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European Journal of Pharmacology 584 (2008) 185–191
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Olanzapine inhibits glycogen synthase kinase-3β: An investigation by docking simulation and experimental validation

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Received 25 August 2007; received in revised form 21 December 2007; accepted 15 January 2008
Available online 30 January 2008

Abstract

Olanzapine was investigated as an inhibitor of glycogen synthase kinase-3β (GSK-3β) in an attempt to evaluate its effect on blood glucose level. The investigation included simulated docking experiments to fit olanzapine within the binding pocket of GSK-3β followed by *in vitro* enzyme inhibition assay as well as *in vivo* subchronic animal treatment. Olanzapine was found to readily fit within the binding pocket of GSK-3β in a low energy orientation characterized with optimal attractive interactions bridging the tricyclic thienobenzodiazepine nitrogen and sulfur atoms of olanzapine and the residue of VAL-135 of GSK-3β. *In vivo* experiments showed a significant decrease in fasting blood glucose level in Balb/c mice at 1.0, 2.0 and 3.0 mg/kg dose levels ($P<0.05$) and 6 fold increase in liver glycogen level at the 3 mg/kg dose level ($P<0.001$). Moreover; olanzapine was found to potently inhibit recombinant GSK-3β *in vitro* (IC_{50} value=91.0 nM). Our findings strongly suggest that olanzapine has significant GSK-3β inhibition activity that could justify some of its pharmacological effects and glucose metabolic disturbances.

Keywords: Olanzapine; Glycogen synthase kinase-3β; Docking; Glucose; Glycogen; Balb/c

1. Introduction

A number of drugs exhibit unexpected side effects related to metabolic changes in humans. Determining the molecular mechanism of these side effects might lead to new targets or new therapies for the treatment of different disorders. In addition, such research may reveal ways to design new drugs with fewer side effects. Well-known examples of drug side effects are seen in atypical antipsychotics. Increased weight gain, obesity, diabetes mellitus, hyperglycemia, hypertension, osteoarthritis, and lipid abnormalities are the most common threats associated with long-term treatment with atypical antipsychotics (Fertig et al., 1998; Taylor and McAskill, 2000; Wetterling, 2001; Newcomer, 2005). Despite their major clinical impact, the mechanisms underlying the etiology of these side effects associated with atypical antipsychotics administration are still poorly understood, and several hypotheses related to increased appetite, insulin resistance, caloric intake, and/or decreased energy expenditure have been suggested (Baptista et al., 2002).

Antipsychotic medications are a mainstay in the treatment of schizophrenia and are widely used in other psychiatric conditions. Atypical antipsychotics are increasingly replacing first-generation antipsychotic agents, mainly due to a decreased risk for extrapyramidal symptoms, better overall tolerability, as well as efficacy (Sartorius et al., 2002; Sartorius et al., 2003). Among these, olanzapine (Fig. 1) induces the most significant weight gain. The olanzapine-induced weight gain in patients was attributed to the increase in body fat (Eder et al., 2001) or increase caloric intake (Gothelf et al., 2002). Moreover; olanzapine has been associated with disturbances in glucose metabolism. Numerous reports have documented the onset and/or exacerbation of diabetes, in some cases with development of hyperglycemic crisis or diabetic ketoacidosis, following the initiation of

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doi:10.1016/j.ejphar.2008.01.019

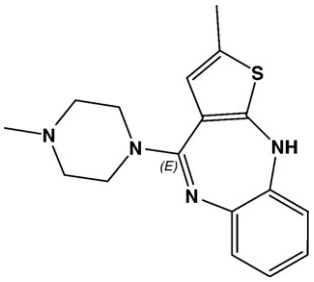

Fig. 1. Molecular structure of olanzapine.

therapy with some of the atypical antipsychotics including olanzapine (Jin et al., 2002; Liebzeit et al., 2001; Mir and Taylor, 2001; Newcomer et al., 2002). The pathogenesis of olanzapine-induced disturbances of glucose homeostasis is unclear. It has recently been demonstrated that olanzapine, in contrast to first-generation antipsychotic agents, increases basal insulin release *in vitro* (Melkersson et al., 2001; Melkersson, 2004). Furthermore, acute administration of olanzapine to cultures of L6 rat skeletal muscle cells was found to impair glycogen synthesis and insulin signaling in L6 skeletal muscle cells through decreasing the phosphorylation of GSK-3 and increasing the phosphorylation of glycogen synthase (Engl et al., 2005). Moreover, olanzapine was reported to increase gluconeogenesis in brain and muscle by similar mechanisms (Fatemi, 2006). However, *in vivo*, olanzapine did not alter beta-cell function in healthy volunteers studied in hyperglycemic clamp experiments (Sowell et al., 2002). One possible pathomechanism for hyperglycemia induced by olanzapine may occur through reducing the function of glucose transporters, impairment in the suppression of hepatic glucose production and restriction of the normal beta-cell response to insulin resistance (Shulman, 2000; Kahn and Flier, 2000). However; the information so far available does not establish whether the increasing risk of developing diabetes reported is a function of the psychiatric illness itself or is induced by the antipsychotic treatment (Bushe and Holt, 2004).

Recently, a study carried out on mice has revealed that olanzapine, in addition to mood stabilization and antidepressant action, can inhibit the activity of GSK3 *via* rapidly increasing the level of brain phospho-Ser9-GSK3 in the brain in a dose-dependent manner (Li et al., 2007). Findings from this study suggest that olanzapine has an acute inhibitory effect on mouse brain GSK3, and that the effect is delivered through increased N-terminal phosphorylation of GSK3. Thus, GSK3 may play a role as a therapeutic target of atypical antipsychotics (Li et al., 2007). Glycogen synthase kinase-3 (GSK-3) is a multifunctional serine/threonine kinase found in all eukaryotes. It was discovered over 20 years ago as one of several protein kinases that phosphorylated and inactivated glycogen synthase, the final enzyme in glycogen biosynthesis (Embi et al., 1980). Insulin stimulates the dephosphorylation of glycogen synthase *via* inhibition of GSK3 activity (Welsh et al., 1998). Molecular cloning revealed that there are two closely related isoforms of GSK3; GSK3α and GSK3β, and their activities are primarily regulated by the phosphorylation of an N-terminal serine (Stambolic and Woodgett, 1994). The latest findings about the key functions of GSK3 have generated an enormous amount of interest in the development of drugs that inhibit GSK3 and which may have therapeutic potential for the treatment of diabetes, stroke and Alzheimer's disease (Martinez et al., 2002).

No studies were conducted to investigate the direct effect of olanzapine on GSK-3β whether in the brain or in the peripheral tissues. Thus we were prompted to further evaluate the effect of olanzapine on GSK-3 in addition to its effects on glucose and glycogen levels in animals. To investigate if olanzapine can bind to GSK-3β active site, an initial molecular docking was performed as a preliminary in-silico screening test. Exploring the effect of olanzapine on such pivotal enzyme and its effect on glucose and glycogen levels could be useful for designing new analogues that could be used to manage different disorders.

2. Materials and methods

2.1. Molecular modeling

2.1.1. Software and hardware

The following software packages were utilized in the present research.

- CS ChemDraw Ultra 7.01, Cambridge Soft Corp. (http://www.cambridgesoft.Com), USA.
- Omega, OpenEye Scientific Software (www.eyesopen.com), USA.
- Fred (Version 2.1.2), OpenEye Scientific Software (www.eyesopen.com), USA (Fred, 2006).

2.1.2. Docking experiment

The chemical structure of olanzapine was sketched in Chemdraw Ultra (7.01), and saved in MDL molfile format. The drug was docked into the binding site of GSK-3β (PDB code: 1Q5K, resolution = 1.94 Å) (Bhat et al., 2003) employing FRED software (FRED, version 2.1.2 Users' Manual, 2006) (Fred, 2006). This docking engine takes a multiconformer database of one or more ligands, a target protein structure, a box defining the active site of the protein based on the co-crystallized ligand and several optional parameters as input. The ligand conformers and protein structure are treated as rigid during the docking process. FRED's docking strategy is to exhaustively score all possible positions of each ligand in the active site (Fred, 2006). The exhaustive search is based on rigid rotations and translations of each conformer. Therefore, it avoids sampling issues associated with stochastic methods; semi random method. The

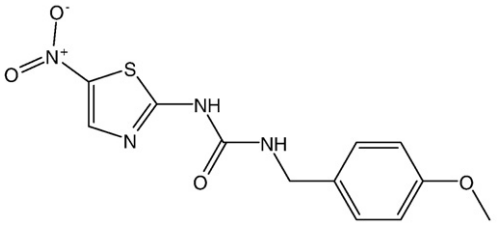

Fig. 2. Molecular structure of co-crystallized structure AR-A014418.

conformational space of olanzapine was explored using OMEGA software. The best docking conditions utilized were those succeeded to retrieve the pose of the co-crystallized ligand (AR-A014418, Fig. 2). In the current docking experiment the following docking parameters was employed

- *Addbox*: Optional parameter that adjusts the geometry of the box defining the active site by extending each edge of the box by the specified number of Angstroms. (Type = float, default = 0). The value was set to 4.00.
- *num_poses*: this parameter specifies the number of poses to be returned by the exhaustive search. Poses will be the top scoring poses selected from the list of all poses and scored by the scoring functions specified by the exhaustive scoring. Any number greater than 0 is a valid setting, although 10–1000 is a recommended reasonable range. (Type = integer, default = 100). The used value was 1000.
- *Num_alt_poses*: This flag specifies how many alternate poses, in addition to the top consensus structure pose, will be passed out of the consensus structure step. Legal values are between 0 and 99.The value was set to be 10.
- The docked poses were scored by the Chemscore scoring function and the highest ranking poses were retained for evaluation.

2.2. Animals and drug treatment protocols

The animal experiments confirm with the Guide for the Care and Use of Laboratory Animals published by the US National Institutes of Health (NIH Publication no. 85-23, revised 1996).

Female Balb/c mice (20–22) g (~10 weeks old), obtained from Jordan University animal house, Jordan, were housed in temperature controlled cages (20–22 °C) with a 12-h light–dark cycle, and given free access to water and formulated diet. Control and treated groups were matched for body weight in all experiments. Animals were habituated to the housing environment for 7 days prior to the drug or vehicle treatment. Olanzapine (a gift from Jordan Sweden Medical Company (JOSWE), Jordan) was dissolved in a minimum amount of acetic acid, then brought up to volume with sterile isotonic saline (0.9%) and adjusted to pH 6 with 5 M NaOH. For control animals, a comparable pH-adjusted saline was prepared. Three doses of olanzapine were used; 1, 2 and 3 mg/kg. Dosages of olanzapine were chosen from previously published effective dose ranges in animal studies (Kapur et al., 2003; Ballard et al., 2007). The drug was dissolved to a concentration (mg/ml) that when injected at 0.2 ml/mouse yielded the desired final dosage (mg/kg). Animals were randomly assigned to each of the 4 treatment groups (6 animals each). The first three groups received the three doses given by one daily intraperitoneal injection for 3 weeks, while the fourth group was given the vehicle.

2.3. Determination of blood glucose levels in mice

After 3 weeks of treatment, on day 22, fasting blood glucose level was determined using blood samples withdrawn from mice tail vein employing glucose kit (SYRBIO, France) (Lott and Turner, 1975). Glucose is oxidized into gluconate and hydrogen peroxide using glucose oxidase enzyme. Subsequently phenol oxidase enzyme catalyzes both the reduction of hydrogen peroxide into water and the condensation of 4-aminoantipyrine with phenol into a colored material (quinoneimine) which gives a maximum absorption at wavelength of 505 nm (Spectroscan 80D-UV–VIS spectrophotometer, USA).

2.4. Determination of liver glycogen

The animals were scarified by cervical dislocation after taking the blood sample, and their livers were removed immediately for glycogen determination. Liver glycogen content was determined quantitatively following reported procedure (Carroll et al., 1956). Briefly: livers were removed immediately after scarifying the animals and were homogenized by a blender (Ultra-Turrax, Janke & Kunkel GmbH & CoKg, Germany) with appropriate volume of 5% trichloroacetic acid over 5 min. The homogenate was centrifuged (Hettich zentrifugen, Germany) at 1110 g for 5 min. The supernatant fluid was taken and filtered using acid-washed filter paper and the residues were homogenized again with another volume of 5% trichloroacetic over 1–3 min to insure better extraction of glycogen. The glycogen of 1.0 ml of this filtrate was precipitated using ethanol (95%, 5 ml), incubated in water bath (Raypa water bath, Germany) at 37–40 °C for 3 h and centrifuged at 1110 g for 15 min. The clear liquid is gently decanted from the packed glycogen and the tubes were allowed to drain in an inverted position for 10 min. The glycogen was dissolved in distilled water (2 ml) and mixed with 10 ml of the anthrone reagent (0.05% anthrone (Sigma-Aldrich, USA), 1.0% thiourea (Sigma-Aldrich, USA) in 72% sulfuric acid). The mixture was incubated in boiling water over 30 min, subsequently, the absorbance was measured at λ_{620} nm by spectrophotometer (Spectroscan 80D-UV–VIS spectrophotometer, USA). Blank and standard solution were prepared by adding 10 ml of anthrone reagent to 2 ml water and to 2 ml of glucose solution containing 0.1 mg of glucose in saturated benzoic acid, respectively. The liver glycogen content is estimated using the following formula:

$$\text{Amount (mg) of Glycogen Liver Tissue} = \frac{\text{DU}}{\text{DS}} \times \frac{\text{Volume of Extract (ml)}}{\text{Weight of Liver Tissue (g)}} \times 0.09$$

where, DU is the absorbance of the unknown sample, DS is the absorbance of the standard (Carroll et al., 1956).

2.5. In vitro GSK-3β enzyme inhibitory assay

GSK-3β (Upstate Biotechnology, U.S.A) was dissolved in a buffer solution (pH 7.2) containing the following: 40 mM HEPES; 5 mM MgCl2; 5 mM EDTA; 100 μM ATP and 50 μg/ml heparin to reach a final enzymatic solution of 10 pg/ml. Subsequently, 50 μl aliquots of the enzymatic solution were pipetted into 0.5 ml vials. Thereafter, appropriate volumes of

olanzapine stock solution were pipetted into the enzymatic solution to yield 10 μM, 1 μM, 100 nM and 10 nM and the solutions were completed to 75 μl. The drug was incubated with the enzyme over 30 min at room temperature, then 25 μl of 2000 pg/ml Tau protein solution in HEPES were added to give final Tau protein concentration of 500 pg/ml. This mixture was incubated over 1 h at room temperature.

The detection of Tau phosphorylation was performed as follows: The GSK-3β reaction mixtures were diluted 1:1 with sodium azide aqueous solution (15 mM) to achieve a final Tau protein concentration of 250 pg/ml. Then 100 μl aliquots of this solution were pipetted into the wells of the Tau [pS396] phosphoELISA kit (Biosource, USA). Subsequently, the wells were incubated for 2 h at room temperature. Then they were aspirated and washed (with the washing solution provided in the kit). Thereafter, 100 μl aliquots of rabbit detector antibody solution were pipetted in the wells and incubated for 1 h at room temperature. Thereafter, the wells were aspirated and washed with the wash buffer. Then, 100 μl aliquots of goat (polyclonal) anti-rabbit IgG-HRP were added to the wells and incubated for 30 min at room temperature. Subsequently, the wells were aspirated and washed with the wash buffer. Finally, 100 μl TMB substrate chromogen solution aliquots were added to each well and incubated for 20–30 min. After the termination of the HRP reaction in each well, the solution absorbances were measured spectrophotometrically at λ of 450 nm. A standard GSK-3β inhibitor (TDZD-8 from Biosource, USA) was employed as positive control (Martinez et al., 2002).

2.6. Data analysis

Data are presented as means±S.E. Statistical comparisons were performed using one-way ANOVA or unpaired Student's *t* test. In all cases, $P<0.05$ is considered statistically significant.

3. Results

3.1. Molecular modeling

Our efforts to investigate the effect of olanzapine on GSK commenced by evaluating the possibility of binding *via* computer-aided molecular modeling techniques. Accordingly, we docked olanzapine into the binding pocket of GSK-3β (PDB code: 1Q5K).

The molecular interactions of the highest ranking binding mode can be summarized in Fig. 3. Clearly from the figure, the (NH) group of olanzapine interacts with the carbonyl moiety of VAL-135 (2.6 Å). On the other hand, the sulfur makes a possible weak hydrogen bond with the NH of the same amino acid (2.7 Å). Furthermore, the nitrogen atom of the diazepine moiety was bridged by two water molecules (H_2O-749 and H_2O-875) to IlE-62. Interestingly, these three interaction signals are the same hot spots provided by the co-crystallized ligand (Fig. 3), where the nitrogen atom of the thiazole ring and amidic NH group adjacent to thiazole are hydrogen bonded to VAL-135, while the amidic carbonyl is bridged to ILE-62 by the same water molecules (H_2O-749 and H_2O-875). Moreover, the

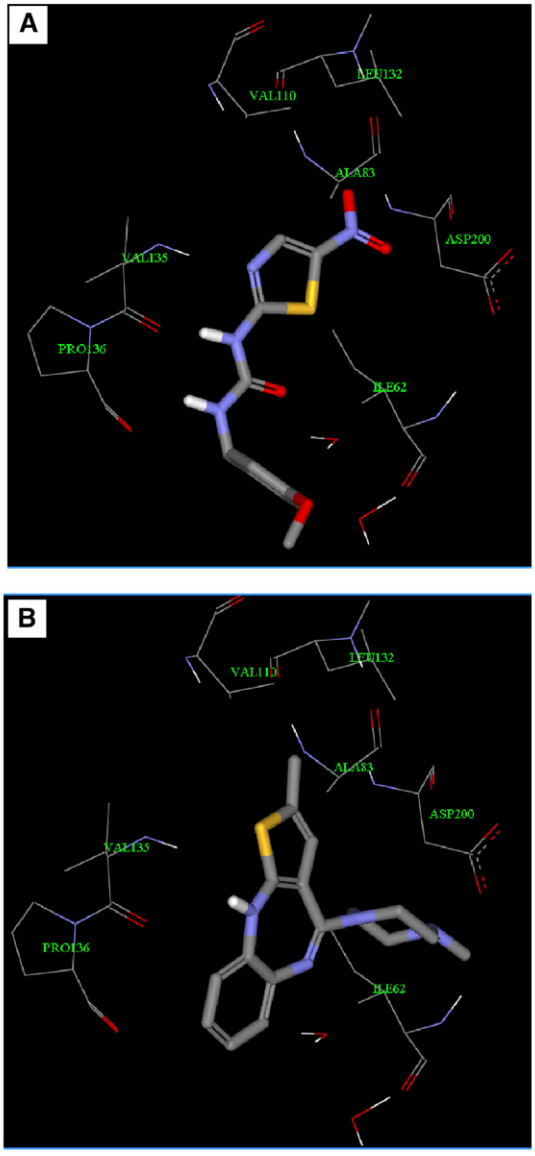

Fig. 3. (A) Detailed view of the co-crystallized structure (AR-A014418) and the corresponding interacting amino acids within the binding site of GSK-3β. (B) Detailed view of the docked olanzapine structure and the corresponding interacting amino-acid moieties within the binding site of GSK-3β.

methyl group attached to the thiophene moiety in olanzapine occupies a hydrophobic pocket assembled from VAL-110, LEU-132 and ALA-83, while; the nitrogen of the piperazine ring is situated at around 4.8 Å from the carboxyl moiety of ASP-200 suggesting potential electrostatic interaction. Overall, these attractive interactions cooperate in stabilizing the proposed complex.

3.2. Effect of olanzapine on blood glucose level, liver glycogen and glycogen synthase kinase activity

After subchronic administration, the three doses of olanzapine (1, 2 and 3 mg/kg) didn't significantly change the body weight as compared to vehicle treated group (data not shown). Fig. 4 shows that olanzapine significantly decreases the fasting blood glucose level in mice at the three dose levels used in the

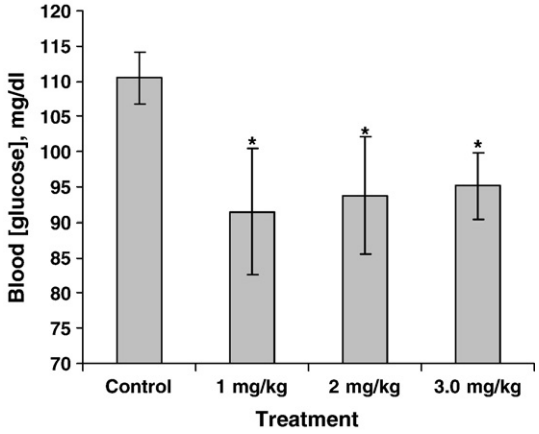

Fig. 4. Effect of chronic olanzapine exposure on blood glucose level in mice. (* $P<0.05$).

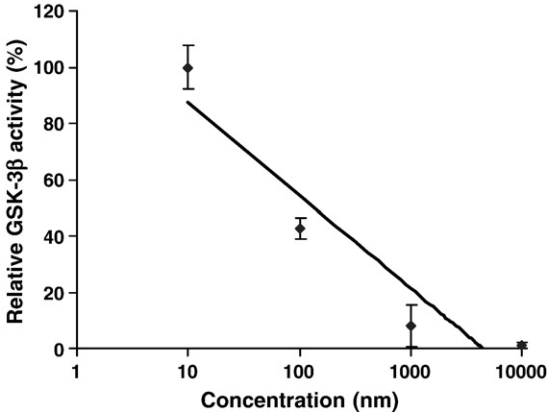

Fig. 6. Effect of olanzapine concentrations on the relative activity of GSK-3β. Data are expressed as means of duplicates ± standard error of the measurements.

study (1, 2, 3 mg/kg) compared to the control group ($P<0.05$). A significant elevation of liver glycogen storage (6 folds increase) was found in animals treated with higher doses of olanzapine, 3 mg/kg, compared to the control group (Fig. 5).

To evaluate the inhibitory effect of olanzapine against GSK-3β, an *in vitro* GSK-3β inhibitory assay was conducted. In this inhibitory assay; the concentration of olanzapine that inhibits 50% of the enzyme, IC_{50}, was measured. Fig. 6 shows the effect of different concentrations of olanzapine on the relative activity of GSK-3β. The drug produced potent inhibition with IC_{50} value of 91.0 nM. The validity of the test was established by testing the inhibitory action of the standard inhibitor TDZD-8 (Martinez et al., 2002) on GSK-3β, which showed an IC_{50} value of 1.5 μM that is comparable to the published value (Martinez et al., 2002).

4. Discussion

Based on the findings that olanzapine has inhibitory activity on brain GSK-3β in mice (Li et al., 2007), through increasing its inhibitory N-terminal serine phosphorylation, this study sought to further identify the effects of olanzapine on blood glucose level and liver glycogen storage as well as on GSK-3β activity. The conducted preliminary molecular modeling study has shown that olanzapine can be successfully docked within the binding pocket of GSK making several significant interactions with key hot spots within the GSK-3β binding pocket. The validation for our docking–scoring procedure was performed through employing the same conditions to dock a well-known GSK inhibitor (AR-A014418, Fig. 2) (Bhat et al., 2003) into the binding pocket of this enzyme. The docking simulation resulted in a close model to the crystallographic structure, which supports our conclusions regarding olanzapine/GSK-3β binding (Fig. 7). Furthermore, three of the important interactions are shared between the co-crystallized ligand and olanzapine (Fig. 3), which increases the confidence in the docking configuration and results.

The preliminary docking study has supported our hypothesis that olanzapine has an inhibitory activity against GSK-3β and encouraged us for further investigation to evaluate the effects of olanzapine on blood glucose and liver glycogen levels. The data presented in this article demonstrate that olanzapine treatment for three weeks did not increase the body weight of the test animals to any significant degree (data not shown), while significantly decreased blood glucose level at the three dose

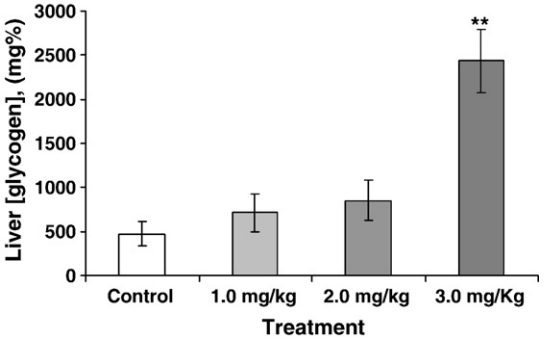

Fig. 5. Effect of chronic olanzapine treatment on liver glycogen storage in mice. (** $P<0.001$).

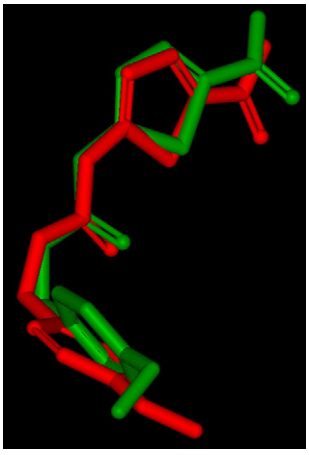

Fig. 7. Comparison between the docked conformer/pose of inhibitor AR-A014418 (red) as produced by the docking simulation and the crystallographic structure of this inhibitor within GSK-3β (green, PDB code: 1Q5K). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

levels used in the study ($P<0.05$, Fig. 4). These findings go in line with a previous study conducted on C57B1/6J, A/J mice and male rats which had shown that olanzapine has no effect on body weight after chronic administration. In contrast, increased body weight as well as mild insulin resistance was observed in female rats. The study also found that acute administration of olanzapine tend to lower blood glucose values compared to the control animals at all time points tested after oral glucose tolerance test (Albaugh et al., 2006). Furthermore, olanzapine-induced low blood glucose level is consistent with at least one clinical report where the drug administration correlated with hypoglycemia (Budman and Gayer, 2001). These findings were contradictory to the reported effects of the drug on blood glucose level in humans (Fertig et al., 1998; Taylor and McAskill, 2000; Wetterling, 2001; Newcomer, 2005). The hyperglycemic effect of olanzapine seen in psychiatric treatment could not be directly linked to the drug as the information so far available does not establish whether the increasing risk of developing diabetes reported is a function of the psychiatric illness itself or is induced by the antipsychotic treatment (Bushe and Holt, 2004). It has been shown that in the retrospective studies analyzed, a number of methodological flaws in the experimental design and data collection do not allow firm conclusions on this issue to be drawn (Bellantuono et al., 2004). In a clinical study to evaluate hyperglycemia in patients with anorexia nervosa it was found that hyperglycemia improved after weight restoration despite continuous use of olanzapine, which indicates that undernutrition itself might be a risk factor for olanzapine-induced hyperglycemia (Daisuke et al., 2007).

Other animal studies found that chronic olanzapine treatment increases body weight and glucose level only at the high concentration level used (Coccurello et al., 2006). This could be partially attributed to the different animal models used in the studies where they exhibit different genetic susceptibilities to diet-induced overweight or drug-induced metabolic changes, and different treatment methodology (e.g., route of administration and period of treatment).

Consistent with the current results of glucose lowering effect of olanzapine, we found that subchronic olanzapine treatment with 3 mg/kg (i.p.) dose significantly increased the glycogen storage in the liver of the mice (Fig. 5). To explain such increase in liver glycogen storage; the inhibitory activity of olanzapine against GSK-3β was evaluated, a hypothesis supported by the initial docking study.

The role of GSK3 as a therapeutic target of atypical antipsychotics and the possible links between GSK3 and mood disorders were investigated by Li et al. It was shown that the effects of atypical antipsychotics, including olanzapine, on GSK3 are shared with the previously identified effects of lithium and other GSK3 inhibitors in that they all inhibit GSK3 (Li et al., 2007).

GSK-3β affects the glycogen synthesis through phosphorylation and thus inhibition of the activity of glycogen synthase. Glycogen synthase is a key metabolic enzyme in the glycogen synthesis pathway and inhibiting the activity of this enzyme leads to decrease glycogen synthesis and storage. Inhibitors of GSK-3β could improve insulin sensitivity, glycogen synthesis and glucose metabolism in human skeletal muscles of diabetic patients and thus it is considered an important target for controlling hyperglycemia (Dokken et al., 2005). To test our GSK-3β inhibition postulation, *in vitro* GSK-3β inhibitory assay was conducted. Unsurprisingly, the results indicated that olanzapine is a potent GSK-3β inhibitor (IC_{50} 91.0 nM) (Fig. 6). Therefore, it improved glucose disposal in these animals due to increase in the activity of glycogen synthase leading to increased rates of glycogen synthesis in the liver. In insulin-responsive tissue, insulin exposure leads to a transient inhibition of GSK-3β *via* protein kinase B phosphorylation of a serine residue (Ser9) (Plyte et al., 1992). The inhibition of GSK-3β activity leads to net dephosphorylation and hence activation of glycogen synthase. Although, GSK-3 inhibitors may engage alternative targets, stimulated glycogen synthase activity was assumed to be the primary mechanism by which GSK-3 inhibition enhanced glucose disposal in animals (Cline et al., 2002). Although, olanzapine has been associated with several side effects including; hyperphagia, chronic hyperleptinemia, weight gain and hyperinsulinemia, these effects could be explained by the activity of olanzapine on numerous targets including; dopamine receptor (D2), serotonin receptor (5-HT2A) in addition to G-protein-coupled receptors for several other biogenic amines including cholinergic, adrenergic, histaminergic, and other dopaminergic receptors (Albaugh et al., 2006). Furthermore, this study revealed that olanzapine has GSK inhibitory activity which could explain some of its various activities.

5. Conclusion

In conclusion, subchronic olanzapine treatment resulted in potent GSK-3β inhibition which was associated with a significant decrease in fasting blood glucose level in Balb/c mice, which in turn could mostly be attributed to enhanced liver glycogen synthesis and storage in the liver of the mice. These data suggest that olanzapine-induced GSK-3β inhibition may represent an important new trace for the various metabolic changes associated with olanzapine treatment.

Acknowledgements

This project was sponsored by the Deanship of Scientific Research at the University of Jordan (grant No. 1102). The authors wish to thank the Deanship of Scientific Research and Hamdi-Mango Center for Scientific Research at the University of Jordan for providing funds towards purchasing O2 and Octane2 Sgi workstations and CERIUS2w software package. The authors would like to thank also the OpenEye Scientific Software for providing us a free license of FRED software (FRED, version 2.1.2).

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