

## A High-Throughput Screen for Aggregation-Based Inhibition in a Large Compound Library

Brian Y. Feng,<sup>†,§</sup> Anton Simeonov,<sup>‡,§</sup> Ajit Jadhav,<sup>‡</sup> Kerim Babaoglu,<sup>†</sup> James Inglese,<sup>‡</sup> Brian K. Shoichet,<sup>\*,†</sup> and Christopher P. Austin<sup>\*,‡</sup>

Department of Pharmaceutical Chemistry & Graduate Group in Chemistry and Chemical Biology, 1700 4th Street, University of California San Francisco, San Francisco, California 94158-2330, and NIH Chemical Genomics Center, National Human Genome Research Institute, National Institutes of Health, Bethesda, Maryland 20892-3370

Received November 13, 2006

High-throughput screening (HTS) is the primary technique for new lead identification in drug discovery and chemical biology. Unfortunately, it is susceptible to false-positive hits. One common mechanism for such false-positives is the congregation of organic molecules into colloidal aggregates, which nonspecifically inhibit enzymes. To both evaluate the feasibility of large-scale identification of aggregate-based inhibition and quantify its prevalence among screening hits, we tested 70 563 molecules from the National Institutes of Health Chemical Genomics Center (NCGC) library for detergent-sensitive inhibition. Each molecule was screened in at least seven concentrations, such that dose–response curves were obtained for all molecules in the library. There were 1274 inhibitors identified in total, of which 1204 were unambiguously detergent-sensitive. We identified these as aggregate-based inhibitors. Thirty-one library molecules were independently purchased and retested in secondary low-throughput experiments; 29 of these were confirmed as either aggregators or nonaggregators, as appropriate. Finally, with the dose–response information collected for every compound, we could examine the correlation between aggregate-based inhibition and steep dose–response curves. Three key results emerge from this study: first, detergent-dependent identification of aggregate-based inhibition is feasible on the large scale. Second, 95% of the actives obtained in this screen are aggregate-based inhibitors. Third, aggregate-based inhibition is correlated with steep dose–response curves, although not absolutely. The results of this screen are being released publicly via the PubChem database.

### Introduction

High-throughput screening (HTS) is the most widespread technique used to identify new candidate leads for drug and chemical probe discovery. Although screening has had notable successes,<sup>1,2</sup> it can also generate a crippling number of false-positive “hits”. Lipinski’s well-known rules<sup>3</sup> were a first step to avoiding such misleading “hits”; subsequently, computational filters have been widely deployed to flag problematic compounds.<sup>4–7</sup> These filters attempt to capture artifactual causes of nonspecific inhibition such as molecules that interfere with assay read-out,<sup>9</sup> oxidize or chemically modify the target,<sup>9–11</sup> or form colloidal aggregates.<sup>12–22</sup> In this latter mechanism, small molecules self-aggregate into a suspension of large particles that indiscriminately associate with proteins and sequester enzymes from substrate. Recent work has suggested that aggregate-based inhibition may explain a large number of promiscuous inhibitors, although exactly how common they are remains unclear.

Aggregate-based inhibition has several characteristic features. Perhaps the most exploitable of these is the sensitivity of this inhibition to non-ionic detergents.<sup>18,20</sup> Moderate concentrations (0.01–0.1%) of such detergents not only disrupt aggregate formation, but can dissociate the protein–aggregate interaction and reverse inhibition. In earlier work, we exploited this characteristic to design a detergent-based counter-screen for aggregation, where detergent-sensitive, aggregate-based inhibition is isolated from detergent-resistant inhibition.<sup>12</sup> We found

that this counter-screen was sufficiently reliable to evaluate aggregation in a small, 1000-compound library. Here, we test this method in a 1536-well format screen of 70 563 molecules from the NCGC small-molecule library. For each molecule, a dose–response curve encompassing 7–15 points from 3 nM to 30  $\mu$ M was calculated according to the recently described qHTS technique.<sup>23</sup> As explained in Inglese et al.,<sup>23</sup> curves were classified 1–4 according to quality, with Class 1 curves being the highest quality fits and displaying a top and a bottom asymptote. Class 2 curves contain a single asymptote, and Class 3 curves show significant inhibition only at the highest concentration point. Class 4 curves are assigned to inactive molecules. These dose–response curves also allowed us to investigate the frequent coincidence of aggregate-based inhibition and steep dose–response curves. Curves with such high Hill slopes are frequently a harbinger of pathological behavior,<sup>24</sup> and we wished to understand if they were an indicator of aggregate-based inhibition.

Here, we address the following questions: Is the detergent-dependent assay for detecting aggregators robust in a low-volume, high-throughput format? How prevalent are aggregates in a well-curated screening library, and how common are they as compared to other types of inhibitors? Is aggregate-based inhibition correlated with steep dose–response? Because we have tested every compound at no less than seven concentrations across a large range, we anticipate that these studies will offer useful guides to what may be expected in screening campaigns, at least against enzyme targets.

### Results

**Library Composition.** The NCGC library that was screened consisted of 70 563 molecules, most of which were part of the

\* To whom correspondence should addressed. (B.K.S.) Phone: (415) 514-4126. Fax: (415) 514-4260. E-mail: shoichet@cgl.ucsf.edu. (C.P.A.) Phone: (301) 217-5725. Fax: (301) 217-5736. E-mail: austinc@mail.nih.gov.

<sup>†</sup> University of California San Francisco.

<sup>‡</sup> National Institutes of Health.

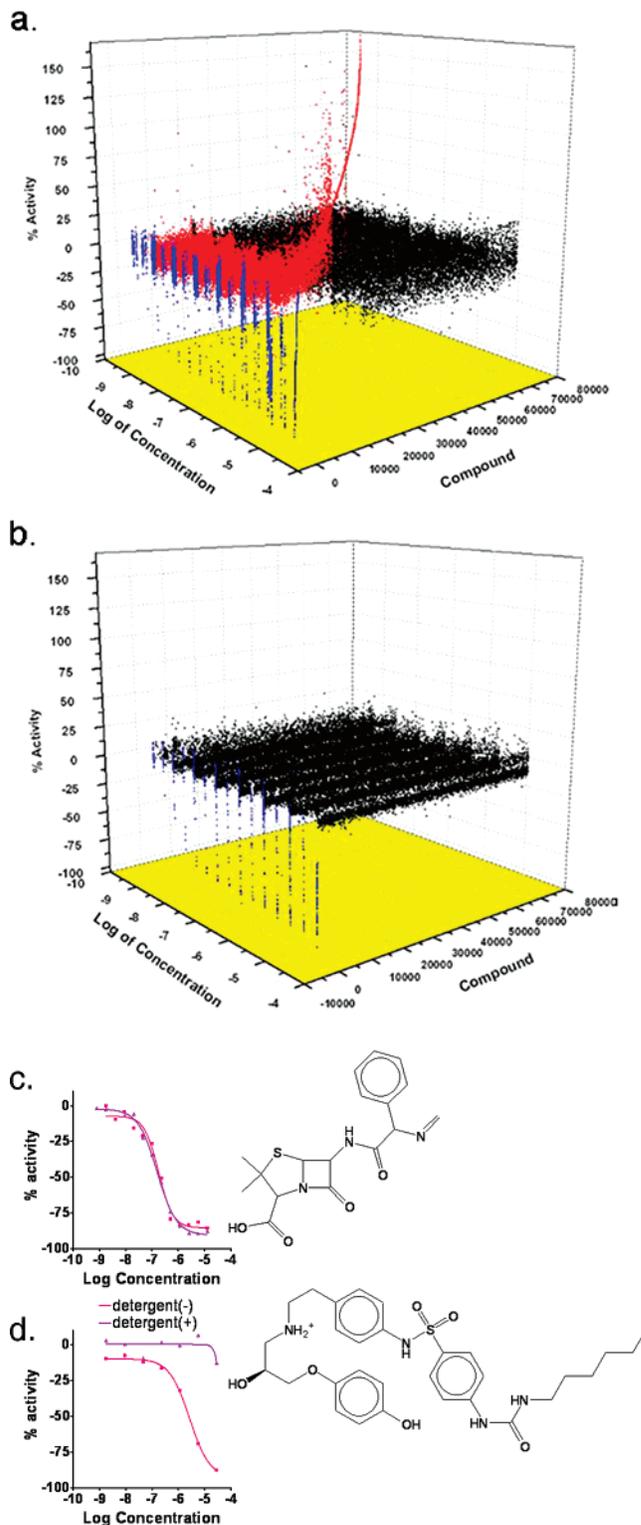
<sup>§</sup> These authors contributed equally to this work.

NIH Molecular Libraries Small Molecule Repository (MLSMR, [http://mlsmr.discoverypartners.com/MLSMR\\_HomePage/](http://mlsmr.discoverypartners.com/MLSMR_HomePage/), ~59 000 molecules). Compounds from this library generally display lead-like<sup>25</sup> or drug-like<sup>3</sup> properties. The MLSMR was additionally supplemented by small collections from various vendors and non-commercial sources (see Materials and Methods).

**Screening Results.** After optimizing enzyme, detergent, and substrate concentrations (see Materials and Methods), we screened 70 563 compounds from the NCGC small-molecule library against  $\beta$ -lactamase under both detergent(-) and detergent(+) conditions (Figure 1a,b). The detergent(-) screen was run continuously in 65 h, while the detergent(+) screen was finished in 51 h. Acceptable signal-to-noise levels were achieved in both cases, as measured by the  $Z'$  score, a standard metric of HTS quality.<sup>26</sup> The detergent(-) screen had an average  $Z'$  of 0.77, whereas the detergent(+) screen had an average  $Z'$  of 0.82. For both assays, the  $Z'$  is well above 0.5, the standard cutoff score for assay reliability. Kinetic data for each reaction were collected, although for this analysis only the first and last points were used to calculate percent inhibition (we are making the full time courses of each reaction publicly available, as these may be a useful source of data for further study). We considered any molecule that displayed dose-dependent inhibition to be an active in the detergent(-) screen. Two criteria were used to describe detergent-sensitivity: first, each dose-response curve was qualitatively classified (the curve class<sup>23</sup>) on the basis of the completeness of the curve and the quality of the fit ( $r^2$ ). We required the curve class of putative aggregators to be more defined (lower in curve class value) in the absence of detergent than in its presence. For example, a molecule that has a dose-response curve of Class 1 in the detergent(-) screen, but of Class 4 in the detergent(+) screen, would satisfy this criterion (Figure 1c,d). Second, the maximum inhibition observed across the titration series had to decrease in the detergent(+) screen relative to the detergent(-) screen. We took a significant decrease to be 3 standard-deviation units from the mean difference in activity upon addition of detergent for the whole library. By these criteria, the assay results fell into four categories: detergent-sensitive inhibition, detergent-insensitive inhibition, inconclusive results, where only one of the criteria for detergent-sensitive inhibition was satisfied, and compounds for which no inhibition was observed in either assay.

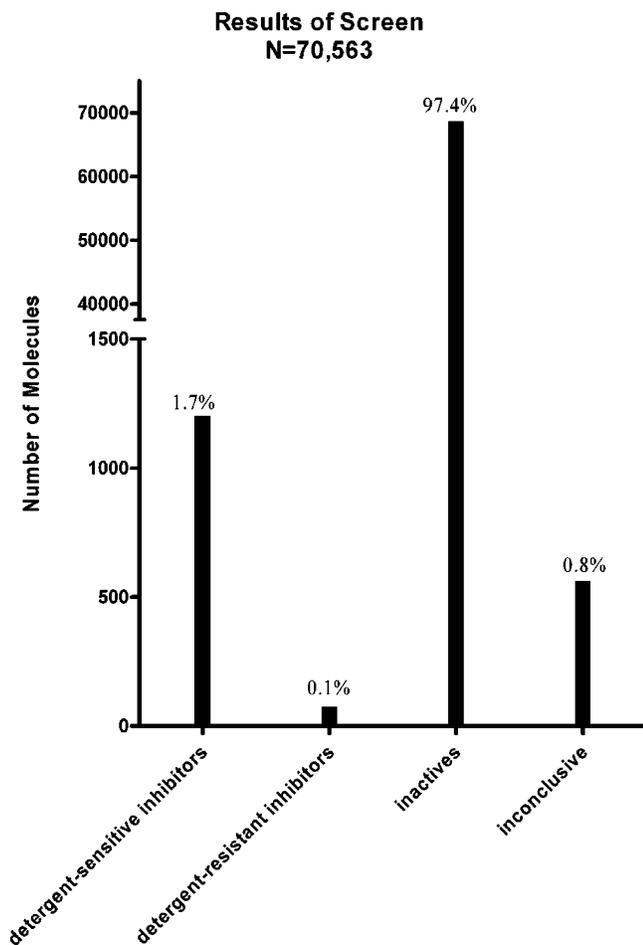
Overall, 1204 molecules (1.7%) met both the inhibition and the curve criteria and were identified as aggregation-based inhibitors, whereas only 70 (0.1%) were identified as detergent-insensitive. These 70 included 25 known  $\beta$ -lactam inhibitors or substrates of  $\beta$ -lactamase. Of the remaining molecules, 562 were unable to be categorized, exhibiting only one of the criteria for detergent-sensitive inhibition (Figure 2). These consisted mostly of molecules that exhibited marginal levels of inhibition, and, while most did show some response to detergent, they fell outside the range of statistical confidence. Aggregators varied in their ability to inhibit  $\beta$ -lactamase, but the most potent had  $IC_{50}$  values near 1  $\mu$ M. The concentration-dependence of these molecules was consistent with a colloidal mechanism of inhibition; few aggregators were detected below 1  $\mu$ M, but by 5  $\mu$ M 60% of the aggregate-based inhibitors were apparent.

**Secondary Assays.** To test the reliability of these measurements, 31 molecules were independently purchased (i.e., resourced from the vendors) and retested in secondary assays. These molecules, identified in the primary screen as 17 aggregators and 14 inactives, were examined in the same  $\beta$ -lactamase assay, conducted in a one-at-a-time cuvette-format. Although the conditions of these experiments were similar to



**Figure 1.** A 3D scatter plot of qHTS data. Concentration-response relationships for all 70 563 molecules are shown, colored as: no relationship (black), inhibition (blue), or activation (red). Comparison between detergent(-) (a) and detergent(+) (b) screening reveals the presence of aggregation-based activity in the detergent(-) assay. Example dose-response curves obtained from qHTS data for detergent-resistant (c) and detergent-sensitive (d) inhibitors.

those of the primary screen, these experiments varied the concentration of DMSO, enzyme, and detergent, as well as the reaction volume (1 mL vs 8  $\mu$ L) and path length. Of the 17 molecules identified as aggregators in the primary screen, 15 exhibited aggregate-based inhibition upon retesting in the



**Figure 2.** Classification and quantification of inhibitors found in the screen. 1204 molecules were identified as detergent-sensitive inhibitors, based on two difference criteria between the detergent(−) and detergent(+) conditions: deterioration in the curve class of the molecule, and a decrease in the maximum observed inhibition across the dilution series. Seventy molecules were detergent-insensitive inhibitors, 68 727 showed no activity, and 562 were ambiguous, only fulfilling one of the two criteria necessary to be identified as an aggregation-based inhibitor.

**Table 1.** Results of Low-Throughput Secondary Assays

type of molecule	number tested	number of aggregators – stringent screening conditions <sup>a</sup>	number of aggregators – relaxed screening conditions <sup>b</sup>
primary screen aggregator	17	15	15
primary screen nonaggregator	14	0	10

<sup>a</sup> Primary screening conditions. <sup>b</sup> Less-stringent conditions (4-fold less enzyme, 5-fold less stabilizing detergent).

secondary assay. Likewise, of the 14 inactive molecules retested, none inhibited under screening conditions. However, 10 of these exhibited aggregate-based inhibition at higher concentrations or under relaxed assay conditions, that is, using decreased enzyme concentration and less detergent in the detergent(−) condition of the primary high-throughput assay (Table 1).

A curious feature of the primary screening data was that some molecules appeared to activate  $\beta$ -lactamase in the detergent-free screen; this activation was eliminated on detergent addition. Six of these putative activators were independently purchased and retested under several conditions in low-throughput, cuvette-based assays (Table 1). In contrast to the aggregators and

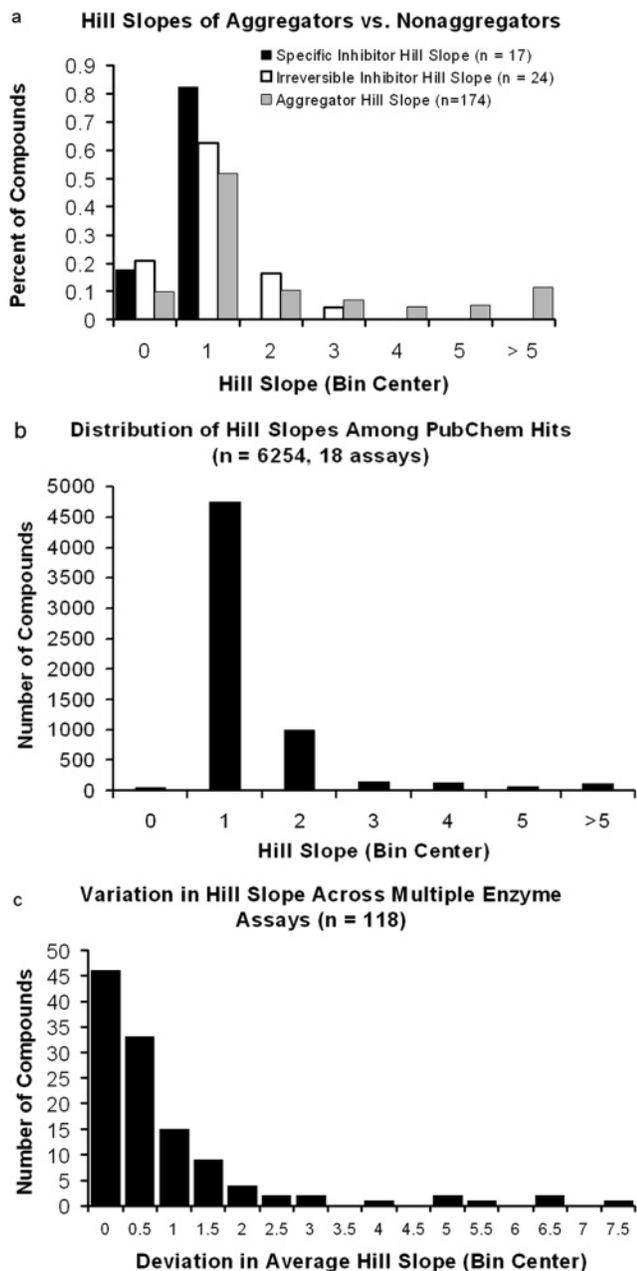
**Table 2.** Sensitivity to Assay Conditions among “Nonaggregators”

Structure	IC <sub>50</sub> ( $\mu$ M)		Detergent Sensitive?	Aggregator?	PubChem SID
	Stringent conditions	Relaxed conditions			
	> 300 <sup>b</sup>	130	Yes	Yes	7973586
	> 100 <sup>b</sup>	> 100 <sup>b</sup>	Yes	Yes	7975152
	> 200 <sup>b</sup>	120	Yes	Yes	7973072
	40	20	Yes	Yes	4259752
	> 66 <sup>b</sup>	20	Yes	Yes	4265457
	> 66 <sup>b</sup>	13 <sup>a</sup>	Yes	Yes	4255506
	150 <sup>a</sup>	35	Yes	Yes	11110962
	100 <sup>a</sup>	20	Yes	Yes	11112813
	82 <sup>a</sup>	46 <sup>a</sup>	Yes	Yes	11112318
	126 <sup>a</sup>	22 <sup>a</sup>	Yes	Yes	11113780

<sup>a</sup> Extrapolated IC<sub>50</sub> values. <sup>b</sup> Highest soluble concentration.

nonaggregators identified in the primary screen, activation was not reproducible in these secondary assays. Subsequently, another 100 of these apparent activators were re-sourced from the vendors, and stocks were remade directly from solid powder. These compounds were then tested under medium throughput conditions, such as 96- or 384-well formats. Because of the larger volume of these assays, as compared to the 1536-well formats, it was often possible to observe the compound immediately after addition to the assay. In these cases, we often observed what appeared to be precipitants, typically at the bottom of the wells at the solvent–polymer interface. Also, when the compounds were tested at these higher volumes, much of the former activation was attenuated, although many of these compounds did still lead to activation. Taken together, these results suggest that activation is a phenomenon peculiar to the very low volume, high-throughput formats used in the primary screen. We will not consider it further here.

**Hill Slope Analysis.** The dose–response information obtained in this screen allowed us to investigate the correlation between aggregate-based inhibition and steep dose–response curves, which are common among high-throughput screening hits.<sup>19,24</sup> We analyzed the Hill slopes of the highest-quality dose–response curves, consisting of 174 aggregate-based inhibitors, 24  $\beta$ -lactam-based irreversible inhibitors, and 17 reversible inhibitors of  $\beta$ -lactamase (Figure 3). The reversible inhibitors displayed standard, single-site dose response curves with slopes near 1, whereas the  $\beta$ -lactam inhibitors were biased toward somewhat steeper curves, as expected for very potent inhibitors.<sup>27,28</sup> However, the aggregators displayed a mixed tendency: many had Hill coefficients close to 1, but many others showed steep curves, even steeper than the covalent  $\beta$ -lactam inhibitors, and similar to those seen for other aggregating inhibitors.<sup>17</sup> Of the 174 aggregators in this set, 70 had Hill slopes steeper than 1.5.



**Figure 3.** (a) Correlation between aggregation-based inhibitors and those with high Hill slopes. Compared to a set of specific, reversible  $\beta$ -lactamase inhibitors and a set of irreversible inhibitors, aggregators are more likely to have steep dose–response curves. (b) Distribution of Hill slopes among the active molecules discovered from 18 enzyme screens currently annotated in PubChem. (c) Variation in the slopes of high Hill slope molecules that were tested in at least two enzyme assays. Hill slopes appear consistent for most of these molecules.

**Data Sets.** The full results of these screens are publicly available through PubChem (<http://pubchem.ncbi.nlm.nih.gov/>) and the Shoichet Lab website (<http://shoichetlab.compbio.ucsf.edu/>).

## Discussion

Perhaps the most practical conclusion to be drawn from this screen is that the detergent-sensitive assay can reliably identify aggregate-based enzyme inhibitors in a genuinely high-throughput format. A counter-screen consisting of 0.01% Triton X-100 effectively separated 1274 assay actives into 1204 aggregators and 70 molecules that act by other mechanisms

(Figure 2). This assay may be used to characterize other libraries, or may be adapted to other enzymes for direct use as a counter-screen.

Many mechanisms have been proposed to explain promiscuous inhibition, including interference in assay read-out,<sup>9</sup> compound oxidation,<sup>10</sup> and chemical modification of the target.<sup>9–11</sup> A striking result of this screen is that 95% of the actives can be attributed to a single one of these: aggregate-based inhibition. How the remaining 5% of actives are distributed among other mechanisms, including true specific, reversible inhibition, is currently under investigation in our laboratory; few of these mechanisms are as rapidly and decisively identified as promiscuous aggregation. What we can say at this point is that 25 of these 70 actives are  $\beta$ -lactam-based inhibitors of  $\beta$ -lactamase, which act by covalently modifying the catalytic nucleophile, Ser64.<sup>29,30</sup> Also, 5–10 of these appear to be aggregates that are resistant to 0.01% Triton X-100, but are sensitive at 0.1%, the concentration used in the original screen.<sup>12</sup> This leaves only about 35 molecules that act by all other mechanisms.

Although the vast majority of hits in this screen inhibited through aggregate formation, the overall percentage of aggregators identified in the library is smaller than what we found in an initial, smaller-scale screen. This previous study suggested that as many as 19% of 298 randomly selected drug-like molecules behave as aggregators at 30  $\mu$ M.<sup>12</sup> Two factors contribute to this discrepancy. The first relates to the sensitivity of aggregate-based inhibition to assay conditions. For reasons of enzyme stability in the 1536-well format, a low level of detergent (0.0001%) was present in the detergent(–) screen; this concentration of detergent is 5-fold greater than the amount required in the original 96-well assay.<sup>12</sup> Additionally, the concentration of enzyme was increased 4-fold relative to the original assay, while the concentration of detergent used in the detergent(+) screen was reduced 10-fold to 0.01%. These changes reduced the number of aggregators found under detergent(–) conditions, resulting in a more stringent screen. Among the 31 compounds retested in low-throughput secondary assays, the original conditions were more sensitive to aggregate-based inhibition than the new high-throughput conditions. At least a 2-fold difference in potency was observed for most aggregators tested under both conditions (Table 2). Finally, we cannot discount the possibility of compound bias in our original set of 298 rule-of-5-compliant molecules. The much larger NCGC library not only allows for more robust statistics, but its careful curation may have also contributed to the lower rate of aggregate-based inhibition.

Steep dose–response curves are often harbingers of pathology in HTS results.<sup>24</sup> Because many aggregators also have such curves, it is tempting to correlate the two phenomena. The qHTS titrations allowed us to investigate this possibility. We selected the highest quality dose–response curves, consisting of 174 aggregators, 17 previously known specific, reversible inhibitors of  $\beta$ -lactamase (from our previous work with this enzyme) and 24 known specific, covalent inhibitors (Figure 3). The classical, competitive inhibitors all had dose–response curves consistent with single-site inhibition, with Hill coefficients near 1. In contrast, the aggregators were biased toward steeper curves, with an average slope of 2.2 versus an average slope of 0.7 for the reversible inhibitors. The aggregator curves were also on average steeper than a set of irreversible  $\beta$ -lactam inhibitors, which had an average Hill slope of 1.2. The steep dose–response curves for the  $\beta$ -lactams are expected, given their generally low  $K_d$  values.<sup>27,28</sup> Overall, 40% (70/174) of the aggregators had Hill coefficients greater than 1.5. Also, every compound with a high

Hill slope was either an aggregator (93% of the time) or a covalent modifier (7% of the time). Thus, increased Hill coefficients may be reliable predictors of aggregation-based or, less frequently, potent covalent inhibition, although these predictions will also miss some aggregators. The high Hill slopes of both potent covalent and aggregation-based inhibitors, and indeed those of potent reversible inhibitors, have the same mechanistic origin: all are stoichiometric inhibitors with very low  $K_d$  values. For such molecules, inhibition is only observed when the inhibitor concentration approaches that of the enzyme. Because the latter is well above the true  $K_d$  of the inhibitor, inhibition rises very quickly in this concentration range, leading to steep curves. Thus, for both aggregators and certain covalent inhibitors, high Hill slopes have nothing to do with cooperative binding and everything to do with high enzyme-to- $K_d$  ratios.<sup>28</sup>

A question that naturally arises is how often are the aggregators found in this screen also found against other enzyme screens of the MLSMR. More generally, do aggregates turn up with the same frequency in other screens? It is difficult to answer this question directly at this point: few of the enzyme screens reported in PubChem test all or even most of the MLSMR molecules; also the conditions of the screen and the definition of “actives” differ from assay to assay (many use detergent, for instance). However, one criterion that may be directly compared is the Hill slopes of active molecules. As of this writing, 18 enzyme screens with Hill slopes for each active are available in PubChem. For these 18 screens, 6254 actives (hits) were reported. Of these, 1452 (23%) have Hill slopes greater than 1.5 (Figure 3b). For those high Hill slope molecules that were tested in two or more assays, the Hill coefficient was fairly stable, suggesting that these measurements are reliable and transferable (Figure 3c). In the  $\beta$ -lactamase screen, all molecules that had steep dose-response curves were either aggregators (93%) or covalent modifiers (7%). If this pattern extends to the other enzyme screens, it suggests that, despite the use of detergents and other adjuvants, more than 20% the actives in these screens are also aggregators. Because only about 45% of the aggregators identified in this study had high Hill slopes, the percentage of aggregation-based artifacts among the enzyme screens is likely to be higher still.

Our interest in undertaking this study was to test an assay that could quantify the presence of aggregate-based inhibitors in a high-throughput setting. Several caveats deserve mention. A surprising conclusion from this study is that so many of the actives were aggregators, and so few can be attributed to other mechanisms of inhibition. Whereas we suspect that similar patterns might occur in other enzyme assays, this inference is tentative, as enzymes with greater liabilities to chemical reactivity, or indeed libraries with different compounds, may show different patterns. Also, we note that identification of an aggregating molecule does not disqualify that molecule from future activity. Whereas aggregators should be flagged for future screens, aggregation is concentration- and condition-dependent, and a molecule that aggregates under one condition and concentration may behave well under different conditions or lower concentrations. More importantly, counter-screens for aggregation should always be considered; one lesson of this study is that such assays may be straightforward.

Perhaps the greatest liability of HTS is the occurrence of false-positive hits, and many mechanisms have been proposed to explain these. A key result of this study is that, at least for robust enzymes like  $\beta$ -lactamase, 95% of the artifactual hits are due to a single mechanism: colloidal aggregation followed by enzyme sequestration. Only 5% can be attributed to all other

mechanisms of inhibition put together, including covalent modification, oxidation, and assay interference. For well-behaved enzymes, the chemical reactivity mechanisms about which the field most worries may be rare in well-curated libraries. Instead, it may be the physical behavior of organic molecules that most contributes to false-positive hits in screening. Certainly, assays that ignore this effect risk drowning in a sea of artifacts. By the same coin, an encouraging aspect of aggregation is that it is a physical phenomenon that we can hope to understand and for which we can control. Pragmatically, we can deploy a simple high-throughput assay to detect it; this assay can be applied to most screening collections, in the highest-format assays, and can do much to prioritize molecules for follow-up experiments.

## Materials and Methods

**Compound Library.** The 70 563-member library was collected from several sources: 1280 pharmacologically active compounds from LOPAC (Sigma-Aldrich), 1120 compounds from Prestwick Chemical (Illkirch, France), 280 purified natural products from TimTec (Newark, DE), three 1000-member combinatorial libraries from Pharmacopeia (Princeton, NJ), 1106 compounds from Tocris (Bristol, U.K.), 59 684 compounds from the National Institutes of Health Molecular Libraries Small Molecule Repository (MLSMR, [http://mlsmr.discoverypartners.com/MLSMR\\_HomePage/](http://mlsmr.discoverypartners.com/MLSMR_HomePage/)), 1981 compounds from the National Cancer Institute (the NCI Diversity Set, [http://dtp.nci.nih.gov/branches/dscb/diversity\\_explanation.html](http://dtp.nci.nih.gov/branches/dscb/diversity_explanation.html)), 148 NCGC internally generated compounds, 20 control compounds from the Shoichet laboratory (UCSF), 96  $\beta$ -peptides from the Gellman laboratory (University of Wisconsin, Madison), 726 compounds from University of Pittsburgh Center for Chemical Methodology and Library Development, and 1121 compounds from Boston University Center for Chemical Methodology and Library Development. The library was deployed as DMSO solutions (7  $\mu$ L each in 1536-well Greiner polypropylene compound plate) at initial concentrations ranging between 2 and 10 mM. Plates were serially diluted, and compounds were assayed at final concentrations ranging from 4 nM to 30  $\mu$ M. Plate-to-plate (vertical) dilutions and 384-to-1536 compressions were performed on an Evolution P3 dispense system equipped with a 384-tip pipetting head and two RapidStak units (Perkin-Elmer, Wellesley, MA). Additional details on the preparation of the compound library are provided elsewhere.<sup>23</sup>

**Assay Implementation.** The detergent-dependent screen was adapted from a previously described, 96-well format assay for the identification of promiscuous inhibitors.<sup>16</sup> Moving to a 1536-well format demanded changes to ensure a high signal-to-noise ratio; both a 4-fold increase in enzyme and a 2-fold increase in substrate concentration were required to maintain a high signal-to-noise ratio. Also, a 5-fold increase in the concentration of Triton X-100, to 0.0001%, in the detergent(-) screen was needed to stabilize the enzyme. Meanwhile, the Triton X-100 concentration in the detergent-(+) screen was lowered from 0.1% to 0.01% to avoid excess bubble formation. Technical adjustments to the liquid handling systems were also necessary to deal with this problem. Finally, serial dilutions were carried out in 5-fold intervals, resulting in DMSO percentages of 0.3%.

**$\beta$ -Lactamase Assays.** AmpC  $\beta$ -lactamase was purified and assayed as described,<sup>17,31</sup> unless otherwise noted. The enzyme was present at 4 nM in a final reaction volume of 8  $\mu$ L. Reactions were conducted in 1536-well Greiner black clear bottom plates, and liquids were handled using a solenoid-based dispenser. Compounds and controls (23 nL) were transferred via a Kalypsys PinTool equipped with a 1536-pin array and inline washing stations. The plates were incubated for 15 min at room temperature (22–23 °C), and reactions were initiated by the addition of substrate (dissolved in buffer, final concentration 400  $\mu$ M). The plates were immediately transferred to a ViewLux (Perkin-Elmer) high-throughput CCD imager and read every 20 s for 4 min at 480 nm. During liquid handling, reagent bottles (AmpC, buffer, and nitrocefin solution)

were kept submerged in a 4 °C water bath. All screening operations were performed on a Kalypsys robotic system (Kalypsys Inc., San Diego, CA) containing one RX-130 and two RX-90 anthropomorphic robotic arms. The absorbance difference between the last and first time points was used to compute the reaction progress.

Cuvette-based assays (1 mL reaction volume) either replicated the above conditions (stringent conditions) or contained 1 nM AmpC  $\beta$ -lactamase, 200  $\mu$ M nitrocefin, and 0.00002% Triton X-100 (relaxed conditions). Cuvette-based assays contained between 1% and 2% DMSO. Compound and enzyme were incubated for 5 min before the reaction was initiated by addition of substrate. Nitrocefin hydrolysis was monitored at 482 nm on a HP8453 UV-visible spectrophotometer.

**Data Analysis.** Initial curve-fitting and data analysis were conducted as previously described.<sup>23</sup> Briefly, concentration-effect relationships were derived using the GeneData Screener software package. Curves were categorized according to fit quality, response magnitude, and degree of measured activity. Manual curve refitting was performed using Graphpad 5.0. The results of these screens are available through PubChem (<http://pubchem.ncbi.nlm.nih.gov/>) and the Shoichet Lab website (<http://shoichetlab.compbio.ucsf.edu/>).

## References

- Proudfoot, J. R. Drugs, Leads, and Drug-Likeness: An Analysis of Some Recently Launched Drugs. *Bioorg. Med. Chem. Lett.* **2002**, *12*, 1647–1650.
- Druker, B. J.; Lydon, N. B. Lessons Learned from the Development of an Abl Tyrosine Kinase Inhibitor for Chronic Myelogenous Leukemia. *J. Clin. Invest.* **2000**, *105*, 3–7.
- Lipinski, C. A.; Lombardo, F.; Dominy, B. W.; Feeney, P. J. Experimental and Computational Approaches to Estimate Solubility and Permeability in Drug Discovery and Development Settings. *Adv. Drug Delivery Rev.* **1997**, *23*, 3–25.
- Rishton, G. M. Nonleadlikeness and Leadlikeness in Biochemical Screening. *DDT* **2003**, *8*, 86–96.
- Oldenburg, K. High Throughput Sonication: Evaluation for Compound Solubilization. *Comb. Chem. High Throughput Screening* **2005**, *8*, 499–512.
- Martin, Y. C. A Bioavailability Score. *J. Med. Chem.* **2005**, *48*, 3164–3170.
- Roche, O.; Schneider, P.; Zuegge, J.; Guba, W.; Kansy, M.; Alanine, A.; Bleicher, K.; Danel, F.; Gutknecht, E. M.; Rogers-evans, M.; Neidhart, W.; Stalder, H.; Dillon, M.; Sjogren, E.; Fotouhi, N.; Gillespie, P.; Goodnow, R.; Harris, W.; Jones, P.; Taniguchi, M.; Tsujii, S.; Vvon Der Saal, W.; Zimmermann, G.; Schneider, G. Development of a Virtual Screening Method for Identification of “Frequent Hitters” in Compound Libraries. *J. Med. Chem.* **2002**, *45*, 137–142.
- Hopkins, A. L.; Mason, J. S.; Overington, J. P. Can We Rationally Design Promiscuous Drugs? *Curr. Opin. Struct. Biol.* **2006**, *16*, 127–136.
- Walters, W. P.; Ajay, A.; Murcko, M. A. Recognizing Molecules with Drug-Like Properties. *Curr. Opin. Chem. Biol.* **1999**, *3*, 384–387.
- Huth, J.; Mendoza, R.; Olejniczak, E.; Johnson, R.; Cothron, D.; Liu, Y.; Lerner, C.; Chen, J.; Hajduk, P. Alarm NMR: A Rapid and Robust Experimental Method to Detect Reactive False Positives in Biochemical Screens. *J. Am. Chem. Soc.* **2005**, *127*, 217–224.
- Rishton, G. M. Reactive Compounds and in Vitro False Positives in Hts. *DDT* **1997**, *2*, 382–384.
- Feng, B. Y.; Shelat, A.; Doman, T. N.; Guy, R. K.; Shoichet, B. K. High-Throughput Assays for Promiscuous Inhibitors. *Nat. Chem. Biol.* **2005**, *1*, 146–148.
- Seidler, J.; McGovern, S. L.; Doman, T.; Shoichet, B. K. Identification and Prediction of Promiscuous Aggregating Inhibitors among Known Drugs. *J. Med. Chem.* **2003**, *46*, 4477–4486.
- Liu, H.; Wang, Z.; Regni, C.; Zou, X.; Tipton, P. A. Detailed Kinetic Studies of an Aggregating Inhibitor; Inhibition of Phosphomannomutase/Phosphoglucomutase by Disperse Blue 56. *Biochemistry* **2004**, *27*, 8662–8669.
- Feng, B.; Shoichet, B. K. Synergy and Antagonism of Promiscuous Inhibition in Multiple-Compound Mixtures. *J. Med. Chem.* **2006**, *49*, 2151–2154.
- Feng, B. Y.; Shoichet, B. K. A Detergent-Based Assay for the Detection of Promiscuous Inhibitors. *Nat. Protocols* **2006**, *1*, 550–553.
- McGovern, S. L.; Caselli, E.; Grigorieff, N.; Shoichet, B. K. A Common Mechanism Underlying Promiscuous Inhibitors from Virtual and High-Throughput Screening. *J. Med. Chem.* **2002**, *45*, 1712–1722.
- McGovern, S. L.; Helfand, B. T.; Feng, B. Y.; Shoichet, B. K. A Specific Mechanism of Nonspecific Inhibition. *J. Med. Chem.* **2003**, *46*, 4265–4272.
- McGovern, S. L.; Shoichet, B. K. Kinase Inhibitors: Not Just for Kinases Anymore. *J. Med. Chem.* **2003**, *46*, 1478–1483.
- Ryan, A. J.; Gray, N. M.; Lowe, P. N.; Chung, C.-w. Effect of Detergent on “Promiscuous” Inhibitors. *J. Med. Chem.* **2003**, *46*, 3448–3451.
- Frenkel, Y. V.; Clark, A. D., Jr.; Das, K.; Wang, Y.-H.; Lewi, P. J.; Janssen, P. A. J.; Arnold, E. Concentration and pH Dependent Aggregation of Hydrophobic Drug Molecules and Relevance to Oral Bioavailability. *J. Med. Chem.* **2005**, *48*, 1974–1983.
- Reddie, K. G.; Roberts, D. R.; Dore, T. M. Inhibition of Kinesin Motor Proteins by Adociasulfate-2. *J. Med. Chem.* **2006**, *49*, 4857–4860.
- Inglese, J.; Auld, D.; Jadhav, A.; Johnson, R.; Simeonov, A.; Yasgar, A.; W, Z.; CP, A. Quantitative High-Throughput Screening: A Titration-Based Approach That Efficiently Identifies Biological Activities in Large Chemical Libraries. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 11473–11478.
- Walters, W. P.; Namchuk, M. Designing Screens: How to Make Your Hits a Hit. *Nat. Rev. Drug Discovery* **2003**, *2*, 259–266.
- Hann, M. M.; Oprea, T. I. Pursuing the Leadlikeness Concept in Pharmaceutical Research. *Curr. Opin. Chem. Biol.* **2004**, *8*, 255–263.
- Zhang, J.; Chung, T.; Oldenburg, K. A Simple Statistical Parameter for Use in Evaluation and Validation of High Throughput Screening Assays. *J. Biomol. Screening* **1999**, *4*, 67–73.
- Straus, O. H.; Goldstein, A. Zone Behavior of Enzymes: Illustrated by the Effect of Dissociation Constant and Dilution on the System Cholinesterase-Physostigmine. *J. Gen. Physiol.* **1943**, *26*, 559–585.
- Shoichet, B. K. Interpreting Steep Dose-Response Curves in Early Inhibitor Discovery. *J. Med. Chem.* **2006**, *49*, 7274–7277.
- Usher, K. C.; Blaszcak, L. C.; Weston, G. S.; Shoichet, B. K.; Remington, S. J. Three-Dimensional Structure of Ampc Beta-Lactamase from Escherichia Coli Bound to a Transition-State Analogue: Possible Implications for the Oxyanion Hypothesis and for Inhibitor Design. *Biochemistry* **1998**, *37*, 16082–16090.
- Chen, Y.; Minasov, G.; Roth, T. A.; Prati, F.; Shoichet, B. K. The Deacylation Mechanism of Ampc Beta-Lactamase at Ultrahigh Resolution. *J. Am. Chem. Soc.* **2006**, *128*, 2970–2976.
- Weston, G. S.; Blazquez, J.; Baquero, F.; Shoichet, B. K. Structure-Based Enhancement of Boronic Acid-Based Inhibitors of Ampc Beta-Lactamase. *J. Med. Chem.* **1998**, *41*, 4577–4586.

JM061317Y