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Reporter Enzyme Inhibitor Study To Aid Assembly of Orthogonal Reporter Gene Assays

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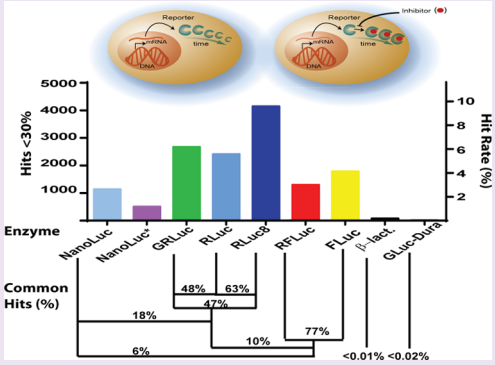
Supporting Information

ABSTRACT: Reporter gene assays (RGAs) are commonly used to measure biological pathway modulation by small molecules. Understanding how such compounds interact with the reporter enzyme is critical to accurately interpret RGA results. To improve our understanding of reporter enzymes and to develop optimal RGA systems, we investigated eight reporter enzymes differing in brightness, emission spectrum, stability, and substrate requirements. These included common reporter enzymes such as firefly luciferase (*Photinus pyralis*), *Renilla reniformis* luciferase, and *β*-lactamase, as well as mutated forms of *R. reniformis* luciferase emitting either blue- or green-shifted luminescence, a red-light emitting form of *Luciola cruciata* firefly luciferase, a mutated form of *Gaussia princeps* luciferase, and a proprietary luciferase termed "NanoLuc" derived from the luminescent sea shrimp *Oplophorus gracilirostris*. To determine hit rates and structure−activity relationships, we screened a collection of 42,460 PubChem compounds at 10 *μ*M using purified enzyme preparations. We then compared hit rates and chemotypes of actives for each enzyme. The hit rates ranged from <0.1% for *β*-lactamase to as high as 10% for mutated forms of *Renilla* luciferase. Related luciferases such as *Renilla* luciferase mutants showed high degrees of inhibitor overlap (40−70%), while unrelated luciferases such as firefly luciferases, *Gaussia* luciferase, and NanoLuc showed <10% overlap. Examination of representative inhibitors in cell-based assays revealed that inhibitor-based enzyme stabilization can lead to increases in bioluminescent signal for firefly luciferase, *Renilla* luciferase, and NanoLuc, with shorter half-life reporters showing increased activation responses. From this study we suggest strategies to improve the construction and interpretation of assays employing these reporter enzymes.



Identification of high quality leads from chemical library screening efforts is one of the critical steps in drug discovery. Cell-based assays now compose a large portion of the assays used to screen compound libraries. One of the most widely used cell-based formats is the reporter-gene assay (RGA) wherein a biological pathway or event is monitored by an enzyme that produces either a fluorescent or bioluminescent product. Firefly luciferase derived from the North American firefly *Photinus pyralis* (FLuc) is the most commonly used reporter enzyme for construction of RGAs. Although widely employed and highly sensitive, FLuc has been shown to be susceptible to inhibition by low-molecular weight (LMW) compounds found in many typical screening libraries.[1−4] Retrospectively, this is not surprising given that FLuc is known to bind two LMW substrates, D-luciferin and ATP. Although FLuc inhibitors comprise only ∼4−10% of typical compound screening libraries (e.g., ≥90% of the library is inactive against the enzyme at screening concentrations of ∼10 *μ*M), these inhibitors are found to be greatly enriched in hit lists derived from FLuc-cell-based assays. Enrichments ranging from 40% to 98% have been noted; this level of interference can confound the interpretation of structure−activity relationships (SAR) and the identification of compounds affecting the pathway of interest.[4−7]

The use of counter-screen databases composed of FLuc-based RGAs often have limited utility in flagging FLuc inhibitors due to idiosyncratic effects these inhibitors have on the assay signal, which can range from strong inhibition to strong activation depending on the mechanism of inhibition, levels of reporter expression, incubation time, choice of detection reagent, and other differences between assay protocols.[4] This high level of interference suggests that susceptibility of the reporter enzymes to inhibitors should be a primary consideration when choosing a reporter enzyme to

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Table 1. Properties of Reporter Enzymes in the Study

enzyme	species	MW (kDa)	Em (nm)	[E][a]	K_M[b]	ATP?	substrate	stability
FLuc	*Photinus pyralis*	62	550−570	10	10	yes	D-luciferin	3 h (cell)
RLuc	*Renilla reniformas*	36	480	12	3.5	no	coelenterazine	4.5 h (cell)
RLuc8	*Renilla reniformas (mutant)*	36	480[f]	3	2.0	no	coelenterazine	4.5 h (cell)
GRLuc[c]	*Renilla reniformas (mutant)*	36	530	2	2.0	no	coelenterazine	>48 h (cell)
β-lactamase[d]	*E. coli (TEM-1)*	29	520	0.3	36	no	CCF2	3.5 h (cell)
RFLuc[c]	*Luciola cruciata (mutant)*	62	620	20	22	yes	D-luciferin	3.0 h (cell)
NanoLuc[e]	*Oplophorus gracilirostris*	19	450	0.2	0.04X	no	furimazine	T_M >60 °C
GLuc-Dura[c]	*Gaussia princeps mut.*	20	485	0.2X	2	no	coelenterazine	60 days in supernatant

[a]All enzyme concentrations are in nM except for the GLuc Dura enzyme that was from the supernatant of cells secreting this enzyme. [b]Apparent K_M values were determined in 1536-well plates in a common buffer system. All concentrations are in μM except for furimazine where the substrate stock was supplied as "50X"; molar concentration not provided. [c]GRLuc, GLuc-Dura, and RFLuc are mutant forms of the native enzyme commercialized by Thermo Fisher Scientific. [d]Fluorescent reporter enzyme using the FRET substrate CCF2, excitation wavelength 450 nm, emission wavelength 520 nm. [e]NanoLuc developed by Promega Corp. [f]The emission of RLuc8 can be blueshifted to 395 nm using DeepBlueC (coelenterazine-400a).

construct an assay, although other factors such as response dynamics and sensitivity will also be important.

A good strategy to identify interference in any assay is to employ an orthogonal assay, *i.e.*, an assay measuring the same biology but using a different detection strategy.[8] For FLuc-based RGAs, the most desirable orthogonal reporter enzyme is one that is phylogenetically unrelated to FLuc, uses a different catalytic mechanism, and shows a different compound inhibition profile.[9,10] Ideally, alternative reporter enzymes are also stable and monomeric and possess equivalent or improved sensitivity relative to FLuc. Several reporter enzymes exist that could fulfill one or more of these criteria, including a few new bioluminescent reporters, but little is known about their propensity for inhibition by LMW compounds. Given the observations stated above, examination of the inhibition profile for a panel of reporter enzymes seems warranted to help develop new strategies for the construction orthogonal RGA systems.

In this study we investigated eight reporter enzymes (Table 1) to determine the degree of inhibition and SAR between different reporter enzyme inhibitors. To determine inhibition, we obtained purified enzyme preparations and developed enzyme assays using substrate concentrations near the apparent K_M value of the enzyme to sensitize the assays to competitive inhibition. The study included a variant of *Luciola cruciata* firefly luciferase (LcLuc) containing a one-amino-acid change from the wild-type enzyme in the D-luciferin (D-LH_2) binding pocket yielding red-shifted luminescence (Thermo Fisher Scientific, RFLuc). Additionally, we investigated several non-ATP-dependent enzymes that utilize coelenterazine as substrate. These included the commonly employed *Renilla reniformis* luciferase (RLuc),[11] mutated forms of RLuc providing green bioluminescence (GRLuc, Thermo Fisher Scientific) or blue luminescence (RLuc8),[12] and a mutated form of the luciferase from *Gaussia princeps* (Thermo Fisher Scientific; *Gaussia*-Dura, GLuc-Dura). We also examined a new luciferase derived from the luminescence decapod shrimp *Oplophorus gracilirostris*[13] termed NanoLuc (Promega Corp.[14]). Additionally, we investigated β-lactamase,[15] which cleaves a cephalosporin-based substrate containing fluorophores for detection of fluorescence resonance energy transfer (FRET).

To characterize the enzymes we screened a 42K compound library available in PubChem using a 10 μM screening concentration. We then compared primary hit rates, and following determination of potency values, we compared the SAR of inhibitors to identify enzymes that are less susceptible to inhibition and/or exhibit a different inhibitor SAR. The bioluminescent spectra and relative signal strength were also compared, and representative inhibitors were examined in RGAs. On the basis of these results we provide recommendations of suitable reporter enzyme pairs that can be used to construct orthogonal RGA systems aimed at readily distinguishing compounds of interest from those that interfere with reporter enzyme signal.

RESULTS AND DISCUSSION

RGA Enzyme Assay Development. Purified enzyme preparations were employed to develop 1536-well microtiter plate assays suitable for HTS. For GLuc-Dura, we used supernatant from cells secreting this enzyme. In all assays we used substrate concentrations that were near the apparent K_M of the enzymes that we determined in a defined assay buffer (Table 1). For the assay buffer, we decided on a common buffer system, which did not appear to impair the activity of the majority of the reporter enzymes, so that the screening results could be easily compared (Supplementary Table S1). The one exception was GLuc-Dura, for which we needed to employ a commercial buffer that contained stabilizers of the bioluminescent signal, although the coelenterazine substrate concentration was held at the apparent K_M value. Titration of enzymes was performed to choose a concentration that was within a range where the signal was proportional to enzyme levels, which we found to be between 0.2 and 20 nM for the enzymes in this study (Table 1). The DMSO concentration was 0.5% for all assays, a concentration that was found not to affect any of the enzymes.

The emission spectra of the luciferases were measured (Figure 1A). The peak luminescent emissions for the various luciferases covered the visible range from blue to red. For the HTS assays, a clear filter was used to capture the bioluminescence signal on PE ViewLux (CCD imager), and exposures were adjusted to keep in the linear range of the detector.

Screening of 42K PubChem Library. To profile the reporter enzymes we used a 42K library available from PubChem.[16] Compounds were screened at a 10 μM screening concentration. All of the assays showed robust performance in 1536-well plates with Z' >0.6 (Supplementary Table S2). Hits were chosen using a −30% threshold, and the overall hit rates and overlap of hits were determined (Figure 2A). The hit rates varied from as low as 0.02% for GLuc-Dura to nearly 10% for RLuc8. Among the reporters, the hit rates for GLuc-Dura, β-

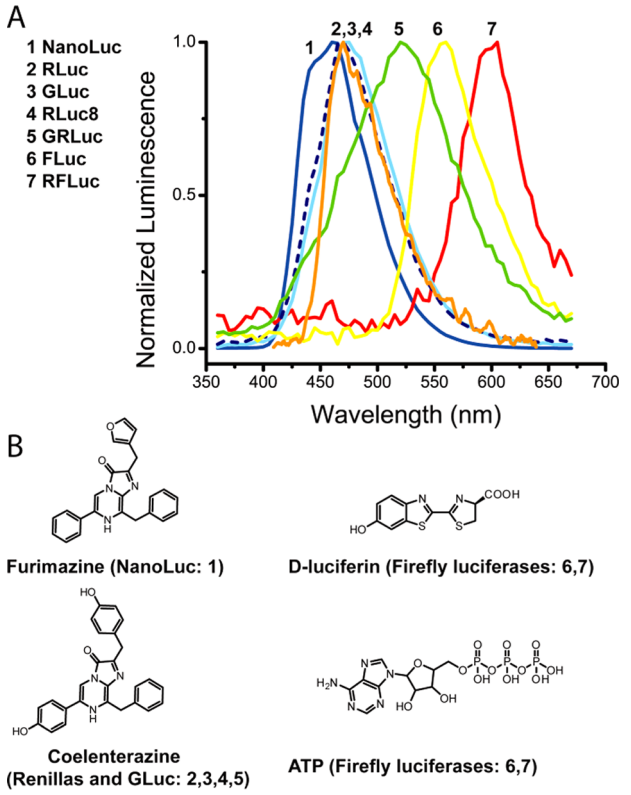

Figure 1. Bioluminescent emission analysis. (A) The normalized bioluminescent spectrum for each enzyme is shown. Peak emissions were 460 nm (**1**, NanoLuc), ~480 nm (**2**, **3**, **4**; RLuc, blue dotted line; GLuc-Dura, orange line; RLuc8, light blue line), 520 nm (**5**, GRLuc), 565 nm (**6**, FLuc), and 600 nm (**7**, RFLuc, mutant). (B) Structures of luciferase substrates.

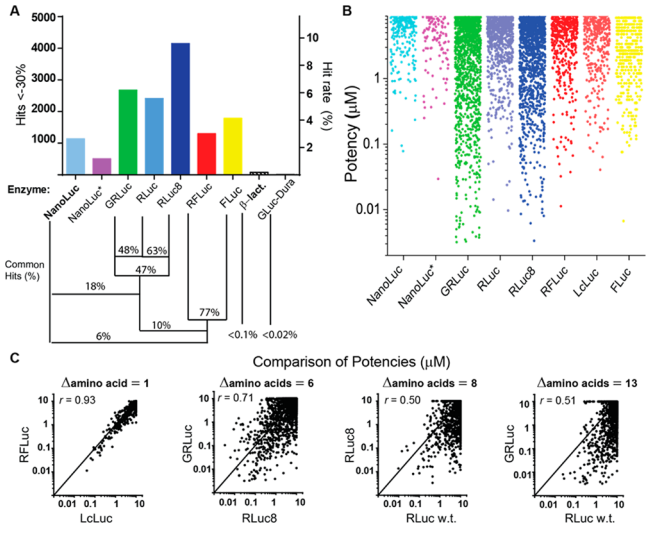

Figure 2. Summary of luciferase inhibitor primary hit rates and potency. (A) Bar chart for the number of primary hits (left *y*-axis) or hit rate (right *y*-axis) for the reporter enzymes. A 30% inhibition threshold was used for the analysis. All assays used K_M levels of substrate in the same buffer system except for a NanoLuc optimal detection buffer (NanoLuc*), which contains saturating furimazine, and GLuc-Dura, which used a different buffer but with K_M levels of coelenterazine. The dendrogram shown below the bar graph represents the percentage of common hits from the primary screening data found between hit lists for each reporter enzyme. (B) Potency distribution (absolute IC_{50} values) for enzyme inhibitors. Only data for compounds that showed an absolute IC_{50} <10 μM are shown. For follow-up, we also used the wild-type form of RFLuc. For comparison, published potency data from PubChem on FLuc[4] is also shown. (C) Comparison of IC_{50}'s between different luciferase variants.

lactamase (<0.1%), NanoLuc (2.7%), and RFLuc (3.1%) were all lower than that of FLuc (4%). The few hits identified for GLuc-Dura all showed weak potency in the primary screen. However, while the substrate concentration in the GLuc-Dura assay was at the apparent K_M value, we had to use a different buffer for this enzyme (with a proprietary composition) to promote a glow response signal that was suitable for our automation. Therefore, we cannot rigorously compare these results to those of other enzymes that were performed in the same buffer.

High confirmation rates were observed for the reporter enzyme actives (average ~85%, Supplementary Table S2). The firefly luciferase assay in particular shows high reproducibility with excellent agreement between independent runs (r^2 >0.95[17]). Examining primary hits using a 30% inhibition threshold revealed little overlap between hits for unrelated reporter enzymes, while related enzymes showed a high degree of overlap (Figure 2A). RFLuc and FLuc show 67% identity overall and 76% identity within the region covering the D-LH_2 binding pocket (approximately residues 220–345). RFLuc differs from the LcLuc by a single amino acid (S286Y) in the active site (Supplementary Figure S1A). Not surprisingly, 77% of the hits were common to both of these enzymes. The RLucs showed the highest hit rates: (6–10%) using a −30% threshold value. RLuc and RLuc8 (8 amino acid differences; Supplementary Figure S1B) showed a 63% overlap, while GRLuc and RLuc (13 amino acid differences; Supplementary Figure S1B) showed a ~47% overlap. However, specific inhibitors were identified (see below), a result that is consistent with the finding that many FLuc inhibitors are competitive with the luciferin substrate[4] and that all the variants assayed here had mutations in the luciferin binding pocket. For GRLuc and RLuc8, the hit rates were ~2.6% at a −80% threshold, while RLuc showed a 0.8% hit rate at this threshold value, suggesting less potent inhibitors for RLuc. Upon confirmation of potency values, GRLuc and RLuc8 indeed showed more potent inhibitors than RLuc (Figure 2B). Potency data for all the enzymes is supplied in Supplementary Table S3.

The inhibitors identified for GLuc-Dura and NanoLuc were generally of weaker potency than those found for any of the RLuc variants or firefly luciferases, with the majority of the NanoLuc inhibitors exhibiting IC_{50}'s > 1 μM (Figure 2B). Few GLuc-Dura inhibitors were identified (<17). NanoLuc hits showed <10% overlap with either of the firefly luciferases. Conversely, NanoLuc showed a higher number of common hits with RLuc and its variants (~18%), a result likely due to the fact that both of these enzymes use coelenterazine-based substrates (Figure 1B). We also tested NanoLuc in an optimized buffer formulation containing excess furimazine substrate (NanoLuc*, Figure 2A,B) and found that this buffer weakened the potency of many inhibitors, consistent with a competitive inhibition mode for these inhibitors.

Comparison of the inhibitor potencies between related luciferase variants further demonstrates how only a few amino acid changes can cause large potency variations (Figure 2C). The highest correlation in potency values was observed between LcLuc and RFLuc (r = 0.93, Figure 2C). GRLuc and RLuc8, which differ by six amino acids, showed a poorer correlation (r = 0.71, Figure 2C), while either RLuc8 or GRLuc

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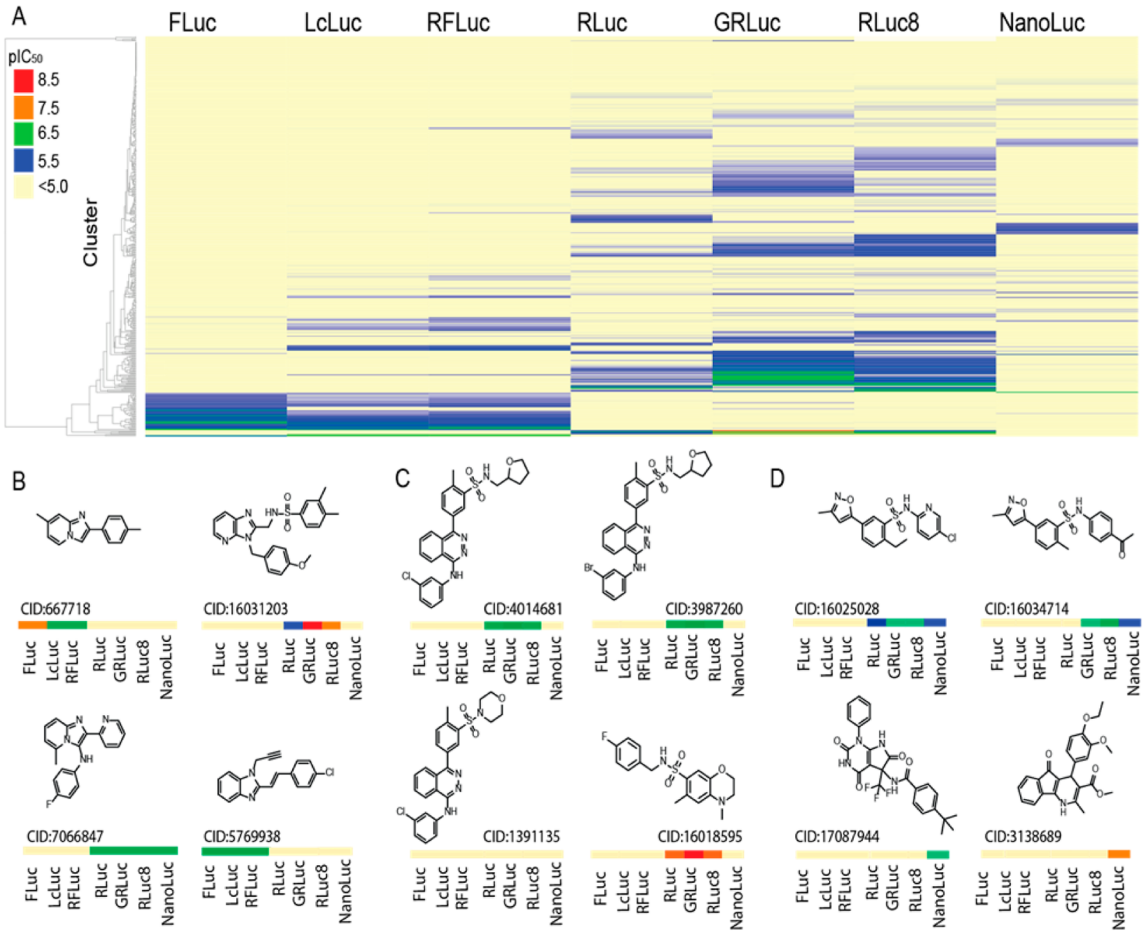

Figure 3. Orthogonal SAR for luciferase inhibitors. (A) Heatmap of inhibitor potency among clusters of related compounds (Tanimoto = 0.75). A total of 3,587 compounds with confirmed potency were used to construct the heatmap. (B) Examples of similar structures showing pan-activity for related luciferases, firefly, NanoLuc, and RLuc/variants (color scheme as in panel A). (C) Examples of potent and selective RLuc/variant inhibitors. (D) Examples of potent NanoLuc inhibitors.

showed large differences in potency values (r = 0.5) despite these variants showing >95% amino acid identity to wt RLuc. The majority of these amino acid differences are in the luciferin binding pocket, and these results suggest a competitive inhibition mode for the majority of *Renilla* luciferase inhibitors. Consistent with this notion, we found that variation of coelentarizine substrate modulated the IC_{50} for a subset of potent *Renilla* luciferase inhibitors with the majority (70%) showing much less potent IC_{50}'s at high substrate concentration (Supplementary Figure S2). The high hit rate for RLuc and variants was surprising given that this enzyme does not utilize ATP. However, perhaps the larger luciferin substrate for RLuc provides an active site that is more susceptible to LMW inhibitors.

Determination of Reporter Enzyme Inhibitor SAR. Structure–activity relationships between luciferase inhibitors were examined by clustering the confirmed actives by chemical similarity. For confirmation, potency values were determined for all samples showing >50% inhibition in the primary screen (Figure 2B, Supplementary Table S2), and the identity and purity of the compounds were confirmed by LC–MS analysis. For each enzyme inhibitor in the analysis, compounds showing IC_{50}'s ≤10 μM were taken as active compounds.

Hierarchal clustering of the confirmed inhibitors by chemical similarity and inhibition profile reveals orthogonal SAR between unrelated luciferases (Figure 3A). Inhibitors of firefly luciferase largely cluster together with little inhibition of RLuc and variants or NanoLuc. However, some exceptions exist where similar structures are present in unrelated luciferases as shown by the benzoimidazole- and imidazopyridine/pyridazine-containing structures that were found as either selective FLuc or RLuc inhibitors. Inhibitors of firefly luciferases containing these scaffolds had smaller substituents off the central imidazole ring (Figure 3B, CID 667718 and CID 5769938), while inhibitors of *Renilla* variants showed larger substitutions (Figure 3B, CID 16031203 and CID 7066847 and Figure 3C). One of these compounds (CID 7066847) showed pan-activity for RLuc/variants and NanoLuc. Representative structures associated with highly potent inhibitors of RLuc and variants were in some cases accompanied by closely related inactive analogs (Figure 3C, CID 1391135), supporting a defined SAR. All of the NanoLuc inhibitors were found to be selective against FLuc. Overlapping inhibition of NanoLuc with RLuc did occur as shown for CID 16025028 and CID 16034714. (Figure 3D), which also showed pan activity for RLuc and variants but not firefly luciferases. The benzenesulfonamide scaffold found in compounds such as CID 16018595 (Figure 3C) was often associated with potent *Renilla* luciferase inhibitors. We found that the benzenesulfonamide scaffold was frequently found among inhibitors for RLuc and variants, overall composing ~20% of the active compounds found for *Renilla* luciferase variants. This represents approximately a 2-fold enrichment

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over what is found in the entire 42K library. One of the more potent RLuc inhibitors showing a potency of <10 nM against GRLuc (CID 16031203; Figure 3C) contains this substructure. This substructure is also found among known RLuc inhibitors, which include H89 and methyl-*N*-(phenylmethyl)-benzenesulfonamide.[9,18,19]

Cell-Based Activity of Luciferase Inhibitors. FLuc inhibitors are often found as activators in FLuc-based RGAs and are highly enriched in hit lists derived from these assays, especially in assays involving prolonged incubation times and detection reagents such as BrightGlo (Promega) containing high substrate concentrations.[2,20] We have noted that RLuc inhibitors can show a similar behavior,[9] and we further examined several pan-active RLuc inhibitors identified in this study in an FLuc/RLuc-based RGA. In this assay both RLuc and FLuc are expressed by a bidirectional tetracycline-inducible promoter. We found that these inhibitors exemplified an activity consistent with specific stabilization of the RLuc reporter (Figure 4), with an activation response covering the

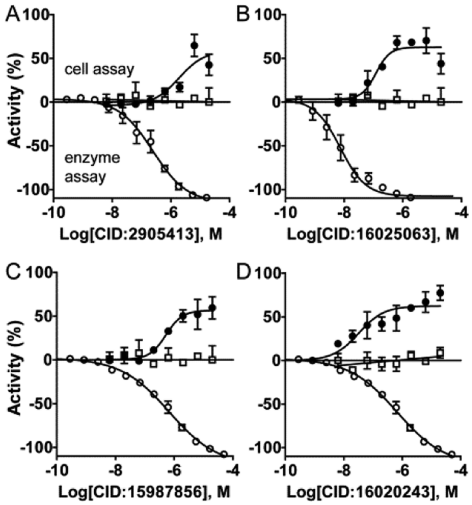

Figure 4. Behavior of RLuc inhibitors in a FLuc/RLuc-based RGA. Graphs show data from both reporters in the RGA (data at or above origin; solid circles RLuc activity; open squares FLuc activity, used as an indicator of cell viability in the assay) and the purified RLuc enzyme assay (curves, below origin). Data from RLuc-based RGA (n = 2) or enzyme assay (n = 4), error bars are SD. All compounds were inactive on FLuc up to 57 μM (FLuc inhibition values taken from PubChem, AID 588342).

concentration range where strong inhibition was observed using purified RLuc enzyme. As well, for compounds that were determined to be inactive against purified FLuc enzyme, no FLuc activity was observed in the cell-based assay. Additionally, none of these compounds appear to be generally cytotoxic based on PubChem data, which show them to be inactive at a testing concentration of 46 μM in cytotoxocity counter-screen assays (PubChem AIDs: 902, 903, and 504648). To examine if such inhibitors indeed stabilize RLuc, we measured the T_M of RLuc using differential-scanning fluorimetry (DSF, Figure 5). The ΔT_M values between the apo enzyme and compound-containing enzyme preparations exhibited values as high as 10 °C and were concordant with the measured IC_{50}'s against RLuc (Figure 5A). Examination of the more stable GRLuc enzyme in cells (Table 1[21]) shows that only an inhibitory response is observed in cells that constitutively expressed this reporter (Figure 6). In fact, a pan inhibitor of RLuc and GRLuc (CID

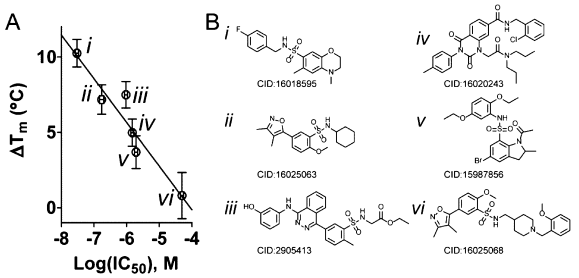

Figure 5. Structures and thermal melt data for RLuc inhibitors. DSF was used to measure the T_M values of representative RLuc inhibitors differing in potency against the enzyme. (A) Correlation of ΔT_M values (taken as the difference between the T_M at 25 μM compound and apoenzyme) and the measured IC_{50}'s for the *Renilla* inhibitors (structures shown in panel B). Compound ***vi*** showed an estimated IC_{50} >50 μM in the enzyme assay. Data shown is from n = 3, error bars are the SD.

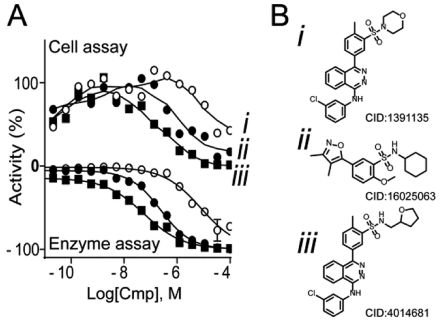

Figure 6. Effect of GRLuc inhibitors in cells. (A) Response from cells stably expressing the GRLuc reporter (top graph) or the purified enzyme assay (bottom graph) after treatment with compound for 48 h. Detection of bioluminescence used the 1X Flash Buffer (Thermo Fisher Scientific) in both the cell and enzyme assays. (B) Structures of the inhibitors.

16025063) shows an activation response in an assay using RLuc as the reporter (Figure 4A) but inhibition in cells that use GRLuc as the reporter (Figure 6). These observations demonstrate the different affects that reporter inhibitors can have on cell-based assays that employ related reporters that differ in half-life.

We also examined representative inhibitors of NanoLuc and FLuc in cell-based assays to investigate the orthogonal nature of these reporters in a cellular system. In this case, we compared both wt and destabilized forms (fused to a PEST sequence) of the reporters in separate HEK293 cell lines stably expressing these reporters under the control of CMV promoters (Figure 7). NanoLucP and NanoLuc refers to a PEST fused form of NanoLuc ($t_{1/2}$ ~30 min) or wt NanoLuc ($t_{1/2}$ ~6 h), respectively. Luc2P and Luc2 refers to a PEST fused form of FLuc ($t_{1/2}$ ~1 h) or wt FLuc ($t_{1/2}$ ~4 h), respectively. We found that selective inhibitors of either FLuc or NanoLuc showed little or no activity when cells were treated with the compounds inhibiting the either stabilized or destabilized variants of the alternative reporter. Some effects were observed at higher compound concentrations in some experiments, which may be due to effects on cell health (see Figure 7B NanoLucP and Luc2P data, compounds ***i*** and ***ii***, for example). In contrast, when we examined a compound (CID 16008501, Figure 7A) that showed some inhibition of both FLuc and NanoLuc enzymes, we observed activity in both reporter-expressing cells

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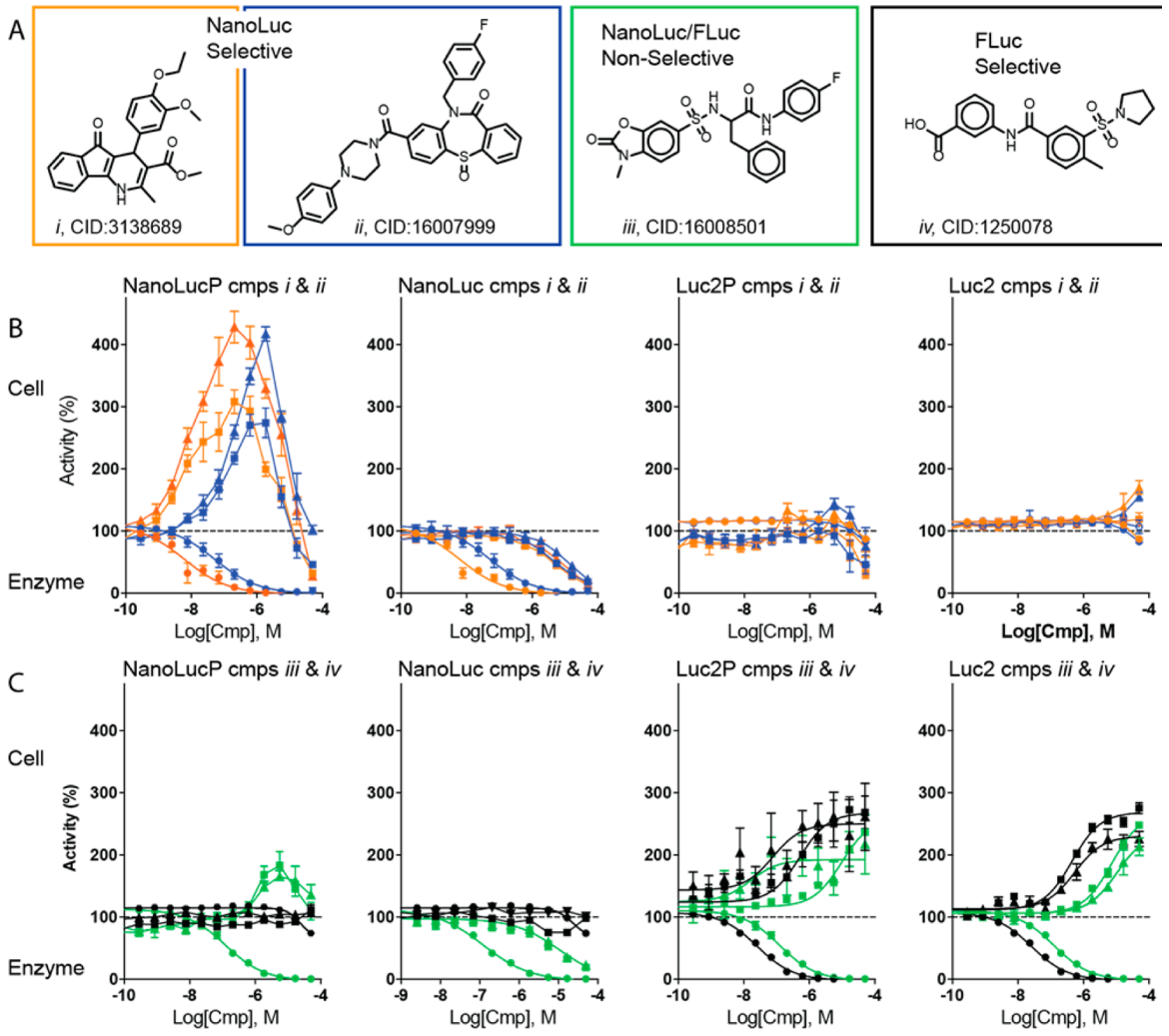

Figure 7. Effect of NanoLuc and FLuc enzyme inhibitors in cells. (A) Structures of reporter enzyme inhibitors characterized in the cell-based assays. (B) Data is color-coded as shown for the boxes in panel A, showing the purified enzyme inhibition data (solid circles in all graphs, always at or below 100% in all graphs) and the cell-based data at either a 24 h (solid triangles) or 48 h (solid squares) incubation time. The cell-based data used detection reagents containing excess substrates. Data shown is from n = 3 or 4, error bars are the SD.

lines. Interestingly, we noted that the FLuc selective inhibitor (CID 1250078) consistently showed activation in FLuc-expressing cells, while the NanoLuc-selective inhibitors (CID 3136689 and CID 16007999) showed an inhibitory response with NanoLuc-expressing cells but a strong activation response when cells expressed NanoLucP. As mentioned above for RLuc vs GRLuc, this is likely due to the intracellular half-life of the reporters where NanoLuc has a relatively stable half-life (6 h[14]), followed by FLuc > FLucP > NanoLucP. Taken together these results demonstrate the orthogonal nature of the reporter inhibitors of NanoLuc and FLuc and suggest that these responses are best distinguished when compounds are validated using concentration−response curves.

Implications and Recommendations for Construction of Orthogonal Reporter Assays. Reporter inhibitors can complicate the interpretation of activity arising from RGAs. Thorne et al.[4] found a hit rate of 5% using a 11 μM screening concentration using an enzyme assay for FLuc against the PubChem collection. This leads to large enrichments in hit lists derived from RGAs requiring careful strategies to efficiently identify reporter inhibitors and focus efforts on viable leads.

The SAR of FLuc inhibitors that have been described (1, 2, 4) can be used to identify compounds interfering with FLuc-RGAs and to prioritize compounds for analysis in secondary assays. Compounds showing strong inhibition against the FLuc enzyme and activity in the RGA should be deprioritized. However, less distinct situations exist, e.g., when compounds show a structure that is consistent with FLuc inhibitors but weak inhibition against the FLuc enzyme compared to the RGA. In other examples, the compounds could undergo cell-based metabolism to yield potent FLuc inhibitors, for example, the conversion in cells of a compound containing an aryl ester to one containing an aryl acid, which can lead to potent inhibition through FLuc-mediated adenylation of the compound.[4,22] In all cases, one is unable to determine how much of the bioluminescence signal modulation is due to FLuc reporter interference versus the cellular pathway of interest without an orthogonal assay. With this in mind, this study was designed to identify enzyme pairs with orthogonal inhibitor SAR.

In a recent report aimed at finding compounds for a gene-dosage disease,[10] orthogonal RGAs were constructed to measure expression of either FLuc or β-lactamase. Cross-validation of the actives derived from each RGA showed that

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apparently >90% of the actives were due to a reporter-based effect. Our study confirms that β-lactamase is a suitable choice as an orthogonal reporter enzyme for FLuc, as β-lactamase shows a low hit rate with no overlap of inhibitor chemotypes with FLuc. Another study aimed at identifying compounds that inhibit β-lactamase through formation of a compound aggregate confirmed the low hit rate (0.1%) for true enzyme inhibitors, similar to what we observed in this study.[23] The low hit rate for the β-lactamase might be due its substrate, containing a β-lactam ring, and inhibitors of this type are not typically found in LMW compound libraries. However, employing the β-lactamase reporter alone will lead to enrichments in compounds that affect the fluorescent signal. Therefore, for assays where down-regulation of reporter expression is the aim, a good orthogonal reporter pair is FLuc and β-lactamase.

In cases where the goal is to up-regulate the reporter response, basal levels of reporter expression often result in a feeble signal. Here the most desirable reporter enzyme is one producing a strong luminescent signal due to the high sensitivity of luminescence assays.[24] From our study, suitable orthogonal bioluminescent reporters would be any combination of FLuc, NanoLuc, or GLuc. The PEST-destabilized form of NanoLuc (NanoLucP) will likely show more interference by apparent activation due to the shorter half-life of NanoLucP. Alternatively, far-red-emitting luciferase reporters such as RFLuc could be multiplexed with blue-emitting luciferase reporters such as NanoLuc or GLuc. The GRLuc reporter would also be a good choice due to its high cell-based stability rendering it less likely to show an activation response upon inhibition. The low hit rate observed for secreted GLuc-Dura and the high stability of this enzyme in media could also make this enzyme a good choice for assays measuring luminescent increases.

Our study also points to the optimal implementation of RGAs in which two reporter enzymes are co-expressed. RLuc has been coupled with FLuc in "dual-reporter" assays where one reporter is used to measure a pathway-specific response and the other is used to measure nonspecific effects such as cytotoxicity and cell health.[25] However, given the prevalence of luciferase inhibitors and the divergent SAR between RLuc and FLuc inhibitors, it may be complicated to determine cell-health/pathway responses from specific reporter enzyme inhibition. For example, the RLuc inhibitor H89[18] is a known PKA inhibitor that could function in the pathway of interest but is also a potent RLuc inhibitor. Conversely, compounds that are simply FLuc inhibitors will appear as noncytotoxic actives as these compounds will likely have no effect on the RLuc signal. To interpret the results, a full understanding of the inhibition profile for both FLuc and RLuc would be required using the enzyme assays described here. In the converse situation, the use of highly related reporter enzymes as orthogonal systems or to identify general cell health from specific effects will also be complicated. This is due to the fact that although there is a high degree of inhibitor overlap between related enzymes (40−80% demonstrated here), there is also a significant proportion of inhibitors that selectively inhibit only one of the enzymes. Therefore, the use of highly related reporters to identify interfering compounds will likely be confounded by the presence of both nonselective and selective inhibitors. Mutations in the luciferin substrate binding pocket have been used to create two color dual luciferase assays,[26] but the contrasting SAR we observed here between variants such as GRLuc and RLuc8 will complicate the interpretation of these results.

We also examined the reporter inhibitors in a cell-based context. The cell-based activation responses for the reporter inhibitors are consistent with inhibitor-based stabilization of the reporter enzyme, as we observe the effect only in cells that express the reporter that the compounds inhibit. For reporters that were destabilized with a PEST sequence (FLuc2P and NanoLucP), we found that reporter inhibitors lead to an activation response in cell-based assays. This suggests that these reporter inhibitors can stabilize the enzymes to degradation by the proteasome system. The different cellular responses for inhibitors that we observed between NanoLuc vs NanoLucP or RLuc vs GRLuc suggest that using reporters with a different half-life could be a way to rapidly identify reporter inhibitors, although application of this strategy would have to carefully consider the response dynamics of the biology under measurement.

Conclusions. (1) For compound screening purposes, RGAs are best implemented using orthogonal reporter pairs where the same biology is measured with two orthogonal reporters, instead of using one for normalization purposes. For assays aimed at measuring luminescence increases, more stable reporter enzymes should be used as reporter inhibitors will likely show an inhibitory response in the RGA and therefore will not be scored as actives. (2) Use of the enzyme assays described here should also facilitate focusing on the most promising leads derived from RGAs. Recently, it has been shown that two reporter enzymes can be co-expressed from the same transcript at near equal levels in cells using a sequence that efficiently induces ribosomal skipping.[19,27] This allows for expression of two orthogonal reporter enzymes in the same cells, thereby providing a single system for rapidly identifying reporter-based interferences. The compounds described here are commercially available (see Supplementary Table S3 and PubChem for information) and can be used as controls to judge the sensitivity of the assay protocol to reporter inhibitors and should aid in assay optimization.

METHODS

Reagents. ATP, bovine serum (BSA), coelenterazine, D-luciferin, magnesium acetate, Tris acetate, and Tween 20 were purchased from Sigma-Aldrich. CCF2-FA was purchased from Life Technologies. Dimethylsulfoxide (DMSO), certified ACS grade, was purchased from Fisher. NanoLuc, furimazine, and the optimized detection buffer were gifts from Promega. See Supporting Information Methods for sources of luciferases enzymes.

Preparation of Compound Libraries and Validation Plates. For the primary screen, a 42K portion of the PubChem library was collected from the NIBR compound library. Compounds were stored in U-bottom cycloolefin (COC) 1536-well plates (Nexus Biosystem) at a concentration of 2 mM in 90% DMSO. For confirmation assays, compounds were cherry picked from the NIBR solution archive in U-bottom COC 1536-well plates. To measure concentration−response curves, compounds were cherry picked and diluted in DMSO as 8-point dilution series using a 1:3.16 dilution factor starting from 2 mM stock solutions. Details on the collection of mass identity and purity are given in Supporting Information Methods.

Luciferase Assays. A total of 3 μL/well of substrate/buffer concentrate (10 μM ATP, 0.05% BSA, 13.3 mM magnesium acetate, 50 mM Tris acetate, 0.01% Tween 20, pH 7.3, final concentration) was dispensed using a Flexdrop (Perkin-Elmer) into polystyrene white 1536-well high base plates obtained from Greiner Bio-one. A total of 20 nL/well of compound solution was transferred to the assay plate using a Janus pin tool equipped with 1536-pin array containing 20 nL

slotted pins (Perkin-Elmer, 0.45 mm diameter, 38.2 mm long). After compound transfer, 1 μL/well of luciferase/buffer (1x PBS pH 7.3, 0.05% BSA, 0.01% Tween 20) was dispensed into the assay plate using a Flexdrop dispenser, and the assay plate was spun down for 1 min at 1000 rpm using a VSpin (Agilent Technology). After a 10 min incubation at RT, luminescence was measured in a ViewLux CCD imager (Perkin-Elmer) using a 10−120 s exposure time and 1X-2X binning. All screening operations were performed by using a fully integrated Dim4 robotic system (Thermo Scientific) running Polara system software (Thermo Scientific; Supplementary Table S1).

Data Analysis. Primary screening data and concentration−response data were processed using in-house-developed software. Activity at >30% inhibition was used to define the primary hit list and for analysis. This level of inhibition was >3 SD of the average signal obtained for any of the luciferase assays. A consensus hit list of approximately 4K compounds was used for validation using eight concentration points. SAR was examined in TIBCO Spotfire using standard clustering by Tanimoto coefficient. See Supporting Information Methods for additional information.

Bioluminescent Spectra. Purified enzymes and substrates were mixed in FLuc buffer and the spectra were recorded on a SpectraMax. The assay was prepared in 384-well white plates with enzyme and substrate at the same concentrations used in the HTS.

Generation of Stable NanoLuc and FLuc Expressing HEK293 Cell Lines. NanoLuc cell lines were generated in HEK293 cells using the mammalian expression vector pF5A-Nluc containing the ORF for Nanoluc under the control of a CMV-D1 promoter.[28] See Supporting Information Methods for additional information.

Cell-Based Assays. All cell-based assays were performed in white solid-bottom tissue culture-treated 1536-well microplates (Corning cat. no. 431306). The cells were treated with 20 nL of compound or DMSO using a Janus pin tool. This allowed a 16-point titration of each compound over a concentration range of 40 μM to 2.8 pM. Assay plates were incubated for 24 or 48 h, whereupon the appropriate detection reagent was added. The RLuc-based RGA used HEK293 cells expressing FLuc and RLuc (driven by a bidirectional tetracycline-inducible promoter). GRLuc was stably expressed in HEK293 cells. See Supporting Information Methods for additional information.

DSF Data Collection. Differential scanning fluorimetry was performed using the fluorescent dye Sypro Orange (cat. S6650, Invitrogen). The thermal denaturation assay was performed using 10X SYPRO dye, 75 μg/mL of a recombinant RLuc protein (NanoLight Technology, cat. no. 314) in a 20 mM Hepes buffer, pH 7.5 containing 150 mM NaCl. The final reaction volume was 10 μL, and 384-well plates were used (Roche, cat. no. 04729749001). The fluorescence emission from the dye was captured using a CFX 384 thermal cycler from BioRad at 0.5 °C intervals along the temperature ramp from 25 to 75 °C. Further details are given in the Supporting Information Methods.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

FLuc, *Photinus pyralis* firefly luciferase; RLuc, *Renilla reniformis* luciferase; GLuc, *Gaussia princeps* luciferase; NanoLuc, optimized form of *Oplophorus gracilirostris* luciferase; RFLuc, red-shifted mutant of *Luciola cruciata* firefly luciferase (LcLuc); CID, compound identifier number for PubChem; D-LH_2, D-luciferin

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