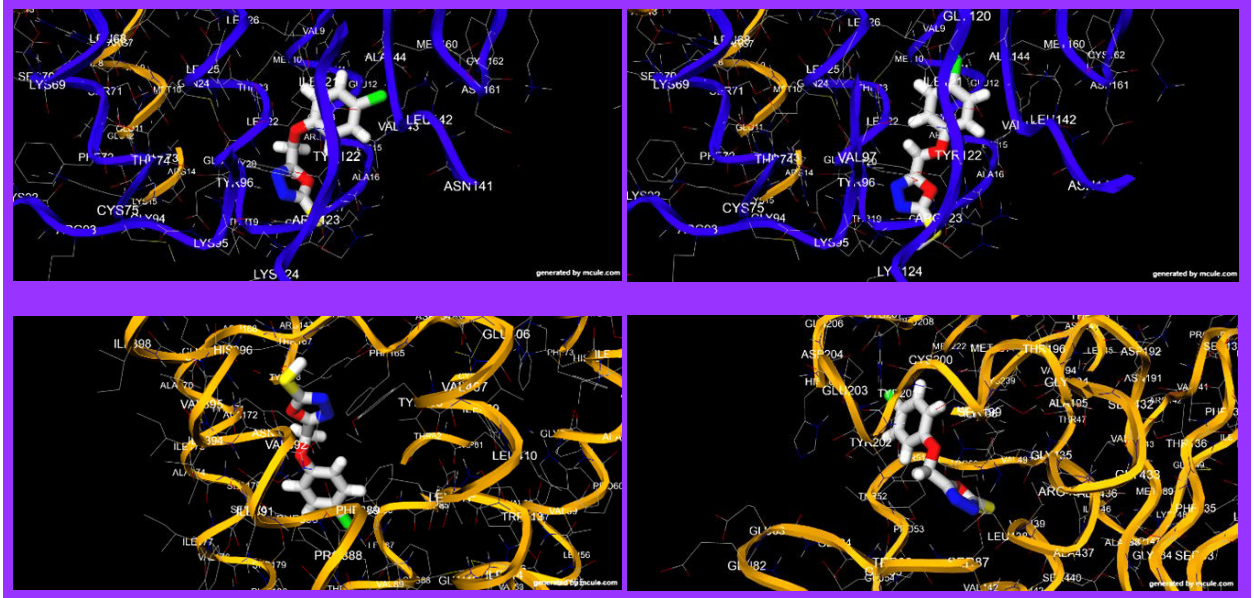

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Hypoglycemic, hepatoprotective and molecular docking studies of 5-[(4-chlorophenoxy) methyl]-1,3,4-oxadiazole-2-thiol

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Abstract

The present study aimed at the evaluation of anti-hyperglycemic and hepatoprotective potential of a new drug candidate, 5-[(4-chlorophenoxy) methyl]-1,3,4-oxadiazole-2-thiol (OXCPM) through *in vitro* and *in vivo* assays, respectively. The compound displayed excellent dose-dependent α-amylase (28.0-92.0%), α-glucosidase (40.3-93.1%) and hemoglobin glycosylation (9.0%-54.9%) inhibitory effects and promoted the uptake of glucose by the yeast cells (0.2 to 26.3%). The treatment of the isoniazid- and rifampicin- (p.o., 50 mg/kg of each) intoxicated rats with OXCPM (100 mg/kg, p.o.) resulted in restoring the normal serum levels of the non-enzymatic (total bilirubin, total protein and albumin) and bringing about a remarkable decrease in the levels of enzymatic (alanine transaminases, aspartate transaminases and alkaline phosphatase) biomarkers. The molecular docking studies indicated high binding affinity of the compound for hyperglycemia-related protein targets; fructose-1,6-bisphosphatase, beta$_2$-adrenergic receptors and glucokinase. The results indicate that OXCPM may not only reduce hyperglycemia by enzyme inhibition but also the disease complications through protection of hemoglobin glycosylation and hepatic injury.

Introduction

Diabetes mellitus is one of the most common and prevalent endocrine disorders, characterized by chronic hyperglycemia due to relative or absolute deficiency of insulin (Alberti and Zimmet, 1998). The American Diabetes Association classify diabetes mellitus into four types i.e. type 1 or insulin dependent diabetes mellitus (IDDM) (genetic deficiency in insulin production as a result of allergic reactions which destroy the pancreatic β-cells), type 2 or non-insulin dependent diabetes mellitus (nIDDM) (combined resistance to insulin-action and insulin-secretory response), type 3 or gestational (carbohydrate intolerance with first recognition during pregnancy) and type 4 (genetic defect- or medication-induced). Among all the types, nIDDM accounts for approximately 90% of the diabetes cases globally (ADA, 2005).

A stepped approach towards management of nIDDM starts with non-pharmacological interventions – dietary manipulations, exercise and average weight reduction – and ends at pharmacotherapies to achieve optimum metabolic control (Venn and Mann, 2004). Some conventionally available treatment options include insulin, amylin analogs (pramlintide), insulin pump- and islet-transplantation, inretin mimetic (exenatide) and oral hypoglycemic agents such as insulin secretagogues

(sulfonylureas and meglitinides), insulin sensitizers (biguanides and thiazolidinediones), α-amylase and α-glucosidase inhibitors (acarbose and miglitol), and dipeptidyl peptidase IV inhibitors (sitagliptin) (Rang et al., 2003).

To date, oral hypoglycemic agents are used either alone or in combination with insulin to control post-prandial hyperglycemia (PPH), however, being non-specific, such agents produce various side effects including abdominal fullness and pain, bloating, flatulence, diarrhea, hepatic injury and renal tumors (Konya et al., 2015; Fujisawa et al., 2005). Furthermore, these oral hypoglycemic agents fail to alleviate the disease's complications. This situation warrants the development of some alternative oral anti-hyperglycemic agents devoid of severe adverse effects.

The compound, 5-[(4-chlorophenoxy) methyl]-1, 3, 4-oxadiazole-2-thiol (OXCPM) (Figure 1), contains a pharmacophore (oxadiazole) that is extensively reported for a broad spectrum of biological activities. Moreover, our findings on the bioactivity of the compound established through ligand-based VS depicted its potential as ion-channel modulator and enzyme inhibitor (Shehzadi et al., 2016). More specifically, it is predicted to target various diseases-classes chiefly those involving oxidative stress, inflammation, metabolic disorders, uncontrolled growth and microbial infections. In continuity to our quest for developing the compound as a novel drug candidate, the present study describes anti-hyperglycemic, hepatoprotective and molecular docking studies of OXCPM.

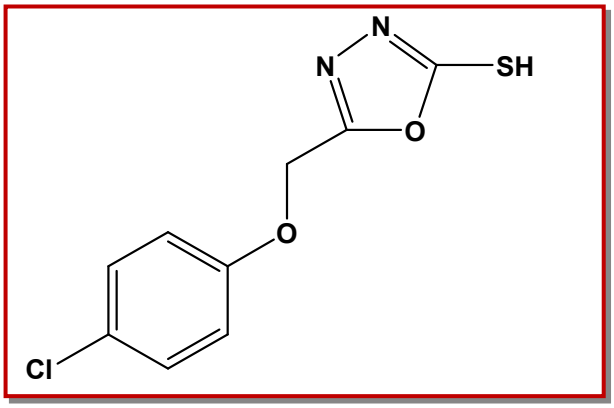

Figure 1: Chemical structure of 5-[(4-chlorophenoxy) methyl]-1, 3, 4-oxadiazole-2-thiol (OXCPM)

Materials and Methods

Chemicals, solvents and other supplies

The materials used included analytical grade phosphate buffer saline tablets (PBS404.100, BioShop Canada Inc., Burlington), sodium hydroxide, potassium iodide, potassium dihydrogen phosphate and sodium dihydrogen phosphate (Merck KG aA, Germany), 3, 5-dinitrosalicylic acid (DNS), disodium hydrogen orthophosphate, hydrochloric acid and sodium chloride (BDH Laboratory Supplies, England), α-amylase (*Bacillus* sp., 3-5 U/mg, UNI-CHEM Chemical Reagents, Beograd), D-(+) anhydrous glucose (Riedel-deHaen AG. D-3016, Germany), bovine RBC's hemoglobin powder (94%, RM238 -100G) by HiMedia Laboratories Pvt. Ltd., India, yeast, ascorbic acid, α-glucosidase and p-nitrophenyl-glucopyranoside (Sigma-Aldrich Chemie, Germany), acarbose (Glucobay®, 50 mg, Bayer Pakistan Ltd, Pakistan), gentamicin (Refobacin®, 40 mg/mL, Merck Pharmaceuticals Ltd., Pakistan), and metronidazole (400 mg, Sanofi-Aventis Ltd., Pakistan). Solvents used in the study included methanol (HPLC grade, Tedia Com-pany, Inc., USA) and in-house prepared double distilled water. The compound (OXCPM) was synthesized and gifted by SZS.

α-Amylase inhibition assay

The method of Xiao et al. (2006) based on the starch-iodine test was used to evaluate α-amylase inhibition potential of the compound. A reaction mixture composed of 1 mL of each of sodium phosphate buffer (20 mM, pH 7.2, containing 6.7 mM sodium chloride), 1 unit/mL of α-amylase solution in buffer and sample in concentration ranges from 0.1-1.0 mg/mL, was incubated at 37°C for 20 min followed by addition of potato starch (1%, w/v). After an incubation time period of 15 min at 37°C, 1 mL of 1 N hydrochloric acid was added in the mixture to stop the enzyme action followed by the addition of 5 mL of iodine reagent (5 mM iodine in 5 mM potassium iodide). A dark-blue color of the reaction mixture indicated the presence of starch whilst a yellow/brown color indicated the absence/partially hydrolyzed starch. The absorbance of the colored mixture was recorded at 620 nm against DDW as the blank. The positive control consisted of acarbose (1.0 mg/mL) and negative control representing 100% enzyme activity contained methanol in place of the compound. The percent inhibition of amylase was calculated using the following formula:

$$\%\text{Anti-amylase activity} = \frac{\text{Sample} - \text{Control}}{\text{Sample}} \times 100$$

α-Glucosidase inhibition assay

The assay was performed according to the method of Abbasi et al. (2005) with slight modifications. Briefly, a reaction mixture containing 70 µL of phosphate buffer saline (50 mM, pH 6.8), 10 µL of different concentrations of the test compound (0.003-0.200 mg/mL) and 10 µL of α-glucosidase was incubated for 10 min at 37°C and the absorbance was recorded at 400 nm. The reaction was initiated by addition of 10 µL of 0.50 mM p-nitrophenyl glucopyranoside and after incubation at 37°C for further 30 min, the absorbance was recorded. Acarbose was used as a standard anti-glucosidase

agent. The percent inhibition was calculated by using the following formula:

$$\%\text{Anti-glucosidase activity} = \frac{\text{Control - Sample}}{\text{Control}} \times 100$$

IC_{50} of the sample was calculated using EZ-FIT enzyme kinetic software (Perrella Scientific Inc., USA).

Glucose uptake by the yeast cells

Yeast cells suspension was prepared as described by Cirillo (1962). Briefly, commercial baker's yeast was washed with ice-cold doubled distilled water by repeated centrifugation at 4,200 rpm for 5 min until the supernatant was clear. The packed cells were reconstituted and a 10% (v/v) suspension was prepared in doubled distilled water.

Then, the method of Bhutkar and Bhise (2013) was used for determination of the activity of OXCPM in promoting glucose uptake by the yeast cells. Briefly, one milliliter of different test concentrations of OXCPM (0.1-1.0 mg/mL) were mixed with equal volume of glucose solution (2, 10 and 20 mg/mL) and incubated at 37°C for 10 min. One hundred milliliters of yeast cell suspension (10%, v/v) was added in the above mixture, vortex for 5 min and incubated at 37°C for further 60 min followed by centrifugation at 3,800 rpm for 5 min. The content of glucose in the supernatant was estimated by DNS method (1 mL of supernatant + 1 mL DNS heated at 85°C for 5 min). The absorbance of the colored mixture was recorded at 540 nm against doubled distilled water as the blank. Metronidazole, at concentration level 1.0 mg/mL, was used as a standard and methanol in the place of the sample/standard served as the negative control. The percent increase in glucose uptake by the yeast cells was calculated using the following formula:

$$\%\text{Increase in glucose uptake} = \frac{\text{Control - Sample}}{\text{Control}} \times 100$$

Non-enzymatic hemoglobin glycosylation

Determination of optimum conditions for estimation: To determine the optimum conditions (incubation time and glucose con-centration) for estimation of the degree of hemoglobin glycosylation, the method of Parker et al. (1981) modified by Adisa et al. (2010) was used. Briefly, one milliliter both, glucose (1-20 mg/mL) and hemoglobin (0.6 mg/mL), prepared in phosphate buffer saline (pH 7.4) were mixed together and incubated in dark at room temperature for 72 hours. The mixture containing buffer in place of glucose served as the control. The amount of hydroxymethyl furfural released upon reaction of glucose with hemoglobin was estimated by measuring the absorbance of the mixture at 443 nm at different incubation periods (0, 24, 48 and 72 hours) against distilled water as the blank.

Physiological glucose concentrations: A mixture containing equal volume of hemoglobin (0.6 mg/mL), gentamycin (0.2 mg/mL), glucose (1-20 mg/mL) and sample/standard (1.0 mg/mL, OXCPM/ascorbic acid) was incubated in the dark at room temperature for 72 hours. The concentration of glycolsylated hemoglobin was determined from the absorption of the reaction mixture at 443 nm at 0, 24, 48 and 72 hours.

Concentration-dependent effect: Concentration-dependent activity of the compound (0.1-1.0 mg/mL) at three glucose concentrations viz. 2, 10 and 20 mg/mL, was evaluated by the method described above. The decrease in the amount of hydroxymethyl furfural release was estimated after 72 hours of incubation. Ascorbic acid, at concentration 1.0 mg/mL, was used as standard and the reaction mixture containing methanol in place of sample/standard was served as the negative control.

Hepatoprotective activity

Twenty four male Wistar rats, aged 15 weeks, weight 190 ± 15 g, were obtained from the Animal House, Punjab University College of Pharmacy, University of the Punjab, Lahore, Pakistan. The animals were allowed to acclimatize to the laboratory conditions i.e. 12 hours light/dark cycle at room temperature with constant humidity for one week. During the housing, standard pellet diet was provided and tap water was supplied *ad libitum*. Before experimentation, the animals were randomly distributed into four groups, six rats each. Group I served as the un-treated vehicle control and received double distilled water; Group II served as the intoxicated control and received hepatotoxic drugs (isoniazid + rifampicin, 50 mg/kg of each, p.o.); Group III served as a standard and received hepatotoxin + silymarin (50 mg/kg each + 200 mg/kg, p.o.); Group IV served as the test group and received hepatotoxin + OXCPM (50 mg/kg each + 100 mg/kg, p.o.). The hepatotoxin and the standard/test drugs or double distilled water were administered to the animals once daily, at different hours, for a period of 30 days (Sankar et al., 2015).

After the completion of experimentation period, the animals were given a short fasting period of 5 hours and anesthetized by diethyl ether to collect blood by cardiac puncture. The serum was separated from the blood by centrifugation at 3,000 rpm for 15 min and stored for biochemical analysis including alanine transaminases (ALT), aspartate transaminases (AST), total protein (TP), albumin (ALB), total bilirubin (TB) and alkaline phosphatase (ALP).

Binding interactions with hyperglycemia-related protein targets

Binding affinities of the compound for various protein targets related to hyperglycemia i.e. glucokinase (GLK), fructose-1,6-bisphosphatase (FBP), beta-2 adrenergic receptors (B2AR), corticosteroid 11-beta-dehydrogenase isozyme 1 (CBDI), dipeptidyl peptidase-4 (DDP-4),

pancreatic α-amylase (pAA), insulin receptor (IR) and α-amylase 1 (AA-1) were determined using an online software "Mcule 1-Click docking (Mcule, Inc. USA, https://mcule.com/apps/1-click-docking/)". In short, the compound's SMILE was entered in the input section and the structure was refined in the drawer. The software-provided data bank was used for selection of the proteins and docking was performed. The binding poses conforming to the highest binding affinities were saved as "pdb" and used later for determination of the pocket composition using a software "COACH (Zhang Lab, University of Michigan, http://zhanglab.ccmb.med.umich.edu/COACH/)".

Statistical analyses

The experiments were performed in triplicates, independent repetitions, not the repetitions of the same reading and data were presented as mean ± standard deviation (SD). The data were analyzed using one-way ANOVA and post-hoc Tukey's HSD test using SPSS version 12.00. A p value below 0.05 was considered as a significant difference.

Results

In vitro anti-hyperglycemic activity

The dose-dependent inhibitory effects of the standards and different concentrations of OXCPM (0.1-1.0 mg/mL) on α-amylase and hemoglobin glycosylation are shown in Table I. The inhibitory responses of the treatment groups were significantly higher ($p<0.05$) compared to the control. The standard and all the test concentrations of the compound also enhanced the uptake of glucose in the yeast cell and the activity increased linearly with increase in the concentration.

At a similar concentration, 1.0 mg/mL, the inhibitory effects of OXCPM on α-amylase and protein glycosylation were comparable to the standard. In contrast to this, a great difference was observed in the response of both OXCPM and the standard on glucose uptake by yeast cells. OXCPM markedly enhanced the glucose uptake by the yeast cells as compared to metronidazole (26.3 ± 0.3% and 7.9 ± 0.3%, respectively). However, in the glucose uptake and hemoglobin glycosylation assays, the activity of both, standards and OXCPM, decreased with gradual increase in the concentration of glucose. From the linear regression analysis of the dose-response curves of OXCPM in α-amylase (59.8 + 41.7, 0.7), hemoglobin glycosylation (17.0 + 38.6, 0.9 at 2 mg/mL, 22.7 + 10.5, 0.9 at 10 mg/mL and 20.0 + 7.3, 0.9 at 20 mg/mL glucose), yeast cell uptake (17.6 +10.0, 0.9 at 2 mg/mL, 3.6 + 3.2, 0.9 at 10 mg/mL and 0.8 + 0.3, 0.7 at 20 mg/mL glucose) and α-glucosidase assays (162.7 + 65.6, 0.5), the EC_{50} were found to be 0.1 mg/mL, 0.7-2.1 mg/mL, 2.3-65.3 mg/mL and 4.2 µg/mL, respectively (Figure 2).

Hepatoprotective activity

An increase in the TB, ALB, ALT, AST and ALP levels,

Table I

Anti-hyperglycemic effect of 5-[(4-chlorophenoxy) methyl]-1,3,4-oxadiazole-2-thiol by various methods

Groups	α-Amylase inhibition assay	Inhibition of hemoglobin glycosylation			Glucose uptake by yeast cell		
		2.0 mg/mL	10.0 mg/mL	20.0 mg/mL	2.0 mg/mL	10.0 mg/mL	20.0 mg/mL
Control	0.1 ± 1.0 (NA)	0.1 ± 0.0	0.2 ± 0.2	0.2 ± 0.1	0.4 ± 0.1	1.9 ± 0.1	2.1 ± 0.9
OXCPM	*OXCPM*	*OXCPM*	*OXCPM*	*OXCPM*	*OXCPM*		
0.10 mg/mL	0.1 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.4 ± 0.1	1.8 ± 0.1	2.1 ± 0.1
0.20 mg/mL	0.2 ± 0.0	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.4 ± 0.1	1.8 ± 0.1	2.1 ± 0.1
0.40 mg/mL	0.3 ± 0.1	0.3 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.4 ± 0.1	1.8 ± 0.1	2.0 ± 0.1
0.60 mg/mL	0.5 ± 0.1	0.3 ± 0.0	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.2	1.8 ± 0.2	2.0 ± 0.0
0.80 mg/mL	0.6 ± 0.7	0.3 ± 0.0	0.2 ± 0.1	0.2 ± 0.0	0.3 ± 0.2	1.8 ± 0.2	2.0 ± 0.0
1.00 mg/mL	0.9 ± 0.1	0.3 ± 0.1	0.2 ± 0.0	0.2 ± 0.0	0.3 ± 0.3	1.8 ± 0.1	2.0 ± 0.0
Standard drugs							
Acarbose	1.8 ± 0.8	-	-	-	-	-	-
Ascorbic acid		0.3 ± 0.0	0.3 ± 0.0	0.3 ± 0.0	-	-	-
Metronidazole	-	-	-	-	0.4 ± 0.3	1.8 ± 0.2	2.1 ± 0.0

Values are presented as mean ± standard deviation (SD, n = 3)

Table II

Serum enzymatic and non-enzymatic biochemical markers for liver function

Groups	Total bilirubin (TB)	Total proteins (TP)	Albumin	Alanine transaminase (ALT)	Alkaline phosphatase (ALP)	Aspartate transaminase (AST)
I	0.5 ± 0.1	7.7 ± 0.1	3.5 ± 0.1	75.0 ± 1.2	200.7 ± 2.0	137.8 ± 1.5
II	0.7 ± 0.1	7.0 ± 1.1	3.7 ± 0.6	98.3 ± 2.3	276.8 ± 1.7	245.0 ± 1.8
III	0.6 ± 0.3	7.8 ± 0.3	3.3 ± 0.3	63.0 ± 1.1[b]	162.0 ± 3.3	66.3 ± 1.3[a]
IV	0.5 ± 0.2	7.5 ± 0.7	3.5 ± 0.1	43.3 ± 1.5[a, b]	91.0 ± 1.5[b]	37.3 ± 2.5[a]

Values are presented as mean ± standard deviation (SD, n = 6), Statistical treatment of data includes one-way ANOVA (analysis of variance) followed by Tukey's HSD (honest significance difference) test, Group I: un-treated/un-compromised vehicle control, Group II: intoxicated control, Group III: standard and Group IV: test, [a]p<0.05 as compared to vehicle control, [b]p<0.05 as compared to intoxicated control

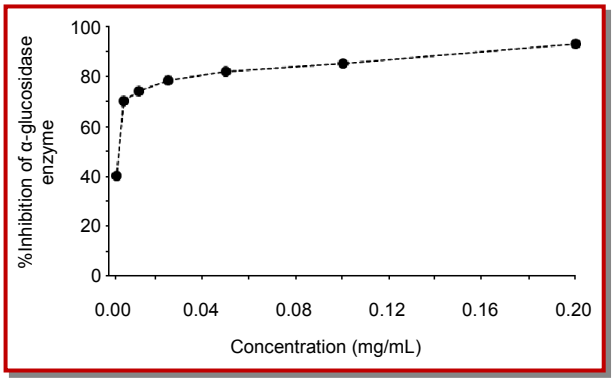

Figure 2: Dose-dependent inhibitory effects of 5-[(4-chlorophenoxy) methyl]-1, 3, 4-oxadiazole-2-thiol (OXCPM) against α-glucosidase

and a decrease in the TP level were observed in the intoxicated rats, however, treatment with silymarin and OXCPM resulted in restoring the normal serum levels of the non-enzymatic and bringing about a decrease in the levels of enzymatic biomarkers (Table II). A remarkable decrease in the ALT and ALP levels was observed in the test group compared to the intoxicated control ($p<0.05$). Moreover, a non-significant difference was observed in the hepatoprotective response of the standard and OXCPM.

Binding interaction with protein targets

Binding affinities: Binding affinities of the compound for hyperglycemia-related protein targets were found to be FBP (5.1-7.6), B2AR (6.6-7.4), GLK (4.9-7.1), CBDI (5.9-6.9), DDP-4 (5.4-6.6), pAA (6.0-6.4), IR (5.5-6.2) and AA-1 (6.1). The highest binding affinity of the compound was observed for FBP: 3kbz (7.6) and 3kc0 (7.1) followed by B2AR 2rh1 (7.4) and GLK 3fr0 (7.1).

Binding conformations: The binding conformation of OXCPM relative to the best binding poses for proteins where affinity was the highest is shown in Figure 3.

Binding pocket composition: The composition of the binding pocket was determined for only those proteins for which the compound showed the highest binding affinity (7.1-7.6). The amino acid residues in the pocket included ASN155 for 2rh1, SER138, PHE139, PRO140, THR155, LYS156, ASN191, ASP192, THR193, ILE212, GLY216, CYS217, ASN218, ASN241, GLU243, GLN274 and GLU277 for 3fr0. The composition of the binding pocket for 3kc0 and 3kbz was similar and included amino acids: GLU105, GLU106, GLU107, GLY196, GLY199, GLN228, GLN230, GLN231, GLN232, LEU248, ASN258, ASN259, ASN260 and ASN264.

Discussion

A plenty of literature report that the anti-hyperglycemic agents may produce their effect by following one or more mechanisms e.g. regeneration of β-cells, enhancement of insulin secretions, promotion of glucose absorption and utilization, manipulation of glucose transporters, retardation of glucose diffusion, uptake of glucose by cells through facilitated diffusion and inhibition of activity of carbohydrate-hydrolyzing enzymes (Rang et al., 2003). One of the most common approaches to manage the post-prandial hyperglycemia is to prevent glucose absorption by hampering the activity of carbohydrate-hydrolyzing enzymes e.g. α-amylase and α-glucosidase (Shobana et al., 2009). The results of α-amylase and α-glucosidase assays reveal that OXCPM may effectively inhibit the initial steps of hydrolysis of the dietary starch to smaller oligosaccharides. It is well-reported that the activity of pancreatic α-amylase correlates to post-prandial hyperglycemia and inhibitors of the enzyme may, therefore, play a pivotal role in treatment and control of type 2 diabetes.

Yeast cells model of glucose uptake is one of the *in vitro* methods used to investigate the anti-hyperglycemic effect of various compounds. In the present study, OXCPM increased uptake of glucose, at various physiological concentrations, by the yeast cells. The amount of glucose in the reaction mixture after 60 min of incubation served as an indicator of glucose uptake by the cells. After the work of Cirillo (1962), it is very well established that the metabolizable and non-metabolizable sugars, and glycosides are transported across the cell membrane by facilitated diffusion. Since, the

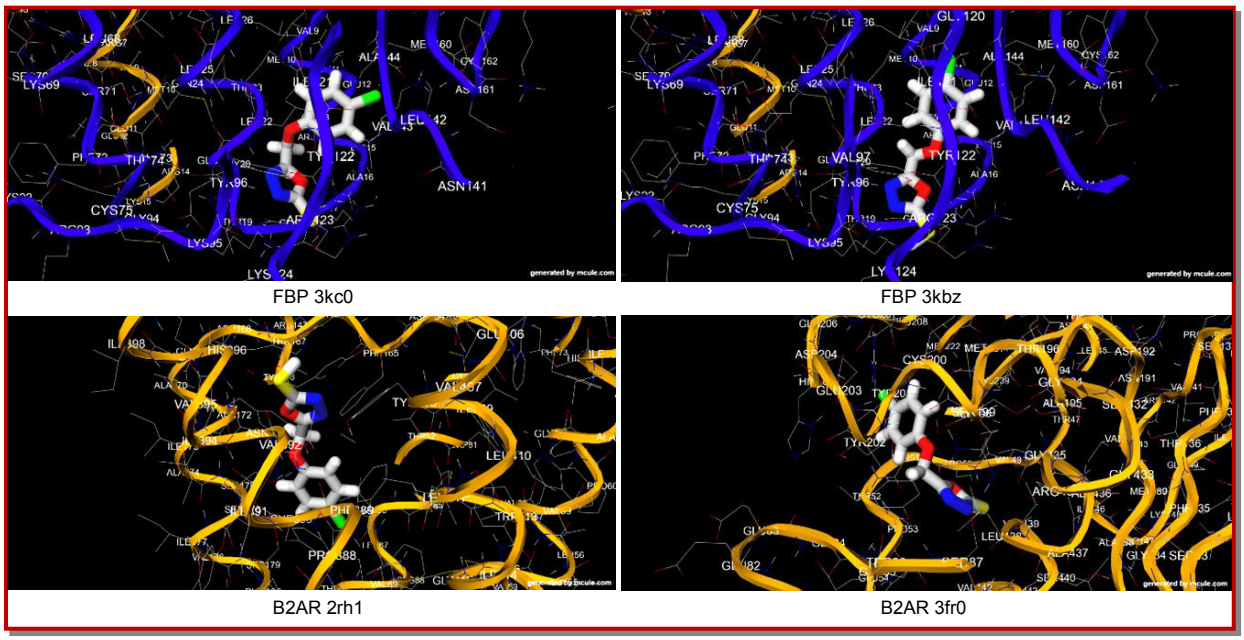

Figure 3: Binding conformation of 5-[(4-chlorophenoxy) methyl]-1,3,4-oxadiazole-2-thiol (OXCPM) relative to the best binding poses for proteins: fructose-1,6-bisphosphatase FBP (3kc0 and 3kbz), beta-2 adrenergic receptor B2AR (2rh1) and glucokinase GLK (3fr0)

process occurs down the concentration gradient, it indicates that transport is possible only when the intracellular glucose levels are reduced (Teusink et al., 1998). The results of the present study reveal that the compound, at all the concentrations under investigation, is capable of enhancing the uptake and hence utilization of glucose by the yeast cells.

Glycosylation of protein (Maillard reaction) is a non-enzymatic process which involves formation of a Schiff's base adduct or aldimine as a result of a) condensation of the free amino group on the N-terminus of the α- and β-chains or b) nucleophilic addition of certain lysine residues, with a sugar aldehyde or ketone followed by Amadori rearrangement and conversion to a stable ketoamine i.e. amino-1-deoxyfructose (Eble et al., 1983). The rearrangement products are further degraded to highly reactive carbonyl compounds (3-deoxyglucosone and sugar fragmentation products) which react with other amino groups in the protein to form several intermediates and advanced glycosylation products (AGP). A variety of proteins – red blood cell membrane proteins, lens crystalline, myelin, collagen, albumin, hemoglobin and insulin – is vulnerable to non-enzymatic glycosylation (NEG) and is believed to contribute to the long-term complications of the disease (Bucala and Vlassara, 1995). The NEG of proteins is a slow and nearly irreversible process which occurs continuously during the life-cycle of red blood cells (RBCs), however, the process is markedly accelerated at least 2-folds in diabetics (Goldstein et al., 1995).

Several literature reports are available on the role of chronic hyperglycemia in the generation of oxygen free radicals (OFR) which disrupt the cell membrane by lipid peroxidation (Tesfamariam, 1994). The lipid peroxidation products e.g. malondialdehyde may also hinder the anti-oxidant potential of the cells by anchoring between sugar and protein or inactivating the natural anti-oxidant enzymes. Furthermore, the tautomeric shift of enediol → dicarbonyl form of the glucose is thought to be facilitated by oxidants (Lyons, 1992). Hence, the supplementation with anti-oxidants which inhibit lipid peroxidation can modulate the glycosylation of cellular proteins (Vinson and Howard, 1996).

Since the glycosylation of protein involves oxidation, therefore, anti-oxidants should be able to prevent the reaction. OXCPM contains conjugated system, thiol (hydrogen donor) and electron–rich atoms, therefore, it is hypotheses to prevent the glycosylation of the proteins by reducing action. In the present study, we used hemoglobin as the target protein to investigate the anti-glycosylation effect of OXCPM. Firstly, the variables in the development of glycosylated proteins, time for incubation and concentration of reducing sugar, were optimized. The glycosylation of hemoglobin was shown to increase with an increase in the concentration of glucose. Furthermore, a linear trend in glycosylation of the protein was observed over the studied time period (72 hours). It is noteworthy that the compound demonstrated anti-glycosylation activity at all physiological glucose concentrations under investigation, however, the response was found linear at 2, 10 and 20 mg/mL. Therefore, these concentration levels were used for investigating the concentration-dependent anti-glycosylation activity of the compound after incubation for 72 hours. The anti-glycosylation effect of

the compound was found to be decreasing with increase in the glucose concentration i.e. 54.9 ± 0.1% at 2 mg/mL, 32.1 ± 0.0% at 10 mg/mL and 27.1 ± 0.0% at 20 mg/mL. A similar trend was observed in the response of the standard i.e. response was maximum at 2 mg/mL (57.3 ± 0.0%) and decreased at 10 mg/mL (42.9 ± 0.0%) and 20 mg/mL (31.8 ± 0.0%). The results of the present study indicated that the non-enzymatic glycosylation of hemoglobin may be prevented by the compound. Molecular docking studies indicated that GLK, FBP and B2AR may be the potential target site of OXCPM to produce anti-hyperglycemic effect.

The relationship between hepatic injury and diabetes remained unclear before the work of El-Serag and Everhart (2002) who, in a prospective cohort study, suggested that diabetic population was twice as likely to suffer from acute hepatic failure compared to normal patients. In 2004, the researchers further added that the disease may increase the risk of hepatocellular carcinoma and chronic liver diseases (El-Serag et al., 2004). The complication was paid more attention after the emergence of hepatotoxicity as a common clinical side-effect of various classes of oral antihyperglycemic drugs e.g. sulfonylureas, α-glucosidase inhibitors, biguanides and thiazolidinediones. Hence, this situation warrants the development of anti-diabetic drugs having hepatoprotective effects too. In addition to the anti-hyperglycemic effects, the compound demonstrated promising hepatoprotective activity in the intoxicated animals. This indicates that the OXCPM do not produce hepatoxicity after oral administration.

Conclusion

OXCPM has excellent enzyme inhibition potential to target post-prandial hyperglycemia. Moreover, the effects of the compound on the liver indicated that it may be advantageous over the available treatments owing to its ability to target the secondary complications of nIDDM due to anti-oxidant and hepatoprotective activities.

Ethical Issue

The experimental procedures used in the study were sanctioned, authorized and approved by the Animal Research and Ethics Committee, Vide reference No. 5034/Ph, University of the Punjab, Lahore, Pakistan. The study was conducted as per the recommendations of the ARRIVE guidelines for animal research and *in vivo* experimentation.

Conflict of Interest

There is no conflict of interest to revel.

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