

Full-length article

Insulin-sensitizing effects of a novel α -methyl- α -phenoxypropionate derivative *in vitro*¹

Hao-shu WU^{2,4}, Juan-hong YU^{3,4}, Ying-yin LI², Yu-she YANG³, Qiao-jun HE², Yi-jia LOU^{2,5}, Ru-yun JI³

²Department of Pharmacology and Toxicology, College of Pharmaceutical Sciences, Zhejiang University, Hangzhou 310058, China; ³State Key Laboratory of Drug Research, Shanghai Institute of Materia Medica, Shanghai Institutes for Biological Sciences, Graduate School of the Chinese Academy of Sciences, Shanghai 201203, China

Key words

YY20; peroxisome proliferator-activated receptor γ ; insulin sensitivity; glucose consumption

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⁴ These authors contributed equally to this paper.

⁵ Correspondence to Prof Yi-jia LOU.
Phn/Fax 86-571-8820-8403.
E-mail yijialou@zju.edu.cn

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Abstract

Aim: To examine the insulin sensitizing effects of a novel α -methyl- α -phenoxypropionate derivative YY20 in insulin-sensitive cell lines. **Methods:** The peroxisome proliferator-activated receptor γ (PPAR γ) agonist bioactivities of YY20 were detected by a preadipocyte differentiation assay. RT-PCR and Western blotting analysis were used to detect the expression of the target gene or protein. The effects of YY20 on insulin-mediated glucose consumption were determined in the HepG2 human hepatocellular carcinoma line. **Results:** YY20 could enhance the differentiation of preadipocytes to adipocytes and upregulate the gene expression of PPAR γ_2 , as well as the protein expression of insulin receptor substrate-1 (IRS-1), glucose transporter-4 (GLUT4), and adiponectin (ACRP30). The effects on GLUT4 and ACRP30 could be reversed by the PPAR γ inhibitor SR-202. Furthermore, YY20 efficiently reduced glucose consumptions in HepG2 cells after 24 h culture, and the effects were related to insulin and YY20 concentrations. **Conclusion:** YY20, a potential insulin-sensitizing agent like rosiglitazone, could enhance glucose consumption in HepG2 cells in a concentration- and insulin-dependent manner. It may improve the insulin resistance associated with type 2 diabetes.

Introduction

Type 2 diabetes is a chronic disease, often characterized by insulin resistance, which can lead to several secondary complications, such as hypertension, atherosclerosis, coronary artery disease, and hyperlipidemia^[1]. Approximately 150 million people worldwide are afflicted with the disease at present, and with a projection of 300 million people being affected by the year 2025, it has become a serious public health problem, particularly in developed countries^[2]. Research of an effective antidiabetic agent would be of great interest for the treatment of type 2 diabetes.

Thiazolidinediones (TZD), which are peroxisome proliferator-activated receptor γ (PPAR γ) agonists, have been demonstrated to have a variety of clinical effects, including improving insulin sensitivity and glucose tolerance^[3,4]. PPAR are ligand-activated transcription factors that belong to the

nuclear receptor superfamily. They have specific tissue distribution and play a pivotal role in regulating the expression of a large number of genes involved in glucose and lipid metabolism^[5]. PPAR γ is mainly distributed in adipose tissue and skeletal muscle, and regulates glucose metabolism. Moreover, PPAR γ_2 is specific in adipose tissue and essential for adipocyte differentiation^[6]. PPAR γ agonists could enhance the differentiation of preadipocytes into adipocytes which is relative to their antidiabetic activities^[7,8]. Because of the adverse reactions of TZD, such as hepatic toxicity and fluid retention, the research of a non-thiazolidinedione insulin sensitizer has attracted much more attention in recent years^[4].

YY20, a novel synthesized non-TZD compound named 2-(3-furan-2-yl-acryloylamino)-3-{4-[2-(5-methyl-2-phenyloxazol-4-yl)-ethoxy]-phenyl}-propionic acid (Figure 1), was a α -methyl- α -phenoxypropionate derivative^[9,10]. The acti-

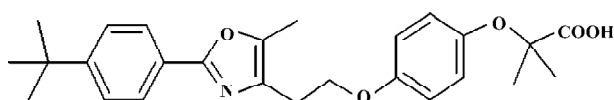


Figure 1. Structure of α -methyl- α -phenoxypropionate derivative YY20.

vation effects of rosiglitazone and YY20 on human PPAR γ were tested by using a transient transfection assay. According to the luciferase activity, YY20 activated PPAR γ 14 ± 2.4 times to the control at $10 \mu\text{mol/L}$, whereas a TZD compound rosiglitazone was 8.7 ± 1.4 times to the control at the same concentration. Although YY20 exhibited potent PPAR γ agonist activity, like rosiglitazone in the report gene system, it is essential that the insulin sensitizing effects are proved in the insulin-sensitive cell lines. In this study, the enhancement effects of YY20 on the differentiation of 3T3-L1 preadipocytes into adipocytes were examined and compared with that of rosiglitazone. As adipocyte differentiation markers^[6], PPAR γ_2 , glucose transporter-4 (GLUT4), insulin receptor substrate-1 (IRS-1), and adiponectin (ACRP30) expressions were studied in 3T3-L1 preadipocytes treated with YY20. In order to further confirm the insulin-sensitizing effects of YY20, the enhancement effects of insulin-mediated glucose consumption were also investigated in the HepG2 human hepatocellular carcinoma line.

Materials and methods

Materials 3T3-L1 mouse preadipocytes and the HepG2 human hepatocellular carcinoma line were purchased from the Cell Center of the Chinese Academy of Medical Sciences, Shanghai, China. Insulin was the product of Eli-Lilly & Co (Indianapolis, IA, USA); isobutylmethylxanthine (IBMX), dexamethasone (DEX), SR-202, Oil red O, methylthiotetrazole (MTT), and bovine serum albumin (BSA) were purchased from Sigma (St Louis, MO, USA). GLUT4, IRS-1, ACRP30, and the β -actin primary antibody were obtained from Santa Cruz Biotechnology (Santa Cruz, CA, USA). Trizol, M-MuLV reverse transcriptase, *Taq* polymerase, and cell culture mediums were the products of Gibco BRL (Gaithersburg, MD, USA). The PCR primers were synthesized by Sangon (shanghai, China). Enhanced chemiluminescent (ECL) substrate was from Pierce (Rockford, IL, USA). YY20 and rosiglitazone were synthesized in our laboratory and were dissolved in dimethyl sulfoxide (DMSO) to prepare the stock solution.

Adipocyte differentiation assay 3T3-L1 cells were maintained at 37°C in an atmosphere of 5% CO_2 in Dulbecco's

modified Eagle's medium (DMEM) containing 10% fetal bovine serum (FBS). The 3T3-L1 preadipocytes were grown in 96-well plates until 2 d postconfluence. The differentiation was induced by addition of $5 \mu\text{g/mL}$ insulin, 0.5 mmol/L IBMX and $1 \mu\text{mol/L}$ DEX (INS-IBMX-DEX cocktail). The induction medium was removed 2 d after incubation. After an additional 2 d of incubation in DMEM supplemented with 10% FBS and $5 \mu\text{g/mL}$ insulin, the medium was changed every other day with DMEM supplemented with 10% FBS. Cells were challenged during the whole period of differentiation with different concentrations of YY20, with rosiglitazone as the positive control and 0.1% DMSO as the vehicle control.

After 7 d differentiation, cells were fixed with 10% formaldehyde for 1 h and then stained with Oil red O (0.1 mg/mL) for 2 h at room temperature. The medium in each well was then removed, and isopropyl alcohol was added to dissolve the precipitate. The optical density (OD) at a wavelength of 510 nm was determined by ELISA spectrometry^[11]. Moreover, 3T3-L1 adipocytes treated with YY20 or rosiglitazone for 2 d or for 6 d, together with the vehicle control, were collected for RT-PCR and Western blot analysis.

PPAR γ inhibiting assay Two days after confluence, 3T3-L1 cells were treated with $100 \mu\text{mol/L}$ SR-202 or vehicle for 0.5 h, then $1 \mu\text{mol/L}$ YY20 or $1 \mu\text{mol/L}$ rosiglitazone was added. The cells were challenged for 4 d. After that, the cells were cultured in DMEM supplemented with 10% FBS for an additional 2 d and collected for Western blot analysis.

RT-PCR analysis The total RNA from the 3T3-L1 adipocytes treated with increasing concentrations of YY20 or rosiglitazone for 2 d or untreated was isolated using Trizol reagent in accordance with the manufacturer's instructions. After extraction, mRNA was precipitated by recommended procedures and dissolved in 0.1% diethylpyrocarbonate solution. The RNA content was quantified by using an ultraviolet spectrophotometer at 260 nm. To synthesize first strand cDNA, $7 \mu\text{L}$ total RNA was incubated with $0.5 \mu\text{g}$ of oligo (dT) 6 primer and $5 \mu\text{L}$ deionized water at 65°C for 15 min. RT of $20 \mu\text{L}$ was performed with 200 units of M-MuLV reverse transcriptase, $4 \mu\text{L}$ of $5\times$ reaction buffer (250 mmol/L Tris-HCl; pH 8.3 at 25°C , 375 mmol/L KCl, 15 mmol/L MgCl_2 , and 50 mmol/L dithiothreitol) and 1 mmol/L deoxynucleoside triphosphate (dNTP) mixture for 1 h at 42°C . PCR of $50 \mu\text{L}$ contained $1 \mu\text{L}$ of the RT reaction product, $5 \mu\text{L}$ of $10\times$ PCR buffer (100 mmol/L Tris-HCl, pH 8.3 at 25°C , 500 mmol/L KCl, and 15 mmol/L MgCl_2), 25 units of *Taq* polymerase, $1 \mu\text{L}$ of 10 mmol/L dNTP mixture, and 30 pmol of each primer. PCR for the amplification of PPAR γ_2 was performed with primers (420 bp product; sense: 5'-ATGGGTGAACTCTGGGA-3', anti-sense: 5'-TCGGCACTCAATGGCCAT-3'), and GAPDH (560

bp product; sense: 5'-ATCTTCTTGTGCAGTGCCAGCC-3', antisense: 5'-GGTCATGAGCCCTCCACAATG-3') was used as the internal control. Each amplification cycle consisted of 10 s of denaturation at 94 °C, 20 s of annealing at 55 °C, and 30 s of extension at 72 °C. Following amplification, the PCR products were separated by 1.5% agarose gel electrophoresis, stained with ethidium bromide, and visualized under ultraviolet light with a bio-imaging analyzer (Bio-Rad, Hercules, CA, USA).

Western blot analysis 3T3-L1 adipocytes treated with different concentration of YY20 for 6 d or untreated were washed with PBS, collected in RIPA buffer (50 mmol/L Tris-HCl, 150 mmol/L NaCl, 2 mmol/L EDTA, 2 mmol/L EGTA, 25 mmol/L NaF, 25 mmol/L β-glycerolphosphate, pH 7.5, 0.2% Triton X-100, 5 mmol/L EDTA, 1 mmol/L PMSF, 10 μg/mL leupeptin, and 10 μg/mL aprotinin) and lysed for 30 min on ice. Samples were clarified by centrifugation for 30 min at 13 000×g at 4 °C. An aliquot of 40 μg of the supernatant protein from each sample was heated with 4×SDS sample buffer at 95 °C for 5 min, and separated electrophoretically on a 7.5% or 12% SDS-PAGE. Subsequently, proteins were transferred onto a 0.45 μm pore size nitrocellulose membranes for 1 h and blocked for 1 h. Nitrocellulose membranes were then exposed to GLUT4, IRS-1, ACRP30, or the β-actin primary antibody in blocking buffer at 1:500 dilution overnight at 4 °C. Then the membranes were incubated with the anti-rabbit IgG or anti-goat IgG secondary antibody conjugated with horseradish at 1:10 000 dilution for 1 h. The proteins were visualized autoradiographically with ECL, and scanned using a bio-imaging analyzer (Bio-Rad, Hercules, CA, USA).

Glucose consumption assay^[12] HepG2 cells were grown in RPMI-1640 (11.1 mmol/L glucose) containing 10% FBS. Two days before the experiments, the cells were plated into 96-well, tissue culture plates with some wells left blank. After the cells reached 80%–90% confluence, the medium was replaced by RPMI-1640 supplemented with 0.2% BSA. Two hours later, the medium was removed and the same culture medium containing YY20, metformin or rosiglitazone, with or without insulin, was added to all wells, including the blank wells. After 24 h of treatment, the medium was removed and its glucose concentrations was determined by the glucose oxidase method^[13]. The amount of glucose consumption was calculated by the glucose concentrations of blank wells subtracting the remaining glucose in the cell plated wells.

Following the glucose measuring in the medium, a MTT assay was used to monitor the cell proliferation and to adjust the glucose consumption values^[14].

Statistical analysis Data were shown as mean±SD. Differences between individual groups were analyzed by using

the *t*-test. A difference with a *P* value of <0.05 was considered to be significant.

Results

Effect of YY20 on adipocyte differentiation After treatment with YY20 and rosiglitazone at 0.1, 1, and 10 μmol/L in the 3T3-L1 cells during 7 d differentiation, the cells full of lipid droplets increased significantly compared with that of the vehicle control (Figure 2A–2D). By Oil red O staining and OD value determination, it was found that YY20 and rosiglitazone could promote adipocyte differentiation of 3T3-L1 cells significantly (*P*<0.05, *P*<0.01; Figure 2E).

Effect of YY20 on PPAR_γ2 gene expression To elucidate the pattern of PPAR_γ2 gene expression after 2 d induction in

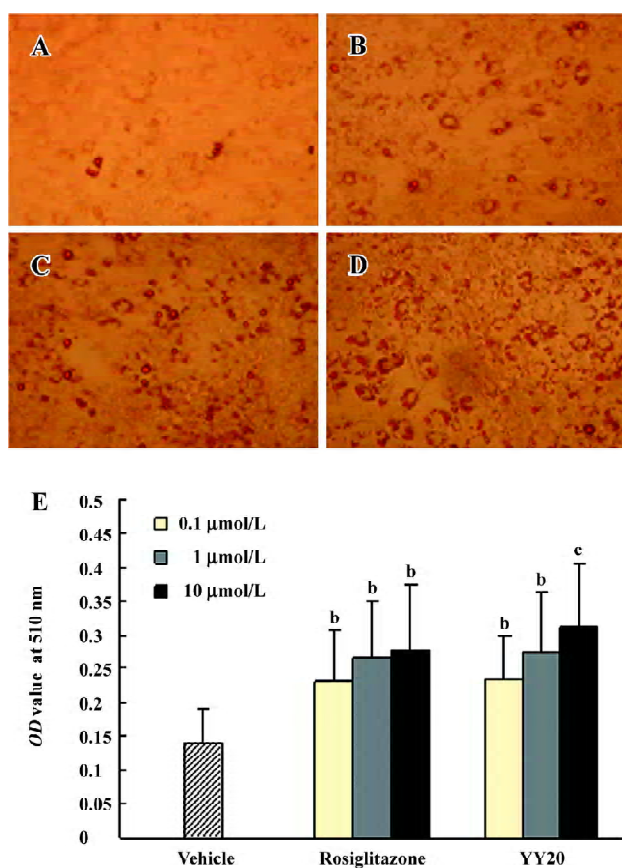


Figure 2. Enhancement of adipocyte differentiation by rosiglitazone or YY20 in 3T3-L1 cells. 0.1, 1, and 10 μmol/L of rosiglitazone or YY20 were added during the 7 d of differentiation. Cells were stained with Oil red O for 2 h. (A) vehicle; (B) 0.1 μmol/L YY20; (C) 1 μmol/L YY20; (D) 10 μmol/L YY20; (E) OD values of Oil red O which dissolved in isopropyl alcohol and were determined at 510 nm. Data are presented as the mean±SD in triplicate. ^b*P*<0.05, ^c*P*<0.01 vs vehicle.×300.

the 3T3-L1 cells, RT-PCR was analyzed. The results suggested that the expression of PPAR γ_2 mRNA increased in the cells challenged with YY20, as well as rosiglitazone (Figure 3).

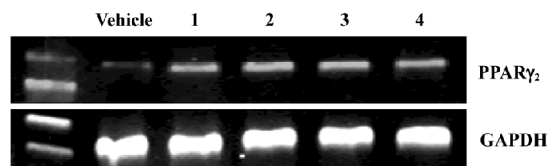


Figure 3. RT-PCR analysis of PPAR γ_2 mRNA in 3T3-L1 cells. Cells were treated and allowed to differentiate for 2 d with an inducing medium containing 5 $\mu\text{g}/\text{mL}$ insulin, 0.5 mmol/L IBMX and 1 $\mu\text{mol}/\text{L}$ DEX (INS-IBMX-DEX cocktail). PCR products were analyzed on 1.5% agarose gel and stained with ethidium bromide. The housekeeping gene GAPDH was expressed at equal levels in all samples. 1, 0.1 $\mu\text{mol}/\text{L}$ YY20; 2, 1 $\mu\text{mol}/\text{L}$ YY20; 3, 10 $\mu\text{mol}/\text{L}$ YY20; 4, 1 $\mu\text{mol}/\text{L}$ rosiglitazone. The figure represents 1 of 3 independent experiments. All 3 experiments showed similar results.

Effect of YY20 on IRS-1 and GLUT4 protein expression

The regulation of IRS-1 and GLUT4 expression by increasing concentrations of YY20 was investigated after 6 d of differentiation in the 3T3-L1 cells. Western blot analysis revealed that YY20 could obviously increase the expression levels of IRS-1 and GLUT4 in a concentration-dependent manner (Figure 4A).

Effect of YY20 on ACRP30 and GLUT4 protein expression, and the influences of the PPAR γ inhibitor After 4 d treatment and 6 d differentiation, YY20, as well as rosiglitazone at a concentration of 1 mmol/L, could upregulate the expression of the ACRP30 and GLUT4 protein; this effect could be reversed by the PPAR γ specific inhibitor, SR-202. And the increase of ACRP30 expression caused by YY20 was completely reversed by SR-202 (Figure 4B).

Effect of YY20 on glucose consumption After treatment in medium with 11.1 mmol/L glucose for 24 h, a glucose-lowering effect of YY20 was observed. With different levels of insulin added in the culture medium, YY20 exhibited different efficacy of the glucose-lowering effect (Figure 5). When the medium was absent of insulin, the glucose consumption of metformin at 1 mmol/L increased by 59% compared to the vehicle control ($P < 0.05$), whereas there were only 16% and 21% increases by 1 mmol/L of rosiglitazone and YY20, respectively ($P > 0.05$). However, with 1 nmol/L insulin present in the medium, the glucose consumption of cells treated with metformin, rosiglitazone, and YY20 were 75%, 45%, and 63%, respectively ($P < 0.05$); the effect of YY20 was related to the concentration (Figure 6). The effects

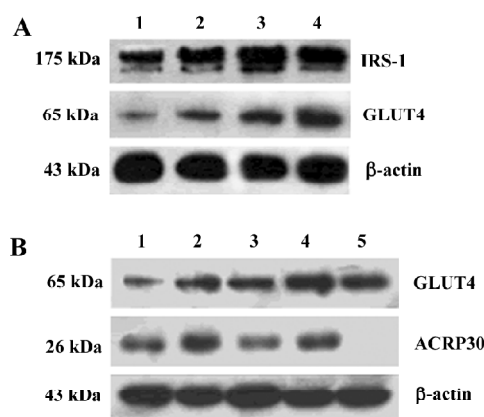


Figure 4. Western blot analysis of IRS-1 and GLUT4 expression in 3T3-L1 cells. Cells treated with YY20 were allowed to differentiate for 6 d. Total proteins were separated electrophoretically on a 7.5% (A) or 12% (B) SDS-PAGE and transferred onto nitrocellulose membranes, exposed to primary and secondary antibodies successively, and visualized autoradiographically with ECL. The housekeeping protein β -actin was expressed at equal levels in all samples. (A) 1, vehicle; 2, 0.1 $\mu\text{mol}/\text{L}$ YY20; 3, 1 $\mu\text{mol}/\text{L}$ YY20; 4, 10 $\mu\text{mol}/\text{L}$ YY20. (B) 1, vehicle; 2, 1 $\mu\text{mol}/\text{L}$ rosiglitazone; 3, 0.1 $\mu\text{mol}/\text{L}$ SR202+1 $\mu\text{mol}/\text{L}$ rosiglitazone; 4, 1 $\mu\text{mol}/\text{L}$ YY20; 5, 100 $\mu\text{mol}/\text{L}$ SR-202+1 $\mu\text{mol}/\text{L}$ YY20. Figure shown here represents 1 of 3 independent experiments. All 3 experiments showed similar results.

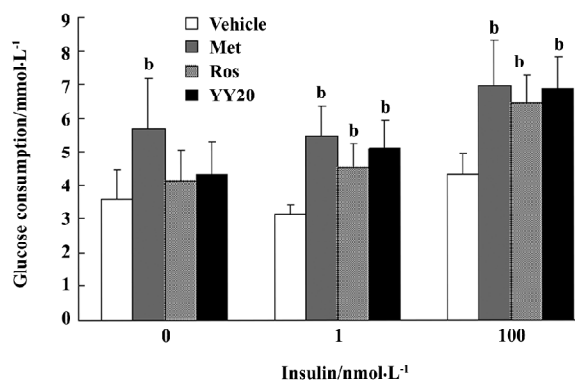


Figure 5. Effects of YY20 on glucose consumption in HepG2 cells with different levels of insulin. Cells were incubated with 1 $\mu\text{mol}/\text{L}$ YY20, with DMSO as the vehicle control and 1 mmol/L metformin (Met) or 1 $\mu\text{mol}/\text{L}$ rosiglitazone (Ros) as positive control in the presence of 11.1 mmol/L glucose and 0, 1, and 100 nmol/L insulin, respectively, for 24 h. Data are presented as the mean \pm SD in triplicate. ^b $P < 0.05$ vs vehicle.

under 100 nmol/L insulin were similar to that of under 1 nmol/L insulin. According to the MTT results, we found that in the presence of 1 mmol/L metformin and less than 1 mmol/L YY20 or rosiglitazone could not affect cell proliferation after 24 h culture (data not shown). When the glucose consump-

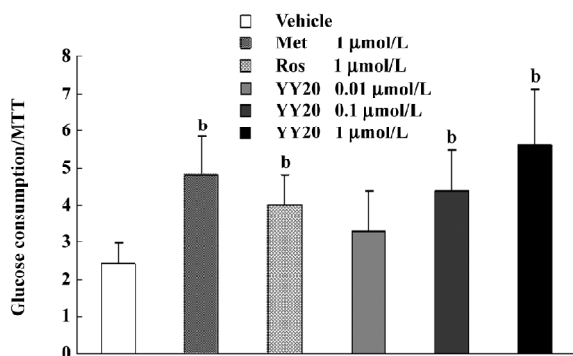


Figure 6. Dose-dependent glucose-lowering effects of YY20 in HepG2 cells. Glucose consumption and MTT OD values were measured after 24 h of incubation with increasing concentrations of YY20 in the presence of 11.1 mmol/L glucose and 1 nmol/L insulin, with DMSO as the vehicle control and 1 mmol/L metformin (Met) or 1 μmol/L rosiglitazone (Ros) as the positive control. Data are presented as the mean±SD in triplicate. ^b*P*<0.05 vs vehicle.

tion values were adjusted with the MTT OD values, the results changed insignificantly.

Discussion

It has been widely known that PPAR γ agonists are dominant regulators of adipocyte development, and PPAR γ activation improves insulin resistance^[15-17]. As a TZD, rosiglitazone is a potent agonist of PPAR γ and could significantly improve the adipocyte differentiation in 3T3-L1 cells. Activation of PPAR γ can increase the number of small adipocytes, but reduce the number of large adipocytes in white adipose tissues. Because small adipocytes are more sensitive to insulin, an increased number of small adipocytes and a decreased number of large adipocytes in white adipose tissues can alleviate insulin resistance^[18]. Furthermore, adipocyte differentiation leads to the expression of adipocyte-specific genes, such as GLUT4 and IRS-1, which are important components of the insulin receptor signal transduction pathway^[19-21]. In this study, we found that the novel non-TZD compound YY20 could enhance 3T3-L1 cell differentiation as effectively as rosiglitazone. YY20 could increase lipid accumulation in differentiated cells and upregulate PPAR γ mRNA, IRS-1, GLUT4, and ACRP30 protein expression in 3T3-L1 adipocytes, indicating that it might activate PPAR γ and enhance insulin sensitivity. Besides an adipocyte differentiation mark, ACRP30 is also deemed as an adipocytokine which can improve insulin resistance^[22]. In our study, the PPAR γ inhibitor could completely reverse the ACRP30 expression upregulated by YY20, but not rosiglitazone, suggesting that the effect of YY20 on ACRP30 expression is

more dependent on PPAR γ activation than rosiglitazone. What this means to the therapy is still unknown.

Liver, adipose tissue, and skeletal muscles are target tissues of insulin and major sites of glucose and lipid metabolism. In our study, the HepG2 human hepatocellular carcinoma line was used to elucidate the glucose-lowering effect of YY20 *in vitro*. According to the results, metformin could elevate the glucose consumption of cells regardless of the insulin levels, whereas PPAR γ agonist rosiglitazone and YY20 were somewhat dependent on insulin, suggesting the different mechanisms of the glucose-lowering effect between these two kinds of antidiabetic drugs. Since PPAR α , not PPAR γ , is predominantly expressed in the liver^[23], we wonder if the mechanism of the glucose-lowering effect in HepG2 hepatocytes is related to the activation of PPAR α .

In summary, as a potential activator of PPAR γ , YY20 could enhance preadipocyte differentiation and upregulate vital molecules in the insulin signaling pathway, and enhance glucose consumption in HepG2 cells in a concentration- and insulin-dependent manner. These results suggest that further studies should be carried out to develop YY20 as a substitute of TZD for diseases with insulin resistance, such as type 2 diabetes.

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