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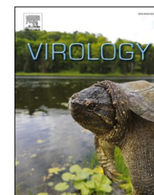

Review

Harnessing viral internal proteins to combat flu and beyond

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ABSTRACT

This mini-review examines the strategy of combining viral protein sequence conservation with drug-binding potential to identify novel antiviral targets, focusing on internal proteins of influenza A and other RNA viruses. The importance of combating viral genetic variability and reducing the likelihood of resistance development is emphasised in the context of sequence redundancy in viral datasets. It covers recent structural and functional updates, as well as drug targeting efforts for three internal influenza A viral proteins: Basic Polymerase 2, Nuclear Export Protein, and Nucleoprotein. The review discusses new insights into protein interactions, potential inhibitors, and recent drug discovery efforts. Similar approaches beyond influenza including Hepatitis E, SARS-CoV-2, Dengue, and the HIV-1 virus are also covered briefly.

1. Introduction

Viruses are molecular parasites that depend on host cells for replication (illustrated in Fig. 1). They possess genetic material encased in a protein coat, sometimes encircled by a lipid membrane. The SARS-Cov-2 viruses contain non-segmented positive-sense RNA as its genetic material, while the influenza A virus contains segmented negative-sense RNA; both viruses have a lipid-enveloped membrane. The influenza RNA exhibits a high mutation rate due to the lack of proofreading ability in the viral RNA polymerase, yet the SARS-Cov-2 virus contains a proofreading exoribonuclease activity as part of non-structural protein 14. Furthermore, in case of segmented RNA, genetic variability is enhanced through reassortment, a process where gene segments mix in cells infected by different virus strains (Lowen, 2018). This process occurs for example in Influenza viruses, Rotaviruses, Bunyaviruses but not in SARS-Cov-2. However, in SARS-Cov-2 infected cells a recombination between the RNAs of co-circulating strains is possible that adds to variability. The high genetic variability allows the virus to rapidly adapt to environmental pressures, such as immune responses or antiviral drugs in human pathogens.

The time-consuming and costly process of drug development may be rendered ineffective within a few years if the virus acquires resistance. Therefore, it is crucial at the beginning of a drug discovery project to analyze the variability of protein drug targets, focusing on evolutionarily conserved regions. Assessing sequence conservation of viral sequences necessitates careful consideration of sequence redundancy, as they are often derived from the same species and have a sampling bias towards human isolates. Including virus sequences from a broader range of species, for example Influenza A, B and C sequences, can alleviate this problem, but in most cases the most pathogenic human isolate dominates the available sequence data. This redundancy problem can be addressed by various means, for example by using phylogenetic trees as implemented in the ConSurf method (Ashkenazy et al., 2016a), by incorporating redundancy of a sequence alignment into a mathematical formula as implemented in the Valdar scoring method (Valdar, 2002a), or by clustering a multiple sequence alignment and replacing each cluster with a representative sequence as implemented in the UCLUST algorithm of the USEARCH tool (Edgar, 2010) or the CD-HIT algorithm (Huang et al., 2010). This mini-review will address studies, where the biological sequence conservation was combined with the chemical drug-binding potential of viral protein targets as pioneered by Darapaneni et al. (2009a). By combining biological sequence data with chemical information, suitable drug target sites on viral proteins have been identified, reducing the likelihood of resistance development. At the same time, this approach improves the prediction of meaningful ligand binding sites as was shown by a systematic evaluation of 348 ligand-bound and ligand-unbound protein structures (Tsujikawa et al., 2016). To ensure targeted binding of drug molecules, an in-silico molecular docking approach must be used to predict potential drug interactions. While wet-lab methods can identify inhibitors of drug targets, they typically cannot probe individual sites. Structural biology techniques can retrospectively identify the bound ligand, but the ligand may not bind to the desired conserved site identified in earlier analyses (Kukol, 2017).

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Zoonotic viruses that can be transmitted from animals to humans, present a particular threat to human health, as the virus can circulate and mutate in the animal reservoir until a major outbreak occurs, as it was the case with past influenza pandemics and the more recent Covid19 pandemic. In case of influenza, even the seasonal outbreaks unrelated to zoonosis continue to present a major global health threat. According to WHO data published in 2023 three to five million cases of severe illness occur annually and 290,000–650,000 deaths result from respiratory diseases related to seasonal influenza worldwide every year (WHO, 2023). As human influenza pandemics primarily originate from zoonotic transmission of the influenza A virus, particularly from swine and avian species, the avian influenza A subtypes H5N1 and H7N9 are currently of significant concern due to their high pathogenicity. Due to the substantial threat posed by the Influenza viruses, prevention and control measures are essential. Vaccines and antivirals remain an important component of pandemic preparedness. Whilst therapeutic options are currently available, they are subject to certain limitations, such as initiation within two days of infection in the case of neuraminidase inhibitors (NAIs), instant availability and the issue of resistance re-emerging or developing at alarming rates (Bialy et al., 2024).

Influenza antiviral drug discovery strategies have focused on a broad range of targets, particularly internal viral proteins which tend to display higher levels of evolutionary conservation. Popular targets include the nucleoprotein (NP) (Kukol and Hughes, 2014), the nonstructural protein 1 (NS1) (Trigueiro-Louro et al., 2019) and the viral polymerase constituents, PB1, PB2 and PA (Nannetti et al., 2019; Chen et al., 2022) which encapsidate viral RNA and regulates efficient genome replication.

This mini review focusses on selected internal influenza A proteins relevant to antiviral discovery as well as other viruses, where the strategy of combining sequence conservation with drug binding potential was used to predict and/or identify ligands to viral proteins that may be used as inhibitors of viral entry, replication or exit.

2. Basic Polymerase 2 (PB2)

2.1. Structure/function updates

The PB2 polymerase subunit plays a role in generating the cap structure for viral mRNA from the 5' end of 7-methyl guanosine triphosphate (mGTP) capped host mRNA during transcription (Reich et al., 2014). The interaction between the H5N1 polymerase cryo-EM structures with the host protein ANP32B have offered new insights into the structural basis of viral genome replication mechanisms (Staller et al., 2024). In this work, the binding interaction sites and mammalian adaptive mutations in avian origin viruses have been identified. Furthermore, the structural and functional aspects of the interaction between the PA subunit of the influenza A polymerase and the C-terminal domain of host RNA polymerase II have also been elucidated and is suggested to be an antiviral target (Keown et al., 2024).

2.2. Drug targeting efforts

The antipsychotic medication Paliperidone (marketed as Invega), amongst other compounds, was identified as a potential PB2 inhibitor through a virtual screening approach (Fig. 2a) (Patel and Kukol, 2017). As Paliperidone seemed to be a promising anti-influenza drug candidate due to its predicted high binding affinity to a conserved region of PB2, experimental validation was subsequently performed, whereby the inhibitory effect on virus replication against three flu A strains; A/PuertoRico/8/1934(H1N1), A/Hamburg/4/2009(H1N1), and A/WSN/1933(H1N1) was carried out (Panagiotidis et al., 2023). Results showed clearly that paliperidone transiently inhibited strain PR/8/34 following early infection; but neither HAM/2009 nor WSN/33. The PB2-NP binding interaction was also interrupted. A multiple sequence allignment of the PB2 protein regions contributing to the paliperidone binding site did reveal only two minor differences between PR/8/34 and the other two strains (supplementary figure). Possibilities as to why

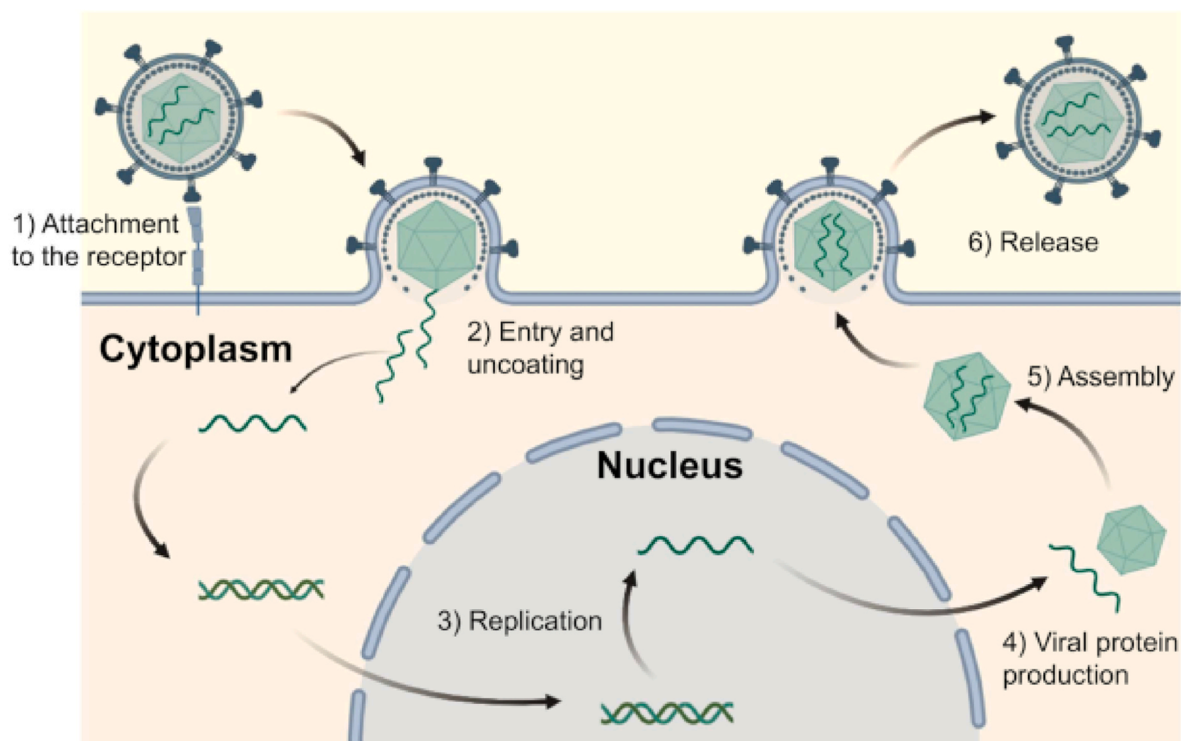

Fig. 1. The typical life cycle of an RNA virus. Replication for a positive sense RNA genome proceeds through double-stranded RNA intermediates, while the positive sense RNA can act directly as mRNA for protein production. Negative-sense RNA is first transcribed to positive-sense RNA, which serves as mRNA and as a template for replication of the negative-sense RNA.

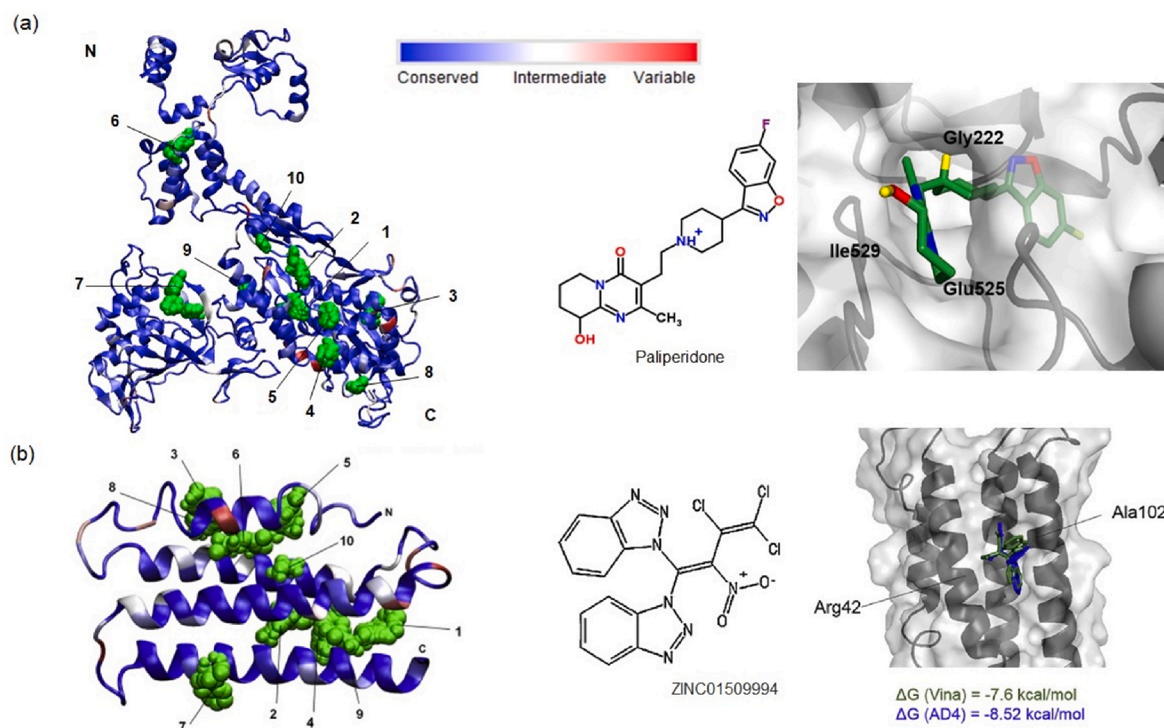

Fig. 2. Conservation, binding site prediction and computational docking of a top-ranked predicted ligand for (a) the PB2 protein and (b) the nuclear export protein. Figure combined from previous publications in this journal (Patel and Kukol, 2017, 2019b).

Paliperidone did not show a broader range of activity against different flu A strains could be due to genetic variability in other regions of the PB2 gene affecting the binding site and/or NP genes conferring resistance, adaptive compensatory mutations in other proteins restoring replication, or the drug binding to a different target site than expected from the prediction. Therefore, investigations whether Paliperidone may function as a multi-target compound may be worth pursuing. It is also suggested that Paliperidone may be more effective when used in combination with current NAIs or the newer polymerase inhibitor Baloxavir Marboxil, or compounds which affect the later stages of influenza virus replication.

Other compounds claimed to display potent antiviral activity by targeting the PB2 subunit include a group of 7- azaindole compounds, such as HAA-09 (Wang et al., 2023), and 7–51A (Sun et al., 2023). Both compounds target the conserved PB2 cap-binding domain. Detailed analysis of HAA-09 biological activity revealed key interactions and binding affinities; the compound was also proven to be effective in vitro and in mouse models against H1N1 strain A/WSN/33 (H1N1) and the Oseltamivir resistant variant (Wang et al., 2023). Likewise, the activity of 7–51A was validated by isothermal titration calorimetry assay and unique interactions between 7 and 51A and PB2 have been elucidated through co-crystal structure analysis; in vivo studies are yet to be conducted (Sun et al., 2023).

3. Nuclear export protein (NEP)

3.1. Structure/function updates

The nuclear export protein (NEP) primarily functions as an adaptor protein to facilitate nucleocytoplasmic transport of the viral ribonucleoproteins during the infectious life cycle (O'Neill et al., 1998). Aside from this role, recent experimental findings based on amino acid substitution screening at the last NEP residue (ILE121) demonstrate the critical role that Ile121 plays in promoting viral genome replication as well as wild-type NEP enhancing polymerase activity (Zhang et al., 2024). This work follows on from an earlier NEP functional study, which included experiments to investigate the effect of N-terminal truncations on viral RNA transcription. Results showed that NEP N-terminal amino acids 1–20 have a regulatory role by mediating transcription inhibition; whilst Ile121 mediated replication upregulation (Zhang et al., 2023). Furthermore, this study also discusses the contradictory results in relation to the role of NEP in vRNA transcription and replication from previous functional studies. For clarification, vRNA transcription refers to the production of mRNA from the RNA genome, while replication refers to process of synthesising a complementary RNA strand that serves as a template for the sythesis of new viral RNA genomes. The host G Protein Pathway Suppressor 2 (GPS2) which can act as a negative regulator of vRNA replication through inhibition of polymerase assembly has been identified as a new NEP binding partner, furthering our understanding of NEP functions (Gong et al., 2021). This direct NEP-GPS2 interaction involving NEP amino acids at position 32–41, promotes the nuclear export and degradation of GPS2 via the proteosome pathway, thereby facilitating virus replication. These findings may be worth considering in context of future drug discovery studies.

3.2. Drug targeting efforts

The NEP has been explored as a drug target through computational studies (Darapaneni et al., 2009b; Patel and Kukol, 2019a), (Fig. 2b) but in comparison to other proteins, there are very few inhibitors identified through experimental research which directly target the NEP. This may be due to the intrinsically disordered nature of the protein and the slower synthesis and late accumulation of NEP during infection (Chua et al., 2013) making it a challenging protein to investigate.

However, the coordinated process of nuclear export, which is achieved through the combined actions of various host cell and internal influenza proteins, has also been suggested as a viable target. Compounds targeting the process of nuclear export have been explored with chalcone-like derivatives identified through phenotypic screening as successful viral replication inhibitors (Li et al., 2023). Specifically,

compound IIB-2, an optimised chalcone-derivative showed inhibitory effects on the exportation of the nucleoprotein with reduced toxicity levels against A/PR/8/34 (H1N1), A/Brisbane/10/2007 (H3N2), and the Oseltamivir resistant strain H1N1 pdm09. Furthermore, the activity of a compound targeting a host protein indirectly implicated in vRNP export has been reported (Yang et al., 2023). The derivative of the antimalarial drug, Artesunate is proposed to inhibit the host enzyme phosphodiesterase 4 (PDE4), consequently limiting nuclear export of vRNP complexes and reducing influenza replication. Yang et al. (2023) showed that broad antiviral activity existed against H5N1, H1N1 and H3N2 strains following treatment with Artesunate in cell-based assays, as well as increased survival rates in infected mice when administered in combination with Peramivir, compared to untreated mice.

4. Nucleoprotein (NP)

4.1. Structure/function updates

The nucleoprotein is the most abundant internal influenza protein and performs several important functions. The NP structure consists of a head domain, body domain and tail loop. Multiple copies of the NP assemble to encapsidate genomic vRNA segments to form the viral RNP complex forming the nucleocapsid. A Cryo-EM structure of the nucleocapsid in a helical conformation has uncovered further NP-NP and NP-RNA interactions involving conserved residues as a model for genome encapsidation (Chenavier et al., 2023). This work builds on several previous structural studies and provides a useful model illustrating the NP-NP interfaces and the proposed pathway of RNA along one strand. Upon binding, the NP has also been shown to have a significant impact on the structure of the vRNA segment and vRNA-NP complex, suggesting a chaperone activity role for the NP (Quignon et al., 2024). The packaging of the eight vRNA gene segments during the life cycle is a highly coordinated process and relies on signals in the NP. A study investigating this packaging functionality, involved viruses with mutated terminal packaging signals combined with mutations at highly conserved acetylated lysine residues (184 and 229) in the NP RNA-binding groove; results showed that the amino acid charge states can influence the genome packaging process with certain mutations leading to defects in the process (Ciminski et al., 2024).

4.2. Drug targeting efforts

Similar to the polymerase, NP inhibitors are also continually being identified. A phenotypic screening investigation has led to the identification of compound A4 with robust broad spectrum anti-influenza activity against A/Puerto Rico/8/1934 (H1N1), A/Brisbane/10/2007 (H3N2) and flu B/Yamagata strains (Li et al., 2023). A4 is an Imidazo[1, 2-a]pyrazine derivative which can cause oligomerisation of NP monomers, prevent its nuclear accumulation and interfere with the transcription and replication stages. Molecular dynamics simulations also revealed the predicted binding mode within a conserved region of the NP, with two A4 molecules bridging two NP units. No significant toxicity issues were identified in mice following oral administration over a seven-day period. The natural product Allopregnanolone, which is currently approved for use as an anti-depressant drug has shown inhibitory activity on influenza replication from phenotypic screening (Dong et al., 2023). The mechanism of action of Allopregnanolone was investigated and the results demonstrated that export of newly synthesised NP in the form of viral ribonucleoproteins was drastically impaired. Direct binding to the NP was confirmed by thermal shift assays and binding modes were predicted through molecular docking; some of the amino acid residues lining the binding pocket included M448, L266, N395, L418, D455. Antiviral potency was evaluated in mice and reduced virus replication (without statistical significance) was observed.

Structural analogs of the anti-inflammatory drug Antrafenine are a new class of compounds found to have anti-influenza activity against A/WSN/33 (H1N1), A/HongKong/1/68 (H3N2) and B/Florida/04/2006 strains (Tang et al., 2023). Specifically, compounds '34' and '41', were shown to be dual inhibitors with the ability to bind both the NP and the acid polymerase (PA) subunit of the RNP. Both compounds did not show any significant cytopathic toxicity at concentrations up to 100 μM. Amino acid residues involved in the NP-34 interaction include those forming the tail loop insertion groove: His272, Glu339 and Thr390, while NP-41 include Ile388, Glu339 and Phe458 based on molecular docking to the PDB 2IQH structure. MD simulations were subsequently performed on the protein-drug complexes and showed that the bound 34-NP complex remained stable (Tang et al., 2023). The research team who previously identified Nucleozin as a NP inhibitor (Amorim et al., 2013), have since identified other novel NP inhibitors, such as compound FA-6005 (Yang et al., 2021). FA-6005 interacts with the conserved NP I41 region and the Y289 pocket, and has an interesting mechanism of action, as it coats the external surface of NP thus inhibiting vRNP functions and various steps of the viral life cycle. FA-6005 antiviral activities against five different flu A strains and B/Wisconsin/01/2010 were demonstrated by plaque reduction assays and the toxicity and survival rates were evaluated in infected mice. In 2023, Via Nova Therapeutics announced FDA clearance of a new investigational NP inhibitor, VNT-101 which is reported to be in clinical development (Via Nova Therapeutics, 2023). Data to support the mechanism of action and NP target site is yet to be published.

This review focusses on selected examples, where sequence conservation and drug-targeting was combined to predict or identify inhibitors, hence we have not included many other compounds that are in the early stages of research as antiviral agents. Overall, many of the studies reviewed in sections 2-4 and summarised in Table 1 offer exciting opportunities for further therapeutic developments as initial in vitro studies have been completed, as well as in many cases preliminary evaluations of anti-viral resistance profiles and in vivo safety profiles.

5. Beyond influenza

The strategy of combining sequence conservation with drug binding potential was applied to the capsid protein of the hepatitis E virus encoded by the open reading frame 2. The sequence conservation was mapped onto the predicted 3D-protein structure using the ConSurf method (Ashkenazy et al., 2016b) and combined with the protein-protein docking of a monoclonal antibody against the capsid protein. The results showed the conservation of epitopes on the capsid protein (Zhang et al., 2018).

One of the first studies using combined conservation and binding site analysis applied to SARS-CoV-2 proteins revealed near 100% conservation of binding sites on various non-structural proteins (Srinivasan et al., 2020). It was, however, not clearly described how the conservation was determined and if sequences from other coronavirus types were included, which would have been necessary to obtain meaningful results

Table 1
Anti-influenza compounds in various stages of research mentioned in the text.

Drug Target	Compound	Stage (early/late/ repurposed)
PB2	Paliperidone	repurposed
	HAA-09, 7–51A	early
NEP	chalcone-like derivatives	early
(via host enzyme)	Artesunate	repurposed
NP	Imidazo[1,2-a]pyrazine derivative A4	early
	Allopregnanolone	repurposed
	Antrafenine analogs	early
	Nucleozin	early
	FA-6005	early
	VNT-101	late

as the analysis was timed quite early in the evolution of the human SARS-CoV-2 virus and many other variants have developed since.

Two other studies focussed on single proteins of SARS-CoV-2, namely the spike protein (Trigueiro-Louro et al., 2020) and the nsp12 polymerase (Figueiredo-Nunes et al., 2023). In those studies the sequence conservation was calculated by the Valdar scoring method (Valdar, 2002b), which incorporates sequence redundancy into a mathematical formula to calculate a conservation score. The drug binding potential in the SARS-CoV-2 studies was determined by a consensus of two or more druggability prediction methods. Results for nsp12 showed that the top-ranked sites, where conservation and druggability overlapped, were in fingers and palm subdomains of nsp12. While those studies show promising results in terms of overlap between high conservation and binding potential, no actual ligands were computationally predicted or tested in viral replication assays. Another interesting approach is based on a protease with a substrate binding site that is structurally conserved among 12 different coronaviruses as well as picornaviruses and noroviruses. Through replication assays broad spectrum antivirals were identified long before the Covid19 pandemic (Kim et al., 2012; Tiew et al., 2011). Among those GC376 was specifically targeted at the SARS-CoV2 protease 3CL and further chemically modified resulting in a number of analogs with IC_{50} of up to 13 nM (Liu et al., 2022). Another recent drug-repurposing study by Romeo et al. (2022) targeted the SARS-CoV-2 nsp13 helicase with an initial conservation analysis based on a multiple sequence alignment of 5 million sequences, aparently without consideration of sequence redundancy. This analysis was followed by prediction of drugable binding sites and molecular docking-based virtual screening of FDA approved drugs. Five of the their top ranked molecules were tested in enzyme assays and one experimental drug, PF-03715455, that was used previously in clinical trials for asthma or pulmonary disease, was able to inhibit the unwinding and ATPase activity of nsp13 with IC_{50} of 3.0 μM and 9.3 μM respectively (Romeo et al., 2022). While research studies mentioned so far used conservation analysis and binding site prediction as separate steps initially and combined the information later, an integrated machine learning approach was chosen to predict novel binding sites for the dengue virus NS2b-NS3-protease. A neural-network type model based on 14 input features for each amino acid residue was used; the features included for example residue type, position in the sequence, hydrophathy and most importantly predicted ligand binding potential and degree of conservation among different related viral proteases. The network was trained against the effect of mutational studies on different dengue virus serotypes and different related viruses extracted from the literature (Aguilera-Pesantes et al., 2017). While the research identified several clusters of residues as candidates for binding sites and was able to identify a known antibody epitope, the usefulness of this approach to identify new inhibitors remains to be seen. Furthermore, due to the necessity for training the neural network, this approach depends on large volume of mutational studies that are more time consuming to perform than sequencing of virus isolates. For further information on sequence conservation and inhibitor identification across SARS-Cov-2 non-structural proteins, readers are referred to the review by Kandwal and Fayne (2023) in this journal.

An alternative approach of protein stability analysis and sequence conservation was used to identify important sites in the capsid protein of the HIV-1 virus (Manocheewa et al., 2015). All possible single point mutations of the 147-residue long capsid protein were generated computationally by introducing all 19 different amino acids at each position in the sequence. The stability of predicted 3D structures of the mutants was evaluated with the protein design software FoldX (van Durme et al., 2011). Analysis of almost 6000 capsid protein sequences revealed that mutations predicted as destabilising were rarely found in those sequences, thus those sites are conserved. More than half of these sites were targeted by known capsid inhibitors or known as epitopes that induce immune response. Based on these results Manocheewa et al. (2015) developed a combined measure of sequence conservation and protein structure stability that was superior in predicting deleterious mutations than any of the two measures alone. In the opinion of the authors, this conservation/stability measure combined with drug binding site prediction and docking-based virtual screening could lead to further improvement in the identification of inhibitors of rapidly mutating viruses.

6. Conclusion

This review underscores the critical importance of targeting internal viral proteins for the development of effective and long-lasting antiviral therapies, particularly for fast mutating RNA viruses. By determining evolutionarily conserved regions of these proteins, researchers can mitigate the rapid emergence of drug resistance, a significant challenge in antiviral drug development. The review highlights several promising targets for the influenza A virus, including the polymerase basic protein 2 (PB2), nuclear export protein (NEP), and nucleoprotein (NP), each playing a vital role in the viral life cycle and offering unique opportunities for therapeutic intervention. The use of computational approaches to combine biological sequence conservation with chemical drug-binding potential has proven effective in identifying potential drug targets and predicting meaningful ligand binding sites. This integrated strategy enhances the likelihood of discovering robust antiviral compounds with broad-spectrum activity and reduced resistance potential. From the research on other RNA viruses, the review has highlighted some alternative and complementary strategies that could be combined with aforementioned approaches in order to determine conserved binding sites. As zoonotic viruses continue to pose a significant threat to global health, ongoing research and innovation in targeting internal viral proteins remain essential for pandemic preparedness and the development of next-generation antiviral therapies.

CRediT authorship contribution statement

Hershna Patel: Writing – review & editing, Writing – original draft. **Andreas Kukol:** Writing – review & editing, Writing – original draft, Conceptualization.

Future perspective

The ongoing battle against viral infections, particularly those caused by RNA viruses, necessitates innovative strategies to stay ahead of rapidly evolving pathogens. Looking forward, the integration of advanced computational tools and virtual screening methods will likely accelerate the identification of conserved viral sequences with high drug-binding potential. Incorporating computationally predicted protein stability as an additional determinant could yield more robust results. Focusing on internal viral proteins may, in fortunate cases, lead to the discovery of broad-spectrum inhibitors for RNA viruses within the same genus, thereby enhancing our preparedness for future pandemics. Such discoveries could significantly bolster our arsenal against a wide range of viral threats. To translate these findings into viable therapeutic options, collaborations between computational and lab-based virologists, structural biologists, and pharmacologists will be essential. These interdisciplinary efforts will be crucial in bridging the gap between theoretical predictions and practical applications, ultimately leading to the development of effective antiviral therapies.

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Declaration of competing interest

The authors declare that they have no known competing financial

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.virol.2025.110414.

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