Amino Acid Conjugates of 2-Mercaptobenzimidazole Ameliorates High-Fat Diet-Induced Hyperlipidemia in Rats via Attenuation of HMGCR, APOB, and PCSK9

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ABSTRACT: Purpose: This study was designed to explore the antihyperlipidemic effects of amino acid derivatives of 2-mercaptobenzimidazole (4J and 4K) in high-fat diet (HFD)-fed rats. Methods: Male Sprague-Dawley rats were divided into nine groups which received either standard diet or HFD for 28 days. Blood samples were taken on 27th day from HFD-fed rats to ensure hyperlipidemia. HFD-induced hyperlipidemic rats later received daily dosing of either vehicle or simvastatin (SIM; 20 mg/kg) or 4J/4K compounds (10, 20, and 30 mg/kg) for 12 consecutive days. On 40th day, animals were sacrificed, and blood samples were collected for the determination of serum lipid profile and liver function parameters. Liver samples were harvested for histopathological, antioxidant, and qPCR analyses. Molecular docking of tested compounds with HMGCR was also performed to assess the binding affinities. Results: 4J and 4K dose dependently decreased serum total cholesterol, triglycerides, low-density lipoprotein, very low-density lipoproteins, alanine transaminase (ALT), and aspartate aminotransferase (AST) levels while significantly alleviated high-density lipoproteins. However, SIM failed to reduce AST and ALT levels. Moreover, tested compounds displayed antioxidant effects by inducing superoxide dismutase and glutathione levels. Histopathology data also displayed protective effects of 4J and 4K against HFD-induced fatty changes and hepatic damage. In addition, 4J and 4K downregulated transcript levels of HMGCR, APOB, PCSK9, and VCAM1, and molecular docking analysis also supported the experimental data. Conclusion: It is conceivable from this study that 4J and 4K exert their antihyperlipidemic effects by modulating multiple targets regulating lipid levels.

INTRODUCTION

Cardiovascular diseases (CVDs) account for one-third of deaths worldwide, and it is projected that CVD would be the leading cause of morbidity and mortality in the developing world.1 The risk factors such as hyperlipidemia, diabetes, and hypertension are prevalent in individuals who are affected by CVDs.2,3 People with hyperlipidemia are considered at high stake for contracting CVDs. Hyperlipidemia is a state of increased levels of cholesterol, triglycerides (TG), or both in the blood which is usually caused by a variety of genetic or acquired disorders.4,5 These lipids accumulate in the walls of the arteries and increase the chances of atherosclerosis that can lead to life-threatening CVDs. Moreover, plasma lipoproteins also play an important role in the transport of cholesterol and TG to the site of absorption, catabolism, or elimination. The five major classes of lipoproteins are the chylomicrons, very low-density lipoproteins (VLDLs), low-density lipoproteins (LDLs), intermediate-density lipoproteins, and high-density lipoproteins (HDLs).1,4,6 The specific biomarkers for hyperlipidemia include high levels of total cholesterol (TC), TG, and LDL and low levels of HDL.6,7 Moreover, certain apolipoproteins (APOs) which are the constituents of the various lipoprotein classes also regulate the metabolism of plasma lipoproteins. They play three major functions, that is, (I) stabilization of micellar structures of lipoprotein particles, (II) acting as cofactors or activators of various enzymes or lipid-transfer proteins that participate in “remodeling” of lipoproteins, and (III) serving as a ligand for cell surface lipoprotein receptors. Therefore, LDL, HDL, and APO have been the targets of therapy for improving the outcomes in hyperlipidemic patients.8–10

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Although a large number of drugs therapies are available for the treatment of hyperlipidemia like niacin, fibrates, statins, and bile acid binding resins but they are associated with lots of side effects. Statin therapy has long been a mainstay in the treatment of hypercholesterolemia, but it also has adverse effects that usually lead to patient noncompliance. Hence, various medicines are being developed for the management of hyperlipidemia. Recently, Food and Drug Administration approved two medications "Evolocumab and Alirocumab" which target a novel pathway to reduce LDL. These are monoclonal antibodies that inactivate proprotein convertase subtilisin-kexin type 9 (PCSK9). PCSK9 regulates LDL receptor (LDLR) degradation and is therefore regarded as a potential target for modulating LDLR expression and consequently LDL levels. However, the safety profile of these monoclonal antibodies is similar to statins. Therefore, there is a need to develop safer and efficacious antihyperlipidemic agents.

In recent years, benzimidazole derivatives have gained importance due to their diverse pharmacological activities which include antimicrobial, antiviral, anticancer, anti-inflammatory, antioxidant, antihypertensive, anticoagulant, immunomodulator, antihyperlipidemic and antidiabetic effects. Benzimidazole nucleus is now considered an indispensable pharmacophore for the development of new therapeutic agents. Keeping in view the wide range of biological effects, we also evaluated the antihyperlipidemic activity of amino acid derivatives of 2-mercaptopbenzimidazole (4J and 4K) in rats.

## MATERIALS AND METHODS

**Chemicals.** Cholesterol and cholic acid were purchased from Sigma-Aldrich (St. Louis, Missouri, USA), and simvastatin was gifted by Medpak Pharmaceuticals (Lahore, Pakistan). Standard kits were purchased from BioLabs (Boston, USA) and Zokeyo (Wuhan, China). Coconut oil, banaspati ghee, and all other chemicals used in this study were of analytical grade.

**Animals.** Male Sprague-Dawley rats weighing around 180–200 g were purchased from University of Veterinary and Animal Sciences, Lahore, Pakistan. The animals were kept under standard conditions in the animal house of Faculty of Pharmacy, The University of Lahore and were acclimatized to the laboratory conditions prior to the start of experiments. All the experiments were performed according to the guidelines of organization for economic co-operation and development (OECD), and the experimental protocols were approved by Institutional Research Ethics Committee of Faculty of Pharmacy, The University of Lahore (approval no.: IREC-2020-42).  

**High-Fat Diet-Induced Hyperlipidemia.** Hyperlipidemia was induced by feeding high-fat diet (HFD) to animals for 28 days. HFD was prepared by homogeneous mixing of cholesterol (2% w/w), cholic acid (1% w/w), banaspati ghee, coconut oil (3:2 w/w), and egg yolk powder (5% w/w) with standard rat chow. After 27 days of HFD, blood samples were taken for the analysis of serum TC, TG, HDL, LDL, and VLDL. Animals having TC levels greater than 280 g/dL were selected for further study. The hyperlipidemic rats were later treated once a day with either simvastatin (SIM; 20 mg/kg) or with various doses (10, 20, and 30 mg/kg) of MBIZ, that is, 4J and 4K for 12 consecutive days. On 40th day, animals were sacrificed, and blood samples were collected for subsequent analyses of abovementioned lipid levels and liver function tests (LFTs). Liver samples were harvested in Trizol and 10% buffered formalin for mRNA and histopathological analyses, respectively.

**Experimental Design.** The experimental animals were divided into nine groups, each containing three rats (n = 3). The division of groups was in the following manner:

1. Control—normal diet
2. Disease group—HFD
3. SIM (20 mg/kg)—HFD followed by SIM
4. 4J (10 mg/kg)—HFD followed by 4J (10 mg/kg)
5. 4J (20 mg/kg)—HFD followed by 4J (20 mg/kg)
6. 4J (30 mg/kg)—HFD followed by 4J (30 mg/kg)
7. 4K (10 mg/kg)—HFD followed by 4K (10 mg/kg)
8. 4K (20 mg/kg)—HFD followed by 4K (20 mg/kg)
9. 4K (30 mg/kg)—HFD followed by 4K (30 mg/kg)

**Histopathological Analysis.** Formalin fixed liver samples were processed by successive dehydration with ethanol baths and later embedded in paraffin. Paraffin blocks were sectioned at 5 μm using a rotary microtome and stained with hematoxylin (H) and eosin (E) using standard procedures.

**Antioxidant Assay.** Liver samples from disease and treated groups were harvested in 1× PBS. After washing, samples were homogenized and stored overnight at −20°C. The samples were freeze–thaw two times and centrifuged to obtain the supernatants. Supernatants were later processed according to manufacturer's instructions to measure superoxide dismutase (SOD) and glutathione (GSH) levels using standard ELISA kits.

**Real-Time qPCR Analysis.** Total RNA was extracted from liver samples using the Trizol reagent and was reverse transcribed using the WizScript cDNA synthesis kit (Wizbio solutions, New Mexico, USA). The relative transcript levels of various genes were measured by the ddCT method using SYBR Green qPCR mix (Zokeyo, Wuhan, China). mRNA levels were measured using the following conditions: initial denaturation at 94°C for 2 min followed by 40 cycles of denaturation at 94°C for 1 min and annealing at 60°C for 2 min. Hypoxanthine–guanine phosphoribosyltransferase mRNA was used as an internal control. Sequences of primers are given in Table S1.

**Molecular Docking Analysis.** The three-dimensional (3D) structure of HMGCR was accessed from the Protein Data Bank (PDB) (www.rcsb.org) with PDB ID of 1T02. The target protein was prepared for docking analysis using Autodock Tools program. The protein was energy minimized, and Gasteiger charges were added and saved in the pdbqt format. The hydrophobicity and Ramachandran graphs were generated by Discovery Studio 4.1 Client (2012). The protein architecture and statistical percentage values of helices, β-sheets, coils, and turns were accessed by VADAR I.8.

The compounds (SIM, 4J, and 4K) were drawn in Discovery Studio Client and saved in the pdb format as ligands after energy minimization. Autodock tools were used for the preparation of ligands in their most stable conformation. The ligands were saved in the pdbqt format after addition of the Kolman and Gasteiger charges. The molecular docking experiment was used for all the synthesized ligands against HMGCR by the PyRx virtual screening tool with the Auto Dock VINA Wizard approach. The grid box center values were as follows (center X = 15.861, center Y = −8.806, and center Z = −22.278), and size values were adjusted (X = 88, Y = 60, and Z = 96) for better conformational position in the
active region of the target protein. Ligands were docked individually against target protein with default exhaustiveness value of 50. The predicted docked complexes were evaluated based on the lowest binding energy values (kcal/mol). The 2D and 3D graphical depictions of all the docked complexes were
Figure 3. Histopathological analysis revealed a protective effect of MBIZ against HFD-induced fatty changes. Hyperlipidemia was induced by feeding HFD for 28 days, and these rats were later treated with either vehicle or SIM/4J/4K for 12 days. On 40th day, animals were sacrificed, and liver samples were harvested for the histopathology examination. (A) Control specimens showed normal hepatic architecture, while the disease group (B,C) exhibited severe fatty changes. Treatments with SIM (D), 4J (E), and 4K (F) reduced HFD-induced pathological changes.

Figure 4. Antioxidant effects of tested compounds. Hyperlipidemia was induced by feeding HFD for 28 days, and rats were later treated with either vehicle or SIM/4J/4K for 12 days. On 40th day, animals were sacrificed, and liver samples were harvested in 1X PBS. After thorough washing, samples were homogenized and stored overnight at −20 °C. The samples were freeze-thaw two times and later centrifuged to obtain the supernatants, which were used to measure soluble proteins by standard kits. 4J and 4K effectively reduced the levels of (A,B) SOD and (C,D) GSH. One-way ANOVA followed by Tukey’s multiple comparison test, $n = 3$, ***) = <0.001.
accomplished by Discovery Studio (Discovery Studio Visualizer Software, Version 4.0., 2012).

Statistical Analysis. Results were expressed as the mean ± standard deviation (SD), and data were analyzed by one-way analysis of variance (ANOVA) followed by Tukey’s multiple comparison test using GraphPad prism 5.1 (Graphpad Software, Inc., San Diego, USA). Probability values of less than 0.05 were considered as statistically significant using the following abbreviations. *** = <0.001, ** = <0.01, and * = <0.05.

RESULTS

4J and 4K Reduced HFD-Induced Hyperlipidemia in Rats. Our findings demonstrated that HFD significantly induced TC, TG, LDL, and VLDL levels and reduced HDL levels in the disease group. 4J and 4K treatments showed beneficial effects on the lipid profile as they remarkably restored all the lipid parameters. In addition, they displayed a dose-dependent improvement in the lipid profile, and at higher dose (30 mg/kg), their effects were comparable to the SIM-treated group (Figure 1).

4J and 4K Attenuated HFD-Induced AST and ALT Levels. HFD is known to alter liver functions and can cause fatty liver disease;27 we therefore measured aspartate aminotransferase (AST) and alanine transaminase (ALT) levels. As expected, the disease group and SIM-treated group showed prominent increase in AST and ALT levels indicating liver dysfunction. Treatment with 4J and 4K exhibited a dose-dependent reduction in HFD-induced AST and ALT levels, suggesting their hepatoprotective effects as well (Figure 2).

Histopathological Examination Revealed Antihyperlipidemic Effects of 4J and 4K. Histopathology of liver samples showed that control group had normal hepatocytes, and there were no signs of vacuolization. Hepatic cords also displayed perfect architecture with normal portal triads and central veins. Disease group specimens exhibited severe fatty changes which included cytoplasmic vacuolization, derangement of hepatic cords, inflammation, necrosis, and extra-medullary hemopoieses. Treatment with SIM reduced most of the pathological changes and showed moderate degree of fatty changes. The cells around the central vein showed normal cytoplasm. 4J treated group displayed mild degree of cytoplasmic vacuolization in hepatocytes. However, the hepatic architecture (hepatic cords) was intact, and no inflammatory or necrotic changes were noticed. Similarly, the liver sections of 4K-treated groups showed mild fatty change. Most of the hepatocytes displayed normal parenchyma with minimal to no vacuolization. Central vein and portal area also showed normal histological appearance. The findings clearly indicate the protective effects of 4J and 4K against HFD-induced hyperlipidemia (Figure 3).
Ameliorated HFD-Induced Oxidative Stress. Prolong use of HFD induces oxidative stress, so we therefore measured antioxidant levels in the homogenates of liver samples. As anticipated, HFD drastically reduced the levels of SOD and GSH. Treatments with SIM or tested compounds significantly restored the levels of antioxidants, indicating their antioxidant potentials as well (Figure 4).

Reduced HFD-Induced Hyperlipidemia by Downregulating HMGCR, APOB, PCSK9, and VCAM1 Transcript Levels. In order to identify the possible molecular mechanism behind antihyperlipidemic effects of MBIZ, we measured the transcript levels of lipid-regulating genes (HMGCR, APOB, APOE, PCSK9, LDLR, and VCAM1). RT-qPCR data showed that HFD significantly induced HMGCR, APOB, and VCAM1 and reduced APOE levels which are normally associated with hyperlipidemia. In addition, HFD did not alter the normal levels of PCSK9 and LDLR. Treatment with MBIZ (4J and 4K) significantly reduced the levels of HMGCR, APOB, and PCSK9. SIM on the other hand failed to reduce PCSK9 levels, which is a well-known effect of HMGCR inhibitors. These findings indicate that the antihyperlipidemic effects of 4J and 4K are due to inhibition of multiple lipid-regulating targets (Figure 5).

Molecular Docking of 4J and 4K with HMGCR Reinforced the Experimental Findings. Structural analysis of target protein showed that HMGCR (PDB ID: 1T02) consisted of 45% helices (338 residues), 24% β-sheets (180 residues), 30% coils (227 residues), 8% turns (60 residues), and a total of 805 amino acid residues. The R-value of selected protein appeared to be 0.251, and the resolution was 2.50 Å. Unit cell dimensions for the lengths were observed to be as follows: a = 228.5, b = 228.5, and c = 228.5 with 90° angle for α, β, and γ. The Ramachandran plot confirmed that 97% amino acids were in the allowed regions for the phi (φ) and psi (ψ) angles (Figure 6).

The affinity among the target protein and the ligands was investigated using molecular docking. AutoDock Vina program was used for the docking analysis through the PyRx user interface. The E-value (kcal/mol) was used to assess the affinity of protein and best docked pose complex. It provided prediction of binding free energy and binding constant for docked ligands. The results obtained from the docking studies of compounds against HMGCR were in conjunction with their pharmacological activities. Binding affinities of 4J and 4K with HMGCR were −5.0 and −6.9 kcal/mol, respectively, which were comparable to SIM (−7.4 kcal/mol) (Table 1; Figures 7 and 8).

Table 1. Binding Affinities of Ligands for HMGCR

| compounds | binding affinities (kcal/mol) |
|-----------|-----------------------------|
| 4J        | −5.0                        |
| 4K        | −6.9                        |
| SIM       | −7.4                        |
DISCUSSION

Hyperlipidemia is a metabolic disease with elevated levels of lipid and/or lipoproteins in the blood. The long-term consumption of HFD and unhealthy lifestyle have contributed to the prevalence of hyperlipidemia. Patient non-compliance for medications is another major contributing factor for its prevalence. Although the current therapies effectively reduce plasma lipid levels, most of these treatments cause unwanted adverse effects. Therefore, scientists are looking for better and safer alternatives for the treatment of hyperlipidemias. In this study, we evaluated the antihyperlipidemic potential of MBIZ (4J and 4K) against HFD-induced hyperlipidemia.

Previous studies have shown that these MBIZ exhibited strong analgesic and anti-inflammatory activities with better safety profile. In our study, we also did not witness any signs of apparent toxicity or lack of physical activity in animals. Both 4J and 4K prominently reduced TC, TG, LDL, and VLDL levels, and they were equally effective in enhancing HDL levels.
Moreover, LFT analysis also revealed a hepatoprotective effect of these compounds, and similar findings were observed in histopathological examinations of liver samples. 4J and 4K effectively prevented HFD-induced vacuolization, inflammation, and necrosis of hepatocytes, and all the specimens of MBIZ-treated groups displayed normal hepatic architecture. The tested compounds also effectively raised the levels of SOD and GSH, demonstrating significant antioxidant effects.

In addition, 4J and 4K demonstrated prominent inhibitory activities. HMGCR plays a pivotal role in the cholesterol biosynthetic pathway, and in fact, it is the rate-limiting step of cholesterol biosynthesis. The conversion of HMG-CoA to mevalonate is the final step in the synthesis of cholesterol, which is inhibited by HMGCR. The inhibition of HMGCR can reduce LDL-cholesterol by 20−35%, a decrease which has been linked to a reduction in the incidences of CVDs. The transcription of HMGCR gene and the rate of synthesis of this enzyme are controlled by the sterol regulatory element-binding proteins (SREBPs). The SREBPs also promote LDLR expression and thereby regulate the cellular uptake, transport, and utilization of cholesterol. PCSK9 is another modulator of the cellular LDLR and plasma cholesterol levels. Studies have shown that gain-of-function mutations of PCSK9 cause hyperlipidemia and early onset coronary heart disease, whereas loss-of-function mutations result in low plasma cholesterol levels and protection against coronary heart disease without apparent negative consequences. PCSK9 interacts with LDLR epidermal growth factor-like repeat A domain and induces its lysosomal degradation, thereby regulates plasma cholesterol levels. Therefore, inhibition of PCSK9 is being pursued as an approach to reduce plasma LDL cholesterol and TG levels. 4J and 4K have clearly demonstrated prominent inhibition of aforementioned hyperlipidemic factors indicating their strong lipid lowering effects. Moreover, these compounds also reduced liver toxicity which is normally observed with the chronic use of statins. Since they have suppressed VCAM1 levels as well, it is plausible that they might also be effective in reducing or preventing atherosclerosis.

**CONCLUSIONS**

Based on recent findings, it can be concluded that the antihyperlipidemic effects of 4J and 4K could be attributed to the inhibition of HMGCR, APOB, PCSK9, and VCAM1.

Atherogenic lipoproteins of hepatic origin carry one molecule of APOB100 per lipoprotein particle, whereas APOB48, a truncated form of APO B100, is found in chylomicrons synthesized in the intestine. Mouse and human genetic models have shown that inhibition of hepatic APOB production may be a therapeutic approach for the treatment of dyslipidemia. The effects of antisense inhibition of APOB synthesis on lipid metabolism have been extensively studied across a number of species. The species-specific antisense APOB inhibitors have been reported to reduce LDL, VLDL, and TC. VCAM1 is another well-recognized marker of atherosclerotic plaque vulnerability. Its overexpression has been observed over the complete course of plaque development. VCAM1 expression promotes the adhesion of leukocytes to the endothelial cells, accelerates the migration of adherent leukocytes along the endothelial surface, and facilitates the proliferation of smooth muscle cells. Therefore, VCAM1 is considered a key player in the pathogenesis of atherosclerosis. Studies on animal models have shown that increased LDL levels promote the expression of VCAM1 in endothelial cells, and hypercholesterolemia can cause atherosclerosis-related pathophysiological changes in the arteries. 4J and 4K have clearly demonstrated prominent inhibition of aforementioned hyperlipidemic factors indicating their strong lipid lowering effects. Moreover, these compounds also reduced liver toxicity which is normally observed with the chronic use of statins. Since they have suppressed VCAM1 levels as well, it is plausible that they might also be effective in reducing or preventing atherosclerosis.

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Figure 8. Molecular docking analysis of SIM with HMGCR. (A,B) 2D and 3D structures of SIM–HMGCR interactions.
However, further studies are still required to validate these findings in vitro and in vivo hyperlipidemic models.

**ASSOCIATED CONTENT**

* Supporting Information
The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.2c05735.

- List of primers used for qPCR (PDF)

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**Author Contributions**
R.S. and M.Z. performed the experiments. I.A. analyzed histopathological data, whereas S.J. and A.Y. analyzed qPCR data. All authors participated in editing of the manuscript. RS wrote the first version of the manuscript. M.N.H.M. designed and supervised the project and edited the final version of the manuscript.

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**Notes**
The authors declare no competing financial interest.

Ethical approval: Experiments were performed according to guidelines of OECD and were approved by Institutional Research Ethics Committee of Faculty of Pharmacy, The University of Lahore (Approval number: IREC-2020-42).

Any other relevant material or data can be provided on request.

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