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The essential roles of chemistry in high-throughput screening triage

It is increasingly clear that academic high-throughput screening (HTS) and virtual HTS triage suffers from a lack of scientists trained in the art and science of early drug discovery chemistry. Many recent publications report the discovery of compounds by screening that are most likely artifacts or promiscuous bioactive compounds, and these results are not placed into the context of previous studies. For HTS to be most successful, it is our contention that there must exist an early partnership between biologists and medicinal chemists. Their combined skill sets are necessary to design robust assays and efficient workflows that will weed out assay artifacts, false positives, promiscuous bioactive compounds and intractable screening hits, efforts that ultimately give projects a better chance at identifying truly useful chemical matter. Expertise in medicinal chemistry, cheminformatics and purification sciences (analytical chemistry) can enhance the post-HTS triage process by quickly removing these problematic chemotypes from consideration, while simultaneously prioritizing the more promising chemical matter for follow-up testing. It is only when biologists and chemists collaborate effectively that HTS can manifest its full promise.

HTS & medicinal chemistry as partners in lead discovery

An increasing number of screening centers are influencing the discovery of potential new therapeutics. These screening centers are being established in the academic environment, modeling themselves on their industrial counterparts [1–5]. However, this can result in unexpected challenges when it comes to later stages in the therapeutic discovery process. To ensure that high-throughput screening (HTS) output will be as fruitful as possible requires the collaboration and (often) early intervention of medicinal chemists trained in the art and science of HTS triage. Typically, industry has centralized these early stages of drug discovery into groups like the Lead Discovery Group at the former Pfizer site in Ann Arbor (MI, USA). That group consisted of approximately 30 experts in medicinal chemistry, cheminformatics, and analytical chemistry and purification sciences. One of the strategies behind having a single group perform unified HTS triage was to allow for

rapid knowledge sharing. The same, or similar, library was constantly being interrogated by a wide variety of targets, and the recognition of actives and hits across these targets was continually bringing potentially nonselective and other so-called ‘bad-actor’ compounds to light. Grouping experts together led to a more facile sharing of this information and other, making the art of HTS triage more efficient. To our knowledge, all major pharmaceutical companies engaged in HTS have some sort of dedicated triage operation, large investments which should testify to the essential nature of this discipline in early drug discovery.

‘Triage’, as commonly understood, applies to the classification of human trauma patients into those who are likely to live, those who are likely to die, and those for whom immediate care might make a significant difference in their health outcome. This system may have originated on the battlefields of the first world war [6,7]. The ability to triage patients is a combination of art and science, some-

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thing that is learned with extensive field experience. In a somewhat analogous process (although, admittedly, the outcomes are far less significant in human terms), HTS triage involves the classification, or prioritization, of hits from screening campaigns into compounds that are likely to survive further investigation, those that probably have no chance of succeeding either as probes (tool compounds), and those that fall into an intermediate classification where intervention could make a significant difference in their survival. Like its hospital and battlefield counterpart, HTS triage is also a combination of science and art, a process that is learned from extensive laboratory experience. Not every active can be saved, some cases will always be recognized as hopeless, while a third group will have structures and calculated or measured physicochemical properties where even seasoned experts are hard pressed to guess the potential outcomes offered by extensive screening and medicinal chemistry follow-up. Triage always has as its bottom line limited resources, and a major task of those performing HTS triage is directing the 'best' use of these finite resources [8]. With unlimited resources, all HTS actives could be carried on through increasingly expensive active-to-hit or lead assays and synthetic chemistry. However, unlimited resources are rarely the case in the pharmaceutical industry and even rarer in the academy. That being said, even medicinal chemistry experts are not in agreement over what constitutes a 'beautiful' compound or what will ultimately progress to a bona fide lead [9]. Therefore, the more potential resources are limited, the more sense it may make to err on the side of caution and apply higher bars to continuation of work on hits. Of course, the nature of the target (e.g., value, novelty and difficulty) plays an important role in this triage, sometimes lowering the bar to the follow-up of active compounds.

In our opinion some of the key places in the process of 'library-to-lead' where expertise in HTS triage can make a significant impact on the success of a drug discovery project are shown in Figure 1. Note that these contributions can begin well before an HTS is completed, and well after the 'cherry-picking' of screening hits, meaning the triage process has essential roles throughout the early discovery process. We should note that the definition of success may be a rapid acknowledgement by the project team that the search for chemical matter was unsuccessful, thereby preventing the consumption of resources on a project with little or no odds of succeeding. In the same fashion that the aforementioned 'Lead Discovery Group' was composed of experts in multiple fields, we believe the most effective HTS triage takes place in the hands of scientists with solid expertise in medicinal chemistry (with its grounding in synthetic chemistry and biological processes), cheminformatics

and purification sciences [10]. This partnership of chemistry with the biological and biophysical assay methods of HTS offers the best chances of probe and drug discovery [11]. In this article, we highlight the importance of this partnership in HTS triage and lead discovery, and provide vignettes of our experiences in this area.

The importance of library design in HTS triage: you will only find what you screen

The goal of all screening methods is to find active compounds that can provide the starting points for compound optimization with respect to target binding, and physicochemical and toxicological properties. Of these, binding and physicochemical properties are often of primary concern in the early stages of drug discovery, given that toxicological challenges can often not typically be predicted accurately *a priori* and will most likely need to be remediated in the optimization process [12–14]. That being the case, the more the compounds that populate the screening library match the desired parameters in each of these attributes (e.g., drug/lead-like, appropriate physicochemical properties), the shorter, typically, will be the optimization of the compound to preclinical candidate status. Therefore, the principles of medicinal chemistry should be considered in assembling a high-quality chemical library within the context of what is commercially available or imaginable.

Two methods of discovering active compounds are commonly employed: high-throughput and fragment-based screening. A third method of finding actives, virtual HTS (vHTS), produces recommendations of subsets of compounds to test in HTS or lower-throughput assays. The rough spectrum of libraries available to researchers is shown in Figure 2, with some examples of specific libraries that have been found to be useful. The GDB-13 is a computationally enumerated and curated collection of small organic molecules containing up to 13 atoms of C, N, O, S and Cl or less (~977 M) and ZINC is combination of several commercial libraries (35 M) [15,16]. The CAS (CAS Registry) library (81 M substances) is shown here as bridging 'virtual' and 'tangible' designations because many of the compounds in that collection are commercially available [17]. The CAS library is not currently available for vHTS, but is extraordinarily helpful in sifting through potential actives with respect to their 'natural histories' (*vide infra*). eMolecules (~6 M) contains compounds that are largely commercially available and are curated on a routine basis [18]. The GPHR (gopher) library is our in-house screening set of approximately 0.25 M compounds, close to the size and chemical composition of many typical academic libraries [DAHILIN JL, WALTERS MA, UNPUBLISHED DATA].

Figure 1. Primary impact points for chemistry in high-throughput screening and early lead discovery. The location of impact points covering the pre- and post-HTS stages highlights the importance of forming early partnerships between chemists and biologists in order to increase the chances of project success. HTS: High-throughput screening; vHTS: Virtual high-throughput screening.

HTS is based on the premise that active compounds (e.g., $IC_{50} < 25 \mu M$) can be discovered by interrogating targets with a large number of real, diverse and quality substances. The term ‘real’ here means compounds that are physically present in a screening collection. Other gross library descriptors can be used. ‘Tangible’ refers to compounds that are either commercially available or known to be amenable to facile preparation. ‘Virtual’ refers to a spectrum of compounds that can be easily prepared (overlaps with tangible), have been prepared, or might not even be capable of being prepared. This premise raises the questions of how large a library is large enough, how diverse is diverse enough, and what quality is good enough? Typical industrial screening libraries of small molecules contain on the order of 1–5 M compounds, while most academic screening libraries are on the order of 0.5 M compounds [19]. At one point it was hypothesized that a library of <25–50 K compounds (‘Holy Grail’ Library) would be sufficient to find weak actives against any target [20]. At the other extreme, a library of up to 10 M compounds was proposed during the ‘combinatorial era’ by at least one major pharmaceutical company. Neither of these extremes worked in practice and the current thinking is that all of the commercially available compound space (~6–8 M compounds) can be adequately covered by a library of around the size of most academic or non-for-profit screening groups [21]. Real compound library size is dictated by cost, storage and equipment throughput, among other considerations. Chemical diversity is best

insured by having several representatives of each compound scaffold in the library to help validate actives (validated hits are more likely to arise when several compounds containing the same scaffold are found in the initial HTS). Quality can be judged by a number of filters such as rapid elimination of swill (REOS), Pan-assay interference compounds (PAINS), physico-chemical properties, number of sp^3 atoms, RO5 and RO3, among others [22–24]. It has been documented that compound beauty (quality) is truly in the eyes of the beholder. Moreover, these studies typically have used as the beholders several expert medicinal chemists who have had extensive experience in compound optimization and drug discovery and development [25–27]. Therefore, if the number and expertise of those reviewing the libraries that constitute an HTS collection (or screening output) are necessarily limited in expertise (e.g., as at an academic screening center), the use of standard, industrial-strength compound filters allows the collective wisdom of many medicinal chemistry experts to be virtually and effectively brought to the table.

Key term

PAINS: Pan-assay interference compounds; a series of compounds identified by Baell *et al.* that showed bioactivity in multiple AlphaScreen HTS campaigns, irrespective of the biological target. Compounds containing the PAINS substructures should generally be considered unsuitable as leads for chemical probes or medicinal chemistry optimization. The chemical mechanism(s) behind their promiscuous bioactivities vary depending on chemotype.

Figure 2. Libraries, their uses and the development of a quality screening set. Placement of representative libraries in this space is necessarily subject to debate. For example, virtual libraries of real compounds can be used in vHTS, and the GDB contains commercially available compounds.

HTS: High-throughput screening; vHTS: Virtual high-throughput screening.

A comparison of size and quality parameters for a few typical libraries is shown in Table 1. It is important to note that even the most carefully tended screening libraries contain approximately 5% PAINS, although this is not appreciably more than what exists in the universe of commercially available compounds (eMolecules). This must be kept in mind when performing ‘active’ triage. It is probably worth rigorously removing these types of interference compounds from libraries designed for virtual screening campaigns [28]. Academic libraries tend to be composed primarily of vendor-supplied compounds (although some institutions have developed proprietary libraries) while industrial libraries are largely compounds that are proprietary to the company. These so-called ‘legacy’ compounds have been prepared during project-related medicinal chemistry campaigns or specifically in file enrichment endeavors (Box 1). Legacy compounds naturally have an understanding of medicinal chemistry principles ‘baked’ into them; we suspect that this is not typically the case with many vendor-supplied compounds.

Fragment-based screening (FBS) has been successfully applied to find active compounds for many years [34,35]. In this process, libraries of compounds with molecular weights less than approximately 250 and con-

taining on the order of 5–10 K compounds are assayed at high concentrations in order to detect weak binding. Small fragments can sample a chemical space the size of a much larger library, and there are several commercially available fragment libraries. Three important benefits of FBS are: libraries are much smaller, thereby requiring fewer technical resources; since the compounds must be soluble to be screened at high concentrations, good physicochemical properties are typically already present in the actives; and the method of detection (e.g., XRD and SPR, among others) typically insures target engagement. In this case, fragment optimization to full hit compound typically consists of fragment growing, linking or merging. These processes are heavily dependent on medicinal chemistry. The major challenges in FBS are the specialized equipment involved, the (relatively) higher compound concentrations used for screening and their potential for nonspecific target engagement, and the potentially challenging optimization processes, but many examples of success in this area have been reported [36,37]. The same fundamental principles used in conventional HTS triages are applicable to FBS, such as the elimination of problematic chemotypes, although the continued growth of fragment-based methods and the

Table 1. Four representative chemical libraries.

Collection	Compounds (n)	HTS	REOS	PAINS	PAINS (%)
MLSMR	314,651	7160	79,695	18,654	6
Academic A	208,887	5082	59,041	10,098	5
Academic B	234,304	3287	41,134	9831	4
eMolecules	6,580,176	200,805	NC	366,939	6

Each library was analyzed for the number of compounds, and for 'HTS' (Pipeline Pilot), REOS and PAINS (Canvas) noncompliance [29,30].
 HTS: High-throughput screening; MLSMR: NIH Molecular Libraries Small Molecule Repository; NC: Not calculated; PAINS: Pan-assay interference compounds; REOS: Rapid elimination of swill.

expansion of knowledge will undoubtedly shed light on triage-related nuances specific to FBS.

vHTS can greatly expand the universe of compounds for screening since it allows the exploration of real, tangible and virtual space. As contrasted with the screening of real compounds, the major bottleneck in virtual screening is dictated by throughput of the computational method. The more computationally demanding the method, the smaller the number of compounds that can be screened in a reasonable amount of time. The typical workflow for virtual screening, therefore, involves development of a model for the target (or ligand), creation of the chemical space of choice by filtering (target or ligand based) of large virtual libraries, the actual computations (simulations), and then computational prioritization of compounds to enter into the 'real' assay. vHTS can effectively enrich the output of HTS by focusing that process on the compounds that are most likely to engage the target. Several examples of this type of enrichment exist [38]. However, many caveats apply to virtual screening [39], and here, as in HTS, the compounds that emerge are only as good as the skills of the scientists that choose to screen, test and triage them. While many practitioners of vHTS are also medicinal chemists, we feel it is imperative for experts in HTS triage be involved in evaluating the libraries to be used in the vHTS process (including the selection of compounds cherry-picked to be tested experimentally) and that vHTS be heavily influenced by the application of known quality filters prior to screening. Eventually compounds that arise from vHTS will need to be confirmed experimentally and possibly optimized at later stages, and the better these compounds adhere to the established norms for compound quality, the more efficient the optimization process may be.

Other approaches to chemical matter

Chemical matter can be defined as the collection of compounds that will be assayed, at any level of throughput, for a given biological target. While lead discovery is often predicated on HTS, other starting points for chemical matter should always be considered

when exploring a new or previously screened target (Figure 3). This multifaceted approach is the hallmark of true lead discovery and is typically performed in parallel with HTS. These methods can be considered to be complementary to HTS, but are knowledge- rather than 'haystack'-based. Diverse approaches to chemical matter have been reviewed, vHTS being one of them [40]. Other approaches involve the use of known compounds from the literature (both journals and patents) that act at the target, other reported screens for the same target, and the reuse of preclinical pharmaceutical or biotechnology company compounds (Box 2).

The critical roles of medicinal chemistry in successful HTS triage

One of the most important aspects of a successful screening campaign is the post-HTS workflow design, or 'screening tree' (Figure 4). A well-designed screening tree helps the project team sift through the hundreds to thousands of HTS actives to prioritize **tractable**, drug/lead-like compounds for further downstream studies such as determining the mechanism(s) of action, hit-to-lead (H2L) chemistry and higher-order experiments (e.g., cell based, *in vivo*). Ideally, this should be done as efficiently and rigorously as possible to arrive at a quick 'go/no-go' decision regarding the drug discovery project at hand. At the root level, the triage process has two opposing yet complementary principles: eliminate from consideration compounds with unfavorable properties; and identify the subsets of compounds with the most favorable properties for follow-up testing (we note that the emphasis on each of these principals depends on the

Key terms

Screening tree: The collection of assays and workflow in an (v)HTS campaign, including pre-HTS steps, such as library design, the HTS itself, and post-HTS triage operations such as counterscreens, orthogonal assays, cross-validating assays, cheminformatics and analytical chemistry.

Tractable/tractability: Hits whose activity has been confirmed using fully characterized compounds that have been synthesized and purified in house.

Box 1. Evolution of chemical libraries.

My (MA Walters') industrial career at Parke-Davis began in 1997 when I joined its Combinatorial Chemistry group. This was in the middle of the 'combinatorial era' when the Diversomer apparatus was being developed, and the details of scavenger and quench reagents were being reported from the group in Ann Arbor (MI, USA) [31,32]. Over the course of the next 10 years at Parke-Davis (and later Pfizer, Ann Arbor Laboratories) I was involved with library design and synthesis, the CNS therapeutic area and the aforementioned Lead Discovery Group. These were the days of compressed libraries (multiple compounds per well) and the proposed 10 million compound library. I chaired the LIFE (Library Idea and File Enrichment) Committee in Ann Arbor along with Dr Jack Hodges. This committee was charged with the evaluation of ideas for focused library synthesis that would then be outsourced to companies like Tripos (St Louis, MO, USA) and ChemRx (San Francisco, CA, USA). These companies would typically prepare libraries of more than 2500 compounds from each accepted library proposal. These efforts across the Pfizer sites lead to an increase in the corporate library of over 1 million compounds. Prior to these efforts, the corporate library that was screened in Ann Arbor was primarily 'legacy' compounds that were the result of medicinal chemistry programs at the site. After extensive file enrichment, the number of compounds prepared by combinatorial chemistry began to outnumber the legacy collection. This became an important consideration as compounds prepared by combinatorial methods began to turn up in high-throughput screening (HTS) campaigns more frequently. This marked a turning point in the practice of HTS triage. In the early days, probably up to about 2000 compounds produced by combinatorial chemistry at vendors (each given an -R suffix in the corporate database) were not typically purified and biologists often refused to screen these potential mixtures. This -R suffix was later dropped and biologists would then often only reluctantly screen compounds that fell in sequential order as represented by their database numbers. This reluctance to screen compounds prepared in parallel changed when mandatory purity requirements of >90% were imposed on all synthesized compounds and when biologists realized that HTS actives from combinatorial libraries could be easily and rapidly followed up in medicinal chemistry. This often reduced the focus on so-called singletons, which were primarily the 'legacy' compounds prepared specifically for medicinal chemistry projects. Coupled with the observation by Baell *et al.* [33] that many classes of PAINS arise from easily synthesized scaffolds (i.e., 'combiphilic' scaffolds. While at the Lead Discovery Group, the term 'combiphilic' was coined by Dr GL Bolton to described scaffolds and processes that could easily be used in combinatorial chemistry [MA WALTERS, PERSONAL DATA]) one wonders what effect this trend has had on the output of HTS in the pharmaceutical industry and, perhaps more telling, in academic laboratories [WALTERS MA, PERSONAL DATA].

nature of the target, assays and data). Since the determinants of project success are fundamentally linked to chemical structure and its accompanying properties, we believe a chemocentric approach, as opposed to a strictly potency-based approach, is essential for prioritizing chemical matter for follow-up experiments. Naively (in our opinion) choosing compounds based solely on activity can be problematic for several reasons, as it could lead to the selection of **artifacts**, non-diverse sampling and unfavorable chemotypes in terms of chemical synthesis, physicochemical properties or intellectual property [48]. The major strength of HTS triage is that it can help channel a finite set of resources into the compounds with better chances of real success further along the discovery pipeline. We contend this is best accomplished by: working together with biologists and screeners to design an efficient screening tree and robust assays; incorporating cheminformatic methods to efficiently process and analyze large data sets; and maintaining a chemocentric (medicinal chemistry) approach to data analysis.

Triages are an integral component of nearly every successful screening campaign. Unless every active from

an HTS campaign was chosen for follow-up and then subjected to every secondary screen in the post-HTS screening tree, then by definition some type of triage occurred. It is difficult to objectively compare one triage to another, given the subjective components of HTS triage and the nuances surrounding each particular project. Triages could be evaluated for both their overall efficiency and their ultimate ability to identify useful chemical matter. Like many scientific fields, two triage experts may design completely different strategies, both of which may ultimately answer the same scientific question. However, finite resources mean there will always be 'known unknowns' and 'unknown unknowns', and an HTS campaign and its associated triage that fails to identify a chemical probe or drug lead does not necessarily mean the HTS or triage was 'flawed'. As we see it, one weakness of HTS triage is that it is difficult to evaluate the process in a rigorously objective manner, as this would require complete knowledge of a system. However, we point out there are several success stories of HTS triage we believe are particularly informative for readers [49–54]. One trend we notice is that the actual triage processes are not emphasized or described in-

Figure 3. Alternative approaches to chemical matter other than high-throughput screening. PubChem and ChEMBL are examples of publicly available chemical databases [41–43].

depth in the scientific literature [55]. Oftentimes, the details of the triage process are mentioned in passing, or described briefly in supplemental materials. This can make it difficult for readers to evaluate how a project team decided on a particular compound or chemotype for lead optimization, for instance. As we will describe later on, it is often when some critical component of a triage fails or is omitted that the significance of HTS triage becomes obvious.

Depending on the active criteria and size of the screen, HTS campaigns can have hundreds to thousands of primary active compounds, although the actual number of true positives is almost certainly a much smaller subset due to **false-positives** and assay artifacts. This number can dwindle even further when promiscuous bioactive compounds are triaged. A typical screening tree in either academia or industry consists of post-HTS statistical analyses, cheminformatics analyses, assay-specific **counterscreens**, **orthogonal assays**, selectivity counterscreens and perhaps some preliminary mechanistic studies, SAR exploration and cell-based assays (while this arrangement is typical for many cell-free HTS campaigns, we note that many of the same core principles can be applicable to cell-based or phenotypic screens, but are not discussed in more detail here for sake of brevity).

The exact order and composition of the follow-up screens and cheminformatic analyses varies from project to project. Factors such as the target, the size and

Key terms

Artifact: An assay readout that is not a direct result of the target phenomenon being assayed; often the result of experimental conditions, assay method and/or instrumentation. A straightforward example would be a compound that fluoresces in the same wavelength as the readout of a fluorescence intensity assay.

False positive: An assay readout by a known inactive compound that is erroneously classified as an active compound during the triage process, usually the result of experimental anomalies, poor assay robustness or an artifact source. Nonspecific enzymatic inhibitors like chemical aggregators have been described in the literature as ‘false positives’, but by strict definition in enzymatic inhibition assays, they are actually true positive inhibitors, albeit by nonspecific and therapeutically uninteresting mechanism(s).

Counterscreen: An assay in which compounds are tested versus a target or assay modification for undesired attributes such as nonselective inhibition (e.g., selectivity) or assay interference (e.g., artifacts).

Orthogonal assay: An assay that interrogates the same biological phenomenon as the primary or HTS assay through an independent assay readout (e.g., a radiolabelled substrate assay to follow-up a fluorescence-based HTS).

Box 2. Parallel approaches to the development of potential PKA inhibitors.

In a recent project related to the use of PKA inhibitors as potential therapeutics to treat SCA1 (spinocerebellar ataxia 1), we employed each of these methods profitably [44]. There is no evidence that PKA has ever been targeted by a major pharmaceutical company. While our screen of approximately 200 K compounds using Caliper technology was being performed (which eventually resulted in six confirmed active compound series, *vide infra*), we also examined the synthesis and activity of structurally simplified derivatives of the known PKA inhibitor, balanol [45,46]. Additionally, we mined a reported screen for PKA inhibitors in PubChem (Bioassay AID = 524), which yielded a further series for exploration. Finally, we prepared compounds based on numerous Akt programs (Akt [also known as PKB] is a close structural relative of PKA, lying on the AGC branch of the kinome [47]) that had been executed in the pharmaceutical industry. In this case, many potent and selective PKA inhibitors had been made and discarded in the process of optimization of scaffolds as selective Akt inhibitors. We resynthesized and tested some of these compounds following published procedures and eventually developed a collaboration with a major pharmaceutical company. They mined their repository to help us find compounds that would assist us with *in vivo* confidence-in-mechanism studies. These compounds came primarily from published work, but the hope is that further collaboration will lead to the sharing of unpublished compounds with better potency, selectivity and physicochemical properties [WALTERS MA, UNPUBLISHED DATA].

composition of the chemical library, the nature of the HTS assays and screening data, the available resources, project timelines and also the available expertise can greatly influence the order and composition of the screening tree. Typically, some form of cheminformatics and statistical analyses are performed with the HTS data to assist in cherry-picking the primary screening actives (Figures 4 & 5). From there, follow-up confirmatory experiments and key counterscreens can be performed. As compound attrition mounts and more data is generated for each compound, the surviving compounds can be subjected to higher-level analyses and increased scrutiny. These types of analyses can help select the most promising compounds for purchasing and the more resource-intensive experiments. In our screening trees, we prefer to have a mix of cheminformatics and knowledge-based analyses (e.g., PAINS filters, calculated descriptors, general medicinal chemistry considerations) and actual experimental screens, including some redundancies when resources permit. This is both an acknowledgement to the limitations of performing ‘armchair chemistry’ and our own preferences for key triage decisions to be supported by several lines of experimental evidence.

One question that is often asked of HTS campaigns is ‘who should pick the compounds for follow-up?’ We contend that those typically in the best position to prioritize compounds for HTS campaigns are experienced medicinal chemists trained in actual HTS triage, supplemented with input from members of the drug discovery team. This expertise ideally requires a diverse and integrated knowledge base consisting of organic synthesis, medicinal (and analytical) chemistry, ADMET principles, cheminformatics, molecular pharmacology, biochemistry, assay design and biological structures. Despite the wealth of accumulated knowledge regarding HTS and medicinal chemistry,

ranking the most tractable compounds is as much an art as a science, as there can be considerable variation in the ranking of compounds among medicinal chemists [25,26]. However, without a strong background in synthetic organic and medicinal chemistry, compound selection would presumably be made mostly on activity profiles or other factors that may not be related to or predictive of the chemical or bioactive nature of the compounds themselves.

Data quality & hand-off can influence HTS triage

An oft-overlooked facet of the post-HTS process is the data hand-off between HTS and medicinal chemistry. Data mining for less obvious ‘hits’ (e.g., less potent but more ligand-efficient compounds) is an increasingly utilized strategy [56,57]. A major obstacle to employing this and related strategies with confidence can be data quality. The data being delivered to those in charge of post-HTS triage and follow-up prioritization are inevitably linked to the HTS assay robustness. To mine data with more confidence and to have better odds of success, especially to find compounds that hover near the chosen activity cut-offs, assays with robust Z’ factors are preferable as well as other sound statistical descriptors [58–61]. We believe drug discovery teams taking the extra time and effort to optimize HTS assays for even slightly-improved Z’ factors and other measures of assay quality can pay dividends down the road, including a reduction in false positives and an overall increase in confidence of the screening data. Data mining can also be enhanced by statistical corrections for the HTS results, which can stem from row and column effects, temporal considerations, and different production runs, to name a few variables [61–63]. Other strategies to increase confidence in screening data are to initially screen compounds in replicates or at multiple concen-

Figure 4. Sample post-high-throughput screening triage screening tree. The process begins with data hand-off from the HTS, from which a series of cheminformatics operations can be performed to standardize, filter and annotate the data. As compounds are progressed, more detailed and time-consuming ('higher-level') analyses and resource-intensive experiments can be performed. We note that the order and details of each operation can vary depending on multiple factors (e.g., target, assay methods, library size and composition, available resources, expertise, project timeline and data volume). For instance, some groups may elect to test all actives by dose-response and counterscreens, and then perform more detailed chemocentric and cheminformatic analyses with the resulting data.

DR: Dose-response; H2L: Hit-to-lead; HTS: High-throughput screening; QC: Quality control.

trations ('quantitative HTS'), although the equipment and other resources needed for these approaches can be prohibitive to many academic groups [58,64]. To ensure the long-term success of an early drug discovery project, we recommend employing sound statistical corrections to the HTS data prior to hand-off to medicinal

chemists. To employ such corrections, certain controls, reference compounds and control plates may need to be included throughout the screening campaign, and therefore it is highly desirable to insist and agree on these types of corrections prior to initiating the full-fledged HTS campaign [65]. After screening a large

Figure 5. Example of a cheminformatics-assisted core analysis during a post-high-throughput screening triage. See also Box 4. The annotated high-throughput screening data are from a PKA screen performed in the ITDD. (A) Singletons; (B–F) representative compounds from three core classes. (B) is an obvious PAINS (substructure alert bolded). Compound (C) is fasudil, a known kinase inhibitor. Compound (D) has a structure reminiscent of known kinase inhibitors (note that the core containing compound [D] has several examples of active and inactive analogs, a promising early pattern that shows potential for early SAR exploration and argues against many of these compounds being statistical false positives). Core13, which contains compounds (E & F), has a profile we typically view with skepticism (note that while [E] is deemed active, all remaining compounds in this core are inactive, including the close analog [F]). This example also shows that effective use of modern cheminformatics software allows for large amounts of complex data to be analyzed efficiently.

number of compounds, portions of compounds are termed active by cut-off criteria, often three standard deviations about the mean percent inhibition (3σ), although there are alternative forms of compound selection besides employing strict, sometimes arbitrary cut-offs, but these methods typically require additional expertise in biostatistics [48,66]. Usually numbering in the hundreds to a few thousand compounds, this number of actives must be skillfully culled to arrive at a much smaller subset for follow-up experiments. As described earlier in the introduction, this process requires an integrated (and artful) application of statistics, cheminformatics and chemical intuition that is honed by repetition and knowledge sharing.

The good, the bad & the PAINS

One of the key roles medicinal chemists can play in the prioritization of chemical matter is the triage of potentially intractable and 'risky' compounds with unfavorable properties. After defining the activity criterion, one of the steps we employ is a series of cheminformatics filters to weed out undesirable substructures or compounds with poor, nonlead-like calculated physicochemical properties. One such step is the computational identification and removal of promiscuous compounds (aka 'frequent hitters' and 'bad actors') such as PAINS [33,67–69]. While several hundred substructures are described in the seminal paper, the chemical mechanism(s) of bioactive promiscuity for many of these scaffolds are uncharacterized or matters of speculation. Scores of these problematic chemotypes have appeared in many of our institutions' HTS campaigns [70,71]. Similar classifications have been reported, such as REOS and others [72], although some of these filters may be too draconian for academic HTS campaigns since they are designed to ensure long-term survival of actives as they progress to preclinical candidates. It is the overwhelming consensus among seasoned medicinal chemists that these compounds (i.e., PAINS and REOS) be avoided for follow-up chemistry or touted as lead compounds or even chemical probes. Despite the awareness of PAINS among reviewers and investigators, compounds flagged as PAINS still manage to make their way into the scientific literature, one of the more notorious and debated examples of these PAINS being rhodanines [73,74]. Medicinal chemists can also flag compounds for potential sources of promiscuous enzymatic inhibition such as **thiol-reactive compounds** (i.e., electrophiles), **redox-active compounds** or covalent modifiers by substructure or by first principles. In terms of thiol reactivity, there are several substructure alerts reported in the literature besides the more obvious reactophores (e.g., acid chlorides) [75–78]. Finally, medicinal chemists are optimally suited to spot

compounds with poor physicochemical properties that are unlikely to advance far in the discovery pipeline. This filtering can often be done by many commercial software packages, but it is our opinion that having a solid background in hands-on medicinal chemistry (with its accompanying tacit knowledge) can be an important user-provided quality control for the interpretation of such calculated descriptors. For example, substructure filters often flag actives as objectionable even though experience would suggest they are perfectly reasonable. This is a reflection of the challenges of substructure definition, and should be taken into account before throwing out actives without considering the substructure in the context of the whole molecule. Given this, we tend to prefer to 'flag' rather than discard compounds outright. This practice is both a nod to the challenges of using hard cut-offs in an art that should at least be multiparametric in its decision-making and to leaving the vial, as it were, open to the examination of chemical matter for tough-targets that typical filters close the cap on.

Chemists and assay designers must work together early on to design screening trees with selectivity, orthogonal assays and counterscreens appropriate for the therapeutic hypothesis. Cost is often a factor. However, the often unexpected costs associated with pursuing promiscuous or artifact-based actives must always be factored into this equation. Selectivity assays are an important component of any post-HTS screening tree, as they can provide clues about the bioactive promiscuity of a given compound. What is a good cut-off for selectivity from a medicinal chemistry viewpoint? While it is possible (but more difficult) to optimize for selectivity starting from a pan-active compound, a good general rule should be greater than five- to ten-fold difference in IC_{50} values, although the actual threshold depends on the therapeutic hypothesis and expert examination of the target and available data. Often times, IC_{50} values from independent experiments can vary several fold, so medicinal chemists can help caution against apparent selectivity when there is

Key terms

Thiol-reactive compounds: Compounds that are capable of forming covalent bonds with biological thiols such as coenzyme A, glutathione and protein cysteine(s) under assay or biological conditions. These compounds are generally avoided in most drug discovery campaigns because of promiscuous bioactivity profiles and ADMET liabilities.

Redox-active compounds: Compounds capable of generating hydrogen peroxide (H_2O_2) under certain assay conditions (often in the presence of strong reducing agents like DTT or TCEP), which can alter biological processes, usually through oxidation of biological thiols.

Key term

Promiscuous inhibitors: Compounds that show apparent bioactivity versus multiple, unrelated biological targets and/or processes, often independent of assay format.

only a few-fold difference in IC_{50} values between different enzymes. We speculate that the apparent selectivity for many promiscuous bioactive compounds, including many PAINS, may be the different intrinsic susceptibilities of different targets to various forms of nonspecific enzymatic inhibition. For instance, two closely-related enzymes may have different reactivity profiles with respect to a particular electrophilic compound series, which could have the unfortunate effect of leading to a false sense of selectivity that is often invoked as evidence of mechanism-based activity [DAHLIN JL, WALTERS MA, PERSONAL DATA]. Selectivity counterscreens should aim to use some biomolecules closely related to the screening target, especially those that may be predictive of off-target effects. Additionally, it may be useful to assay a completely unrelated enzyme or biomolecule to gauge promiscuity effects. In practice, this may be done by including the candidate compounds in another unrelated HTS campaign. Whenever possible, reference compounds and appropriate controls (positive and negative compound controls) should be included in these assays to judge the quality of the assays and measure inter- and intra-experiment variability. Input regarding the appropriate orthogonal assay and counterscreens is also an important contribution that medicinal chemists can make to decorating the screening tree. The underlying chemical basis of the HTS assay should be carefully considered so that an orthogonal method to cross examine the biological phenomenon can be designed, even at the expense of being lower-throughput, as is often the case with orthogonal assays. There should be some type of correlation between the activity in the primary HTS method and the orthogonal methods, and in our experience it is worth the investment of time and resources to design, optimize and finally corroborate the primary and orthogonal assays before beginning the HTS production run.

Medicinal chemistry concepts can be used advantageously to help design orthogonal and counterscreens to identify assay artifacts and **promiscuous inhibitors**. Medicinal chemists can be a significant asset to HTS campaigns by triaging compounds with known liabilities such as redox activity, chemical aggregation and thiol reactivity (some specifics of problematic substructures will be discussed in the next section). Compounds that produce H_2O_2 , often fueled by the presence of reducing agents such as DTT and TCEP in many biological buffers, can nonspecifically inhibit

enzymes or alter biological processes. This can occur by oxidizing protein cysteines or other critical amino acid side chains, for instance, and can mimic a well-behaved inhibitor [79]. However, we have found the use of a facile counterscreen such as the HRP-PR assay can easily identify such compounds for triage [80,81]. Compounds that nonspecifically inhibit protein activity by chemical aggregation can also be identified by several different counterscreens, such as a surrogate β -lactamase assay (our current assay of choice), Hill slope analysis, DLS or NMR-based methods. Medicinal chemists should emphatically insist that HTS assays and any follow-up experiments include detergents (unless prohibited by experimental considerations) to minimize the chance of enriching chemical aggregators [70,82–90]. Another area where medicinal chemists can greatly assist the HTS process is the triage of thiol-reactive compounds, which can nonspecifically react with protein thiols and lead to promiscuous enzymatic inhibition and potentially toxicity [78]. These compounds (or certain metabolites) can also react with other biological thiols such as glutathione and coenzyme A, all of which can have important ADMET implications. Multiple experimental methods are available to probe thiol reactivity, including glutathione-based competition and HPLC-based methods, NMR, fluorescent probes, peptide probes and ALARM NMR/LC-MS [78,91–97]. Other sources of promiscuous inhibition or assay artifacts include chelation, fluorescence, fluorescence quenching, singlet-oxygen production or capture, compound–reporter interactions (e.g., luciferase reporters) and apparent compound–antibody interference [85,98–110]. We note that many of these mechanisms such as redox activity and thiol reactivity are particularly relevant to many cell-based assay readouts, including cell proliferation and reporter systems [PERSONAL DATA]. Medicinal chemists can strongly influence the identification of such compounds by correlating the structures of HTS actives with the predicted chemical mechanism(s) of assay interference. Finally, promiscuous compounds can be checked by careful literature and database searches, including PubChem and SciFinder [111–113]. It is our experience that the screening tree must be in place as early in the biology–chemistry HTS collaboration as practical. Strict decision points should be established and enforced. In our observations, projects sometimes build up so much momentum that it is difficult to admit defeat. At that point it is tempting to invoke the faulty logic of sunk-costs and continue down a less-than-fruitful pathway.

Detailed inspection of raw dose–response data is yet another layer of the triage process of target-based assays. Besides simply examining the IC_{50} values derived from curve-fitting regressions, we find additional value in examining the shape and quality of the

curves [114]. Such analysis can provide important clues about the nature of chemical inhibition and data quality. Dose–response curves can be grouped into categories by quality of the curve fit to the data, efficacy and the number of asymptotes [64]. Such descriptors can help prioritize compounds with full dose–responses, for instance. Hill slope analysis is another important consideration, as it can give clues about potential mechanisms (e.g., positive and negative cooperativity) [115]. During one of our recent post-HTS triages, steep Hill slopes were also useful for flagging potential chemical aggregators [70,116]. The consideration of dose–response data can be incorporated at several points in the triage process, depending on when compound titration experiments are performed, the specifics of the HTS organization and the number of compounds to be analyzed. In practice, we have found it useful to display curve descriptors alongside the IC_{50} values once dose–response data are available and to triage compounds with steep Hill slopes prior to more detailed cheminformatics analyses. We find the more nuanced analyses of dose–response data is increasingly useful as the number of compounds to be considered is whittled down by the triage process.

The role of cheminformatics in HTS triage

The cornerstone of cheminformatics in HTS is the ready availability of digital structure data associated with digital compound attribute data. This combination of data allows the rapid handling of millions of data points and calculation of physicochemical properties, similarities, shapes, substructures, and so on to go along with activity data that have been collected in the HTS. This compound attribute data can then be used to generate information (inference of relationships) and finally, knowledge, the understanding of what these relationships mean as applied to the task at hand. Cheminformatic methods also enable the rapid searching of the scientific and patent literature by

chemical structure (Box 3), a critical but underutilized tactic that we will describe later. As throughput continues to increase, and as data and analyses become more complex, there is always the possibility for information overload to stymie HTS triage – especially as more data accumulates throughout the course of a multistage triage. However, coupled with a well-designed screening tree, modern cheminformatic tools and vigilant data management can enable large data sets to be quickly processed and analyzed effectively by experienced users.

There is a large body of literature available on the application of cheminformatics to HTS triage [118–122]. Essentially, cheminformatic methods help the medicinal chemist rapidly sift through the large number of active and inactive compounds that arise in a typical HTS (Box 4). On large data sets, this work usually consists of the calculation of physicochemical properties (e.g., molecular weight, clogP, rotatable bonds, among others) [24,123–125], ligand efficiency metrics (a combination of properties and activity) [126–130], structural attributes [33,72,131–132] and annotation of the HTS data set with these values or flags. This is typically followed by strict cut-off filtering (e.g., only compounds with a molecular weight ≤ 500 are kept), multiparametric filtering [133,134] or flagging of the actives (or a combination of these methods) to help prioritize and reduce the number of compounds that need to be considered further. It is imperative that similar schemes be applied either pre- or post-vHTS since in these cases bioactivity is not used as filter until after the bulk of the chemical matter has been selected [39,135–137].

The contribution of purification & analytical sciences to HTS triage

Another critical (but often overlooked) contribution of medicinal chemistry in the post-HTS screening tree involves hit confirmation by analytical chemistry techniques and chemical synthesis. After confirming dose–response with library samples, typically the top

Box 3. Structural landscapes.

In the mid-2000's I (MA Walters) proposed to download structures from project-related articles and patents that could be found in the Chemical Abstracts Service (CAS) [177] database and then use cheminformatics to analyze this data. A group of us (including Dr Chris Kibbey, Pfizer Scientific Computing) envisioned that visualization of the structural landscape encompassed by compounds in the literature related to our chosen target could lead to a rapid understanding of the medical chemistry explorations that had preceded our work. As it is still the state of affairs, downloading and exporting large numbers of structures from CAS at that time was impossible. In fact, it became apparent that the power of digital structural information tied to literature knowledge was highly underappreciated. We were eventually able to download approximately 10 K structures (at approximately US\$1 per structure) and view a project chemical landscape where links to the literature were associated with structure, and structures were grouped by similarity. This project eventually died from lack of senior management support, although apparently similar ideas did surface elsewhere within the organization, as structures from patents were used to gauge the relative importance of claimed compounds [117].

Key term

Structure–interference relationship (SIR): The misguided correlation of chemical structure with supposed biological activity, when in fact the presumed bioactivity is actually a composition of assay interference, false-positives and/or artifacts.

prioritized compounds are supplied by commercial vendors. This is all but guaranteed by the nature of the library employed in screening. Depending on the resources and synthetic expertise on-hand, one can also choose to resynthesize the top compounds in-house. The decision to synthesize in-house or purchase compounds is typically a time versus money issue that must be balanced by the goals and resources of the project team. Most screening compounds are available in milligram quantities from international vendors. The amount of compounds chosen for resupply, budget issues, and types of experiments and quality-controls all dictate the amount of compound needed for resupply. In our experience, we order 10–25 mg of compound, which is enough for making several aliquots for multiple independent experiments and internal quality controls. Typically, most screening compounds from reputable vendors can be supplied within 2–4 weeks. Close structural analogs can also be purchased at the same time to perform a focused SAR ('SAR-by-commerce'). One caveat is that if the choice of actives to pursue is unwise, this may actually degenerate into a **structure–interference relationship** study that is related to SAR but doesn't really measure target engagement [104].

In our collective experience, there are many times where the vendor-supplied structure is not compatible with the spectroscopic data. Additionally, compounds can isomerize, degrade or oxidize during their shelf-life or during the course of an experiment [138–141]. For this reason, careful sample and batch tracking by bar-coded vials and an electronic database is very useful. Another related issue is the hydration of compounds stored in dimethyl sulfoxide (DMSO) and the effect of freeze-thaw cycles [142–144]. We strongly recommend re-checking vendor-supplied samples for purity (e.g., HPLC-MS) and structural characterization (^1H and ^{13}C NMR) [145,146]. Ideally, this would be done for every commercial re-supply, but in practice this is difficult for smaller academic labs, and therefore we recommend this be a priority for those compounds that have high probabilities of being recommended for mechanism-of-action studies or analog expansion. The purity of any compound, commercial or synthesized, is nontrivial. In our experience, most vendor-supplied screening compounds have approximately 95% purity according to ultra performance liquid chromatogra-

phy-mass spectrometry (UPLC-MS) analyses [FRANCIS S, UNPUBLISHED DATA]. However, compound samples with even trace impurities can lead project teams astray. These impurities can be remnants from the chemical synthesis, including transition metal catalysts, left-over starting materials, reactive intermediates, oxidative products, rearrangements or enantiomers, to name a few [146–153]. Therefore, medicinal chemists should insist on in-house HPLC purification (or comparable method) of any compound making its way into critical experiments. Often commercial samples are sold as racemic mixtures, so care should be taken to isolate the active enantiomer responsible for biological activity, such as the use of chiral chromatography or recrystallization, and eventually confirm with an enantiopure sample. Typically, little, if any, information is provided by a chemical vendor about the synthetic scheme of a compound, and it is rather difficult to predict or identify any bioactive impurity *a priori*. Therefore, after vendor resupply and subsequent HPLC-purification, a third step we employ in the chemical resynthesis and complete characterization of the top compounds [98]. This safeguard ensures the compound identity, purity and bioactivity of a potential lead compound, and gives clues about its synthetic feasibility, scale-up and analog expansion.

A demand for compound natural histories

Lastly, we propose that during hit and active follow-up it is essential to investigate the 'natural history' of prioritized compounds. This is not only to keep in mind the potential for intellectual property (IP) in the future, but to understand the potential for off-target reactivity, promiscuity and interference. Because all of these are so intimately related to chemical structure, this process is best done by a scientist with an appreciation for medicinal chemistry. Chemical filters and rules can be used to help reduce the number of compounds screened and to prioritize actives for future follow-up. A 'natural history' examination can use the same rules and filters, but also should include searches of relevant data, information and knowledge bases. An example of this is shown in Table 2. Five common core structures were selected based on compounds we have seen repeatedly in assays against a variety of targets. The natural history shows the results from searching these compound classes in SciFinder Scholar (relevant to target, IP, promiscuity, and interference), compound libraries (eMol, an academic library, and the MLSMR), and PubChem. These compound classes have also been flagged as PAINS. Nevertheless, note that these compounds continue to be reported in manuscripts purportedly linked to biological activity, calling into question the validity of these research reports.

Box 4. Cheminformatics in early high-throughput screening triage.

- A careful analysis of an high-throughput screening (HTS) will help the project team focus on what are most the attractive actives and can help find compounds that have good ligand efficiency. This analysis typically involves four steps: data standardization; property annotation; substructure sorting; and visualization and selection. The complexity of this analysis is typically proportional to the number of compounds assayed. For typical full-file screens (all of the compounds in a collection), we employ the following procedure. We obtain a structure-data file from our screening group that also contains our unique compound identifier (GPHR number) and the percent inhibition (or other readout) from the primary assay. This structure-data file contains data for all of the compounds screened. This data file is first standardized with respect to molecular representation, duplicate removal, among others, and then annotated with physicochemical property descriptors (clogP, molecular weight, number of H-bond donors, number of H-bond acceptors, topological polar surface area), a pan-assay interference compounds (PAINS) flag (0 = no; 1 = yes), a Lipinski filter flag, and the PEI (percent inhibition/molecular weight). We use a combination of Pipeline Pilot and Canvas to perform this annotation, but other software can also be employed. We then examined the top ~100 actives (by percent inhibition and then by PEI) looking for common core structural motifs [11,120]. These motifs are used to create substructure filters for these cores. This core generation can be done manually or with software like Canvas. Finally, all compounds, both actives and inactive, are annotated with a core designator (core1, core2, core3, core[n]) or with a singleton designator (typically n + 1, where n is the number of cores found in the first pass analysis of common cores. No numerical filtering is performed at this point. This comprehensive data package is then examined using Spotfire Lead Discovery and the best compounds are prioritized for cherry-picked IC₅₀ determination from the original screening plates. Activity is typically defined as having a percent inhibition $\geq 3\sigma$ from the average activity found in the assay, although this cut-off can be varied depending on the assay and other factors. In our experience, percent inhibition is subject to large errors. However, we consider it a confirmation of preliminary activity if several compounds containing the same core structure appear as actives in our first analysis. Our first-pass prioritization is based on a consideration of activity and a balance of calculated physicochemical properties as described elsewhere. All PAINS compounds are deprioritized unless our experience suggests that they have been flagged inappropriately.
- An example of this type of analysis is shown in Figure 5. An HTS targeting the discovery of PKA inhibitors was performed on our screening library (at that time 196 K compounds) and gave an initial data file that included 470 actives as defined by a $\geq 3\sigma$ criterion from the average activity (0.2% actives). The top 100 actives were deconstructed into core structures and each compound in the HTS data set was assigned to a core. Core structures were found in 17,741 structures in the HTS (9%) while 87 of the 470 actives were in core structures (19%). PAINS core structures containing compounds like B were included here for illustrative purposes. Structures like C and D confirm that the assay was selecting compounds of interest. Compounds for follow-up would be selected from cores that showed multiple compounds above the 'active' cut-off (highest priority), and both the singletons (381/470 actives) and compounds with relatively high PEI would be considered for dose-response confirmation. Compounds such as those from core13 are viewed with skepticism. Notice that while compound E is active, all of the related analogs in this series are inactive, including many close structural analogs such as compound F.
- Following cherry-picked IC₅₀ determinations and perhaps other follow-up assays, compounds exhibiting sigmoidal-shaped dose-response curves and having IC₅₀ ≤ 30 μ M are usually prioritized for repurchase using the same criteria discussed above. Typically, 10 mg of every prioritized, commercially available compound is purchased (some close analogs may also be purchased to help establish preliminary SAR), and these samples undergo quality-control tests (LC/MS/PDA/ELSD) to help establish identity and purity, and is then retested. Quality control will often include compound purification, as it is becoming increasingly clear that some assays are very sensitive to trace impurities (e.g., metals, electrophilic reagents, and decomposition products) in the commercially available samples. Additionally, NMR methods may be used to confirm identity in some cases. Confirmation (IC₅₀ ≤ 30 μ M; sigmoidal-shaped dose-response curves; $0.8 \leq$ Hill slopes ≤ 1.2) of these more well-characterized compounds in the primary assay helps instill confidence that the selected chemical matter is worth spending more time and money investigating during both the hit- and series-validation stages of the project. As a matter of course, we annotate the entire data package with information for each compound as it is generated, and continually review this data to insure prioritized compounds are properly followed-up.
- Larger data sets of actives from HTS that are found in industry will typically require a more detailed set of filters (absolute or multiparametric) to whittle them down to a manageable size. In our experience, the number of actives from a typical academic library full-file screen is <1 K and highly automated triage is not necessary. Often this number can be quickly culled after cherry-picking, dose-response confirmations and some key follow-up screens. At this point in the triage process we also perform fairly extensive literature and database searches to determine the natural history of our best series. The expansive amount of data available on some commercially available compounds can be overwhelming. However, we find that a search of Scifinder (using either exact or similarity searches) can often offer us a quick readout on a compound series. For example, the number, document type, and abstract of the available references can often allow us to bin series with respect to their priority for follow-up.

Natural histories like this can further prioritize compounds for follow-up, allowing the most efficient use of limited resources. Such searches may also turn up previous medicinal chemistry follow-up on the same

structural classes, saving the time that could be lost following the same SAR.

A recent publication underscores the importance of performing some detective work prior to reporting or

Table 2. Compound natural history.						
Examples and substructures		SciFinder (substructure search)			eMol/Academic/ MLSMR† (number of compounds)	PubChem‡ (compounds/tested/ actives/probes) (n)
		Number of substances	‘Screening’ references containing those substances	‘Screening’ references reporting these substances since 2011 to present
1	218,934	152	62	35,529/1765/1684	213,282/13,720/4345/1
2	2482	20	12	274/51/24	2932/392/130/0
3	13,831	24	15	1105/146/22	1233/90/63/0
4	238	2	1	38/12/7	75/15/11/0
<p>†Number of substances containing this substructure in eMolecules, a representative Academic library, and the MLSMR.</p> <p>‡PubChem analysis: number of compounds containing substructure; number of these compounds that have been tested in an assay; number of these compounds that have shown activity in at least one assay; number of probe molecules containing this substructure.</p> <p>MLSMR: Molecular Libraries Small Molecule Repository.</p> <p>Compound 1 [154], 2 [155], 3 [156], 4 [157], 5 [158].</p>						

Table 2. Compound natural history (cont.).					
Examples and substructures		SciFinder (substructure search)		eMol/Academic/ MLSMR† (number of compounds)	PubChem‡ (compounds/tested/ actives/probes) (n)
		Number of substances	'Screening' references containing those substances	'Screening' references reporting these substances since 2011 to present
3362	31	11	319/79/22
5					2719/253/177/0
†Number of substances containing this substructure in eMolecules, a representative Academic library, and the MLSMR. ‡PubChem analysis: number of compounds containing substructure; number of these compounds that have been tested in an assay; number of these compounds that have shown activity in at least one assay; MLSMR: Molecular Libraries Small Molecule Repository. Compound 1 [154], 2 [155], 3 [156], 4 [157], 5 [158].

Compound	Exact	Sources	≥90%	Biol	Ref.
1 (S6)	0	4	79	5	[160–164]
2 (S40)	1	4	48	1	[165]
3 (S47)	0	4	19	5	[156,166,167,168,169]
4 (HS26)	0	10	26	6	[168,169,170,171,172,173]

Figure 6. The natural history of PAINS compounds reported in a recent manuscript. Searches performed in SciFinder. ≥90%: Compounds that are greater than 90% similar; Biol: Compounds that were reported in a ‘biological study’ from a journal article [155,159–172]; Exact: Exact substructure found; Sources: Commercial sources of exact compound.

otherwise following up on HTS results. In this manuscript, the compounds **1–4** are cited as the ‘most potent compounds’ with respect to inhibiting Keap1–Nrf2 interactions (Figure 6). These compounds were identified by structure-based virtual screening and their activity was confirmed in standard biological assays. Compounds **1**, **3**, and **4** are classified as PAINS and **2** is closely related to a PAINS substructure. This is not acknowledged in the manuscript.

Admittedly, in the past not all researchers had access to software capable of screening for PAINS substructures. However, almost all authors and reviewers should have access to the CAS database and there are now freeware versions of PAINS filters available [68]. Figure 5 shows an analysis of these compounds in SciFinder, software that is readily available at our institutions. We performed a simple literature ‘screen’ on the exact structure of each ‘most potent compound.’ Although each of the compounds **1–4** is commercially available from several vendors, there are 0–1 references of any kind mentioning the exact structures shown. This, and the names of the vendors selling these compounds, suggests that they were all prepared for sale in screening libraries. In and of itself this is not a problem, although the fact that they have received such scant interest in the literature may be a warning sign. What about related compounds? Have they been reported in the literature? We used an arbitrary cut-off of ≥90% similarity (equivalent

to a regioisomeric shift of an aromatic substituent) to look for closely related compounds. The number of substances we found for **1–4** is shown in the ≥90% column. To better place these substances into context, we then retrieved all of the references for these compounds that were classified by the term ‘Biological Study’ and refined this list by the ‘Document Type’ equivalent to ‘Journal’. The number of references retrieved by this protocol is listed in the Biol column of the figure. Each of the references is listed in the references column. These references typically report the activity of the closely related compounds against other targets, and some describe analog synthesis resulting in extensive apparent SAR.

None of these references appear in this recent publication. Moreover, reference [168], which we found in this relatively conservative exercise in SciFinder, is the seminal manuscript by Soares *et al.* that describes the potential for redox interference by compounds essentially (≥90%) identical to **3** and **4**. This apparent disregard or lack of knowledge of the preceding literature is not an isolated phenomenon. The two other most recent publications, references [165] and [169], make no mention of PAINS and no mention of the work on similar compounds reported in references [155,165–168] and [167–172], respectively (admittedly, reference [169] does reference an earlier paper by that describes the protocol for detecting compounds capable of redox interference [81]).

While the knowledge of PAINS substructure might not yet be part of the lexicon of information readily available to researchers, some relatively straightforward literature exercises may have led the researchers to contemplate the potential polypharmacology of their ‘hits’ and might have raised red flags with respect to compounds 3–4. This exercise in ‘natural history’ should be done when clusters or series of actives are discovered during HTS triage and well before SAR-by-commerce or extensive assays resources are devoted to the project. In our opinion, a rigorous analysis of the literature can influence where resources can most effectively impact the ultimate success of a project, be they for selecting which are the best series for follow-up, development of counterscreens, or making a go/no-go decision. It is also worthwhile mentioning that the aforementioned triage best practices and screening trees we describe would have, in all likelihood, flagged these problematic chemotypes at several stages. To us, these case studies, and many others like them where highly problematic compounds are published in reputable journals, further argue for the essential roles of chemistry in HTS triage.

Conclusion & future perspective

HTS is an important component of drug discovery. However, we believe that the literature will continue to be overrun by questionable ‘hits’ until the art and science of chemistry-driven compound triage is more widely practiced and appreciated. This art requires a constant review of the screening literature, knowledge-sharing among those in the field, and triage

experience. This science sits on the three-legged stool whose legs are medicinal chemistry, cheminformatics and the purification sciences, and benefits from input from biostatisticians, biologists, screening teams and molecular pharmacologists. In our opinion, the practice of HTS triage has improved over time with: the realization that activity is not the only piece of data that should be considered; the focus on more ligand-efficient hits and the understanding that smaller may be better for starting points; and the increased accessibility and sophistication of cheminformatic resources. The field should continue to improve with regards to efficiency and the ability to prioritize tractable chemical matter as: HTS becomes even more accessible, screening throughput increases, assay methods and screening libraries evolve, and more screening data are deposited in databases for data mining and knowledge-sharing. This availability of more screening data will hopefully drive the development of more predictive models of favorable (and also unfavorable) chemical properties, thereby positively enhancing the cheminformatics capabilities of HTS triage. Roadblocks to progress in HTS triage will be the continued availability of PAINS and other nontractable chemical matter in vendor and screening libraries, and what we see as a continued lack of awareness of these nefarious compounds among many medicinal chemists and screeners. This will only improve if the customers – screeners and medicinal chemists – stop purchasing and publishing these types of compounds.

We believe the future can be positively shaped by at least three straightforward mandates regarding

Box 5. The Institute for Therapeutics Discovery and Development.

There is a long and rich history of academic drug discovery and development in the academic setting. The University of Minnesota (UMN; MN, USA) shares in this rich history, most notably by the discovery of Ziagen® (ViiV Healthcare, Brentford, Middlesex, UK), an anti-AIDS compound that has prolonged millions of lives and brought the university over US\$600 million in royalty payments over the past 15 years. That compound was discovered by Dr Robert Vince, based on a rational drug design approach [175]. His Center for Drug Design remains a key player in the drug discovery at the UMN. Seven years ago, the university decided to embark on an experiment that would involve the organization of drug discovery in an institute that would serve all of the biomedical researchers at the UMN and also support translational projects developed by our close collaborators at the Mayo Clinic. The Institute, which is directed by Dr Gunda Georg, is comparable to a mini-biotech in its capabilities, and to a small pharmaceutical company in the scope of the projects it engages. The projects primarily arise from the biomedical laboratories at the UMN, but projects from other institutions and industry are becoming more prevalent in our portfolio. The Institute consists of five core groups: HTS, Lead and Probe Discovery (LaPD), Medicinal Chemistry, Chemical Process Discovery and Development (including GMP synthesis), and Pharmacology. Each of these groups is lead by a director with industrial experience. The capabilities of the Institute have led to the purposeful organization of other drug discovery and development groups within the confines of the UMN and an overarching interest at the university of accelerating bench-to-bedside processes (the capabilities of the ITDD have recently been more extensively reviewed [176]). The LaPD is intentionally modeled after the aforementioned industrial-based Lead Discovery Group. We have expertise spanning medicinal chemistry, cheminformatics and purification services, and routinely handle the triage of three to five high-throughput screening campaigns per year.

publication of assay results: that assay development manuscripts be required to provide evidence of target engagement; that PAINS and other interference compounds be appropriately referenced and flagged; and that authors provide relatively straightforward 'natural histories' on all reported active compounds including a discussion of the relevance of these natural histories to the screening method and the target under investigation. The recommendation that authors upload machine-readable structural information (e.g., SMILES) of reported bioactive molecules will facilitate the initial review and later analysis of these manuscripts [173]. We recommend that all journals consider requiring this as supporting information. Another related recommendation is the inclusion of more detailed descriptions of the post-HTS triage process in manuscripts describing HTS results. This should allow for more critical examinations of the science by readers and call much-needed attention to this critical component of successful HTS campaigns. Such recognition may spur further advances in the field, such as the development of new tools and data/evidence-driven methodologies to render the triage process more effi-

cient and successful in terms of identifying tractable chemical matter.

Of course, while the application of these mandates will do much to assure the quality of the science reported, care should be taken to recognize and acknowledge the importance of covalent or irreversible inhibitors, since these will often fail simple and simplistic cheminformatic filters [174]. Should these types of compounds be reported, the relevance of such inhibitors to the target in question should be adequately rationalized. We have no doubt that HTS will continue to provide new starting points for discovery of biochemical probes and drugs. The best way to insure that this occurs in the most effective manner, we believe, is through a partnership between HTS and chemistry that begins early in the discovery process (Box 5).

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Executive summary

Medicinal chemistry in high-throughput screening organization and assay design

- Chemists and high-throughput screening (HTS) personnel must work together to obtain high-quality and robust HTS data. Appropriate control plates and reference compounds to correct for systematic errors arising from the screening method can help in this process.
- Plans for confirming bioassay activity should be established early by a combination of assay-specific counterscreens, orthogonal assays and selectivity assays.
- Virtual HTS projects should form early partnerships with biological scientists and medicinal chemists experienced with assay design and post-HTS triage, respectively.

Post-HTS triage

- Post-HTS triage combines the elimination of risky hits (false-positives, artifacts, frequent hitters and unfavorable physicochemical properties) and the prioritization of more promising hits for follow-up experiments.
- Post-HTS triage combines elements of cheminformatics, medicinal chemistry and the purification sciences (a 'chemocentric' approach).
- The evaluation of chemical 'beauty' (e.g., HTS triage) is both an objective and subjective process.
- Potential active compounds should be evaluated for bioassay promiscuity and natural history by conducting thorough literature, database and patent searches.
- Post-HTS triage should include assays to eliminate redox-active compounds, chemical aggregators and promiscuous thiol-reactive compounds, even if these nefarious mechanisms are not initially suspected.
- The structural identity and purity of promising bioactive compounds should be confirmed, and bioactivity verified by chemical re-supply and independent chemical synthesis.

Recommendations for reporting HTS results

- Compounds flagged as pan-assay interference compound should be pursued with extreme caution, and should be considered by reviewers and researchers alike as unsuitable as leads or chemical probes unless proven otherwise by rigorous experimentation.
- More drug discovery teams should publish examples of assay artifacts or 'no-go' decisions, to serve as lessons to others and to prevent wasted follow-up by independent groups.
- Detailed descriptions of the post-HTS triage process should be included in manuscripts reporting novel chemical matter derived from screening campaigns. This should raise awareness of this critical component of successful drug discovery campaigns, increase overall scientific transparency, and hopefully inspire the development of more data/evidence-based triage practices.

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