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ORIGINAL ARTICLE / ÖZGÜN MAKALE

ANTIVIRAL ACTIVITY OF BETAFERON FOR COVID-19

BETAFERON'UN COVID-19 İÇİN ANTİVİRAL AKTİVİTESİ

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ABSTRACT

Objective: *SARS-CoV-2 infection has been spread worldwide since 2019 and declared a pandemic infection. Unfortunately, humanity is still trying to deal with the infection. Under these circumstances, scientists head towards drug repurposing studies as the fastest solution for combatting SARS-CoV-2 viral infection. Betaferon (Interferon beta-1b) is a member of interferons, and its mechanism of action is the same as naturally produced interferon beta-1a in the immune system.*

Material and Method: *In this study, the antiviral effect of Betaferon on SARS-CoV-2 infection in vitro and in silico was analyzed. The drug toxicity, gene expression, and docking calculations are evaluated.*

Result and Discussion: *Betaferon showed significant antiviral activity against COVID-19. Furthermore, Betaferon decreased the expression of both viral entries mediating proteins such as ACE2 and TMRPSS2. Betaferon decreases not only the expression of TMPRSS2 but also the enzymatic activity of TMPRSS2. Furthermore, in silico analyses revealed that Betaferon interacts with viral Spike protein. Hence, a decrease in the expression of viral entry mediating proteins, inhibition of the activity of TMPRSS2, and interaction with viral Spike protein indicate that Betaferon has an antiviral activity for COVID-19 virus through inhibition of viral entry pathway.*

Keywords: *Betaferon, COVID-19, drug-repurposing, SARS-CoV-2*

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ÖZ

Amaç: *COVID-19 enfeksiyonu 2019'dan beri dünya çapında yayılmış ve pandemik enfeksiyon olarak ilan edilmiştir. Maalesef, insanlık hala bu enfeksiyonla başa çıkmaya çalışıyor. Bu koşullar altında bilim insanları, SARS-CoV-2 viral enfeksiyonu ile mücadelede en hızlı çözüm olarak ilaç yeniden konumlandırma çalışmalarına yönelmiştir. Betaferon (İnterferon beta-1b), interferon ailesinin bir üyesidir ve immün sistem tarafından doğal olarak üretilen interferon beta-1a aynı etki mekanizmasına sahiptir.*

Gereç ve Yöntem: *Betaferon'un SARS-CoV-2 enfeksiyonuna karşı antiviral etkisi in vitro ve in siliko olarak araştırıldı. Antiviral aktivitenin tayini için ilaç toksisitesi, gen ifadesi ve docking (kenetlenme) hesaplamaları yapılarak değerlendirildi.*

Sonuç ve Tartışma: *Betaferon, SARS-CoV-2 viral enfeksiyonuna karşı önemli antiviral aktivite göstermiştir. Ayrıca Betaferon, hem virüsün girişinde rol oynayan ACE2 ve TMRPSS2 proteinlerinin ifadesini azaltmıştır. Betaferon, yalnızca TMPRSS2 ifadesini değil, aynı zamanda TMPRSS2'nin proteolitik aktivitesini de doza bağlı bir şekilde azaltmıştır. Bununla birlikte Betaferon'un viral Spike protein ile etkileşime girdiği in silico analizlerle gösterilmiştir. Dolayısıyla, ACE2 ve TMPRSS2 ifadesinin azalması, TMPRSS2 aktivitesinin düşmesi ve SARS-CoV-2'nin Spike proteini ile etkileşimi, Betaferon'un viral giriş yolağını engellenmesi yoluyla SARS-CoV-2 virüsüne karşı antiviral aktiviteye sahip olduğunu göstermiştir.*

Anahtar Kelimeler: *Betaferon, COVID-19, ilaç yeniden konumlandırma, SARS-CoV-2*

INTRODUCTION

Chinese authorities found the first case of COVID-19 in Wuhan, where it was first reported in December of this year. There has been an ongoing pandemic since the disease spread around the world [1]. Globally, pre-clinical and clinical trials for the treatment of COVID-19 have been ongoing. Different therapeutic strategies have been studied, including antiviral, antimalarial, and immunomodulatory approaches [2]. However, there is still no definitive therapeutic strategy. One of the methods to introduce new therapeutics is drug repurposing which significantly reduces the time and cost of drug studies [3].

Interferon beta-1b (Betaferon), the active ingredient in Betaferon, is a member of a class of medications known as interferons. Betaferon was originally indicated for Multiple Sclerosis (MS) treatment and its active ingredient is Interferon beta-1b [4]. Interferons are naturally occurring chemicals that the body produces to aid it in the battle against attacks such as infections caused by viruses, among other things [5]. It has been shown that Interferon beta-1b has immune modulator activity, and anti-inflammatory and antiviral activity [6]. It is possible to generate interferon beta-1b by a process known as 'recombinant DNA technology. The interferon beta-1b is produced by bacteria that has been implanted with a gene (DNA) that allows it to manufacture the protein [7]. Interferon-beta-1b is the non-glycosylated version of interferon beta-1a and the replacement interferon beta-1b functions in the same way as naturally produced interferon beta-1a in the immune system [8,9].

Moreover, interferon beta-1a inhibited the replication of the SARS-CoV-2 virus [10]. Furthermore, Interferon 1 beta reduced lung fibrosis [11]. In addition, ex vivo lung tissue samples revealed that viral infection did not induce the production of interferons hence, usage of Interferon beta-1b can have antiviral

activity against the COVID-19 [12,13]. Herein, the antiviral effect of Interferon beta-1b to combat SARS-CoV-2 was studied particularly via computational and molecular biology analyses.

MATERIAL AND METHOD

In silico calculations

All reagents were analytical grade and used without processing. Betaferon was kindly provided by Bayer Pharmaceutical Company (Istanbul-Turkey). Betaferon structure (PDB ID:1au1), spike protein closed form (PDB ID:6vxx) and spike protein open form (PDB ID:6vyb) structures were used. HDock server [14] was used for docking calculations. For both forms, the 4 lowest-energy bonds were examined. The protein structures obtained from the PDB databank are visualized via BIOVIA Discovery Studio 2021 software [15].

MTT Assay

The MTT assay was carried out the determination of the toxic doses of Betaferon (Millipore, CT02). Vero E6 cells detached with trypsinization. Detached cells were counted and 10.000 cells per well were seeded on a 96-well plate and incubated for 24 hours. For the next 48 hours, the cells were treated with 0.96, 0.48, 0.24, 0.12, and 0.06µg/ml Betaferon for 48 hours. Betaferon had its solvent, it was applied as solvent control. Absorption was measured at 570 nm using EpochTM Microplate Spectrophotometer. The OD values were analyzed relative to the control hence, the percentage of cell viability was determined.

Flow Cytometry for Protein Expression

TMPRSS2 expression of Betaferon treated cells, solvent control cells, and control cells were examined by flow cytometry (NovoCyte Flow Cytometry). Cells were harvested with trypsinization. Collected cells were fixed with 4% Paraformaldehyde, permeabilize, and stained using an antibody against TMPRSS2 (Santa Cruze, sc-515727) respectively. Novo Express 1.3.0 Software (ACEA Biosciences, Inc) is used for flow cytometry.

Evaluation of Gene Expression Through qRT-PCR

TMPSS2 and ACE2 mRNA expression level alterations after Betaferon treatment were assessed with quantitative real-time PCR (qRT-PCR). RNA isolation was carried out with RiboEx (GeneAll® Ribo Ex, 301-001) by following the manufacturer's protocol. cDNA synthesized with 1µg of isolated mRNA (TONBO Biosciences, 31-5300-0100R). qRT-PCR was carried out with instructions of the manufacturer by using CYBER FastTM qPCR Hi-ROX Master Mix (TONBO Biosciences). Gene expression normalized to the expression of GAPDH. Raw data was analyzed with the $2^{-\Delta\Delta CT}$ method.

Western Blotting

The impact of Betaferon on ACE2 protein levels was evaluated through Western Blotting. Cells are harvested with a cell scraper and collected cell pellet lysed with protein lysis buffer. Concentrations of proteins were measured with BCA Protein Assay Reagent (Pierce, Rockford, IL). 60 μg protein for each treatment group loaded with polyacrylamide gel. Polyacrylamide gel was transferred to polyvinylidene difluoride (PVDF) membrane (TransBlot Turbo, Bio-Rad) with a semi-dry transfer technique due to directives of the manufacturer (Bio-Rad, Hercules, CA). Monoclonal anti-ACE2 antibody from ProSci (1:250) and goat anti-rabbit IgG-horseradish peroxidase-conjugated antibody from Santa Cruz Biotechnology (1:2500) were used as primary and secondary antibodies respectively. Monoclonal anti-β-actin antibodies from Santa Cruz and goat anti-mouse HRP conjugated from Pierce were used as primary and secondary antibodies respectively for β- actin. All of the antibodies were diluted in a 5% BSA containing TBST blocking solution. Membrane developed and images were taken with ChemiDoc MP Imaging System (Bio-Rad, Hercules, CA).

Proteolytic Activity Assay to Evaluate the Enzymatic Activity of TMPRSS2

In Vero E6 cells, the proteolytic activity of TMPRSS2 after Betaferon treatment was examined. To assess the proteolytic activity of TMPRSS2, 20.000 cells/well were seeded into a 96-well plate in 100 μl of cell culture maintenance medium. Seeded cells were treated with varied Betaferon concentrations (0.96μg/ml, 0.48μg/ml, 0.24μg/ml and, 0.12μg/ml) for 48 hours. The cells were washed with 1X PBS and incubated with fluorogenic synthetic peptide Boc-Gln-Ala-Arg-AMC (Enzo, BML-P237-0005) at a final concentration of 200 μM at 37°C for 2 hours. Supernatants in the wells were collected individually. Low-rate centrifugation was performed at 3000 rpm, 10 min at 4°C. A new 96-well plate was used to replace the cleared supernatants. Fluorescence intensity was measured through a fluorescence spectrometer at 380 nm (excitation) and 460 (reflection).

Statistical Analysis

Each piece of the data was subjected to statistical analysis to determine its importance. We conducted in vitro experiments excluding qRT-PCR were performed in 3 biological replicates, whereas qRT-PCR was performed in 6 biological replicates to obtain the most accurate results. Paired two-tailed t-tests were used to compare the control and drug-treated group. Significance was defined as p values less than 0.05. To be considered significant p-value must fall below 0.05. Graph Pad Prism 8.01 was used to analyze the data.

RESULT AND DISCUSSION

MTT assay was carried out for the assessment of the viability of Vero E6 cells after the Betaferon treatment as a concentration gradient (0.06, 0.12, 0.24, 0.48, and 0.96 µg/ml). We did not observe a significant decrease in cell viability accompanied by an increasing concentration of Betaferon. According to these results, the concentration of 0.96 µg/ml was determined to use in the forward experiments including anti-viral as 0.96 µg/ml is the topmost dose while the viability of the cell is resembling with the control group. Vero E6 cells were infected with SARS-CoV-2 in which were pretreated with Betaferon with concentration gradient (0.06, 0.12, 0.24, 0.48, and 0.96 µg/ml) and after 48-hour viral copies in the supernatant were collected and analyzed through qRT-PCR for determination of the antiviral activity of Betaferon. Based on anti-viral activity results and MTT results (Figures 1 and 2), it was determined that 0.96 µg/ml Betaferon concentration for further molecular experiments.

We performed molecular analysis to understand the downstream impacts of Betaferon on the viral life cycle. Since TMPRSS2 and ACE2 proteins play a crucial role in enabling virus entry, we tested whether Betaferon has a repressive effect on both of these proteins. Firstly, we analyzed gene expression levels of TMPRSS2 after the 0.96 µg/ml Betaferon treatment by qRT-PCR. We verified that the application of Betaferon significantly lowers TMPRSS2 expression compared to solvent control after 48 hours (Figure 3A). To evaluate the alterations in TMPRSS2 protein expression with the application of Betaferon, we performed a flow cytometry analysis. TMPRSS2 protein level was reduced in the Betaferon treated group after 48 hours due to the control. (For Vero-E6; Control cells 91.8 ± 0.3 %, Solvent treated cells 98.8 ± 0.45%, Betaferon treated cells 84.68 ± 0.31%) (Figure 3B). These data suggest that the application of Betaferon lowers the expression of TMPRSS2 both at protein and mRNA grade in 48 hours.

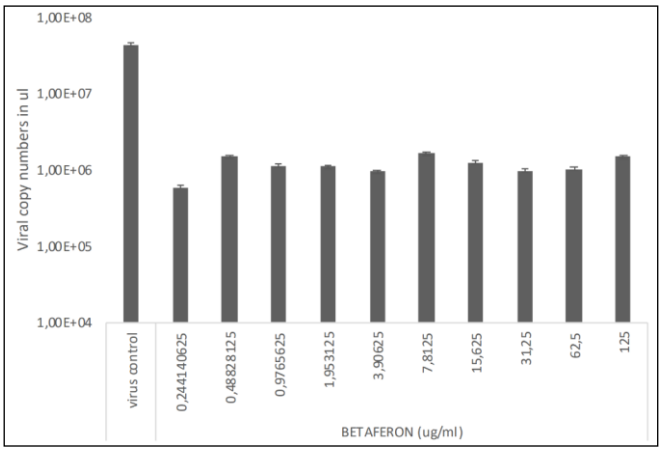

Figure 1. Antiviral activity of Betaferon. Pre-treated Vero E6 cells with Betaferon at different concentrations infected with SARS-CoV-2. After 48-hour viral copies in the supernatant were analyzed through qRT-PCR.

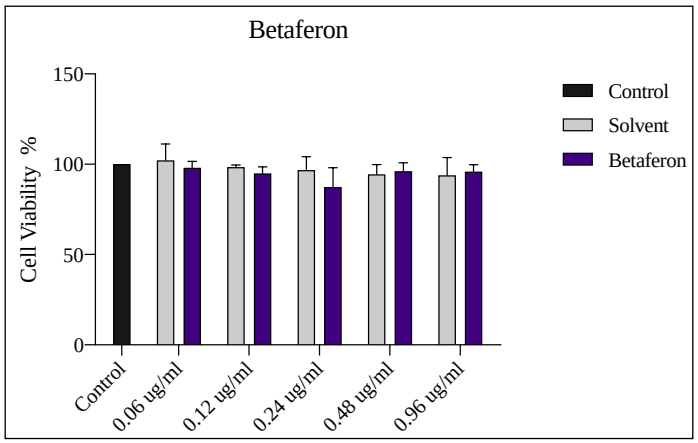

Figure 2. Cytotoxic effect of Betaferon at the concentrations of 0.06, 0.12, 0.24, 0.48, and 0.96 µg/ml on Vero E6 for 48 hours. Data show the mean and the SD of samples. The black bar represents the cells cultured with the maintenance medium (Control). The data obtained from the cells treated with the maintenance medium including Betaferon's solvent represented as grey bars in the graph (Solvent Control). Purple bars show the cells treated with Betaferon. Data was interpreted as the mean ± SD (n= 4).

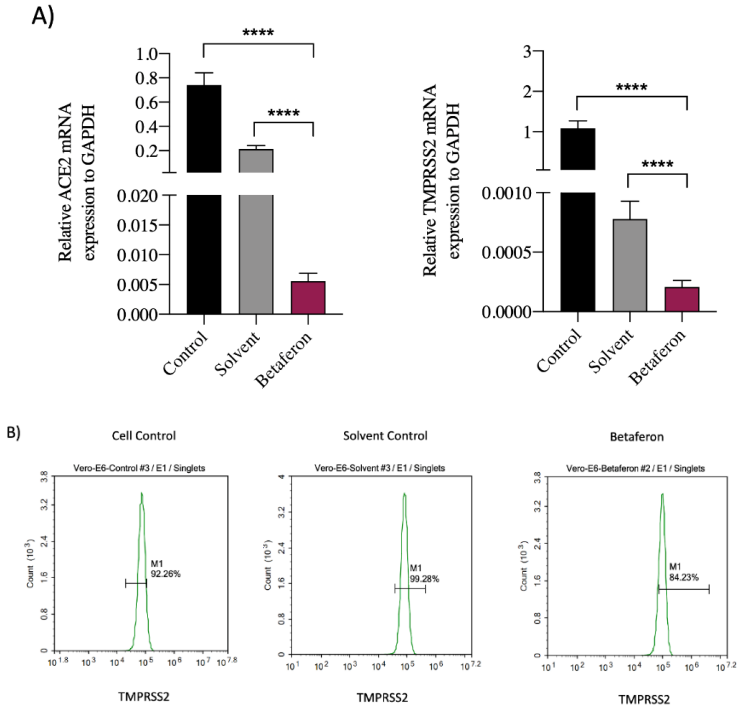

Figure 3. qRT- PCR manifests relative fold differences of TMPRSS2 mRNA expression after 48 h Betaferon treatment in **A)** Vero-E6 cells ($p<0.0005$). **** indicates $p<0.0005$. Data was interpreted as the mean ± SD (n= 6). **B)** Illustration represents flow cytometry graphs of Betaferon (0.96 µg/ml) treated cells. The Betaferon treated cells show a decrease in the TMPRSS2 expression (Paired Two-tailed T-test, $P<0.05$). * indicates $p<0.05$. Data was interpreted as the mean ± SD (n= 3).

To realize our suggestion about the anti-viral activity of Betaferon, and to reveal the impact of Betaferon in the regulation of ACE2 at protein and transcript grades, we carried out western blotting and qRT-PCR. Our qRT-PCR results showed that Betaferon significantly decreases the mRNA level of ACE2 after 48-hour treatment (Figure 3A). Consequently, we observed that Betaferon has a significantly diminishing impact on the regulation of ACE-2 protein in Vero-E6 (Figure 4).

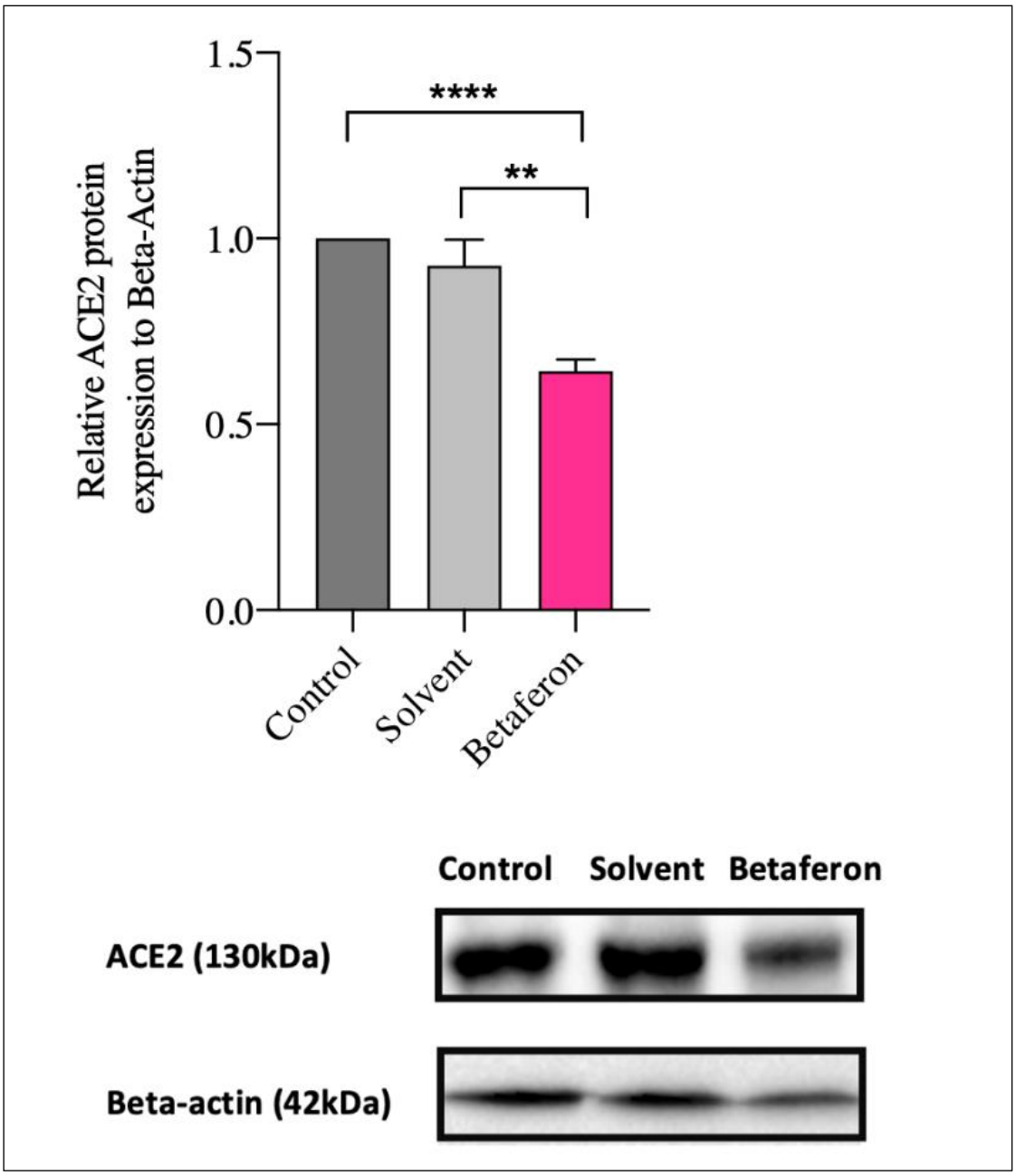

Figure 4. Betaferon decreases ACE2 protein expression in Vero E6 cells. Analysis of band intensities shows alterations in protein levels of ACE2 in Betaferon (0.96 μg/ml) treated Vero-E6 cells. The Betaferon treatment results in a significant decrease in cells. ** indicates $p<0.005$. Data was interpreted as the mean ± SD (n= 3).

As we know, SARS-CoV-2 penetrates the host cell through its surface spike protein for binding on the host cell. Moreover, SARS-CoV-2 priming with the host cell results in fusion. TMPRSS2 has a cleavage domain that cuts the spike protein to induce fusion of membrane. Our previous study about the anti-viral activity of Ribavirin on SARS-CoV-2 revealed that the inhibitory impacts on the proteolytic activity of TMPRSS2 are valuable as a symbol of the anti-viral effect of the drug at the molecular grade [17]. The TMPRSS2 proteolytic activity is measured with the addition of synthetic commercial protease substrate into treated Vero-E6 cells with 0.48 and 0.96 μg/ml Betaferon for 48 hours [17]. We determined the proteolytic activity of TMPRSS2 due to the detection of soluble forms of proteins liberated from cells. Our results indicated that the application of Betaferon significantly diminishes TMPRSS2 activity in a dose-dependent manner (Figure 5). This finding indicates that especially 0.96 μg/ml Betaferon treatment has an inhibitory impact on the proteolytic activity of TMPRSS2.

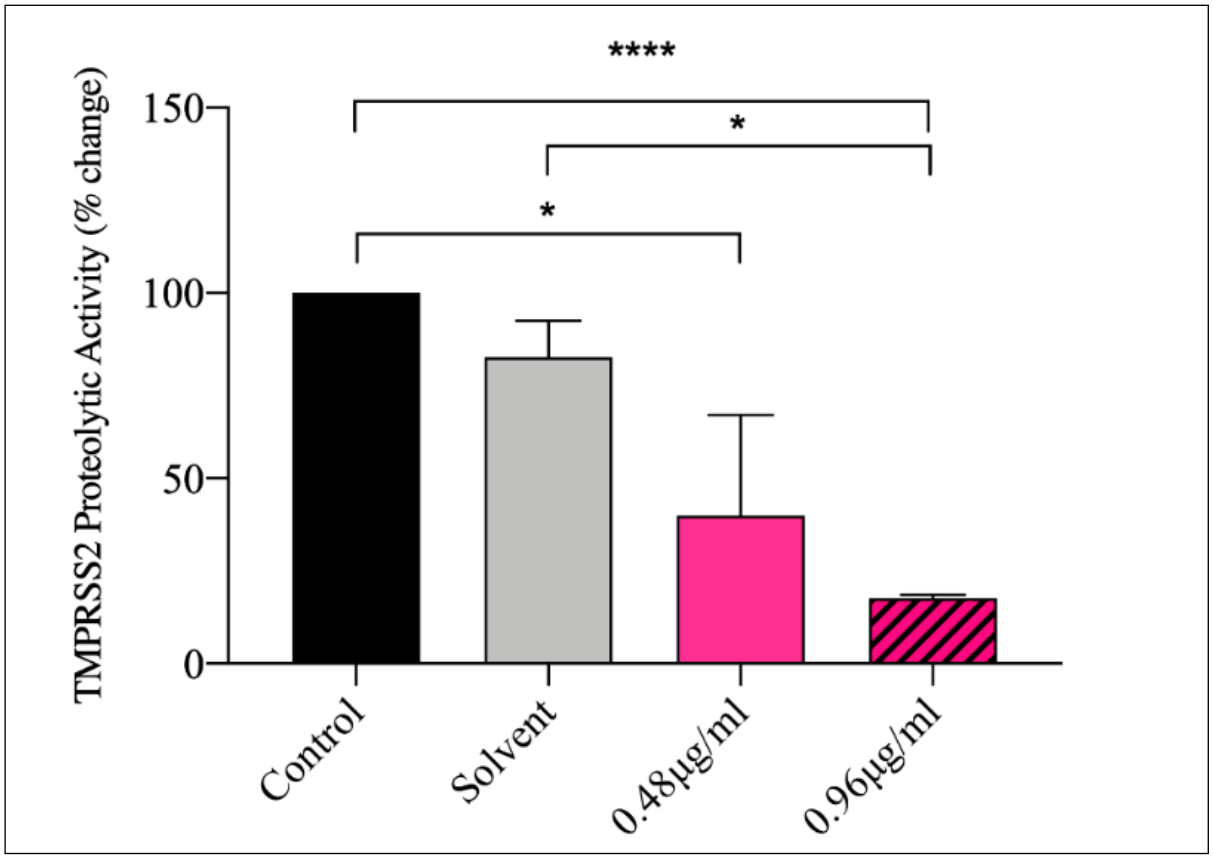

Figure 5. Examination of the proteolytic activity of TMPRSS2 carried out through substrate assay analysis. Betaferon treatment causes a remarkable reduction of the primed substrate by TMPRSS2. This data demonstrates that Betaferon suppresses the enzymatic activity of TMPRSS2. * indicates $p<0.05$; **** indicates $p<0.005$. Data interpreted as the mean ± SD (n= 3)

Concerning the analysis of the drug's mechanism of action against viruses in-depth, we performed *in silico* analyzes. *In silico* analyses revealed that the lowest energy binding score to the closed form of

Spike protein is -418.13 kcal/mol. All 4 bindings to this region are in the RBD region (Figure 6). Binding to the RBD region is critical for virus attachment to the ACE2 receptor and inactivating the mechanism of entry into the cell.

The lowest energy of the bonds to the open form of Spike protein is -339.66 kcal/mol. There are differences between the patterns of attachment to this form; 1st and 2nd pattern binding to the RBD region, 3rd pattern binding to the S2 subunit, and 4th pattern binding are to the N-terminal domain (Figure 7). Regions other than RBD are also used in antibody targets and are effective in inactivating the virus.

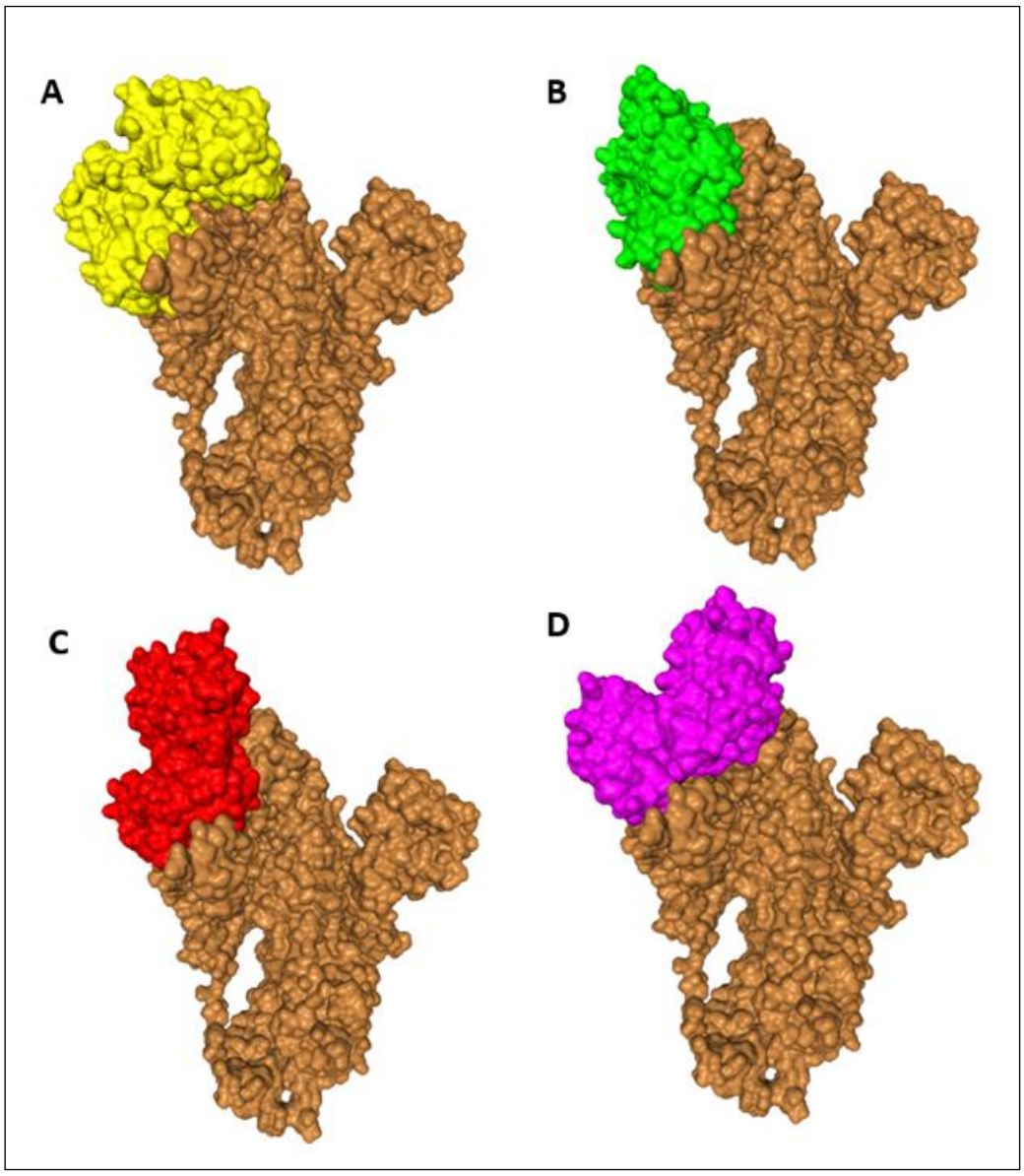

Figure 6. Docking results of Betaferon and spike protein (close form). All docking has been to the RBD region. The binding energies are respectively A: -418.13 kcal/mol, B: -337.87 kcal/mol, C: -324.85 kcal/mol, D: -322.86 kcal/mol.

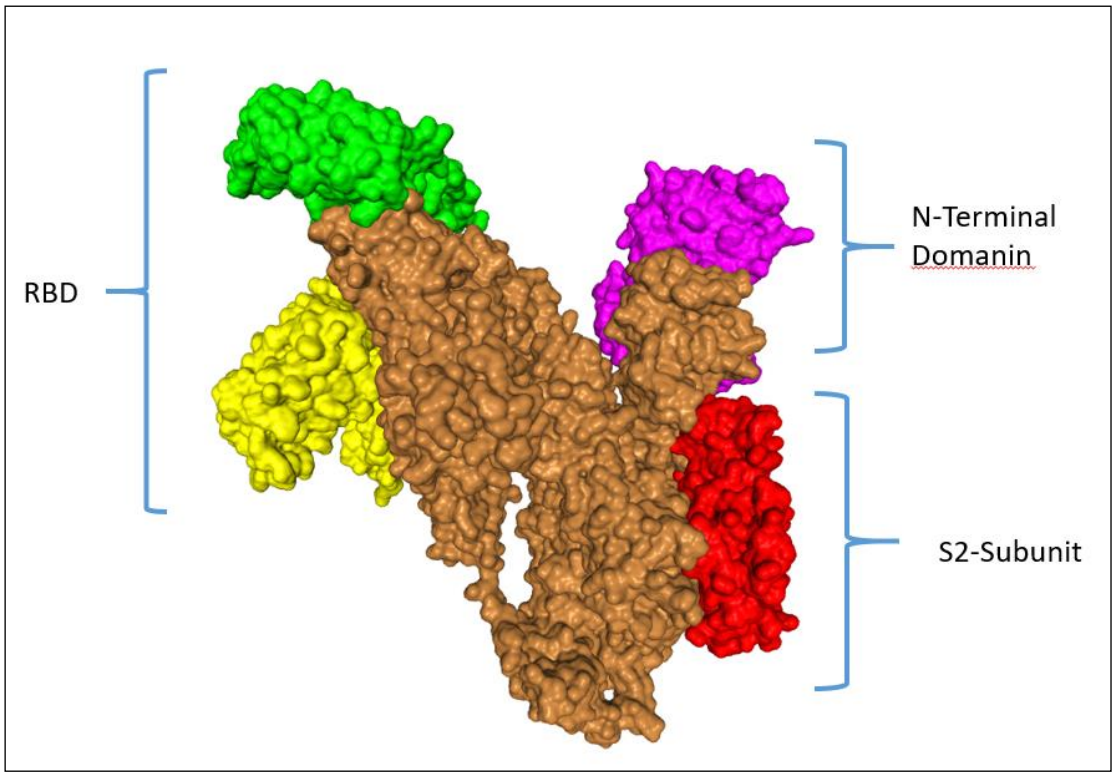

Figure 7. Docking results of Betaferon and spike protein (open form). The first two binding to the open form are to the RBD sites (binding energy: -339.66 kcal/mol and -317.65 kcal/mol), the others are the S2- subunit (binding energy: -316.42 kcal/mol) and N-Terminal (binding energy: -316.42). kcal/mol) area. These bindings to different regions of the protein are important for virus inactivation.

When the bond profile of spike protein (closed form) and betaferon is examined, it is seen that there are many hydrogen bonds. In addition to these, electrostatic interactions are also observed, albeit slightly. The interaction of betaferon with the open form was observed in 4 different regions. Hydrophobic and electrostatic interactions are observed with hydrogen bonds in the 1st region. The connection profile here shows a high similarity with the closed form. When the interactions in the 2nd region are examined, it is seen that the pi-sulfur bond forms a pi-sulfur bond with the residue CYS141. While hydrophobic interactions increase in the 3rd region, the interaction profile in the 4th region shows a high similarity with the 2nd region.

In conclusion, Betaferon showed significant antiviral activity against the SARS-CoV-2 virus. *In vitro* and computational studies revealed that Betaferon actively inhibits the viral entry pathway. It decreases in expression of ACE2 and TMPRSS2 proteins in both protein and mRNA grades. In addition to the decrease in the expression of TMPRSS2 protein Betaferon also inhibits the proteolytic activity of TMPRSS2. Furthermore, the interaction of Betaferon with the SARS-CoV-2 Spike protein indicates that Betaferon shows Betaferon is effective not only in the host but also in the SARS-CoV-2 virus. Hence,

Betaferon has significant antiviral activity against the COVID-19 virus via inhibition of the viral entry pathway by targeting both host and viral proteins. In our study, it is seen that the experimental data were confirmed by *in silico* results. The interaction of betaferon with both the closed and open form of the Spike protein increases its inhibition capacity. interaction with different regions of the open form increases the effect of the drug despite the high binding energies. The fact that it is larger than the small molecule structurally affects the binding profile considerably. In this way, it increases its affinity by establishing many hydrogen bonds with different residues. Since the spike-ace2 interface is very large, inhibiting this region is only possible with large molecules such as betaferon. The most important result of this is that the probability of being affected by mutations in the Spike protein decreases.

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AUTHOR CONTRIBUTIONS

Conception: *F.B., K.C.A.;* Design: *F.B., K.C.A., S.A.O;* Supervision: *K.C.A., S.A.O;* Resources: F.B., *K.C.A., S.A.O;* Materials: *F.B. K.C.A.;* Data collection and/or processing: *F.B., K.C.A., S.A.O;* Literature search: *F.B.;* Writing manuscript: *F.B.; K.C.A., S.A.O.*; Other:-

CONFLICT OF INTEREST

The authors declare no conflict of interest.

ETHICS COMMITTEE APPROVAL

The authors declare that ethics committee approval is not required for this study.

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