Bicyclic imidazolium inhibitors of Gli transcription factor activity

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Abstract

Gli transcription factors within the Hedgehog (Hh) signaling pathway direct key events in mammalian development and promote a number of human cancers. Current therapies for Gli-driven tumors target Smoothened (SMO), a G protein-coupled receptor-like protein that functions upstream in the Hh pathway. Although these drugs can have remarkable clinical efficacy, mutations in SMO and downstream Hh pathway components frequently lead to chemoresistance. In principle, therapies that act at the level of Gli proteins, through direct or indirect mechanisms, would be more efficacious. We therefore conducted a screen of 325,120 compounds for their ability to block the constitutive Gli activity induced by loss of Suppressor of Fused (SUFU), a scaffolding protein that directly inhibits Gli function. Our studies reveal a family of bicyclic imidazolium derivatives that can inhibit Gli-dependent transcription without affecting the ciliary trafficking or proteolytic processing of these transcription factors. We anticipate that these chemical antagonists will be valuable tools for investigating the mechanisms of Gli regulation and developing new strategies for targeting Gli-driven cancers.

Entry for the Table of Contents

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Conflict of Interest
The authors declare no conflict of interest.

Supporting information for this article is given via a link at the end of the document.
A high-throughput phenotypic screen has identified a new class of Hedgehog pathway antagonists that act downstream of Suppressor of Fused. These bicyclic imidazolium derivatives inhibit full-length, activated Gli transcription factors without altering their trafficking or stability. The small-molecule antagonists serve as useful tools for investigating the mechanisms of Gli regulation and developing new strategies for targeting Gli-driven cancers.

**Keywords**

Hedgehog signaling; Gli; transcription factor; cancer; small-molecule inhibitor

Hh signaling is a key driver of metazoan development, regulating morphogenetic processes as diverse as fruit fly body segmentation\(^1\) and vertebrate digit formation\(^2\). In mammals, the Hh pathway acts through three zinc finger transcription factors: GLI1, GLI2, and GLI3 (Fig. 1A). GLI2 and GLI3 are the primary mediators of physiological Hh signaling, and their functions are posttranslationally controlled\(^3\)–\(^5\). In quiescent cells, Gli factors are proteolytically processed into N-terminal transcriptional repressors (GLI2R and GLI3R) through interactions with the scaffolding protein Suppressor of Fused (SUFU), protein kinase A, and the proteasome\(^6\). Secreted morphogens such as Sonic Hedgehog (SHH) activate the pathway by binding the 12-pass transmembrane protein Patched1 (PTCH1), thereby abrogating its suppressive effects on the 7-pass transmembrane receptor Smoothened (SMO). Activated SMO induces Gli-SUFU dissociation, blocking Gli repressor formation and allowing full-length Gli proteins to adopt transcriptionally active states (GLI2A and GLI3A)\(^6\). GLI2A and GLI3A exhibit altered subcellular trafficking, accumulate at the tip of the primary cilium\(^7\), and translocate to the nucleus to drive gene expression\(^8\). Hh target genes include those that encode canonical pathway components such as PTCH1 and a constitutively active Gli isoform, GLI1, establishing negative and positive feedback loops, respectively\(^9\),\(^10\).
Dysregulation of these biochemical and cellular processes can lead to several human cancers\cite{11}. For example, loss-of-function mutations in \textit{PTCH1} are frequently found in basal cell carcinoma and medulloblastoma, and these tumor subtypes are susceptible to SMO antagonists\cite{12}. However, SMO-targeting chemotherapies often lead to the emergence of drug-resistant tumor cells, many of which carry SMO mutations that abrogate inhibitor binding\cite{13}. SMO antagonists are also ineffective against Gli-dependent cancers that arise from mutations in \textit{SUFU}, other downstream pathway components, or non-canonical drivers of Gli activation. These clinical challenges could be addressed with Hh pathway inhibitors that act at the level of Gli proteins, and a number of small molecules that can suppress Gli-dependent transcription have been reported. Gli antagonists include the synthetic compounds (e.g., GANT61 and HPI-1)\cite{14,15} and natural products (e.g., arcyriaflavin C, cyananbugeigenin C, and glabrescione B, 5’-O-methyl-3-hydroxyflemingin A, and physalin F)\cite{16–21}. In addition, arsenic trioxide has been shown to reduce the stability and ciliary accumulation of GLI2, and this agent is currently in clinical trials for treatment of SMO inhibitor-resistant basal cell carcinoma\cite{22,23}. Glabrescione B and 5’-O-methyl-3-hydroxyflemingin A bind directly to the GLI1 zinc finger region, but how the other chemical reagents inhibit Gli function remains unclear. Moreover, the small-molecule antagonists were identified or validated in cell-based assays utilizing exogenous Gli proteins, potentially bypassing endogenous regulatory processes, and most are limited by their mid-micromolar potencies and cytotoxicity. Novel potent inhibitors of endogenous Gli function would help uncover the biochemical and cellular mechanisms that govern these transcription factors and guide the development of Gli-targeting drugs.

To discover small-molecule antagonists of Gli function, we established a \textit{Sufu}−/− murine embryonic fibroblast (MEF) line that is stably transfected with a Gli-dependent firefly luciferase reporter (SUFU-KO-LIGHT cells; Fig. 1B). Since SUFU negatively regulates Gli proteins, SUFU-KO-LIGHT cells exhibited constitutive Hh pathway activity driven by endogenous Gli factors. Hh pathway activity in these cells was insensitive to the SMO antagonists cyclopamine\cite{24}, SANT-1\cite{25}, and vismodegib\cite{26} (Fig. S1). In comparison, the Gli antagonist GANT61 could reduce luciferase reporter activity, albeit with mid-micromolar potency.

We tested 325,120 compounds from a structurally diverse library on the SUFU-KO-LIGHT cells using a screening concentration of 5 μM. Among the inhibitory compounds that emerged from this screen was a bicyclic imidazolium derivative 1 with N1 and C4 aryl substituents (Fig. 1C). This compound inhibited luciferase expression in the reporter line (IC50 = 1.4 μM) but did not suppress cytomegalovirus (CMV) promoter-driven luciferase activity (Fig. 1D–E). Nor did imidazolium 1 inhibit the Wnt pathway, which has transduction and regulatory mechanisms similar to those of Hh signaling\cite{27}; the compound had no inhibitory effect on cells stably transfected with a TCF/LEF-dependent firefly luciferase reporter (WNT-LIGHT cells) and treated with WNT3A-conditioned medium (Fig. 1F). We also evaluated the ability of compound 1 to block Hh signaling in C3H10T1/2 cells, a pluripotent mesenchymal line that differentiates into alkaline phosphatase-expressing osteoblasts in a SHH-dependent manner (Fig. 1G)\cite{28}. We observed that imidazolium 1 suppressed alkaline phosphatase activity in SHH-treated C3H10T1/2 cells (IC50 = 0.54
µM). Taken together, our findings establish inhibitor 1 as a Hh pathway antagonist that is potentially active in multiple cell types.

We next sought to explore the structure-activity relationship (SAR) landscape of imidazolium-based Gli antagonists and to develop more potent analogs. We first assessed the activities of imidazolium derivatives in the SUFU-KO-LIGHT assay, exploring various N1 and C4 substituents and ring fusions. These studies utilized both commercially available imidazoliums with this core scaffold and others we could synthetically access from aniline and lactam fragments (Table 1). Briefly, the imidazolium derivatives could be rapidly prepared through O-methylation of the corresponding lactam, amidine formation with a substituted aniline, and condensation with a substituted α-bromo acetophenone (Fig. 2A).

Compound potency could be significantly enhanced by replacing bromine atom in imidazolium 1 with other groups. A variety of structures were tolerated at the para position of the C4 aryl substituent, with dialkylamine or phenyl derivatives exhibiting the greatest potency (Table 1; 2–5). In comparison, modifying the N1 aryl ring had modest effects on potency, and both para- and ortho-alkoxy derivatives were active in the SUFU-KO-LIGHT cells, indicating that coplanarity of the N-aryl substituent and imidazolium ring is not necessary for activity (Table 1; 4 and 5).

We further observed that imidazolium derivatives with tricyclic scaffolds remained active (Table 1, 9 and 10), but those incorporating heteroatoms in the saturated ring had dramatically reduced inhibitor potency (Table 1, 11 and 12).

Together, these data indicate that the C4 substituent and the imidazolium ring fusion are important factors in inhibitory potency. Interestingly, exchanging the N1 and C4 substituents to yield isosteric analogs had varying effects. For example, the isosteres 7 and 8 showed an 8-fold difference in activity, but the biaryl analogs 4 and 6 were nearly equipotent (Table 1). These results suggest that the nitrogen atoms within the imidazolium ring, which have different configurations between isosteres, do not directly influence target binding but can influence the conformation of ring substituents. We also investigated whether the permanent positive charge of imidazolium ring system—a unique electronic property of the core bicyclic scaffold—contributes to compound activity. We synthesized uncharged isosteres of imidazolium 5, which has an IC50 of 150 nM in the SUFU-KO-LIGHT assay (Fig. 2B). Exchange of the positively charged imidazolium ring with a neutral indole 13 or pyrazole 14 dramatically reduced inhibitor potency. From these SAR studies, we identified a class of N1 ortho-methoxy-substituted aryl and C4 biaryl imidazoliums that show 10-fold greater potency in our SUFU-KO-LIGHT assay versus the screening hit, and we selected compound 10 as an optimized scaffold for cell-based studies.

We proceeded to investigate the effects of imidazolium 10 on key aspects of Hh signal transduction. We first assessed its activity in NIH-3T3 cells, a Hh pathway-competent murine fibroblast line. SHH stimulation of these cells induces the expression of endogenous Hh target genes such as Pch1, and 10 blocked this activity in a dose-dependent manner (Fig. 3A). The inhibitor also reduced SHH-dependent GLI1 protein expression in these cells with comparable potency (Fig. 3B). These results corroborate our previous findings with the
synthetic Gli reporter in SUFU-KO-LIGHT cells and the C3H10T1/2 differentiation assay. We note that the IC50 of 10 in the NIH-3T3 cell-based assay was significantly higher than that observed in SUFU-KO-LIGHT cells. While the mechanistic basis for that difference is unknown, we speculate that it could reflect the constitutive Hh pathway activity in SUFU-KO-LIGHT cells, which might influence expression of the imidazolium target(s), its regulators, or downstream effectors. Alternatively, the compounds could target additional Gli regulatory mechanisms in SUFU-KO-LIGHT cells that are not operative in NIH-3T3 cells.

We then examined effects of inhibitor 10 on two posttranslational steps in Gli activation. In response to SHH signaling, GLI2 accumulates at the distal tip of the primary cilium, likely due to altered trafficking kinetics of its transcriptionally active form[6]. SHH stimulation also blocks GLI3R formation and reduces total GLI3 levels, presumably due to instability of GLI3A. We first treated NIH-3T3 cells with SHH to induce ciliary GLI2 and investigated whether the compound affected GLI2 trafficking (Fig. 3C–D). As visualized by immunofluorescence microscopy, GLI2 accumulation within the primary cilium was unchanged by 10, whereas cyclopamine reduced ciliary GLI2 levels to that of unstimulated cells. We also analyzed GLI3 protein states by western blot to ascertain whether 10 alters the proteolytic processing of this factor (Fig. 3E). While cyclopamine could reverse SHH-dependent posttranslational regulation of GLI3, 10 had no apparent effect.

The ability of compound 10 to block Hh pathway activity without affecting known posttranslational aspects of Gli regulation suggests that bicyclic imidazoliums can abrogate the function of full-length, activated Gli proteins. To investigate this possibility, we studied the effects of 10 on the constitutively active isoform GLI1, which does not undergo proteolytic processing. We transduced exogenous, epitope-tagged GLI1 (FLAG-GLI1) into NIH-3T3 cells, added the inhibitor for 24 hours at varying concentrations, and used qRT-PCR to monitor the resulting Ptch1 distribution of FLAG-GLI1 as ascertained by immunofluorescence (Fig. 3H–I) and subcellular fractionation (Fig. S2).

We next assessed whether the imidazoliums specifically disrupt GLI1 function by comparing the activity of inhibitor 10 in wild-type and Gli1−/− MEFs[29]. We stimulated the cells with SHH and quantified Ptch1 transcript levels. The compound retained its ability to suppress Ptch1 expression in both cell lines, supporting a model in which the inhibitor can also suppress the function of GLI2A and/or GLI3A (Fig. 3I). Thus, the bicyclic imidazoliums appear to target a functional step that is epistatic to activator formation and conserved across Gli isoforms. expression. Compound 10 suppressed Ptch1 transcription induced by FLAG-GLI1 (and by any endogenous Gli1 activated by the exogenous protein) in a dose-dependent manner without altering FLAG-GLI1 levels (Fig. 3F–G). Inhibitory doses of the imidazolium also did not perturb the nuclear-cytoplasmic period

To gain additional insights into the mechanisms of inhibitor action, we profiled one of the compounds (imidazolium 5) against the NCI-60 cancer cell line collection. Two of the lines have significantly upregulated GLI1 expression and are resistant to SMO inhibitors[30], and their growth was suppressed by the bicyclic imidazolium (Fig. S3). However, 5 also inhibited the proliferation of other cancer cell types in a dose-dependent manner, suggesting it targets a more general cellular process that can influence Gli function. We used these data
to conduct a NIH COMPARE analysis, which employs a Pearson’s $t$-test to identify compounds with similar cellular activity profiles. Inhibitor 5 had marked similarities ($R^2 > 0.85$) with several other small molecules or biological agents screened against these lines. Compounds with the highest correlation also bear a permanent positive charge (Fig. 4A), providing further evidence that this feature plays a major functional role.

Previous studies have shown that hydrophobic cations can preferentially accumulate in mitochondria\cite{31}, and the most correlated “hit” in our COMPARE analysis, NSC 236620, inhibits mitochondrial respiration in breast cancer cells\cite{32}. Informed by these findings, we examined the effects of bicyclic imidazoliums on mitochondrial health and function. We first tested whether imidazolium 10 altered mitochondrial membrane potential by monitoring uptake of the voltage-dependent dye tetramethylrhodamine methylester (TMRM). The Gli antagonist blocked mitochondrial TMRM uptake at similar doses to the IC50 for Gli inhibition, indicative of membrane potential loss (Fig. S4). Further imaging of mitochondrial morphology with the voltage-localized dye MitoTracker Deep Red revealed that these organelles undergo extensive morphological changes upon treatment with comparable concentrations of imidazolium 10 (Fig. 4B–C). In comparison, cyclopamine had no effect on mitochondrial membrane potential (Fig. S4) or dynamics (Fig. 4B), indicating that these mitochondrial effects are not a general consequence of Hh pathway inhibition.

We next determined if the imidazolium derivatives disrupt mitochondrial ATP production via oxidative phosphorylation (OXPHOS). We measured the OXPHOS capacity of NIH-3T3 cells using the Seahorse Mito Stress assay, which quantifies oxygen consumption rates associated with the electron transport chain. Inhibitor 10 blocked OXPHOS at concentrations slightly lower than its Hh pathway IC50 in the same cell line (Fig. 4D), but cyclopamine had no significant effect on mitochondrial respiration at doses up to 10 $\mu$M (Fig. S5).

Finally, we examined whether inhibition of Gli function requires OXPHOS suppression. We depleted NIH-3T3 cells of mitochondrial DNA by treating them with ethidium bromide\cite{33} (Fig. 4E), eliminating key components of the electron transport chain. The resulting $\rho^0$ cells were OXPHOS-deficient, and their responsiveness to SHH was moderately reduced, as gauged by $Pch1$ expression (Fig 4F). However, the resulting $\rho^0$ Hh pathway activity remained sensitive to inhibitor 10 indicating that imidazolium action does not require loss of mitochondrial respiration. Consistent with this mechanistic scheme, the ATP synthase antagonist oligomycin A had no effect on $Pch1$ levels in the wild-type or $\rho^0$ NIH-3T3 cells. Taken together, these observations demonstrate that the inhibitory effects of imidazoliums on Gli function and mitochondrial respiration are separable activities.

In summary, we have discovered bicyclic imidazoliums as a new class of Hh pathway inhibitors that interfere with the transcriptional activity of Gli proteins without affecting their subcellular localization or protein stability. Through SAR studies, we have identified structural elements that are essential for inhibitory activity and developed derivatives with sub-micromolar potencies in cells. To our knowledge, our studies are the first to identify small-molecule Hh pathway antagonists with confirmed epistasis to the activated, full-length
state of multiple Gli isoforms. While the compounds also perturb mitochondria at these
effective concentrations, our studies demonstrate that oxidative phosphorylation
dysregulation does not account for Gli inhibition. Further investigations will be required to
determine whether other aspects of mitochondrial function influence Gli activity, and the
imidazoliums will be useful tools for uncovering novel mechanisms of Gli regulation.
Development of these Gli antagonists through medicinal chemistry could also provide leads
for new anti-cancer therapies.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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References

[1]. Yang Y, Drossopoulou G, Chuang PT, Duprez D, Marti E, Bumcrot D, Vargesson N, Clarke J,
[9]. Regl G, Neill GW, Eichberger T, Kasper M, Ikram MS, Koller J, Hintner H, Quinn AG, Frischauf
2015, 6, DOI 10.1038/ncomms7709.
[12]. Tang JY, Mackay-Wiggan JM, Asztterbaum M, Yauch RL, Lindgren J, Chang K, Coppola C,
[13]. Sharpe HJ, Pau G, Dijkstra GJ, Basset-Seguin N, Modrusan Z, Januario T, Tsui V, Durham AB,
1092. [PubMed: 18357592]


Figure 1.
Bicyclic imidazoliums inhibit Hh pathway activity downstream of SUFU. a) Diagram of the Hh pathway in the absence (off) or presence (on) of SHH. b) Scheme of the Gli-dependent luciferase reporter in SUFU-KO-LIGHT cells. c) Parent imidazolium 1 identified in the high-throughput chemical screen. d-g) Activities of 1 in SUFU-KO-LIGHT cells (n=3), cells stably transfected with a CMV-driven luciferase reporter (n=2), WNT-LIGHT cells (n=2), and C3H10T1/2 cells (n=3). Data are the average reporter activities for the indicated number of biological replicates ± s.e.m., normalized as the percentage of a DMSO control.

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Figure 2.
Structure-activity relationship analyses of bicyclic imidazoliums. a) Synthesis of bicyclic imidazolium analogs: (a) 85 °C, 6 days; (b) THF, 78 °C, 16 h; (c) Ac2O, 120 °C, 5 h. b) Uncharged isosteres of imidazolium 5 exhibit reduced potency. IC50 values ± s.e.m. in the SUFU-KO-LIGHT cell assay are shown (n ≥3 biological replicates for each compound).
Figure 3.
Bicyclic imidazoliums suppress the function of full-length Gli activators. **a)** Hh pathway activity after 24 h of compound and SHH co-treatment, as ascertained by *PtcH1* qRT-PCR. Data are the average of three biological replicates ± s.e.m. **b)** Representative GLI1 western blot analyses from the same cells described in (a). Data are the average of three biological replicates ± s.e.m., normalized to the unstimulated DMSO control. **c–d)** Immunofluorescence micrographs illustrating ciliary GLI2 levels after 24 h SHH pre-treatment and subsequent 6 h compound treatment. Scale bar: 5 μm Quantified ciliary GLI2 levels are shown with mean and 95% confidence intervals (>150 cilia per condition from two biological replicates). Kruskal-Wallis test for pairwise comparison to the DMSO + SHH condition. **e)** Representative western blot showing GLI3 forms in cells treated with 10 and
SHH for 24 h. f) *P*ch*1* levels in FLAG-GLI1-expressing NIH-3T3 cells measured by qRT-PCR after 24 h of compound treatment. Data are the average of three biological replicates ± s.e.m. One-way ANOVA relative to the DMSO + FLAG-GLI1 condition. g) Western blot of FLAG-GLI1 expression in same cells from (e). h-i) Immunofluorescence imaging and quantification of the FLAG-GLI1 cytoplasmic:nuclear ratio after 4 h of compound treatment (>100 cells from two biological replicates for each condition). Scale bar: 20 μm. j) *P*ch*1* expression in WT and *Glil*^−/−^ MEFs after 24 h of compound treatment as measured by qRT-PCR. Data are the average of three biological replicates ± s.e.m. One-way ANOVA relative to the DMSO + FLAG-GLI1 condition. Statistics: * = *P*<0.01; ** = *P*<0.001; *** = *P*<0.0001.
Figure 4.
Gli inhibition and oxidative phosphorylation suppression by bicyclic imidazoliums are separable activities. a) Compounds in the NIH COMPARE library (>88,000 molecules) with cancer cell activity profiles most similar to imidazolium 5. b-c) Representative fluorescence micrographs of the mitochondrial morphology of NIH-3T3 cells treated with 10 or cyclopamine (Cyc) for 24 h, subsequently stained with Mitotracker Deep Red, and quantified by mitochondrial phenotype (>240 cells per condition). Mitochondria with elongated networks or rounded structures were scored as healthy or disrupted, respectively. The 5 μM 10 condition was not quantified due to loss of Mitotracker staining. Scale bar: 10 μm. d) Seahorse Mito Stress assay for oxygen consumption rate (OCR) in NIH-3T3 cells incubated with varying doses of 10 and tracked for 15 min. e) Seahorse Mito Stress assay of...
wild-type and \( \rho^0 \) cells treated with 100 ng/mL ethidium bromide for 9 days. f) Hh pathway activity measured by qRT-PCR of \( \text{Ptch1} \) expression in wild-type and \( \rho^0 \) NIH-3T3 cells treated with compounds for 24 h. Data shown in e-f are the average of three biological replicates \( \pm \) s.e.m.
Table 1.
Inhibitory activities of imidazolium derivatives.

<table>
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<th>Compound</th>
<th>R$_1$</th>
<th>R$_2$</th>
<th>IC$_{50}$ (μM)</th>
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<tr>
<td>1</td>
<td>p-OEt</td>
<td>p-Br</td>
<td>1.6 ± 0.1</td>
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<tr>
<td>2</td>
<td>p-OEt</td>
<td>p-N$^0$PrMe</td>
<td>0.10 ± 0.02</td>
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<tr>
<td>Compound</td>
<td>( R_1^{[a]} )</td>
<td>( R_2^{[a]} )</td>
<td>IC50 (( \mu \text{M} ))(^{[b]} )</td>
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<tr>
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<td>-----------------</td>
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</tr>
<tr>
<td>3</td>
<td></td>
<td></td>
<td>0.12 ± 0.01</td>
</tr>
<tr>
<td>4</td>
<td></td>
<td></td>
<td>0.13 ± 0.01</td>
</tr>
<tr>
<td>5</td>
<td></td>
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<td>0.15 ± 0.05</td>
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\( ^{[a]} \) Substituent positions as indicated.

\( ^{[b]} \) IC50 values are averages of three determinations.
<table>
<thead>
<tr>
<th>Compound</th>
<th>R₁[a]</th>
<th>R₂[a]</th>
<th>IC₅₀ (μM)[b]</th>
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<tbody>
<tr>
<td>6</td>
<td></td>
<td>p-OEt</td>
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<td>7</td>
<td>o-OMe</td>
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<tr>
<td>Compound</td>
<td>$R_1^a$</td>
<td>$R_2^a$</td>
<td>IC50 (μM)$^b$</td>
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<td>----------</td>
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<tr>
<td>9</td>
<td></td>
<td></td>
<td>0.20 ± 0.02</td>
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<tr>
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<tr>
<td>11</td>
<td>H</td>
<td>$p$-OMe</td>
<td>&gt;100</td>
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$^a$ Notation for $R_1$ and $R_2$.
$^b$ IC50 values are measured in micromolar (μM).

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<table>
<thead>
<tr>
<th>Compound</th>
<th>R₁[^a]</th>
<th>R₂[^a]</th>
<th>IC₅₀ (μM)[^b]</th>
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<tr>
<td>12</td>
<td>p-OMe</td>
<td>p-OMe</td>
<td>16 ± 3</td>
</tr>
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</table>

[^a]: All non-alkoxy substituents are on the para position of the aryl ring.

[^b]: SUFU-KO-LIGHT cells were treated with various doses of the indicated compound for 24 h, and the resulting luciferase reporter activities were assessed. IC₅₀ values represent the average of at least three biological replicates ± s.e.m.