Review

Journal of the Brazilian Chemical Society

https://dx.doi.org/10.21577/0103-5053.20240112

J. Braz. Chem. Soc. **2024**, *35*, 10, e-20240112, 1-29
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Structure-Based Virtual Screening: Successes and Pitfalls

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Structure-based virtual screening (SBVS) is an important approach that makes the first stages of drug development and repurposing processes faster and more efficient. Advances in experimental techniques and *in silico* computational modeling have contributed significantly to the characterization of diverse biological targets. Combined with the rapidly growing number of chemical compounds available in virtual databases, these advances enable the effective application of SBVS for prioritization of putative bioactive compounds to treat a wide range of pathologies. Techniques such as molecular docking, along with the utilization of pharmacophore models, are commonly employed for screening large databases of compounds, providing a solid foundation for employing SBVS in drug development pipelines. This review comprehensively analyzes recent advancements and strategies employed in the field of SBVS and explores methodologies for validation, limitations, and challenges associated with this approach. Through a series of case studies across different therapeutic targets, we demonstrate SBVS's versatility and efficacy in identifying potential therapeutic agents. However, challenges remain, and understanding these is crucial for maximizing SBVS's potential. We address these challenges, offering insights into the current limitations and future prospects of SBVS in drug development.

Keywords: drug design, computer-aided drug discovery, virtual screening methods, structure-based pharmacophore modeling, and computational chemistry in drug development

1. Introduction

The traditional drug development process is complex, requires high economic costs, time, and multidisciplinary efforts.[1] Fortunately, advances in technology have revolutionized the drug development process, moving away from the "trial and error" method and starting to use modern tools such as automated assays and artificial intelligence.[2]

In the past, medicine was primarily based on the use of herbs and potions with healing effects, and only in the mid-19th century were the first efforts made to isolate and purify the chemical compounds responsible for their medicinal properties, known as active ingredients.[3,4] These efforts led to the discovery of several important molecules by the first half of the 19th century through vegetal extracts, such as opioids (e.g., morphine) for use in chronic pain, salicylates (e.g., salicylic acid) for use in inflammation and as antipyretics, and tropane alkaloids (e.g., cocaine) for use in anesthesia.[5,6] This period also saw the birth of the first pharmaceutical companies, where chemists made several chemical analogues in an attempt to improve the properties of natural compounds.[5-7] Furthermore, some drugs were discovered by serendipity, such as antibacterial penicillin derived from fungi and the antipsychotic chlorpromazine (Figure 1).

However, according to Pasteur: "...In the fields of observation, chance favors only the prepared mind".[8,9] These early discovery processes, though they achieved some successes, were expensive, inefficient, and relied heavily on manual trial and error methods, lacking the efficiency of automated assays like high-throughput

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Editor handled this article: Brenno A. D. Neto
In memory of Professor Eliezer Barreiro, renowned in the field of Medicinal Chemistry, whose contributions will continue to inspire future generations.

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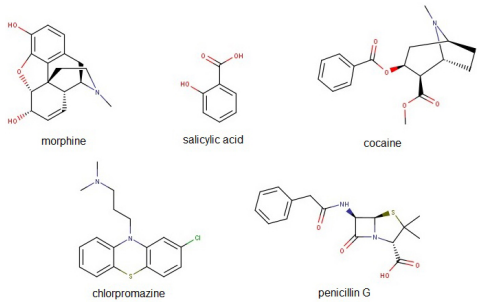


Figure 1. Morphine, salicylic acid and cocaine are examples of drugs originating from vegetal extracts. Chlorpromazine and penicillin were discovered by serendipity.

screening (HTS). Furthermore, the methods employed often resulted in the discovery of hit compounds (e.g., molecule that produces reproducible activity above a defined threshold in a biological assay and whose structural identity has been established) with little or no understanding of their pharmacokinetics, toxicity, or mechanisms of action.[10-12]

The 20th century was a golden era in the pharmaceutical field, providing most of the current repertoire of drugs currently used in therapeutics.[13] The rapid discovery of drugs during this period can be attributed, in part, to advances in the fields of microbiology and parasitology, which allowed the understanding of infectious processes and etiological agents of various diseases.[14,15] Significant contributions have also come from the fields of biochemistry and physiology, allowing detailed understanding of the underlying physiological and biochemical disorders that lead to the phenotype of various diseases, as well as notable advances in the field of organic chemistry, further refining synthetic processes.[16,17]

With these advances, the rational design of drugs could be primarily achieved through exploration and utilization of molecules with known activity, including the application of concepts such as structure-activity relationships and the development of "me-too" drugs and drug repurposing.[18-20] Additionally, approaches such as phenotypic screening assess the effects of potential drugs on cultured cell lines (*in vitro*), isolated tissues/organs (*ex vivo*), or whole animals (*in vivo*), allowing for the refinement of lead compounds through testing of the effects of series of analogues. Moreover, target-based screening of molecules on purified target proteins *in vitro* allowed the elucidation of mechanisms of action at the molecular level.[21] The search for lead compounds is not limited to plant extracts alone, but also includes sources in venoms, toxins, and metabolites (from animals and microorganisms), as well as screening in molecular virtual libraries.[22,23]

Even given advances in organic synthesis and this diversity of sources, the available chemical space is still far from being comprehensively explored in the field of drug discovery. The chemical space is a metaphor that can be likened to the cosmological universe in its vastness, with chemical compounds populating space instead of stars, making its exploration a challenging task.[24,25] To contextualize the complexity of the task, consider a modest list of 150 substituents, ranging from mono-substituted to 14-substituted hexanes. Given this list, it is possible to generate more than 10^{29} derivatives of *n*-hexanes. However, not all theoretically possible compounds fall within the limits of what is synthetically viable to produce, even with the extensive current knowledge of organic chemistry.[24] This limitation led to the emergence of combinatorial chemistry, a strategy characterized by the rapid and efficient construction of a variety of structurally related compounds, resulting in the creation of combinatorial libraries.[26] Starting from a single known bioactive molecule as a model, it is possible to assemble a set of theoretically isofunctional molecules.[26] The process of generating combinatorial libraries, whether through experimental or virtual means, can be based on synthetic routes or scaffold-based strategies, providing powerful tools to explore broad areas of chemical space.[27]

The first combinatorial libraries were used in HTS techniques, which employ automatic robotic technology to experimentally screen a large number of molecules in search of those that induce the desired biological effect.[28,29] However, the growing number of compounds available for screening added to other limitations and costs involved in the HTS approach, leading to the development of *in silico* approaches, such as virtual screening (VS), to complement HTS.[30,31]

The integration of the aforementioned techniques (combinatorial chemistry and HTS) significantly optimized the process of developing new drugs. However, the HTS hit rate is often extremely low.[32,33] Further, many hits can be "artifacts", yielding false signals across a variety of assays because their activity does not depend on a specific drug-like interaction.[34,35] The low hit rate and high technology investment involved in HTS has constrained its usage to advanced research programs and pharmaceutical companies.[36,37] Therefore, VS emerges as a valuable rational approach to guide the selection of new hits. VS can be used in integration with HTS, with combinatorial libraries, or applied for screening in large databases for private use (in the case of pharmaceutical companies), research groups, or public use (such as the Zinc,[38,39] PubChem,[40,41] ChEMBL[42,43] and DrugBank[44,45] databases). VS might reduce costs associated with purchasing biological and chemical materials and analyzing HTS results.[46,47] Additionally, synthetic modeling techniques and artificial intelligence have led to an explosion in the number of compounds in large databases.[48] For example, the Zinc database, which is

widely used in large-scale VS, contained less than 1 million molecules in 2004, while the latest version (Zinc22) has surpassed 37 billion unique chemical entries, representing a 50,000-fold increase in just 18 years.[38,49,50]

Combinatorial chemistry (improved by computational methods) and the explosion in the number of molecules available in databases can promote the idea that "bigger is better" because expansion in chemical space brings some advantages, such as rapid and cost-effective identification of drug-candidate ligands and protein targets, as well as the selection of new molecular scaffolds, which are crucial for patents and drug discovery.[51] However, the idea of "bigger is better" needs to be examined with caution, as the expansion in chemical space can increase the occurrence of false positive hits.[50] This is due to the fact that the diversity of compounds in an expanded chemical space can lead to non-specific interactions or artifacts. Moreover, the complex structures and as yet poorly documented chemical properties of new entities, combined with a limited understanding of their structure-activity relationships (SAR), exacerbates this risk. Furthermore, more complex molecules exhibit poorer fits with protein targets, suggesting that inclusion of ever more complex molecules in databases is unlikely to yield proportional gains in drug discovery.[52] To address this challenge, researchers focus on improving our understanding of SAR. Evidently, the growth of virtual libraries requires efficient strategies to avoid wasted efforts.[51,53]

To assist in choosing strategies, computer-aided drug design (CADD) offers a set of chemical and physical strategies to discover, design, and develop promising compounds using mathematical and computational tools in the areas of chemoinformatics and bioinformatics.[54,55] On October 5, 1981, Fortune magazine[56] published a cover article entitled "The Next Industrial Revolution: Designing Drugs by Computer at Merck". Some have credited this as being the start of intense interest in the potential for CADD. The technique can be categorized into three distinct approaches: (*i*) ligand-based drug design (LBDD), where data related to active ligands play a central role; (*ii*) structure-based drug design (SBDD), which requires experimental information or molecular modeling data of target macromolecules; and (*iii*) fragment-based drug design (FBDD), which utilizes molecular fragments as potential innovative starting points for drug development.[57,58] However, it is worth noting that while CADD can assist in FBDD, FBDD itself is considered a distinct approach from CADD techniques.[57] These strategies offer diverse methods in the quest for identification of new therapeutic substances, allowing integrative approaches based on the nature, availability, and quality of the accessible data.[59-61]

In the face of paradigm shifts in drug discovery processes and significant advances in structural biology, the exploration of structure based virtual screening methods is of great interest to the scientific community and the pharmaceutical industry.

2. Virtual Screening

VS performs a search *in silico* for biologically active molecules within large compound databases using CADD techniques.[62-64] VS is often used to predict the ability of molecules to bind to a macromolecular target of known 3D structure, allowing estimation of promising hits even before the biological assay and thus optimizing cost-effectiveness compared to the HTS approach for a hit detection.[62,65] Current estimates of the Research and Development (R&D) costs required to bring a new drug to market vary from $113 million to just over $6 billion.[66-69] VS has as its main advantage the reduction of costs and time in the early stages of R&D,[46] as it allows the intelligent exploration of vast virtual libraries, sourced from combinatorial libraries and large commercial databases containing billions of compounds, such as Zinc,[39] PubChem,[41] DrugBank,[45] and ChEMBL.[43]

VS can be further subdivided into ligand-based virtual screening (LBVS), where information from ligands with known activity is employed to search for similar compounds through the generation of molecular fingerprints and ligand-based pharmacophore modeling, and structure-based virtual screening (SBVS), where prior knowledge of the target structure information is crucial.[70,71] Based on structural information of the biological target, molecular docking and/or structure-based pharmacophore modeling can be employed in the search for ligands that exhibit high complementarity to the target. Within this context, we focus on explaining the uses, methods, and advantages of SBVS.[72]

2.1. SBVS

SBVS is a VS strategy, with the main premise being structural knowledge of the target macromolecule, focusing mainly on chemical complementarity between possible ligands and the studied structure.[73-75] A good target must be validated as a crucial component in the pathophysiology of the studied disease, and must also be "druggable." In other words, the target must be able to be therapeutically modulated by small molecules.[76,77] Furthermore, the target should be accessible to the potential drug molecule, and, upon binding, the potential drug must elicit a biological response that may be measured both *in vitro* and *in vivo*.[78]

The human genome was completely described in in the early 2000s, bringing a wealth of opportunities for SBVS applications and the potential for discovery of new drug targets.[79] However, the vast majority of currently well-explored biological targets belong to so-called target superfamilies (e.g., G-protein-coupled receptors, kinases, proteases, ion channels, nuclear hormone receptors), which comprise less than 500 of the 10,000 potential targets in drug discovery, indicating the vastness of possibilities that remain to be explored through this strategy.[16] Targets can be characterized both through experimental techniques (i.e., nuclear magnetic resonance, crystallography, cryo-electron microscopy) or computational protein structure modeling to search for ligands in large databases using SBVS.[80-83] The Universal Protein Resource (UniProt)[84,85] includes an expert-curated core of around 568,000 reviewed UniProt/Swiss-Prot protein sequence entries and over 229 million unreviewed UniProt/TrEMBL entries that are annotated by automatic systems. Protein Data Bank (PDB) currently has more than 200,000 experimentally determined 3D structures of proteins and nucleic acids (DNA and RNA) and their complexes with one another and with small-molecule ligands (e.g., enzymes, cofactors, inhibitors, peptides, and drugs) an extremely low number in view of the number of amino acid sequences available in Uniprot.[84-87] The difficulty of characterization through experimental techniques, which can take months or even years to conduct, makes computational modeling a crucial tool in the study of protein structures.[88]

SBVS tools offer significant benefits to research and pharmaceutical companies. To analyze the impact of these strategies on scientific publications, a search was performed in the Web of Science platform using the term "structure based virtual screening". The results show an enormous increase in the number of publications and citations related to this term, as shown in Figure 2.

The continuous evolution of SBVS and its significant progress in bibliometric descriptors motivated us to explore which areas employed it most. The Treemap (Citations Topic Meso) in Figure 3 indicates that the most common

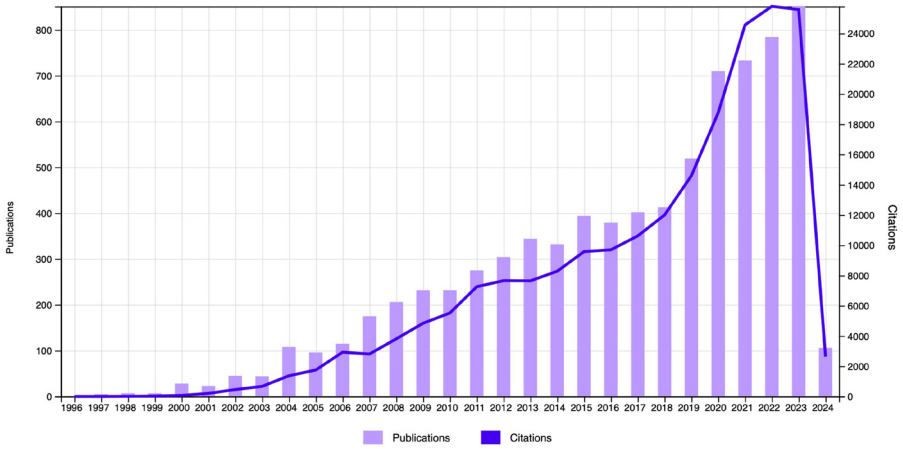


Figure 2. Result of a search on the Web of Science for the term "structure based virtual screening", showing rapid growth in the number of publications and citations since the 1990s. The data were obtained using the Web of Science platform.

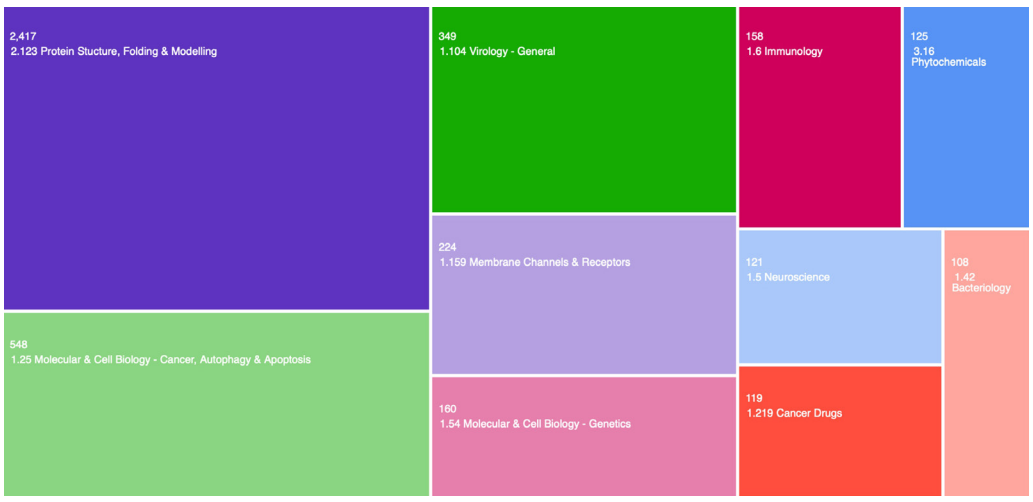


Figure 3. Most common research areas using SBVS. The largest is protein structure, folding and modeling (data obtained from Web of Science).

research areas employing SBVS are protein structure, folding, and modeling, molecular and cell biology (cancer, autophagy, and apoptosis), and general virology.

Computational protein structure modeling has greatly benefited from advances in artificial intelligence (AI) techniques such as deep learning (DL), which has significantly improved the ability to generate good virtual models. Before the rise of AI, structure prediction methods were categorized as template-based modeling (TBM, also known as comparative modeling) or template-free modeling (FM). Until recently, TBM was considered the most reliable approach to predict protein structures, but these methods faced a long period of stagnation due to the absence of appropriate templates, drastically decreasing the accuracy of molecular modeling.[74]

With the employment of AI programs such as AlphaFold2[89-91] in protein modeling platforms, model prediction offers vast coverage of distinct protein structures with relatively good accuracy. AlphaFold2 uses the end-to-end protein structure prediction method to forecast the tridimensional protein structure from the amino acid sequence, focused on the relationship between input sequence and output structure.[92] The DL architecture employs a neural network incorporating multiple sequence alignments (MSAs) and outputs protein structures, enabling accurate end-to-end structure prediction.[93,94] AI geometric optimization modeling methods infer protein structure spatial characteristics (i.e., contacts, distances, orientation, hydrogen bonds between residues), and these deductions are combined with force fields (parameter sets used to calculate the potential energy of a system at the atomistic level) and optimized for sampling the conformation with the lowest energy using Monte Carlo methods.[95,96] Tools such as D-I-TASSER[97,98] and trROSSETA[99,100] also employ these methods to evaluate the target, and such data assists in exploring the topology of the binding site, sub-cavities, and physicochemical and/or electrostatic properties.[101] These data enable greater understanding of target characteristics, which is essential in SBVS, allowing for more efficient exploration of the target of interest, including the search for agonist molecules, receptor antagonists, competitive enzymatic inhibitors, and allosteric modulators.[102,103] After the examination of structural data, SBVS strategies can be properly employed, primarily through methods such as molecular docking and structure-based pharmacophore modeling.[104,105]

2.2. Molecular docking-based virtual screening

Molecular docking is a computational simulation technique that stands out as a versatile and comprehensive tool within the drug discovery process.[87,106] Molecular docking plays an important role in analyzing the interaction between ligand and biological target, applicable not only to small molecule-receptor interactions but also to protein-protein interactions.[74,107,108] This capability contributes significantly to the utility of molecular docking in elucidating complex biological processes, searching for hits, optimizing hits to leads, and drug repositioning, providing valuable insights into potential therapeutic interactions (Figure 4).[110,111]

Over recent decades, the evolution of molecular docking has been driven by improved algorithms and advances in computational capacity.[112] Previously, molecular docking

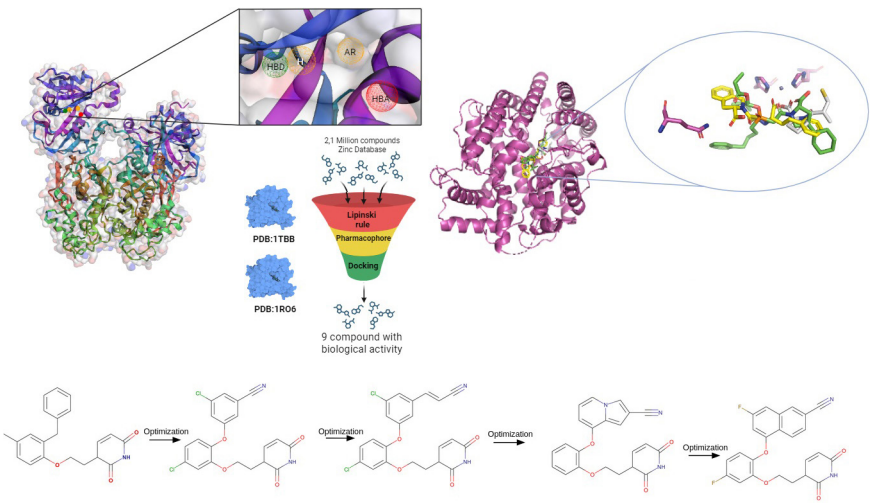


Figure 4. Strategies employed in the SBVS approach and optimization of hits. (Bottom) Hit optimization steps are adapted from Jorgensen;[109] (left) an example of pharmacophoric model is shown, with four features: hydrogen bond donor (HBD); hydrogen bond acceptor (HBA); aromatic (AR); and hydrophobic area (H), PDB accession code for the DHFR protein: 3K2H; (right) conformations generated by a search algorithm and selected by a scoring function for different ligands are shown (captopril is shown as pale cyan lines, quinaprilat is shown in yellow lines and fosinopril is shown in green line segments), PDB accession code for the ACEII protein: 1O86.

was limited to a rigid approach, in which static geometric and physicochemical complementarities between ligands and the biological target were taken into account.[113] This approach disregarded flexibility and induced fit binding models, focusing only the fixed structural characteristics of the molecules.[114-116] Semi-flexible docking introduces flexibility, allowing only the small molecule to adjust its conformation during the docking process and covering all degrees of freedom, including rotation, translation, and conformational changes, while maintaining the macromolecule as a rigid structure. This enables a more dynamic consideration of interactions, although still disregarding altogether the flexibility of the biological target molecule.[117]

With advances in computational capacity, it has become possible to create fully flexible docking tools, representing a more comprehensive approach where both the target and ligand are considered fully or partially flexible, allowing for the implementation of induced fit binding models where both molecules can modify their conformations to optimize their binding.[118-120] This approach is particularly valuable when dealing with complex biomolecular interactions, where flexibility is an essential feature for the efficient formation of complexes.[121] In summary, the choice between these approaches will depend on the specific nature of the molecular system in question and the goals of the research in molecular docking.[122] Regardless of the approach (e.g., rigid, semi-flexible, flexible) molecular docking involves two challenges. The first is the search for ligand conformations through search algorithms. The second is the prediction of binding affinity through a scoring function, which quantifies the thermodynamics of the specific molecular interactions that are formed between receptor and ligand and ranks the compounds poses with a score representing chemical affinity.[123,124]

2.2.1. Search algorithm

In docking-based VS, an SBVS strategy, search algorithms are used to predict the conformation of ligands, exploring the possible conformational space that a given ligand can assume at the target binding site. Docking-based VS may be characterized as systematic, stochastic or deterministic.[125-127]

Systematic algorithms perform exhaustive searches in all conformational spaces, exploring all degrees of freedom of the ligand in a combinatorial manner, rotating all dihedral angles of the ligand according to a predetermined range of values and a set of initial restraints (e.g., geometrical and chemical constraints), focusing on regions of conformational space that are likely to have high scoring ligand poses.[75,128] Another way that the systematic algorithms perform conformational search is through incremental construction, which fragments the ligand into rigid and flexible segments (sub-molecules). This approach begins by introducing the rigid segment of the molecule into the binding site and incorporating the flexible segments in subsequent iterations while conducting a systematic search for torsion angles between the segments.[129,130]

Stochastic search algorithms introduce random changes to ligand degrees of freedom. One common implementation of stochastic algorithms is the so-called genetic algorithm (GA).[131] The GA is based on the theory of biological evolution, which can be either Darwinian or Lamarckian, wherein the arrangement of the ligand in the protein can be defined as a set of values describing variables (translation, orientation, and conformation) of the ligand in the protein, with each variable representing a gene, the set of variables representing the genotype, and the atomic coordinates forming the phenotype. The combination of all these data represents a chromosome.[132,133] The genetic algorithm generates an initial population of chromosomes randomly, from which the capacity and total energy of interactions between the ligand and protein are assessed through an energy function. The fittest populations are manipulated using genetic operators (i.e., crossover, mutation), forming new populations of chromosomes that are then reevaluated.[134,135]

In deterministic algorithms, searches for conformations are conducted with explorations directed towards lower energy states compared to the initial state, implying that the initial state, along with its associated energy level, serves as a fundamental guide for the generation of subsequent energy states. Essentially, these algorithms follow a trajectory predetermined by the initial configuration and the energy associated with that configuration.[136,137]

2.2.2. Scoring function

Score functions commonly estimate the binding energy between ligand and the target, assuming that binding affinity is described as the sum of independent terms. Applications in molecular docking-based VS involve the evaluation of conformations previously generated by the search algorithm in the target's binding site, estimating the binding affinity and thus assisting in the process of selecting hits for a specific molecular target.[138] Score functions can be grouped into three main approaches: force field-based functions, empirical functions, and knowledge-based functions.[139] Over the years, several studies have evaluated and compared different scoring functions, and each has its virtues and disadvantages. None of the available scoring functions outperforms the others on all tasks, but each scoring function may perform better than others on specific

tasks.[140] To overcome the limitations that each scoring function may present, the consensus scoring approach is commonly used, which combines results from different docking programs by calculating the average score or classification of each molecule obtained in individual programs.[141] Softwares like Gold[142,143] offer empirical scoring functions (e.g. Chemscore)[144] and force field-based scoring functions (e.g. Goldscore)[144] in their package, while programs like DockThor[145,146] offer empirical scoring functions (e.g. DockT score[147]).[148]

Force field-based scoring functions estimate bond energy by summing the contributions of bonded terms (bond elongation, angular bending, and dihedral variation) and unbonded terms (electrostatic and van der Waals interactions) into a general master function. This type of scoring function calculates the energy associated with each term of the function using the equations of classical mechanics.[148,149] Force field score functions are appropriate to compute binding free energy between proteins and ligands with relatively greater predictive accuracy than other types of score functions due to their consideration of the enthalpy, solvation energy, and entropy.[150,151]

Empirical score functions focus on estimating the binding affinity in a molecular complex by evaluating a specific set of atomic interactions within that complex, employing a training database to enhance predictive capability.[152,153] This database includes structures with known affinities, allowing the model to assign appropriate weights to the various atomic interactions based on their contributions to the total binding affinity. In essence, empirical functions leverage existing data to make predictions about the binding affinity.[154]

Knowledge-based scoring functions are constructed through rigorous statistical analysis of target structures that have been characterized by experimental techniques.[155] The underlying principle is to identify interatomic distances that occur more frequently than a statistical average, interpreting these occurrences as indicative of favorable contacts in molecular interactions.[156,157] An illustration of the processes of searching for and scoring ligand poses in a binding site is exemplified in Figure 4.

2.3. Pharmacophoric model-based VS

Pharmacophoric models represent one the most promising approaches in VS. The technique is applicable to both SBDD and LBDD for hit discovery and optimization of lead compounds to final drug candidate, among other applications.[158,159] The term "pharmacophore" was first introduced in the early 1900s and commonly attributed to Paul Ehrlich, to refer to the molecular framework that carries (from the Greek "*phoros*") the essential characteristic responsible of a drug (from the Greek "*pharmacon*") to express the desired biological response.[160,161] Before the advent of CADD techniques in the 1940s, initial considerations regarding SAR, coupled with knowledge of bond distances and van der Waals sizes, allowed the construction of simple two-dimensional pharmacophoric models. These models were employed, for instance, in the search for analogs of para-aminobenzoic acid (PABA) and the estrogenic agent trans-diethylstilbestrol.[162,163] The three-point model, proposed by Easson and Steadman,[164] contributed significantly to a better understanding of ligand-receptor interactions and to the construction of pharmacophoric models by accounting for the chirality of molecules. When an asymmetric center is present in a compound, substituents on the chiral carbon atom are believed to make a three-point contact with the receptor.[165,166] In the 1960s, Kier[167] was the first to calculate the pharmacophoric model in a study of muscarinic agonists using the quantum mechanics of extended Hückel theory, establishing the beginning of computationally generated pharmacophores. This spurred the development of various algorithms, which in turn led to the software tools widely used in drug design today.[168,169]

Historically, many authors have used the term "pharmacophore" to define structural or functional groups possessing biological activity. However, the International Union of Pure and Applied Chemistry (IUPAC) definition of the term, given in Wermuth *et al.*,[170] says that a pharmacophore is the ensemble of steric and electronic features that is necessary to ensure the optimal supramolecular interactions with a specific biological target structure and to trigger (or to block) its biological response. This definition discards a misuse often found in the medicinal chemistry literature,[170,171] which consists of naming as pharmacophores simple chemical functional groups such as guanidines, sulfonamides, or dihydroimidazole (formerly imidazolines), or typical structural skeletons such as flavones, phenothiazines, prostaglandins, or steroids. Therefore, a pharmacophore does not represent a real molecule or a real association of functional groups, but rather a purely abstract concept that accounts for the common molecular interaction capacities of a group of compounds toward their target structure.[170] Pharmacophore models are based on the premise that the presence of common chemical features, coupled with a specific spatial arrangement, will lead to similar biological activity on the same target.[172]

Pharmacophoric models can be generated through ligand-based or structure-based approaches.[173] In structure-based methodologies, one strategy to define the locations

of pharmacophoric features in a binding site involves identifying "hotspots", which are regions of the protein cavity where the most favorable contacts occur between residues and probe molecules. Hotspots may be obtained by mapping the interactions that these "probes" (virtually inserted small molecule fragments with specific chemical properties) form in the binding site.[174,175] Hotspots serve as excellent guides in constructing pharmacophoric models based on the target structure.[176]

The chemical features of molecules capable of interacting with the receptor are represented by spheres and vectors. The most important types of pharmacophoric features include hydrogen bond acceptors (HBA), hydrogen bond donors (HBD), hydrophobic areas (H), positively and negatively ionizable groups (PI/NI), aromatic groups (AR), and metal-coordinating areas. Additional size restrictions in the form of shape or exclusion volumes (XVOL), representing forbidden areas, can be incorporated to reflect the size and shape of the binding pocket, as shown in the Figure 4.[177]

Pharmacophoric models based on the structure of the target can use data derived from experimental techniques or computational modeling. In this approach, it is crucial to conduct a thorough analysis of the input data quality, as this directly impacts the pharmacophoric model quality.[177,178] Checking the protonation states of individual amino acid residues, the positioning of hydrogen atoms, the presence and relevance of non-protein substances (cofactors, solvent molecules) that may have a functional role, the *apo* or *holo* conformation of proteins, and identifying gaps in the target structure are critical considerations that must be examined to ensure the quality of the pharmacophoric model.[179] Pharmacophoric models must undergo validation to quantitatively assess their selectivity and specificity before being employed in VS. This validation can be conducted using statistical metrics such as receiver operating characteristic (ROC) curve and enrichment analysis.[180,181]

Pharmacophoric approaches constitute one of the most interesting SBVS techniques, defining molecular functional characteristics necessary for the binding of a molecule to a given receptor and then using this information to direct the SBVS of large collections of compounds to select ideal candidates.[182] Although the pharmacophoric model is a broad and complex technique with several potential applications, it can also be used as a filtering method in an initial phase of SBVS.[183,184] Since pharmacophoric models do not depend on specific atoms or functional groups, molecules that meet the pharmacophoric criteria not only possess potential activity but also may exhibit greater chemical diversity.

2.4. Recent advancements in docking engines and pharmacophore modeling tools

In recent years, molecular docking has seen some major advancements, largely thanks to the integration of AI and machine learning (ML) techniques. One of the standout developments is the emergence of AI-driven docking algorithms capable of accurately predicting the binding affinity between a small molecule and its protein target. These algorithms use deep learning methods, such as convolutional neural networks (CNNs) and recurrent neural networks (RNNs), to analyze large datasets of molecular structures and their corresponding binding energies. By learning from these datasets, AI-powered docking algorithms can quickly and effectively screen millions of compounds, significantly accelerating the drug discovery process.[62,104,147,155,185]

Moreover, the combination of ML techniques with molecular docking has led to the creation of hybrid models that combine the strengths of both approaches. These hybrid models integrate physics-based scoring functions with ML-based scoring functions, resulting in more accurate predictions of ligand-protein interactions. Additionally, advancements in reinforcement learning have enabled the development of adaptive docking protocols capable of dynamically adjusting their search strategies based on real-time feedback. This adaptability not only enhances docking accuracy but also improves overall efficiency. Collectively, these innovations represent a transformative phase in molecular docking, offering unprecedented opportunities for the rapid and cost-effective discovery of novel therapeutics.[62,104,147,155,185]

In pharmacophore modeling, recent advancements owe much to the integration of AI and ML techniques. AI-powered algorithms have revolutionized the process of pharmacophore identification by efficiently analyzing complex molecular structures and pinpointing key features necessary for ligand binding. Machine learning models, particularly deep learning models like CNNs and graph neural networks (GNNs), have been particularly helpful in recognizing subtle patterns and relationships within large pharmacological datasets. This has resulted in the development of pharmacophore models that accurately capture the interactions between ligands and their target proteins with unprecedented precision.[186-191]

Furthermore, the integration of machine learning approaches has facilitated the development of dynamic and adaptable pharmacophore models. These models can adjust in real-time based on feedback from experimental data, enabling continuous refinement and optimization. AI techniques have also boosted the speed and scalability

of pharmacophore modeling, enabling researchers to efficiently screen vast compound libraries and prioritize promising drug candidates. With the power of artificial intelligence and machine learning, pharmacophore modeling is set to transform drug discovery by accelerating the prioritization of novel therapeutic agents with improved specificity and efficacy.[186-191]

2.5. SBVS validation examples

Rigorous validation is essential to ensure that SBVS is a reliable tool in drug discovery, providing results aligned with the underlying structural biology and thus proving useful for subsequent optimization of the selected compounds. Continuous improvement of methods not only enhances the reliability of SBVS, but also increases its effectiveness, solidifying it as a valuable tool in the drug discovery process.[192,193] However, the application of validation methods in SBVS studies is notably heterogeneous, ranging from situations where it is entirely absent, to inappropriate applications, to meticulous and thoughtful procedures. Inappropriate validation methods can include overfitting to known structures or neglecting ligand flexibility, leading to unreliable performance assessments. Conversely, thoughtful validation approaches involve, for example, blind testing with experimental data and cross-docking validation to assess robustness across diverse receptor conformations.[194-196] Nevertheless, even when appropriate methodologies are employed in various forms of SBVS, there is a notable lack of uniformity and clearly defined reference methods for this technique.[192,194-196]

Validation in SBVS involves assessing the method's ability to reproduce plausible results that are compatible with previously reported experiments, while ensuring that identified drug candidates are biologically relevant.[104,197] An example of this is retrospective docking validation, which typically requires benchmarking against known ligand-receptor complexes to evaluate accuracy and reliability in reproducing experimental binding modes and affinities. Prospective validation, on the other hand, focuses on predicting the binding modes and affinities of newly discovered ligands, often through blind tests against experimental data.[195] The evolution of docking engines has seen significant advancements in various aspects, including scoring functions, conformational sampling algorithms, and incorporation of ML techniques. While early docking engines struggled with accuracy and speed, recent advances have led to improved performance, with enhanced scoring functions and more efficient sampling methods enabling better prediction of ligand-receptor interactions.[48] These advancements have significantly impacted the reliability and applicability of virtual screening approaches in drug discovery, allowing for more effective identification of lead compounds with desired biological activity.[48,198,199] Within this context, we present in this section two of the most widely implemented techniques commonly found in SBVS validations.[104,200]

2.5.1. ROC curve

The ROC curve is a graphical representation that demonstrates the performance of a binary classifier system as the discrimination threshold varies. Also known as the relative operating characteristic curve, its construction involves plotting the ratio of true positives to total positives against the ratio of false positives to total negatives for different threshold values. These ratios correspond to sensitivity (true positive rate) and specificity (false positive rate). ROC analysis provides tools for selecting possibly optimal models and discarding less optimal ones.[201]

The plotting of the ROC curve and the evaluation of the area under the ROC curve (AUC-ROC) in SBVS involves the use of true positives compounds (TPCs) and false positive compounds (FPCs), known as decoys.[202,203] TPCs are compounds with known biological activity against the molecular target of interest, and are accessible in databases such as ChEMBL.[42] In certain cases, a small number (> 2) of known TPCs is required to calculate the AUC-ROC.[204] On the other hand, decoys are molecules that are intentionally included in a screening dataset but are known not to interact with the target of interest. They serve as a control to help assess the performance of the screening method by mimicking the properties of active compounds while lacking their specific biological activity. When a screening method incorrectly identifies a decoy as a hit, it results in a false positive, indicating a potential flaw in the method's ability to distinguish between active compounds and non-interacting molecules.[104] DUD-E[205,206] and ZINC[38] are examples of databases that provide decoys. The use of decoys aims to improve the reliability of VS results, as the program can distinguish TPCs from FPCs that have similar physical properties but are known to be inactive.[193,207]

After generating decoys, the SBVS process is carried out using known TPCs and decoys against a target of interest (in the case of docking-based VS) or a pharmacophore based on the structure of a target.[202,208] In docking-based VS, for each ligand-target complex, an affinity energy is calculated. It is expected that TPCs, known to bind to the target of interest, will exhibit higher affinity energies compared to inactive compounds or decoys. In pharmacophore-based VS, results are based on the model's feature overlap with molecules, leading to a similarity ranking of screened compounds, with TPCs typically being selected.[209,210] In

this scenario, the ROC curve represents the distribution of true and false results on a graph, while the AUC-ROC allows the evaluation of the probability of a result being false. Thus, the AUC-ROC reflects the probability of retrieving an active compound rather than inactive ones, enabling the verification of the sensitivity of the SBVS experiment concerning its specificity. The larger the area under the curve, the better the ability to have TPCs and fewer FPCs.[104,201]

AUC values can range from 0 to 1, and the practical interpretation proposed by Hamza *et al*.[211] establishes categories such as excellent (AUC between 0.90 and 1.00), good (AUC between 0.80 and 0.90), fair (AUC between 0.70 and 0.80), poor (AUC between 0.60 and 0.70), and failure (AUC between 0.50 and 0.60). Therefore, the closer the AUC value is to 1, the greater the ability of the SBVS tool to distinguish between TPCs and FPCs, with values above 0.7 considered acceptable.[104] However, there are several critiques regarding the application of the ROC curve as a method for evaluating the performance of VS. The primary concern is that the method does not effectively highlight the most accurately classified active compounds that should be prioritized for *in vitro* experiments, a phenomenon referred to as early recognition.[104,193,202] In response to this challenge, Truchon and Bayly[200] proposed refining the ROC curve for VS through the enhanced discrimination of ROC by Boltzmann (BEDROC), which incorporates exponential weighting to assign greater significance to early classifications of active compounds relative to later ones. However, Nicholls[212] argued that both AUC-ROC and BEDROC show a high level of correlation in VS simulations, concluding that the ROC curve alone is a sufficient metric for performance evaluation.[212,213]

2.5.2. Enrichment analysis

Enrichment analysis in the context of SBVS is of crucial importance for assessing the capability of SBVS to increase the proportion of active molecules among the top candidates selected during the procedure. Enrichment analysis involves comparing the distribution of TPCs to inactive ones (FPCs) along the ranked list generated by VS. The premise is that, as one progresses through the list, active compounds should be selected in proportions higher than expected by chance alone.[104,214]

Common metrics in enrichment analysis include cumulative enrichment and the enrichment factor (EF). The first represents the cumulative increase in the proportion of active compounds as the list progresses, visualizing results through cumulative enrichment curves. The EF, on the other hand, is determined by the number of active compounds found in a specific fraction relative to those that would be selected in a random search, often calculated for specific percentages of the database, as exemplified by EF10% for 10% of the database.[104,200,215]

Despite the simplicity of the EF, its limitations include dependence on the assigned fraction value and the number of true positives and true negatives, making it more of an experimental performance metric (reflecting how well it performs in real-world experimental conditions) than a method performance metric, which would evaluate its inherent capabilities under standardized conditions or benchmarks.[216,217] An additional disadvantage is the equal weighting of active compounds beneath the cutoff, making it impossible to differentiate between algorithms that place all active compounds at the beginning of the list, a phenomenon known as the saturation effect.[200,218] To overcome these limitations, the relative enrichment factor (REF), proposed by von Korff *et al*.,[219] normalizes EF by the maximum possible enrichment, providing well-defined boundaries and reducing sensitivity to the saturation effect.

Enrichment analysis plays a crucial role not only in assessing the ability of SBVS models to select active compounds, but also in enabling the comparison of different approaches and parameter optimization. Furthermore, successful enrichment evaluations support SBVS in efficiently directing its efforts toward more promising regions of the compound list, resulting in a more effective selection of molecules for subsequent experimental testing.[104,200,214]

In conclusion, both ROC curve and EF serve as validation tools in SBVS, offering distinct yet complementary insights into the performance and effectiveness of screening methods.[200] While the ROC curve provides a comprehensive assessment of discrimination between active and inactive compounds across various thresholds, making it applicable in both pharmacophore-based and docking-based approaches, the EF offers valuable information on the early enrichment of active compounds. This is particularly beneficial in pharmacophore-based screening for selecting compounds for experimental validation.[199,201,207] By utilizing these tools judiciously, researchers can better evaluate and enhance the reliability and efficacy of SBVS methods in identifying potential drug candidates.

3. SBVS Pitfalls

Elaborating a VS protocol is a challenging task that can lead medicinal chemists into several pitfalls. Scior *et al*.[220] summarize almost all pitfalls that occur in VS, and here, we describe the most common errors in the employment of SBVS techniques.

Definitions based on erroneous expectations can lead to errors in the interpretation of docking results. Considering

potency as the main criterion in the search for hits should also be avoided, because affinity and potency are optimized in the hit-to-lead stages of the development project.[220,221] Instead, the emphasis should be on maximizing structural diversity and drug-likeness as well as minimizing false-positive rates. This multifaceted approach ensures that the compounds selected for biological testing not only exhibit potency, but also possess diverse chemical scaffolds and favorable properties for further drug development.[222] Additionally, methods such as clustering or scaffold decomposition can be employed to reduce costs by decreasing the number of compounds tested while maintaining structural diversity.[223] Moreover, scoring functions do not have sufficient precision for predicting the binding affinity of compounds with very different structures and different binding modes.[224] In initial steps, the VS scoring function should be seen as a tool for creating hit libraries, rather than attempting to rank compounds by greater potency.[220]

Molecular recognition is a dynamic and highly complex process, involving a large number of intermolecular interactions between the ligand, the receptor molecule and the system's solvent.[225,226] Search algorithms seek for conformations that have better complementarity with the binding site. However, these conformations in a biological assay are uncertain due to various contributing factors. These factors include the dynamic nature of proteins, solvent effects, binding pocket flexibility, and entropy effects.[224] Furthermore, the flexibility of the ligand and protein, especially in highly flexible targets that require induced fitting, coupled with the difficulty in determining the bioactive conformation, can lead to protocol errors.[227,228] The notion that the bioactive conformation is invariably the lowest energy conformation has been challenged by empirical observations and theoretical considerations, indicating that this assumption may not always hold true.[229] Considering this in conformational analysis can also lead to errors.[230,231] The use of flexible molecular docking approaches and molecular dynamics for refining results of VS are alternatives in these cases, although they have a high computational cost.[224,232] Semi-flexible docking with different target conformations can be used in cases of limited computational power.[224] Therefore, it is crucial for the medicinal chemist to maintain a certain skepticism when analyzing results, and to use knowledge about possible molecular interactions (e.g., hydrogen bonds, van der Waals) to analyze the results conscientiously.[233]

In the construction of pharmacophoric models, the choice of features, although commonly guided by specific criteria such as physical-chemical properties of the cavity and protein hot spots, can be arbitrary, requiring a researcher's intuition.[173,175,177] Therefore, caution is needed. Including a greater number of features in the model in order to faithfully represent the binding site or achieve high selectivity can lead to overly stringent and complex models. Applied in VS, these models may result in low structural diversity and increase the likelihood of selecting false positives. Therefore, the researcher's experience and knowledge play a crucial role at this stage of the project.[220]

The use of physicochemical property filters also has its pitfalls, such as dependence on "drug-like" compounds and Lipinski's rule of five.[234,235] Although these filters are interesting and valuable in drug discovery, several drugs approved by the Food and Drug Administration (FDA) in recent years deviate from the rules of these filters, indicating that they must be used judiciously to avoid restricting the VS project to a relatively narrow fraction of the chemical space.[236]

Errors in the interconversion of different molecular formats are also common in VS. Since molecular modeling represents a niche market and not all software adheres to the same quality control standards, it is common for information to be lost or altered when converting a file format to another, or even when using the same format across different softwares.[220,237] The information that can be distorted through these technical limitations ranges from benign annotations to more serious issues, such as atomic coordinates, chirality, hybridization, and protonation states. These distortions can impact the reliability and integrity of molecular data.[216,220] The knowledge and ability of the medicinal chemist to properly interpret the results is a crucial factor in the discovery of drugs.[238]

4. SBVS Success Cases

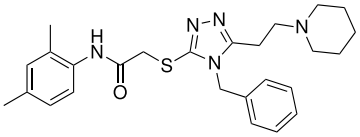
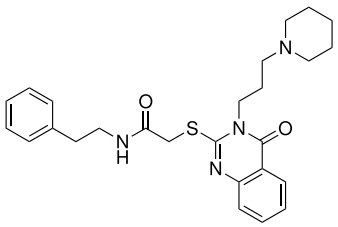
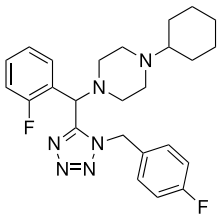
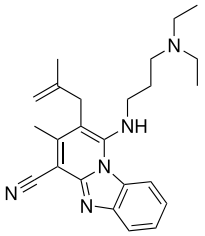
In this section, we describe seven successful cases of the application of SBVS to discover promising hits and their particularities.

4.1. CCR5 receptor agonists

The C–C chemokine receptors type 5 (CCR5) are G protein-coupled transmembrane receptors that play a crucial role in immunological processes. They are expressed in almost all leukocytes and act as receptors for the β-chemokine group, and are thus implicated in the chemotaxis of these cells, driving them to inflamed regions in need of their physiological action. This family of receptors is also involved in the modulation of other less well-understood inflammatory mechanisms, and additionally play a role in some types of cancers such as breast and prostate cancers, inhibiting their migration and

metastasis and enhancing the effectiveness of chemotherapy in some kinds of cancer stem cells when inhibited by antagonists.[239,240] Furthermore, CCR5 together with the C-X-C motif chemokine receptor 4 (CXCR4) are used by the human immunodeficiency virus (HIV) as co-receptors for cell entry. Therefore, Kellenberger *et al*.[241] took interest in developing CCR5 receptor antagonists which led them to employ a SBVS methodology to select promising hits from a library the group filtered according to criteria such as drug-likeness, probable toxicity, reactive compounds and pan-assay interference compounds (PAINS). The study screened 44,524 different compounds in parallel in both the softwares Gold[144] and Surflex,[242] with a subset of the compounds extracted by a 2D pharmacophoric filter based on all known CCR5 antagonists at the time, which the research group used as a validation dataset. This framework led to the selection of 5% of the screened dataset in both softwares employed, and among those, less than one hundred compounds were commonly selected by both programs. The researchers then employed a graph-based maximum common substructures (MCS) post-processing procedure to classify the selected compounds, followed by visual inspection of the binding poses, which resulted in 77 final hits. Among the 77 hits, 59 were commercially available for purchase, and were acquired for conducting *in vitro* receptor functional response in an aequorin luminescence assay, with radiolabeled MIP1-β (CCL4) as a tracer in a competition binding assay, with the compounds tested in two concentrations. Ten compounds exhibited detectable binding affinity to the CCR5 receptor. Nine out of the ten binding hits showed agonist activity in the conducted assay, with the most potent one showing a value of half maximal effective concentration (EC_{50}) of 1.9 μM (Table 1). This work illustrates how an SBVS strategy can be effectively used to select a few promising hits from a large library of compounds, making it possible to find active compounds with much less money, time and work expenditure. However, it also illustrates that virtually prioritizing compounds with affinity for the desired target does not necessarily mean they will have the desired activity profile on said target.[241]

Table 1. CCR5 receptor agonists found by SBVS of a library of 44,524 compounds

Compound code in the original work	PubChem CID	Compound chemical structure	EC_{50} / μM
8	1167892		3.0
9	4080740		antagonist
10	3209888		> 100
11	3258407		1.9

EC_{50}: half maximal effective concentration. Data obtained from Kellenberger *et al*.[241]

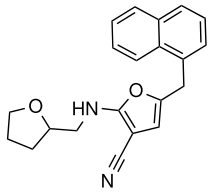
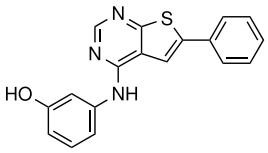
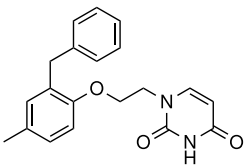
4.2. Reverse transcriptase inhibitors

One of the most effective therapeutic strategies in combating the HIV infection involves inhibition of its reverse transcriptase (RT) enzyme, which represents a crucial target in the physiology of the virus. Non-nucleoside reverse transcriptase inhibitors (NNRTI) play an important role in HIV antiretroviral therapy. These inhibitors bind to the allosteric site of the reverse transcriptase, promoting the inhibition of the catalytic activity of this enzyme and thus preventing virus replication. Nichols *et al.*[243] virtually screened a library of over two million compounds from the Zinc database[38] using three crystal structures in complex with NNRTIs, one with the Y181C mutation (PDB ID 1JLA), a conventional wild type (WT) structure (PDB ID 1RT4), and another WT structure with an alternative Y181 conformation (PDB ID 2BE2).[38,87] After screening the library with Glide,[126,244-246] nine compounds were acquired and tested for their antiviral activity against the WT HIV-1 and Y181C variants using infected human T cells.[243] Despite the small number of compounds tested, two compounds inhibited WT or Y181C at low micromolar concentrations. This suggests that use of ensemble docking, which considers multiple conformations of the same target, can improve SBVS results. This approach can be applied in selecting new leads with antiviral activity against mutant RT strains. Subsequently, Jorgensen[109] utilized a hit with an EC_{50} value of 4.8 μM, employing thermodynamic integration (TI) and free energy perturbation (FEP) (both alchemical perturbation methods based on statistical mechanics theory) methods alongside molecular dynamics (MD) or Monte Carlo (MC) simulations (which compute the relative binding free energy between congeneric series of ligands towards a target). These techniques guided the optimization of a series of catechol-based NNRTIs. First, the author determined the optimal substitution pattern for the phenyl rings and evaluated the possibility of replacing the methylene between the rings with an oxygen. In good agreement with FEP predictions, the synthesized compounds showed improved antiviral activity in an MTT-based assay in cell culture. The addition of a cyano vinyl group at position 5 of the terminal ring yielded the most potent NNRTI reported in cell culture assays, with an EC_{50} value against WT HIV of 55 pM. Additionally, this series of compounds exhibited nanomolar activity against clinically important HIV variants Y181C and Y181C/K103N and low cytotoxicity. The accuracy of their model was further supported by good agreement between the predicted binding positions of the catechols and experimentally determined crystal structures (PDB ID 4H4M). However, the cyano vinyl group can act as a Michael acceptor, leading to potential covalent modifications of proteins or nucleic acids. To avoid toxic risks in future development, the cyano vinyl phenyl group was replaced by a bicyclic substructure (indole, indolizine and benzofurans). Using MC/FEP predictions, several indolizines showed good antiviral potency, confirming the feasibility of substituting the cyano vinyl group. One compound exhibited a half-maximal inhibitory concentration (IC_{50}) of 380 pM against WT HIV and improved antiviral activity against the Y181C/K103N variant compared to parent compounds, although its potency decreased against the single Y181C mutant. This loss of potency was explained by the loss of aryl-aryl interaction between the bicyclic heterocycle and Y181 observed in the WT RT crystal structure (PDB ID 4MFB). Additional work[247] determined the antiviral activity of 20 against a wider range of mutant variants in an alternative single-round infectivity assay using $CD4^+$ T cells from blood donors. Compound **5** showed an excellent profile, with low nanomolar activity against all tested HIV variants. It exhibited an EC_{50} of 1 nM against the K101P mutant, which is a low-frequency viral mutation but confers resistance to both etravirine and rilpivirine, as shown in Table 2.[247]

4.3. Phosphodiesterase 4B inhibitors

Chronic obstructive pulmonary disease is one of the most predominant diseases globally. Phosphodiesterase 4 (PDE4), a hydrolytic enzyme, has been proposed as a promising target in asthma and chronic obstructive pulmonary disease. PDE4B selective inhibitors are desirable to reduce the dose limiting adverse effect associated with non-selective PDE4 inhibitors, and the group of Gangwal *et al.*[248] developed ligand-based pharmacophore models for a diverse class of PDE4B and PDE4D inhibitors. The best pharmacophore models, Hypo1 for PDE4B and Hypo1 for PDE4D, were validated using different methods to assess their predictive power over the diverse test set compounds. The highly predictive models were further employed in virtual screening to prioritize selective PDE4B inhibitors. Three diverse chemical databases containing 748,822 compounds, post-filtered according to Lipinski's rule of five, were utilized in VS. The hits from the VS were filtered based on estimated activity, FitValue, QED value, and molecular docking analysis using the software Glide 5.5.[126,245] The docking protocol was validated by re-docking the co-crystallized ligand, rolipram, in the active site of PDE4B (PDB ID 1RO6) and PDE4D (PDB ID 1TBB) with a root-mean-square deviation (RMSD) value of 0.328 and 0.356 Å, respectively.

Table 2. Reverse transcriptase inhibitors found by SBVS of a library of over two million compounds from the Zinc database[243]

Compound code in the original work	PubChem CID	Compound chemical structure	EC_{50} / nM	
			WT	Y181C
3	–		6.2	12.0
4	733568		NA	7.5
5	5304849		4.8	NA

EC_{50}: half maximal effective concentration; NA: not applicable. Data obtained from Nicholls *et al.*[243] and Jorgensen.[109]

Ten compounds were selected and purchased for testing in biological assays, using rolipram as a reference. Compound ZINC33106106 have shown potent and selective PDE4B inhibitory activity, as shown Table 3.[248]

4.4. FTO inhibitors

The fat mass and obesity-associated protein (FTO) belongs to the family of Fe^{2+} and α-ketoglutarate (α-KG)-dependent oxygenases. It demethylates sites modified with N6-methyladenosine (m6A) and mRNA sites modified with N6,2'-*O*-dimethyladenosine (m6Am), influencing various mRNA-related processes, including transcriptional stability, alternative splicing, mRNA translocation, and protein translation.[249]

To discover potential FTO inhibitors, a structure-based virtual screening VS strategy was employed by the group of Peng *et al.*[249] on a library of FDA-approved drugs. The binding site was defined as both the substrate-binding site and the *N*-oxalylglycine cofactor site, as in the crystal structure of FTO (PDB accession code 3LFM). The Fe^{2+} ion was processed according to a previously published protocol,[250,251] with reduced van der Waals interaction and partial atomic charge parameters. The group docked 1323 FDA-approved drugs obtained from Zinc against FTO using DOCK 3.5.54 and generated the top 500 docking poses for each compound. Subsequently, these docking poses were filtered based on three predefined structural descriptors, according to the FTO crystal complex structure bound to N3-methylthymidine. After filtering, 332 compounds remained. The docking poses were energy minimized and re-scored using a more sophisticated scoring method, molecular mechanics/generalized born surface area (MM/GBSA), using the software PLIP.[252,253] The refined poses were further filtered and visually inspected, and 19 compounds were selected for experimental validation. Following experimental validation, entacapone, a catechol-*O*-methyltransferase (COMT) inhibitor used to treat Parkinson's disease, demonstrated the capability to inhibit FTO demethylation activity with an IC_{50} of 3.5 μM, and thus is a potent chemical inhibitor of FTO (Table 4). Entacapone competed with both m6A-containing oligonucleotide substrates and the FTO cofactor α-KG and bound to FTO with a dissociation constant (K_d) of 234 nM in the presence of Fe^{2+} or 1,072 nM without Fe^{2+}, suggesting it forms a complex with the cation.[249]

4.5. Triose phosphate isomerase inhibitors

Infectious diseases caused by intestinal protozoa such as *Entamoeba histolytica* and *Giardia lamblia* are a worldwide public health problem, affecting over 70 million people each year. They colonize the intestines, primarily causing diarrhea, and can lead to more severe complications.[254] The treatment of choice, metronidazole, is associated with adverse side effects and drug resistance. In a drug-repurposing study, the research group of Juárez-Saldivar *et al.*[254] conducted a molecular

Table 3. Phosphodiesterase 4B inhibitors found by SBVS of a library of 748,822 compounds

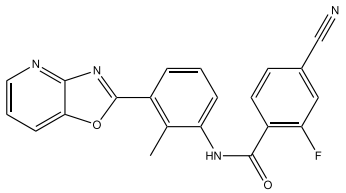
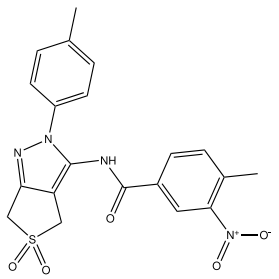
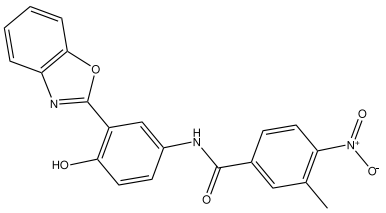
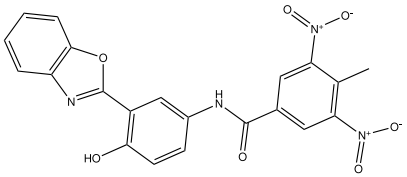
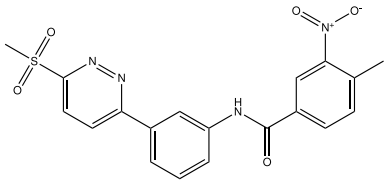
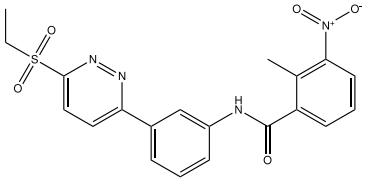
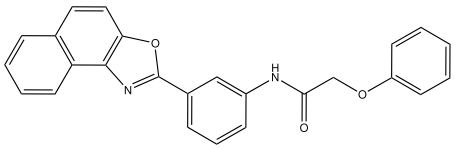
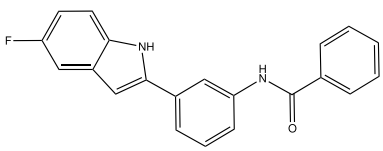
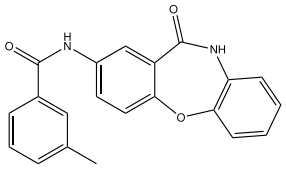
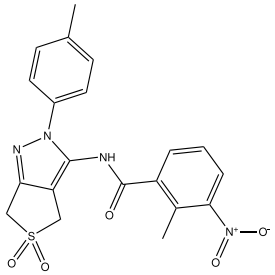
Compound code in the original work	Zinc ID	Compound chemical structure	EC_{50} / nM	
			PDE4B	PDE4D
1	ZINC05612594		813	672
2	ZINC03008558		2,202	1,253
3	ZINC01210083		378	3,056
4	ZINC27499887		790	> 10,000
5	ZINC15880786		140	1,792
6	ZINC15880845		216	1,136
7	ZINC01212527		174	2,440
8	ZINC33106106		2	2,116

Table 3. Phosphodiesterase 4B inhibitors found by SBVS of a library of 748,822 compounds (cont.)

Compound code in the original work	Zinc ID	Compound chemical structure	EC_{50} / nM	
			PDE4B	PDE4D
9	ZINC09827866		461	136
10	ZINC03008557		140	209

EC_{50}: half maximal effective concentration. Data obtained from Gangwal *et al.*[248]

Table 4. Phosphodiesterase 4B inhibitors found by SBVS of a library of 748,822 compounds

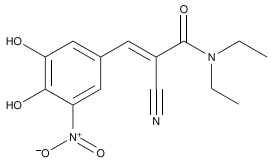
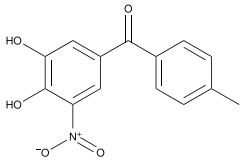
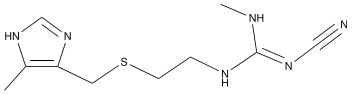
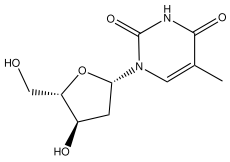
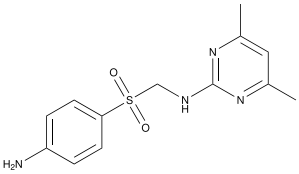
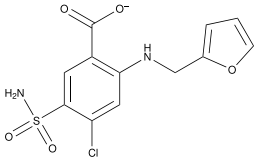
Compound code in the original work	PubChem CID	Compound chemical structure	Inhibition (at 10 μM) / %
1	5281081		93
2	4659569		85
3	2756		< 20
4	159269		< 20
5	5327		< 20
6	4739262		< 20

Table 4. Phosphodiesterase 4B inhibitors found by SBVS of a library of 748,822 compounds (cont.)

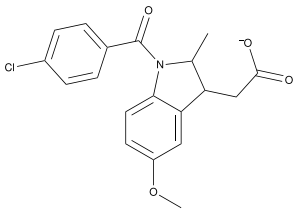
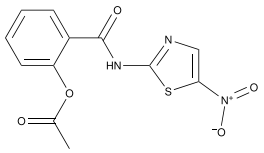
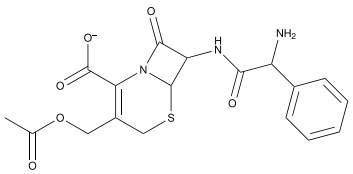
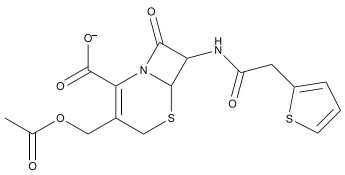
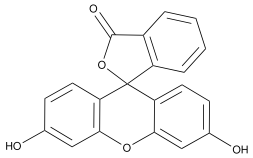
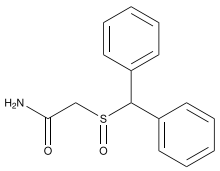
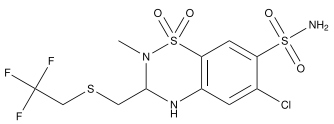
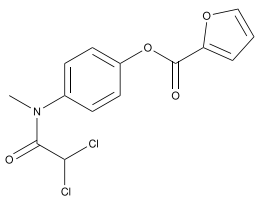
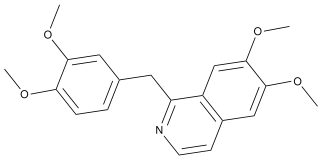
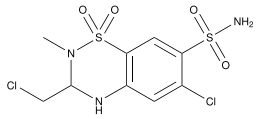
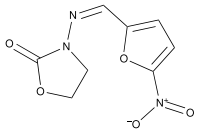
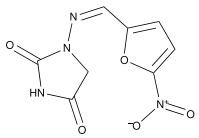
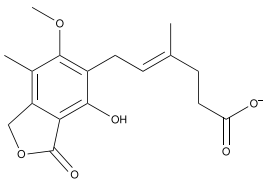
Compound code in the original work	PubChem CID	Compound chemical structure	Inhibition (at 10 μM) / %
7	35025669		< 20
8	41684		< 20
9	19150		< 20
10	7048634		< 20
11	16850		< 20
12	4236		< 20
13	12876779		< 20
14	19529		< 20
15	4680		< 20

Table 4. Phosphodiesterase 4B inhibitors found by SBVS of a library of 748,822 compounds (cont.)

Compound code in the original work	PubChem CID	Compound chemical structure	Inhibition (at 10 μM) / %
16	12309468		< 20
17	5323714		< 20
18	6604200		< 20
19	6918995		< 20

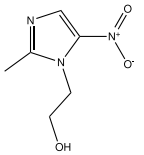
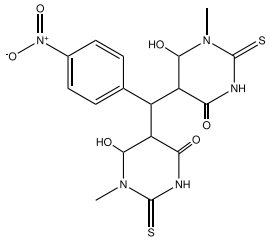
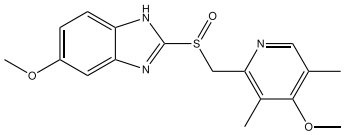
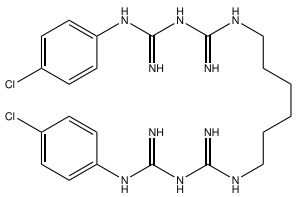
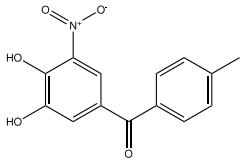
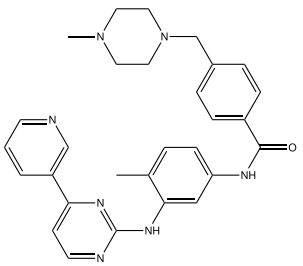
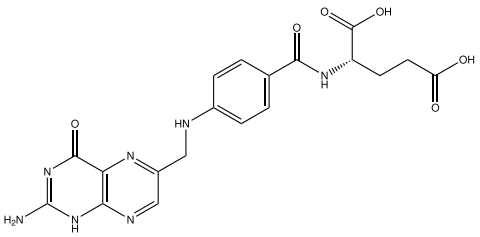
Data obtained from Peng *et al.*[249]

docking-based virtual screening campaign with a library of 1,466 FDA-approved drugs, with a molecular weight between 100 and 900 Da. These were docked against the glycolytic enzyme triose phosphate isomerase (TIM) from *E. histolytica* (TIMEh) and from *G. lamblia* (TIMGl) by means of the AutoDock Vina software.[255,256] The systems were prepared for the docking procedure through the UCSF Chimera[257,258] and AutoDock tools[255] packages and the results analyzed with the PLIP tool.[252] Beforehand, known inhibitors of the enzymes (compound D4 and omeprazole, for TIMEh and TIMGl, respectively) were docked against their crystallographic structures obtained from the Protein Data Bank (PDB accession codes 1M6J for TIMEh and 4BI7 for TIMGl) to be used as guides for determining the docking experiment parameters, to validate the protocol and to serve as control references for the VS procedure. The 1,466 screened compounds were then ranked based on their docking score, and the ten best ranked compounds for each enzyme had their molecular interactions with them further investigated. Through a comparison of the formed molecular interactions of the best ranked compounds with that of the reference compounds, and using a merged *per*-compound docking score for both enzymes, four of the docked compounds were selected for *in vitro* trophozoites growth inhibition assays, specifically, chlorhexidine, tolcapone, imatinib and folic acid, as well as the standard treatment for those parasitoses, metronidazole. The *in vitro* growth inhibition assays demonstrated that all compounds had IC_{50} values in the microgram range for *G. lamblia*, with folic acid showing the weakest inhibition and tolcapone the strongest, with values of 5.34 and 0.05 μg mL^{-1}, respectively. The latter was close to the reference compound (omeprazole) value of 0.025 μg mL^{-1}. All selected and assayed compounds were able to inhibit the growth of *G. lamblia* trophozoites with IC_{50} values below the standard treatment, metronidazole (IC_{50} = 7.8 μg mL^{-1}). On the other hand, only folic acid and metronidazole were effectively capable of inhibiting *E. histolytica* growth, with IC_{50} values of 0.186 and 0.205 μg mL^{-1}, respectively, which were more effective than the reference compound (D4), which had a IC_{50} value of 8.306 μg mL^{-1}.[254] This interesting study demonstrates the power and versatility of VS techniques in selecting from a large library the compounds with greater probability of showing desired pharmacological properties. Although further studies are needed to suggest possible repositioning of the mentioned drugs, the SBVS strategy used by the authors proved effective in the search for compounds with antiprotozoal activity (Table 5).

4.6. Proteasome inhibitors

Polyphenols, an important class of natural products, are widely distributed in plant foods. These compounds possess diverse biological activities and exert protective effects in various pathophysiological contexts, such as cardiovascular, circulatory, neurodegenerative diseases, and cancer. In

Table 5. Triose phosphate isomerase inhibitors found by SBVS of a library of 1,466 FDA-approved drugs

Compound code in the original work	FDA approved drug name	Compound chemical structure	IC_{50} / (μg mL^{-1})	
			E. histolytica	*G. lamblia*
1	metronidazole		0.205	7.8
2	D4		8.306 ± 1.616	–
3	omeprazole		–	0.025
4	chlorhexidine		> 100	4.93 ± 0.005
5	tolcapone		> 100	0.05 ± 0.002
6	imatinib		> 100	3.46 ± 0.005
7	folic acid		0.186 ± 0.003	5.34 ± 0.007

FDA: Food and Drug Administration; IC_{50}: half-maximal inhibitory concentration. Data obtained from Juárez-Saldivar *et al.*[254]

cancer, polyphenols impair cell proliferation, tumor growth, angiogenesis, inflammation, and apoptosis activation. Armed with this information, Marchese *et al.*[259] decided to investigate the potential interference of polyphenols

in the activity of the proteasome. The proteasome plays a fundamental role in the degradation of oncogenic proteins and the regulation of cellular pathways. Thus, the authors conducted a SBVS using polyphenolic compounds already approved by the FDA to identify polyphenolic compounds with potential proteasome inhibitory activity. In the initial phase, molecular docking-based virtual screening was performed using the Glide software,[245] where, considering the highest theoretical binding affinity, two flavone glycosides were selected, diosmin and hesperidin.[126,245] To confirm the *in silico* results, *in vitro* assays were performed using the purified catalytically active β5 subunit of the proteasome. Incubation of the recombinant β5 proteasome subunit with hesperidin or diosmin resulted in inhibition of β5 enzymatic activity, which was particularly evident at a concentration of 200 μM. Next, in the evaluation of multiple myeloma cells treated with diosmin or hesperidin, protein accumulation could occur because of proteasome inhibition. Western blot analysis 48 h after treatment with hesperidin or diosmin showed a significant positive regulation of poly-ubiquitin species after treatment with both compounds, thus confirming, in a cell-based assay, the results obtained in an SBVS strategy (Table 6).[259]

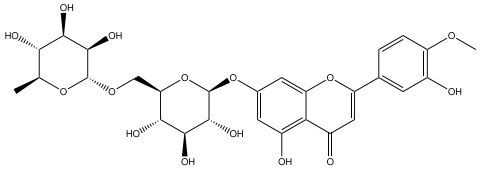
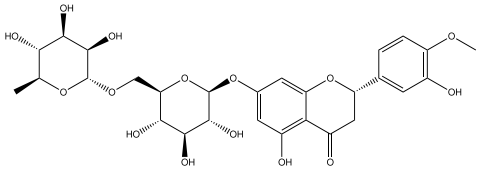
4.7. PD-1/PD-L1 interaction inhibitors

The programmed cell death protein 1 (PD-1) helps prevent the development of autoimmune diseases by negatively regulating the immune system. When this immune checkpoint binds to its ligand (programmed cell death ligand 1 (PD-L1)), the resulting interaction inhibits immune responses, stimulates cytokine release, and triggers cytotoxic reactions, suppressing T cell functions. PD-L1 expression enables various types of tumor cells (e.g., melanoma, colorectal, and renal cells) to evade immune system attacks. The advent of immune checkpoint therapy, specifically designed to block the PD-1/PD-L1 interaction, either directly targets tumor cells or nonspecifically reinvigorates the immune system, relying on the negative regulation of the immune mechanism induced by PD-L1 and the resulting suppression of cancer cell growth. In a study conducted by Lu *et al.*,[260] the authors conducted a SBVS utilizing the available human PD-1 protein in the PDB (PDB code: 5B8C), to find small molecule compounds capable of blocking the PD-1/PD-L1 interaction. They selected 208,023 compounds from the National Cancer Institute (NCI) compound database, which were then subjected to a filtering step (Lipinski's rule of 5) before SBVS using iGemdock software.[261] The top 100 compounds from molecular docking were subjected to a reassessment of interaction residues and physical-chemical properties of the binding site using the SiMMap server.[262] The compounds were reordered according to the SiMMap score, and six compounds were selected for *in vitro* assays. Compound CH4 exhibited inhibitory capacity for the PD-1/PD-L1 interaction at a dose of 10 μM, indicating that CH4 and CH5 may be hit compounds that act by blocking the PD-1/PD-L1 interaction (Table 7). Through this process, SBVS proved to be an excellent strategy for prioritizing promising hits to block PD-1/PD-L1.[260]

5. Conclusions

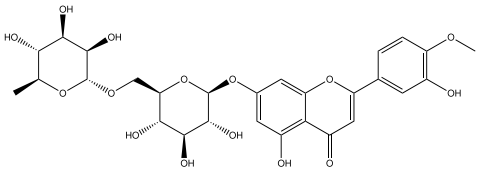
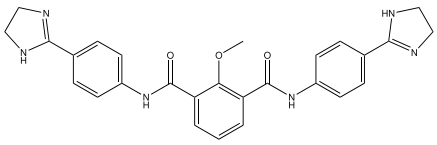
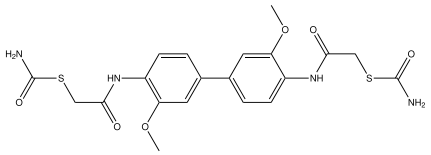
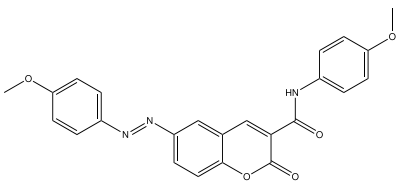
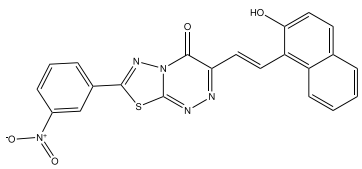
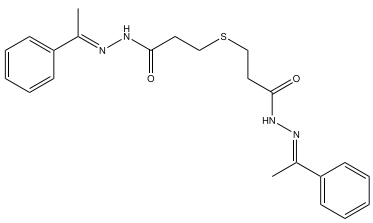
SBVS is an important strategy in the prioritization of putative bioactive compounds from vast chemical databases. This approach accelerates the exploration of potential drug candidates and facilitates drug repositioning, streamlining drug development pipeline and reducing costs.

Table 6. Proteasome inhibitors found by SBVS of a library of FDA-approved polyphenolic compounds

Compound code in the original work	Compound common name	Compound chemical structure	Concentration / (μg mL^{-1})	β5 proteasome activity / %
1	diosmin		200	> 60
2	hesperidin		200	40

Data obtained from Marchese *et al.*[259]

Table 7. Programmed cell death protein 1 (PD-1) inhibitors found by SBVS of a library of 208,023 compounds from the National Cancer Institute (NCI) compound database

Compound code in the original work	PubChem CID	Compound chemical structure	Concentration / (μg mL^{-1})	Cell viability / %
CH-1	376635		10, 20, 40, 80	N.A.
CH-2	248395		10, 20, 40, 80	N.A.
CH-3	–		10, 20, 40, 80	N.A.
CH-4	379775		20	30-40
CH-5	–		10	30-40
CH-6	5355233		80	> 40

N.A.: not aplicable. Data obtained from Lu *et al.*[260]

Advances in macromolecular characterization and target validation have further enhanced the utility of SBVS in medicinal chemistry. The shift towards using 3D target structures for pharmacophore models offers improved hit prioritization by enhancing selectivity. Nevertheless, the adoption of consensus scoring across many platforms bolsters the reliability of hit prioritization. It is important to emphasize that SBVS serves as an initial screening tool in drug discovery, acting as a means to explore chemical space. Hits prioritized through SBVS require thorough optimization in parameters such as binding affinity and selectivity, toxicity, pharmacokinetics, and bioavailability to become viable drug candidates. The cited successful applications across various therapeutic areas, from HIV treatment to cancer immunotherapy and chronic obstructive pulmonary disease, highlight SBVS's significance as a cornerstone in modern medicinal chemistry. Future advancements could harness ML to refine scoring functions and integrate hybrid models for a more holistic approach. Additionally, exploring multi-target screening may enhance therapeutic outcomes. In conclusion, as SBVS continues to evolve, its integration with advanced technologies promises to further revolutionize drug discovery, offering renewed hope for tackling complex medical challenges.

Acknowledgments

The authors thank the School of Pharmaceutical Sciences, University of São Paulo (FCF-USP), for support and infrastructure. We are grateful to the Brazilian agencies CNPq (436791/2018-8, 310232/2017-1 and 441004/2023-7), FUSP (403605), FAPESP (2017/25543-8) and CAPES (finance code 001).

Author Contributions

Adenilson G. Lima was responsible for researching the studies cited in this work and for writing (original draft, review, and editing); André B. Penteado researched the cited studies and contributed to writing (original draft, review, and editing); Jullyane Gabryelly de Jesus researched the cited studies and contributed to writing (original draft, review, and editing); Vanessa J. R. de Paula contributed to writing (original draft, review, and editing); Wítor R. Ferraz researched the studies cited in this work and contributed to writing (original draft, review, and editing); Gustavo Henrique G. Trossini was responsible for conceptualization, project administration, and supervision.

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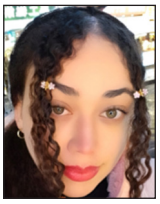

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Submitted: February 29, 2024
Published online: June 26, 2024