Hypothesis-Driven Screening

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Chapter 13

Hypothesis-Driven Screening

Ulrich Schopfer, Caroline Engeloch, Frank Höhn, Hervé Mees, Jennifer Leeds, Fraser Glickman, Günther Scheel, Sandrine Ferrand, Peter Fekkes, and Martin Pfeifer

Summary

Phenotypic chemogenomics studies require screening strategies that account for the complex nature of the experimental system. Unknown mechanism of action and high frequency of false positives and false negatives necessitate iterative experiments based on hypotheses formed on the basis of results from the previous step. Process-driven High Throughput Screening (HTS), aiming to "industrialize" lead finding and developed to maximize throughput, is rarely affording sufficient flexibility to design hypothesis-based experiments.

In this contribution, we describe a High Throughput Cherry Picking (HTCP) system based on acoustic dispensing technology that was developed to support a new screening paradigm. We demonstrate the power of hypothesis-based screening in three chemogenomics studies that were recently conducted.

Key words: High throughput screening, Compound management, Hypothesis-driven screening, High throughput cherry picking, Bacterial growth inhibition, Biosynthetic pathway, Yeast growth modulation

1. Introduction

High Throughput Screening (HTS) is often regarded as the main culprit for the decline in research productivity at large pharmaceutical companies *(1)*. It is portrayed as mindless screening of large compound collections with unsuitable properties. Disappointingly, this impression overlooks the positive developmental trajectory that the young science of screening has taken over the last 15 years. However, the negative view does highlight the two most critical factors for a successful HTS: a carefully planned and executed screening strategy and a high-quality, diverse compound

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library. Therefore, focusing on the investments in automation as intellectually inferior attempts to replace the scientific method with massive screening overlooks an important distinction; automation increases the speed and throughput of experimentation, but it is not a replacement for a well-validated experimental design, which ultimately determines the usefulness of the results. Similar to previous technological developments, for example, combinatorial chemistry, it is the initial euphoria that, when followed by disappointment, leads to frustration. Only later, when the technology is applied to commensurate problems and when it is combined with other approaches does the technology show its true potential.

One of the most exciting new themes in this context is the positioning of HTS in the repertoire of chemogenomic approaches *(2, 3)*. The goal of chemogenomics is to elucidate gene function by perturbation of the gene product or associated pathway with a small molecule. The ability of HTS to employ over a million effector molecules to investigate individual components of a larger cellular system provides science with a powerful, rational strategy to discover target–ligand pairs. A phenotypic approach that screens for perturbations of cellular systems, e.g., in antibacterial discovery, can be defined as a forward chemogenomic strategy (function → gene). Likewise, a biochemical screen for compounds that bind to a purified protein, e.g., a nuclear receptor, constitutes a reverse chemogenomic approach (gene → function) *(4)*.

Forward chemogenomic approaches are utilized when little is known about a potential target, and approaches such as docking and virtual screening cannot be used to identify ligands. HTS methods are ideally suited to tackle such problems since they can initiate, e.g., in the case of a cellular phenotypic screen, with very little information about targets. Once a target has been identified, the chemistry space can be explored in greater depth through subsequent rounds of screening to discover viable small molecule leads for chemical optimization. Further rounds of screening will determine SAR around the previously identified chemotype.

The development of HTS was recently reviewed by Hertzberg et al. and Macarron et al. *(5, 6)*. The early days of HTS were characterized by a focus on quantity and speed. Compound collections were assembled mainly from internal sources, e.g., from agricultural or dye programs, without giving too much consideration to the suitability of compounds for drug discovery. Combinatorial chemistry was in its infancy, using mainly amide formation to produce large libraries of compounds. However, this period also saw the development of some of the essential tools of today. Under the auspices of the Society for BioMolecular Screening *(7)*, the standards for high-density microplates were developed. This triggered rapid development of laboratory automation, with the most impressive results in multiparallel liquid

handling. Homogeneous assays were developed that supported the "mix-and-read" mode which enables throughput in excess of 100,000 wells per day.

Since the 1990s, an intense focus on quality has eliminated many of the earlier weaknesses in HTS. A strong emphasis on physicochemical properties of compounds led to the development of concepts such as drug-like and lead-like properties *(8–10)* which had a large impact on the direction of compound libraries. The statistical analysis of HTS data allowed a better interpretation of primary data and quality parameters emerged that guided assay development and execution *(9, 10)*. Efforts to "industrialize" HTS were directed at reducing variability at all stages of the process *(11)*. Not only the practice of HTS improved, also the interfaces to target biology and chemistry were optimized. Upstream, target validation and "druggability" were assessed, and downstream, a new hit-to-lead process *(12)* emerged that attempted to generate a few high quality lead compounds from large hit lists. Looking back over the last 20 years, a powerful set of technologies has been developed that allows massive, parallel experimentation to produce high quality data. While there continues to be further development of automation and screening, HTS can be considered to be a mature technology today.

The focus on performance and standardization has led to a process-driven screening paradigm that is mainly governed by the capabilities of large, automated screening factories. While the process-driven HTS approach is typically described as a linear sequence of steps, future lead finding strategies will incorporate a rational selection of parallel and iterative experiments.

HTS practitioners will shift their focus from technology development to skillful application of screening technologies to investigate biological problems. A well-defined scientific question leads to the formation of a robust experimental strategy which is designed to answer that specific question. The results, regardless of whether they support or reject the hypothesis, in turn help to define the next round of experiments. This iterative strategy is very different from the historical HTS paradigm, which was largely process-driven and guided by the capabilities of the automation and logistics processes of a screening operation **(Fig. 1)**.

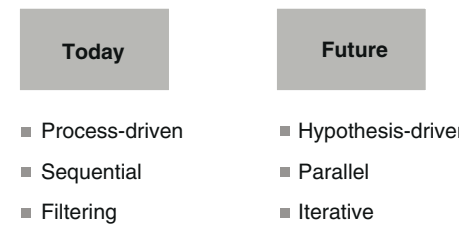

Fig. 1. Process-driven versus hypothesis-driven screening.

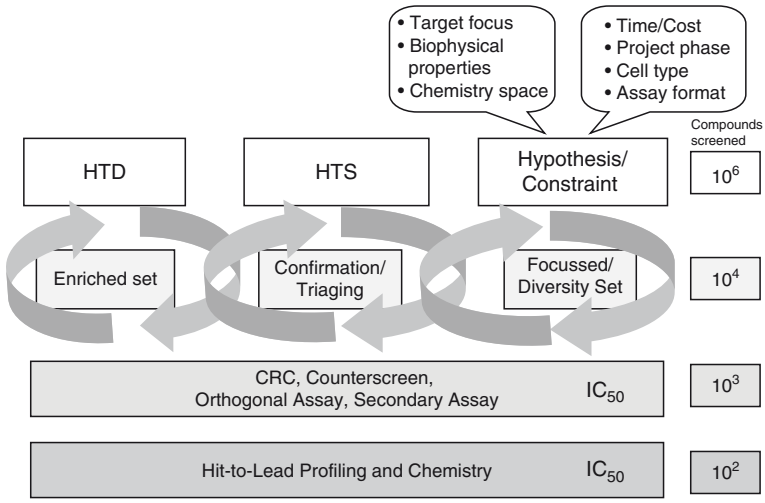

Fig. 2. Hypothesis-driven screening combines iterative and parallel elements. *H2L* Hit-to-Lead.

Increasingly, full-deck screens will be followed, preceeded or even replaced by Medium Throughput Screens (MTS) of preselected compound sets **(Fig. 2)**. Cheminformatics methods will be used to a much larger extent to customize compound selections for more diverse, or more focused, sets. The scope of cheminformatics will expand beyond the current focus on reduction of hitlists ("triaging") to a broader panel of activities that guide hitlist follow-up, including prediction of pharmacophores, expansion of chemotypes, and prediction of false positives. High Content Screening (HCS) or Fragment Based Screening (FBS) will be used routinely in lead discovery projects alongside HTS activities.

Technical feasibility and financial constraints e.g., in assays with primary cells or Imaging/HCS, limit the number of compounds that can realistically be screened. In these cases, iterative or focused strategies can offer a useful alternative to whole-library HTS. Pilot screens that are geared towards helping to develop and validate follow-up assays, or focused screens that deliver tools for target validation and assay development are useful approaches to increase the likelihood of a successful downstream HTS. The use of High Throughput Docking (HTD) to predict targeted libraries or combining screens with multiple readouts are other ways to enhance the information content of screening programs.

Logistics, automation and data handling systems will have to be adapted to support the new paradigm, which necessitates follow-up such as orthogonal screens, counter screens and other iterative interrogations of a primary hitlist. HTS systems have to be designed to support experimentation, such that one addresses

a scientific hypothesis, rather than simply conforming to an industrialized process.

As a prototype for next generation automation which supports this paradigm, we discuss the design of a High Throughput Cherry Picking (HTCP) system based on acoustic dispensing. In the examples illustrated below, we demonstrate how hypothesis-based screening combines input from virtual screening, structural biology and other approaches to support a powerful strategy for both target and lead discovery via chemogenomics.

2. Methods

2.1. High Throughput Cherry Picking: System Design

Traditional plate logistics systems produce copies of preformatted 384-well or 1536-well master plates. This allows the efficient supply of standardized sets in excess of one million compounds. These systems also have a "cherry-picking" capability, allowing random access to individual samples that are typically stored in individual tubes *(13)* and providing 1,000–10,000 samples per day. However, the aforementioned systems offer only limited flexibility for the assembly of focused compound sets. Hypothesis-based screening requires logistics that allow to select large compound sets to be assembled with flexibility of plate layout. By increasing the number of samples that can be picked, large hit-lists from HTD or from primary screening can be tested. Flexible plate layouts allowing for repeats and multiple concentrations of compounds enable the researcher to tailor the experiment to the scientific question. For the design of a HTCP system the following key requirements were identified: (1) Storage of approximately 1,000 source plates in 1536-well format, (2) Maintenance of sample integrity for at least 6 months, (3) 40,000 picks per 24 h in 20–1,000 nL volumes, (4) Dispensing quality with CVs ≤10% and a bias ≤10%.

In collaboration with Velocity11 *(14)*, a concept was developed having as key components a 1536-well plate store and the innovative acoustic dispensing technology. A full copy of the Novartis screening deck, as 2 mM DMSO stock solutions in 1536-well source plates, is housed in an integrated cold storage unit. The major technological advance in this new system is the ECHO device (Labcyte), an acoustic dispenser that is capable of ejecting solution droplets. Volumes from 2.5 nL up to 1 μL can be transferred with high accuracy over repeated dispensing. The HTCP system is built on the BioCel 1800 platform, integrating a number of specialized modules designed for specific tasks **(Table 1)**.

Table 1
High throughput cherry picking, main system components

Instrument	Provider	Description
BioCel 1800	Velocity 11	Automation platform
VSpin	Velocity 11	Microplate centrifuge
VStack	Velocity 11	Labware stacker
Nanodrop I	Innovadyne	Automated liquid pipettor for low volume back-fill dispensing
ECHO 550	Labcyte	Compound reformatter for low volume compound transfer and low volume back-fill DMSO dispensing
Cytomat 44	Thermo	Controlled climate automated storage system for microplates

2.2. Acoustic Dispensing

The ECHO device is a reliable liquid handler designed to dispense in the nanoliter range. The solution transfer is based on a contact-free technology that relies upon droplet ejections e.g. elicited by acoustic energy. An ultrasonic transducer circulated by a temperated immersion fluid is placed below the bottom of the source plate. An ultrasonic wave is transmitted through the immersion fluid, across the bottom of the source plate, into the solution in the well, and is refracted at the meniscus of the solution (**Fig. 3**).

The time of flight of the wave echo, recorded by the transducer, is calculated and gives exact information regarding the solution level in the well and the DMSO content of that solution. This calculation defines the exact amount of energy that is necessary to eject a droplet of e.g. 2.5 nL from the liquid surface and have it reach the bottom of a well in the inverted destination plate. The desired dispense volume is ultimately achieved by repeatedly exciting droplets with a frequency of close to 500 Hz.

2.3. Compound Storage

For the storage of low volumes (typically 6 µL per well) over extended periods of time, evaporation poses a significant challenge. A 1536-well COC plate *(15)* which was optimized to minimize evaporation by reducing laminar flows between lid and plate was developed jointly by Novartis and Greiner. In a long term storage study, the advantage of this optimized plate design could be shown (**Fig. 4**). The volume of the source plates stored in a Cytomat 44 *(16)* incubator was monitored using a volume audit functionality of the ECHO 550. These audits report the DMSO concentration and the liquid level per source plate well. The source plates were filled with 5 µL of 90% DMSO solution and stored at 4°C and at 15% relative humidity. The plates were loaded and unloaded from the store at daily

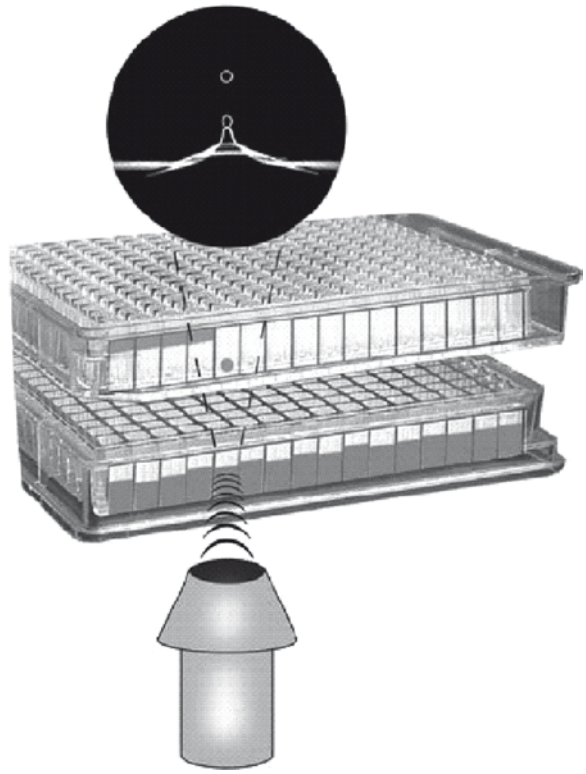

Fig. 3. Principle of acoustic dispensing. An ultrasonic transducer ejects droplets from the source well by the application of focussed sound energy. Figure reproduced with permission of Labcyte.

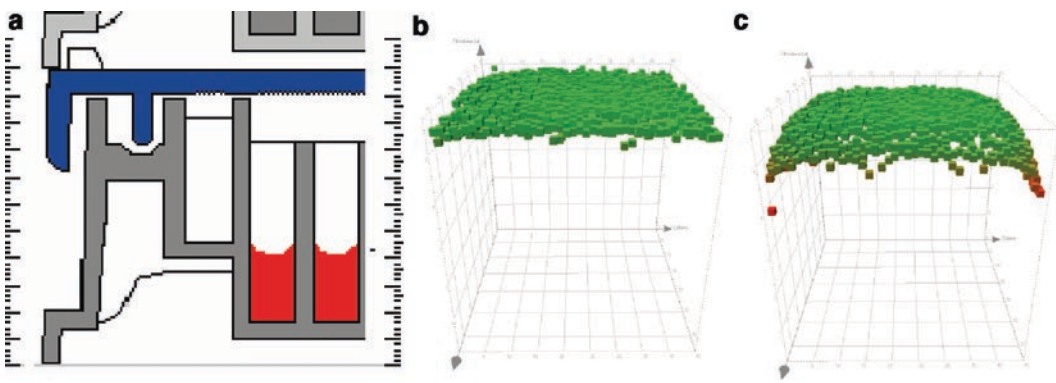

Fig. 4. Compound storage. (**a**) Scheme of the Novartis/Greiner 1536w COC storage plate. (**b**) Evaporation profiles of a source plate audited after loading and (**c**) after 3 months storage. Less than 1 μL volume loss was detected in the corner wells. Each point represents a well volume at the time of the audit.

intervals in order to simulate frequent plate access. After 10 weeks the average volume loss in the corner wells of these plates was less than 1 μL.

The Novartis approach of storing stocks solutions at 2 mM concentration in a 90:10 DMSO:water mixture *(15, 17)* provides a critical advantage for the HTCP system. With the addition of 10% water to DMSO, the freezing point of the mixture drops from 18 to below 4°C. This freezing point depression avoids the potential for repeated freeze/thaw cycles which could impact the solubility of some compounds. Since no melting step is required, sample access is rapid. At a storage temperature of 4°C and a relative humidity of 15–20% the water content of a 90:10 DMSO:water mixture is close to equilibrium so that very little further water is absorbed from the atmosphere.

2.4. Accuracy and Precision

Source plates (1536-well format) filled with 5 μL of a freshly prepared 10 μM fluorescein solution (obtained from a 10 mM stock solution) were loaded onto the ECHO device. Protocols with single volume dispense commands (50 nL, 40 nL, 10 nL) were then generated in order to produce three 1536-well black plates per test volume. The requested volume was dispensed to all wells except to columns 45–48 which were reserved for calibration and blank samples (1,408 data points per plate). Five microliters of diluent (buffer solution of PBS with 0.1% CHAPS) were then transferred to columns 1–44. After the addition of calibration solution to columns 45–48 the plate was measured on an Envision plater reader *(18)* (**Table 2**). The accuracy and precision values are excellent and in accordance with specifications reported by Labcyte *(19)*.

2.5. System Throughput

The throughput of the HTCP system was found to be dependent of a number of parameters. The picking rate of the acoustic dispenser ECHO 550 determines the maximum picking rate that can be achieved. However, with a cycle time of 2 min for each source plate that needs to be accessed, the retrieval of source plates from the store can become a bottleneck. Therefore, the

Table 2
Accuracy and precision of liquid transfers from a 1536-well source plate to a 1536-well destination plate on the ECHO 550 acoustic dispenser

Transfer volume (nL)	CV (%)	Bias (%)
2.5	6.2	1.1
10	5.3	1.6
40	5.2	1.4
50	4.5	1.1

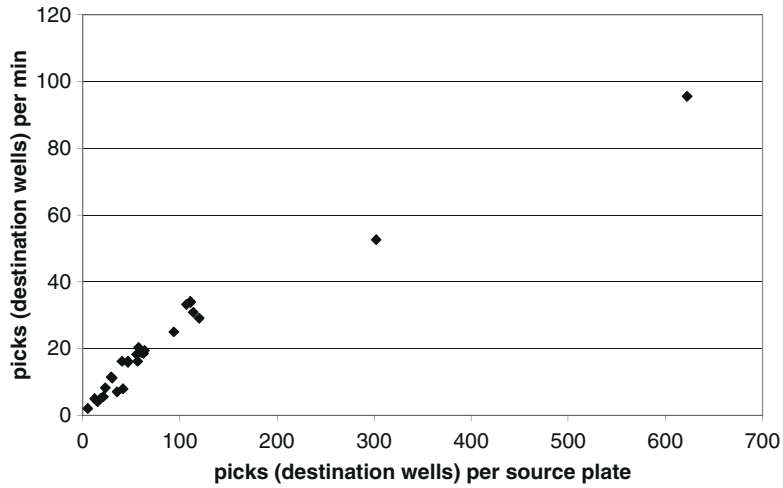

Fig. 5. Dependency of HTCP pick rates on source plate density. The pick rate (number of produced wells per minute) depends on the number of picks that can be taken from the same source plate.

number of compounds picked from the same source plate and the number of replicates and concentrations derived from one source well can have a dramatic influence on the pick rate **(Fig. 5)**. The amount of stock solution transferred from one source well is also an important parameter. The time needed to transfer volumes greater than 200 nL can have a measurable impact on the overall pick rate.

3. Results and Discussion

In this section, we exemplify the power of hypothesis-driven screening. Three chemogenomics projects, and the impact of flexible experimental design enabled by HTCP Picking capabilities are discussed. Two of these examples are drawn from the area of infectious diseases; the third example describes a neuroscience project.

Infectious diseases continue to be one of the major causes of global morbidity and mortality. Nevertheless, there have only been two truly novel antibiotic chemical entities approved by the FDA for human use in over 25 years; the oxazolidinones including linezolid, and the lipopeptide class exemplified by daptomycin. Notably, both of these "new" drug classes were identified by forward-chemogenomics, cell-based screening against bacterial pathogens during the "golden era" of antimicrobial discovery *(20)*. The "new era" of drug discovery emphasized the screening

of enormous synthetic chemical libraries against purified target enzymes. The challenge was to achieve potent antibacterial activity from inhibitors obtained via in vitro enzymatic screens, largely through empirical medicinal chemistry programs. The outcome of the in vitro target/synthetic chemical entity screening approach has, thus far, not led to new market introductions. Most compounds currently in clinical trials are derivatives of known chemical classes for which antimicrobial resistance mechanisms already exist in the clinical setting *(21)*. To improve our chances of discovering quality leads, we complemented the target-based antimicrobial discovery programs with cell-based high throughput antibacterial screens. This allowed us to harness the power of chemogenomics, a time-honored tool in the study of bacterial genetics and microbial physiology, to discover new targets and inhibitors. Combining large scale screening with rapid follow-up studies that were guided by cheminformatics to assist in chemical triaging, target identification, and liability assessment, we were able to take a more systematic approach to the otherwise "blind" process that characterized early cell-based antimicrobial drug discovery *(22)*.

3.1. Bacterial Growth Inhibition

Two cell-based antibacterial HTS programs were run at Novartis, separated by a 3-year interval **(Fig. 6)**. In the first HTS 600,000 compounds were tested and 700,000 additional compounds were assayed in the second HTS. In both HTS, growth of the Gram positive pathogen *Staphylococcus aureus* was assessed at a single compound concentration (40 μM). Bacterial growth inhibition was monitored by measuring the reduction of the nonfluorescent substrate resazurin to the fluorescent product resorufin *(23)*. A comparison of the throughput and timelines following each of the primary screens illustrates the impact of the HTCP system as an enabling technology for rapid, hypothesis-driven iterations of testing.

The first HTS yielded approximately 50,000 hits capable of imparting >95% inhibition of *S. aureus* growth at 40 μM. Since cherry-picking capacities were limited at this time, a strategy was designed to select the most promising candidates from this hit list. The hypothesis was that the application of strict *in silico* filters could achieve an enrichment of novel compounds with favorable "drug-like" properties, and the least potential for promiscuity *(24, 25)*.

Broad clustering algorithms were then applied to capture the largest diversity from the smallest number of compounds. Compounds containing overtly toxic pharmacophores, known to be unstable or impure, containing features considered unsuitable for further development, or with similarity to marketed antibacterial scaffolds were removed from further consideration. Having removed over 90% of the compounds from the primary hit list,

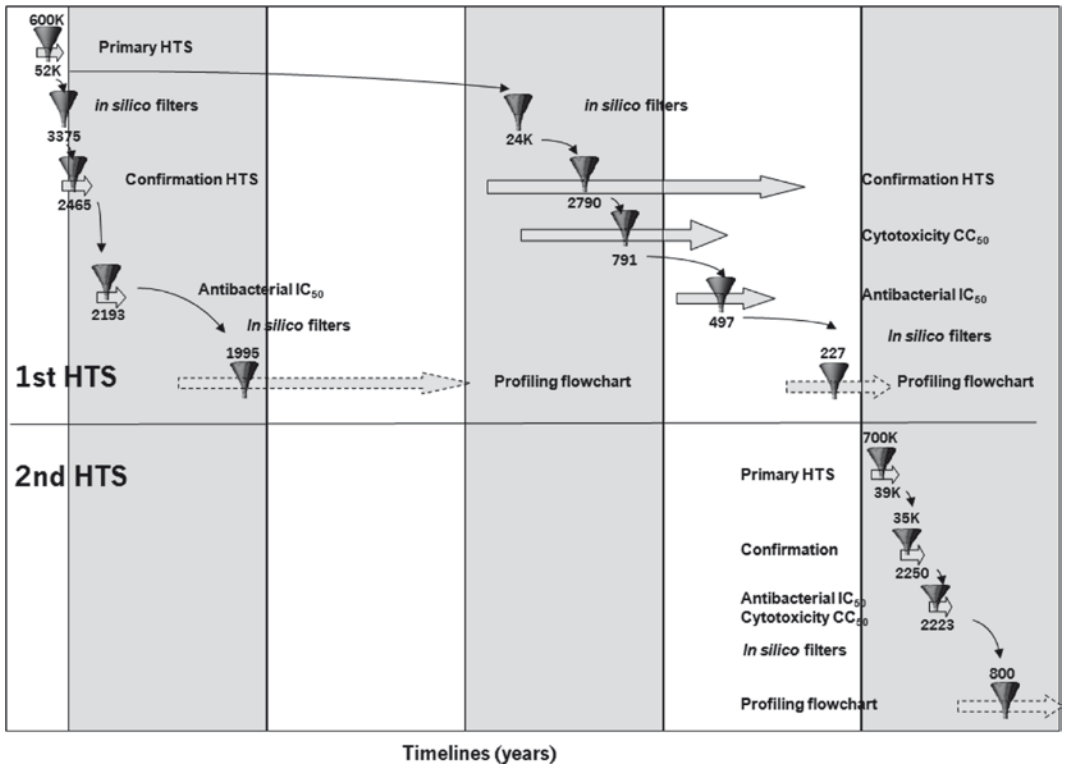

Fig. 6. Timelines and compound throughput for two Novartis HTS for bacterial growth inhibitors. The *top* and *bottom* portions depict the compound flows in two HTS campaigns against *S. aureus* in a five-year interval.

only 3,375 compounds were selected for confirmation screening. Of those, 2,465 were confirmed to inhibit bacterial growth by at least 50% at 40 μM, and were selected for dose-response assays. At the completion of the 4-month HTS campaign, 1,995 validated compounds were advanced into the downstream flowchart. While potent compounds emerged from this strategy, and lead optimization programs ensued, it also became apparent that the initial selection criteria were too stringent. Many antibacterials fall outside of the "drug like" profile because, for example, they are large, lipophilic molecules that either do not have to enter the cell cytoplasm, or have dedicated transporters *(24)*.

In an effort to cast a wider net for attractive antibacterial starting points, the hit list was submitted to a second iteration of testing. Approximately 24,000 compounds, after removal of the most undesirable scaffolds, were investigated. By cherry-picking 2,000 compounds per month, antibacterial confirmation screens against six organisms were performed at a single compound concentration (4 μM). 2,790 compounds inhibited growth of at least one test strain by 50% or greater. Over the course of 1 year, these antibacterial compounds were assayed for mammalian cytotoxicity. Compounds that had CC_{50} greater than 24 μM (the top

concentration achievable in the MTS cytotoxicity assays) were then tested for antibacterial IC_{50} via semiautomated methods. When the entire cell-based data set was available, cheminformatics tools were applied, and downstream flowchart activities on the 227 selected hits were initiated. Several additional novel compound:target pairs were discovered from this phase of the screening follow-up, which validated the expanded approach. To summarize, hit follow-up from the first antibacterial HTS began by looking first at compounds that fulfilled traditional drug-like criteria. In a second iteration, the remainder, and majority, of the hits were tested in time-consuming effort that took nearly 2 years. Despite the lengthy timelines, the efforts were rewarding, and multiple novel compound:target pairs were identified and progressed through the discovery pipeline.

A second antibacterial HTS was performed 3 years after the first HTS (**Fig. 6**, *bottom*). The second HTS benefited from the lessons learned from the first program, and, most critically, from the dramatic increase in cherry-picking capacity. With the introduction of HTCP, nearly the entire set of HTS hits (35,000 compounds) was quickly assessed at 4 μM against two bacterial species. Immediately following the confirmation screen, dose-response assays on the 2,250 active compounds were performed in parallel against five bacterial strains and three mammalian cell lines. The consolidation of bacterial and mammalian cell assays into a single process shortened the dose-response screen timeline from 1 year, following the first HTS, to 1 month following the second HTS. HTCP afforded the rapid processing of compounds through multiple biological tests, thereby enabling the selection of biologically interesting hits while reducing the reliance on criteria based on calculated properties to reduce hit lists to manageable sizes. Significant improvement in the timeline and throughput of the HTS, and the quality and quantity of data, were achieved with the HTCP capacity. Because of the enhanced screening capacity, the compounds moved quickly into downstream triaging and liability assessment which led to rapid decision making. One outcome from the first screening effort which supported the HTCP approach in the second screen was the observation that antibacterial IC_{50} against *S. aureus* was predictive of the Minimum Inhibitory Concentrations (MIC) measured by standard low-throughput, manual-read methods *(26)* (**Fig. 7**). This data provided us with the confidence to use HT methods to rapidly and accurately assess new antibacterial compounds, and to plan "top up" screens at regular intervals with minimal startup effort.

The comprehensive dataset resulting from high-throughput antibacterial screens provides investigators with an easily retrievable catalog of all antibacterial compounds in the collection. Aside from driving cell-based antibacterial programs and new target/mechanism

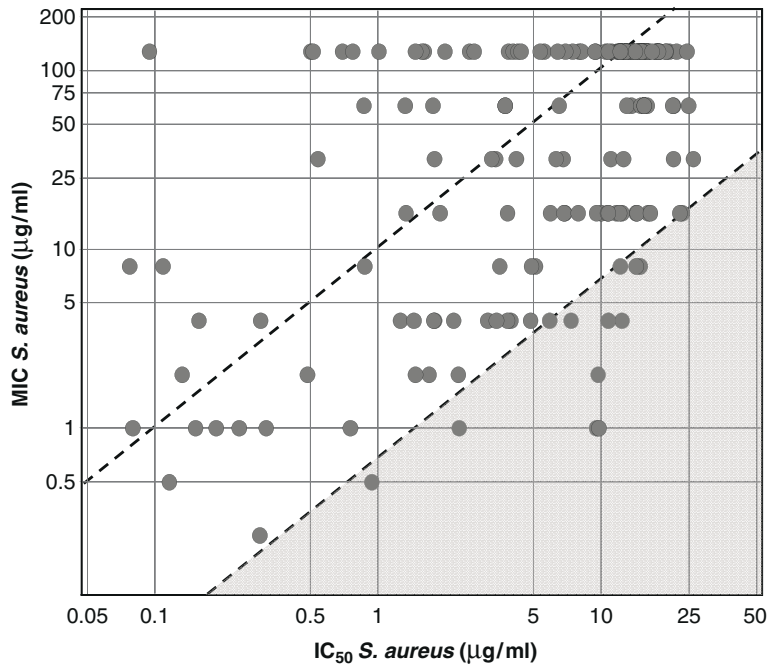

Fig. 7. Correlation between anti-*S. aureus* IC_{50}s generated in high-throughput and MICs generated by standard low-throughput methods. The graph demonstrates that the HTS antibacterial IC_{50} data generated using compound solutions from the HTCP system are a robust predictor of antibacterial MICs of compounds from the powder samples. The false negative rate is low, indicating that few truly antibacterial compounds would fail to be detected via the high throughput method.

of action identification, this information is powerful when triaging a hit list from an in vitro target-based screen. One can now use the antibacterials database in combination with cheminformatics to form hypotheses about cell or target based activity, and HTCP to iteratively test these. Some examples where this process has been instrumental are target validation, mechanism of antibacterial action, synergy screening, antibacterial resistance profiling, and specificity and selectivity assays.

3.2. Biosynthetic Pathway Screen

Whole-cell, bacterial growth inhibition screens can be challenging because the hit lists are typically very large and are composed of nonspecific toxic compounds, compounds which interfere with the detection steps, compounds with unknown mechanisms of action, and compounds that inhibit well-described targets. Hit lists are often too large for simultaneous liability identification and target deconvolution via mechanistic assays *(24)*. On the other hand, target-based HTS approaches often failed to identify novel molecules with potent, broad-spectrum antibacterial activity. The paucity of enzyme inhibitors with antibacterial activity is due to several factors including the limited permeability of compounds

through the cell membrane, the efflux of compounds back out of the cell, the metabolism of the compounds by the bacteria, or the presence or induction of compensatory pathways which restore bacterial metabolism even in the presence of a specific enzyme inhibitor.

In order to develop a lead-finding approach that extracts the most value from the downstream flowchart, we employed a hybrid between target-based screening and cell-based screening, using "sensitized" strains of bacteria. Such sensitized strains are useful for screening because they render the bacteria more sensitive to growth inhibition by a compound that inhibits the down-regulated enzyme or potentially other enzymes in the same pathway. The expression of the target enzyme can be regulated by cloning a suitable promoter/operator sequence upstream of the coding sequence, and then modulating the expression by adjusting the level of inducer. By replacing the wild-type copy of the essential gene with a recombinant, inducible copy, the strains cannot grow in the absence of the exogenously supplied inducer. By measuring growth as a function of inducer concentration, it is possible to identify the minimal amount of inducer (and, therefore, enzyme concentration) that supports growth **(Fig. 8a)**. By utilizing this condition, the cell line can become highly sensitized to compounds that would not otherwise be identified as growth inhibitors. By comparing the IC_{50} of compounds against a highly induced cell line versus the sensitized cell line, one can select hits that may specifically inhibit the down-regulated enzyme or pathway.

Figure 8b depicts the IC_{50} of a compound against a *S. aureus* strain sensitized for an essential, biosynthetic enzyme. At low concentration of the inducer IPTG (isopropyl-β-D-thiogalacto-pyranoside), low enzyme expression renders the bacteria more

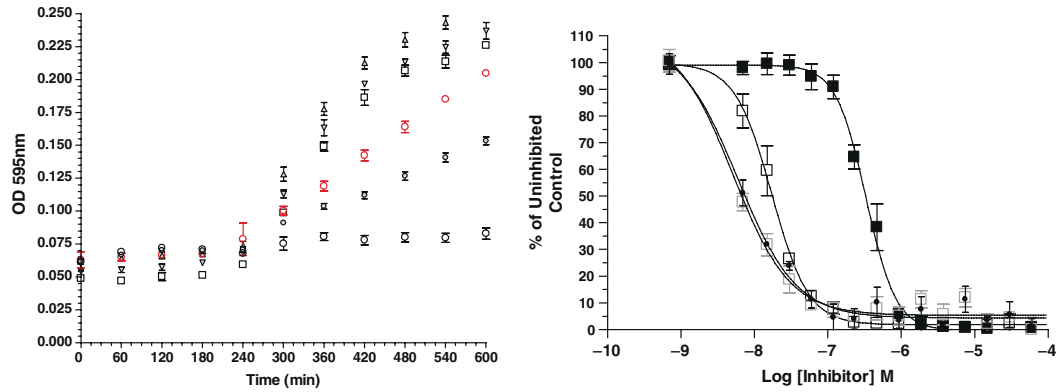

Fig. 8. **a** IPTG-dependent growth of *S. aureus* sensitized strain. Inoculum ≈7,000 bacteria/μL, Δt = 37°C, ΔT = 0–10 h, Assay volume 30 μL, n = 4. [IPTG] = (□) 1 mM, (▼) 500 μM, (▲) 50 μM, (○) 5 μM, (●) 2.5 μM, (○) 0. **b** IPTG-dependent Inhibition of sensitized *S. aureus* Growth by a specific enzyme inhibitor. Inoculum ≈7,000 bacteria/μL, Δt = 37°C, ΔT = 8 h, Assay volume 30 μL, n = 4. [IPTG] = (■) 1 mM, (□) 5 μM, (□) 0.75 μM, (■) 0 gave IC_{50} values of 332, 16, 5 and 2.5 nM respectively.

sensitive to an enzyme inhibitor, whereas under high IPTG concentration, high enzyme expression renders the bacteria less sensitive to the inhibitor.

A 1.5 million compound HTS was performed using this recombinant, inducible *S. aureus* strain cultured in clear bottom 384-well microtiter plates containing compounds at a final concentration of 10 μM. Growth was assessed by measuring culture density (absorbance at 595 nm) following 8 h incubation. The growth phenotype allowed to sort the compounds into two distinct populations **(Fig. 9)** and to thus identify nearly 25,000 hits. Using HTCP, the active compounds were cherry picked and diluted serially to four concentrations in two separate 384-well microtiter plates. These compounds were then tested in two parallel microbial assays. In the first assay, the cells were grown in the presence of high concentrations of IPTG (1 mM), where the growth rate is not limited by enzyme concentration. In the parallel assay, the cells were grown in the presence of a low concentration of IPTG (10 μM), where the growth rate was limited by the low concentration of the enzyme.

When the data was fit to a sigmoid dose response curve, an IC_{50} value could be obtained for each compound under conditions of high and low IPTG. In this experiment, 1,300 of the 25,000 cherry-picked and serially diluted compounds were at least fivefold more potent against the sensitized strain. These compounds were selected as potential enzyme inhibitors with demonstrated antibacterial activity. In the end, more than 50% of these compounds were shown to inhibit the purified enzyme. The hit list represented a variety of chemical structural classes that were capable of inhibiting growth of wild-type *S. aureus*, but also other Gram positive bacteria that depend on the same biosynthetic pathway for survival.

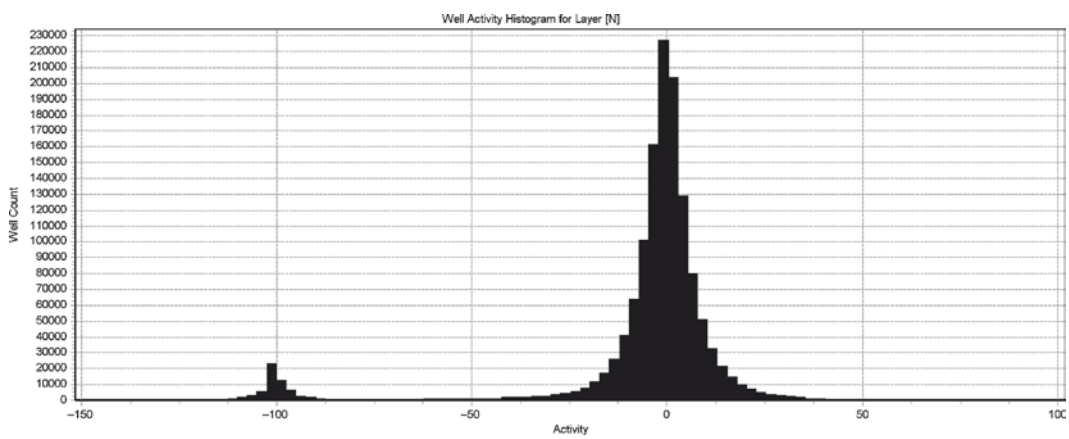

Fig. 9. Histogram of compound activities. Compound number distribution versus their activity in the *S. aureus* growth assay. Populations of active and inactive compounds can clearly be distinguished.

The HTCP system allowed us to rapidly array four point DRC libraries on a list of 25,000 primary hits allowing for a complete retesting of primary hits for cell-based selectivity measurements. Prior to the institution of the hybrid target:cell-based screening system, this rapid deployment of the downstream flowchart would have been impossible due to the unmanageably large number of hits that are identified by a cell-based screening approach. The HTCP system provided flexibility to explore large numbers of compounds to identify compounds of interest, and opens further possibilities for a less biased approach towards selecting hits for further testing in selectivity and dose-response assays.

The ultimate success of the approach was based on four features: (1) direct label-free measurement of bacterial growth in a 384-well format using optical density at 595 nm, (2) the use of a sensitized bacterial strain, in which growth was dependent upon IPTG-inducible expression of an essential enzyme in a biosynthetic pathway, (3) the development of a panel of high throughput enzyme and cell-based assays to rapidly profile the hits for specificity and selectivity, (4) the ability of the HTCP System to rapidly generate focused libraries at multiple concentrations to support the selectivity and mechanism of action profiling of the compounds.

3.3. Yeast Growth Modulation Screen

It had been mentioned in the previous section that potential cytotoxic effects are an inherent challenge with cell-based screening assays especially at higher compound concentrations in the micromolar range. Hitlists of assays for inhibitors of a cellular response will also contain compounds that reduce the measured signal due to an unspecific toxic effect on the cells. During subsequent follow-up screening these false positives have to be discriminated from hits acting on the target under investigation. Usually this discrimination is achieved by retesting the hits in a range of concentrations against the specific target readout and in parallel against a general cytotoxicity measure. Such counter screens detects e.g., cellular protein or DNA content, cellular ATP levels, activity of a cellular housekeeping enzyme or cell count if the assay format allows for cell proliferation to be observed. An appropriate concentration window between target readout and cytotoxicity measurement indicates a target-specific mode of action. If the cellular system is sensitive and the primary screening concentration is in the micromolar range, unspecific cytotoxicity mechanisms may raise primary hit rates up to several percent of the screening library. In this case the hit confirmation against a cytotoxicity counter screen can become a very demanding task that cannot be accomplished without a HTCP system.

In screening projects that are looking for stimulatory compounds this problem is even more pronounced, although this may not be obvious at first glance. Cytotoxic compounds suppress

cellular responses and should not give rise to false positives in a stimulation setting. However, stimulation assays can lead to false negative results when the stimulatory target-specific effect of an active compound turns into unspecific cytotoxic – and thus inhibitory – activity at higher concentrations. Depending on the width of such biphasic, bell-shaped concentration response curves and the choice of screening concentration relative to the ascending and descending parts of the concentration response curve **(Fig. 10)**, a target-specific hit may appear as stimulatory (a), inactive (b) or inhibitory (c). Unfortunately, the most potent hits tend to be most prone to this effect and will get lost unless they exhibit a large window against cytotoxicity. Furthermore, low hit rates in stimulatory assays tempt scientists to increase the primary screening concentration, thereby increasing the false negative rate even further. Ideally, stimulatory screens should be performed over a broad range of compound concentrations which is usually not affordable. Here, another solution is described for minimizing false negative rates in stimulatory assays which heavily relies on HTCP during hit follow-up and retesting all active samples irrespective of whether they are stimulatory or inhibitory.

This is best illustrated with a recent screening project based on a genetically engineered *S. cerevisiae* yeast cell line that expresses a toxic protein under the transcriptional control of the Gal1–10 promoter. This toxic protein is implicated in a number of diseases, and inhibitors of this protein are potential therapeutic modalities. Upon switching from a glucose-containing to a galactose-containing medium the expression of the toxic protein is turned on and the yeast cells stop to grow. The primary

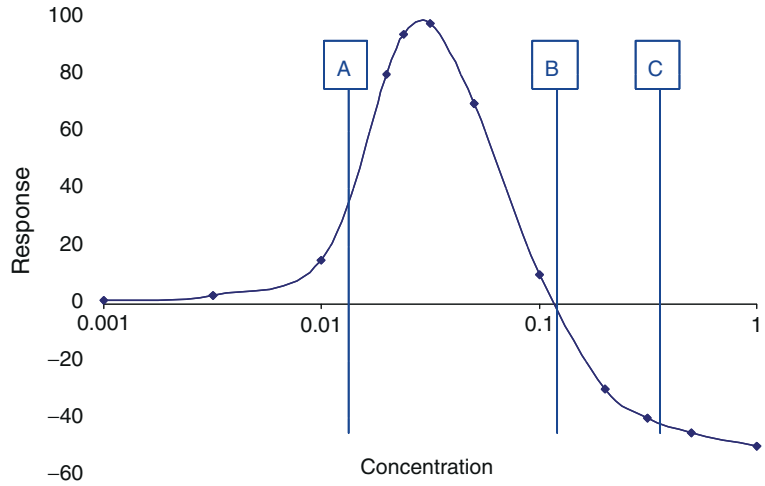

Fig. 10. Yeast growth modulation screen. Biphasic concentration response curve for target-specific stimulatory compounds that are cytotoxic at higher concentrations. A target-specific hit may appear as stimulatory (**A**), inactive (**B**) or inhibitory (**C**).

screening assay quantifies yeast growth via luciferase-mediated determination of ATP content of the yeast lysate. This method is very sensitive and runs robustly in the 1536-well format. During the downstream flowchart an independent orthogonal readout was used for confirmation of compound activity which quantifies yeast growth directly by culture density (absorbance at 850 nm) in 384-well format. Active compounds with the desired profile would interfere with the toxic mechanism of the protein, revert the growth arrest and lead to an increase in yeast cell number over the incubation period of 20 h, irrespective of the readout method used.

Primary screening of 1.2 million compounds at a concentration of 20 μM led to 2,000 stimulatory hits **(Fig. 10**, type A**)** based on a threshold of 10% increase in cell number as measured by ATP content. Another 5,900 compounds proved to be inhibitory **(Fig. 10**, type C**)** during primary screening based on a threshold of 10% reduction of cell number. Compounds of type B **(Fig. 10)** where a balance of stimulatory and cytotoxic activities leads to an inactive readout, can not be discriminated from truly inactives. However, a screen for general inhibitors of yeast growth inhibition had been run with the parental *S. cerevisiae* strain. This screen had identified 25,000 inhibitory hits, 17,500 of which did not overlap with the 2,000 stimulatory nor the 5,900 inhibitory hits from the target-based yeast growth modulation screen. The HTCP made it feasible to combine these three hit lists and retest all 25,400 hits as four point concentration response curves, covering compound concentrations from 33 to 1.2 μM. A small subset of compounds exhibited only inhibitory or neutral activity in this concentration range and was retested at a lower concentration range from 1.3 μM to 11 nM. Any compound that stimulated growth by at least 10% anywhere in the concentration range (1,743 compounds) were tested once more as eight point dilution series in the concentration range of 20 μM–6 nM in four replicates. Based on the higher statistical significance of these data, a more stringent selection threshold of at least 35% stimulation was now applied which was met by 118 compound solutions. These 118 compounds were freshly dissolved from powder samples and validated in the orthogonal culture density assay (turbidity of the culture at 850 nm), again as eight point dilution series and in four replicates. In parallel, compound solutions were checked by LC-MS and UV absorption for their chemical integrity and purity. Finally, 26 compounds remained which increased ATP content of the yeast culture by at least 35%, which increased culture turbidity by at least 20%, which had the right mass and a UV purity of at least 50% and which exhibited a concentration window of at least tenfold between ascending and descending part of the concentration response curve.

A retrospective analysis was performed for the 26 finally validated hits in order to find out from which primary hit list they originated. While 18 compounds were found as stimulatory hits in the yeast growth modulation screen, additional five compounds could be rescued from the inhibitory hits in this screen. Additional five compounds were found in the screen of the parental yeast strain. If only the stimulatory hits from the primary target screen would have been followed up, eight (31%) compounds of the final hits would have been missed. In conclusion, the HTCP enabled us to minimize the false negative rate by adopting a broad and inclusive hit follow-up strategy at early screening stages when data power and quality is relatively poor (e.g., single point determinations in primary screening) and becoming more and more stringent towards later screening stages when data power has increased (e.g., concentration response curves, replicates, orthogonal target readout).

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