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Analysis of *Fasciola hepatica* HDM-1 mRNA Expression across Developmental Stages and Computational Simulation of Its Internalization Pathway

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Abstract

FhHDM-1(*Fasciola hepatica* Helminth Defense Molecule-1) plays a role in the ability of *Fasciola* spp., to evade the host's immune system. Here, the transcription of FhHDM-1 was shown in the developmental stages of *Fasciola hepatica* (*F.hepatica*). We simulated peptide initialization by the Linux operating system using GROningen Machine for Chemical Simulations (GROMACS) and Coarse-grained molecular dynamics (CGMD) Simulation. The final structure of the peptide was extracted from the last frame of the simulation and used as the initial structure for the simulation of protein initialization into the plasma membrane. All HDM clade sequences showed amino acid sequence identity in the sequence part of HDMs, consisting of an N-terminal signal peptide, and a C-terminal amphipathic motif when compared with orthologues from other trematodes in databases. Stereo chemical structures and biochemical features of peptides, were provided for protein biological expression systems. Our findings revealed the presence of FhHDM-1 mRNA in *F. hepatica* adult flukes and the lack of mRNA in its eggs and miracidia, indicating its central role in the evolution of the parasitic life cycle. The internalization of the peptide into the plasma membrane lipid rafts by the polar heads of lipid molecules and interaction with phospholipids, in turn, results in creating curvature, leading to endocytosis pathways. The possible presenting antigens by MHC classes I and II were identified as overlapping fragments. Its capability to enter macrophages and suppress lysosomal acidification indicates its potential as a therapeutic agent for inflammatory diseases, alongside its ability to present epitopes through MHC-I and MHC-II pathways.

Introduction

Fascioliasis as an emerging food-borne parasitic disease affected humans and animals and is associated with losses in animal production (1, 2). *F. hepatica* is capable of secreting a helminth defense molecule, a homolog of the mammalian cathelicidin-like host defense molecule, namely FhHDM-1. Of course, its C-terminal amphipathic

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helix (a 34-residue) is conserved among the HDMs of some human trematodes and has a CAP18/LL-37 like structural property. This peptide shows not only immune-modulatory effects, that could favor survival, but also have parasite-intrinsic functions as it is capable of binding and detoxifying heme (heme-binding properties and maintain of heme homeostasis), (3-5). On the other hand, the MF6p/HDM-1 in *F. hepatica* and *Fasciola gigantica* (*F. gigantica*) is not found to be an appropriate antigen for serodiagnosis of *Fasciola* (6).

Regarding the role of FhMF6p/HDM in immunomodulation, the entire FhHDM-1, and/or the C-terminal region consisting of MF6 and the heme-binding area has attracted attention for providing therapeutic drugs and chimeric or multicomponent vaccines (6, 7). Live helminths or helminth products can be capable of changing susceptibility to immune-mediated disease or inflammatory diseases (8-12). This study aimed to investigate the expression levels of FhHDM-1 in various developmental stages of *F. hepatica*, including adult flukes, eggs, and miracidia. Additionally, the study attempted to characterize the stereochemical properties of FhHDM-1 through bioinformatics analysis. Another key objective was to elucidate the mechanism of internalization of FhHDM-1 into macrophages via the endocytic pathway.

Materials and Methods

Sample collection

To study the expression of the *F. hepatica* HDM-1 (*FhHDM-1*) gene in different life stages (egg, miracidium, and adult), eight livers naturally infected with *F. hepatica* were collected from sheep from licensed slaughterhouses in Tehran, Iran. Livers were immediately transported to the laboratory on ice, and processed within 3 hours of collection. Each liver was dissected and massaged in 500 mL of sterile distilled water under aseptic conditions. Adult flukes were extracted manually by pressing the liver tissue. Viable adult flukes were transferred to sterile 10cm Petri dishes for further egg collection.

Differential Diagnosis of F.hepatica based on the morphomolecular characteristics

Morphological identification was performed by measuring the length and width of adult flukes under a Zeiss Stereo microscope (Carl Zeiss AG, Göttingen, Germany) in the taxonomy lab in Iran Parasitology Museum (Faculty of Veterinary Medicine, Tehran, Iran). For molecular confirmation, DNA was extracted using a DNA extraction kit following the manufacturer's procedure (MBST, Iran). PCR amplification of a 234 bp fragment of the mitochondrial cytochrome oxidase I (COI) gene was carried out using universal primers synthesized by Sinaclon Company (Iran). The primer sequences were as follows:

Forward—5′-TTGATTGGGGGTTTTGGTAA-3′ and Reverse—5′-CCCAGCCAAATGAAGAGAAA-3′.

Thermal cycling was carried out using a Bio-Rad Thermal Cycler (Bio-Rad S1000™, USA) under the following conditions: initial denaturation at 95 °C for 5 min; followed by 35 cycles of 94 °C for 30 s, 55 °C for 30 s, 72 °C for 30 s; and a final extension at 72 °C for 7 min.

Egg Culture and Miracidia Collection

Adult *F. hepatica* specimens were washed thoroughly with sterile distilled water, and eggs were manually extracted by gently pressing flukes between two sterile microscope slides. Approximately 10,000 eggs were transferred to two, 10 cm sterile culture Petri dishes containing 10 mL of sterile distilled water. Cultures were incubated at 27 °C under natural light conditions. Egg counts were conducted using a McMaster counting chamber (VWR-USA). From days 17 to 20 post-incubation, plates were examined daily under a light microscope (Olympus SZX16, 10× magnification) to observe the emergence of miracidia in the eggs. *F. hepatica* Eggs containing miracidia were collected and then induced to hatch

in a laboratory setting, under study-light during 15±5 minutes (13).

RNA Extraction, cDNA Synthesis, and RT-PCR

Total RNA was extracted separately from eggs, miracidia, and adult flukes using the SinaPure™ RNA Extraction Kit (Sinaclon, Iran) following the manufacturer's protocol. RNA Quantity and quality was assessed via NanoDrop (Thermo Scientific, USA), and agarose gel electrophoresis respectively. cDNA synthesis was performed using the Sinaclon First Strand cDNA Synthesis Kit with oligo(dT) primers. RT-PCR amplification of a 153 bp fragment of the *FhHDM-1* gene was performed using specific primers: **Forward**: 5′-GAAAGCGGAAGGAAAATGGT-3′ and **Reverse**: 5′-ATCGGTGAGCCGATTCAGTA-3′. COI was amplified in parallel as an internal control. PCR products were visualized using 1.5% agarose gel electrophoresis and sequenced by Bioneer Corporation (Seoul, South Korea) via the Sanger method. Sequences were analyzed using NCBI-BLAST and aligned using ClustalW. The 3D structure of the FhHDM-1 peptide was modeled using the I-TASSER server. Structural validation was conducted using PROCHECK to assess stereochemical quality (14-16), and Ramachandran plots were generated. Surface hydrophobicity and charge distribution were analyzed using Protein-sol patches (17, 18) at pH 6.3 to evaluate solubility and guide potential modifications for future expression studies (18).

Molecular Dynamics Simulation for Endocytosis Prediction

The production simulations were analyzed for Peptide insertion depth and orientation, Lipid rearrangement around inserted peptides, Changes in membrane thickness, Diffusion coefficients of lipid species, and Potential peptide-induced membrane curvature.

Simulation Setup and Parameters

Molecular dynamics simulations were performed using GROMACS 2022 on a Linux platform to investigate peptide-membrane interactions. The MARTINI coarse-grained force field was employed to reduce computational costs by 2-3 orders of magnitude compared to atomistic approaches while maintaining chemical specificity.

Membrane Model Construction

A heterogeneous lipid bilayer was constructed with physiologically relevant composition consisting of: 50% DOPC (dioleoyl phosphatidyl choline), 37.5% POPS (palmitoyl oleoyl phosphatidyl serine), and 12.5% cholesterol.

Peptide Model Preparation

Peptide structures were initially generated using PEP-FOLD and positioned in proximity to the membrane surface. The system was fully solvated and neutralized with Na^+ /Cl^- counter ions to maintain physiological conditions.

Prediction of Antigenicity and T Cell Epitopes

Antigenicity of the FhHDM-1 protein was predicted using the VaxiJen v2.0 server (threshold = 0.4, accuracy 70–89%) (19). T cell epitope prediction was performed using the IEDB analysis resource, evaluating proteasomal cleavage, TAP transport, and MHC-I binding affinity (20, 21). The MHC-II NP tool was also used to predict class II-presented epitopes by Giguère et al., 2013 (22).

Results

Determination of F. hepatica based on the morphomolecular characteristics

In the present study, worms with an average length and width of 30×13 mm were identified as *F. hepatica* (132 liver fluke), and worms with an average length and width of 75×12 mm were considered *F. gigantica* (53 liver fluke) and excluded from this study. The amplification of the 234 bp fragment of *F. hepatica* cytochrome c oxidase subunit-1based upon morphological characters.

Cultivation of F. hepatica eggs and miracidia hatching

Miracidia of *F.hepatica* developed within 17-20 day's period as out of 10000 *F.hepatica* cultured eggs, 48.75% (1425) completely developed into miracidia. On the day 19 after culture, 11.9% (170

miracidia) and on the day 21, 40% (570 miracidia) of eggs were hatched.

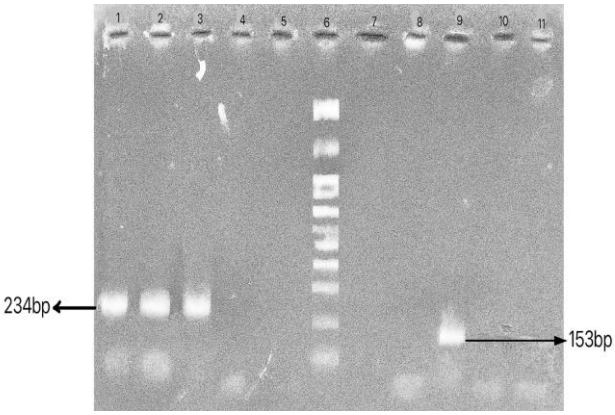

Fig. 1. Agarose gel electrophoresis (1.5%) of RT-PCR products for HDM-1(153bp) and cytochrome c oxidase subunit-1 (234 bp). The figure displays the following amplifications: 1. 234 bp fragment of the cytochrome c oxidase subunit-1 from adult *F. hepatica*; 2. 234 bp fragment from *F. hepatica* miracidia; 3. 234 bp fragment from *F. hepatica* eggs; 4. Negative control (The Sample without template DNA); 5. Negative control (The Sample with *Wolbachia* spp. DNA as a template DNA); 6. 100 bp DNA marker (Sinaclon, Iran); 7. Negative result of amplification for *F. hepatica* eggs HDM-1 mRNA; 8. Negative result of amplification for *F. hepatica* miracidia HDM-1 mRNA; 9. 153 bp fragment of adult *F. hepatica* HDM-1 mRNA; 10. Negative control (The Sample without template DNA); Negative control (The Sample with *Wolbachia* spp. DNA as a template DNA).

CDNA synthesis and amplification of the HDM-1 gene from eggs, miracidia, and adult F. hepatica flukes

The 234bp amplified fragment of COX1 in the synthesized cDNA from total RNA of eggs, miracidia and, adult flukes played the role of an internal control. The 153bp amplified fragment of *F. hepaticaHDM*-1 in the synthesized cDNA of adult flukes could indicate the presence of mRNA and HDM-1 expression in this sample as the lack of amplified fragment in the synthesized cDNA of eggs and miracidia was considered that they could be related to the deficiency of mRNA and HDM-1 expression (Figure1). The sequencing result of *F. hepaticaHDM*-1 adult flukes was registered in the gene bank with access numbers MN786462, and it was translated into amino acid sequences.

Membrane simulation

To determine the reliability of the lipid bilayer simulation results, analyzes were performed at the end of the membrane simulation, the results of which are shown in Figure 2a- 2i. These results all confirm the stability of the lipid bilayer membrane and the complete formation of lipid molecules together. To simulate the peptide in the presence of the membrane, the peptide was placed parallel to and perpendicular to the lipid bilayer membrane by designing a suitable program file using the Gromex program (Figure 2g- 2i).

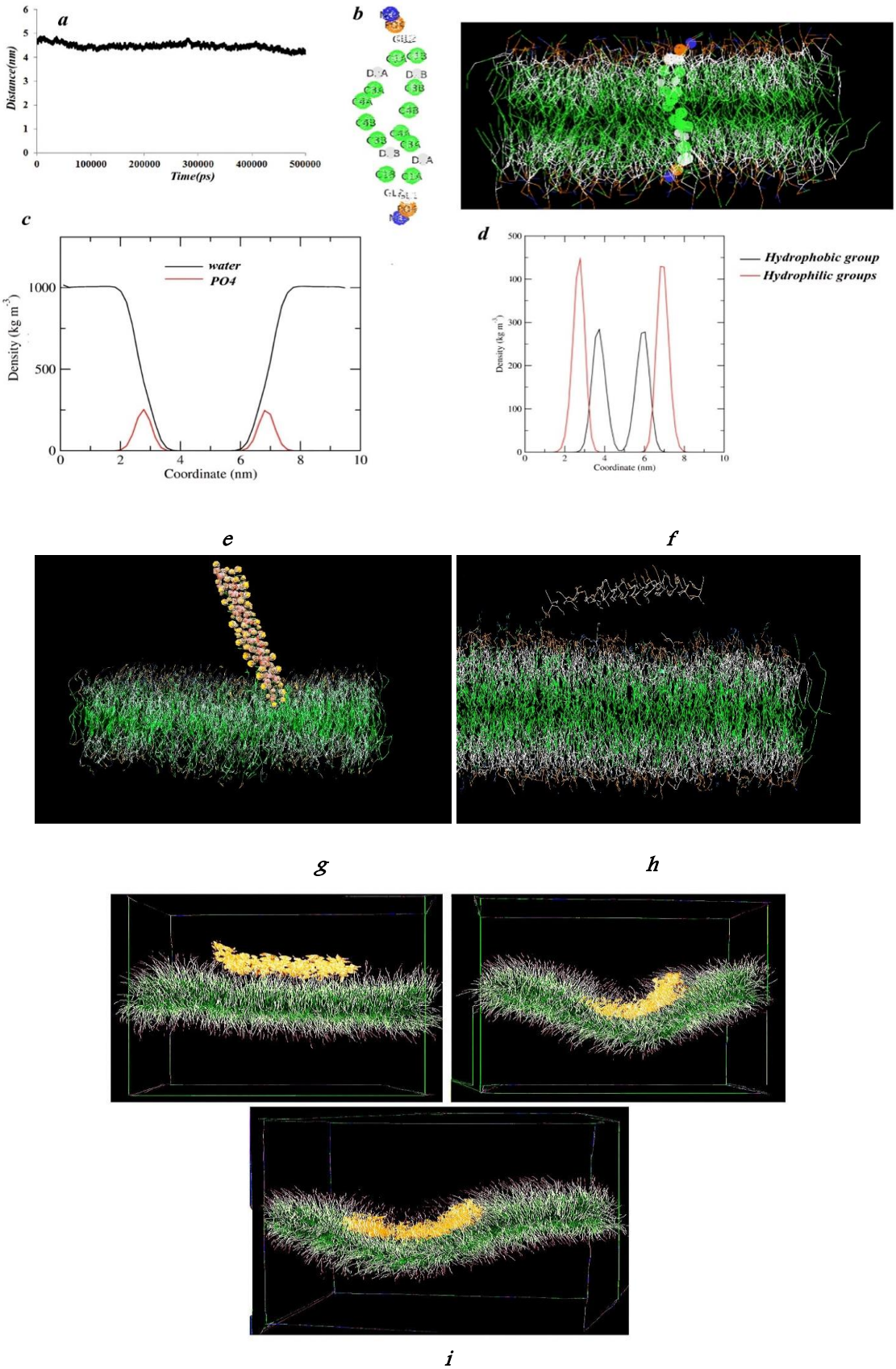
a
Distance(nm)
Time(ps)
b
c
water
PO4
Density (kg m-3)
Coordinate (nm)
d
Hydrophobic group
Hydrophilic groups
Density (kg m-3)
Coordinate (nm)
e
f
g
h
i

Fig. 2. Different characteristics of pure membrane. Figure 2a includes changes in the distance (membrane thickness) between the phosphate atoms of each membrane layer. The electron density of the polar heads of lipid molecules at the two outer surfaces of the membrane can also be observed (Figure 2b). Another diagram of this figure is related to the density of the phosphate group and the density of water molecules on the outer surface of the two layers of the membrane (Figure 2c), which clearly shows that the polar heads, hydrophilic, are located outwards during the simulation, and the two outer sides of the membrane have the highest density (Figure 2d). Figure 2e and 2f show placement of peptide in the presence of membrane. The image illustrates the positioning of a peptide within a biological membrane. It highlights the internalization process, where the peptide interacts with lipid molecules, causing curvature in the membrane. Figure 2g, 2h, and 2i show initiation of endocytosis and peptide initialization that were stimulated by the Linux operating system using GROningen MAchine for Chemical Simulations (GROMACS) and Coarse-grained molecular dynamics (CGMD) Simulation based on Martini force field. The results suggest that the peptide can be internalized into the membrane through endocytosis within 1000 nm of simulation.

Prediction of antigenicity and presenting epitopes to T cells

The sequence of FhHMD-1 was predicted antigenic with an overall score of 0.5093 (threshold of 0.5). The possible T cell epitopes on the sequence of protein was scanned against a panel of most frequently occurring alleles using the IEDB recommended prediction method (Net MHC pan). The MHC-I epitopes were evaluated based upon the proteasome cleavage score, the TAP score, and the MHC binding score. The total score was calculated as the sum of these scores, indicating the ratio of the amount of peptides presented by MHC molecules on the cell surface. The predicted epitopes were screened for IC50 less than 50 nM as candidates with higher binding affinity (Table 1). The top ranked MHC II binding epitopes are presented in Table 2, where the higher value of cleavage probability score and the lower amount of the percentile rank predicts the greater possibility of antigen presenting by MHC II molecules.

Table 1. Evaluation of the MHC I antigen processing

Allele	Position	Peptide	Proteasome Score	TAP * Score	MHC Score	Total Score	IC50 (nM)
HLA-A*02:03 HLA-A*02:06	16-25	KMVKALRDAV	0.85	0.33	-1.13	0.05	13.5
HLA-B*15:01	20-29	ALRDAVTKAY	1.29	1.41	-1.34	1.36	21.9
HLA-A*30:01	31-40	KARDRAMAYL	1.37	0.43	-1.24	0.57	17.2
HLA-A*68:01	50-59	TEVITILLNR	1.04	0.61	-1.29	0.37	19.4

* Transporter associated with antigen processing (TAP)

Table 2. Evaluation of the MHC II antigen processing

Position	Peptide	Peptide length	Cleavage probability score	Cleavage probability percentile rank
34-48	DRAMAYLAKDNLGEK	15	0.85345	0
43-56	DNLGEKITEVITIL	14	0.61552	0.18
23-35	DAVTKAYEKARDR	13	0.54836	0.36
23-36	DAVTKAYEKARDRA	14	0.46804	0.53
42-56	KDNLGEKITEVITIL	15	0.46326	0.71

Discussion

The study of FhHDM-1 (*Fasciola hepatica* Helminth Defense Molecule-1) in the context of fascioliasis pathogenesis is critical, particularly concerning the immune response and potential therapeutic applications.In the present study, RT-PCR analysis revealed that FhHDM-1 was not expressed in *Fasciola hepatica* eggs and miracidia, in contrast to its expression in adult flukes (Figure1). MF6p/FhHDM-1 has been previously identified as a protein in adult excretory/secretory (E/S) products and somatic soluble newly excysted

juveniles extract (NEJ) of *F. hepatica* (23). The current research emphasizes the expression of FhHDM-1 primarily in adult flukes, eggs, and miracidia, which may inadvertently overlook significant expression patterns in other critical life-cycle stages, such as metacercariae. This study focused on eggs, miracidia, and adult flukes; other developmental stages (e.g., metacercariae) were not analyzed. Future research should therefore include all key life cycle stages. We recommend performing quantitative real-time PCR (qRT-PCR) and *in situ* hybridization to map the stage-specific mRNA expression pattern of FhHDM-1 across all developmental stages of *F. hepatica*, including metacercariae. Furthermore, western blotting and immunohistochemistry using specific antibodies against FhHDM-1 should be employed to verify protein expression and localization in tissues from these stages.

Expression of *Fh*HDM-1 in developmental stages of *F. hepatica* indicated its key biological function in the parasitic life-cycle (24). These discrepancies highlight the need for more standardized and sensitive methodologies in future studies to ensure the reliability of results. It would be beneficial to compare methodologies across studies to better understand why variations in expression patterns occur. This could lead to improved experimental designs and a clearer understanding of FhHDM-1's biological significance.

The evolutionary relationships of HDM cDNA sequences from trematode pathogens alongside cDNA sequences derived from *F. hepatica* in this study were assessed using the neighbor-joining method. The resulting phylogenetic tree revealed significant clades that included *Fasciola* HDMs, the Chinese or oriental liver fluke, as well as *schistosoma* and *paragonimus* HDMs. Notably, all sequences within the HDM clades exhibited amino acid sequence identity in specific regions, including an N-terminal signal peptide, a predicted α-helical secondary structure, and a highly conserved amphipathic C-terminal motif. This 34-residue region serves as a heme- and LPS-binding site (24), and the amphipathic C-terminal residue of *F. hepatica* demonstrated a high degree of similarity with those found in other helminths. Our amino acid sequences showed an impressive 100% similarity with the *F. hepatica* sequences available in databases.

Our stimulation indicated that the density of the desired peptide among the hydrocarbon chains of the membrane lipids increased, confirming the internalization of the peptide into the membrane (Figure 2a-2b). The distribution of lipid groups and ions around the peptide was calculated for quantifying the position of peptide internalization in to biological membranes and understanding of the internalization mechanism. Our findings demonstrated the peptide has moved away from the aqueous environment over time and enters the plasma membrane lipid rafts by the polar heads of lipid molecules and interaction with phospholipids, in turn results in creating curvature, leading to endocytosis pathways (Figure 2d). Additionally, a decrease in the ion radial distribution function around the peptide revealed that the peptide was moving away from the aqueous environment, while an increase was simultaneously observed in the distribution of polar groups (hydrophobic molecules) and their density around the peptide (Figure 2c and 2d). Our analysis revealed that the peptide can be internalized into the membrane with interaction of polar groups, when is surrounded by hydrocarbon tails of the membrane, implying that negatively charged phosphate groups are involved in the internalization of peptide into membrane. A distance of 1 nm was predicted between peptide and the lipid phosphate groups for internalization of peptide (Figure 2a – 2d).

Here, we stimulated internalization of FhHDM-1 into macrophage plasma membrane, where our findings revealed that the peptide can be internalized into the membrane via endocytosis within 1000 nm of simulation, suggesting rapid internalization into macrophages (Figure 2e and 2f).

Furthermore, the thickness and membrane diffusion coefficient increase when comparing with

the pure membrane, where the gradual internalization of the peptide into the membrane was stimulated (Figure 2g- 2i). FhHDM-1 has been reported to be capable of binding LPS, and its interaction with the surface of macrophages (Figure 2g- 2i). FhHDM-1 and its short C-terminal peptide was found to be capable of beneficial against LPS-induced inflammation in mice by decreasing inflammatory mediators (24). Furthermore, FhHDM-1 has been suggested as an adjuvant anti-arthritis therapy for inhibiting rheumatoid arthritis-induced osteopenia (25), where its C-terminal amphipathic helix with 34-residue has been defined to be able to suppress lysosomal acidification and RANKL-induced osteoclast formation via overexpression of sequestome1/p62 (25). FhHDM-1 has been shown to be able to binds to macrophages plasma membrane lipid rafts through selective interaction with phospholipids and/or cholesterol, result in internalization via endocytic pathway, processed by lysosomal cathepsin L, releasing a conserved amphipathic helix, C-terminal peptide, which in turn is able to prevent vacuolar ATPase activity by proteases. As a result, endolysosomal alkalization is capable of inhibiting antigen processing and MHC class II presentation (3) and impairing activation of NLRP3 inflammasome through lysosomal cathepsin B protease which in turn inhibits IL-1β and T helper 1 immune responses (5), suggesting modulation of host immunity via a pathogen-derived protein by which prolonged parasite survival.

The analysis of the FhHMD-1 protein sequence has yielded promising results regarding its potential as an immunogenic target. With an overall antigenicity score of 0.5093, FhHMD-1 demonstrates a significant likelihood of eliciting an immune response. This finding is essential for identifying potential vaccine candidates or therapeutic agents. Utilizing the IEDB-recommended prediction method (Net MHC pan), we identified several T cell epitopes that could be presented by MHC-I molecules (26). The evaluation of these epitopes based on proteasome cleavage scores, TAP scores, and MHC binding scores provides a comprehensive framework for understanding their presentation efficiency on the cell surface (26). The total score derived from these metrics indicates the relative abundance of peptides available for T cell recognition, which is crucial for mounting an effective immune response. In addition to MHC-I epitopes, our analysis also revealed top-ranked MHC-II binding epitopes, as presented in Table 2. The higher cleavage probability scores and lower percentile ranks associated with these MHC-II epitopes suggest a strong potential for antigen presentation by MHC-II molecules. This dual capability—effective presentation by both MHC-I and MHC-II—positions FhHMD-1 as a candidate for inducing both CD8+ cytotoxic T cell responses and CD4+ helper T cell responses. To robustly validate the transcriptional, structural, functional, and immunological insights of this study, we propose the following experimental approaches; investigate the membrane-remodeling capacity of FhHDM-1 by assessing its ability to induce curvature and endocytosis in host cells via fluorescence and electron microscopy. Determine the immunomodulatory properties of recombinant FhHDM-1 by analyzing its impact on immune cell function, including cytokine secretion, surface marker expression, and phagocytic activity. These experiments are critical for establishing the protein's therapeutic and immunological relevance in fascioliasis.

It is important to recognize that computational models provide only hypothetical insights. To validate these findings and ensure their reliability, additional in vitro and in vivo experiments are essential. Future research should emphasize empirical data collection to either confirm or challenge the computational predictions presented in this study. An integrated approach—combining computational analyses with experimental evidence—will offer a more balanced and comprehensive understanding. While this study provides novel insights into the transcriptional

profile and molecular features of FhHDM-1 in *F. hepatica*, the analyses were limited to eggs, miracidia, and adult flukes. Notably, other developmental stages—particularly metacercariae, the infective stage for definitive hosts—were not included. Given the critical role of metacercariae in zoonotic transmission, future investigations into their gene expression and structural biology may reveal further details about parasite survival strategies, host adaptation, and potential targets for intervention. Expanding research to encompass these stages would contribute to a comprehensive understanding of the parasite's life cycle and its impact on zoonotic disease dynamics.

Conclusions

The investigation into FhHDM-1 (*F. hepatica* Helminth Defense Molecule-1) highlights its significant role in modulating immune responses during fascioliasis pathogenesis, particularly through its expression in adult flukes and its conserved structural features. The ability of FhHDM-1 to internalize into macrophages via endocytosis and inhibit lysosomal acidification suggests its potential as a therapeutic agent for managing inflammatory diseases. Additionally, its capacity to present epitopes through both MHC-I and MHC-II pathways positions, leads as a promising candidate for vaccine development. Overall, the findings support FhHDM-1's potential as an immunogenic target and emphasize the need for further research to validate its mechanisms and explore clinical applications in treating autoimmune and inflammatory disorders. Incorporating additional empirical data, expanding the analysis of developmental stages, standardizing methodologies, and critically evaluating the limitations of computational approaches are essential steps for advancing the field and ensuring the reliability of future research.

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Ethics approval

Ethical approval for the study was obtained from the Ethics Clearance Committee of the Tehran University of Medical Sciences (No. IR.TUMS.REC.1400.033).

Conflict of Interests

The authors have declared that no conflict of interest exists.

Artificial Intelligence Statement

The authors confirm that no generative artificial intelligence (AI) tools were used for the writing, or data analysis of this manuscript. AI was utilized for language editing and grammar correction.

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