DEVELOPMENT AND VALIDATION OF A GENERIC ASSAY TO DETECT COMPOUNDS ACTING VIA AN AGGREGATION-BASED MECHANISM

SUKRITI MALPANI
(B. Sc. (Hons.) Biological Sciences)
National University of Singapore

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Summary

High throughput screening (HTS) has emerged as a reliable component of the drug discovery process. It is now recognized that a large number of compounds inhibit their target enzyme via an aggregation-based binding mechanism leading to false positive results in HTS assays. Aggregate-forming compounds act non-competitively; show little relation between structure and activity; have steep dose-response curves and are reported to inhibit multiple unrelated enzymes (McGovern et al. 2002; McGovern et al. 2003; Feng et al. 2007). Removal of these compounds from screening hit lists is desirable as they are not good starting points to initiate medicinal chemistry programs. There are many techniques currently in use to identify aggregation-based inhibition such as dynamic light scattering (DLS), testing sensitivity of inhibition potency to detergent or enzyme concentration, and measurement of meniscus curvature changes in high density multi-well plates associated with colloidal changes in solution.

To evaluate the feasibility of large-scale identification of aggregate-based inhibition, hits from three enzyme screens (β-Lactamase, DENV RdRp and Pantothenate kinase) were analysed for signs of aggregate-based inhibitions using various techniques. For a majority of non-specific hits, characteristic features of aggregate-based inhibition such as steep dose-response curves, presence of aggregate particles in solution and inhibition of unrelated enzymatic targets were not found to be associated with detergent or enzyme-concentration sensitive inhibition. Particle size measurements by DLS were inconsistent for many compounds. Steepness of dose response curves depended on buffer composition and assay format employed.

Aggregate-based inhibitors displayed target specificity towards their respective target enzymes rather than 'promiscuous' inhibition of multiple targets.

Different detergents often yielded conflicting results and required derivation of new cut-offs for different enzyme systems or different assay conditions. For example, while the sensitivity of inhibition potency to detergent was not dependent on the nature of the detergent for hits of β-Lactamase, this was not the case for hits of the DENV RdRp enzyme. The inhibition potencies of the hits of DENV RdRp were found to have different degrees of sensitivity to different detergents. Furthermore, the results of the enzyme-concentration sensitivity tests for the DENV RdRp hits did not seem to correlate with the detergent-sensitivity results. It was observed that the interaction between the enzyme and its substrate possibly confounded the effect of varying the enzyme concentration.

The measurement of changes in meniscus curvature, as a means of identification of aggregate-forming small molecule compounds, has been used for the first time in an actual HTS campaign, as reported in this study. The meniscus measurements of hits from all screens correlated well with detection of aggregation-based inhibition based on measurement of changes in inhibition potency. A classification scheme is presented that can be used to rapidly characterize the hits from high throughput screens and eliminate compounds with a non-specific mechanism of inhibition. In summary, the meniscus-based aggregation assay is simple, cost-effective, and a reliable method to identify and eliminate compounds that inhibit a specific target enzyme via an aggregation-based mechanism.

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List of Abbreviations

Acetyl CoA	Acetyl coenzyme A
BBT	2′-[2-benzothiazoyl]-6′-hydroxybenzothiazole
BCS	Biopharmaceutical Classification System
BSA	Bovine Serum Albumin
BZBTH2B	Benzo(b)thiophene-2-boronic acid
CIP	Calf Intestinal Alkaline Phosphatase
CMC	Critical Micelle Concentration
DENV	Dengue virus
DLS	Dynamic Light Scattering
EC_{50}	Half maximal Effective Concentration
HTS	High Throughput Screening
IC_{50}	50% Inhibitory Concentration
LC-MS	Liquid chromatography-mass spectrometry
NMR	Nuclear Magnetic Resonance
NS5	Non-structural protein 5
PanK	Pantothenate Kinase
RdRp	RNA-dependent RNA polymerase
RNA	Ribonucleic acid
SAR	Structure Activity Relationship
TEM	Transmission Electron Microscopy
UTR	Untranslated region

1. Introduction

1.1 Introduction to High Throughput Screening

High throughput screening (HTS) is a widely used approach to discover novel chemical entities for drug design. In concert with the generation of large libraries of chemically diverse small molecules, the advancements in automation technologies have lead to growth of HTS programs in academia and industry (Inglese et al. 2007; Shelat and Guy 2007). A recent worldwide study involving 58 HTS laboratories has reported increasing numbers of leads identified by HTS over the years (Fig. 1) and documented 104 clinical candidates and four marketed products that have emerged from these leads (Fox et al. 2006).

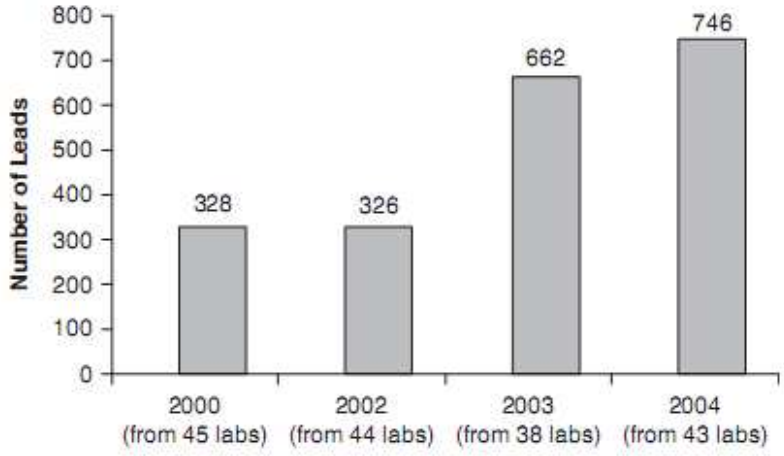

Figure 1: Historical comparison of number of leads found by HTS study participants. Reprinted with permission from "High-throughput screening: update on practices and success" by Fox et al in J Biomol Screen, 2006 11(7):864-869. Copyright 2006 by Sage Publications.

HTS methodology enables expeditious screening of sizeable chemical libraries to identify leads that act on a biological target of interest, e.g., as inhibitors of target enzymes, as competitors for binding of a natural ligand to its receptor, as agonists or antagonists of receptor-mediated intracellular processes, and so forth. HTS assays involve a variety of strategies such as the measurement of catalytic activity from a purified enzyme (Zhang et al. 1999), a reconstituted complex of a

signalling pathway (McDonald et al. 1999), a cellular extract (Verma et al. 2004), or measurement of phenotypic changes (Hodder et al. 2004) in intact cells. Configuring assays to function within the constraints imposed by high-throughput settings differentiates an HTS assay from traditional laboratory assays, as outlined in Table 1.

Table 1: Differences in allowed parameters between laboratory "bench top" and HTS assays. Reprinted with permission from "High-throughput screening assays for the identification of chemical probes" by Inglese et al. in Nat Chem Biol 2007;3(8):466-479. Copyright 2007 by Macmillan Publishers Ltd.

Parameter	Bench top	HTS
Protocol	May be complex with numerous steps, aspirations, washes	Few (5–10) steps, simple operations, addition only preferred
Assay volume	0.1 ml to 1 ml	<1 µl to 100 µl
Reagents	Quantity often limited, batch variation acceptable, may be unstable	Sufficient quantity, single batch, must be stable over prolonged period
Reagent handling	Manual	Robotic
Variables	Many-for example, time, substrate/ligand concentration, compound, cell type	Compound, compound concentration
Assay container	Varied-tube, slide, microtiter plate, Petri dish, cuvette, animal	Microtiter plate
Time of measurement	Milliseconds to months. Measurements as endpoint, multiple time points, or continuous	Minutes to hours. Measurements typically endpoint, but also pre-read and kinetic
Output formats	Plate reader, radioactivity, size separation, object enumeration, images interpreted by human visual inspection	Plate reader-mostly fluorescence, luminescence and absorbance
Reporting format	"Representative" data; statistical analysis of manually curated dataset	Automated analysis of all data using statistical criteria

1.2 Steps involved in setting up a high throughput screen

1.2.1 Assessment of assay quality

Large screens involving hundreds of thousands of compounds are expensive in time and resources. Thus before starting a large screen, it is important to assess the suitability or quality of the assay to be used in screening and ascertain if the assay would be useful in a high-throughput setting. A statistical term, called the Z or Z'-factor (Zhang et al. 1999), is commonly used to evaluate the quality of assays.

$$\text{Z-factor} = 1 - \frac{3(\sigma_p + \sigma_n)}{|\mu_p - \mu_n|}.$$

The Z or Z'-factor is defined in terms of four parameters: the means and standard deviations of both the positive (p) and negative (n) controls (μ_p, σ_p, and μ_n, σ_n). A Z-factor of 1 is considered ideal. This value is approached when there is a huge dynamic range (large difference between the signal means of the positive and negative controls) with small standard deviations. Z-factors can never be greater than 1. A value between 0.5 and 1 is aspired for in HTS settings. A Z-factor between 0 and 0.5 is considered sub-optimal. If an assay has a Z-factor that is less than 0, it implies that the signals from the positive and negative controls could overlap, making the assay essentially useless for screening purposes.

1.2.2 Primary screen

The goal of any HTS campaign is to identify active compounds ("hits") and choose the best candidates for lead optimization. This is achieved through a multitude of steps (Fig. 2). After an assay has been developed and validated, entire chemical libraries (hundreds of thousands to millions of compounds) are screened against the target of interest. Primary screening usually involves single measurements of the activity of each small-molecule compound. These single data points of unknown

samples are compared to positive and negative control samples to determine which compounds are active against the biological target. A robust assay with a Z-factor between 0.5 and 1 is conducive to single point testing. An assay with a sub-optimal Z-factor between 0 and 0.5 would require multiple data points for each compound to ensure reproducibility of the assay readout.

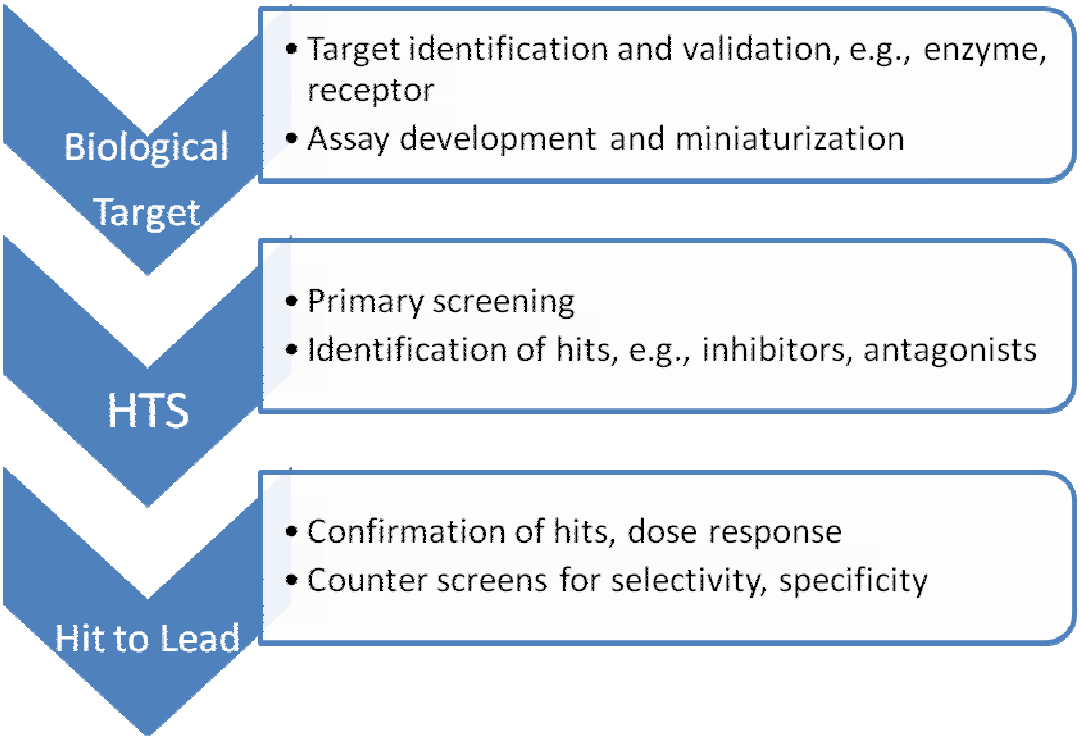

Figure 2: Illustration of steps involved in the initial drug discovery process

1.3 Hit to lead phase

The hit rate from a primary screen can vary between 0.1 and 1% (Eisenthal and Danson 2002) depending on the target, the assay format and the cut-off used to decide if a compound is considered 'active' or not. After selecting hits from compounds tested in the primary screen, the next step is to confirm the activity of these hits. Establishing a dose-response relationship is an important step in hit confirmation. It routinely involves a secondary screen in which a range of compound concentrations usually prepared by serial dilution are tested in an assay to assess the concentration or dose dependence of the assay's readout. Typically, this dose-

response is expressed as the 50% inhibitory concentration (IC_{50}) in enzyme-, protein-, antibody-based assays; or as the half maximal effective concentration (EC_{50}) in cell-based experiments. Compounds that display potency in a dose-dependent manner are chosen for further analysis.

1.3.1 Selectivity

Lead candidates should ideally interfere with only the chosen target, and not other, related targets. Selectivity toward a drug target decreases the risk of off-target toxicity that might occur in the clinical trial stage. Screens for selectivity usually include drug targets of the same protein or receptor family, for example, panels of G protein-coupled receptors (Swanson and Beasley 2010) or kinases (Fabian et al. 2005; Goldstein et al. 2008; Karaman et al. 2008). In cases where selectivity between subtypes is important, screens might include a panel of homologous enzymes, different protein complexes, or heterooligomers. Selectivity screens enable profiling of the action of a confirmed hit on a defined spectrum of biological target classes. Ideally, only those compounds which are highly selective towards the target of interest will progress to the next stage.

1.3.2 Evaluation of potential lead candidates

It has been studied that more often than not, marketed drugs are similar to the leads from which they originate (Proudfoot 2002). Therefore it is of utmost importance to choose the best hits to promote to lead status. The most desirable binding characteristics a ‘lead’ like compound should have are: non-covalent, high affinity ligand binding; reversible, competitive binding; and tractability in structure–activity relationship (SAR) of a series of structural analogues of the binder (Rishton 2003). Furthermore, it has been well established that potency alone is a false predictor of ‘lead’ likeness (Wunberg et al. 2006) and that an ideal lead molecule must exhibit a balance of potency, selectivity, and favourable physicochemical properties.

Therefore, merely re-confirming target inhibition is an inadequate measure of the quality of a hit as it doesn't necessarily ensure that the compound satisfies the required criteria. Many compounds can appear to possess 'lead' like characteristics in a HTS assay. However, false positives can result from multiple mechanisms, including: non-specific hydrophobic binding, poor solubility (protein or substrate precipitation), reactive functional groups, low purity, assay interference, aggregation-based enzyme inhibition and experimental errors (Keseru and Makara 2006). Some concerns, such as false positivity due to reactive functional groups, can be addressed by triaging of hit lists by medicinal chemists and elimination of compounds with undesirable chemical structures (Rishton 1997).

Other concerns such as assay interference require more intensive probing. Therefore hits are subjected to a battery of follow-up assays or counter screens to identify those that don't exhibit the intended biological interaction or falsely appear active due to confounding factors. The number and stringency of counter screens can vary widely and depend on the drug target. The next section provides an overview of some of the ways a compound can appear active in a biochemical assay without possessing any biological activity and strategies to identify these false positives.

1.4 Sources of false positives in high throughput screening

1.4.1 Interference in assay readout

Current HTS technologies are largely based on sensitive light based detection methods, such as fluorescence or luminescence, to quantify the effect of a compound on a target enzyme, receptor or signalling pathway (Inglese et al. 2007). These assay types are preferred because of their high sensitivity, flexibility across multiple homogeneous formats, ease of miniaturization, and applicability across a wide range of targets. However, they are highly sensitive to spectral artifacts (Shapiro et al.

2009). For instance, false negatives can occur due to light scattering, coloured, or fluorescent compounds that contribute to the net fluorescence signal. Small-molecule compounds are able to interfere with the fluorometric readout in many cases. The most straightforward interference results from spectral overlap between screening compounds and the assay system in optical and fluorescence assay formats (Gribbon and Sewing 2003). Compounds may falsely be identified as inhibitors if they absorb light at the detection wavelength of the fluorogenic substrate. In such cases, the net fluorescence signal measured in the assay will be attenuated by the compound to be tested. As a result, a reduction of the fluorescence signal is detected even in the absence of any interaction of the compound with the enzyme (Liu et al. 1999; Birdsall et al. 1983).

A recent study profiling the fluorescence spectral properties (Simeonov et al. 2008) of about 70,000 compounds (PubChem Assay IDs – 587-594,709) found that 2–5% of the compounds in the library fluoresced in the blue spectral region (~350-500 nm) and that for several fluorescence-based assays involving excitation in the blue spectral region, up to 50% of the hits identified in the screen were actually fluorescently active. The study further reported that when excited at red-shifted wavelengths (~600 nm); only 0.004–0.01% of the library fluoresced, indicating that use of red-shifted fluorophores is one way to reduce this mode of generation of false positives. Other methods to counter spectral interference are: inclusion of a pre-read after compound addition but prior to fluorophore addition to the reaction; inclusion of a time delay after excitation of fluorophore (time-resolved); use of a ratiometric fluorescence output; and use of an alternative assay to confirm the activity (Thorne et al. 2010).

1.4.2 Inhibition of detection system

Assay set-ups that employ enzyme-coupling systems are another example of a complex system that may suffer from detection interference. Many enzymes form reaction products that are not amenable to direct detection in an *in vitro* biochemical assay. To obtain a convenient spectral readout, the target enzyme's activity may be monitored by coupling its product to the reaction of an additional enzyme or auxiliary enzymes. The coupling reaction utilizes the target enzyme reaction product to produce a colorimetric (e.g., lactate dehydrogenase-coupled NADPH oxidation to detect pyruvate formation) or fluorescent (e.g., horseradish peroxidase-coupled fluorescent dye oxidation to detect H_2O_2 formation) or luminescent (e.g., luciferase-coupled detection of ATP production by kinases) signal. However, the coupled enzyme itself may be susceptible to inhibition by small molecules. For example, a profiling effort of a 70,000 compound library (PubChem Assay ID - 411) determined that at least 3% of the library inhibited firefly luciferase activity in a concentration dependent manner (Auld et al. 2008) demonstrating that HTS hit lists may contain a large number of compounds that inhibit the coupled enzyme rather than the target enzyme.

Direct assays can be carried out to test if apparent compound activity is due to inhibition of the coupling enzyme. Inhibitors of the coupling system can also be eliminated by counter screening hits using the same coupling system, but with a different target enzyme that produces the same reaction product as the original target enzyme (Seethala and Zhang 2009). If the other enzyme is related to the original target enzyme or from the same family, selectivity considerations can be addressed at the same time. Any compound that is positive in this counter screen may then be eliminated from consideration regardless of whether it inhibits the coupling enzyme or the undesired counter screening enzyme. Another method to distinguish between assay format-dependent inhibition and target-specific inhibition is to re-test the

activity of hits in an orthogonal assay, i.e. an assay that has a different readout compared to the format used in the original screening methodology (e.g., use of fluorescence readout as opposed to absorbance).

1.4.3 Aggregation-based enzymatic inhibition in biochemical assays

Compound aggregation, through self association of organic molecules in aqueous media, was recently discovered to be one of the main causes for false positives in HTS (McGovern et al. 2002). The study by Brian Shoichet's group reported that above a certain concentration some small-molecule compounds self-associate to form aggregate particles. These particles, at 30–400 nm in size, strongly scattered light detectable by dynamic light scattering and could be visualized by transmission electron microscopy (Fig. 3).

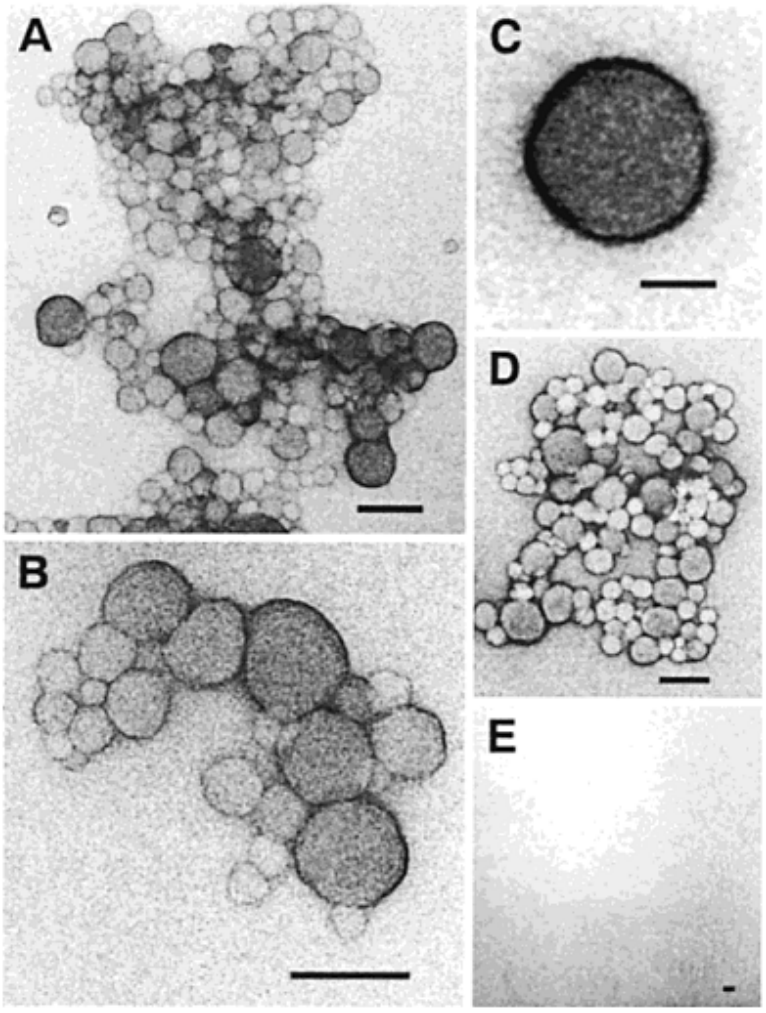

Figure 3: Aggregating compounds visualized by transmission electron microscopy (McGovern et al. 2002). A to C- 100 µM tetraiodophenolphthalein in 20 mM Tris; D- 50 µM Congo Red in 20 mM Tris; E- 625 µM ANS in 20 mM Tris. Bar = 100 nm. ANS – negative control. Reprinted with permission from "A common mechanism underlying promiscuous inhibitors from virtual and high-throughput screening" by McGovern et al. in J Med Chem 2002;45(8):1712-1722. Copyright 2002 by American Chemical Society.

These 'aggregators' that were initially identified as inhibitors of enzyme targets such as dihydrofolate reductase, thymidylate synthase, insulin receptor, tyrosine kinases, etc; were also found to inhibit several unrelated model enzymes such as β-Lactamase, β-Galactosidase and chymotrypsin. Decreased inhibition in the presence of bovine serum albumin suggested a non-specific mechanism of action and implied that inhibition by these molecules could be attenuated in the presence of excess protein. The compounds also showed sensitivity to the molar ratio of inhibitor to enzyme. Increasing the concentration of the model enzymes by 10-fold

significantly decreased the inhibition potency (increased the IC_{50}) of these 'aggregators' but not of classical, well-behaved inhibitors. To investigate if an aggregate-based inhibition model could explain the lack of specificity of many kinase inhibitors; 15 widely used known kinase inhibitors were analyzed for traits of non-specific behaviour (McGovern and Shoichet 2003). It was found that more than half of the kinase inhibitors also inhibited unrelated model enzymes, displayed sensitivity to enzyme concentration and formed aggregates of 100-1000 nm diameter as observed by dynamic light scattering. Due to their propensity to inhibit a panel of unrelated enzymes, inhibitors that act via an aggregation-based inhibition are often called 'promiscuous' inhibitors.

On the basis of the pilot studies, it was proposed that aggregate-forming compounds may be common in pharmaceutical screening libraries; and that such non-specific inhibitors could artificially inflate hit rates in screening for new drug leads. Since these compounds act non-competitively, show little relation between structure and activity (flat SAR), and have poor specificity, their elimination from hit lists could potentially save a great deal of effort that would otherwise be spent in trying to optimize their apparent activity (Borchardt et al. 2004). Therefore, Shoichet et al. have studied these aggregate-forming inhibitors in great detail and provided a better understanding of how they work; how frequently they occur in screening libraries; and techniques that can be used to detect aggregate-based inhibition; as described below in this section.

In an effort to understand the mechanism of aggregation-based inhibition, Shoichet's group studied the interaction of aggregate-forming inhibitors with model proteins like β-Lactamase. By using centrifugation and gel electrophoresis-based approaches, it was found that inhibition occurred via the direct binding of enzyme to aggregate (McGovern et al. 2003). β-Lactamase mutants with increased or decreased

thermodynamic stability relative to wild-type enzyme were equally inhibited by aggregate-forming compounds, suggesting that denaturation by unfolding was not the primary mechanism of action of aggregate-forming inhibitors. However, visualization by electron microscopy revealed that enzyme did associate with the surface of aggregated molecules. Interestingly, β-Lactamase inhibition by compound aggregation was found to be reversible by non-ionic detergents such as Triton X-100 (McGovern et al. 2003; Ryan et al. 2003). Since the enzyme was thought to be sequestered by the aggregated compounds, it was inferred that the presence of detergents either prevented formation of aggregates or interfered in the binding of enzymes by aggregated compounds.

Recently, the stoichiometry of binding of enzyme to aggregates was elucidated to be as high as 10,000 enzyme molecules per aggregate particle (Coan and Shoichet 2008). Given the size of the aggregates and the stoichiometry of binding, the aggregation model suggests that all sequestered enzyme can be accommodated on the surface on the aggregate (Fig. 4). This deviation from the classical 1:1 enzyme to inhibitor stoichiometry also explains another phenomenon generally associated with aggregate forming inhibitors, namely steep dose-response curves (Shoichet 2006;Feng et al. 2007). In the case of a classical, single-site inhibitor, inhibition usually increases from 10% to 90% over a large (81-fold) concentration range, whereas for compounds displaying steep dose-response curves the same increase in inhibition is observed within a 10-fold range of compound concentration. Since aggregate-forming inhibitors are known to form aggregates only above a certain concentration, usually in the micromolar range (Coan and Shoichet 2008), many aggregate-forming compounds are found to have steep dose-response curves with high Hill coefficients.

Shoichet and co-workers recently suggested that partial unfolding of the protein occurs upon aggregate binding (Coan et al. 2009). They examined changes in solvent accessibility of the β-Lactamase enzyme upon binding to an aggregate-forming inhibitor using hydrogen-deuterium mass spectrometry and noted that binding to aggregate particles increased deuterium exchange by the enzyme. This global increase in proton accessibility upon aggregate binding suggested a model consistent with partial denaturation of the protein (Fig. 4). This mechanism was confirmed by the observation that enzyme-aggregate complexes were more susceptible to tryptic proteolysis compared to free enzyme molecules.

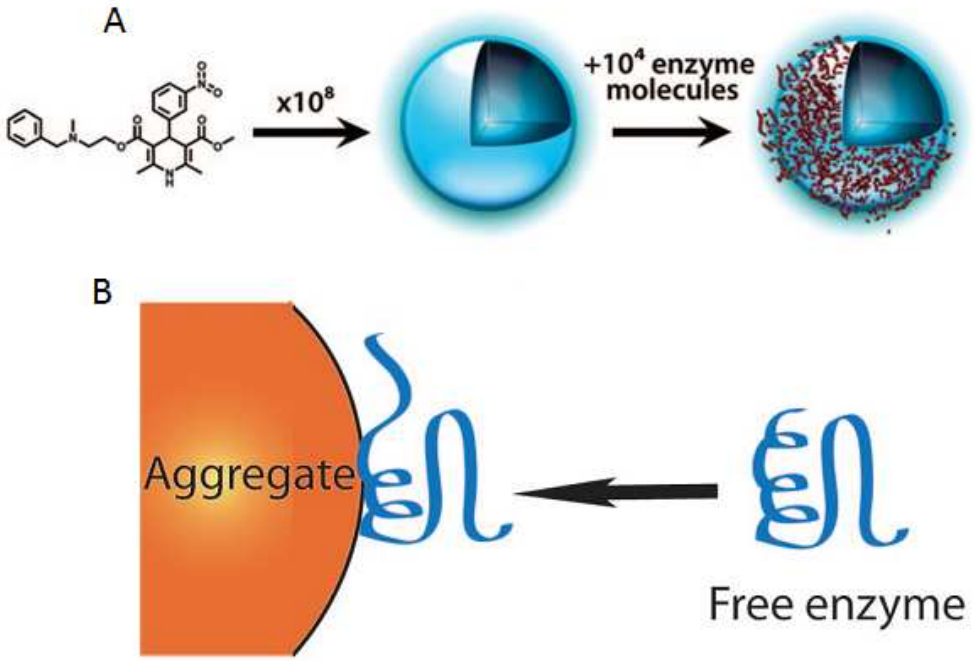

Figure 4: (A) Model of aggregate and enzyme binding. Reprinted with permission from "Stoichiometry and physical chemistry of promiscuous aggregate-based inhibitors" by Coan and Shoichet in J Am Chem Soc 2008;130(29):9606-9612. Copyright 2008 by American Chemical Society. (B) Mechanism of action of small-molecule aggregators – binding to the aggregate promotes a partial unfolding event. Reprinted with permission from "Promiscuous aggregate-based inhibitors promote enzyme unfolding" by Coan et al. in J Med Chem 2009;52(7):2067-2075. Copyright 2009 by American Chemical Society.

Subsequent to the initial studies on aggregate-forming inhibitors of β-Lactamase, aggregate-forming false positives have been discovered among inhibitors

of kinesin motor proteins (Reddie et al. 2006), phospho-mannomutase/phosphoglucomutase (Liu et al. 2004), and reverse transcriptase (Frenkel et al. 2005); establishing the incidence of this spurious mode of inhibition among inhibitors of various enzymes.

In an effort to estimate the prevalence of detergent-sensitive inhibition for a typical HTS involving a biochemical assay, investigators have tested various small-molecule libraries for enzyme inhibition sensitive to Triton X-100 using β-Lactamase as a model enzyme. In a 96-well format assay, it was found that 19% of the 1030 'drug-like' compounds tested demonstrated detergent-dependent inhibition when screened against β-Lactamase at 30 μM (Feng et al. 2005). For a library of ~ 70,000 compounds (PubChem Assay Ids- 584, 585), screened in a 1536-well assay format, 95% of the actives identified in the screen against β-Lactamase were Triton X-100 sensitive (Feng et al. 2007; Babaoglu et al. 2008). A screen of 200,000 compounds against the cysteine protease cruzain (PubChem Assay ID- 2249) revealed that approximately 1.9% of the library or 90% of the actives were detergent-sensitive inhibitors (Jadhav et al. 2010), indicating that the prevalence of this type of assay interference is neither library-specific nor limited to a particular type of enzyme, as cruzain and β-Lactamase are structurally and functionally different. Another study on cruzain inhibitors reported divergent modes of inhibition (competitive or aggregation-based) dependent on assay conditions, within a homologous structure-activity series, demonstrating that aggregate-based inhibition could be responsible for multiple logs of apparent(interpretable) SAR (Ferreira et al. 2009).

Recent studies have provided evidence that small-molecule aggregation exists in more biological contexts and is not just an artifact of in vitro high throughput biochemical assays. A study investigating the behaviour of aggregates in high protein concentrations found that aggregates appear to be more stable in *'in vivo'* like

conditions where serum protein is present in abundance (Coan and Shoichet 2007). Another study illustrating the ability of chemical aggregators to block amyloid fiber formation by yeast prion proteins and prevent infection of yeast cells by Sup35 prions (Feng et al. 2008) also points to the fact that aggregates have potentially widespread effects in biological systems of varying complexity.

Given the fact that many drug-like molecules and some known drugs (Seidler et al. 2003) are capable of forming colloidal aggregates there has been speculation that aggregation may affect the bioavailability of drugs within the body. To address this concern, researchers tested Biopharmaceutics Classification System (BCS) class II and class IV drugs for aggregate formation in a buffer mimicking conditions in the small intestine (Doak et al. 2010). It was found that six of these drugs formed colloids at concentrations equal to or lower than the concentrations reached in the gut, suggesting that aggregation may have an effect on the absorption and *in vivo* distribution of these drugs.

In a nutshell, screening hit lists appear to be inundated by aggregate-forming inhibitors. These hits are deceptive as the inhibition is reproducible (i.e., these compounds will consistently inhibit the target under the same experimental conditions) and dose-dependent. However, their mode of activity is undesirable; and the lack of sensitivity of their biological activity to structural changes (flat SAR) makes them poor starting points for medicinal chemistry.

1.4.3.1 Detection of aggregation-based inhibition

This section provides an overview of the different methods currently is use for detection of aggregation-based inhibition; and their advantages and limitations. Some methods of aggregation detection rely on characteristics of aggregate-based inhibition such as steep dose-response curves; sensitivity to detergent, enzyme

concentration or presence of bovine serum albumin (BSA). While many aggregate-forming inhibitors have steep dose-response curves (Feng et al. 2007); this phenomenon is not exclusive to aggregate-forming inhibitors and could simply be a sign of potent inhibition (Straus et al. 1943; Shoichet 2006). Addition of BSA to the assay buffer is practicable in most cases, but BSA has binding sites for various drug classes and should be used with care as it can interfere with the binding of the compound to the target enzyme by sequestering small drug-like molecules (Bi et al. 2009).

It has been established that addition of 0.01-0.1% Triton X-100 to the assay reagents leads to significant attenuation of aggregate-based inhibition (McGovern et al. 2003; Feng et al. 2005; Feng et al. 2007). The most rapid method for identifying aggregate-based inhibitors is therefore to repeat screening in the presence of a detergent such as Triton X-100 and check for loss of potency. However, high amounts of detergent can have a deleterious effect on enzymatic activity (Manandhar et al. 2007; Nishiya et al. 1998) or influence reporter enzymes such as firefly luciferase (Simpson and Hammond 1991); limiting the utility of detergents in investigation of aggregation-based inhibition in such scenarios.

It is possible to detect aggregation-based non-specific inhibition by determining the IC_{50} value of a compound at different enzyme concentrations. If the enzyme kinetics follows the Michaelis-Menten model, the IC_{50} should be invariant with respect to enzyme concentration, since the latter is negligible compared to substrate and inhibitor concentration. However this does not hold good for 'aggregators' as the effective concentration of the inhibitory species would be much lower when compared to a classical inhibitor that binds with a stoichiometry of 1:1 (Shoichet 2006). Thus increasing the enzyme concentration would cause a decrease in compound potency as the number of enzyme molecules would no longer be

significantly lower than the number of aggregate particles. Testing sensitivity to enzyme concentration is a convenient means of identifying aggregation-based inhibition if the assay format is sensitive enough to measure enzyme activity over a large range of enzyme concentration and enzyme kinetics are well characterized.

Researchers have suggested that it might be useful to flag or eliminate aggregate-forming inhibitors from compound libraries by either testing for detergent-sensitive inhibition against a model enzyme such as β-Lactamase or testing for inhibition of a panel of unrelated enzymes. But a compound that inhibits one target non-specifically might well be a potent, specific inhibitor of another target and a compound that aggregates at a higher concentration may have legitimate biological activity at lower concentrations (McGovern et al. 2003; Seidler et al. 2003). In addition, whether or not a compound will act via an aggregation-based mechanism is dependent on the properties of the compound itself, the assay conditions (Ferreira et al. 2009; Jadhav et al. 2010) and the protein target (Giannetti et al. 2008). For these reasons, and because compounds that aggregate are structurally diverse (McGovern et al. 2002), interference due to aggregate-based inhibition might need to be empirically determined for a given assay (Inglese et al. 2007).

Techniques such as dynamic light scattering or electron microscopy have been in use to directly observe or measure aggregate particles. However, these methods are typically low throughput (McGovern et al. 2003; Frenkel et al. 2005). There have been other approaches such as NMR-based detection (Dalvit et al. 2006), surface plasmon resonance based biosensors (Giannetti et al. 2008), and photonic crystal biosensor microplates (Chan et al. 2009) to identify aggregate-forming inhibitors by measuring binding of compounds to the target enzyme. In addition to the lack of validation of these methods in an actual HTS campaign, they are resource intensive and require use of specialized apparatus.

1.5 Aim of the project

The early and relatively less costly elimination of undesirable or intractable lead classes such as aggregate-forming inhibitors is of significant value before extensive medicinal chemistry and pharmacokinetic profiling efforts are initiated. The aim of this project, therefore, is to investigate generic mechanisms to detect aggregation-based inhibition.

While there are many techniques being used currently such as sensitivity to detergent or enzyme concentration; as outlined in the previous section, there are circumstances under which they may not be useful. It would be of interest to apply these techniques to actual HTS programs to establish viability of application. Furthermore, a generic assay that could be applied to any HTS campaign to eliminate inhibitors acting via an aggregation-based mechanism would be of great benefit.

Recently, a potential generic assay for detection of aggregation-based false positives based on the pronounced capillarity of colloidal solutions in the high-density, multiwell plates used in HTS has been developed (Cai and Gochin 2007). Unlike a regular spectrophotometer, where the light path is horizontal and does not pass through an air-water interface, the principle of this assay is based on the effect of curved meniscus on spectrophotometric measurement using a plate reader with vertical light path. The shape of the meniscus has a significant effect on fluorescence intensity when detected using a top read fluorescence plate reader due to the light path of the device being dependent on the whether the liquid surface is curved or flat (Cottingham et al. 2004). The effect is normally avoided in HTS by adding a small amount of non-denaturating surfactant to the assay reagents, but if no detergent is present, the colloidal particles reduce the surface tension and the resulting change in the shape of the meniscus can then be quantified. The viability of this approach was demonstrated with a handful of known ‘aggregators’ and ‘non-aggregators’. Good

separation was observed between the two classes of compounds and Z'-factor of 0.76 was reported for the assay (Cai and Gochin 2007).

The aim of this project is to evaluate the above described meniscus-based assay with regard to its applicability in real-life HTS of enzyme targets and assess predictability and correlation with more frequently used methods such as sensitivity to detergent or enzyme concentration, dynamic light scattering, multiple enzyme inhibition etc. As test cases, inhibitors of three different enzymes: *E. Cloacae* β-Lactamase, *M. tuberculosis* Pantothenate kinase (PanK), and Dengue virus RNA-dependent RNA polymerase (DENV RdRp); are used in this study.

2. Materials and Methods

2.1 β-Lactamase primary screen and secondary assays

2.1.1 Primary screen

Purified *E. cloacae* P99 β-Lactamase (Sigma) was used in all experiments. The assay buffer consisted of 25mM PIPES/KOH, pH 7, 10% (v/v) glycerol, 1mM dithiothreitol, and 2mM $MgCl_2$ (Ryan et al. 2003). The compound library Novartis2008 (Novartis Institute for Tropical Diseases), which was used in the β-Lactamase screen, consisted of 8272 compounds. Benzo(b)thiophene-2-boronic acid (BZBTH2B), purchased from Sigma, served as the reference inhibitor for the enzyme. Required volumes of compounds and controls were transferred to the assay plates from stock solutions stored in 96-well polypropylene plates (Corning Costar) using the Mosquito liquid handling system (TTP Labtech). All compounds were screened at 20μM in single point. Assays were performed in 384-well clear plates (Corning Costar). Each assay plate contained compounds in columns 1-22; and 16 wells each of the total (DMSO vehicle) and blank (100μM BZBTH2B) controls in columns 23 and 24. Enzyme and substrate concentrations were optimized to obtain linear reaction progress curves within a 5 min time course. The enzyme was present at 2.5nM in a final reaction volume of 50μl. Reactions were initiated by addition of the chromogenic substrate CENTA (Invitrogen) at a final concentration of 25μM. CENTA hydrolysis was monitored at room temperature by measuring absorbance at 405nm on plate reader (Safire2, Tecan). The enzyme activity was calculated as mean OD/min.

2.1.2 Secondary assays using chromogenic substrate

Using the same assay conditions as described above (enzyme and substrate present at 2.5nM and 25μM respectively in a final reaction volume of 50μl), chosen compounds were subjected to dose-response studies. Each assay plate contained

compound dilutions in columns 2-23, and 16 wells each of the total (DMSO vehicle) and blank (100μM BZBTH2B) controls in columns 1 and 24. Dose-response curves contained 8 concentrations of compounds obtained using 3-fold serial dilution. Freshly prepared solutions of Tween-20 (Sigma), Triton X-100 (Thermo Scientific) and CHAPS (Amresco) were added to the enzyme preparation at the specified concentrations in the detergent (+) dose-response studies.

2.1.3 Secondary assays with fluorometric readout

For the fluorometric procedure, soluble fluorocillin green (Invitrogen) was used as substrate in the same assay buffer used in the primary screen. The solid fluorocillin substrate was dissolved in DMSO as per manufacturer's instructions and then diluted in assay buffer. Enzymatic hydrolysis of the lactam ring of fluorocillin yields a green fluorescent product which can be measured at wavelengths of 495 nm (excitation) and 525 nm (emission). Dose-response curves containing 8 concentrations of 3-fold serially diluted compounds were obtained in 384-well black plates (Corning Costar) under the following reaction conditions: (1) 0.5nM enzyme in assay buffer with no detergent, (2) 0.5nM enzyme with assay buffer containing 0.005% Tween-20 and (3) 5nM enzyme in assay buffer with no detergent. In all reactions, substrate was present at 2.5μM in a final reaction volume of 50μl. Each assay plate contained compound dilutions in columns 2-23, and 16 wells each of the total (DMSO vehicle) and blank (100μM BZBTH2B) controls in columns 1 and 24. Enzyme activity was measured at room temperature over the course of 5 min and 100s for 0.5nM and 5nM of enzyme, respectively, on an Infinite M1000 plate reader (Tecan).

2.1.4 Data analysis

Primary screen data were analyzed in IDBS ActivityBase. Z-factors were calculated based on the means of the total and blank controls using the formula

described previously (see Introduction). Percent inhibition was computed from the mean values of the total (uninhibited) and blank (100% inhibition) controls using the formula: % Inhibition = 100*(1-((mean$_{sample}$- mean$_{blank}$)/(mean$_{total}$- mean$_{blank}$))). Dose-response curves were analyzed using GraphPad Prism 5 (GraphPad Software). IC_{50} values were determined using a nonlinear regression fit assuming a sigmoidal dose–response model with variable slope.

2.1.5 Dynamic light scattering analysis

Measurements were performed using a Zetasizer Nano (Malvern Instruments) with a He-Ne laser (633 nm) and 173° collecting optics. The software used to collect and analyze the data was the Dispersion Technology Software version 5.03 (Malvern Instruments). β-Lactamase assay buffer (25mM PIPES/KOH, pH 7, 10% (v/v) glycerol, 1mM dithiothreitol, and 2mM $MgCl_2$) was filtered using a 0.2-micron pore size filtration unit (Millipore) before using it to dilute compounds. Disposable solvent resistant cuvettes (ZEN0040, Malvern) were used for measurements. The solvent builder feature of the software was used to estimate viscosity and refractive index of the assay buffer. Samples were equilibrated for 2 min before measurements at room temperature. The number of scans (ranging from 12-25) was determined by the DLS software based on the quality of the sample. The fluctuations in scattering intensity for each sample were averaged by the software, neglecting outliers due to contaminants such as dust, to yield the size distribution for that sample. For each compound, three independent measurements were made.

2.2 DENV RdRp assay principle, hit selection and follow-up assays

2.2.1 Assay principle, compound screening and hit selection

A novel fluorescence-based alkaline phosphatase-coupled polymerase assay was recently developed at the Novartis Institute for Tropical Diseases (Niyomrattankit et al. 2011) to discover new inhibitors of dengue virus RNA-

dependent RNA polymerase (DENV RdRp). The assay involves use of an adenosine nucleotide modified by attaching the 2′-[2-benzothiazoyl]-6′-hydroxybenzothiazole (BBT) fluorophore group to the γ-phosphate (BBT-ATP); and 3'UTR-U_{30} RNA as substrates. During polymerase reaction, adenosine monophosphate is incorporated into the RNA resulting in the release of non-fluorescent BBT-PP_i, the RdRp reaction by-product. Subsequent treatment of the reaction with Calf Intestinal Alkaline Phosphatase (CIP) in high pH buffer terminates RdRp activity and liberates highly fluorescent BBT molecule from BBT-PP_i. Measurement of the final reaction product serves as an indirect measure of RdRp activity.

A compound library of diverse structures selected from various vendors, comprising 40,572 compounds; was used in a pilot screen to find compounds active against DENV RdRp (Niyomrattanakit et al. 2011). The DENV non-structural protein 5 (NS5) protein of Dengue virus serotype 4 containing the RdRp domain was expressed as described. BBT-ATP was synthesized by and purchased from Jena Bioscience GmbH. Reference inhibitor 3'dATP, which functions as a chain terminator, was purchased from Trilink Biotech. RNA substrate 3'UTR-U_{30} was purchased from Dharmacon. The CIP enzyme was purchased from New England Biosciences. The screen was performed in 384-well black plates in a total of 118 plates. Each plate contained compounds in columns 1-22 and 16 wells each of the total (DMSO vehicle) and blank (20μM 3'dATP) controls in column 23 and 24. The polymerase reaction was run in optimized buffer for NS5 RdRp consisting of 50mM Tris-Cl (pH 7.0), 1mM $MnCl_2$, and 0.01% Triton X-100. The NS5 protein and the 3'UTR-U_{30} substrate were mixed in assay buffer at concentrations of 40nM and 100nM respectively and incubated at room temperature for 30 min. To each well of the assay plate containing either compound or control, 5μl of the above solution was added. Reaction was initiated by addition of 5μl of the BBT-ATP substrate at a concentration of 4μM. The final reaction volume of 10μl containing 20nM RdRp,

50nM 3'UTR-U_{30} RNA substrate and 2μM BBT-ATP was incubated for 1hr at room temperature followed by addition of 10μl of stop buffer (25nM CIP, 200mM NaCl, 25mM $MgCl_2$, 1.5M deoxyethanolamine) to inactivate RdRp and to hydrolyze the BBT-PPi. Plates were read after 1 hr incubation at room temperature to ensure complete hydrolysis of BBT-PPi by CIP. The fluorescence was measured at wavelengths of 422 nm (excitation) and 566 nm (emission) on an Infinite M1000 plate reader.

The Z-factor averaged from 118 plates was found to be 0.81 with SD value at 0.05. Compounds with greater than 30% inhibition (calculated from 3×SD of sample) were selected as hits for reconfirmation (407 compounds in total). All compounds that auto-fluoresced or inhibited the coupling enzyme CIP were eliminated from the hit list. The remaining compounds were subjected to liquid chromatography-mass spectrometry (LC-MS) analysis to confirm compound purity. A total of 30 compounds passed all the required selection filters and these 30 compounds are the subject of the various follow-up assays performed in this study.

2.2.2 Testing inhibition potency of hits in different detergents

The 30 compounds chosen for follow-up were subjected to dose-response studies in the presence of different detergents in the assay buffer. Desired amounts of freshly prepared Triton X-100, Brij-35 (Thermo Scientific) and CHAPS were included in the assay buffer (in place of the 0.01%Triton X-100 present in the assay buffer used in the pilot screen). Dose-response curves containing 10 concentrations of 3-fold serially diluted compound were obtained in the presence of low and high amounts of the above mentioned detergents in the assay buffer (2 concentrations of each detergent giving 6 dose-response curves for each compound). Each assay plate contained compounds in columns 1-11 and 14-23, and 32 wells each of total (DMSO vehicle) and blank (20μM 3'dATP) controls in columns 1, 12, 13 and 24. All other

reaction parameters such as RdRp, 3'UTR-U_{30} and BBT-ATP concentrations, incubation times, and reaction termination procedure were identical to conditions used in the pilot screen.

2.2.3 Testing inhibition potency of hits at varying enzyme concentrations

Dose-response curves were obtained at 10nM and 100nM RdRp in order to test the effect of enzyme concentration on inhibition potency. The concentration of the BBT-ATP substrate was kept constant at final concentration of 2μM and the buffer composition was the same as the pilot screen except for the Triton X-100 concentration (0.002% instead of 0.01%) in all reactions described below. For reactions involving 10nM RdRp, compounds were tested separately at two different RNA concentrations. At concentrations of 50nM and 150nM 3'UTR-U_{30} RNA, reactions were incubated at room temperature for 60 min and 100 min respectively before inactivation by CIP. For reactions involving 100nM RdRp, 150nM of 3'UTR-U_{30} RNA was used and reaction was allowed to progress for 20 min before termination by CIP. Dose-response curves contained 10 concentrations of each compound obtained by 3-fold serial dilution. Each assay plate contained compounds in columns 1-11 and 14-23; and 32 wells each of total (DMSO vehicle) and blank (20μM 3'dATP) controls in columns 1, 12, 13 and 24.

2.2.4 Effect of Triton X-100 on kinetic constants of DENV RdRp

Apparent K_m and V_{max} values for 3'UTR-U_{30} RNA substrate were obtained at different Triton X-100 concentrations by plotting the observed BBT production as a function of RNA concentrations. RNA concentrations ranging from 0-70 nM were assayed at 10nM RdRp with BBT-ATP concentration at 2μM. Time course of the reaction were analyzed by linear regression to obtain the slopes in RFU/min. These values were then converted to pmole/min using a standard curve of BBT obtained as described previously (Niyomrattanakit et al. 2011). Using GraphPad Prism 5

software, initial rates (v) of enzyme activity were fit into the Michaelis–Menten equation $v = V_{max} \{[S]/([S]+K_m^{app})\}$ where the V_{max} and K_m^{app} are the maximum velocity and Michaelis–Menten constant, respectively, and [S] is the substrate concentration.

2.3 Selection of compounds from PanK hit list

A high throughput screen of 1.2 million compounds from the Novartis Compound Archive was performed recently against the *M. tuberculosis* Pantothenate Kinase (PanK) enzyme (Habig et al. 2009). Briefly, compounds were tested for inhibition of PanK activity in a luminescence based assay (using the Kinase-Glo Plus kit by Promega). To eliminate readout artifacts, primary hits were re-tested in a polarization-based assay for detection of ADP (employing the Transcreener KINASE Plus Assay kit by BellBrook Labs). For both formats, standard reaction buffer contained 50 mM HEPES (pH 7.5), 4 mM $MgCl_2$, 2 mM EGTA, and 50 mM NaCl. The 2022 compounds that showed a defined dose-response curve in both readouts were tested for shift in potency on variation of enzyme concentration and addition of detergent to the assay buffer. Based on the assumption that ATP-competitive inhibitors interact specifically with the enzyme, the authors used ATP-competitive inhibitors among the hits to assess the feasibility of using detergent or enzyme-concentration sensitivity to identify non-specific inhibitors. A cross-comparison revealed that the potency of 535 ATP-competitive compounds was sensitive to detergent concentration whereas the potency of only 103 of 788 ATP competitive compounds was substantially affected by an increase in enzyme concentration. Since enzyme concentration sensitivity appeared to be more predictive than detergent sensitivity in identification of non-stoichiometric inhibitors of PanK, only those hits that were found to be insensitive to enzyme concentration were analyzed by NMR to confirm binding to enzyme.

Due to the fact that these compounds have been the subject of detailed analyses, their mode of inhibition was known. This made them ideal candidates for determination of predictive value of different techniques to detect non-specific inhibition. Based on compound availability, a subset of 15 enzyme-concentration sensitive and 26 enzyme-concentration insensitive PanK hits were chosen from hit list and obtained from the Novartis Compound Archive. These 41 compounds and a reference inhibitor of PanK, acetyl coenzyme A (Acetyl CoA); were used in other assays as described below.

2.4 Measurement of change in meniscus

The assay was first optimized in the β-Lactamase assay buffer with dyes such as fluorescein (Fluka) or lucifer yellow (Invitrogen); which allowed quantification of change in meniscus shape due to the effect light-path length on fluorescence emission through a curved liquid surface. For both dyes, the fluorescence of the dye measured in assay buffer containing 1mM Triton X-100 or 1mM Tween-20 (control buffer) was found to be between 40-50% lower than fluorescence of the dye in assay buffer without detergent. Fluorescein dye was chosen for compound measurements based on the recommendation of the authors who developed this assay (Cai, personal communication).

A concentration of 0.1μM fluorescein was found to be optimal for use in all assay buffers tested in this study (β-Lactamase, DENV RdRp and PanK assay buffers). Hits of all three enzymes (14 hits from β-Lactamase primary screen, 30 DENV RdRp hits and 41 hits chosen from the PanK hit list) were tested at 20μM in their respective assay buffers. Reference inhibitors for each enzyme were also tested (β-Lactamase - BZBTH2B, DENV RdRp - 3'dATP and PanK- Acetyl CoA). Compounds were added to 384-well black plates followed by addition of 30μl of either assay buffer or control buffer (assay buffer with 1mM Triton X-100 or 1mM Tween-20). After allowing the signal to stabilize for 30min, fluorescence was

measured at wavelengths of 490 nm (excitation) and 514 nm (emission) on an Infinite M1000 reader. Relative fluorescence was measured as a ratio of the observed fluorescence of dye in assay buffer to that in control buffer. Each compound was assayed in triplicate.

3. Results

3.1 β-Lactamase primary screen and follow-up assays

3.1.1 Hit Selection and re-confirmation

A total of 8272 compounds from compound library Novartis2008 were screened against *E. cloacae* β-Lactamase in detergent-free conditions in 24 assay plates. Z-factor remained above the 0.5 cut-off across all plates (Fig. 5). Average Z-factor for the 24 assay plates was 0.71 with an SD value at 0.05.

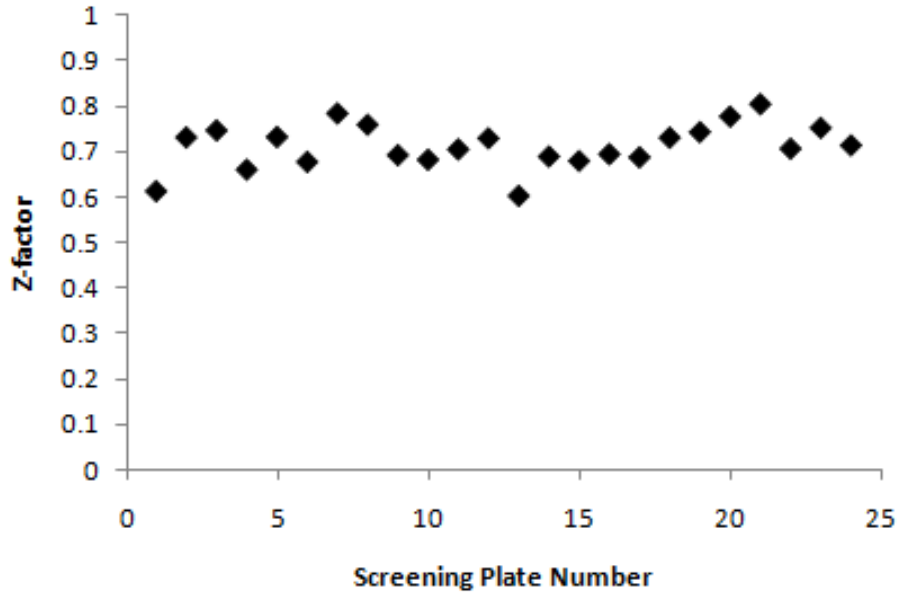

Figure 5: Z-factor trend across assay plates used in the primary β-Lactamase screen

The distribution of β-Lactamase inhibition for this library was found to be right skewed (Fig. 6). The interquartile range for the distribution of percent inhibition (range containing 50% of compounds) was found to show a difference of 10.6 percentage points (-5 to 5.6%), indicating screen quality was good. Based on a 40% inhibition cut-off (see Discussion), 19 compounds were selected as hits, corresponding to a hit rate of 0.23%. Fourteen compounds were available upon re-ordering from the Novartis Compound Archive. All 14 hits were found to inhibit β-Lactamase in a dose-dependent manner, giving a re-confirmation rate of 100%. Of

the 14 hits, 11 had steep dose-response curves, indicated by a Hill co-efficient lower than -2 (Table 2).

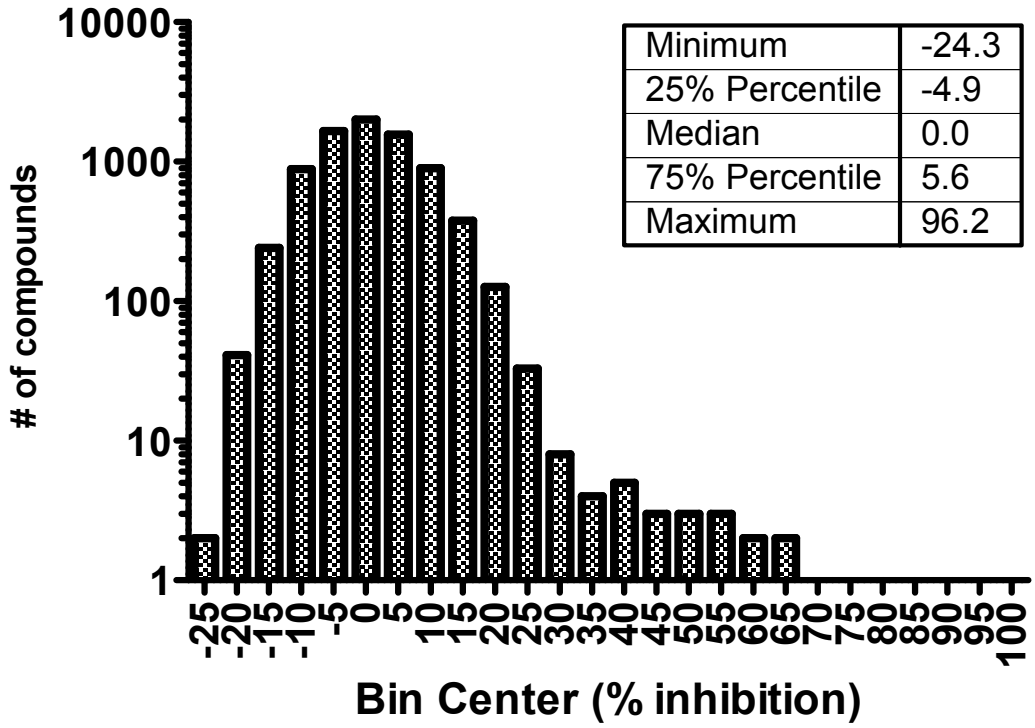

Figure 6: Histogram of normalized inhibition data of compound library tested against β-Lactamase. Distribution was analyzed using GraphPad Prism 5.

3.1.2 Detergent sensitivity of inhibition potency of β-Lactamase hits

Hits were subjected to dose-response analysis in the presence of detergent (Tween-20, Triton X-100 and CHAPS) to determine if inhibition potency was sensitive to detergent. Of the 14 hits, 13 were found to display detergent-sensitive inhibition in all three detergents tested (Table 2). In the presence of detergent (0.004% Tween-20, 0.007% Triton X-100, 0.3% CHAPS) at concentrations below CMC; these 13 compounds either failed to inhibit the enzyme or had a greater than 3-fold increase in their IC_{50} values.

Table 2: IC_{50} values of hits from β-Lactamase screen in the absence and presence of detergent. Concentration of enzyme and substrate in all assays was 2.5nM β-Lactamase and 25μM CENTA respectively. Tween-20, Triton X-100 and CHAPS concentrations were 0.004%, 0.007% and 0.3% respectively.

Compound code	IC_{50} vs. β-Lactamase (μM)	Hill co-efficient	IC_{50} shift in detergent		
			Tween-20	Triton X-100	CHAPS
BZBTH2B	7.11	-1.00	No change	↑1.2-fold	↓1.4-fold
BLAC-1	15.23	-1.02	↑ >3-fold	↑ >3-fold	↑ >3-fold
BLAC-2	2.00	-14.34	↑ >3-fold	↑ >3-fold	↑ >3-fold
BLAC-3	3.86	-3.90	↑ >3-fold	↑ >3-fold	↑ >3-fold
BLAC-4	8.00	-4.99	↑ >3-fold	↑ >3-fold	↑ >3-fold
BLAC-5	19.37	-4.09	↑ >3-fold	↑ >3-fold	↑ >3-fold
BLAC-6	4.81	-2.47	↑ >3-fold	↑ >3-fold	↑ >3-fold
BLAC-7	7.02	-13.31	↑ >3-fold	↑ >3-fold	↑ >3-fold
BLAC-8	>20	-0.97	↑ >3-fold	↑ >3-fold	↑ >3-fold
BLAC-9	18.40	-3.48	↑ >3-fold	↑ >3-fold	↑ >3-fold
BLAC-10	6.45	-14.06	↑ >3-fold	↑ >3-fold	↑ >3-fold
BLAC-11	14.10	-1.38	↑2.6-fold	↑2-fold	↑1.1-fold
BLAC-12	7.01	-12.43	↑ >3-fold	↑ >3-fold	↑ >3-fold
BLAC-13	3.03	-3.78	↑ >3-fold	↑ >3-fold	↑ >3-fold
BLAC-14	7.30	-11.91	↑ >3-fold	↑ >3-fold	↑ >3-fold

The inhibition potency of the reference inhibitor BZBTH2B was not affected by the presence of detergent. BZBTH2B displayed well-defined dose response curves in the presence of all detergents (Fig. 7A). A known aggregate-former tetraiodophenolphthalein (McGovern et al. 2002), as expected, did not inhibit the enzyme in the presence of detergent (Fig. 7B). Among the hits only one compound, BLAC-11, inhibited the enzyme in a dose-dependent manner in the presence of all three detergents (Fig. 8A). All other hits displayed tetraiodophenolphthalein-like behavior in the presence of detergent (Fig. 8B).

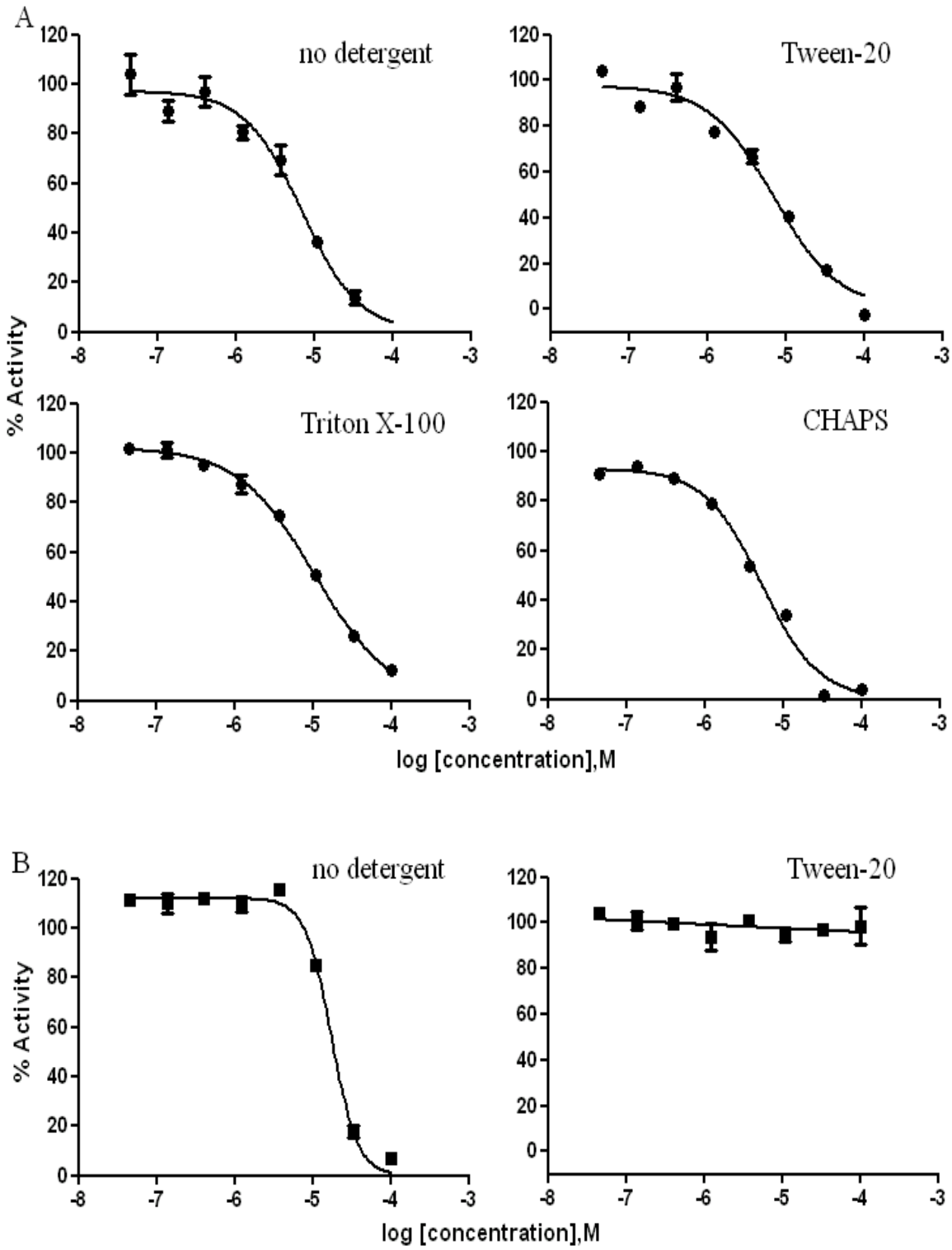

Figure 7: Dose-response curves of A) BZBTH2B, a reference inhibitor of E. cloacae β-Lactamase and B) Tetraiodophenolphthalein, a known aggregate-forming inhibitor of β-Lactamase. All dose-response curves contained 8 concentrations from highest concentration of 100μM diluted serially 3-fold. Each data point represents the average from duplicated wells.

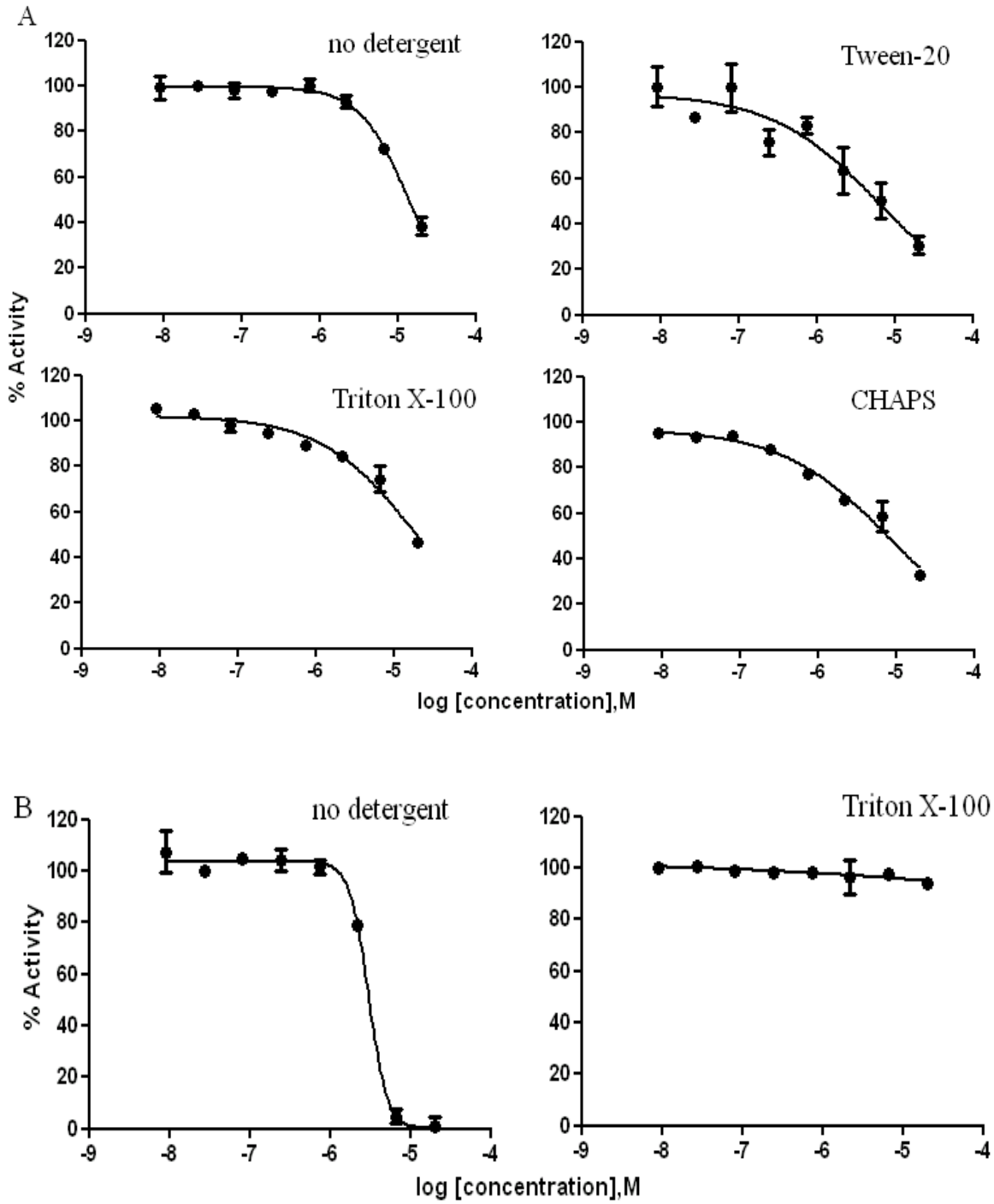

Figure 8: Dose-response curves showing inhibition of β-Lactamase by A) BLAC-11 and B) BLAC-13. All dose-response curves contained 8 concentrations from highest concentration of 20μM diluted serially 3-fold. Each data point represents the average from duplicated wells.

3.1.3 Enzyme-concentration sensitivity of inhibition potency of β-Lactamase hits

When enzyme concentration was increased 10-fold (2.5nM to 25nM), Z'-factors with chromogenic substrates such as CENTA and Penicillin-G were less than 0 (data not shown). The chromogenic assay format was not found to be sensitive enough to allow accurate quantification of reaction rate at higher enzyme concentrations. Therefore, a fluorometric assay format was used to test sensitivity of inhibition potency at increased enzyme concentrations. At β-Lactamase concentrations of 0.5nM and 5nM, the assay could be performed using identical substrate concentrations. Of the 14 hits, 10 hits had greater than 3-fold IC_{50} shifts and one hit had a fold-change very close to 3. The sensitivity of compound potency to enzyme concentration corresponded well to detergent sensitivity measured under the same assay format (Table 3).

Table 3: IC_{50} values of hits from β-Lactamase screen in the fluorometric assay format. Concentration of Fluorocillin in all assays was 2.5uM. Tween-20 concentration was 0.005%.

Compound code	IC_{50} vs. 0.5nM β-Lactamase (μM)	Hill co-efficient	IC_{50} shift using	
			10X (5nM) β-Lactamase	Tween-20 (at 0.5nM β-Lactamase)
BZBTH2B	0.43	-1.05	↑1.22-fold	↑1.33-fold
BLAC-1	0.61	-0.51	↑ >3-fold	↑ >3-fold
BLAC-2	1.87	-0.81	↑ >3-fold	↑ >3-fold
BLAC-3	0.93	-1.69	↑ >3-fold	↑ >3-fold
BLAC-4	5.05	-2.14	↑2.55-fold	↑ >3-fold
BLAC-5	13.85	-1.67	↑ >3-fold	↑ >3-fold
BLAC-6	1.90	-1.26	↑ >3-fold	↑ >3-fold
BLAC-7	6.83	-2.17	↑2.60-fold	↑ >3-fold
BLAC-8	52.38	-0.43	↑ >3-fold	↑ >3-fold
BLAC-9	9.86	-2.77	↑ >3-fold	↑ >3-fold
BLAC-10	2.53	-3.75	↑2.92-fold	↑ >3-fold
BLAC-11	13.32	-0.86	↑2.19-fold	↑2.35-fold
BLAC-12	1.71	-3.18	↑ >3-fold	↑ >3-fold
BLAC-13	1.47	-1.42	↑ >3-fold	↑ >3-fold
BLAC-14	3.56	-1.30	↑ >3-fold	↑ >3-fold

Only 5 of 14 compounds displayed steep dose-response curves in the fluorometric assay format opposed to 11 of 14 hits in the assay with the chromogenic substrate indicating that the Hill co-efficients of dose-response curves of the hits were

not in complete agreement between the two assay formats. Detergent sensitivity of inhibition potency, measured in the form of IC_{50} changes, was found to be consistent for all hits in both assay formats (Tables 2 and 3); with 93% of hits found to display detergent-sensitive inhibition in the chromogenic and fluorogenic assays.

3.1.4 Dynamic light scattering analysis of β-Lactamase hits

β-Lactamase hits were subjected to DLS analysis in order to measure the size of aggregate particles in solution. For some of the hits, particle sizes were found to be between 200-1000 nm in diameter. However, the software reported accompanying error messages indicating poor data quality. In addition, for the same compound, the variation in measured particle size was observed to be high as 40% (e.g., 250 nm in one measurement and 600nm in another measurement). The measurements were also confounded by high signal to noise ratios.

Since fitting algorithms cannot always distinguish high from low quality raw data, they generally give a size distribution for every sample, even for raw data that does not fit DLS criterion. Thus, instead of relying solely on particle size distributions, autocorrelation functions (plotted as correlograms) should be scrutinized as they are reliable indicators of data quality. A high quality correlogram can be described as having high amplitude (Y intercept) and a smooth exponential decay to a single, flat, and zero baseline (Malvern, Technical Support Library). Correlograms that deviate from the norm usually indicate that the raw data was of sub-optimal quality. For a majority of the β-Lactamase hits, quality of raw data was found to be poor as deciphered from the correlograms plotted by the software.

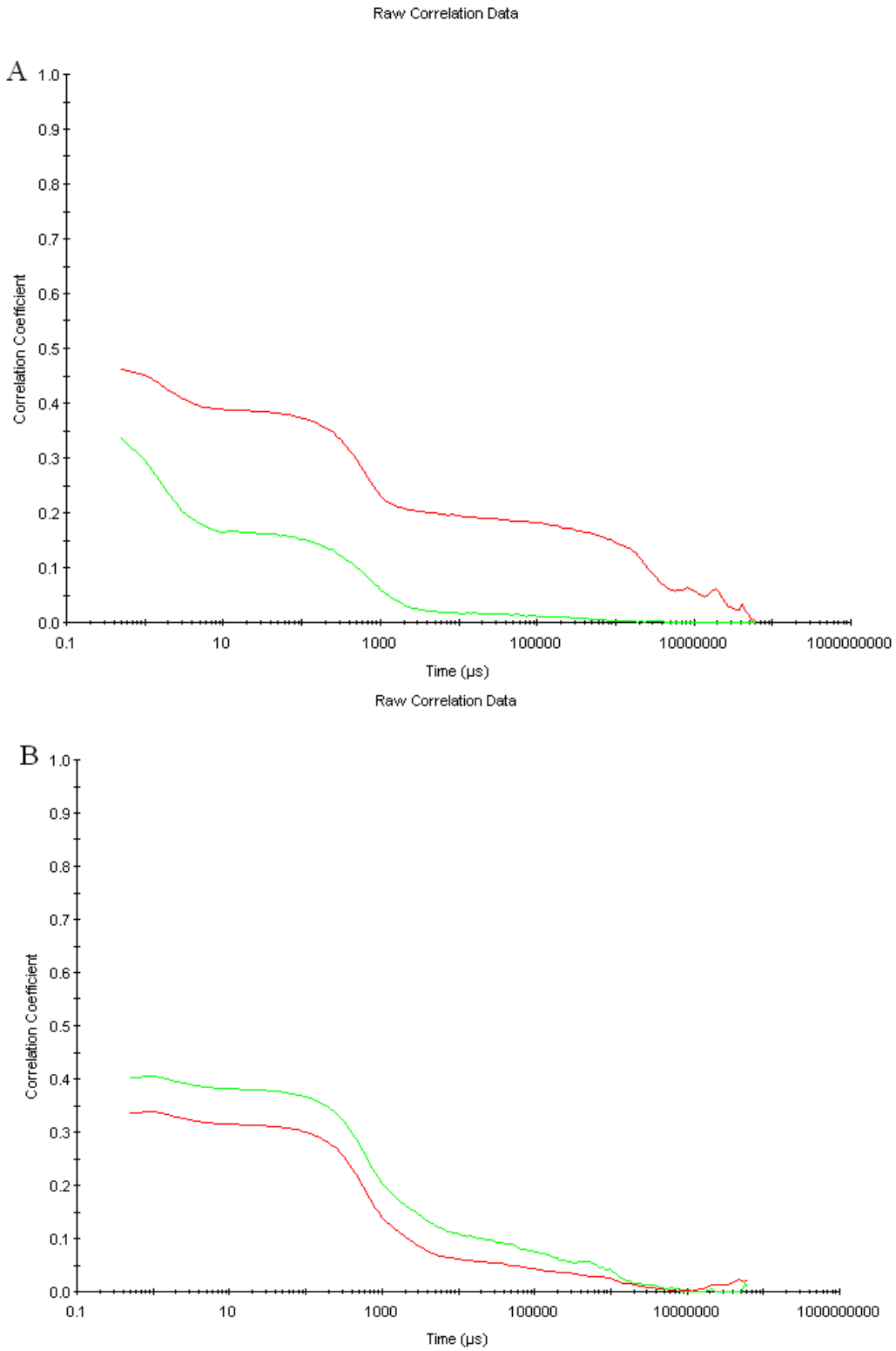

Figure 9: DLS correlogram of BLAC-1 at A) 20µM and B) 66µM as measured with a Malvern Zetasizer Nano ZS dynamic light scattering instrument in assay buffer. Red and green lines represent independent measurements of the sample.

For example, repeated measurements of compound BLAC-1 at 20µM and 66µM gave inconsistent results with particle size diameters ranging from 200-950

nm. The correlogram of BLAC-1 (Fig. 9) was suggestive of low intensity scattering even at a compound concentration of 66μM. In the dose-response assay, the maximum compound concentration used was 20μM and the compound BLAC-1 had both enzyme-concentration and detergent-dependent potency shifts indicative of aggregation-based inhibition. It is possible that the amount of scattered light from the aggregate particles was not sufficient to make successful measurements in a DLS assay.

Other compounds had noisy correlograms indicative of high signal to noise ratios. As a representative of these, the correlograms of compound BLAC-2 are depicted in Figure 10. The correlograms showed presence of noise and had elevated baselines suggestive of either large particle sizes outside the range of the instrument or number fluctuations during the measurement. Despite optimizing run time and collecting data for extended periods, the correlogram quality could not be improved. Either the particles formed are too large to be detected by the instrument or the nature of the compound impeded accurate measurement of particle size.

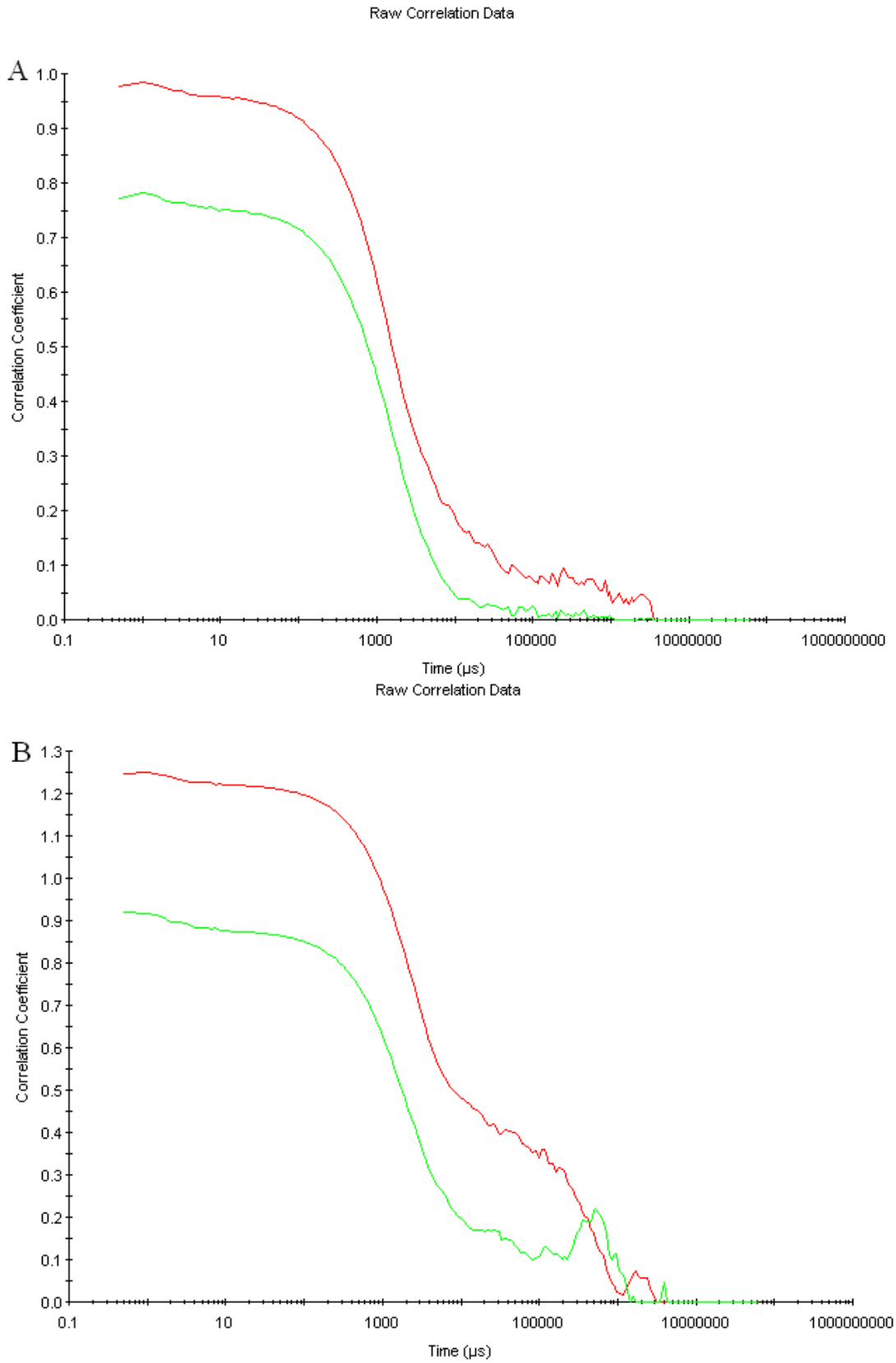

Figure 10: DLS correlogram of BLAC-2 at A) 20µM and B) 66µM as measured with a Malvern Zetasizer Nano ZS dynamic light scattering instrument in assay buffer. Red and green lines represent independent measurements of the sample.

3.2 Follow-up of DENV RdRp pilot screen

3.2.1 Detergent sensitivity of inhibition potency of DENV RdRp hits

As DENV RdRp displayed poor activity in the absence of detergent, the pilot screen was run in assay buffer containing 0.01% Triton X-100 (Niyomrattanakit et al. 2011). To ensure that the nature of detergent did not affect inhibition, the hits were re-tested in low concentrations of three detergents – Triton X-100, Brij-35 and CHAPS. The re-testing was done at the minimum concentration of detergent (below its CMC) that was required to observe a good signal window in the RdRp assay.

The inhibitory potency of a specific inhibitor of the DENV RdRp, 3'dATP, did not vary significantly in the different detergents. Additionally, it was found that the 30 compounds retained activity in all three detergents (Table 4), indicating that inclusion of a low concentration of detergent in the assay buffer did not affect the inhibitory potency of RdRp hits. However, some hits (RDRP-1, 2, 8, 16, 20 and 23) appeared to be a lot more potent in buffer containing CHAPS compared to Brij-35.

It was observed that almost all of the hits had dose-response curves with good Hill co-efficients (greater than -2) in the presence of every detergent tested. The value of the Hill-coefficient varied for the same compound tested in buffer containing different detergents. No compounds had steep dose response curves in assay buffer containing CHAPS. While 5 compounds had high Hill co-efficients in the presence of Brij-35, only one compound had a high slope in buffer containing Triton X-100.

Table 4: IC_{50} values of DENV RdRp hits in the presence of different detergents in the assay buffer. The concentrations of the detergents were 0.004% Brij-35, 0.002% Triton and 0.03% CHAPS. Enzyme and substrate concentrations were the same as the pilot screen.

Compound code	Brij-35		Triton X-100		CHAPS	
	IC_{50} (µM)	Hill co-efficient	IC_{50} (µM)	Hill co-efficient	IC_{50} (µM)	Hill co-efficient
3' dATP	0.15	-0.75	0.30	-0.86	0.38	-0.78
RDRP-1	>20	-1.90	20.13	-11.81	7.42	-1.52
RDRP-2	>20	-0.87	12.91	-0.81	10.43	-0.99
RDRP-3	17.30	-1.43	14.07	-0.85	9.07	-0.98
RDRP-4	13.07	-1.41	11.00	-0.80	7.54	-0.95
RDRP-5	11.60	-1.43	7.60	-0.80	5.59	-0.99
RDRP-6	7.21	-1.13	4.81	-0.72	3.63	-0.83
RDRP-7	14.29	-1.62	14.97	-0.77	9.70	-1.08
RDRP-8	>20	-0.70	15.77	-0.67	7.77	-1.00
RDRP-9	14.97	-1.09	8.38	-0.99	5.84	-1.09
RDRP-10	22.14	-1.63	17.44	-0.99	9.12	-1.04
RDRP-11	10.71	-1.33	9.61	-0.85	4.61	-1.39
RDRP-12	18.50	-1.54	16.47	-0.89	8.45	-1.46
RDRP-13	18.34	-2.25	18.59	-1.23	12.53	-1.54
RDRP-14	14.09	-1.62	13.15	-1.38	10.41	-1.53
RDRP-15	17.57	-3.92	26.00	-1.23	14.10	-1.67
RDRP-16	13.48	-1.17	7.13	-0.96	3.07	-0.99
RDRP-17	9.65	-1.62	4.55	-0.75	2.92	-0.92
RDRP-18	17.53	-1.05	11.63	-0.95	9.36	-1.44
RDRP-19	19.90	-1.06	10.80	-1.15	6.31	-1.15
RDRP-20	20.25	-11.66	10.19	-0.79	1.84	-0.96
RDRP-21	13.48	-1.22	8.99	-0.94	6.39	-1.10
RDRP-22	4.74	-0.70	3.43	-0.55	2.06	-0.60
RDRP-23	17.50	-3.38	15.48	-0.60	3.12	-0.87
RDRP-24	12.95	-1.44	15.47	-0.95	7.55	-1.14
RDRP-25	13.99	-3.04	9.24	-0.99	4.02	-1.12
RDRP-26	21.96	-1.47	11.58	-1.62	7.04	-1.69
RDRP-27	11.84	-1.45	11.88	-1.08	7.20	-1.22
RDRP-28	6.27	-1.24	6.90	-1.10	4.19	-1.28
RDRP-29	15.42	-1.47	6.40	-1.18	4.59	-1.51
RDRP-30	9.63	-1.19	10.54	-0.98	5.72	-1.11

Detergent-dependent potency shifts were investigated by obtaining the IC_{50} values of the hits in increased amounts of detergent. To account for minor variations of the IC_{50} values of compounds in the presence of different detergents (Table 4), a 5-fold increase in IC_{50} was used as a cut-off was used to assign whether or not a compound experienced a significant decrease in potency at increased concentrations of detergent. For a total of 30 hits investigated, inhibition of 43% of the compounds was sensitive to Brij-35, 54% to Triton X-100 and 20% to CHAPS (Table 5).

Prevalence of hits displaying detergent-sensitive inhibition of DENV RdRp was found to be lower compared to 93% prevalence of detergent-sensitive inhibitors among β-Lactamase hits.

Table 5: Changes in IC_{50} values of DENV RdRp hits at higher concentrations of detergent. Reaction conditions were identical to those in the protocol followed for the pilot screen.

Compounds	Brij-35 (Fold change-IC_{50} 0.11%/0.0044%)	Triton X-100 (Fold change-IC_{50} 0.1%/0.002%)	CHAPS (Fold change-IC_{50} 0.37%/0.025%)
3' dATP	1.6	1.4	1.5
RDRP-1	>5	>5	3.22
RDRP-2	2.88	>5	4.12
RDRP-3	3.69	4.43	>5
RDRP-4	>5	>5	3.81
RDRP-5	>5	3.17	4.71
RDRP-6	>5	>5	>5
RDRP-7	3.45	>5	4.56
RDRP-8	>5	>5	3.54
RDRP-9	1.68	>5	4.30
RDRP-10	>5	>5	4.14
RDRP-11	3.17	3.92	2.96
RDRP-12	2.74	1.51	3.81
RDRP-13	4.52	1.31	>5
RDRP-14	3.91	>5	4.28
RDRP-15	>5	>5	1.19
RDRP-16	>5	>5	3.99
RDRP-17	>5	>5	4.22
RDRP-18	1.24	0.76	1.53
RDRP-19	2.69	1.90	2.56
RDRP-20	>5	>5	>5
RDRP-21	1.95	1.65	1.95
RDRP-22	>5	>5	1.88
RDRP-23	>5	>5	>5
RDRP-24	1.22	0.97	1.50
RDRP-25	3.59	>5	>5
RDRP-26	>5	0.06	2.12
RDRP-27	2.38	1.61	1.77
RDRP-28	1.60	1.20	1.42
RDRP-29	1.11	4.42	1.54
RDRP-30	1.80	1.18	2.34

3.2.2 Enzyme-concentration sensitivity of inhibition potency of DENV RdRp hits

Table 6: Enzyme-concentration dependent changes in IC_{50} values of DENV RdRp hits. All reactions were performed in assay buffer containing 0.002% Triton and at 2µM BBT-ATP substrate concentration.

Compound code	IC_{50} (µM) [Enzyme: RNA - 10nM :50nM]	IC_{50} (µM) [Enzyme: RNA - 10nM :150nM]	IC_{50} fold change (Enzyme: RNA 100nM:150nM/ 10nM:50nm)	IC_{50} fold change (Enzyme :RNA 100nM:150nM/ 10nM:150nm)
3' dATP	0.58	0.84	1.1	0.76
RDRP-1	18.96	>20	1.36	0.67
RDRP-2	7.86	3.22	>5	>5
RDRP-3	8.31	3.14	>5	>5
RDRP-4	6.92	2.41	>5	>5
RDRP-5	5.47	1.37	>5	>5
RDRP-6	3.30	1.60	>5	>5
RDRP-7	11.67	4.36	4.25	>5
RDRP-8	5.84	2.29	>5	>5
RDRP-9	4.45	1.94	>5	>5
RDRP-10	8.15	4.86	>5	>5
RDRP-11	4.52	1.84	>5	>5
RDRP-12	12.15	4.61	3.47	>5
RDRP-13	18.22	6.29	2.45	>5
RDRP-14	15.15	4.75	1.80	>5
RDRP-15	18.38	4.58	4.30	>5
RDRP-16	4.30	0.91	4.91	>5
RDRP-17	1.86	0.68	>5	>5
RDRP-18	5.22	4.63	>5	>5
RDRP-19	3.64	3.03	>5	>5
RDRP-20	3.57	1.35	>5	>5
RDRP-21	4.05	3.22	>5	>5
RDRP-22	0.83	0.59	>5	>5
RDRP-23	6.60	1.52	>5	>5
RDRP-24	5.55	2.94	>5	>5
RDRP-25	6.75	3.58	>5	>5
RDRP-26	9.23	2.45	3.22	>5
RDRP-27	5.49	2.93	>5	>5
RDRP-28	3.32	3.23	>5	>5
RDRP-29	5.45	3.97	4.06	>5
RDRP-30	4.75	2.91	>5	>5

Sensitivity of inhibition potency to varying enzyme concentrations was performed at 10nM and 100nM RdRp under 2 sets of conditions: 1) different concentrations of 3'UTR-U_{30} RNA substrate at each enzyme concentration, and 2) identical concentrations of 3'UTR-U_{30} RNA substrate used at both enzyme concentrations. Under the first condition, inhibition potency of 21 out of the 30 hits appeared to be sensitive to enzyme concentration while under the second condition;

the inhibition of 29 out of 30 hits was significantly attenuated (IC_{50} increased by greater than 5-fold) at the higher enzyme concentration (Table 6).

3.2.3 Effect of Triton X-100 concentration on enzyme kinetics

Effect of Triton X-100 was tested as it was the detergent used in the assay buffer in the pilot screen and in testing sensitivity of inhibition potency to enzyme concentration. The apparent K_m and V_{max} values of DENV RdRp for the 3'UTR-U_{30} RNA substrate were determined in the presence of varying amounts of Triton X-100 to examine whether the detergent affected the enzyme kinetics. Concentration of the second substrate BBT-ATP was kept constant at 2μM in all reactions.

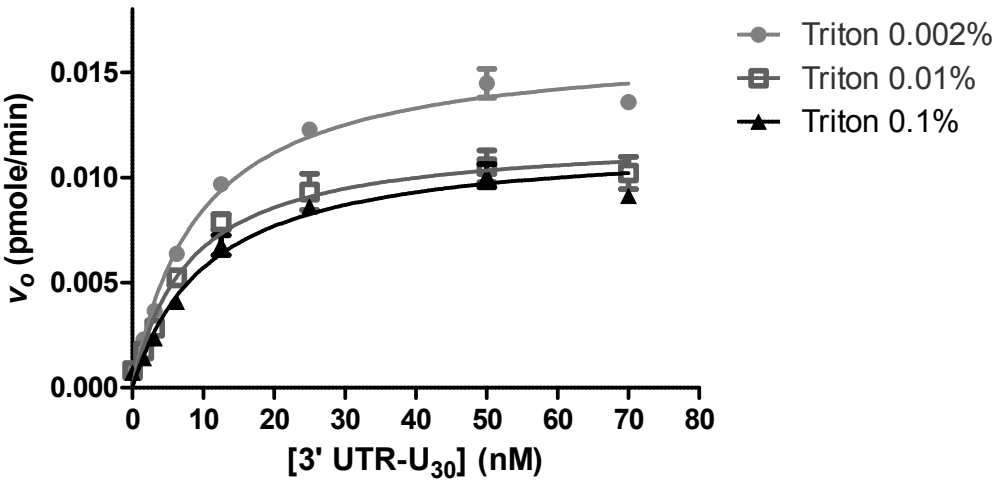

Figure 11: Effect of Triton X-100 on apparent K_m and V_{max} values of DENV RdRp. Different 3' UTR-U_{30} RNA concentrations (0–70nM) were used to determine DENV RdRp activity in the presence of varying amounts of Triton X-100. Concentration of BBT-ATP substrate was kept constant at 2μM in all reactions. Enzyme concentration in all experiments was 10nM. Each data point represents the average of 4 measurements.

As shown in Table 7, the K_m^{app} values did not differ significantly with detergent concentration but the V_{max}^{app} was slightly higher at decreased Triton X-100 concentrations (0.016 pmole/min in 0.002% Triton X-100 as opposed to 0.012 pmole/min in both 0.01% and 0.1% Triton X-100). At all three concentrations of Triton X-100, the rate of reaction began to drop at RNA concentrations higher than

50nM (Fig. 11). The drop in activity therefore did not appear to be influenced by the amount of detergent; rather it seemed to be an effect of RNA concentration (see Discussion).

Table 7: The apparent K_m and V_{max} of the 3'UTR-U_{30} RNA substrate at different Triton X-100 concentrations. Concentration of BBT-ATP substrate was kept constant at 2µM in all reactions. Enzyme concentration in all experiments was 10nM.

Michaelis-Menten	0.002% Triton X-100	0.01% Triton X-100	0.1% Triton X-100
Best-fit values			
Vmax (pmole/min)	0.016	0.012	0.012
Km (µM)	9.36	7.96	10.61
95% Confidence Intervals			
Vmax (pmole/min)	0.015 to 0.018	0.011 to 0.013	0.010 to 0.013
Km (µM)	7.24 to 11.51	5.25 to 10.66	7.10 to 14.12

3.3 Investigation of inhibition of unrelated enzymes or a model enzyme as means of identification of aggregation-based inhibition

Percentage target inhibition of enzymes *E. Cloacae* AmpC β-Lactamase, *M. tuberculosis* Nicotinamide adenine dinucleotide synthetase, *M. tuberculosis* Pantothenate Kinase and Dengue virus serotype 4 RNA-dependent RNA polymerase by compounds from library Novartis2008 were compared to determine if any compounds inhibited more than one target enzyme (Fig. 12).

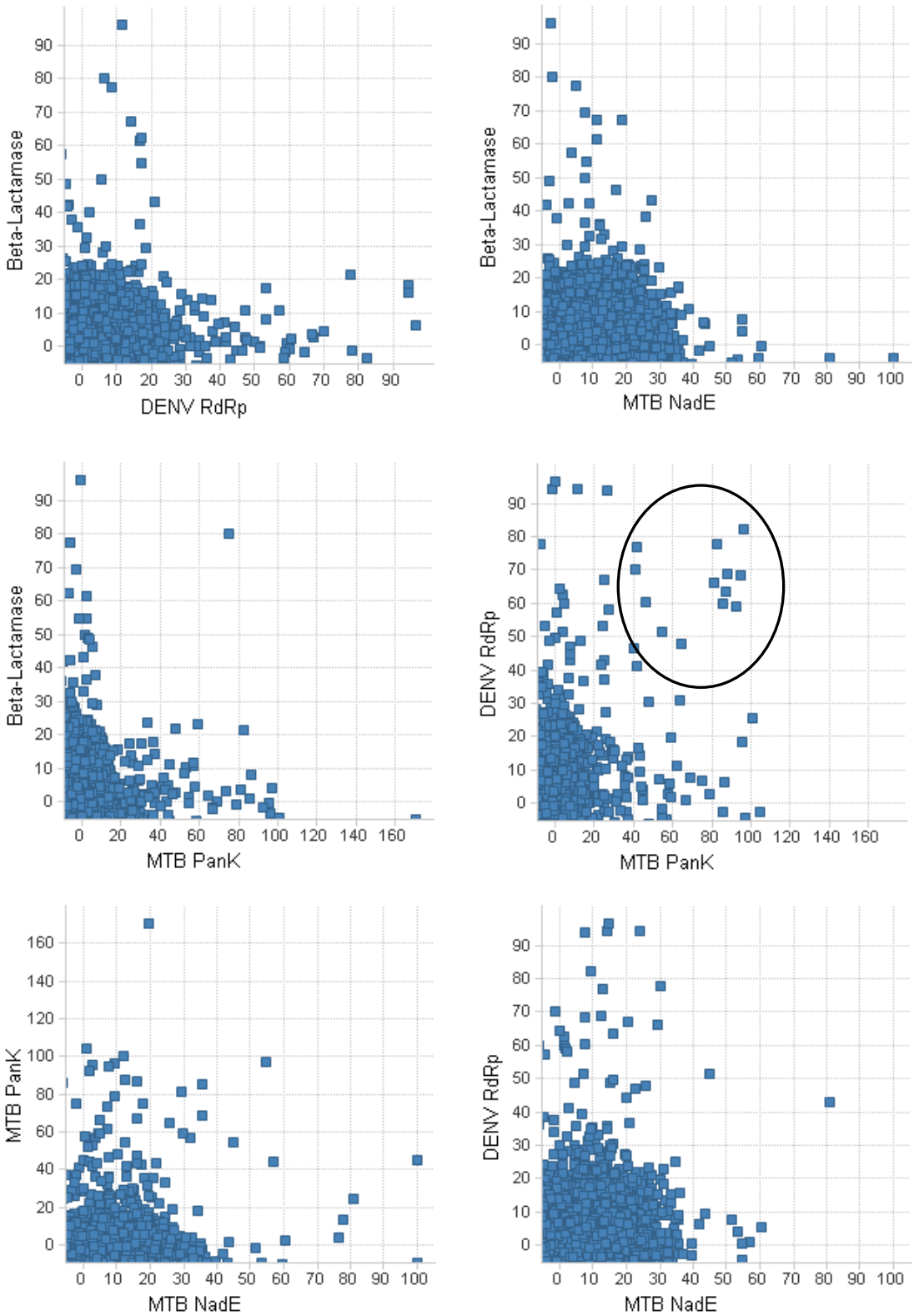

Figure 12: Comparison of primary screens of various enzymes – *E. Cloacae* AmpC β-Lactamase (Beta-Lactamase), *M. tuberculosis* Nicotinamide adenine dinucleotide synthetase (MTB NadE), *M. tuberculosis* Pantothenate Kinase (MTB PanK) and Dengue virus serotype 4 RNA-dependent RNA polymerase (DENV RdRp). All axes represent percent inhibition.

β-Lactamase inhibition data for compounds from library Novartis2008 was obtained using a chromogenic assay format as described earlier (see Materials and Methods). For all other enzymes, the inhibition data for compound library Novartis2008 was retrieved from previous Novartis records. Dengue virus serotype 4 RNA-dependent RNA polymerase activity was measured in an end point read-out using a fluorescence-based assay and both *M. tuberculosis* Nicotinamide adenine dinucleotide synthetase and Pantothenate Kinase assays involved a fluorescence polarization-based end point read-out. Enzyme assays with different detection technologies were chosen to ensure the comparison was not influenced by assay readout. As can be seen from Figure 12, except for a slight overlap between hits of *M. tuberculosis* Pantothenate Kinase and Dengue virus serotype 4 RNA-dependent RNA polymerase; each enzyme appeared to have a distinct set of inhibitors.

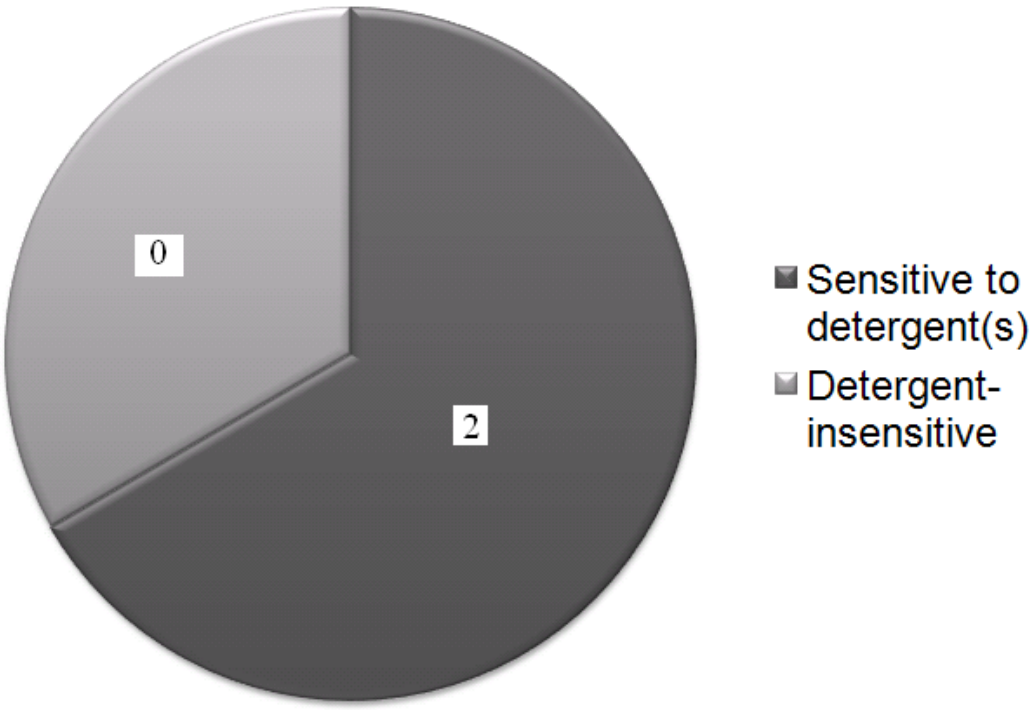

Figure 13: Distribution of DENV RdRp hits. Of all 30 hits, inhibition of 20 hits was sensitive to at least one of the detergents tested and the other 10 hits were detergent-insensitive inhibitors. The numbers on the pie chart fractions represent how many compounds in that category inhibited β-Lactamase.

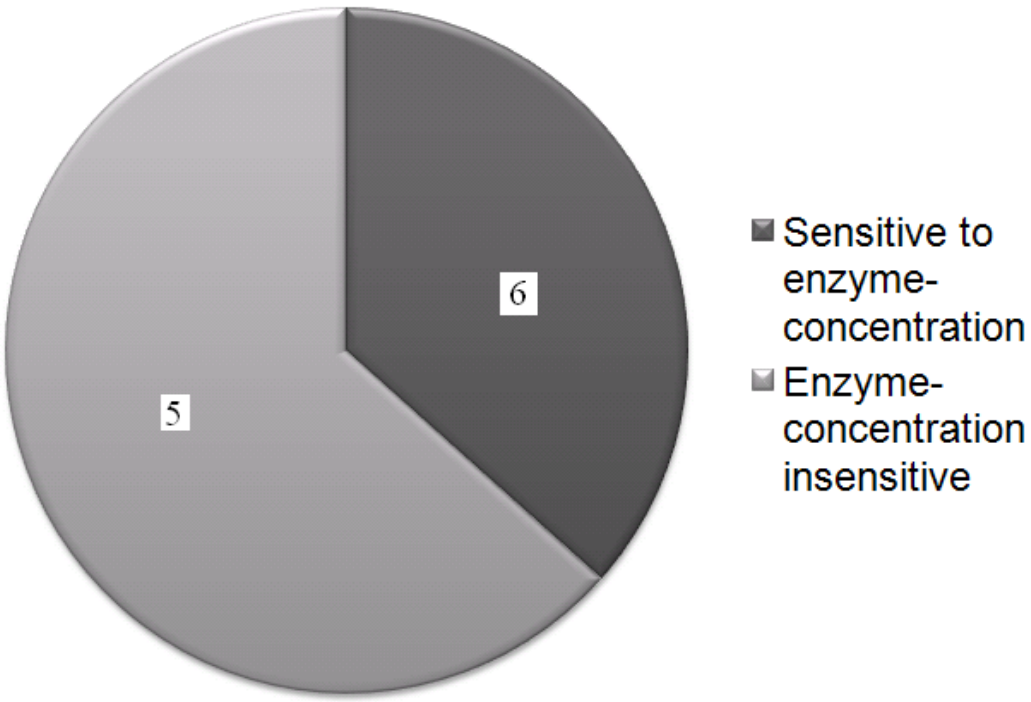

Figure 14: Distribution of Pantothenate Kinase hits. Of the chosen 41 compounds, 26 were insensitive to enzyme concentration and 15 compounds were significantly less potent (i.e non-specific) when assayed at higher enzyme concentrations. The numbers on the pie chart fractions represent how many compounds in that category inhibit β-Lactamase.

Hits from the DENV RdRp and PanK screens (see Materials and Methods) were assayed for inhibition of β-Lactamase enzyme activity using identical reaction conditions as employed in the β-Lactamase primary screen (see Materials and Methods). They were classified as β-Lactamase inhibitors if they inhibited enzyme activity by greater than 40%, which was the % cut-off used for hit selection in the primary β-Lactamase screen. As can be seen from Figures 13 and 14, inhibition of β-Lactamase was not a hallmark of compounds that inhibited their respective target enzyme non-specifically (determined by sensitivity of inhibition potency to detergent or enzyme concentration). Among the PanK hits, five compounds that satisfied the stoichiometric considerations for specific inhibition of PanK (IC_{50} unchanged at different PanK concentrations) were found to be inhibitors of β-Lactamase. Among the DENV RdRp hits, only 2 of 20 detergent-sensitive hits inhibited β-Lactamase. Thus, assaying inhibitory potency of compounds against β-Lactamase did not appear

to be indicative of whether a compound inhibited its target enzyme via an aggregation-based mechanism.

3.4 Development and validation of change in meniscus shape as a generic assay for detection of aggregate formation

In agreement with previous reports (Cai and Gochin 2007), it was observed that the liquid surface was nearly flat for buffer solution without detergent and formed a concave meniscus upon addition of detergent at a concentration above its CMC, an effect that could be observed by the naked eye when the plates were examined. The assay signal was found to be robust; fluorescence intensity of the fluorescein dye remained stable for up to 4 hours. The gain setting of the fluorescence reader was optimized to give a consistent signal window in all assay buffers (β-Lactamase, DENV RdRp and PanK). Relative fluorescence was measured as a ratio of the observed fluorescence of fluorescein dye in the presence of test compound in assay buffer to that of the compound in control buffer (assay buffer containing 1mM Triton X-100 or 1mM Tween-20).

Since this assay has never been used in an actual HTS campaign prior to this study, cut-offs to classify compounds as 'aggregators' had to be derived. For all three enzymes, it was found that the reference inhibitors used as controls in the primary screens served as useful benchmarks in the measurement of changes in meniscus curvature. As expected, by virtue of being known non-aggregating inhibitors, these reference inhibitors did not influence the shape of the liquid meniscus and hence did not affect the relative fluorescence of the dye.

Therefore, hits could be classified on the basis of deviation of their relative fluorescence ratios from the reference inhibitor ratio. A ratio lower than 3 standard deviations from the mean of the ratio derived from multiple measurements of the reference inhibitor was used as the cut-off to classify which compounds might be

inhibiting the target via an aggregation-based mechanism. Additionally, in combination with data from an independent method to determine aggregation-based inhibition (such as sensitivity to detergent or enzyme concentration), the cut-offs of 1, 2 and 3 SD's away from the reference ratio could potentially be used to assign a rank or prioritize which hits to chose for lead optimization.

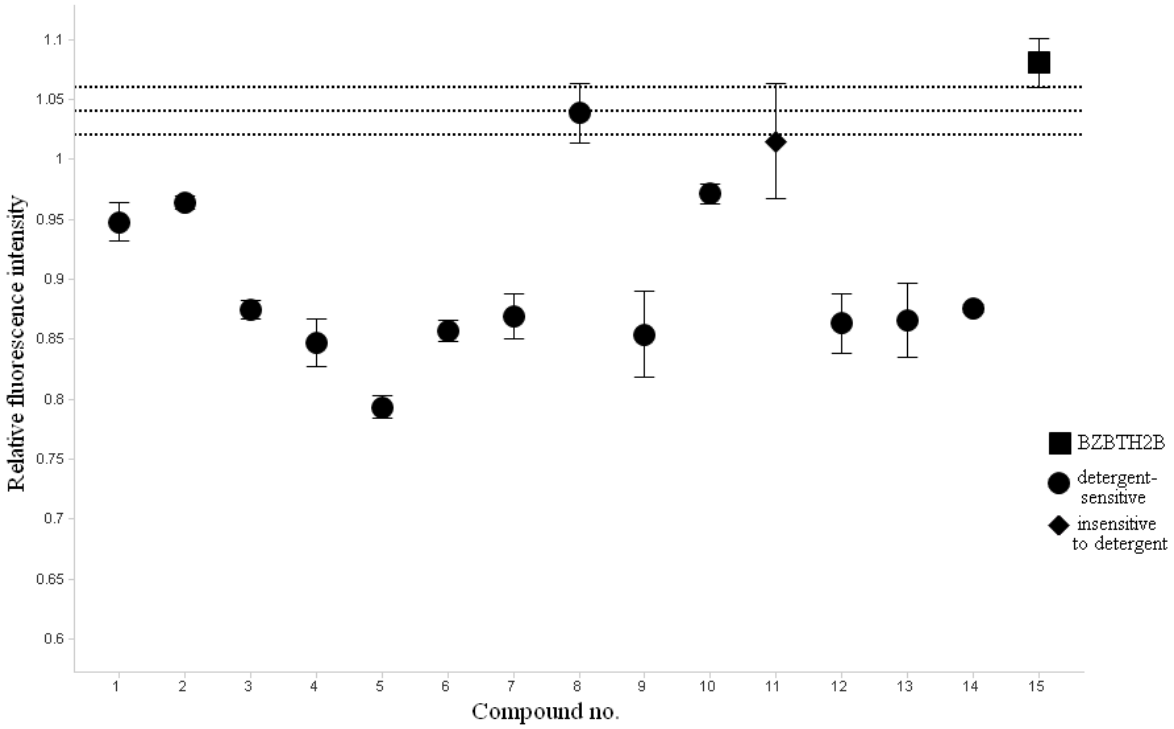

Figure 15: Relative fluorescence of β-Lactamase hits measured as the ratio of top-read fluorescence intensity in assay buffer to control buffer. Compounds were assayed at a concentration of 20µM. Compound no. corresponds to compound code in Table 2. Sensitive to detergent (●) means IC_{50} increased by more than 3-fold in the presence of detergent and vice versa in case of insensitivity to detergent (◆) Compound no. 15 (■) is BZBTH2B (specific inhibitor of β-Lactamase). From top to bottom, the dotted lines represent 1, 2 and 3 SD's respectively from the mean of the BZBTH2B ratio.

Among the β-Lactamase hits (Fig. 15), 12 of 14 compounds had relative fluorescence ratios smaller than 3 SD's less than the mean of ratio obtained with BZBTH2B, indicating that these compounds formed colloidal particles in the assay buffer. The data was in good agreement with the detergent sensitivity tests in which all compounds except BLAC-11 (compound no. 11 in Fig 15) displayed characteristics of aggregate-based inhibition. Only compound 8 (corresponding to

compound code BLAC-8 in Table 2) did not appear to form colloidal particles in spite of lack of β-Lactamase inhibition in the presence of detergent in the enzyme assay.

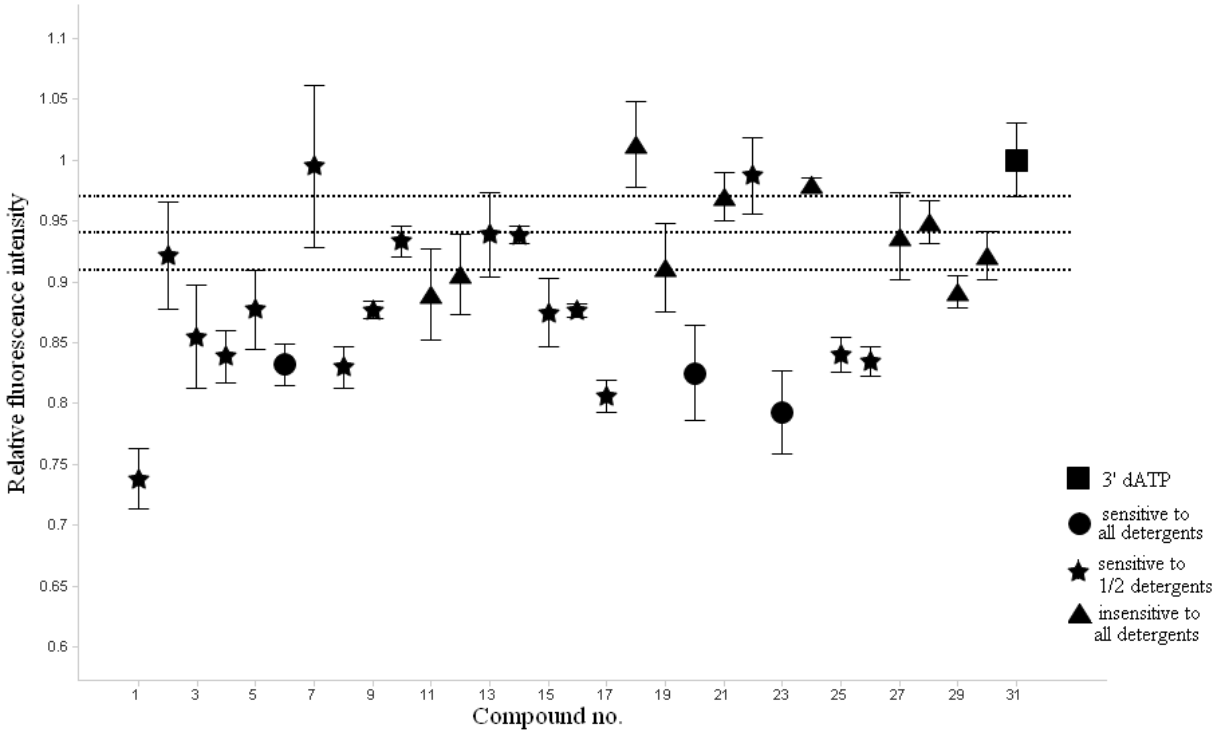

Figure 16: Relative fluorescence of DENV RdRp hits measured as the ratio of top-read fluorescence intensity in assay buffer to control buffer. Compounds were assayed at a concentration of 20μM. Compound no. corresponds to compound code in Table 4. Sensitive to all detergents(●) implies >5-fold IC_{50} increase in Triton X-100, Brij-35 and CHAPS; and vice versa for compounds insensitive to all detergent (▲) . Sensitive to 1/2 detergents (★) implies >5-fold IC_{50} in one or two but not all of the above mentioned detergents. Compound no. 31(■) is 3' dATP (specific inhibitor of DENV RdRp). From top to bottom, the dotted lines represent 1, 2 and 3 SD's respectively from the mean of the 3'dATP ratio.

Among the DENV RdRp hits (Fig. 16), the three compounds which were found to have inhibition potency that was sensitive to all detergents (Triton X-100, Brij-35 and CHAPS), had relative fluorescence ratios much lower than 3 SD's from the mean of the 3'dATP ratio. Of the 10 hits that did not have significant changes in their IC_{50} values at higher concentrations of detergent, 7 compounds had ratios within the 3 SD window and the remaining 3 compounds had ratios in the borderline region. Of the 17 hits that had significant changes in their IC_{50} values in at least one of the

three detergents tested; 10 compounds had ratios lower that the cut-off; 2 compounds had ratios in the borderline and the remaining 5 compounds had ratios that were not indicative of change in meniscus shape due to colloid formation.

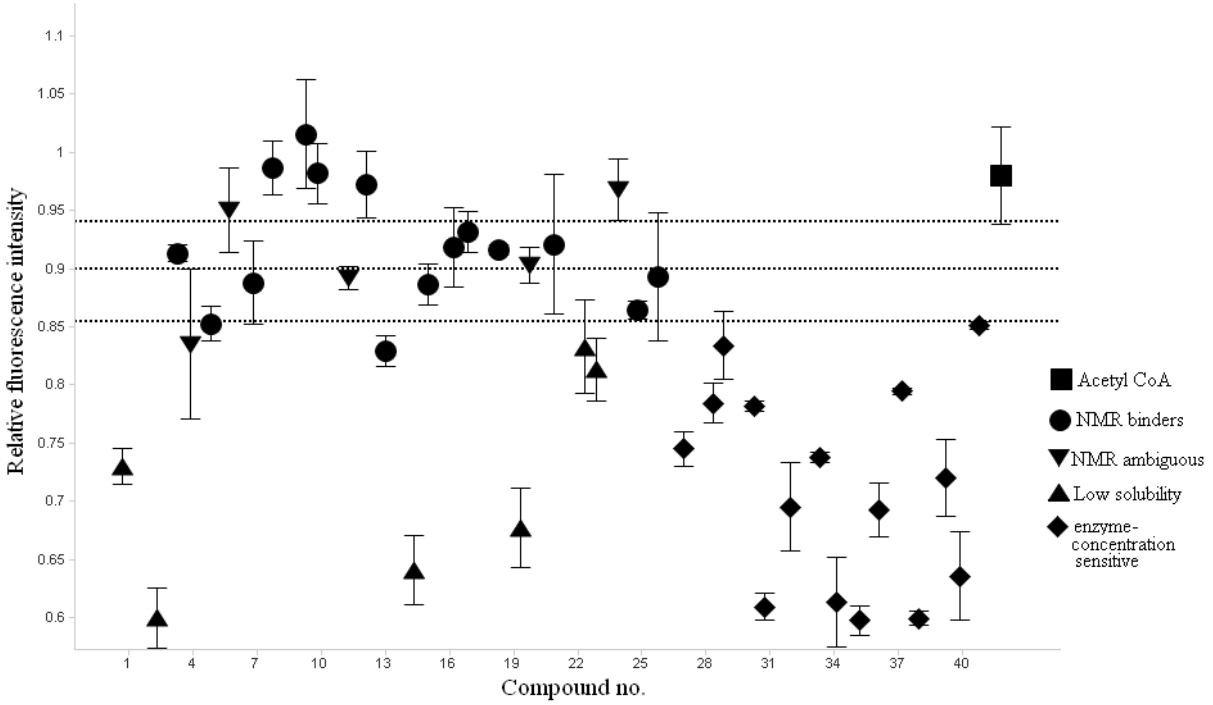

Figure 17: Relative fluorescence of MTB PanK hits measured as the ratio of top-read fluorescence intensity in assay buffer to control buffer. Compounds were assayed at a concentration of 20µM. IC_{50}'s of compound no. 1-26 were not significantly affected by enzyme concentration and these compounds were further characterized by NMR studies. IC_{50}'s of compound no. 27-41(◆) were significantly increased at higher PanK concentrations and these compounds were not subjected to NMR analysis. NMR binders (●) - binding to PanK confirmed; NMR ambiguous (▼) - binding to enzyme could not be confirmed; Low solubility (▲) - compound not soluble enough for NMR analysis. Compound no. 42 (■) is Acetyl CoA (a specific inhibitor of PanK). From top to bottom, the dotted lines represent 1, 2 and 3 SD's respectively from the mean of the Acetyl CoA ratio.

It has been previously established that testing enzyme concentration sensitivity was more predictive detergent sensitivity in identifying non-stoichiometric enzyme inhibitors from a PanK hit list without overly affecting stoichiometric enzyme inhibitors (Habig et al. 2009). All compounds represented in Figure 17 were chosen from the hit list mentioned above based on their sensitivity to enzyme concentration (see Materials and Methods). Among the 15 enzyme-concentration

sensitive (i.e. non-specific) PanK hits, 13 compounds had ratios much lower than 3SD's from the mean of the Acetyl CoA ratio, indicating aggregate formation in the assay buffer; and 2 compounds had ratios that fell in the borderline regions (Fig. 17). Among the 26 hits that were chosen for NMR analysis based on their enzyme-concentration insensitive inhibition, 20 hits did not appear to form colloids as indicated by unaffected meniscus. Of the remaining 10, the 6 hits that had ratios lower than the cut-off were found to have solubility issues due to which they could not be characterized by NMR-based techniques; and the other 4 hits of which 3 were confirmed binders, had ratios in the borderline region.

4. Discussion

4.1 Choice of β-Lactamase as model enzyme

β-Lactamase was set-up as a model enzyme system to study aggregate-forming inhibitors because it has been extensively studied. Since all mechanistic

studies by Shoichet et al. investigating the mode of action of aggregation-based inhibitors were performed with this model enzyme, inhibition of β-Lactamase by aggregate-forming inhibitors is well characterized. Large compound libraries have been tested to determine the feasibility of different methods of identifying aggregate-forming inhibitors of β-Lactamase (McGovern et al. 2003; Feng et al. 2005; Feng et al. 2007). It has been well established that detergent-sensitive β-Lactamase inhibitors consistently display characteristic features of non-specific inhibition such non-stoichiometric binding of the enzyme (verifiable by increasing the enzyme concentration by 10-fold) and form aggregate particles in solution (verifiable by DLS measurements of particle size). Results from numerous studies have validated detergent sensitivity as a convenient proxy for determination of aggregation-based inhibition.

Furthermore, hits from a β-Lactamase screen were likely to be a good source of aggregate-forming inhibitors. Many drug like molecules from screening libraries have been observed to inhibit the enzyme (McGovern et al. 2002, McGovern and Shoichet 2003; Seidler et al. 2003). Further, a screen of 70,563 compounds against AmpC β-Lactamase highlighted aggregate forming compounds as the major source of false positives. Approximately 95% of the screening hits (1204 out of 1274) were reported to be colloidal aggregators (Feng et al. 2007). The utilization of a kinetic readout ensured that the interference from coloured substances on the assay readout was negligible. In addition, very few actives were found to be attributable to reactive compounds, with aggregators outnumbering the reactive hits by a ratio of over 100:1 (Babaoglu et al. 2008). These observations prompted the inference that non-specific covalent inhibition may be a minor issue where β-Lactamase is concerned and that the enzyme was possibly more vulnerable to inhibition by aggregate particles. Given the fact that β-Lactamase appears to have an increased susceptibility towards aggregate-based inhibition that is easily verifiable by the methods described above,

compounds that inhibit β-Lactamase would be ideal to substantiate whether or not an alternative technique for determination of compound aggregation has good predictive value.

4.2 Design and implementation of compound library screening for inhibitors of β-Lactamase

Shoichet's group used *E. Coli* AmpC β-Lactamase in their assays, whereas *Enterobacter Cloacae* P-99 AmpC β-Lactamase was used for all experiments in this study due to its commercial availability. Though it has been established that *E. cloacae* β-Lactamase has a similar propensity to detergent-reversible inhibition by aggregate-forming compounds (Ryan et al. 2003); there have been no reports of large scale screens for inhibitors of this enzyme. Before screening compounds against *E. Cloacae* P-99 β-Lactamase in this study, it was verified with known aggregators of *E. Coli* AmpC β-Lactamase (McGovern et al. 2002, McGovern et al. 2003) and specific inhibitors of the AmpC β-Lactamase family; that detergent sensitive inhibition of *E. Cloacae* P-99 β-Lactamase functioned in a manner similar to that of *E. Coli* β-Lactamase (data not shown).

A small compound library (Novartis2008), comprising approximately 8000 compounds, was chosen to screen for inhibitors of *E. Cloacae* P-99 β-Lactamase. The primary screen was performed in detergent-free conditions utilizing a chromogenic assay format. One major deviation from the assay procedure followed by Shoichet et al. was the concentration of the chromogenic CENTA substrate. Shoichet's group used a high concentration of CENTA (Coan and Shoichet 2007) in their screens to avoid interference from coloured compounds; which was found to be at least 10 times higher than the reported K_m (10μM) of this substrate (Bebrone et al. 2001). In this study, the primary screen was run at a final CENTA concentration of 25μM, which was five times lower than the concentration in previously reported screens and was

closer to the published K_m of 10μM. Since the enzyme has a tendency to stick to plastic surfaces as noted previously (Ryan et al. 2003), 25μM was also observed to be the minimum substrate concentration required to obtain a good signal window in detergent-free assay conditions. Running the primary screen in completely detergent-free conditions did not influence screen quality; as the Z-factor associated with each plate remained above the required 0.5 cut-off (Fig. 5).

A threshold of mean ± 3SD is often used to select hits if data is normally distributed. Since in this study, the distribution of compound activity was found to be skewed to the right (Fig. 6), hits with the highest percent inhibition were chosen instead. A cut-off of 40.0% inhibition was selected based on normalized responses of all compounds in the library. Based on this cut-off, a hit rate of 0.23% was observed. This hit rate was found to be considerably lower than the 1.8% hit rate (1274 hits from a library of 70,563 compounds; Feng et al. 2007) obtained in a previous screen against *E. Coli* AmpC β-Lactamase. It is possible that the high substrate concentration (> 10-fold over K_m) used in that screen biased the screen towards detection of non-competitive inhibitors and lead to an inflated hit rate.

4.2.1 Prediction of aggregation-based inhibition by β-Lactamase hits based on sensitivity to detergent

To address concerns that aggregate-based inhibition might be influenced by the nature of the detergent; due to detergent affecting either enzyme activity or interaction between enzyme and compound or both; sensitivity to three different detergents was tested. One zwitterionic detergent (CHAPS) and two non-ionic detergents (Triton X-100 and Tween-20) were chosen. The concentration used was kept well below the CMC of each detergent to prevent possible entrapment of the compound within detergent micelles. The enzyme was found to have higher activity in the presence of detergent, possibly because addition of detergent to the buffer prevented the enzyme from binding to the assay plate (Ryan et al. 2003). The three

detergents impacted β-Lactamase activity differently. As observed from the controls on the assay plates, the enzyme was found to be most active in Tween-20 and Triton X-100 and least active in CHAPS. None of the detergents appeared to affect the inhibitory potency of a specific, reversible inhibitor of β-Lactamase, BZBTH2B; as evidenced from the unchanged IC_{50}'s in the detergent (+) screens (Table 2, Fig. 7).

In the case of Tween-20, Triton X-100 or CHAPS addition to the assay buffer; 13 of the 14 hits displayed a dramatic decrease in potency (either complete loss of inhibition or more than a 3-fold increase in IC_{50}). Only one compound, BLAC-11, was found to inhibit β-Lactamase in the presence of detergent (Table 2, Fig. 8), suggesting that it did not inhibit via aggregate formation. The results were consistent with the notion that a majority of β-Lactamase inhibitors act via an aggregation-based mechanism that can be detected by sensitivity of inhibition potency to detergent. The data suggested that although the various detergents influenced the enzymatic activity differently; attenuation of inhibition of β-Lactamase by aggregate-forming inhibitors was not found to depend on the type of detergent used in the counter screen.

4.2.2 Prediction of aggregation-based inhibition by β-Lactamase hits based on sensitivity to enzyme concentration

Testing the sensitivity of inhibition potency of the 14 hits to enzyme concentration was a challenging task. Ideally, the only variable in this counter screen should be concentration of the enzyme. However, upon increasing the enzyme concentration by 10-fold (2.5nM to 25nM) while keeping the concentration of the chromogenic substrate constant at 25μM; it was found that the substrate was depleted too quickly to allow measurement of reaction rate. The Shoichet laboratory uses a custom-made, slower-acting form of the chromogenic substrate at higher enzyme concentrations (McGovern et al. 2002). In our laboratory, a commercially available slower-acting substrate, Penicillin G, failed to achieve a suitable signal window and gave an unacceptable Z'-factor

To enable measurement of change in potency of β-Lactamase inhibitors upon increasing enzyme concentration, a more sensitive assay format was sought. Fluorocillin green, a non-fluorescent substrate for β-Lactamase that yields a green fluorescent product on enzymatic or chemical hydrolysis of the lactam ring, has been used in recent years by research groups that work with *E. Cloacae* AmpC β-Lactamase (Shukla and Krag 2009). In comparison to chromogenic substrates, it has been reported as a more sensitive means of assaying P99 β-Lactamase activity (personal communication, Shukla).

The fluorogenic assay format was found to have a broad, dynamic signal range. This facilitated measurement of enzyme activity at two enzyme concentrations 10-fold apart while keeping the substrate concentration constant. The sensitivity of compound inhibition to enzyme concentration was tested in detergent-free conditions, in the same buffer that was used in the primary screen. Whereas with the chromogenic substrate, a minimum of 2.5nM of enzyme was required to observe a reaction signal, fluorocillin allowed quantification of enzyme activity with as low as 0.5nM β-Lactamase. At this low concentration of 2.5nM, most hits had well defined dose-response curves with Hill co-efficients in the acceptable range (Table 3).

To validate the applicability of this assay format in determining aggregate-based inhibition, the detergent sensitivity counter screen was repeated with the fluorocillin substrate. The result was the same as that obtained in the determination of detergent sensitivity of hits using a chromogenic substrate; 13 of the 14 hits tested were found to display (Tables 2 and 3). The confirmation of detergent sensitivity by an orthogonal assay with a different readout established that neither method is biased towards detection of aggregate-forming inhibitors.

4.2.3 Prediction of aggregation-based inhibition on the basis of particle size measurements of β-Lactamase hits using Dynamic Light Scattering

The particle sizes obtained by DLS were inconclusive in determining aggregation due to of the lack of reproducibility, sub-optimal data quality and high signal to noise ratios (Fig. 9, 10). The poor quality of data can be partly attributed to the inherently low resolution nature of DLS and its consequent inability in dealing with samples that have high degrees of polydispersity. Recent studies have illustrated the limited ability of current DLS technology in providing reliable data on polydisperse samples (Filipe et al. 2010). These limitations have largely to do with the DLS particle-sizing models. The first order result from a DLS experiment is the intensity distribution of particle sizes. The DLS particle-sizing model assumes scattering from uniformly sized spherical particles. Drug-like compounds can aggregate in non-spherical forms (McGovern et al. 2002; Feng et al. 2008; Chan et al. 2009, Doak et al. 2010). In addition, it is possible that aggregating compounds display size heterogeneity in solution and that the different aggregate species have irregular shapes (Fig. 2). These factors could lead to large fit errors (Malvern Instruments Technical Support, personal communication). The intensity distribution is weighted according to the scattering intensity of each particle fraction. Whereas particle-sizing models are capable of generating reliable particle size distributions for species with widely differing molecular masses, they are ill-equipped to resolve samples whose particle fractions don't differ greatly in mass or size. Furthermore, the particle scattering intensity is proportional to the square of the molecular weight. As such, the intensity distribution can be somewhat misleading, in that larger particles can dominate the distribution.

The presence of unwanted signals in the scattered light is another major limitation to the quality of light scattering results. Studies have reported inconsistencies in particle size measurements due to elevated signal to noise ratios (Ryan et al. 2003; Chan et al. 2009). The occurrence of noise in DLS could be due to various factors such as fluctuations in the intensity of the laser beam, light scattering

by the solvent, convection currents in the scattering cell, scattering at the wrong angles due to reflections in the cell, or presence of foreign particles such as dust (Pecora 1985). These factors can be a great hindrance in achieving reproducible measurements.

Other factors such as optical and chemical properties of the test compound can influence the measurement. If a compound forms aggregates with dimensions comparable to the wavelength of light, intra-aggregate interference effects can dramatically reduce the apparent size of the aggregates (Bloomfield 2000). Further, compounds that absorb light at or near the wavelength used in the DLS measurement (between 500-650nm depending on instrument) may escape detection due to interference. For example, Congo red, a compound that is known to be an inhibitor of β-Lactamase and forms aggregates observable by TEM, does not form aggregates detectable by light scattering (McGovern et al. 2002; Feng et al. 2005).

Signals from aggregates can be weak depending on the type of aggregate and the nature of the aggregating compound, leading to inconclusive results. A study on promiscuous inhibitors of β-Lactamase revealed that 27% of a set of randomly selected drug-like molecules were 'ambiguous' light scatterers and therefore could neither be classified as non-aggregators nor aggregators (Feng et al. 2005). The same study also noted discrepancies between DLS data and the tendency to inhibit enzyme activity via an aggregation-based mechanism. Some detergent-sensitive inhibitors did not scatter light by DLS and conversely all light-scattering compounds were not found to inhibit β-Lactamase. It was noted that light-scattering precipitates lacking inhibitory activity accounted for a significant proportion of the discrepancies, highlighting the inability of light scattering techniques to distinguish between precipitation and aggregation.

For particle size measurements of β-Lactamase hits in this study, it was found that DLS analysis was a not suitable approach to get quantitative information about size of aggregate particles formed in solution. The measurements were time-consuming; each compound often required repeated measurements due to lack of reproducibility. The measurement settings had to be adjusted for each compound; and despite efforts at optimization the raw data quality still remained sub-standard. There are research groups that use DLS successfully to measure sizes of aggregates; therefore we can only speculate that either the hits from the β-Lactamase screen had qualities that interfered in the DLS measurements, or the equipment used was unsuitable for measurements of aggregates of drug like-molecules.

4.3 Determination of specificity of DENV RdRp hits

4.3.1 Assessment of classification of specificity of DENV RdRp hits based on detergent sensitivity of inhibition potency

The hits from the pilot screen that passed both the auto-fluorescence and CIP inhibition counter screens (see Materials and Methods) were tested for sensitivity to detergent. The major difference compared to the β-Lactamase primary screen was that whereas β-Lactamase activity could be measured in the absence of detergent; some amount of detergent was needed to achieve a good signal window in the DENV RdRp assay. Therefore for the β-Lactamase detergent sensitivity counter screens, detergent was added to the buffer and the IC_{50}'s in the presence and absence of detergent were compared; whereas for the DENV RdRp hits sensitivity to detergent was determined by testing the compounds at varying concentrations of detergent.

To investigate whether the nature of detergent affected the inhibitory potencies of hits against DENV RdRp, the hits were re-tested for activity in the presence of Triton X-100, Brij-35 and CHAPS (Table 4). The IC_{50} values of the hits appeared to be highest in Brij-35, followed by Triton X-100 and then CHAPS;

however in many cases the IC_{50}'s of the compound remained were not significantly changed. The fact that Brij-35 and Triton X-100 are non-ionic whereas CHAPS is zwitterionic might account for the difference in potencies in the presence of different types of detergent. The hits were subsequently tested at higher amounts of detergent, allowing quantification of change in IC_{50} as a measure of aggregate-based inhibition. The change in potency upon increasing detergent concentration was not consistent among the various detergents (Table 5). For many compounds, it was found that they displayed detergent-sensitive inhibition in only one of the detergents tested. It is possible that each detergent interacts differently with the compound or the enzyme-inhibitor complex.

Detergent has been known to affect the interaction between enzymes and their inhibitors, depending on the type and the concentration used. In a study on West Nile virus protease, it has been observed that non-ionic detergents like Triton X-100 and Tween-20 interfered with the inhibitory activity of a specific inhibitor of the protease at concentrations above 0.001%; whereas zwitterionic detergent CHAPS did not affect the inhibitory potency of the compound (Ezgimen et al. 2009). In another study, 0.05% Triton X-100 was found to disrupt the inhibition of the reverse transcriptase of human immunodeficiency virus type 1 by a specific inhibitor (Debyser et al. 1992). A study on Rce1p protease reported that disruption of aggregates was dependent on the concentration and type of detergent (Manandhar et al. 2007). It was found that aggregates of certain compounds were not dissociated by Triton X-100 below 0.04%; and that certain aggregates could be dissociated by Triton X-100 but not Tween-20 at a concentration near its CMC. This implies that specific inhibitors could be mis-identified as non-specific if detergent interferes in the interaction of the inhibitor with the enzyme. Thus determining specificity solely based on testing sensitivity of inhibition potency to detergent can be misleading. While in this study, the inhibition of DENV RdRp by its specific inhibitor, 3'dATP,

appeared to be unaffected by detergent type and concentration; this may not hold good for other specific inhibitors of the enzyme. Thus in a search for novel inhibitors of an enzyme such as DENV RdRp, it is necessary to be aware of fallible nature of detergent sensitivity in classifying whether or not a compound is a specific inhibitor.

Another observation of interest was the frequency of detergent-sensitive inhibitors. Whereas for the non-ionic detergents Triton X-100 and Brij-35, of the 30 hits, 43 and 54% respectively were detergent-sensitive inhibitors; only 20% of the hits had inhibition potency that was sensitive to zwitterionic CHAPS. Given that IC_{50} values were lower in buffer containing a low concentration of CHAPS, IC_{50} shifts were conceivably less pronounced when compounds were subjected to a higher concentration of CHAPS; as opposed to shifts in Triton X-100 and Brij-35. Additionally, in comparison to previous reports on large numbers of detergent-sensitive hits (as high as 95%) in screens against β-Lactamase (Feng et al. 2007) and cruzain (Jadhav et al. 2010), the number of hits that inhibited DENV RdRp in a detergent-sensitive manner was found to be much lower in all detergents tested. This could be because DENV RdRp is not as prone to sequestration by aggregating compounds as other enzymes. Since the binding between the DENV RdRp and its RNA substrate appears to be strong (~10nM K_m^{app} for RNA substrate, Table 7); it is possible that the active enzyme which is in the form of an enzyme-RNA complex, is less susceptible to inhibition via an aggregation-based mechanism.

4.3.2 Assessment of classification of specificity of DENV RdRp hits based on sensitivity of inhibition potency to enzyme concentration

For inhibitors of DENV RdRp, testing sensitivity to enzyme concentration was complicated by the fact that it is a multi-substrate enzyme in which the substrates are charged. As mentioned earlier, the ideal scenario would be one where substrate

concentration was kept constant over the range of enzyme concentrations tested so that the only variable would be the amount of enzyme. Keeping the amount of the BBT-ATP substrate constant (at 2μM), inhibition was tested at 10nM and 100nM DENV RdRp. RNA concentrations of 50nM and 150nM were used for 10nM and 100nM enzyme respectively to ensure that a saturated amount of RNA template was available to the enzyme for it to be able to initiate the reaction. Nine hits appeared to have inhibition that was insensitive to enzyme concentration (Table 6), but seven of these (RDRP- 1, 7, 13, 14, 15, 16, 26) were found to have attenuated inhibition in high amounts of at least one detergent (Table 5). Eight of the ten hits that had inhibition potencies insensitive to detergents appeared to lose potency at increased enzyme concentrations; indicating very poor agreement between these two methods of identifying non-specificity.

Besides the enzyme concentration, amount of 3'UTR-U_{30} RNA used in the reactions was the only other variable that might have influenced inhibition potency. Therefore effect of RNA concentration on DENV RdRp inhibition was examined (Fig. 11). Compounds were re-tested at 10nM RdRp using an increased RNA concentration of 150nM, to match the substrate concentration used at 100nM enzyme. IC_{50} values were found to be lower than when 50nM RNA was used. In other words, the inhibitors appeared to be more potent when increased concentrations of RNA were used. Therefore, under the new conditions where RNA concentrations were now the same, 29 out of 30 hits had IC_{50}'s that were more than 5-fold higher at 100nM compared to 10nM RdRp. An RNA concentration of 150nM was found to be more than 10-fold higher than the K_m^{app} (~10nM) of the RNA substrate determined at 10nM DENV RdRp (Table 7). It was also observed that the rate of enzyme reaction began to drop at RNA concentrations above 50nM (Fig. 11). Thus it is possible that high concentrations of RNA had an adverse effect on the enzyme activity; causing the inhibitors to possibly appear more potent due to the enzyme being less active rather

than due to true inhibitory potency. Additionally, since RNA has a negative charge, it might bind to the enzyme non-productively (i.e. at non-catalytic sites) and lead to reduction in enzyme activity. Therefore changes in IC_{50} values at different enzyme concentrations could possibly be a DENV RdRp assay artifact caused due to interaction between the enzyme and substrate; rather than a reflection of non-specific inhibition.

4.4 Steepness of dose-response curves as an indicator of aggregate-based inhibition

Steep dose-response curves have been found to be associated with aggregate-forming inhibitors of β-Lactamase (Feng et al. 2007). However, in this study, it was observed that the dose-response curves of the hits from the β-Lactamase screen were dependent on the assay format used to test the compounds. Of the 14 hits tested, it was observed that 13 hits had inhibition potencies that sensitive to detergent in both chromogenic and fluorometric assay formats. Whereas in the chromogenic format, 11 compounds had steep dose-response curves with high Hill co-efficients, only 5 compounds displayed steep dose-response curves in the fluorometric format (Tables 2 and 3).

There have been no reports on the nature of the dose-response curves of inhibitors of the DENV RdRp enzyme. A recent study on the association of dose-response curves and inhibitory potential of anti-HIV drugs reported that different classes of anti-viral drugs were found to be typically associated with specific slope values (Shen et al. 2008). It was found that non-nucleoside and nucleoside inhibitors of the reverse transcriptase had dose-response curves associated with slopes values greater than 1 and ~1 respectively and that slopes were indicative of anti-viral activity of the compounds *in vivo*.

In this study, among the DENV RdRp hits, most compounds had slope values close to 1 (Table 4) and consequently the hits could not be separated into different slope classes. Furthermore, the steepness of dose-response curves of the RdRp hits found to be dependent on which detergent was added to the assay buffer (Table 5). None of the 30 hits had steep curves in buffer containing CHAPS, despite 10 hits displaying detergent sensitive inhibition at a higher concentration of CHAPS. Among the 16 hits that had lower inhibition potencies at a higher concentration of Triton X-100, only 1 had a steep dose-response curve. Among the 13 hits that had higher IC_{50} values at increased concentrations of Brij-35, only 5 hits had dose-response curves indicative of non-specific aggregation-based inhibition. Hence, slopes of the dose-response curves were indicative of neither nature of inhibitor nor nature of inhibition among hits of the DENV RdRp enzyme.

4.5 Target specificity of aggregate-forming inhibitors

Many reports have claimed that inhibition of a panel of unrelated enzymes is a sign that that a compound is acting via an aggregation-based mechanism (McGovern et al. 2002; McGovern et al 2003). If aggregate-forming inhibitors were capable of inhibiting several targets by simply by virtue of sequestering/denaturing the enzyme, these compounds would frequently appear on hit lists from various screens. Since a majority of the hits from the β-Lactamase screen performed in this study were detergent-sensitive and therefore assumed to be aggregate-formers, these compounds should cause aggregation-based interference in other enzyme assays if non-specific aggregate-based inhibition was purely a characteristic of the respective compound. As can be seen in Figure 12, a retrospective analysis revealed that this does not appear to be the case. The compounds that inhibited β-Lactamase were not found to inhibit any other enzyme and vice versa. Since the inhibition of β-Lactamase by these hits was confirmed in a fluorometic assay format, it cannot be argued that a

particular assay format is predisposed to interference by aggregate-forming inhibitors. Extremely low frequency of overlap between the hits of two enzymes (Pantothenate Kinase and Nicotinamide adenine dinucleotide synthetase) for which exactly the same assay format was used further corroborates the dependence of aggregate-based assay interference target protein properties and assay conditions employed.

The results of assaying hits from other enzyme assays for inhibition of β-Lactamase, suggest that it cannot be used as a convenient proxy for aggregate-based promiscuity. If aggregation-based inhibition was dependent solely on the properties of the compound, we would expect more compounds that inhibited their target enzyme non-specifically to inhibit β-Lactamase; an enzyme known to be susceptible to aggregate-based inhibition (Ryan et al. 2003; McGovern et al 2003). However, only 10% of detergent-sensitive RdRp hits were found to inhibit the enzyme (Fig. 13). In addition, compounds that specifically inhibit their respective target enzyme should not inhibit β-Lactamase enzyme as was found with some specific inhibitors of PanK (Fig. 14). As can be observed from hits of the RdRp and PanK enzyme, β-Lactamase inhibition is not a good indicator of non-specificity. Thus inhibition of a model enzyme like β-Lactamase cannot be used as a generic assay to detect aggregation-based inhibition. The results from this study further highlight the assay dependent and conditional nature of aggregate-based inhibition.

4.6 Viability of change in meniscus assay as a generic assay for detection of aggregation

The assay was initially validated using 14 known aggregators and 8 non-aggregators (Cai and Gochin 2007). There have been no reports of its application in a HTS campaign. As demonstrated in this study, measurement of change in meniscus

shape as an indicator of compound aggregation appears to have good predictive value. For the β-Lactamase hits, the classification of aggregate formers based on changes in meniscus was in good agreement with detection of aggregation-based inhibition based on detergent sensitivity tests (Fig. 15). For the DENV RdRp hits (Fig. 16), it is difficult to determine the predictive value of the meniscus measurements as for more than 50% of the hits; detergent sensitivity results were inconclusive due to lack of conformity between IC_{50} changes in different detergents (Table 5). Among the compounds for which sensitivity of compound inhibition to detergent was consistent in all 3 detergents tested, the meniscus data was generally found to be in agreement. Among the PanK compounds, the assay accurately identified a majority of the enzyme-concentration sensitive (i.e. non-specific) inhibitors as aggregate formers. However, for the PanK hits that were designated as specific based on insensitivity of compound inhibition to enzyme concentration; there were a few compounds that had ratios normally associated with colloid formation (Fig. 17). Additionally, the specific compounds among the PanK hits with ratios much smaller than 3SD's from the mean of the reference inhibitor (Acetyl CoA) ratio could not be confirmed as binders by NMR analysis due to poor solubility. Thus either they were false positives in the enzyme-concentration sensitivity tests or false negatives in the assay measuring changes in meniscus.

As with any other assay, there is the possibility that the meniscus assay can incorrectly classify compounds. Interaction of compounds with the fluorescent dye included in the buffer can influence the fluorescence intensity without a corresponding change in liquid meniscus (Cai and Gochin 2007). Cloudy aggregate solutions or coloured compounds that strongly absorb light and affect the readout are other sources of assay interference (personal communication, Cai). Formation of aggregate particles by a compound does not always translate to enzyme inhibition (Feng et al. 2005). Therefore even if a compound was found to bring about a change

in the shape of the liquid meniscus due to colloid formation, it may not necessarily be found to inhibit the target in an assay measuring enzyme activity. Furthermore, there is a caveat to detection of aggregation by a compound in the absence of enzyme. Some compounds only form aggregates in the presence of enzyme (Reddie et al. 2006) and therefore would escape detection by in the meniscus assay but can be detected in an assay involving measurement of enzyme activity.

As demonstrated in this study, an assay measuring changes in meniscus shape can be applied to study inhibitors of any enzyme. Since the change in meniscus shape due to formation colloidal particles is based on pronounced capillarity observable in high density (384, 1536) multi-well plates, the assay is utilizable in high throughput settings. As opposed to testing sensitivity of compound inhibition to detergent or enzyme concentration, the assay directly detects aggregate formation by the compound and does not involve either enzyme or substrate. Thus no knowledge of enzyme kinetics or interaction of detergent with the target enzyme is required. The assay is not limited by choice of buffer and allows measurement of compound aggregation in the buffer used to measure enzyme activity. As aggregation of small molecule compounds is partially dependent on the composition of the aqueous medium (Augustijns and Brewster 2007), detecting aggregate formation in the assay buffer is more relevant than testing whether or not a compound inhibits a model enzyme like β-Lactamase. While this assay involves use of detergents such as Triton X-100 and Tween-20, the purpose of the detergents is not to prevent aggregation or prevent interaction of the target enzyme with aggregate particles. Rather, it exploits the ability of detergent micelles to induce a change in the shape of the liquid meniscus, allowing assay buffer containing detergent at concentrations higher than its CMC to serve as a control. A ratio of observed fluorescence of compound in assay buffer to that of compound in the same assay buffer containing detergent thus ensures alleviation of any compound-specific absorption or fluorescence.

4.7 Concluding remarks

As seen from the lack of correlation between hits from screens of different enzymes, aggregate-forming inhibitors appear to be target specific rather than 'promiscuous'. The ability of a compound to inhibit a target via an aggregation-based mechanism appears to depend on factors such as nature of the target enzyme, enzyme kinetics, assay conditions employed to measure enzyme activity and the nature of the compound itself. A compound that inhibits its target enzyme specifically could potentially inhibit another enzyme non-specifically. The results advise against a one-off characterization of library compounds and suggest that identification of aggregation-based inhibition needs to be addressed for each new target separately.

Additionally, as seen from this study, testing sensitivity of compound inhibition to enzyme concentrations requires a good understanding of the enzyme kinetics and measurement of change in inhibition potencies in the presence of detergent can be influenced by the type of detergent used. To avoid falsely labelling compound inhibition as detergent-sensitive as a result of interaction between the detergent and enzyme or enzyme/inhibitor complex; ideally the effects of more than one detergent should be tested. Hence, measuring changes in inhibition potency at varying detergent or enzyme concentrations can be a tedious process.

The meniscus-based aggregation assay, on the other hand, is simple to implement and provides a quantitative direct measurement of formation of aggregates in solution. The assay is practicable in high throughput settings without the requirement of specialized equipment or time-consuming data-analysis. In future studies, the concentration dependence of aggregation could easily be followed using a dose-response curve in the fluorescence assay. In addition, formation of aggregate particles could be monitored in the presence of the target enzyme or active form of

the enzyme in the form of a complex (e.g., active form of DENV RdRp is in the form of DENV RdRp-RNA complex). Since this assay directly measures aggregate formation rather than enzyme activity, no knowledge of enzyme kinetics would be required. Furthermore, the assay would require no additional optimization, merely an additional step of inclusion of enzyme in the assay buffer.

Therefore, using meniscus measurements as an indication of aggregate formation; large numbers of HTS hits could conceivably be prioritized efficiently for subsequent characterization by methods such as isothermal calorimetry, NMR, surface plasmon resonance and X-ray crystallography, which allow direct measurements of binding but require dedicated equipment and have much lower throughput.

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