A high-throughput screen of the GTPase activity of *Escherichia coli* EngA to find an inhibitor of bacterial ribosome biogenesis

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Abstract

The synthesis of ribosomes is an essential process, which is aided by a variety of trans-acting factors in bacteria. Among these is a group of GTPases essential for bacterial viability and emerging as promising targets for new antibacterial agents. Herein, we describe a robust high-throughput screening process for inhibitors of one such GTPase, the *Escherichia coli* EngA protein. The primary screen employed an assay of phosphate production in 384-well density. Reaction conditions were chosen to maximize sensitivity for the discovery of competitive inhibitors while maintaining a strong signal amplitude and low noise. In a pilot screen of 31,800 chemical compounds, 44 active compounds were identified. Further, we describe the elimination of non-specific inhibitors that were detergent-sensitive or reactive as well as those that interfered with the high-throughput phosphate assay. Four inhibitors survived these common counter-screens for non-specificity but these chemicals were also inhibitors of the unrelated enzyme dihydrofolate reductase, suggesting that they too were promiscuously active. The high-throughput screen of the EngA protein described here provides a meticulous pilot study in the search for specific inhibitors of GTPases involved in ribosome biogenesis.

Introduction

In prokaryotes, the 70S ribosome is composed of a 30S small subunit and a 50S large subunit. Bacterial ribosome biogenesis involves the coordinated production, folding and assembly of 54 proteins and 3 rRNAs [1]. Ribosomal subunit assembly is aided by several classes of ribosome biogenesis factors (RBFs), including RNases, helicases, methyltransferases, pseudouridylases, folding chaperones and GTPases [1]. Deletion of the genes coding for ribosome biogenesis factors often leads to promising phenotypes from a drug discovery perspective, including strains that are non-viable or exhibit slow growth phenotypes. Nevertheless, our understanding of the pathway for assembly of ribosomal subunits *in vivo* is in its infancy and there are no specific inhibitors of the process. Indeed, a chemical inhibitor of ribosome biogenesis would have exciting potential as an antibacterial lead with a novel mechanism of action and it would also be a useful probe of this complex process. Where bacterial ribosomes are assembled on a time-scale of minutes, a chemical probe would be an elegant alternative to perturbing the system by genetic means.

Four GTPases, EngA, CgtA, YihA and Era, are among the most important ribosome biogenesis factors. Each of these factors was found to be essential for bacterial viability [2]. These factors were also demonstrated to bind to ribosomal subunits and depletion of each protein led to the accumulation of 30S and 50S subunits as well as unprocessed rRNA [2, 3]. Where the precise roles of these proteins in ribosome biogenesis remain obscure and the indispensable phenotypes present an obstacle to genetic perturbation, the GTPases are a particularly exciting area of focus for new probe discovery. Likewise, the essential nature of these genes makes them ideal targets for drug discovery. Nevertheless, there are challenges to effective inhibitor screens of these factors that include slow GTPase activity and relatively high affinities for GTP [2]. In the

work reported here, we present a high-throughput screen and follow up assays toward the identification of specific inhibitors of GTPase function where the prototype is the ribosome biogenesis factor EngA.

EngA is a broadly conserved bacterial GTPase that lacks a human orthologue and has been shown to be indispensable to a variety of Gram-positive and Gram-negative organisms [4, 5]. EngA appears to be important for maturation of the 50S subunit. Polysome profiles of EngA-depleted cells revealed a decrease in the level of 70S ribosomes and an accumulation of 30S and 50S ribosomal subunits compared to wild type cells [4-6]. Depletion of EngA also led to accumulation of the unprocessed rRNA precursors pre-23S and pre-16S [6]. When lysates of wildtype *E. coli* were fractionated on sucrose gradients, EngA cofractionated with the 50S subunit [4, 6]. In *Bacillus subtilis*, the 50S subunit from cells that were depleted of YphC (the EngA orthologue) migrated slower than mature 50S on a sucrose cushion [5]. These slower-migrating subunits had reduced levels of the ribosomal proteins L16, L27 and L36 [5]. Although EngA is important for producing mature 50S subunits, its precise role in the process is not known.

The EngA protein contains tandem GTP-binding domains. We previously characterized extreme cooperativity in these domains where mutations targeting either G-domain abolished the *in vitro* GTPase activity of EngA and led to an inviable phenotype [4]. Thus, an inhibitor of either G-domain will inevitably inactivate the entire protein. Here, we describe the optimization of a 384 density high-throughput assay for GTP hydrolysis, designed for favorable sensitivity, signal and noise characteristics. A pilot screen of 31,800 compounds validated this primary screening assay. Also presented is a thorough collection of secondary assays aimed at the elimination of

false positives and non-specific inhibitors. This screen of EngA may represent a prototypic approach for discovery of specific inhibitors of GTPases involved in ribosome biogenesis.

Materials and Methods

Materials. We screened a collection of 31,800 diverse chemical compounds derived from the Custom Library of 16,000 compounds (Maybridge), the DIVERSet of 9,989 compounds (ChemBridge), the Prestwick Chemical Library of 1,120 compounds (Prestwick), the Natural Products Library of 361 compounds (BioMol), the Lopac^{1280} (International Version) library of 885 compounds (Sigma), the Spectrum Collection of 1,214 compounds (MicroSource), a synthetic library of 1,200 compounds (in-house) and a targeted Kinase Library of 1000 compounds (Chemical Diversity Labs). Guanosine triphosphate sodium salt (GTP), TrisHCl, $MgCl_2$, KCl, malachite green oxalate and ammonium molybdate tetrahydrate were from obtained from Sigma (Oakville, Ontario). High purity OmniSolv dimethylsulfoxide (DMSO), 98% sulfuric acid and KH_2PO_4 were from EMD Biosciences (Gibbstown, New Jersey).

Phosphate-based GTPase Assay. All enzymatic assays were carried out with recombinant untagged EngA that was purified by Q-Sepharose Fast Flow anion exchange chromatography (Amersham Biosciences, Baie D'Urfe, Quebec) [4]. One micromolar EngA was incubated with 300 μM GTP in assay buffer (100 mM TrisHCl, 20 mM $MgCl_2$ and 400 mM KCl, pH 7.5) containing 5% *vol*/*vol* DMSO in a final reaction volume of 50 μL in 384-well microplates. Reactions were mixed well and incubated for 25 minutes at ambient temperature before addition of 20 μL malachite reagent containing 1.65 M sulfuric acid, 0.99% *wt/vol* ammonium molybdate tetrahydrate, 0.066% *wt/vol* malachite green oxalate and 0.1% Tween-20 (detection method adapted from Reference [7]). This quenched assay was incubated for 25

minutes at ambient temperature for color development. Optical density at 600 nm was read in an EnVision™ multilabel plate reader (Perkin Elmer Life Sciences, Waltham, Massachusetts). The OD_{600} was related to the amount of phosphate produced using the equation of the line of a phosphate standard curve. The standard curve contained a 2-fold serial dilution of 5 – 80 μM KH_2PO_4.

Primary Screen. EngA was screened in high-throughput using the GTPase assay described above. We tested 31,800 compounds in duplicate in 384-well plates. Reactions were set up on a SAMI EX Assay Workstation (Beckman Coulter Inc., Fullerton, CA). Compounds dissolved in DMSO were added to empty wells to final concentrations of 20 μM compound and 2% *vol/vol* DMSO. An equivalent volume of DMSO was added to the controls. Next, assay buffer containing GTP was added to each well. Reactions were initiated with 10 μL of an enzyme stock that was kept on an integrated static Peltier cooling block. Each 384-well plate contained 32 high activity controls (uninhibited reaction) and 32 low activity controls (lacking enzyme) in the first two and last two columns with high and low controls alternating from top to bottom.

The Z`-factor is a statistical parameter that reflects the quality of a high-throughput screen [8]. Z` was calculated for the high and low activity controls using Eq. 1, where SD represents the standard deviation and H and L represent high and low controls, respectively.

$$Z` = 1 - \left(\frac{3(SD_H + SD_L)}{|Mean_H - Mean_L|} \right) \qquad \text{(Eq. 1)}$$

The percent activity of each well was calculated from the absorbance data using Eq. 2, where C represents the compound value and H and L represent the averages of the high and low controls on the same plate as the compound.

$$\%Activity = \left(\frac{C\text{-}L}{H\text{-}L} \right) \times 100 \qquad \text{(Eq. 2)}$$

A hit was defined as a compound with a residual activity of less than three standard deviations below the average of the compound data in either replicate.

Secondary Screen. The 44 compounds that were selected from the primary screen were retested at 50 μM using the GTPase assay described above in the presence or absence of 0.01% Triton X-100 or 2 mM DTT. Susceptibility to Triton X-100 or DTT was defined as restoration of the GTPase activity of EngA to > 70% compared to uninhibited controls. The compounds that were not sensitive to Triton X-100 or DTT were checked for interference with the malachite green assay by measuring the signal produced by 30 μM phosphate (KH_2PO_4) in the presence of 50 μM compound. Compounds that reduced the signal of the phosphate standard by > 70% were eliminated.

HPLC analysis of GTPase activity. The effect of the four candidate inhibitors on the enzymatic conversion of α-^{32}P-GTP to α-^{32}P-GDP was monitored by paired ion chromatography (PIC) on a Waters 600 HPLC (Milford, MA). Reactions were quenched with two volumes of 8M urea and loaded onto an Inertsil ODS-3 column (4 mm x 150 mm, 5 μm) (GL Sciences Inc, Torrance, California). Resolution and elution of GTP and GDP were achieved with a two minute linear gradient from Pic A (15 mM dibasic potassium phosphate and 10 mM tetrabutylammonium hydrogen sulfate, pH 7.0) to Pic B (Pic A containing 30% *vol/vol* acetonitrile), followed by five minutes of Pic B. Analytes were visualized by in-line scintillation

counting and quantified by integration of GTP and GDP peaks using the Waters Millenium® software.

Dose-response Assays. Various concentrations of the four active molecules were tested in the GTPase assay containing 0.5 μM EngA and either 100 μM or 1 mM GTP. To obtain dose-response curves, SigmaPlot™ software was used to fit the data to a four parameter logistic model using Eq. 3, where min and max are the fitted minimum and maximum activities, Hillslope is the slope of the curve at its midpoint and IC_{50} is the concentration of compound producing 50% inhibition.

$$\%Activity = \min + \frac{\max - \min}{1 + \left(\frac{[inhibitor]}{IC_{50}} \right)^{-Hillslope}} \qquad \text{(Eq. 3)}$$

To calculate the expected change in IC_{50} at low (100 μM) and high (1 mM) substrate concentrations, Eq. 4 was used, where K_i is the inhibition constant of a competitive inhibitor, S is substrate concentration and K_M is the Michaelis-Menten constant.

$$K_i = \frac{IC_{50}}{1 + \frac{[S]}{K_M}} \qquad \text{(Eq. 4)}$$

The K_i of a competitive inhibitor is constant and does not depend on the substrate concentration, therefore

$$(K_i)_{low} = (K_i)_{high}$$

$$\frac{(IC_{50})_{low}}{1 + \frac{[S]_{low}}{K_M}} = \frac{(IC_{50})_{high}}{1 + \frac{[S]_{high}}{K_M}}$$

Substituting substrate concentrations of 100 μM or 1000 μM GTP and a K_M of 150 μM yielded,

$$4.6x\left(IC_{50}\right)_{low} = \left(IC_{50}\right)_{high}$$

Dihydrofolate Reductase (DHFR) Assay. The assay was carried out with the DHFR assay kit (Sigma Aldrich, St Louis, MI) in a 96-well microplate. Each reaction contained 0.001 units of DHFR, 150 μM NADPH and 30 μM dihydrofolate in 50 mM Tris, pH 7.5 in a final reaction volume of 200 μL. Compound (or an equivalent volume of DMSO) was added to a final concentration of 100 μM. The absorbance at 340 nm was monitored continuously for 5 minutes in a SpectraMax® spectrophotometer. The rate of each reaction was expressed as a percentage of the rate of the uninhibited reaction.

Results and Discussion

Defining an assay for the primary screen that is sensitive to inhibition.

While whole cell active inhibitors of protein translation are commonplace, a specific inhibitor of ribosome biogenesis has never been reported. We present a pilot chemical screening study of the bacterial ribosome biogenesis factor EngA from *E. coli*. To measure the rate of production of phosphate by EngA, variations of the ammonium molybdate/malachite green assay and commercial kits were compared. The protocol published by Baykov *et al* [7] produced the greatest amplitude between the signals from the high and low activity controls, which afforded a more substantial screening window. Linear detection of phosphate was observed from 5 μM to 80 μM (Figure 1A).

Previous kinetic characterization of EngA revealed a Michaelis-Menten constant (K_M) of 150 μM [4]. Reactions are more sensitive to competitive inhibition at lower substrate concentrations where the reaction rate varies linearly with substrate concentration [9]. The screen

would ideally be carried out at a substrate concentration that is $\leq K_M$; however, due to the relatively high affinity of EngA for GTP, a concentration of 2 x K_M (300 μM) of GTP was used. This substrate concentration afforded a reasonable compromise between sensitivity to competitive inhibitors and sufficiently high signal amplitude in the enzyme assay.

We chose conditions where the reaction was linear with both time and enzyme concentration in order to ensure that the rate of the reaction was first order and, thus, sensitive to inhibition. In a reaction progress curve containing 300 μM GTP, the amount of phosphate produced was fairly linear up to 25 minutes, which corresponded to 8% depletion of substrate (Figure 1B). Due to the relatively slow catalytic rate of EngA (k_{cat} of 70 h^{-1} [4]), 1 μM enzyme was necessary to achieve this amount of substrate conversion. Comfortable that compounds screened at 20 μM would be far in excess of this enzyme concentration, we verified that EngA at 1 μM was within the linear range of enzyme concentrations for product formation (Figure 1C). In all, these assay development efforts afforded primary screening conditions that were sensitive to inhibition while remaining practical for high-throughput chemical screening.

Primary Screen of EngA

EngA was screened against 31,800 diverse chemical compounds that were a compilation of synthetic molecules, known natural products and a kinase-directed collection that was chosen because EngA is a nucleotide-binding protein. The high activity controls contained uninhibited GTPase reactions while the low activity controls lacked enzyme (Figure 2A). The Z`-factor, which accounts for the separation between the high and low controls and the noise of the assay, was 0.68. A Z`-factor of 0.5 to 1.0 describes a robust primary screening assay with good signal amplitude and relatively low noise [8].

Each compound was screened in duplicate at 20 μM. A replicate plot of the primary screen showed strong reproducibility between the two replicates, yielding an R^2 value of 0.77 (Figure 2B). A hit was defined as a molecule with residual activity that was more than 3 standard deviations away from the mean of all compounds, which corresponded to 63% residual activity. There were 44 hits, representing an overall hit rate of 0.15%. For compounds that were sourced from Chembridge, Biomol, Prestwick or the Kinase Library, each collection had a hit rate around 0.2% while compounds from Maybridge had a lower rate (0.03%) and those sourced from Microsource had a comparatively higher rate (1%). The Microsource collection comprises previously approved drugs, molecules with known biological activity and natural products.

Thus, this pilot screen of 31,800 compounds had a robust Z`-factor and showed good correspondence between the two replicates of the screening data. Similar methodology might be extended to other bacterial ribosome biogenesis GTPases to find specific inhibitors of ribosome biogenesis. There are no reports of ribosome-stimulation of the GTPase activity of EngA, but for ribosome-stimulated GTPases, this biochemical trait may be exploited in primary or secondary screens to identify inhibitors of ribosome-stimulated GTPase activity.

Secondary screen of EngA to eliminate false positives.

Figure 3 shows an overview of the secondary screen. The structures and re-tested activities of the 44 hits from the primary screen and the reasons for elimination are provided (see Supplemental Table 1). Inhibition was confirmed for 38 of the 44 compounds. The majority of inhibitors that are identified in biochemical high-throughput screens show detergent-sensitive inhibition that is thought to be due to non-specific adsorption to and inactivation of enzymes [10]. For 18 of the 38 compounds, inhibition was reversed by addition of 0.01% Triton X-100. Another mechanism of nonspecific inhibition is covalent modification of enzymes by

promiscuous electrophilic compounds, which can be reversed in the presence of a sacrificial nucleophile such as dithiothreitol (DTT) [11]. For 5 of the remaining 20 molecules, inhibition was reversed by addition of 2 mM DTT. To test for interference with phosphate detection by malachite green, the signal from 30 μM of a phosphate standard was measured in the presence of 50 μM of each compound. Eleven of the 15 compounds decreased the signal from the phosphate standard by > 30%.

Chemical structures and dose-response curves of the four inhibitors of EngA.

Of the four remaining compounds, MAC-0174809 and MAC-0080023 are synthetic molecules that are structural analogs while MAC-0182099 and MAC-0182344 are natural products with more complex structures (Figure 4A). The minimum inhibitory concentrations yielding 50% inhibition (IC_{50}) were 13.3 μM for MAC-0174809, 12.5 μM for MAC-0080023, 14.3 μM for MAC -0182099 and 4.6 μM for MAC-0182344 (Figure 4B). All four compounds produced steep slopes in the dose-response curves with Hill values close to 4 (Figure 4B). Reversible and specific inhibitors that bind stoichiometrically to enzymes reduce activity from 90% to 10% over an 81-fold change in inhibitor concentration [12]. This yields a slope in the dose response curve that is assigned a Hill slope value of 1. In some cases, high Hill slopes may indicate that the inhibitor is acting by a nonspecific mechanism such as colloidal aggregation, enzyme denaturation or micelle formation [13, 14].

To test if each inhibitor was competitive with GTP, the dose-response of the inhibitor was obtained at two concentrations of substrate. According to Eq. 4, which only applies to competitive inhibitors, an increase in substrate concentration from 100 μM GTP to 1 mM GTP should cause a 4.6-fold increase in the IC_{50}. For these four compounds, no increase in the IC_{50}

was observed at the higher substrate concentration, indicating that they are not competitive with GTP (Figure 4B).

Specificity of Inhibition.

Although high Hill values and lack of competition with substrate are common traits of promiscuous inhibitors, specific inhibitors may also display these traits [14]. More definitive evidence of the lack of specificity of these four compounds was obtained by testing them against dihydrofolate reductase (DHFR) in a spectrophotometric assay. DHFR activity was chosen as a counter-screen because this is a well-characterized, commercially available enzyme, which acts in an unrelated pathway. DHFR reduces dihydrofolate to tetrahydrofolate using the cofactor nicotinamide adenine dinucleotide phosphate (NADPH), where the consumption of NADPH can be monitored by the decrease in absorbance at 340 nm. At 100 μM, all of the compounds reduced the activity of DHFR to 20% - 40% compared to uninhibited controls (data not shown).

The four most promising inhibitors were not sensitive to detergent or DTT, however, due to the lack of competition with substrate, steep IC_{50} curves and coincident activity against DHFR, we have concluded that all four molecules are likely inhibiting by a non-specific mechanism. MAC-0174809 and MAC-0080023, both contained a rhodanine ring. An analysis by Baell *et al* to identify nuisance compounds, which they termed PAINS (Pan Assay Interference compounds), revealed that 40% of compounds containing the rhodanine substructure were inhibitors in at least 2 of the 6 screens analyzed [15]. In future screening campaigns, it would be useful to include 0.01% Triton X-100 and 2 mM DTT to reduce the selection of promiscuous actives. We also recommend prioritizing the test for assay interference in secondary screening.

The GTPase activity of EngA was shown to be an amenable target for a chemical screen using a convenient low cost assay that was practical for high-throughput experiments. The assay was also optimized for low noise and good separation between the high activity controls and low activity controls to maximize the screening window. The robust Z` and high replication suggests that this high throughput assay can be used with larger compound libraries to continue the search for an inhibitor of ribosome biogenesis.

Supplementary Files

Supplemental Table 1 (.pdf) is a table of the "Structures and retested activities of the 44 hits from the screen of EngA".

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Figure Legends

Figure 1. Defining conditions for a GTPase assay of EngA that is sensitive to inhibition. (A) Standard curve of phosphate (Pi) with detection by malachite green. A two-fold serial dilution of KH_2PO_4 (5 μM – 80 μM) in GTPase assay buffer was combined with a solution containing malachite green, ammonium molybdate and Tween-20 and incubated for 25 minutes. Optical density at 600 nm was read in an EnVision™ multilabel plate reader. The equation of the line of the standard curve was $y = 0.036x + 0.157$ and the R^2 value was 0.997. (B) Reaction progress curve of EngA. One micromolar EngA was incubated with 300 μM GTP in GTPase assay buffer containing 5% *vol/vol* DMSO for various times. The production of phosphate was detected by the malachite green method and the measured absorbance values were related to [Pi] using the equation of the line of the standard curve. (C) Reaction rate dependence on enzyme concentration. Various concentrations of EngA were incubated with 300 μM GTP in GTPase assay buffer containing 5% *vol/vol* DMSO for 25 minutes. The production of phosphate was detected by the malachite green method and the measured absorbance values were related to [Pi] using the equation of the line of the standard curve.

Figure 2. The signal:noise ratio and reproducibility of a pilot screen of EngA. (A) Evaluation of the controls of the screen. The high activity controls (light gray circles) contained the uninhibited GTPase reaction while the low activity controls (dark gray circles) lacked enzyme. Solid lines represent the mean of each sample set while hashed lines represent three standard deviations above and below each mean. Eq. 1 yielded a Z' factor of the controls of 0.68, where a range of 0.5 – 1.0 indicates a good screening window. (B) Replicate plot of the primary screen of EngA. A pilot screen of 31,8000 compounds was carried out in duplicate against the GTPase activity of EngA. Twenty micromolar compound was incubated with 1 μM

EngA and 300 μM GTP in assay buffer for 25 minutes at room temperature. Phosphate production was detected by malachite green by measuring absorbance at 600 nm. The percent activity was calculated using Eq. 2 and plotted for each replicate. The diagonal line represents perfect replication. An inhibitor (black circle) had a percent residual activity that was more than 3 standard deviations away from the mean of all the compounds.

Figure 3. Elimination of false positive inhibitors. The inhibitors that were identified in the primary screen were tested at 50 μM in four secondary assays to eliminate false positives. The follow-up tests were (i) confirmation of inhibition in the GTPase assay, (ii) inhibition in the presence of 0.01% Triton X-100 to check for aggregation, (iii) inhibition in the presence of 2 mM dithiothreitol to check for reactivity and (iv) interference with detection of a 30 μM K_2HPO_4 phosphate standard.

Figure 4. Chemical structures and dose-response curves of the four inhibitors of EngA. The effect of various concentrations of inhibitor on the GTPase activity of 1 μM EngA was tested at 100 μM GTP (open circles) and 1 mM GTP (closed circles). GDP and GTP were resolved by paired ion chromatography HPLC. SigmaPlot™ software was used to fit the data to a four parameter logistic model using Eq. 3. (A) MAC-0174809 and (B) MAC-0080023, are synthetic molecules that both contain rhodanine and chlorobenzene rings. (C) MAC-0182099 (also known as garcinol) is a polyisoprenylated benzophenone compound from the *Garcinia indica* plant. (D) MAC-0182344 (also known as theaflavin monogallate) is a polyphenol found in tea leaves.

Figure 1.

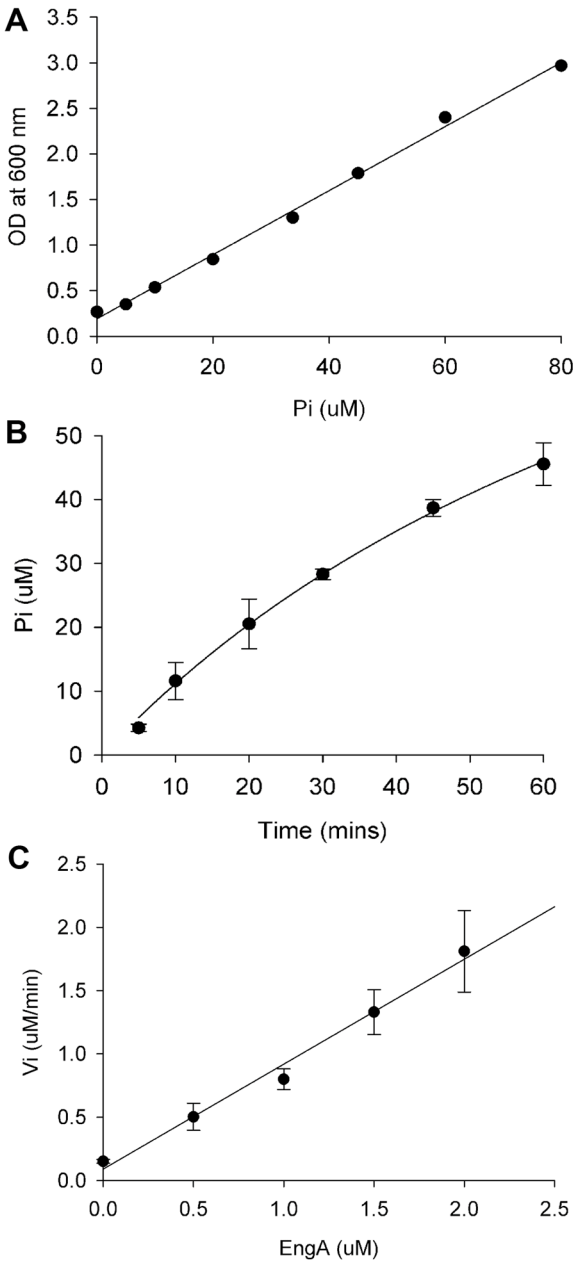

Figure 2.

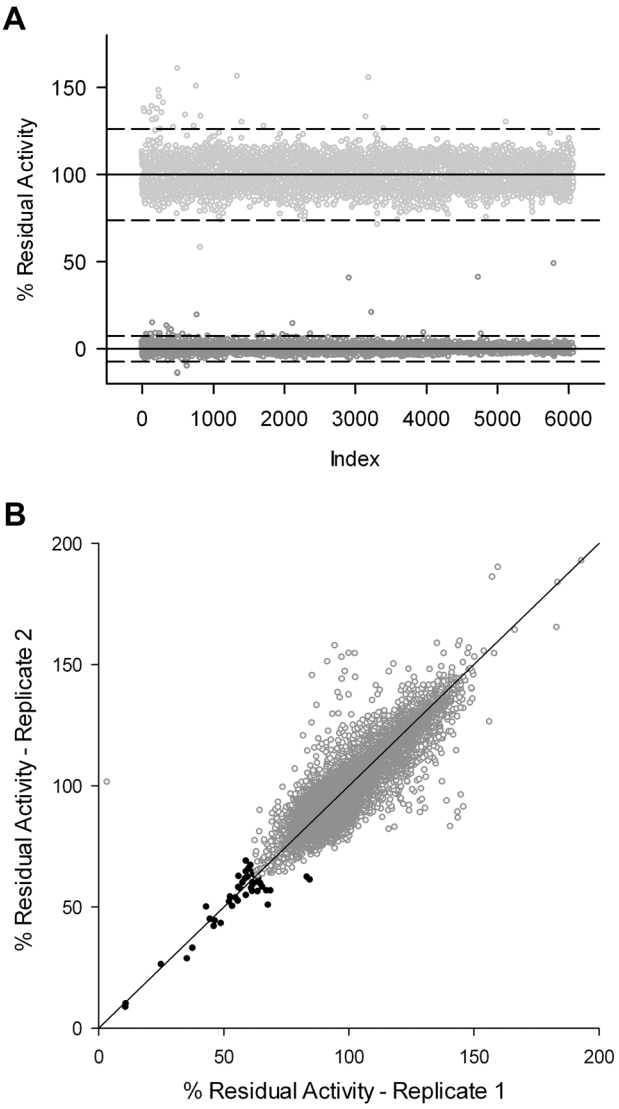
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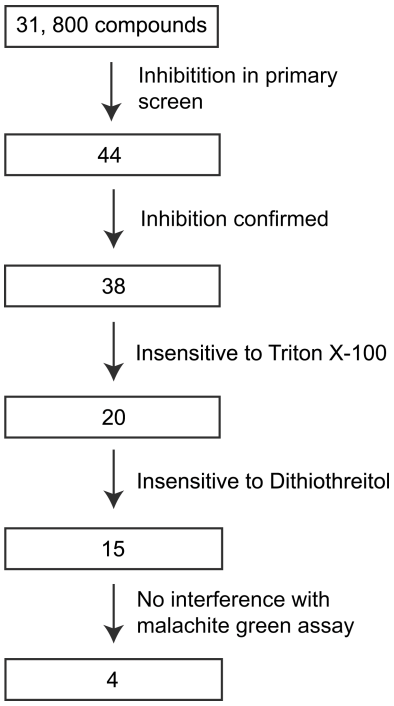
Figure 3.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No interference with malachite green assay
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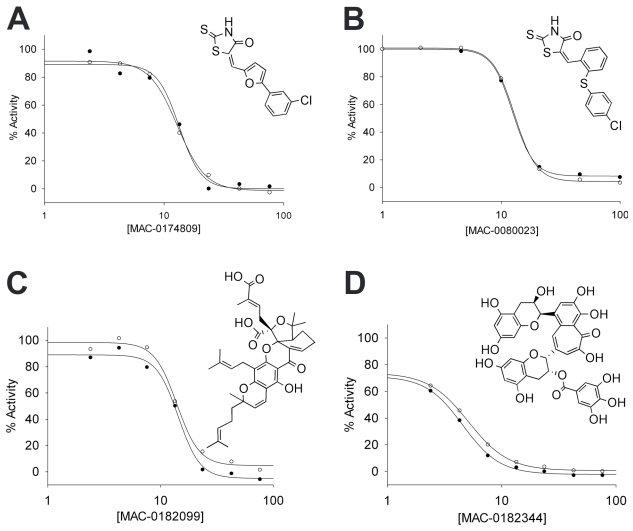
Figure 4.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