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High-throughput assays for promiscuous inhibitors

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High-throughput screening (HTS) searches large libraries of chemical compounds for those that can modulate the activity of a particular biological target; it is the dominant technique used in early-stage drug discovery. A key problem in HTS is the prevalence of nonspecific or 'promiscuous' inhibitors. These molecules have peculiar properties, act on unrelated targets and can dominate the results from screening campaigns[1]. Several explanations have been proposed to account for promiscuous inhibitors, including chemical reactivity[1,2], interference in assay read-out[2], high molecular flexibility[3] and hydrophobicity[2,4]. The diversity of these models reflects the apparently unrelated molecules whose behaviors they seek to explain. However, a single mechanism may explain the effects of many promiscuous inhibitors: some organic molecules form large colloid-like aggregates that sequester and thereby inhibit enzymes[5]. Hits from HTS, leads for drug discovery and even several drugs appear to act through this mechanism at micromolar concentrations[5–9]. Here, we report two rapid assays for detecting promiscuous aggregates that we tested against 1,030 'drug-like' molecules. The results from these assays were used to test two preliminary computational models of this phenomenon and as benchmarks to develop new models.

To investigate the severity of the problem posed by promiscuous aggregation, and to allow others to examine their own libraries, we developed two rapid assays using 96-well-plate format to detect this behavior. The first assay screens for the detergent-sensitive nature of aggregate-based inhibition[5,10]. Inhibition of β-lactamase[7] was measured in the presence and absence of 0.1% Triton X-100; molecules that inhibit only in the absence of detergent are considered likely promiscuous aggregators. The second uses a dynamic light scattering (DLS) plate reader to measure particle formation. Control experiments suggested that both assays behaved comparably to their low-throughput counterparts[5,7,9] and could distinguish known aggregators from known nonaggregators and known promiscuous inhibitors from specific inhibitors (**Supplementary Figs. 1** and **2** online).

We selected 1,030 molecules to test these assays, purchased from Chemical Diversity, Inc., a major supplier of HTS libraries. The molecules were chosen by several criteria, including full Lipinski compliance[11] and chemical diversity; overall, the molecules covered the same physical property space as did the Comprehensive Medicinal Chemistry (CMC) database (MDL Information Systems, Inc., 2004) of drugs (**Supplementary Table 1** online). They comprised three subsets: 298 were chosen at random, 493 were predicted aggregators and 239 were predicted nonaggregators. The latter two classes were selected with two preliminary computation models for aggregation (see later discussion and **Supplementary Methods** online). All molecules were screened at 30 μM in the DLS assay and at both 30 and 5 μM in the detergent-dependent enzyme assay.

In the detergent-dependent inhibition assay, an unexpected 19% of the randomly selected molecules were detergent-sensitive inhibitors at 30 μM. Of the predicted aggregators, 39% showed detergent-sensitive inhibition, whereas only 6% of predicted nonaggregators did so (**Fig. 1a**). Some of these molecules were visibly insoluble; after these were removed from consideration, 21% of the random set, 40% of the predicted aggregators and 7% of the predicted nonaggregators showed detergent-sensitive inhibition. For all molecules, any inhibition shown in the absence of detergent was largely or completely attenuated in the presence of 0.1% Triton X-100, a property characteristic of aggregate-based inhibition[10].

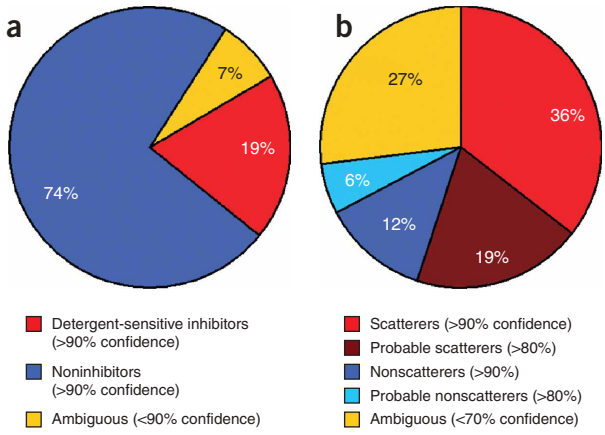

Figure 1 Results of the high-throughput assays applied to random drug-like molecules. (**a**,**b**) Behavior of 298 randomly selected molecules in (**a**) the detergent-dependent inhibition screen and (**b**) the DLS screen. The statistical percent confidence of the assignments is given in parentheses. Both assays were conducted at 30 μM.

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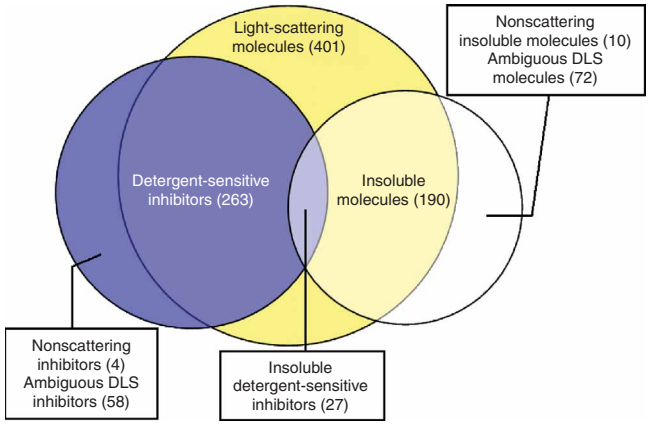

Figure 2 Comparison of the two high-throughput assays, showing overlap of positive results from the DLS and the detergent-dependent enzyme assays at 30 μM.

To examine the reliability of these results, we tested molecules from the putative inhibitor and noninhibitor populations by a more sensitive, low-throughput version of the assay. Nine molecules from each population were tested one at a time; their behavior in the two assays was well correlated. All putative inhibitors also inhibited chymotrypsin at 100 μM, consistent with the observation that detergent sensitivity is a good proxy for aggregate-based promiscuity (**Supplementary Table 2** online).

In the DLS assay, 36% of the 298 randomly selected molecules scattered light at intensities high enough to indicate particle formation (**Fig. 1b**); 52% of the predicted aggregators and 15% of the predicted nonaggregators did so as well. As in the enzyme assay, 13 molecules from the putative scattering population and 12 from the nonscattering population were tested in a more rigorous, low-throughput version of the assay (**Supplementary Table 3** online). All behaved much the same in this more sensitive assay as in the high-throughput assay. Thus, the identification of particle formation by the DLS plate reader seems reliable, with three caveats. First, other aggregative phenomena, such as precipitation, can also lead to light scattering. Second, because of the size heterogeneity of aggregates in solution, many data acquisitions were required to obtain sufficient data for DLS analysis; DLS assays sometimes required up to 3 h of acquisition per plate. Third, many molecules scattered light with intermediate intensity, with over one-quarter not clearly a member of either population. The presence of particles could not be determined for these cases.

There are notable discrepancies in the results of the two screens. Whereas 39% of tested molecules scatter light, only 26% promiscuously inhibit (**Fig. 2**). Insoluble, light-scattering precipitates that lack inhibitory activity contribute to this discrepancy. Indeed, among visibly insoluble molecules, only 14% inhibited, suggesting that precipitation and aggregation are distinct phenomena. Conversely, four detergent-sensitive inhibitors did not scatter light by DLS. These molecules may have optical properties that interfere with observation in DLS; this is the case for many known aggregators such as Congo red, which can only be studied by DLS at concentrations 100 times its half-maximal inhibitory concentration (IC_{50}) versus β-lactamase[7]. Overall, our view is that the detergent-dependent enzyme assay gives the fastest and most reliable single indication of aggregate-based inhibition.

To investigate the concentration dependence of aggregate-based inhibition, all 1,030 molecules were rescreened in the enzyme assay at 5 μM. Detergent-dependent inhibition among the random molecules dropped to 1.4% (from 19%); 8.6% of predicted aggregators and none of the predicted nonaggregators from either model inhibited (**Fig. 3**).

Until now, we have referred to the computational predictions of promiscuous aggregating and nonaggregating molecules as coherent sets. In fact, two models were used to select these molecules: a naive Bayesian (NB) method and a previously described recursive partitioning (RP) method[9] (**Supplementary Methods**). Whereas both models successfully enriched for promiscuous inhibitors among their predicted aggregators and noninhibitors among predicted nonaggregators, both also predicted many false positives and false negatives (**Fig. 3**). Overall, the NB model seemed better at predicting aggregate-based inhibition, although both scored comparably at predicting light scattering.

To improve model accuracy, the experimental results from the predicted molecule sets were used to retrain the two models; the 298 molecules from the random subset were withheld as a test set. Originally, both the NB and RP models had a misclassification rate (MR) of 26% for the random set. Upon retraining, the MR of the refined NB model improved to 20%. A random forest model[12] replaced the RP model; upon training with the larger set, the MR for this model was 11% (**Supplementary Table 4** online). Notably, both computational models were more accurate at predicting inhibitors than DLS (MR = 46%), suggesting that aggregation-based inhibition involves more than particle formation alone. A caveat to these computational models is that they remain too crude to capture the concentration dependence of aggregate formation.

Four noteworthy results emerge from these studies. First, a high percentage (19%) of randomly selected, drug-like molecules form promiscuous aggregates at screening-relevant concentrations (here, at 30 μM) in simple biochemical buffers. This percentage is so high as to dominate any high-throughput screen not controlling for this effect. Even at 5 μM, 1.4% of randomly selected molecules acted as promiscuous aggregates, a number large enough to complicate many screens. Second, and more optimistically, promiscuous aggregates may be detected rapidly and robustly. The detergent-dependent enzyme assay, in particular, should be applicable to libraries much larger than that described here. Third, this phenomenon has a steep concentration dependence, possibly reflecting an underlying critical-point phenomenon. Fourth, predictive models show some potential for predicting aggregation-based promiscuity in large libraries. To

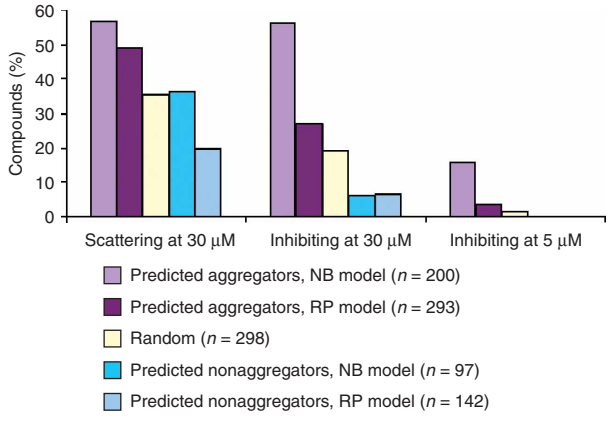

Figure 3 Accuracy of preliminary predictive models. Percentage of positive results among the subsets of test molecules predicted by the NB and RP models in the DLS and detergent-dependent inhibition screens.

allow for further development, we have made our experimental results for all 1,030 molecules available (**Supplementary Table 5** online and http://shoichetlab.ucsf.edu). In summary, promiscuous inhibition induced by aggregation is common among drug-like molecules at micromolar concentrations, and simple assays may be used to rapidly and reliably detect such inhibitors.

METHODS

DLS plate-reader and low-throughput assays. Plate-reader assays were conducted on a Proterion DynaPro Plate Reader. The Dynamics Software package version 6.0 was used to analyze the data. All compound solutions were made up in filtered 50 mM potassium phosphate, pH 7.0. Samples were diluted from 10 mM stocks (neat DMSO) and analyzed in 10-s data acquisitions at 30% laser power. Samples were analyzed in Corning 96-well UV-transparent plates (model no. 3679). Low-throughput DLS assays were conducted on a DynaPro MS/X light-scattering instrument as previously described[9].

β-lactamase assays. AmpC β-lactamase was purified and assayed as described[7,13].

Reactions contained 1 nM AmpC β-lactamase, 100 μM nitrocefin in 50 mM potassium phosphate, pH 7.0, at room temperature (22 °C). These reactions also contained 0.00002% Triton X-100 to stabilize the enzyme. Compound and enzyme were incubated together for 5 min before the reaction was initiated by the addition of substrate. Nitrocefin hydrolysis was monitored at 482 nm on a SpectraMax 340 UV-visible plate reader for high-throughput assays and an HP8453 UV-visible spectrophotometer for the low-throughput assays. To test for detergent effects on putative aggregators, reactions were conducted as described here with the exception that buffer containing 0.1% Triton X-100 was added before the introduction of compound.

Chymotrypsin assays. All chymotrypsin assays were conducted as previously described[7].

Calibration of high-throughput assays. The high-throughput enzyme assay was calibrated on a set of 19 known aggregators and 27 known nonaggregators. The high-throughput DLS assay was calibrated with a set of 16 known aggregators and 33 known nonaggregators. In both assays, the behavior of these control compounds was used to develop statistical cutoffs for classifying unknown molecules (**Supplementary Methods** online).

Test set selection. The 1,030 test set molecules were purchased from Chemical Diversity, Inc. Prediction set molecules were classified as either promiscuous aggregators or nonaggregators by one of two models: a previously described RP model[9] or an NB model (**Supplementary Methods** online). The predicted set contained 493 predicted aggregators (200 NB and 293 RP) and 239 predicted nonaggregators (97 NB and 142 RP). The random set contained 298 molecules. All compounds were prepared as 10 mM stocks in neat DMSO.

All selected compounds satisfied the following Lipinski criteria: (Nitrogen_Count + Oxygen_Count) <= 10, Molecular_Weight <= 500, Num_H-Bond_Donors <= 5, and AlogP <= 5.6[11]. An upper bound of 5.6 is more appropriate for the AlogP-based estimation of logP[14]. To further ensure that our test sets were reasonable representations of drug-like molecules, we compared common physical property distributions in our prediction set, the random set and the CMC database. The CMC was filtered to remove compounds that were unlikely to be orally bioavailable, such as contrast agents, solvents and pharmaceutical aids; the filtered database was denoted CMC*[14]. The interquartile ranges of chemical properties for both the prediction and random sets are similar to those of the CMC* (**Supplementary Methods** online).

Statistical analyses. Population normality and confidence intervals for the enzyme inhibition data were calculated with GraphpadPro (Prism Software). The NB DLS classifier and the NB predictive model for aggregate-based inhibitors were developed with the NB classifier algorithm in Pipeline Pilot 4.5.0 (Scitegic, Inc). The algorithm calculates the sum of conditional probabilities for the occurrence of a given feature in a set of molecules representing two classes. The random forest model was developed with the Arbor software package (**Supplementary Methods** online).

Other methods. Assay development procedures, the predictive models, sample DLS data, screening results and a complete list of the 1,030 molecules (**Supplementary Table 5**) are available online and at http://shoichetlab.ucsf.edu.

Accession codes. BIND identifiers (http://bind.ca/): 266517–266779.

Note: Supplementary information is available on the Nature Chemical Biology website.

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COMPETING INTERESTS STATEMENT

The authors declare that they have no competing financial interests.

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