Finding small molecular compounds to decrease trimethylamine oxide levels in atherosclerosis by virtual screening

Abstract: The intestinal microbial metabolite trimethylamine oxide (TMAO) affects the formation and development of atherosclerosis (AS). The design and development of an effective targeted drug to reduce serum TMAO levels may provide new avenues for inhibiting AS morbidity. Target genes that may elevate TMAO levels in patients with AS were explored using bioinformatics, virtual screening, and molecular dynamics (MD). We expected these genes to indicate potential TMAO inhibitors. Of these genes, hFMO3 was responsible for increasing TMAO levels. Four small-molecule compounds (SMC-1, SMC-2, SMC-3, and SMC-4) with the lowest binding energy and CGenFF penalty < 10 were connected to the main binding pocket of hFMO3 by hydrogen and/or cation–pi interactions. A 100 ns MD simulation showed that the four systems quickly reached equilibrium. The root mean square deviation of all four small-molecule compounds was less than 0.35 nm, that of the four ligand complexes was less than 0.40 nm, and the average deviations of each amino acid residue from the reference position over time did not differ. Molecular mechanics Poisson–Boltzmann surface area analyses showed that SMC-2, SMC-3, and SMC-4 bound very well to hFMO3, and the energy contribution of the key residues LEU40 and GLU32 was more remarkable in SMC-2, SMC-3, and SMC-4. These four small-molecule compounds may be useful as targeted drugs to reduce serum TMAO levels, inhibiting atherosclerosis formation.

Keywords: atherosclerosis, TMAO, hFMO3, virtual screening

1 Introduction

Atherosclerosis (AS), a chronic disease of the artery wall characterized by endothelial dysfunction, inflammatory cell recruitment, and foam cell formation, is the leading cause of death and shortened life expectancy worldwide [12]. In China, the incidence of AS has shown an upward trend, and it is more common in middle-aged and older people, with the fastest development at the age of 40–49 years. Risk factors such as hyperlipidemia, hypertension, diabetes, smoking, and alcohol consumption are closely related to the onset of AS [3–7]. In particular, a diet high in cholesterol and fat can cause increased plasma cholesterol levels and promote the formation and development of atherosclerotic plaques [8]. When AS causes 50% narrowing of the arteries supplying vital organs or thromboses detach and block blood vessels, it may lead to reduced or interrupted blood flow, resulting in myocardial infarction or ischemic stroke [9]. Active intervention and control of relevant risk factors are important to reduce the incidence of AS.

By in-depth exploration of the mechanism of AS formation, it was found that trimethylamine-N-oxide (TMAO) is promising as a potential therapeutic target for inhibiting AS development [10]. TMAO is an intestinal microbial metabolite that is mainly metabolized by the intestinal microbiota through the intake of compounds such as choline and carnitine to produce trimethylamine (TMA). Furthermore, TMAO is oxidized in the liver by flavin-containing monooxygenases (FMOs), which play the main catalytic role. The diet–gut microbiome–liver pathway is the main
biosynthesizer of TMAO [11]. Precursors that produce TMA include TMAO, choline, phosphatidylcholine, carnitine, γ-buty1 betaine, betaine, croton betaine, and glycero1 phosphorylcholine, which are abundant in fish, beef, and eggs [12]. There are four main enzymes involved in the production of TMA: choline-TMA lyase (cutC/D), carnitine monoxygenase (cntA/B), betaine reductase, and TMAO reductase (torTCAD) [13]. In addition, the cntA/B homologous enzyme yeaW/X can also use carnitine, choline, γ-buty1 betaine, and betaine to produce TMA [14]. After TMA enters the liver, it is oxidized to TMAO by human FMO (hFMO3) [11,15]. hFMO3 requires flavin adenine dinucleotide (FAD) and nicotinamide adenine dinucleotide phosphate oxidase (NADPH) as cofactors and molecular oxygen as a co-substrate to complete the catalytic process. First, the cofactor NADPH binds to FAD and rapidly reduces it to FADH2. The reduced FAD that received two electrons rapidly binds to molecular oxygen to form a stable C4-position flavin peroxidation intermediate, before binding to the appropriate nucleophilic substrate RSH. Through the nucleophile attack of FADH-OOH by the substrate, one oxygen atom is transferred to the substrate to form an oxidation product (RSOH), and the other oxygen atom forms water, thereby reducing FADH-OH to FAD and releasing NADP+ for the next catalytic cycle [16].

TMAO is distributed throughout the human body, excreted in urine, sweat, and respiration within 24 h, and more than 95% is cleared by kidneys as a prototype [11,15,17,18]. The residual TMAO can inhibit the degradation of cholesterol in the blood, so that cholesterol can only be deposited in the arterial blood vessel wall, resulting in thickening and hardening [11,15]. TMAO was identified as an atherosclerotic promotor by enhancing the formation of foam cells and atherogenic plaques in mice [19,20]. TMAO induces vascular inflammation and endothelial dysfunction in vivo and in vitro by activating NFκB [21]. In addition, studies have revealed the activation of the ROS/TXNIP/NLRP3 inflammasome in TMAO-induced inflammation and endothelial dysfunction. TMAO also enhances monocyte adhesion, which then promotes AS [22]. In addition, the accumulation of oxidatively modified low-density lipoprotein (ox-LDL) in macrophages is also critical for the development of AS. These macrophages become foam cells and begin to accumulate within the lining of blood vessels, participating in lesion progression. TMAO induces macrophages to take up more LDL and form foam cells by activating CD36/ MAPK/JNK and SR-A1 expression, promoting AS [21,23–25].

Reducing circulating TMAO levels has attracted widespread attention. Combined with TMAO metabolism and its mechanism of action, microorganisms, FMOs, and TMA lyases may be potential targets for reducing TMAO levels in patients with AS [26,27]. The choline analogue 3,3-dimethylbutanol (DMB) has been found to have an inhibitory effect on choline TMA lyase activity, and DMB has been developed as a competitive inhibitor of CutC to reduce TMAO production, thereby weakening the promoting effect of choline in AS. The inhibitor has a non-lethal effect on human intestinal commensal bacteria containing CutC, can inhibit the production of TMA in cultured commensal bacteria, and reduce plasma TMAO levels in mice with choline-supplemented diets, slowing the development of AopE μ mice with AS. DMB is a natural product found mainly in certain types of balsamic vinegar, red wines, cold-pressed extra virgin olive oil, and grapeseed oils. DMB has not been found to have any adverse effects on liver or kidney functions, even when mice drank water with up to 1% DMB [28].

In this study, we aimed to identify novel TMAO inhibitors to inhibit the formation and development of AS. To this end, statistical/mathematical models were established using machine learning techniques that were able to predict and quantify purchasable small-molecule compounds as potential TMAO inhibitors using virtual screening [29–31]. The Uniprot [32] and ZINC [33] databases served as sources of known biological and structural data for further molecular dynamics (MD) simulations.

### 2 Methods

To find small-molecule compounds that could be direct inhibitors of TMAO, we used bioinformatics, virtual screening, and MD simulation, aiming to ultimately discover inhibitors that effectively reduce TMAO levels in patients with AS (Figure 1).

#### 2.1 Potential target prediction

Human genes involved in AS and TMAO production, respectively, were obtained by bioinformatics analysis and the NCBI gene database (https://www.ncbi.nlm.nih.gov/gene/) [34], and Venn diagrams were drawn (http://bioinformatics.psb.ugent.be/webtools/Venn/) [35] to identify genes that jointly influence AS and TMAO. Potential target genes related to elevated TMAO levels in patients with AS were identified in the Reactome database (https://reactome.org/) [36] using pathway analysis, expression analysis, and literature mining. The target gene structure was obtained using Uniprot, and Pymol2.5.3, UCSF Chimera1.16, and AutoDock Tools 1.5.6 were used for structural conversion and image processing.
for subsequent combined pocket prediction and molecular docking [32,37–39].

### 2.2 Virtual screening

#### 2.2.1 Determining the combined pocket

We obtained the hFMO3 protein structure using the Uniprot platform [32] and used the Proteins Plus Server online tool (https://proteins.plus) [40] to predict and describe potential combined pockets in the hFMO3 protein structure, thereby predicting the probable binding regions of small-molecule compounds. The Proteins Plus Server tool [40] detected potential combined pockets in specific proteins and sorted them based on their volume, surface area, and druggability scores, using UCSF Chimera1.16 to process the images of hFMO3 potential combined pockets [38].

#### 2.2.2 Molecular docking

Before screening for receptors, the hFMO3 protein was treated with a detectable water molecule and residue ligand in Pymol software [41]. The Biogenic library was selected as a potential hit resource for virtual screening, including primary metabolites (metabolites) and secondary metabolites (natural products). The Biogenic library was downloaded from the ZINC16 database (https://zinc.docking.org) [33] on August 03, 2022, and contained 135,335 small-molecule compounds. The AutoDock tool automatically added Gasteiger charges and polar hydrogen atoms to the uploaded hFMO3 protein and small-molecule compounds [42,43]. These small-molecule compounds were virtually screened with hFMO3 protein primary binding pockets using AutoDock Vina1.2.0, and the screening results were sorted according to the minimum binding energy of the molecule docking [44,45].

The top 1% of the small-molecule compounds in the virtual screening output were selected, and these optimal results for hFMO3 protein were repeatedly screened to confirm the accuracy of the AutoDock Vina1.2.0 output [44]. The screening process was run using UCSF Chimera1.16 on local machines. AutoDock Tools1.5.6 was used to process and store the 3D conformational PDB file with the smallest binding energy of the small-molecule compounds, and proteins and ligands with the least binding energy were saved as complex PDB files using Pymol2.5.3 [41,42]. Pymol2.5.3 was further used to clarify the specific chemical bonds of interactions between complexes [41].

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**Figure 1:** Flow chart of small-molecule compounds found to reduce TMAO levels in patients with AS.
2.3 MD simulation

MD simulation is an effective method for predicting ligand–target interactions. Atoms and molecules throughout the complex are allowed to move and interact for a specific time. The trajectories of these atoms and molecules are determined using Newton’s equations of motion. Different fields of action determine the potential energy of interacting atoms through molecular mechanics [46]. The specific MD simulation steps are as follows (http://www.mdtutorials.com/gmx/) [47]:

1. Generate topology: The small-molecule compound PDB file was hydroxylated in the Avoqadro program [48], and the small-molecule compound topology was generated using the CGenFF online platform (https://cgenff.umaryland.edu) [49]. A penalty value lower than 10 was used, and the topology under the charmm36 position was converted into the available form of GROMACS. The topology of the hFMO3 protein under the charmm36 position was constructed using the GROMACS platform, and the complex topology was further prepared;

2. Define box and solvate: The simulation box was set to a cube 1 nm away from the perimeter of the molecular protein complex, and a water molecular solvent was added to the simulation box;

3. Add ions: Na⁺ and Cl⁻ were added to neutralize the charge in the simulation box, and Cl⁻ was used to replace other solvent molecules in the simulation box;

4. Energy minimization: To ensure stable operation of the power simulation system, it is necessary to minimize the energy. The energy was less than 1,000 kJ/mol/nm, and the maximum step size was set to 50,000 steps;

5. Equilibration: The temperature of the equilibrium system was 300 K at room temperature, and the pressure of the equilibrium system was 4.5 × 10⁻⁵ bar;

6. Production MD: The power simulation step size was set to 50,000,000 steps, and each step was 2 fs. The total simulation time was 100 ns, and other settings were set to defaults;

7. Analysis [50]: After protein centralization, the root mean square deviation (RMSD) of ligand and complex were calculated respectively, evaluating the deviation of ligand and complex conformation from the original conformation at different time points during the 100 ns simulation process. The fluctuation of amino acid residues was inferred by root mean square fluctuation (RMSF) parameters. Meanwhile, the average deviation of each amino residue in the complex with time from the reference position was explained.

2.4 Molecular mechanics

Poisson–Boltzmann surface area (MM-PBSA)

Poisson–Boltzmann Surface Area is open-source software, and g_mmpbsa was primarily used to calculate the free energy of binding between the receptor and the inhibitor after MD [51]. As a scoring function, MM-PBSA has been used in computational methods for drug design. In this study, MM-PBSA was used to determine the binding free energy of hFMO3 with molecules SMC-1, SMC-2, SMC-3, and SMC-4, respectively.

The following equation describes the binding free energy:

\[
G_{\text{binding}} = G_{\text{complex}} - (G_{\text{protein}} + G_{\text{ligand}}).
\]

The free energy of the protein–inhibitor complex is represented by the \(G_{\text{complex}}\), the free energy of the protein in the solvent is represented by \(G_{\text{protein}}\) and the free energy of the inhibitor in the solvent is represented by \(G_{\text{ligand}}\).

2.5 Exploration of possible mechanisms of inhibition of small-molecule compounds

The figures of the chemical structures of FAD and NADPH were downloaded from the PubChem database (https://pubchem.ncbi.nlm.nih.gov/) [52]. Using AutoDock software, hFMO3 was shown to molecular dock with FAD and NADPH. The specific docking method is given in detail in Section 2.2.2. Furthermore, the polarity interactions between coenzyme, prosthetic group, and hFMO3 were clarified using Pymol software and visualized. The relationship between the interaction sites between the coenzyme prosthetic group of hFMO3 and hFMO3 was compared with each small-molecule compound to analyze the possible mechanism by which small-molecule compounds can inhibit hFMO3 activity.

2.6 Cloud server support

Due to the serial nature of the AutoDock Vina algorithm, there have been no reports of GPU parallel acceleration success. Currently, the acceleration of AutoDock Vina usually relies on the overlay of computing power. Therefore, we split
the batch docking into ten datasets and performed parallel computing on ten high-performance computing servers under the same parameters for docking conditions to achieve acceleration. Batch molecular screening relied on the C6.2X LARGE16 Tencent Cloud Server (https://cloud.tencent.com/), and ten virtual machines accelerated molecular screening in parallel. The MD analysis was performed on the GN8.3XLARGE112 14-core p40 Tencent Cloud Server.

3 Results

3.1 hFMO3 as a potential therapeutic target

A total of four human target genes were screened for AS and TMAO crossover, namely NLRP3, NEA1, TXNIP, and FMO3 (Figure 2a), of which NLRP3 can be activated by TMAO to cause inflammation and cannot regulate TMAO levels [21,23,24]. NEA1 and TXNIP genes may influence the oxidative stress response, but their role in regulating TMAO levels is unclear. Human FMO3 encodes a key enzyme for the biological oxidation of TMA to TMAO [11], which directly affects plasma TMAO levels in patients with AS (Figure 2c); therefore, hFMO3 was defined as a potential therapeutic target gene for reducing TMAO levels. The key active regions of the hFMO3 protein can be used in structure-based screening studies to design a targeted drug to reduce plasma TMAO levels. The 3D molecular formula of hFMO3 is shown in Figure 2b.

### 3.2 Combined pockets and active areas

The first three combined pocket locations and related information are shown in Figure 2b and Table 1 by volume, surface area, and druggability scores. The main binding regions to FAD in the hFMO3 protein sequence were 9–13, 32, 40–41, and 61–62, and those to NADPH were 60–61 and 195–198. The first combined pocket contained almost all the active regions of hFMO3. In order to improve the docking efficiency and reduce the docking cost, according to the first combined pocket position, UCSF Chimera 1.16 was used to set up the molecular docking grid box with a size and position of (22 × 30 × 28), (X: −11.867, Y: 15.851, Z: 68.917).

### 3.3 Virtual screening and molecular docking

The 135,335 small-molecule compound mol2 structures downloaded from the ZINC 16 database [53] were split into 304,226 small-molecule mol2 files, which allowed allotting one or

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**Figure 2:** Target gene identification and targeted inhibitor analysis. (a) Identified novel target genes that inhibit AS by reducing TMAO levels; (b) hFMO3 structure and active region. Structure of hFMO3: the C-terminal is shown in red and the N-terminal is shown in blue; combined pockets of hFMO3: purple, Pocket1; cyan, Pocket2; yellow, Pocket3; (c) structure of four small-molecule compounds and their possible mechanisms of action to inhibit hFMO3. The four small molecules are represented as SMC-1, SMC-2, SMC-3, SMC-4; O2: oxygen; H+: hydrogen ions; TMA: trimethylamine; NADPH: reduced nicotinamide adenine dinucleotide phosphate, a coenzyme in the oxidation process; hFMO3: human flavin-containing monooxygenase 3, a key enzyme for the oxidation of TMA to TMAO; FAD: flavin adenine dinucleotide, a prosthetic group in the oxidation process; H2O: water molecules; TMAO: trimethylamine oxide; NADP+: nicotinamide adenine dinucleotide phosphate; ×: blocking; ↓: lower; –: competing binding sites; plotted by Biorender online platform (https://app.biorender.com/).
more different names for each small molecule; we excluded the duplicate small molecules from the top 1% of small molecules with the smallest binding energy and selected the four small-molecule compounds SMC-1, SMC-2, SMC-3, and SMC-4, with the smallest binding energy and CGenFF penalty values <10. Their 2D structure and basic information are shown in Figure 2c and Table 2.

We found that hydrogen bonds were formed between SMC-1 and TYR-331, ASN-154, and GLN-373 amino acid residues in the binding pocket of the hFMO3 protein. Cation–pi bonds were formed between SMC-2 and HIS-150 and TRP-41 amino acid residues in the hFMO3 protein-binding pocket, and hydrogen bonds formed between SMC-2 and ASN-61, SER-62, GLY-376, TRP-41, HIS-149, LEU-40, GLY-38, ARG-51, THR-108, VAL-110, CYS-146, SER-13, and GLY-370 amino acid residues in the hFMO3 protein-binding pocket. Cation–pi interactions formed between SMC-3 and TRP41 amino acid residues in the hFMO3 protein-binding pocket, and hydrogen bonds were formed between SMC-3 and ASP-198, TRY-55, PHE-59, TRP-41, GLY-38, LEU-40, ARG-51, GLU-32, VAL-110, PHE-31, SER-13, and ASN-61 amino acid residues in the hFMO3 protein-binding pocket. Cation–pi interactions were formed between SMC-4 and HIS-150 and TRP-41 amino acid residues in the hFMO3 protein-binding pocket, and hydrogen bonds formed between SMC-4 and VAL-110, PHE-31, ARG-51, LEU-40, GLY-38, CYS-146, SER-13, HIS-149, GLY-370, ASN-61, GLU-281, ASN-194, SER-195, and TYR-55 amino acid residues of hFMO3 (Figure 3a).

### Table 1: hFMO3 protein structure binding prediction

<table>
<thead>
<tr>
<th>Pocket no.</th>
<th>Volume (Å³)</th>
<th>Surface area (Å²)</th>
<th>Druggability score</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>3530.4</td>
<td>3638.92</td>
<td>0.81</td>
</tr>
<tr>
<td>2</td>
<td>317.61</td>
<td>408.02</td>
<td>0.59</td>
</tr>
<tr>
<td>3</td>
<td>104.94</td>
<td>163.77</td>
<td>0.29</td>
</tr>
</tbody>
</table>

3.4 MD simulation

The initial conformation of the hFMO3 inhibitor ligand complex by the docking procedure was subjected to MD simulation. The MD simulation of the four complex systems was performed for 100 ns by the GROMACS MD simulation analysis tool to explore the conformational dynamics of the four systems [46]. Using the initial ligand structure as a reference, the RMSD of different small-molecule compounds was calculated (Figure 3a), showing that the four small-molecule compounds reached equilibrium rapidly within 100 ns, the conformation changes of each molecule were less than 0.35 nm compared with the initial conformation, and SMC-2 and SMC-4 changes were relatively small. Using the initial hFMO3 inhibitor ligand complex structure as a reference, the RMSFs of different complexes were calculated (Figure 3b); the four complexes reached equilibrium rapidly within 100 ns, the changes in each conformation were less than 0.40 nm compared with the initial conformation, and the changes in the hFMO3 inhibitor SMC-2 were relatively greater. In addition, the RMSFs of each amino acid residue of the hFMO3 protein were determined to explore the stability of the four small-molecule compounds binding to hFMO3 (Figure 3c). Overall, each amino acid residue in the four hFMO3 inhibitor ligand complexes deviated approximately the same from the reference position on average over time, and the four complexes were less flexible and more stable.

### Table 2: Binding characteristics of the four most promising small-molecule compounds

<table>
<thead>
<tr>
<th>No.</th>
<th>Name</th>
<th>Molecular formula</th>
<th>Affinity</th>
<th>Penalty</th>
</tr>
</thead>
<tbody>
<tr>
<td>SMC-1</td>
<td>ZINC0000257455376</td>
<td>C_{60}H_{39}N_{10}O_{17}</td>
<td>−12.2</td>
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<tr>
<td>SMC-2</td>
<td>ZINC000085426282</td>
<td>C_{60}H_{39}N_{10}O_{17}P_{3}</td>
<td>−12.1</td>
<td>0</td>
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<tr>
<td>SMC-3</td>
<td>ZINC000096014977</td>
<td>C_{60}H_{39}N_{10}O_{17}P_{4}</td>
<td>−12</td>
<td>0</td>
</tr>
<tr>
<td>SMC-4</td>
<td>ZINC000096085195</td>
<td>C_{60}H_{39}N_{10}O_{17}P_{5}</td>
<td>−12</td>
<td>0</td>
</tr>
</tbody>
</table>

3.5 MM-PBSA analyses

For MM/PBSA is an efficient and reliable method for calculating the free energy of small inhibitors bound to their protein targets. In general, low binding energy values indicate that the binding between the ligand and the target is good, and the results of the g.mmpbsa are shown in Figure 4a and b. In Figure 4a(i), we can see that for the key residues GLU281, PHE160, and THR425, the energy contributions of GLU281, PHE160, and THR425 are −8.74, 17.93, and −11.36 kJ/mol, respectively. In Figure 4a(iv), the energy contributions of the key residues LYS33, LEU40, and GLU32 are −12.953, −11.807, and 18.132 kJ/mol. In Figure 4a(ii), we can see that for the key residues SER147, GLU32, and LEU40, these values are −8.74, 17.93, and −11.36 kJ/mol, respectively. The energy contributions of LEU40 and GLU32 are more remarkable in SMC-2, SMC-3, and SMC-4. In addition, in Figure 4b(i), the binding is −100.972 kJ/mol, the VDW is −183.698 kJ/mol, the PB is 148.178 kJ/mol, and the SA is −29.512 kJ/mol. In Figure 4b(ii), the binding is −163.898 kJ/mol, the VDW is −343.52 kJ/mol, the PB is 443.983 kJ/mol, and the SA is −42.106 kJ/mol. In Figure 4b(iii), the binding is −158.31 kJ/mol, the VDW is −394.526 kJ/mol,
the PB is -548.011 kJ/mol, and the SA is -46.366 kJ/mol. In Figure 4b (IV), the binding is -117.814 kJ/mol, the VDW is -382.344 kJ/mol, the PB is -537.973 kJ/mol, and the SA is -48.824 kJ/mol. Overall, SMC-2, SMC-3, and SMC-4 bind very well to hFMO3.

3.6 Possible mechanisms of inhibition of small-molecule compounds

FAD and NADPH were separately molecularly docked with hFMO3 to clarify the interaction between the four small-molecule compounds and hFMO3 coenzyme prosthetics. The results indicated that FAD in the first three conformations was linked to hFMO3 through polar hydrogen bonds, among which FAD-1 was linked to ASN-61, CYS-146, HIS-149, HIS-150, GLU-281, TYR-331, GLN-373, and ALA-378 of hFMO3. FAD-2 was connected to hFMO3’s ASN-194, SER-216, SER-218, ASN-275, GLU-281, ALA-328, TYR-331, and GLN-427. FAD-3 was connected to hFMO3’s SER-13, ASN-61, HIS-150, ASN-194, SER-195, GLU-281, GLN-373, and GLN-427. The NADPH of the first three conformations was connected to hFMO3 by polar hydrogen bonding, and NADPH-1 was connected to SER-53, ASN-61, HIS-150, SER-173, LEU-192, and GLN-373.
ASN-194, SER-195, SER-218, GLY-330, TYR-331, and GLN-373 of hFMO3; NADPH-2 was connected to hFMO3’s SER-53, TYR-55, ASN-61, SER-173, ASN-194, SER-195, GLU-281, and GLN-373; and NADPH-3 was linked to hFMO3’s SER-13, ASN-61, SER-62, HIS-150, GLU-281, GLN-373, and ALA-378 (Figure 5b).

Visual analysis with Pymol software showed that SMC-1 bound to hFMO3 as well as FAD, while SMC-2, SMC-3, and SMC-4 bound to hFMO3, FAD, and NADPH, respectively (Figure 5c). Specifically, SMC-1 and FAD-1 competed to be linked to TRY-331 and GLN-373 of hFMO3; SMC-1 and FAD-2 competed to be linked to hFMO3’s TRY-331; SMC-2 and FAD-3 were not competitive; SMC-2, SMC-3, and SMC-4 were similar, and these are similar to the chemical structure of the hFMO3 coenzyme NADPH (C21H26N7O17P3) during TMAO generation (Figure 2c, Table 2), which may be the principle of their similar inhibition mechanisms.

![Figure 4: MM-PBSA analyses results. (a) Residue-wise decomposition of binding free energies obtained from the MM-PBSA analyses. I: SMC-1; II: SMC-2; III: SMC-3; and IV: SMC-4. (b) The binding energy of the binding of the protein complexed with different ligands. I: SMC-1; II: SMC-2; III: SMC-3; and IV: SMC-4.](image-url)

4 Discussion

The effects of the intestinal microbial metabolite TMAO on the mechanisms of AS provide a new direction for targeted...
AS therapy. Based on the metabolic mechanisms of TMAO, the present study explored new therapeutic pathways for treating AS with its key enzyme hFMO3 as the target and used machine learning-guided virtual screening for new inhibitors that could reduce serum TMAO levels and hinder the formation of AS. We obtained stable MD simulation results of four novel inhibitors, and preliminary exploration of the inhibition mechanisms has been carried out. Currently, we are performing follow-up experiments to verify the effectiveness of the small-molecule compounds and explore the biochemical mechanisms of inhibition of AS formation.

The virtual screening yielded four small-molecule compounds SMC-1, SMC-2, SMC-3, and SMC-4 that were closely linked to the hFMO3 protein with different types and numbers of chemical bonds. Among them, SMC-1 is only connected to hFMO3 through three hydrogen bonds to form a complex, while the other three small-molecule compounds are connected to the hFMO3 protein by two interaction forces, hydrogen bonds, and cation–pi bonds. However, none of these four small-molecule compounds showed interaction forces such as pi–pi bonds or salt bridges, and the specific bond forms and quantities still need to be determined. MD was applied to simulate the microscopic evolution of the ligand complexes of the four hFMO3 inhibitors at the atomic level under the premise of closest experimental conditions [54]. Combined with RMSD and RMSF, the conformational changes of small-molecule compounds, hFMO3 inhibitor ligand complexes, and protein amino acids were evaluated, concluding that the MD simulation showed good results, and

**Figure 5:** Binding of the four small-molecule compounds to hFMO3. (a) Different small-molecule compounds bind to hFMO3; dotted line, interaction bonds; purple, hydrogen bonds; and green, cation–pi bonds. (b) Interaction of FAD and NADPH with hFMO3 in different conformations. I: FAD-1 and NADPH-1, II: FAD-2 and NADPH-2, III: FAD-3 and NADPH-3; the yellow dotted line indicates polar hydrogen bonds. (c) Four small-molecule compounds bind to FAD and NADPH. Gold, four different small-molecule compounds; green, the optimal conformational 3D structure of FAD molecules after docking; violet, the optimal conformational 3D structure of NADPH molecules after docking, and the hFMO3 protein is the gray background.
experiments could be carried out to further verify their effectiveness and safety. However, by combining the four small-molecule compounds with different hFMO3 binding sites and using different types of interactions/bonds, the three NADPH analogues SMC-2, SMC-3, and SMC-4 may be more robust and inhibit better than SMC-1.

In the process of TMAO formation, the key enzyme hFMO3 does not directly bind to the substrates O2, H+, and TMA, but does so only by combining with coenzymes and prosthetic groups (NADPH, FAD) to catalyze the reaction generating TMAO. This is carried out in the liver, and the generated TMAO is transported to target organs via the bloodstream to play a role in the health of the body [55]. The present study found that SMC-1 affected the activity of FAD binding to hFMO3 by competitively inhibiting FAD, while the NADPH analogues SMC-2, SMC-3, and SMC-4 inhibited activity by simultaneously competitively inhibiting FAD and NADPH binding to hFMO3, reducing serum TMAO levels, and thereby inhibiting AS formation (Figure 2c). However, the same small-molecule compounds compete with different conformations of FAD and NADPH to bind to hFMO3 active sites, so the specific mechanisms of inhibition of the four small-molecule compounds need to be further verified.

However, hFMO3 produced by the liver is not only a key enzyme in the conversion of TMA to TMAO, but it is also an essential liver enzyme that catalyzes the oxygenation of various nitrogen- and sulfur-containing compounds, including pharmaceutical and dietary compounds [56]. In the present study, the reduction of serum TMAO levels was shown to be mainly due to the competitive binding of FAD and/or NADPH to hFMO3 to inhibit its activity, but whether this affects the other oxidation reactions with hFMO3 as a key enzyme in liver function needs further study. In addition, inhibiting the conversion from TMA to TMAO will increase the relative levels of TMA in the body, and this may have other adverse effects on the body, such as causing fishy odor in urine and sweat [57]; this requires further verification.

Compared with traditional experimental drug screening, virtual screening–molecular docking may increase the risk of false positives, but it can reduce the number of actual screening compounds and improve the efficiency of lead discovery, which can discover potential targeted drugs more efficiently, economically, and predictably [58]. The advantages of pre-experimental simulation have been continuously highlighted, and simulation is the link between theory and experiments and plays a key role in explaining experimental phenomena and predicting theoretical results [59,60]. Indeed, simulation results still require verification via systematic experiments to select the safest and most effective targeted drugs.

5 Conclusions

By virtual screening and MD simulation, we found four small-molecule compounds (SMC-1, SMC-2, SMC-3, and SMC-4) that had the potential to inhibit the activity of the TMAO key enzyme hFMO3. Notably, the main suppression mechanism of the four small-molecule compounds is likely competition for the binding of NADPH and/or FAD to hFMO3. At present, our research group is verifying the effectiveness and feasibility of these results through experiments and obtaining good preliminary results. We believe that these small-molecule compounds have great promise as new effective targeted drugs to inhibit the formation and progression of AS by reducing plasma TMAO levels.

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References


[58] Dauber-Osguthorpe P, Hagler AT. Biomolecular force fields: Where have we been, where are we now, where do we need to go and how do we get there? J Comput Aided Mol Des. 2019;33:133–203.
