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Structure–activity relationship of 2,2-dimethyl-2*H*-chromene based arylsulfonamide analogs of 3,4-dimethoxy-*N*[(2,2-dimethyl-2*H*-chromen-6-yl)methyl]-*N*-phenylbenzenesulfonamide, a novel small molecule hypoxia inducible factor-1 (HIF-1) pathway inhibitor and anti-cancer agent

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Abstract

We have discovered that 3,4-dimethoxy-*N*[(2,2-dimethyl-2*H*-chromen-6-yl)methyl]-*N*-phenylbenzenesulfonamide, a novel small molecule HIF-1 pathway inhibitor, can antagonize tumor growth in animal models of cancer, but the treatment necessitates its delivery in a formulation, due to poor water solubility (<15 µg/mL; pH 7.4), evidencing that the chemotype needs further exploration of its amenability to additional chemical modifications for ultimate optimization of function and pharmacology.

As a first step towards this goal we investigated the structure–activity relationships of 15 lipophilic 2, 2-dimethyl-2*H*-chromene based arylsulfonamide analogs of 3,4-dimethoxy-*N*[(2,2-dimethyl-2*H*-chromen-6-yl)methyl]-*N*-phenylbenzenesulfonamide to find out strategies of modification. A 3,4-dimethoxybenzenesulfonyl group in region 1 showed the strongest inhibition among five arylsulfonyl groups tested. The presence of propan-2-amine in region 2 conferred the strongest inhibitory effect of the compound on HIF-1 activated transcription in a reporter assay.

These findings are important as they help define the structural motifs where the 3,4-dimethoxy-*N*-

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Supplementary data

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[(2,2-dimethyl-2*H*-chromen-6-yl)methyl]-*N*-phenylbenzenesulfonamide can be chemically modified to improve its pharmacological properties towards development as a cancer therapeutic.

Keywords

Hypoxia inducible factor-1; Small molecule HIF-1 transcription factor inhibitors; Tumor hypoxia; 3,4-Dimethoxy-*N*[(2,2-dimethyl-2*H*-chromen-6-yl)methyl]-*N*-phenylbenzenesulfonamide; Structure–activity relationships; Anti-cancer drugs; Brain cancer

1. Introduction

Fast tumor cell proliferation in the context of a dysfunctional vasculature generates a hypoxic microenvironment in solid tumors, which hinders the efficacy of chemo- and radiotherapies. Therefore, developing novel therapeutics to target hypoxic tumor is an active area of investigation.^{1–3} Tumor cells respond to the hypoxic stress by activating the hypoxia inducible factor (HIF) family of transcription factors, which bind to DNA sequences called hypoxia response elements (HREs) in target genes that encode proteins regulating a variety of biological functions, including erythropoiesis, angiogenesis, glycolysis, and metastasis. Hypoxia inducible factors (HIF-1 and HIF-2), are heterodimers consisting of an oxygen-sensitive subunit (HIF-1 α or -2 α), and a constitutively expressed subunit HIF-1 β . HIF- α subunits are stabilized under hypoxic conditions, while they undergo proteasomal degradation in an oxygenated environment.⁴

The best-studied family member in cancer is HIF-1. High expression of HIF-1 α in solid tumors has been associated with treatment failure and mortality,⁵ which prompted the development of HIF-1 inhibitors as anti-cancer drugs, either alone or in combination with chemo- or radiotherapy.^{6–16}

We previously screened a library of 10,000 small molecules, containing 2,2-dimethyl-2*H*-chromene as a structural motif,^{17,18} for inhibition of HIF activity using a cell-based reporter assay.^{14,15,19–22} The inhibition of HIF-1 transcriptional activation under hypoxia was measured by the activity of a genetically engineered reporter gene, which was stably transfected in a human glioma cell line. The library screening with this bioassay revealed 3,4-dimethoxy-*N*[(2,2-dimethyl-2*H*-chromen-6-yl)methyl]-*N*-phenylbenzenesulfonamide (**6e**, Fig. 1), which showed strong inhibition of reporter activity under hypoxia.^{19–21} Further testing of **6e** revealed that it has potent anti-cancer properties in animal models for brain cancer (glioblastoma), eye cancer (uveal melanoma) and pancreatic cancer (manuscripts in preparation). However, the treatment necessitated delivery of **6e** in a formulation, due to poor water solubility (<15 $\mu\text{g/mL}$; pH7.4), suggesting that further pharmacological optimization is necessary before this family of compounds can be translated to the patient. This structural optimization requires the exploration of chemical routes for modification of the compounds.

As the first step, we synthesized a series of lipophilic analogs of **6e** and compared their activities. We focused on lipophilic congeners that contain a privileged structural motif, 2,2-dimethyl-2*H*-chromene group, and systematically diversified the arylsulfonamides. The goal

was to discover effect of variations in sulfonamide groups on hypoxia-induced reporter activity, which would provide useful information for further modification. The inhibition of HIF-1 transcriptional activity under hypoxia by each compound was measured independently in an HRE-luciferase reporter cell-based assay in a dose-dependent manner. Finally, to probe the molecular mechanisms underlying the biological action of the inhibitors, we investigated the HIF-1 α protein levels under hypoxia in human glioblastoma cells in the presence of analogs of **6e** by a Western blot analysis.

2. Results and discussion

2.1. Design of analogs of 3,4-dimethoxy-*N*-[(2,2-dimethyl-2*H*-chromen-6-yl)methyl]-*N*-phenylbenzenesulfonamide

Fifteen *N*-[(2,2-dimethyl-2*H*-chromen-6-yl)methyl]-*N*-*R*-arylsulfonamides were designed, based on the structure of **6e**. Analogs of **6e** were designed with subtle variations with the hope that they would not change its presumable molecular pose. The 2,2-dimethyl-2*H*-chromene motif (region 3) was preserved in all compounds as a privileged structure, while the arylsulfonamide group was diversified to investigate structure–activity relationships (Fig. 1). Propan-2-amine (**5a–5e**), aniline (**6a–6e**) and 4-fluoroaniline (**7a–7e**) were chosen as alternate amines in region 2. This strategy allowed us to investigate the effect of substituting aniline with an alkylamine or a functionalized aniline on the inhibition of hypoxia/HIF-activated reporter expression. 2-Methylpropan-1-amine in region 2 produced five potent analogs in our previous study,¹⁹ so we further investigated propan-2-amine in region 2 to see if the potency would be preserved with minor chain length change. 4-Fluoroaniline was selected due to small size and the electron-withdrawing property of fluorine; the latter would also give an insight into the potential behavior of future compounds with a heteroarylaniline such as pyridin-2-amine and pyridin-4-amine in region 2. Benzenesulfonyl (**5a**, **6a**, **7a**), 2,5-dichlorobenzenesulfonyl (**5b**, **6b**, **7b**), 4-fluorobenzenesulfonyl (**5c**, **6c**, **7c**), 4-nitrobenzenesulfonyl (**5d**, **6d**, **7d**), and 3,4-dimethoxybenzenesulfonyl (**5e**, **6e**, **7e**) groups were used to investigate the effect of substituents on a benzene ring of the arylsulfonyl group in region 1 (Table 1).

2.2. Syntheses of analogs of **6e**

Fifteen analogs of **6e**, *N*-[(2,2-dimethyl-2*H*-chromen-6-yl)-methyl]-*N*-*R*-arylsulfonamides, were synthesized from *N*-[(2,2-dimethyl-2*H*-chromen-6-yl)methyl]amine and commercially available arylsulfonyl chlorides (Scheme 1).

4-[(2-Methylbut-3-yn-2-yl)oxy]benzaldehyde (**1**) was synthesized from an alkylation of 4-hydroxybenzaldehyde with 3-chloro-3-methyl-1-butyne using sodium hydroxide as a base in aqueous dimethylformamide (DMF) (water/DMF (v/v) = 8:5). 4-Hydroxybenzaldehyde was used in excess, since it was the less expensive reagent, and easily eliminated from the crude reaction mixture in diethyl ether by washing with aqueous potassium hydroxide solution and then performing column chromatography. 2,2-Dimethyl-2*H*-chromene-6-carbaldehyde (**2**) was formed from **1** by Claisen cyclization in refluxing *N*-methyl-2-pyrrolidone (NMP). NMP was selected as a Claisen cyclization solvent due to its high boiling point (202 °C), and ease of removal from a diethyl ether solution by washing with water.^{23–25} *N*-[(2,2-

dimethyl-2-*H*-chromen-6-yl)methyl]propan-2-amine (**4a**) was synthesized from **2** and propan-2-amine by imine formation, and then reduction with diisobutylaluminum hydride (DIBAL). The final sulfonamide, *N*-[(2,2-dimethyl-2-*H*-chromen-6-yl)methyl]-*N*-(propan-2-yl)benzenesulfonamide (**5a**), was synthesized by combining **4a** with a benzenesulfonyl chloride in the presence of triethylamine. All other sulfonamides were synthesized in the same manner. The reaction yields were not optimized.

2.3. HRE-mediated luciferase reporter assays

We measured the inhibition of HIF-1 transcriptional activity under hypoxia by analogs of **6e** using a cell-based reporter assay. A hypoxia/HIF-responsive luciferase gene reporter plasmid driven by a mini cytomegalovirus (CMV) gene promoter and an enhancer element made of 6 tandem repeats of the hypoxia response element (HRE) of the vascular endothelial growth factor gene (*VEGF*) was stably transfected in LN229 human glioblastoma cells to generate LN229-V6R cells (Fig. 2).^{19,27} LN229-V6R cells were cultured in an oxygenated ambient air environment (21% O₂, normoxia) for 24 h, the analogs of **6e** added and after 1 hour, the cell culture flasks transferred to a hypoxic cell culture incubator (1% O₂) for 24 h, after which a cell extract was prepared. Hypoxia induced the intracellular stabilization of the HIF-1 α subunit, HIF transcriptional complex assembly, binding to the HRE on genomic DNA in the nucleus and transcription of the luciferase reporter gene. To measure the amount of luciferase enzyme synthesized in the cells following HIF activation in the presence or absence of inhibitors, we used a chemiluminescence assay as we previously described.^{14,15} Briefly, the amount of luciferase present in the cell extracts was quantified by exposing it to luciferine substrate in vitro and measuring the light emitted in the reaction with a luminometer. Analogs of **6e** were diluted serially to measure reporter inhibition in a dose-dependent fashion at concentrations of 0.5, 1, 2, 5, 10 and 25 μ M. Three independent measurements for each concentration of each compound were performed and the experiment was repeated twice independently to obtain six measurements in total. The average percent of remaining luciferase activity in extracts from treated cells versus that found in extracts from untreated hypoxic cells as a control was plotted for each compound in a dose-dependent fashion. The IC₅₀ values of each compound were obtained by fitting the data of luciferase activities and concentrations by a four-parameter nonlinear regression model, in which the IC₅₀ value was one of the parameters (Table 2). The statistical analysis was conducted in R statistical software, as we previously described.¹⁹

Fifteen analogs of **6e** showed inhibition of HIF-1 transcriptional activity under hypoxia in a dose-dependent fashion with IC₅₀ values ranging from 1.5 to 17 μ M. Among the three amines tested for region 2, propan-2-amine showed the strongest inhibitory activity when combined with four arylsulfonyl groups: benzenesulfonyl (**5a**), 4-fluorobenzenesulfonyl (**5c**), 4-nitrobenzenesulfonyl (**5d**) and 3,4-dimethoxybenzenesulfonyl (**5e**). Among five sulfonyl groups tested for region 1, the 3,4-dimethoxybenzenesulfonyl group showed the strongest HIF-1 inhibitions when combined with all three amines: propan-2-amine (**5e**), aniline (**6e**) and 4-fluoroaniline (**7e**).

2.4. HIF-1 α Western blot analyses

The activity of the HIF transcription factor is tightly regulated under normoxia. In the presence of oxygen, the HIF-1 α subunit is constitutively hydroxylated at proline –402 and –564 by oxygen-dependent prolyl hydroxylases (PHDs), which triggers the interaction of HIF-1 α with the VHL tumor suppressor protein, an E3 ubiquitin-protein ligase that targets it for proteosomal degradation.^{4,5,28} Under hypoxic conditions, as occurs in solid tumors, the HIF-1 α subunit becomes stabilized due to lack of PHD binding, binds to HIF-1 β and a stable HIF transcriptional complex is formed in the nucleus with co-factors p300/CBP.^{28–30} To determine whether analogs of **6e** may block HIF activity by preventing HIF-1 α protein accumulation under hypoxia or destabilizing the protein, HIF-1 α protein levels under hypoxia were measured by Western blotting in the presence of analogs of **6e** (Fig. 3). **5e**, **6e**, and **7e** showed the strongest HIF-1 inhibition for each amine series in region 2 (less than 5% remaining activity at 10 μ M), and **5c** contains a 4-fluorobenzenesulfonyl group in region 1 that could predict the behavior of electron-withdrawing heteroarylsulfonyl groups (e.g. pyridine-4-sulfonyl group). HIF-1 α Western blot analyses were performed on cell extracts from LN229-V6R cells incubated with 10 μ M of the compounds under hypoxia for 24 h. A modest 30% reduction in the level of HIF-1 α protein was observed in extracts from hypoxic cells treated with **5e**, whereas little change was observed for **5c**, **6e**, and **7e** treated cells. The batch-to-batch variation between **6e** and **6e*** was minimal as well. While this modest reduction in HIF-1 α protein level induced by **5e** might contribute to the strong inhibition of HIF transcriptional activity, it is unlikely to be the primary cause, because at 10 μ M **5e** almost entirely abolished HIF-1 activity in the luciferase reporter assay (less than 5% remaining activity, see Supplementary data 4). Overall, the western blot analysis showed that the compounds had little activity on the synthesis and stability of HIF-1 α .

3. Conclusions

Fifteen analogs of **6e**, *N*–[(2,2-dimethyl-2*H*-chromen-6-yl)-methyl]–*N*–*R*-arylsulfonamides, were synthesized to possess 2,2-dimethyl-2*H*-chromene as a privileged structural motif, and a sulfonamide group composed of three different amines and five different sulfonyl groups. The inhibition of HIF-1 transcriptional activity mediated by the 15 analogs was measured in LN229-V6R, a human glioblastoma cell line containing a hypoxia/HIF activated luciferase reporter gene. All 15 *N*–[(2,2-dimethyl-2*H*-chromen-6-yl)methyl]–*N*–*R*-arylsulfonamides inhibited the reporter gene expression under hypoxia in a dose-dependent fashion with IC₅₀ values in the range of 1.5–17 μ M. The substitution of propan-2-amine in region 2 of **6e**, showed the strongest inhibition among three tested amines in the sulfonamide group. The presence of a 3,4-dimethoxybenzenesulfonyl group in region 1 was most potent in inhibiting reporter activity among the five sulfonyl groups tested in the sulfonamide group.

To probe the molecular mechanisms underlying the inhibition of HIF transcriptional activity by the compounds, we measured HIF-1 α protein levels under hypoxia. A modest 30% decrease in HIF-1 α protein level was observed with **5e** and little decrease with **5c**, **6e**, and **7e** in comparison to the almost complete abrogation in transcriptional activity of the reporter at 10 μ M. This result indicates that the mechanism of HIF inhibition by the analogs of **6e** is not by preventing accumulation of HIF-1 α protein or destabilizing it under hypoxia. Inhibition

of HIF activity may occur at any step in its activation cascade, in which hypoxia-stabilized cytoplasmic HIF-1 α translocates to the nucleus where it binds to HIF-1 β and p300/CBP to form a stable HIF transcriptional complex, which can bind to the HREs of target genes and activate their transcription. Our ongoing investigations with **6e** are focused on the hypothesis that **6e** might bind the CH1 domain of the p300/CBP co-factors and thereby antagonize their interaction with the C-terminal activation domain of HIF-1 α .

In summary, *N*[(2,2-dimethyl-2*H*-chromen-6-yl)methyl]-*N*-*R*-arylsulfonamides are novel small molecules that inhibit the function of an important transcription factor necessary for cancer cell growth under hypoxia. These compounds have molecular weights around 400 g/mole, and can be synthesized in 4–5 steps with reasonable yields. The structure–activity relationship studies in this Letter, demonstrated that we could modify 3,4-dimethoxy-*N*[(2,2-dimethyl-2*H*-chromen-6-yl)methyl]-*N*-phenylbenzenesulfonamide (**6e**), using either of two strategies. One is to introduce hydrophilic amines in region 2 with a 3,4-dimethoxybenzenesulfonyl group in region 1, which is suggested from results with **5e**, **6e**, and **7e**. In this strategy, simple electron withdrawing heteroaryl amines, such as pyridine-2-amine, pyridine-4-amine, might decrease HIF-1 pathway inhibition, if they behave the same way as 4-fluoroaniline. The other strategy is to introduce hydrophilic arylsulfonyl groups in region 1 with a propan-2-amine group in region 2 as suggested from results with **5a**, **5c**, **5d**, and **5e**. In a follow-up study, we plan on using the latter strategy to introduce heteroarylsulfonyl groups in region 1 with simple alkyl groups in region 2. Since the molecules can be diversified at the last step of a synthesis, this approach is more advantageous than the former strategy.

4. Experimental

4.1. Chemistry

Glassware was oven-dried (200 °C) overnight and cooled under argon, and all reactions were performed under argon. All reagents were purchased from commercial sources. Column chromatography was performed with Silica Gel 60 (particle size 40–63 μ m) with less than 4 psi of argon pressure. TLC was performed with TLC Silica gel 60 F₂₅₄ and visualized by UV (254 nm) and staining with 10% (w/w) of phosphomolybdic acid hydrate solution in ethanol.

NMR spectroscopy was recorded by Varian INOVA 400, Mercury 300 and VNMRs 400 spectrometers. Mass spectroscopy was obtained by JEOL JMS-SX102/SX102A/E. HPLC was measured with a Waters 1525 Binary HPLC pump coupled with a Waters 2487 Dual λ Absorbance Detector. HPLC data was processed by Waters Breeze GPC Software. Two HPLC systems were used to measure the purity of each analog by % area of a UV absorbance peak at 254 nm: WAT045905 (Symmetry C₁₈, 5 μ m, 4.6 $\times$ 150 mm), 70:30:0.1 = methanol/water/triethylamine, 2 mL/min of a flow rate; WAT011695 (Nova-Pak C₁₈, 4 μ m, 3.9 $\times$ 300 mm), 70:30:0.1 = 90% ethanol, 5% methanol, 5% isopropanol/water/triethylamine (**5a–5d**, **6a–6d**, and **7a–7d**) or 60:40:0.1 = 90% ethanol, 5% methanol, 5% isopropanol: water: triethylamine (**5e**, **6e**, **7e**), 0.8 mL/min of a flow rate. The peaks after 3.5 min were considered as peaks of compounds. As an eluent, we used a mixture of 95% ethanol, 5% methanol, and 5% isopropanol instead of 100% of ethanol due to an institutional regulation.

4.1.1. 4-[(2-Methylbut-3-yn-2-yl)oxy]benzaldehyde (1)—A solution of 2.4 g (20 mmole) of 4-hydroxybenzaldehyde and 1 g (10 mmole) of 3-chloro-3-methyl-1-butyne in 8 mL of DMF, was mixed with 5 mL of 4 N aqueous sodium hydroxide solution. The bi-phase mixture was stirred vigorously at 60 °C for 8 h. After cooling, 20 mL of water was added to the reaction mixture, which was extracted with 30 mL of diethyl ether three times. The combined organic layer was washed with 40 mL of 1 N aqueous sodium hydroxide solution and brine. The organic portion was then dried with magnesium sulfate and concentrated in vacuo. A pale yellow viscous liquid was produced as a crude product. A small portion of the crude product was purified by column chromatography using ethyl acetate/hexane (1:8) as an eluent, and the rest of the crude product was used in the next reaction.

^1H NMR (CDCl_3): δ 1.73 (s, 6H), 2.67 (s, 1H), 7.34 (d, J = 8.8 Hz, 1H), 7.83 (d, J = 8.8 Hz, 1H), 9.91 (s, 1H).

4.1.2. 2,2-Dimethyl-2H-chromene-6-carbaldehyde (2)—A crude **1** from the previous reaction was dissolved in 20 mL of *N*-methyl-2-pyrrolidone, NMP (bp 202 °C), and refluxed for 8 h. After cooling, a small portion of the crude mixture was extracted with diethyl ether to confirm complete conversion to the desired product by NMR. Forty milliliters of water was added to the reaction mixture, and then extracted with 60 mL of diethyl ether three times. The combined organic layer was washed with brine, dried with magnesium sulfate, and then concentrated in vacuo. The crude product was purified by column chromatography using ethyl acetate/hexane (1:8) as an eluent, which afforded 565 mg (3 mmole) of 2,2-dimethyl-2H-chromen-6-carboxaldehyde as a pale-yellow viscous liquid (15% in two steps, based on 20 mmole of 4-hydroxybenzaldehyde).

^1H NMR (CDCl_3): δ 1.46 (s, 6H), 5.68 (d, J = 9.9 Hz, 1H), 6.36 (d, J = 9.9 Hz, 1H), 6.85 (d, J = 8.05 Hz, 1H), 7.50 (d, J = 2.37 Hz, 1H), 7.63 (dd, J = 8.52, 1.89 Hz, 1H), 9.83 (s, 1H).

HRMS m/z M^+ calcd 189.09101, found 189.09071.

4.1.3. Imine synthesis (3a, 3b, 3c)—A mixture of 102 mg (0.54 mmole) of **2**, 20 mg of *p*-toluenesulfonic acid monohydrate and 1 mL of propan-2-amine (**3a**) (0.1 mL of aniline (**3b**) or 0.1 mL of 4-fluoroaniline (**3c**)) in 2 mL of methylene chloride, was refluxed overnight. After cooling, a small portion of the crude product was concentrated in vacuo to confirm complete conversion to the desired imine by NMR. The indication was the disappearance of the aldehyde peak (9.83 ppm) and the appearance of the imine peak (8.18 ppm). After confirming the complete conversion to an imine, the crude mixture was concentrated in vacuo and then, used for the next reaction without purification.

4.1.3.1. 1-(2,2-Dimethyl-2H-chromen-6-yl)-*N*-(propan-2-yl)methanimine (3a): ^1H NMR (CDCl_3): δ 1.24 (d, J = 6.15 Hz, 6H), 1.43 (s, 6H), 3.48 (septet, J = 6.1 Hz, 1H), 5.63 (d, J = 5.6 Hz, 1H), 6.35 (d, J = 10 Hz, 1H), 6.78 (d, J = 8.2 Hz, 1H), 7.39 (dd, J = 8.2, 2.1 Hz, 1H), 7.45 (d, J = 1.76 Hz, 1H), 8.18 (s, 1H).

^{13}C NMR (CDCl_3): δ 24.4, 24.5, 28.3, 61.7, 77.0, 77.3, 77.5, 116.5, 121.5, 122.2, 122.3, 125.6, 125.6, 129.6, 130.0, 130.0, 131.3, 155.3, 157.9, 158.0.

4.1.3.2. 1-(2,2-Dimethyl-2H-chromen-6-yl)-N-phenylmethanimine (3b): ¹H NMR (CDCl₃): δ 1.49 (s, 6H), 5.69 (d, *J* = 9.67 Hz, 1H), 6.41 (d, *J* = 9.67 Hz, 1H), 6.70–7.61 (m, 8H), 8.35 (s, 1H).

4.1.3.3. 1-(2,2-Dimethyl-2H-chromen-6-yl)-N-(4-fluorophenyl)methanimine (3c): ¹H NMR (CDCl₃): δ 1.48 (s, 6H), 5.69 (d, *J* = 9.96 Hz, 1H), 6.40 (d, *J* = 9.96, 1H), 6.60–7.62 (m, 8H), 8.32 (s, 1H).

4.1.4. Amine synthesis (4a, 4b, 4c)—The crude **3a** from the previous reaction was dissolved in 3 mL of toluene, to which 1 mL (1 mmole) of diisobutylaluminum hydride (1 M solution in toluene) was added slowly. The reaction mixture was stirred overnight and poured slowly into 10 mL of 1 N aqueous hydrochloric acid solution with stirring. The mixture was extracted with 20 mL of ethyl acetate three times. The combined organic layer was washed with brine, dried with magnesium sulfate, and then concentrated in vacuo. *N*-[(2,2-dimethyl-2H-chromen-6-yl)methyl]methanamine was purified by column chromatography using triethylamine/methanol/methylene chloride (1:3:100) as an eluent to afford 111 mg (0.5 mmole) of a pure product (**4a**) (89% in two steps). *N*-[(2,2-Dimethyl-2H-chromen-6-yl)methyl]aniline (**4b**) (85% in two steps) and *N*-[(2,2-dimethyl-2H-chromen-6-yl)methyl]-4-fluoroaniline (**4c**) (80% in two steps) were synthesized by the same method.

4.1.4.1. *N*-[(2,2-Dimethyl-2H-chromen-6-yl)methyl]propan-2-amine (4a): ¹H NMR (CDCl₃): δ 1.10 (d, *J* = 6.35 Hz, 6H), 1.42 (s, 6H), 2.86 (septet, *J* = 6.35 Hz, 1H), 3.67 (s, 3H), 5.60 (d, *J* = 9.85 Hz, 1H), 6.31 (d, *J* = 9.85 Hz, 1H), 6.72 (d, *J* = 8.26 Hz, 1H), 6.95 (d, *J* = 2.22 Hz, 1H), 7.04 (dd, *J* = 7.94, 1.9 Hz, 1H).

HRMS *m/z* M⁺ calcd 232.16959, found 232.17035.

4.1.4.2. *N*-[(2,2-Dimethyl-2H-chromen-6-yl)methyl]aniline (4b): ¹H NMR (CDCl₃): δ 1.44 (s, 6H), 4.21 (s, 2H), 5.63 (d, *J* = 9.67 Hz, 1H), 6.31 (d, *J* = 9.67 Hz), 6.64–6.77 (m, 4H), 7.00–7.22 (m, 4H).

HRMS *m/z* M⁺ calcd 266.15394, found 266.15385.

4.1.4.3. *N*-[(2,2-Dimethyl-2H-chromen-6-yl)methyl]-4-fluoroaniline (4c): ¹H NMR (CDCl₃): δ 1.44 (s, 6H), 4.16 (s, 2H), 6.31 (d, *J* = 9.96 Hz, 1H), 6.54–6.61 (m, 2H), 6.76 (d, *J* = 8.21 Hz, 1H), 6.85–7.12 (m, 4H).

HRMS *m/z* M⁺ calcd 284.14452, found 284.14434.

4.1.5. Sulfonamide synthesis (5a–5e, 6a–6e, 7a–7e)—A mixture of 22 mg (0.093 mmole) of **4a**, 0.1 mL (164 mg, 0.93 mmole, 10 equiv) of benzenesulfonyl chloride, and 1 mL of triethylamine in 2 mL of methylene chloride was refluxed overnight. After cooling, the reaction mixture was concentrated in vacuo, and purified by column chromatography using ethyl acetate/hexane (1:6) as an eluent to afford 9 mg (0.024 mmole) of *N*-[(2,2-dimethyl-2H-chromen-6-yl)methyl]-*N*-(propan-2-yl)benzenesulfonamide (**5a**, 26%). All 15 sulfonamides were synthesized by the same method.

4.1.5.1. *N*-[(2,2-Dimethyl-2*H*-chromen-6-yl)methyl]-*N*-(propan-2-yl)benzenesulfonamide (5a): Yield: 26%, a column chromatography eluent: ethyl acetate/hexane (1:6).

¹H NMR (CDCl₃): δ 0.97 (d, *J* = 7.03 Hz, 6H), 1.43 (s, 6H), 4.12–4.19 (m, 1H), 4.30 (s, 2H), 5.61 (d, *J* = 9.67 Hz, 1H), 6.30 (d, *J* = 9.96 Hz, 1H), 6.70 (d, *J* = 8.20 Hz, 1H), 7.02 (s, 1H), 7.08 (d, *J* = 8.20 Hz, 1H), 7.46–7.56 (m, 3H), 7.79–7.82 (m, 2H).

HRMS *m/z* (M+Na)⁺ calcd 394.14474, found 394.14566.

HPLC (1) *R*_T = 10.246 min, Purity = 97%.

HPLC (2) *R*_T = 5.614 min, Purity = 91%.

4.1.5.2. 2,5-Dichloro-*N*-[(2,2-dimethyl-2*H*-chromen-6-yl)methyl]-*N*-(propan-2-yl)benzenesulfonamide (5b): Yield: 12%, a column chromatography eluent: ethyl acetate/hexane (1:6).

¹H NMR (CDCl₃): δ 1.16 (d, *J* = 6.74 Hz, 6H), 1.41 (s, 6H), 4.23–4.28 (m, 1H), 4.39 (s, 2H), 5.58 (d, *J* = 9.96 Hz, 1H), 6.22 (d, *J* = 9.96 Hz, 1H), 6.60 (d, *J* = 8.20 Hz, 1H), 6.87 (d, *J* = 2.05 Hz, 1H), 6.99 (d, *J* = 8.20 Hz, 1H), 7.38 (d, *J* = 8.50 Hz, 1H), 7.54–7.63 (m, 2H).

HRMS *m/z* (M+Na)⁺ calcd 496.05114, found 496.05160.

HPLC (1) *R*_T = 19.756 min, Purity = 96%.

HPLC (2) *R*_T = 7.325 min, Purity = 96%.

4.1.5.3. *N*-[(2,2-Dimethyl-2*H*-chromen-6-yl)methyl]-4-fluoro-*N*-(propan-2-yl)benzenesulfonamide (5c): Yield: 17%, a column chromatography eluent: ethyl acetate/hexane (1:6).

¹H NMR (CDCl₃): δ 1.12 (d, *J* = 7.03 Hz, 6H), 1.55 (s, 6H), 4.17–4.32 (m, 1H), 4.40 (s, 2H), 5.74 (d, *J* = 9.67 Hz, 1H), 6.41 (d, *J* = 9.67 Hz, 1H), 6.82 (d, *J* = 8.20 Hz, 1H), 7.11 (s, 1H), 7.18 (d, *J* = 8.50 Hz, 1H), 7.27 (t, *J* = 8.50 Hz, 2H), 7.88–7.93 (m, 2H).

HRMS *m/z* (M+Na)⁺ calcd 412.13532, found 412.13553.

HPLC (1) *R*_T = 9.684 min, Purity = 99%.

HPLC (2) *R*_T = 5.019 min, Purity = 99%.

4.1.5.4. *N*-[(2,2-Dimethyl-2*H*-chromen-6-yl)methyl]-4-nitro-*N*-(propan-2-yl)benzenesulfonamide (5d): Yield: 26%, a column chromatography eluent: ethyl acetate/hexane (1:6).

¹H NMR (CDCl₃): δ 1.074 (d, *J* = 6.74 Hz, 6H), 1.43 (s, 6H), 4.21–4.26 (m, 1H), 4.31 (s, 2H), 5.62 (d, *J* = 9.96 Hz, 1H), 6.24 (d, *J* = 9.96 Hz, 1H), 6.68 (d, *J* = 8.20 Hz, 1H), 6.91 (s, 1H), 7.04 (d, *J* = 8.20 Hz, 1H), 7.88 (d, *J* = 8.79 Hz, 2H), 8.28 (d, *J* = 8.50 Hz, 2H).

HRMS m/z (M+Na)⁺ calcd 439.12982, found 439.13048.

HPLC (1) R_T = 9.791 min, Purity = 99%.

HPLC (2) R_T = 4.976 min, Purity = 99%.

4.1.5.5. 3,4-Dimethoxy-*N*-[(2,2-dimethyl-2*H*-chromen-6-yl)methyl]-*N*-(propan-2-yl)benzenesulfonamide (5e): Yield: 45%, a column chromatography eluent: ethyl acetate/hexane (1:3).

¹H NMR (CDCl₃): δ 1.05 (d, J = 6.74 Hz, 6H), 1.47 (s, 6H), 3.94 (s, 3H), 3.99 (s, 3H), 4.14–4.24 (m, 1H), 4.33 (s, 2H), 5.66 (d, J = 9.67 Hz, 1H), 6.35 (d, J = 9.67 Hz, 1H), 6.76 (d, J = 8.20 Hz, 1H), 6.96 (d, J = 8.50 Hz, 1H), 7.07 (s, 1H), 7.144 (dd, J = 8.20, 2.05 Hz, 1H), 7.28 (d, J = 2.05 Hz, 1H), 7.47 (dd, J = 8.50, 2.05 Hz, 1H).

HRMS m/z (M+Na)⁺ calcd 454.16587, found 454.16570.

HPLC (1) R_T = 6.042 min, Purity = 98%.

HPLC (2) R_T = 6.367 min, Purity = 94%.

4.1.5.6. *N*-[(2,2-Dimethyl-2*H*-chromen-6-yl)methyl]-*N*-phenylbenzenesulfonamide (6a): Yield: 71%, a column chromatography eluent: ethyl acetate/hexane (1:6).

¹H NMR (CDCl₃): δ 1.39 (s, 6H), 4.63 (s, 2H), 5.57 (d, J = 9.67 Hz, 1H), 6.23 (d, J = 9.96 Hz, 1H), 6.59 (d, J = 8.79 Hz, 1H), 6.87–6.98 (m, 4H), 7.21–7.23 (m, 3H), 7.47–7.68 (m, 5H).

HRMS m/z (M+Na)⁺ calcd 428.12909, found 428.12915.

HPLC (1) R_T = 8.910 min, Purity = 93%.

HPLC (2) R_T = 4.974 min, Purity = 97%.

4.1.5.7. 2,5-Dichloro-*N*-[(2,2-dimethyl-2*H*-chromen-6-yl)methyl]-*N*-phenylbenzenesulfonamide (6b): Yield: 28%, a column chromatography eluent: ethyl acetate/hexane (1:6).

¹H NMR (CDCl₃): δ 1.41 (s, 6H), 4.91 (s, 2H), 5.59 (d, J = 9.96 Hz, 1H), 6.26 (d, J = 9.96 Hz, 1H), 6.63 (d, J = 8.79 Hz, 1H), 6.88–7.49 (m, 9H), 7.82 (d, J = 2.05 Hz, 1H).

HRMS m/z (M+Na)⁺ calcd 496.05114, found 496.05160.

HPLC (1) R_T = 22.911 min, Purity = 99%.

HPLC (2) R_T = 4.985 min, Purity = 99%.

4.1.5.8. *N*-[(2,2-Dimethyl-2*H*-chromen-6-yl)methyl]-4-fluoro-*N*-

phenylbenzenesulfonamide (6c): Yield: 62%, a column chromatography eluent: ethyl acetate/hexane (1:6).

¹H NMR (CDCl₃): δ 1.39 (s, 6H), 4.62 (s, 2H), 5.58 (d, *J* = 9.67 Hz, 1H), 6.23 (d, *J* = 9.67 Hz, 1H), 6.60 (d, *J* = 8.50 Hz, 1H), 6.86–7.24 (m, 9H), 7.66 (dd, *J* = 8.79, 5.27 Hz, 2H).

HRMS *m/z* (M+Na)⁺ calcd 446.11967, found 446.11967.

HPLC (1) *R*_T = 9.330 min, Purity = 98%.

HPLC (2) *R*_T = 5.164 min, Purity = 98%.

4.1.5.9. *N*-[(2,2-Dimethyl-2*H*-chromen-6-yl)methyl]-4-nitro-*N*-

phenylbenzenesulfonamide (6d): Yield: 23%, a column chromatography eluent: ethyl acetate/hexane (1:6).

¹H NMR (CDCl₃): δ 1.45 (s, 6H), 4.72 (s, 2H), 5.65 (d, *J* = 9.96 Hz, 1H), 6.28 (d, *J* = 9.96 Hz, 1H), 6.67 (d, *J* = 7.91 Hz, 1H), 6.91–7.02 (m, 4H), 7.30–7.33 (m, 3H), 7.88 (d, *J* = 9.08 Hz, 2H), 8.38 (d, *J* = 8.79 Hz, 2H).

HRMS *m/z* (M+Na)⁺ calcd 450.12494 found 450.12551.

HPLC (1) *R*_T = 10.179 min, Purity = 98%.

HPLC (2) *R*_T = 5.202 min, Purity = 97%.

4.1.5.10. 3,4-Dimethoxy-*N*-[(2,2-dimethyl-2*H*-chromen-6-yl)methyl]-*N*-

phenylbenzenesulfonamide (6e): Yield: 31%, a column chromatography eluent: ethyl acetate/hexane (1:3).

¹H NMR (CDCl₃): δ 1.39 (s, 6H), 3.80 (s, 3H), 3.97 (s, 3H), 4.57 (s, 2H), 5.58 (d, *J* = 9.96 Hz, 1H), 6.23 (d, *J* = 9.67 Hz, 1H), 6.60 (d, *J* = 7.91 Hz, 1H), 6.85–6.95 (m, 8H), 7.00 (d, *J* = 2.05 Hz, 1H), 7.32 (dd, *J* = 8.50, 2.05 Hz, 1H).

HRMS *m/z* (M+Na)⁺ calcd 488.15022 found 488.15029.

HPLC (1) *R*_T = 7.372 min, Purity = 99%.

HPLC (2) *R*_T = 7.730 min, Purity = 97%.

4.1.5.11. *N*-[(2,2-Dimethyl-2*H*-chromen-6-yl)methyl]-*N*-(4-

fluorophenyl)benzenesulfonamide (7a): Yield: 61%, a column chromatography eluent: ethyl acetate/hexane (1:6).

¹H NMR (CDCl₃): δ 1.39 (s, 6H), 4.59 (s, 2H), 5.58 (d, *J* = 9.67 Hz, 1H), 6.23 (d, *J* = 9.67 Hz, 1H), 6.60 (d, *J* = 8.50 Hz, 1H), 6.84–6.90 (m, 6H), 7.51–7.68 (m, 5H).

HRMS *m/z* (M+Na)⁺ calcd 446.11967, found 446.11995.

HPLC (1) R_T = 8.941 min, Purity = 96%.

HPLC (2) R_T = 5.082 min, Purity = 88%.

4.1.5.12. 2,5-Dichloro-*N*-[(2,2-dimethyl-2*H*-chromen-6-yl)methyl]-*N*-(4-fluorophenyl)benzenesulfonamide (7b): Yield: 22%, a column chromatography eluent: ethyl acetate/hexane (1:6).

^1H NMR (CDCl_3): δ 1.44 (s, 6H), 4.16 (s, 2H), 5.63 (d, J = 9.96 Hz, 1H), 6.31 (d, J = 9.96 Hz, 1H), 6.58 (dd, J = 9.08, 4.40 Hz, 2H), 6.75 (d, J = 8.21 Hz, 1H), 6.89 (t, J = 8.79 Hz, 2H), 6.98 (d, J = 2.05 Hz, 1H), 7.10 (dd, J = 7.91, 1.76, 1H).

HRMS m/z ($\text{M}+\text{Na}$) $^+$ calcd 518.38138, found 518.38149.

HPLC (1) R_T = 8.951 min, Purity = 98%.

HPLC (2) R_T = 5.723 min, Purity = 91%.

4.1.5.13. *N*-[(2,2-Dimethyl-2*H*-chromen-6-yl)methyl]-4-fluoro-*N*-(4-fluorophenyl)benzenesulfonamide (7c): Yield: 54%, a column chromatography eluent: ethyl acetate/hexane (1:6).

^1H NMR (CDCl_3): δ 1.39 (s, 6H), 4.59 (s, 2H), 5.59 (d, J = 9.96 Hz, 1H), 6.23 (d, J = 9.96 Hz, 1H), 6.61 (d, J = 7.91 Hz, 1H), 6.84–6.92 (m, 6H), 7.18 (t, J = 8.50 Hz, 2H), 7.67 (dd, J = 8.79, 4.98 Hz, 2H).

HRMS m/z ($\text{M}+\text{Na}$) $^+$ calcd 464.11024, found 464.11058.

HPLC (1) R_T = 10.205 min, Purity = 91%.

HPLC (2) R_T = 5.102 min, Purity = 99%.

4.1.5.14. *N*-[(2,2-Dimethyl-2*H*-chromen-6-yl)methyl]-*N*-(4-fluorophenyl)-4-nitrobenzenesulfonamide (7d): Yield: 33%, a column chromatography eluent: ethyl acetate/hexane (1:6).

^1H NMR (CDCl_3): δ 1.40 (s, 6H), 4.63 (s, 2H), 5.60 (d, J = 9.96 Hz, 1H), 6.23 (d, J = 9.67 Hz, 1H), 6.62 (d, J = 8.21 Hz, 1H), 6.82–6.97 (m, 6H), 7.83 (d, J = 8.79 Hz, 2H), 8.34 (d, J = 9.08 Hz, 2H).

HRMS m/z ($\text{M}+\text{Na}$) $^+$ calcd 469.12280, found 469.12403.

HPLC (1) R_T = 10.882 min, Purity = 91%.

HPLC (2) R_T = 5.325 min, Purity = 95%.

4.1.5.15. 3,4-Dimethoxy-*N*-[(2,2-dimethyl-2*H*-chromen-6-yl)methyl]-*N*-(4-fluorophenyl)benzenesulfonamide (7e): Yield: 34%, a column chromatography eluent: ethyl acetate/hexane (1:3).

^1H NMR (CDCl_3): δ 1.37 (s, 6H), 3.74 (s, 3H), 3.95 (s, 3H), 4.59 (s, 2H), 5.55 (d, J = 9.96 Hz, 1H), 6.21 (d, J = 9.96 Hz, 1H), 6.58 (d, J = 9.08 Hz, 1H), 6.86–7.01 (m, 6H), 7.19–7.34 (m, 5H).

HRMS m/z ($\text{M}+\text{Na}$) $^+$ calcd 506.14079, found 506.14080.

HPLC (1) R_T = 6.565 min, Purity = 99%.

HPLC (2) R_T = 7.219 min, Purity = 95%.

4.2. Biology

4.2.1. Generation of hypoxia/HIF reporter cell line (LN229-V6R cells)—The LN229-V6R cell line was constructed by stably transfecting LN229 human glioblastoma cells²⁸ with a vector, pBI-GL *VEGF*HRE V6R, which was engineered by modifying the commercial pBI-GL (TET) mammalian expression vector, so as to replace the tet-responsive elements driving reporter gene expression with 6 tandem copies of the *VEGF* gene HRE.²⁶ pBI-GL *VEGF*HRE V6R contains luciferase and lacZ reporter genes under a bi-directional hypoxia/HIF-responsive promoter. These cells synthesize luciferase and β -galactosidase under hypoxic (1% oxygen) conditions and can be used as a bioassay for the detection of HIF transcription inhibitors.¹⁹ The LN229-V6R cells were cultured in DMEM medium supplemented with 10% fetal bovine serum and penicillin/streptomycin at 37 °C under normoxic (21% O_2 , 5% CO_2 , 74% N_2) or hypoxic (1% O_2 , 5% CO_2 , 74% N_2) conditions.

4.2.2. Assays to measure HIF-1 transcriptional activity using HRE-driven luciferase reporter gene expression (luciferase assays)—LN229-V6R cells were seeded in 48-well plates (3×10^4 cells per well in 300 μL DMEM medium) and cultured for 24 h under normoxia before addition of new medium with chemical compounds. The synthesized compounds were dissolved in 100% of DMSO at 10 mM and a 100 μM solution of each compound was prepared in complete DMEM with 1% DMSO to prepare the compounds for cell treatment. The desired concentrations of *N*-[(2,2-dimethyl-2*H*-chromen-6-yl)methyl]-*N*-*R*-arylsulfonamides were prepared by serial dilutions in culture medium with 1% DMSO, and 300 μL was distributed to each well in triplicate for each concentration and compound. The 48-well plates were incubated at 37 °C under normoxia for one hour, then transferred to hypoxic conditions in a CO_2 incubator for 24 h. The cells were washed with PBS and then lysed with lysis buffer (50 μL /well) that was prepared by a 1/5 dilution of a passive lysis buffer 5 $\times$ (Promega, E194A) in distilled water. 20 μL of the protein suspension from each well was mixed with 25 μL of luciferase assay substrate and luminescence was measured using a 20/20n luminometer (Turner BioSystems Inc.).

4.2.3. Western blot analysis to determine HIF-1 α protein levels—LN229-V6R cells (2×10^5) plated in 2 mL of complete DMEM culture media in 35 mm dishes were cultured under normoxia for 24 h, and then the media were removed by vacuum suction. Ten micromolars of the compounds (**5c**, **5e**, **6e**, and **7e**) in culture medium with 1% of DMSO were added to each dish. Dishes were incubated under normoxia for one hour, and then transferred into a hypoxic incubator at 37 °C for 24 h. The cells were lysed with 1 $\times$ loading buffer (62.5 mM Tris-HCl (pH 6.8 at 25 °C), 2% (w/v) SDS, 50 μM 2-mercaptoethanol,

10% glycerol, 0.01% bromophenol blue) buffer, and the proteins were separated by electrophoresis in a 12.5% Tris–HCl polyacrylamide gel, before transfer onto a nitro-cellulose membrane.^{14,15} The membranes were immunoblotted using primary antibodies against HIF-1 α (1:500 dilution, BD biosciences, San Diego, CA) and β -actin (1:3000 dilution, Santa Cruz Biotechnologies, Santa Cruz, CA). The secondary horseradish peroxidase-conjugated antibodies were added and horseradish peroxidase activities were measured by enhanced chemiluminescence as previously described.¹⁵ Densitometry of the Western blot was processed by ImageJ 1.44p. (Wayne Rasband, National Institutes of Health, USA, <http://imagej.nih.gov/ij>)

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Abbreviations

HIF-1	hypoxia inducible factor-1
HREs	hypoxia response elements
HRE-AP assay	hypoxia response element-mediated alkaline phosphatase assay
HRE-Luc assay	hypoxia response element-mediated luciferase assay

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Figure 1.

Chemical structure of 3,4-dimethoxy-*N*-[(2,2-dimethyl-2*H*-chromen-6-yl)methyl]-*N*-phenylbenzenesulfonamide (**6e**) and design of *N*-[(2,2-dimethyl-2*H*-chromen-6-yl)methyl]-*N*-*R*-arylsulfonamides. Regions were defined according to Ref. 19.

Figure 2.

pBI-GL HRE V6R plasmid structure. The pBI-GL HRE V6R plasmid was generated from the mammalian expression vector pBI-GL (TET) and is redrawn here from Figure 1 in Ref. 29. The arrows indicate the direction of transcription of both genes (luciferase and LacZ) that are under the control of the 6 copies of the *VEGF* gene HREs.

Figure 3.

Protein immunoblot (Western blot) analysis of HIF-1 α expression under hypoxia. **6e*** is previously synthesized **6e**¹⁹. Densitometry of the Western blot was processed by ImageJ 1.44p (Wayne Rasband, National Institutes of Health, USA, <http://imagej.nih.gov/ij>), and band intensity expressed as percent of untreated control cells under hypoxia normalized for β -actin.

Scheme 1.

Synthesis of 15 analogs of **6e**. Reagents and conditions: (a) 3-chloro-3-methyl-1-butyne, aq NaOH, DMF, 60 °C, 8 h; (b) *N*-methyl-2-pyrrolidone, reflux, 8 h (15% in two steps); (c) amine (propan-2-amine, aniline, 4-fluoroaniline), *p*-toluenesulfonic acid monohydrate, methylene chloride, reflux, overnight; (d) DIBAL, toluene, overnight (89% in two steps); (e) arylsulfonyl chloride, triethylamine, methylene chloride, reflux, overnight (12%–71%).

Table 1Structures of 15 analogs of **6e**

Region 2 (N-R)	Region 1 (SO ₂ -Ar)
	5a	5b	5c	5d	5e
6a	6b	6c	6d	6e
7a	7b	7c	7d	7e

Propan-2-amine (**5**), aniline (**6**), and 4-fluoroaniline (**7**) in region 2, were combined with benzenesulfonyl (**a**), 2,5-dichlorobenzenesulfonyl (**b**), 4-fluorobenzenesulfonyl (**c**), 4-nitrobenzenesulfonyl (**d**), and 3,4-dimethoxybenzenesulfonyl (**e**) group in region 1 to generate 15 different arylsulfonamide analogs. Alterations in region 1 are shown horizontally, while alterations in region 2 are shown vertically.

Table 2

IC₅₀ values of HIF reporter gene inhibition under hypoxia for 15 *N*[(2,2-dimethyl-2*H*-chromen-6-yl)methyl]-*N*-*R*-arylsulfonamides

Compound name	IC ₅₀ (μM)	Number of repeats	Standard error	95% Confidence interval
5a	2.2	6	0.7	0.7, 3.6
5b	16.8	6	9.0	−0.9, 34.5
5c	3.8	6	0.8	2.2, 5.4
5d	3.0	6	1.0	1.1, 4.9
5e	1.5	6	0.3	0.9, 2.1
6a	2.7	6	0.6	1.6, 3.9
6b	3.3	6	0.6	2.1, 4.6
6c	4.8	6	1.0	2.9, 6.7
6d	4.2	6	1.0	2.1, 6.3
6e ^a	1.6	6	0.2	1.2, 1.9
7a	7.2	6	1.9	3.5, 10.8
7b	8.0	6	2.2	3.7, 12.4
7c	6.6	6	1.9	2.9, 10.4
7d	4.8	6	1.8	1.3, 8.3
7e	3.2	6	0.7	1.8, 4.6

^aWe previously reported the IC₅₀ values for **6e** in the ~0.6 μM range (*n* = 42).^{19,21} In the current experimental set the IC₅₀ value of **6e** (1.6 μM) was larger than the previous values; this variation might be related to the particular batch of cells used and the smaller number of repeats (*n* = 6). Because this compound was used as an internal standard we used the latter value for comparison with the other compounds tested under the same conditions.