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Imine Derivatives of Benzoxazole Attenuate High-Fat Diet-Induced Hyperlipidemia by Modulation of Lipid-Regulating Genes

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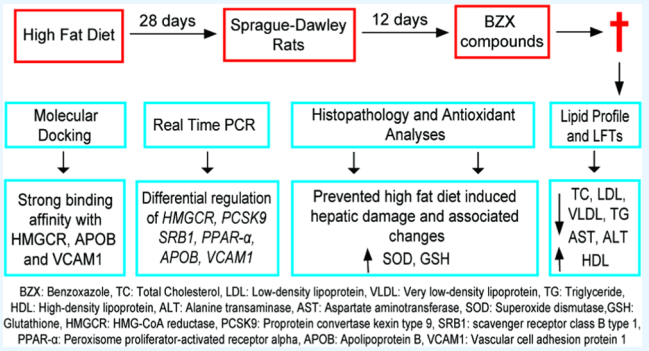
ABSTRACT: *Purpose*: Hyperlipidemia being the prominent risk factor of cardiovascular diseases and side effects associated with the current lipid-lowering drugs have attracted the interest of scientists in the quest for new alternatives. In view of the diverse pharmacological potentials of benzoxazole (BZX) compounds, this study was designed to evaluate the antihyperlipidemic activity of imine derivatives of BZX in high-fat diet (HFD)-fed rats. *Methods*: Hyperlipidemia was induced in Sprague−Dawley rats by using HFD for 28 days. On the 28th day, blood samples were collected, and animals having serum triglycerides (TG) greater than 400 mg/dL and total cholesterol (TC) greater than 280 mg/dL were selected for further study. Hyperlipidemic rats were daily treated with either a vehicle or simvastatin (SIM; 20 mg/kg) or BZX compounds (10, 20, and 30 mg/kg), for 12 consecutive days. After the specified time duration, hyperlipidemic biomarkers were evaluated in the blood samples of sacrificed rats. Liver samples were collected for histopathological and mRNA analyses. Binding affinities of BZX derivatives with different targets were assessed by molecular docking. *Results*: The present study revealed that the BZX derivatives dose-dependently reduced the serum levels of TC, TG, low-density lipoprotein, and very low-density lipoprotein along with improvement in high-density lipoprotein levels. Similarly, all the compounds reduced HFD-induced alanine transaminase and aspartate aminotransferase levels except BZX-4. Histopathology of liver samples demonstrated mild to moderate fatty changes upon treatment with BZX-1, BZX-2, and BZX-4. The hepatic architecture of the BZX-3-treated samples was close to normal, and only mild inflammation was witnessed in these samples. Moreover, all the compounds significantly increased superoxide dismutase and glutathione levels, indicating their antioxidant potentials. Gene expression data showed that BZX-1 and BZX-3 reduced lipid levels by inhibiting *HMGCR*, *APOB*, *PCSK9*, *SRB1*, and *VCAM1* and via improving *PPAR-α* and *APOE* mRNA levels. BZX-2 demonstrated its antihyperlipidemic effects mainly due to inhibition of *APOB*, while BZX-4-mediated effects appeared to be due to attenuation of *APOB*, *PCSK9*, and *SRB1*. BZX derivatives displayed strong binding affinities with HMGCR, APOB, and VCAM1, which suggested that some of the interactions might be required for inhibition of these target proteins. *Conclusions*: Based on the current findings, it can be concluded that BZX derivatives exert their antihyperlipidemic effects via modulation of multiple lipid-regulating genes.



INTRODUCTION

Cardiovascular diseases (CVDs) are the leading cause of morbidity throughout the Asia-Pacific region and account for nearly 400 000 deaths/year worldwide.[1,2] The frequency of cardiovascular risk factors such as dyslipidemia is on the rise in many Asian countries.[2] According to "The American Heart Association", the estimated cost for annual cardiovascular treatments is approximately at $863 billion globally and is expected to be around $10 44 billion till 2030. Among several cardiovascular (CV) risk factors, hyperlipidemia is one of the top ten expensive medical conditions in 2008 that is directly correlated with the risk of developing coronary heart disease (CHD) and future CV events.[3] Therefore, hyperlipidemia is regarded as a topic of concern and major contributing factor to clinical and economic burdens of CVDs.[3]

Hyperlipidemia is characterized by the abnormal levels of one or more plasma lipids, such as triglycerides (TG), total cholesterol (TC), cholesterol esters, phospholipids, and plasma lipoproteins.[4] Elevated levels of low-density lipoprotein (LDL) present an increased risk of development of atherosclerotic

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plaques. On the contrary, high-density lipoprotein (HDL) aids in cholesterol regulation to prevent imbalances that could be the leading cause of atherosclerotic vascular diseases.[5] Lipids are transported together with protein complexes called lipoproteins, whose transport and metabolism are regulated by specific apolipoproteins (APO). Among different APOs, APOE, APOB, APOA-I, APOA-II, APOA-IV, APOC-I, APOC-II, and APOC-III are considered important in redistribution of lipids among various cells and tissues.[1,6] APOB is the primary protein required for the formation of LDL particles, and elevated levels of APOB containing LDL have been linked with the development and progression of atherosclerosis.[7,8] APOE plays a role in homeostasis of plasma lipids by removing excess lipoproteins from the bloodstream. It modulates plasma lipoprotein levels through its interaction with LDL-R, facilitating the internalization of chylomicrons and LDL from cells to tissues.[9,10] Therefore, overproduction of liver-derived apoB and downregulation of apoE can be the leading causes for the predominance of LDL particles.[8]

Besides life style modification such as healthy diet, weight reduction, and physical exercise, several treatments have been used to manage and treat hyperlipidemia. Although treatment options such as statins, ezetimibe, bile acid sequestrants, and fibrates considerably lower lipid levels, their associated side effects such as myopathies and rhabdomyolysis have limited their use.[11,12] New strategies for the management of hyperlipidemia are evolving with the better understanding of the underlying pathophysiology.[5] Recent clinical trials have focused on several targets including proprotein convertase subtilisin kexin type 9 (PCSK9), 3-hydroxy-3-methylglutaryl-coA reductase (HMGCR), vascular cell adhesion molecule (VCAM-1), scavenger receptor-class B type 1 (SRB1), peroxisome proliferator-activated receptor α (PPAR-α), APOB, and APOE. PCSK9 plays an essential role in the metabolism of LDL particles by inhibiting LDL receptor recirculation to the cell surface. Administration of newly approved monoclonal antibodies such as "canakinumab, evolocumab, and alirocumab" shows significant capability in reducing LDL levels by inhibiting PCSK9.[12] SRB1 is a multiligand membrane receptor protein that primarily promotes the uptake of HDL-derived cholesteryl esters into cells and tissues.[13] VCAM-1, a member of the immunoglobulin family, is highly expressed in vascular endothelial cells where it accelerates leukocyte adhesion and migration to endothelial surfaces and has closed linkage with the pathogenesis of hyperlipidemia-associated atherosclerosis.[14] PPAR-α is a member of the steroid/thyroid hormone receptor superfamily involved in the transcription of genes responsible for transport and metabolism of lipids as well as mitochondrial and peroxisomal fatty acid oxidation in the liver. It has been observed that hepatic steatosis can be alleviated in mice by administering PPAR-α agonists, such as fibrates, which are commonly used to treat hyperlipidemia, leading to enhancements in mitochondrial fatty acid oxidation.[15] The side effects of the current lipid-lowering drugs have increased the tendency to move toward traditional and alternative treatments.[16]

Scientists are now focusing on pharmacophores having diverse biological activities with fewer side effects. The diverse oxazole analogues with multidirectional potential stand out in current organic chemistry and provide alternatives to tackle future therapeutic difficulties.[17] In medicinal chemistry, benzoxazole being the most important heterocyclic chemical class has been incorporated into numerous pharmaceutical compounds, making it a flexible heterocyclic compound with a wide range of biological functions.[18] Structural resemblance of benzoxazoles to the bases adenine and guanine provides a strong basis for its interaction with biopolymers in the living systems. Modifications in the structure of BZX are attributed to its enhanced and diverse biological profiles. Many substituted BZX are considered potent anti-inflammatory, hypoglycemic, antibacterial, antitubercular, anticancer, and antifungal agents.[19] Considering the wide biological profile of BZX compounds, we assessed the antihyperlipidemic activity of novel imine derivatives of benzoxazoles (BZX-1, BZX-2, BZX-3, and BZX-4) in high-fat diet (HFD)-induced hyperlipidemic rats.

MATERIALS AND METHODS

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

Animals Used. Adult male Sprague−Dawley rats (7 weeks old; 180−200 g) were obtained from the University of Veterinary and Animal Sciences, Lahore, Pakistan. All animals were kept under standard conditions in the animal house of Faculty of Pharmacy, University of Lahore, and acclimatized for 1 week prior to the start of experiments. Animal care was provided in accordance with the guidelines of OECD.[20] All the experimental protocols were approved by the Institutional Research Ethics Committee, Faculty of Pharmacy, University of Lahore (Approval No: IREC-2020-47).[21]

High-Fat Diet-Induced Hyperlipidemia. Hyperlipidemia was induced by using high-fat diet (HFD) consisting of a homogeneous mixture of cholesterol (2% w/w), cholic acid (1% w/w), banaspati ghee and coconut oil (3:2 w/w), and egg yolk powder (5%) in powdered standard rat chow for 28 days.[22−24] Rats had free access to feed and water throughout the study.[20] On the 28th day, blood samples of rats were evaluated for TC and TG levels. Elevated levels of these biomarkers confirmed development of the model for further experiment. Animals having TC and TG levels greater than 280 and 400 mg/dL, respectively, were selected for further study.[25,26] Hyperlipidemic rats were treated once daily with either simvastatin (20 mg/kg) or with various doses (10, 20, and 30 mg/kg) of BZX, i.e., BZX-1, BZX-2, BZX-3, and BZX-4 for 12 consecutive days. After 12 days of treatment, animals were sacrificed and blood samples were collected for the analysis of the lipid profile and liver function tests (LFT). Serum lipid levels and LFT were assayed automatically using commercially available kits.[20] Liver tissues were also harvested in 10% buffered formalin and Trizol for histopathological and mRNA analyses, respectively.

Molecular structures of BZX derivatives are provided in Figure S1.

Experimental Protocol. Experimental animals were divided into the following groups, each having three rats (n = 3).

1. Normal control (NC): normal diet
2. HFD: high-fat diet
3. SIM (20 mg/kg): HFD followed by SIM
4. BZX-1 (10 mg/kg): HFD followed by BZX-1 (10 mg/kg)

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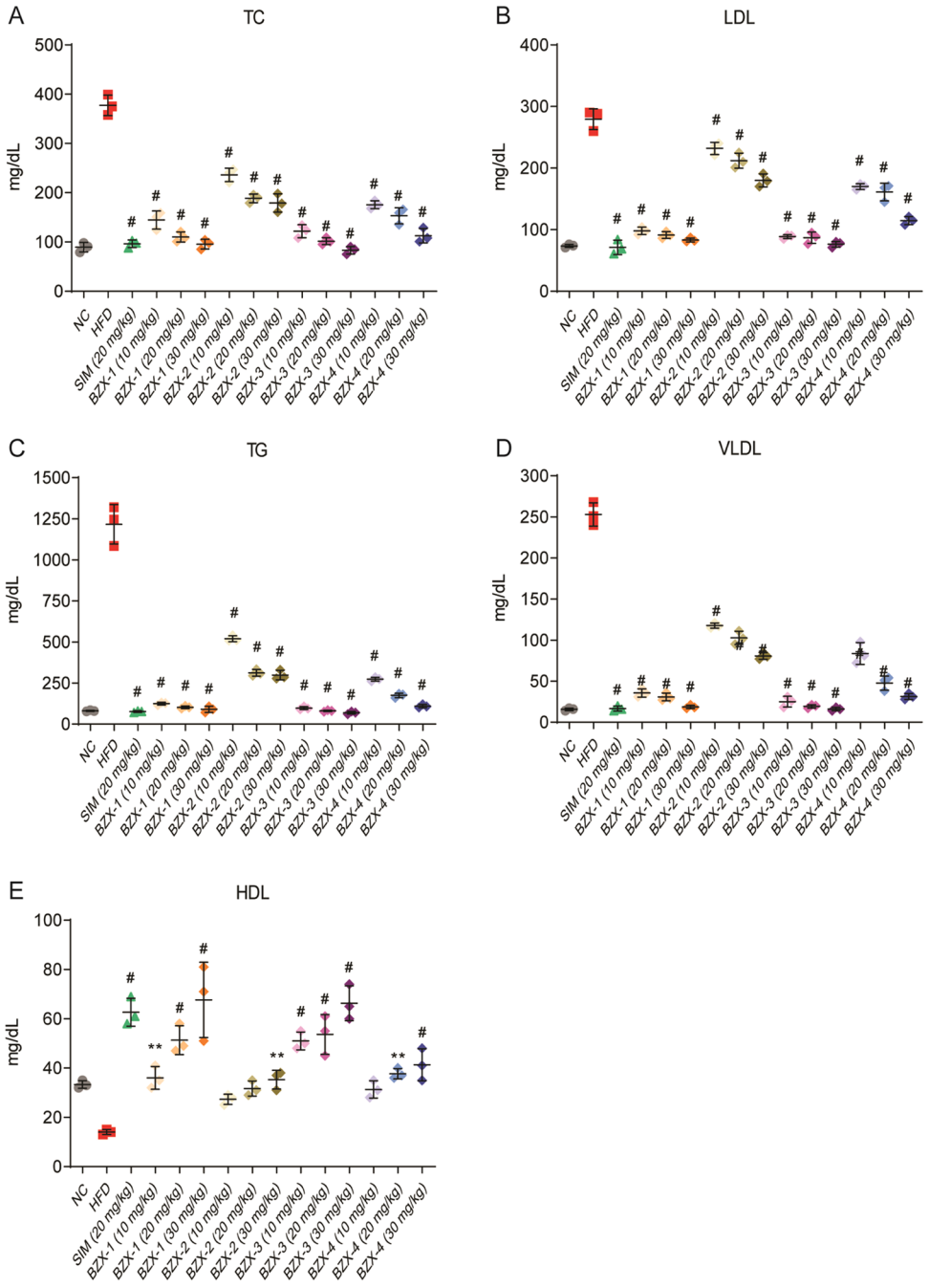

Figure 1. BZX derivatives improved the lipid profile of HFD-fed rats in a dose-dependent manner. Hyperlipidemia was induced in rats using a HFD, and hyperlipidemic rats were later treated with either SIM or BZX compounds. BZX derivatives reduced HFD-induced (A) TC, (B) LDL, (C) TG, and (D) VLDL and increased (E) HDL levels. # ≤ 0.001, ** ≤ 0.01; one-way Anova followed by Tukey's multiple comparison test (n = 3).

5. BZX-1 (20 mg/kg): HFD followed by BZX-1 (20 mg/kg)
6. BZX-1 (30 mg/kg): HFD followed by BZX-1 (30 mg/kg)
7. BZX-2 (10 mg/kg): HFD followed by BZX-2 (10 mg/kg)
8. BZX-2 (20 mg/kg): HFD followed by BZX-2 (20 mg/kg)
9. BZX-2 (30 mg/kg): HFD followed by BZX-2 (30 mg/kg)
10. BZX-3 (10 mg/kg): HFD followed by BZX-3 (10 mg/kg)
11. BZX-3 (20 mg/kg): HFD followed by BZX-3 (20 mg/kg)
12. BZX-3 (30 mg/kg): HFD followed by BZX-3 (30 mg/kg)
13. BZX-4 (10 mg/kg): HFD followed by BZX-4 (10 mg/kg)
14. BZX-4 (20 mg/kg): HFD followed by BZX-4 (20 mg/kg)
15. BZX-4 (30 mg/kg): HFD followed by BZX-4 (30 mg/kg)

Histopathological Analysis. Formalin-fixed liver samples were embedded in paraffin after successive dehydration with

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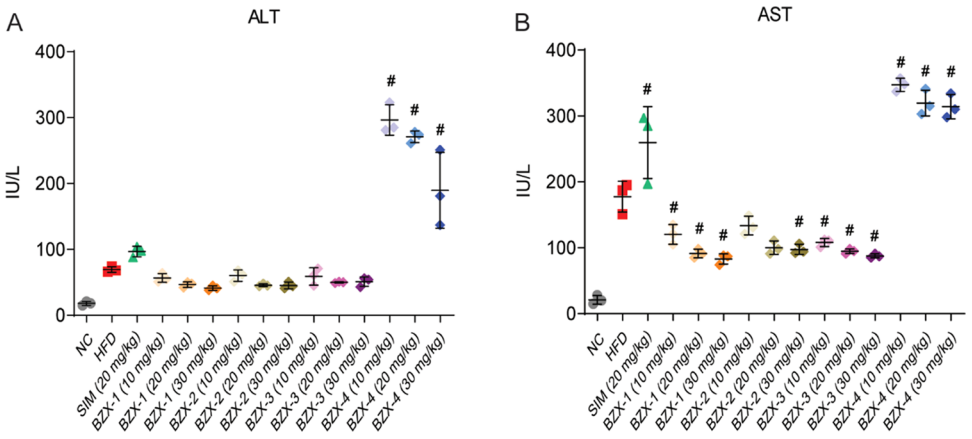

Figure 2. BZX-1, BZX-2, and BZX-3 reduced HFD-induced serum AST and ALT levels. Hyperlipidemia was induced in rats using a HFD, and hyperlipidemic rats were later treated with either SIM or BZX compounds. BZX derivatives reduced HFD-induced (A) ALT and (B) AST levels except BZX-4. # ≤ 0.001; one-way Anova followed by Tukey's multiple comparison test (n = 3).

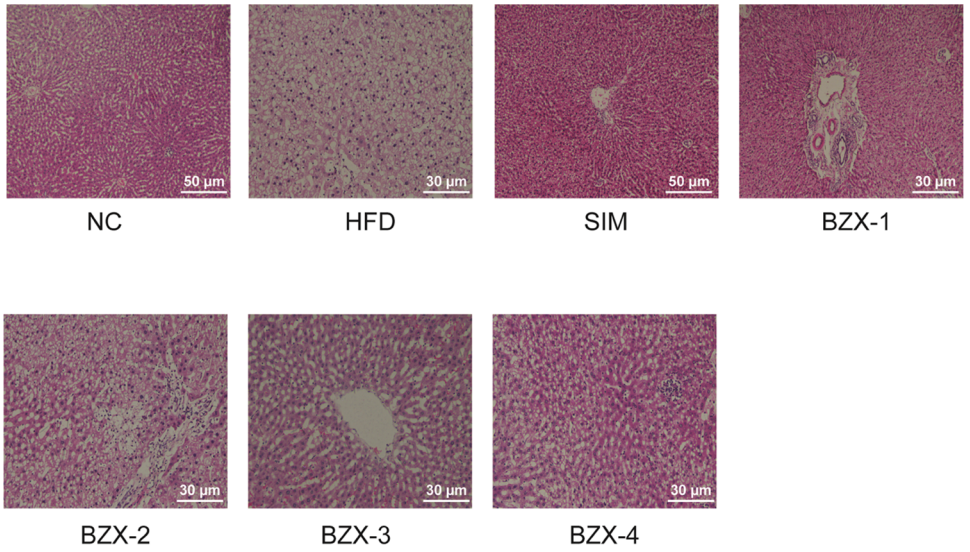

Figure 3. BZX compounds prevented HFD-induced hepatic damage and fatty changes. Hyperlipidemia was induced in rats using a HFD, and hyperlipidemic rats were later treated with either SIM or BZX compounds. BZX compounds displayed a prominent reduction in HFD-associated fatty changes/damage in liver specimens. BZX-3 showed superior effects, as all the specimens displayed a perfect hepatic architecture with minimal inflammation.

ethanol. Paraffin blocks were sectioned at 5 μm thickness by using a rotary microtome and later stained with hematoxylin−eosin dye using standard histological techniques.[27]

Antioxidant Assay. Liver samples were harvested in 1× PBS. Samples were homogenized and later stored overnight at −20 °C. Supernatants were separated and processed according to the manufacturer's instructions to measure superoxide dismutase (SOD) and glutathione (GSH) levels using standard enzyme-linked immunosorbent assay (ELISA) kits.

Real-Time PCR Analysis. Liver samples were homogenized, and total RNA (2 μg) was extracted using the standard Trizol method. Reverse transcription of RNA was performed according to the manufacturer's instructions using the WizScript cDNA synthesis kit (Wizbio solutions, New Mexico). The relative expressions of various genes were measured by the ΔΔCT method using SYBR Green qPCR mix (Zokeyo, Wuhan, China) and primers reported in our previous study.[28] The following RT-PCR conditions were used: pre-denaturation at 94 °C for 2 min, followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 60 °C for 1 min, and extension at 72 °C for 15 s. Hypoxanthine−guanine phosphoribosyltransferase (HPRT) mRNA was used as a housekeeping control.

Molecular Docking Analysis. Three-dimensional (3D) structures of HMGCR (1CKT), APOB (1ICM), and VCAM1 (1IJ9) were retrieved from the Protein Data Bank (PDB) (www.rcsb.org). Target proteins were prepared for docking analysis using the Autodock Tools program. Proteins after energy minimization and addition of Gasteiger charges were saved in the pdbqt format. The protein architecture and statistical values of helices, β-sheets, coils, and turns were accessed using VADAR 1.8.[29] Ligands (BZX-1, BZX-2, BZX-3, and BZX-4) were drawn in Discovery Studio Client and saved in the pdb format. Kolman and Gasteiger charges were later added, and ligands were then saved in pdbqt. The molecular docking experiment was used for all the synthesized ligands against target proteins using the PyRx[29] virtual screening tool with the Auto Dock VINA Wizard approach.[30,31] The two-dimensional (2D) and 3D graphical depictions of all the

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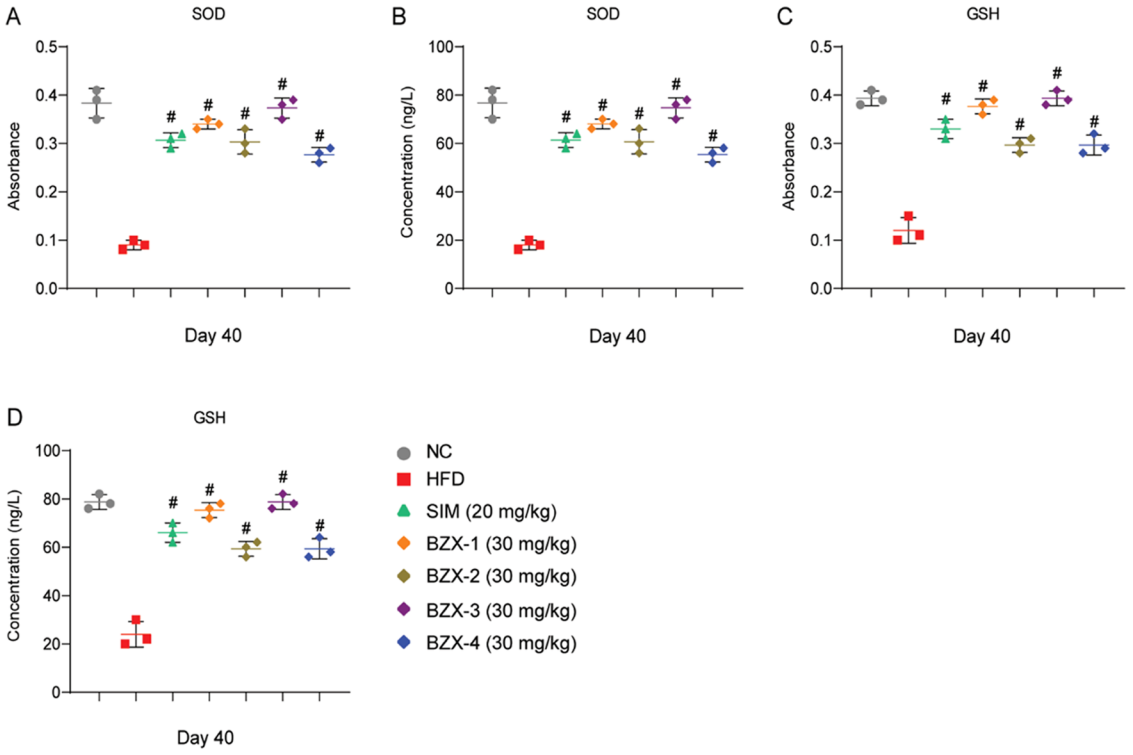

Figure 4. BZX derivatives improved serum antioxidant levels in HFD-fed rats. Hyperlipidemia was induced in rats using a HFD, and hyperlipidemic rats were later treated with either SIM or BZX compounds. BZX derivatives increased (A, B) SOD and (C, D) GSH levels in hyperlipidemic rats. # ≤ 0.001; one-way Anova followed by Tukey's multiple comparison test (n = 3).

docked complexes were accomplished using Discovery Studio (Discovery Studio Visualizer Software, Version 4.0., 2012).

Statistical Analysis. Results were expressed as mean ± standard deviation (SD), and data were analyzed by one-way analysis of variance (ANOVA) followed by post-hoc test using GraphPad prism 9.0 (Graphpad Software, Inc., San Diego). P < 0.05 was considered statistically significant. The following abbreviations were used to denote the level of significance: # ≤ 0.001, ** ≤ 0.01, and * ≤ 0.05.

RESULTS

BZX Derivatives Restored Hyperlipidemia-Induced Body Weight. Animals fed with a HFD demonstrated an increase in body weight, while groups treated with BZX derivatives displayed a dose-dependent reduction in body weight. The effects of a higher dose of BZX-3 were comparable to those of the SIM-treated group (Figure S2).

BZX-1 and BZX-3 Significantly Reduced HFD-Induced Hyperlipidemia in Rats. An abnormal increase in the serum levels of TC, TG, and LDL is the critical marker that leads to the progression of cardiovascular complications. Multiple studies have demonstrated that chances of further cardiovascular events can be reduced by lowering these markers.[32] Our findings also demonstrated that HFD-induced hyperlipidemic rats displayed a significant decrease in the serum levels of HDL and elevation in the serum levels of TC, TG, LDL, and VLDL compared to the control group. All BZX compounds clearly showed dose-dependent reduction in the levels of TC, TG, LDL, and VLDL along with improvement in HDL levels. Serum levels of TG (90 ± 18.5 mg/dL), TC (95.6 ± 9.71 mg/dL), LDL (83.3 ± 3.21 mg/dL), and VLDL (18.6 ± 2.08 mg/dL) were found to be significantly (P < 0.001) lowered in rats treated with the BZX-1 compound (30 mg/kg) as compared to TC (377.3 ± 20.6 mg/dL), TG (1217 ± 120.0 mg/dL), LDL (279.3 ± 16.7 mg/dL), and VLDL (253.0 ± 14.1 mg/dL) levels of the untreated disease (HFD) group (Figure 1A–D), whereas HDL (67.6 ± 15.2 mg/dL) levels improved significantly (P < 0.001) compared to HDL (14.0 ± 1.00 mg/dL) levels of the disease group (Figure 1E). BZX-2 (30 mg/kg) demonstrated significant (P < 0.001) reduction in TG (298.3 ± 28.5 mg/dL), TC (179.0 ± 18.5 mg/dL), LDL (180.0 ± 10.5 mg/dL), and VLDL (80.6 ± 4.04 mg/dL) levels, while it showed elevation in HDL (35.3 ± 3.78 mg/dL) levels in comparison to the disease group. Similarly, a marked reduction in TG (69.3 ± 8.02 mg/dL), TC (83.3 ± 7.63 mg/dL), LDL (76.3 ± 4.72 mg/dL), and VLDL (16.0 ± 2.00 mg/dL) levels was observed along with elevation of HDL (66.3 ± 7.09 mg/dL) in rats treated with BZX-3 (30 mg/kg). BZX-4 (30 mg/kg) also remarkably reduced TG (110.0 ± 10.1 mg/dL), TC (112.7 ± 14.5 mg/dL), LDL (114.7 ± 6.50 mg/dL), and VLDL (31.3 ± 3.51 mg/dL) levels, together with elevation in serum levels of HDL (41.3 ± 6.51 mg/dL). Moreover, comparable antihyperlipidemic activities of BZX1 and BZX3 compounds were witnessed compared to the gold standard—SIM (Figure 1A–E).

BZX-1, BZX-2, and BZX-3 Attenuated HFD-Induced AST and ALT Levels. Hyperlipidemia is considered a prominent risk factor of fatty liver and plays a critical role in liver dysfunction.[33] Prominent elevation in hepatic biomarkers such as AST (177.7 ± 23.4) and ALT (69.3 ± 4.16) was observed in HFD-induced hyperlipidemic rats as compared to the normal group. All BZX compounds dose-dependently reduced the plasma levels of AST and ALT levels except BZX-4. The BZX-1-treated group showed a significant reduction in ALT (41.3 ± 3.51) and AST (83.0 ± 7.81) levels, and similarly, BZX-2 and BZX-3 decreased LFT in a manner

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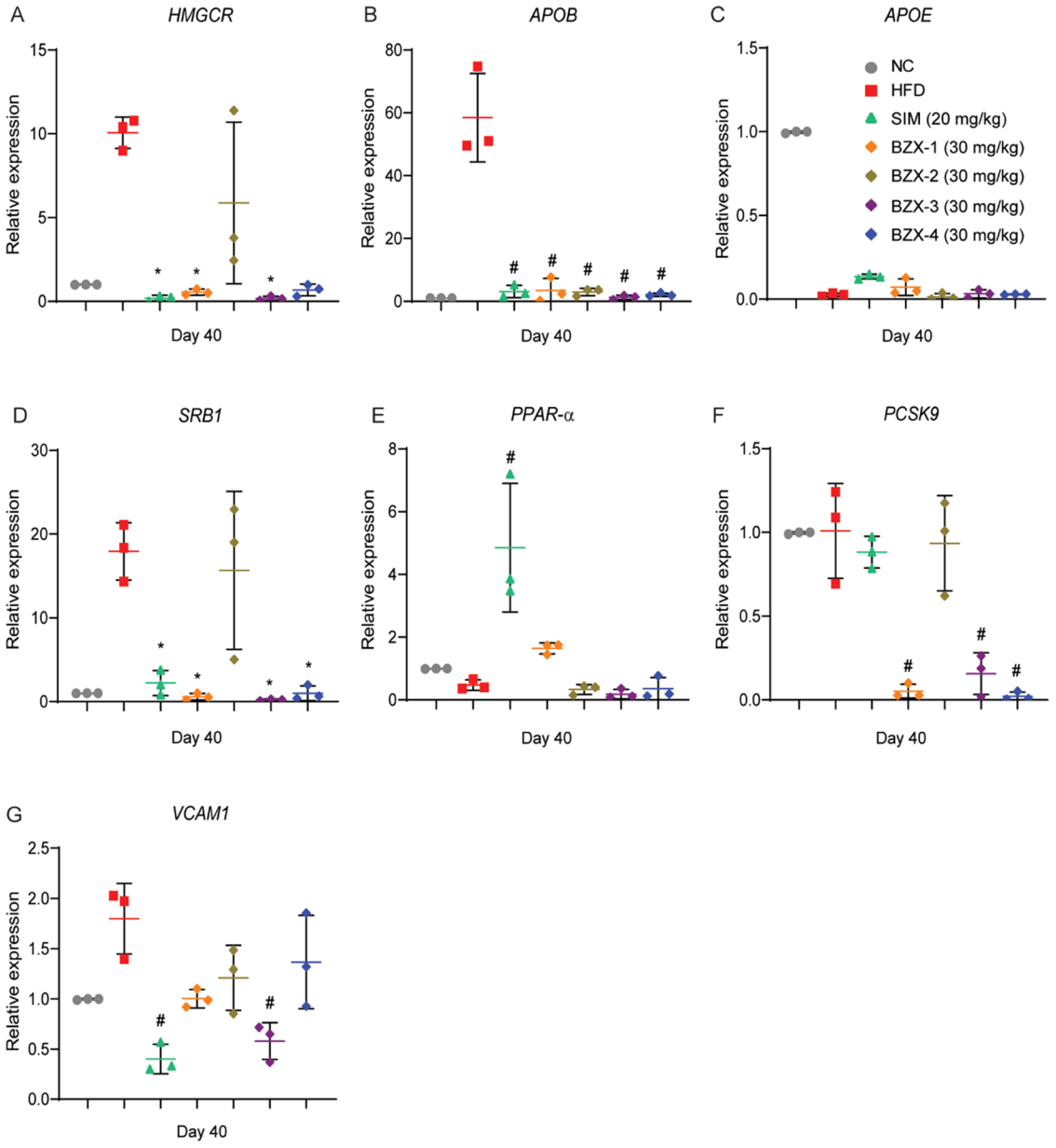

Figure 5. BZX derivatives exert antihyperlipidemic effects by differential regulation of lipid-regulating genes. Hyperlipidemia was induced in rats with a HFD, and hyperlipidemic rats were later treated with either SIM or BZX compounds. BZX compounds improved the lipid profiles of hyperlipidemic rats by differentially regulating mRNA levels of (A) HMGCR, (B) APOB, (C) APOE, (D) SRB1, (E) PPAR-α, (F) PCSK9, and (G) VCAM1. # $\leq$ 0.001, * $\leq$ 0.05; one-way Anova followed by Tukey's multiple comparison test (n = 3).

Table 1. Binding Affinities of BZX Derivatives with Target Proteins

compounds	HMGCR (1CKT)	APOB (1ICM)	VCAM-1 (1IJ9)
BZX-1	−7.4	−9.0	−7.9
BZX-2	−7.9	−8.9	−8.1
BZX-3	−7.5	−9.3	−8.3
BZX-4	−7.1	−7.1	−7.5

comparable to that of BZX-1. Interestingly, BZX-4-treated rats demonstrated an increase in ALT (189.7 ± 57.5) and AST (314.0 ± 18.3) levels, indicating that it might have hepatotoxic effects. SIM as expected strongly increased AST levels along with a slight increase in ALT levels when compared to HFD-fed rats (Figure 2A,B).

BZX-3 Prominently Prevented HFD-Induced Fatty Changes in Hyperlipidemic Rats. Fatty liver has been the focus of many researchers, as it is associated with poor liver functions. Steatosis is the first stage of fatty liver disease, which can progress to steatohepatitis, fibrosis, cirrhosis, and even cancer if left untreated.[32]

Histopathological analysis of liver samples showed that the HFD induced vacuolization, inflammation, necrosis, and extramedullary hemopoieses in liver samples, while the control group showed nonvacuolated hepatocytes with a normal architecture of the hepatic cords, portal triads, and central veins. BZX-treated groups showed effectiveness in reversing fatty changes. BZX-1-, 2-, and 4-treated groups showed mild to moderate fatty changes, while the reversal of fatty changes was significantly evident in BZX-3-treated hyperlipidemic rats. Hepatocytes of BZX-3-treated rats showed relatively normal

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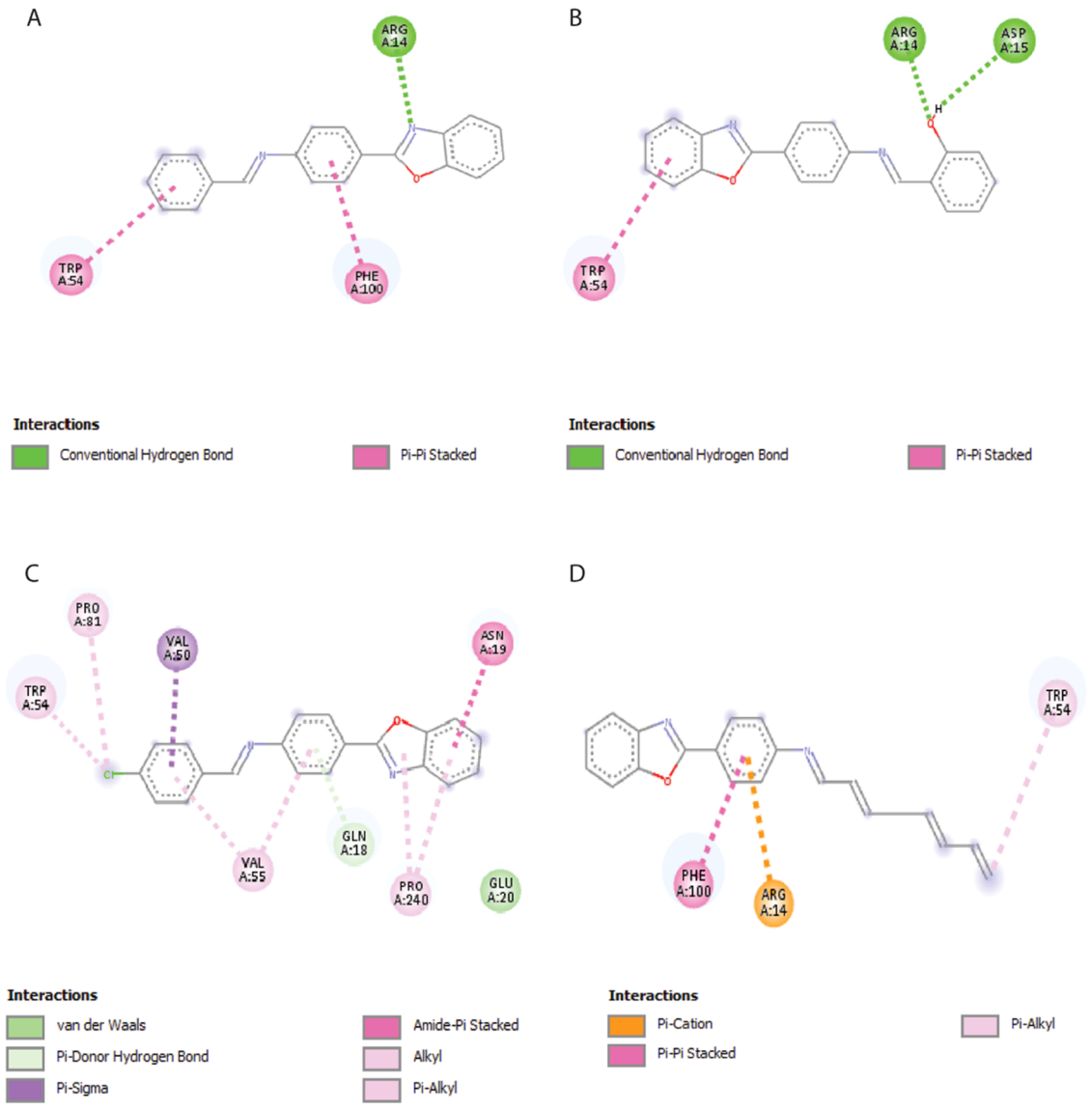

Figure 6. BZX derivatives displayed strong binding affinities with HMGCR. 2D structures of docked complexes; (A) BZX-1−HMGCR, (B) BZX-2−HMGCR, (C) BZX-3−HMGCR, and (D) BZX-4−HMGCR.

morphology with minimal fatty changes and mild inflammation, and it was comparable to the SIM-treated group. In addition, the architecture of the hepatic cords was immaculate, with regular portal triads and central veins. Our results clearly demonstrated a superior hepatoprotective effect of BZX-3 when compared to other BZX compounds (Figure 3).

BZX Compounds Reduced HFD-Associated Oxidative Stress. Studies have shown that HFD induces oxidative stress;[34] we therefore measured antioxidant markers. As anticipated, HFD significantly decreased superoxide dismutase (SOD) and glutathione (GSH) levels. Treatment with SIM and BZX derivatives restored SOD and GSH levels, indicating their antioxidant potentials (Figure 4A−D).

Modulation of Lipid-Regulating Genes by BZX Compounds. In order to elucidate the mechanism behind the antihyperlipidemic potential of BZX compounds, the transcript levels of different lipid-modulating genes (*HMGCR, VCAM-1, APOB, APOE, PCSK9, PPAR-α*) were measured by RT-qPCR. The results demonstrated a marked elevation in the transcript levels of *HMGCR* in HFD-induced hyperlipidemic rats. BZX-1- and BZX-3-treated groups showed a significant ($P < 0.001$) downregulation in the *HMGCR* expression compared to the disease group. Reduction of the *HMGCR* expression by BZX-1 and BZX-3 was comparable to that of the SIM-treated group (Figure 5A). The HFD also significantly increased the mRNA levels of *APOB, PCSK9, SRB1*, and *VCAM*-1 compared to those of the normal group (Figure 5B,D,F,G). Animals treated with BZX compounds showed a prominent reduction in the transcript levels of *APOB*, compared to the untreated HFD-induced hyperlipidemic rats. Similarly, a significant inhibition of *PCSK9* was witnessed in BZX-1-, BZX-3-, and BZX-4-treated groups, whereas BZX-2 and SIM failed to inhibit the expression of *PCSK9* (Figure 5B,F). BZX-3 only displayed inhibition of *VCAM-1*, which was comparable to that of the SIM-treated group. Moreover, downregulation in the transcript levels of *PPAR-α* and *APOE* was observed in HFD-induced hyperlipidemic rats. Treatment with SIM effectively improved the levels of *PPAR-α* and *APOE*, while only BZX-1 was able to slightly induce these genes (Figure 5C,E).

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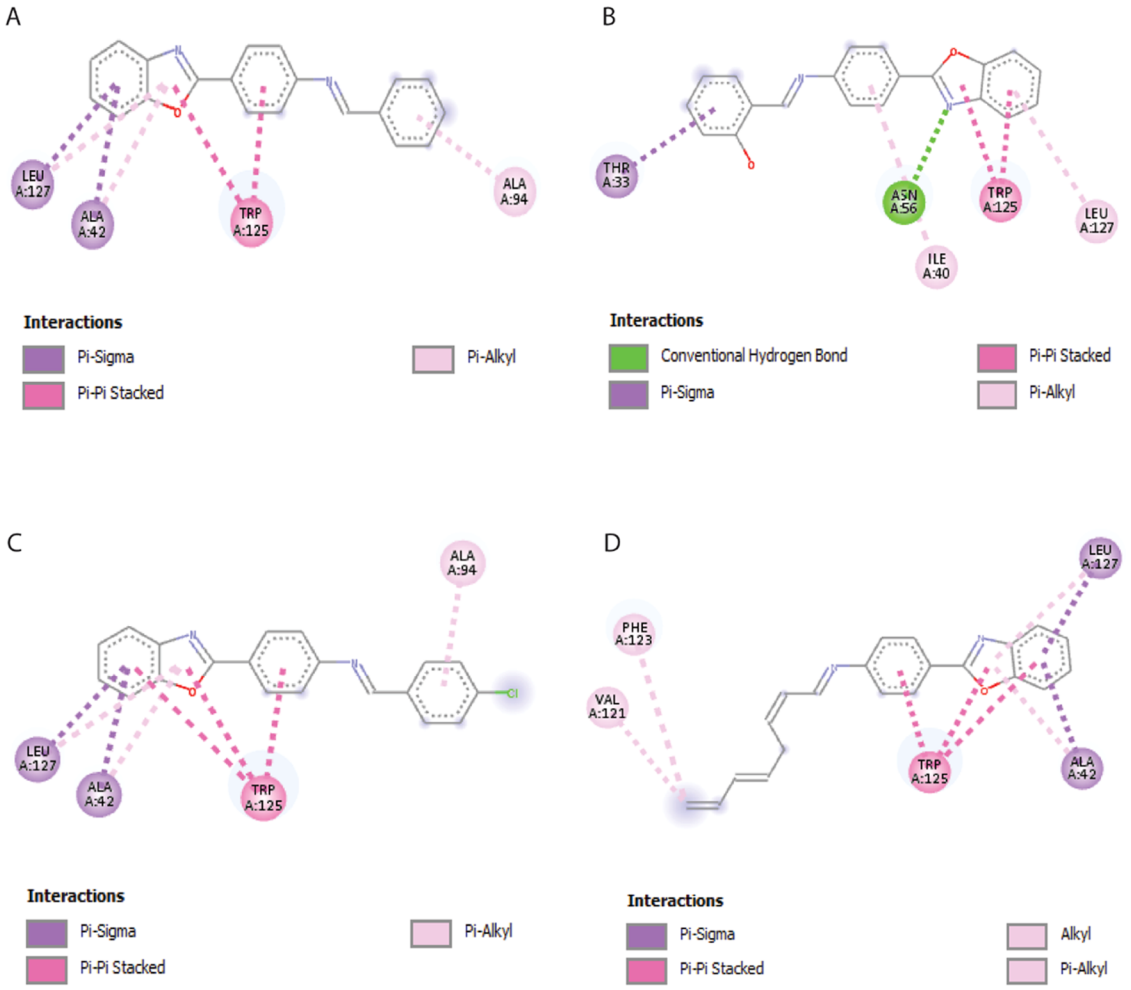

Figure 7. BZX derivatives displayed strong binding affinities with APOB. 2D structures of docked complexes; (A) BZX-1−APOB, (B) BZX-2−APOB, (C) BZX-3−APOB, and (D) BZX-4−APOB.

BZX Compounds Displayed Strong Binding Affinities with HMGCR, APOB, and VCAM1. The *E*-value (kcal/mol) was used to assess the affinity of protein and the best-docked pose complex. It provided an estimation of the binding free energy and binding constant for docked ligands. The results revealed that all the BXZ compounds displayed strong binding affinities with HMGCR, APOB, and VCAM1 (Table 1). The present docking analysis data suggest that some of the interactions might be required for strong antihyperlipidemic effects, which are lacking in the case of BZX-2 and BZX-4 (Figures 6, 7, 8 and S3−S5).

DISCUSSION

Hyperlipidemia is a multifaceted disorder associated with increased levels of free fatty acids, TG, and LDL as well as reduced plasma HDL concentration that accelerates other cerebrovascular diseases including the development of atherosclerosis thrombosis and coronary heart diseases.[35,36] Noticeable side effects and limitations associated with antihyperlipidemic drugs have retained the interest of scientists in the discovery of new, safer, and better alternative candidates.[37] Approximately 75% of the "Food and Drug Administration"-approved molecules having cholesterol lowering potential contain a common nitrogen heterocyclic moiety, such as atorvastatin (pyrrole derivative) and chloroquine (quinoline derivative).[38] Heterocyclic compounds possessing an oxazole ring have been significantly considered for the development of new chemical entities in the recent years.[39] Diverse pharmacological activities of benzoxazoles have been attributed to their improved solubility and the ability to form bonds with a variety of enzymes and receptors.[40]

In this study, BZX derivatives were investigated for their cholesterol lowering potential in HFD-induced hyperlipidemic rats. The present findings showed that BZX-1 and BZX-3 robustly decreased the serum levels of TC, TG, LDL, and VLDL, and they were equally effective in increasing HDL levels. LFT analysis demonstrated a hepatoprotective action of these compounds, and histological studies of liver samples revealed similar results. Gene expression studies revealed divergent effects of these derivatives on lipid-modulating targets. BZX-1- and BZX-3-targeted multiple genes due to their similar structures and their lipid lowering effects appeared to be due to the downregulation of *HMGCR*, *APOB*, *PCSK9*, *SRB1*, and upregulation of *APOE* and *PPAR-α*. BZX-2 displayed its lipid lowering effects mainly due to reduced mRNA levels of *APOB*. BZX-4-mediated lipid regulation could be attributed to its *APOB*, *SRB1*, and *PCSK9* inhibitory activities.

HMGCR is a well-known rate-limiting transmembrane enzyme, which is regulated by multiple feedback mechanisms and involved in catalyzing the conversion of HMG-CoA to

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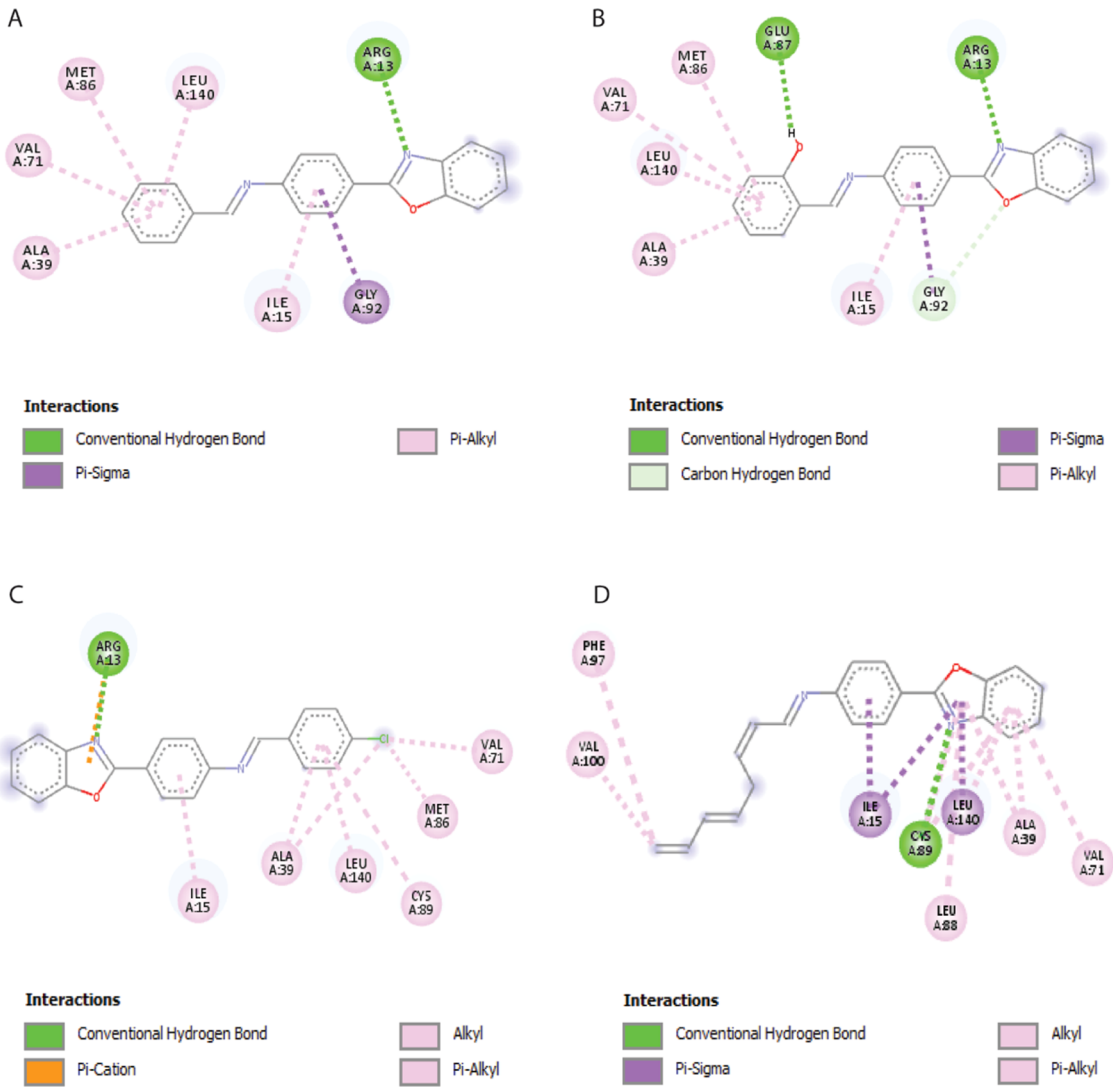

Figure 8. BZX derivatives displayed strong binding affinities with VCAM-1. 2D structures of docked complexes; (A) BZX-1−VCAM-1, (B) BZX-2−VCAM-1, (C) BZX-3−VCAM-1, and (D) BZX-4−VCAM-1.

mevalonate, a precursor of sterols including cholesterol synthesis.[41,42] Inhibition of cholesterol biosynthesis by blocking HMGCR in the liver results in the increased clearance of LDL from plasma by multiple methods of reverse cholesterol transport[43] and through activation of sterol regulatory element-binding protein-2 (SREBP-2).[44] PCSK9, a serine protease, is another target that mediates the absorption of LDL via LDLR, which is principally accountable for eliminating plasma cholesterol. When LDL binds to LDLR, clathrin-coated pits are formed, which are internalized and degraded in the lysosomes.[45] PCSK9 acts as a catalyst and directs the endocytosis and lysosomal degradation of LDLR, hence decreasing LDLR recycling and its involvement in clearing plasma LDL.[46] Therefore, PCSK9-inhibiting interventions are being employed to drastically reduce plasma LDL levels.[47]

Studies have also revealed the involvement of APOB, a major component of LDL and VLDL, in the formation of atherogenic plaques.[48−51] Elevated levels of APOB in the bloodstream result in LDL retention in the arterial walls and are characteristic of the events that can spark inflammatory responses and CVS events.[52] Moreover, APOE, a multifunctional protein, plays critical roles in lipid metabolism and atherosclerotic lesions have been linked to its deficiency or dysfunction.[53,54] It stimulates the internalization of lipoprotein particles by interacting with large heparan sulfate proteoglycans on the surface of hepatocytes. Atheroprotective effects of APOE include its role in cholesterol efflux, suppression of smooth muscle cell proliferation, protection against lipid peroxidation, and limitation of platelet aggregation[53] through interacting with several receptors including LDL receptor-related protein 1 (LRP1), the VLDL receptors, and the ApoE2 receptor.[55] SRB1 is another multiligand membrane receptor protein whose primary role is to control selective cholesterol trafficking.[56] SRB-1 mediates both the influx of HDL-derived cholesteryl esters into cells and tissues by reverse cholesterol transport and efflux of cholesterol from peripheral tissues, including macrophage foam cells, back to the liver for bile acid formation, with subsequent biliary secretion or transport to steroidogenic tissues, particularly the adrenal gland and ovary, for steroid synthesis and storage. Therefore, upregulation of the SRB1 levels in the visceral fat tissues and downregulation in the liver lower the plasma lipid levels.[13] PPAR-α is a well-known key regulator of the several genes that are involved in controlling lipid metabolism through fatty acid uptake,

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oxidation of fatty acids, triglyceride turnover, and transport of lipids.[57] Activation of PPAR-α dramatically increases hepatic fatty acid metabolism, while genetic inactivation leads to large lipid buildup in the liver and increased plasma free fatty acid levels.[58] The present data indicate that our findings are in accordance with the previous studies.

VCAM-1, a key atherosclerotic marker, emerges on the surface of endothelial cells in the areas prone to lesion formation due to exposure to inflammatory cytokines and is normally upregulated in hyperlipidemia.[59] VCAM-1 mediates cell adhesion by interacting with adhesion molecules and facilitates cell−cell contacts vital to immune function through interactions with its integrin counter receptors that are constitutively expressed on lymphocytes, monocytes, and eosinophils.[60,61] Lymphocyte transmigration through the vascular endothelium requires VCAM-1-mediated signaling, including ROS generation, RhoA activation, and phosphorylation of target proteins.[62] Therapies aimed at targeting lymphocyte adhesion by antagonizing VCAM-1-mediated signaling have been proved effective in preventing chemical vapor depositions (CVDs). This study also showed VCAM1 inhibitory potentials of BZX-1 and BZX-3, indicating that they might be effective in preventing atherosclerosis and other CV comorbidities. The current study establishes benzoxazole as a promising therapeutic scaffold that may lead to the development of target-based drugs for the treatment of hyperlipidemia and other CVDs.

CONCLUSIONS

This study clearly indicates that BZX derivatives can effectively reduce serum lipid levels by targeting multiple lipid regulatory genes. Furthermore, these compounds except BZX-4 did not show any signs of liver toxicity, which is typically seen with long-term use of statins, indicating their better safety profile. As BZX-1 and BZX-3 targeted several lipid regulatory genes including VCAM1, it is therefore plausible that these derivatives might be effective in the prevention/treatment of other CV disorders. Based on the current data, future studies can be designed to validate these findings in human models and to investigate the role of BZX derivatives in various CHDs.

ASSOCIATED CONTENT

Data Availability Statement

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

Supporting Information

The Supporting Information is available free of charge at https://pubs.acs.org/doi/10.1021/acsomega.3c00443.

Molecular docking and structures of BZX compounds (PDF)

(PDF)

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Author Contributions

M.Z. and R.S. performed the experiments. I.A. analyzed histopathological data, and W.Y., S.J., and M.N.M. analyzed qPCR oxidative stress and LFT data. H.A.A.K. performed molecular docking analysis. I.A. participated in editing of the article. M.Z. wrote the first version of the article. M.N.H.M. designed and supervised the project and edited the final version of the article.

Notes

The authors declare no competing financial interest.

Experiments were performed according to the guidelines of OECD and were approved by the Institutional Research Ethics Committee of the Faculty of Pharmacy, University of Lahore (Approval Number: IREC-2020-47).

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