Therapeutic p28 peptide targets essential H1N1 influenza virus proteins: insights from docking and molecular dynamics simulations

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Abstract

The H1N1 influenza virus causes a severe disease that affects the human respiratory tract leading to millions of deaths every year. At present, certain vaccines and few drugs are used to control the virus during seasonal outbreaks. However, high mutation rates and genetic reassortment make it challenging to prevent and mitigate outbreaks, leading to pandemics. Thus, alternate therapies are required for its management and control. Here, we report that a bacterial protein, azurin, and its peptide derivatives p18 and p28 target critical proteins of the influenza virus in an effective manner. The molecular docking studies show that the p28 peptide could target C-PB1, NS1-ED, PB2-CBD, PB2-RBD, NP, and PA proteins. These complexes were further subjected to the simulation of molecular dynamics and binding free energy calculations. The data indicate that p28 has an unusually high affinity and forms stable complexes with the viral proteins C-PB1, PB2-CBD, PB2-RBD, and NP. We suggest that the azurin derivative p28 peptide can act as an anti-influenza agent as it can bind to multiple targets and neutralize the virus. Additional experimental studies need to be conducted to evaluate its safety and efficacy as an anti-H1N1 molecule.

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Graphic abstract

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Introduction

Influenza A virus (IAV) is a global health concern that causes high morbidity and mortality in humans, swine, and various other mammalian and avian species. To date, the virus has caused several seasonal epidemics and occasional global pandemics [1–4]. Genetic reassortment of the virus is one of the main factors causing severe human pandemics with high casualty rates. RNA viruses have the potential to develop genetic variations in a short time [5, 6]. Several subtypes of influenza infection are currently known, and the variation between the subtypes is primarily due to the development of genetic variations through rearrangement in the viral genome [7]. In 1918–1919, the influenza pandemic was caused by H1N1 strain; in 1957, the causative strain was H2N2, the 1968 pandemic was due to H3N2 strain, and the recent 2009 pandemic was due to the severe relapse of H1N1 strain [8, 9]. IAVs are subtyped based on two of its viral surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA). At present, there are 16 HA subtypes and 9 NA subtypes known for the virus. Additional subtypes have been discovered in bats that are of uncertain significance for humans. The most diverse

viral subtypes are found in aquatic waterfowl and are thus regarded as the natural reservoir of IAVs [10]. The wide range of subtypes and the appearance of new subtypes of IAVs could cause future pandemics. At present, several IAV subtypes, including H1N1 and H3N2, co-circulate between humans, animals, and birds as seasonal recurrences throughout the world [11].

Understanding the IAV life cycle and pathogenesis is vital to design and develop therapeutic molecules. IAV is an enveloped virus of the family Orthomyxoviridae. Its genome consists of eight segments of negative-strand RNA that code for a minimum of 10 proteins: hemagglutinin (HA), neuraminidase (NA), matrix protein 1 (M1), matrix protein 2 (M2), nucleoprotein (NP), non-structural protein 1 (NSP1), non-structural protein 2 (NS2, also known as nuclear export protein, NEP), polymerase acidic protein (PA), polymerase basic protein 1 (PB1), polymerase basic protein 2 (PB2) [4, 12–14]. These RNA genome segments are associated with copies of NP and the viral polymerase (P3) complex, which consist of PA, PB1, and PB2 fragments. The RNA, along with its associated proteins together forms the viral ribonucleoprotein complex (vRNP). The envelope of the virus is made up of a lipid

bilayer and contains three viral transmembrane proteins: HA, NA, and M2, all of which are crucial for viral entry into the host cell [1].

Currently, standard methods for developing therapeutic strategies are either the conventional design of drugs or the generation of vaccine candidates against the target parasite. A less explored method is the assessment of bacterial peptides against the diseases. Peptides do not accumulate in the body and have high specificity and low toxicity rendering them one of the most promising candidates for designing and developing novel drugs [15]. Azurin is a well-known copper-containing redox-active protein produced by the bacterium *Pseudomonas aeruginosa* [16]. Structurally, azurin consists of an immunoglobulin-like β -sandwich core formed by eight antiparallel strands, similar to the variable domain of an immunoglobulin. Several in vitro and in vivo studies have demonstrated the potential of azurin in the treatment of cancer, various pathogenic infections (such as toxoplasmosis, malaria), and viruses (such as HIV-1) [17–19]. The therapeutic activity of the azurin is associated with its 28 amino acid α -helical region known as the fragment p28 (L50–D77) [20]. In the USA, the fragment p28 has crossed the phase-I clinical trials for cancer therapy. It presented no signs of toxicity and regressed significant tumors in patients [21]. Moreover, a fragment within p28, known as p18 (L50–G67), is the protein transduction domain (PTD) responsible for the preferential entry of azurin and p28 into human cancer cells. Recently, we reported that the p28 derivative of azurin could bind strongly toward specific SARS-CoV-2 proteins [22].

The current study was focused on analyzing the anti-H1N1 potential of azurin and its derived peptides through high-end computational methods. We observed that p28 could bind and form stable complexes with specific IAV proteins.

Materials and methods

Target proteins and structure preparation

For docking, the structures of the H1N1 IAV proteins were retrieved from the RCSB protein database [23]. The target proteins chosen for the study were NS1 and all the protein members of the vRNP complex, i.e., NP, PB1, PB2, PA, and M2. The structures of the effector domain (ED) of NS1 (PDB ID: 3M5R), the extracellular domain of M2 (PDB ID: 5DLM), NP (PDB id: 6J1U), NA (PDB ID: 6G02), HA (PDB ID: 3UBQ), N-terminal of PA (PDB ID: 4AVQ), N-terminal of PB1 (PDB ID: 2ZNL), C-terminal of PB1 (PDB ID: 3A1G), RNA-binding domain (RBD) of PB2 (PDB ID: 3CW4) and cap-binding domain (CBD) of PB2 (PDB ID: 3WIO) were taken. The obtained structures were then processed using UCSF Chimera v.1.13.1 [24], which

involved removing the water molecules and non-peptide residues, if any, and refining structural clashes. Structures that contained broken residue fragments were modeled with their respective complete structures using SWISS-Modeller and energy minimized (steepest descent) using UCSF Chimera [25].

Preparation of the structures of azurin, p18, and p28

The structure of azurin homotetramer was retrieved from RCSB PDB (PDB ID: 1E5Z). The structure was processed using UCSF Chimera, chain A was extracted from the tetramer, and all non-peptide residues were removed. For preparing the derived fragments of azurin protein, L50–D77 (for p28) and L50–G67 (for p18) were extracted, and the rest of the amino acid residues were deleted.

Docking studies using ClusPro

Once the structures were processed, the ligand peptide azurin and its derivatives p18 and p28 were docked against the selected viral proteins, i.e., NS1-ED, NP, N-PB1, C-PB1, PB2-CBD, PB2-RBD, PA, and M2. For protein–protein docking, ClusPro server 2.0 was used, with default docking parameters. ClusPro 2.0 uses three-step computation for docking; in the first step, a rigid body docking of receptor and ligand with around 70,000 conformations each, followed by which, with the help of root-mean-square deviation (RMSD), 1000 lowest energy structures were selected, and in the final step, those structures are subjected to final refining and energy minimization [26, 27]. After the docking step, all the results from the balanced scoring scheme were obtained, and the receptor–ligand complex was visualized and studied for interactions using BIOVIA Discovery Studio Visualizer [28].

Molecular dynamics simulations

After docking, the azurin/derivatives that displayed a strong affinity toward viral proteins were used for all-atom molecular dynamics (MD) simulations. GROMACS v5.1.4 package was employed to run the MD simulations of the viral protein–peptide complexes [22, 29]. The AMBER-ff99SB-ILDN force field was used, and the TIP3P water model was used to fill water molecules inside the dodecahedral periodic boundary box with the distance between complex and the box wall fixed at 1.2 nm [30]. 1-nm periodic boundary was set for Lennard-Jones and Coulomb interactions [31]. With the help of modified Berendsen thermostat, the temperature of 310 K was fixed along with Parrinello–Rahman pressure coupling with 1.0 bar reference pressure [32]. The neutralization of complex structures was done by adding the Na^+/Cl^- ions. For removing the initial steric clashes,

the whole complex minimization was performed using the steepest descent algorithm via a tolerance of 10 kJ/mol/nm for 1000 steps. NVT and NPT ensembles were performed with the minimized systems for 0.1 and 0.5 ns, respectively [33]. For the determination of the stability of the complex systems, the equilibrated complex systems were processed for the production simulation with a timescale of 100 ns each. The binding free energy of the complex systems was assessed by *g_mmpbsa* calculations for a period of 15 ns (75–90 ns), where the system attained the lowest energy during the simulation [34].

Results

Docking of azurin, p18, and p28 to viral proteins

The docked complexes were retrieved from the ClusPro server, and the models were studied for interactions. All types of interactions between the viral proteins and azurin and its derivatives were investigated. The distances between the interacting residues were calculated and compared with

the available literature on functional sites of the viral proteins. Throughout the manuscript, all repeated interactions should be considered as either second or third interaction between the residues.

Analysis of the effector domain of NS1

NS1 protein of IAV is one of two NSPs and contains 230–237 amino acid residues depending on the strain (~26 kDa) [35]. It consists of two domains, (1) N-terminal RNA-binding domain, which helps protect IAV against host antiviral mechanism via blocking INF α/β , and (2) the 74 residues C-terminal effector domain (NS1-ED). The ED contains cleavage and polyadenylation specificity factor (CPSF)-binding domain (K175–G210), poly(A)-binding protein (PABP) domain (E218–T225), nuclear localization signal 1 (D34–R38), nuclear localization signal 2 (R211–P216), nuclear export signal (A132–L141) and eIF4G1 domain (I81–G113), through which it binds to several RNA and proteins to regulate host and viral gene expression [36–40] and alter signaling pathways [41], leading to downregulation of host apoptosis [42] and antiviral defense [43, 44].

Fig. 1 The docked complex of NS1-ED with azurin/derivatives: Azurin and its peptides p18 and p28 docked to a different binding pocket of NS1-ED. Color code: NS1-ED (red), azurin (blue), p18 (yellow), and p28 (green)

The NS1-ED was docked with azurin, p18, and p28. Figure 1 shows azurin, p18, and p28 bound to the e1F4G1 domain of NS1-ED. Docking of azurin with NS1-ED gave a cluster of 147 members and the lowest energy of -585.9 weighted score. Through the analysis of the docked structure, a total of 23 interactions were recorded between azurin and NS1-ED (16 hydrogen bonds and 7 other bonds, including pi-anion, alkyl, and pi-alkyl bonds). Hydrogen bonds were observed between the functionally important interactive residues (viral protein-azurin/derivative): I81–N18 (2.91 Å), S83–T21 (3.08 Å), V84–A1 (2.73 Å), T86–C3 (2.53 Å), C3–V84 (1.89 Å), K27–T86 (1.65 Å), S83–T21 (3.20 Å), and S135–Q28 (2.88 Å). Electrostatic and hydrophobic interactions were also recorded at critical sites such as R88–E2, R88–E2, I81–N18, S83–T21, V84–A1, T86–C3, and S165–D76, among several other interactions.

The NS1-ED/p18 docked complex had the lowest weighted score energy of -632.5 and displayed a relatively low number of hydrogen bonds (nine) with crucial interactions such as K110–S51 (2.15 Å), K110–T52 (2.86 Å), K110–T52 (3.04 Å), V194–G67 (1.99 Å), W203–G63 (2.67 Å), L50–V180 (1.90 Å), S51–V180 (1.99 Å), and S51–G179 (2.98 Å). Besides hydrogen bonds, the complex contained only hydrophobic interactions between the active-site residues: R108–L50, K110–L50, V180–M56, W203–M64, and W203–M64.

The NS1-ED/p28 docked complex showed binding patterns similar to that of azurin, with 12 out of 26 interactions being hydrogen bonds. The model with the lowest energy of -752 weighted score was chosen. The hydrogen bonds were present between the functionally important residues: R108–L50 (2.59 Å), R108–D55 (2.55 Å), R108–D77 (2.28 Å), R108–D77 (2.06 Å), K110–S51 (2.94 Å), N188–M56 (2.18 Å), L50–R108 (2.651 Å), and K110–L50 (2.96 Å). Compared to azurin, NS1-ED/p28 docked complex had a lower number of hydrogen bonds but higher electrostatic and hydrophobic interactions spread across 21 key sites such as R108–D77, R108–D55, K110–D55, R108–D77, R108–L50, R108–D55, R108–D77, R108–D77, K110–S51, and N188–M56. Azurin and p28 formed a higher number of hydrogen bonds and electrostatic/hydrophobic interactions with the NS1-ED than p18.

Analysis of polymerase proteins PB1, PB2, and PA

Along with the negative-sensed vRNA, the vRNP complex of IAV contains the nucleoprotein and trimeric RNA-dependent RNA polymerase (RdRp), comprising of PB1, PB2, and PA proteins. PB1 is the third and one of the functionally active proteins of the RdRp tetramer and contains the polymerase domain [45, 46]. As mentioned, PB1 binds to PB2 through its C-terminal region and PA through its N-terminal region [47]. The PB1 also contains nucleoside

triphosphate-binding region; nuclear localization signals as well as RNA-binding regions [46, 48, 49]. PB2-CBD sandwiches the 7-methylguanine (m7G) cap between two aromatic residues like other cap-binding proteins, with a cation– π interaction between F404 and the hydrophobic side chain of H357, with a completely different but conserved protein fold [50, 51]. The m7G cap-binding cleft is formed by H357, F404, E361, and K376 in the cap-binding domain. PB2 also contains an RNA-binding domain (RBD) that binds to the viral RNA through its RNA binding groove containing a functional residue of L627 along with the ϕ -region containing 589–605 helix, encircled by 623–635 loop [52]. Two conserved domains of PFXXXXP and GXYSXhhR (“h” denotes a hydrophobic amino acid and “X” denotes any amino acid) can also be seen in the PB2-RBD, which are expected to contribute toward essential function.

The influenza RdRp does not have a defined proofreading activity, resulting in around 10,000 new viral mutants during the infection course [53, 54]. Several experiments suggested that various PA residues participate in polymerase and other activities. Residues K102 and H510 participate in the cap-binding activity, whereas residues T157, L226, and multiple C-terminal residues play their role in replication activity [47]. Through various studies, it is seen that N-PA is responsible for numerous functions, including endonuclease and protease activity, protein stability as well as cap and promoter binding activities [55, 56]. Structural and functional assays on PA have shown that PA contains the endonuclease active site with a putative P107, D108, X10, E119, and K134 active site motif. PA also contains E154, K158, D160, E165, E166, R168, and R170, which are highly conserved across influenza species, suggesting that this region may be necessary for polymerase activity.

p28 binds effectively to the PB1 C-terminal non-polymerase region

Azurin formed a total of 15 interactions (including 7 hydrogen bonds) when docked to the C-terminal non-polymerase region of PB1 (C-PB1). The complex showed the lowest weighted score -456.7 . The hydrogen bonds in the cluster were distributed in the functionally important sites S712–N42 (2.26 Å), S720–V43 (2.14 Å), I724–Q12 (2.23 Å), and E731–Q14 (2.68 Å), while other bonds at important sites were R727–D11, R727–D11, R727–D11, and I724–M13. p18 formed 19 interactions with C-PB1, and the minimum weighted score was -667.4 . H-bonds were present in the functional sites V715–S51 (2.45 Å) and S713–S51 (3.54 Å). The non-hydrogen bonds were between E716–L50, V715–M56, V715–V59, I746–M64, and I750–V60. When compared to azurin and p18, the p28 formed a much higher number of interactions with C-PB1 with a total of 22 bonds (including 8 hydrogen bonds)

Fig. 2 The docked complex of C-PB1 with azurin/derivatives: Both azurin and p28 bound to the same pocket, thereby exhibiting similar interaction patterns. Color code: C-PB1 (red), azurin (blue), p18 (yellow), and p28 (green)

(Fig. 2). Four of these 8 hydrogen bonds lie in the functional sites R723–D71 (2.31 Å), D725–S51 (2.70 Å), E716–Y72 (2.48 Å), and R723–D71 (3.09 Å). The non-hydrogen bonds interactions were R723–D71, D725–L50, R723–D71, R727–D76, R727–D77, D729–L50, R721–M56, S712–M64, R721–M56, R723–L73, and I724–V59.

The N-terminal PA-binding region of PB1 (N-PB1) showed a total of 8 interactions (including 4 hydrogen bonds) with azurin. N-PB1 and p18 formed 5 interactions (including 3 hydrogen bonds). Similarly, N-PB1 and p28 formed 13 interactions (including 3 hydrogen bonds). Thus, compared to C-PB1, azurin and its derivatives formed lesser interactions with N-PB1. Also, the interactions N-PB1 were formed in non-functional sites, thereby deeming the azurin and its derivatives inactive against the viral PB1.

p28 preferentially binds to CBD and RBD of PB2

Azurin formed a total of 29 interactions when docked to the PB2-CBD (including 20 hydrogen bonds and 9 electrostatic and hydrophobic interactions). The complex showed the lowest weighted score -497.6, with a maximum

of 94 members. The hydrogen bonds were distributed throughout the functionally essential sites K339–Q57 (2.05 Å), R355–A119 (2.64 Å), and R355–S118 (2.66 Å), while other bonds in the active site were R355–K122, A119–R355, and H357–A119. p18 formed 22 interactions (including 14 hydrogen bonds) with C-PB1, and the minimum weighted score was -646 with 168 members. The interactions were formed between S337–T52 (2.27 Å), S337–D55 (2.90 Å), L50–R355 (2.02 Å), T52–S337 (2.84 Å), T52–E361 (2.55 Å), and H357–D55 (3.67 Å). Other interactions, such as M56–F404, H357–L50, and F404–L50, were also present. Similar to azurin, p28 interacted with PB2-CBD at S337–D55 (2.60 Å), R355–K74 (2.63 Å), L50–R355 (1.97 Å), and H357–D55 (3.18 Å) and formed a total of 29 interactions. It showed a minimum weighted score of -811 with 283 members. Other non-hydrogen bonds interactions in the functional sites included K339–D55, R332–D55, K339–D77, R355–D62, R355–D77, L50–F404, R355–L73, and H357–L50, the majority of the interactions being either electrostatic or hydrophobic. It was observed that H357, which is a major catalytic site, formed interactions in all three complexes.

Although p18 and p28 displayed favorable hydrogen bonds with m7G binding cleft residues with hydrophobic interactions, p28 formed higher interactions than p18 (Fig. 3).

Docking of azurin to PB2-RBD showed a total of 25 interactions (12 hydrogen bonds and the rest being electrostatic and hydrophobic interactions). The complex exhibited the lowest weighted score of -480.3 , with a maximum of 224 members. The hydrogen bonds in the cluster were distributed at the functionally important sites S634–M64 (2.86 Å), S709–D62 (2.80 Å), N711–Q57 (4.46 Å), N711–T61 (2.10 Å), N711–D62 (2.89 Å), S634–M64 (1.86 Å), S635–Y72 (2.30 Å), and S635–G67 (1.99 Å), while the other interactions in the important sites were formed between P585–V43, I710–A65, F633–M64, and F633–P115. p18 formed 20 interactions (including 8 hydrogen bonds) with PB2-RBD, and the minimum weighted score was -624.8 with 385 members. The hydrogen bonds were present in the functional sites R630–G67 (2.62 Å), R630–A65 (1.99 Å), S634–M56 (2.52 Å), S634–S51 (2.83 Å), N711–V59 (2.43 Å), Q632–L50 (2.21 Å), and G727–S51 (2.23 Å), while non-hydrogen bonds were found in the interaction sites S634–M56, F633–M56,

R630–M64, M631–M64, V690–L50, L691–L50, I726–L50, M631–V60, and I710–V60. Compared to azurin and p18, p28 formed a higher number of interactions (total 25); however, the number of hydrogen bonds was relatively lesser (total 7), out of which 6 were in the functional sites R630–Y72 (2.63 Å), S634–S51 (2.69 Å), S635–M56 (2.29 Å), Q632–L50 (2.01 Å), I726–S51 (2.01 Å), and G727–S51 (2.01 Å) (Fig. 4). A minimum weighted score of -614.9 with a maximum of 161 members was observed for the complex. Other non-hydrogen bond interactions in the functional sites were R630–D76, R630–D71, R630–D71, R630–D76, S634–M56, S635–M56, F633–M56, R630–L73, M631–L73, V690–L50, L691–L50, I726–L50, M631–V59, and M631–V60 that were mostly hydrophobic interactions.

p28 specifically binds to the PA endonuclease active site

Docking of azurin with PA resulted in a cluster of a maximum of 96 members and the lowest energy of -536 weighted score. Out of a total of 18 interactions, 9 interactions were hydrogen bonds spread across 3 functional sites

Fig. 3 The docked complex of PB2-CBD with azurin/derivatives: All three ligands bound to the same binding site and interacted with residues present in the active site. Color code: PB2-CBD (red), azurin (blue), p18 (yellow), and p28 (green)

with PB2-RBD, while p18 formed relatively lesser interactions. Color code: PB2-RBD (red), azurin (blue), p18 (yellow), and p28 (green)

R170–M13 (1.96 Å), R170–Q12 (1.51 Å), and R170–M13 (2.29 Å). While the other interactions were observed in 4 functional sites K122–E166, K122–E166, R170–M13, and A119–R170. On docking PA with p18, a lower number of interactions were observed. A total of 15 interactions were formed, out of which 10 interactions were hydrogen bonds present in the functional site R170–S51 (1.83 Å), R170–T52 (2.27 Å), and R170–T52 (1.79 Å). The maximum members in the cluster were 392, with the lowest weighted score energy of –654. Besides hydrogen bonds, the cluster contains only one interaction present in the functional site of R170–D55. The docking of p28 with PA showed similar types and number of bonds as of the azurin complex. A total of 18 interactions were observed, of which 8 hydrogen bonds were present in the functional sites S51–E80 (2.08 Å) and S51–E80 (2.71 Å). The docked complex had a cluster with a maximum of 132 members with the lowest energy of –757.8 weighted score. The chosen model also contained two non-hydrogen bonds present in the active sites L50–E80 and H41–A53. Compared to the azurin cluster, p18 exhibited a higher number of interactions, including hydrogen bonds

in the conserved domain. p28 cluster interacted with two of the cation-binding endonuclease catalytic domain residues (D108 and E119) (Fig. 5).

Analysis of the viral nucleoprotein

Encoded by genome segment 5, NP plays a central role in the viral replication/transcription cycle [57] and is the most abundant viral protein in the infected cells. NP has no intrinsic active enzymatic site [58], but it has a recognized function of RNA packaging, vRNP transport, and interaction with cellular proteins like actin [59, 60]. NP contains several nuclear localization signals and nuclear export signals to transport the vRNP across the host nucleus through importin- α and the CRM-1-dependent pathway [61, 62], as well as a nuclear accumulation signal (Q327–S345), potentially to store NP in the nucleus [63].

Docking of azurin with NP showed a total of 35 interactions (including 25 hydrogen bonds, and the rest were salt bridges, electrostatic and hydrophobic interactions). The complex showed the lowest weighted score of -556.2 .

Fig. 5 The docked complex of PA with azurin/derivatives: azurin and p18 bound to the same pocket in the conserved domain, while p28 interacted with the endonuclease domain of PA. Color code: PA (red), azurin (blue), p18 (yellow), and p28 (green)

with a maximum of 93 members. The hydrogen bonds were formed between R156–Q12 (2.28 Å), R156–M13 (2.03 Å), R174–G116 (2.72 Å), R174–P115 (2.22 Å), R175–Y72 (1.95 Å), R195–M13 (2.93 Å), R199–Q57 (2.34 Å), R199–Q57 (1.94 Å), Q57–R199 (2.63 Å), D71–D72 (2.74 Å), R174–P115 (3.11 Å), R195–G116 (3.40 Å), and D71–D72 (3.78 Å), while other interactions in the active sites were R156–L120, R174–P115, A119–R195, K113–D71, K113–D71, R65–E91, and K41–D72. The docking of p18 with NP showed the model had a minimum weighted score of -832.9 with 323 members and a total of 26 interactions, of which 19 were hydrogen bonds, present in the functional sites R174–Q57 (2.54 Å), R174–M56 (2.39 Å), R175–V59 (3.05 Å), R175–D62 (2.00 Å), R195–A53 (1.72 Å), R199–Q57 (3.05 Å), R199–Q57 (3.09 Å), R174–M56 (3.16 Å), R175–V59 (3.41 Å), and R195–Q57 (3.63 Å). The non-hydrogen bonds in the interaction sites were R175–D62, R175–D62, R174–M56, and R174–V60. Like azurin, p28 formed 19 hydrogen bonds but a higher number of total interactions (32 bonds). Nine hydrogen bonds were present in the functional sites R156–D76 (2.22 Å), R156–D77 (1.94 Å), R156–D76

(2.14 Å), R156–D77 (1.90 Å), R174–A53 (3.00 Å), R195–S51 (2.68 Å), R195–A53 (2.53 Å), L68–R361 (2.63 Å), and Y72–R361 (2.10 Å). The chosen cluster had a minimum weighted score of -915.9 , with a maximum of 362 members. Other non-hydrogen bond interactions recorded were R156–L50, R361–L68, A53–R174, R156–D76, R195–D55, R361–D69, R156–D76, and R361–D69 and the interactions were mostly salt bridges, electrostatic, and hydrophobic. In all three complexes, the azurin/derivatives formed interactions with the RNA-binding groove, a critical interaction site in the NP protein. It was observed that p28 interacted with Arg residues, which azurin and p18 could not. Therefore, p28 docked NP complex formed a more stable interaction and was the most favorable of all three docked complexes (Fig. 6).

Analysis of viral surface proteins

IAV contains three proteins HA, NA as well as M2, on its surface. The IAV strain type is derived from the combination of various subtypes of HA and NA. HA binds to sialic acid for host recognition and viral entry, whereas NA binds

Fig. 6 The docked complex of NP with azurin/derivatives: All the three ligands, i.e., azurin, p18, and p28, bound to the RNA-binding groove of the NP. Color code: NP (red), azurin (blue), p18 (yellow), and p28 (green)

and cleaves the sialic acid for successful viral budding [12]. M2 is a proton channel that acidifies the viral core for vRNP release into the host cytoplasm. These three proteins are crucial drug targets for treating IAV infections [13] and the design of potential vaccine candidates.

Azurin, p18, and p28 did not show any significant interaction with any of these three proteins. Azurin, p18, and p28 formed 19, 17, and 22 interactions, respectively, with HA. Even though the number of interactions was moderate, none of them were in the active site of HA, and thus, these interactions may not alter HA's functional properties. With NA, azurin formed 25, while p18 and p28 formed 21 interactions each. Like HA, in NA and azurin/derivative complexes, none of the interactions were present in the active site. Due to its smaller size, the number of interactions formed with M2 and azurin and its derivatives was also less (15 bonds with azurin, 5 bonds with p18, and 7 bonds with p28). Due to fewer interactions, M2 could not form stable complexes with azurin and its derivatives. Thus, it can be concluded that none of the surface proteins of the IAV H1N1 strain exhibited an effective and stable interaction with azurin and

its derivatives. A summary of the number of bonds between viral proteins and azurin derivatives is presented in Table 1.

MD simulations of viral protein–peptide complexes

The docking suggested that both azurin and p28 peptide derivative could bind well to the functional sites of the viral proteins. Interestingly, in certain docked complexes, p28 was able to form more number of interactions and better complexes than azurin. Thus, we used p28-interacting complexes for further MD simulation analysis.

The complexes were subjected to MD simulation for 100 ns each. Following the simulation, the trajectories were analyzed in detail. The analysis of the complexes of p28 with C-PB1, NS1-ED, PB2-CBD, PB2-RBD, NP, and PA showed an average RMSD value of $0.4857 \pm 8.2e^{-4}$ nm, $0.1981 \pm 5.02e^{-4}$ nm, $0.2461 \pm 4.16e^{-4}$ nm, $0.2156 \pm 3.30e^{-4}$ nm, 0.6306 ± 0.0013 nm, and $0.2218 \pm 4.18e^{-4}$ nm, respectively (Fig. 7a). During MD simulation, the p28 complexes with proteins NS1-ED, PB2-CBD, PB2-RBD, and PA showed fewer deviations in their structure, while C-PB1 and NP showed higher RMSD

Table 1 The number of hydrogen bonds (HB), electrostatic (ES), hydrophobic (HP) and other bonds (O) formed between viral proteins and azurin/p18/p28

	Azurin				p18				p28			
	HB	ES	HP	O	HB	ES	HP	O	HB	ES	HP	O
NS1	16	3	4	0	9	0	12	0	12	4	7	3
PB1	4	0	14	0	3	2	9	1	3	1	8	1
PB2-CBD	20	2	7	0	14	2	4	2	12	7	8	2
PB2-RBD	5	1	4	1	4	0	2	3	8	4	10	3
PA	9	3	6	0	10	3	2	0	11	3	7	2
NP	25	5	5	0	19	3	4	0	15	11	6	0
HA	8	2	8	1	13	0	3	1	9	4	10	0
NA	15	2	8	0	9	0	9	3	9	3	8	1
M2	2	0	4	0	1	0	4	0	5	0	2	0

values. To understand the compaction in the complexes upon interaction, the radius of gyration (R_g) was calculated. The R_g values of the p28 complexes with C-PB1, NS1-ED, PB2-CBD, PB2-RBD, NP, and PA were found to be $1.3654 \pm 7.28e^{-4}$ nm, $1.3851 \pm 1.28e^{-4}$ nm, $1.6069 \pm 2.10e^{-4}$ nm, $1.7235 \pm 2.62e^{-4}$ nm, $2.4581 \pm 6.48e^{-4}$ nm, and $1.7374 \pm 2.30e^{-4}$ nm, respectively (Fig. 7b). The structural fold of p28 complexes with NS1-ED, PB2-CBD, PB2-RBD, and PA was following the RMSD values, while complex with NP showed relatively higher R_g values. The p28 complex with C-PB1 was very compact with a low R_g value; the complex showed compaction in the structure after 25 ns that was maintained till the end of the simulation. The R_g data of the C-PB1 complex were in close corroboration with the RMSD value (Fig. 7a). To check whether the structures of C-PB1 and NP complexes were stable throughout the simulation, the frames were visualized, and the intramolecular hydrogen bonds of the complexes were calculated. It was observed that the number of intramolecular hydrogen bonds in these complexes was constant during the simulation, and no instabilities were visualized during the 100-ns period. In addition to the RMSD analysis of the complexes, the RMSD of the p28 peptide was also calculated. The RMSD values of only p28 peptide in the complex C-PB1, NS1-ED, PB2-CBD, PB2-RBD, NP, and PA were found to be $0.4857 \pm 8.28e^{-4}$ nm, $0.1981 \pm 5.02e^{-4}$ nm, $0.24 \pm 4.16e^{-4}$ nm, $0.2156 \pm 3.30e^{-4}$ nm, 0.6306 ± 0.0013 , and $0.2218 \pm 4.18e^{-4}$ nm, respectively (Fig. 7c). Furthermore, the RMSF analysis of p28 peptide in the complexes revealed that the p28 complexed with NS1-ED and PA showed higher fluctuations in almost all the residues. In contrast, in other complexes, p28 had fewer fluctuations in localized regions (Fig. 7d).

Once the stability of the complexes was confirmed, the affinity of the peptide p28 toward PB1, NS1-ED, PB2-CBD, PB2-RBD, NP, and PA was studied. Initially, the number of hydrogen bonds between the viral proteins and the p28 peptide was calculated. As represented in Fig. 7e, f, the number of hydrogen bonds was in the following series:

NP > C-PB1 > PB2-CBD > PB2-RBD > NS1-ED > PA. Next, using the g_mmpbsa tool, the energy distributions were calculated. Among the simulated complexes, the affinity of p28 toward NP strongest with a binding energy value of -2255.27 kJ/mol, followed by PB2-CBD and PB2-RBD with binding energies of -1284.22 kJ/mol and -1130.47 kJ/mol, respectively (Table 2). The binding energies data were in corroboration with the hydrogen bonds calculated for these complexes. The complexes with positive binding energy recorded sparse hydrogen bonds between the proteins and the p28 peptide. Also, in the RMSF analysis of p28, higher per residue fluctuations were observed for these complexes. We propose that p28 can stably and favorably bind to NP, PB2-CBD, PB2-RBD, and C-PB1, while binding of p28 to NS1-ED and PA was relatively less effective.

Discussion

RNA viruses have a very high tendency to mutate and evolve. They can develop resistance within a short time and after very few mutations [64]. The mutation rates are not fixed and evolve in response to drug or immune pressures. The resistance against the nucleoside analog azidothymidine (AZT) is a classic example of the importance of viral mutation. AZT was the first approved drug for human immunodeficiency virus (HIV) treatment, but HIV rapidly developed AZT-resistance mutations that led to the failure of AZT monotherapy. Two classes of anti-IAV drugs, adamantanes, and neuraminidase inhibitors targeting the M2 and NA protein, respectively, are used to treat IAV infections. However, IAV has acquired resistance against both classes of drugs by mutating M2 and NA proteins [65]. Thus, evaluation and development of new therapeutics against the influenza virus are needed. Though several studies have proved the efficacy of specific compounds against H1N1, no standard regimen against these viruses is available today [66]. In recent years, azurin has been shown to be effective against

Fig. 7 Analysis from the MD simulation trajectories: **a** RMSD of the complexes of p28 with C-PB1, NS1-ED, PB2-CBD, PB2-RBD, NP, and PA. **b** The radius of gyration of the complexes. **c** RMSD of p28 peptide derivative in C-PB1, NS1-ED, PB2-CBD, PB2-RBD, NP, and

PA. **d** RMSF analysis of p28 peptide derivative in C-PB1, NS1-ED, PB2-CBD, PB2-RBD, NP, and PA. **e, f** Number of hydrogen bonds between p28 peptide derivative and C-PB1, NS1-ED, PB2-CBD, PB2-RBD, NP, and PA proteins

Table 2 Binding energy calculations

	C-PB1	NS1-ED	PB2-CBD	PB2-RBD	NP	PA
Van der Waals energy (kJ/mol)	-356.72 ± 21.95	-137.65 ± 23.51	-321.8 ± 24.6	-246.26 ± 34.56	-339.96 ± 36.17	-326.03 ± 27.31
Electrostatic energy (kJ/mol)	-1011.15 ± 110.24	-220.03 ± 71.19	-1971.43 ± 104.4	-1242.48 ± 132	-3277.32 ± 115.29	-20.23 ± 162.71
Polar solvation energy (kJ/mol)	988.74 ± 131.97	522.08 ± 92.07	1052.31 ± 123.77	388.24 ± 133.09	1409.61 ± 102.25	722.552 ± 222.64
SASA energy (kJ/mol)	-45.35 ± 1.96	-20.23 ± 2.2	-43.31 ± 2.21	-29.97 ± 3.44	-47.59 ± 4.41	-40.36 ± 3.15
Binding energy (kJ/mol)	-424.47 ± 60.21	144.16 ± 64.38	-1284.22 ± 62.06	-1130.47 ± 75.87	-2255.27 ± 79.08	335.91 ± 90.33

Distribution of energies between NP, C-PB1, PB2-CBD, PB2-RBD, NS1-ED, and PA and p28 peptide derivative complexes. Standard deviation values are mentioned in the table

several parasitic diseases and cancer [17–19, 22, 67]. Based on these data, the present study was conceived to explore the anti-IAV activity of azurin and its peptides.

The study was initiated with the docking of H1N1 IAV proteins, i.e., NS1-ED, NP, C-PB1, N-PB1, PB2-CBD, PB2-RBD, PA, and M2 against azurin and its derivative peptides p18 and p28. Based on previous literature, the functional sites of these proteins were identified, and the interactions of azurin and its peptides were validated by the functionally active residue interactions. It was observed that C-PB1, NS1-ED, PB2-CBD, PB2-RBD, NP, and PA were targeted well by the peptide ligands. The docking studies also revealed that p28 could form strong and stable interactions with these proteins, whereas p18 could not generate similar interaction patterns. These results were in accordance with previous studies where p28 peptide elicited similar, if not better, interactions with the viral proteins and cancer [18, 67]. p28 also has the capability to enter cells and elicit favorable responses. Considering the docking results from this study and earlier reports, we decided to simulate and understand the stability, dynamics, and interactions between p28 and the potential target IAV proteins. p28 interacted with the NP, C-PB1, PB2-RBD, and PB2-CBD proteins with good binding energies and formed an average number of hydrogen bonds. The lowest energy structure extracted from the simulation was the best complex with strong interactions. It was surprising that NS1-ED and PA showed strong interaction in docking studies, while simulation studies contradicted it. The number of hydrogen bonds between p28 and NS1-ED and PA was also relatively less. Though both the viral protein and the peptide remained structurally stable throughout the simulation period, the p28 peptide did not interact well with NS1-ED and PA, as evident from the RMSF analysis of p28. We believe the higher residue fluctuations were recorded due to the non-favorable interactions in the interface leading to higher RMSF and fewer intermolecular hydrogen bonds.

We hypothesize that p28 peptide derived from azurin can be a potential macromolecule of therapeutic interest against

the H1N1 virus. With its targeting capabilities against NP, C-PB1, PB2-RBD, and PB2-CBD, it is highly possible that p28 could exhibit anti-H1N1 activity. Further effort should be focused on testing the IAV neutralizing ability of p28 using experimental in vitro and in vivo methods. At this point, it is essential to emphasize that p28 is under clinical trials as an anti-cancer drug (NSC745104). If it clears the phase 3 levels as an anti-cancer drug, it is possible to repurpose p28 as an anti-H1N1 peptide drug. Using multiple in silico approaches, this study establishes and validates the binding ability of p28 to these viral targets and examines data on the stability and interactions. The therapeutic peptide could be a revolutionary drug if found effective against H1N1 and can help fight other influenza infections. We advocate p28 must be synthesized and tested urgently as a public health priority.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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