Chapter 7: Proteomics

Olanzapine inhibits body weight gain in male Wistar rats, which is amplified by adjunctive Topiramate treatment; hepatic proteome analysis points towards a disruption in growth hormone regulation.

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Abstract

Humans chronically treated with the atypical antipsychotic Olanzapine (OLZ) often experience weight gain and insulin resistance, and co-administration of the anticonvulsant drug Topiramate (TPM) has been reported to inhibit these effects. It is unclear whether TPM can prevent OLZ-induced insulin resistance in a weight gain independent fashion. For this reason, we chose to investigate these interactions in male Wistar rats feeding a medium high fat diet, in which OLZ has been documented to reduce weight gain. In a 21-day oral treatment protocol, we found that TPM (25 mg/kg b.i.d.) alone reduced adiposity and improved glucose homeostasis assessed by an intragastric glucose tolerance test (IG-GTT). In addition, TPM exaggerated weight loss in rats treated with OLZ (5 mg/kg b.i.d.). Despite decreased adiposity in OLZ and particularly in OLZ+TPM treated rats, glucose and insulin profiles during the IG-GTT were comparable to control animals which did gain weight, but were increased when corrected for skeletal muscle weight. Hepatic proteomic analyses revealed that aldose reductase-related protein 1 and glucose-6-phosphate dehydrogenase levels were increased in the OLZ and OLZ+TPM treated groups relative to untreated controls. This indicates an up-regulation of the polyol pathway, which would normally be found in cases of hyperglycemia. TPM did not influence hepatic protein expression related to OLZ treatment, but specifically increased hepatic UDP-glucuronosyltransferase 2B11 (UGT2B11) expression, a protein also involved in OLZ metabolism. Furthermore, a down-regulation of cytochrome P450 CYP2C11 and an up-regulation of CYP2C12 were observed in OLZ treated rats. In combination with the lack of body weight gain in the OLZ-treated rats, this indicates that OLZ inhibits growth hormone action in male Wistar rats. Therefore OLZ treated male rats do not gain weight and have decreased muscular mass compared to Control, which may affect total body insulin sensitivity.

Keywords:
Olanzapine, Topiramate, proteomics, insulin sensitivity, glucose response, liver, aldose reductase, G6PD, CYP2C11, CYP2C12, UGT2B11, growth hormone, male Wistar rat
Introduction

Chronic treatment with the a-typical antipsychotic Olanzapine (OLZ) in humans has been reported to underlie weight gain and to increase the risk of developing insulin resistance [1-4]. The anticonvulsant Topiramate (TPM) has opposing effects; it decreases body weight and improves insulin sensitivity [5-7]. The mechanisms by which OLZ and TPM influence body weight (BW) regulation and insulin sensitivity are still a matter of discussion.

OLZ is classed as a second-generation antipsychotic (SGA), which is characterized by an a-typical receptor binding profile. Similar to the first generation antipsychotics (FGA) OLZ has an antagonistic affinity to the dopamine D1 and D2 receptor, but has an additional antagonistic affinity to predominantly the 5-HT2A/C receptors, and in a lesser extent to the H1, M3, and α-adrenergic receptors [8,9]. OLZ is clinically preferred over FGAs, because it induces less extrapyramidal side effects (e.g. akinesia, akathisia) and has better clinical efficacy in treating the negative symptom dimensions of schizophrenia (e.g. anhedonia, avolition, and apathy). On the other hand, OLZ treatment is highly associated with weight gain and insulin resistance, which undermines treatment compliance and increases morbidity [10,11].

Based on meta-analyses of human clinical trials in patients and healthy subjects, Newcomer et al (2006) found that the level of weight gain liability related to a-typical agents positively correlates to the risk of developing insulin resistance, dyslipidemia, and hyperglycemia. In fact, insulin resistance caused by antipsychotic treatment may primarily be a result of weight gain. However, OLZ treatment has also been associated with the development of insulin resistance and type 2 diabetes in the absence of weight gain. Kim et al (2010) showed that, regardless of the population, insulin resistance worsens as weight increases, but additionally reported that OLZ treatment was also associated to insulin resistance independent of adiposity [12]. These interactions are more complex in patient populations because both insulin resistance and type 2 diabetes have been reported to occur at higher rates in drug-naïve schizophrenic populations compared to the general population [13,14]. These findings suggest an increased risk within the patient population per se, which possibly is enhanced by OLZ treatment.

Controlled human studies have not observed an apparent difference between male and female responsiveness related to OLZ-induced weight gain. In contrast, rodent studies have demonstrated consistently that an increase in BW due to OLZ treatment is only apparent in female rats [15]. Nonetheless, Cooper et al (2007) did observe increased adiposity and decreased muscle mass in male rats after chronic OLZ
treatment without the occurrence of weight gain. Furthermore, they observed an increase in fasting insulin levels, indicative of a decline in insulin sensitivity [16]. Due to the similar response of humans and male rats in terms of OLZ-induced insulin resistance, it can be suggested that male rats can serve as a feasible model for OLZ-induced insulin resistance that develops in a weight gain independent manner.

TPM’s insulin sensitizing effect is predominantly based on its adiposity-reducing effect by stimulating lipolysis and inhibiting lipogenesis [17,18]. Multiple human studies have been performed and demonstrated the ability of TPM to attenuate OLZ-induced weight gain and maintain insulin sensitivity [19-21]. The idea that TPM is an effective adjunctive during OLZ treatment was further supported by Vieta and colleagues, who found that co-administration of TPM reduced the scores on the brief psychiatric rating scale (BPRS), thereby improving the clinical effectiveness of OLZ [19].

In a randomized controlled OLZ study including 1336 schizophrenic patients, Kinon et al (2005) found that 15% of the subjects demonstrated severe rapid weight gain (>7% BW gain) irrespective of gender, another 55% of subjects displayed weight gain less than 7% of their initial BW, whereas approximately 30% kept a stable weight or lost weight over a treatment period of 52 weeks. Furthermore, they reported that improvements in the BPRS score were particularly found in cases where OLZ treatment induced rapid and severe weight gain [22]. Based on these findings, Kinon and co-workers suggested that rapid BW gain could be used as a marker for OLZ’s clinical efficacy [22]. From the perspective of OLZ-induced insulin resistance that arises independent of BW gain, it is interesting to investigate if TPM remains effective during OLZ treatment in subjects without OLZ-induced weight gain.

Previously, we demonstrated the effect of chronic oral OLZ treatment on weight gain in female Wistar rats [23]. Here, we introduced the same drug administration protocol to a group of male Wistar rats, and additionally included groups treated with TPM and OLZ+TPM. To study the effects of OLZ and/or TPM on insulin and glucose regulation, we performed an intragastric-glucose tolerance test (IG-GTT). In order to investigate the role of the liver in the metabolic states of the different groups after chronic drug treatment, we extended the study with a hepatic proteomic analysis.

Materials and Methods

2.1 Animals

All procedures involving animal welfare have been approved by the Animal Experimentation Committee of the University of Groningen. Twenty-four male Wistar
rats (408 ± 3.4 g; day-7), obtained from Harlan (Horst, NL) were individually housed in clear Plexiglas cages (25x25x30 cm) on a plastic floor with wood chip bedding. Room temperature was controlled at 22 ± 2 °C, under a 12:12 hr light-dark cycle (lights off at 11:00AM; CT12). Animals had one week to acclimatize before undergoing surgery. Baseline measurements started one week after surgery when all animals had surpassed their pre-surgical BW. Prior to drug treatment, animals had ad lib access to standard chow (3.8 kcal/g, Arie Blok Diets, Woerden, NL) and water. To facilitate weight gain, a western style diet (medium fat diet with lard: 4.7 kcal/g; 45% fat, Arie Blok Diets, Woerden, NL) was provided ad libitum to all animals from the start of drug treatment (day 0).

2.2 Drugs and administration protocol

OLZ and TPM were kindly provided by Abbott (OLZ: Fournier Laboratory, France; TPM: Hannover, Germany). To obtain a daily administration of 10 mg/kg OLZ and 50 mg/kg TPM, OLZ (5 mg/ml) and TPM (25mg/ml) were diluted in 0.9% NaCl (saline). OLZ was first dissolved in saline using 1M HCl and adjusted to pH 6.5-7 using 1M NaOH. TPM was diluted in saline (SAL) using 1M NaOH and adjusted to pH 7.4 using 1M HCl. All animals received separate oral administrations consisting either of OLZ+SAL, TPM+SAL, OLZ+TPM, or SAL+SALat circadian time (CT)12 and CT18 over a 21-day period. Seven days prior to the start of drug treatment all animals received saline administrations (2ml/kg) twice a day to habituate to the drug administration protocol.

2.3 Experimental set-up

Animals were divided into four weight-matched experimental groups: 1) Control (SAL+SAL; n=5), 2) OlZ (+SAL; n=6), 3) OLZ+TPM (n=6), 4) TPM (+SAL; n=6). At day -1 during baseline and at day 14 of treatment an intragastric-glucose tolerance test (IGGTT) was performed. At day 21 animals were sacrificed by perfusion of cold saline (4 °C) via the left ventricle (app. 300 ml saline/animal). After perfusion, livers and other tissues were collected and directly frozen with liquid nitrogen and stored at –80°C for further analyses (see below). Abdominal adipose tissue depots (epididymal and retroperitoneal) were carefully removed and weighed. Skin was separated from the carcass to measure total skeletal muscle weight.

2.4 Surgical Procedure

During surgery (under high O2-low CO2 isoflurane inhalation anesthesia) a gastric cannula (1.40mm OD, 0.80mm ID) was inserted in the corpus of the stomach [23] and a second cannula (1.40mm OD, 0.80mm ID) was inserted into the right jugular vein [24]. The jugular vein cannula allowed stress free blood sampling and the intragastric
cannula was used for controlled drug delivery. Both cannulas were subcutaneously guided towards the head, where they were exteriorized via a bent 19G stainless steel metal sleeve, which was fixed to the skull by surgical stainless steel screws and dental cement. Post-surgery analgesia (0.1 mg/kg Finadyne diluted in 0.1 ml/kg saline) was administered s.c. 15 minutes before animals were taken off anesthesia. Exteriorized jugular vein and gastric cannula were closed by plastic caps made of a piece of flame-sealed PE100 tubing, and were rinsed twice a week starting 2 days after surgery to prevent blockage. The gastric cannula was rinsed with 0.5 ml saline; obstruction of the jugular vein cannula was prevented by using a 55% PVP solution in between blood sampling experiments.

2.5 Intragastric-glucose tolerance test (IG-GTT)

During the study, two separate IG-GTTs were performed at day -1 and day 14 at the start of the dark phase (CT12). Prior to the start of IG-GTT, animals were fasted for 4 hours. A baseline blood sample was drawn at 60 minutes (t=-60) before the start of glucose infusion (t=0) followed by an intragastric drug or saline administration (2ml/kg). At day -1 all animals received an intragastric saline administration 60 minutes prior dark onset. Intragastric glucose infusion started at t=0 and stopped after 9 minutes at a rate of 1ml/min. In total animals received 9ml of 150mg/ml glucose. Blood samples (0.2 ml each) were taken at time points -60, 0, 2.5, 5, 10, 15, 20, 25, 30, and 40 minutes. Blood samples were immediately put on ice in vials containing 10μl EDTA (0.09 g/ml). Whole blood samples of 50µl diluted in 450µl 2% heparin solution were stored at 20°C until analysis of glucose concentrations by the ferricyanide method [25] in a Technicon auto analyzer. The remaining blood samples were centrifuged (15min, 2500 rpm, 4°C) and plasma was collected and stored at -20°C until insulin determination. Plasma insulin levels were measured in duplicates using a commercial radioimmunoassay kit (Rat Insulin, 125I-Insulin Cat# RI-13K, Linco Reasearch, Nucli Lab, NL). From the gathered data glucose and insulin responses were transformed into area under the curve (AUC) and were consecutively used to determine the relative change between baseline and treatment responses (AUC (%)). To further explore the effects on glucose and insulin regulation the Glucose Index was determined by multiplying the AUCGluc with the AUCIns (G*I).

2.6 Hepatic lipid isolation.

Liver tissues (100 mg) were homogenized using ice-cold phosphate-buffered saline. Hepatic concentrations of triglycerides (Roche Diagnostics, Mannheim, Germany), free and total cholesterol were measured using commercial kits (DiaSys Diagnostic Systems, Holzheim Germany) after lipid extraction according to Bligh and Dyer [26].
2.7 Hepatic RNA isolation

Total liver RNA was isolated using the TRI Reagent method (Sigma, St. Louis, MO) according to the manufacture’s protocol. Concentration of RNA was evaluated using a Nanodrop 2000c Spectrophotometer (Thermo Fisher Scientific, Rockford, IL, USA). RNA integrity was verified by northern blot. Samples were considered suitable for hybridization when they showed intact bands of 18S and 28S ribosomal RNA subunits at a ratio of approximately 2. cDNA was obtained using the reverse transcription procedure with Moloney Murine Leukemia Virus-RT (Thermo Fisher Scientific, Rockford, IL, USA) with random primers according to protocol of the manufacturer. cDNA levels for Acetyl-CoA Carboxylase 1 (Acc1), mitochondrial Acetyl-CoA Carboxylase 2 (Acc2), and Malonyl-CoA Decarboxylase (Mlycd) were measured by real-time quantitative PCR amplification using a Step One Plus Real-Time PCR system (Applied Biosystems, Foster City, USA). Expression levels were normalized to 36b4. Sequences of primers and probes are listed in the table below.

2.8 Proteomics

Frozen liver tissue (50mg) was lysed in 350µL 100mM Tris-HCl pH 7.6, 4% SDS, 0.1M DTT (lysis buffer) by letting the mixture thaw for a few seconds and placing it in a TissueRuptor (9001272; disposable probes: 990890, QIAGEN) rotor-stator homogenizer set at medium speed for 30s at room temperature followed by sonication with a power setting of 50 for 5-25 times for 20s at room temperature. The homogenate was further diluted with 650µL lysis buffer, vortex mixed for 30s and denatured for 3min at 95°C with 700rpm shaking in an Eppendorf thermomixer. The resulting homogenate was centrifuged at 16000× g for 5min at 20°C. The supernatant was removed and stored at -80°C in 100 or 400µL aliquots. Ten µL were kept to determine the total protein concentration at 562nm using the Micro BCA assay according to the manufacturer’s (ThermoFisher) instructions.

<table>
<thead>
<tr>
<th>Gene</th>
<th>acidic ribosomal phosphoprotein</th>
<th>acetyl-Coenzyme A carboxylase alpha (Acaca)</th>
<th>acetyl-Coenzyme A carboxylase beta (Acacb)</th>
<th>malonyl-CoA decarboxylase</th>
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<td>Abbreviation</td>
<td>36b4</td>
<td>Acc1</td>
<td>Acc2</td>
<td>Mlycd</td>
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<tr>
<td>Forward</td>
<td>GCT TCA TTG TGG GAG CAG ACA</td>
<td>CCA TCC AAA CAG AGG GAA CAT C</td>
<td>CCC AGG AGG CTG CAT TGA</td>
<td>GCT GTG ATG TGG CGT ATC AAC</td>
</tr>
<tr>
<td>Reverse</td>
<td>CAT GGT GTT CTT GCC CAT CAG</td>
<td>CTA CAT GAG TCA TGC CAT AGT GGT T</td>
<td>AGA CAT GCT GGG CCT CAT AGT A</td>
<td>CTT GGA GCC CAG GTA GGA GAT</td>
</tr>
<tr>
<td>Probe</td>
<td>TCC AAG CAG ATG CAG CAG ATC CGC</td>
<td>ACG GTA AAC AGA ATG TCC TTT GCC TCC AAC</td>
<td>CAC AAG TGA TCC TGA ATC TCA CGC GC</td>
<td>CAG CAG CCT CAA AGG CCT CAC CAG</td>
</tr>
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Lysates containing 100µg protein were alkylated with 15µL 0.55M iodoacetamide, 50mM NH$_4$HCO$_3$, 8M urea and 0.1M Tris-HCl, pH 8.5 for 30min in the dark with shaking (750rpm, 23°C). Proteins were subsequently precipitated by adding 80µL water followed by 600µL methanol (vortex mix), 150µL chloroform (vortex mix) and 450µL water (vortex mix). Precipitated proteins were recovered by centrifugation at 13000 rcf for 5min at room temperature and the upper water phase above the protein ring was removed. Another 450µL methanol were added (vortex mix) and the protein pellet was recovered by centrifugation at 16000 rcf for 5min at room temperature. After removing the supernatant another 450µL methanol were added (vortex mix) and the final protein pellet recovered by centrifugation at 16000 rcf for 5 min at room temperature after removing the supernatant.

The pellet was dissolved in 10µL 8M urea, 0.1M Tris-HCl, pH 8.5 buffer containing LysC (2µg, 1:50 enzyme to protein ratio) and proteins were digested for 6h at 25°C by mixing at 750rpm. Thirty µL trypsin in 50mM NH$_4$HCO$_3$ (2µg, 1:50 enzyme to protein ratio) were added and the sample mixed by up-and-down pipetting. Digestion was carried out overnight at 25°C and mixing at 750rpm. The pH was adjusted by adding 10µL digest to 90µL 0.1% formic acid + 0.5µL 100% formic acid directly in the auto sampler vial inserts (mix digests very well until no crystals are visible). This resulted in a solution of 10µg digested protein in 40µL. These aliquots were stored at -80°C until analysis.

Two-dimensional peptide fractionation was performed by connecting a strong cation-exchange (SCX) cartridge (Zorbax SCX Bioseries, 35 x 0.3mm, 3.5µm, Agilent) to a C$_{18}$ reversed-phase LC-chip (160nL trap column, 150mm x 75µm separating column, Zorbax 300SB-C18, 5µm, Agilent) via an automatic switching valve. For peptide fractionation prior to LC-MS/MS one aliquot (10µg digested protein in 40µL solution) was injected on the strong cation-exchange cartridge that had been conditioned by washing with 0.1% formic acid in water followed by 10 injections of 40µl 1M NaCl and equilibration in 3% acetonitrile, 0.1% formic acid in water for 15min at 15µL/min without connecting to the LC-chip. Peptides were eluted from the SCX cartridge with the following salt steps (40µL each): 5-10-15-20-25-30-40-60-90-120-300 and 500mM NaCl in 3% acetonitrile/0.1% formic acid at 4µL/min flow rate. Eluted peptides were transferred to the C$_{18}$ LC-chip by automated valve switching. The flow rate of 4µL/min was reduced to 1µL/min during peptide separation in the second dimension. An Agilent 1200 nanoLC chromatography system was used at 250nL/min flow rate for all analyses. For reversed-phase LC separations, the following solutions were used: A, ultrapure water (conductivity 18.2 MΩ, Sartorius) with 0.1% formic acid (LC-MS grade, Fluka); B, acetonitrile (HPLC-S gradient grade, Biosolve) with 0.1% formic acid.
Peptides were analyzed by LC-MS/MS on a quadrupole-time-of-flight (QTOF) mass spectrometer (Agilent 6520). The instrument was operated under the MassHunter Data Acquisition software (version B.01.03; Build 1.3.157.0; Agilent) in the 2GHz mode. Peptides were separated on the LC-chip with a 4min linear gradient from 3 to 10% B, a 65min linear gradient from 10 to 45% B and a 5min linear gradient from 45 to 90% B followed by a hold of 7min at 90% B, a 2min linear gradient from 90 to 3% B and equilibration of the column at 3% B for 16min. MS settings were the following: mass range, 280-2000 m/z; acquisition rate, 3 spectra/s; data storage, profile; fragmentor, 175V; skimmer, 65V; OCT 1 RF Vpp, 750V. The spray voltage was adjusted between 1800-2000V; drying gas (N\textsubscript{2}), 325°C; drying gas flow, 5L/min. Mass correction was done during analysis using internal standards (methyl stearate m/z, 299.294457 and HP-1221 m/z, 1221.990637) continuously evaporating from a wetted wick inside the spray chamber. For protein identification, automatically selected peptide ions were fragmented by collision-induced dissociation in the data-dependent acquisition mode using the following settings: precursor setting, maximum 3 precursors/cycle with a static mass-exclusion range of 200-400 m/z; absolute threshold, 1000; relative threshold, 0.01% of the most intense peak; active exclusion enabled after 2 selections, release of active exclusion after 0.4min, precursors sorted by abundance only. The MS/MS settings used were the following: fragmentor, 175V; skimmer, 65V; OCT 1 RF Vpp, 750V; width precursor ion selection, medium (4 m/z); mass range, 50-2000 m/z; acquisition rate MS, 3 scans/s; acquisition rate MS/MS, 2 scans/s; collision energy, 3.8V/100Da; offset, 0V. The MS/MS files were stored in profile mode with a threshold of MS, 200/0.01 (absolute/relative threshold) and of MS/MS, 30/0.01.

**Peptide identifications.**

Raw spectra were preprocessed by the Agilent MassHunter Qualitative Analysis software (version B.02.00). MS/MS spectra were converted to mgf format and exported to the Phenyx local database search tool (Geneva Bioinformatics, version 2.5/2.6). The databases searched were IPI.rat (version 20090202) and IPI.rat_rev (20090202) (the later database is a reversed version of IPI.rat and serves to determine the false positive rate). Taxonomy, rat; scoring model, ESI-QTOF (QTOF); parent charge, +2, +3, +4 (trust the charge: medium). The search was done in one round with the following search parameters: enzyme, trypsin (KR_noP); missed cleavage, 2; cleavage mode was set to ‘normal’ with conflict resolution ‘yes’; parent tolerance, 0.1Da. Peptide/AC score, 6; peptide length, 6; p-value <0.0001. The following amino acid modifications were selected: Cys_CAM (carboxy methylation) variable; ACET_nterm (acetylation N-terminal) variable; Oxidation_M (oxidation of
methionine), variable.

Quantitative data preprocessing.

Relative quantification of peptides was performed using single stage data and msCompare (PMID 22318370) framework using OpenMS (PMID: 18366760 and 20013373) homogenous pipeline. Parameters were set according to CSF dataset reported in Hoekman et al. (PMID 22318370). Integration of the various SCX fractions were performed by completing one dimensional LC-MS quantitative data processing workflow with additional module, which matched peaks using OpenMS peak matching module of msCompare framework and summed up the matched peaks in all SCX fractions of one samples creating a final quantitative table for each identified peptides in each samples.

2.9 Data analyses

All data is expressed as the average ± standard error of the mean (sem), unless otherwise stated. Differences between measured parameters over the duration of treatment (ΔBW, FI, WI) were analyzed using repeated measures (rm-) ANOVA post hoc LSD. Statistical comparisons between averages were analyzed using oneway-ANOVA post hoc LSD; within group comparisons were analyzed using the paired t-test. Correlations between variables were tested using linear regression ANOVA. Differences were considered to be significant when P<0.05. Statistical analyses were performed using SPSS20.0. Graphs were designed using Graphpad Prism 5.0.

Results

Body weight and food intake regulation:

Over the treatment period, BW changes, and food and water intake, are illustrated in figure 1. At the start of the treatment at day 0, average BW was comparable between groups (Control=418±3.5g, OLZ=420±5.2g, OLZ+TPM=424±7.1g, TPM=421±7.0g). Delta BW changes during treatment (fig.1a) were different between all treatment groups (rmANOVA post hoc LSD; P<0.001, F_{63,399}=40.440), and resulted after 21 days of treatment in ΔBWs of: Control=60±5.4g > TPM=46±3.4g (P<0.05) > OLZ=-3.5±3.7g (P<0.001) > OLZ+TPM=-25±3.8 (P<0.01; F_{3,22}=99.467, post hoc LSD). Daily caloric intake (fig. 1b) was different (rmANOVA; F_{63,399}=1.858, P<0.001) between the OLZ and OLZ+TPM groups (P<0.05, post hoc LSD) as well as between the OLZ+TPM group compared to Control (P<0.001, post hoc LSD) and TPM (P<0.01, post hoc LSD). Control and TPM groups did not differ in daily caloric intake between groups. During the first week of treatment, cumulative caloric intake was decreased in TPM (P<0.05,
post hoc LSD) and OLZ+TPM (P<0.001, post hoc LSD) compared to Control (oneway-
ANOVA post hoc LSD; $F_{3,22}=6.421$, P<0.01), whereas cumulative intake during the first
week of treatment was comparable in the OLZ treated group compared to Control.
After 14 days of treatment, cumulative intake was increased (oneway-ANOVA,
$F_{3,22}=16.038$, P<0.001) in Control compared to OLZ (P<0.01 post hoc LSD), OLZ+TPM
(P<0.001 post hoc LSD), and TPM (P<0.05, post hoc LSD). The cumulative intake after
fourteen days did not differ between the OLZ and TPM group, but both were
increased compared to the OLZ+TPM group (P<0.01, post hoc LSD). After 21 days of
treatment, total cumulative intake was different between all groups: Control=2116±36 kcal > TPM=2006±71 kcal > OLZ=1764±11 kcal > OLZ+TPM=1614±49 kcal (oneway-ANOVA post hoc LSD; $F_{3,22}=21.695$, P<0.001).

Daily water intake (fig. 1c) during 21 days of treatment was different (rmANOVA,
$F_{63,399}=2.140$, P<0.001) in the TPM group compared to Control (P<0.05) and OLZ
(P<0.001, post hoc LSD). A similar response was observed in the OLZ+TPM in which
daily water intake was different compared to the OLZ group (P<0.01). Cumulative
water intake after 7 days (oneway-ANOVA, $F_{3,22}=4.031$, P<0.05) and 14 days (oneway-
ANOVA, $F_{3,22}=6.263$, P<0.01) of treatment was increased in TPM compared to
Control (P<0.05, post hoc LSD) and OLZ (P<0.01, post hoc LSD). Increased cumulative
water intake after 14 days was also observed in the OLZ+TPM group compared to the
OLZ group (P<0.05). Cumulative water intake after 21 days of treatment (oneway-
ANOVA, $F_{3,22}=8.244$, P<0.01) resulted in: TPM=592±25ml > OLZ+TPM=543±27ml >
Control=490±27ml > OLZ=427±22ml. Total 21 day cumulative water intake was
increased in TPM compared to control (P<0.05, post hoc LSD) and OLZ (P<0.01, post hoc LSD); OLZ+TPM treatment increased 21 day cumulative water intake compared to
OLZ treatment (P<0.01).

The higher BW of Control (478±6g) and TPM (466±13g) compared to OLZ (417±8g)
and OLZ+TPM (400±6g) was partly the result of a difference in total skeletal muscle
weight (SMW), which in the Control (241±4g) and TPM (243±7g) was higher
compared to OLZ (217±4g) and OLZ+TPM (211±3g; $F_{3,22}=18.226$, post hoc LSD,
P<0.001). Other components contributing to weight loss were visceral adipose tissue
stores. Cumulative epididymal and retroperitoneal adipose depot weights (fig. 1D)
were higher in the Control group (=20±2g; oneway-ANOVA, $F_{3,22}=9.827$, P<0.001)
compared to the OLZ (=13±1g, P<0.001, post hoc LSD), the OLZ+TPM (=12±1g,
P<0.001, post hoc LSD), and the TPM group (=13±1g, P<0.001, post hoc LSD). Visceral
adiposity was correlated with circulating leptin levels ($R^2=0.635$, ANOVA, $F_{1,19}=31.313$, P<0.001). Leptin levels were also increased in the Control group (=13.0±2.3ng/ml;
oneway-ANOVA, $F_{3,22}=7.321$, P<0.01) compared to the OLZ (=5.9±0.7ng/ml; P<0.01,
post hoc LSD), the OLZ+TPM (=5.7±0.8ng/ml; P<0.01, post hoc LSD), and the TPM group (=7.6±0.9ng/ml; P<0.01, post hoc LSD).

Glucose and Insulin responses:

A summary of the results from the IG-GTTs is presented in Table 1. Basal glucose levels at day-1 were similar among groups. At day 14 of treatment, basal glucose levels were increased (oneway-ANOVA; F$_{3,22}$=4.703, P<0.05) in the Control group.
compared to the OLZ (P<0.05, post hoc LSD) and the OLZ+TPM groups (P<0.01, post hoc LSD). Animals in the TPM group showed increased basal glucose levels at day 14 of treatment compared to OLZ+TPM (P<0.05, post hoc LSD). Within-group analyses revealed an increase of basal glucose levels in the Control (P<0.05, t=-3.737, paired t-test) and the TPM (P<0.05, t=-4.562, paired t-test) treated groups, which was absent in the OLZ and OLZ+TPM groups. Glucose responses as a result of the IG-GTT (expressed as area under the curve; AUC) were not different between groups at day 14 of treatment, nor within groups between baseline and treatment responses. No differences in the relative change of glucose responses (AUC (%)) between baseline and treatment were measured between groups. In addition, between-group analyses did not reveal a difference in insulin AUC in response to the IG-GTT at baseline and at day 14 of treatment. Nonetheless, the relative insulin AUC (%) after 14 days of treatment was lower in the TPM group compared to Control (one-way-ANOVA, F_3,18=1.681, P<0.05), and within-group analysis revealed a reduction of insulin AUC after 14 days of treatment compared to baseline exclusively in the TPM group (P<0.05, t=4.566, paired t-test). The Glucose Index (G*I) revealed no significant difference between groups (one-way-ANOVA post hoc LSD), but a decreased G*I almost reached significance in the TPM group compared to the Control group. Within-subject analysis revealed a decrease of G*I in the TPM group compared to its baseline G*I (P<0.05, t=5.798). Finally, we decided to correct the AUC_{Ins} measured at day 14 of treatment for the SMW and found increased AUC/SMW responses in the OLZ and OLZ+TPM treated groups compared to the Control (P<0.01) and TPM treated group (P<0.05; F_3,19=7.676, post hoc LSD; see Table 1).

**Hepatic lipid profile:**

Lower hepatic TGC levels in the OLZ (P<0.001, post hoc LSD), OLZ+TPM (P<0.001, post hoc LSD), and TPM groups (P<0.001, post hoc LSD) were found compared to

<table>
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<th>Basal Glucose (mM)</th>
<th>AUC (%)</th>
<th>G*I (%)</th>
<th>Ins/SMW (AUC/g)</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>Baseline</td>
<td>Treatment</td>
<td>Glucose</td>
<td>Insulin</td>
</tr>
<tr>
<td>Control</td>
<td>3.5±0.1</td>
<td>4.3±0.1</td>
<td>99.1±3.3</td>
<td>108.7±4.5</td>
</tr>
<tr>
<td>OLZ</td>
<td>3.4±0.1</td>
<td>3.6±0.1</td>
<td>94.5±3.2</td>
<td>96.1±8.7</td>
</tr>
<tr>
<td>OLZ+TPM</td>
<td>3.2±0.1</td>
<td>3.4±0.2</td>
<td>104.2±4.3</td>
<td>91.8±6.1</td>
</tr>
<tr>
<td>TPM</td>
<td>3.4±0.1</td>
<td>4.1±0.3</td>
<td>97.8±3.1</td>
<td>85.7±8.2</td>
</tr>
</tbody>
</table>

Percentage express the relative difference between baseline and treatment measurements.
*P<0.05, paired t-test; **P<0.05, ***P<0.01, one-way-ANOVA post hoc LSD.
G*I: Glucose Index; AUC: Area under the curve; SMW: Skeletal muscle mass
Controls (fig. 2A; oneway-ANOVA, $F_{3,20}=20.581$, $P<0.001$). In addition, hepatic TGC levels were increased in the TPM group compared to the OLZ+TPM group ($P<0.05$, post hoc LSD). Total cholesterol levels (fig. 2B; oneway-ANOVA, $F_{3,19}=10.837$, $P<0.001$) were decreased in the OLZ ($P<0.001$, post hoc LSD), OLZ+TPM ($P<0.001$, post hoc

**Fig 2:** Hepatic lipogenic profile, A) Triglycerides, B) Total cholesterol, and C) Free cholesterol levels were reduced in the drug treated groups compared to control (**P<0.001, **P<0.01, **P<0.05; oneway ANOVA post hoc LSD). Quantitative RT-PCR did not reveal a difference in D) Acetyl-CoA Carboxylase 1, E) Acetyl-CoA Carboxylase 2, or F) Malonyl-CoA Decarboxylase mRNA expression between groups.
Free cholesterol levels (fig. 2C; one-way-ANOVA, $F_{3,19}=6.897$, $P<0.01$) were also decreased in the OLZ (P<0.01, post hoc LSD), OLZ+TPM (P<0.01, post hoc LSD), and TPM groups (P<0.01, post hoc LSD) compared to Controls. In addition, adipose depots weights were correlated to hepatic TGC ($R^2=0.646$, ANOVA, $F_{1,19}=32.866$, P<0.001), total cholesterol ($R^2=0.483$, ANOVA, $F_{1,19}=16.845$, P<0.01), and free cholesterol ($R^2=0.221$, ANOVA, $F_{1,19}=5.109$, P<0.05); similar correlations were found between circulating leptin and hepatic TGC ($R^2=0.540$, ANOVA, $F_{1,19}=21.102$, P<0.001), total cholesterol ($R^2=0.353$, ANOVA, $F_{1,19}=9.828$, P<0.01), and free cholesterol ($R^2=0.206$, ANOVA, $F_{1,19}=4.657$, P<0.05).

Quantitative rt-PCR did not reveal any difference between groups for ACC1 (fig. 2D; one-way-ANOVA, $F_{3,20}=0.659$, P=0.586), ACC2 (fig. 2E; one-way-ANOVA, $F_{3,20}=0.626$, P=0.608), or MCD RNA expression (fig. 2F; one-way-ANOVA, $F_{3,20}=0.355$, P=0.786).

Hepatic proteomics:

Hepatic proteomic analyses (Table 3) revealed that aldose reductase-related protein 1 and glucose-6-phosphate dehydrogenase levels were increased in the OLZ and OZL+TPM treated groups. This indicates an up-regulation of the polyol pathway. Furthermore, a down-regulation of cytochrome P450 CYP2C11 and an up-regulation of CYP2C12 were found in the OLZ treated groups. TPM did not influence overall hepatic protein expression related to OLZ treatment except for an increase in hepatic UDP-glucuronosyltransferase 2B11 (UGT-2B11) expression, a protein also involved in OLZ metabolization. To adequately judge the relation between the hepatic protein expression profiles and the observed physiological effects, it is necessary to perform a full protein network analysis. For now, we decided that a log2 ratio above 0.807 (corresponding with a 1.75 fold change to Control) or below -0.807 (corresponding with 0.57 (1/1.75) fold change to Control) is a conservative approach to consider an up or down-regulation of protein expression as relevant. In addition, we added the expression ratios of ACC1 and MCD to the table as a comparative to the quantitative rt-PCR data. Whereas no direct correlation was visible between outcomes, both methods did not observe a change compared to Control. ACC2 protein expression is missing in Table 3, because ACC2 could not be identified from the proteomic data set.

Conclusions and discussion

In the present study we confirmed that OLZ treatment in male Wistar rats reduces BW gain and caloric intake. Others have previously reported increased adiposity in male rats treated with OLZ [16], but this difference is likely due to differences in
Table 3: Hepatic proteome profile of chronic TPM, OLZ+TPM, or OLZ treated male Wistar rats compared to control.

<table>
<thead>
<tr>
<th>Protein Name</th>
<th>Uniprot Accession#</th>
<th>TPM/Cont</th>
<th>log2 ratio Both/Cont</th>
<th>OLZ/Cont</th>
</tr>
</thead>
<tbody>
<tr>
<td>Aldose reductase-related protein 1</td>
<td>Q5RUPO</td>
<td>0.518</td>
<td>2.750 *</td>
<td>2.946 *</td>
</tr>
<tr>
<td>Glucose-6-phosphate 1-dehydrogenase (G6PD) [CHAIN 0]</td>
<td>P05370_CHAIN_0</td>
<td>0.487</td>
<td>1.079 *</td>
<td>1.008 *</td>
</tr>
<tr>
<td>Fatty acid synthase</td>
<td>P12785</td>
<td>0.229</td>
<td>0.988 *</td>
<td>0.758</td>
</tr>
<tr>
<td>Alcohol sulfotransferase A (AD-ST) (STA) (ST2A2) [CHAIN 0]</td>
<td>P24789_CHAIN_0</td>
<td>-0.073</td>
<td>1.580 *</td>
<td>2.112 *</td>
</tr>
<tr>
<td>Acyl-coenzyme A amino acid N-acyltransferase 2</td>
<td>Q5FVR5</td>
<td>-1.292 *</td>
<td>-</td>
<td>-2.573 *</td>
</tr>
<tr>
<td>Phytanoyl-CoA dioxygenase, peroxisomal (PhyH) [CHAIN 0]</td>
<td>P5T793_CHAIN_0</td>
<td>-0.298</td>
<td>-0.830 *</td>
<td>-0.932 *</td>
</tr>
<tr>
<td>UDP-glucuronosyltransferase 2B1 (UDPGT 2B1) [CHAIN 0]</td>
<td>P09875_CHAIN_0</td>
<td>1.422 *</td>
<td>1.838 *</td>
<td>0.714</td>
</tr>
<tr>
<td>Retinal dehydrogenase 1 (RALDH 1) (RALDH1) [CHAIN 0]</td>
<td>P5T647_CHAIN_0</td>
<td>-0.262</td>
<td>0.974 *</td>
<td>0.362</td>
</tr>
<tr>
<td>Bile salt sulfotransferase (ST) (ST2A1) [CHAIN 0]</td>
<td>P15709_CHAIN_0</td>
<td>-0.183</td>
<td>0.492</td>
<td>0.983 *</td>
</tr>
<tr>
<td>Ferritin heavy chain (Ferritin H subunit) [CHAIN 0]</td>
<td>P19132_CHAIN_0</td>
<td>0.839</td>
<td>1.067</td>
<td>0.654</td>
</tr>
<tr>
<td>Alanine aminotransferase 1 (ALT1) (GPT 1) [CHAIN 0]</td>
<td>P23409_CHAIN_0</td>
<td>-0.236</td>
<td>0.702</td>
<td>0.210</td>
</tr>
<tr>
<td>Carbonic anhydrase 3 (CA III) [CHAIN 0]</td>
<td>P14141_CHAIN_0</td>
<td>-0.483</td>
<td>-1.042 *</td>
<td>-1.428 *</td>
</tr>
<tr>
<td>3-alpha-hydroxysteroid dehydrogenase (3-alpha-HSD)</td>
<td>P23457</td>
<td>0.088</td>
<td>0.565</td>
<td>0.636</td>
</tr>
<tr>
<td>3-beta-hydroxysteroid dehydrogenase type 5 (3-beta-HSD V) [CHAIN 0]</td>
<td>P24364_CHAIN_0</td>
<td>0.230</td>
<td>-1.522 *</td>
<td>-2.025 *</td>
</tr>
<tr>
<td>3-oxo-5-alpha-steroid 4-dehydrogenase 1 (SSAR 1) [ISOFORM Short]</td>
<td>P24008_ISOFORM_Short</td>
<td>-0.102</td>
<td>0.979</td>
<td>1.252 *</td>
</tr>
<tr>
<td>3-oxo-5-beta-steroid 4-dehydrogenase</td>
<td>P31210</td>
<td>-0.160</td>
<td>-0.307</td>
<td>-0.653</td>
</tr>
<tr>
<td>Hydroxyacid oxidase 2 (HAO2) [CHAIN 0]</td>
<td>Q07523_CHAIN_0</td>
<td>-0.390</td>
<td>-2.284 *</td>
<td>-2.604 *</td>
</tr>
<tr>
<td>Cytochrome P450 2C6</td>
<td>P05178</td>
<td>0.185</td>
<td>0.889</td>
<td>0.414</td>
</tr>
<tr>
<td>Cytochrome P450 2C11</td>
<td>P08683</td>
<td>-0.619</td>
<td>-1.440 *</td>
<td>-2.319 *</td>
</tr>
<tr>
<td>Cytochrome P450 2C12, female-specific</td>
<td>P11510</td>
<td>-0.135</td>
<td>1.292 *</td>
<td>0.810 *</td>
</tr>
<tr>
<td>Acetyl-CoA carboxylase 1 (ACC1) [ISOFORM 2]</td>
<td>P11497_ISOFORM_2</td>
<td>-0.047</td>
<td>0.215</td>
<td>0.139</td>
</tr>
<tr>
<td>Malonyl-CoA decarboxylase, mitochondrial (MCD) [ISOFORM_Cytoplasmic]</td>
<td>Q920F5_ISOFORM_Cytoplasmic</td>
<td>-0.229</td>
<td>0.063</td>
<td>-0.220</td>
</tr>
</tbody>
</table>

* Protein expression considered to be up- or down-regulated compared to Control.
* in Bold proteins expressing a reversed regulation in male rats during growth hormone treatment [AHluwalia et al, Molecular Endocrinology 18(3):747–760, 2004].
dosage (i.e., which in fact may have an inverted U-shaped relation with BW). The reduction in BW gain was reflected by a major reduction of abdominal adiposity depots combined with a striking reduction of skeletal muscle weight. The absence in weight gain is, in all likelihood, a result of decreased caloric intake. We previously showed that, under the same administration protocol, female Wistar rats did gain weight on 5mg/kg OLZ b.i.d. [23]. Hence, we emphasize that the results presented here are typical for male Wistar rats and a major gender difference in the mechanisms underlying this discrepancy has yet to be discovered. The observed weight gain in female Wistar rats was partly due to compensatory food intake during the light phase. Male rats, however, did not compensate for decreased food intake during the dark phase (see supplementary data Fig. S1), which resulted in decreased daily caloric intake and weight loss.

TPM treatment attenuated BW gain relative to Controls. The difference in BW can be explained by a reduction of caloric intake, primarily during the first week of treatment, resulting in a reduction of abdominal adiposity, whereas skeletal muscle mass was not different compared to Controls. In addition, TPM administration led to an increase in water intake, which was most likely a result of increased renal output (based on an observation in a previous study, not published) through its carbonic anhydrase inhibiting property at the renal proximal tubule blocking CO$_3^-$ reabsorption [27]. Nonetheless, the major reduction of BW was observed during the first day of treatment and persisted throughout the duration of the study.

The use of TPM as an adjunctive to OLZ treatment resulted in additive weight loss and a further reduction in caloric intake compared to the administration of OLZ or TPM alone was underlying this effect. In contrast, water intake in the OLZ+TPM group was increased compared to the OLZ treated group. Although adjunctive TPM treatment promoted weight loss, no additive effect was observed for adipose tissue weights or circulating leptin levels. These factors remained comparable to both the OLZ as well as the TPM treated group.

Because of subjection to the HF diet, fasting basal glucose levels increased in the Control and TPM treated animals compared to their baseline levels, but also compared to OLZ and OLZ+TPM treated groups at day 14 of treatment, the latter probably reflecting the more profound semi-fasted state of OLZ or OLZ+TPM treated rats. During the IG-GTT, rats treated with TPM showed a reduced insulin response compared to both their baseline response and compared to the Control group after 14 days of treatment. This reduced insulin response while maintaining glycemia comparable to the Controls is indicative of an increase in insulin sensitivity. In contrast, rats treated with OLZ or OLZ+TPM did not display a change in glucose-
insulin responsiveness, despite decreased abdominal adiposity. Therefore, we performed an additional study (see supplementary data fig. S2) and observed that a BW-matched control group - through food restriction - indeed reduced the insulin response. Accordingly, this implies that, in relation to the level of developed adiposity, the insulin sensitivity had in fact decreased in the OLZ and OLZ+TPM treated groups compared to the ad lib fed Control group. Moreover, the OLZ and OLZ+TPM group revealed increased levels of AUC_{Ins} per gram SMW, meaning that both groups had an increased insulin response relative to its skeletal muscle mass. This dissociation could not be made for the body weight matched control group, which showed a similar AUC_{Ins}/SMW-ratio as the ad lib Control group (see supplementary data fig. S3). The latter showed that lower muscle mass was not directly related to a reduced capacity of glucose uptake. Moreover, OLZ has been reported to reduce skeletal muscle glucose uptake and glycogen synthesis in vitro via the inhibition of an insulin dependent pathway [28], possibly involving 5HT_{2A} receptor antagonism [29]. The possibility that the reduction in skeletal muscle mass in OLZ treated rats could underlie their relative reduction in whole body insulin sensitivity is, however, speculative and deserves further investigation. Especially, because we haven’t measured intramuscular lipid storage, which, besides total skeletal muscle mass, may be another factor influencing muscular insulin sensitivity [30].

We found that abdominal adiposity and circulating leptin levels strongly correlated to the level of hepatic TGC, tChol, and fChol. All levels were higher in the Control group compared to the drug-treated groups. In the OLZ and OLZ+TPM treated groups, the reduced hepatic TGC and total and free cholesterol levels were presumably a result of the observed reduction in caloric intake and associated weight loss. A similar reduction of hepatic TGC and cholesterol was observed in the TPM treated rats, which could not be solely attributed to a reduction in food intake over 21 days of treatment. In agreement, Richard et al (2000) reported a similar reduction of food intake and adiposity in TPM treated rats [17]. However, the reduction in food intake was not accompanied by a decrease of energy expenditure, therefore animals were not in a fasting state and the decrease of adiposity was primarily a result of increased lipolysis [17]. In addition, Wilkes et al (2005) observed that TPM treatment led to enhanced insulin sensitivity in female Zücker rats, especially in liver and adipose tissue, which resulted in increased insulin-induced glucose uptake [31]. In accordance with these studies, we found that TPM treatment reduced palatable diet intake, adiposity, and the insulin response to an IG-GTT. A reduction in hepatic TGC accumulation and diminished visceral body fat content has been pin-pointed as leading causes underlying the improvement of insulin sensitivity [32] in the TPM treated animals. The results here indicate that this relation is disconnected by OLZ.
treatment, and adjunctive TPM treatment is not able to restore this effect.

After observing the impact of OLZ and adjunctive TPM treatment on physiological and behavioral parameters, we set out to study the hepatic protein expression levels (Table 3) to reveal possible protein pathways relevant to the observed treatment outcomes. Proteomic analysis revealed that TPM irrespective of OLZ treatment significantly elevated the hepatic level of UDP-glucuronosyltransferase (UGT) 2B1 compared to Control. UGTs are predominantly involved in phase II metabolism of xenobiotics or drugs. More specifically, UGTs are responsible for the addition of glucuronic acid to their substrate, which increases solubility, and allowing elimination of the altered substrates via the kidneys. This corresponds with studies showing that the major part of TPM is cleared from the circulation via the kidneys [33]. Although UGT 2B1 was not considered to be up-regulated in the OLZ treated group, OLZ is also metabolized via a UGT dependent pathway [34,35]. Hence, it should be noted that TPM may affect the metabolization of OLZ, which could relate to the additive increase of hepatic UGT 2B1 levels in the OLZ+TPM treated group. Unpublished data from our group in male rats demonstrated that co-administration of TPM indeed increased circulating OLZ levels, suggesting that TPM blocked OLZ metabolization. Since drug doses used in this study were relatively low (compared to our unpublished study), therefore additional studies are needed to reveal any relevant dose dependent drug-drug interactions during chronic treatment. Although we considered the increase below relevant levels, the increase of alanine aminotransferase in the OLZ+TPM group may be a sign of drug-induced liver damage [36].

Besides differential expression of UDPs in TPM treated rats, the proteomic analysis specifically showed an up-regulation of aldose reductase related protein 1 (AR) and glucose-6-phosphate dehydrogenase (G6PD) in OLZ and OLZ+TPM treated groups. We did not find any additive effect of TPM on OLZ-related increases of hepatic AR and G6PD, indicating that TPM does not affect glucose and insulin responsiveness via a hepatic pathway, but presumably is a consequence of reduced adiposity. The increase of AR and G6PD induced by OLZ is also observed during the development of uncontrolled diabetes mellitus, in which AR is the first enzyme in the polyol pathway that is predominantly activated under hyperglycemic conditions during which glucose is converted into sorbitol [37]. In concordance, the observed increase of G6PD implies that at least the first part of the polyol pathway is up-regulated [37]. High intercellular sorbitol concentration is thought to lead to biochemical alterations in the cell, e.g. increased osmolality, causative of nephropathy, retinopathy, and neuropathy. These conditions are all related to diabetes [38].

Aldose reductase inhibitors are effective in reducing sorbitol accumulation and
therefore have been developed as an adjuvant treatment to decrease complications related to diabetes [39,40]. Some aldose reductase inhibitors, like tolrestat, are withdrawn from the market due to an increased risk of liver toxicity, while others, such as epalrestat, are still marketed [41]. Chandra et al (2002) showed that nitric oxide (NO) is also able to attenuate AR, and therefore prevents sorbitol accumulation in the cell [42].

Interestingly, and related to our study, several studies exist that link OLZ treatment to changing NO levels. Atmaca et al (2007) observed a reduction of serum NO after 6 weeks of OLZ treatment in schizophrenia patients [43]. Hou et al (2006) observed that OLZ significantly inhibited NO release by LPS-stimulated N9 microglial cells and suggested that this effect might be a new mechanism through which OLZ exhibits its therapeutic effect in the treatment of schizophrenia [44]. Both studies suggest a causal relation between OLZ-treatment and low serum NO, which may lead to increased AR levels and accumulation of sorbitol in both liver and neuronal cells. This seems to be contradictory to OLZ’s proposed neuroprotective effects [45].

Besides the effects of OLZ on hepatic AR and G6PD, we found a major reduction of CYP2C11 and 3-beta-hydroxy-steroid dehydrogenase 5, and an up-regulation of Alcohol SulfoTransferase A (ST2A2) and CYP2C12 levels in the OLZ-treated rats. Daskalopoulos et al (2012) demonstrated that sulpiride administration (i.e., classed as an a-typical antipsychotic, but predominantly antagonistic at the dopamine receptors 1, 2, 3, and 4) resulted in a strong decrease of hepatic CYP2C11 in rats, which was associated with a reduction in growth hormone (GH) levels [46]. A strong link between fluctuating GH levels and activated transcription of various P450 cytochromes was later established; which are especially important in the metabolism of xenobiotics. Hence, D2 receptor antagonism, which influences GH release, also influences the metabolism of the prescribed drug and its adjuvant [46]. Secondly, Ahluwalia et al (2004) used microarray analysis to study the role of GH levels combined with gender differences on rat liver gene expression and found that male rats treated with GH showed an up-regulation of 3-beta-hydroxy-steroid dehydrogenase 5 and CYP2C11, and a down-regulation of ST2A2 and CYP2C12 [47], which are exactly the opposite results compared to our study (see Table 3). CYP2C12 and ST2A2 are considered female-predominant genes [47], but are up-regulated in male Wistar rats after chronic OLZ treatment. Remarkably, it is the pulsatile secretion of GH in male rats that is considered to effectively reduce CYP2C12 via a GH activated STAT5 pathway [48]. In contrast, CYP2C11 is considered a male-predominant gene, and was found to be down-regulated due to OLZ treatment in our study. The importance of GH in masculinization was already demonstrated by Waxman et al
[1991], who showed that the growth inhibition in male hypophysectomized (HPX) rats could be specifically reversed by a pulsatile administration of GH [49]. Furthermore, Waxman et al (1991) showed restoration of CYP2C11 in the livers of male HPX rats, whereas down-regulation of CYP2C12 was reported due to GH treatment in intact male rat livers [49]. Interestingly, CYP2C11 expression becomes down-regulated at high glucocorticoid concentrations that can be reversed by mifepristone [50], which has shown to be effective in reducing OLZ-induced weight gain [51].

One of the striking outcomes in the study presented here is the lack of BW gain in the OLZ and OLZ+TPM treated male Wistar rats. Although we did not assess GH levels in the present study, it may be speculated that the significantly lower amount of total skeletal muscle weight is the result of disrupted structural growth in the OLZ treated groups. Interestingly, the deletion of the dopamine D2 receptor causes dwarfism in male mice, but not in female mice [52]. The dwarfism observed in male D2 knockout mice is related to a reduction of growth hormone (GH), which is associated with a reduction of insulin-like growth factor-1 (IGF-1). In accordance, serum IGF-1 levels are positively correlated to hepatic CYP2C11 expression and down-regulation of CYP2C11 results in low serum IGF-1 levels [53]. IGF-1 increases insulin sensitivity of the skeletal muscle in a complex interaction with circulating GH [54] and may be an additional pathway of skeletal muscle insulin desensitization in male rats treated with OLZ. On the other hand, male D2 knockout mice have increased prolactin (PRL) levels. Both PRL and GH are secreted by the anterior pituitary and regulated via hypothalamic dopaminergic neurons. Whereas PRL secretion is inhibited by dopamine, GH secretion is stimulated by dopamine via D2 receptors. The difference between male and female GH release is that GH is released in a steady state fashion in female rats, but is pulsatile and peaks approximately every 3-4 hours in male rats [52]. In a previous study, we did find OLZ-induced increases of PRL levels in female Wistar rats. Prolactin in females has orexigenic effects [55] and, therefore, partly explains the OLZ-induced weight gain observed in female rats, whereas disruption in GH regulation in male rats relates to the observed weight inhibition by OLZ. Moreover, it indicates that OLZ is able to affect hormonal secretion from the anterior pituitary.

To further support the hypothesis of an OLZ-induced disruption of GH regulation in male rats; Ueda et al (1997) also demonstrated that GH influences the expression of sulfotransferases ST2A1 (table 3: bile salt sulfotransferase) and ST2A2 (table 3: Alcohol sulfotransferase A) [56]. In their study, ST2A2 expression was absent in males, whereas it was abundant in female rat livers, similar to expression patterns of CYP2C12. Hypophysectomy caused an increase of ST2A2 expression in male rat livers.
This effect could be reduced by pulsatile GH administration, but not by continuous GH infusion. ST2A1 expression was found in both genders, but at a much lower level in male rats compared to female rats. In this case, hypophysectomy resulted in an absence of ST2A1 expression in both male and female rats. Continuous infusion of GH resulted in an increase of ST2A1 in the male rat liver, which was absent after pulsatile GH administration [56]. Hence, the data of Ueda and colleagues demonstrate that the gender difference of ST2A1 and ST2A2 expression is due to a difference in diurnal GH secretion. ST2A2 down-regulation appeared especially dependent of a pulsatile GH secretion [56]; this corresponds to our data showing that ST2A2 is the main up-regulated protein in OLZ treated male rats. Expression of the hepatic ST2A subfamily is up-regulated by glucocorticoids [57,58] and is gender dependent [59]. The expression levels of ST2A are interesting because TPM appears to attenuate the expression of ST2A1 and ST2A2 in the OLZ+TPM group. The administration of TPM alone, however, did not considerably reduce expression of ST2A1 or ST2A2. Nonetheless, these results do relate to the findings of Runge-Morris et al (1998), who demonstrated that the anticonvulsant phenobarbital, that similarly as TPM potentiates the action of GABA_A, reduces the overall hepatic expression of the ST2A subfamily [60].

The gender specificity of OLZ-induced weight gain in rats has been thoroughly described by Cooper et al (2005, 2007), who showed an increase of BW in female Han Wistar rats [15], but a dose-dependent reduction in BW gain in male Han Wistar rats [16]. While male Han Wistar rats showed a decrease of BW-gain due to OLZ-treatment, a relative increase of adiposity was observed as well. Similar to our results, male Han Wistar rats showed a reduction in gastrocnemius muscle mass. In both male and female rats, an increase of prolactin was observed, whereas estradiol (females) and testosterone (males) levels were decreased. Testosterone is synthesized from cholesterol via a β-hydroxysteroid dehydrogenase (β-HSD) involved pathway, and the activation of testosterone production in the testis is related to increased testicular CYP2C11 levels [61]. Our data revealed a decrease of hepatic cholesterol, β-HSD, and CYP2C11, which all together may explain the reduction of testosterone in OLZ treated male rats observed by Cooper and colleagues [16]. In humans, free testosterone levels increase, but do not normalize, after switching from typical antipsychotics (specific D2 antagonist) to OLZ [62].

In summary.

In agreement with previous studies, we show that male Wistar rats do not gain weight due to OLZ-treatment. Female Wistar rats, however, do gain weight under the same treatment paradigm as illustrated in our previous study [23]. The increase of
hepatic AR and G6PD protein expression in OLZ treated male rats suggest an up-regulation of the polyol pathway, which is associated with hyperglycemia. Furthermore, the hepatic proteomic profile of the male Wistar rat treated with OLZ, together with the low skeletal muscle weight, insinuate a reduction of GH levels. Future studies are required to demonstrate whether OLZ indeed has an effect on the pulsatile GH secretion typically present in male rats.

Because OLZ inhibits BW gain in male Wistar rats, it is hard to directly dismiss TPM as an effective adjuvant to reduce OLZ-induced weight gain. TPM did not affect OLZ-related increases of AR and G6PD hepatic protein expression; neither did it affect the expression of CYP2C11 and CYP2C12. Therefore, the effectiveness of TPM as an adjunctive treatment seems to be primarily based on the reduction of adiposity and, therefore, improvement of insulin sensitivity, but only under conditions of OLZ induced weight gain as is observed in several human studies [19-21].

**Acknowledgements**

This work was supported by Top Institute Pharma project T2-105. We would like to thank J. Bruggink and F. Calcagnoli for their valuable technical support.
Fig. S1: A) Diurnal food intake. Food intake during the dark phase is higher in both the Control and TPM group compared to the OLZ and OLZ+TPM group (\#P<0.01, \(F_{3,22}=10.622\), oneway-ANOVA post hoc LSD). OLZ+TPM shows higher light phase food intake compared to TPM (+P<0.05, \(F_{3,22}=2.877\), oneway-ANOVA post hoc LSD). Within-group testing showed higher food intake during the dark phase compared to the light phase in the Control (*P<0.05, \(t_4=3.102\), paired t-test) and TPM (**P<0.01, \(t_5=4.740\), paired t-test) group, whereas food intake in the OLZ and OLZ+TPM group was similar during the dark compared to the light phase. B) Diurnal water intake. Dark phase water intake is higher in both the Control and TPM group compared to the OLZ and OLZ+TPM group (\#P<0.01, \(F_{3,22}=8.194\), oneway-ANOVA post hoc LSD). OLZ+TPM shows higher light phase water intake compared to all other groups (++P<0.01, \(F_{3,22}=6.627\), oneway-ANOVA post hoc LSD). Within-group analyses show higher water intake during the dark phase compared to the light phase in the Control (*P<0.05, \(t_4=4.391\), paired t-test) and TPM (**P<0.01, \(t_5=4.223\), paired t-test) group, whereas in the OLZ+TPM group light phase water intake is higher compared to dark phase (**P<0.01, \(t_5=-4.298\), paired t-test), no difference within the OLZ treated group was measured between dark and light phase water intake. Dark phase intake is represented by dark bars, light phase is represented by white bars.
**Fig. S2:** A) Body weight prior and post 21 days of treatment showed an increase in the Control group (**P<0.001, t₄=-13.840, paired t-test), but no increase of body weight was observed in the OLZ or body weight matched (BWM)-control group. B) Relative glucose and insulin response (AUC) at day 14 of treatment compared to baseline response prior to treatment. Glucose responses in all three groups did not change after 14 days of treatment. Only within the BMW-control group a decrease of treatment compared to baseline insulin response was observed (*P<0.05, t₃=3.184, paired t-test). C) Adiposity after 21 days of treatment was higher in the Control group compared to both the OLZ and BWM-control group (*P<0.05, ***P<0.01, F₂,2₃=12.088, oneway-ANOVA post hoc LSD). D) Circulating leptin levels after 21 days of treatment are higher in the Control group compared to both the OLZ and BWM-control group (**P<0.01, ***P<0.001, F₂,2₃=9.966, oneway-ANOVA post hoc LSD). No differences between BWM and OLZ treated groups have been observed. BWM-Body Weight Matched control group. AUC: Area under the curve.
Fig. S3: Insulin area under the curve corrected for skeletal muscle weight. The ratio $\text{AUC}_{\text{Ins}}/\text{SMW}$ was increased in the OLZ and OLZ+TPM groups compared to the Control and TPM treated groups ($^\#P<0.05$, $^{##}P<0.01$; $F_{4,25}=7.676$, post hoc LSD). The body weight matched (BWM) control group revealed a similar ratio between $\text{AUC}_{\text{Ins}}$ and SMW as the ad lib fed Control group, but was lower compared to both the OLZ and OLZ+TPM treated groups ($^{##}P<0.01$; $F_{4,24}=5.170$, post hoc LSD). B) Skeletal muscle weight. Decreased skeletal muscle weight was observed in the OLZ, OLZ+TPM, and BWM group compared to both the Control and TPM groups ($^{##}P<0.01$; $F_{4,30}=12.599$, post hoc LSD). 

AUC: Area under the curve; SMW: Skeletal muscle weight; BWM: Body weight matched.
References


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