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Chimeric protein consisting of 3M2e and HSP as a universal influenza vaccine candidate: from in silico analysis to preliminary evaluation

Behrokh Farahmand[1] · Najmeh Taheri[1] · Hadiseh Shokouhi[1] · Hoorieh Soleimanjahi[2] · Fatemeh Fotouhi[1]

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Abstract

The 23-amino acid ectodomain of influenza virus M2 protein (M2e) is highly conserved among human influenza virus variants and represents an attractive target for developing a universal vaccine. Although this peptide has limited potency and low immunogenicity, the degree of M2e density has been shown to be a critical factor influencing the magnitude of epitope-specific responses. The aim of this study was to design a chimer protein consisting of three tandem repeats of M2e peptide sequence fused to the Leishmania major HSP70 gene and evaluate its characteristics and immunogenicity. The structure of the deduced protein and its stability, aliphatic index, biocomputed half-life and the anticipated immunogenicity were analyzed by bioinformatics software. The oligonucleotides encoding 3M2e and chimer 3M2e-HSP70 were expressed in *Escherichia coli* and affinity purified. The immunogenicity of the purified recombinant proteins was preliminary examined in mouse model. It was predicted that fusion of HSP70 to the C-terminal of 3M2e peptide led to increased stability, hydropathicity, continuous B cell epitopes and antigenic propensity score of chimer protein. Also, the predominant 3M2e epitopes were not hidden in the chimer protein. The initial in vivo experiment showed that 3M2e-HSP chimer protein stimulates specific immune responses. In conclusion, the results of the current study suggest that 3M2e-HSP chimer protein would be an effective universal subunit vaccine candidate against influenza infection.

Keywords Fusion protein · Influenza virus · M2e · HSP70 · in silico · Universal vaccine

Introduction

Despite availability of antiviral drugs and inactivated vaccines, influenza is still a highly contagious, acute and febrile respiratory disease caused by different types of Influenza viruses. These enveloped viruses belong to Orthomixoviridae family with negative-sense segmented ribonucleic acid genome. All three types of influenza A, B and C viruses can cause epidemics, whereas only type A may lead to pandemics and has greater proportion of human infectivity [1]. Influenza A viruses are classified into diverse subtypes based on the differences in surface glycoproteins, hemagglutinin and neuraminidase [2]. Influenza A viruses undergo genetic reassortments (antigenic shift) and point mutations (antigenic drift). Accumulation of point mutation in the surface antigenic proteins can lead to annual epidemics. On the other hand, genetic reassortment between human and animal influenza viruses might cause pandemics [3]. These genetic changes enable influenza viruses to evade immune system. The best strategy to prevent the flu is by getting a flu vaccine each year.

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✉ Fatemeh Fotouhi
fotouhi@pasteur.ac.ir

Behrokh Farahmand
b_farahmand@pasteur.ac.ir

Najmeh Taheri
n.taheri62@gmail.com

Hadiseh Shokouhi
shokohi.hadis@gmail.com

Hoorieh Soleimanjahi
soleim_h@modares.ac.ir

[1] Department of Influenza and Other Respiratory Viruses, Pasteur Institute of Iran, 69, Tehran 1316943551, Iran

[2] Department of Virology, Tarbiat Modares University, Tehran, Iran

However, CDC data show that flu vaccination reduces the risk of flu illness by between 40 and 60% among the overall population during seasons when most circulating flu viruses are well matched to the flu vaccine [4].

Therefore, conventional influenza vaccines are not effective enough to prevent influenza virus infection. Obviously, an efficacious vaccine against a variety of circulating virus strains is needed for emergent threats. Researches on universal influenza vaccines focus on the influenza virus-conserved proteins, such as matrix protein, nucleoprotein and hemagglutinin stalk domain. The matrix protein 2 (M2) is a crucial component of the viral envelope which is essential for viral replication. It is encoded by a separate open reading frame of the 7th segment of the influenza virus genome. M2 protein has an important role in virus cellular entry and maturation. The external region of M2 which consists of 23 amino acids (M2e) is recognized by the host's immune system [5]. Hence, M2e peptide is considered as a promising candidate for a broad-spectrum recombinant influenza A vaccine and in recent years several researchers have focused on this peptide. Researchers at university of Ghent have widely researched on influenza subunit vaccine containing M2e exposed on HBC particles and showed that mucosal vaccination using this chimer protein requires effective and safe adjuvant [6]. Denis and his colleagues showed that a monomeric form of M2e peptide was not immunogenic [7]. A close correlation between epitope density of M2e and epitope-specific humoral immune responses in mouse model has been shown [8]. The immunogenicity of different M2e epitope densities was tested in several studies [9, 10]. A variety of heat shock proteins (HSPs) and molecular chaperones have been suggested as adjuvant for cross-priming with antigenic peptides. HSP70 acts as an adjuvant when co-administered with peptide antigens or given as fusion proteins. Several reports have shown that Leishmania Major HSP70 is a major target of humoral immune responses [11–13].

In the present study, three tandem copies of M2e sequence with appropriate flexible linker were designed for cloning into the pET28a vector, upstream of $HSP70_{(221-604aa)}$ gene. The structure of the deduced protein and its physicochemical properties, stability, half-life and immunogenicity were analyzed by bioinformatics software. Recombinant 3M2e and 3M2e-HSP chimer protein were expressed in prokaryotic system and affinity purified. The immunogenicity of the purified proteins was preliminary examined in mouse model to understand if 3M2e sequence genetically fused to a desirable carrier such as Leishmania major HSP70 exhibit enhanced immunogenicity.

Materials and methods

Construct design of 3M2e-HSP

The sequences of M2 extracellular domain of influenza A virus (accession number: ACF41880) and Leishmania major HSP70 gene (accession number: Q07437) were obtained from NCBI database (National Center for Biotechnology Information http://www.ncbi.nlm.nih.gov).

A chimer protein was designed consisting of three tandem repeats of M2e peptide sequence fused to the C-terminal of Leishmania major $HSP70_{(221-604aa)}$ gene. In order to prevent formation of disulfide bonds in 3M2e peptide, two cysteine at positions 16 and 18 were replaced with serine. Using NEB cutter Web site analysis result, BamHI restriction enzyme site (GGATCC) was chosen and added at both end of the 3M2e gene sequence. Three tandem repeats of M2e peptide were arranged alongside with different linkers and analyzed using Expasy Proteomics Web site. The oligonucleotide encoding 3M2e peptide with Gly–Gly–Gly–Lys–Gly–Gly in abbreviation form of GGGKGG linker was codon optimized according to the codon usage table of *Escherichia coli* strain K12 using OPTIMIZER server (http://genomes.urv.es/OPTIMIZER/) and joined to the C-terminal of Leishmania major HSP70 gene and analyzed with Swissprot server (http://www.bioinformatics.org/sms2/rev_trans.html). The immunogenicity of the selected epitopes was checked using the Vaxijen online server (http://www.ddg-pharmfac.net/vaxijen/VaxiJen/VaxiJen.html). VaxiJen v2.0 allows antigen variety based on the physicochemical qualities of proteins without the use of sequence alignment [14]. Arrangement of fragment junctions is shown in Fig. 1.

In silico analysis of protein structures

The protein structure of 3M2e-HSP70 chimer protein sequence was analyzed using several bioinformatics tools to determine physicochemical properties. The computation of various physical and chemical parameters, theoretical isoelectric point (pI), molecular weight, total number of positive and negative residues, extinction coefficient, half-life, instability index, aliphatic index and grand average hydropathy

Fig. 1 Schematic diagram of recombinant chimeric protein containing three tandem repeats of M2e and $HSP70_{(221-604aa)}$ sequences bound together by appropriate linkers and 6xHis tag in the both ends of the construct

(GRAVY) for the target protein was done using the Expasy-ProtParam Tool (http://us.expasy.org/tools/protparam.html). Details concerning hydrophobicity and flexibility distribution along the chain are offered by the scales Kyte-Doolittle and Average Flexibility Index, respectively (http://web.expasy.org/protscale/). The secondary structure analysis was done using various online servers such as Advanced Protein Secondary Structure Prediction Server PHD (https://npsa-prabi.ibcp.fr/cgi-bin/secpred_phd.pl) and TMHMM online server (http://www.cbs.dtu.dk/services/TMHMM) [15].

Tertiary structure prediction was performed using 3D viewer software such as Phyre (http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index), WeblabViewer (http://www.scalacs.org/TeacherResources) and Swiss-Pdb-Viewer4.1.0 (https://spdbv.vital-it.ch) free available online software [16]. The PDB format files of chimeric protein were extracted from iterative treading assembly refinement (I-TASSER) server. I-TASSER modeling starts from the structure templates identified by LOMETS from the PDB library [17]. Top patterns were predicted in I-TASSER, and structural analysis was performed for the best model among them. The selection of pattern was done using three indicators: C-Score, DFIRE2 energy profile and stereochemical qualities. Structural feature prediction of the amino acid sequences such as prediction of solubility upon overexpression and antigenicity of desired proteins were performed using online programs SOLpro and ANTIGENpro (http://scratch.proteomics.ics.uci.edu/). Stereochemical qualities of obtained 3D models were evaluated through ERRAT (http://services.mbi.ucla.edu/ERRAT/). Procheck and Verify 3D in protein structure validation server (http://psvs-1-5-dev.nesg.org/) were used for verification of the progress of crystallographic model building and refinement [18].

Prediction of immunogenic epitopes

The immunogenicity of desired sequences were analyzed using web-based B cell epitope prediction tools where criteria were set to have 75% specificity and 12 aa epitope length (immuneepitope.org; Bcepred http://www.imtech.res.in/raghava/bcepred and http://www.imtech.res.in/raghava/cbtope). The discontinuous B cell epitopes were determined from 3D structure proteins PDB format, based on the solvent-accessibility and flexibility (http://tools.immuneepitope.org/tools/ElliPro/iedb_input) [19, 20].

Antibody epitopes were predicted using the Parker's scale in the Hydrophilicity Prediction program (http://tools.immuneepitope.org/tools/bcell/iedb_input) and (http://tools.iedb.org/ellipro/).

T cell epitopes were screened using ProPred software (imtech server) and IEDB for predicting MHC Class-I and II alleles. ProPred and IEDB also allow the prediction of the standard proteasome and immune proteasome cleavage sites in an antigenic sequence. The simultaneous prediction of MHC binders and proteasome cleavage sites leads to the identification of potential T cell epitopes (http://www.imtech.res.in/raghava/web.php) [21, 22].

Construction, expression and purification of recombinant 3M2e and chimeric proteins

Designed three tandem repeats of M2e was synthesized (Biomatik, Denmark) and cloned in two expression vectors (pET28a and pET28a-HSP70). The constructions were analyzed using PCR amplification, enzymatic digestion and sequencing. The confirmed expression vectors encoding 3M2eand 3M2e-HSP70 (with GGGKGGflexible linker) were transformed into the competent *E.coli* BL21 (DE3). The protein expression was evaluated in different conditions (IPTG, temperature and times after induction). Purification of recombinant protein was performed using Ni-TED 2000 (MN, Germany) column according to the manufacturer's instruction.

SDS–PAGE and western blotting

Samples from all the stages of extraction and purification (pellets and supernatants) were analyzed for size and purity by SDS–PAGE and Western blotting. For SDS–PAGE analysis, the expressed target protein was lysed in loading buffer and then separated by SDS–PAGE on 12% gel [23]. The gels were stained with Coomassie brilliant blue R-250 (CBB) and scanned with a conventional scanner.

For Western blotting, the expressed target protein separated by SDS–PAGE was transferred to nitrocellulose membrane (Sartorius, 11327.41Bl). The membrane was blocked and then incubated with anti-His HRP-conjugated monoclonal antibody (Qiagen, 34460). Binding signals were visualized with DAB substrate (Sigma-Aldrich, D5637).

Preliminary study of 3M2e and 3M2e-HSP immunogenicity

Six-week-old female BALB/c mice ($n = 5$ for each protein) were immunized subcutaneously with three doses of 15 µg recombinant proteins every other week. Two groups were injected with PBS or HSP70 as controls. Before immunization and 2 weeks after the last injection, the mice were bled through the orbital sinus and the sera were kept at − 20 °C for further use.

To evaluate total anti-M2e IgG, 96-well ELISA plate (Greiner Sigma-Aldrich) was coated overnight with 10 ng/well of M2e synthetic peptide (RP20206, GeneScripte) at 4 °C. Mice sera were diluted as 1:1000, HRP-conjugated anti-mouse IgG was used as secondary antibody (Sigma-Aldrich, A8924), and the optical density was measured at

450 nm wavelength. The specific anti-M2e subclasses were investigated using goat anti-IgG1 or IgG2a subtype antibodies (Sigma-Aldrich, 122M4781) as secondary antibodies [24].

Virus neutralization test (VNT) was performed to show the protectivity of anti-M2e antibodies in vitro. MDCK cells were seeded at 3×10^4 cells/well into 96-well flat bottom plates 1 day before the experiment. The mice sera were heat-inactivated at 56 °C for 30 min, serially diluted and mixed with an equal volume of viral inoculum, A/PR8/34 (100 $TCID_{50}$/well) in DMEM supplemented with 1 µg/mL TPCK-treated trypsin. The mixture was incubated for 1 h at room temperature and then added to confluent MDCK monolayers in duplicate and incubated at 37 °C and 5% CO_2 for more 48 h. The supernatant was then harvested and added to an equal volume of 0.5% chicken red blood cells and incubated for 1 h at room temperature in a 96-well U-bottom plate. The absence of hemagglutination was considered as neutralization.

Results

Biostructure analysis and validation

Linker sequence composition could affect folding stability and epitope exposing of a fusion protein. In silico analysis was used to investigate different linkers in 3M2e and chimer proteins. In order to select a suitable linker for joining M2e and HSP sequences, three different flexible linkers, GGGKGG, GGGGS and GSGSG, were studied using bioinformatics software.

The integrated results using ProtParam, ProtScale, SOLpro, ANTIGENpro, Bcepred and CBTOPE showed that flexibility of 3M2e-HSP chimeric protein increased by adding all three linkers compared with non-linker ones (Table 1).

The physicochemical properties of 3M2e peptide and 3M2e-HSP protein were analyzed using ProtParam and ProtScale software. From this point of view, the average molecular weight of 3M2e and 3M2e-HSP was 14.13 and 56.65 kDa, and their theoretical isoelectric pH values were 6.33 and 5.98, respectively. Isoelectric point below seven indicates a negatively charged protein.

The instability and aliphatic index of 3M2e and 3M2e-HSP is shown in Table 1. The high aliphatic index of chimer protein (70.87) indicates this protein is stable in wide range of temperatures. The biocomputed half-life of 3M2e and 3M2e-HSP were > 10 h and the average hydropathicity were − 1.04 and − 0.7, respectively. The minus grand average hydropathicity (GRAVY) value means that these molecules are nonpolar.

The hydrophilic character predicted by ProtParam was in good agreement with the hydrophilicity plot given by ProtScale software (Hopf & Woods scale). It was shown that chimer protein has a decreased hydrophilicity compared to 3M2e alone. The secondary structure elements of 3M2e-HSP (helices, sheets and random coils) were predicted using online servers as shown in Fig. 2.

Solubility of proteins is the most important factor and outstanding index for protein functionality. The solubility and probability were predicted using SOLpro software. The results showed that 3M2e-HSP was soluble with probability of 0.66 upon overexpression.

Tertiary structure prediction results from I-TASSER modeling were compared for four 3D models of 3M2e-HSP with or without different flexible linkers. The best predicted tertiary structure of 3M2e-HSP with the maximum confidence score (c-score: 0.43) was selected from a set of top models (Table 2).

The data generated by a Ramachandran plot confirmed the structural stability of the 3M2e and chimer proteins (Fig. 3). The Ramachandran plot analysis of chimer protein using PROCHEK revealed that 89.4% amino acid residues from modeled structure were incorporated in the favored regions 9.2% in additionally allowed regions, 1.4% in generously allowed regions and 0.0% in disallowed regions of the related plot. Overall quality factor was calculated with ERRAT analysis, and the modeled structure was found to have 59.55% quality factor. The global quality *Z*-scores calculated using Verify 3D (− 1.77), ProsaII (− 0.79), Qmean (− 3.63) and Procheck (− 0.55) confirmed model stability.

The overall PROCHECK G-factor for 3M2e and 3M2e-HSP homology modeled structures was − 0.33 and 0.07, respectively. The scores indicate that the modeled structures for two recombinant proteins were satisfactory (acceptable value> − 0.50).

Table 1 Physicochemical parameters of designed proteins predicted by ProtParam, ProtScale, SOLpro and ANTIGENpro server

	Disulfide bonds	Probability of solubility upon overexpression	Probability of antigenicity	Instability index	Aliphatic index	Flexibility
3M2e-HSP (non-linker)	1	0.75 (insoluble)	0.90	43.69	73.27	0.51
3M2e-HSP (GGGKGG)	1	0.66 (insoluble)	0.91	43	70.87	0.53
3M2e-HSP (GGGGS)	1	0.67 (insoluble)	0.91	44.87	71.14	0.53
3M2e-HSP (GSGSG)	1	0.67 (insoluble)	0.91	42.03	71.28	0.52

Fig. 2 Secondary structure of 3M2e-HSP proteins with respect to protein sequence. H/h: alfa helix (35.26%); E/e: extended strand (14.26%); C/c: random coil (50.48%)

Table 2 Comparison of four 3D models of 3M2e-HSP (with different flexible linkers) in I-TASSER server

Model name	C-score	TM-score	RMSD (Å)
S372373 non-linker	0.24	0.75 ± 0.11	6.8 ± 4.0
S372537 (GSGSGS) linker	− 0.07	0.70 ± 0.12	7.5 ± 4.3
S372521 (GGGGS) linker	0.18	0.74 ± 0.11	7.0 ± 4.1
S371918 (GGGKGG) linker	0.43	0.77 ± 0.10	6.4 ± 3.9

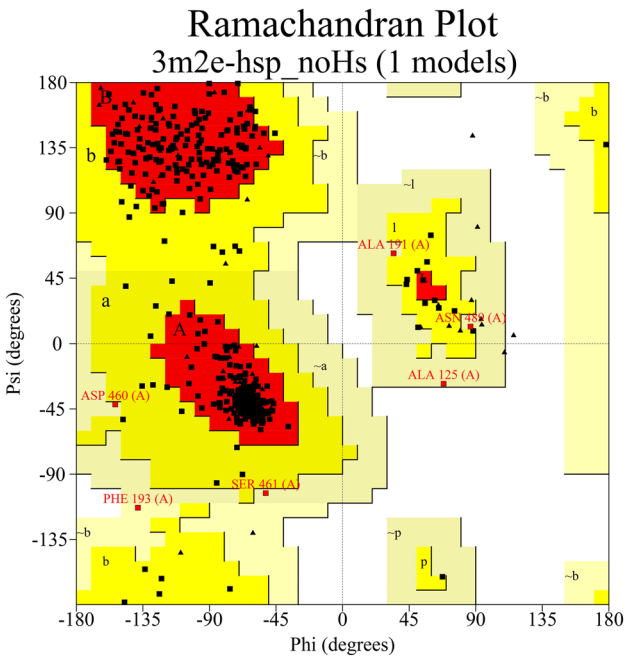

Fig. 3 Modeling structure of chimer protein was validated and plotted by Ramachandran plot

The quality of model was assessed by atoms root mean square deviation (RMSD). The RMS deviation for covalent bonds relative to the standard dictionary was 0.017 and 0.057 Angstroms for 3Me2 and 3M2e-HSP chimer protein, respectively. A model can be considered reliable or accurate when its RMSD is < 4 Å or < 2 Å, respectively.

B cell epitopes prediction

B cell epitopes are important for protection against viral infections. The predicted epitopes were candidates for inducing humoral or cell-mediated immunity against influenza in BALB/c mouse model. B cell epitope of chimer protein was predicted, and the epitopes having cutoff values > 0.3 were selected. The main discontinuous B cell epitopes of 3M2e-HSP having high antigenicity are shown in Table 3. Three-dimensional protein structure is very important in understanding the protein interactions, functions, localization and conformational antigenic sites. The result of tertiary structure of 3M2e-HSP chimer protein showed that predominant epitope of 3M2e has not been masked by HSP.

According to data presented in Table 4, linear epitopes related to M2e sequence peptide which were indicated by balls (Fig. 4) were predicted to be surface accessible, thus, could be easily exposed to the immune B cells.

T cell epitope prediction

Prediction of T cell epitopes is a crucial step in influenza epitope-based vaccine design. IEDB was used to predict T cell epitopes for the 3M2e-HSP chimer protein. IEDB was first used to predict the highest scoring epitopes of chimer which binds with maximum number of MHC Class-I alleles. The chimer sequence was entered to the IEDB server, selecting all the BALB/c mice alleles extracted from mouse alloantigens (http://www.biolegend.com). The antigenic score of each epitope was evaluated using VaxiJen 2 server. The chimer sequence was also used to predict MHC class II binding regions using IEDB online server. The antigenicity

Table 3 Predicted discontinuous epitopes of 3M2e-HSP using Ellipro

No.	Residues	Number of residues	Score
1	A:K298, A:K299, A:K300, A:G301, A:E302, A:N303, A:R304	7	0.863
2	A:M1, A:G2, A:S3, A:S4, A:H5, A:H6, A:H7, A:H10, A:S11, A:S12, A:G13, A:L14, A:V15, A:P16, A:R17, A:G18, A:S19, A:H20, A:M21, A:A22, A:S23, A:M24, A:T25, A:G26, A:G27, A:Q28, A:Q29, A:M30, A:G31, A:R32, A:G33, A:S34, A:S35, A:L36, A:L37, A:T38, A:E39, A:V40, A:E41, A:T42, A:P43, A:I44, A:R45, A:N46, A:E47, A:W48, A:G49, A:S50, A:R51, A:S52, A:N53, A:D54, A:S55, A:S56, A:D57, A:G58, A:G59, A:G60, A:K61, A:G62, A:G63, A:S64, A:L65	63	0.84
3	A:K305, A:K306, A:K308, A:I309, A:Q310, A:Q311, A:I312, A:G313, A:E314, A:I315, A:K317, A:T318, A:S321, A:T322, A:R325, A:E326	16	0.748
4	A:G128, A:Y151, A:N155, A:E156, A:Q157, A:G158, A:K243, A:N244, A:Y246, A:E247, A:K248, A:V249, A:R250, A:S251, A:Q252, A:L253, A:K254, A:N255, A:N256, A:A257, A:K258, A:E259, A:I260, A:G261, A:N262, A:G263, A:F265, A:E266, A:F267, A:Y268, A:H269, A:C271, A:D272, A:N273, A:T274, A:C275, A:M276, A:E277, A:S278, A:V279, A:K280, A:N281, A:G282, A:T283, A:Y284, A:D285, A:Y286, A:P287, A:K288, A:Y289, A:S290, A:E291, A:E292, A:A293, A:K294, A:K295, A:K296, A:K297	58	0.689
5	A:V193, A:G194, A:K195, A:E196, A:F197, A:N198, A:H199, A:L200, A:E201, A:K202, A:R203, A:I204, A:E205, A:N206, A:L207, A:N208, A:K209, A:K210, A:V211, A:D213, A:G214, A:D217, A:I218	23	0.612
6	A:C337, A:S338, A:N339, A:T362, A:R365, A:M366, A:V367, A:A370, A:F371, A:E373, A:R374, A:R375, A:N376, A:K377, A:Y378, A:L379, A:E380, A:E381, A:H382, A:P383, A:S384, A:A385, A:G386, A:K387, A:D388, A:P389, A:K390, A:K391, A:T392, A:G394, A:I396, A:Y397, A:R398, A:R399, A:V400, A:N401, A:G402, A:K403, A:W404, A:M405, A:R406, A:E407, A:L408, A:I409, A:L410, A:Y411, A:D412, A:K413, A:E414, A:E415, A:I416, A:R418, A:D427, A:S441, A:L443, A:N444, A:D445, A:A446, A:T447, A:Y448, A:Q449, A:R450, A:L451, A:E452, A:H453, A:H454, A:H455, A:H456, A:H457, A:H458	70	0.591
7	A:E70, A:T71, A:P72, A:I73, A:R74	5	0.552
8	A:S123, A:S126, A:R127	3	0.542

Table 4 Predicted linear epitopes of 3M2e-HSP using Ellipro

No.	Chain	Start	End	Peptide	Number of residues	Score
1	A	1	64	MGSSHHHHHHSSGLVPRGSH-MASMTGGQQMGRGSSLLTEVET-PIRNEWGSRSNDSSDGGGKGGS	64	0.848
2	A	265	322	FEFYHKCDNTCMESVKNGTYDYP-KYSEEAKKKKKKKGENRKKK-KIQQIGEIYKTRHST	58	0.751
3	A	396	416	IYRRVNGKWMRELILYDKEEI	21	0.699
4	A	372	386	DERRNKYLEEHPSAG	15	0.661
5	A	243	263	KNLYEKVRSQLKNNAKEIGNG	21	0.65
6	A	196	214	EFNHLEKRIENLNKKVDDG	19	0.638
7	A	444	458	NDATYQRLEHHHHHH	15	0.592
8	A	70	78	ETPIRNEWG	9	0.578
9	A	350	354	LDYEG	5	0.533

score for total epitopes with percentile rank under 1 was analyzed using VaxiJen Server (Table 5).

Construction and production of recombinant proteins

The synthesized 3M2e was successfully cloned in pET28a and pET28a-HSP70 upstream of HSP70 gene. The correctness of 3M2e gene subcloning at valid sites, downstream of T7 promoter and in frame with 6xHis tag and HSP fragment was confirmed using enzymatic digestion (Fig. 5a, b) and sequencing analysis (Eurofins MWG—Germany and chromas software version 1.45—Australia).

The protein expression was done in different temperatures, IPTG concentrations and times of harvest. The best condition (37 °C, 4 h and 0.5 mM IPTG) was selected for further protein expression. Since the recombinant proteins had histidine tags in both ends, proteins were purified using

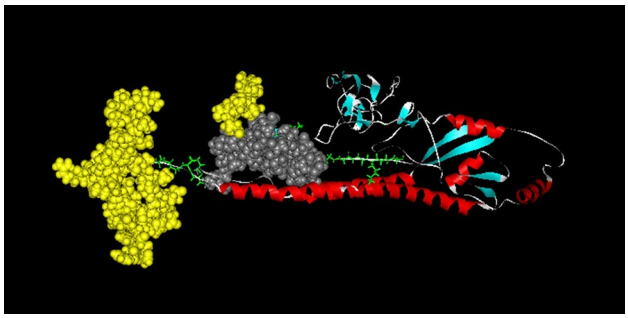

Fig. 4 Three-dimensional structure of 3M2e-HSP70 chimer protein. In this model, gray balls are 3M2e residues, yellow balls are the residues of predicted epitopes in 3M2e, green sticks indicate linkers region and flat ribbon shows HSP70 segment

Nickel affinity chromatography column. The immunoblotting results of purified recombinant proteins, 3M2e and 3M2e-HSP, using anti-M2 monoclonal antibody (ab5419, Abcam) indicated that antigenic properties of M2e have not been impaired in prokaryotic system (Fig. 5c).

Antibody responses

Mice were immunized with 3M2e and chimer 3M2e-HSP proteins in three doses. The mice sera samples were subjected to ELISA to measure total anti-M2e IgG and subclasses 2 weeks after the last injection. ELISA results showed higher levels of specific antibody induced in mice received 3M2e-HSP, compared to 3M2e recipients (> 3 times). No anti-M2 IgG was detected in PBS or HSP control groups. The ratio of IgG2a/IgG1 antibodies, as representative of Th1/Th2 cell activity, revealed that in 3M2e-HSP recipients Th1 cells were more involved compared to the 3M2e receivers (Table 6).

To evaluate whether anti-M2e antibodies detected by ELISA could protect cells against viral infection in vitro, the MDCK cells were inoculated with a mixture of influenza A/PR8/34 virus and serum dilutions. Anti-PR8 antibody prepared in rabbit was used as positive control. As expected, the results showed that anti-M2e antibodies were not capable of neutralizing the virus and inhibiting its propagation in MDCK cell line.

Discussion

Influenza virus matrix protein 2 which is a tetrameric type III membrane protein is expressed in the plasma membrane of infected cells as well as viral envelope; functions as a proton-selective channel. The N-terminal region of M2 is remarkably conserved in the first human influenza strain isolated in 1933 up to now. It may provide a novel alternative to the permissive influenza vaccines, trivalent inactivated and cold adapted, that require updates per annum [25, 26]. Antibodies produced against M2e can limit influenza virus replication and decrease illness and mortality rate of influenza infection [27–29]. High M2e-specific serum IgG antibody titers were obtained following either intraperitoneal or intranasal administration of purified M2e peptide in mouse [30]. Numerous studies proved that passive delivery of anti-M2e antibodies resulted in significant reduction in virus replication in the lung and protected mice from lethal infection. Fan and his groups showed that passive transfer of sera from monkeys immunized with M2e conjugate peptide protected mice against lethal viral challenge [28]. Also,

Table 5 BALBC/mice MHC-I & II binding prediction

Start position	Peptide	Length	Allele	Percentile rank	Antigenicity score	Antigenicity
MHC-I						
383	GVPQIEVTF	9	H-2-Dd	0.2	1.2324	+
325	ALIKRNTTI	9	H-2-Kd	0.5	0.4447	+
458	YAYSMKNTL	9	H-2-Kd	0.5	0.6254	+
27	SHMASMTGG	9	H-2-Kd	0.6	0.5903	+
332	TIPTKKSQI	9	H-2-Dd	0.7	1.1284	+
283	AYGAAVQAF	9	H-2-Kd	0.9	− 0.0517	−
284	YGAAVQAFI	9	H-2-Kd	0.9	− 0.3536	−
66	GGGKGGSLL	9	H-2-Dd	0.9	0.9761	+
95	GGGKGGSLL	9	H-2-Dd	0.9	0.9761	+
MHC-II						
159	ASSHRALRRLRTACE	15	H2-IAd	0.74	0.3418	−
160	SSHRALRRLRTACER	15	H2-IAd	0.76	0.211	−
158	LASSHRALRRLRTAC	15	H2-IAd	0.94	0.479	+

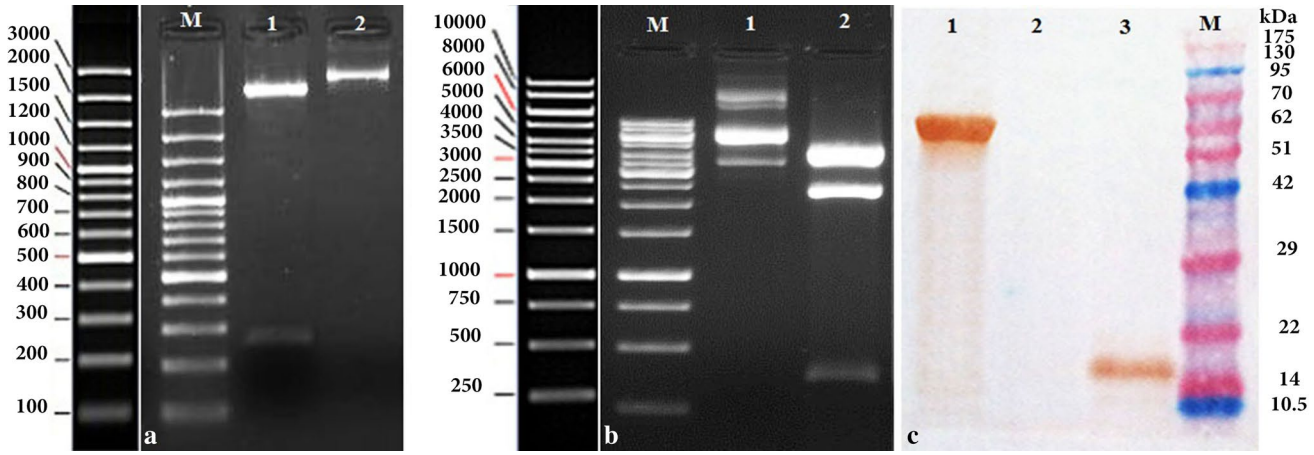

Fig. 5 Analysis of vector construction and recombinant protein expression. **a** Restriction enzyme analysis of recombinant plasmid pET28a/3M2e using BamHI. Lane 1: digested plasmid resulted in 270 bp fragment, Lane 2: undigested plasmid, M; 100 bp DNA ladder. **b** Restriction enzyme analysis of recombinant plasmid pET28a/3M2e -HSP using BamHI and XhoI. Lane 1: undigested plasmid, Lane 2: digested plasmid resulted in 270 bp and 1152 bp fragments, M; 1Kb DNA ladder. **c** Western blot analysis of recombinant 3M2e and 3M2e-HSP70 using monoclonal anti-M2 antibody. lane 1: chimer protein (3M2e-HSP70); lane 2: negative control; lane 3: 3M2e protein; lane M: protein marker

Table 6 Titer and the ratio of secreted IgGs in different groups

Vaccine group	No. of responders (%)	Total specific anti-M2e IgG	Specific anti-M2e IgG1	Specific anti-M2e IgG2a	IgG2a/IgG1 ratio
3M2e	100	0.82 ± 0.15	0.96 ± 0.17	0.52 ± 0.16	0.54
3M2e-HSP	100	1.75 ± 0.22	1.85 ± 0.35	2.20 ± 0.40	1.19
PBS	0	0.14 ± 0.02	ND	ND	ND
HSP	0	0.11 ± 0.02	ND	ND	ND

The mice were immunized with M2e or chimer protein three times, at 15-day intervals. On day 14 after the last immunization, serum samples were collected and evaluated for M2e specific antibody in triplicate by ELISA. The data presented in the table are the mean of OD 450 nm in each group ± SD. The differences between cases and control groups were statistically significant. Also, the results indicate statistically significant difference between the 3M2e alone and chimer group ($P < 0.001$)

Wang and his colleagues demonstrated that M2e potently inhibited influenza A virus replication in MDCK cells [26].

Due to the small size and proximity to the membrane, M2e is inadequately immunogenic and distant from reaching the immune system. Many studies have confirmed that influenza infection in mouse and human induces a poor M2e specific antibody response [8, 31]. Therefore, desirable approaches to enhance immunogenicity of M2e protein immunization stay to be established. Appropriate adjuvant and increasing epitope density of M2e are considered valuable ways to improve M2e-based vaccines efficacy [6, 9].

Extensive earlier studies determined that epitope density played a significant role in the humoral responses. Liu and Chen explained that enhanced antibody titers against M2e using high epitope density resulted in increasing of the survival rate [32]. Babapoor et al. [33] determined that tetrameric M2e stimulates more specific humoral immune responses compared to the monomeric presentation which induces a significant protection against homologous virus challenge [33].

It has been shown that the immunogenicity of small peptide antigen can be enhanced by using desirable carriers and adjuvants such as HSP70. Heat shock proteins play important roles in recognizing and binding to nascent polypeptide, assembly of protein complexes and translocation of proteins across cellular compartments [34]. Also, HSP70 can regulate the immune system, nonspecifically enhance the innate immune responses, promote activation of immune cells including T Lymphocytes and releasing of cytokines. In modern vaccines, HSP70 could also be used as adjuvant when it is fused to or co-injected with target gene [12]. Ebrahimi and his team showed that fusion of M2e to the N-terminal region of Mycobacterium tuberculosis HSP70 enhanced immunogenicity of M2e in animal models [35].

In another study, researchers examined different parts of Leishmania major (Lm) HSP70 and showed that the

complete open reading frame of HSP70 (amino acids 221–604) induced more antibody responses and showed the highest immunogenicity compared to the other truncated forms of HSP70, which was not suitable in the case of Leishmania [36].

In this study, three tandem repeat of M2e was fused to Lm. HSP70 using minimum linkers to prepare a chimer protein in prokaryotic system. Linkers are short peptide sequences inserted between different proteins, make it more likely fold independently and behave as expected. Studies showed that not only the structures and characteristics of target molecules are taken into consideration, but the composition, length and flexibility of the inter-peptide linker must also be considered [37, 38]. Using statistical study of natural linkers, Argos suggested that pentapeptides consisting of only Gly, Ser and Thr would make the best linkers for gene fusion, as these residues were most strongly preferred within natural linkers. He also found that Thr, Ser, Pro, Asp, Gly, Lys, Gln, Asn and Ala (in order of decreasing preference) are the preferred residues in linkers [39]. Three tandem repeats of M2e sequence were connected to each other and to HSP70 with flexible linkers and analyzed bioinformatically. In silico analysis of protein sequence showed that adding glycine linker (GGGKGG) resulted in more flexible and stable recombinant protein.

According to physichochemical information obtained from software, we predicted that fusing $HSP70_{(221-604)}$ to 3M2e peptide, led to increased stability, hydropathicity and antigenic propensity score of 3M2e- HSP70 chimer protein. To avoid protein aggregation, we introduced two Cys to Ser substitution at positions 16 and 18 of M2e peptide. This ensures that cysteine could not lead to potential protein aggregation under oxidative conditions by the formation of inter particle disulfide bonds, improving the uniformity of M2 peptide conjugates [40]. As expected, maximum flexibility was observed in position of linkers in 3M2e peptide sequence. Moreover, it is revealed that joining HSP to 3M2e would not affect its flexibility.

Structure of the M2 protein in natural state has free N-terminal [25]. By using online software and determining three-dimensional structure of 3M2e and 3M2e-HSP70 chimer proteins, we found that fusion of HSP70 to the C-terminal of 3M2e did not make the major epitopes of 3M2e peptide out of reach. Therefore, it could be a strong immunogen as a subunit vaccine.

Inactivated influenza vaccines and subunit vaccines usually induce Th2-type immune response in BALB/c mice which is characterized with the stimulation of IgG1 antibodies. However, in the sera of survived mice upon viral challenge, the major antibody subtype is IgG2a, which is stimulated during Th1-type immune responses [41]. It has been confirmed that antibodies, besides neutralizing properties, can mediate host effector functions and aid viral removal from a host. Interaction of the Fc portion of immunoglobulin IgG2a antibodies with complement components and activatory Fc receptors results in the stimulation of antibody-dependent cell-mediated cytotoxicity and phagocytosis which associates in the clearance of viral infection [41, 42]. As it was predicted by in silico analysis, the genetically fusing HSP to the C-terminal of 3M2e did not mask the predominant epitopes and the initial in vivo experiment showed that 3M2e-HSP chimer protein stimulates immune responses strongly. Analyses of antibody subtypes suggested that chimer protein-induced both IgG1 and IgG2a antibodies that may contribute to the improved protective immunity. However, the results of VNT showed that anti-M2e antibodies could not inhibit virus entry and propagation in vitro. Since influenza virus attaches to the host cell via hemagglutinin and there are only a few M2 proteins on the virion envelope, we did not expect anti-M2e antibody efficacy in VNT. On the other hand, during virus infection in animal, anti-M2e responses could interfere with virus propagation and decline virus infection.

The results of our other study revealed that 3M2e—HSP chimer protein elevated specific antibody responses and induced lymphocyte proliferation and IFN-γ secretion. Moreover, the chimer protein protected mice against lethal influenza challenge which confirmed in silico predicted results [43]. Thus, 3M2e-HSP chimer protein would be an effective universal subunit vaccine candidate against influenza infection.

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Compliance with Ethical Standards

Conflict of interest The authors declare that they have no conflict of interest.

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