# The holistic integration of virtual screening in drug discovery

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**During the past decade, virtual screening (VS) has come of age. In this review, we document the evolution and maturation of VS from a rather exotic, stand-alone method toward a versatile hit and lead identification technology. VS campaigns have become fully integrated into drug discovery campaigns, evenly matched and complementary to high-throughput screening (HTS) methods. Here, we propose a novel classification of VS applications to help to monitor the advances in VS and to support future improvement of computational hit and lead identification methods. Several relevant VS studies from recent publications, in both academic and industrial settings, were selected to demonstrate the progress in this area. Furthermore, we identify challenges that lie ahead for the development of integrated VS campaigns.**

## Introduction

The identification of novel lead structures is a central task at the beginning of a drug discovery campaign. There are many ways to identify hits, which can then be used as starting points for hit-to-lead optimization. The systematic experimental testing of large compound libraries (i.e. HTS) has been established since the 1980s. The costs of HTS experiments are tremendous and, thus, VS, an *in silico* analog of HTS, was developed ten years later. Comparison of the appearance of literature related to VS and HTS highlights this development (Fig. 1).

Notably, the most cited HTS-related publication, that by Lipinski *et al.* [1], discusses the application of both HTS and VS to estimate the solubility and permeability of chemical compounds. Although VS was initially seen as a cost-saving substitute for HTS, both techniques are of a more complementary nature and recent developments in the area of lead identification approaches make use of the advantages of both. In this article, we do not intended to review the exhaustive applications of VS; instead, we present and analyze the evolution of VS over the past two decades. VS is currently maturing as a hit identification strategy, as occurred with HTS a decade before. This process becomes more evident as we observe the development of VS from a more isolated procedure toward a fully integrated technique for hit and lead identification [2]. Experimental data are no longer only collected after a VS campaign but are instead incorporated into the process.

Ten years ago, a trend toward the integration of VS and HTS had been documented [3], for which a classification has been proposed recently [4]. Here, we emphasize current progress in VS from selected recent publications and give an overview of the emerged integral strategies in drug discovery. We suggest a categorization of the global VS technique according to its level of integration into: classic VS, parallel VS, iterative VS and integrated VS (Fig. 2). We provide a definition of each category and focus on the benefits and bottlenecks of each.

## Classical applications of virtual screening

VS is often compared to a funnel, where a large number of molecular compounds, often referred to as a VS library, is reduced by a computational algorithm to a smaller number that will then be tested experimentally (Fig. 2a). The screening library often contains $10^5$–$10^7$ molecules, whereas the desirable output of these protocols is in the range of $10^0$ to $10^3$, depending on the study. The role of VS algorithms is to enrich active compounds in the highly reduced output. The protocol often comprises several 'filtering layers', which hold back inactive or undesired molecules or prioritize compounds according to their predicted activity (so-called

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 1359-6446/06/$ - see front matter  http://dx.doi.org/10.1016/j.drudis.2013.01.007

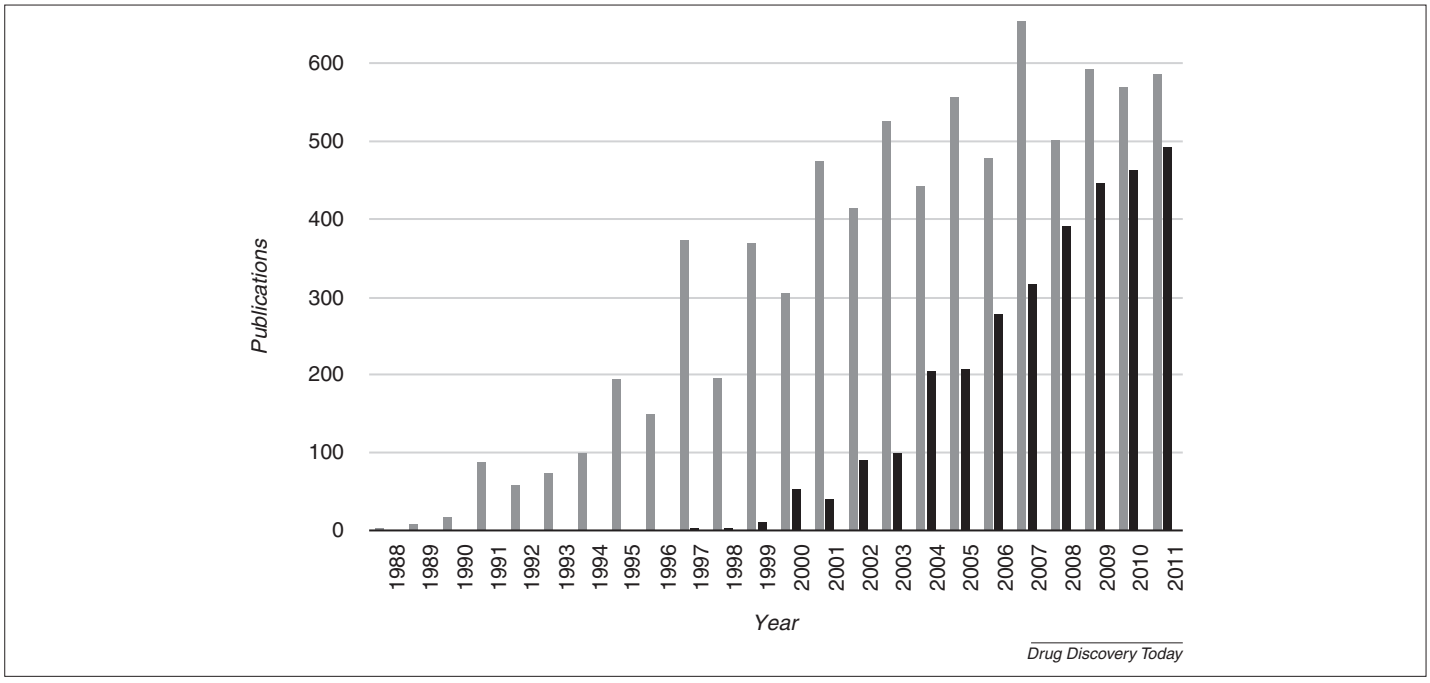


**FIGURE 1**

Chronological overview of the number of high-throughput screening (HTS; gray bars) and virtual screening (VS; black bars) publications according to ISI Web of Knowledge (Thomson Reuters, http://www.isiknowledge.com).

'ranking'). Often the layers are arranged according to the computational time required; however, the growth in computational power resulted in a tendency to apply computationally expensive methods even to large databases (e.g. high-throughput molecular docking). The final step usually comprises manual selection of compounds by experts, often referred to as 'cherry picking'.

Numerous classic VS studies have been extensively reviewed by Bajorath and coworkers [5,6]. The interested reader is referred to those articles, because here we emphasize the maturation of VS strategies.

## Parallel applications of virtual screening

Another VS strategy is to apply multiple protocols in parallel and to combine the results (Fig. 2b). Often, these protocols cover various methods from different domains, including two- (2D) and three-dimensional (3D), ligand- and structure-based, similarity searching, machine learning and molecular modeling methods. The fundamental idea behind this parallelization is that each single method is complementary to the others in terms of the resulting virtual hit lists. Each single protocol is considered to be a classic approach, as described above. The fusion of multiple results helps to improve the overall performance by increasing the number of true positives and decreasing the number of false positives in the final selection [7]. Although the beneficial effect on the enrichment of true positive compounds has been studied thoroughly, the effect on true and/or false negatives remains largely unclear. The broad application of parallel VS emerged originally with the appearance of high-performance computational clusters in cheminformatics and computational chemistry working groups boosting the available processor time.

In general, parallel VS is a valid strategy to increase the enrichment rates. Thus, it is important to select the most suitable data fusion strategy for merging resulting virtual hit lists. Various fusion models (e.g. similarity or group fusion) have been described [7]. Furthermore, the application of an additional VS method as the last step of a fully parallelized approach has been observed. Here, we summarize selected studies exemplifying the use of parallel VS.

In 2005, coworkers from Sanofi-Aventis reported the discovery of blockers of the voltage-dependent potassium channel Kv1.5 by multiple VS approaches [8]. Given the lack of biological assays suitable for an HTS approach and the 3D protein structure, they used homology modeling to produce a receptor-based pharmacophore model. This was then used as a query in a VS of the compound library of the company, where 244 molecules had been selected for *in vitro* validation. In total, 19 were successfully confirmed as hits (a hit rate of 7.8%), and five compounds had an $IC_{50}$ in the range of $<10\ \mu M$ up to 900 nM. Intermolecular pairwise distance measurements based on UNITY fingerprints (Tripos International, http://www.tripos.com) showed that if one of the five hits was used as query, none of the remaining hits would have been found, because of high structural dissimilarity. Repeating the same experiment based on Feature Trees [9] revealed only a single compound, because all the others had distances of less than a suggested similarity cutoff [10]. Interestingly, two additional VS approaches using 2D similarity searching and a ligand-based pharmacophore had been run previously. Both approaches also resulted in successful identifications of novel Kv1.5 blockers. However, the number of chemotypes identified was lower compared with the number of chemical classes identified via the receptor-based pharmacophore approach (five chemotypes). In addition, none of the identified hits was found by more than one of the VS approaches. This clearly shows the complementarity of VS techniques in terms of the identified hits. As a consequence, it was not necessary to apply more complex data fusion methods to

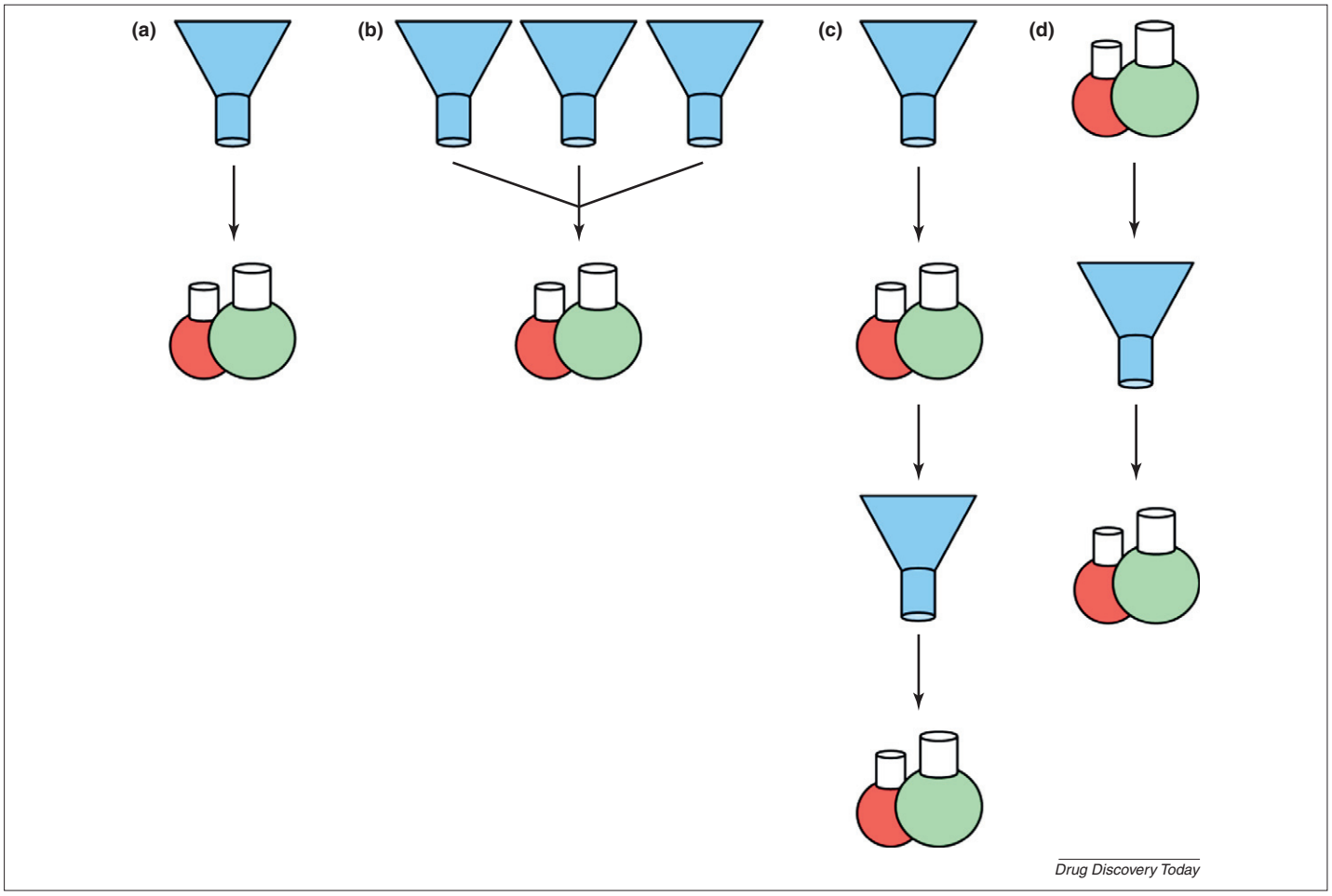


**FIGURE 2**

Schematic overview of virtual screening (VS) categories explained in the main text: classical VS **(a)**, parallel VS **(b)**, iterative VS **(c)** and integrated VS **(d)**. Main differences are observed in the alignment of applied VS protocols (blue funnels) and wet experiments (colored flasks).

combine the results of each VS task. A simple union of all hits increased the number of starting points considered to be favorable for subsequent optimization.

A more recent study by Tömöri *et al.* emphasized the complementarity of 2D and 3D VS methods, illustrated using a hit-finding task for novel phosphodiesterase type V (PDE5) inhibitors [11]. The study was split into two parts, with the first part comprising a classic similarity-based VS and a second part in which follow-up hits were found by both 2D- and 3D-based methods.

In the first part of their study, 27 reference inhibitors were collected from the literature and from clinical data. A database with more than 25 million commercially available compounds was reduced to 8655 molecules by similarity searching against the reference compounds. After filtering by physicochemical properties, diversity selection and cherry picking, 97 compounds were obtained, resulting in eight hits with a PDE5 inhibition of >55% at 10 μM (hit rate of 8.2%), two of which had an $IC_{50}$ of <1 μM. Based on their novelty and efficacy, six of the eight compounds were selected for the second part, which comprised three rounds: (i) individual similarity searches; (ii) group-fused similarity searches; and (iii) docking of next neighbors.

In the first round of the second part, each of the initial six hits selected was used as a query for similarity searching via JChem fingerprints (ChemAxon, http://www.chemaxon.com) individually. For each query, ten nearest neighbors and ten most diverse molecules up to a Tanimoto coefficient [12] of >0.8 have been selected for purchase (120 compounds in total). Of these, 104 could be obtained for testing, with 22 molecules showing an inhibition of >55%, whereas nine had an $IC_{50}$ of <1 mM.

In the second round, the similarity ranked lists of the first round were fused, and the highest rated compounds, plus an additional set of ten diverse compounds, were selected for purchase. In total, 14 could be obtained for testing, and three showed an inhibition of >55%. Notably, these molecules had not been found by the approaches run previously, showing the potential of group fusion.

In the last round, 1810 nearest neighbors of the initial six hits (Tanimoto distance up to >0.75) were selected for GOLD [13] docking experiments into the binding pocket of a crystal structure of PDE5. Out of the top-ranking 60 compounds, 48 molecules could be obtained for biological validation and 11 compounds showed a significant inhibition at 10 μM, whereas three compounds had an $IC_{50}$ of ≤1 μM. Interestingly, although all three had also been identified in the other rounds, they were not ranked as high as in the 3D-docking experiment. This indicates that ligand- and structure-based VS methods highlight different aspects of screening molecules, which is the basis for the complementarity

of their results. However, overlapping hit lists are possible and sometimes even desired for hit prioritization.

Xia *et al.* used solely 3D-based VS for novel inhibitors of 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) [14]. The foundation of their experiment was given by 15 co-crystallized inhibitors (ligand queries), using the structure of the enzyme as docking target, and a commercial screening database with multiple conformations generated using OMEGA (OpenEye Scientific Software, http://www.eyesopen.com).

In the first part of their study, ligand-based ROCS [15] (OpenEye Scientific Software, http://www.eyesopen.com) screenings with the queries against the screening database resulted in 1000 top-ranked virtual hits. The second part was a structure-based approach, where the screening database was docked into the target protein structure, also resulting in 1000 top-ranked molecules. Combination of both lists was performed using a more thorough docking procedure of all 2000 top-ranked virtual molecules using the Glide docking [16]. Out of the top 200 ranked molecules in this list, 70 compounds were purchased and 14 showed significant inhibition of >50% at 1 μM in a scintillation proximity assay for 11β-HSD1. Of these compounds, eight showed differing scaffolds and $IC_{50}$ values of <100 nM in follow-up binding affinity measurements, with the best value being 3.7 nM.

This study showed that the resulting virtual hit list of ligand- and structure-based methods can vary immensely, and that both must be performed in parallel to cover correct true positives. Although fusing the results with another method is legitimate, one could argue that the final results are massively biased toward structure-based docking. Unfortunately, it was not noted how many of the 14 true positives source were from the ligand-based approach or from the parallel structure-based approach. Yet, the identified hits exhibited extraordinary well-validated measurements and demonstrate furthermore the capability of VS.

To evaluate the performance of different data fusion strategies, Svensson *et al.* [17] benchmarked five algorithms (sum rank, rank vote, sum score, Pareto ranking and parallel selection) on 14 targets of the DUD data set [18]. Retrospective VS tasks were performed using docking (Glide), pharmacophore search [Phase (Schrödinger, http://www.schrodinger.com)], shape similarity (ROCS) and electrostatic similarity searching [EON (OpenEye Scientific Software, http://www.eyesopen.com)] spanning a broad selection of ligand- and structure-based methods. The authors investigated whether data fusion could increase the enrichment compared with running each task alone. They observed that data fusion in general leads to a higher recovery rate of known actives compared with the best single procedure. However, the fusion algorithms performed differently well, led by parallel selection as the best algorithm over all 14 targets, followed closely by Pareto ranking and rank voting. Therefore, data fusion constitutes a necessary procedure in VS tasks where manifold screening methods are applied in parallel.

## Iterative applications of virtual screening

Other recent prospective studies demonstrate the maturation of VS to a third category, namely iterative VS. Here, VS is not an isolated task before the experimental evaluation; instead, it is sequentially integrated, hence iteratively, into the hit identification, hit expansion and hit-to-lead optimization processes. The information obtained from *in vitro* screening experiments subsequently flows back into VS and helps to improve the *in silico* model (Fig. 2c). The idea behind adaptive optimization is that compounds for *in vitro* evaluations are selected from the *in silico* compound library and are subsequently subjected to experimental evaluation. The most active compounds are then used to choose similar novel structures as new starting points. However, the similarity threshold applied is not constant but adaptive and decreases with every screening round. This approach assumes that, although first hits found by a VS procedure are perhaps not the best ones that are present in the available compound library, the subsequent 'narrowing in' will result in more potent hits. The first prospective application of a genetic algorithm was reported by Weber and coworkers [19], where a thrombin inhibitor ($K_i$ = 0.22 μM) was evolved from 160,000 possible Ugi-type reaction products by 18-fold application of the optimization strategy. Less optimization rounds are required if more target knowledge in terms of a more sophisticated screening model is used for input. Zander and coworkers [20] demonstrated that the potency of initial hits from the first *in silico* screening round can be optimized by simple substructure search. Their prospective study aimed to develop bacterial thymidine kinase inhibitors for treatment of methicillin-resistant *Staphylococcus aureus* (MRSA) infections in which a VS cascade was used to retrieve initial hits. The first round of VS comprised a clustering step using the topological pharmacophore descriptor CATS [21] and self-organizing maps [22]. Then, three methods were applied sequentially: PHAST [23] (molecular comparison by string alignment), pseudoreceptor modeling PRPS [24] (automatic receptor-based pharmacophore screening) and ShaEP [25] (3D similarity search using shape and electrostatic potential comparison). All three methods were able to retrieve weak hits. From 14 compounds screened *in vitro*, seven exhibited MICs between 128 mg/l and 32 mg/l. Subsequently, substructure searches have been performed using the two most potent compounds, which led to two compound series with improved potencies showing MIC values up to 0.25 mg/l.

The cascaded study design is not necessarily restricted to narrowing in on the structural diversity of the screening hits toward higher potency. At each step, a broader search can be performed to modify the scaffold toward better chemical accessibility or more promising ADME and/or toxicological properties. The broader search requires application of different screening protocols at each step. A comprehensive study was performed by Hofmann and coworkers [26]. Here, a charge-based descriptor similarity search was performed to find novel 5-lipoxygenase (5-LO) inhibitors based on a published reference structure. Seven out of 11 compounds selected for biological evaluation exhibited low micromolar $IC_{50}$ values on 5-LO. Two hit series were selected for substructure search and both yielded compounds with submicromolar potency [27,28].

Experimental information available *a priori* is valuable for such sequential VS campaigns and enables the first biological evaluation step to be skipped. A study by de Graaf and coworkers [29] demonstrated the impact of the availability of this information on the hit rate of the structure-based VS campaign. In this study, the X-ray structure of histamine H1 receptor (H1R) with a co-crystallized antagonist was used for structure-based VS by docking. A large number of active H1R antagonists available from literature

were used to validate the docking protocol and to adjust scoring thresholds for the selection of compounds. After a collection of commercially available fragment-like ligands was docked into the binding site of H1R, 26 compounds were selected for experimental validation. The hit rate of this study was high (73% of compounds exhibited a $K_i < 15$ μM), which underlines the importance of *a priori* experimental information.

## Integrated applications of virtual screening

Most recently, a fourth category of advanced applications of VS emerged, which demonstrates full integration of computational techniques in the hit retrieval process, hand in hand with HTS. The most simple liaison is described as the use of HTS results in subsequent VS approaches (Fig. 2d). However, differing sequential arrangements have been observed to highlight complementary effects, such as reduction of false positive HTS hits or focusing chemical space.

In an effort to combine HTS-derived activity data to train *in silico* models aiming at the prospective discovery of further starting points, scientists from Vanderbilt University [30] performed a high-throughput screening of a diverse library of approximately 140,000 screening compounds to identify positive allosteric modulators (PAMs) of the metabotropic glutamate receptor 5 (mGluR5). The potencies of a total of 1382 primary PAM hits were used to train quantitative structure–activity relationship (QSAR) models using artificial neural networks (ANN) to predict $EC_{50}$ potencies. Both continuous and classification predictors were trained on numerical structural descriptors. The resulting ANNs were applied in a prospective VS of a virtual database of approximately 450,000 drug-like screening compounds, leading to a set of 282 potentially active virtual hits, out of which 232 (82.2%) were confirmed as true active potentiators and partial agonists. This result constitutes an enrichment factor of 23 for PAM activity and 30 for the overall modulation of the mGluR5 compared with a 1% hit rate in the original screening deck. Although 72% of the confirmed hits turned out to be close derivative PAMs from the training data set, which could also have been identified by more simple 'analoguing' approaches, 28% were considered to be non-trivial modifications (e.g. not sharing a common scaffold with ligands from the training data set).

This approach could be a particularly interesting strategy in cases where discovery efforts are aimed at further chemical space not yet covered by available HTS libraries (e.g. targeting vast virtual libraries or additional compound libraries available from commercial sources).

The value of cheminformatics as a particular VS method applied post-HTS was shown by a group at Novartis in an effort to elucidate the potential of screening artifacts in HTS assays [31]. The authors retrospectively analyzed the molecular properties of a comprehensive number of hits obtained from a high-throughput screening platform on 26 targets, including signal-transduction proteins, enzymes, protein–protein interactions, kinases and other non-membrane proteins, over a period of several years. The size of a typical primary hit list resulting from this proprietary affinity-based HTS technology was reported to range from several hundreds to up to more than 10,000 compounds from a library of approximately 500,000 screening compounds. The study aimed to identify sublibraries of frequent hitters and to discriminate between compartments of screening space yielding high and no hit rates. To guide this effort, an analysis was performed on the number of unique active molecules and scaffolds, represented by their topological molecular fingerprints and Bemis-Murcko scaffolds [32]. The authors observed no substantial frequent hitter problem, because most hits (70–90%) annotated by their molecular fingerprints were identified in only a single assay, leading to the conclusion that the hits might be selective for their corresponding target over the range of screening campaigns evaluated. A similar finding was observed for the analysis of molecular scaffolds. Here, only a minor increase in frequency of individual molecular frameworks being potential frequent hitters was found.

An additional investigation of compliance to the Rule-of-Five [33] revealed that a considerable number of molecules (42%) from the validated hit lists violated none of the rules, whereas most satisfied at least two of the four criteria, demonstrating the potential developability of the identified starting points into leads. Additionally, the authors reported that both random subsets and subsets assembled by rational selection tended to show comparable coverage of structure space in the corresponding screening library, with a marginal superiority of the rationally selected subsets. In brief, this study demonstrates the value of cheminformatics in HTS triaging for early identification of potential frequent hitters and over-represented scaffolds making computational methods an integral part of postscreening protocols.

A case study to improve the accuracy of primary HTS hit lists and, thus, reduce the efforts required for hit follow-up in confirmatory screens was carried out by Jenkins and colleagues [34]. Here, VS using two different docking methods was applied in parallel with a HTS of two diverse screening libraries aiming to discover angiogenin inhibitors. As a particular challenge, the authors were faced with a high percentage of false positives from HTS observed in follow-up assays, prompting a resource-saving computational triaging step after the initial screening. It was shown that a consensus selection of HTS hits also identified by VS could considerably improve the enrichment of primary assay results, reducing the rate of false positives, and was particularly effective at identifying ligands with targeted mid-micromolar dissociation constants. To assess the value of VS as a preprocessing step to their HTS campaign, the authors also analyzed the ability of VS, in particular molecular docking, to identify subsets of the HTS library with an increased likelihood of containing actives, thus reducing the number of compounds to be screened *in vitro*. One of the two applied VS schemes reduced the number of compounds required for screening by 50 times (retaining 42% of the actives). However, to increase the percentage of actives substantially, the fusion of two VS methods was required, which led to a reduction of the library size by four times.

The discovery of protein–protein interaction (PPI) inhibitors is especially challenging owing to the nature of the binding site itself, where the mechanism of ligand–protein interaction is largely unclear. Betzi and coworkers [35] successfully applied VS and experimental screening to identify inhibitors for the human immunodeficiency virus type 1 (hiv-1) negative regulatory factor (Nef) protein without prior knowledge of the reference inhibitors. The group combined high-throughput docking using the available

apo-structure of the protein and applied pharmacophore constraints in addition to an analog-searching strategy and compared the results with a systematic HTS of the same library, demonstrating significant enrichment could be obtained by *in silico* screening. In this study, the authors could validate a drug-like VS hit as being a tractable reference compound for their cell-based *in vitro* assay. In a subsequent step, the complete library of screening compounds was tested in the same *in vitro* assay, revalidating the best-scoring VS hit. Thus, the combined approach of VS and HTS could demonstrate not only the value of computational prefiltering of libraries with the goal of enrichment, but also the true complementary interplay of *in silico* and *in vitro* studies mutually validating each other, especially in a challenging scenario with little previous knowledge to start from.

In another publication, a group at Gedeon Richter [36] demonstrated not only the effectiveness of VS in a head-to-head comparison with experimental HTS of the same library in terms of enrichment, but also their complementarity. Here, hit identification for inhibitors of the serine/threonine kinase glycogen synthase kinase-3β (GSK-3β) was performed. A VS campaign using high-throughput docking with pharmacophore prefiltering was validated by screening the same library of compounds *in vitro*. Similar enrichment factors at 1% of the screening library were obtained for both the *in silico* and *in vitro* screen, whereas a considerable increase in the overall hit rate was observed for VS (12.9%) compared with HTS (0.55%). Notably, analysis of the resulting validated hits by clustering and multidimensional scaling [37] of their corresponding molecular fingerprints as structural descriptors indicated that the majority of chemical space identified by HTS could also be explored by VS and vice versa. The authors concluded that, owing to the comparably large number of false positives and negatives in VS, it might constitute a viable strategy to design focused screening libraries for HTS or to prefilter commercially available target-directed VS libraries.

## Concluding remarks

In the literature, the evolution of VS strategies is an evident trend. Not only the maturation of computational methods, but also an increasing degree of their integration into the discovery process can be observed over the past decade. It is noteworthy that the prospective applicability of VS *per se* is no longer a matter for debate, but rather the question is how to maximize its outcome. At present, the community is focusing on best VS practices to ensure the delivery of attractive chemical starting points. Methodological improvements can still be observed, and there appears not to be a single best way to tackle the different challenges faced by computational approaches with during drug discovery. Perhaps this question will never be answered, because the outcome of VS campaigns depends on the respective biological target, level of prior knowledge and the aim of the study.

The limitations of VS have been comprehensively described elsewhere [38,39] and the current discussion highlights the need to create community-wide standards of setting-up and reporting VS studies, as done for HTS. A precedent-setting step has already been taken by the new publication guidelines for computational studies in the *Journal of Medicinal Chemistry* [40]. We believe that the definition of VS categories as proposed in this review is a helpful step toward documenting the past evolution and supporting future improvement of methods.

In the case studies discussed, it appears that VS is almost as relevant as HTS as a hit-delivery method. However, it is still inferior in terms of budget and resources. Especially in times of increasing pressure on research and development (budget cuts, site closures and lay-offs) [41], we believe that there is a huge opportunity for computational groups to prove themselves as cost savers and risk minimizers. The classic role of computational drug designers seems to have evolved beyond a supporting function in medicinal chemistry departments, because closer interaction of virtual and wet screeners might be a key aspect for future drug discovery success.

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