Short Review - Informatics

Integrating molecular modelling methods to advance influenza A virus drug discovery.

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Teaser: Molecular modelling is being extensively applied to identify and evaluate potential drug compounds against the influenza A virus, which continues to pose a threat to public health worldwide.

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Abstract

Since the discovery of the anti-influenza drugs Oseltamivir and Zanamivir using computer aided drug design methods, there have been significant applications of molecular modelling methodologies applied to influenza A virus drug discovery, such as molecular dynamics simulation, molecular docking and virtual screening. This review provides a brief general introduction to molecular modelling in the context of drug discovery and then focuses on the advances and impact of integrating these methods with specific reference to potential influenza A antiviral drug targets.

Introduction

The influenza A virus is capable of causing severe respiratory illness or health complications in humans and can result in high transmission and infectivity rates amongst different species. Out of the four antigenic types of Influenza viruses (A, B, C and D), type A viruses infect the widest host range and have been the cause of pandemics due to zoonoses, hence the focus of this review is on anti-influenza A drug discovery. Whilst vaccines are formulated annually based on global surveillance data, immunisation cannot guarantee extensive or long-term protection due to the continuously evolving nature of the virus. For many years there has been great focus on discovering novel anti-influenza compounds which target various proteins of the virus life cycle as well as drug resistant mutants [1]. Current influenza A drug targets are shown in Figure 1. To date, only a small number of influenza A antivirals have been licensed for use throughout the world including the neuraminidase inhibitors (NAIs) and the M2 protein proton channel inhibitors [2]. However, widespread antiviral resistance has developed against the M2 inhibitors which are no longer recommended for use. Although there have been concerns over high levels of resistance to NAIs against certain strains in previous years, data from the USA and the UK has shown that fortunately, seasonal strains in current circulation remain susceptible [3]. Most recently the polymerase inhibitor Favipiravir gained approval with a limited license in Japan only [4], as well as the acid polymerase inhibitor Baloxavir marboxil which was approved for use in Japan and the USA in 2018 [5].

The basic polymerase 2 (PB2) inhibitor Pimodivir (JNJ-63623872; previously VX-787) has shown promising results in phase II clinical trials [6] and is recruiting for phase III clinical trials. In addition, monoclonal antibody preparations such as MEDI8852 which target the major surface glycoprotein haemagglutinin (HA) have also been clinically evaluated [7,8]. Despite this, the urgent need to develop novel effective antiviral therapeutics against seasonal, pandemic and NAI and M2 inhibitor resistant strains continues to be widely reported. It is also crucial that there is more than one treatment option available in the occurrence of a serious outbreak to overcome the possibility of resistance emerging. Drug discovery is aided immensely by the availability of 3D protein structures. For some potential drug target proteins, the structures are not or only partially known. Even if a protein structure is available, it may not be in a conformation accessible to drug binding or potential binding sites may not be known. Here the techniques of molecular modelling can predict binding sites and through molecular simulation reveal hidden binding sites or previously unknown conformations. Once models of protein structures have been developed and analysed, research can proceed to molecular docking of inhibitors and calculation of binding affinities using free energy calculations or simplified methods for selected inhibitors.

Molecular modelling methods are closely integrated with wet-lab research. Molecular modelling can be applied in the initial stages of the drug discovery process followed by wet-lab experiments, in the later stages of refinement of hits identified in high-throughput screening, or even in optimising ADMET (Absorption-Distribution-Metabolism-Excretion-Toxicity) properties. Molecular modelling methods are comparatively cheap compared to wet-lab based high-throughput screening that requires access to expensive equipment and maintenance of a large library of chemical compounds. Recently, more and more new drugs originate from academic or small biotech companies that have access to limited financial resources. Intelligent approaches based on a combination of molecular modelling and small-scale wet-lab based experiments can help to optimise the resources and may even lead to results in a shorter timeframe [9].

Molecular modelling – general overview

Molecular modelling broadly covers the theoretical and computational methods used to model the structure, properties and dynamic behaviour of molecules at the atomic level. In the context of drug discovery, molecular modelling methods offer the important advantages of saving time and cost by allowing for detailed characterisation and analysis of predicted drug binding modes and strength of protein-ligand interactions without biological risks involved.

Since the historically successful identification of the NAI Zanamivir by structure based computer aided drug design [10], numerous studies have emerged which incorporate innovative molecular modelling and structure based methods. These include molecular dynamics (MD) simulation, molecular docking and ligand or receptor based virtual screening, or a combination of all three using various software and programs [11]. In drug discovery projects, MD simulations are typically performed to provide a dynamic view of protein structure over time and analyse protein conformation with or without bound ligands; cryptic binding sites (sites that are only visible when a ligand is bound to the protein) can also be revealed, whilst docking and screening of chemical libraries are applied to investigate interactions and calculate binding free energies between two molecules [12]. The receptor-based (also known as structure-based) virtual screening method requires knowledge of the target protein structure to predict the best binding conformation of small molecules to a specific site. These techniques have proven to be useful in discovering and designing new antiviral drugs [13–15]. MD simulations are more time-consuming at setup- and runtime level compared to molecular docking. Hence molecular docking can be used as a high-throughput method to screen ten to hundred thousands of ligands. In molecular docking, the protein-ligand complex is given a score that correlates with or represents the predicted binding affinity. This score can be computed from a physics-based, empirical-based or knowledge-based potential energy function. More recently, grid-based convolutional neural network methods have shown increased scoring accuracy [16–18]. However, in critical evaluations these performance gains were attributed to overfitting of the model to the training data [19] or from detecting common differences between active and decoy compounds used for training [20]. Free energy calculation (FEC) based on MD simulations is considered the most accurate method of predicting the binding affinity of a ligand to its target. However, FECs are not used in systematic search procedures to find the best binding pose of a ligand, as this would be too time consuming. A complication of FEC based on MD simulations is the assignment of the force field parameters for the ligand that must be evaluated in separate simulations. First a docking is carried out followed by a small number of FECs that can be used to predict the binding affinity of a few compounds[21]. MD simulations can also be used to estimate

the kinetics of drug binding and un-binding as well as residence time, which is an estimation of how long a drug remains in a binding site and a potential determinant of efficacy; both of these parameters are considered to be key factors in the development of new therapeutics [22].

MD has assisted many drug discovery studies and now with more accurate forcefields and practical simulation run times being achieved through use of graphical processor unit (GPU) acceleration, the interest in applying MD is certainly growing. However, regardless of these methods being well-established, the accuracy of force fields used, simulation times and scoring functions remain a limitation. The number of experimentally resolved influenza A protein structures available is ever increasing enabling researchers to undertake innovative drug discovery studies without having to generate models through prediction methods. In the case of influenza A, there is also the specific need to address the issue of drug resistance, therefore, studies which integrate sequence based analysis and consider evolutionary information at residue level can enhance the impact of the overall findings [23,24]. This review will summarise advances in applications of molecular modelling based studies and highlight selected recent examples of these techniques in practice.

Discovery of influenza A inhibitors and drug targets

Given the essential and multi-functional roles of influenza A proteins, there are many options when it comes to selecting an antiviral drug target(s), with almost all major proteins having been the subject of extensive investigation. Furthermore, molecular dynamics, docking and virtual screening protocols have also been combined as integrative strategies to identify influenza A inhibitors, and identify binding pockets [25–28] as shown in figure 2. These methods have also been applied after initial experimental work to analyse molecular interactions. An overview of studies that have used molecular modelling methods is shown in Table 1.

The specific biology of the influenza A virus present further challenges to the process illustrated in figure 2. The negative-strand RNA genetic material of the influenza virus has a high mutation rate due to the absent proofreading capability of the viral RNA polymerase. It has been estimated that no two flu viruses in an infected individual are 100% genetically identical, hence the term quasispecies was introduced [37]. A further complication is the mixing of gene segments in a cell infected by two different virus strains, a process known as reassortment, that adds to genetic variability. This may render the process of drug development fruitless within a couple of years, if the virus becomes resistant. Thus, for the influenza virus in particular, it is important to analyse protein drug target variability not only among a specific virus strain and host, but overall strains and host ranges. Only, when a drug binding site and high conservation overlap, it is likely, albeit not guaranteed, that the virus does not develop resistance through mutations or genetic reassortment. For viral surface exposed proteins, overlap between conservation and a drug binding site may not always be found, but internal viral proteins usually show a high degree of conservation, as shown in figure 3 for the nuclear export protein [27].

Nucleoprotein

One successful example is the discovery that the nonsteroidal anti-inflammatory drug Naproxen can be repurposed as an inhibitor of the nucleoprotein (NP), which was identified through virtual screening of compounds filtered from a database initially containing 100,335 compounds into the RNA binding groove. The docked complex obtained from the screening was then subjected to MD

simulations and further docking to assess the stability of the complex and the binding interactions. Naproxen was also verified *in vitro* and *in vivo* and showed reduced levels of virus replication [26]. Although, since this discovery, more potent NP inhibitors have been discovered from high-throughput or cell-based screening assays [38].

Neuraminidase targeting 430-loop with MM-GBSA/ MM-PBSA methods

A new NA inhibitor known as *6a* has been identified to target the 430-loop [30], in this work, virtual screening of 670,000 compounds was initially performed and the top 30 compounds were docked to explore binding modes. MD simulations were then performed for more precise binding predictions and to calculate binding free energy via the molecular mechanics generalised born surface area (MM-GBSA) and molecular mechanics Poisson Boltzmann surface area (MM-PBSA) method. Compound 6a showed the lowest binding free energy, as well as good inhibitory *in vivo* activity and provided a basis for the design of six other NA inhibitor compounds. One advantage of structurally refining post-docking complexes through MD simulation in a solvated environment is that effects of water molecules can be accounted for in drug binding, and it can also be observed if a compound leaves or remains in the binding site. It is therefore suggested that the dynamics of the target protein with (or without) the bound compound should be investigated where possible to account for receptor flexibility [39].

By using MD in conjunction with docking or virtual screening, the limitation of the receptor being considered as a rigid structure can also be overcome. Furthermore, a multiple receptor conformation docking approach combined with virtual screening can be applied to account for receptor plasticity and distinguish active compounds from decoys and improve overall docking results [40,41]. Consequently, end point analysis methods such as MM-PBSA and MM-GBSA methods can be applied to calculate the free energy of binding which could improve virtual screening and docking results. These calculations have been proven to be useful in drug design [42] prior to experimental validation. Free energy methods also allow focused optimisation to increase the inhibitory potency of drug leads by assessing the effects of modifications to a chemical structure [43].

Nuclear Export Protein (NEP) and Non-Structural Protein 1 (NS1)

As well as potential drug compounds, new drug target sites are also being discovered. In a recent study, the full length nuclear export protein (NEP) was modelled and MD simulations were performed with partial restraints. The simulations were analysed through clustering to extract representative structures for prediction of binding hot spots which were mapped to conserved regions of the NEP. This was followed by consensus virtual screening which involved using two docking software [44] to screen over 50,000 drug-like compounds from two chemical libraries against a selected target site [27]. It would be of considerable interest to know if any of the identified compounds were capable of inhibiting virus replication *in vitro*. Another protein considered an attractive antiviral target is the multi-functional non-structural protein 1 (NS1) as it plays an essential role in counteracting the hosts interferon based immune response [45,46]. In a similar study to[27], the flexibility of the RNA binding domain of NS1 was explored through MD simulations followed by clustering and root mean square fluctuation (RMSF) analysis. Subsequently, the suitability of all estimated NS1 drug binding pockets were thoroughly evaluated for their potential to bind drug-like ligands [35]. Though both of these studies account for protein flexibility to

some extent through conventional MD simulations, the sampling of conformations can be improved with enhanced sampling methods [47]. These methods have emerged to overcome the limitation of standard MD simulation to sample conformations by providing an effective boost in terms of potential energy, thus moving the system over high energy barriers so that molecular conformations may be sampled more extensively, compared to unbiased simulations. A few examples of such methods include replica exchange MD, metadynamics, accelerated MD and simulated annealing; each having their own advantages and limitations, as described by[48,49].

Neuraminidase 150-loop

Molecular modelling methods have also given further insight into well established drug targets such as the neuraminidase. An enhanced sampling approach was applied to the NA enzyme, where replica exchange MD simulations were used to explore the structural flexibility of the 150-loop cavity associated with NAI binding. The simulations sampled open, intermediate and closed conformations. RMSD, volumetric, clustering and principle component analyses were performed to identify residues involved with inter-conversion of the loop region to facilitate NA specific drug design and to help understand the mechanism of antiviral resistance [29]. This work also builds on previous research based on standard MD simulation demonstrating that the NA 150-cavity of the 2009 H1N1 pandemic virus exists in an open state, which became a new target for drug design not visible in the crystal structure [50].

M2 proton channel

MD simulations have been applied to rationally design inhibitors of M2 carrying drug-resistant mutations such as V27A, L26F, and S31N [51], as well as to elucidate the mechanism of action of novel dual inhibitors targeting the wild-type and Amantadine-resistant M2 [34], which represent promising inhibitors for further development. Recently, MD simulations have also revealed insights into the drug resistance mechanism of M2-S31N inhibitors through widening of the channel pore [52].

The well-tempered form of the metadynamics technique has also been exploited to investigate the stability and binding pathways of amantadine and its derivative adamantyl bromothiophene to the S31 and N31 mutant M2 [33]. The simulation free energy profiles suggest how the ligands are positioned and move through the proton channel pore during entry. Despite valuable insights enhanced sampling methods could offer to analyse protein conformational dynamics, there are a limited number of recent influenza A related drug discovery studies published which apply enhanced sampling methods, presumably due to the difficulty of implementing them.

Polymerase

The PA subunit of the viral polymerase complex has been the subject of intensive modelling and docking studies. This was aided by the initial determination of the structure of the N-terminal part (residues 1-195) of PA [53,54], which contains an active site involved in cleaving the 5'-cap of host pre-mRNA in order to be used for the synthesis of primers for viral mRNA. In subsequent studies the PA N-terminus was successfully co-crystallised with seven inhibitors which resulted in seven atomic structures of PA with different inhibitors bound (PDB-IDs: 4E5E to 4E5J and 4E5L) [55]. This lead

eventually to the approved drug Baloxavir marboxil. Research presented in a study on 19 mutated models of the cap – dependent endonuclease proposed the mechanism by which reduced sensitivity to Baloxavir marboxil occurs [36]. In this analysis, MD simulations of the endonuclease in complex with Baloxavir revealed different modes of interaction between the aromatic ring of the drug and the endonuclease binding site I38 (shown in Figure 1b). Binding free energy was also lower for 13 out of 19 mutation models calculated using MM-GBSA method compared to the wild type endonuclease. Through computational mutagenesis, this study clearly illustrates how loss of molecular recognition due to mutations correspond with reduced drug sensitivity and drug resistance. MD simulations and docking have also been successfully applied to identify inhibitors targeting the PA-PB1 subunit interactions [28], and PA-PB1 inhibitors with a cycloheptathiophene-3-carboxamide scaffold have shown broad-spectrum antiviral activity and a high barrier to drug resistance [56].

Neuraminidase with QM/MM methods

Another useful approach for drug discovery is the combined quantum mechanics/molecular mechanics (QM/MM) method which must be used to study chemical and enzyme reactions with greater accuracy and to support binding affinity calculations. The QM/MM approach can be used to simulate bond breaking or formation through a quantum mechanical treatment of a subset of atoms, such as the drug binding site, whilst a molecular mechanical description is used for the rest of the system without explicit consideration of electrons. This method has been applied to gain further insights into the binding interactions of H1N1 NA with Oseltamivir, Zanamivir, Laninamivir octanoate and Laninamivir [31] and it was reported that the predicted binding free energies were in good agreement with previous experimental results. Although, in comparison to other (MM) methods, this technique does not appear to be widely applied for influenza A drug discovery, as simulations are very time consuming and expert knowledge in quantum mechanics in setting up and analysing simulations is required.

Multi-scale modelling studies

Protein structure prediction and protein docking algorithms have also been used to model the additional NA domains including the stalk, transmembrane and intravirion domains. From subsequent MD simulations of the NA protein modelled with the stalk embedded in the lipid bilayer with and without amino acid deletions in the stalk, it was shown how the length of the NA stalk influences the dynamics of sialic acid binding pocket residues in the NA surface region, and therefore binding affinity to the host sialic acid substrate. In addition to these important features, new druggable hot spots on the NA surface head were also identified through computational mapping and suggest opportunities for virtual screening and docking [57]. These results reflect how overall structural changes in areas other than the active/binding site impact ligand binding affinity.

Moving beyond analyses of single proteins, pioneering results from the first all-atom MD simulations of the entire influenza A viral lipid envelope model including water, lipids, 30 NA tetramers and 236 HA tetramers have been published [58], highlighting the significant progress achieved in simulating large, complex biomolecular systems. Thorough conformational sampling in these simulations enabled characterisation of a secondary sialic acid binding site of the NA enzyme that participates in initial substrate binding, followed by the substrate sliding into the active site. Principle component

analysis revealed notable insights into the transitions between the open and closed states of the 150-loop cavity and the primary binding site, including changes to catalytic-site volume, which can strongly influence drug binding. This study paves the ground for new types of NA inhibitors, that can be available, if resistance emerges. An interesting aspect was that improved sampling of binding pocket states was achieved, in contrast to studies focusing on single proteins. This was explained by either the molecular crowding effect of the whole lipid envelope, or alternatively the number of NA molecules (30x4) over which conformations were sampled. This work also presents a novel integrative modelling strategy for antiviral drug development targeting enveloped viruses.

Likewise, in a previous integrative structural modelling study, sialic acid (SIA) association rates to the HA and NA active site and secondary binding site to the entire viral surface were modelled and simulated. Based on the predicted association results from the SIA simulation complexes, the authors outline how targeting the secondary NA site presents new opportunities for drug development by disrupting the HA/NA functional balance [59].

Conclusion and Future Perspective

The studies reviewed highlight some of the latest applications and impact of molecular modelling over the years in antiviral discovery. Examples of combined approaches that researchers have taken increase the chance of identifying the most promising targets and compounds for drug development. Many of the aforementioned studies have also elucidated atomic level detail on structure and dynamics of influenza A protein targets and emphasise how drug discovery could benefit from incorporating these techniques. In addition to the physico-chemical considerations of macromolecular conformations and molecule binding, the rapid rate of evolution of the influenza virus was addressed focussing drug discovery efforts at conserved binding sites.

Given the various molecular modelling methods available to choose from, it raises the question of how to implement the most suitable for a drug discovery project. Furthermore, the software and parameters to use also need to be carefully selected. On one hand, sufficient (enhanced) sampling followed by accurate binding affinity calculations of protein-ligand complexes could be considered as best practice and theoretically should provide better estimates and assessment of biomolecular motion and interactions. On the other hand, a simple and more computationally efficient procedure such as docking a ligand to a static protein structure with prior knowledge of an appropriate binding site could possibly be sufficient for antiviral discovery. In order to address this question, positive and negative controls should be included into the modelling process. While controls are standard practice in wet-lab experiments, published computer-lab experiments have not widely applied this principle. In either case, predictions need to be validated experimentally to provide proof of binding and antiviral activity in order to justify the choice of method and guide further work.

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Table 1. An overview of selected studies using MD/Docking including a list of structures and
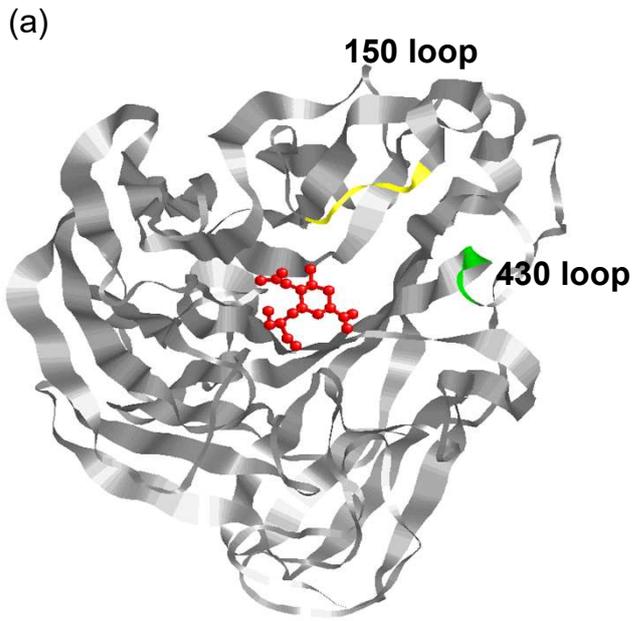identified antiviral inhibitors.

Figure 1. Current influenza A antiviral targets a) Neuraminidase (NA) (PDB: 2HU0 [60]), b) Acid
Polymerase (PA) (PDB: 6FS6 [61]), c) Basic polymerase 2 (PB2) (PDB: 4P1U [62] and d)
Haemagglutinin (HA) (PDB: 5JW4 [63] in complex with inhibitor compounds in red. This figure was
made with Rasmol [64].

Figure 2. Flow chart illustrating the integration of some of the most consistently used molecular
modelling approaches to predict and discover potential antiviral drugs using molecular docking.
(PDB, Protein Data Bank; PCA, Principle Component Analysis; MSM, Markov State Modelling, MM-
PBSA, Molecular Mechanics Poisson Boltzmann Surface Area; MM-GBSA, Molecular Mechanics
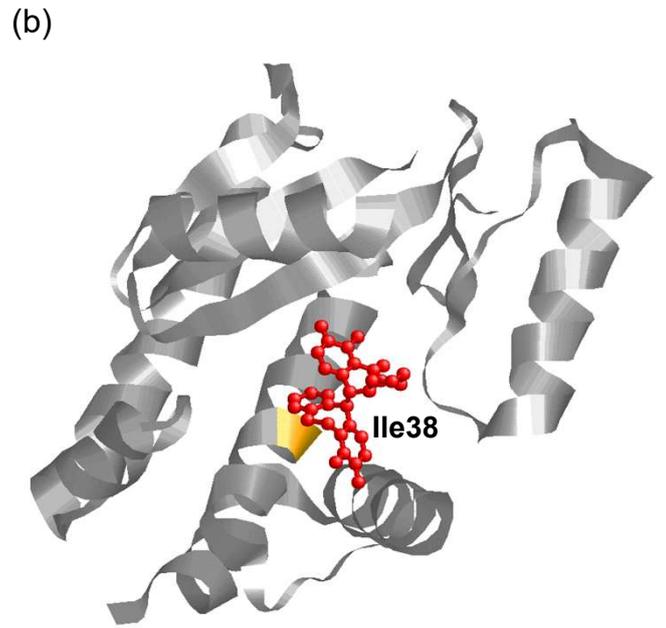Generalised Born Surface Area).

Figure 3. Overlap between predicted binding sites (green spheres) and amino acid sequence
conservation for a partially modelled structure of the influenza A nuclear export protein. Reprinted
from [27] with permission from Elsevier under STM guidelines.

Table 1. An overview of selected studies using MD/Docking including a list of structures and identified antiviral inhibitors.
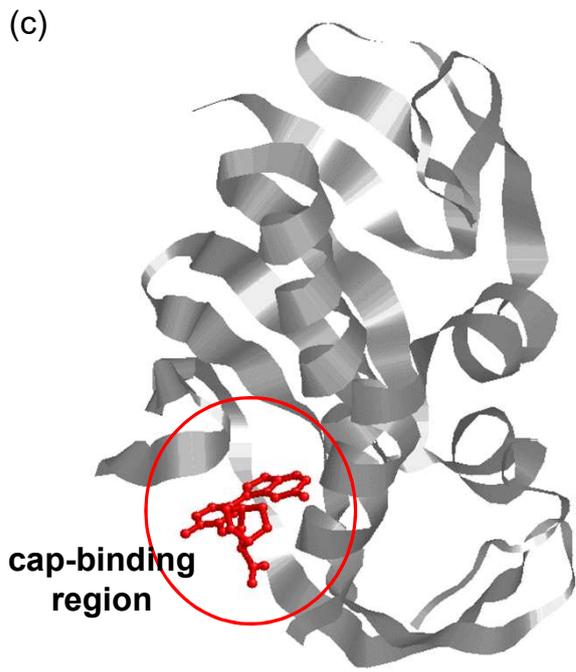
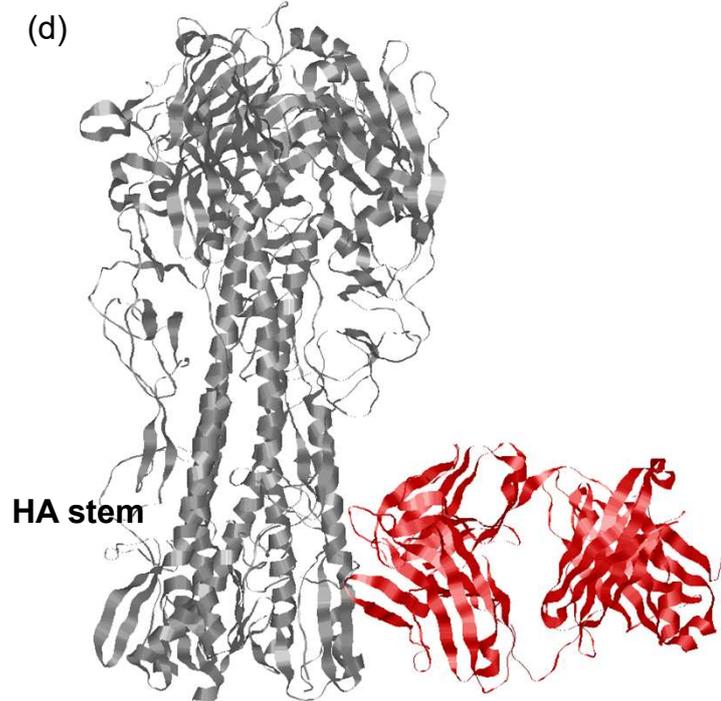| Target protein	MD simulation/screening protocol(s)	Antiviral drugs/ identified compounds	Compounds tested in wet-lab assay	Refs
NP	Virtual screening followed by NP-ligand MD simulation	Naproxen	Yes	[26]
NA	REMD with RMSD, volumetric, clustering and principle component analyses			[29]
	Virtual screening, NA-ligand MD simulation, MM-GBSA and MM-PBSA	6a	Yes	[30]
	MD with QM/MM			[31]
M2	MD simulation of wild type and drug-resistant M2	Spirane amine compounds	Yes	[32]
	Metadynamics			[33]
	M2-ligand MD simulation	Compound 11	Yes	[34]
NEP	Standard MD, clustering, RMSF, virtual screening	ZINC01564229, ZINC01717023, Nandrolone phenylpropionate	No	[27]
NS1	Standard MD, clustering, RMSF			[35]
PA	PA-ligand MD simulation with MM-GBSA			[36]
PA-PB1	PA-PB1 MD simulation with MM-GBSA, virtual screening	Compounds of the 3- cyano-4,6-diphenyl-pyridine family	Yes	[28]

(a) NA-Oseltamivir — 150 loop, 430 loop

(b) PA-Baloxavir Acid — Ile38

(c) PB2-Pimodivir — cap-binding region

(d) HA-MEDI8852 — HA stem

Amino acid conservation

Conserved   Intermediate   Variable

Influenza A Nuclear export protein

N-ter

C-ter

Binding site 1

3   6   5

8

10

2   4   9

7