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Article

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Abstract: Ionic liquid MIE-NH_2 displays a new role of development of modification of glycoproteins of lactoferrin through a reductive amination mechanism to synthesize versatile pharmaceuticals. This work introduces a new strategy of modification of Lactoferrin by using methylimidazolium N-ethylamine, this ionic liquid MIE-NH_2 linked to N-glycans in Lactoferrin derivatives. Using UPLC/ESI-QTOF and MALDI-TOF mass spectrometry to perform and detect the modifying of ionic liquid-linked glycoproteins. Relevantly, modifying the lactoferrin by MIE-H_2 as a small molecule of ion liquid lactoferrin (IL-Lf), which could be a potential antiviral drug and it is achieved by inhibiting various targets. The probability of the lactoferrin modified as a small IL-Lf-molecule to inhibit any of these targets was investigated to find out its potency as a SARS-CoV-2 inhibitor. Molecular docking disclosed the activity of modifying glycoproteins - small IL-Lf-molecules containing amino groups and interaction with targeted Mpro, RdRp, TMPRSS2, and PLpro. Clinically, this study shows the a volubility to provide small IL-Lf-molecules as significantly important drugs that target main protease (Mpro), RNA dependent RNA polymerase (RdRp), transmembrane protease serine 2 (TMPRSS2), and Papain-like protease (PLpro).

Keywords: ionic liquid; infections; lactoferrin; N-glycans; mass spectrophotometry

1. Introduction

Lactoferrin (Lf) is a member of the transferrin, that binds and transfers iron in the blood and it can also remove excess iron from the body, which is a most abundant glycoprotein in human and ruminant milk resources [1]. The previous studies of lactoferrin disclosed a wide display with different functions including anticancer activity, anti-inflammatory, and cognitive function enhancement in patients with Alzheimer's. Lactoferrin has 1–4 glycans with single-chain polypeptides of about 80,000 Da. Lactoferrin is present in large scales in milk with multifunctional

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glycoprotein including N-glycans, which are active with functional groups and depending on the species, and it makes a significant contribution to the host that defines the system. [2–4]. In addition, lactoferrin carries many important biological functions, including N-glycans bonding to iron or others, being bioactive in cell explosion and diversity, as an anti-parasitic protein. Lactoferrin is known to generate host protective responses against Mycobacterium tuberculosis, anti-bacterial and anti-viral. These functions diverge from lactoferrin considerable attention as the primary nutritional contribution to iron-binding by the role of glycosylation[5,6]. The molecular structure of human Lactoferin and amino acids were discovered in two globular lobes of ~700 amino acids stabilized by disulfide bonds, which are linked by a flexible alpha helix- amino and carboxy, N-lobe and C-lobe [7].

Ionic liquids containing N-active group, that's make the critical role of carbonyl groups of glycan binding in many biological processes very easily. The chain of saccharides-glycan moieties in lactoferrin is likely to contribute significantly to the N-ionic liquids roles by carbonyl of saccharides. Despite the high amino group of ionic liquid sequence homology in different with excellent results, which exhibits a unique N-glycosylation for heterogeneity of the biological properties and Lactoferrin is chosen as a good example source of N-glycans [6,8,9].

Exploring and identifying the new characterization of novel ionic liquid delivers reacting with oligosaccharides of glycoproteins. Several interesting studies are encouraging for discovers the new application of ionic liquids as potential drugs antimicrobial including anti- coronavirus disease 2019 [10]. Studying the activity of glycoproteins is the assessment of the contributions of individual glycans to the observed bioactivities. This work examines how the study of N-link glycosylation in Lactoferrin which reacted with ionic liquid MIE-NH_2 increases the understanding of ionic liquid functionality [2,11,12]. Since 2019, researchers have revealed various structural and non-structural targets of SARS-CoV-2 that have been utilized in drug repurposing in the fight against COVID-19. In this study, some of such targets that have been investigated well were selected for the computational study. There are clinically available drugs that target main protease (Mpro), RNA-dependent RNA polymerase (RdRp), Transmembrane protease serine 2 (TMPRSS2), and Papain-like protease (PLpro) [13–15]. Drugs like favipiravir and remdesivir, which have been used for the treatment of COVID-19, are examples for RdRp inhibitors. Lopinavir and ritonavir are PLpro and M inhibitors whereas nafamostat and camostat act on TMPRSS2 [16]. These drugs (IL-Lfs) have small molecular structures with various scaffolds. The probability of the synthesized molecule to inhibit any of these targets was investigated to find out its potency as a SARS-CoV-2 inhibitor.

2. Materials and Methods

2.1. Materials,

All chemicals including Tert-butyl N-(2-bromoethyl) carbamate and N-methylimidazole were purchased from J&K (Shanghai, China). Acetonitrile and solvent used for HPLC were purchased from Merck (Ankara, Turkey). Lactoferrin-free iron and all other chemicals used in this study were bought at the highest grade from commercial suppliers without further purification or modification.

2.2. Synthesis of ionic liquid-[1-(2-aminoethyl)-3-methyl-1H-imidazole-3-ium] MIE-NH_2][BF_4^-])

Tert-butyl N-(2-bromoethyl) carbamate (225 mg, 1.1 mmol) was reacted with N-methylimidazole (82 mg, 1 mmol) in an anhydrous mixture solvent of CH_3CN and t-BuOH [5 mL (3/2, v/v)], reaction mixture refluxing at 80 ºC for 2 days. Removing unreacted materials by washing with ethyl acetate three times and the product was dried under reduced pressure and obtained the light yellow viscous liquid (scheme 1,a) [17–19]

The viscous liquid (1) IL-Br-1 (289 mg g, 85.7 mmol) was generated by stirring with KBF4 (1.1 equiv.) in water solution for 24 h at room temperature. Then, the reaction mixture was filtered and vacuum distilled, and washed the product by dichloromethane and ethyl acetate, respectively. The product was vacuum-dried by a rotary evaporator at 55 ºC to remove the traces of dichloromethane

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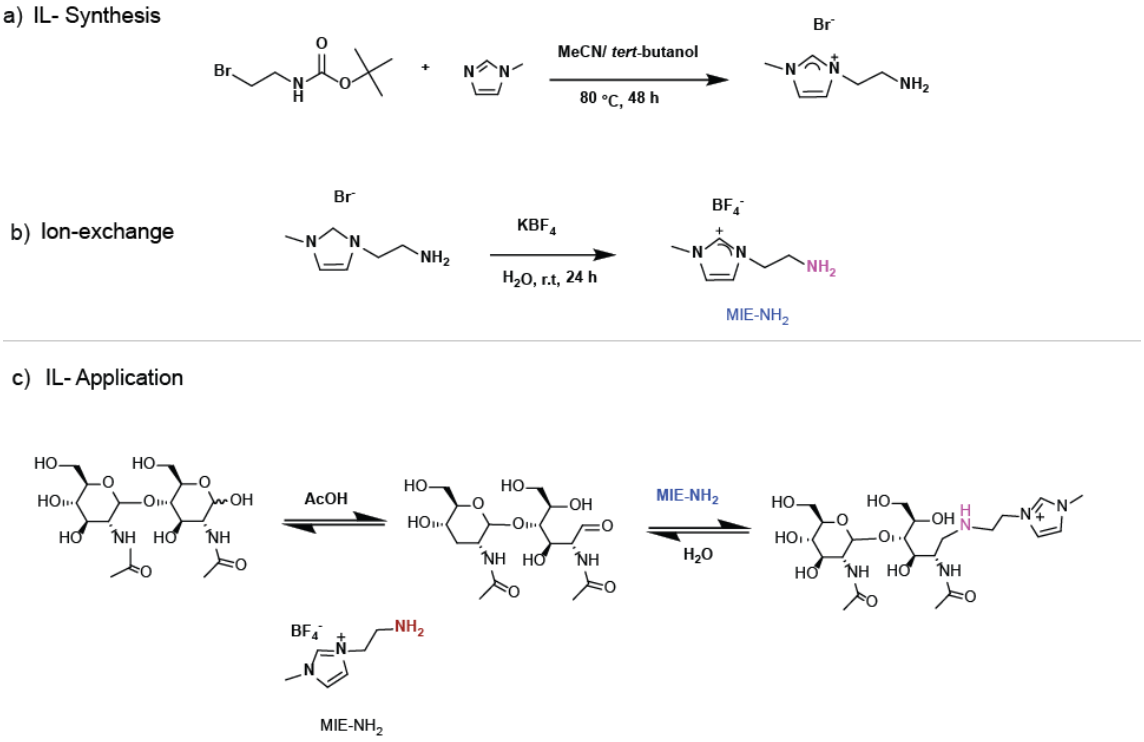
and ethyl acetate. After drying for 6 h under vacuum at 80 °C, the expected ionic liquid [MIE-NH_2][BF_4^-] was obtained (Scheme 1, c) [17,20,21]



Scheme 1. Synthetic route of methylimidazolium N-ethylamine and derivation of aminoglycosides with GlcNAc extracted from lactoferrin [15,17].

2.3. Derivatization N-glycans of Lactoferrin with ILs-NH_2

Derivatization solution contains 70 mM MIE-NH_2 and 0.1 M sodium cyanoborohydride in dimethyl sulfoxide/acetic acid solution (7:3,v/v)) was added to a sample of Lactoferrin until completely dissolved. The derivatization mixture was mixed by ultrasonic for about 30 minutes and incubated at 70 -90 °C for 4 h [17,18].

2.4. Molecular Docking

The crystal structure of the selected targets was retrieved from the protein data bank (PDB). The selected structures were downloaded from the database and a grid box was determined for each one. Crystal structures of main protease (PDB ID: 8GFN) [22], RNA-dependent RNA polymerase (PDB ID: 7B3B) [23], TMPRSS2 (PDB ID: 7MEQ) [24], and Papain-Like protease (PDB ID: 7LLF) were selected and then they were made ready for docking [25]. The molecular docking was performed through AutoDock Vina as described in previous [26,27].

3. Results

The glycoproteins in bovine lactoferrin (BLf) were chosen as good substrates source to prepare N-glycans with high structure including oligosaccharides [28]. The high proportion of glycosylation verifies the methodology of MIE-NH_2 following reductive amination, it was used for labelling of N-glycans [17]. According to our previous studies, ionic liquids drive lactoferrin iron free (IL-Lf-iron free)[18]. There was found proximity 42 types of N-glycans with diverse potential sites N-glycosylation in bovine Lactoferin (BLf), the different N-glycans with all structures sites [29,30]. The lactoferrin IL-Lf molecules modified by using ionic liquid, there are 14 different MIE-NH_2 derivative lactoferrin-N-glycans were deduced according to UPLC profile and MS spectrum see in figure 1. The corresponding structures of Lactoferrin-MIE-NH_2 were assigned as shown in Figures 1 and 2. The results of the detection in figure 1 suggested the possible structures of compounds were modified by

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IL-MIE-NH_2 and this result was confirmed by MALDI ToF analysis. The m/z values of structures either with mono-charge or di-charge were calculated related to the signals of MIE-NH_2 linked to N-glycans was observed. In the extracted ion chromatogram of the products of N-glycans linked to MIE-NH_2 from Lactoferrin by HPLC two peaks exhibited the same m/z value of new products 1716.50 which assigned and identified with theoretical m/z = 1716.70 $[m]^+$). In this case, we suggested the new product is MIE-NH_2 linked monofucosylated monogalactosylated bi antennary complex N-glycan isoforms [17,31]. For example, from LC-MS analysis, it was found the peak of 13.7 min was assigned as MIE-NH_2-FA2G1 and the peak at 14.6 min was derived as MIE-NH_2-A2G1F. This work demonstrated the catalytic mechanism of the derivatization of Lactoferrin-N-glycans with ionic liquid MIE-NH_2 following the reductive amination. The free aldehyde realized in the acidic medium and by reducing ligand as sodium cyanoborohydride, which possesses significant converted the carbonyl to an imine by the NH_2 group of MIE-NH_2 (Scheme 1 , b)[18].

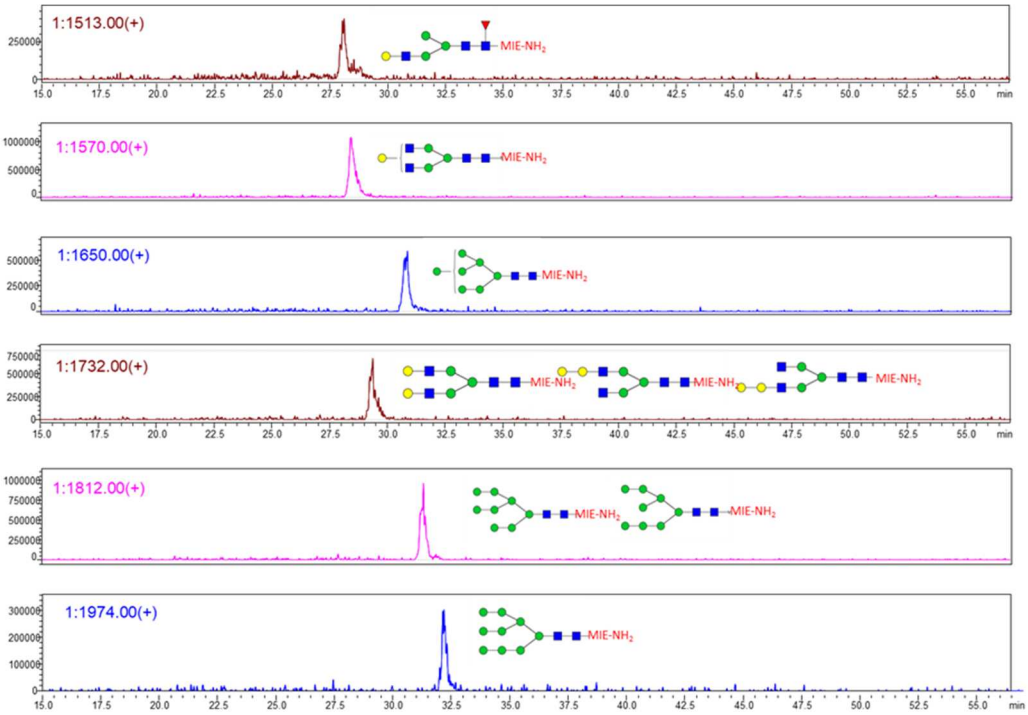

Figure 1. LCMS result of derivatization of different N-Glycans from lactoferrin and linked with MIE-NH_2.

Recent scientific research focused on the progress in protein-based nanomedicine, albumin-paclitaxel as nanoparticles have been introduced in novel therapeutics and used for the treatment of cancer and viral infections [10, 29]. However, specific drug targeting of SARS-CoV-2 is almost challenging and absent until now, premature drug release and supports the poor pharmaceutical stores for resistance COVID-19 and its mutations. Therefore, some studies with alternative protein-based nanomedicines have opening the eyes to the use ionic liquids for extend and developing a novel of small molecules form glycoproteins. Regarding to this challenge, lactoferrin (Lf-iron free) offers a promising bioactive well as potentials therapeutic and drug nano carrier. In this work, we focused on the major pharmacological actions of modified glycoproteins form lactoferrin with ionic liquids to produce new molecules including antiviral, anti-cancer, and/ or improve immunology.

To enhance the efficacy of glycoproteins as potential drugs anti SARS-CoV-2 it was functionalization of N-glycans with an emphasis of lactoferrin. Besides this technique wide application, it's depended on the recent advances of ionic liquids-Lf-based small molecules as efficient platforms for delivering novel drugs anti-viral drugs, particularly for treating the COVID-19 infections.

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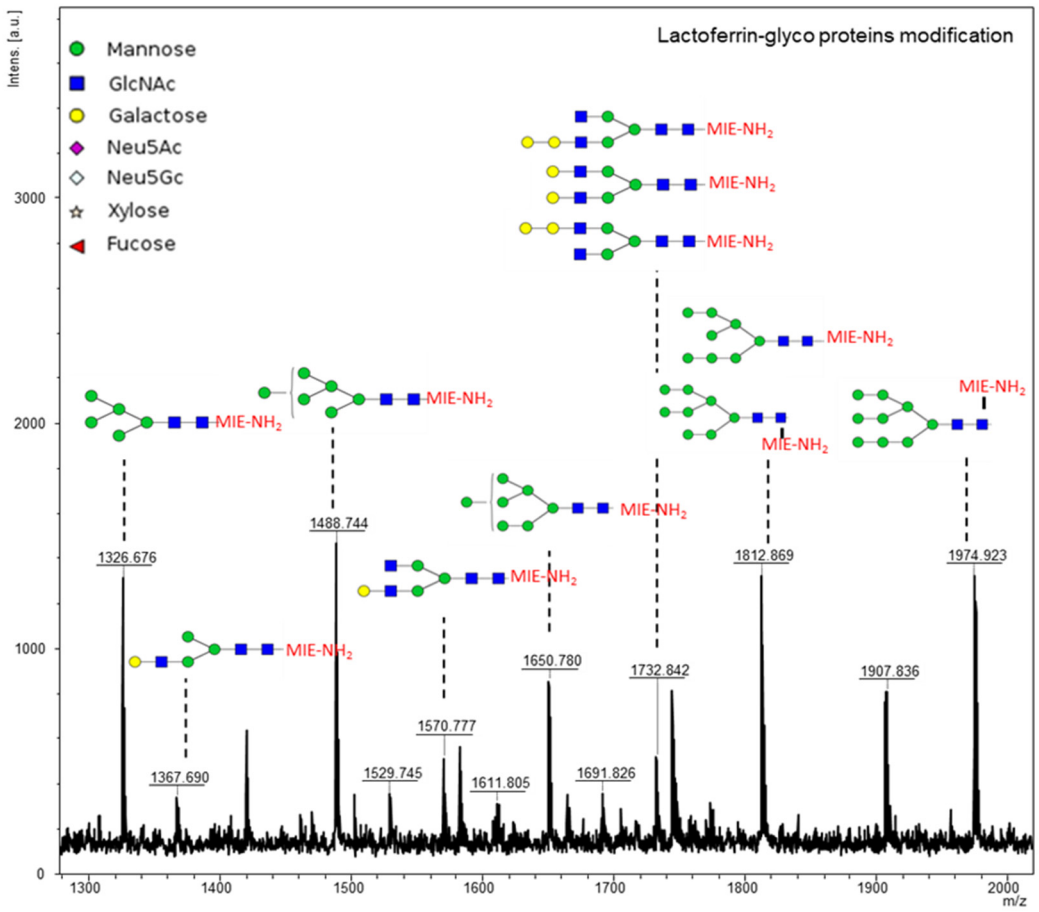

Figure 2. MALDI-TOF-MS results confirm the modulation of N-glycans from lactoferrin linked with MIE-NH_2 [18].

The binding potential of the new IL-Lf molecule to four targets (Mpro, RdRp, TMPRSS2, PLpro) was investigated via molecular docking. The resulting interaction was compared to a standard drug, remdesivir, which is one of the drugs that has been used in the fight against COVID-19 and approved by the FDA for this indication. The crystal structure of RdRp utilized in this study has remdesivir inside it. A previous study suggested that remdesivir exhibited its activity by binding to RdRp [23]. As a result, we investigated the binding potential of remdesivir to RdRp first in order to validate the docking process. The docking investigation demonstrated that remdesivir interacted to the enzyme with ten conventional hydrogen bonds Urd7 (2), Ade8, Gua10, Cyt11, Cyt12, Gua13, Asn496, Asn497 (2)) and one other interaction (Ade10). The compound interacted with the enzyme very well with -8.9 kcal/mol binding energy. The ligand had interactions mostly on the nucleotide residues (Table 1, Figure 3). Similarly, in the previous crystallographic analysis, remdesivir had interactions with the nucleotides to exert remdesivir-induced RdRp stalling [23]. The high level of binding observed for remdesivir leads us to assume that the docking protocol would give reliable interaction of the compounds with the targets. Thereafter, molecular docking of the modified molecule and remdesivir to the targets was pursued.

Table 1. Binding residues of the derivatized IL-Lf molecule and remdesivir with the targets.

Compounds	Target	Binding energy (kcal/mol)	Hydrogen bonding points	Other interaction points
Molecule	8GFN	-7.5	Thr26, Asn142, Ala145, His163, His164, Glu166, Val186, Arg188(2)	Thr25[a], Met49[b], Gln189[c]

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Remdesivir	8GFN	-8.0	Gly143, Ser144, Ala145, His164, Glu166	His41[d], His41[e], Ala145[e], Met165[c], Met165[e], Pro168[c]
Molecule	7B3B	-7.7	Urd7, Ade8(2), Cyt9(2), Cyt12, Gua13, Asn497(3)	-
Remdesivir	7B3B	-8.9	Urd7(2), Ade8, Gua10, Cyt11, Cyt12, Gua13, Asn496, Asn497(2)	Ade10[c]
Molecule	7MEQ	-5.5	Glu299, Lys300, Gly439, Ser441, Ser460, Gly462, Gly464	His296(2)[c], Gln438[c]
Remdesivir	7MEQ	-7.0	Val280, Ser436, Gly439, Ser441, Ser460, Gly464	Val278[e], His279[c], Val280[c]
Molecule	7LLF	-5.4	Leu162, Glu167, Gln269(2)	Glu161[c], Glu167[c], Gln269[c]
Remdesivir	7LLF	-6.5	Ser212, Tyr213, Glu214, Gln215, Tyr251, Glu252	Tyr213[e], Lys218[f], Tyr251[c], Tyr305[e]

[a]pi-sigma, [b]pi-sulphur, [c]carbon-hydrogen bond, [d]pi-pi, [e]alkyl/pi-alkyl, [f]pi-ion.

The derivatized molecule had a good level of interaction with the main protease. It interacted with the enzyme through nine conventional hydrogen bonds (Thr26, Asn142, Ala145, His163, His164, Glu166, Val186, Arg188 (2)) and one other interaction types (Thr25, Met49, Gln189) (Figure 3, Table1). It interacted with the enzyme stronger than remdesivir as it formed four more conventional hydrogen bonds. The binding energy of remdesivir was slightly lower than that of the molecule. Therefore, the two compounds are expected to have a similar affinity to the enzyme with a slightly higher affinity for remdesivir. A previous crystallographic study revealed that ligands had interactions with the enzyme at His41, Ala145, His163, His164, Met165, Glu166, and Gln189 [22]. In the computational study, the interactions at all of these residues were observed (Figure 3, Table 1). In this regard, the computational study gave similar interaction points with the experimental study. The modified compound interacted with RdRp very well. It formed ten conventional hydrogen bonds (Urd7, Ade8 (2), Cyt9(2), Cyt12, Gua13, Asn497(3)). Together with this, its interaction was weaker than that of remdesivir as the later had one more carbon hydrogen bond interaction (Figure 3, Table 1). In addition to this, remdesivir had lower binding energy than the molecule that implicated a better affinity for it. Therefore, remdesivir interacted with RdRp stronger and had a higher affinity towards the enzyme. The interactions detected were mostly with the nucleotide residues of the enzyme for both of them as observed in a previous study[23].

The derivatized IL-Lf molecule had strong interaction with TMPRSS2 with seven conventional hydrogen bonds (Glu299, Lys300, Gly439, Ser441, Ser460, Gly462, Gly464) and three other interactions (His296(2), Gln438) (Figure 3, Table 1). The molecule had better interaction with TMPRSS2 in relative to remdesivir as it formed more hydrogen bonds. However, the binding affinity of remdesivir is expected to be slightly higher than the molecule as it had lower binding energy. An experimental study reported that TMPRSS2 had interactions with ligands at Ile256, His296, Asp345, Asp435, Ser436, Gly439, and Ser441 residues [24]. In this computational study, the interactions at His296, Ser436, Gly439, and Ser441 were observed. There was the similarity between the experimental and computational studies as more than half of the residues were common for both methods. In addition to this, the computational study gave strong interaction to TMPRSS2 for the two compounds. The molecule interacted with PLpro through four conventional hydrogen bonds (Leu162, Glu167, Gln269 (2)) and three other interactions (Glu161, Glu167, Gln269) (Figure 3, Table 1). The interaction was good but weaker than the interaction of remdesivir with the enzyme. The binding affinity of remdesivir was also better as it had lower binding energy [33]. Therefore, remdesivir is expected to have better interaction with PLpro. Furthermore, the synthesized molecule had the weakest interaction with this enzyme. A previous crystallographic study reported that various ligands had interactions with Asp164, Arg166, Glu167, Tyr264, Tyr268, and Gln269 [23,34]. In this study, the synthesized small-molecule ionic liquid-Lf had interactions with Glu167 and Gln269 residues. The molecule had some level of interaction similarity with the previous study. On the other hand, remdesivir didn't have any common interaction residue with the experimental study. This has implicated that it could interact with the enzyme but at a different binding region.

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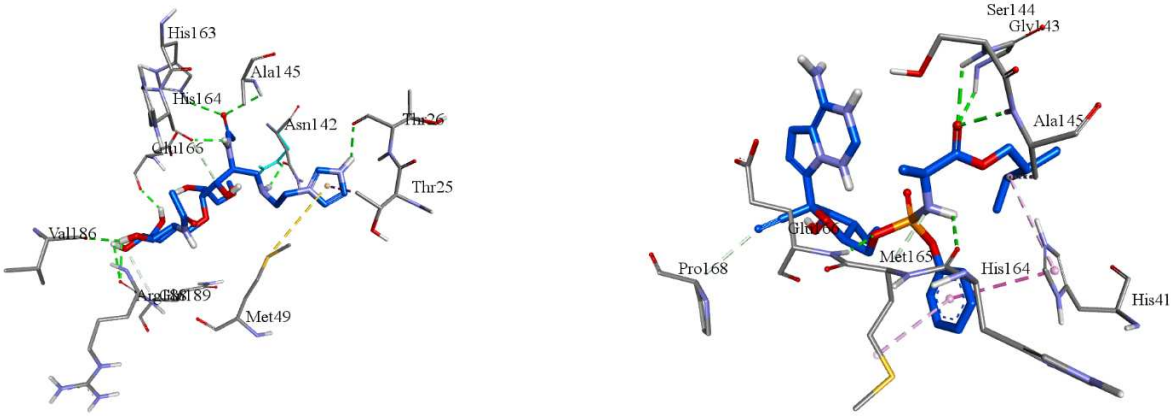
The computational study revealed that the synthesized molecule interacted with the targets very well generally. The strength of the interaction was different from each other with a decreasing order of RdRp, Mpro, TMPRSS2, and PLpro. The molecule had a stronger interaction than remdesivir with Mpro and TMPRSS2. Together with this, remdesivir had slightly lower binding energy than the molecule with the four target structures. Overall, the computational study demonstrated that the designed IL-Lf molecule had high binding potential toward the targets, especially towards Mpro and TMPRSS2.



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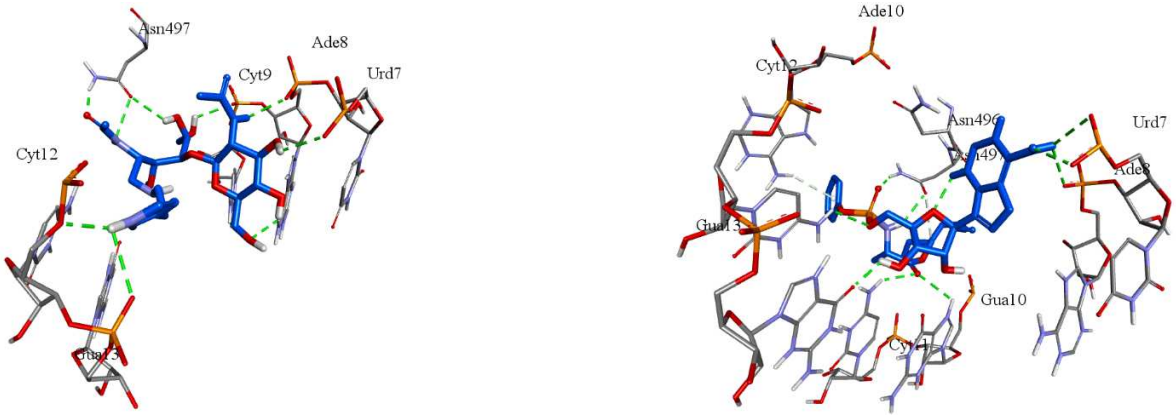

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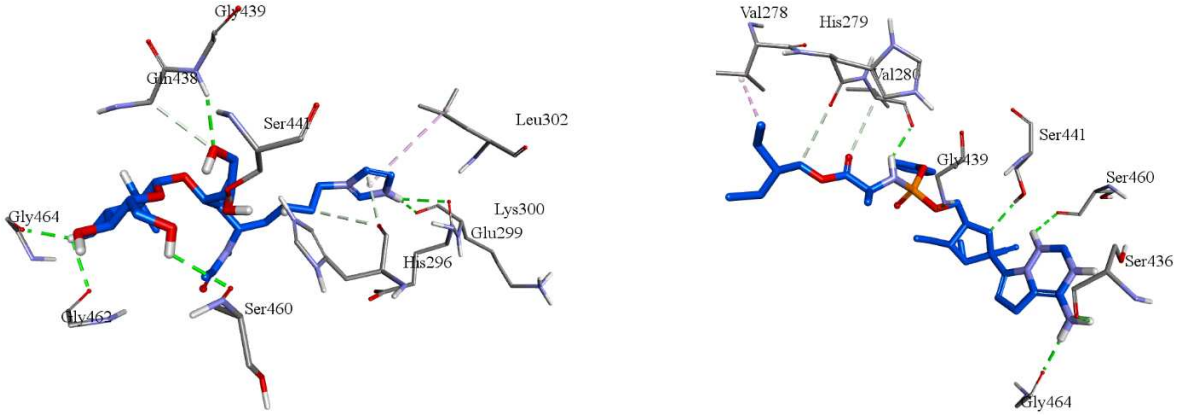

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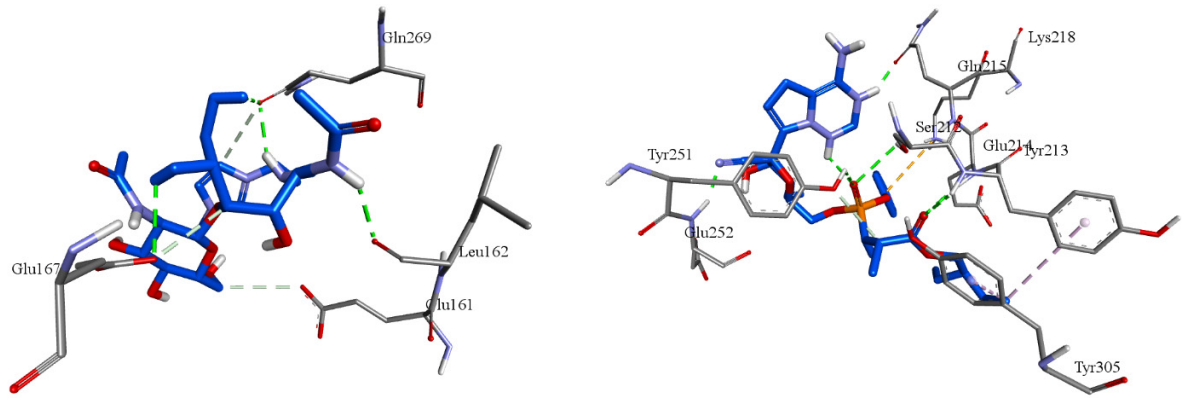

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Figure 3. 3D interaction profiles of the molecule and standard drug (remdesivir) with A) main protease (8GFN), B) RNA dependent RNA polymerase (7B3B), C) TMPRSS2 (7MEQ), D) Papain-Like protease (7LLF). The interactions of the IL-Lf molecule were presented in the left side and that of remdesivir was presented in the right side for each target.

4. Discussion

4.1. Pharmacological and antiviral activities of IL-lactoferrin molecule

The antiviral of SARS-CoV-2 treatments and the potential drug benefits remain controversial. Lactoferin is known for a wide array of different functions including anti-inflammatory, anticancer activity, and cognitive function improvement in patients with Alzheimer's disease [2]. Furthermore, several studies of lactoferin showed positive antiviral efficacy against HCV, HIV, HBV, HPV, Influenza viruses, retroviruses (AIDS, T-cell leukemia), and poliovirus, following the binding to the surface of the virus which inhibits the production of DNA copy of the viral RNA. In this study, ionic liquid replaced iron on lactoferin which scavenges or by competition for binding to host cells According to relevant previous studies, the interaction of remdesivir with main protease (8GFN), RNA-dependent RNA polymerase (7B3B), TMPRSS2 (7MEQ), and Papain-Like protease (7LLF)[35]. A computational study illustrated the activity of IL-Lf small molecule of lactoferin formulated IL-Lf against SARS-CoV-2. Similarly, this study proposed the mechanism of the interactions of the IL-Lf molecule with the targets. Modifying Lactoferin is a good candidate for increasing the production of pro-inflammatory cytokines IL-6, IL-8, TNF-α and MIP-1α [34,36]. In cell-based assays, cysteine protease enzyme cathepsin L (catL) activates and receptors are required for viral entry while Calu-3 cells use A Plasma membrane-resident serine proteases are pH-independent Enzymes (TMPRSS2). The new candidate of modified lactoferrin with ionic liquid and their derivatives could change PH-acidity in the endosomal-lysosomal system. Therefore, several compounds modified from lactoferrin by MIE-NH_2 in Figures (1, 2), suggest preventing SARS-CoV-2 infection and preventing infection of Calu-3 cells. In this study, we suggest of examining the antiviral activity of MIE-NH-Ft-compounds that target host cell-dependent functions. The ionic liquid supplemented with glycans (lactoferrin) is also able to inhibit pro-inflammatory cytokines (eg, IL-1b, TNF-α and IL-2) which constitute major components of the cascade of events leading to acute respiratory distress syndrome in COVID-19 patients, a combination of ionic liquid with glycoproteins was considered as a potential treatment of SARS-Cov-2.

4.2. Mechanistic study of the inhibition of coronavirus activity by small molecules of lactoferrin (IL-Lf)

This study illustrates one possible strategy to block the function of SARS-CoV-2 Main Protease (Mpro) by the ionic liquid- -lactoferrin as small molecules. Main Protease (Mpro) is an enzyme that plays a significant role in the replication of SARS-CoV-2 [37]. To design of novel inhibitors, we proposed the novel small molecule of lactoferrin modified by ionic liquid MIE-NH_2 as covalent

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inhibitors, which released N-glycans form lactoferrin and Ionic liquids as top-ranking inhibitors were selected for mechanistic investigations; one with reduction amination that has amine group and the other with an aldehydes. Cleavage of the structured protein (Mpro) from the SARS-CoV-2 reference, MD study shown thecandidate more stable enzyme-inhibitor decreasing in the order of RdRp, Mpro, TMPRSS2, and PLpro by desired small molecule IL-lf, which had high binding potential towards the targets, especially with RdRp and Mpro. Towards the provision of novel therapeutics agents for current and emerging viruses, depends on the targeting of proteins either by forming a covalent bond or non-bonding interaction [38–40]. A significant need exists for the development of small-molecule Ionic liquid-Lf inhibitors that directly target proteases. We decided the performing of a virtual screening of the inhibitors of the SARS-CoV-2 Mpro. In tartrates, screening work-fow has been employed as an extensive dataset of antiviral compounds to generate new candidates with a 3D-Scafold deep learning model [41,42]. In this research, we found that small molecule modified from lactoferrin with ionic-L show promise to be consider as a covalent inhibitor of Mpro. This mechanism of alkylation-amination of a non-structural glycoprotein was also studied for reference. MD simulations discovered that the presence of the inhibitor candidates in the active site is affected the global dynamics of Mpro; two regions near the active site and shown the new structure of the upon substrate binding. A previous study reported that various ligands had interactions with Asp164, Arg166, Glu167, Tyr264, Tyr268, and Gln269, the other simulation proposed a concerted nucleophilic-attack of the deprotonated CYS145 to the carbonyl group of (NSP) [37]. In contrast and similarly, the small molecule-IL-Lf reacts with Mpro in a stepwise manner. It is found that both activated groups of carbonyl, amines and or hetero-oxygens had interactions with Glu167 and Gln269 residues. This is a clear requirement for efficient inhibition by the activated ionic liquids- N-glycans from lactoferrin, which could be reversible as the resulting, alkylation amination/ amidation and or can be hydrolysed to regenerate the free enzymes, whereas inhibition by deported functional group is instead expected to be irreversible. These results highlight that both activated N-glycans and lactoferrin alkyl amine-based candidates can serve as inhibitors of Mpro.

5. Conclusions

In this study, Molecular docking confirmed the modification and application of ionic liquid methyl imidazole ethyl amine MIE-NH_2 by interaction with lactoferrin (IL-Lf-iron free). The detection by UPLC/ESI-QTOF and MALDI-TOF mass spectrometry performs the result of interaction between lactoferrin and ionic liquid; these results promote the new strategy for producing small molecule-IL as a novel drug antiviral. A molecular docking study explored the available strategy for modifying glycoproteins by ionic liquids following the reductive amination mechanism, this study suggested new drugs by modifying carbohydrates in ionic liquid with potential bioactive, the separation of MIE-NH_2 shows the high selectivity of the carbonyl group of sugars which could be accomplished by the hydrophilic interaction chromatography. This study suggested that new small molecules of lactoferrin (IL-Lf) containing methyl imidazole ethylamine promote antimicrobial, and antiviral including SARS-CoV-2 treatment. Molecular docking enhancing ionic liquid functionalized of N-glycans with an emphasis on lactoferrin and the modified molecule of IL- lactoferrin molecule interacted with the targets very well generally, with a decreasing order of RdRp, Mpro, TMPRSS2, and PLpro; the desired small molecule had high binding potential towards the targets, especially with RdRp and Mpro.

Author Contributions: methodology, writing, and analysis by AMS and MTM, SA methodology, formal analysis, and software; review and funding and editing, EAAS and SkA; supervision, AHA; project administration, AMS and RA.

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