ABSTRACT

Objective: Ivermectin is an FDA-approved, broad-spectrum anti-parasitic agent. It was originally identified as an inhibitor of interaction between the human 29 immunodeficiency virus-1 (HIV-1) integrase protein (IN) and the Importin (IMP) α/β1 30 heterodimers, which are responsible for nuclear import. Recent studies demonstrate that ivermectin is worthy of further consideration as a possible SARS-CoV-2 antiviral.

Methods: We built the pathogen-host interactome and analyzed it using PHISTO. We compared Ivermectin and plant molecules for their interaction with Importin α3 (IMA3) using molecular docking studies.

Results: A phytochemical ATRI001 with the lowest binding energy-7.290 Kcal/mol was found to be superior to Ivermectin with binding energy-4.946 Kcal/mol.

Conclusion: ATRI001 may be a potential anti-SARS-CoV-2 agent; however, it requires clinical evaluation.

Keywords: Ivermectin, SARS-CoV-2, IMA3, Phytochemical and Molecular docking

INTRODUCTION

Severe acute respiratory syndrome coronavirus-2 (SARS-CoV-2), the causative agent of COVID-19 pandemic, is a single-stranded positive-sense RNA virus, which is closely related to earlier SARS-CoV [1]. Available reports on SARS-CoV proteins have demonstrated a potential role of IMPα/β during infection in the signal-dependent nuclear-cytoplasmic shuttling of the SARS-CoV Nucleocapsid protein (2-5), that may cause significant impact on host cell division [6, 7]. Additionally, the SARS-CoV accessory protein ORF6 has been shown to antagonize the antiviral activity of the STAT1 transcription factor by sequestering IMP α/β on the rough ER/Golgi membrane [8]. These reports suggest that nuclear transport inhibitory activity of ivermectin may be effective against SARS-CoV-2.

Seven human isoforms of IMA mediate nuclear import of cargo into the tissue in an isoform-specific manner. Active transport of proteins from the cytoplasm to the nucleus is mediated by a family of nuclear transport receptors known as importins (or karyopherins), together with several ancillary proteins, including nucleoporins and Ran [8-10]. The classical nuclear import pathway is initiated by a unique NLS on importin β [11]. The cargo-IMA complex gets transported through the nuclear pore by building a heterotrimeric complex with importin β (IMB), necessitating interactions with FG repeat regions on nucleoporin proteins [12, 13].

Once the complex traverse the nuclear envelope, the RanGTP dissociates the complex, and the import receptors get recycled back to the cytoplasm for the next rounds of transport [14-17]. IMA includes importin β-binding (IBB) domain at its N-terminal and NLS binding domain towards C-terminal featuring ten armadillo (ARM)-repeat motifs [18]. Most commonly, the cargo NLS binds on the concave site of the ARM repeats and involves interactions at either the major site through ARM repeats 2–4 or minor site ARM repeats 6–8. The classical monopartite NLSs (e.g., SV40T-ag [19]) are known to interact with the major site, while, human phospholipid scramblase [20] and TPX2 [21] with the minor site. The classical bipartite NLSs like nucleoplasmin interact with both the major and minor sites [19]. Although this process has been well characterized for the importin α1 adaptor protein, many nuclear proteins exhibit specificity for other importin α isoforms.

For example, both RCC1 [22], HIV-1 integrase [23], W protein of Nipah virus (NiV) [24, 25], avian influenza PB2 viral polymerase subunit [26] and SARS-CoV bind specifically to importin α3. Whereas, STAT1, a signaling molecule in the innate immune system response, binds specifically to the convex C-terminal surface of importin α5, α6, and α7 [27, 28].

A wide variety of active phytochemicals have been found to have therapeutic applications against viruses. The antiviral mechanism of these agents may be explained by their antioxidant activities, scavenging capacities, inhibition of DNA, RNA synthesis, or blocking of viral reproduction. Epidemiological and experimental studies have revealed that a large number of phytochemicals have promising antiviral activities [29].

More than 220 Phyto-compounds evaluated by others for activity against anti-severe acute respiratory syndrome-associated coronavirus (SARS-CoV) using a cell-based assay measuring the SARS-CoV-induced cytopathogenic effect on Vero E6 cells and compounds [30–32] showed excellent activities [33, 34]. The bioactive compounds with anti-SARS-CoV activity in the mmol range included abietane and labdane-type diterpenes sesquiterpenes and lupane-type triterpenes [34].

The current study is aimed to screen a library of plant small molecules library against IMA3 using molecular docking studies. The plant small molecule library developed in-house, consists of 4,08,000 small molecules, which are classified using physicochemical parameters as major classifiers.

MATERIALS AND METHODS

Topological analysis of pathogen-host interactome (PHI)

The drug target identification and validation were carried out using a network-based topological analysis method using a web-based application Pathogen-Host Interaction Search Tool (PHISTO) available at the URL: http://www.phisto.org/browse.xhtml#. PHISTO is the most com-
prehensive pathogen-human protein-protein interaction database on the web. It is used to explore molecular connectivities between the pathways in SARS-CoV and Human interaction through topological analysis [35, 36]. The information over the interaction of human IMA3 (Uniprot ID: O00629) was retrieved from PHISTO.

**Protein preparation**

The crystal structure of IMA3 was pre-processed for docking studies using the Protein Preparation Wizard [37] available in Schrödinger suite 2019-2. Crystallographic water molecules (water molecules without 3 H bonds) were deleted and hydrogen bonds corresponding to pH 7.0 were added, considering the appropriate ionization states for both the acidic and basic amino acid residues. Missing side-chain atoms were added, and breaks present in the structure were built using Prime v4.0, Schrodinger 2019-2 [38]. Using the OPLS-2005 force field [39] energy of the modeled structure was minimized.

**Binding site prediction**

The crystal structure of Hendra virus W protein C-terminus in complex with IMA3 crystal form 2 (PDB ID: 6BVW) and IMA3 in cargo free state (PDB ID: 6BVZ) was superimposed to understand the conformational differences between cargo bound state and free state. The protein structure alignment and superimposition was performed concerning backbone atoms using Schrodinger package Maestro ver93.

**Ligand preparation**

The three-dimensional conformers of Ivermectin and 4,08,000 small plant molecules in our library were subjected for ligand minimization using the Ligprip application provided in Schrödinger Maestro [39]. The ligand minimization was performed by assigning force field OPLS_2005 and stereochem was calculated retaining specific chiralities. The ADME (absorption, distribution, metabolism, and excretion) predictions were done for all ligands using the QikProp package (version 4.6 Schrodinger, LLC, New York, NY, 2015) [40].

**Molecular docking**

The crystal structure of human IMA3, an adaptor protein involved in the transport of viral protein from the cytoplasmic compartment of an infected cell into a nuclear compartment through NPCs, was prepared using the Protein Preparation Wizard [41]. The major NLS binding site (137–229) was defined with a 10 Å radius around the selected residues (Asn141, Ser144, Trp179, Asn183, and Asn219) present in the crystal structure which are identified as key residues in SARS_nCoV-2 protein and host IMA3 complex formation and a grid box 20X20X20 Å was generated at the centroid of the active site for docking. The molecular docking of prepared small molecules over IMA3 was performed using Grid-Based Ligand Docking with Energetics Glide v7.8, Schrodinger 2019-2 [42] in ‘High Throughput Virtual Screening’ HTVS mode without applying any constraints.

Considering the glide score 13,000, molecules were shortlisted and subjected for docking in ‘standard precision’ SP mode. The top molecules with high glide score and Ivermectin were further screened using in ‘extra precision’ mode. The final best-structured docking was selected using a Glide score function, Glide energy, and Glide E mode energy. Finally, the lowest-energy docked complex of three plant molecules and Ivermectin were interpreted to derive the conclusion.

**RESULTS AND DISCUSSION**

**Topological analysis of pathogen-host interactome and drug target identification**

A thorough analysis of virus-host interactomes may reveal insights into viral infection and pathogenic strategies [43, 44]. In the current study, IMA3-centric Virus-Human interactome was built by screening domain interactions between Virus-Human protein-protein interactions (PPIs), as shown in table 1. The list of human viruses, including SARS-CoV is reported to transport their cargo protein through IMA3 (Uniprot ID: 000629) mediated nuclear transport mechanism. Due to the lack of experimental interaction data on SARS-CoV-2, the significant identity between SARS-CoV (Taxonomy ID: 227859) and SARS-CoV-2 (Taxonomy ID: 2697049) proteomes has encouraged us to considered and the SARS-CoV-Human interactome. Through PHI analysis, it is understood that the transportation of SARS-CoV-2 cargo protein N6S from the cytoplasmic compartment to the nuclear compartment of the infected host cell is mediated by IMA3 [2,3]. Hence, IMA3 is identified as a potential target and molecular docking was performed.

**Binding site prediction**

The human IMA3 has its significant function in nuclear protein import as an adapter protein for nuclear receptor KPNB1. IMA3 binds specifically and directly to substrates containing either a simple or bipartite NLS motif [45]. Docking of the importin/substrate complex to the nuclear pore complex (NPC) is mediated by KPNB1 through binding to nucleoporin FxFG repeats and the complex is subsequently translocated through the pore by an energy-requiring, Ran-dependent mechanism. At the nucleoplasmic side of the NPC, Ran binds to IMB, and the three components separate to get re-exported from the nucleus to the cytoplasm where GTP hydrolysis releases Ran from importin [46]. Hence, the NLS site on IMA3 is very important in nuclear protein import.

As tabulated in table 2, IMA3 consists of an N-terminal hydrophilic region, a hydrophobic central region composed of 10 ARM repeats, and a short hydrophilic C-terminus. The N-terminal hydrophilic region contains the IBB domain, which is sufficient for binding IMB and essential for nuclear protein import [47]. The IBB domain is thought to act as an intragenic autoregulatory sequence by interacting with the internal autoinhibitory NLS as shown in fig. 1.

![Fig. 1: Simplified representation of IMA3 and nuclear pore complex of cargo containing a classical NLS occurs via importin-α/importin-β heterodimer. As noted IMA3 has major and minor NLS binding sites specific to cargo type](image)
Table 1: Interaction of human importin α3 (Uniprot ID: 000629) with diverse pathogen proteins

| Pathogen                                    | Taxonomy ID | Uniprot ID | Pathogen protein | Experimental method            | Pubmed ID |
|----------------------------------------------|-------------|------------|------------------|-------------------------------|-----------|
| Bovine papillomavirus TYPE I (BPV 1)         | 10559       | P03116     | VE1_BPV1         | CIP                           | 17192311  |
| Deltapapillomavirus 4                       | 337052      | P03116     | VE1_BPV1         | CIP                           | 17192311  |
| Hendra virus ISOLATE                        | 928303      | P0C1C6     | W_HENDH          | TAP                           | 22810585  |
| HORSE/ AUSTRALIA/ HENDRA/1994               |             |            |                  |                               |           |
| Human herpesvirus 8                         | 37296       | Q04917     | O4917_HHV8       | Other Methods                 | 25544563  |
| Human herpesvirus 8 STRAIN G1K8             | 868565      | Q2HRC8     | ORF11_HHV8P      | ACT                           | 25544563  |
| Human immunodeficiency virus 1 (HIV1)        | 11676       | B9A2Q4     | B9A2Q4_9HIV1     | Y2H, CIP, NMR                 | 8659115,  |
|                                               |             |            |                  |                               | 9549947   |
| HIV1                                         | 11676       | Q79822     | Q79822_9HIV1     | Y2H, CIP, NMR                 | 8659115,  |
|                                               |             |            |                  |                               | 9549947   |
| HIV1                                         | 11676       | Q72874     | Q72874_9HIV1     | FT, imaging technique         | 103656569 |
| HIV1                                         | 11676       | Q77YF8     | Q77YF8_9HIV1     | pull down                      | 22174317  |
| HIV1 ISOLATE BRU                             | 11686       | P04620     | REV_HV1R         | pull down                      | 22174317  |
| HIV1 ISOLATE HXB2                           | 11706       | P04618     | REV_HV1H2        | ACT                           | 22174317  |
| HIV1 ISOLATE HXB2                           | 11706       | P04585     | POL_HV1H2        | ACTC, pull down                | 20554775  |
| HIV1 ISOLATE HXB2                           | 11706       | P069726    | VPR_HV1H2        | ATC                           | 20554775  |
| Influenza A virus STRAIN A/AICH/2/1968 (H3N2) | 387109     | I6TAH8     | I6TAH8_16A0      | ATC                           | 28169297  |
| Influenza A virus STRAIN A/PUERTO RICO/8/1934 (HI N1) | 211044   | P03466     | NCAP_J34A1       | pull down, ATC                 | 12740372  |
| Influenza A virus STRAIN A/PUERTO RICO/8/1934 (HI N1) | 211044   | P03438     | PB2_J34A1        | ATC                           | 28169297  |
| Influenza A virus STRAIN A/PUERTO RICO/8/1934 (HI N1) | 211044   | P03433     | PA_J34A1         | ABC                           | 26789921  |
| Influenza A virus STRAIN A/UDORN/1972 H3N2 | 385599      | Q20MD0     | Q20MD0_9HNA      | Other Methods                 | 17376915  |
| Influenza A virus STRAIN A/VICTORIA/3/1975 H3N2 | 392809  | H9XJ5      | H9XJ5_175A3      | Molecular sieving, pull down, x-ray crystallography | 25599645 |
| Influenza A virus STRAIN A/Wilson-Smith/1933 (H1N1) | 381518   | P03427     | PB2_J33A0        | CIP                           | 25464832  |
| Influenza A virus STRAIN A/Wilson-Smith/1933 (H1N1) | 381518   | P15682     | NCAP_J33A0       | CIP                           | 25464832  |
| Influenza A virus STRAIN A/Wilson-Smith/1933 (H1N1) | 381518   | P03470     | NRAM_J33A0       | CIP                           | 25464832  |
| Influenza A virus STRAIN A/Wilson-Smith/1933 (H1N1) | 381518   | B4URF7     | B4URF7_9HNA      | ACT, ATC                      | 26651948, |
| JC polyomavirus                              | 10632       | Q9DIUG7    | Q9DIUG7_P0VJC    | TAP                           | 28169297  |
| Macaca mulatta polyomavirus 1               | 1891767     | P03070     | LT_SV40          | ELISA, TAP, pull down          | 9168958,  |
|                                               |             |            |                  |                               | 22810586, |
|                                               |             |            |                  |                               | 26701745  |
| Macaca mulatta polyomavirus 1               | 1891767     | Q9DH70     | Q9DH70_SV40      | Y2H                           | 9168958,  |
|                                               |             |            |                  |                               | 12740372  |
| Merkel cell polyomavirus                     | 493803      | B8ZX42     | B8ZX42_9POLY     | TAP                           | 22810586  |
| Murād herpesvirus 4 (Marine herpesvirus 68)  | 33708       | O41946     | O41946_MHV68     | TAP                           | 22028648  |
| Nipah virus                                  | 121791      | Q997F2     | V_NIPAV          | TAP                           | 22810585  |
| Nipah virus                                  | 121791      | P0C1C7     | W_NIPAV          | TAP                           | 22810585  |
| Plasmodium yoelii yoelii                     | 73239       | P06914     | CSP_PLAYO        | pull down                      | 17891117  |
| Severe acute respiratory syndrome (SARS)     | 227859      | P59634     | NSc_CVH5A       | Y2H                           | 17596301  |
| Yersinia pestis                              | 632         | Q8D1P8     | Q8D1P8_YERPE     | Y2H, Pooling approach         | 20711500  |

Table 2: List of molecular features and functional sites on human IMA3

| Feature key | Description | Position(s) on 000629 |
|-------------|-------------|-----------------------|
| Domain      | IBB         | 2–58                  |
| Repeat      | ARM 1, Truncated | 66–106               |
| Repeat      | ARM 2       | 107–149               |
| Repeat      | ARM 3       | 150–194               |
| Repeat      | ARM 4       | 195–233               |
| Repeat      | ARM 5       | 234–278               |
| Repeat      | ARM 6       | 279–318               |
| Repeat      | ARM 8       | 319–360               |
| Repeat      | ARM 9       | 361–400               |
| Repeat      | ARM 10: Atypical | 401–443              |
| Region      | NLS binding site (major) | 447–485              |
| Region      | NLS binding site (minor) | 137–229              |
| Motif       | Nuclear localization signal | 306–394              |
Binding of KPNB1 probably overlaps the internal NLS and contributes to a high affinity for cytoplasmic NLS-containing cargo substrates [48]. After dissociation of the importin/substrate complex in the nucleus, the internal autoinhibitory NLS contributes to a low affinity for nuclear NLS-containing proteins. The major and minor NLS binding sites are mainly involved in recognition of simple or bipartite NLS motifs. As described by Elena et al., in 1998 [49], structurally located within a helical surface groove, they contain several conserved Trp and Asn residues of the corresponding third helices (H3) of ARM repeats which mainly contribute to binding as shown in fig. 1. The secondary structure superimposition of the two IMA3 structures in cargo bounded and Free State reveals a global root-mean-square deviation (RMSD) of 1.27 Å. And the major RMSD contribution was observed at the ARM2-3 position because of the remarkable conformational change in the loop at the NLS site, as shown in fig. 2A-2C. Hence the amino acids at the ARM2-3 region (Asn141, Ser144, Trp179, Asn183, and Asn219) were identified as a key binding site residue.

Fig. 2: A: The crystal structure of IMA3 in cargo free state with an open loop at the NLS Major Site (PDB ID: 6BVZ). B: The crystal structure of Hendra virus W protein C-terminus (the loop in golden color) in complex with IMA3 crystal form 2 rendering closed loop at NLS Major Site (PDB ID: 6BWA). C: The superimposed conformers of cargo bounded and free structures

Fig. 3: Molecular interaction of ligands with at NLS major binding site on human IMA3 (PDB ID: 6BWA). A: Two-Dimensional (2D) representation of ATRI001 interaction with IMA3 facilitated by 6 H-bonds shown in pink arrow. B: Three dimensional (3D) illustration of ATRI001 interaction with IMA3 facilitated by 6 H-bonds shown in yellow dotted lines. C: Three dimensional (3D) illustration of Ivermectin interaction with IMA3 facilitated phobic enclosures but no H-Bonds
Molecular docking

The molecular docking study was performed to understand the molecular interaction of plant molecules with human IMAS. Initial HTVS screening suggested 13,000 molecules with reasonable interaction with IMAS, and further, the shortlisted molecules were docked in the standard precision mode where the accuracy of prediction is better than the HTVS mode [50].

The docking in SP mode has suggested 20 top molecules as lead molecules. As iVermectin is reported to inhibit IMAS mediated cargo Nuclear Import, it was also subjected to subsequent docking in extra precision (XP) mode. All 20 plant molecules showed better interaction than iVermectin with IMAS. However, the lowest energy ligand-bound conformers are always energetically favorable [51]. Hence, the plant molecule ATRI001 with the lowest binding energy-7,290 Kcal/mol was identified as a potent inhibitor of IMAS by displaying better interactions with NLS site on human IMAS by forming 6 H-bonds with Asp 102, Asn141, Ser 144, Trp179, Asn183 and Asn219 as shown in fig. 3A and 3B.

ATRI001 is a glycoconjugate having (2R,3R,4S,5S,6R)-6-Ethylhexole-2,3,4,5-tetrol as a monosaccharide sugar group and connected to phtyo-moiety [11, 2R,4S]-1-{GR}-3-Hydroxybut-1-enyl]-2,6,6-trimethylcyclohexane-1,2,4-triol through glycosidic linkage. The interaction of sugar group with IMAS is facilitated by 3 H-bonds, two of them were formed by donating electrons to side-chain atoms of Asn141 and Ser 144, one of 3 H-bonds was formed by accepting the electron from backbone atoms of Asp102. Hence, the interaction between photo-moiety and IMAS was established by means of 3 H-bonds; two of them were formed by donating electrons to side-chain atoms of Asn183 and Asn219, remaining H-bond found formed by accepting the electron from backbone atoms of Asp102. iVermectin, a known IMAS inhibitor, showed its lowest binding energy-4,946 Kcal/mol at its lowest energy conformation without any hydrogen bonds, as shown in fig. 3C. Hence, the interaction of ATRI001 was found better than iVermectin by using three parameters: fitting at NLS binding site, low interaction penalties, and a good number of bonded interactions.

CONCLUSION

The Comparative analysis to evaluate the IMAS inhibition activity of iVermectin and plant small molecules using in silico approaches suggested that a plant molecule ATRI001 is superior to iVermectin. Our in silico experiment shows ATRI001 can block the nuclear import of SARS-CoV-2 cargo. These predictions, however, require further investigations.

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Nil

AUTHORS CONTRIBUTION

All the authors have contributed equally.

CONFLICT OF INTERESTS

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