Published in final edited form as:
J Med Chem. 2010 January 14; 53(1): 37–51. doi:10.1021/jm901070c.

Quantitative Analyses of Aggregation, Autofluorescence, and Reactivity Artifacts in a Screen for Inhibitors of a Thiol Protease

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Abstract

The perceived and actual burden of false positives in high-throughput screening has received considerable attention; however, few studies exist on the contributions of distinct mechanisms of non-specific effects like chemical reactivity, assay signal interference, and colloidal aggregation. Here, we analyze the outcome of a screen of 197,861 diverse compounds in a concentration-response format against the cysteine protease cruzain, a target expected to be particularly sensitive to reactive compounds and using an assay format with light detection in the short-wavelength region where significant compound autofluorescence is typically encountered. Approximately 1.9% of all compounds screened were detergent-sensitive inhibitors. The contribution from autofluorescence and compounds bearing reactive functionalities was dramatically lower: of all hits, only 1.8% were autofluorescent and 1.48% contained reactive or undesired functional groups. The distribution of false positives was relatively constant across library sources. The simple step of including detergent in the assay buffer suppressed the nonspecific effect of approximately 93% of the original hits.

INTRODUCTION

High-throughput screening (HTS) remains the dominant technique for small molecule discovery. After remaining confined to biopharmaceutical companies for decades, high-throughput screening has recently entered the public domain via the efforts of a growing number of non-profit institutions, including the Molecular Libraries Initiative[1] of the NIH Roadmap under whose program the structures of the library compounds as well as the primary and secondary screening results are being made available to researchers via the PubChem database (http://pubchem.ncbi.nlm.nih.gov/). In addition to providing a wealth of data on the interaction between chemical space and novel target space, the unprecedented public availability of compound structure and screening results allow one to ask a series of

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COMPETING INTERESTS STATEMENT
The authors declare no competing financial interests.

SUPPLEMENTAL INFORMATION **PROVIDED**
Methods and results from the flow cytometry study of 30 small molecules, reactive and undesirable functional group filters used in this study. This material is available free of charge via the Internet at http://pubs.acs.org.

fundamental questions about the different sources of compound interference in HTS and their relative contributions.

Three major categories of compound-originating interference can lead to confounding assay results and the inadvertent selection of false positives[2] on which precious personnel, material, and time resources can be pointlessly spent. Colloidal aggregation of small molecules has gained prominence recently as a universal mode by which many small molecules can act on enzymatic targets to yield reproducible yet irrelevant inhibition.[3-6] Two key properties ascribed to aggregators allow the facile identification of at least a large subset of these false positives. On the experimental side, aggregators are detergent-sensitive and as little as 0.01% of a reagent like Triton X-100 effectively disrupts the promiscuous inhibition by more than 95% of the potential aggregators[6-8]. Analytically, inhibition by colloidal aggregates may often be detected by high Hill coefficients in the concentration-response curves of screening hits.[6, 9]

While aggregation is largely viewed as a fundamental assay- and target-independent compound property determined by the compound structure and medium properties such as assay pH and buffer composition, the other two major sources of compound interference appear to manifest themselves very differently depending on the assay format and the nature of the target. Interference from compound spectral density, in general, and autofluorescence in particular has plagued both miniaturized and traditional assays which use a range of detection formats, with direct fluorescence intensity and fluorescence polarization modes being the most severely affected. Our recent profiling of the Molecular Libraries Small Molecule Repository (MLSMR) for compound autofluorescence10 unambiguously identified the spectral regions most susceptible to interference. Assays based on the common coumarin reporters were especially sensitive to library interference, while red-shifting the reporter fluorophore to dyes such as rhodamine reduced interference several hundred-fold.10 While fluorescence itself (defined by parameters such as extinction coefficient, quantum yield, and fluorescence lifetime) is a fundamental compound property, the relative magnitude of fluorescence interference depends on the target environment, specifically the strength of assay signal.2 For example, enzymatic reactions associated with relatively high K_m values in the mid- to high-micromolar range by necessity consume or generate high concentration of reporter fluorophore which makes them more resistant to autofluorescence,[2, 11] while fluorescence polarization assays of tight-binding ligand-receptor pairs (i.e., associated with single or double digit nanomolar Kd values) are the most susceptible to interference due to the low tracer concentration employed.[2]

The third source of interference is compound reactivity. Compounds with obvious reactive groups have also been called hot compounds and their identification in some screens has led to debates whether such compounds should be summarily excluded from the screening deck[12, 13]. However, there is no consensus on what exactly constitutes a reactive functionality; moreover, the chemical reactivity of many functional groups depends on their concentration, the pH of the reaction medium, and the exact nature of the protein targets. At present, there is no universal scale to judge reactivity and only a few reports on strategies to screen for it.[14-16]

Our recent studies using AmpC β-lactamase as a reporter system (utilizing a chromogenic assay format by following the release of orange-colored product as a result of the hydrolysis of the nitrocefin substrate) highlighted aggregation as the major source of interference: approximately 95% of the screening hits were promiscuous colloidal aggregators[6]. Due to the relatively high concentration of substrate used and the utilization of kinetic read, the interference from colored substances on the assay readout was negligible. Surprisingly, few actives were attributed to reactive compounds, with aggregators outnumbering the reactive

hits by a ratio of over 100:1. Meanwhile, almost all library members containing presumed hot functionalities were inactive.17 These results prompted the provocative conclusion that non-specific covalent inhibition may in fact be a minor issue in HTS. To date, the beta-lactamase study represents the only large-scale profiling of a diverse compound library with respect to aggregators and other false positives, thereby providing the only publicly available reference set of aggregating compounds. To probe the generalizability of such conclusion, herein we profile another enzyme, cruzain, which has a structure and function different from that of β-lactamase but which represents an interesting boundary test case: being a cysteine protease, with a "hot" thiol nucleophile, it should be especially sensitive to electrophilic covalent inactivation. Cruzain is a key protease in *Trypanosoma cruzi*, the protozoan parasite responsible for Chagas disease. The enzyme is essential for the parasite survival and replication, and has been validated as a drug target for this organism.[18, 19] The fluorogenic assay routinely employed to measure cruzain activity[20] utilizes a coumarin-type detection label and as such falls in the blue-shifted spectral region most susceptible to compound fluorescence interference;[10] we thus expected to encounter a sizeable number of autofluorescent false positives.

We describe the development and quantitative high-throughput screening of a kinetic fluorogenic assay for cruzain. Each compound was tested at a minimum of 7 concentrations following our previously-reported quantitative high-throughput screening (qHTS) approach.[21] We compared the rate and nature of hits in screens performed in a "low-detergent" (0.00005%) and a "high-detergent" (0.01%) condition. The use of kinetic read, the application of detergent-sensitivity screening, and a chemoinformatics analysis of the library allowed us to evaluate the effects of the three major modes of compounds interference - aggregation, autofluorescence, and reactivity. Comparing hit lists between the low-detergent and high-detergent screens allowed us to identify active colloidal aggregators. Analysis of the fluorescent signal evolution pattern for each sample led to the identification of autofluorescent compounds. Lastly, we queried the screening hits against a list of suspected reactive functionalities in order to evaluate the fraction of purported reactives among the non-aggregating and non-fluorescent cruzain hits.

MATERIALS AND METHODS

Reagents

Triton X-100, sodium acetate, and DTT (dithiothreitol) were purchased from Sigma-Aldrich. DMSO Certified ACS Grade was from Fisher. The cruzain model fluorogenic substrate Z-Phe-Arg-AMC was from Bachem. The screening assay was performed in 100 mM acetate buffer pH 5.5 containing 5 mM dithiothreitol. The detergent-present screen contained 0.01% Triton X-100, while the detergent-free assay contained 0.00005% of the same detergent (this turned out to be important for enzyme stability). Black solid bottom polystyrene assay plates were from Greiner Bio-one (Monroe, NC). The entire screening library (Galapagos Biofocus DPI, South San Francisco, CA) was subjected to purity analysis before plating by using an eight-channel MUX high-throughput parallel chromatographic system (Micromass Ltd, Manchester, UK and Waters, Milford, MA) and separating the sample on Phenomenex Gemini 5 μm C18 column (2 × 50 mm) and a linear gradient of 0.1% aqueous formic acid to 90% acetonitrile in 0.1% aqueous formic acid over 3 min. Compounds used in the followup experiments were reanalyzed for purity. The purity analysis was performed via liquid chromatography- mass spectrometry (LCMS) on a Waters ACQUITY reverse-phase UPLC system and a 1.7 μm BEH column (2.1 × 50 mm) by using a linear gradient in 0.1% aqueous formic acid (5% ACN in water increasing to 95% over 3 min). Compound purity was measured on the basis of peak integration (area-under-the curve) from both UV/vis absorbance (at 214 nm) and evaporative light scattering detection (ELSD), and compound

identity was determined on the basis of mass analysis; all compounds passed purity criteria (>95%).

Compound library

The 197,861 member library comprised two main subsets: 139,740 compounds from the NIH MLSMR, prepared as 10 mM stock solutions in 384-well plates and delivered by Galapagos Biofocus DPI (South San Francisco, CA, http://mlsmr.glpg.com), and NCGC internal exploratory collection of 58,121 compounds which consisted of several commercially available libraries of known bioactives (1,280 compounds from Sigma-Aldrich (LOPAC^{1280} library), 1,355 compounds from Prestwick Chemical Inc. (Washington, DC), 1,271 compounds from Tocris (Ellisville, Missouri), 2,031 known actives from Microsource (Gaylordsville, CT), 419 purified natural products from TimTec (Newark, DE), 1,980 compounds from the National Cancer Institute (the NCI Diversity Set), and 1,408 toxins from the National Institute of Environmental Health Sciences. Additional libraries included collections from other commercial and academic collaborators (three 1,000-member combinatorial libraries from Pharmacopeia (Cranbury, NJ), 42,240 diverse drug-like molecules, 704 compounds from Boston University Center for Chemical Methodology and Library Development, 473 compounds from University of Kansas Center for Chemical Methodologies and Library, 96-member peptide library from Prof. Sam Gelman's lab, University of Wisconsin, Madison, 1,143 compounds from the University of Pittsburgh Center for Chemical Methodology and Library Development), and 20 boronic acid AmpC β-lactamase inhibitors from the Shoichet lab. The remaining samples were known actives acquired from various commercial suppliers and compounds produced via internal chemistry efforts. Details on the formatting of the compound library for qHTS are provided elsewhere. [21, 22]

Control plate

Titration of the vinyl sulfone inhibitor K11777 (4-methyl-N-((2S,5R,E)-3-oxo-5-phenethyl-1-phenyl-7-(phenylsulfonyl)hept-6-en-2-yl)piperazine-1-carboxamide, **1**) [23, 24] was delivered via pin transfer of 23 nL of solution per well from a separate source plate into column 2 of each 1,536-well assay plate. The starting concentration of the control was 1 mM, followed by twofold dilution points in duplicate, for a total of sixteen concentrations, resulting in final assay concentration range from 5.7 μM to 0.175 nM, corresponding to the dilution of 23 nL stock into 4 μL assay reaction.

Cruzain Assay Miniaturization and qHTS

To measure the enzymatic activity of cruzain, we used a fluorogenic substrate Z-Phe-Arg-AMC, which is converted to a highly fluorescent 7-amino-4-methylcoumarin reporter upon hydrolysis.[24] Assay optimization was performed directly in 1,536-well format at a final reaction volume of 4 μL. For the detergent-present screen, Triton X-100 was used at 0.01% and the cruzain was present at 1.5 nM. During the optimization of the detergent-free assay, low stability and high variability in the specific activity of cruzain was noted, likely due to protein adsorbtion on polystyrene assay plates. To stabilize the enzyme, its final concentration was raised to 3 nM and a trace of Triton X-100 (final concentration of 0.00005%) was included in the detergent-free assay; we note that a similar adjustment step was needed for the AmpC β-lactamase aggregation screen described earlier.[6] At the conditions selected - 1.5 nM or 3 nM final cruzain concentration and 2 μM final substrate concentration (the latter chosen to match previously reported conditions and being close to the K_m value for this substrate) [24, 25] - the signal evolution was robust and low substrate conversion could be conveniently monitored over the course of 1 minute, making the assay highly sensitive to cruzain inhibitors. All assay components were tested and found stable for at least 24 hours when formulated as stock solutions at their working concentrations in both

the detergent-present and the detergent-free buffers (data not shown). Such demonstrated stability permitted the implementation of an unattended overnight screening operation.

To perform the high-throughput screen, 3 μL of reagents were dispensed into 1536-well Greiner black solid-bottom assay plate. Compounds and controls (23 nL) were transferred via Kalypsys pintool equipped with 1,536-pin array (10 nL slotted pins, V&P Scientific, Palo Alto, CA). The final concentrations of the screening compounds ranged from 57.5 μM to 3.7 nM, with seven concentrations assayed across a five-fold dilution series. The plate was incubated for 15 min at room temperature, and then a 1 μL aliquot of 8 μM substrate solution was added to start the reaction. The plate was transferred to ViewLux high-throughput CCD imager (Perkin-Elmer, Waltham, MA) where kinetic measurements (4 reads, one read every 30 seconds) of the AMC fluorescence were acquired using standard 340 nm excitation and 450 nm emission filter set. During dispense, reagent bottles were kept submerged into 4 °C recirculating chiller bath and all liquid lines were covered with aluminum foil to minimize fluorophore degradation. All screening operations were performed on a fully integrated robotic system (Kalypsys Inc, San Diego, CA) as described elsewhere.[26]

Screening data were corrected and normalized and concentration–effect relationships derived by using in-house developed publicly available algorithms; a four parameter Hill equation[27] was fitted to the concentration-response data as described elsewhere (http://www.ncgc.nih.gov/pub/openhts/curvefit/). Percent activity was computed from the median values of the uninhibited, or neutral, control (32 wells located in column 1) and the no-enzyme, or 100% inhibited, control (64 wells, entire columns 3 and 4), respectively. Plates containing DMSO only (instead of compound solutions) were included approximately every 50 plates throughout the screen to monitor any systematic trend in the assay signal associated with reagent dispenser variation or decrease in enzyme specific activity. Reactive functionality queries were implemented as Daylight SMARTS (Daylight SMARTS – A Language for Describing Molecular Patterns, http://www.daylight.com/dayhtml/doc/theory/theory.smarts.html) (see Table 3 and the full list provided in the Supporting Information, Table S2) and the ChemAxon Java toolkit (http://www.chemaxon.com/) was used to process the library and annotate compounds with problematic functional groups.

Secondary Assay in 96-well plate format

Forty-two compounds active in the qHTS were repurchased from commercial vendors (ChemBridge, Enamine, ChemDiv, IBScreen, Sigma and Tocris) and re-tested in a higher-volume, lower-throughput format. In particular, we were interested to re-test compounds that appeared to be non-aggregators in the cruzain qHTS, discussed here, but had been aggregators in a similar qHTS screen against AmpC β-lactamase.[28] Thrity-two compounds from this category were purchased, ten of which were active only at the highest concentration tested in that screen (30 μM), whereas 22 showed full dose-response curves in the absence of detergent. We were also interested in investigating compounds that were not screened in the previous β-lactamase HTS, but were inconclusive aggregators in the cruzain qHTS. To this end we re-tested ten compounds. 20 mM stock solutions of compounds were prepared in DMSO from dry powders. Assays were performed in Sodium Acetate 0.1 M pH 5.5, 5 mM DTT, either in the presence and absence of 0.01% Triton X-100. The final concentration of cruzain was 0.4 nM, and the substrate concentration was 2.5 μM (K_m = 2 μM). One μL of a 200x DMSO stock of compound was added to each well, followed by addition of 100 μL of buffer solution containing 0.8 nM cruzain. After 15 minutes incubation, 100 μL of a solution of 5 μM Z-FR-AMC in buffer was added to the enzyme-compound mixture to start the reaction. Cruzain activity was measured in a Flexstation microplate spectrofluorometer (Molecular Devices, Sunnyvale, CA) and assays were

followed for five minutes. Percentages of enzyme inhibition were calculated based on initial rates of substrate cleavage, compared to a DMSO control. All assays were performed at least twice. Each compound was initially tested at 11.5 μM. If detergent sensitivity was not observed at this concentration, compounds were further tested at 30, 60 and 120 μM.

RESULTS AND DISCUSSION

Cruzain Quantitative High-throughput Screens

The screens of the 197,861-compound collection (Figure 1) were completed within a two separate workweeks. During the first week, approximately 60% of the 1,107 library 1,536-well plates were screened in the detergent-free assay first; this was immediately followed by a screen of the same set of library plates in the detergent-present assay. The remaining 40% of the library was screened in the same manner during the second week. The screening of each compound against the two assays in close succession minimized the possibility for sample-age related differences in results. Overall, two sets of 1,107 1,536-well assay plates were run under the detergent-free and detergent-present conditions, respectively, leading to the generation of 197,861 concentration responses consisting of at least seven points per compound per assay and corresponding to a total of approximately 1.5 million samples tested per detergent condition.

The Z′ screening factors associated with each plate and each screening condition remained high and stable throughout the two screens (Figure 2): the average Z′ for the detergent-free screen was 0.78, while the corresponding average for the detergent-present screen was 0.93. Of the 2,214 plates tested in both screens, only six failed; these were re-screened immediately using the same batches of enzyme and substrate. As a further quality control, we included a concentration response of the known vinyl sulfone cruzain inhibitor K11777 **1** [20, 24] added as a 16-point dilution series in duplicate between 5.7 μM and 0.175 nM into the second column of every assay plate. The shape and quality of the concentration response remained consistent throughout both screens (Figure 3, green data points) with the associated minimum significant ratios (defined by Eastwood et al29) of 2.5 and 1.4 for the detergent-free and detergent-present conditions, respectively, further indicating stable runs.

The cumulative effect of all library compounds on the cruzain activity at each screening condition is shown in Fig. 3. On the plots, concentration responses were color-coded and positionally sorted based on activity with inactive samples (flat concentration responses) represented by the black dots, activators in red, and inhibitors in blue; green points in the very front of the 3D plots represented the 1,107 duplicate responses of the intra-plate control titration of **1**. The outcomes from the detergent-free versus detergent-present screens were strikingly different: the detergent-free screen yielded over 15 times more hits than its detergent-present counterpart. Similar to our experience with the β-lactamase profile,[6] we observed a large number (12,746) of apparent activators in the detergent-free cruzain screen which turned inactive in the detergent-present screen (indicated as Category A compounds in Figure 4). Over 85 % of the activators were associated with partial or single-point top concentration responses indicating that the condition-dependent activation was being observed only at the highest compound concentrations where complicating phenomena such as transient precipitation, light scatter, and compound aggregate-assisted enzyme stabilization have been known to lead to false positive effects.[30] We did not consider those compounds further. During the following steps of our analysis, we categorized the active hits based on detergent sensitivity, autofluorescence, and presence of reactive functionalities or undesirable chemical features, to arrive at a final filtered set of cruzain inhibitors.

Detergent-sensitive Hits (Aggregators)

Of the compounds showing concentration-dependent inhibition in the detergent-free screen, almost 90% appeared to be detergent-sensitive hits, or aggregators. The precise assignment of aggregation phenotype for *every* library compound was complicated by the many detergent-sensitive compounds (10,399 samples) that exhibited only a weak and noisy inhibitory response in the detergent-free screen, making their categorization as inhibitors problematic. We categorized these compounds as weak or inconclusive detergent-sensitive inhibitors (Category B in Figure 4).

The many inconclusive responses in the detergent-free screen stood in contrast with the approximately 550 inconclusive samples (or 20-fold fewer) observed in the detergent-present experiment (Category F in Figure 4, described in detail in Reactive Functionalities Analysis). This observation, combined with the lower and more variable Z′ factor associated with the low-detergent screen (Figure 2), demonstrates a secondary benefit that a detergent can have on assay performance, stabilizing a protein by minimizing its adsorption to the plastic walls, a phenomenon well-known among enzymologists.

In turn, detergent-sensitive inhibitors which exhibited complete concentration-response curves and had a maximum response greater than 40% inhibition in the low-detergent condition were considered conclusive aggregators; a total of 3,844 compounds met these more stringent criteria (Category C in Figure 4). Overall, 1.94% of the library members exhibited detergent-sensitive inhibition. With the exclusion of categories A, B, and C compounds, there remained 1,978 detergent-insensitive actives, which were further analyzed with respect to autofluorescence and the presence of reactive and undesirable functionalities (see below).

96-well plate aggregator assays

To probe the reproducibility of aggregation and its dependence on assay format, 42 compounds were re-purchased as dry powders and tested for aggregation effect in the standard 96-well plate cruzain assay. The criterion for aggregation in this format was the shift in percent inhibition greater than 35% upon inclusion of 0.01% Triton X-100. Among the compounds retested, ten had been inconclusive cruzain qHTS aggregators. These are molecules which showed detergent-sensitivity in the qHTS, but for which classification as aggregators was less robust due to weak cruzain inhibition even in the absence of Triton, with reported activity only at the highest concentration screened by qHTS (Category B in Figure 4). Nine out of ten “inconclusive” aggregators from the cruzain qHTS displayed typical aggregation behavior when tested in the 96-well plate assay. The 10th compound did not show reproducible cruzain inhibition in this assay format, even in the absence of detergent (Table 1). These results suggest that most detergent-sensitive compounds would be confirmed as aggregators in follow up assays. That is true even for weak inhibitors, for which the assignment as aggregators was less evident based on the qHTS results. Therefore, the classification of 3,844 compounds as aggregators is a conservative estimate, and the number of artifacts present in this category is likely much higher.

The other 32 compounds retested in this format were previously-identified as aggregators in a β-lactamase screen[6, 9] but were inactive against cruzain in the present screen. Out of these, 22 displayed aggregation behavior in the 96-well plate assay, though four of them only did so at 120 μM, a level twice that used in the qHTS (Table 2). The mismatch in compound phenotype between testing in miniaturized settings and traditional format highlights the conditional nature of aggregation: compounds that inhibit through colloidal aggregation in one setting may fail to do so detectably in a different setting.

Unexpectedly, ten compounds which showed aggregation effect against β-lactamase (also confirmed by AmpC cuvette-based assays6) were tested here but exhibited no aggregation phenotype in *both* the HTS and the 96-well plate cruzain assays (Table 2). These ten compounds did not belong to any particular structural class and were diverse with respect to their size and functional groups. Thus, it appears that the assay format variations aside, there are genuine differences in at least some compounds' propensity to form aggregates and for those aggregates, if formed, to interfere with the enzymatic reaction depending on the target identity and buffer conditions: at least a subset of the library behaves as conditional aggregators even when traditional reaction formats are compared.

The apparent assay-to-assay variability of the aggregator phenotype prompted us to examine a set of compounds by flow cytometry using an instrument platform designed to characterize compounds with respect to solubility and aggregation potential.[31] Thirty compounds, of which ten were positive in the present cruzain aggregation screen, while the remaining twenty were inactive in the cruzain detergent-free screen but were classified as aggregators in the β-lactamase study, were tested in different buffers systems in order to characterize their innate propensity for aggregation and precipitation, and to compare those properties with the corresponding behavior in the enzymatic screens. No concordance was evident between the flow cytometry assignments and the HTS-derived aggregation phenotypes (Supporting Information Table S1). The divergence of aggregation as detected by a physical technique using well-mixed and equilibrated samples with particle-size analysis from the aggregation as defined by an inhibitory effect in a miniaturized assay parallels the earlier noted differences between aggregation assignments from HTS and 96-well plate assays. It thus appears more appropriate to refer to the aggregation seen through the effect of the sample on an enzyme reporter as a *functional* aggregation, as distinct from and not always overlapping with *physical* aggregation, the latter being the property of a compound to form colloidal particles in a certain solution medium under bulk equilibrated conditions. It is worth noting that such subdivision of aggregation parallels the assignment of often significantly different kinetic versus equilibrium, or thermodynamic, solubility values to compounds tested in otherwise identical solvents.[32]

The lack of concordance between aggregation observed in miniaturized settings and traditional assay format points to the fact that aggregates do not form at the same rate at all conditions but their formation rather depends on process-related factors such as the mode of compound delivery and mixing, and the surface-to-volume ratio of the reaction vessel. The conditional nature of aggregation observed here is in agreement with the recently published study[33] of aggregating compounds conducted by surface plasmon resonance. In it, Giannetti et al. describe a number of instances of compounds which exhibited aggregation-type binding to a certain protein target but completely failed to interact with others. If the same compound behaves so differently against different proteins when tested under the well defined parameters of an established biophysical technique, it does not come as a surprise that assay context dependency of aggregation is observed in miniaturized assay screens. Further, while there were inter-library variations, we did not observe any significant distribution bias of the aggregators among library types: the makeup of the aggregators (Figure 1B) largely mirrored that of the collection screened (Figure 1A). Thus, at present it appears more prudent not to assemble lists of permanent aggregators, or to computationally predict which compounds will display aggregation-based enzyme inhibition, but to adopt assay protocols and post-screen studies such that the burden of aggregators on the overall process is reduced. For example, a simple inclusion of detergent in the assay buffer has been shown repeatedly to significantly reduce the interference from aggregators[6-8] and our present study confirms this notion once again. The inclusion of 0.01% Triton X-100 is unlikely to be fully curative, however: in support of the notion that the detergent effect is a continuum, rather than a step function, our previous β-lactamase study highlighted the

occurrence of a small number of detergent-hardy aggregators - compounds whose inhibitory potency is right-shifted upon addition of 0.01% detergent but whose effect only disappears completely when detergent is added to a considerably higher 0.1% level.

Autofluorescent Hits

For every assay well, fluorescence intensity increase was measured in a real-time kinetic mode by acquiring four reads every 30 seconds. The enzyme activity and the corresponding effect of library compounds were computed from the sixty-second initial rate. The uniform collection of such a short time course on 1,536 samples was enabled by the use of the CCD-based ViewLux reader. As described previously,[10, 34] the starting fluorescence intensity of each well was noted and used to flag compounds which interfered with the assay signal by exhibiting high level of autofluorescence. This process is illustrated with the example in Figure 5. An example plate titration series is shown as a sequence of plate heatmaps (A). Each rectangle represents a 1,536-well plate, with 7 such plates comprising a concentration series. Individual plate wells in blue show signal decrease and in red are wells that cause signal increase. The first kinetic read reveals auto-fluorescent compounds which appear as active on that heatmap (red wells). In turn, the fluorescence intensity difference within the first minute of reaction monitoring is used to calculate activity. The full kinetic reads of a library sample in panel B, left plot show a dramatic increase in initial raw fluorescence units as a function of increasing compound concentration, consistent with compound autofluorescence. The activity calculation of such samples often leads to false inhibition assignment such as that seen for the oxazole-4-nitrile **42** shown in Figure 5C, left plot, due to the fact that for samples possessing inherent fluorescence significantly over the assay average the noise or drift in fluorescence signal collected over the time course completely overtakes the signal contributed by the assay itself and dominates in the calculation of the slope and rise of the reaction progress curve. In contrast, a non-fluorescent inactive compound **43** (Figure 5B and 5C, right plots) was characterized with reaction time-course trends which varied very little with compound concentration (Figure 5B, right plot) and as a consequence the corresponding concentration-response curve was relatively flat (Figure 5C, right plot).

The above analysis was applied to the 1,978 compounds exhibiting inhibitory concentration responses in the detergent-present screen (detergent-insensitive hits). In order to balance the threshold for statistical significance and the need to flag artifacts as rigorously as possible, we selected an arbitrary raw starting fluorescence intensity cutoff of 400 RFU which was approximately 10% below the value of 446 corresponding to the typically selected three standard deviations level. Based on this criterion, 507 compounds were categorized as detergent-insensitive autofluorescent false positives, representing approximately 1.75% of all screening hits (Category D in Figure 4). We note that if only end-point data were to be collected, the percentage of autofluorescent compounds interfering with the readout would likely have increased due to the absence of the subtracting-out effect which kinetic data collection has on the assay signal. Similar to the distribution observed for aggregators, there was no significant bias in the origin of autofluorescent hits as a function of library source (Figure 1C). The only outlier (the Pharmacopeia set) represented an example of a focused combichem-derived collection within which the source of large number of autofluorescent compounds was confined to just two cores, pteridin-7(8H)-ones and quinazolines.

In addition to Category D fluorescent compounds, members of Category A, B, and C also contained fluorescent molecules. For example, 108 out of the 3,844 aggregators and 357 out of the 10,399 inconclusive aggregators were also fluorescent. When the above raw-fluorescence cutoff filter was applied to the entire collection of 197,861 compounds without regard to compound activity in the enzymatic assay, approximately 5.6% of the collection was scored as autofluorescent. The high percentage of autofluorescent members as a fraction

of the entire library determined here is consistent with our fluorescence spectral profiling of a smaller library within the same coumarin light detection region[10] and further supports the need for development of red-shifted fluorogenic assays.

Reactive Functionalities Analysis

After the above triaging of screening artifacts, 1,471 detergent-resistant non-fluorescent inhibitors of cruzain remained, representing 0.74% of the screened collection. Considering the composition of the chemical library with its inclusion of known actives collections (Figure 1 and Methods) and compounds included for small molecule probe development, we expected many reactive functionalities and undesirable groups to be present in the screening deck. To this end, we implemented 243 substructure queries using i) previously reported exclusion filters 13, 17, 35, [36] and ii) queries performed on the MLSMR collection specifically (NIH Molecular Libraries Small Molecule Repository Excluded Functionality Filters, https://mli.nih.gov/mli/compound-repository/mlsmr-compounds/).

The queries were grouped into two tiers of filters: reactive functionalities and medicinal chemistry exclusions. Reactive functionalities included aldehydes, expoxides, alkyl halides, Michael acceptors, metals, 2-halo pyridines, phosphorous nitrogen bonds, alpha-chloroketones, beta-lactams, and others (Table 3, see the full list provided in the Supporting Information, Table S2). Medicinal chemistry exclusions included groups such as flavanoids, crown ethers, hydrazines, poly phenols, oximes, primary halide sulfates, multiple nitro groups, etc (see Table S2). Members of the latter set are not compounds directly reactive with potential enzyme active sites but have been deemed otherwise unfit for medicinal chemistry optimization - and have often been discussed together with the reactive-functionality hits.13 Additionally, electrophilic nitriles were included in the medchem exclusion list due to the likelihood of covalent modification of the active site cysteine in cruzain.[37] Using these tiers, 10,600 compounds were found to be potentially reactive in the entire screening deck. For the medchem exclusions, 18,842 compounds were found to be potentially problematic within the library. Accounting for overlapping samples, a total of 27,047 library compounds met one or both sets of the exclusion filter lists; thus, 13.7% of the entire screening collection contained potentially problematic compounds as defined by these two tiers. Although risky compounds represented a significant fraction (13.7%) of the entire screening deck, more than half (55%) of that subset of compounds contained just three most frequently occurring functional groups: hydrazines, electrophilic nitriles, and free thiols. The top 10 substructure queries accounted for 80% of the filtered compounds. These included alpha dicarbonyls, Michael acceptors, bis-trisubstituted olefins, 2-halopyridines, non-organic unacceptable atoms, quaternary C, Cl, I, P, S, and alphatic C6 chains. Table 3 shows examples of some frequently occurring filters in the detergent resistant inhibitors, while Supplemental Table S2 contains all 243 filter definitions as implemented in SMARTS (complete SMARTS queries are available at the following URL: http://ncgc.nih.gov/projects/cruzain). Hydrazines, nitriles, and thiol-containing reactives accounted for a majority of the problematic compounds. Next on the list of frequent actives were Michael acceptors, metals, bis-trisubstituted olefins, aldehydes, and 2-halopyridines.

Applying these criteria, we identified 428 out of the 1,471 detergent-insensitive non-fluorescent inhibitors to be carriers of reactivity or medchem liability (Category E in Figure 4). Thus, only 428 compounds, or 1.56%, out of 27,047 total suspected problematic library members inhibited cruzain. In a further breakdown, out of the 428 problematic compounds, only 112 belonged to the reactives category and did not meet any of the medchem exclusion criteria. An additional 91 compounds met both the reactive functionality and the medchem exclusions criteria and, finally, 225 compounds were identified in response to the medchem exclusion query but did not contain reactive functionalities.

Hydrazines were the most prevalent class of category E compounds, accounting for 139 out of the 428 filtered actives. However, only 2% of the total number of hydrazines contained within the screening collection were active against cruzain; it is likely that only the ones prone to acylation displayed inhibition. The next highest in actives frequency were nitriles, which are known covalent but reversible inhibitors of cysteine proteases.38 Nitriles were included in our exclusion list strictly as context specific filters due to likelihood of their occurrence among the actives. However, nitriles often are not promiscuous even against proteases,[37] and indeed several are being progressed through late-stage clinical trials for thiol protease targets. Only 128 out of 4,276 (3%) of all such compounds were active against cruzain.

Disulfide bond formation is a common type mechanism of covalent modification of proteins, in particular of cysteine proteases. Among the functional groups that could potentially attack cysteine to form sulfur-sulfur bond, there were 98 free thiols that were potent detergent resistant inhibitors of cruzain. These accounted for 23% of all the Category E compounds. However, despite their abundance among the actives, only 2.6% of all free thiol containing compounds were active in the present screen. Next in this group of filters, we found that 31% of the hydrazothioureas containing compounds were active against cruzain. Among the reactive thio-hydroxamates within the library, 6 out of 66 were detergent resistant inhibitors. However, none of the 20 regular hydroxamate warhead compounds present in the collection was active, thus suggesting covalent modification of the active site cysteine by some of the thio-hydroxamates. Several additional miscellaneous thio containing filters captured another 45 compounds - many of which were potent - that could potentially fall under the same mechanism.

Some non-reactive substructures such as polyphenol containing compounds were also frequent hitters in the detergent-present cruzain assay. Polyphenol moieties occur in many natural products such as flavonoid-based antioxidants and tannins. However, they are known to be promiscuous and have atypical high activity rates in biological assays.[35] A PubChem search for compounds similar to quercetin, for example, shows hit rates ranging from 16% to 38% (data not shown) in diverse types of biochemical and cell-based assays. Polyphenols are also known to be metal chelators that have slow off-rates and rely on non-covalent but strong interactions.[38] Due to their general promiscuity and to the lack of elucidating meaningful structure-activity-relationships from their optimization, these moieties were included as part of our functional group filters. In the detergent-present assay, we observed 37% and 71% activity rate, respectively, for compounds that contained multiple phenolic hydroxyls or polyhalophenols substructures.

Among the categories of functional groups queried associated with little or no cruzain inhibition, there were over 250 phospho- containing filters such as various types of phosphonates, thio-phosphines, phosphor-containing warheads. Additional reactive groups such as benzylic quaternary nitrogens and quaternary nitroxy groups were also inactive despite 72 library compounds containing one of these substructures. Additionally, the library contained 508 oximes which were all inactive. Some nitrile containing compounds such as cyanamides (34 compounds) and cyanohydrins (39) were also all inactive. The library contained 41 trifluoromethyl ketones that also yielded flat response in the HTS. Only 1 of 158 perhalo ketones was active. Known alkylating agents such as epoxides had only two compounds result in inhibition. Lastly, all azeridines and thioepoxides were inactive.

The identification of 203 screening hits bearing reactive functionalities does not automatically equate to those 203 hits in fact acting on cruzain by non-specific irreversible covalent modification (although this mechanism of action would be highly likely for the compounds bearing true “hot” functionalities, such as the thiol-containing members): a

detailed investigation of all 203 hits would be required to answer this question, an undertaking which is outside the scope of this work. However, it is noteworthy that even if all 203 suspected reactives were to indeed act by that mechanism, their relative contribution (and similarly, that of the medchem exclusions types of hits) to the total hit rate observed here would be very small, far-outweighed by aggregation. Finally, as was noted for the aggregators and autofluorescent hits, relatively flat distribution of reactive and problematic hits was observed across library sources (Figure 1D). The greater contribution from two bioactives libraries (the LOPAC and Tocriscreen stets) likely reflects the provenance of most members which were selected for inclusion based on activities observed in cell-based or animal settings. On the other hand, significantly below average contribution of reactive hits was noted for the NCGC diversity collection because of the stringent exclusion of reactive compounds during its design and acquisition. Of the remaining 1,048 inhibitors, 550 were considered inconclusive (Category F in Figure 4, see 'inconclusives' description above). And finally, 493 compounds were categorized as top inhibitors of cruzain (Category G in Figure 4). This final list of inhibitors was 1.72% of the total number of actives found in the qHTS and 0.25% of the entire screening deck. The complete lists of compounds belonging to categories C-G are available at the following URL: http://ncgc.nih.gov/projects/cruzain. Further analyses and chemical elaboration of select top inhibitors will be described elsewhere.[39]

Hill Coefficient Analysis

Steep dose response curves for HTS hits, characterized by a Hill coefficient of greater than 1.5 or 2.0, may be harbingers of artifactual behavior.[9] As in our β-lactamase profile, the collection of concentration-response data at the primary screen level allowed us to evaluate an unprecedented number of dose responses (197,861 in the present study) in one experiment. The Hill coefficient distribution of the hits associated with the highest-quality dose-response curves and belonging to category C (3,844 conclusive aggregators), E (428 detergent-resistant inhibitors possessing reactive or undesirable functional groups), and G (493 filtered detergent-resistant conclusive inhibitors) is shown in Figure 6 A, with example screen-derived dose-response curves for members of each category shown in panels B-D. Compounds belonging to categories E and G (reactive and undesirable, and filtered inhibitors, respectively) were centered around Hill slopes of 1 and 2, with approximately 85% of the hits in each category belonging to those bins. In that regard, the reactive hits did not appear to be particularly biased toward steep concentration responses. On the other hand, the aggregators (category C) were spread more evenly across all bins, with slightly over a quarter of all aggregators displaying slopes of 3.0 or greater. Consistent with previous findings,[6] on the average the aggregators possessed steeper Hill slopes than both the filtered hits and the hits marked by reactive/undesired functionalities. Approximately 9% of the top filtered inhibitors within Category G were associated with steep Hill slopes of 3 and above. This set of compounds displayed no trend with respect to chemical structure or concentration-response curve characteristics other than the high Hill slope. It is possible that these compounds are borderline cases that fall near the cutoffs for aggregation phenotype.

CONCLUSIONS

This study is one of very few that undertakes a large scale, comprehensive analysis of artifactual "hits" in high-throughput screening, and the first to compare the major mechanisms side-by-side. Three key observations merit emphasis. First, aggregators once again far-outnumbered the actives acting by both the autofluorescent and reactive mechanisms. Thus, even for a system deliberately selected to be exquisitely sensitive to fluorescence interference and covalent modifiers the contribution of these two mechanisms remained minor. Furthermore, out of the many library members possessing suspected

reactive functionalities, very few were detected as inhibitors, indicating that those types of compounds do not contribute to the burden of post-HTS hit validation to the extent that is often assumed and in overall agreement with the notion advanced by many that even seemingly reactive compounds must first recognize the target and bind in an orientation relevant for a reaction. It appears that for a number of targets and assay formats compounds interfering with the assay signal are likely to create a greater burden on follow up than that associated with reactive library members.

A second observation that may surprise many investigators is that the occurrence of the interfering compounds was relatively constant among different compound sources. Thus, aggregates made up 2.17% of MLSMR compounds, and whereas they were less common among the “bioactive” collections deriving from Tocris, LOPAC and others, at between 0.5 and 2.1% they were still substantial. Whereas there are only 420 natural products in our library, making reliable quantification difficult, even these had a 1.2% of aggregates among them. This confirms previous studies, though on a much larger scale, that have suggested that at screening-relevant concentrations, few types of compounds may be considered to be immune from this property of colloidal aggregation in biochemical buffers. The same relatively flat distribution across library type was observed for the other major sources of interference, autofluorescence and problematic functionalities. Thus, when it comes to the physical behavior of molecules in HTS, one cannot count on the source of compound library to provide a “safe place”.

Finally, this study suggests that aggregation-based inhibition is context dependent. In parallel studies of aggregators using different enzymes, assay settings, and detection formats -the correlation between detergent-sensitive inhibition in different settings is far from perfect. Thus, many of the compounds that were found to act as aggregate-based inhibitors of AmpC β-lactamase were not inhibitors of cruzain. Much of this reflects the physical variables of assays performed at very low volumes, with high surface to volume ratios, the steep concentration dependence of aggregation, its stoichiometric inhibitory properties,[31] and its sensitivity to buffer conditions. It has remained tempting to assemble lists of permanent aggregators or unwanted compounds for use in triaging HTS hits or to pre-score a library. However, the present studies argue the opposite point: aggregation is highly variable and condition-dependent phenomenon, and compounds with purported reactive functionalities do not always react, casting doubt on the use of “blacklisted” compound filters, though no doubt some compounds really are ugly and should be removed. A simple and effective step toward reducing the post-HTS follow-up burden becomes obvious from this work, and from earlier studies: inclusion of detergent in the assay medium (whenever tolerated). Whereas the most reliable strategy may be parallel screens with and without detergent (in order to best annotate detergent-hardy aggregators), simply including detergent effectively leads to the disappearance of over 90% of the low micromolar false positives.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We thank Sam Michael, Adam Yasgar, and Paul Shinn with assistance with the robotic screens and compound management. This research was supported in part by the NIH Roadmap for Medical Research and the Intramural Research Program of the NHGRI, NIH (to CPA) and by NIH grant GM63815 (to BKS). The authors wish to thank the anonymous reviewers for their valuable comments during the manuscript review process.

Abbreviations

HTS	high-throughput screening
MLSMR	Molecular Libraries Small Molecule Repository
qHTS	quantitative high-throughput screening
DMSO	dimethyl sulfoxide
DTT	dithiothreitol, RFU, relative fluorescence units

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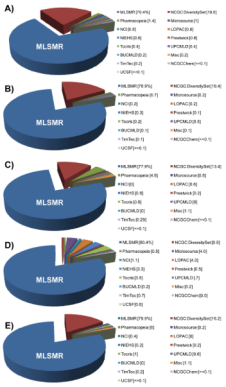


Figure 1.

Composition of the 197,861-sample library screened against cruzain (A) and similarity between the distribution of major screening hit categories across compound sources (artifacts [aggregators (B), autofluorescent (C)], reactive (D) and conclusive inhibitors (E) as defined in the text and Figure 4) and the library makeup.

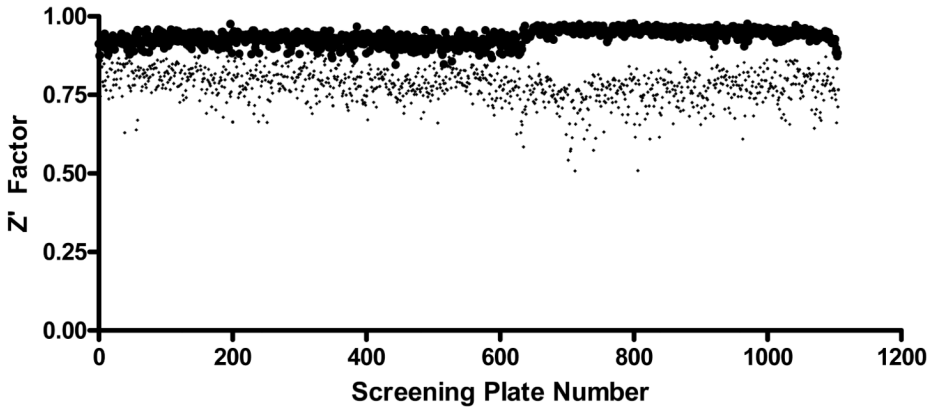


Figure 2.

Z′ trend of 1,106 plates screened in the detergent-free (small rhombs) and detergent-present (large circles) assays. The average Z′ of screen without detergent was 0.78 and improved to 0.93 with addition of detergent.

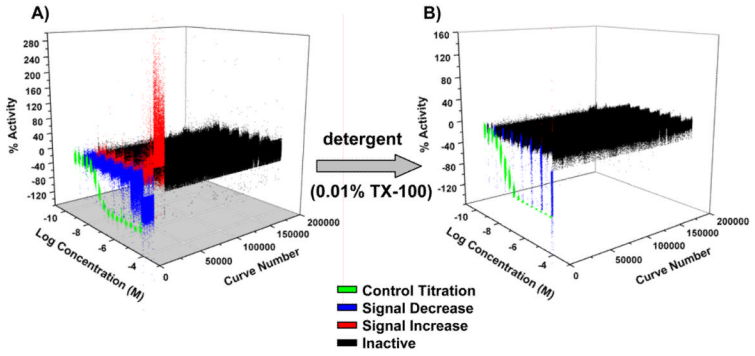
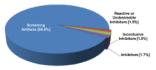


Figure 3.

Cruzain concentration-response data of control titrations and all library samples tested in the detergent-free (A) and detergent-present (B) assays. Control titrations (in green) were included on each 1,536-well plate screened. Apparent inhibitors are shown in blue, apparent activators are in red, and inactives are in black. A total of 14% of samples gave signal increase or signal decrease in the detergent-free while only 1% of samples resulted in any activity response in the detergent-present assay.



Category	Comment	Description	Number of Samples	% of all 'actives'	% of all screened
A	Screening Artifact	Detergent-sensitive 'activators'	12,746	44.00	6.44
B	Screening Artifact	Detergent-sensitive weak or inconclusive 'inhibitors'	10,389	35.90	5.26
C	Screening Artifact	Detergent sensitive 'inhibitors' (aggregators)	3,844	13.27	1.94
D	Screening Artifact	Detergent-resistant 'inhibitors' that are fluorescent artifacts	507	1.75	0.26
E	Problematic Inhibitors	Detergent-resistant inhibitors with reactive or undesirable groups	428	1.48	0.22
F	Inconclusive Inhibitors	Detergent-resistant filtered but weak or inconclusive inhibitors	550	1.90	0.28
G	Inhibitors	Detergent-resistant filtered inhibitors	493	1.70	0.25

Figure 4.

Categorization of hits. Signal-increase compounds lead to false ‘activation’ artifact (Category A) which disappeared completely upon inclusion of detergent. Among the signal decrease-compounds, a majority of the apparent inhibition was due to aggregation (category B and C). Kinetic reads during screening helped identify fluorescent false positives (Category D), while reactive and problematic functional group filtering eliminated additional promiscuous inhibitors (Category E). The remaining weak (Category F) or potent (Category G) inhibitors represented 3.6% of the total actives found in the detergent-free qHTS.

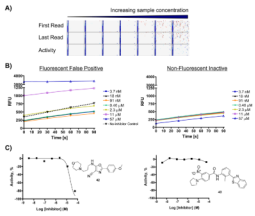


Figure 5.

Fluorescence Interference Analysis. A) Plate heatmaps associated with one 7-concentration compound series in 1,536-well format. Shown are the first and last fluorescent reads and the activity calculated from the 60-second initial rates. Subtracting out the higher-than-average but steady fluorescence of some compounds (red dots on heatmaps associated with first and last reads) leads to the significant reduction of interference (small number of blue dots in the activity heatmap). However, for compound **42** which is highly fluorescent (left plots in B and C), the drift in inherent fluorescence within the reaction time course (indicated within panel B) can lead to the erroneous calculation of concentration-response effect (C, left plot). A non-fluorescent inactive compound **43** (right plots in B and C) displays nearly overlapping reaction time courses (B, right plot) and as a consequence the corresponding concentration-response trend is relatively flat, resulting in the correct assignment of inactive phenotype (C, right plot).

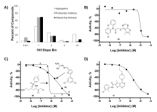


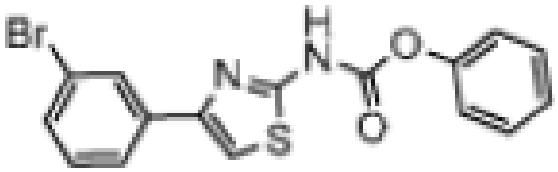
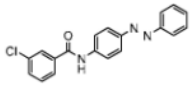
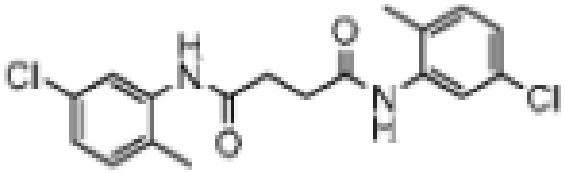
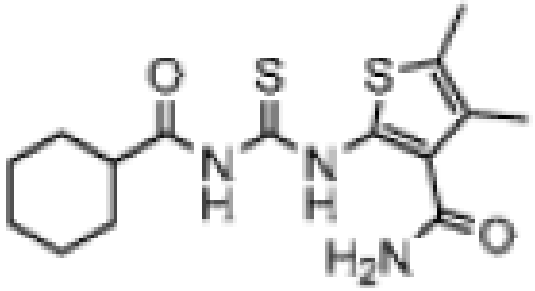
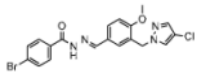
Figure 6.

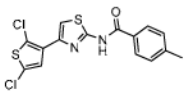
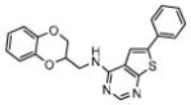
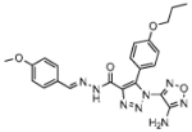
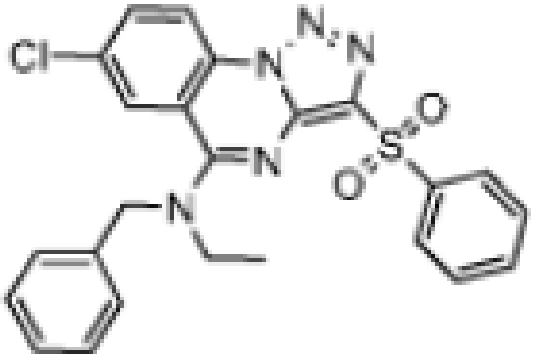
(A) Hill slopes of 3,844 Category C aggregators vs. 228 of Category E reactive or problematic inhibitors vs. 493 Category G top detergent sensitive inhibitors. (B) qHTS detergent-free curve of an aggregator SID 17508642 (**44**) with a Hill slope of 4. (C) Detergent resistant inhibition curves of two reactive compounds, SID 50107106 (**45**) (steep Hill slope) and SID 14739064 (**46**) (Hill slope of 0.5). (D) Detergent resistant cruzain specific inhibitor SID 24406445 (**47**) with Hill slope of 1.

J Med Chem. Author manuscript; available in PMC 2010 November 29.

Table 1

Retesting aggregation-based inhibition among compounds showing weak activity in the cruzain qHTS screen. ND, not determined, NC not conclusive (cruzain HTS category B).

Compound	Compound Structure and ID (ZINC/MLS)		Apparent aggregation IC_{50} (uM)		Aggregation in cruzain secondary assay (uM)
			qHTS AmpC	qHTS cruzain	
2		937394/ 000582777-01	ND	NC	30
3		5061076/ 000571688-01	ND	NC	12
4		670958/ 000535625-01	ND	NC	12
5		358914/ 000579888-01	ND	NC	12
6		8683095/ 000584672-01	ND	NC	12

Compound	Compound Structure and ID (ZINC/MLS)		Apparent aggregation IC_{50} (uM)		Aggregation in cruzain secondary assay (uM)
			qHTS AmpC	qHTS cruzain	
7		4076934/ 000564778	ND	NC	12
8		2632466/ 000334999	ND	NC	12
9		8684633/ 000331442	ND	NC	NC
10		8604460/ 00112246-01	ND	NC	12

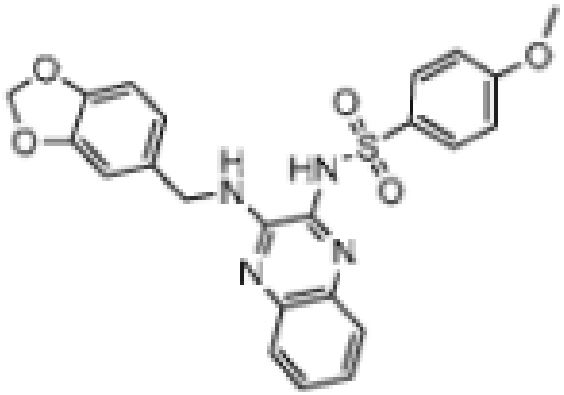
Compound	Compound Structure and ID (ZINC/MLS)		Apparent aggregation IC_{50} (uM)		Aggregation in cruzain secondary assay (uM)
			qHTS AmpC	qHTS cruzain	
11		2416530/ 000580367	ND	NC	12

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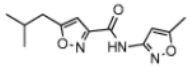
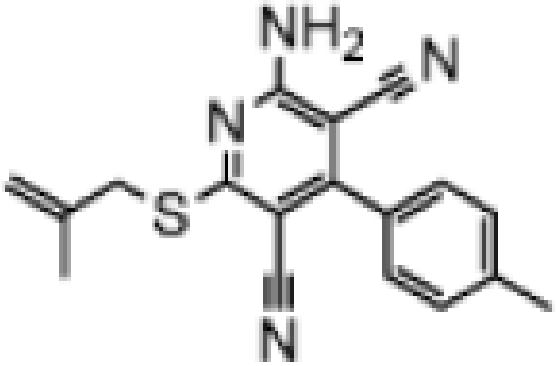
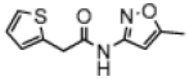
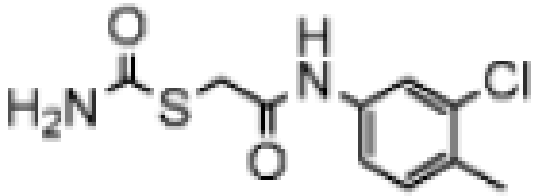
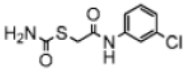
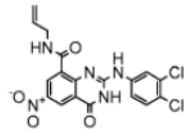
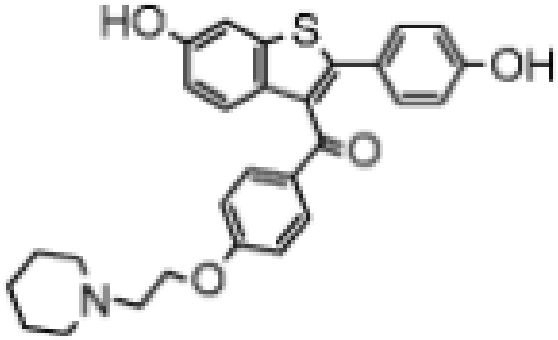
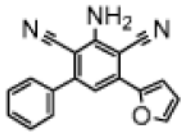
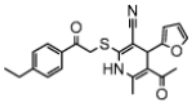
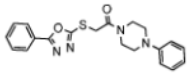
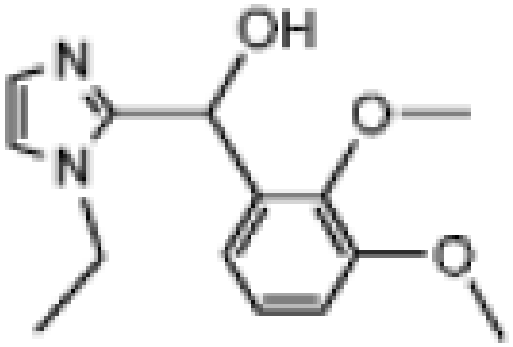
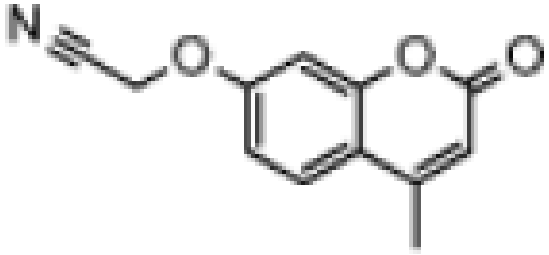
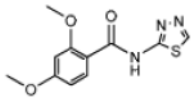
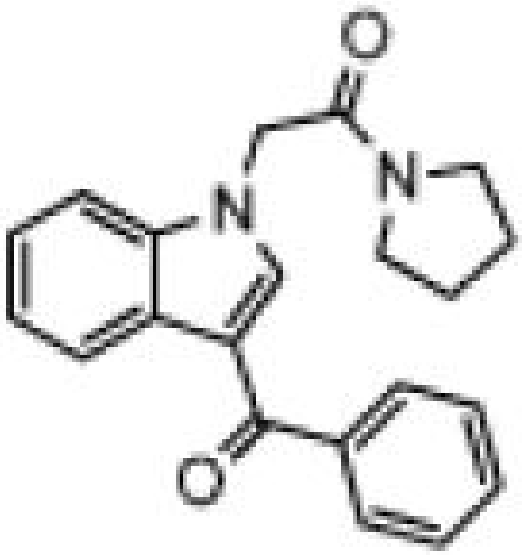
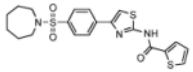
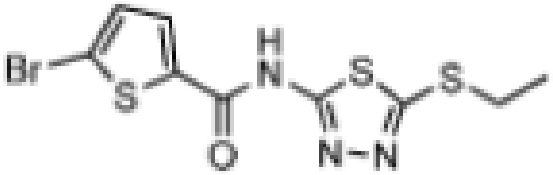
Table 2

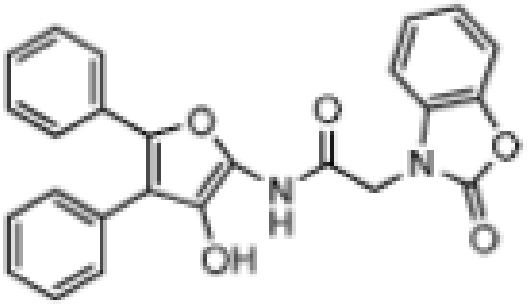
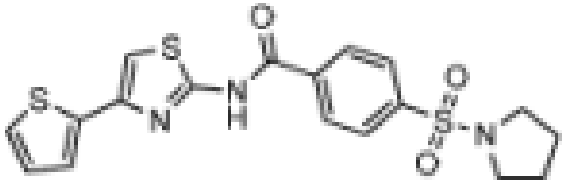
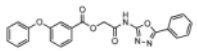
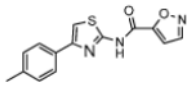
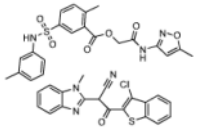
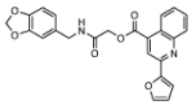
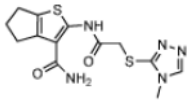
Retesting aggregation-based inhibition among compounds showing no activity in the cruzain qHTS screen, but active in the AmpC β-lactamase screen. ND, not determined, NC not conclusive.

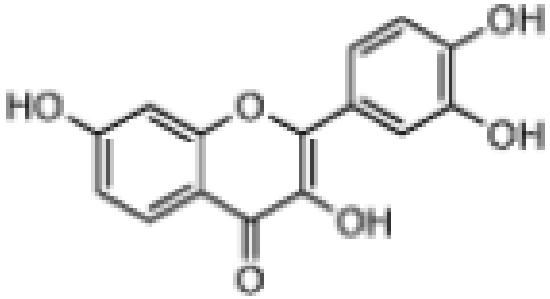
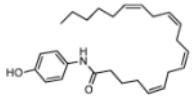
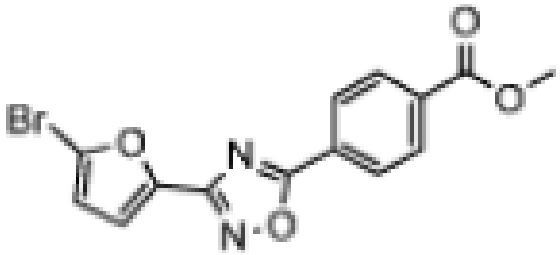
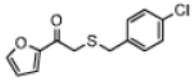
Compound	Compound Structure and ID (ZINC/MLS)		Apparent aggregation IC_{50} (uM)		Aggregation in cruzain secondary assay (uM)
			qHTS AmpC	qHTS cruzain	
12		2498105/ 000116408-01	4	> 60	120
13		4278501/ MLS000088971	6	> 60	12
14		116298/ NCGC00054143-01 MLS000062277	13	> 60	30
15		441313/ NCGC00078622-01 MLS000873495	13	> 60	> 120
16		441325/ NCGC00078398-01 MLS000108274	8	> 60	**> 120**

Compound	Compound Structure and ID (ZINC/MLS)		Apparent aggregation IC_{50} (uM)		Aggregation in cruzain secondary assay (uM)
			qHTS AmpC	qHTS cruzain	
17		3939153/ NCGC00092331-01	3	> 60	12
18		8022522/ NCGC00092320-01	2	> 60	NC
19		3952356/ NCGC00092316-01	13	> 60	> 120
20		538275/ NCGC00015889-01	5	> 60	NC
21		28097/ MLS000104455	6	> 60	12

Compound	Compound Structure and ID (ZINC/MLS)		Apparent aggregation IC_{50} (uM)		Aggregation in cruzain secondary assay (uM)
			qHTS AmpC	qHTS cruzain	
22		5020062/ MLS000107936	13	> 60	30
23		2720269/ 000064654-01	13	> 60	30
24		233889/ 000114196-01	4	> 60	> 120
25		1896423/ 000114241-01	4	> 60	> 120
26		338636/ 000114245-01	4	> 60	> 120

Compound	Compound Structure and ID (ZINC/MLS)		Apparent aggregation IC_{50} (uM)		Aggregation in cruzain secondary assay (uM)
			qHTS AmpC	qHTS cruzain	
27		573681/ 000115404-01	4	> 60	120
28		913228/ 00061701-01	8	> 60	12
29		1055160/ 00058956-01	10	> 60	12

Compound	Compound Structure and ID (ZINC/MLS)		Apparent aggregation IC_{50} (uM)		Aggregation in cruzain secondary assay (uM)
			qHTS AmpC	qHTS cruzain	
30		1071667/ 0060528-01	13	> 60	12
31		3204843/ 000054451	13	> 60	12
32		2663624/ 000097727	16	> 60	12
33		4144714/ 000081820	8	> 60	> 120
34		2654246/ 000081812	10	> 60	30
		1235408/ 000081882	13	> 60	12
35		3251243/ 000081948	13	> 60	30
36		3396507/ 000098416	10	> 60	120

Compound	Compound Structure and ID (ZINC/MLS)		Apparent aggregation IC_{50} (uM)		Aggregation in cruzain secondary assay (uM)
			qHTS AmpC	qHTS cruzain	
37		3472917/ 000056697	3	> 60	60
38		39111/ 00017344-01	13	> 60	30
39		3871503/ 00025010-01	10	> 60	30
40		116787/ 000119208-01	4	> 60	30
41		3265502/ 000098321-01	10	> 60	120

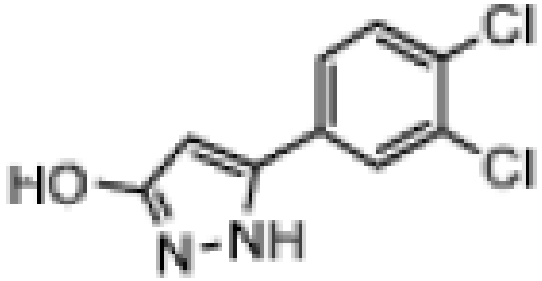
Compound	Compound Structure and ID (ZINC/MLS)		Apparent aggregation IC_{50} (uM)		Aggregation in cruzain secondary assay (uM)
			qHTS AmpC	qHTS cruzain	
42		5028122/ 000066902-01	13	> 60	12

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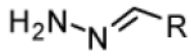
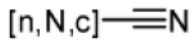
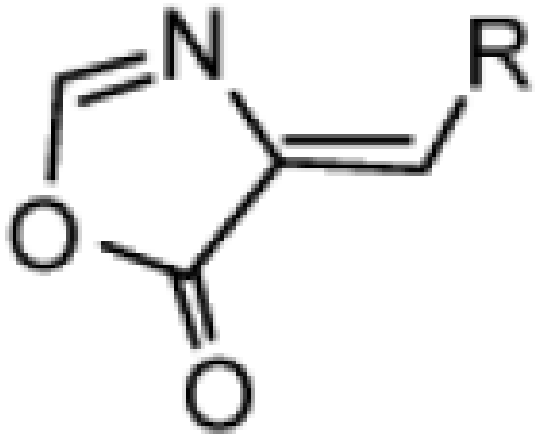
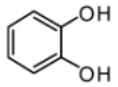
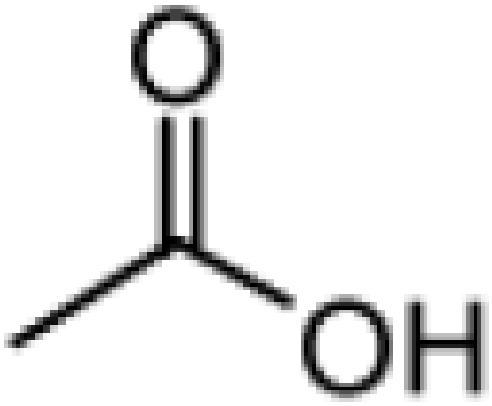
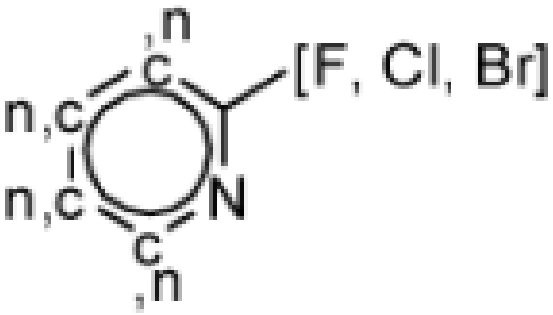
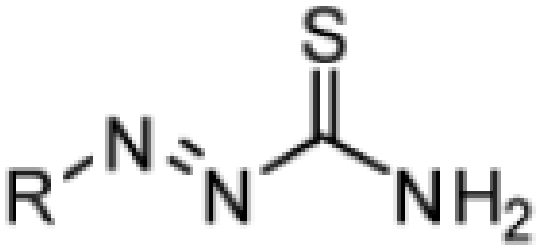
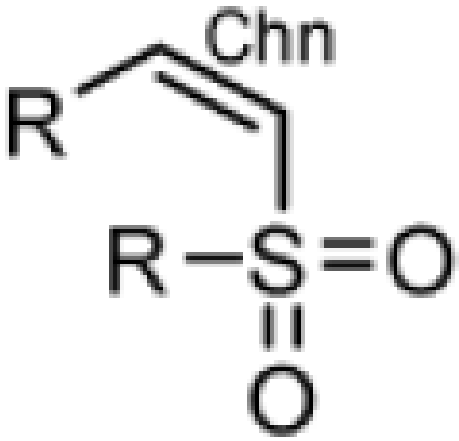
Table 3

Partial list of reactive or problematic functional groups. The number of detergent resistant cruzain inhibitors is provided along with total number of compounds that contain the substructure query. See Supporting Information Table S2 for full list of 234 queries.

Exclusion Filter	# Inhibitors	# Screeened
	139	6,999
	128	4,276
—SH	98	3,799
	21	1,208
	16	48
 (> 4 phenolic OH's)	13	35
Metals	12	157

Exclusion Filter	# Inhibitors	# Screeened
	11	360
	9	746
	6	19

Exclusion Filter	# Inhibitors	# Screeened
	0	15