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Deciphering antiviral efficacy of malaria box compounds against malaria exacerbating viral pathogens- Epstein Barr virus and SARS-CoV-2, an in silico study

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ABSTRACT

In malaria endemic countries, coinfections and cotransmissions of different viral pathogens are widely reported. Prior studies have shown that malaria can trigger the Epstein-Barr virus (EBV) reactivation in the body. Besides, the altered immunity due to malaria could increase susceptibility to acquire co-circulating viruses like SARS-CoV-2 or vice versa during pandemic times. The dual burden of pathogens can deteriorate health by inducing disease severity. There are no or limited antiviral therapies available against EBV and SARS-CoV-2. Exploring the novel antimalarials for checking antiviral efficacy and using them in such cases could be the efficient approach of ‘hitting two birds with one stone’. We investigated the antiviral potency of medicine for a malaria venture’s malaria box containing 400 drug-like or probe-like compounds with experimentally proven antimalarial activity. We utilized a molecular docking approach to screen these compounds against crucial proteins- EBNA1 of EBV and RdRp of SARS-CoV-2 respectively. Based on binding affinity we shortlisted the top three compounds for each protein. Further, for validation of complex stability and binding, the protein–ligand complex is subjected to 100 ns molecular dynamic simulation. All the compounds showed stable binding with respective proteins. Based on binding free energies, involvement of important residues from target sites, and ADMET properties of compounds, the top ligand for each protein is selected. Ligand B (MMV665879) for EBNA1 ($\Delta G_{\text{bind}} = -183.54 \text{ kJ/mol}$) and Ligand E (MMV665918) for RdRp ($\Delta G_{\text{bind}} = -172.23 \text{ kJ/mol}$) could act as potential potent inhibitors. These antimalarial compounds can hence be utilized for further experimental investigation as antivirals against EBV and SARS-CoV-2.

1. Introduction

In malaria-endemic regions, along with malaria parasite, people often get exposed to several other pathogens. Previous studies have shown the cotransmission and coinfection cases of various viral pathogens with malaria. In malaria positive individuals, underlying viruses like Epstein Barr virus (EBV) and Human immunodeficiency virus (HIV) may get triggered at cellular level and develop an opportunistic infection [1,2]. While co-circulation of disease-causing viruses, like dengue or severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) in malaria patients, could increase the host susceptibility to develop coinfections due to altered immune status [3]. These opportunistic viral infections could deteriorate overall health and may generate fatal outcomes. Overlapping symptoms during coinfections put forward diagnostic as well as therapeutic challenges [4,5]. In coinfection conditions, the presence of one pathogen may get neglected and the health condition may worsen. This negligence could be more in case of viral infections due to factors like unknown episodes of etiology and the requirement of sophisticated testing tools. In such a scenario the broad-spectrum drugs which can target both the coinfecting organisms could help in effective treatment (Fig. 1).

Many studies have reported the coinfection or reactivation of the EBV during malaria onset. Both the pathogen altogether are responsible for the development of Burkitt’s lymphoma. Additionally, during the recent pandemic, the SARS-CoV-2 also affected malaria-endemic areas. Various studies reported the cases of SARS-CoV-2 coinfection with Plasmodium sp. which is a causative agent of malaria [5,6]. Interestingly, like malarial parasite, SARS-CoV-2 was also reported to cause

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EBV reactivation [7]. However, there is limited or no approved antiviral therapy available which could be effective against these viruses [8,9].

EBV is a pervasive virus that infects ~90–95% of the world’s population by adulthood [10]. The virus establishes persistent infection by resting in latent form. It can develop infectious mononucleosis in immunocompetent individuals [10]. While in immunocompromised situations or under the influence of coinfections, it may lead to cancers like Burkitt’s lymphoma, nasopharyngeal carcinomas or neurological complications associated with multiple sclerosis, Alzheimer’s disease, etc. [11,12]. The primary targets of the virus are B-cell and epithelial cells but it is also capable of infecting cells of the blood–brain barrier and central nervous system [13–17]. In the reservoir B-cells, the virus has been reported to establish different types of latency stages like type III, II, and I based on its differential gene expression pattern. However, EBNA1 is the only viral protein expressed through all these latent stages [18], which highlights its importance for viral existence. It tends to bind DNA at specific sites like oriP, which can initiate viral DNA replication; additionally, it plays a role in viral episome maintenance, cell survival, and proliferation [18]. The protein has 641 amino acid long sequence and functions actively in homodimeric form. The C-terminal region of protein from 459 to 607 amino acids is involved in DNA binding and is necessary for all known functions [19]. It comprises two domains, a flanking (470–503 amino acids) and a core (504–604 amino acids) domain (Fig. 2a). The region of the flanking domain is majorly involved in determining contacts with the DNA [19]. To date, no available drugs have proved effective in targeting EBV latency. The clinical drugs used against herpesviral infection (targeting viral DNA polymerases) have partial inhibitory activity against EBV lytic phase. In recent studies, attempts to inhibit EBNA1 using peptides or small molecules have been made [20–22]. The inhibitory compounds targeted residues around the flanking domain (Fig. 2a) and subsequent blocking its interaction with DNA, have been shown to alter viral infection inside cells [20,22]. Repurposing the known drug-like molecules targeting these regions could aid in controlling and/or diminishing the EBV infection as well as reactivation in the infected cells.

In recent times, the pandemic caused by the novel coronavirus, SARS-CoV-2 also drastically affected malaria endemic regions. The need for antiviral therapy against this virus became a global concern [9,23,24]. Over time, various strains of SARS-CoV-2 evolved and put forward new challenges to control the high transmission of the virus. Although vaccines are available, there has been reports of breakthrough infections due to waning humoral response and possible immune escape brought by the emergence of variants of concerns [25,26]. Thus, the need to discover novel therapeutics is of utmost importance to address symptoms due to acute infections. Various studies utilized computational tools to put forward potent SARS-CoV-2 inhibitors to accelerate the further research [27–31]. SARS-CoV-2 is an RNA virus that encodes various structural and non-structural proteins (nsp) to establish successful infection. The virus utilizes nsp12 which is RNA-dependent RNA polymerase (RdRp) for replication and transcription of the viral genome. The other proteins like nsp7, nsp8, nsp9, and nsp14 work in coordination with RdRp to carry out the designated molecular events. Compared to other proteins of SARS-CoV-2, the RdRp has a conservative sequence. The nsp12 comprises different domains like a nidovirus RdRp-associated nucleotidyltransferase domain (NiRAN), an interface domain, N-terminal β-hairpin, and right-hand RdRp domain [32]. This right-hand domain includes other subdomains namely fingers, palm, and thumb (Fig. 2b). These subdomains contain conserved polymerase motifs A to G (Fig. 2b) which forms the active site of RdRp. The entry of the RNA template is expected via motifs A and C through groove formed by motifs F and G. The thumb subdomain and motif E bed the primer strand. The product-template hybrid leaves the active site from the RNA exit tunnel at the contrary site of template entry [32,33]. Due to the vital role
of RdRp in replication and for the viability of RNA viruses inside cells, there are statistically fewer chances of mutations occurring in this gene. Hence, the RdRp has been explored as an ideal therapeutic target for antiviral drugs. Being nucleoside analogs, prodrugs like Remdesivir and favipiravir have been shown to inhibit RdRp activity, as they get incorporated into the growing chain and terminate the further process. However, the nsp14, working in coordination with RdRp, has the proofreading activity, which may remove the misincorporation of pro-drug moieties in the nascent chain. Hence, the previous studies have utilized an interesting approach to target RdRp with non-nucleoside inhibitors or drugs. Investigation of various known drug-like compounds for potency to use as non-nucleoside inhibitors could reveal and put forward numerous molecules as effective antivirals.

In the vision of accelerating drug discovery research for malaria and neglected diseases, the Medicines for Malaria Venture (MMV) compiled the Malaria Box having a collection of 400 drug-like or probe-like compounds with proven in-vitro antimalarial activity. Looking towards concerns regarding the association of EBV and SARS-CoV-2 with malaria in endemic regions, we aimed to screen the novel antimalarials from the malaria box against these two pathogens. This approach could be used as ‘hitting two birds with one stone’ in cases of malaria-EBV or malaria-COVID-19 coinfection and can be implemented as antivirals against lone viral infections. Based on efficacy, these compounds were shortlisted from around structurally unique 20,000 antimalarial molecules in three phenotypic (high throughput screening) campaigns undertaken by GlaxoSmithKline (GSK), Novartis-GNF, and St. Jude Children’s Research Hospital. The compounds from the malaria box have been repurposed and proven to be effective against various other pathogens in numerous studies.

Considering the important role of EBNA1 of EBV and RdRp of SARS-CoV-2 in infection, we utilized an in-silico approach to target these proteins with malaria box compounds. Using the molecular docking approach we shortlisted the top 3 compounds, based on their binding affinity, as potent small molecule inhibitors against respective proteins. Further, we validated the stable binding and interactions of these compound-protein complexes using molecular dynamic (MD) simulations. All the protein ligand complexes showed stable interactions. Based on binding energies, involvement of important residues, and ADMET properties we put forward one compound for each protein as a potent inhibitor. These compounds can further be utilized in experimental approaches to inhibit the EBV or SARS-CoV-2 in infections as well as in malaria coinfection situations.

2. Methods

2.1. Structure retrieval and protein-ligand preparation

For EBNA1, the structure with the 1.35 Å resolution (PDB ID: 6NPP) while for the RdRp, the structure with a resolution of 2.90 Å (PDB ID: 6M71) was utilized. EBNA1 was not having any missing amino acid residues in the given structure and was used by removing...
attached ligands for further operations. In the case of RdRp, the chain A denoting actual RdRp activity was considered and chain B/C/D comprising cofactors were eliminated as referred from previous studies \[42,43\]. The terminal missing amino acids from chain A of RdRp were excluded while to fill missing residues in the range of 51–68, 75 and 103–111, the structure was modeled using the Modeller tool \[44\]. The obtained structure with the lowest RMSD and Z-score was considered and taken for loop refinement. GalaxyWEB was used for loop refinement and the loop refined structure was used for further analysis \[45\]. A list of antimalarial compounds was retrieved from the MMV portal, and each compound 3D structure was downloaded from PubChem. The three test compounds for which 3D structure was unavailable (MMV006545, MMV007092, and MMV019881), were developed using Chimera and energy minimized using Schrödinger suite. For EBNA1 three reference compounds (positive control), previously reported to have inhibitory activity, \[20\] were considered namely VK0044, VK0064, and VK0497. Similarly, for RdRp the Remdesivir was used as a reference compound (positive control). For protein preparation the water molecules were removed, the addition of polar hydrogens and Kollman charges was done using AutoDock Vina 1.1.2. Ligands were also subjected to preparation by merging nonpolar hydrogens, adding Gasteiger charges and identifying aromatic carbons and rotatable bonds using AutoDock Vina 1.1.2.

### 2.2. Target site prediction and molecular docking

For EBNA1 the target residues were selected from literature as well as our previous study \[20,22\] and for RdRp the active site was selected from literature \[46\]. The active site residues were further validated using CASTp and FT-Map servers \[47,48\]. The details of target site residues are provided in Fig. 2. For EBNA1 the gridbox of dimensions center \(x = -8.0948\), \(y = -32.6590\), \(z = -15.1687\) and size \(x = 17.5751\), \(y = 15.0108\), \(z = 17.0003\) was considered enclosing the target residues Lys477, Asn480, Ile481, Gly484, Ser516, Asn519, Leu520, Leu582, Thr585, Lys586, and Thr590. For RdRp, the gridbox of dimensions center \(x = 118.52\), \(y = 114.27\), \(z = 133.44\) and size \(x = 20.87\), \(y = 30.20\), \(z = 29.75\) was considered enclosing active site motifs with respective amino acid range viz. Motif G: 499–511, Motif F: 544–560, Motif A: 612–626, Motif B: 678–710, Motif C: 753–767, Motif D: 771–796, Motif E: 810–820. Each protein was then subjected to 400-ligand docking using

| Sr. no. | Ligand MMV Box ID | Pubchem CID | Name | 2D structure | Targetprotein | Binding energy (kcal/mol) |
|---------|-----------------|-------------|------|--------------|----------------|--------------------------|
| 1. A    | MMV 665,883     | 2,192,323   | N-[3-[3-(1H-benzimidazol-2-yl)phenyl]carbamoyl]phenyl|![Image](link) | EBNA1         | -9.3                     |
| 2. B    | MMV 665,879     | 1,271,219   | N-[3-(1H-benzimidazol-2-yl)phenyl]-| ![Image](link) | RdRp          | -10.8                    |
| 3. C    | MMV 665,954     | 45,176,114  | N-[7-pyrrolin-4-yl-2,3-dihydro-1-benzofuran-2-yl]methyl]-4,5,6,7-tetrahydro-1-benzothiophene-3-carboxamide | ![Image](link) |              | -9.1                     |
| 4. D    | MMV 000,753     | 135,403,990| 4-(4-{8-[2-(4-hydroxyphenyl)-4-phenyl-1H-imidazol-5-yl]dibenzo[b,| ![Image](link) |              | -10.8                    |
| 5. E    | MMV 665,918     | 16,815,088  | N-(benzo[d][1,3]dioxol-5-yl)-2-(3-(3-fluorophenyl)-1,2,4-triazolo[4,3-b]pyridazin-6-y]thio)acetamide | ![Image](link) |              | -9.5                     |
| 6. F    | MMV 011,895     | 135,444,435| 4,4'-[benzene-1,4-diylbis(oxy-1H-benzimidazole-5,2-diyl)]dianiline | ![Image](link) |              | -9.3                     |
2.3. Molecular dynamic simulation

The molecular dynamics simulations analysis was performed on GROMACS 2020.4 version [50]. GROMOS 54A7 parameterized force field was used for simulation. Docked structure of ligands with its coordinate was separately saved and hydrogen was added in the structure using Chimera to save the PDB files of the ligands having docked conformation. These structures were used to generate the force field parameters using ATB version 3 server and ITP files generated by the server were used for simulation studies [51]. In our study, a total of eight simulations were performed, one each for the proteins alone (as control) and three complexes of each protein using different ligands. SPC water model (Simple Point Charge) was used to solvate all the systems, all of this (Protein/Protein-ligand complex, ions) was done within the solvation box of cubic shape that was 1.0 nm away from protein/protein-ligand complex. All directions Periodic Boundary Conditions was utilized; the LINCS algorithm was used to apply the bond constraints [52]. Before going for simulation, all systems were made overall neutral, systems with protein EBNA1 had 4 net positive charges whereas RdRp based systems had 12 net negative charges. Therefore, 4Cl and 12Na⁺ were introduced in the respective systems before simulation. This was followed by energy minimization and two-step equilibrations. The systems had more than 72,000 atoms and 2.5 lakhs atoms for proteins EBNA1 and RdRp respectively. For energy minimization, the steepest descent algorithm was set at a tolerance of 1000 kJ/mol/nm. Now, equilibration’s first step was performed with volume and temperature constant for 2 ns (NVT), and in the second step pressure and temperature were kept constant for 5 ns (NPT) [53,54]. The final production run was of 100 ns. Trajectories after the final run were subjected to analysis using in-built tools and additional plug-ins of GROMACS. Simulation depends on a bunch of factors right from the number of atoms to the size of box to technology used like HPC and parallelization, which have made long time-scale simulations possible [55]. All the simulations were performed using the GPU-based server with 16 cores, Intel Xeon CPU with two NVIDIA Tesla graphic cards.

2.4. Binding energy calculations

Free binding energy was calculated using the g_mmpbsa tool [56]. The free binding energy was determined for the three complexes of both the proteins. The binding energy for protein–ligand complexes present in the solvent is given by:

\[
\Delta G_{\text{bind}} = \Delta G_{\text{complex}} - \left( \Delta G_{\text{protein}} + \Delta G_{\text{ligand}} \right)
\]

Where, \(\Delta G_{\text{complex}}\) is the total free energy of the complex (Protein-Ligand), \(\Delta G_{\text{protein}}\) is the total free energy of protein in solvent, and \(\Delta G_{\text{ligand}}\) is the total free energy of ligand in solvent. \(\Delta G_{\text{SA}}\) is the energy contributed by molecular mechanics interaction, \(\Delta G_{\text{vap}}\) is the polar solvation energy, \(\Delta G_{\text{elec}}\) is the non-polar solvation energy, \(\Delta S\) is the energy contributed by entropy and \(\Delta G_{\text{inc}}\) is the energy contributed by electrostatic interactions. The g_mmpbsa tool calculates all the parameters of energy and gives the total free binding energy.
2.5. ADMET and physicochemical property screening

The properties of shortlisted compounds with respect to ADMET i.e. adsorption (A), digestion (D), metabolism (M), excretion (E), toxicity (T) and other physicochemical properties were analyzed to evaluate drug-likeness of the compounds using pKCSM platform [57]. The platform utilizes graph-based signatures to produce predictive models of central ADMET properties for drug development. The canonical SMILES of each compound retrieved from PubChem were used as input for the analysis.

3. Results and discussion

3.1. Modelling the missing residues and loop refinement

The 3D structure of 932 amino acid long RdRp (6 M71: chain A) had small range of intermediate missing residues (at position 51–68, 75 and 103–111), hence those residue regions were modelled using MODELLER 10.1. It produced five model structures as output. Based on the RMSD, DOPE score Rama favored and Z-score values model 5 was considered for further procedures (Supplementary Table S1). As the modelled part was mostly forming a loop structure, it was subjected for loop refinement using a GalaxyWEB server. GalaxyRefine tool was used to refine the selected model structure, five models were generated by the server. According to RMSD, Rama favored, Clash score and Poor rotamers score model 4 (Supplementary Table S2) was selected and used for further analysis.

3.2. Molecular docking and shortlisting of potent ligands

To shortlist the potent molecules out of 400 antimalarial compounds from the malaria box, the virtual screening against EBNA1 and RdRp was performed in comparison with suitable reference mole-
The docked protein–ligand complexes were visualized on the PyMOL platform to confirm the molecular arrangement at the target site. The resulting docked complexes provided various poses/confirmations. Further, the confirmations with the highest docking scores and lowest RMSD values were considered for comparative analysis with other complexes. For EBNA1, the reference molecules VK0064, VK0044, and VK0497 showed binding energies $-6.4$, $-7.5$, and $-8.9$ kcal/mol respectively (Supplementary information Table S3). The VK0497 was considered for further comparison due to higher affinity in accordance with the previous report [20,22].

The three compounds from the malaria box namely MMV665883 (Ligand A), MMV665879 (Ligand B), and MMV665954 (Ligand C) showed lower binding energies (>9.0) that is higher affinities than the reference molecule VK0497 (Table 1). The ligands were found to be situated at target sites in chain B, interacting through various hydrogen bonds or hydrophobic interactions (Supplementary Fig. S1). These three compounds interacting with EBNA1 were selected for further screening. For RdRp, the remdesivir showed binding energy $-7.2$ kcal/mol. Surprisingly, many compounds (~210) showed higher affinity than remdesivir towards RdRp. The top 3 compounds namely MMV000753 (ligand D), MMV665918 (ligand E), and MMV011895 (ligand F) were situated in active sites of RdRp and interacted with various residues of the conserved motif (Supplementary Fig. S2). The ligands D, E, and F bound the RdRp with binding energies of $-10.8$, $-9.5$, and $-9.3$ kcal/mol respectively. These three RdRp-ligand complexes were taken into account for further study (Table 1). Notably, the two compounds MMV665794 and MMV020912 were observed in the top 10 ligand list of both the proteins (EBNA1 and RdRp) (Supplementary information Table S3). However, the two compounds were having less affinity towards EBNA1 compared to the most potent reference compound VK0497, therefore were excluded from further study.

3.3. MD simulation and trajectory analysis

3.3.1. Structural stability and flexibility analysis of EBNA1 and RdRp complexes with respective ligands

MD simulation is a well-known technique to assess the protein and protein-ligand complex structural and functional stability [58–60]. It reveals the dynamic behavior of the proteins and their interactors in...
a virtual intracellular environment [61,62]. To investigate the binding stability of docked protein–ligand complexes and elucidate the related energetics the 100 ns MD-simulations were performed. The root-mean-square deviation (RMSD) values of protein backbone atoms in each system were evaluated for qualitative analysis of system stability and overall system convergence. In the case of EBNA1, the RMSD for each system was elevated in the initial quarter with fluctuations at a subsequent time up to the beginning of later quarter of the simulations. Post fourth quarter, all the systems reached equilibrium (Fig. 3a). The protein from the EBNA1-ligand B complex showed higher RMSD than control (EBNA without ligands) as well as other complexes. Its RMSD difference with other complexes was around 0.2 nm. The RMSD graphs

Fig. 6. The 3D and 2D interaction details of RdRp in complex with a) ligand D, b) ligand E and c) ligand F respectively after achieving equilibrium. The non-covalent interactions like van der Waals, Pi-donor hydrogen bonds Pi-sigma, Pi-alkyl, Pi-cation, and amide-Pi stacked were found to be prevalent to stabilize the binding of all the ligands with RdRp.
for complexes of EBNA1 with ligand A and B respectively were converged with control in the later half. The radius of gyration ($R_g$) of complexes was studied to check the structural compactness of protein post interaction with ligands. The EBNA1 in complex with ligand B showed a slight drop in $R_g$ up to the first quarter and variation throughout but ultimately lay near the $R_g$ values of other systems at the end of the simulation. The overall $R_g$ of all the systems of EBNA1 with or without ligands showed very less deviation ($<0.05$ nm) compared to their initial poses as well as with each other throughout the simulation (Fig. 3b). To decode the residue level fluctuations of protein bound or unbound to ligands, the root means square fluctuations (RMSF) were calculated. The loop regions in the range of amino acids 490–500, 540–550 showed more fluctuations around 0.5 nm. Unlike chain A, the S82–S93 loop region from chain B (near the site of ligand interactions), showed comparatively more fluctuation than the EBNA1 without any ligand (apo form) (Fig. 3c). In the case of RdRp, all the systems equilibrated post $~50$ ns as depicted from the RMSD plot (Fig. 4a). RdRp in apo form or in a complex with ligand E or F showed similar $R_g$ (Fig. 4b). But, RdRp in complexes with ligand D showed slightly reduced $R_g$ ($<0.05$ nm). The loop regions in amino acid ranges between 50 and 76 and 895–920 in RdRp and these were the areas with highest level of fluctuations going up to 1 nm. All the residues showed a similar fluctuation pattern in apo or ligand bound form (Fig. 4c).

### 3.3.2. Affinity energetics of EBNA1 and RdRp in complex with ligands:

To estimate the affinity of ligands to respective proteins- EBNA1 and RdRp, $\Delta G_{bind}$ was evaluated using the MMPBSA approach. It also elucidated the components involved like $E_{	ext{bonded}}$, $E_{	ext{vdw}}$, $E_{	ext{elec}}$, $G_{	ext{polar}}$ and $G_{	ext{non-polar}}$. The summary of MMPBSA analysis for all protein–ligand complexes are enlisted in Table 2. The binding energies of all the complexes were highly negative indicating strong binding and better structural stability. For EBNA1, ligand B showed the highest negative binding energy ($-183.54$ kJ/mol) compared to other ligands. This denotes that ligand B can strongly and stably bind the target site of EBNA1. The $E_{	ext{vdw}}$ induced the complex formation for lig A and ligand C while in the EBNA1-ligand B complex the involvement of $E_{	ext{elec}}$ was more. For RdRp, the binding energies of ligand E and F were closer to each other but ligand D was having lower binding energy. The involvement of $E_{	ext{elec}}$ as well as $E_{	ext{vdw}}$ was highest for the RdRp-ligand E complex. Considering the $\Delta G_{bind}$ value, the EBNA1-ligand B ($-183.54$ kJ/mol) and RdRp-ligand E ($-172.23$ kJ/mol) complex were most stable compared to the other two ligands.

### 3.3.3. Essential residues involved in EBNA1 and RdRp protein–ligand complexes:

To decode the residues of importance in protein–ligand complex formation the decomposition of binding energy was performed using the MMPBSA scheme (Supplementary Table S4). We also supplemented the above results by analyzing the final confirmation of each complex after 100 ns simulation in 3D as well as 2D confirmations as shown in Fig. 5 and Fig. 6. The change in residues involved EBNA1-ligand interaction in initial docked complex (Supplementary Fig. S1) and final equilibrated complex (Fig. 5) denotes that the ligand acclimatize themselves to fit potently at binding site by interacting with different residues. For all the residues involved in the interaction, molecular mechanic (MM) energy favored the complex formation as depicted from highly negative energy values than other parameters. For all EBNA1-ligand complexes (Fig. 5a, b, and c) the amino acid residues Asn480, Ile481, Asn519, Leu582, Val583, Lys586 and Thr590 were commonly observed in the interactions. The non-covalent interactions were predominant in all the complexes. In EBNA1-ligand A complex, the Lys486 ($-10.07$ kJ/mol), Leu482 ($-9.30$ kJ/mol) and Ile481 ($-9.70$ kJ/mol) contributed higher energies than other molecules. The involvement of Lys477, Asn519, and Lys586 was very much higher in EBNA1-ligand B interaction as depicted from their contribution of $-19.24$, $-13.06$ and $-12.67$ kJ/mol. The EBNA1-ligand C showed Ile481 ($-10.17$ kJ/mol) and Pro589 ($-9.08$ kJ/mol) as major contributors in complex formation. The Lys586 residue showed prominent dedication in interaction with all ligands as referred from its higher energy contributions. Asn519 and Thr590 residues are critical for EBNA1-DNA interactions and their lack of contact could inhibit binding with DNA [20]. Previously, reference compound VK-0497 has been shown to inhibit EBNA1 by utilizing these residues along with critical contacts with Lys477 and Lys586 [20]. The interaction of Ligand B with EBNA1 includes all these residues as top energy contributors as shown in Supplementary Table S4. Besides, all the ligands fit in the parameter of Lipinski’s rule of five (Supplementary Table S5) considering higher stability and involvement of important amino acids, ligand B could be better to use against EBV by targeting EBNA1.

The RdRp-ligand complexes were majorly involved many noncovalent interactions (Fig. 6a, b, and c). The ligands observed to be dynamically oriented themselves to fit better into the binding cavity as depicted from differences in interacting residues of initially docked complexes (Supplementary Fig. S2) and complexes after attaining equilibrium (Fig. 6). In the RdRp-ligand D complex, the major energy contributor residues were Lys545 ($-12.08$ kJ/mol) and Arg555 ($-11.22$ kJ/mol). Interestingly, in case of RdRp-ligand E interaction, the Arg555 stood out as a sole major energy contributor with $-17.79$ kJ/mol while the second one was Lys545 ($-8.2$ kJ/mol). The RdRp-ligand F complex was stabilized through majorly hydrophobic interactions. The Arg555 ($-18.95$ kJ/mol), Lys798 ($-10.81$ kJ/mol), Lys545 ($-9.78$ kJ/mol), and Arg624 ($-9.727$ kJ/mol) were denoting the major shares in RdRp-ligand F interaction. The hydrophilic residues Lys545, Arg553, and Arg555 from motif F form the NTP entry channel. The highly stable interactions of ligands D, E, and F could hinder the RNA template entry into the polymerase and may efficiently inhibit the subsequent event due to lack of template itself. Out of all three ligands, ligand E was found to be interacting more with conserved motif residues from motifs F (Lys545, Ala550, Lys551, Arg553, Ala554, Arg555, Lys593) as well as motif A (Tyr619, and Arg624) with major energy contributions. Additionally, Ligand E also showed a comparatively better fit in Lipinski’s rule parameters compared to ligand D and F (Supplementary Table S5).

### 4. Conclusion

There is a crucial need to identify drug candidates with antiviral potency against viruses like EBV and SARS-CoV-2, which are associated with worsened cases of malaria pathology [5,13]. Repurposing the antimalarial compounds against these viruses could help treat such virus and malaria coinfection cases. Additionally, compounds showing antiviral potency can be used against viral monoinfection. Our study investigated the inhibitory potential of malaria box molecules against EBV and SARS-CoV-2 by targeting crucial proteins EBNA1 and RdRp, respectively. Using a molecular docking approach, we shortlisted the top 3 compounds out of 400 molecules for each protein. A subsequent MD simulation run for 100 ns was used to assess the compound stability and affinity. All the ligands showed stable binding with the respective protein as referred from RMSD, RMSF, and $R_g$ analysis. We further selected one out of the three shortlisted compounds against each viral protein based on binding energy analysis, involvement of important functional residues, and ADMET analysis. Ligand B (MMV665879) was most stably bound and showed the highest participation of crucial residues in interaction with EBNA1. Hence, ligand B could potently inhibit EBNA1 activity and subsequent blocking of viral pathways. For SARS-CoV-2 RdRp, ligand E (MMV665918) was found to be the more stable binder than other ligands. Interestingly, this interaction involved the residues of conserved motifs as top energy contributors. Hence, by binding at the site of RNA processing, the
ligand E could efficiently hinder the RdRp activity. As both of these ligands are already proven to possess antimalarial activity, their use in the case of respective viral coinfection with malaria could be beneficial. Our study opens the gateway for utilizing multi-targeting compounds as drugs to combat malaria and viral coinfection in further preclinical and clinical studies.

Declaration of Competing Interest
The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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