

# Design, Synthesis and Pharmacological Evaluation of 2-(3-Benzoyl-4-Hydroxy-1,1-Dioxido-2H-Benzo[e][1,2]thiazin-2-yl)-N-(2-Bromophenyl) Acetamide as Antidiabetic Agent

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**Purpose:** The present study is based on screening new and potent synthetic heterocyclic compounds as anti-diabetic drugs using various computational tools, lab experiments, and animal models.

**Methods:** A potent synthetic compound 2-(3-benzoyl-4-hydroxy-1,1-dioxido-2H-benzo[e][1,2]thiazin-2-yl)-1-(2-bromophenyl) acetamide (FA2) was checked against diabetes and screened via enzyme inhibition assays, enzyme kinetics against alpha-glucosidase and alpha-amylase. Protein–ligand interaction was analyzed via molecular docking and toxicological analysis via ADMET. Experimental animals were used to examine the compound FA2 safety, delivery, and check various biochemical tests related to diabetes like fasting glucose sugar, cholesterol, triglyceride, HbA1c, creatinine, and insulin level. Histography of liver, kidney, and pancreas was also performed.

**Results:** Results showed that FA2 had binding energy of -7.02 Kcal/mol and -6.6 kcal/mol against  $\alpha$ -glucosidase (PDB ID: 2ZE0) and  $\alpha$ -amylase (PDB ID: 1B2Y), respectively. Moreover, in vitro enzyme inhibition assays and enzyme kinetics against  $\alpha$ -glucosidase and  $\alpha$ -amylase were performed, and FA2 showed IC<sub>50</sub> at  $5.17 \pm 0.28 \mu\text{M}$  and  $18.82 \pm 0.89 \mu\text{M}$  concentrations against  $\alpha$ -glucosidase and  $\alpha$ -amylase, respectively. Kinetics studies showed that the FA2 compound impeded  $\alpha$ -glucosidase and  $\alpha$ -amylase as a non-competitive mode of inhibition with  $K_i'$  values  $-0.320 \pm 0.001$  and  $0.141 \pm 0.01$ , respectively. FA2 was further analyzed on alloxan-induced mice for 21 days. Biochemical tests (fasting glucose sugar, cholesterol, triglyceride, HbA1c, creatinine, and insulin levels) and histological examination of liver and kidney showed that the FA2 compound showed better results than acarbose. Histology of pancreas found to show the maintenance of normal pancreatic acini and Langerhans islets in FA2 treated mice compared to acarbose and nontreated diabetic controls.

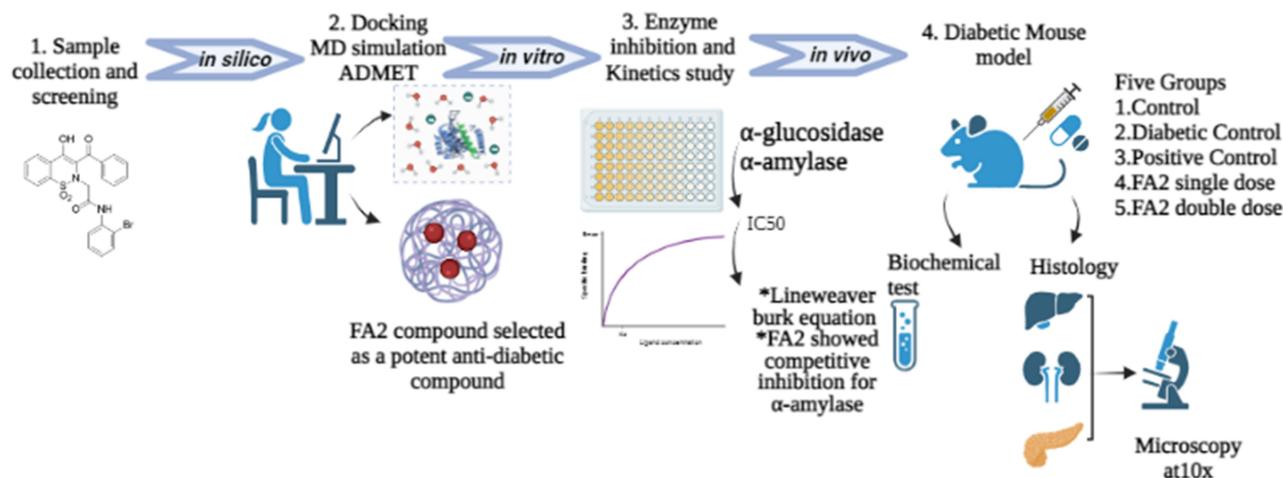
**Conclusion:** Investigating anti-diabetic potential of FA2 compound showed that the selected benzothiazine derivative has tremendous importance in reducing dose concentration and side effects.

**Keywords:** diabetes, benzothiazine, enzyme kinetics, mice model, drug discovery

## Introduction

Diabetes is a well-known chronic condition characterized by hyperglycemia due to inadequate insulin synthesis. Diabetes affects an estimated 220 million individuals globally. The death toll with diabetes will be more than double between 2005 and 2030, according to ADA.<sup>1</sup> As an international diabetes federation (IDF), three common types of diabetes are observed in the human population: T1DM, T2DM, and gestational diabetes. Type 1 and 2 are most common in the human population: type 1 is defined by inadequate insulin level, and type 2 is marked by insulin futility. About 90% of diabetic patients globally have Type 2 diabetes due to an imbalanced diet and physical activities. Thus, the rising prevalence of

## Graphical Abstract



type 2 DM has become a major clinical issue worldwide.<sup>2</sup> T2DM is a metabolic syndrome caused by a deficiency in insulin production and activity.<sup>3</sup> T2DM is also related to carbohydrate metabolism abnormalities and is defined by hyperglycemia, a health-threatening concurrent disease worldwide.<sup>4</sup> Hyperglycemia also induces oxidative stress (OS) via numerous metabolic ways, such as the overdose of superoxide negative ion ( $O^{-2}$ ) produced via mitochondrial electron transport chain (ETC).<sup>5</sup> OS is also thought to be the key reason for various long-term metabolic diseases, such as heart diseases, diabetes, and the onset of multiple cancers.<sup>6</sup> The high glucose level in the blood may disturb redox status, which damages several organs and cause various health issues, such as inducing hyperglycemia and hyperlipidemia. Therefore, maintaining ROS status could be a promising way to prevent diabetes-related difficulties, such as cardiomyopathy, renal disorder, retinopathy, skin diseases, alveolar bone loss, and cardiac problems.<sup>6-8</sup> The regulation of blood glucose levels and a balanced lifestyle is considered an ideal therapeutic mode for diabetic patients,<sup>9</sup> and this therapy is also used to recover diabetic patients with COVID-19.<sup>10</sup>

The hyperglycemic effect can be reduced with limited intake and digestion of nutritional carbohydrates (by inhibiting the  $\alpha$ -glucosidase and  $\alpha$ -amylase enzyme activities) in the gastrointestinal track.<sup>3,11</sup> The  $\alpha$ -glucosidase enzyme is essential for its ordinary physical role. The  $\alpha$ -glucosidase contributes to glycolysis by hydrolyzing the  $\alpha$ -D-glucopyranosyl bond to generate  $\alpha$ -D-glucose (reducing sugar) from sucrose (non-reducing sugar).<sup>12</sup> The pancreas secretes amylase to hydrolyze starch into maltose, isomaltose, and oligo-maltose monomers that the colon cannot absorb. Gastrointestinal  $\alpha$ -glucosidases, such as maltase, iso-maltase, and sucrase are membrane-bounded proteins found in the small intestine epithelium. Gastrointestinal  $\alpha$ -glucosidases are engaged in completing sugar breakdown to produce absorbable sugar monosaccharides.<sup>11,13</sup> The anti-postprandial hyperglycemia effect of acarbose, miglitol, and voglibose were verified by suppressing  $\alpha$ -glucosidase function and doctors prescribed these medicines for treatment of T2DM. They are frequently claimed to produce diarrhea, flatulence, abdominal pain, and liver issues, the most prevalent reason for noncompliance. Thus, synthesizing new  $\alpha$ -glucosidase antagonists with negligible side effects is a critical approach.<sup>14</sup> Researchers are now focusing on finding new heterocyclic compounds as promising candidates for glycosidase inhibitors to counteract these adverse effects. The majority of pharmacists are tempted to perform research in heterocyclic systems. Because of the diverse and adaptable biological features of heterocyclic systems, many heterocyclic scaffolds are used as vital components in many therapeutic molecules. Heterocyclic-derived compounds have the potential to be anti-diabetic medicines.<sup>15</sup>

Heterocyclic chemistry research accounts for over half of all organic chemistry research worldwide. Many bioactive organic molecules with heterocyclic frameworks serve an important role in medicine.<sup>16</sup> Researchers have paid great attention to synthesizing small molecular weight compounds to increase their solubility and excretion rate; for instance,

benzoimidazol, benzothiophen, and benzothiazol are reported as good  $\alpha$ -glucosidase inhibitors.<sup>17</sup> Noori et al<sup>18</sup> have reported benzoimidazol with acetamide derivatives as an  $\alpha$ -glucosidase inhibitor. Benzothiazine acetamide derivatives are well-known anti-diabetic,<sup>16</sup> anti-inflammatory,<sup>19</sup> anti-leukemic,<sup>20,21</sup> anti-microbial,<sup>22</sup> monoamine oxidase (MAO) inhibitors,<sup>23</sup> endothelin receptor antagonists (ERA),<sup>24</sup> antiallergic and antioxidant compounds.<sup>20,25</sup> Benzothiazine derivative FA2 was selected because of their great role in the pharmaceutical field; for instance, benzothiazine carboxamides, sudoxicam, piroxicam, and many other potent benzothiazine oxides have clinical importance. The present study is based on screening FA2 as a potent anti-diabetic compound via computational tools and experimental approaches.<sup>20,21,26–28</sup> T2DM can be managed by lowering postprandial hyperglycemia (PPHG) by postponing the activity of  $\alpha$ -amylase and  $\alpha$ -glucosidase, which are managed the carbohydrate ingestion and glucose absorption in the gastrointestinal tract.<sup>29</sup> Thus, this study is planned to explore the potential of benzothiazine acetamide derivative against diabetes.

## Materials and Methods

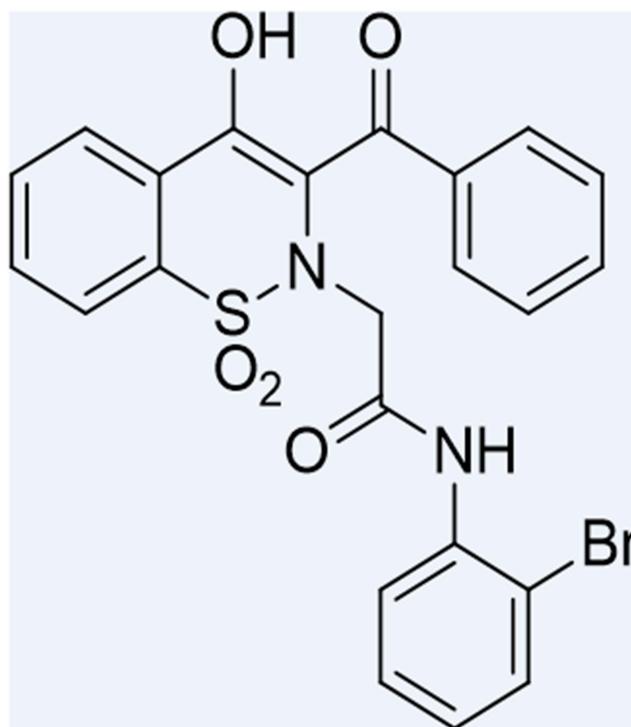
### Chemistry

The selected compound (Figure 1), 2-(3-benzoyl-4-hydroxy-1,1-dioxido-2*H*-benzo[*e*][1,2]thiazin-2-yl)-1-(2-bromophenyl) acetamide (FA2) was synthesized by reported methodology (Scheme 1).<sup>30</sup> The 3-benzoyl-4-hydroxy-1,2-benzothiazine 1,1-dioxide (0.5 g, 1.66 mmoles), 2-bromo-*N*-(2-bromophenyl) acetamide (0.49 g, 1.67 mmoles) and K<sub>2</sub>CO<sub>3</sub> (0.46 g, 2.33 mmoles) were mixed in dimethyl formamide (10 mL). The reaction mixture was vortex for 10 minutes at 28–30 °C, then heated at 100 °C for 120 minutes. The contents of the flask were poured into ice-cold distilled water to obtain the precipitates of the product (0.64g, 75% yield). Finally, FA2 was recrystallized from ethanol for purification.

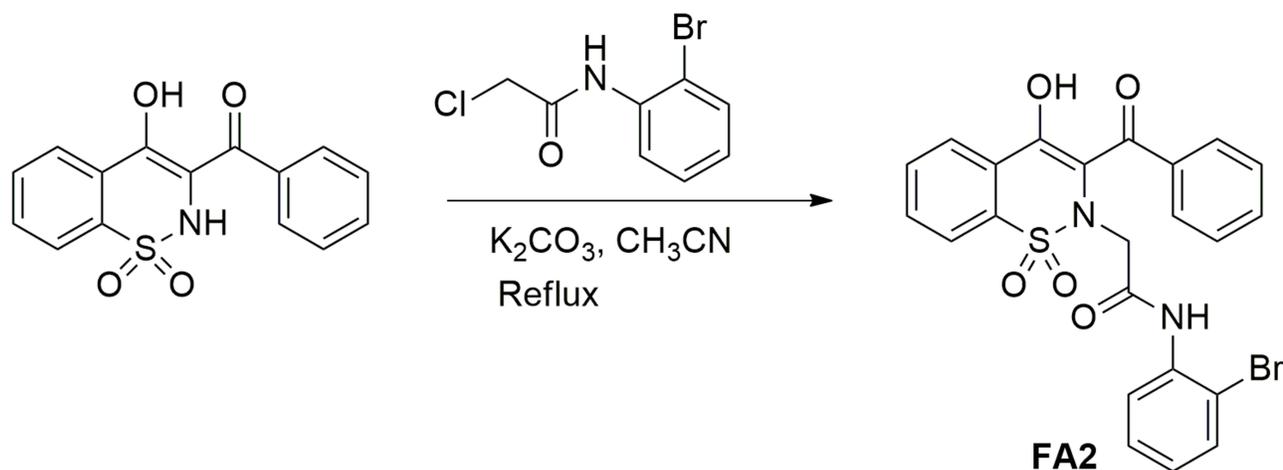
### In silico Study

#### Target Protein and Benzothiazine Ligands Preparation

The 3D crystalline structures of  $\alpha$ -glucosidase (PDB ID: 2ZE0) and  $\alpha$ -amylase (PDB ID: 1B2Y) were fetched from the PDB (<https://www.rcsb.org/>). The 2D construction of FA2 was drawn out via ChemDraw Ultra (Figure 1), minimized its energy by Chimera 1.16, and saved as a .pdb format.



**Figure 1** Structure of 2-(3-benzoyl-4-hydroxy-1,1-dioxido-2*H*-benzo[*e*][1,2]thiazin-2-yl)-1-(2-bromophenyl) acetamide (FA2).



**Scheme 1** Synthesis of targeted 2-(3-benzoyl-4-hydroxy-1,1-dioxido-2H-benzo[e][1,2]thiazin-2-yl)-N-(2-bromophenyl) acetamide.

### Energy Optimization

The UCSF Chimera tool was utilized to optimize the PDB coordinates of the targeted enzymes and the FA2 molecule for better docking results. These coordinates have the least amount of energy and the most stable configuration.

### Molecular Docking Analysis

The FA2 molecule was docked with 2ZE0 and 1B2Y. The ligand and protein structures were saved in .pdbqt format. PyRx-python software was used to calculate the binding scores of the molecules and target enzymes, and discovery studio (DS) visualizer was used to display the protein/ligand interactions. The results were compared with acarbose (PubChem CID: 41774) with the targeted enzymes.

### Drug-Likeness Evaluation

Examining FA2 drug-likeness properties is an important step in drug design to check the compound's properties. Physicochemical parameters such as molecular mass, log P water (miLogP), the number of OH<sup>-</sup>, and H<sup>+</sup> were analyzed via swissADME software (<http://www.swissadme.ch/>) to evaluate the remedy ability of the best-docked ligand models.<sup>31,32</sup> The pharmaceutical drug properties of the synthetic compound FA2 were evaluated according to the Lipinski's rule of five (RO5).<sup>33</sup>

### ADMET Analysis

Chemical absorption, distribution, metabolism, excretion, and toxicity (ADMET) all show essential shares in the pharmaceutical industry. A potent medicinal compound should be effective without any lethal side effects and have suitable ADMET characteristics at an acceptable dose quantity. Additionally, the pharmacokinetic features of the successful compounds were investigated to forecast their ADMET in the animal body.

The ADMET properties of the FA2 compound were predicted via computational tools, eg, SwissADME (<http://www.swissadme.ch/>)<sup>34</sup> and PreADMET (<https://preadmet.bmdrc.kr/>).<sup>31</sup> Cardiotoxicity was also predicted via predherg software (<http://predherg.labmol.com.br>).

### In vitro Anti-Diabetic Activity

#### Sample Preparation

FA2 compound was synthesized at the chemistry department, Government College University Faisalabad (GCUF).<sup>25</sup> The compound was weighed at 1.903 mg, prepared the stock solution (10 mM) into 500 μL DMSO, and kept at room temperature.

### $\alpha$ -Glucosidase Inhibition Assay

The  $\alpha$ -glucosidase inhibition assay was conducted to evaluate the conversion of PNPG into p-nitrophenol by  $\alpha$ -glucosidase and yellow color was measured at 405 nm via spectrophotometer.<sup>35</sup> For this assay, acarbose was used as a reference compound and the reactions were conducted on 96 well plates. A mixture of 10  $\mu$ L of 10 mM test sample (FA2) and 40  $\mu$ L of 0.5 U  $\alpha$ -glucosidase from *S. cerevisiae* (sigma Aldrich, Merck) was prepared in 100  $\mu$ L of 1x PBS (pH 7.4) and reaction plate was incubated at 37 °C for 5–10 minutes. After incubation, 40  $\mu$ L of 5 mM p-nitrophenyl- $\alpha$ -D-glucopyranoside (PNPG, sigma Aldrich, Merck) was added as a substrate to start the reaction and kept at 37 °C for 20–30 minutes. The reaction was ended by using 40  $\mu$ L of 1 M sodium carbonate. The quantity of p-nitrophenol released from PNPG was observed at 405 nm via spectrophotometer (Bio-Tek with GEN5 software). The percentage inhibition was calculated with the following formula:

$$\% \text{Inhibition} = \frac{[(\text{Absorbance}(\text{control}) - \text{Absorbance}(\text{Sample}))]}{\text{Absorbance}(\text{Control})} \times 100 \quad (1)$$

In the control reaction, DMSO was used instead of the sample. The 50% inhibition concentration (IC<sub>50</sub>) of FA2 and acarbose were calculated with the microdilution method and analyzed results through Microsoft Excel software 2013.

### $\alpha$ -Amylase Inhibition Assay

The spectrophotometric method was used to perform  $\alpha$ -amylase inhibition assay in 96 well plates<sup>35</sup> with slight modifications. A mixture of 40  $\mu$ L of 2 U  $\alpha$ -amylase from porcine pancreas (Sigma Aldrich, Merck) and 1  $\mu$ L of 10 mM FA2 solution (or 10  $\mu$ L of PBS as a control) was incubated at 37 °C for 5–10 min. After incubation, 40  $\mu$ L of 1% (w/v) soluble potato starch (Sigma Aldrich, Merck) solution (prepared in 20 mM PBS, pH 6.9) was added as a substrate. The mixture was kept at 37 °C for 20–30 minutes and stopped by adding 40  $\mu$ L of 3,5-dinitrosalicylic acid (DNS). The reaction mixture was kept at 95 °C for 5 min followed by rapid cooling. Acarbose was used as a reference, and reading was taken at 540 nm wavelength.

The following formula was used to calculate the percentage inhibition:

$$\% \text{Inhibition} = \frac{[(\text{Absorbance}(\text{control}) - \text{Absorbance}(\text{Sample}))]}{\text{Absorbance}(\text{Control})} \times 100 \quad (2)$$

In the control reaction, DMSO was used instead of the test sample. The 50% inhibition concentration (IC<sub>50</sub>) of FA2 and acarbose were calculated with the microdilution method and analyzed using Microsoft Excel software 2013.

### DPPH Activity

The free radical donating activity of the FA2 compound was measured using DPPH (1,1-diphenyl-2-picrylhydrazyl) radical scavenging method as described by Okawa, Kinjo<sup>36</sup> with some modifications and carried out in 96 well plates. A reaction mixture contained 10  $\mu$ L of 500  $\mu$ M FA2 (prepared in DMSO) and 190  $\mu$ L of 300  $\mu$ M DPPH (Sigma, Merck) was placed in an incubator at 37 °C for 30 minutes. After that plate was read at 517 nm wavelength via a microtiter plate reader (Molecular Devices, USA). Ascorbic acid was used as a positive control.

The following formula was used to calculate the percentage inhibition:

$$\% \text{Inhibition} = \frac{[(\text{Absorbance}(\text{control}) - \text{Absorbance}(\text{Sample}))]}{\text{Absorbance}(\text{Control})} \times 100 \quad (3)$$

In control, DMSO was added instead of the test sample. The experiment was conducted in the dark and in triplets (n=3). The 50% inhibition concentration (IC<sub>50</sub>) of FA2 and ascorbic acid were calculated with the microdilution method and analyzed through Microsoft Excel software 2013.

## MTT Assay

### Cell Culture

The human hepatocarcinoma cell line (HepG2) cells (ATCC, Manassas, USA) were cultured in Dulbecco's modified Eagle medium (DMEM) containing 10% fetal bovine serum (FBS) and 1% streptomycin–penicillin (100 units/mL and 1% 100 µg/mL) at 37 °C with 5% CO<sub>2</sub> incubator. Cell lines were treated with compounds in DMSO (< 1%).

### Determination of Cell Viability

The cytotoxicity was checked via MTT assay. HepG2 cells were grown in 96-well plates with 100µM and 200 µM dilutions of FA1. The plate was incubated at 37 °C for overnight. The next day, the treated plate with 80–90% confluency was used for treatment with 20 µL of MTT (5 mg/mL) and kept at 37 °C for 240 min. After incubation, formazan crystals were dissolved in 100 µL of control (DMSO). The absorbance was quantified in a microplate reader at the test wavelength of 570 nm, and a reference wavelength 630 nm. The percentage cell viability was calculated using the following formula:

$$\% \text{cell viability} = \frac{[(\text{Absorbance at 570 nm}(\text{test sample}) - \text{Absorbance at 630 nm}(\text{test Sample}))]}{\text{Absorbance at 570 nm}(\text{Control}) - \text{Absorbance at 630 nm}(\text{Control})} \times 100 \quad (4)$$

## Inhibitory Kinetics Study

Lineweaver Burk equation was used to describe the inhibition mechanism of the compound FA2 against  $\alpha$ -glucosidase and  $\alpha$ -amylase. For this, inhibitor [V] and substrate [S] were used as variables, and enzyme concentration was constant. In case of  $\alpha$ -glucosidase, the concentration of substrate (PNPG) was used as 1, 3, 5, 7 and 9 mM and the concentration of potato starch as substrate was used as 0.25%, 0.5%, 1%, 1.5% and 2% for  $\alpha$ -amylase. The concentration of inhibitor (FA2) was used as 0.1 mM, 0.25 mM, 0.5 mM, 0.75 mM, 1 mM. The inhibition mechanism and Km value were calculated through Lineweaver-Burk and Michaelis Menten equations.

$$V_0 = \frac{V_{\max}[S]}{K_m + [S]}$$

$$\frac{1}{v_0} = \frac{K_m + [S]}{V_{\max}[S]} = \frac{K_m}{v_{\max}} \frac{1}{s} + \frac{1}{v_{\max}}$$

Vo =initial reaction rate,

Km =Michaelis Menten constant,

Km/Vmax =slope,

1/Vmax =-intercept, and

S = substrate

The secondary graph was plotted to evaluate the inhibition constant and intersecting line between Y-intercept and [V] was represented the Ki and Ki' values. GraphPad Prism software was used to study the enzyme kinetics.

## In vivo Anti-Diabetic Activity

### Animals

This study used albino laboratory-bred mice (strain balb/c) as an anti-diabetic model. For this purpose, mice were categorized into five groups, and each group contained five mice (25–40 g). Group 1 was designated for untreated mice (positive control). Group 2 comprised alloxan-induced mice without treatment (diabetic control). Group 3 contained diabetic mice treated with acarbose (reference control). Group 4 represented diabetic mice treated with FA2 compound (single dose). Group 5 had diabetic mice treated with FA2 (double dose). The mice were subjected to overnight hunger conditions and the initial fasting blood glucose was measured from the mice's tail vein. Alloxan was dissolved in normal saline, and intraperitoneal dose of alloxan (120 mg/kg) was induced in overnight starved mice. The mice were fed in hygienic polypropylene cells and kept in a temperature-controlled animal house, faculty of pharmacy, GCUF (approved from ethical review committee) that was well-ventilated and had a steady 12 hours light/dark schedule. A higher level of

blood glucose (hyperglycemia) was detected after 72 hours and only those mice with > 250 mg/dl blood glucose concentration were selected for the further ant-diabetic study. During the experiment, acarbose and test compound (FA2) were dissolved in sterile normal saline and injected into mice. Fasting blood glucose level in fasting mice was measured from the mice's tail vein on the first, seventh, fourteenth-, and twenty-first days during treatment.

### Assessment of Blood Glucose Level

The blood sample was taken from the mouse tail vein, and blood sugar level was measured via glucose oxidase-peroxidase reactive strips and a glucometer (on call-ACON LABS INC.).

### Biochemical and Histological Examinations

On day 21, mice were anesthetized with chloroform and whole blood from the anesthetic mouse's submandibular was collected in BD vacutainer plus blood collection tubes (K2EDTA) and serum separator tubes (SST). HbA<sub>1c</sub>, insulin and cholesterol were measured via VITRO SCIENT kit, and triglycerides and creatinine were checked through Clinical Chemistry Reagents SRL (CCR) kit. These parameters were studied in controls and treated diabetic mice under Anim diagnostic and collection center, Pakistan.

About 2 mice from each group were dissected via a dorsal midline incision, removed organs (pancreas, kidneys, and liver), and fixed in 10% formalin. After fixation, selected organs were embedded with paraffin and sliced to obtain 6µm thick paraffin sections. These slices were stained with Harris, hematoxylin, and eosin (H&E). The stained tissue samples were examined under the compound microscope at 100X resolution.

### Statistical Analysis

Comparison of the control group with the treated group was assessed through two-way ANOVA through GraphPad prism v 8.0.2. The P-value < 0.05 indicates that the data is more significant than the control group. All data were analyzed in triplicate (n=3).

## Results

### Chemistry

<sup>1</sup>H NMR (600 MHz, DMSO-d<sub>6</sub>), δ: 4.04 (s, 2H), 7.06 (t, 1H, J = 7.80 Hz, Ar-H), 7.18–7.24 (m, 3H, Ar-H), 7.55 (d, 1H, J=7.9 Hz, Ar-H), 7.63–7.72 (m, 3H, Ar-H), 7.87–7.89 (m, 2H, Ar-H), 8.03–8.14 (m, 3H, Ar-H), 9.49 (br-s, 1H, NH). <sup>13</sup>C NMR (150 MHz, DMSO-d<sub>6</sub>) δ: 53.9 (1C), 113.0 (1C), 116.2 (1C), 118.0 (1C), 122.0 (1C), 125.0 (1C), 127.1 (1C), 128.4 (1C), 128.70 (2C), 128.74 (2C), 130.1 (1C), 132.9 (1C), 133.0 (1C), 134.0 (1C), 135.2 (1C), 138.4 (1C), 139.1 (1C), 147.8 (1C), 165.6 (1C), 168.0 (1C), 189.2 (1C). LC-MS (ESI) m/z calcd. for C<sub>23</sub>H<sub>17</sub>BrN<sub>2</sub>O<sub>5</sub>S [M+H]<sup>+</sup>, 513.00; found, 513.08 (see [Figures S1–S3](#)).

### Molecular Docking

In silico analysis revealed that the FA2 has the best RMSD value and binding energy against 2ZE0 and 1B2Y proteins. The relative molecular docking analysis has been performed for acarbose as a control and FA2 as a potent antidiabetic inhibitor. The top three complexes were selected based on the highest binding residue interactions, drug properties, and highest binding energy. The docking results against FA2 showed significant binding energies as -7.2 kcal mol<sup>-1</sup> and -6.6 kcal mol<sup>-1</sup> for 2ZE0 and 1B2Y, respectively, compared with acarbose ([Table 1](#)). Almost all the docked compounds are bound on a similar binding pocket as the control ([Figure 2](#) and [Table 1](#)).

### Druglikeness and ADMET Analysis

The drug-like properties of the FA2 compound were evaluated in silico based on RO5 and FA2 compound showed < 500 molecular weight, < 10 nON, < 5 nOHNH, and ~5 miLogP, as mentioned in [Table 2](#). The FA2 compound fulfilled the RO5 and showed excellent drug-likeness characteristics compared to acarbose. Acarbose showed some variations of the RO5 ([Table 2](#)).

**Table 1** Docking Results of  $\alpha$ -Glucosidase and  $\alpha$ -Amylase (2ZE0 and 1B2Y)

Compound	2ZE0 ( $\alpha$ -Glucosidase)			1B2Y ( $\alpha$ -Amylase)		
	Binding Energy	RMSD (Å)	Interacting Residues	Binding Energy	RMSD (Å)	Interacting Residues
<b>Acarbose</b>	-7.4 kcal mol <sup>-1</sup>	4.8	TRY 299, SER323, PHE 325, ASP 159, GLU 202	-7.1 kcal mol <sup>-1</sup>	4.01	ALA 416, LEU 288, ASP 284, ARG 417, SER 329, GLT 377
<b>FA2</b>	-7.0 kcal mol <sup>-1</sup>	4.2	TYR 299, PHE325, VAL 302, TRP 329, ALA 203, TRP207	-6.6 kcal mol <sup>-1</sup>	3.1	ALA 416, ASP 284, LEU 288, ARG 417, SER 378

The pharmacokinetics parameters were evaluated via PreADMET and SwissADME online tools to predict the ADME and toxicity (T) of the FA2 compound (Table 3). The data mentioned in Table 3 were collected from both online tools. These parameters were evaluated based on the solubility, human gastrointestinal absorption, blood–brain barrier interactions, P-glycoprotein, and cytochrome P450 (CYP450) inhibitors. The FA2 compound did not cross the blood–brain barrier and showed high gastrointestinal absorption and inhibitor of enzyme CYP450 as compared to the reference compound (acarbose). Toxicity also showed that FA2 compound fulfilled the safety criteria like Ames toxicity, lethal dose limit (LD50) (Table 3), and cardiotoxicity, as shown in Figure 3. Pred-hERG results showed that FA2 compound was non-cardiotoxic with 60% confidence.

### $\alpha$ -Glucosidase and $\alpha$ -Amylase Inhibition Activities

The synthetic compound FA2 was screened and checked for its inhibitory effect on  $\alpha$ -glucosidase,  $\alpha$ -amylase and DPPH activity at 500  $\mu$ M concentration (Table 4). The FA2 compound was a potent inhibitor of  $\alpha$ -glucosidase and  $\alpha$ -amylase with 69.4% and 70.8% inhibition.

It was found that FA2 inhibited  $\alpha$ -glucosidase and  $\alpha$ -amylase activities in a dose–response manner. Both enzymatic activities were significantly ( $p < 0.05$ ) decreased with the increment of FA2 concentration. In the case of  $\alpha$ -glucosidase activity, compound FA2 and acarbose (reference control) showed 50% inhibition at  $18.82 \pm 0.89 \mu$ M and  $58.8 \pm 2.69 \mu$ M concentrations, respectively. Similar results were also observed with the  $\alpha$ -amylase inhibition assay. Synthetic compound FA2 was inhibited at  $5.17 \pm 0.28 \mu$ M concentration with compared to acarbose. The results showed that synthetic compound FA2 has significant inhibition against  $\alpha$ -glucosidase and  $\alpha$ -amylase compared with acarbose.

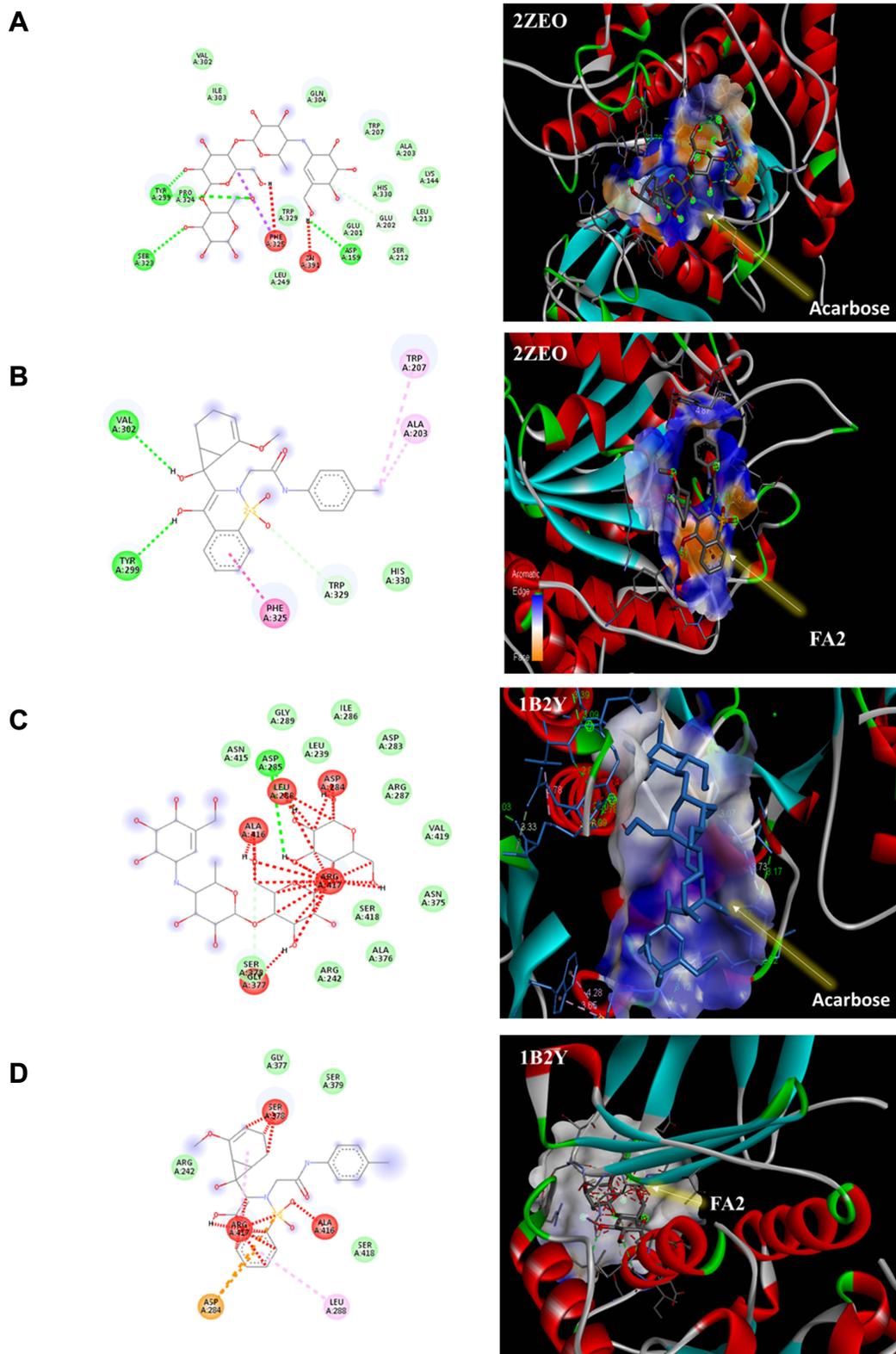
The antioxidant capacity of the FA2 compound was evaluated via DPPH assay. It was found that FA2 showed non-significant results compared with ascorbic acid (control).

### Cytotoxicity Analysis

The cytotoxicity of tested compound FA2 was checked on HepG2 cell line. FA2 compound showed no toxicity when compared with control (DMSO) as shown in Figure 3. The percentage cell viability of different concentrations (100  $\mu$ M, 200  $\mu$ M, 400  $\mu$ M and 500  $\mu$ M) of FA2 was checked and maximum cell viability (98–80%) was observed at different concentrations of FA2 compound.

### Kinetics Study

The type of inhibition against  $\alpha$ -glucosidase and  $\alpha$ -amylase by synthetic compound FA2 was examined via the Lineweaver-Burk equation. According to the Lineweaver-Burk equation, the graph was plotted between the reciprocal of inhibitor velocities  $[1/V]$  on the y-axis and the reciprocal of substrate concentrations  $[1/S]$ . The results showed that FA2 compound inhibited  $\alpha$ -glucosidase and  $\alpha$ -amylase in a non-competitive manner (Table 5). Secondary graphs were plotted to calculate the inhibitory constant ( $K_i$ ) and dissociated constant ( $K_i'$ ). FA2 showed  $K_i 0.168 \pm 0.02$  and  $K_i' -0.32 \pm 0.001$  against  $\alpha$ -glucosidase and  $K_i 0.287 \pm 0.07$  and  $K_i' 0.141 \pm 0.01$  against  $\alpha$ -amylase.  $K_i$  value is the indication of affinity for the enzyme inhibitor site, as shown in Figure 4. This type of inhibition shows that FA2 binds



**Figure 2** 3D docking model of acarbose and FA2 against  $\alpha$ -glucosidase (2ZE0: **A** and **B**) and  $\alpha$ -amylase (1B2Y; **C** and **D**); 2D structure representing interaction of molecules with enzyme pocket residues.

**Table 2** Druglikeness Properties of FA2 Compared with Acarbose

Compound	Molecular Weight (g/mol)	Number of HBA* (nON)	Number of HBD** (nOHNH)	MlogP***
Lipinski's rule of five	<500	<10	<5	<5
FA2	513.36	5	2	4.24
Acarbose (Control)	645.61	19	14	-5.51

**Abbreviations:** \*HBA, hydrogen bond acceptor; \*\*HBD, hydrogen bond donor; \*\*\*MlogP, Moriguchi octanol-water partition coefficient.

**Table 3** In silico Pharmacokinetic Analysis: Absorption, Distribution, Metabolism, Excretion and Toxicity of FA2 and Acarbose

Properties	Compounds	Acarbose	FA2
<b>Absorption</b>	Water solubility	Soluble	Moderate
	P-glycoprotein substrate	No	No
	Log Kp (skin permeation) cm/s	-16.29	-6.40
	Blood-Brain Barrier	No	No
	Gastro- Intestinal Absorption	Low	High
<b>Distribution</b>	Subcellular localization	Mitochondria	Mitochondria
<b>Metabolism</b>	CYP 3A2 Inhibitor	No	No
	CYP 2C9 Inhibitor	No	Yes
	CYP 2D6 Inhibitor	No	No
	CYP 2C19 Inhibitor	No	Yes
	CYP 3A4 Inhibitor	No	No
<b>Excretion</b>	Renal OCT2 substrate	No	No
<b>Toxicity</b>	Rat acute toxicity (LD50,mol/kg)	1.46	2.515
	Oral Toxicity classification	VI	III
	Ames toxicity	No	No

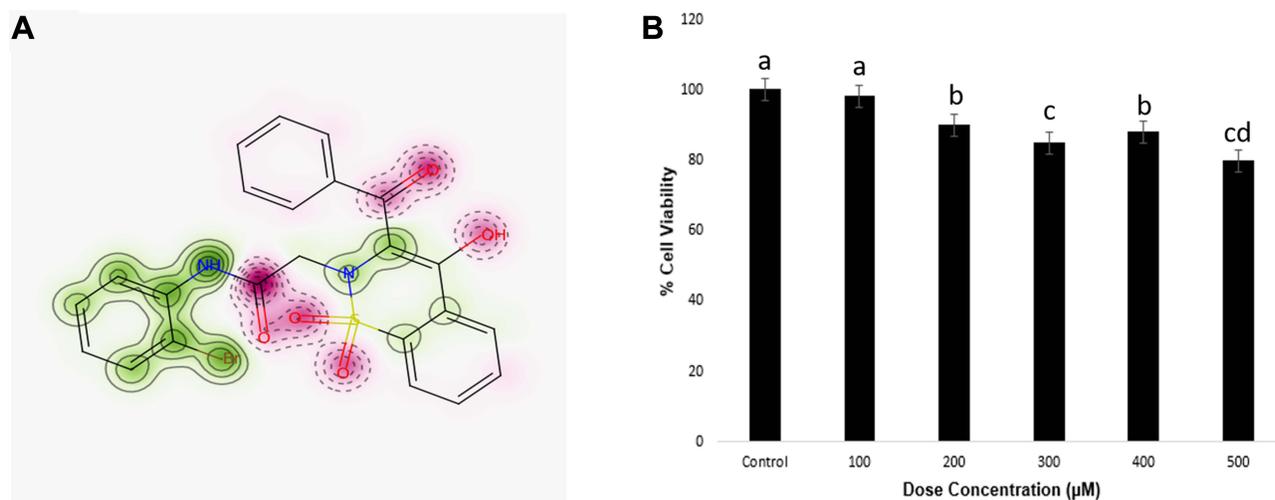
at the allosteric site instead of the active site. Thus, FA2 compound has the potential to bind  $\alpha$ -glucosidase and  $\alpha$ -amylase and inhibit the enzyme activity, whether the substrate is present or not.  $K_i$  and  $K_i'$  values of acarbose against  $\alpha$ -glucosidase and  $\alpha$ -amylase were published earlier and used in this study.<sup>37</sup>

## In vivo Study

### Effect of FA2 Compound Within a Living Body

#### Effect on Fasting Blood Sugar Level (mg/dL)

In the current study, the effect of synthesized compound (FA2) on fasting blood sugar (FBS) levels in alloxan-induced diabetic mice was monitored from Day 1 to day 21 as shown in [Figure 5A](#). Graphic presentation of FBS has demonstrated a comparable decline rate in FBS with the positive control (acarbose) and diabetic control. The maximum decline rate was observed on the 21st day of treatment. As shown in [Figure 5](#), the FA2 compound showed a significant ( $p < 0.05$ ) decline rate in FBS level at high concentration (double dose 2x) when compared with controls.



**Figure 3** Cardiotoxicity prediction of FA2 (A) was analyzed via using computational tool pred-HERG and Cytotoxicity activity of FA2 compound (B) was checked against hepatocarcinoma cell line (HepG2) at different concentrations of FA2 (100  $\mu$ M, 200  $\mu$ M, 300  $\mu$ M, 400  $\mu$ M and 500  $\mu$ M). All data points and error bars represent the mean  $\pm$  SD of three replicates (n=3). Different letters represent significant differences ( $P < 0.05$ ) among various treatments and control (DMSO).

### Biochemical Analysis

To demonstrate the effect of synthesized compounds within a living body, FA2 was injected in alloxan-induced mice from day 1 to day 21 under fasting conditions. Cholesterol, serum creatinine, triglyceride, HbA1C level, and Serum insulin were checked in control, diabetic control, reference control, and treated diabetic animals as shown in Figure 5.

### Effect of FA2 Compound on the Liver (Cholesterol, Triglycerides)

#### Cholesterol and Triglycerides are Two Forms of Lipids Found in the Blood

Triglycerides are fats that store unused calories and supply energy to your body. Cholesterol is required for the formation of cells and the production of some hormones. Normal cholesterol and triglyceride ranges are 200 mg/dL and 40–160 mg/dL, respectively. In this study, cholesterol and triglyceride content in blood were significantly ( $p < 0.05$ ) low in treated animals compared to diabetic control. Among treated groups, FA2 with a high dose showed a significant ( $p < 0.05$ ) decline rate in cholesterol and triglyceride compared with the reference compound acarbose as in Figure 5B and C).

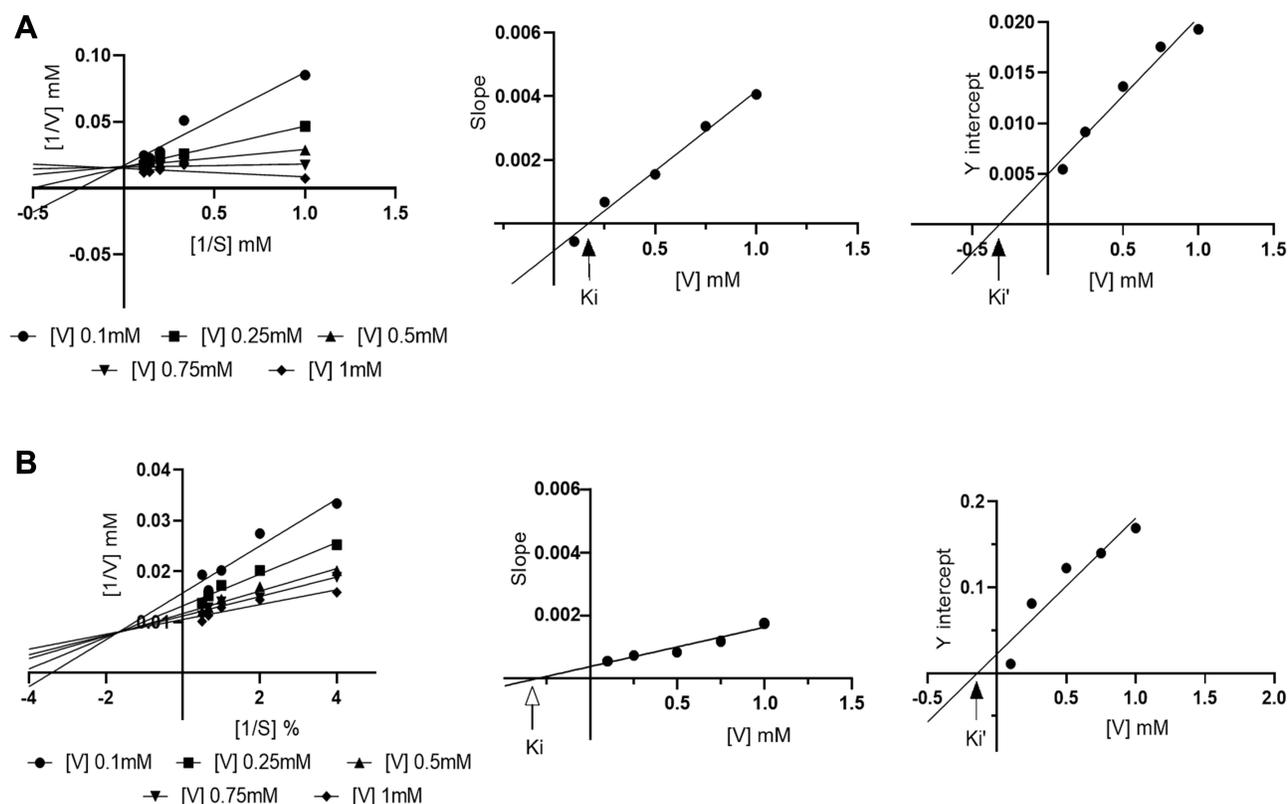
**Table 4** In vitro Anti-Diabetic and Anti-Oxidant Effect of FA2. The Data is Represented in Triplicate (n=3) and Values are Mean  $\pm$  SD. All Data Was Repeated in Triplicate (n=3)

Sample Name	$\alpha$ -Amylase (IC50) $\mu$ M	$\alpha$ -Glucosidase (IC50) $\mu$ M	DPPH Activity
FA2	5.17 $\pm$ 0.28	18.82 $\pm$ 0.89	81.29 $\pm$ 3.9
Acarbose	10.12 $\pm$ 0.42	58.8 $\pm$ 2.69	NA*
Ascorbic Acid	NA*	NA*	78.08 $\pm$ 2.98

Abbreviation: \*NA, not applicable.

**Table 5** Type of Inhibition Against  $\alpha$ -Glucosidase and  $\alpha$ -Amylase

Compounds	Ki	Ki'	Mode of Inhibition
FA2 ( $\alpha$ -glucosidase)	0.168 $\pm$ 0.022	-0.3203 $\pm$ 0.001	Non competitive
FA2 ( $\alpha$ -amylase)	0.2872 $\pm$ 0.07	0.1412 $\pm$ 0.010	Non competitive
Acarbose ( $\alpha$ -glucosidase)	-0.046 $\pm$ 0.015	-0.042 $\pm$ 0.053	Competitive
Acarbose ( $\alpha$ -amylase)	0.896 $\pm$ 0.009	1.67 $\pm$ 0.043	Competitive



**Figure 4** Lineweaver–Burk plots of inhibition kinetics of  $\alpha$ -glucosidase (**A**) and  $\alpha$ -amylase (**B**) by FA2 compound. Each point represents the average of triplicate ( $n=3$ ).

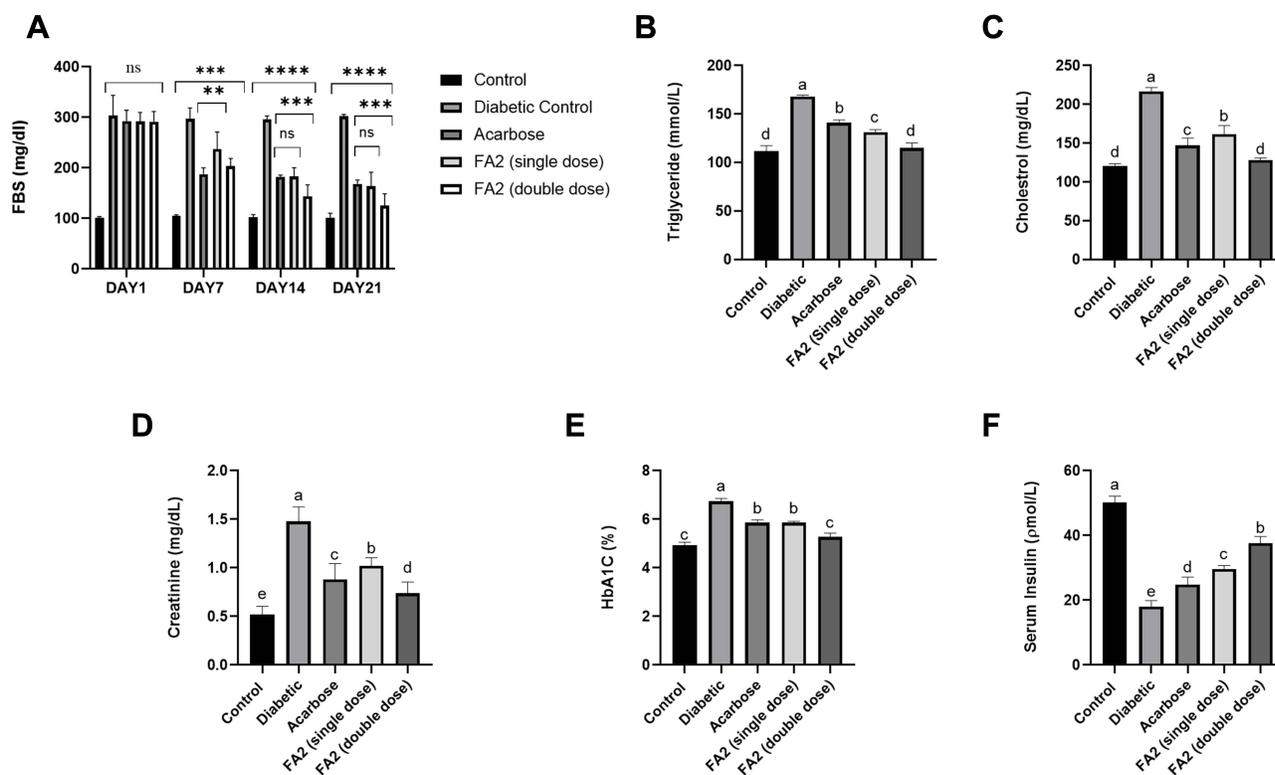
### Effect of FA2 Compound on Kidneys

Creatinine is a byproduct of creatine, a chemical component that helps muscles obtain the required energy. Creatinine is removed from the blood by the renal as a waste product and excreted in the urine. A creatinine test determines the level of this substance in the blood or urine. Normal creatinine range is from 0.8 to 1.4 mg/dL. In the current study, treated animals with acarbose and FA2 compound showed a significant ( $p < 0.05$ ) decrease in creatinine content compared with diabetic control. Furthermore, when treated groups (FA2) were compared with the acarbose, a significant ( $p < 0.05$ ) decrease in creatinine level was also observed in high doses of FA2 (Figure 5D).

HbA1c is a measure of erythrocyte hemoglobin glycation, as erythrocytes have a life span of around 120 days, and it represents total blood glucose levels for 2–3 months. It is also used to monitor diabetic treatment. It has been established that HbA1c is an important complement to regular self-blood glucose testing in achieving the best possible glycemic management. Renal failure can have a variety of effects on HbA1c formation and measurement. When employing some HbA1c assays, nitrogenous waste isocyanate can form carboxylated Hb, which can be indistinguishable from HbA1c. The normal range of HbA1c is up to 6.5%. HbA1c levels in treated animals with FA2 showed a significant ( $p < 0.05$ ) low percentage compared with diabetic control. Treated group FA2 with low dose showed non-significant results, but FA2 with double dose showed significantly ( $p < 0.05$ ) lower HbA1c level when compared with acarbose, as shown in Figure 5E.

### Effect of FA2 Compound on the Pancreas

Insulin is a peptide hormone produced by pancreatic  $\beta$ -cells, and it is the body's principal anabolic hormone. It modulates carbohydrate, fat, and protein metabolism by boosting glucose absorption from the bloodstream into hepatic, fat, and skeletal muscle cells. The normal range of serum insulin is up to 150 pmol/L. In the present study, treated animals showed significant ( $p < 0.05$ ) high serum insulin concentration compared with diabetic control. Synthetic compounds



**Figure 5** Biochemical analysis on alloxan-induced diabetic mice compared with acarbose (A–F). A represents fasting blood sugar level (FBS), \*\* $p < 0.05$ , \*\*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$  (B and C) shows testing triglyceride and cholesterol level, (D and E) represents creatine and HbA1c level in blood and F indicates serum insulin level in blood. All groups were kept in hygienic polypropylene cells and temperature-controlled animal house for 21 days. Data points and error bars represent the mean  $\pm$  SD of five replicates ( $n=5$ ). Different letters represent significant differences ( $P < 0.05$ ) among various treatments and controls.

FA2 with high dose showed significant ( $p < 0.05$ ) high level of insulin in serum ( $38 \pm 2$  pmol/L) compared with acarbose ( $25 \pm 3.1$  pmol/L) as shown in Figure 5F.

## Histological Analysis

### Histology of Liver

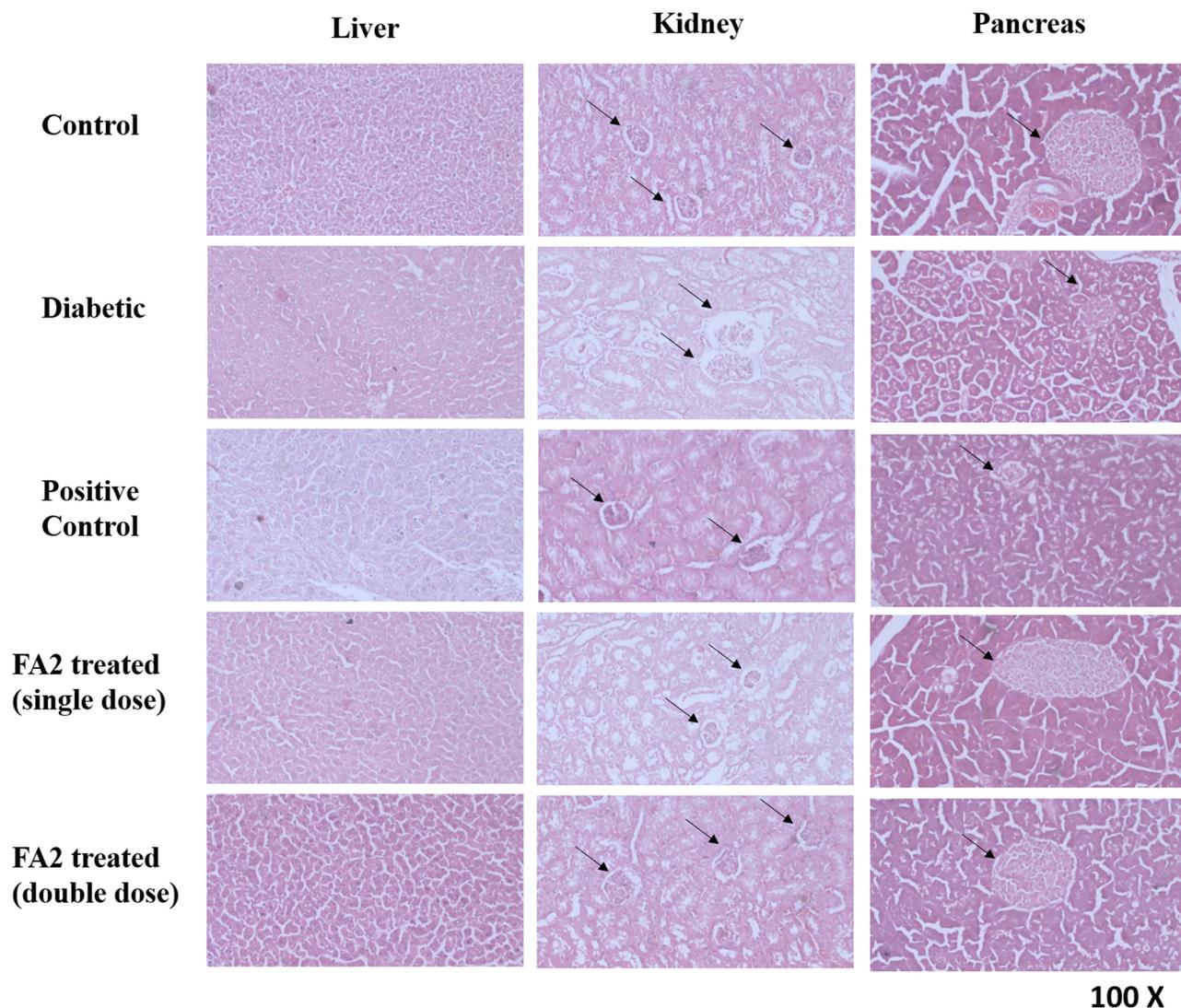
Normal hepatic cells with well-preserved cytoplasm, nucleus, nucleolus, and central vein were observed on micrographs of the liver. All treated groups showed normal lobular structure, prominent central vein and properly congested compared with diabetic control as shown in Figure 6.

### Histology of Kidneys

Histology of kidney (Figure 6) in normal animals showed a normal number of renal capsules, Bowman's space, glomerulus capillary, and the number of mesangium intraglomerular cells compared with diabetic control. A mild increase in glomerulus Bowman's space, decrease in mesangium intraglomerular cells were observed in positive control compared with FA2 compounds. No other significant changes were seen in control and treated animals. However, the treatment groups of FA2 (double dose) showed better glomerulus structure improvements than other groups.

### Histology of Pancreas

Pancreas histology revealed normal pancreatic acinus and islets of Langerhans in normal control. In the diabetic group, extensive damage of pancreatic islets of Langerhans and a reduced number of zymogen granules were observed as shown in Figure 6. A significant improvement in pancreatic islets of Langerhans was observed in FA2 treated mice compared with acarbose treated mice.



**Figure 6** Histopathological studies of synthetic compounds (FA2) on alloxan-induced diabetic mice. All groups were kept in hygienic polypropylene cells and temperature-controlled animal house for 21 days. These micrographs were taken at 100X resolution via a compound microscope. The glomerulus and Langerhans islets were shown via arrow signs in kidney and pancreas micrographs, respectively.

### Post-Experiment Procedures

After treatment, all research animals were anesthetized and disposed of under euthanized procedure approved by NIH and the institutional ethical committee (Ref. No. GCUF/ERC/22 dated 03-12-2021).

### Discussion

Diabetes is a common chronic metabolic disorder characterized by a rise in plasma sugar due to a lack and/or inadequate use of insulin in the body. Treatment for type 2 diabetes requires lowering glycogen synthesis, improving insulin signaling, increasing insulin production from  $\beta$ -pancreatic cells, or inhibiting glucose digesting enzymes such as  $\alpha$ -glucosidase and  $\alpha$ -amylase.<sup>15,38</sup> Thiazines are a class of heterocyclic chemical compounds with many biological activities (anti-diabetic, antifungal, anti-cancer, anti-inflammatory, anti-parasitic, anticonvulsant, and antiviral activities) and play an important role in the treatment of many autoimmune diseases.<sup>16,25</sup> Inhibiting digestive enzymes such as  $\alpha$ -amylase and  $\alpha$ -glucosidase has been suggested to be useful for glycemic control. The present study was designed to screen the potent benzothiazine derivative FA2 via computational tools in vitro screening and in vivo testing. Taj et al,<sup>39</sup> also used molecular docking and

ADMET software to screen out the best potent pyrazolo benzothiazine derivative as  $\alpha$ -glucosidase inhibitor for treating diabetes. The synthetic compounds exhibited significant potential to control the hyperglycemic conditions in diabetic patients as previously reported synthetic derivatives for instance, benzothiazole<sup>40</sup> and pyrrolidines.<sup>41</sup> Taj et al,<sup>37</sup> reported pyrazolo benzothiazine-based derivative as an  $\alpha$ -glucosidase inhibitor at a concentration (IC<sub>50</sub>) of 5.8  $\mu$ M. Rahim et al,<sup>42</sup> also found the potent synthetic benzimidazole derivatives as  $\alpha$ -glucosidase inhibitors.  $\alpha$ -glucosidase and  $\alpha$ -amylase are considered important enzymes in glycemic control and diabetic complications. Selvarasu et al,<sup>43</sup> also found the same results and reported a new synthetic 1-aryl-N-tosyl-1H-tetrazole-5-carboxamide derivative. FA2 showed higher antioxidant activity compared with ascorbic acid. Júnior et al,<sup>44</sup> also reported gallic acid as a good antioxidant and diabetic phenolic compound. Several findings have shown the correlation between the production of free radicals and the pathogenesis of diabetes in the treatment of diabetes.<sup>45–47</sup> Benzothiazine derivatives are considered to have anti-diabetic properties and are used as a therapeutic agents. The enzyme kinetics results also showed that the FA2 compound had non-competitive inhibition with  $\alpha$ -glucosidase/ $\alpha$ -amylase and tended to inhibit the function of the  $\alpha$ -glucosidase.<sup>11</sup> Peng et al,<sup>9</sup> also found mixed-type inhibition to investigate kaempferol for the treatment of diabetes. Rouzbehan et al,<sup>48</sup> also showed the same kinetics results by using medicinal plants and indicated that the non-competitive mode of inhibition might not be affected the activity of the inhibitor in the presence of high substrate dose. Because there is no competition between substrate and inhibitor, a little concentration of inhibitor is enough to maintain the glucose level.<sup>49,50</sup> The role of FA2 was investigated in regulating critical biological pathways involved in type 2 diabetes and the potential of selective benzothiazine agonists as a new therapeutic approach to treat this illness in this work. FA2 compound significantly lowered the blood cholesterol and triglyceride level compared to acarbose in the alloxan-induced diabetic mice model (balb/c strain). Documenting high levels of cholesterol and triglyceride in the blood is considered a direct driver of the thickening of arteries in T2DM patients.<sup>51</sup> During diabetes, the liver absorbs spare fatty acids, esterification with glycerol phosphate, and deposits them as triglycerides. This causes a decline in insulin-mediated metabolic activity and may cause diabetes and other metabolic disorders.<sup>52</sup> Studies on acarbose, sulfonylureas, and metformin showed that these anti-diabetic agents significantly affect lipid profile at high doses<sup>53</sup> and do not rescue the pancreatic  $\beta$ -cells for continual distortion.<sup>54</sup> The FA2 compound showed better results in maintaining the cholesterol, triglyceride, creatinine, and HbA<sub>1c</sub> level than acarbose at the same concentration in the present study. Histopathology of the liver also showed that the FA2 compound has no side effects on hepatic cells. Histology of kidneys also showed that renal capsules and glomerulus structure in FA2 case had the better condition than acarbose treated mice. It has been reported that renal nephropathy is strongly associated with DM.<sup>55</sup> Diabetes-related nephropathy may cause renovascular lesions, renal medulla hypoxia, and cell death due to insufficient oxygen supply to the glomerulus and renal medulla. The decreased oxygen supply alters the stability of the hypoxia-inducible factor (HIF), which may induce kidney fibrosis in diabetic patients.<sup>56,57</sup> Insulin regulates blood glucose by directing the liver, muscle tissue, and adipocytes to absorb glucose from the blood. Hyperglycemia condition is linked with insulin resistance and ketoacidosis, causing abnormal absorption of glucose and the development of advanced glycation end products. This process eventually results in pancreatic beta-cell production dysfunction and cell death.<sup>54</sup> Present study investigated that benzothiazine derivative showed significant effects in maintaining the level of insulin compared with acarbose. The histography also showed that pancreatic  $\beta$ -cells are more prominent in FA2 compound treated mice compared to acarbose treated mice.

## Conclusions

The targeted FA2 compound was synthesized with maximum yield. The anti-diabetic activity of FA2 compound was screened against  $\alpha$ -glucosidase and  $\alpha$ -amylase, and FA2 showed the best antidiabetic activity. Moreover, it also fulfilled drug safety criteria via cytotoxicity, ADMET, and cardiotoxicity. This compound has the potential to bind with the allostatic site of enzymes to inhibit the enzyme's activity. In vivo diabetic mouse model study showed that FA2 could be safe for body tissues and histographics showed no significant side effects to pancreatic beta cells. Current research suggests that the FA2 compound is potentially safe for managing the hyperglycemic impact on diabetic patients and other diabetic-related complications. However, more studies and experiments are required to narrow down the effects of FA2 at the gene level, explore related metabolic pathways, and develop advanced biochemical testing to fulfill the pharmacological checklist.

## Institutional Review Board Statement

The animal study protocol was approved by the Ethical Review Committee of Government College Faisalabad (Ref. No. GCUF/ERC/22 dated 03-12-2021) for studies involving animals. NIH guidelines were followed for the welfare of the laboratory animals.

## Author Contributions

All authors made a significant contribution to the work reported and took part in drafting, revising, or critically reviewing the article; gave final approval of the version to be published; have agreed on the journal to which the article has been submitted; and agree to be accountable for all aspects of the work.

## Disclosure

The authors declare no conflicts of interest in this work.

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