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RESEARCH ARTICLE

The Pathophysiology of COVID-19 and SARS-CoV-2 Infection

In silico investigation of the viroporin E as a vaccine target against SARS-CoV-2

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Abstract

Viroporins, integral viral membrane ion channel proteins, interact with host-cell proteins deregulating physiological processes and activating inflammasomes. Severity of COVID-19 might be associated with hyperinflammation, thus we aimed at the complete immunoinformatic analysis of the SARS-CoV-2 viroporin E, P0DTC4. We also identified the human proteins interacting with P0DTC4 and the enriched molecular functions of the corresponding genes. The complete sequence of P0DTC4 in FASTA format was processed in 10 databases relative to secondary and tertiary protein structure analyses and prediction of optimal vaccine epitopes. Three more databases were accessed for the retrieval and the molecular functional characterization of the P0DTC4 human interactors. The immunoinformatics analysis resulted in the identification of 4 discontinuous B-cell epitopes along with 1 linear B-cell epitope and 11 T-cell epitopes which were found to be antigenic, immunogenic, nonallergen, nontoxin, and unable to induce autoimmunity thus fulfilling prerequisites for vaccine design. The functional enrichment analysis showed that the predicted host interactors of P0DTC4 target the cellular acetylation network. Two of the identified host-cell proteins – BRD2 and BRD4 – have been shown to be promising targets for antiviral therapy. Thus, our findings have implications for COVID-19 therapy and indicate that viroporin E could serve as a promising vaccine target against SARS-CoV-2. Validation experiments are required to complement these in silico results.

acetylation; functional enrichment analysis; immunoinformatics; SARS-CoV-2; viroporin

INTRODUCTION

The severe acute respiratory syndrome-coronavirus 2 (SARS-CoV-2) emerged in China at the end of December 2019. Globally, as of 19 January, 2021, there have been 94,124,612 confirmed cases of the coronavirus disease 2019 (COVID-19), including 929,994 deaths reported to WHO (https://covid19.who.int/). The clinical presentation of COVID-19 is diverse, ranging from asymptomatic infection to mild upper respiratory tract illness to severe interstitial pneumonia with respiratory failure and death (1). A systematic review and meta-analysis of 45 studies with a total number of 4,203 patients reported that the pooled rates of intensive care unit (ICU) admission, mortality, and acute respiratory distress syndrome (ARDS) were 10.9%, 4.3%, and 18.4%, respectively (2).

The COVID-19 disease not only affects the lungs but also has cardiovascular, endothelial, and inflammatory sequelae (3). Hyperinflammation and coagulopathy have been identified as the main contributors to disease extensiveness and death in infected patients (4). Severe cases of COVID-19 have been associated with increased serum levels of several inflammatory cytokines and chemokines, leukocytosis, and lymphocytopenia [reviewed in Merad and Martin (4) and Yap et al.(5)]. The activation of the inflammasome pathway and its contribution in the pathobiology of COVID-19 disease are currently being investigated with possible impacts on therapeutic targeting (5).

Viral ion channels or viroporins are integral viral membrane proteins (6) that have been linked to inflammasomes activation (7). The severe acute respiratory syndrome coronavirus (SARS-CoV) encodes three viroporins, the proteins 3a, E, and 8a of which the first two have been shown to be indispensable for maximal SARS-CoV replication (8). The ion channel activity of the E protein has also been identified as a virulence factor in mouse models (8, 9). The transport of calcium cations through the E protein ion channel has been shown to activate the (NOD)-like receptor protein 3 (NLRP3) inflammasome resulting in severe immuno-pathological effects on the infected host (10). The fact that the amino acid sequences of the E viroporins from SARS-CoV and SARS-CoV-2 have been reported to possess 94.7% identity (5, 11) has led to the hypothesis that the two proteins probably have similar functions (5).

Recently, a machine-learning based analysis of mutation stability showed that mutations in the transmembrane region of

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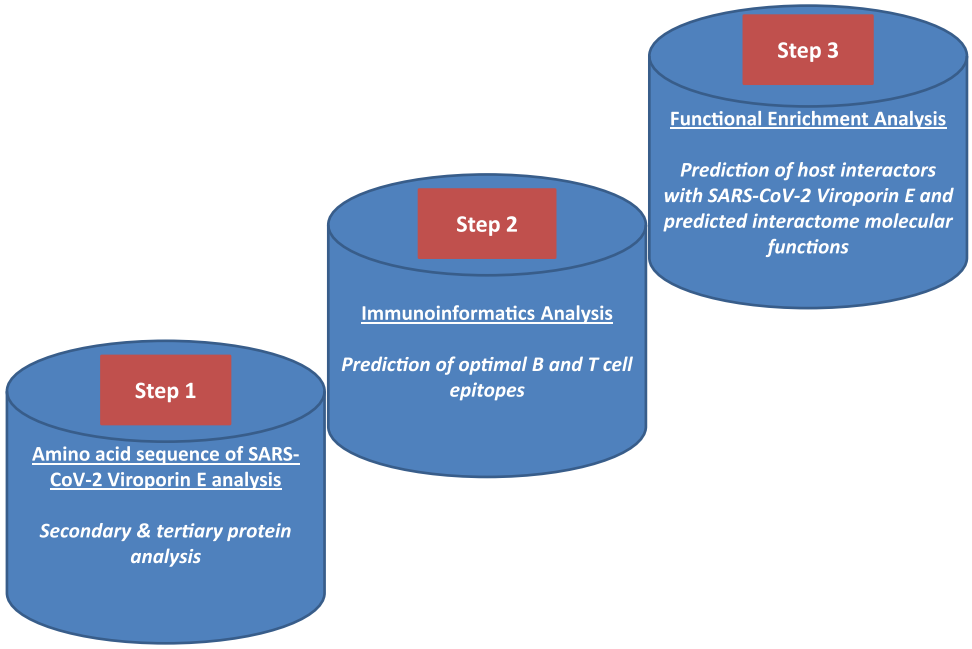

Figure 1. Workflow of the in silico study. The SARS-CoV-2 E protein sequence in FASTA format was obtained from the Uniprot database. Thirteen computational prediction tools were used for the immunoinformatics and functional enrichment analyses.

the E protein – keeping the PDZ-binding motif (PBM) intact – could provide the basis for the development of live-attenuated vaccine and inactivated vaccine (12). Computational approaches for vaccine and drug design–as the one mentioned above–have guided the development of novel therapeutic strategies during the last decade. In silico methodology has widely been used for the identification of drugs and vaccine candidate epitopes against several bacteria and viruses including SARS-CoV-2 (12–19). Most of these studies have focused on the spike proteins and viral RNA-polymerases as potential vaccine and drug targets (12), whereas very few studies involve viroporin E as a vaccine or drug target candidate (12, 14).

Therefore, in this study, we aimed at the complete immunoinformatic analysis of the SARS-CoV-2 E viroporin, the envelope small membrane protein P0DTC4. In addition, we identified the human proteins interacting with P0DTC4 as well as the significantly enriched molecular activities of the corresponding genes highlighting the role of host proteins interacting with SARS-CoV-2 viroporin E in the immunopathophysiology of COVID-19.

MATERIALS AND METHODS

Immunoinformatic Analysis of the Envelope Small Membrane Protein P0DTC4

The SARS-CoV-2 E protein sequence in FASTA format was obtained from the Universal Protein Resource (Uniprot) database (https://www.uniprot.org/) (entry P0DTC4) (20). The whole protein antigenicity was determined using the VaxiJen v. 2.0 server (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html), which enables the classification of antigens solely based on the physicochemical properties of proteins regardless of the sequence length and the need for alignment (21). This initial examination identified P0DTC4 as a probable antigen with a prediction probability equal to

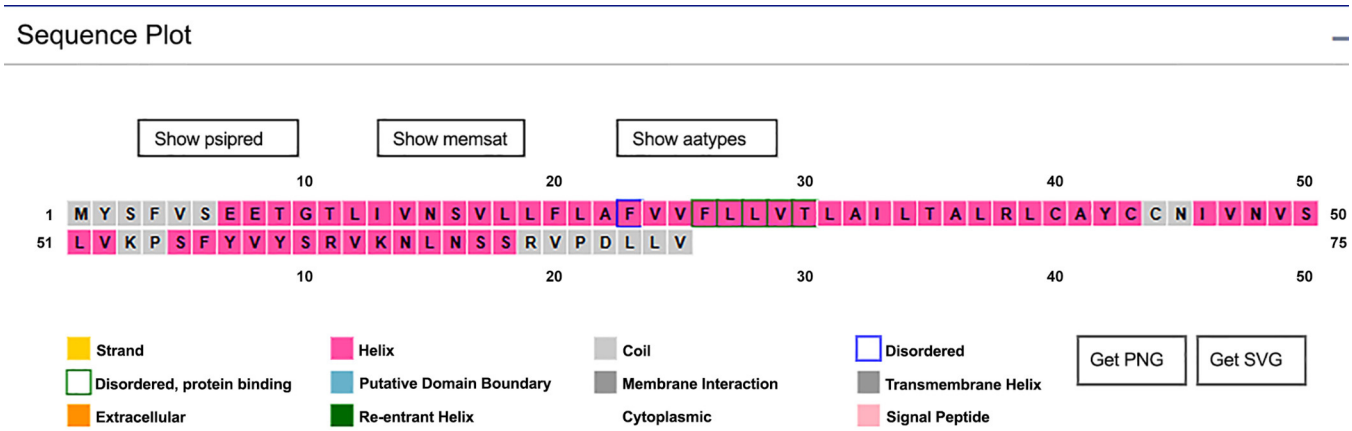

Figure 2. The secondary structure of the SARS-CoV-2 E viroporin as predicted by the PSIPRED 4.0 database.

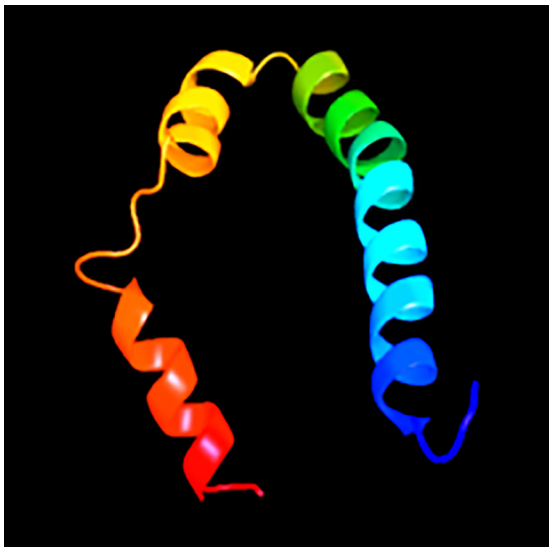

Figure 3. The three-dimensional (3-D) model of the SARS-CoV-2 E viroporin as retrieved by the PHYRE2 server. The model was built on the PDB template c5×29B (NMR structure of the SARS-CoV e protein pentameric ion channel). NMR, nuclear magnetic resonance.

0.5262 (the predefined threshold for the virus model was 0.4). Subsequently, the secondary and tertiary structure of the protein was modeled by the PSIPRED 4.0 (http://bioinf.cs.ucl.ac.uk/psipred/) (22) and PHYRE2 (http://www.sbg.bio.ic.ac.uk/~phyre2/html) (23) servers, respectively. B-cell linear and discontinuous epitopes were predicted by the ElliPro tool (http://tools.iedb.org/ellipro/) of the Immune Epitope Database Analysis Resource (IEDB) (24). The Vaxitop module of the Vaxign system (http://www.violinet.org/vaxign/vaxitop/) was used for the prediction of epitope binding to MHC class I and class II alleles (25). Vaxitop is a vaccine epitope prediction and analysis system based on the principle of reverse vaccinology. The MHC I epitopes were further investigated for the ability to enhance immunogenicity with the class I immunogenicity tool of IEDB (http://tools.iedb.org/immunogenicity/) (26). The antigenicity of the predicted B- and T-cell epitopes was tested via VaxiJen (21) and peptides that were identified as high antigenic were subsequently examined for allergenicity, toxicity, and ability to induce autoimmunity by the AllerTOP v. 2.0 (https://www.ddg-pharmfac.net/AllerTOP/) (27), ToxinPred (http://crdd.osdd.net/raghava/toxinpred/) (28), and Peptide Match (https://research.bioinformatics.udel.edu/peptidematch/index.jsp) (29) servers, respectively. In all of the aforementioned tools, the default parameters were used and where appropriate, *Homo sapiens* was selected as the host species. Finally, MHC class II binders were also screened for the ability to induce IFN-γ using the predict module of the IFNepitope server (http://crdd.osdd.net/raghava/ifnepitope/) and the motif and support vector machine (SVM) hybrid approach (30).

Identification of the Human Proteins Interacting with the Envelope Small Membrane Protein P0DTC4 and Functional Enrichment Analysis of the Corresponding Genes

The Biological General Repository for Interaction Datasets (BioGRID) database (https://thebiogrid.org/) (31) was accessed for the investigation of the human proteins that interact with P0DTC4. The genes encoding the identified proteins were entered as a list in the ToppFun module of the ToppGene database (https://toppgene.cchmc.org/enrichment.jsp) (32) for the detection of the statistically significantly enriched molecular functions. ToppFun performs functional enrichment of input gene list based on transcriptome (gene expression), proteome (protein domains and interactions), regulome (TFBS and miRNA), ontologies (GO, pathway), phenotype (human disease and mouse phenotype), pharmacome (drug-gene associations), and bibliome (literature co-citation). We repeated the analysis with the g:GOSt tool of the g:Profiler server (https://biit.cs.ut.ee/gprofiler/gost) that relies on ENSEMBL as a primary data source and was recently updated with new data (33). The workflow of the methodology followed in the study is displayed in Fig. 1. The applied computational tools were selected on the basis of software documentation, performance, and citation in previous publications (16, 34). All analyses were performed on May 2020.

Predicted Linear Epitope(s):

No.	Chain	Start	End	Peptide	Number of residues	Score	3D structure
1	_	8	14	ETGTLIV	7	0.83	View
2	_	59	65	YSRVKNL	7	0.786	View
3	_	51	56	LVKPSF	6	0.586	View
4	_	32	43	AILTALRLCAYC	12	0.549	View

Predicted Discontinuous Epitope(s):

No.	Residues	Number of residues	Score	3D structure
1	_:G10, _:T11, _:L12, _:I13	4	0.793	View
2	_:S60, _:R61, _:K63, _:N64, _:L65	5	0.79	View
3	_:S50, _:L51, _:V52, _:K53, _:S55, _F56, _:Y59, _:V62	8	0.619	View
4	_:A32, _:I33, _:T35, _:A36, _:L37, _:R38, _:L39, _:C40, _:A41, _:Y42, _:C43	11	0.577	View

Figure 4. The B-cell linear and discontinuous epitopes that were predicted by the ElliPro tool of the immune epitope database analysis resource (IEDB) based on the predicted three-dimensional (3-D) model of the SARS-CoV-2 viroporin E.

Table 1. *The MHC I binding epitopes of the SARS-CoV-2 E viroporin as predicted by the Vaxitope server**

Peptide	Length	Immunogenicity Score	Antigenicity Score	Allergenicity	Toxicity	Peptide Match-Ability to Induce Autoimmunity
VLLFLAFVVFL	**11**	**0.3976**	**0.4012**	**Probable nonallergen**	**Nontoxin**	**No**
LFLAFVVFLL	**10**	**0.32115**	**0.5111**	**Probable nonallergen**	**Nontoxin**	**No**
LLFLAFVVFL	**10**	**0.32104**	**0.6159**	**Probable nonallergen**	**Nontoxin**	**No**
FLAFVVFLLV	**10**	**0.30526**	**0.5651**	**Probable nonallergen**	**Nontoxin**	**No**
FLAFVVFLL	**9**	**0.30188**	**0.5308**	**Probable nonallergen**	**Nontoxin**	**No**
LFLAFVVFL	**9**	**0.29187**	**0.4568**	**Probable nonallergen**	**Nontoxin**	**No**
LLFLAFVVF	**9**	**0.2341**	**0.8144**	**Probable nonallergen**	**Nontoxin**	**No**
FVSEETGTL	9	0.23237	0.3864 Probable nonantigen	Probable allergen	Nontoxin	No
VTLAILTALR	**10**	**0.21765**	**0.8404**	**Probable nonallergen**	**Nontoxin**	**No**
LAFVVFLLV	**9**	**0.2141**	**0.7976**	**Probable nonallergen**	**Nontoxin**	**No**
VTLAILTAL	**9**	**0.21055**	**0.6140**	**Probable nonallergen**	**Nontoxin**	**No**
SEETGTLIV	9	0.2095	0.3052 Probable nonantigen	Probable nonallergen	Nontoxin	No

*Only results with >0.2 immunogenicity score are presented. Bold lines correspond to epitopes which fulfill prerequisites for vaccine design. MHC, major histocompatibility complex.

RESULTS

Immunoinformatic Analysis of the P0DTC4 Protein

The secondary structure and the three-dimensional (3-D) model of the P0DTC4 protein are presented in Figs. 2 and 3, respectively. The tertiary model was built on the SARS-CoV E protein pentameric ion channel template (PDB: c5×29B) with 99.8% confidence. The PDB file of the constructed, final model was entered in the ElliPro tool which identified four linear and four discontinuous B-cell epitopes (Fig. 4). Out of the four predicted linear peptides, only AILTALRLCAYC was found to fulfill the requirements for vaccine design as it was identified as probable *1*) antigen (score 0.7860), *2*) nonallergen, and *3*) nontoxin. In addition, no match for this peptide was identified in the peptide search database.

The Vaxitop method identified 52 and 36 unique MHC I and MHC II alleles, respectively. The findings pertaining to the remaining analyses with respect to the immunogenicity of the MHC I epitopes, the ability of MHC class II binders to induce IFN-γ, and the antigenicity, allergenicity, toxicity, and ability to induce autoimmunity of all predicted T-cell epitopes are shown in Tables 1 and 2. In total, 11 T-cell epitopes were found to fulfill prerequisites for vaccine design.

Identification and Functional Genomic Analysis of the Predicted Host-Cell Proteins

Six human proteins [AP3B1: adaptor-related protein complex 3, β 1 subunit; SLC44A2: solute carrier family 44 (choline transporter), member 2; ZC3H18: zinc finger CCCH-type containing 18; CWC27: CWC27 spliceosome-associated protein homolog (*Saccharomyces cerevisiae*); BRD2&4: bromodomain containing 2&4] were found to form the host interaction network with P0DTC4 (Fig. 5). Lysine-acetylated histone binding and acetylation-dependent protein binding were the most significantly enriched molecular activities of the corresponding genes both in the g:Profiler and ToppFun tools (Fig. 6 and Table 3).

DISCUSSION

In this study, we aimed at the investigation of the SARS-CoV-2 E viroporin as a potential vaccine target. In the past few years, viral ion channels are in the focus of antiviral research development (6, 35). It has been shown that compounds which block the ion channel activity are effective antiviral drugs both in vitro and in vivo (36). Most recently, the SARS-CoV-2 3a protein was proposed as a feasible therapeutic target against the COVID-19 disease (37). Both the 3a and E proteins of SARS-CoV have been reported to induce the activation of the NLRP3 inflammasome via their viroporin activity, by disturbing the ionic concentration within the cells which results in the generation of reactive oxygen species by the damaged mitochondria, but also independently of it, by stimulating NF-κB signaling which in turn promotes chemokine production [reviewed in Yap et al. (5)].

Table 2. *The MHC II binding epitopes of the SARS-CoV-2 E viroporin as predicted by the Vaxitope server**

Peptide	Antigenicity Score	Allergenicity	Toxicity	IFN-γ Induction	Peptide Match-Ability to Induce Autoimmunity
CNIVNVSLV	1.4201	Probable allergen	Nontoxin	Negative	No
YCCNIVNVS	1.3929	Probable nonallergen	Toxin	Negative	No
CCNIVNVSL	1.3710	Probable nonallergen	Toxin	Positive	No
RLCAYCCNI	1.1243	Probable nonallergen	Toxin	Positive	No
VYSRVKNLN	1.0946	Probable nonallergen	Nontoxin	Negative	No
KPSFYVYSR	0.9740	Probable allergen	Nontoxin	Negative	No
SRVKNLNSS	0.9490	Probable nonallergen	Nontoxin	Negative	No
VVFLLVTLA	0.9374	Probable nonallergen	Nontoxin	Negative	No
LAILTALRL	0.8872	Probable nonallergen	Nontoxin	Negative	No
SFYVYSRVK	0.8251	Probable nonallergen	Nontoxin	Negative	No
LLFLAFVVF	**0.8144**	**Probable nonallergen**	**Nontoxin**	**Positive**	**No**

*Only results with >0.8 antigenicity score are presented. Bold line corresponds to the epitope which was also predicted to have the potency to induce IFN-γ. MHC, major histocompatibility complex.

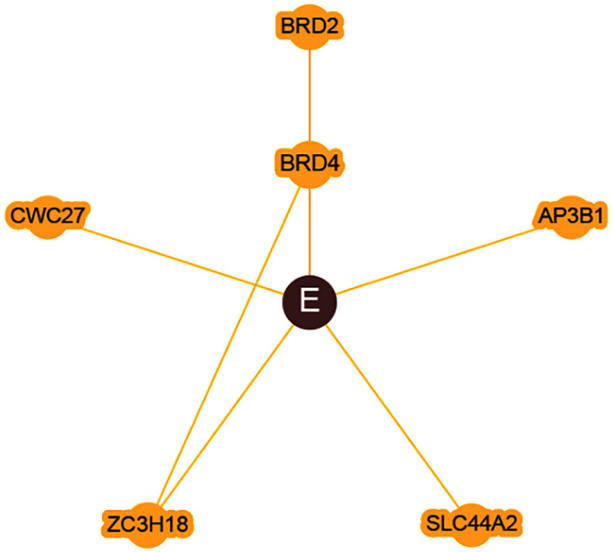

Figure 5. The human proteins interacting with the SARS-CoV-2 E viroporin as retrieved by the BioGRID database.

The primary amino acid sequence alignment of the 3a proteins from SARS-CoV and SARS-CoV-2 detected a sequence identity equal to 72.4% (11). We focused on the viroporin E protein and analyzed both its secondary and tertiary structure. The high degree of identity (94.7%) between the sequences of the E proteins of SARS-CoV and SARS-CoV-2 (11) was reflected in our results regarding the 3-D structure of the two viroporins as confirmed by the predicted 3-D model of P0DTC4. This finding strengthened the hypothesis that the two proteins have a conserved function, as proteins with high sequence identity and high structural similarity are likely to possess functional similarity and evolutionary relationships (38). Subsequently, we tested the whole P0DTC4 antigenicity. The SARS-CoV-2 E viroporin was identified as a probable antigen. The succeeding immunoinformatics analysis resulted in the prediction of four discontinuous B-cell epitopes along with 1 linear B-cell epitope and 11 T-cell epitopes which were found to fulfill the criteria of safety and effectiveness for vaccine design. It should be noted that at present, T-cell epitope prediction is considered more developed and reliable than that of B-cell prediction mainly due to the fact that the majority of B-cell epitopes are conformational, and thus they cannot be isolated from the protein structure (39).

Our findings indicate that the selected B- and T-cell linear peptides are antigenic, immunogenic, nonallergen, nontoxin, and impotent in inducing autoimmunity. The significance of these results lies in the fact that they provide vaccine candidates which could be tested immediately on experimental animal models, thus minimizing the cost and time for vaccine epitope research and development against the COVID-19 disease.

Deciphering the mechanism by which viral-host protein-protein interactions induce the molecular pathogenesis of infection is key to identifying novel drug candidates for clinical trials (40). It has been reported that the observed differences of SARS-CoV-2 and SARS-CoV in terms of their pathogenicity and epidemiology are likely attributed to the complex interactions between various viral and host factors (17). Hence, in our study, we also aimed at the identification of the human proteins interacting with P0DTC4. This was important as it has been suggested that each viroporin can interact specifically with other viral or cellular proteins (41). Functional enrichment analysis (FEA) with respect to the molecular function of the genes encoding the six retrieved proteins showed that lysine-acetylated histone binding and acetylation-dependent protein binding were the most significantly enriched annotations. In fact, the aforementioned molecular activities were attributed to the members of the bromodomain and extra-terminal (BET) family of chromatin binding proteins (BRD2 and BRD4) (42). The function of the bromodomain protein modules as acetyl-lysine binding domains is now well established (43, 44). Bromodomains are found in several transcriptional coregulators and histone-modifying complexes which mediate acetylation (45).

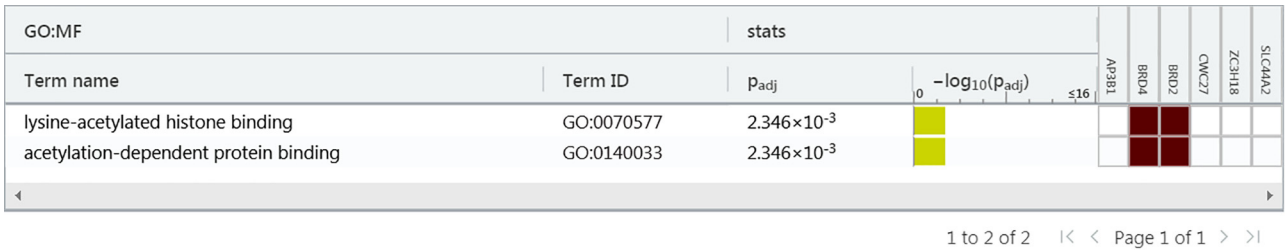

Figure 6. The results of the g:Profiler FEA analysis with respect to the molecular function of the human genes encoding the host proteins interacting with the SARS-CoV-2 E viroporin. FEA, functional enrichment analysis.

Table 3. *The enriched molecular functions of the predicted host-cell proteins interacting with P0DTC4**

ID	Name	*P* Value	FDR B&H	FDR B&Y	Bonferroni	Genes from Input	Genes in Annotation
GO:0070577	Lysine-acetylated histone binding	1.382E-5	2.902E-4	1.256E-3	5.804E-4	2	19
GO:0140033	Acetylation-dependent protein binding	1.382E-5	2.902E-4	1.256E-3	5.804E-4	2	19
GO:0034211	GTP-dependent protein kinase activity	6.235E-4	7.855E-3	3.398E-2	2.619E-2	1	2
GO:0140030	Modification-dependent protein binding	8.751E-4	7.855E-3	3.398E-2	3.675E-2	2	149
GO:0106140	P-TEFb complex binding	9.351E-4	7.855E-3	3.398E-2	3.927E-2	1	3

*B&H, Benjamini and Hochberg; B&Y, Benjamini and Yekutieli; FDR, false discovery rate.

Protein acetylation (PA) is one of the commonest posttranslational modifications (46) and has a key role in transcription regulation.

Increasing evidence has supported the significant contribution of PA in airway inflammation through the epigenetic control of inflammatory genes expression [reviewed in Adcock et al. (47) and Ito et al. (48)]. It has been reported that signaling via the NF-κB/RelA transcription factor/BRD4 axis in distal tracheobronchiolar epithelial cells mediates acute inflammation in response to viral patterns (49). Several viruses have been shown to exploit the host's PA network to ensure their replication (50–52) and maintain or exit latency (46, 53, 54).

Due to the fact that the bromodomain-containing proteins act as epigenetic modulators controlling both cellular functions and the viral life cycle (55), their targeting (especially the targeting of BRD4) via small molecule inhibitors has emerged as a potent therapy for viral infectious diseases (49, 55–58). Our prediction that BRD2 and BRD4 are functional interactors with the P0DTC4 protein was recently verified (59), reinforcing the hypothesis that the SARS-CoV-2 infectivity might also affect cellular acetylation. Considering the antiviral activity of BRD4 inhibitors as well as their ability to reduce neutrophilic airway inflammation (49), we propose the experimental validation of their clinical efficacy against the COVID-19 disease.

In conclusion, in this study we have identified vaccine candidates for SARS-CoV-2 as well as host factors that can be inhibited by highly specific compounds. Our findings have implications for COVID-19 therapy prospects and also indicate that the SARS-CoV-2 viroporin E could serve as a promising vaccine target for this devastating disease. A limitation of this study is that it relies on computational methodology, and therefore experimental data are required to validate the in silico estimates. Still, data mining and immunoinformatics have greatly changed the field of vaccinology providing a time and cost-effective strategy that aims at very focused, evidence-directed experimental investigation of limited epitope candidates (39, 60).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

S.G.Z. conceived and designed research; E.R. performed experiments; E.R. analyzed data; E.R. interpreted results of experiments; E.R. prepared figures; E.R. drafted manuscript; K.I.G. and S.G.Z. edited and revised manuscript; E.R., K.I.G., and S.G.Z. approved final version of manuscript.

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