYLOXADIAZOLE-BASED PPAR LIGANDS

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Abstract – Peroxisome proliferator-activated receptors (PPARs) are nuclear receptors that regulate transcription of genes involved in lipid, glucose, and cholesterol homeostasis in a ligand-dependent manner. PPARs have long been a target of drug development, and evaluation of PPARs activity frequently involves reporter-gene assay, in which luciferase activity is utilized to visualize transcriptional activation by the receptors. Here, we report our experience of the unexpected emergence of luciferase-inhibitory activity during our search for PPAR antagonists. We believe information about negative experiences like this will help to make medicinal chemists more aware of potential pitfalls in SAR studies with luciferase-based assays.

INTRODUCTION

Peroxisome proliferator-activated receptors (PPARs) are nuclear receptors that regulate transcription of genes involved in lipid, glucose, and cholesterol homeostasis in a ligand-dependent manner.[1] Three PPAR subtypes have been identified, and they show distinct tissue distributions and physiological functions despite their high sequence homology. PPARα is mainly expressed in tissues actively engaged in fatty acid catabolism, including skeletal muscle, liver, and adrenal gland, while PPARγ is predominantly expressed in adipose tissues and macrophages, and PPARδ is ubiquitously expressed.

Small-molecular ligands for PPARs have been extensively studied and some of them, including fenofibrate and pioglitazone, are widely prescribed as drugs for type 2 diabetes mellitus or dyslipidemia. Endogenous ligands for PPARs have been proposed to be fatty acids and their metabolites. Correspondingly, most PPAR ligands consist of a hydrophobic tail part and a carboxylic acid or its bioisosteric thiazolidinedione moiety as a critical pharmacophore.

We have previously reported a novel class of acetamide-bearing phenyloxadiazole compounds, which do not have an acidic pharmacophore, as partial agonists of the PPARα/δ subtypes.[2] During the course of structure-activity relationship (SAR) studies of the acetamides using luciferase-based PPARs reporter gene assays,[3] we found that a small change in the structure converted the acetamides to seemingly highly potent "antagonists" for PPARs. As only a few PPARγ antagonists have been reported to date,[4] we set out to examine the structure-activity relationships of the acetamides as "antagonists" for PPARγ. Disappointingly, however, follow-up studies on selectivity over PPAR subtypes and other nuclear receptors showed promiscuity of these "antagonistic" compounds, suggesting the possibility of luciferase inhibition, and this was confirmed by *in vitro* luciferase inhibition assay. In this report, we would like to share our experience, because knowledge of negative experiences like this should help medicinal chemists to avoid similar pitfalls in SAR studies with luciferase-based assays.

RESULTS AND DISCUSSION

Our previous research yielded a class of PPARα/δ partial agonists with acetamide-bearing phenyloxadiazole structure, including **SO031** (**Figure 1A**). This compound showed partial agonistic activity for PPARα and δ subtypes (EC_{50} 33 nM and 46 nM, respectively),[2] while no activation was observed with PPARγ. To further explore the potential of the phenyloxadiazole-based PPAR ligands, we set out to examine the PPAR-antagonistic activity of this class of compounds, anticipating that structural modification might result in activity-switching from agonist to antagonist, or subtype-selectivity switching. We screened our library of phenyloxadiazole compounds, including synthetic intermediates, for PPARγ antagonist activity with a luciferase-based reporter gene assay,[3] and identified **RS013** as a highly potent antagonistic compound (**Figure 1A** and **C**). In the presence of 300 nM **rosiglitazone** as an agonist,[5] the IC_{50} of this compound was estimated to be 80 nM, and **RS013** was only slightly less active than **GW9662**, the most potent PPARγ antagonist known to date.[4] As this compound appeared to be promising lead for developing potent PPARγ antagonists, we set out to confirm its suitability for structural optimization studies. But, during the confirmation assays, we noted promiscuity of this compound among PPAR subtypes (PPARα and PPARδ) and other nuclear receptors (glucocorticoid receptor and liver-X-receptor β), which raised the possibility of interference with the assays. To see

whether **RS013** indeed interfered with luciferase-based reporter assay, we performed a control experiment without nuclear receptor. Our reporter gene assay involves the use of a fusion protein of nuclear receptor ligand-binding domain (LBD) and GAL4N (a bacterial DNA binding domain), which recruits the LBD to the upstream region of the luciferase reporter via binding to the MH100 sequence (**Figure 1B**). Upon addition of agonists, the LBD activates transcription of the luciferase gene. So, to unequivocally determine if the antagonist-like activity of **RS013** is actually mediated by the nuclear receptor LBD, we tested **RS013** in the absence of the LBD. As shown in **Figure 1D**, **RS013** downregulated luciferase activity even in the absence of nuclear receptor LBD, clearly indicating that the action of **RS013** is not mediated by the nuclear receptor LBDs.

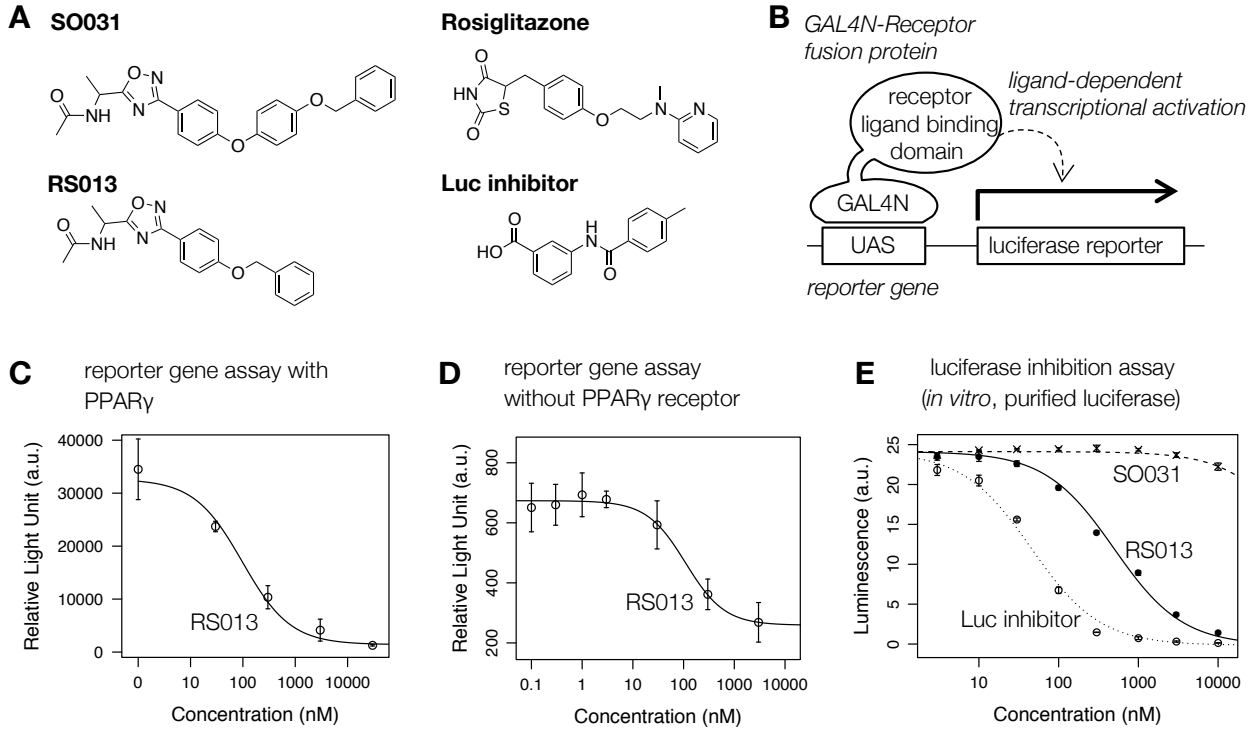
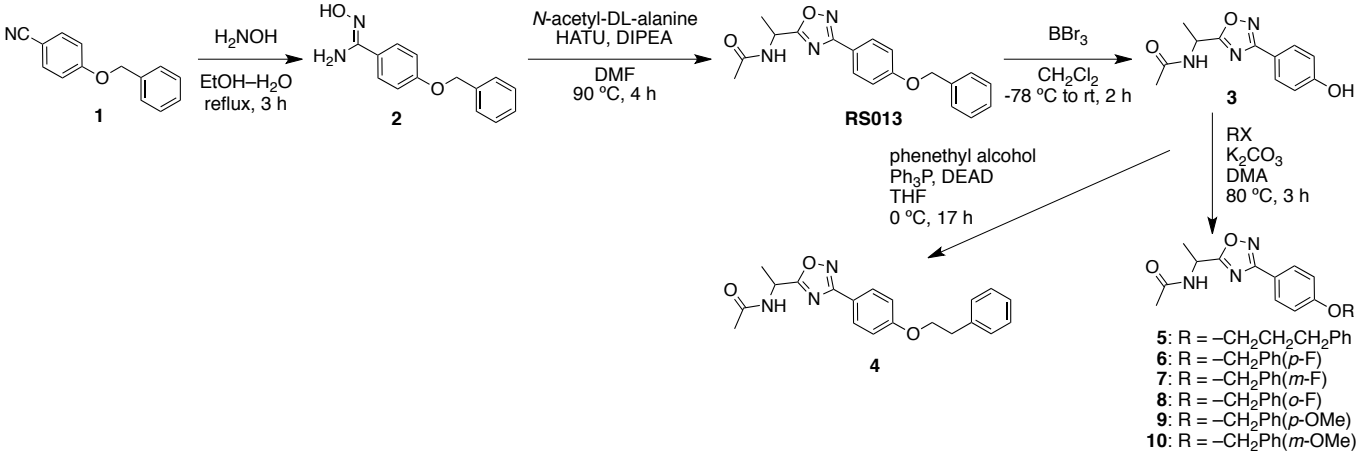


Figure 1. A. Chemical structures of SO031, RS013, PPARγ agonist rosiglitazone, and a luciferase inhibitor. **B.** Schematic representation of our nuclear receptor reporter-gene assay. **C.** PPARγ antagonist assay in the presence of 0.3 μM rosiglitazone. **D.** A control reporter-gene assay without PPARγ-GAL4N construct. **E.** In vitro luciferase inhibition assay using recombinant firefly luciferase. Data points represent average values, and standard deviations are depicted as error bars (n = 3).

One possible mechanism of the interference would be inhibition of the luciferase reporter, as is sometimes encountered in high-throughput screening.[6] To examine this possibility, we performed in vitro luciferase inhibition assay using purified recombinant luciferase and 3-(4-methylbenzamido)benzoic acid (**Luc inhibitor** in **Figure 1A**) as a positive control.[7] As shown in **Figure 1E**, **RS013** potently inhibited luciferase activity while **SO031**, which did not show significant antagonist-like activity in PPAR reporter gene assay, was not inhibitory up to 10 μM. These results clearly demonstrated that the antagonist-like

activity of **RS013** observed in our luciferase-based PPAR reporter gene assay was due to inhibition of the luciferase reporter.

Thus, a relatively small modification to **SO031** unexpectedly gave rise to a potent luciferase inhibitor **RS013**. So, to gain insight into the pharmacophore for luciferase inhibition, we performed a preliminary structure-activity relationship study of **RS013** for luciferase-inhibitory activity.

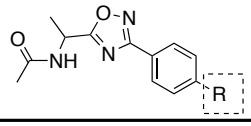


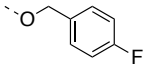
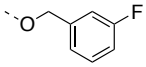
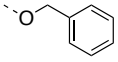
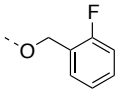
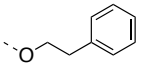
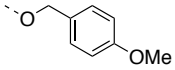
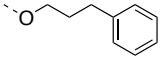
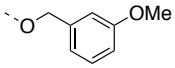
Scheme 1. Synthesis of **RS013** derivatives **3-10**

Structural comparison of **RS013** and **SO031** indicated that the tail part of the phenyloxadiazole was critical for luciferase inhibition. Therefore, we first evaluated structure-activity relationships at this position in more detail (**Table 1**). Synthesis of the **RS013** derivatives is illustrated in **Scheme 1**. When the benzyl group on **RS013** was removed (**3** in **Table 1**), the luciferase-inhibitory activity was greatly attenuated, implying that hydrophobic interaction with the enzyme is important. The length of the methylene linker between phenolic oxygen and the terminal phenyl group also affected the inhibitory activity. Loss of the methylene linker (**11**) resulted in loss of inhibitory activity, and introduction of longer linkers (**4** and **5**) also attenuated the activity; the benzyl substituent (**RS013**) showed the most potent inhibition. These results indicate that the binding region on the enzyme that accommodates the tail part of the inhibitor is limited in size, and not larger than the benzyl group.

To further understand the structure-activity relationships at the tail part, we examined substituent effects at the benzyl group (**Table 1**). A fluorine atom (electron-withdrawing) or a methoxy group (electron-donating) was introduced onto the benzyl group, (**6**-**10**). The derivative with ortho fluorine substitution (**8**) showed comparable activity to **RS013**, but all the other derivatives showed attenuated inhibitory activity. In particular, para substitution (**6** and **9**) resulted in a large loss of activity, while meta (**7** and **10**) or ortho substitution (**8**) followed in that order. These results suggest that the substituent effect was mostly due to steric constraints, and electronic effects did not make a major contribution.

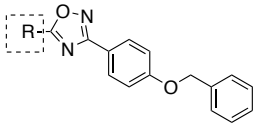
Table 1. Structure-activity relationships of the tail part on luciferase inhibitory activity



Compounds	R	Luciferase (IC_{50}, nM)	Compounds	R	Luciferase (IC_{50}, nM)
3		33,300	**6**		3,760
11		>100,000	**7**		1,210
RS013		495	**8**		424
4		4,340	**9**		>100,000
5		>100,000	**10**		3,020
Luc inhibitor		58.5			

Finally, we assessed the contribution of the acetamide moiety. Removal of the acetyl group (**12**) resulted in a more than 5-fold decrease of the inhibitory activity. This result indicated that the presence of the acetamide moiety is important for luciferase-inhibitory activity.

Table 2. Importance of the head acetamide group on luciferase inhibitory activity



Compounds	R	Luciferase (IC_{50}, nM)
RS013		495
12		2,270

In summary, we report the emergence of a class of luciferase inhibitors during structure-activity relationship studies of a phenyloxadiazole-based PPAR ligand. As a small change of substituent resulted in potent inhibition of luciferase, we examined the structure-activity relationship of this class of compounds as luciferase inhibitors. Luciferases are one of the most common reporter enzymes, and are widely used in many biological assays. Thus, we should be aware of the possibility of encountering

luciferase inhibitors during chemical optimization studies or screening campaigns, and bear in mind the importance of secondary assays to filter out undesired false-positive compounds, including counter assays such as the one used here,[9] or assays with orthogonal reporter enzymes or different principles.[6,7]

EXPERIMENTAL

In vitro luciferase inhibition assay

To a solution of QuantiLum recombinant firefly luciferase (0.1 nM, Promega) in TMBT buffer (50 mM Tris-HCl pH 7.6, 2.5 mM $Mg(OAc)_2$, 0.05% bovine serum albumin, and 0.01% Tween 20) in a 96-well plate (Greiner MicroClear white plate) was added test compounds (0.1% DMSO as co-solvent) and substrates (1 μM D-luciferin, 1 μM ATP). After one minute, the luminescence signal was measured with an EnVision multimode plate reader. Routinely, we mixed 4x QuantiLum working solution (0.4 nM) in TMBT buffer, 4x test compound solution in TMBT buffer containing 0.4% DMSO, and 2x substrate solution (4 μM D-luciferin and 4 μM ATP) to make 50 μL reaction mixtures in 96-well plates. QuantiLum luciferase stock solution (100 nM or 6 μg/mL in TMBT buffer), 10 mM D-luciferin stock solution (in water), and 100 mM ATP stock solution (in water) can be aliquoted and stored at -80 ºC for several months. Under our assay conditions, the K_M value for D-luciferin was estimated to be 50 μM (with 1 μM ATP).

Reporter-gene assay[3]

HEK 293 cells at 20% confluence were plated in a clear-bottomed white 96-well plate. After 24 h, transfection of the plasmids (nuclear receptor pCMX-LBD-GAL4N, MH100-tk-luc, and pCMX-βGal) was performed by the calcium phosphate method. The transfected cells were treated on the next day with test compounds pre-diluted with DMEM containing 10% FBS. After 24 h incubation, the medium was removed and 50 μL of luciferase substrate solution (1% Triton X-100, 250 μM D-luciferin, 1.5 mM ATP, 0.42 mM CoA, 30 mM, 100 mM 3-mercapto-1,2-propanediol, 100 mM tricine pH 7.8, 8 mM $Mg(OAc)_2$, and 0.2 mM EDTA) was added. Luminescence was measured with an ARVO microplate reader (PerkinElmer) after 1 min. Then, 125 μL of β-galactosidase substrate solution (1.25 mM *o*-nitrophenyl-β-D-galactopyranoside, 100 mM sodium phosphate buffer pH 6.8, 100 mM 3-mercapto-1,2-propanediol, 8 mM KCl, and 1.7 mM $MgCl_2$) was added and the absorbance at 405 nm was measured with a microplate reader after incubation at 37 ºC. The luciferase activity of each sample was normalized by the level of β-galactosidase activity and designated as relative light unit.

Dose-response analysis

Dose-response analysis and plotting were performed using the statistical environment R in combination

with the *drc* package.[8]

General Information for Organic Synthesis

Syntheses of compounds **1**, **2**, **3**, **11**, **12**, and **RS013** were previously reported.[2] All chemical reagents and solvents were purchased from Sigma–Aldrich, Kanto Chemical, Tokyo Chemical Industry or Wako Pure Chemical Industries, and used without further purification. Moisture-sensitive reactions were performed under an atmosphere of argon, unless otherwise noted. Reactions were monitored by thin-layer chromatography (TLC, Merck silica gel 60 F254 plate), and spots were visualized under UV light or with appropriate TLC stains. Flash chromatography was carried out with silica gel (Silica gel 60N, 40–50 μm particle size) purchased from Kanto Chemical. NMR spectra were recorded on a JEOL JNM-ECA500 (500 MHz) spectrometer, operating at 500 MHz for ^{1}H-NMR and at 125 MHz for ^{13}C-NMR. Proton and carbon chemical shifts are expressed in δ values (ppm) relative to internal tetramethylsilane (0.00 ppm), residual $CHCl_3$ (7.26 ppm), CHD_2OD (3.31 ppm) or C_2HD_5SO (2.49 ppm) for ^{1}H-NMR, and internal tetramethylsilane (0.00 ppm), $CDCl_3$ (77.16 ppm), methanol-d_4 (49.00 ppm) or dimethyl sulfoxide-d_6 (39.50 ppm) for ^{13}C NMR. Data are reported as follows: chemical shift, multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad), coupling constants (Hz), and integration. High-resolution mass spectrum was recorded on a Bruker micrOTOF II mass spectrometer.

Preparation of a known luciferase inhibitor, 3-(4-methylbenzamido)benzoic acid.

Methyl 3-(4-methylbenzamido)benzoate. A solution of methyl 3-aminobenzoate (172 mg, 1.14 mmol) in pyridine (2 mL) was treated with *p*-tolyl chloride (0.159 mL, 1.20 mmol) at 0 ºC, and the reaction mixture was stirred at room temperature for 2 h. Ice-cold water was added to it, and the resulting pale brown precipitate was collected by filtration. The precipitate was dissolved in hot EtOH and crystallized upon cooling, giving colorless needles (103 mg, 0.382 mmol, 34%). ^{1}H-NMR ($CDCl_3$) δ: 8.14 (t, J = 1.5 Hz, 1H), 8.06 (ddd, J = 8.0, 2.0, 1.0 Hz, 1H), 7.89 (br s, 1H), 7.83 (dt, J = 8.0, 1.5 Hz, 1H), 7.79 (d, J = 8.0 Hz, 2H), 7.47 (t, J = 7.5 Hz, 1H), 7.31 (d, J = 8.0 Hz, 2H), 3.93 (s, 3H), 2.44 (s, 3H).

3-(4-Methylbenzamido)benzoic acid (Luc inhibitor). Methyl 3-(4-methylbenzamido)benzoate (101 mg, 0.375 mmol) was dissolved in THF (4 mL) and MeOH (2 mL), and treated with 5 M NaOH (1mL). The reaction mixture was stirred at room temperature for 24 h and then neutralized with 1 M aqueous HCl. The white precipitate was collected by filtration and washed with 50% aqueous MeOH, giving the title compound (86.9 mg, 0.340 mmol, 91%) as colorless needles. This compound was reported to be a luciferase inhibitor by Thorne et al. (Ref. 7, compound **26**, IC_{50} 40 nM in the presence of a near-K_D concentration of luciferin). ^{1}H-NMR (DMSO-d_6) δ: 10.35 (s, 1H), 8.43 (t, J = 2.0 Hz, 1H), 8.05 (ddd, J = 8.0, 2.0, 1.0 Hz, 1H), 7.91 (d, J = 8.0 Hz, 2H), 7.68 (dt, J = 7.5, 1.5 Hz, 1H), 7.48 (t, J = 7.5 Hz, 1H), 7.35

(d, J = 8.0 Hz, 2H), 2.39 (s, 3H). ^{13}C-NMR (DMSO-d_6) δ: 167.23, 165.48, 141.79, 139.50, 131.74, 131.16, 128.94 (2C), 128.85, 127.75 (2C), 124.37, 124.32, 121.10, 21.01.

Preparation of phenyloxadiazole derivatives.

General procedure A: *O*-alkylation

To a solution of phenol (1.0 eq.) and alkyl bromide in DMA was added K_2CO_3 at room temperature. The mixture was stirred at 80 ºC for 3 h, and then quenched by addition of H_2O. The resulting mixture was extracted with AcOEt. The combined organic layer was washed with brine, dried over Na_2SO_4 and concentrated under reduced pressure. The residue was purified by silica gel column chromatography to afford the target product (95-100%) as a white solid.

***N*-(1-(3-(4-Phenethyloxyphenyl)-1,2,4-oxadiazol-5-yl)ethyl)acetamide (4).**

To a solution of **1** (31 mg, 0.13 mmol), phenethyl alcohol (18 μL, 0.15 mmol) and PPh_3 (38 mg, 0.15 mmol) in anhydrous THF (1.2 mL) was added DEAD (24 μL, 0.15 mmol). The mixture was stirred at 0 ºC for 1 h, and then at room temperature for 16 h. After the reaction, the solvent was removed under reduced pressure. The residue was purified by silica gel column chromatography (hexane:$CHCl_3$:AcOEt = 1:1:1) to afford the product **11** (18 mg, 37%) as a white solid.

^{1}H-NMR (500 MHz, $CDCl_3$) δ 7.91 (d, J = 9.5 Hz, 2H), 7.16-7.27 (m, 5H), 6.96 (d, J = 9 Hz, 2H), 6.14 (d, J = 7.4 Hz, 1H), 5.36-5.42 (m, 1H), 4.16 (t, J = 7.2 Hz, 2H), 3.03 (t J = 7.2 Hz, 2H), 2.02 (s, 3H), 1.56 (d, J = 6.9 Hz, 3H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 179.3, 169.5, 168.0, 161.3, 138.0, 129.1, 129.1, 128.6, 126.7, 118.9, 114.9, 68.8, 42.9, 35.7, 23.2, 20.1; ESI-TOF-HRMS calcd for $C_{20}H_{21}N_3O_3$ (m/z) $[M+Na]^+$ 374.1475, found 374.1473.

***N*-(1-(3-(4-(3-Phenylpropoxy)phenyl)-1,2,4-oxadiazol-5-yl)ethyl)acetamide (5)**

The title compound was prepared according to general procedure A (quant.). ^{1}H-NMR (500 MHz, $CDCl_3$) δ 7.98 (d, J = 8.6 Hz, 2H), 7.31–7.28 (m, 2H), 7.22–7.20 (m, 3H), 6.98–6.95 (m, 2H), 6.17 (d, J = 8.6 Hz, 1H), 5.46 (dq, J = 8.0, 8.0 Hz, 1H), 4.02 (t, J = 6.3 Hz, 2H), 2.83 (t, J = 7.7 Hz, 2H), 2.17–2.11 (m, 2H), 1.63 (d, J = 6.9 Hz, 3H); ^{13}C-NMR (500 MHz, $CDCl_3$) δ 179.17, 169.43, 167.97, 161.50, 141.27, 129.08 (2C), 128.49 (2C), 128.45 (2C), 126.01, 118.69, 114.76 (2C), 66.98, 42.83, 32.07, 30.67, 23.18, 20.04; ESI-TOF-HRMS calcd for $C_{21}H_{23}N_3O_3$ (m/z) $[M+Na]^+$ 388.1632, found 388.1631.

***N*-(1-(3-(4-((4-Fluorobenzyl)oxy)phenyl)-1,2,4-oxadiazol-5-yl)ethyl)acetamide (6)**

The title compound was prepared according to general procedure A (95%). ^{1}H-NMR (500 MHz, $CDCl_3$) δ 8.00 (d, J = 9.2 Hz, 2H), 7.42 (dd, J = 8.6, 5.4 Hz, 2H), 7.09 (t, J = 8.6 Hz, 2H), 7.04 (d, J = 9.2 Hz, 2H),

6.18 (d, *J* = 7.4 Hz, 1H), 5.46 (dq, *J* = 7.4, 7.4 Hz, 1H), 5.08 (s, 2H), 2.09 (s, 3H), 1.63 (d, *J* = 7.4 Hz, 3H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 179.26, 169.45, 167.87, 162.59 (d, *J* = 247.1 Hz), 160.98, 132.08, 129.38 (d, *J* = 7.2 Hz, 2C), 129.16 (2C), 119.24, 115.59 (d, *J* = 21.6 Hz, 2C), 115.10 (2C), 69.39, 42.82, 23.16, 20.00.

***N*-(1-(3-(4-((3-Fluorobenzyl)oxy)phenyl)-1,2,4-oxadiazol-5-yl)ethyl)acetamide (7)**

The title compound was prepared according to general procedure A (quant.). ^{1}H-NMR (500 MHz, $CDCl_3$) δ 8.00 (d, *J* = 8.6 Hz, 2H), 7.36 (td, *J* = 8.0, 5.7 Hz, 1H), 7.20 (d, *J* = 8.0 Hz, 1H), 7.16 (d, *J* = 9.2 Hz, 1H), 7.04 (d, *J* = 8.6 Hz, 2H), 6.23 (d, *J* = 7.5 Hz, 1H), 5.46 (dq, *J* = 7.5, 7.5 Hz, 1H), 5.12 (s, 2H), 2.08 (s, 3H), 1.63 (d, *J* = 6.9 Hz, 3H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 179.29, 169.48, 167.84, 162.96 (d, *J* = 245.9 Hz), 160.83, 138.93 (d, *J* = 7.2 Hz), 130.21 (d, *J* = 8.4 Hz), 129.17 (2C), 122.71 (d, *J* = 3.6 Hz), 119.34, 115.10 (2C), 114.92, 114.22 (d, *J* = 22.8 Hz), 69.19 (d, *J* = 2.4 Hz), 42.81, 23.14, 19.95.

***N*-(1-(3-(4-((2-Fluorobenzyl)oxy)phenyl)-1,2,4-oxadiazol-5-yl)ethyl)acetamide (8)**

The title compound was prepared according to general procedure A (quant.). ^{1}H-NMR (500 MHz, $CDCl_3$) δ 8.01 (d, *J* = 8.6 Hz, 2H), 7.51 (td, *J* = 7.4, 1.7 Hz, 1H), 7.36–7.31 (m, 1H), 7.18 (td, *J* = 7.4, 1.1 Hz, 1H), 7.12–7.08 (m, 1H), 7.07 (d, *J* = 9.2 Hz, 2H), 6.23 (d, *J* = 7.4 Hz, 1H), 5.46 (dq, *J* = 7.4, 7.4 Hz, 1H), 5.19 (s, 2H), 2.09 (s, 3H), 1.63 (d, *J* = 7.4 Hz, 3H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 179.24, 169.47, 167.87, 160.91, 160.43 (d, *J* = 247.1 Hz), 129.94 (d, *J* = 8.4 Hz), 129.68 (d, *J* = 3.6 Hz), 129.15 (2C), 124.31 (d, *J* = 3.6 Hz), 123.52 (d, *J* = 14.4 Hz), 119.27, 115.42 (d, *J* = 20.4 Hz), 115.06 (2C), 63.73 (d, *J* = 4.8 Hz), 42.81, 23.15, 19.97.

***N*-(1-(3-(4-((4-Methoxybenzyl)oxy)phenyl)-1,2,4-oxadiazol-5-yl)ethyl)acetamide (9)**

The title compound was prepared according to general procedure A (quant.). ^{1}H-NMR (500 MHz, $CDCl_3$) δ 8.00 (d, *J* = 8.6 Hz, 2H), 7.38 (d, *J* = 8.6 Hz, 2H), 7.05 (d, *J* = 8.6 Hz, 2H), 6.94 (d, *J* = 8.6 Hz, 2H), 6.23 (d, *J* = 8.0 Hz, 1H), 5.46 (dq, *J* = 8.0, 6.9 Hz, 1H), 5.04 (s, 2H), 3.82 (s, 3H), 2.08 (s, 3H), 1.63 (d, *J* = 6.9 Hz, 3H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 179.19, 169.46, 167.92, 161.24, 159.57, 129.29 (2C), 129.09 (2C), 128.33, 118.95, 115.12 (2C), 114.05 (2C), 69.87, 55.30, 42.81, 23.15, 19.98.

***N*-(1-(3-(4-((3-Methoxybenzyl)oxy)phenyl)-1,2,4-oxadiazol-5-yl)ethyl)acetamide (10)**

The title compound was prepared according to general procedure A (96%). ^{1}H-NMR (500 MHz, $CDCl_3$) δ 8.00 (d, *J* = 8.6 Hz, 2H), 7.31 (t, *J* = 8.0 Hz, 1H), 7.04 (d, *J* = 8.6 Hz, 2H), 7.01 (d, *J* = 8.6 Hz, 1H), 6.99 (t, *J* = 2.0 Hz, 1H), 6.89 (dd, *J* = 8.1, 2.3 Hz, 1H), 6.21 (d, *J* = 7.5 Hz, 1H), 5.46 (dq, *J* = 7.4, 7.4 Hz, 1H), 5.10 (s, 2H), 3.82 (s, 3H), 2.09 (s, 3H), 1.64 (d, *J* = 6.9 Hz, 3H); ^{13}C-NMR (125 MHz, $CDCl_3$) δ 179.21,

169.44, 167.89, 161.12, 159.85, 137.92, 129.72, 129.11 (2C), 119.61, 119.09, 115.13 (2C), 113.60, 112.91, 69.92, 55.24, 42.81, 23.16, 19.99.

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9. Although *in vitro* luciferase inhibition assay can provide direct evidence that a compound is a luciferase inhibitor, we have experienced some situations in which the *in vitro* luciferase assay appears less sensitive than cell-based counter assays (such as the one in Figure 1D). This phenomenon might be due to the different settings of the assays (*in vitro* or *in cellulo*), as well as the complex nature of the luciferase enzyme, which requires multiple co-factors, and sometimes catalyzes the formation of a stronger inhibitor (AMP adduct, for example) from a weaker inhibitor within its substrate-binding pocket (for details, see Refs 6 & 7). So, even if a compound is inactive in *in vitro* luciferase assay, it is important to perform secondary assays that monitor other processes for confirmation.