PERSPECTIVE

ACS chemical biology

A Specific Mechanism for Nonspecific Activation in Reporter-Gene Assays

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ABSTRACT The importance of bioluminescence in enabling a broad range of high-throughput screening (HTS) assay formats is evidenced by widespread use in industry and academia. Therefore, understanding the mechanisms by which reporter enzyme activity can be modulated by small molecules is critical to the interpretation of HTS data. In this Perspective, we provide evidence for stabilization of luciferase by inhibitors in cell-based luciferase reporter-gene assays resulting in the counterintuitive phenomenon of signal activation. These data were derived from our analysis of luciferase inhibitor compound structures and their prevalence in the Molecular Libraries Small Molecule Repository using 100 HTS experiments available in PubChem. Accordingly, we found an enrichment of luciferase inhibitors in luciferase reporter-gene activation assays but not in assays using other reporters. In addition, for several luciferase inhibitor chemotypes, we measured reporter stabilization and signal activation in cells that paralleled the inhibition determined using purified luciferase to provide further experimental support for these contrasting effects.

As high-throughput screening (HTS) gains momentum in academia and public databases grow in size and scope, refining our understanding of target-specific and nonspecific effects within HTS assays will facilitate a more accurate interpretation of screening results. Cell-based reporter-gene assays are designed to measure the influence of a library compound on a cellular process or pathway through the modulation of the reporter gene's transcription and expression levels. The level of reporter is a function of its transcription, expression, and stability. However, enzymes can be stabilized by inhibitors (*1*) when an E·I complex is more resistant to degradation than the free enzyme. In cell-based assays, this can lead to an accumulation of the enzymatic reporter independent of effects on transcription/translation, thus complicating the interpretation of HTS results (*2*). After characterizing and developing a comprehensive profile of luciferase inhibitors (*3*), we were able to search for these compounds in the list of compounds identified as active in the HTS assays found in PubChem. We show here that many of the compounds designated as activators of luciferase-based reporter-gene assays are luciferase inhibitors. Further luciferase inhibitors were not enriched in assays using other reporter types (*e.g.*, GFP and β-lactamase), suggesting luciferase stabilization as the more likely activation mechanism, as opposed to targeted or general activation of gene transcription. Our findings thus show the utility of small-molecule library bioactivity profiles and underscore the value of making such library characterization assays available in PubChem.

The *Photinus pyralis* luciferase is commonly used in cell-based reporter-gene assays because the luminescent response provides a sensitive assay signal with a wide dynamic range due to its relatively short protein half-life (*4*). Not surprisingly, an increase in luciferase

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Received for review April 5, 2008 and accepted June 10, 2008.

Published online July 1, 2008

10.1021/cb8000793 CCC: $40.75

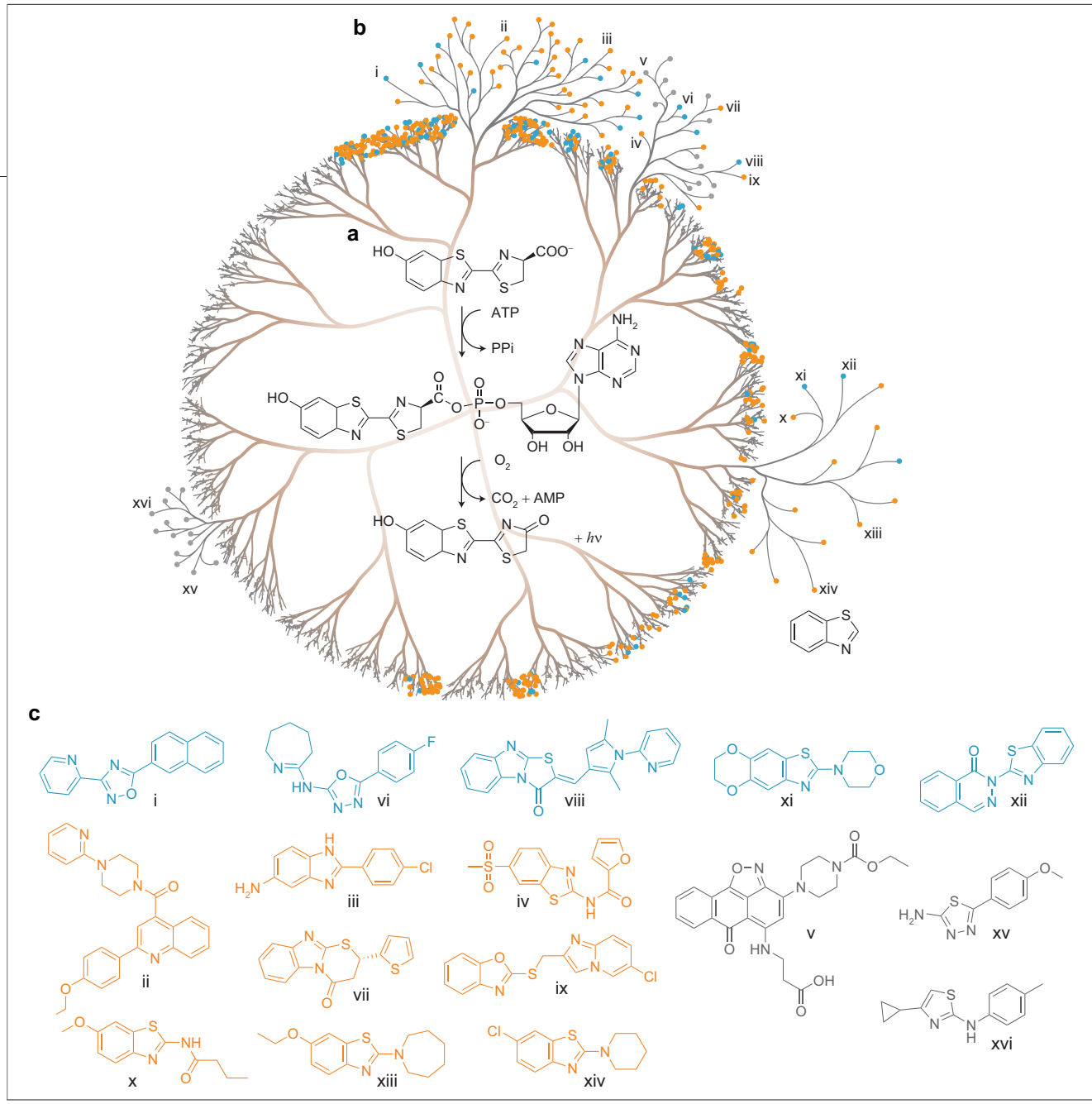

Figure 1. The firefly luciferase subchemome. A hierarchical clustering algorithm based on maximum common substructures was used to group the structures. The dendrogram from the clustering hierarchy was automatically generated using an in-house graph layout algorithm. a) The reaction catalyzed by firefly luciferase. b) Dendrogram representation of luciferase inhibitors identified in the cell-free luciferase qHTS (AID 411) and overlap with actives from a Steroidogenic Factor 1 (SF-1) receptor cell-based reporter-gene assay. SF-1 actives are designated as orange circles (SF-1 activators) and blue circles (SF-1 inhibitors) for the primary cell-based reporter-gene screens (AIDs 522 and 525, respectively). The right hemisphere of the dendrogram contains prominent chemical series previously identified either as competitive or noncompetitive inhibitors of firefly luciferase. c) Example compounds are highlighted that include known noncompetitive luciferase inhibitors such as i and xii that cause apparent inhibition in the reporter-gene assay, as well as compounds that mimic the luciferase substrate (see, *e.g.*, iv or x) that appear as activators in the SF-1 activation assay. A representative quinoline showing activation (ii) that we identified as a potent competitive inhibitor of firefly luciferase is also shown. Compounds inactive in the reporter-gene assay were more diverse and included compounds with probable low cell penetration due to the presence of charged groups and high molecular weight (*e.g.*, compound v).

half-life can have a substantial effect on an assay readout. Using the model described by Hargrove and Schmidt (*5*) and assuming no effect on the rate of protein synthesis or mRNA levels, a modest increase in luciferase protein half-life (*e.g.*, ~30%) can lead to a 150% increase in luciferase levels within 12 h. Signal from the increased levels of luciferase would be detected as this will be well within a reporter-gene assay response window, especially as many of these cell-based assays involve compound incubation times of

18 h or longer (*6*). Further, we noted in our previous study that ATP or luciferin competitive inhibitors demonstrated reduced inhibition or appeared inactive in the presence of luciferin-containing reporter-gene detection reagents, which generally employ an excess of luciferase substrates (*3*). Therefore, in this scenario, it seems possible that luciferase inhibitors could interact with and stabilize the cellular luciferase enzyme during the long cell-based incubation times. However, upon addition of luciferin-containing detection reagent, the inhibitor would be effectively displaced from the enzyme through competition by the excess substrate provided and thus would not inhibit the measured luciferase reaction. If this is the case, one may predict an increase in the reporter levels and thus increased signal characteristic of activation.

We have previously described a cell-free profiling screen for inhibitors of the ATP-dependent luciferase (Figure 1, panel a) from the firefly *P. pyralis* (PubChem AID 411) using quantitative HTS (qHTS), which determined the concentration−response behavior for >70,000 samples in the Molecular Libraries Small Molecule Repository (MLSMR) (*3*). Approximately 3% of the library showed inhibitory activity, and none of the compounds caused a direct activation of luciferase. This comprehensive profile allowed us to define the structure−activity relationship (SAR) for a prominent luciferase inhibitor series (Figure 1, panel b).

To investigate preexisting evidence for this mechanism in HTS, we utilized our understanding of luciferase inhibitor SAR to analyze assays available in PubChem that were screened against the MLSMR. We first examined how luciferase inhibitors were distributed among PubChem assays. We queried PubChem to determine the types of assays associated with these luciferase inhibitors (Figure 2). Nearly 50% of the assays were luminescence assays that used *P. pyralis* firefly luciferase, the same variant present in our qHTS. Among these, we found both biochemical-based assays (including our original luciferase profile qHTS as well as one from another center, AID 1006) and cell-based reporter-gene assays designed to identify either activators or inhibitors. Further, we noted that all of the reporter-gene assays were based on expression of *P. pyralis* luciferase. Luciferase inhibitors were also identified, although not overrepresented among hits (see below), in assays that typically show high hit rates such as those for cellular cytotoxicity and cytochrome P450 inhibition assays.

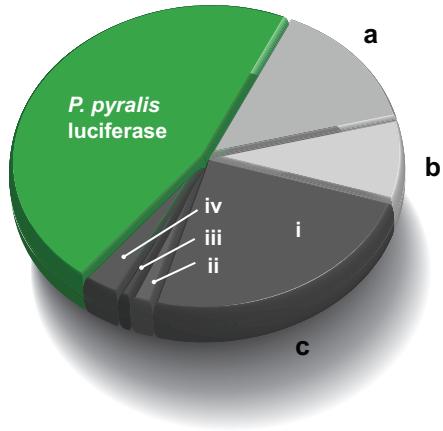

Figure 2. PubChem assays associated with luciferase inhibitors. Sections of the chart are colored as follows: *P. pyralis* luciferase-based assays (green, 46.3%) consisting of cell-based reporter-gene assays for either activators or inhibitors (20.9% and 9.3%, respectively) and biochemical luciferase-coupled assays (16.1%). Also shown are a) cytotoxicity assays (14.4%), b) biochemical cytochrome P450 assays (9.0%), and c) other assay formats (30.3%) consisting of fluorescence-based assays (i, 24.1%), β-lactamase-based assays (ii, 1.8%), Alphascreen or chemiluminescence (iii, 1.3%), and absorbance-based assays (iv, 3.1%). A total of 1879 luciferase inhibitors identified in the qHTS associated with high-quality CRCs were used to query the PubChem assays using the BioAssay Summary feature. Only assays that covered at least 75% of the luciferase inhibitors are shown (64 total).

The next assay category was fluorescence-based assays, followed by a variety of other assay types.

We then compared the enrichment of luciferase inhibitors versus assay format for 100 assays in PubChem. Our luciferase qHTS identified a frequency of luciferase inhibitors of 3% within the MLSMR; therefore, active sets or "hit lists" containing only 3% luciferase inhibitors would not be considered enriched above the expected background. However, an HTS active set found to contain, for example, 30% luciferase inhibitors, is enriched 10-fold. We would thus expect that luciferase-coupled enzyme assays or reporter-gene assays designed to identify compounds that act as inhibitors would be enriched for luciferase inhibitors, and indeed, we noted a high percentage of luciferase inhibitors in these assays (Figure 3). However, we also noted that reporter-gene assays targeting activators also displayed a similar percentage of luciferase inhibitors within ac-

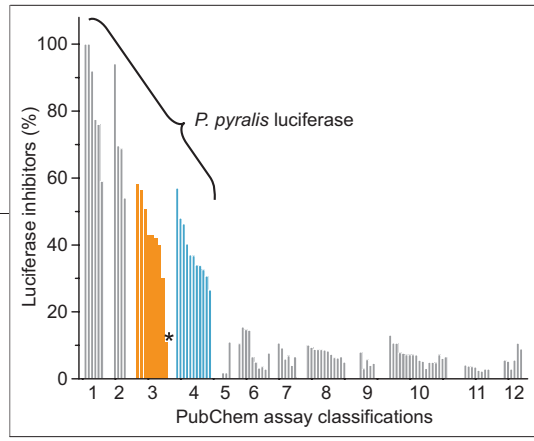

Figure 3. Percentage of luciferase inhibitors within hits from 100 PubChem assays. The PubChem active list from each assay was compared with the luciferase qHTS activity, and all compounds showing inhibitory CRCs in the luciferase assay were used to calculate the percentage. Assays are grouped by assay type and are shown in order as (1) *P. pyralis* luciferase-based biochemical assays; (2) cytotoxicity assays using *P. pyralis* (Perkin-Elmer detection reagent; (3, orange) cell-based reporter-gene assays scored for activation (the last assay in this group (*) is a cell-based luciferase reporter-gene assay scored for activation that used an unusually stringent cutoff to designate the actives, 200% of control; all other assays typically used a cutoff between 27% to 50% of control values); (4, blue) cell-based reporter-gene assays scored for inhibition; (5) cell-based luciferase reporter-gene assays scored for activation where a short compound exposure was used (2.5 h); (6) cell-based reporter-gene assays using β-lactamase; (7) FRET-based assays; (8) luminescent cytotoxicity assays using *Photuris pennsylvanica* luciferase (Promega, CellTiter-Glo); (9) absorbance-based assays; (10) fluorescent-based assays; (11) luminescent cytochrome P450 assays using *P. pennsylvanica* luciferase; and (12) other assays, including AlphaScreen and cell-free chemiluminescence assays. The type of luciferase is important to consider in this analysis. Luminescent cytotoxicity assays using CellTiter-Glo and coupled assays for P450s utilized an optimized variant of *P. pennsylvanica* firefly luciferase (available in formulations from Promega Corp as Ultra-Glo), and from our previous studies we have determined that *P. pennsylvanica* is largely resistant to inhibitors of *P. pyralis* luciferase (*3*). Consistent with this finding, we found little enrichment for luciferase inhibitors in assays using the *P. pennsylvanica* luciferase, while a set of cytotoxicity assays using *P. pyralis* luciferase (Perkin-Elmer reagent, 2) showed large enrichments for luciferase inhibitors.

tive data sets. The enrichment of luciferase inhibitors in these assays varied with the compound incubation time. For example, in a dopamine receptor potentiation assay (see, *e.g.*, AID 641) having a short (2.5-h) compound exposure time, a low (≤3-fold) enrichment was observed, whereas assays with prolonged compound exposure times showed large luciferase inhibitor enrichments of ≥10-fold (see, *e.g.*, AID 560). Furthermore, in one assay for activators of Steroidogenic Factor 1 (SF-1), ~60% of the hits selected for confirmatory concentration–response curve (CRC) determination were luciferase inhibitors (AID 692). Enrichment for luciferase inhibitors was not observed in reporter-gene assays that used β-lactamase, GFP, or other reporters, despite compound exposure times for as long as 20 h and the use of similar hit cutoff criteria (typically 30–50%). Thus, the prevalence of luciferase inhibitors within compound libraries, such as the MLSMR, and their enrichment in luciferase reporter-gene assays provide support for inhibitor-mediated stabilization of this enzyme reporter.

In our previous study, we characterized structure–activity relationships for several prominent chemical series, including compounds that mimicked the luciferin substrate and acted as competitive inhibitors of the enzyme (*3*). An examination of the luciferase inhibitor SAR in relation to the SF-1 reporter-gene assay actives (Figure 1) revealed that the major chemical series previously recognized as containing potent luciferase inhibitors was among either the activators or inhibitors identified in the SF-1 luciferase reporter-gene assays (Figure 1, panel b). For example, potent luciferase inhibitors whose inhibition is not easily relieved by detection reagents (*3*) were identified as inhibitors in the SF-1 inhibition assay (Figure 1, panel c, blue structures). However, compounds that mimic the luciferase substrate were found to be associated with SF-1 reporter-gene activation, consistent with the ability of these compounds to form a stable E·I complex within cells that is later abolished in detection mixes containing excess substrate concentrations (Figure 1, panel c, orange structures). The portion of the luciferase subchemome containing diverse structures inactive in the SF-1 reporter-gene assays (gray areas of the chemome, Figure 1) could be due to multiple factors that affect small-molecule activity, such as the achievable intracellular concentration, serum binding sequestration, or experimental variation between laboratories, which includes preparation of the compound sample, a highly variable step in HTS (*7*).

To further experimentally support an inhibitor-based stabilization mechanism, we examined representative compounds in HEK293 cells expressing *P. pyralis* luciferase. Of note, one of the compounds we examined is a quinoline (Figure 1, ii) that was identified as a competitive inhibitor of firefly luciferase in our previous work (*3*) and as an activator in PubChem luciferase reporter-gene assays (Figure 4, panel b). In these experiments, we measured the CRCs for luciferase activity after treat-

ing cells with compound for 24 h. To rule out the possibility that these compounds influenced the rate of transcription or mRNA stabilization, we also examined the stability of the luciferase signal in compound-treated cells after the addition of cycloheximide (*2*), a small molecule that inhibits eukaryotic translation (*8*). The same compounds were also measured in a cell-free luciferase assay using purified luciferase and K_m levels of substrates to confirm the inhibitory effect of these compounds. In these experiments, we observed apparent activation of the luciferase signal within the relevant screening concentration ranges (1−10 μM) upon addition of a reporter-gene detection cocktail containing excess luciferase substrates (see Figure 4, panels a−c). When we examined the stability of the signal after cycloheximide treatment, we noted a slower rate of decay in activity for wells treated with compound compared with wells without compound (Figure 4, panel d). Further, plots of the relative amount of luciferase activity remaining after 24 h of treatment with cycloheximide (Figure 4, panels a−c, red lines) showed a CRC that mirrored the inhibition of the purified enzyme. These parallel but opposite responses strongly support the observation that increased luciferase activity is due to inhibitor-based stabilization of the luciferase enzyme itself. Further, we found that stabilization can occur regardless of the mode of action of the compound. For example, we have previously shown that quinoline-like compounds (Figure 4, panel b) exhibit competitive inhibition with respect to ATP and luciferin, whereas a 1,2,4-oxadiazole (Figure 4, panel c) is a noncompetitive inhibitor. However, both types of inhibitors, competitive and noncompetitive, appear to stabilize luciferase in the cycloheximide-treated cells.

The activation phenotype for all inhibitors tested was generally characterized by a bell-shaped CRC, with activation increasing from low to high concentrations of less than 10 μM, followed by a gradual decrease in activation with increases in inhibitor compound concentration. The complex bell-shaped CRCs observed in the cell-based assays are due to two opposing responses: activation in the reporter gene assay due to stabilization of the luciferase enzyme, and inhibitory responses that include cytotoxcity or the amount of residual luciferase inhibition in the reporter-gene detection reagent. For example, we noted that the benzthiazole partially inhibited purified luciferase assayed with the reporter-gene detection cocktail at concentrations above 10 μM (Figure 4, panel a; bottom graph, □) resulting in a decreased activity above 10 μM in both the cell and cell-free assays (open circles and open squares). Alternatively, the quinoline did not appear to significantly inhibit purified luciferase in the reporter-gene detection cocktail (Figure 4, panel b; bottom graph, □) but exhibited a bell-shaped CRC in the cell-based assay (Figure 4, panel b; top graph, ○), suggesting cytotoxic effects. For compounds such as the 1,2,4-oxadiazole (Figure 4, panel c) that behave as noncompetitive inhibitors with respect to ATP and luciferin, the factors that influence the ability to observe the activation are more complex. For example, stabilization is clearly seen for this compound when examining the amount of luciferase activity remaining after 24-h treatment with cycloheximide (Figure 4, panel c; red line). In addition, the rate of decay of luciferase activity in the presence of compound is diminished (Figure 4, panel d). However, the activation effect was not observed at relevant screening concentrations, although it was found to be significant at very low concentrations ($\leq IC_{50}$) (Figure 4, panel c; top graph, ○).

Observation of apparent reporter-gene activation due to inhibitor-based stabilization of the reporter will therefore depend on several factors. These factors include the direct inhibition of the enzyme in the detection reagent, effects on cell viability, the degree of cell penetration/retention of the compound, affinity of the compound for the reporter, degree of stabilization, and the chosen screening concentration. In general, whether or not this increase will be detected as an apparent activation will depend on how much E·I is formed within the cells resulting in stabilization and how efficiently the inhibitor is competed off in the presence of detection reagents. Given these factors, this effect will be most readily observed when the amount of free enzyme is maximized during detection, which can occur, for example, with competitive-type inhibitors and prolonged cell incubation times. These complexities help to explain why the luciferase reporter-gene assays mentioned above using a short (2.5-h) incubation time (AIDs: 641, 642, and 647, all related to potentiation of the D1 receptor) did not show enrichment in luciferase inhibitors.

This study is an example of how information from compound profiling and PubChem can be employed, in this case, to make an informed connection between luciferase inhibitors and apparent gene activation in HTS reporter-gene assays. This work also illustrates the value

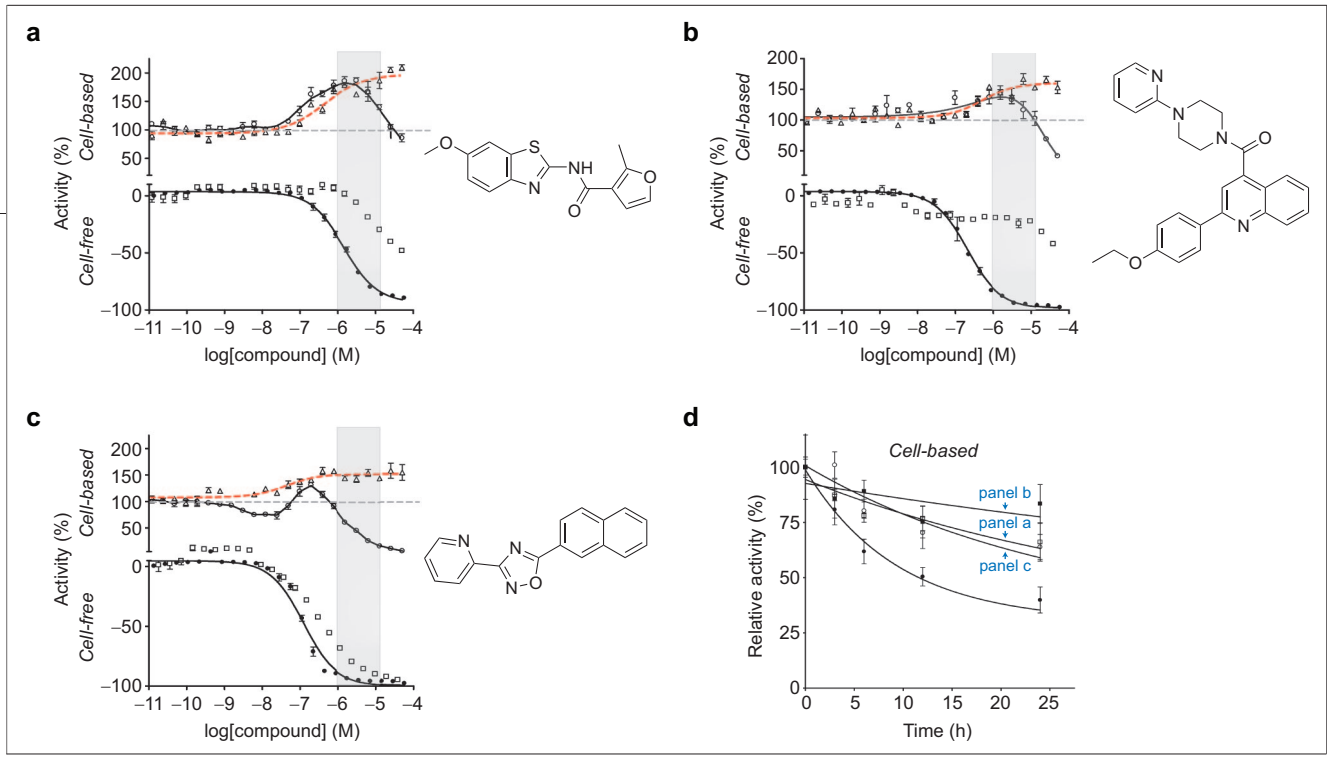

Figure 4. Stabilization of firefly luciferase by inhibitors. a–c) Top graphs show luciferase activity from HEK293 cells expressing luciferase in cells treated with compound for 24 h (○, black fitted line) or the remaining luciferase activity following 24-h treatment with cycloheximide (△, red fitted line). Bottom graphs depict the cell-free luciferase activity determined using purified luciferase assayed with a reporter gene detection cocktail (SteadyGlo, □) or using K_m concentrations of luciferase substrates (●). The gray shaded rectangle shows the typical concentration range used in HTS. The structures of the compounds assayed are also shown within each graph. d) Decay of luciferase activity following cycloheximide treatment in the absence (●) or presence of 6.25 μM compound for the compound shown in panels a (□), b (■), or c (○). The structures of the compounds assayed are also shown within each graph.

of compound library profiling in identifying underlying mechanisms of reproducible "off-target" assay responses that can confound the interpretation of the primary experimental results. The counterintuitive finding that inhibitors of reporters can appear as activators in cell-based reporter-gene assays is a prime example of an "off-target" response that can lead to erroneous interpretations if the underlying mechanism is not appreciated. While the SF-1 assay actives were subsequently retested in a related nuclear receptor counter-screen (RORα) using the same luciferase reporter to identify selective actives, we demonstrate an alternative "counter-screen database" approach to aid in the efficient selection and prioritization of follow-up compounds and ascribe a probable mechanism.

Luciferase assays are often the method of choice for HTS for many reasons, most notably the enormous signal above background these assays can exhibit (*4*). Although this study highlights an artifact inherent to luciferase-based assays, now that it is understood and a profile of luciferase inhibitors has been characterized and described (*3*), researchers can use this information to prevent following uninteresting actives. All assays have artifacts, and many of these are far less well understood than luciferase inhibitors. For example, fluorescence responses are nonlinear and depend on the assay format and detector settings, making artifacts difficult to characterize and identify. To further complicate the matter, we have found that oftentimes "compound" fluorescence may actually be due to fluorescent impurities in the chemical sample (*9*). In contrast, interference with luciferase-based assays can be understood with more standard medicinal chemistry rules that define the SAR of the inhibitor series for the luciferase enzyme. The fact that this same SAR can be used to explain nonspecific activation in luciferase reporter-gene assays underscores the tractability of luciferase-based artifacts compared with other methods. The use of orthogonal assays (*10*), for example, based on β-lactamase reporters where inhibitors are most likely less prevalent (*11*), or substrate-independent reporters such as fluorescent proteins, expressed in a common cell line, would provide a complementary assay to the primary screen. An understanding of the SAR and effects of luciferase inhibitors in both cell-free and cell-based systems should allow more judicial development and application of this important category of bioluminescent assays.

As HTS in academia expands beyond the pharmaceutical industry to address the needs of chemical biology and translational research, the numerous sources of artifacts painstakingly discovered in the pharmaceutical sector will, for the most part, not transition beyond pro-

prietary company databases. Broad and open access to a public chemical biology database can serve to mitigate reinvestigation of common HTS artifacts. The striking occurrence of luciferase inhibitor enrichment in assays designed to detect receptor agonists should reinforce the notion of inhibitor-stabilization as an important consideration in the interpretation of luciferase reporter-gene assays.

METHODS

Construction of the Luciferase Subchemome. The luciferase subchemome dendrogram was generated by an in-house interactive visualization tool called Phylochem. Given the identified list of 1879 luciferase inhibitors, Phylochem first applied a hierarchical clustering algorithm (using a suitable similarity metric based on maximal common substructure) to organize the structures. A depth-first traversal of the resulting dendrogram was then performed to project each node onto a circle with the radius proportional to the node's depth. The embedding of each node in the dendrogram is similar to the layout used by the radial clustergrams of Agrafiotis *et al.* (*12*). The final layout was obtained by the merging of overlapping nonterminal nodes.

Compound Preparations. Compounds tested in this study for luciferase stabilization were initially identified and described by Auld *et al.* (*3*) and included members of a benzthiazole series, a quinoline series, and a noncompetitive luciferase inhibitor, 1,2,4-oxadiazole. Compounds were obtained from ChemBridge and reanalyzed for purity in house. Purity analysis was performed *via* LC−MS analysis on a Waters ACQUITY reverse-phase UPLC system and 1.7 M BEH column (2.1 mm × 50 mm) using a linear gradient in 0.1% aqueous formic acid (5% ACN in water increasing to 95% over 3 min). Compound purity was measured by peak integration from both UV−vis absorbance and ELSD, and compound identity was based upon mass analysis; all compounds passed purity criteria (>95%). These compounds were prepared as DMSO solutions in 1536-well plates at initial concentrations of 10 mM to 1 nM in a 24-point 2-fold titration across the plate. Each compound titration existed in duplicate on each plate, except for the benzthiazole and 1,2,4-oxadiazole compounds, with four titrations on the plate. Four rows of DMSO also existed on the compound plate.

Inhibition of Purified Luciferase. A 20-nM luciferase stock (luciferase from *P. pyralis*, Sigma-Aldrich, L9506) was prepared in PBS pH 7.4 (Invitrogen, 10010) such that upon delivery of 3 μL to the assay well, the final concentration of luciferase was 10 nM in the 6-μL total assay volume. After 3 μL of this luciferase stock was dispensed to assay plates (Greiner 1536-well white, tissue culture, sterile, 789173-F) using a BioRAPTR Flying Reagent Dispenser (FRD), 23 nL of inhibitor compounds were immediately transferred from the compound plate into the assay plate using a Kalypsys pin-tool transfer station, resulting in a final compound concentration of ~38 μM to ~4.6 pM. Three microliters of Promega Steady-Glo Luciferase Assay Reagent (E2520) was dispensed into each well, again using the BioRAPTR FRD. Plates were read within 5 min of assay reagent addition using a PerkinElmer ViewLux CCD Imager with a clear filter and 10-s plate exposure time. Alternatively, the luciferase enzyme activity was measured using 10 μM D-luciferin (Sigma-Aldrich, L9504) and 10 μM ATP (Sigma-Aldrich, A7699), which represents substrate concentrations ≈ K_m. These experiments were performed to reconfirm results described in Auld et al. (*3*), and data plotted from these experiments are the average of two to four compound titrations for a given compound.

Inhibition of Luciferase Degradation in HEK293 Cells Constitutively Expressing Luciferase. HEK293 cells transiently transfected with the pGL3-Control Vector offered by Promega (E1741) that expresses the *P. pyralis* luciferase were plated at a density of 10,000 cells/well using a Multidrop Combi Dispenser (Thermo Electron Corp.) in a 4.5-μL volume. After incubation for 1 h at 37 °C to allow a short recovery, 23 nL of inhibitor compounds were immediately transferred from the compound plate into the assay plate using a Kalypsys pin-tool transfer station, resulting in a final compound concentration of ~50 μM to ~6 pM. Cells were then incubated at 37 °C for 24 h. Subsequently, 23 nL of a 2.25 mg mL^{-1} cycloheximide (Sigma-Aldrich, C0934) stock in DMSO (or DMSO alone) was added into the assay plate using the Kalypsys pin-tool for a final concentration of 10 μg mL^{-1} of cycloheximide in a 4.5-μL total assay volume. Plates were incubated for various times (time 0, 3, 6, 12, or 24 h) at 37 °C before addition of 4.5 μL of Promega Steady-Glo Luciferase Assay Reagent using the BioRAPTR FRD. After a 15-min incubation at RT in the dark, plates were read using a PerkinElmer ViewLux CCD Imager with a clear filter and 10- or 30-s plate exposure time. Data plotted from these experiments are the average of four to eight compound titrations for a given compound.

Data Analysis. Data were plotted using GraphPad Prism 4, and the software's built-in analysis was used to fit nonlinear curves to the data. To generate plots of the relative amount of luciferase activity remaining after 24-h treatment with cycloheximide, the ratio of luciferase activity 24 h post-cycloheximide treatment to luciferase activity at time zero was calculated and normalized to the luciferase activity obtained in the absence of compound at 24 h and then plotted for each concentration of compound tested.

Acknowledgment: This research was supported by the Molecular Libraries Initiative of the National Institutes of Health Roadmap for Medical Research and the Intramural Research Program of the National Human Genome Research Institute, National Institutes of Health. We thank N. Southall for critically reading this manuscript prior to submission and D. Leja for graphical artwork.

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