Development of a High Throughput Lysyl Hydroxylase (LH) Assay and Identification of Small Molecule Inhibitors Against LH2

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Abstract

Lysyl hydroxylase-2 (LH2) catalyzes the hydroxylation of telopeptidyl lysine residues on collagen, leading to the formation of stable collagen cross-links that connect collagen molecules and stabilize the extracellular matrix. High levels of LH2 have been reported in the formation and stabilization of hydroxylysine aldehyde-derived collagen cross-links (HLCCs), leading to fibrosis and cancer metastasis in certain tissues. Identification of small molecule inhibitors targeting LH2 activity requires a robust and suitable assay system, which is currently lacking. Thus, despite being a promising target for these diseases, small molecule inhibitors for LH2 have yet to be reported. Therefore, we developed a luminescence-based strategy to monitor lysyl hydroxylase (LH) activity, and validated its ability to identify new inhibitors in a screen of approximately 65,000 compounds against LH2. Primary hits were confirmed using the same LH assay against mimiviral L230. This newly developed LH assay is robust, suitable for high-throughput screening, and able to identify potent specific inhibitors of LH2.

Keywords

Lysyl Hydroxylase-2 (LH2); luminescence; high throughput screen; succinate detection; cancer

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Introduction

Lysyl hydroxylases (LHs) belong to the large superfamily of 2-oxoglutarate- (aka: alpha-ketoglutarate) dependent oxygenases that requires alpha-ketoglutarate (α-KG) and Fe(II) for activity.[1] These enzymes couple the two-electron oxidation of substrate to the oxidative decarboxylation of α-KG to yield succinate and carbon dioxide.[1] LHs consist of three family members – LH1, LH2 and LH3. While all LHs catalyze the hydroxylation of lysine residues on collagen, only LH2 can modify telopeptidyl lysine residues.[2] Lysine hydroxylation is important in the formation of stable collagen cross-links, that connect collagen molecules and stabilize the extracellular matrix.[3]

LH2-mediated hydroxylation of telopeptidyl lysine residues in collagen has been shown to initiate the formation of hydroxylysine aldehyde-derived collagen cross-links (HLCCs), which are highly stable and resistant to collagenase cleavage.[3] It is reported that high levels of LH2 expression under pathological conditions can lead to the accumulation of these HLCCs in extracellular matrices, causing fibrosis.[4, 5] In addition, high levels of LH2 expression contribute to metastasis in lung cancer,[6] breast cancer,[7, 8] and sarcoma,[9] highlighting the therapeutic potential for inhibitors of LH2 to combat fibrosis and cancer.

However, there are no reported inhibitors of LH2 to date, and no high-throughput system to screen for novel LH2 inhibitors has been described. A robust assay system that is amenable to high throughput is important for the identification of small molecule inhibitors of LH2. Several assay methods for Fe(II)/2-oxoglutarate-dependent dioxygenases have been reported in the literature. One of the earliest and widely adapted methods developed was based on measuring the amount of $^{14}CO_2$ released during decarboxylation of 2-oxo-[1-^{14}C]glutarate. [10] However, the radioactive nature of such assays makes them impractical to use for high throughput purposes. Several non-radioactive assay methods have also been developed. For example, a fluorescence-based assay reported by McNeill et. al. monitors depletion of α-KG by its post-incubation derivatization with o-phenylenediamine to form a fluorescent product. [11] Similarly, Luo et. al. have reported an absorbance assay that couples succinate production to NADH oxidation.[12] However, these assays have limited sensitivity and require large amount of protein and product turnover to generate reliable signal.

Recently we reported a luminescence assay that measures LH2 activity based on the amount of succinate production in the assay.[13] The assay was superior in terms of stability and signal-to-noise ratio and was used in the kinetic characterization of LH2. As mentioned earlier, there is a need for a robust high throughput assay for LH in order to identify small molecule inhibitors for therapeutic purposes. Therefore, in this paper, we utilized this luminescence assay system to develop a robust luminescence-based high throughput assay platform that can facilitate the identification of potent, specific, small molecule inhibitors of LH2.

Materials and Methods

Reagents

Recombinant LH2 and L230 proteins were purified according to the methods explained previously.[13, 14] Unless otherwise stated, all chemicals were purchased from Sigma (St. Louis, MO) and were of highest purity available. Succinate Glo™ JMJC Demethylase Assay kit was purchased from Promega (Madison, WI, catalog #: CS1747A04). Peptide substrates for LH2 (IKGIKGIKG, abbreviated as [IKG]$_3$) and L230 (GTKGETGLKGII, abbreviated as GI-12) were ordered from LifeTein (Somerset, NJ) and the purity was determined to be greater than 98% by HPLC. White Nunc 384-well plates were obtained from Thermo Scientific (Waltham, MA, catalog #: 262360).

Chemical compounds

A total of 65,257 compounds were selected for primary screening. Compound libraries consisted of: NIH clinical collection (674 compounds; obtained from Evotec, San Francisco, CA), Spectrum collection (2,000 compounds; obtained from MicroSource Discovery, Gaylordsville, CT), Lopac (1,280 compounds; obtained from Sigma-Aldrich, St Loise, MO), Fragment sets (18,143 compounds; obtained from Chembridge and Chemdiv, San Diego, CA), Kinase set (11,250 compounds; obtained from Chembridge), and Diversity sets (29,718 compounds; obtained from NCI, Chemdiv, and Maybridge). In addition, compounds also consisted of following 2 library sets: 1) A kinase focused library collection of 600 compounds custom-selected by TxSACT (Texas Screening Alliance for Cancer Therapeutics) from various vendors; and 2) A collection of 2,000 unique molecules with diverse pharmacophores deposited from chemists at the University of Texas at Austin and University of Kansas.

Bioluminescence-based LH Assay

Unless otherwise stated, all LH assays were performed in an assay buffer (50 mM HEPES pH 7.4, 150 mM NaCl, 0.5% DMSO and 0.01% Triton X-100) containing 1 μM LH2, 1000 μM [IKG]$_3$ peptide, 100 μM ascorbic acid, 10 μM α-KG and 10 μM FeCl$_2$. Assays were performed in white Nunc 384-well plates using a 10 μL volume. First, 6 μL of 1.67X LH2 mix was aliquoted to a 384 well plate. Second, 2 μL of 5X additive (either detergent, BSA, DMSO, or compound) was added and incubated for 30 minutes. Third, the reactions were started with 2 μL of 5X substrate mix containing peptide substrate, α-KG, ascorbic acid and FeCl$_2$. FeCl$_2$ solution was always freshly prepared in 10 mM HCl and then diluted to an assay mixture to minimize iron oxidation in aqueous solution. Reactions were allowed to proceed for 90 minutes before adding detection reagent (Succinate Glo™ JMJC Demethylase Assay; Promega Corp.), according to manufacturer's instruction, unless otherwise stated. Briefly, 10 μL of Succinate detection reagent I was added to quench the lysyl hydroxylation reaction and convert succinate product to ATP. After 60 minutes, 20 μL of Succinate detection reagent II was added to convert ATP to light. After 10 minutes incubation, luminescence was read using Envision plate reader (Perkin Elmer, Waltham, MA).

Optimization assays for LH2 and peptide substrate were performed by varying LH2 (0 to 1 μM) and [IKG]$_3$ peptide substrate (0 to 1000 μM) in the assay. Similarly, tolerance tests were performed by varying DMSO (0 – 5%), Triton X-100 (0 – 0.16%), Tween-20 (0 – 0.16%), NP-40 (0 – 0.16%), and BSA (0 – 0.8%) in the assay. In all cases, LH2 mix was incubated with different concentrations of tested reagents at room temperature for 30 minutes. Assays were then initiated with the addition of substrate mix. Succinate detection assays were performed similarly as mentioned above.

Time course experiment for LH2 was performed by varying reaction times. Assays were initiated with the addition of substrate mix. Following 5 min, 60 min, 120 min or 180 min incubations, assays were quenched with the addition of Succinate detection reagent I. Succinate detection assays were then performed as mentioned above.

Finally, stability of LH2 at room temperature was also tested. LH2 mix was incubated at room temperature for 0, 1 or 2 hours. After respective incubation time, reactions were initiated with the addition of substrate mix. Succinate detection assays were performed similarly as mentioned above.

Assay validation

Assay validation was performed by plating columns 1–22 of the 384 well plate with positive controls (reaction containing LH2) and columns 23–24 with negative controls (reaction containing no LH2). First, 6 μL of enzyme mixture containing 1.67X LH2 in 1X assay buffer (columns 1–22) or 1X buffer only (columns 23–24) were dispensed to 384 well plates. Second, 2 μL of 5X DMSO (2.5%) in 1X buffer was added and incubated for 60 minutes. Third, 2 μL of 5X substrate mixture (containing peptide substrate, α-KG, ascorbic acid and FeCl$_2$) in 1X buffer was added to start the reaction and incubated for 90 minutes. Succinate levels were then measured using the Succinate Glo™ JMJC Demethylase Assay as described above. Enzyme mixture and DMSO were dispensed using the JANUS Automated Workstation (Perkin Elmer, Waltham, MA). Substrate mixture, Succinate detection reagent I, and Succinate detection reagent II were added using MicroFlo Select Bulk Dispenser (BioTek, Winooski, VT) equipped with a 1 μL cassette (for substrate dispense) or 10 μL cassette with large bore tips (for Succinate detection reagents I and II dispense). The MicroFlo dispenser is an automated reagent dispenser equipped with an 8 tip cassette allowing speedy dispense of bulk reagents from a reservoir.

Primary Screening and Hits confirmation

High throughput screening was performed as described in 'assay validation', except that compounds were used instead of DMSO in columns 3–22. Columns 1–2 were positive controls (reaction containing LH2 but no compounds) and columns 23–24 were negative controls (reaction with no LH2 or compounds). Columns 3–22 were assayed with library compounds. Hits from primary screen filtered by PAINS and OIDD filters available from FAFDrugs4 (http://fafdrugs3.mti.univ-paris-diderot.fr/references.html) were cherry-picked and confirmed using the same LH assay to eliminate any false hits due to systematic error. Assay interference was tested for the same cherry-picked hits using the same LH assay by replacing LH2 enzyme with succinate at 10 μM concentration. The assay was incubated for

1 hour before continuing with the succinate detection assay. Compound competition with Fe(II) was performed using the LH assay at varying concentrations (high, 100 μM and low, 10 μM) of $FeCl_2$. Selectivity test was conducted using the same LH assay but replacing LH2 enzyme and $[IKG]_3$ peptide substrate with mimivirus L230 enzyme and GI-12 peptide substrate at 1 and 1000 μM concentrations respectively. Compound potency against LH2 was determined by using a 3-fold serial dose response assay consisting of 7 different compound concentrations starting at 100 μM (fragments) or 25 μM (non-fragments). The compound concentrations in all experiments (except dose response assays) were same as that of primary screen (i.e. 100 μM for fragments and 25 μM for non-fragments).

Data analysis

Data calculations were performed using Microsoft Excel whereas all data plots and fittings were performed using KaleidaGraph 4.0. The z' factor was calculated using Eq. (1),[15] where σ_p and σ_n are the standard deviations of the positive and negative controls, respectively, and μ_p and μ_n are the mean counts (RLU) of the positive and negative controls, respectively. Percent inhibition of enzyme activity was calculated using Eq. (2), where μ_p is the average counts of the positive control wells and x_i is the counts of test sample.

$$z' = 1 - \frac{3(\sigma_p + \sigma_n)}{|\mu_p - \mu_n|} \quad (Eq.1)$$

$$\% I = 100 * (\frac{\mu_p - x_i}{\mu_p}) \quad (Eq.2)$$

Results and Discussions

Assay development for HTS application

Recently, we reported a scalable production of active LH2 enzyme and a lysyl hydroxylase (LH) assay to monitor LH2 activity, based on the amount of succinate produced.[13] This LH assay was the first reported luminescence-based strategy for the detection of succinate, by employing a coupled enzymatic assay in collaboration with Promega's science team[16]. The succinate was quantitated in two steps: first, the succinate product is converted to ATP by a succinate recycling enzyme; and second, the ATP is detected by a luminescent readout. We have demonstrated the superiority of this luminescence strategy over previous technologies in terms of sensitivity and robustness in monitoring LH2 activity. Promega launched this new detection platform as Succinate Glo™ JMJC Demethylase assay[16] very recently, claiming that this novel assay technology could be a universal tool for assaying diverse array of Fe(II)/2-oxoglutarate dioxygenases to efficiently profile inhibitors against a panel of enzyme superfamily as leads for drug development. Although this assay is well proven its advantage in sensitivity, robustness, homogeneity, and compound interference, its application to high-throughput screening (HTS) has not been reported yet. Interestingly,

while LH2 is a valid target for fibrotic diseases and cancer, there are currently no inhibitors reported for such important disease targets. Therefore, we sought to develop a suitable and robust HTS platform utilizing this bioluminescence-based assay to identify small molecule inhibitors of LH2 activity.

While this assay was established for small scale, further optimizations were required in order to use this assay for automated small molecule screening. Initially, the assay included α-KG, FeCl$_2$, ascorbate, and catalase at 100 μM, 50 μM, 500 μM, and 1.5 μM, respectively in an assay buffer containing 50 mM HEPES pH 7.4 and 150 mM NaCl.[13] A 4-fold signal to background (S/B) was achieved when utilizing [IKG]$_3$ at its K$_m$ (~179 μM). A higher S/B would increase the robustness of the assay. Thus, in order to achieve this, we first explored if LH2 and the [IKG]$_3$ peptide substrate could be further optimized. Both reagents were varied in the assay optimization experiments. As shown in Figures 1A and 1B, the signal is proportional to the concentrations of LH2 and [IKG]$_3$ peptide. A S/B over 10-fold was obtained at 1 μM LH2 and 1,000 μM [IKG]$_3$ peptide after a 90 minute reaction, and these concentrations were adapted for further assay development.

In order to confirm the optimum assay time, LH2 assay was monitored at various time points (Figure 1C). We observed that the luminescence signal increased linearly and proportionally to the assay time for up to 120 min. To estimate the amount of succinate production in the assay, a standard curve was generated using different concentrations of succinate standards (Figure 1D). It was estimated that about ~5 μM succinate was produced in 90 minutes, which corresponds to ~ 5 % substrate conversion based on 100 μM of α-KG. Therefore, 90 min was confirmed as the optimum assay time.

Since a stable buffer system is an important element for any high-throughput assay, we first explored the buffer components. HTS buffers usually contain small concentrations of additional additives such as BSA, detergents, and DMSO. BSA and detergents are added to minimize non-specific binding of enzyme to the plastics, to prevent non-specific inhibition of enzyme by compounds, and to prevent non-specific inhibition of aggregated compounds. DMSO is the ideal solvent for compounds and so a small percentage is usually included in the assays. Therefore, the assay tolerance against these additives should be validated prior to compound screening.

While BSA is commonly used at a concentration of 10 μg/mL or 0.1% in most enzymatic assays, we found that the luminescence signal dropped sharply with increasing concentrations of BSA (Figure 2A), indicating that the assay is very sensitive to BSA. Moreover, the signal dropped by almost half at 0.1% of BSA. In order to confirm whether BSA is interfering with LH2 activity but not with the Succinate Glo reactions, we performed LH assay utilizing 10 μM succinate in the absence of LH2. We did not observe any significant drop in signal with increasing BSA concentrations (Supplementary Figure S1), confirming that BSA interferes with LH2 activity but not with the Succinate Glo kit components.

Furthermore, we examined whether DMSO or detergents interfere with the assay. As shown in Figures 2B–2E, DMSO (0–5%), Triton X-100 (0–0.16%), Tween-20 (0–0.16%), and

NP-40 (0–0.16%) had no significant effect on the enzyme activity. Consequently, Triton X-100 (0.01%) and DMSO (0.5%) were chosen as additives in the assay, while BSA was excluded from further experiments. The final HTS assay buffer consisted of 50 mM HEPES pH 7.4, 150 mM NaCl, 0.01% Triton X-100, and 0.5% DMSO, unless otherwise stated.

As the assay was originally developed with α-KG, $FeCl_2$, ascorbate, and catalase at 100 μM, 50 μM, 500 μM, and 1.5 μM, respectively,[13] we explored if these concentrations can be reduced further similar to the condition reported by Alves et. al.[16] Achieving similar activity at reduced concentrations will be beneficial in reducing reagent usage, as well as assay interference. Although the absolute luminescence counts were lowered with reduced concentrations of α-KG, $FeCl_2$ and ascorbate, the signal to background (S/B) was not altered when their concentrations were reduced by 5- to 10-fold (data not shown). Using these optimized concentrations at 10 μM α-KG, 10 μM $FeCl_2$, and 100 μM ascorbate, we were able to maintain a robust S/B (~ 12 fold). With the reduced substrate concentrations, a higher product turnover was required to achieve similar level of assay quality. Therefore, we measured the assay at ~ 30% product conversion, which was achieved after 90 minutes (data not shown). As this was above the linear range of the assay, we understand that some weak inhibitors may be missed due to the slight reduction in the assay sensitivity. However, this was necessary to achieve a good assay robustness. We then examined the requirement of catalase, an enzyme that catalyzes the reduction of hydrogen peroxide, in this assay system. Catalase is often utilized in ascorbate/Fe(II) hydroxylase assays, based on its ability to decompose hydrogen peroxide generated by aqueous ascorbic acid and thus, decreases the background noise.[17] Comparing the LH2 activity in the presence and absence of catalase, no change was observed (data not shown), confirming that catalase can be omitted in this assay platform, which will be advantageous for minimizing assay interference. Collectively, we were able to reduce α-KG from 100 μM to 10 μM, $FeCl_2$ from 50 μM to 10 μM, ascorbate from 500 μM to 100 μM, and eliminate catalase from the reaction entirely.

More importantly, for LH2 assay, we previously reported a K_m of 11 ± 2 μM against α-KG and 169 ± 39 μM against $[IKG]_3$ peptide.[13] Therefore, our optimized assay is designed at the K_m range for α-KG, but at the saturating range for $[IKG]_3$ peptide. Hence our assay was designed to include weak competitive inhibitors against α-KG, but eliminate weak competitive inhibitors against $[IKG]_3$ peptide. While this assay setup was suitable for our case, if desired, the concentration of $[IKG]_3$ peptide can also be lowered to Km range, with some sacrifice in S/B ratio and assay robustness.

Finally, a stability test was performed to check how long LH2 can be stored at room temperature before initiating the assay. As shown in Figure 2F, LH2 was fully active at least 2 hours before starting the assay with substrate. For stability purpose, Succinate Detection reagent I was supplemented with 1 mM DTT, which seemed to increase overall signal stability in our assays.

Once the assay condition was established, the assays were validated using robotic liquid handlers because the compatibility of the assay protocol with an automated system is critical in HTS applications. Assay mixture and compounds were dispensed using a Janus Automated Workstation equipped with a 384 MDT head, while other reagents were

dispensed using a Microflo Select bulk dispenser. The assay automation was evaluated in a 384-well plate where columns 1–22 were assayed with positive controls and columns 23–24 with negative controls. The quality of the assays were assessed using a statistical parameter called z' factor, which takes into account the mean and standard deviation values for the positive and negative controls.[15] Notably, there was a left-to-right gradient when succinate detection reagent I was dispensed using a bulk dispenser, resulting in low assay reproducibility and z' values below 0.5. Often times, this can be caused by biochemical reagents undesirably binding to plastic surfaces in disposable plastics such as tips, plates, or tubings. In our case, addition of 0.02% Tween-20 into the Succinate Detection reagent I significantly resolved heterogeneity within the plate and reproducibility between assays, improving the z' over 0.5. The assay produced a reproducible z' factor of 0.78 ± 0.01 from two consecutive experiments, suggesting high confidence level in the assay quality. The average S/B and signal-to-noise (S/N) were 15.04 ± 1.75 and 16.28 ± 0.75 respectively, further supporting the robustness of the assay.

Compounds screening and hit confirmation

Using this validated assay, ~65,000 compounds were screened, which comprised a total of 210 assay plates. Compounds were screened at two different concentrations depending on their molecular weight distribution: 25 μM for diverse library and 100 μM for fragment library. The quality of 210 assay plates were again evaluated by calculating z' values and their distribution is summarized in Figure 3A. As shown, the z' values were greater than 0.5 for all plates with average z' value of 0.81 ± 0.07. Furthermore, the average S/B and S/N from HTS were 17.00 ± 3.66, and 22.51 ± 6.84 respectively. These statistics demonstrated that the assay was robust across all 210 plates. Figure 3B shows the distribution of percent inhibition values of all library compounds presented as a histogram. As shown, the two control groups are well separated and a normal-like bell-shaped distribution can be observed for library compounds with a mean value closer to that of 0 % inhibition control. The overall hit identification and confirmation flow is illustrated in Figure 3C. In our analysis, compounds were ranked by their inhibition percent and a compound was considered a 'hit' if the percent inhibition value was higher than 50%. Using this cutoff, a total of 2,525 hits were identified in the primary screen and then PAINS and OIDD filters available from FAFDrugs4 (http://fafdrugs3.mti.univ-paris-diderot.fr/references.html) were applied to identify and eliminate any potential promiscuous hits. A total of 912 drug-like compounds were selected to confirm their reproducible inhibitory activity using the same assay. In parallel, these 912 compounds were also subjected to a counter-screen against succinate to eliminate any compounds that interfered with the Succinate Glo kit itself. Compounds that showed lower than 50% activity in the rescreen or higher than 10% activity in the counter-screen were eliminated, resulting in a final total of 160 hits.

These 160 hits were further analyzed in a competition assay and a selectivity assay. First, hits were subjected to competition with 10 or 100 μM $FeCl_2$ in an LH assay. Any change in compound activity due to the change in the concentration of $FeCl_2$ was considered that compound is a non-specific hit which acts by sequestering $FeCl_2$. Second, the selectivity assay was performed against a mimivirus L230 enzyme at single compound concentration. L230 enzyme has distinct lysyl hydroxylase and glycosyltransferase domains, which is very

similar to the bifunctional architecture of human LHs.[18] The active site of L230 LH domain is highly conserved and structurally very similar to the human LHs.[14] Moreover, the L230 enzyme also has telopeptidyl LH activity similar to human LH2,[14] and therefore hits are expected to exhibit similar activity profile for both LH2 and L230. We first validated the LH assay using L230 enzyme and viral collagen peptide substrate GI-12 without any modification. It was observed that the L230 showed similar activity to LH2, exhibiting a S/B of ~12, thus confirming its ability to determine compound selectivity. Compounds showing greater than 50% activity for both enzymes were considered further. These steps narrowed down the hits to 37 compounds, comprising 27 fragments and 10 non-fragments. These final 37 compounds were evaluated in a dose response assay against LH2. Inhibition plots for the top 3 compounds with pIC_{50} values above 6 in a LH2 assay are shown in Figure 4.

In conclusion, we developed a lysyl hydroxylase assay for detecting inhibitors of LH2 activity in a 384-well plate system that is robust and suitable for use in high-throughput screening. This new platform was used to screen approximately 65,000 compounds, and identified several potent, specific small molecule inhibitors of LH2, which are in the process of further development and modification. This assay, which is ideal for screening large libraries, will facilitate future drug discovery efforts to identify inhibitors of LH2 activity, which may produce new therapeutic molecules to combat fibrosis and cancer.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgements

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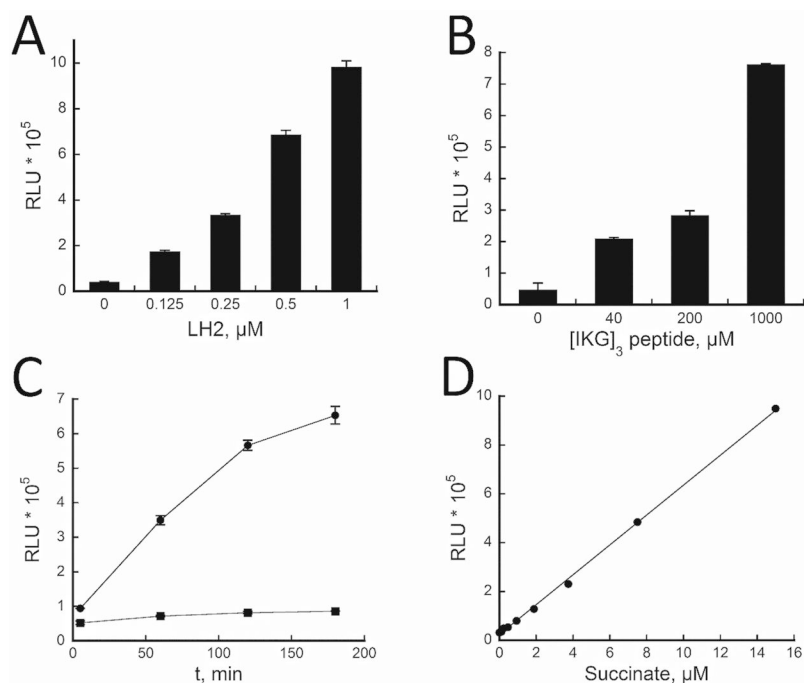
Figure 1. LH2 assay optimization.
Assays were performed by varying A) LH2 concentrations (0 – 1 μM), B) [IKG]$_3$ peptide concentrations (0–1000 μM), and C) time (5, 60, 120 or 180 min; with (●) and without (■) LH2). D) A succinate standard curve fitted linearly (R^2 = 0.9988).
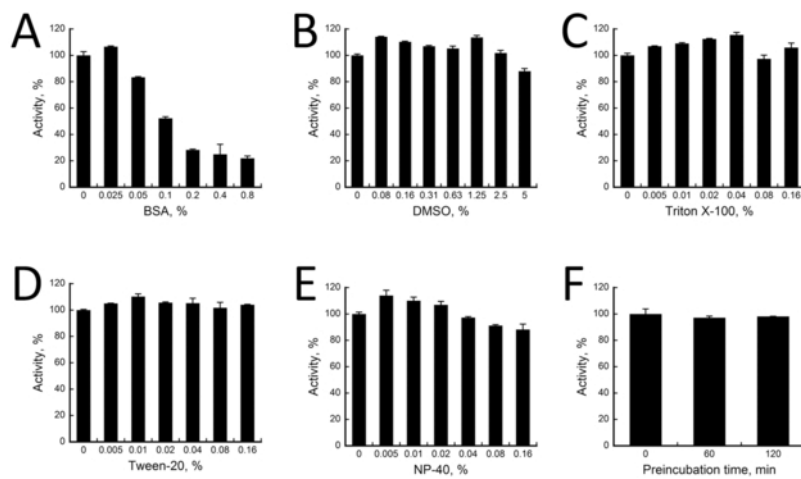
Figure 2. Assay stability against additives or time.
Activity of LH2 (in %) were measured against variations of A) BSA, B) DMSO, C) triton X-100, D) tween-20, E) NP-40, and F) incubation time. In all cases, a positive control with 0% variation is normalized to 100 % activity. % activity of LH2 assays containing variations were calculated according to $[\frac{x_i}{x_0} * 100]$, where x_0 is RLU for positive control and x_i is RLU for varying samples.
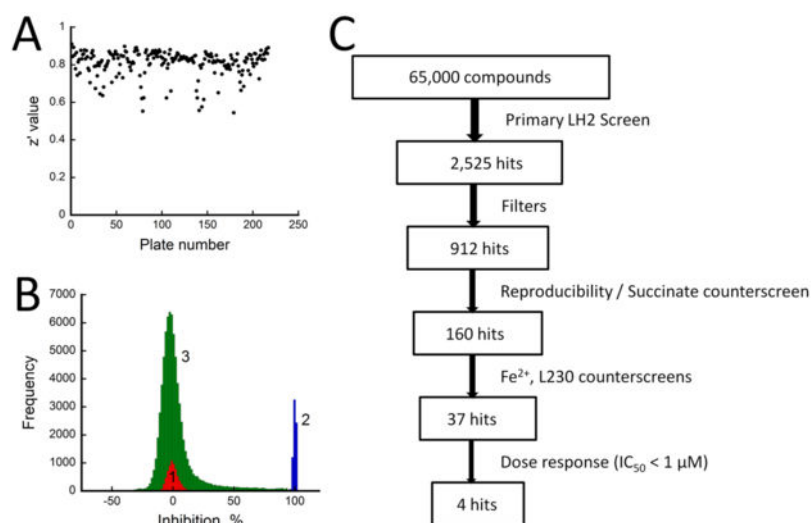
Figure 3. Statistical analysis for the screen.
A) Scattered plot of z' values for all assay plates. B) Histogram showing percent inhibition of LH2 activity against frequency of compounds (red, peak 1, 0% inhibition control; blue, peak 2, 100% inhibition control; green, peak 3, % inhibition by library compounds). C) Flowchart of hits identification and confirmation process.
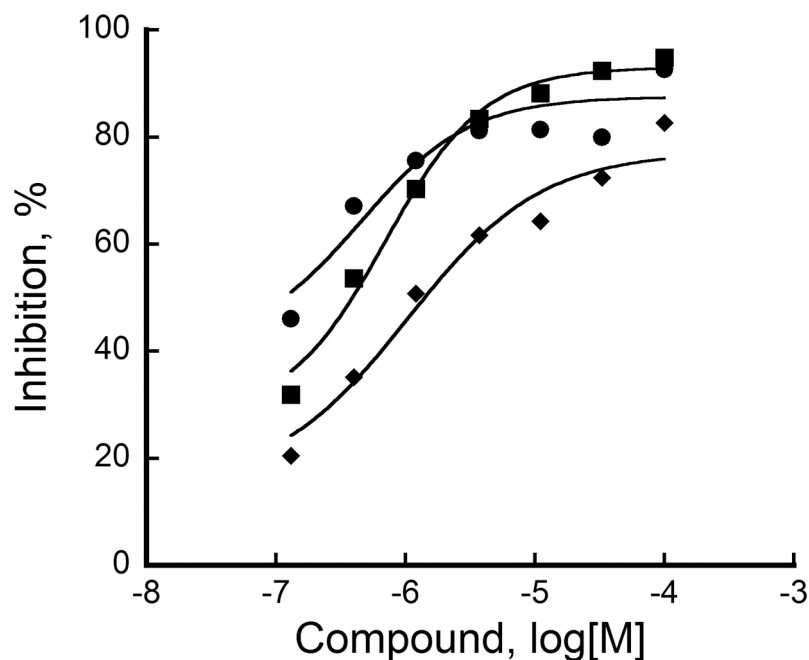
Figure 4. Dose Response activity of top 3 compounds against LH2.
X-axis represents different concentrations of compounds (in log[M]) whereas Y-axis
represents inhibition of LH2 activity (in percent). In all cases, a no compound (0%
inhibition) control was included and used as reference to calculate percent inhibition values.
Percent inhibition was calculated according to equation (2) and data were fitted to four
parameter logistic non-linear regression. Compounds 1 ($\bullet$), 2 ($\blacksquare$) and 3 ($\blacklozenge$) show pIC_{50}
values of 6.94 ± 0.08, 6.52 ± 0.04 and 6.32 ± 0.10, respectively, where $pIC_{50} = -\log(IC_{50})$.