Sudarshan Publications

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Pharma Research

An International Peer Reviewed, Indexed Journal

10.62655/s-epub.2023.v15.i02.pp1-8

STRUCTURE-BASED VIRTUAL SCREENING OF NATURAL PRODUCTS AGAINST SARS-COV-2 MAIN PROTEASE

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Article info

Received date:01-04-2023
Accepted date:09-08-2023
Published date:18-12-2023
ISSN;0975-8216

Keywords:

Virtual screening, SARS-CoV-2, Main protease, Natural products, Molecular docking

Abstract

Structure-based virtual screening was among the fastest responses to the emergence of SARS-CoV-2, and natural product libraries were screened against the viral main protease (Mpro) in dozens of studies. This review summarises, from published work, how Mpro structures are selected and prepared, how natural product libraries are assembled and filtered, and how docking, scoring and molecular dynamics refinement are combined. It tabulates the compound classes reported as hits, notably flavonoids, and compares them with synthetic reference inhibitors. The review reports no new data. The literature shows consistent enrichment of polyphenols, but experimental confirmation is sparse, scoring is unreliable across chemotypes, and drug-likeness is rarely tested against real pharmacokinetic constraints.

1. INTRODUCTION

Severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) was identified in early 2020 as the cause of a cluster of pneumonia cases in Wuhan, and sequence comparison placed it in the same species as the SARS coronavirus of 2003 [1, 2]. Its positive-sense RNA genome of roughly 30 kb opens with two large reading frames that are translated into the polyproteins pp1a and pp1ab. These are inert until they are cut into functional non-structural proteins, among them the RNA-dependent RNA polymerase and the helicase. Two viral proteases do the cutting, a papain-like enzyme and the main protease (Mpro, also called 3C-like protease). Mpro carries most of the load, releasing at least eleven non-structural proteins from the polyprotein [3, 4]. Without it the replication

machinery never assembles, which is why the enzyme drew attention within weeks of the genome sequence being released.

Several features make Mpro a sensible target. The enzyme works as a dimer, with a catalytic dyad of cysteine 145 and histidine 41 sitting in a cleft between two domains. It prefers substrates with glutamine at the P1 position, a specificity that no known human protease shares, so selective inhibition is at least plausible [3, 5]. Experience with the 2003 outbreak gave a head start. Structures of the SARS-CoV enzyme and its inhibitors had already been solved, and the new enzyme proved to be very close to it in sequence [3, 6]. Within months, crystal structures of SARS-CoV-2 Mpro bound to peptidomimetic inhibitors were deposited, and these supplied computational chemists with accurate templates to work from [5, 6, 7].

Drug-like inhibitors followed quickly. Repurposing screens showed that the hepatitis C protease inhibitor boceprevir and the veterinary compound GC-376 block Mpro and reduce viral replication in cell culture [8]. Medicinal chemistry programmes then produced nirmatrelvir, an oral compound with a nitrile warhead that is given with the pharmacokinetic booster ritonavir [9]. All of these are peptide-like covalent binders. Interest has therefore continued in other scaffolds, and natural products are a recurrent source because of their structural diversity and long record as antiviral and anti-infective leads [10]. Structure-based virtual screening, which ranks large collections of compounds by their predicted fit to a crystal structure, is the cheapest way to sift them. This review, which reports no new data, summarises how such screens against Mpro have been designed, what they have reported, and how far the results can be trusted.

2. THE MAIN PROTEASE AS A DOCKING TARGET

The active site of Mpro is a shallow cleft that is usually described in terms of subsites named after the substrate residues they accommodate. The S1 subsite binds the glutamine side chain through hydrogen bonds to histidine 163 and glutamate 166, and this is the most conserved and most exploited interaction. The S2 pocket is hydrophobic and unusually adaptable, able to swell to take larger groups. The S1' region sits next to the catalytic cysteine and is where covalent warheads react [5, 6]. Compounds that do well in docking tend to be those that reach S1 with a hydrogen-bond donor or acceptor, fill S2 with an aromatic or aliphatic ring, and add a few polar contacts along the main chain of the 164 to 168 stretch. Most published natural product hits are described in exactly these terms.

Choosing which structure to dock into is a decision that gets less attention than it deserves. The Protein Data Bank held hundreds of Mpro entries by 2022, including apo forms, covalent complexes and structures with non-covalent ligands [11]. Room-temperature crystallography has shown that the S2 loop and neighbouring residues are more flexible than cryogenic structures suggested, and that the cavities change shape between ligands [12]. A receptor taken from a complex with a large peptidomimetic may therefore reject smaller aromatic compounds that would fit a different conformer. Careful studies dock into several structures, a practice called ensemble docking, and report how rankings change. Studies that use a single template give no way of knowing whether the top hits are real or simply the compounds that fit one frozen shape.

Covalent inhibition complicates matters further. Nirmatrelvir, GC-376 and the compounds developed from N3 all form a bond with the sulfur of cysteine 145 [5, 6, 8, 9]. Natural products screened by docking rarely carry such a warhead, and almost all are treated as reversible binders sitting in the pocket. That is a fair simplification for polyphenols, but it means their predicted affinities cannot be compared with the potencies of covalent reference compounds, which are measured by a different kinetic scheme. Some screening papers do place a covalent control in the same ranked list, and the reader is left to decide what a docking score of a few kilocalories per mole means next to an inhibitor that forms an irreversible bond.

The protonation state of the catalytic dyad is another source of silent error. Calculations

and crystallographic analyses suggest that the dyad may exist partly as a zwitterionic pair, with a deprotonated cysteine and a protonated histidine, and the chosen state alters which poses score well near the S1' site [4, 6]. Conserved water molecules in the S1 pocket and near the oxyanion hole also matter, because a ligand that displaces them pays an energetic penalty that many scoring functions ignore. Preparation protocols that add hydrogens automatically, remove all waters and move on are fast, but they bake in assumptions that should be stated. Reports of the better studies list the software, force field, assigned protonation states and retained waters in enough detail for the work to be repeated.

3. PREPARING NATURAL PRODUCT LIBRARIES

Natural products have supplied or inspired a large share of approved small-molecule drugs, particularly antimicrobials and anticancer agents, which is the usual justification for screening them against a new viral target [10]. The libraries used come from several kinds of source. General-purpose collections such as ZINC include natural product subsets that can be downloaded in ready-to-dock formats [13]. Other groups build their own databases from the phytochemical literature, for example compounds reported from Chinese herbal medicines [14], from African medicinal plants [15], or from marine organisms [16]. These bespoke sets are usually small, from a few hundred to a few thousand structures, and their composition reflects the interests of whoever compiled them rather than any systematic sampling of chemical space.

Ligand preparation is more demanding for natural products than for synthetic libraries. Many records lack defined stereochemistry or contain a mixture of tautomers, and glycosides, macrocycles and peptides are common. Each stereocentre doubles the number of possible isomers, so a compound with five undefined centres may be docked as one arbitrary form that was never isolated. Protonation at physiological pH, tautomer enumeration and low-energy conformer generation all change the outcome. A screening result that depends on the particular isomer is a result about the database entry, not about the plant extract it came from, and the better papers report how undefined centres were handled and whether the enumerated isomers were docked separately.

Most workflows apply property filters before or after docking. Property-based drug-likeness filters, such as Lipinski's rule of five and the quantitative estimate of drug-likeness, are the usual first screen [17], and web tools such as SwissADME and pkCSM add predicted absorption, permeability and toxicity endpoints [18, 19]. Substructure filters for pan-assay interference compounds (PAINS) are used less often, although they ought to be applied routinely, because catechols, quinones and rhodanines are overrepresented among promiscuous screening hits [20]. This matters in the present context, since several of the polyphenols that dominate Mpro hit lists carry catechol or pyrogallol groups. A filter that removes all such compounds would empty many published tables, while skipping the filter leaves open the possibility that the compounds act by aggregation or redox cycling rather than by occupying the site.

The scale of screening has grown steadily. Early papers docked a few dozen compounds chosen by hand, whereas later studies have screened hundreds of millions of purchasable molecules with fast docking engines and then rescored the best fraction [21]. Natural products are a small portion of such collections, and most are represented only by synthetic mimics. A practical consequence is that large-scale screens and natural product screens answer different questions. The first asks which commercially available scaffolds fit the pocket best, the second asks whether a particular family of plant or marine metabolites contains something that does. Mixing the two kinds of result in a single discussion of promising leads, as some reviews do, hides this distinction.

4. DOCKING, SCORING AND REFINEMENT

AutoDock Vina is the most widely used program in the Mpro literature, mainly because it is free, fast and simple to automate [22, 23]. AutoDock4, with its separate grid

maps and Lamarckian genetic algorithm, appears in older work [24], and the commercial Glide package is the usual choice in industry-linked studies, often with a standard-precision pass followed by an extra-precision pass on the best compounds [25]. Reviews of docking and virtual screening describe the shared logic. A search algorithm generates poses inside a defined box around the binding site, a scoring function estimates binding free energy for each pose, and compounds are ranked by the best score [26, 27]. How wide the box is, how thoroughly the search runs and which side chains are kept flexible can all change the ranking.

Scoring functions are the weakest link. They are fitted to reproduce binding data for a training set and capture solvation, entropy and electrostatics only crudely. Larger molecules usually score better simply because they make more contacts, which favours glycosides and biflavonoids over smaller, more ligand-efficient compounds [26, 28]. Scores from different programs are not on a common scale, so one program's value of minus eight cannot be compared with another's. Some groups use consensus scoring, retaining only compounds that rank highly in two or more programs, and this improves reliability a little. What none of these approaches does is convert a docking score into a trustworthy potency, and papers that quote scores as if they were inhibition constants are over-reading the method.

Molecular dynamics (MD) simulation is now routine as a second stage. The top-ranked complexes are solvated, equilibrated and simulated for tens to a few hundred nanoseconds, usually in GROMACS or a similar package [29]. Stability is judged from the root-mean-square deviation (RMSD) of the ligand and protein, the persistence of hydrogen bonds with residues such as histidine 163 and glutamate 166, and the fluctuation of residues around the pocket. Binding free energies are then estimated by the MM/PBSA or MM/GBSA end-point methods, which are cheaper than alchemical approaches but sensitive to the dielectric constant chosen and to the length of the trajectory [30]. A pose that survives 100 ns of simulation is more credible than a raw docking pose, though still a prediction.

A typical published workflow therefore has four tiers. A filtered library is docked with a fast, low-precision setting, the best few percent are re-docked with a more exhaustive search, a few tens of compounds are inspected visually for sensible interactions, and the final handful go into MD with free energy estimates [15, 16, 25]. Each tier trades coverage for accuracy, and each introduces its own cut-off, often chosen by convention. Seen together, the pipeline looks rigorous. Yet no tier is validated against experiment unless the authors include known actives and decoys as controls, and that step is the least often reported.

5. REPORTED HITS FROM NATURAL PRODUCT SCREENS

The main findings from natural product screens against Mpro are collected in table 1, together with the synthetic reference inhibitors that serve as benchmarks. Flavonoids are the best-supported family. Baicalin and baicalein, obtained from Scutellaria roots and used in a traditional Chinese preparation, inhibited SARS-CoV-2 Mpro in enzyme assays, and a crystal structure of baicalein bound to the enzyme was reported [31]. Earlier work on the SARS-CoV enzyme had shown that several flavonoids, including quercetin and its relatives, inhibit the related protease with micromolar potency [32]. The virtual screens that followed repeatedly rediscovered these scaffolds, which is reassuring about the method, and also a reminder that it often returns what is already known.

Library-based studies have reported a wide range of other chemotypes. A docking screen of constituents of Chinese herbal medicines highlighted a number of polyphenols and glycosides as potential direct inhibitors of the enzyme [14]. A virtual screen of marine natural products, followed by molecular dynamics, proposed several compounds with predicted binding comparable to that of known inhibitors [16], and an in silico study of alkaloids and terpenoids from African medicinal plants ranked a different set of leads after ADMET filtering [15]. A very

large docking campaign on commercial compounds, not limited to natural products, nominated several hundred candidates for testing [21]. In all of these papers, the proportion of nominated compounds that were later confirmed in a biochemical assay is small or unreported.

Table 1: Selected natural product and reference inhibitors of SARS-CoV-2 Mpro from published studies

Compound or library	Reported finding	Ref.
Baicalein and baicalin Scutellaria flavonoids	Inhibited Mpro in enzyme assays; crystal structure of the baicalein complex reported	[31]
Quercetin and related flavonols SARS-CoV 3CLpro work	Micromolar inhibition of the closely related SARS-CoV enzyme; basis for later screens	[32]
Chinese herbal constituents in silico library	Docking nominated polyphenols and glycosides as possible direct Mpro inhibitors	[14]
African medicinal plants alkaloids and terpenoids	Docking and ADMET filtering ranked candidate leads; no experimental confirmation	[15]
Marine natural products virtual screen with MD	Several compounds predicted to bind comparably to known inhibitors after simulation	[16]
606 million purchasable compounds ultra-large docking	Hundreds of candidates nominated for testing; mainly synthetic, few natural	[21]
Boceprevir and GC-376 repurposed protease inhibitors	Inhibited Mpro and reduced viral replication in cell culture	[8]
Alpha-ketoamide 13b peptidomimetic	Crystal structure with Mpro; template for later inhibitor design	[5]
N3 and related peptidomimetics covalent inhibitors	Complex structures defined subsites and guided structure-based design	[6, 7]
Nirmatrelvir oral clinical candidate	Nitrile warhead; optimised from peptidomimetics for oral use	[9]

Docking papers describe the proposed binding modes in remarkably similar language, which says something about both the target and the method. Nominated flavonoids and terpenoids are typically placed with a hydroxyl group hydrogen bonded to glutamate 166 or histidine 163 in S1, an aromatic ring stacked against histidine 41, and a second ring wedged into the hydrophobic S2 pocket [5, 6, 14, 16]. Such poses are plausible, because they mimic the contacts made by the peptidomimetic ligands in the crystal structures. The sameness, however, may owe as much to the shape of the cleft and the bias of the scoring functions as to genuine complementarity. When the alkaloids and terpenoids from African plants are examined, their top poses are described in much the same terms as those of the polyphenols [15], although their chemistry is entirely different.

The distribution of the entries in table 1 is shown in fig. 1. The synthetic reference inhibitors form the largest group, because they have the most complete experimental support: crystal structures, enzyme kinetics and cell-based antiviral data [5, 6, 8, 9]. The natural product groups are each represented by a single study or two, and for most of them the evidence is computational. The chart illustrates an imbalance in the field. The compound classes that have been discussed most in virtual screening papers are not the ones with the strongest experimental backing, and the classes with real structural data, flavonoids in particular, are a minority of the nominated hits.

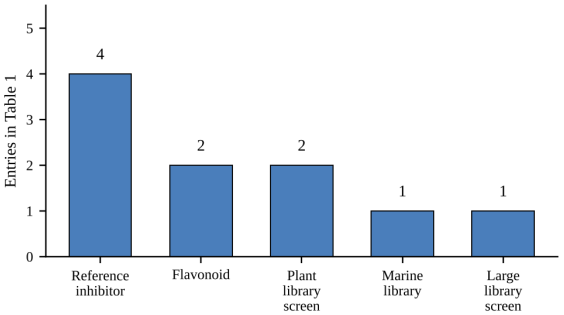


Fig. 1: Number of entries in table 1 by compound group

A cautionary pattern runs through these studies. Among the compounds reported as inhibitors in enzymatic work, potencies for polyphenols are usually in the micromolar range, several orders of magnitude weaker than the optimised synthetic compounds, and the assays are sensitive to detergent, reducing agent and enzyme concentration [8, 31, 32]. Compounds that appear active in one

laboratory can fail to reproduce in another, particularly when aggregation or thiol reactivity contributes to the signal. For this reason, results from screening papers are best treated as hypotheses about where to look, not as discoveries of antiviral agents, and the claims in some abstracts about the potential to treat COVID-19 go well beyond what the data allow.

6. LIMITATIONS AND FUTURE DIRECTIONS

The gap between docking and the bench is the central limitation. Surveys of virtual screening pitfalls point to a familiar list: scoring functions that reward size, receptor structures that are rigid or poorly prepared, libraries with property biases, and the absence of negative data for comparison [26, 28]. Hit rates for structure-based screens in the literature are typically a few percent at best, and for natural product libraries, which contain many assay-interfering motifs, the rate of true, selective binders is probably lower [20, 28]. Reported hit lists that are never followed by a biochemical test therefore carry little information, and journals handling such papers could reasonably ask for at least a single orthogonal assay before claims of inhibition are made.

Even a confirmed inhibitor of the isolated enzyme is far from a drug. Many polyphenols are poorly absorbed, extensively conjugated by glucuronidation and sulfation in the gut wall and liver, and cleared quickly, so concentrations reaching infected tissue may be well below the enzyme IC50 [18, 19]. Selectivity matters as well. Mpro is a cysteine protease, and compounds that react with active-site thiols could also affect human cathepsins and calpains, a point addressed in work on reference inhibitors [8]. Cell-based antiviral assays, cytotoxicity measurements and early pharmacokinetic profiling should follow soon after the first enzyme result, not years later, and ought to be planned into the screening project from the beginning.

Context also matters when judging how much a new lead could add. An effective oral Mpro inhibitor is already available in the form of nirmatrelvir, and its design drew on years of structure-based optimisation of covalent peptidomimetics [9]. A natural product intended to compete would need either a clear advantage, such as activity against variants carrying active-site substitutions, or a different mechanism, such as binding at the dimer interface [12, 33]. The active site appears to be strongly conserved among coronaviruses, which favours all inhibitors of this class, though it leaves open how the enzyme will respond to prolonged drug pressure. Screening studies seldom argue the case for their hits in this way, and doing so would sharpen the questions they ask.

Several practices would make the literature easier to use. Authors can state the receptor entry, protonation states and box dimensions, deposit ligand files, and report enrichment against decoys before making predictions [34]. They can dock into more than one conformer, since the pocket is plastic, and note how mutations seen in circulating variants alter the cavity [12, 33]. And they can pair the computational stage with a simple fluorescence-based enzyme assay and a counter-screen for aggregation. None of these steps is expensive, and together they would let the field separate real chemical starting points from artefacts of scoring. Natural product chemistry has a long record of surprising its computational critics, so the effort of testing the predictions is likely to be justified.

7. CONCLUSION

Virtual screening of natural products against the SARS-CoV-2 main protease has produced a large literature with a small experimental core. The target is well defined and structurally rich, docking engines are accessible, and libraries of plant and marine metabolites are easy to obtain, so the barrier to producing a paper is low. The reliable findings concern flavonoids, where enzyme assays and a crystal structure support what the screens predicted [31, 32]. Most other nominated compounds rest on scores whose accuracy across chemotypes is limited [26, 28]. Taken at face value, the volume of published hit lists overstates what is actually known about the chemistry of the target.

Progress will depend less on bigger libraries than on better discipline. Decoy-based

benchmarking, treatment of receptor flexibility and protonation, filters for assay interference and early experimental confirmation would all raise the value of future studies [12, 20, 34]. Pharmacokinetic reality should be considered at the outset, since polyphenols that inhibit an isolated enzyme may never reach useful concentrations in the body [18]. With these safeguards, natural product screens remain a reasonable way to generate starting points, and the established reference inhibitors offer a clear standard against which any new lead should be judged [5, 8, 9]. Reviewers and readers can help by asking, for each nominated compound, what the evidence is beyond the docking score.

8. ABBREVIATIONS

ADMET-Absorption, distribution, metabolism, excretion and toxicity, MD-Molecular dynamics, MM/GBSA-Molecular mechanics generalised Born surface area, MM/PBSA-Molecular mechanics Poisson-Boltzmann surface area, Mpro-Main protease, PAINS-Pan-assay interference compounds, RMSD-Root-mean-square deviation, SARS-CoV-2-Severe acute respiratory syndrome coronavirus 2, COVID-19-Coronavirus disease 2019, IC50-Half-maximal inhibitory concentration

9. FUNDING

Nil

10. AUTHORS CONTRIBUTIONS

All the authors have contributed equally.

11. CONFLICT OF INTERESTS

Declared none

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