

SHORT THESIS FOR THE DEGREE OF DOCTOR OF
PHILOSOPHY (PhD)

**Novel pharmacological possibilities to influence insulin
resistance**

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Novel pharmacological possibilities to influence insulin resistance

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The Examination takes place at the Library of Department of Pharmacology,
Faculty of Pharmacy, University of Debrecen; 8th Sept 2015. at 11:00

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2015. at 13:00

1. INTRODUCTION

The increasing prevalence of obesity and the insulin resistance associated with leads to the development of type 2 diabetes mellitus (T2DM) and its complications mean a global health problem. Diabetes mellitus (DM) has become the main problem of public health in the 21st century and it is one of the most prevalent non-infectious, so-called „civilization diseases“. According to WHO the estimated number of patients suffering from this disease will reach 552 million by 2030, and it doesn't contain the non-diagnosed cases. In the light of these facts it is evident that the global economic cost of the treatment of the obesity associated with diabetes are the highest one. These costs reach 32,4 billion dollars in the USA. 90% of the people suffering from diabetes all over the world have T2DM and it manifests in adults. These data and the expected increasing incidence correlate with the rapid spreading of metabolic syndrome, in which the insulin resistance and obesity play a central role. In 1997 WHO pronounced obesity an epidemic disease worldwide. In 2008 1,4 billion adults were estimated to be overweight and 500 million to be obese. However, obesity is considered to be the first possible cause of death that can be preventable. There are several factors which play a role in the development of diabetes, eg.: genetic- and, environmental factors, insufficient exercise, and also chronic drug treatment. Typical examples for these drugs are atypical antipsychotics (clozapine, olanzapine), which have been developed to improve the compliance of patients with schizophrenia due to the reduced incidence of the extrapyramidal symptoms, but AAP treatment could be associated with increased appetite with concomitant weight gain and obesity that can later induce T2DM. This progress reduces the efficacy of the therapy and results in more and more patients diagnosed with T2DM. One of the main topics of this dissertation is the investigation of metabolic disorder effects of AAP.

On the other hand our investigation focused on the new therapeutic possibilities of diabetes and insulin resistance because there haven't been introduced any new original drug into the market for a long time. Although there is a growing number of type 2 diabetic patients, the available remedy for this disease is confined to a limited number of drug classes but neither of these drugs get the right solution to the treatment of diabetes. The development of original molecules is a time consuming process which also needs resources. The aim is to search for generic drugs by

computational molecular database screening, find a potential PPAR γ agonist and also investigate it in vitro and in vivo experiments in order to study its insulin sensitizing effect. Another half of this dissertation is the pharmacological influence of insulin resistance.

2. AIMS

1. The primary aim of the present study was to investigate the acute effect of a single oral dose of olanzapine on the MIS. Furthermore, we also aimed to explore the initial biochemical events of the olanzapine-induced metabolic changes, which lead to increased appetite and altered eating behaviour. We investigate the acute effect of a single oral dose of olanzapine on gut hormones (ghrelin, insulin, leptin, GLP-1, GIP, pancreatic polypeptide, peptide YY, amylin) which participate in the regulation of hunger and satiety and modulate insulin sensitivity. In order to study the effect of olanzapine on insulin sensitivity and appetite, we have to exclude some disturbing factors which come from increased adiposity and/or weight gain therefore, olanzapine was used in a single oral dose of 1 mg/kg, which was proven to be effective to induce weight gain in female Sprague-Dawley rats.
2. The aim of the present study was to investigate whether our in silico prediction method could be used for screening generic drugs in order to find new therapeutic indications. To test our methodological approach, the FDA database containing 1,255 generic drugs was screened to predict their PPAR γ activation propensities. Then, the in silico selected drugs were tested in vitro using PPAR γ receptor-expressing cell cultures to determine whether they are able to activate the PPAR γ receptor. Finally, a series of in vivo experiments were carried out by means of hyperinsulinemic euglycemic glucose clamping (HEGC) in a rat model of type 2 diabetes in order to study the PPAR γ receptor-related insulin sensitizing effect of a drug showing the most promising in vitro effect.

3. MATERIALS AND METHODS

Ethical aspects

The present study was performed in accordance with the Guide for the Care and Use of Laboratory Animals (1996, published by National Academy Press, 2101 Constitution Ave. NW, Washington, DC 20055, USA) and the use of animals was reviewed and approved by the Animal Ethics Committee of the University of Debrecen (08/2007 DE MÁB and 16/2007 DEMÁB).

Animals, drug treatment and study design

Twenty-four female, Sprague-Dawley rats weighing 175–200 g (obtained from Innovo Ltd, the local distributor of Charles River Ltd) were used throughout the study. After arrival, animals were habituated to the new environment for a week. Rats were housed in an animal room with 22–24 °C and 50–70 % relative humidity. The lighting was set to 12-h light and 12-h dark periods (lights off at 7 a.m.). The animals were individually placed in metabolic cages (3701 M081, Tecniplast, Italy), and they were allowed to eat standard laboratory chow (S8106-S011 SMR/M-Z+H; ssniff Spezialdiäten GmbH, Germany) and drink tap water ad libitum throughout the acclimatization period. A week later, animals were divided into two main groups, the vehicle-treated/control (n=12) and the olanzapine-treated groups (n=12). Within each main group, rats were randomly assigned to fasted (n=6) or re-fed subgroups (n=6). In both subgroups, the food was removed 16 h before the start of the experiment, but in the re-fed group, the food was replaced for 2 h before the induction of anaesthesia. Then, anaesthesia was induced (thiopental, 50 mg/kg, i.p.) and the surgery was performed. Olanzapine (obtained from Sigma-Aldrich, Hungary) or vehicle was administered via oral gavage before food replacement in re-fed group or 2 h before the anaesthesia induction in fasted group. Olanzapine was used in a single oral dose of 1 mg/kg, which was proven to be effective to induce weight gain in female Sprague-Dawley rats.

Meal size measurement

In order to determine the size and duration of the first meal after drug/vehicle administration, the protocol described by van der Zwaal et al. was followed. Food was removed from rats 16 h before the commencement of the experiment. In the re-fed

groups of both vehicle- and olanzapine-treated rats, the food was replaced in the food hopper and the animals were left to eat ad libitum. A video surveillance system was used to record the eating behaviour, and the weight of the food hopper was recorded continuously. The size and the duration of the first meal were determined as follows. A meal was determined as the duration of food consumption when animals had eaten at least 1 kcal of chow, and the inter-meal interval was at least 5 min. After measuring the first meal size, animals were left to eat ad libitum until induction of the anaesthesia. The total amount of food consumed during this 2-h period was also determined.

General procedures

Rats were anaesthetized with intraperitoneal injection of thiopental sodium (50 mg/kg), the trachea was cannulated and the animals were allowed to breathe freely through the cannula. Continuous anaesthesia was maintained throughout the experiment by succeeding intravenous injection of thiopental sodium of 1.5 mg/100 g body weight through a catheter inserted into the right femoral vein. The left carotid artery was also cannulated for blood sampling and for measurement of blood pressure by means of a Statham P23 DB transducers attached to an electro-manometer (Experimetria Ltd, Budapest, Hungary). The left jugular vein served for insulin and 20 % glucose infusion. To avoid blood coagulation, the animals were injected with intravenous heparin (100 IU/kg i.v.). The body temperature was kept constant at 37 ± 0.5 °C by means of heated operating table (Type 872H with heating controller Type 861, Hugo Sachs Elektronik—Harvard Apparatus GmbH, March-Hugstetten, Germany). At the end of the experiments, the animals were killed by an intravenous overdose of thiopental sodium (100 mg/kg).

Rapid Insulin Sensitivity Test (RIST)

The rapid insulin sensitivity test (RIST) was performed in order to investigate changes in postprandial whole-body insulin sensitivity in response to single oral dose of 1 mg/kg olanzapine. In brief, after a 30-min post-surgery stabilization period, arterial blood samples were taken from the carotid artery at every 5 min for blood glucose determination. When blood glucose value of three consecutive determinations was stable, it was referred to as the control. Then, 50 mU/kg bolus insulin (Humulin R®, obtained from Eli Lilly, USA) was infused i.v. over 5 min. At the same time, a

continuous glucose infusion was also started. Blood glucose level was determined every 2 min and the rate of glucose infusion was adjusted according to maintain the previously determined control value. The total amount of infused glucose (expressed as mg/kg body weight) required to counteract the hypoglycaemic effect of 50 mU/kg insulin produced the RIST index as an indicator of whole-body insulin sensitivity.

HOMA-IR

The homeostasis model assessment of insulin resistance (HOMA-IR) index calculation was used in order to determine the insulin sensitivity during the basal, fasting state. Based on the fasting plasma insulin (FPI) and fasting blood glucose (FBG) levels, the whole-body insulin resistance (HOMA-IR) can be calculated. This HOMA approach has been widely used in clinical research to assess insulin sensitivity. The HOMA-IR was established as the product of the fasting concentrations of glucose (expressed as millimoles per litre) and insulin (expressed as milliunits per millilitre) divided by a constant of 22.5. The main advantage of the HOMA-IR calculation over the FPI or FBG/FPI ratio is that it is compensated for fasting hyperglycaemia. The increased HOMA-IR value indicates insulin resistance.

Determination of blood hormone levels

Before the commencement of the RIST procedure, blood samples (0.1 ml) for metabolic hormone (insulin, glucagon-like peptide-1 (GLP-1), glucose-dependent insulinotropic polypeptide (GIP), pancreatic polypeptide, peptide YY, ghrelin, leptin and amylin) determination were drawn from the carotid artery. Samples were centrifuged at 10,000g for 2 min at 4 °C; then, plasma samples were obtained and stored at -70 °C until subsequent determination. For plasma metabolic hormone level measurement, we used the Rat Metabolism Panel (RGT-88 K-08, EMD Millipore Corp., Billerica, MA, USA) and determined—according to the manufacturer’s instructions—the following hormone levels: insulin, GLP-1 (active), GIP (total), pancreatic polypeptide, peptide YY (total), ghrelin (active), leptin and amylin (active). (Performed by Molecular Medicine Institute.)

One dimensional DPM

The selection of potential PPAR γ antagonist candidates was performed using a simpler version of the recently introduced DPM – a systematic, pattern-based, bioactivity prediction method. This method uses virtual affinity fingerprints of small molecule compounds to predict their medical effects and targets. In DPM, the affinity fingerprint is called IP, which consists of a set of docking scores of a compound against the binding sites of a predefined non target protein set. The main difference between DPM and one-dimensional DPM (oDPM) presented here is that the latter method applies a one-dimensional distance-like metric to compare the IPs that were considered as vectors in a multidimensional space, while DPM generates hyperplanes to separate active and inactive compounds in the multidimensional space. The main reason for applying oDPM instead of DPM includes the limited number of compounds in the reference set. In our earlier work, we found that at least ten members are required for a group to reliably perform multivariate statistical methods of DPM; with under ten members, an alternative evaluation method is required, like oDPM. Since the detailed mechanism of DPM has been introduced earlier, we will only summarize its most important features and point to the differences between oDPM and DPM.

Data collection

A total of 1,255 FDA-approved drug molecules were extracted from the Drug Bank database. In addition, 149 proteins were collected from the Research Collaboratory for Structural Bioinformatics Protein Data Bank, as described in our previous work. (Data collection was performed by Zoltan Simon PhD.)

Docking preparations and calculations

Docking preparations and calculations were performed by AutoDockTools (Molecular Graphics Laboratory, The Scripps Research Institute, La Jolla, CA, USA) and DOVIS 2.0 (DOcking-based VIRTUAL Screening, Biotechnology High Performance Computing Software Applications Institute, Department of Defense, Frederick, MD, USA) software, using the AutoDock4 (Molecular Graphics Laboratory, The Scripps Research Institute) docking engine with its native scoring function. Preparation procedures were the same as presented earlier. Each drug molecule was docked to each protein, and binding free energies were extracted and the minima were imported into a

database. Docking runs were performed on a Hewlett-Packard cluster of 104 central processing units (Hewlett-Packard Company, Palo Alto, CA, USA). In sum, $1,255 \times 149 = 186,995$ dockings were performed, repeated 200 times for each drug–protein pair, and were docked and scored by AutoDock4 (Molecular Graphics Laboratory, The Scripps Research Institute). (Docking preparations and calculations were performed by Zoltan Simon PhD and his research group)

Reference set

Instead of using a medical effect database, a single-target focused reference set of eight molecules was applied. These known PPAR γ antagonists were rosiglitazone, bexarotene, gemfibrozil, pioglitazone, alpha-linolenic acid, fenofibrate, bezafibrate, and clofibrate.

IP similarity calculations

A similarity coefficient (d) based on the angle enclosed by two IP vectors was used to create the IP similarity matrix. The IP vectors are determined by the docking scores as coordinates in a 149-dimensional space created by the 149 members of the protein set. Cosine angle distance coefficient was used to determine the angle between two vectors in the above-described 149-dimensional space as follows:

$$d_{AB} = \arccos \left(\frac{\sum_{i=1}^n a_i b_i}{\sqrt{\sum_{i=1}^n a_i^2} \sqrt{\sum_{i=1}^n b_i^2}} \right) \frac{180}{\pi}$$

where d_{AB} is the IP distance value between molecules A and B, and a_i and b_i are the docking score values of molecules A and B on the i -th protein of $n = 149$, respectively.

Neighbor-based bioactivity prediction

Based on the IP similarity matrix, similarity lists were generated to each member of the reference set. Applying the cut-off value of $d = 3$, the number of appearances of the drugs on each list was calculated, and the most abundant compounds were selected

for further analysis. The maximum number of appearances was three. (Performed by Zoltan Simon PhD and his research group.)

In vitro study

The PPAR γ -activating actions of the in silico-selected generics were tested on a Mono Mac 6 (MM6) cell line. The MM6 is a monoblastic leukemic cell line, which is known to express PPAR γ . The activation of PPAR γ results in the expression of a wide range of genes; among them, some can be activated solely by the PPAR γ . One of them is the fatty acid-binding protein 4/adipocyte protein (FABP4/aP2) gene, which was studied.

The study was carried out on 48-well plate, with 200,000 cells per well. Every treatment was done in triplicate. The cells were pretreated with phorbol 12-myristate 13-acetate, which makes the cells similar to macrophages and sensitizes their PPAR γ response. After 24 hours, the phorbol 12-myristate 13-acetate was washed out, fresh nutrient solution was added, and then the treatment was commenced by increasing the doses (10^{-8} M to 10^{-5} M) of the selected drugs in order to obtain their concentration–response curves. Rosiglitazone (10^{-8} M to 10^{-5} M) was used as a positive control, and the vehicle (dimethyl sulfoxide, ethanol, or water) as a negative control. After 24 hours of incubation, the cells were lysated in TRIzol® reagent (Life Technologies, Carlsbad, CA, USA), and the ribonucleic acid (RNA) was isolated. Then, the samples of RNA were converted into complementary deoxyribonucleic acid by means of reverse transcription polymerase chain reaction, and the PPAR γ activity was determined by measuring the expression of the FABP4/aP2 gene’s messenger RNA (mRNA) by real-time reverse transcription polymerase chain reaction. Beside the FABP4/aP2 gene, the mRNA expression of the cyclophilin A housekeeping gene was also determined in the samples. Then, the data obtained from the FABP4/aP2 gene expression were normalized to the cyclophilin A values by means of the $\Delta\Delta C_t$ method, and these normalized values were evaluated. (In vitro study was performed by Department of Biochemistry Institute of Biology, ELTE Budapest, Hungary.)

In vivo study

Animals and study design

The experiments were carried out on 5-week-old male Wistar rats. The animals were housed in an animal room with a temperature of 22°C–24°C, and with 50%–70% relative humidity. The lighting was set to 12-hour light and 12-hour dark periods.

Forty-eight animals were used throughout the study, and they were divided into three main groups. The first main group of Wistar rats served as healthy, vehicle-treated controls. These animals were held on a standard laboratory diet (ssniff®, EF R/M Control; ssniff Spezialdiäten GmbH, Soest, Germany), and they drank tap water ad libitum. The second main group of the rats was fed with a high-fat diet (HFD, ssniff®, EF R/M with 20% fat; ssniff Spezialdiäten GmbH, Soest, Germany) for 3 weeks. These rats served as the insulin-resistant, nondiabetic, vehicle treated group. In the first two main groups of rats, eight rats per group were used. The third main group of rats was divided into four subgroups with eight rats per subgroup. These rats were fed HFD for 2 weeks, and they were then treated with streptozotocin (STZ; 50 mg/kg, intraperitoneally). After an additional week, the animals showed stable fasting hyperglycemia. This animal model mimics the two characteristic features of type 2 diabetes. The peripheral insulin resistance was induced by the HFD, while impaired glucose-stimulated insulin secretion was induced by the STZ treatment. This type 2 diabetic animal model was originally established and validated by Reed et al. The first subgroup of the third main group was treated with vehicle, and the remaining three subgroups were treated with a daily oral dose of 50 mg/kg, 100 mg/kg, and 200 mg/kg of nitazoxanide, respectively. All treatments lasted for 8 days, and on the final day, 2 hours after the last vehicle/drug administration, the animals were anesthetized in order to execute the HEGC study for determination of whole-body insulin sensitivity.

Dose selection

As our ultimate goal was to find a generic drug that could be reintroduced in new clinical indications, the important aspect of our drug development paradigm was that the drug could improve insulin sensitivity in similar or lower doses than previously used in animal studies, where the drug demonstrated its efficacy in the originally approved indication (such as nitazoxanide as an antiprotozoal agent). Accordingly, we selected

doses of nitazoxanide where the dose range (50–200 mg/kg/day) demonstrated efficacy in reducing oocyst shedding in an immunosuppressed rat model of cryptosporidiosis.

Determination of insulin sensitivity

The HEGC procedure was performed as described previously. In brief, after an overnight fasting, rats were anesthetized with an intraperitoneal injection of 50 mg/kg of sodium thiopental (Thiopental Sandoz®; Sandoz Pharmaceutical PLC, Basel, Switzerland). After a middle sagittal surgical incision on the ventral surface of the neck, the trachea was exposed and a polyethylene tube was introduced into it to allow spontaneous breathing of the animals. Then, the left jugular vein and the left carotid artery were exposed and cleaned from the adhering connective tissues. Insulin and glucose were infused as separate lines of infusion through the two branches of the left jugular vein, while the arterial cannula served for blood pressure monitoring as well as to obtain blood samples (0.1 mL) for subsequent blood glucose and plasma insulin determinations. When the surgery was completed, there was a 30-minute stabilization period; then, a continuous insulin infusion (Humulin R®; Eli Lilly and Company, Indianapolis, IN, USA) at a rate of 3 mU/kg/minute was commenced along with glucose infusion (20% w/v). The rate of glucose infusion was adjusted in order to maintain euglycemia (5.5 ± 0.5 mmol/L). The blood glucose concentration was determined by means of a glucometer (Accu-Chek®; F Hoffmann La-Roche Ltd, Basel, Switzerland) before and at 5-minute intervals during the first 80 minutes, and at 10-minute intervals during the last 40 minutes of the HEGC experiment. In order to determine the fasting and steady-state plasma levels of insulin, additional blood samples were collected (0.5 mL, in 20 μ L of ethylenediaminetetraacetic acid and 10 μ L of Trasylol®; Bayer AG, Leverkusen, Germany) from the carotid artery immediately before the commencement of insulin infusion and during the steady state of the HEGC, respectively. The blood samples were centrifuged (Centrifuge 5415R; Eppendorf AG, Hamburg, Germany) for 2 minutes at 4°C and 10,000g; then, the plasma was aliquoted, frozen, and stored at -70°C for subsequent determinations. The plasma insulin level was determined by means of a commercially available radioimmunoassay kit (RK-400CT, Institute of Isotopes of the Hungarian Academy of Sciences, Budapest, Hungary). (Determination of insulin was performed by József Németh PhD.) The average glucose infusion rate

(GIR; mg/kg/minute) required to maintain the euglycemia during the last 30 minutes of the HEGC characterizes the glucose uptake of the peripheral insulin-sensitive tissues.

Derivative measures regarding the characterization of the insulin action can be calculated by means of plasma insulin and blood glucose values obtained during the fasting state as well during as the steady state of the HEGC. Accordingly, the insulin sensitivity of the peripheral tissues was characterized by the insulin sensitivity index (ISI), a measure of the amount of glucose taken up by peripheral tissues per unit of plasma insulin concentration. Furthermore, the metabolic clearance rate of insulin (MCRI) as an indicator of the rate of disappearance of insulin from the blood was calculated as the insulin infusion rate (mU/kg/minute), divided by the difference between the steady-state and fasting-plasma insulin level, and was expressed as mL/kg/minute.

Statistical analysis

All data were expressed as mean \pm standard deviation (SD) and were analyzed with paired t-tests when possible; otherwise, one-way analysis of variance followed by a modified repeated measures t-test (according to Bonferroni's method) was used.

4. RESULTS

Effect of single oral dose of olanzapine on basal and postprandial whole-body insulin sensitivity.

Basal, fasting insulin sensitivity was determined by means of HOMA equation. Olanzapine treatment increased both fasting plasma insulin and blood glucose levels, which resulted in elevation in the HOMA-IR index indicating fasting insulin resistance. Postprandial insulin sensitivity was characterized by means of RIST index. In fasted, vehicle-treated animals, the RIST index was lower compared to the vehicle-treated re-fed group. Olanzapine administration did not modify the RIST index in neither the fasted nor the re-fed groups compared to vehicle-treated controls indicating that a single dose of olanzapine had no effect on the postprandial increment in whole-body insulin sensitivity.

Effect of single oral dose of olanzapine on metabolic hormone levels

Arterial blood samples (0.1 ml) were taken during the postoperative stabilization period in order to determine the levels of metabolic hormones in fasted (n=12) and postprandial (n=12) states. The plasma levels of amylin, GLP-1, PYY and PP did not differ significantly between the fasted and re-fed groups of either vehicle- or olanzapine-treated rats. In vehicle-treated animals, the plasma insulin, leptin and GIP values were elevated significantly after meal. This postprandial increment in plasma leptin and GIP levels could also be observed in olanzapine-treated animals. However, olanzapine administration caused elevation in fasting plasma insulin compared to vehicle-treated group, but the postprandial insulin response was blunted in olanzapine-treated rodents. After meal, the plasma level of ghrelin decreased significantly in both vehicle- and olanzapine-treated rats. On the other hand, the postprandial ghrelin response was reduced after olanzapine administration.

Effect of single oral dose of olanzapine on first meal size

In the re-fed groups, there was no statistically significant difference in the first meal size between the vehicle- and olanzapine-treated groups. The average meal size was 2.66 ± 0.5 and 2.82 ± 0.4 g in the vehicle- and olanzapine-treated groups, respectively. There was no statistically significant difference in the average first meal time duration between the vehicle- and olanzapine-treated groups, and the respective values were 13.9 ± 1.1 and 16.5 ± 1.2 min. Finally, during the entire re-fed period, there was no statistically significant difference between the total amount of food consumed in the vehicle- and olanzapine-treated rats and the respective values were 4.33 ± 0.5 and 4.05 ± 0.5 g.

One-dimensional DPM

The basis of the oDPM analysis is that the pairwise similarities between two IPs were considered as vectors in a multidimensional space. This approach is referred to as “one-dimensional” since the distance of the two IP vectors is a one-dimensional measure. The advantage of this measure is that it reflects the pattern of the docking score values in the profile more than the actual scores. For instance, if two drugs possess the same IP, but with different average docking scores, their d value will be small, suggesting a high level of similarity. On the other hand, a single miscalculated

docking score can cause significant error in the distance measurement. This disadvantage is eliminated in DPM. It should be mentioned that in the case of small groups (less than ten members), DPM cannot be applied because of the high probability of overfitting. Therefore, PPAR γ predictions can be obtained only by a simpler method like oDPM, regardless of its generally weaker prediction accuracy when compared to DPM. The average area under the curve value of oDPM on 157 effect groups with at least ten members is 0.62 ± 0.12 , while the accuracy of DPM is 0.97 ± 0.03 .

After examining the closest neighbors of the members of the reference set, and after counting the most abundant neighbors of the members, ten generic drugs were selected if they appeared on the list three times: dantrolene, entacapone, ethacrynic acid, ketorolac, tiaprofenic acid, tolmetin, bromfenac, lubiprostone, nitazoxanide, and suprofen. A further 44 compounds appeared on the list twice, while 116 drugs reached one appearance; all of them were discarded. It is notable that the compounds are close to each other and seem to form a fairly coherent group that is similar to the reference set (average IP distance values and standard errors for the reference set and the predicted set are 1.37 ± 0.85 and 1.30 ± 0.77 , respectively). Lubiprostone is the only compound that is distant from the others. Based on these results, no further refinement of the prediction could be performed; therefore, these ten compounds were piped to in vitro screening. (Performed by Zoltan Simon PhD and his research group.)

In vitro study

The effect on FABP4/aP2 gene activity

Six of the ten chemical structures studied did not alter FABP4/aP2 gene expression in the MM6 cell line (data not shown). On the other hand, bromfenac, nitazoxanide, suprofen, and lubiprostone induced significant elevation in the FABP4/aP2 gene transcript. However, the induced elevation in the mRNA expression was distinct among the MM6 cell lines treated with these four chemicals. Suprofen induced a significant elevation in the mRNA expression level, though at its lowest concentration applied (10⁻⁸ M); however, increasing doses were not followed by further elevation in mRNA expression, except at its highest dose (10⁻⁵ M), but this change was not significant

Compared to its lower doses. Lubiprostone also induced significant elevation in the mRNA level at its lowest dose (10⁻⁸ M) applied; however, the effect induced by

increasing its doses was inconsistent, and no dose-dependent elevation in mRNA expression was seen. The other two drugs, bromfenac and nitazoxanide, induced dose-dependent activation of the FABP4/aP2 gene. Bromfenac caused significant elevations in mRNA expression in the two higher doses (10⁻⁶ M and 10⁻⁵ M), while the nitazoxanide was able to induce significant elevation in 10⁻⁷ M concentration, and its effect seemed to be dose-dependent in that its effect on FABP4/aP2 gene activity was nonsignificant in the 10⁻⁶ M concentration. The greater SD can explain the observed nonsignificant alteration.

The percentile changes in FABP4/aP2 gene expression were also determined by comparing the effects of the highest applied dose of these four drugs to the effects elicited by their vehicle. In this comparison, lubiprostone proved to be the most effective, showing an approximate 10 times elevation in the normalized mRNA expression level, while nitazoxanide, suprofen and bromfenac showed 7 times, 6 times and 3 times elevation, respectively.

Rosiglitazone as a reference drug induced a concentration-dependent enhancement in FABP4/aP2 gene expression in the MM6 cell line. Moreover, the stimulating effect of rosiglitazone on FABP4/aP2 gene expression was approximately one order of magnitude higher than that observed after administration of either of the other drugs (data not shown). On the other hand, the vehicles failed to evoke any changes in FABP4/aP2 gene expression in the MM6 cell line. (In vitro study was performed by Department of Biochemistry Institute of Biology, ELTE Budapest, Hungary.)

In vivo study

Determination of whole-body insulin sensitivity

Based on the in vitro data and the available information regarding these generic drugs, we selected nitazoxanide for further study in order to determine its insulin sensitizing effect on type 2 diabetic rats.

Animals held on a HFD became insulin resistant, but not diabetic at the end of the 3-week diet period. Rats treated with intravenous STZ at the end of the second week of the HFD regimen showed not only decreased insulin sensitivity compared to healthy animals, but also marked fasting hyperglycemia (ie, these rats became type 2 diabetic). The vehicle-treated healthy and HFD groups of rats showed significantly lower fasting

blood glucose levels and significantly elevated GIR and ISI compared to the vehicle-treated HFD + STZ rats. The nitazoxanide treatment induced a dose-dependent improvement in the glycemic status of the HFD + STZ-treated type 2 diabetic rats. This improvement in glycemic status was characterized by a significant increase in the GIR needed to maintain euglycemia, as well as by the enhancement of the ISI during the HEGC in rats treated with 200 mg/kg nitazoxanide over 8 days. The fasting blood glucose level of the vehicle-treated HFD + STZ rats was significantly elevated compared to the vehicle-treated healthy or HFD rats, and nitazoxanide treatment showed a dose-dependent reduction in the fasting blood glucose level, reaching a statistically significant level at a dose of 200 mg/kg. The fasting plasma insulin level was elevated in all type 2 diabetic groups (HFD + STZ + vehicle, HFD + STZ + nitazoxanide) and the nitazoxanide treatment did not cause a significant change. Finally, no significant difference among the MCRI could be observed in either treatment groups. In addition, no alteration in the resting mean arterial blood pressure was observed.

5. DISCUSSION

The primary aim of the present study was to investigate the acute weight-independent effect of olanzapine on the MIS in female rats. Furthermore, we also aimed to explore the effect of the single oral dose of olanzapine on the feeding behaviour and the plasma levels of meal-related hormones which could be responsible for the initial biochemical events of the olanzapine-induced metabolic changes. The MIS was first described by a Canadian research group in the middle of the 1990s. Subsequently, intensive research in this field has revealed several details of this mechanism including the role of vagal nerve and muscarinic receptor, the involvement of the sensory effector pathways, its pharmacological susceptibility and the putative trigger role of postprandial CCK release. Clinical significance of the MIS mechanism is emphasized by the observations that long-term deterioration of the MIS mechanism resulted in a metabolic state resembling human type 2 diabetes and that pharmacological activation of this mechanism can improve the MIS in experimental diabetes. These data suggest that the potential pharmacological exploitation of the MIS could improve insulin sensitivity in patients with insulin resistance. Moreover, the present study revealed again the basic

phenomenon of the MIS, i.e. the improved insulin sensitivity in re-fed animals compared to fasted ones.

Olanzapine is one of the AAPs with the greatest liability to induce weight gain. However, olanzapine is able to induce metabolic derangement in the absence of obesity. Here, we corroborated that olanzapine can induce whole-body insulin resistance indicated by the elevated HOMA-IR index in the absence of increased adiposity or obesity and these changes are in agreement with previous preclinical and clinical observations that revealed the olanzapine-induced acute hyperinsulinaemia, hyperglycaemia and insulin resistance without coexisting obesity, increased adipose tissue mass or psychological illness. Surprisingly, the MIS was unaffected after single oral dose of olanzapine in spite of the high affinity of olanzapine to muscarinic M3 receptors.

According to the receptor binding profile of olanzapine and the atropine sensitivity of the MIS, one can speculate that muscarinic M3 receptor does not play a significant role—at least—in the acute effect of olanzapine on MIS. On the other hand, we cannot rule out that olanzapine plasma concentration was reduced below the pharmacological active concentration when the RIST assay was carried out because the plasma half-life of olanzapine is considerably shorter in rodent than in humans and approx. 2 h elapsed from the drug administration until the insulin infusion. Furthermore, we used only one dose of olanzapine, so it cannot be ruled out that in higher doses, it could modulate this adaptive insulinsensitizing mechanism. The lack of effect of olanzapine on meal-related improvement in whole-body insulin sensitivity cannot be explained by its effect on feeding behaviour, since olanzapine failed to influence either the first meal size and duration or the total amount of meal consumed in our experimental set-up. These results are in accordance with previous reports demonstrating that olanzapine treatment did not modify the meal size, but may reduce the inter-meal period thereby causes hyperphagia. However, the changes in meal-related hormone levels observed in olanzapine-treated rats shed light on the initial events of the developing insulin resistance and hyperphagia. Both ghrelin (serves as hunger signal) and insulin (serves as satiety signal) responses were blunted postprandially suggesting an acute impairment in the regulation of feeding behaviour. This in turn could contribute to the reduced inter-meal period.

There is only one study that investigated the acute effect of olanzapine on meal-related hormones. In that study, they found an increased first meal size and significantly elevated pre-prandial plasma total ghrelin level in olanzapine-treated rats, but the postprandial active ghrelin level was similar in the saline- and olanzapine-treated groups. Here, we demonstrated that there was no significant difference in the first meal size and the pre-prandial ghrelin levels in vehicle and olanzapine-treated rats, but the postprandial ghrelin response was blunted in the olanzapine-treated rats. The discrepancy between the two studies could be explained by the different genders and ages of the rats, the different routes of administration and the fact that in the study of van der Zwaal, the rats were trained for the expected meal time. On the other hand, the blunted postprandial ghrelin and insulin response also supports the putative role of impaired ghrelin and insulin action in the development of olanzapine-induced hyperphagia. Although there were no statistically significant differences in the other hormone levels investigated, this cannot rule out that olanzapine treatment could modify these parameters, since in our study, the blood samples for hormone determination were obtained approximately 2 h after meal; so, we have no information about the postprandial levels of these hormones during the first 2 h. Other reports indicate that in rats 2 h after oral glucose tolerance test or after test meal, the plasma level of metabolically important hormones return to the basal level. This could explain why we were unable to detect elevated plasma levels of GLP-1, amylin, PYY or PP 2 h after meal. On the other hand, our results are in accordance with those clinical data which also suggested that short-term oral treatment with olanzapine does not modify the gut hormone secretion.

The aim of the present study was to find generic drug(s) with PPAR γ receptor agonist activity by means of screening the FDA-approved generic molecule database using oDPM methods. As a result of our current work, we have identified ten potential PPAR γ ligands by means of the oDPM method. It had been shown that four out of the selected ten drugs were able to induce the expression of the transcript of the FABP4/aP2 gene, which is specific to PPAR γ receptor activation. Among these four generics, nitazoxanide was chosen to further study its putative PPAR γ receptor agonist action by

means of HEGC in a rodent model of type 2 diabetes in order to explore its insulin sensitizing property.

The drug development process became very expensive during the last decades. There have been several attempts made, trying to shorten the time-to-market as well as to decrease the amount of human and financial resources needed for the drug development process. In order to solve these above mentioned difficulties, the interest of the pharmaceutical industry has turned to the renewal of generics. Using drugs at the same or lower concentrations, as well as for similar treatment periods as were used in their original indications, several steps in the development process can be spared, such as conducting time-consuming and costly toxicity studies; an example of this kind of drug renewal was published previously. We demonstrated that cicletanine, a phosphodiesterase enzyme 1–5 inhibitor (originally developed for the treatment of hypertension), is able to enhance whole-body insulin sensitivity in insulin-resistant rabbits at lower doses than is required for its vascular effects.

The actuality of our present study has been underpinned by the fact that the prevalence of diabetes is increasing globally, and the total number of patients suffering from this chronically devastating condition have reached 347 million. On the other hand, the number of available drugs used to treat diabetes is limited, and the most frequently prescribed medications are thiazolidinediones, which have several side effects such as fluid retention, edema, congestive heart failure, or bone fractures. Although these side effects are due to the activation of the PPAR γ receptor, conformation of the receptor is the key feature that determines the affinity of corepressors and coactivators to the ligand-bound PPAR γ receptor, thereby determining the gene transcription profile and biological response after PPAR γ activation. The diverse regulation of the PPAR γ receptor activation and signal transduction pathways was made feasible to design selective PPAR γ modulator molecules, which retain the insulin-sensitizing effect, but do not possess those unwanted effects mentioned above.

In the present study, we used computational modelling of the receptor–ligand interaction, which ensures high throughput screening of large molecule libraries. By screening the FDA-approved generics molecule database, our results supported the original assumption that the *in silico* oDPM methodology applied could facilitate the initial drug candidate selection if the molecular structure of the target protein is known. Here we combined the advantages of the *in silico* oDPM method and generics-based

drug development strategy. The *in silico* oDPM method used allows us to select candidates from the molecule library within a relatively short time period. Using generics in new therapeutic indication could shorten the development process. On the other hand, the *in silico* prediction method has some limitations. For example, it does not allow us to establish whether the established receptor–ligand interaction corresponds to agonist or antagonist properties on the targeted receptor. In order to answer this important question, both *in vitro* and *in vivo* experiments were carried out.

oDPM was incapable of finding any differences between the four compounds that were active *in vivo*, and the rest of the predicted set, which again underlines the importance of *in vitro* testing and reminds us of the inherent limitations of *in silico* approaches. Our oDPM method is also currently incapable of predicting the side effects of a compound. Side effect prediction is more difficult than the presented prediction process, since the side effect databases are often incomplete and a lot of questions need to be answered (ie, the ambiguous terminology of the adverse events, the handling of different frequencies of adverse events depending on the exact treatment and dosage, and so on). Of course, the list of the effects and targets of the close IP neighbors of a molecule might still yield clues to its potential side effects (for example, a predicted angiotensin-converting enzyme inhibitory effect can point to possible effects on blood pressure). This opportunity will be addressed in future research, using the DPM method because it performs better than oDPM when studying large groups.

The data from *in vitro* experiments revealed those generics from our selection list that are able to stimulate the PPAR γ receptor in a dose-dependent manner. Finally, *in vivo* experiments were carried out in order to study whether PPAR γ activation results in the enhancement of insulin sensitivity among insulin-resistant, type 2 diabetic rats. Whole-body insulin sensitivity was determined by means of HEGC, the gold standard for determining insulin sensitivity in humans. Since the full insulin-sensitizing effect of PPAR γ receptor activation requires several days, we treated the animals for 8 days, which has been shown to be sufficient in inducing insulin-sensitizing effects in rodents.

According to our original concept, we intended to investigate the putative insulin sensitizing action of the selected generics using the same dose-range as was used in its original indication. In the present study, we investigated the insulin-sensitizing effect of nitazoxanide, a generic drug that was originally approved as an antiprotozoal agent. Its established mechanism of action is to inhibit the pyruvate ferredoxin oxidoreductase

enzyme-dependent electron transfer reaction. Beside on its antiprotozoal action, the experimental results suggested that nitazoxanide can also bear antiviral activity due to its blocking properties over the maturation of the viral hemagglutinin, which in turn results in impaired intracellular trafficking and insertion into the host plasma membrane of the hemagglutinin. On the other hand no data regarding its putative PPAR γ receptor-activating action are available in the literature or in the patent databases. In an immunosuppressed rat model of cryptosporidiosis, nitazoxanide was able to effectively inhibit the oocyte shedding at a daily oral dose of 50–200 mg/kg. So, we selected this same dose range and the applied treatment lasted for 8 days, according to the time required for evolution of the effect of PPAR γ activation. Although in clinical practice nitazoxanide is usually administered for only 3–5 days to treat protozoal infections, the available preclinical toxicological data suggest that there are no safety concerns when nitazoxanide is used for a longer term. These features made nitazoxanide an ideal generic drug candidate to be reintroduced in a new clinical indication as an insulin-sensitizing agent.

The main discovery of the present study is the demonstration of the predictive value of the oDPM method in searching for PPAR γ ligands. The positive predictive value of the oDPM method was supported both by *in vitro* measurement of FABP4/aP2 gene activity after dose-dependent exposure of the MM6 cell line to nitazoxanide, and by *in vivo* demonstration of the dose-dependent insulin-sensitizing effect of nitazoxanide using an insulin-resistant type 2 diabetic rat model. Although we did not determine PPAR γ mRNA or protein expression from the tissue samples after the *in vivo* experiments, our results did suggest that the improvement in insulin sensitivity could be a consequence of the enhancement in the glucose uptake of the peripheral insulin-sensitive tissues, since both the GIR and the ISI were increased in response to nitazoxanide treatment. On the other hand, the MCRI and the steady-state plasma insulin level were left unaffected by the nitazoxanide treatment. These data indicate that the putative mechanism of action of nitazoxanide is in the enhancement of the insulin sensitivity, which is similar to what was expected after PPAR γ stimulation. Moreover, our results demonstrated that nitazoxanide failed to induce body weight gain and elevation in mean arterial blood pressure. It should be noted that the treatment period was only 8 days, and thus changes in either weight gain or systemic blood pressure cannot be ruled out, as they may occur after longer treatment periods. A more

comprehensive study investigating the effects of the chronic administration of nitazoxanide on different organ systems will be able to address this issue.

6. SUMMARY

Olanzapine, which is one of the AAP, can acutely induce insulin resistance and metabolic derangement according to previous preclinical and clinical observations. But we do not know (there is no data in the literature) whether it is able to modulate the meal-induced insulin sensitization (MIS). Here we corroborated that olanzapine can induce insulin resistance in the absence of increased adiposity or obesity and these changes are in agreement with previous observations. However, the changes in ghrelin levels observed in olanzapine treated rats suggest an acute impairment in the regulation of feeding behaviour, which can play a role in the chronic olanzapine treatment induced weight gain. It was proven that the MIS was preserved in response to a single oral dose of olanzapine, which is the major original finding of our study (against the effect on the basal insulin sensitivity). The importance of this finding is that pharmacological activation of the MIS can improve insulin sensitivity in patients with olanzapine-induced insulin resistance.

Using *in silico* computational molecular database screening and reintroduction of generic drugs by second medical use patents help to decrease the time and resources needed for drug development. In this study we combined these two methods (ways) in order to identify a generic molecule, which is able to improve insulin sensitivity through its' PPAR γ receptor activation property. The main discovery of the present study is the demonstration of the predictive value of the oDPM method in searching for PPAR γ ligands. Our results presented the usefulness of this method in the screening of the molecule database for lead selection, and we discovered and supported the insulin sensitizing effect of nitazoxanide, in both *in vivo* and *in vitro* experiments. This generic drug can be a prototype chemical structure of the new generation of insulin sensitizers.

7. APPENDIX



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Registry number: DEENK/73/2015.PL
Subject: Ph.D. List of Publications

Candidate: Diána Klára Kovács
Neptun ID: LF1U82
Doctoral School: Doctoral School of Pharmaceutical Sciences

List of publications related to the dissertation

1. **Kovács, D.**, Hegedűs, C., Kiss, R., Sári, R., Németh, J., Szilvássy, Z., Peitl, B.: Meal-induced insulin sensitization is preserved after acute olanzapine administration in female Sprague-Dawley rats.
Naunyn-Schmiedeberg's Arch. Pharmacol. Epub ahead of print (2015)
DOI: <http://dx.doi.org/10.1007/s00210-015-1091-8>
IF:2.36 (2013)
2. **Kovács, D.**, Simon, Z., Hári, P., Málnási-Csizmadia, A., Hegedűs, C., Drimba, L., Németh, J., Sári, R., Szilvássy, Z., Peitl, B.: Identification of PPAR γ ligands with One-dimensional Drug Profile Matching.
Drug Des. Devel. Ther. 7, 917-928, 2013.
DOI: <http://dx.doi.org/10.2147/DDDT.S47173>
IF:3.026



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List of other publications

3. Hegedűs, C., Kovács, D., Drimba, L., Sári, R., Varga, A., Németh, J., Szilvássy, Z., Peitl, B.:
Investigation of the metabolic effects of chronic clozapine treatment on CCK-1 receptor
deficient Otsuka Long Evans Tokushima Fatty (OLETF) rats.
Eur. J. Pharmacol. 718 (1-3), 188-196, 2013.
DOI: <http://dx.doi.org/10.1016/j.ejphar.2013.08.034>
IF: 2.684

Total IF of journals (all publications): 8,07

Total IF of journals (publications related to the dissertation): 5,386

The Candidate's publication data submitted to the iDEa Tudóstér have been validated by DEENK on
the basis of Web of Science, Scopus and Journal Citation Report (Impact Factor) databases.

02 April, 2015

