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Peptide inhibitors of human HMG-CoA reductase as potential hypocholesterolemia agents

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ABSTRACT

Hypercholesterolemia may lead to obesity and cardiovascular diseases. To prevent hypercholesterolemia, many drugs have been developed while searching for better drugs to treat hypercholesterolemia has never been ended. Other than small molecule drugs, peptide drugs are gaining more visibilities in many therapeutic areas. In the present study, we employed phage-display techniques to screen peptide inhibitors against human HMG-CoA reductase. The results indicate that the tetrapeptide PMAS inhibits hHMGR effectively (IC_{50} = 68 μM), could be a lead compound to develop hypocholesterolemic agents.

1. Introduction

Human HMG-CoA reductase (hHMGR) is the rate-limiting enzyme of the mevalonate pathway that produces cholesterol and other isoprenoids [1]. The enzyme contains 888 amino acids with the first 339 residues as the membrane anchor domain located in the endoplasmic reticulum. A linker region is located between residues ranging from 340 to 449, while the catalytic domain, from residues ranging from 450 to 888, resides in the cytoplasm [2]. Cholesterol is an important molecule in living cells. Approximately seventy percent of the total cholesterol in the human body arises from endogenous biosynthesis and the remainder is provided by the diet [3]. Epidemiological evidence has shown a positive relationship between total cholesterol concentration and the mortality rate of the coronary heart disease. For people with very high level of cholesterol, diet alone is insufficient to achieve the desired level of LDL [4]. To treat hypercholesterolemia, hHMGR has been a major target for developing drugs such as statins. The efficacy of these drugs to control blood cholesterol levels has been well recognized and statins are widely prescribed to lower cholesterol levels [2,5]. However, they also cause severe adverse effects, such as distal muscle weakness, headache and acute renal failure [5,6].

Recent nutritional studies have demonstrated that consumption of soybean protein in place of animal protein can lower blood cholesterol level. A meta-analysis of controlled clinical studies concluded that substituting soybean protein for animal protein significantly lowered total cholesterol, low-density lipoprotein (LDL) cholesterol, and triglycerides without affecting high-density lipoprotein (HDL) cholesterol [7,8]. From the enzymatic digestion of food proteins, hypocholesterolemic peptides have been studied and identified [9,10].

In the present study, we employed phage display technology [11,12] to screen for novel short peptides with hypocholesterolemic potentials. Phages tightly bound against recombinant hHMGR was isolated by biopanning (affinity selection) and their binding capability evaluated by ELISA. Then, the phage single-stranded DNAs were prepared, sequenced and analyzed. The potential peptide-hHMGR interactions were visualized through molecular modeling (docking) tools. Finally, some of the best fitted peptides were synthesized to perform enzymatic assays.

2. Materials and methods

2.1. Bacterial strain and phage library

Escherichia coli strain ER2738 (F′ $proA^+B^+$ $lacI^q$ Δ(lacZ) M15 zzf::Tn10(Tet^R)/fhuA2 glnV (lac-proAB) thi-1Δ(hsdS-mcrB)5($r_K^- m_K^-$ McrB$^-$)) was obtained from New England BioLabs (MA, USA) for phage propagation. *E. coli* strain DH5α (fhuA2Δ(argF-lacZ)U169

Abbreviations: ELISA, enzyme-linked immunosorbent assay; HMG-CoA, 3-hydroxyl-3-methylglutaryl coenzyme A; hHMGR, human HMG-CoA reductase; HRP, horseradish peroxidase; IPTG, isopropyl β-D-1-thiogalactopyranoside; LB buffer, Luria-Bertani buffer; LDL, low density lipoprotein; NADPH, nicotinamide adenine dinucleotide phosphate; MTT, 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide; pfu, plaque forming unit; TMB, tetramethylbenzidine.

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phoA glnV44Φ80Δ(lacZ)M15 gyrA96 recA1 relA1 endA1 thi-1 hsdR17) was used to determine the effect of selected peptides on bacterial cell growth. *E. coli* strain BL21Star (F^- ompT hsdS B ($r B^-$ mB^-) gal dcm rne131) obtained from Invitrogen (USA), was used to induce over-expression of the cloned genes. The phage-displayed random peptide library Ph.D.-12 was purchased from New England BioLabs with an original phage titer 1.5×10^{13} pfu/ml, and a library complexity of 2.7×10^9.

2.2. Cloning, over-expression and purification of recombinant hHMGR

The 1380 bp DNA encoding the catalytic domain (residue 417 to residue 888) of human HMGR gene (Genebank ID M15959) was amplified by PCR. The total mRNA molecules of hHMGR gene were collected from HepG2 cells, reverse transcribed to cDNA, and used as templates for PCR using the forward primer: 5′-GGT CTC GAG TAT TCA GGC TGT CTT CTT-3 and reverse primer: 5′-ACA GGA TCC AAC TCC TCC TTA CTC GAT-3′. The obtained DNA fragment was subcloned into the *Xho*I-*Bam*HI site of vector pET28a (Novagen; http://www.novagen.com/petfram.html) to obtain plasmid pET28a/hHMGR. The pET28a vector carries an N-terminal His-tag/thrombin/T7-tag sequence, a *lac*I operator between T7 promoter, and the ribosomal binding site RBS. The plasmid pET28a/hHMGR was used to transform *E. coli* BL21 Star and the expression of the cloned gene was induced by the addition of 1 mM IPTG at OD_{600} = 0.6. After growing for another 4 h, the cells were collected and sonicated with Ultrasonic Processor (Microson/M8726). The recombinant His-hHMGR protein was then purified using the HiTrap Chealth column (Amersham, USA) connected to an AKTA-prime fractional collector (GE Healthcare, USA). The collected protein was further concentrated by passing through Centricon (Millipore, USA) and finally dissolved in 0.1 M KH_2PO_4 buffer [1,12].

2.3. Affinity selection of phages binding with hHMGR

Screening the phage library was performed following the manufacturer's protocol (New England BioLabs) by three rounds of affinity selection in four wells of ELISA plate. Selection of phages bound tightly with recombinant hHMGR was performed by incubating 100 μl hHMGR (100 μg/ml) in an ELISA plate well overnight at 4 °C. The wells were blocked with 200 μl blocking buffer (0.1 M $NaHCO_3$, 5 mg BSA/ml) at 4 °C for 1 h and washed with 200 μl TBST (50 mM Tris–HCl, 150 mM NaCl, and 0.1% v/v Tween-20) six times. Selection was carried out by adding 100 μl phages solution (1×10^{10} pfu/ml) in TBST buffer and incubated for 1 h at room temperature with gentle agitation. The unbound and loosely bound phages were removed and the wells were washed ten times with 200 μl TBST. Then, the tightly bound phages were eluted with 100 μl elution buffer (0.2 M glycine–HCl, pH 2.2, 1 mg BSA/ml), and 85 μl of eluent was collected and neutralized with 15 μl 1 M Tris–HCl (pH 8.0), followed by titering the phages. The second and third biopanning processes were similar to the first round, except that the Tween-20 concentration was increased to 0.5% in the TBST wash buffer. After three rounds of selection, the eluents were amplified and plated. Sixty phage clones were picked randomly for the binding assay.

2.4. Phage-hHMGR binding assay by ELISA

The binding assays were performed by coating the wells with recombinant hHMGR protein (100 μl, 100 μg/ml), incubated overnight at 4 °C, blocked (with 200 μl blocking buffer), and washed with 200 μl TBST (containing 0.5% Tween-20) six times. The selected phages (100 μl, 1×10^9 pfu/ml) were added to each well, incubated at 25 °C for 2 h, and washed with TBST six times. Then, 200 μl rabbit anti-M13 antibodies (1/8000 in the blocking buffer) were subsequently added, incubated at 25 °C for 2 h and washed with 200 μl TBST six times. Then 200 μl horseradish peroxidase-conjugated goat anti-rabbit IgG antibodies (1/8000 in the blocking buffer) were added at 25 °C for 2 h and washed with 200 μl TBST buffer six times. The bound antibodies were detected by adding 100 μl TMB solution and OD_{655} was recorded [13].

2.5. Phage DNA preparation, DNA sequencing and peptide synthesis

The M13 phage single-stranded DNA preparation and sequencing were performed as described previously [13]. DNA sequencing was performed by Genomics BioScience and Tech Company (Taipei, Taiwan). DNA sequences were analyzed using the program Translate and Clustal-W from the ExPASy proteomic web server (http://us.expasy.org/). Peptides were synthesized by an automated PSIII synthesizer (NatureGene Corp, USA) as described previously [12].

2.6. Molecular docking of peptides with hHMGR

The 3D structure PDB 1HWK of human HMG-CoA reductase was downloaded from the Protein Data Bank (http://www.rcsb.org/pdb) as the molecular target [14]. Two of the tetrameric structures were chosen and fixed with the forcefield CHARMm (Chemistry at Harvard Macromolecular mechanics) equipped in DS 2.0 (http://accelrys.com/products/discovery-studio) to add up the hydrogen atoms, partial charges and missing residues, so that it can be used properly for molecular docking processes [15,16]. The peptides were docked into the binding site of hHMGR and their binding affinities were estimated by the mean force (-PMF) and DOCK scores of the LigandFit program [16–18]. The peptide–receptor (1HWK) interactions were further analyzed and the potential types of weak interactions were displayed with DS 4.0 visualizer (Accelrys Software Inc., Discovery Studio Modeling Environment, Release 4.0, San Diego: Accelrys Software Inc., 2013).

2.7. ADMET prediction of selected peptides

Before performing the sophisticated and costly ADMET experiments, the ADMET properties of the selected peptides (four hexapeptides and six tetrapeptides) can be analyzed in silicon prior to their enzyme activity assays. In the ADMET module of DS molecular modeling tool, six models (aqueous solubility, blood–brain barrier penetration, CYP2D6 inhibition, hepatotoxicity, human intestinal absorption, and plasma protein binding) were used to predict the ADMET characteristics of the selected molecules (data not shown). Some of the peptides were also evaluated by DSSTox (Distributed Structure-Searchable Toxicity) Database, which is a computational tool developed by US EPA's National Center for Computational Toxicology for predicting toxicity based on chemical structure (http://epa.gov/dsstox structure browser/).

2.8. hHMGR activity and MTT assays

The HMG-CoA-dependent oxidation of NAPDH was measured for assessing hHMGR activity [19]. The testing peptides (100 μM) were added to the assay mixtures (200 μl final volume) containing 100 μM HMG-CoA, 200 μM NADPH, 10 mM DTT, 10 mM EDTA, 200 mM KCl and KH_2PO_4 buffer, pH 7.4. Then, 20 μg hHMGR was added to each reaction mixture and the reactions were monitored by following OD_{340} (at a 30 min interval) using an EnSpire Multimode ELISA plate reader [10]. One unit (U) of hHMGR was defined as the amount of enzyme that catalyzes the oxidation of 1 mol of NADPH per minute. Protein concentration was determined by the method of Bradford [20].

The potential cellular toxicity of the selected compounds on HepG2 cells was assessed by MTT method [21]. The MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay is based on the conversion of MTT into formazan crystals by the living cells. HepG2 cells were cultured in a 96-well culture plate (1×10^5 cell/ml) for 24 h at 37 °C in atmosphere of 5% CO_2. The cultures were treated with different concentrations of the selected compounds for 24 h. The supernatants were then removed and MTT (2.5 mg/ml) was added and incubated for an additional 4 h. The purple formazan crystals that developed from tetrazolium (MTT) within the cells by the action of mitochondrial succinate dehydrogenase were extracted into DMSO. The optical OD570 was measured using an EnSpire Multimode ELISA Plate Reader (PerkinElmer, USA).

3. Results and discussion

3.1. Preparation of recombinant hHMGR protein and biopanning

The 1380 bp DNA encoding the catalytic domain (residue 460 to residue 880) and part of the linker region (residue 429 to residue 460) of hHMGR gene was subcloned into the vector pET28a, and expressed in *E. coli* DH5α. The recombinant hHMGR was then purified via Ni-NTA column, concentrated by Centricon, and confirmed by Western blot analysis (data not shown). Screening of the phage library against recombinant hHMGR was performed by three rounds of biopanning (affinity selection). The proportion of phages selected from each round was 7.4×10^{-4}, 1.6×10^{-2}, and 2.2×10^{-3}%, respectively, indicating a successful selection process. We then randomly picked 60 phage clones which were potential tight binders toward hHMGR for further binding assay.

3.2. Assays of phage binding capability, DNA sequencing and peptide synthesis

The binding capability of the selected phages with hHMGR protein was evaluated by ELISA. The bound phages were detected by rabbit anti-M13 antibody and horseradish peroxidase-conjugated IgG antibody [12]. As shown in Fig. 1, most of the phages exhibit much higher binding affinity as compared to the original library. Among them, phages E1, E5, E8, E12 and E13, show slightly higher affinity. To determine the amino acid sequence of the tightly bound peptides, the single-stranded DNAs of the phages were prepared and sequenced. As displayed in Table 1, these peptides are all hydrophilic in nature (negative values of xLogP), and most are negatively charged with approximately 30% hydrophobic residues.

Table 1
Characterization of the selected peptides.

Name	Peptide sequence	MW	xLogP	Net charge	Hydrophobic ratio (%)
E1	DHIHWITPSHPG	1384	−8.7	−1	25
E2	DHYSYTWFSWPT	1583	−5.4	−1	25
E3	ACSYHTTRAFVC	1384	−7.5	+1	58
E4	ACLYHTTRAFVC	1384	−5.4	+1	58
E5	GHFKWVPYDSLY	1505	−7.5	0	33
E6	THWNWLNPYMAV	1525	−4.8	0	50
E7	ALKIWPNPPRSN	1374	−4.0	+2	33
E8	WHLEWITPMASD	1479	−8.0	−2	50
E9	THISWMSPQKLW	1507	−7.1	+1	41
E10	THERLYWYSPSE	1561	−6.1	−1	16
E11	DSLRQLPLPVLS	1325	−9.3	0	41
E12	EHMQWMRATDLF	1564	−7.3	−1	50
E13	QLEWSYWPQLSR	1586	−9.5	−1	61

Table 2
The molecular docking values and xLogP of the synthesized peptides.

Name	Peptides	DOCK	-PMF	xLogP	MW
1	DHYSYT	140	88	−6.8	784
2	WFSWPT	117	99	−1.2	816
3	TPMASD	186	173	−8.2	614
4	RATDLF	123	77	−6	721
5	WFSW	137	124	−0.1	624
6	HFKW	146	95	−2.7	617
7	WHLE	165	78	−3.7	582
8	PMAS	129	113	−4.7	404
9	HERL	154	105	−6.3	553
10	ATDL	142	125	−5.9	417

3.3. Molecular docking of selected peptides with hHMGR

To evaluate the binding affinity of the peptides carried by the selected phages, the PDB structure 1HWK was adopted as the receptor to perform the molecular docking analysis [14]. In PDB 1HWK, hHMGR forms a tetrameric structure and the inhibitor (atorvastatin) presented at the interface of two adjacent monomers. Keep that in mind, the present work chose the predicted binding site of hHMGR which also includes residues from subunit A and subunit B of hHMGR, and contain all of the critical residues reported previously [14]. Since the size of dodecapeptides was too

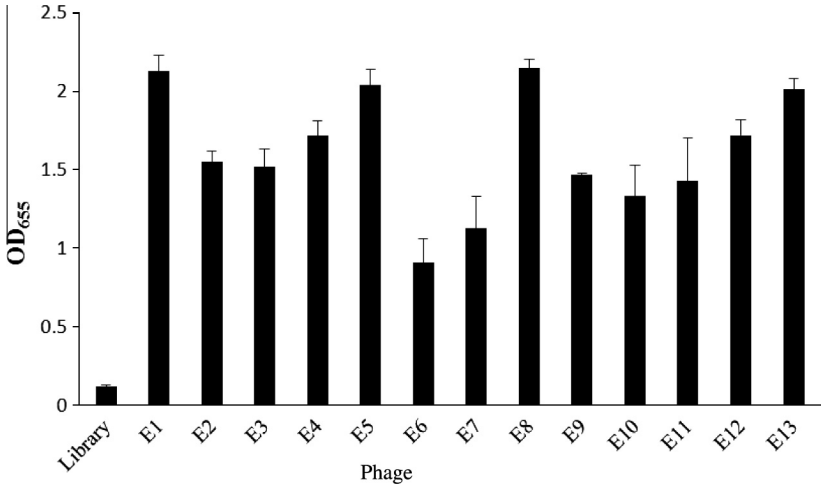

Fig. 1. Binding capabilities of the selected phages toward recombinant hHMGR estimated by ELISA. The original library was served as the control. In each well, same amounts of phages (1×10^9 pfu/well) were added. The experiments were performed in triplicate and presented as mean ± standard deviation.

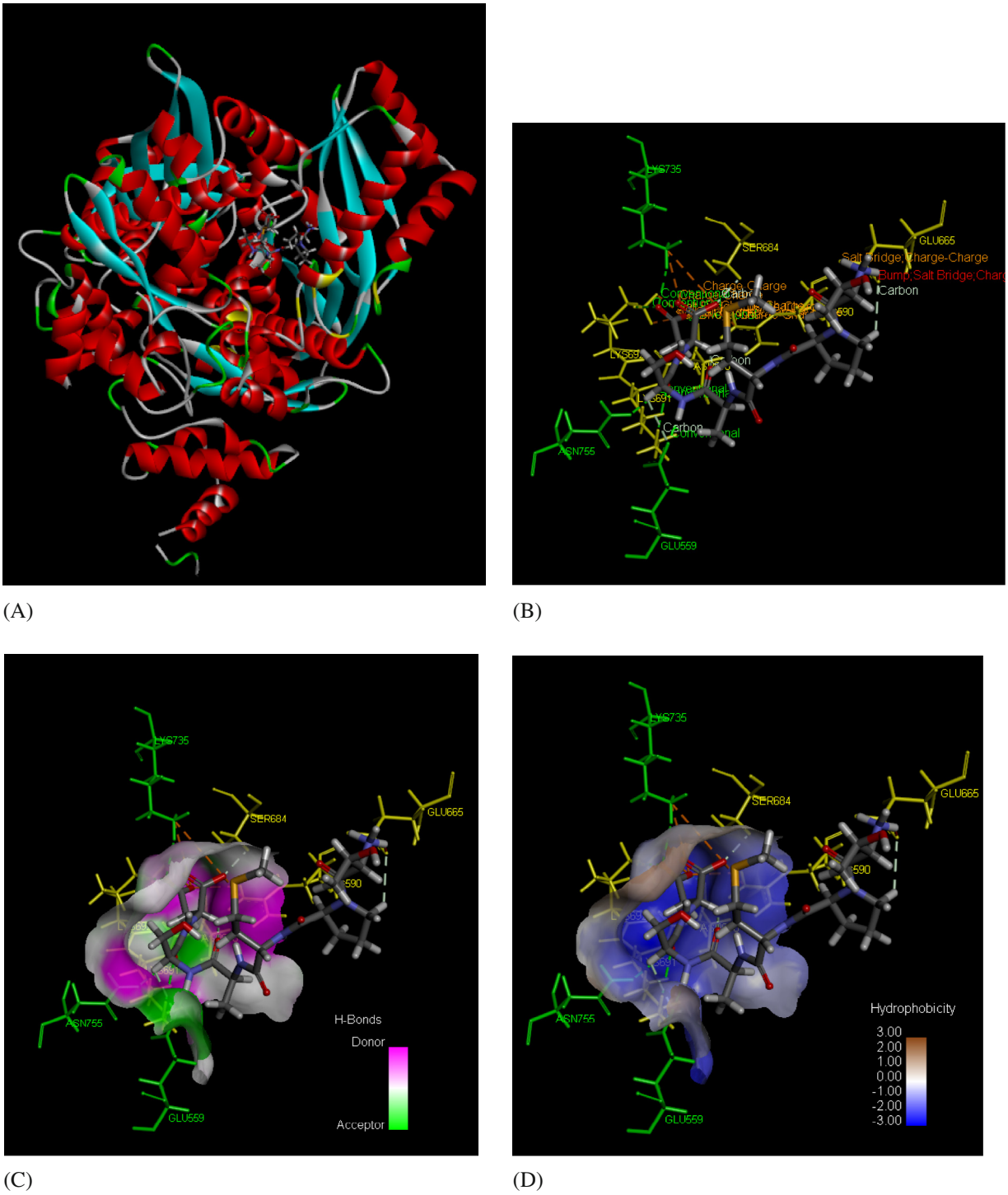

Fig. 2. The peptide #3 (TPMASD) docked at the binding site of hHMGR. (A) The ribbon structure of hHMGR with the peptide. (B) Peptide #3 (TPMASD) interacts with hHMGR at the binding site (residues of subunit A in green, and subunit B in yellow). The types of interactions were also indicated (the details are summarized in the text). (C) The potential hydrogen-bond interactions of peptide with hHMGR. The potential H-bond donor areas and H-bond acceptor areas of the binding site were displayed in pink and green color, respectively. (D) The potential hydrophobic interactions of the peptide with hHMGR. The potential hydrophobic areas and hydrophilic areas of the binding site were indicated in dark brown and blue color, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

large to fit in the binding site, each of them was divided into two hexapeptides to facilitate the molecular docking process and some of them indeed received docking scores (data not shown). After analyzing the docking results, we then designed four hexapeptides and six tetrapeptides to synthesize peptides for further experiments (Table 2). Simultaneously, we found that all of the ten peptides were docked smoothly and received reasonably high docking scores. As shown in Table 2, the –PMF and DOCK scores of hexapeptide #3 (TPMASD), tetrapeptides #5 (WFSW), #8 (PMAS), and #10 (ADTL) are much higher than that of the known statin-like molecules (data not shown). The interaction of peptide #3 (N′-TPMASD-C′) with hHMGR, which received the highest docking

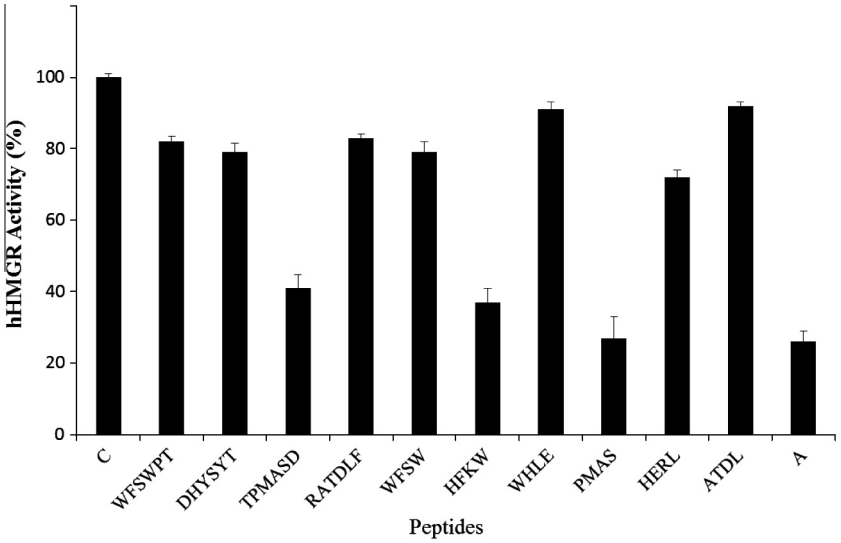

Fig. 3. Inhibition of human HMG-CoA reductase activities by the synthesized peptides. The concentrations of the ten peptides are 100 μM. The positive control (A) uses the same assay mixture with added atovastatin (30 nM). The control reaction (C) contains the same assay mixture except without the addition of peptide. The experiments have been repeated for three times and the standard deviations calculated.

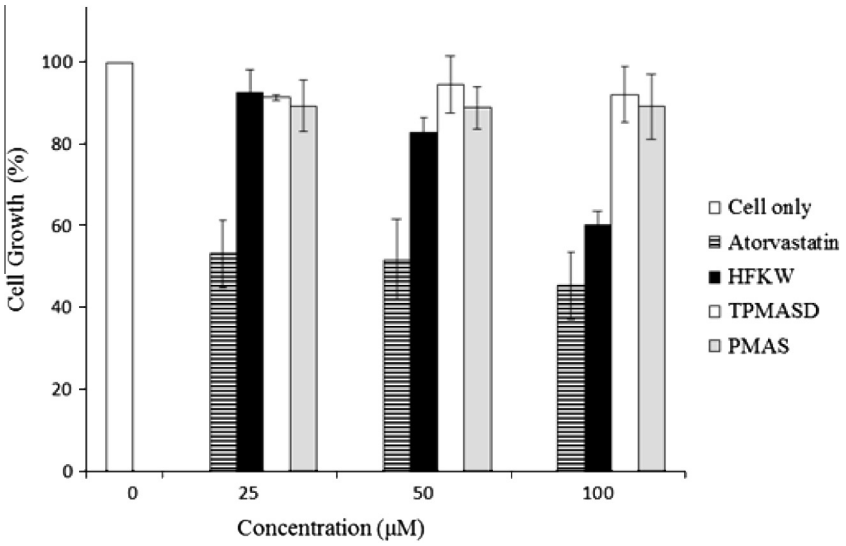

Fig. 4. MTT assays of the selected peptides. The symbols/colors in the columns: horizontal lines, black, white and gray, represent atorvastatin, peptide HFKW, peptide TPMASD and peptide PMAS, respectively.

scores, was chosen for further ligand–protein interaction analysis. As shown in Fig. 2A, peptide #3 (TPMASD) dwelled snugly at the center of the binding site, with its N-terminus stayed in the inner side and the C-terminus extruding out. Besides van der Walls interactions, the type of interactions of peptide #3 with hHMGR was predicted to be mainly electrostatic (Fig 2B). The putative hydrogen bonds were predicted to be between the Thr residue of peptide #3 with Lys691, Lys735, and Asn755 of the subunit A of hHMGR, and the Pro residue of the peptide with Asp690 and Asp692 of the subunit B of hHMGR (Fig 2C). The ligand–protein interaction analysis tool also predicted a minor interaction, which is the salt bridge between the Thr residue of the peptide#3 with Glu665 of the subunit B of hHMGR (Fig 2B). Furthermore, the hydrophobic plot (Fig 2D) confirmed that the nature of the binding site environment is mainly hydrophilic and the hydrophobic interactions between the peptide and hHMGR can be negligible.

3.4. Enzymatic activity assays

The hHMGR enzymatic activities were assessed by measuring the HMG-CoA-dependent oxidation of NAPDH [10]. Using the data from the Lineweaver–Burk plot, the K_m and the V_{max} values of the recombinant hHMGR was calculated to be 103.5 μM, and 10.1 μM NADPH/min/mg respectively. These values were reasonably close to the reported data collected from the similar HMG-CoA-dependent oxidation of NADPH or a radioisotope method [19]. Finally, the potential inhibitory effects of the ten synthesized peptides were measured. As shown in Fig. 3, among the ten peptides, hexapeptide #3 (TPMASD), tetrapeptides #6 (HFKW) and #8 (PMAS) were found to inhibit hHMGR effectively. The IC_{50} of hexapeptide #3 (TPMASD), tetrapeptides #6 (HFKW) and #8 (PMAS) were further determined to be approximately 80 μM, 80 μM and 68 μM, respectively. These IC_{50} values are still much higher than

those of statins-like molecules which have IC50 values in the nM range [2]. The reasons that all of the selected peptides have rather high docking scores but much poor hHMGR inhibitory activities remain to be answered.

From glancing through the molecular structures, we can observe that the known statin-like drugs all possess a HMG-CoA-like moiety on their structural scaffolds which are much more hydrophobic than the ten selected peptides. Upon analyzing the atorvastatin-hHMGR interaction (diagram not shown), Pi-alkyl interactions were predicted between its five-membered ring and the Ala856 residue of subunit A of hHMGR; alkyl-alkyl interactions between the dimethyl groups on the five-membered ring and the Cys561 of the subunit A of hHMGR; a few H-bonds were also predicted to be between the HMG-CoA-like region and Glu559, and Asn755 of the subunit A and also Asp690 and Lys691 of the subunit B of hHMGR. A carbon–fluorine interaction was also noticed between the fluorine at the six-membered ring and Arg 590 of the subunit B of hHMGR. Though more ligand–protein interactions were predicted between atorvastatin and hHMGR, these interaction analyses are basically only descriptive. They are still not enough to explain the discrepancies between the rather high docking scores and the much lower hHMGR inhibitory activities of the selected peptides as compared with the current drug atorvastatin.

3.5. ADMET predictions and MTT assays

The ADMET properties influence the performance and pharmacological activity of the compound as a drug and are the primary factors causing drug attrition. Traditionally ADMET applied at the end stage of drug development, however, it would be better to remove molecules with poor ADMET properties from the drug development pipeline to save R&D costs. Therefore, we have estimated the ADMET properties of the selected peptides and statins. We found that most of the ten peptides exhibited very good ADMET properties, except that peptides #1, #7, #8 and #9 had minor plasma protein binding capabilities (data not shown). However, atorvastatin was predicted to have a weak hepatotoxicity and plasma protein binding capabilities (data not shown). The potential cytotoxic effects of the selected peptides HFKW, TPMASD and PMAS were further examined by MTT assays. As shown in Fig. 4, within the concentration range, atovastatin exhibited a pronounced inhibitory effect upon cell growth. Peptide HFKW also carried a concentration-dependent cytotoxicity effect, while peptides TPMASD and PMAS were found almost unharmed to the cells in the assay. These preliminary experimental results echoed well with the pre-ADMET predictions.

For the past few years, peptide drugs have gained more visibilities in many therapeutic areas. In general, peptide drugs can effectively combine the advantages of small molecules (cost, conformational restriction, membrane and permeability) and those of protein drugs (target specificity and high potency), thus offering new potential opportunities [22]. In the present study, we employed phage-display techniques to screen for peptide inhibitors against human HMG-CoA reductase. From a limited amount of trials, we found that the tetrapeptide PMAS is an effective inhibitor of hHMGR (IC_{50} = 68 μM) and has a nice predicted ADMET properties. Therefore, it is anticipated that it may have a potential to develop hypocholesterolemic agents.

Acknowledgments

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