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D_2-dopaminergic receptor linked pathways: critical regulators of *CYP3A*, *CYP2C* and *CYP2D*

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RUNNING TITLE PAGE

Running Title: D_2-dopaminergic control in CYP3A, CYP2C and CYP2D regulation

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Abbreviations: ACN, acetonitrile; CYP, cytochrome;C, control; CAR, constitutive androstane receptor; CORT, corticosterone; CREB, cAMP responsive element-binding protein; FOXO1, Forkhead box protein O1; GH, growth hormone; HNF1α; hepatocyte nuclear factor 1α; HNF4α, hepatocyte nuclear factor 4α; INS, insulin; JNK, c-Jun N-terminal kinase; PRL, prolactin; PXR, pregnane X receptor; RXR, retinoic X receptor; rCYP, recombinant CYP; STAT5b, signal transducer and transcriptional activator 5b; SULP, sulpiride; T3, triiodothyronine; T4, thyroxine; TSH, thyroid stimulating hormone; WORT, wortmannin.

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ABSTRACT

Various hormonal and monoaminergic systems hold determinant roles in the regulation of several *CYPs* in the liver. Growth hormone (GH), prolactin (PRL) and insulin are involved in *CYP* regulation and their release is under dopaminergic control. Therefore, this study has focused on the role of D_2-dopaminergic systems in the regulation of the major drug-metabolizing *CYPs*, the *CYP3A*, *CYP2C* and *CYP2D*. Blockade of D_2-dopaminergic receptors with either sulpiride (SULP) or L-741,626 markedly down-regulated CYP3A1/2, CYP2C11 and CYP2D1 expression in the rat liver. This suppressive effect appears to be mediated by the insulin/PI3K/Akt/FOXO1 signaling pathway. Furthermore, inactivation of the GH/STAT5b signaling pathway appears to play a role in the D_2-mediated down-regulating effect on these *CYPs*. SULP suppressed plasma GH levels, followed by reduced activation of STAT5b, the major GH pulse-activated transcription factor, which has an up-regulating effect on various *CYPs* in the hepatic tissue. PRL, which possesses a down-regulating control on *CYPs*, was increased by SULP, and may thus also contribute in the SULP-mediated effect. Finally, it appears that the SULP-induced inactivation of the cAMP/PKA/CREB signaling pathway, which is a critical regulator of *PXR* and *HNF1α*, as well as inactivation of the JNK, contribute in the SULP-induced down-regulation of the above mentioned *CYPs*. Taken together the present data provide evidence that drugs acting as D_2-antagonists, could interfere with several major signaling pathways involved in the regulation of *CYP3A*, *CYP2C* and *CYP2D*, critical enzymes in drug-metabolism, thus affecting the effectiveness of the majority of prescribed drugs and the toxicity and carcinogenic potency of a plethora of toxicants and carcinogens.

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INTRODUCTION

Cytochrome P450s (CYPs) hold a major role in the hepatic metabolism of xenobiotics such as drugs, carcinogens and toxic agents. Metabolism can activate pro-drugs to more pharmacologically active metabolites, inactivate pharmacologically active compounds or activate pre-carcinogens, thus triggering the formation of several tumors in humans (Gonzalez and Gelboin, 1994; Ingelman-Sundberg, 2004). CYPs also catalyze the biotransformation of endogenous compounds, such as steroids and fatty acids (Guengerich, 2003; Spatzenegger and Jaeger, 1995). Inhibition of CYP isoforms metabolizing clinically used drugs may lead to increased levels of a drug-substrate in the body, and cause detrimental adverse effects, especially in situations when low therapeutic index drugs are used (Spatzenegger and Jaeger, 1995). Conversely, up-regulation of the critical enzymes may accelerate the metabolism of the drug-substrate, thus leading to reduced pharmacological outcome of the drug (Konstandi et al., 2005; Daskalopoulos et al., 2012). It is therefore of importance, particularly in multi-drug therapies to be aware of the possibilities of drug-drug interactions, leading either to reduced/increased drug efficacy or severe toxicity and/or tumorigenesis (Gonzalez and Gelboin, 1994; Guengerich, 2003; Konstandi et al., 2004; Pelkonen et al., 2008).

Some of the most important CYP enzymes for the hepatic phase I drug metabolism are members of the subfamilies CYP3A, CYP2C and CYP2D (Ingelman-Sundberg, 2004). These cytochromes are responsible for the biotransformation of over than 90% of the most widely prescribed drugs (Guengerich, 2003).

Central/peripheral catecholaminergic systems have been identified to regulate *CYP* expression (Konstandi et al., 1998; Konstandi et al., 2004; Rendic and Guengerich, 2010; Daskalopoulos et al., 2012). In addition, previous studies clearly

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indicated a critical role for hormones, such as the growth hormone (GH) (Waxman and O'Connor, 2006), thyroid hormones (Takahashi et al., 2010) and insulin (Kim and Novak, 2007) in the regulation of various hepatic *CYPs* (Daskalopoulos et al., 2012).

Diabetic rats display increased hepatic CYP2A1, CYP2C6/7, CYP2E1, CYP3A2 and CYP4A3 expression, which can be restored to normal levels by insulin administration (Shimojo et al., 1993). Insulin exerts many of its effects via the PI3K/Akt signaling pathway, which regulates the expression of several genes, including various *CYP* isoforms (Kim and Novak, 2007). Among upstream signaling factors regulating the release of insulin in response to increased plasma glucose levels, dopamine holds a significant role: stimulation of D_2-dopaminergic receptors in the pancreatic β-cells, suppresses insulin release (Rubi et al., 2005). In accordance with this and other earlier studies, we have shown that blockade of D_2-dopaminergic receptors stimulated insulin release and markedly down-regulated *CYP2E1* (Konstandi et al., 2008). It is possible that in this regulation, the insulin/PI3K/Akt signaling pathway holds a determinant role (Woodcroft et al., 2002).

A broad spectrum of drugs prescribed for a variety of disease states including schizophrenia, bipolar disorder, depression and Parkinson's disease; exert their effects mainly via D_2-dopaminergic receptors, and the related signaling pathways (Beaulieu and Gainetdinov, 2011). Given its central role in the regulation of insulin release, it is conceivable that drugs acting via this pathway could alter the expression of various drug metabolizing CYP enzymes, thus affecting the pharmacodynamics and toxicity of drug-substrates during pharmacotherapy (Gonzalez and Yu, 2006).

The aim of this study was to assess the role of the D_2-dopaminergic receptor linked signaling pathways in the regulation of the cytochromes *CYP3A*, *CYP2C* and *CYP2D*. For this purpose, rats were treated with the D_2-dopaminergic antagonist,

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sulpiride (Konstandi et al., 2008), a typical antipsychotic drug used mainly in the treatment of psychosis associated with schizophrenia and major depressive disorder (Maitre et al., 1994). The direct effect of sulpiride on hepatocytes was also assessed *in vitro* using primary hepatocyte cultures. Blockade of the D_2-dopaminergic signaling pathways caused a strong reduction in the expression of cytochromes *CYP3A, CYP2C* and *CYP2D*. Given the fact that the vast majority of the prescribed drugs are metabolized by these CYPs, their down-regulation would lead to strongly increased blood levels of drug-substrates during multi-drug therapy and drug-drug interactions with potentially detrimental side effects.

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MATERIALS AND METHODS

Animals

All experimental animals used in this study (adult male Wistar Kuo/Io/rr rats, 2-3 months old) were in-bred at the Animal House of the University of Ioannina, Greece and were housed in groups of three to four in plastic cages with standard rodent chow and water available *ad libitum*. The animals were maintained on a 12-hr light/dark cycle (lights on at 6am) and were adapted to handling for an adaptation period of 5-7 days prior to the experiment. All procedures involving animals were reviewed and approved by the local ethics committee. They conformed to the International European Ethical Standards (86/609-EEC) for the care and use of laboratory animals.

In vivo **assessment of the role of D_2-dopaminergic receptor-related pathways in the regulation of hepatic drug metabolism**

Controls (group I) received normal saline (s.c., twice daily) for 4 consecutive days. Sulpiride (Sigma Aldrich, 2 μg/kg b.w., SULP) was administered subcutaneously to the animals of group II, twice daily, for 4 consecutive days. The highly selective D_2-dopaminergic receptor antagonist, L-741,626 (SID 50104688 in PubChem; Sigma-Aldrich, 1.5 mg/kg b.w., i.p.) was also administered to rats (group III), twice daily, for 4 consecutive days. All animals were sacrificed 1 hour after the last injection.

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Isolation of microsomes

Microsomal fractions were prepared from liver samples which were homogenized in ice-cold homogenization buffer (0.15 M KCl, 10 mM K_2EDTA, 1mM dithiothreitol, pH 7.4). The homogenates were centrifuged at 14,075 g (4°C) for 20 min. The upper phase was transferred into new vials and was centrifuged for 60 min at 96,552 g (4°C). The microsomal pellet was re-suspended in ice-cold homogenization buffer, homogenized and centrifuged for 45 min at 96,552 g (4°C). The washed microsomal pellet was re-suspended in ice-cold storage buffer (K_2HPO4/KH_2PO4, pH 7.4, 1 mM K_2EDTA, 0.1 mM dithiothreitol, 20% glycerol) and stored at -80°C until assayed (Lang et al., 1981).

Primary hepatocyte cultures

Primary hepatocytes were isolated and used in cultures according to the method of Klaunig et al. (Klaunig et al., 1981). In brief, primary hepatocytes were isolated from rats weighing 250-300 g using a two-step collagenase perfusion method. They were suspended in William's Medium E (Gibco) containing 1% L-glutamine (PAA) and 1% penicillin/streptomycin. The cells were counted in a Neubauer cell chamber and plated at a density of 1 x 10^5 cells per well, in 3.8 square centimeter diameter collagen type I coated dish (BIOCOAT, Cell Environment, Becton Dickinson Labware, UK). The viability of the isolated hepatocytes was checked with trypan blue dye 0.4% exclusion and only cells with viability higher than 85% just before plating were used. Hepatocytes were cultured at 37°C for 24 hours under an atmosphere of humidified 5% CO_2, in order to allow them to adhere to the wells. Time and dose response experiments started 24 hours later. Primary hepatocyte cultures were treated either with SULP (1-25 μM) or insulin (INS), at different doses ranging

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from 1-100 μM in combination with wortmannin, an inhibitor of the PI3K/Akt signaling pathway. The cAMP/PKA inhibitor, H89 (10 μM, 24 hours), and the JNK inhibitor, SP600125 (10 μM, 24 hours), were also added in the primary hepatocyte cultures 30 min before SULP. Time response experiments were also conducted with drug treatment of primary hepatocytes ranging between 4 and 36 hours.

Enzyme activity

Bufuralol 1'-hydroxylation

Bufuralol 1′-hydroxylation reflects the cytochrome CYP2D activity (Matsunaga et al., 1990). Liver microsomal protein (40 μg) was pre-incubated at 37°C for 5 min in a 200 μl reaction mixture containing potassium phosphate (100 mM, pH 7.4) in the presence of 50 μM bufuralol substrate. The reaction was initiated with NADPH (0.5 mM) at 37°C and lasted 7.5 min. It was terminated using 20 μl of perchloric acid 60%. After centrifugation at 14,075 g for 10 min, the concentration of 1'-hydroxy-bufuralol (main metabolite of bufuralol) was determined in the supernatant (100 μl) by HPLC with a fluoroscence detector at 252 and 302 nm. A reversed phase Luna C_{18} column (5 μm, 150 x 3 mm) was used. The mobile phase contained a mixture of 30% acetonitrile (ACN) and 70% perchlorate buffer 20 mM (pH 2.5) and the sample was eluted at a flow rate of 1 ml/min for 15 min.

The positive control of the bufuralol1΄-hydroxylation took place using the following recombinant CYPs (rCYP), the rat CYP2D1 and CYP2D2 + P450 Reductase Supersomes (BD Biosciences-Gentest).In brief, approximately 200 μl of potassium phosphate buffer 0.1 M pH 7.4, containing the substrate Bufuralol (50 μM) were pre-incubated at 37°C for 2 min. Then the rCYP (50 μM) and NADPH (1 mM)

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were added and the mixture was incubated at 37°C for 30 min. The reaction was terminated with 20 µl ACN and the samples were left undisturbed on wet ice for 10min and then they were injected into the HPLC for analysis.

Testosterone hydroxylation

Cytochromes CYP3A1/2, CYP2C11 and CYP2A1 catalyze testosterone hydroxylation in the rat liver. The activities studied were the CYP3A1/2 dependent 6β-testosterone hydroxylation and the CYP2C11-dependent 2α- and 16α-testosterone hydroxylation (Murray et al., 2001). Briefly, liver microsomal proteins (1 mg) were incubated at 37°C for 7.5 min in 500 µl of a mixture containing potassium phosphate (50 mM, pH 7.4) in the presence of 200 µM testosterone (the substrate was diluted in 5 µl ACN). The reaction was initiated with NADPH (1 mM) and terminated adding 2 ml of a (2:1) chloroform/methanol mixture at 4°C. All incubations were performed at conditions under which linearity with time was established. After extraction, centrifugation and separation, the organic phase was removed and evaporated under nitrogen. The residues from evaporation were dissolved in 200 µl of ACN and aliquots of 100 µl were injected onto HPLC. Testosterone metabolites were resolved on a reversed phase Zorbax C_{18} column (5 µm, 150 × 4.6 mm) and eluted over 30 min with the mobile phase A containing 10% ACN and 0.5% acetic acid at a flow-rate 0.8 ml/min, followed by an elution over 35 min with the mobile phase B containing 36% ACN and 0.5% acetic acid.

As a positive control in the detection of the 6β-testosterone hydroxylation, the rat CYP3A1 and CYP3A2 Supersomes containing P450 reductase and Cytochrome b5 (BD Biosciences-Gentest) were used. For the positive control of the 16α-testosterone hydroxylation, the rat CYP2C11 + P450 reductase + Cytochrome b5 Supersomes

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were used (BD Biosciences-Gentest). As previously described, approximately 200 μl of potassium phosphate buffer 0.1 M, pH 7.4, containing the substrate testosterone (5 μM) were preincubated at 37°C for 2 min. Then, the recombinant CYP (50 μM) and NADPH (1 mM) were added and the mixture was incubated at 37°C for 30 min. The reaction was terminated with 20 μl ACN and the samples were left undisturbed on wet ice for 10 min and then they were kept at -20°C for 24 hours before analyzing.

For the negative control of the CYP3A-dependent 6β-testosterone hydroxylation, ketoconazole (Sigma-Aldrich), a CYP3A inhibitor was used. Sulfaphenazole (Sigma-Aldrich), a CYP2C11 inhibitor, was used for the negative control of the CYP2C-dependent 2α- and16α-testosterone hydroxylation. In brief, 200 μl of potassium phosphate buffer 0.1M, pH 7.4, containing testosterone as substrate (5 μM), the CYP inhibitor (1 μM) and microsomes 0.1 mg were preincubated at 37°C for 2 min. Then, NADPH (1 mM) was added and the mixture was incubated at 37°C for 30 min when sulfaphenazole was used as inhibitor or 5 min when ketoconazole was used as inhibitor. The reaction was terminated with 200 μl ACN (sulfaphenazole) or 200 μl methanol (ketoconazole) and the samples were kept on wet ice for 5 min, followed by centrifugation at 100,000 g, at 4°C for 15 min. The supernatant was collected and kept at -20°C for 24 hours. The following day the samples were centrifuged at 10,000 g, at 4°C for 10 min for protein precipitation and analyzed by HPLC.

Western blot analysis

Immunoblot analysis of the cytochrome CYPs, STAT5b and FOXO1 apoprotein levels was carried out using microsomes and nuclear extracts or cytosol of liver samples, respectively. For the preparation of the nuclear extracts and cytosol the

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NE-PER nuclear extraction kit (Pierce, Rockford, IL) was used. The content of the phosphorylated CREB, JNK, Akt and p70S6K was determined by western blot in total cellular proteins, extracted from the liver using RIPA buffer supplemented with protease inhibitors, PMSF (10 μM), BGP (50 μM) and NaF (50 μM). Protein concentrations were determined by the BCA protein assay (Pierce, Rockford, IL). Proteins were subjected to SDS-PAGE gel electrophoresis and immunoblotting using the following antibodies: rat polyclonal CYP3A1, CYP3A2, CYP2C11, CYP2D1 IgGs, mouse monoclonal total STAT5a/b IgG (Santa Cruz Biotechnology) and rabbit monoclonal p-STAT5b IgG (Tyr 694, Cell Signaling Technology). Rabbit polyclonal p-CREB-1 IgG (Ser 133), rabbit polyclonal total JNK2 IgG, mouse monoclonal p-JNK IgG (Thr 183 and Tyr 185) (Santa Cruz Biotechnology), rabbit polyclonal p-p70S6K IgG (Thr 389), rabbit polyclonal total p70S6K IgG (Cell Signaling Technology), rabbit polyclonal p-FOXO1 (Ser 256) and total FOXO1 IgGs (Santa Cruz Biotechnology), as well as rabbit polyclonal p-Akt (Ser 473) and total Akt IgGs (Santa Cruz Biotechnology) were also used. Secondary antibodies, conjugated with horseradish peroxidase (Santa Cruz Biotechnology) were used and the proteins were detected using a chemiluminescence detection kit (ECL, Amersham, GE Healthcare). Immunoblotting with GAPDH or β-actin (Santa Cruz Biotechnology) and anti-mouse IgG horseradish peroxidase conjugated secondary antibody, were used as loading control.

Quantitative real-time PCR

For the isolation of total RNA from liver tissue and primary hepatocytes, the TRIzol reagent was used (Invitrogen) following the manufacturer's protocol. The concentration of total RNA was determined with a spectrophotometric method.

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Quantitative real-time reverse transcriptase PCR (qPCR) was performed with cDNA generated from 1 μg total RNA with a SuperScript II reverse transcriptase kit (Invitrogen). The sequences for the forward and reverse gene-specific primers used are shown in the Table 1. SYBR Green PCR master mix (Applied Biosystems, Warrington, UK) was used for the real-time reactions, which were carried out using the Thermal Cycler Real-Time Detection System C1000 (BioRad, Italy). Relative mRNA expression levels were normalized to *β*-actin (QuantiTect Primer Assay, Qiagen) and values were quantified using the comparative threshold cycle method.

Hormonal determinations

Serum corticosterone concentrations were measured using the Corticosterone RIA kit (Coat-A-Count kit, Diagnostic Products Corporation, USA). The detection limit was approximately 5.7 ng/ml and the intra-assay coefficient of variation (CV) was 4.0%. The GH serum levels were assessed with the rat growth hormone RIA kit (Millipore, MA, USA). The detection limit was 0.5 ng/ml and the intra-assay coefficient of variation was 10%. Prolactin (PRL) serum levels were measured with the rat prolactin RIA kit (MP Biomedicals Europe, France) and the detection limit was 0.5 ng/ml. Serum thyroid hormone concentrations were measured with the Dynatest T3, Dynatest T4 and Dynatest TSH kits (Brahms, Germany). The normal ranges were 80-200 ng/dl (Dynatest T3), 4.5-12 μg/dl (Dynatest T4) and 0.4-4 mg/ml (Dynatest TSH), respectively. The insulin levels were measured using an ELISA kit (Mercodia Rat ELISA kit for insulin, Uppsala, Sweden). The detection limit was 3.3 ng/ml and the intra-assay coefficient of variation was 3.1%. The blood glucose levels were measured with a commercially available kit (Merck, Germany) using the technique of glucose oxidase (Trinder, 1969).

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Statistical analysis

Data were analysed using the one-way analysis of variance (ANOVA) followed by multiple comparisons with Bonferonni's and Tuckey's list honest significant difference methods and is presented as the mean ± SE. In all cases, probability value of $p \leq 0.05$ was considered as significant.

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RESULTS

Involvement of D_2-dopaminergic receptor-linked pathways in *CYP3A* regulation

In vivo administration of the D_2-dopaminergic receptor antagonist, SULP, resulted in the down-regulation of the hepatic *CYP3A1/2* expression. Both the CYP3A-dependent 6β-testosterone hydroxylation, CYP3A1/2 apoprotein and mRNA levels were detected at markedly lower levels in SULP-treated rats compared to controls (Fig. 1). The highly selective D_2-dopaminergic antagonist L-741,626 also repressed CYP3A1/2 expression at mRNA, apoprotein and activity level (Fig. 1).

In contrast, *in vitro* experiments using primary hepatocyte cultures demonstrated that D_2-dopaminergic receptor blockade with SULP markedly increased *CYP3A1* and *CYP3A2* mRNA levels (Fig 2A). This increase was not mediated by the JNK-, cAMP/PKA-, or PI3K- related pathways, as neither the JNK inhibitor, SP600125, the PKA inhibitor, H89,or the PI3K inhibitor, Wortmannin (WORT), prevented it (Fig. 2A). Interestingly, incubation of primary hepatocytes with insulin (INS) strongly suppressed *CYP3A1/2*, whereas this suppressive effect was completely reversed by WORT (Fig. 2B).

Involvement of D_2-dopaminergic receptor-linked pathways in *CYP2C* regulation

In vivo administration of SULP markedly suppressed *CYP2C11* expression in the liver of rats, as assessed at enzyme activity level (2α- and 16α-testosterone hydroxylation), as well as at apoprotein and mRNA levels (Fig 3). L-741,626 also repressed CYP2C11 expression at mRNA, apoprotein and 2α- and 16α-testosterone hydroxylation level (Fig. 3)

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However, SULP significantly increased CYP2C11 expression *in vitro* (Fig 2A), an effect that was not prevented by SP600125, H89 or WORT (Fig. 2A). And, as in the case of CYP3A, treatment of primary hepatocytes with INS markedly decreased CYP2C11 mRNA transcripts, an effect completely blocked by WORT (Fig. 2B).

Involvement of D_2-dopaminergic receptor-linked pathways in *CYP2D* regulation

Blockade of D_2-dopaminergic receptors with SULP markedly decreased the CYP2D-dependent 1´-bufuralol hydroxylation, as well as the CYP2D1 mRNA transcripts and apoprotein, compared to controls (Fig 4). L-741,626 also repressed CYP2D1/2 expression (Fig. 4)

Similarly to the CYP3A and CYP2C, treatment of primary hepatocytes with SULP resulted in an up-regulation of *CYP2D1* and *CYP2D2* (Fig. 2A) and this up-regulation was not prevented by neither SP600125, H89 or WORT (Fig. 2A). In contrast, INS strongly suppressed *CYP2D1* and *CYP2D2*, an effect completely blocked by WORT (Fig. 2B).

Positive and negative control of CYP-dependent activities

Incubation of liver microsomes with Ketoconazole markedly suppressed the CYP3A-catalyzed 6β-testosterone hydroxylation compared to that of microsomes incubated with the vehicle (Supplemental Figure 1A). 2α- and 16α- testosterone hydroxylation was also significantly suppressed by Sulfaphenazole (Supplemental Figure 1B and 1C). In contrast, incubation of liver microsomes with SULP did not modify these activities (Supplemental Figure 1A-1C).

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Incubation of rCYP3A1 with SULP increased the 6β-testosterone hydroxylation, whereas SULP had no effect on rCYP3A2-catalyzed activity (Supplemental Figure 1D). Interestingly, the D_2-antagonist markedly repressed the rCYP2C11-catalyzed 2α- and 16α- testosterone hydroxylation (Supplemental Figure 1E and 1F), whereas SULP had no effect on the rCYP2D1 and rCYP2D2-catalyzed bufuralol 1΄-hydroxylation (Supplemental Figure 1G and 1H).

Assessment of D_2-dopaminergic receptor blockade on hormonal state

Treatment of rats with SULP strongly suppressed plasma GH, triiodothyronine (T3), thyroxin (T4) and corticosterone concentrations (Table 2). In contrast, SULP markedly increased plasma prolactin levels (PRL, Table 2). It is of importance to this study that the drug also increased plasma insulin levels, followed by a reduced plasma glucose concentration (Table 2).

Assessment of D_2-dopaminergic receptor-mediated effect on PI3K/Akt/FOXO1 pathway

The D_2-dopaminergic antagonist SULP markedly increased the phosphorylation of Akt in the liver of rats (Fig. 5A). Moreover, the phosphorylation of p70S6K, a down-stream element in the PI3K/Akt pathway, was also strongly increased following SULP treatment (Fig. 5A). Interestingly, SULP strongly decreased the levels of phosphorylated FOXO1 in the nucleus of hepatocytes (Fig. 5B), whereas they were markedly increased in the cytosol (Fig. 5C). It should be also noted that SULP up-regulated *eNOS* and *iNOS* (fold induction, mean ± SE, Control

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vsSULP, 1.0 ± 0.13 vs 1.8 ± 0.22, n=10, p<0.01, iNOS and 1.0 ± 0.12 vs 2.0 ± 0.14, n=10, p<0.01, eNOS).

L-741,626 had also similar effects with SULP on the PI3K/Akt/FOXO1signal transduction pathway (Fig. 6A-6C).

Assessment of the D_2-dopaminergic receptor-mediated effect on STAT5b activation

It is well established that growth hormone (GH) and the GH-pulse activated transcription factor STAT5b (Holloway et al., 2006) play major roles in the regulation of several *CYPs* (Waxman and Holloway, 2009). Treatment of rats with SULP strongly suppressed STAT5b activation as assessed at nuclear and cytosolic STAT5b phosphorylation level in Tyr 694 (Fig. 5B and Fig. 5C). Similarly, L-741,626 markedly reduced STAT5b activation in the nucleus and cytoplasm compared to controls (Fig. 6B and 6C).

D_2-receptor mediated effect on nuclear transcription factors involved in *CYP* regulation

CAR, PXR, RXRα and HNF1α mRNA levels were detected at markedly lower levels in the liver of SULP- and L-741,626-treated rats compared to controls (Fig. 7A-7D). No significant change though, was observed in the hepatic *HNF4α* expression following SULP, whereas L-741,626 suppressed it (Fig 7E). CREB phosphorylation, a down-stream element in the cAMP/PKA signaling pathway and JNK phosphorylation were also suppressed by both SULP and L-741,626 (Figure 5A and 6A).

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In vitro experiment using primary hepatocyte cultures showed that treatment of the cells with SULP increased CAR mRNA transcripts. This increase is profoundly mediated by the JNK, cAMP/PKA and PI3K-linked signaling pathways as it was prevented by the corresponding inhibitors, SP600125, H89 and WORT (Fig. 7A). RXRα mRNA was also increased following treatment of the hepatocytes with SULP, and this increase was mainly mediated by the JNK-linked pathway (Fig. 7C). PXR, HNF1α and HNF4α mRNA expressions were not affected by a direct exposure of hepatocytes to SULP (Fig. 7B, 7D and 7E).

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DISCUSSION

An increasing body of evidence suggests that central and peripheral catecholaminergic systems play an important role in the regulation of drug metabolizing enzymes including the *CYPs* (Konstandi et al., 2004; Konstandi et al., 2005; Konstandi et al., 2006; Daskalopoulos et al., 2012). In accordance with this, the data of the present study clearly indicated a critical role for the dopaminergic pathways in *CYP* regulation. The investigation has focused on the role of the D_2-dopaminergic receptor related pathways: targets of many therapeutically important drugs prescribed for several neurodegenerative and psychopathological disorders including Parkinson's disease, depression and psychosis (Craig and Lin, 1981; Bonci and Hopf, 2005; Kabbani et al., 2012). These drugs acting either as D_2-agonists or antagonists directly affect the dopamine receptor system by mimicking, blocking or altering the sensitivity to dopamine, thus altering the functional output of the dopaminergic systems (Cooper et al., 1996).

Pharmacological blockade of D_2-dopamine receptors using SULP, a selective D_2-receptor antagonist and typical antipsychotic drug, resulted in a strong down-regulation of *CYP3A1/2*, *CYP2C11* and *CYP2D1* (but not *CYP2D2)*. The involvement of the D_2-dopaminergic receptors in the above mentioned *CYP* regulation has been confirmed using also the highly selective D_2-dopamine receptor antagonist L-741,626. The SULP-induced down-regulation appeared to be indirect, as treatment of primary hepatocytes with SULP had an opposite effect. We hypothesize that the SULP-induced down-regulation of these CYPs is the outcome of a combined effect of the drug on central and peripheral aminergic and hormonal systems that overrides the drug's direct up-regulating effect on hepatocytes. In terms of the CYP2C11, it is of

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interest to note also that *in vitro* experiments using recombinant CYP2C11 revealed a direct down-regulating effect of the drug on this cytochrome.

The role of insulin in the D_2-receptor mediated *CYP* down-regulation appears to be crucial. This hypothesis is based on the notion that insulin release, which holds a negative regulatory control on several *CYPs* (Yoshida et al., 1996; Woodcroft and Novak, 1997; Woodcroft and Novak, 1999) is under dopaminergic control via the pancreatic beta-cell D_2-receptors (Rubi et al., 2005 - Scheme 1).

We have shown before that blockade of D_2-dopamine receptors stimulates the release of insulin (Konstandi et al., 2008). And the current *in vitro* experiments using primary hepatocytes clearly indicated that insulin down-regulates *CYP3A1/2*, *CYP2C11* and *CYP2D1* via the PI3K/Akt signaling pathway, as pre-treatment of hepatocytes with wortmannin, a PI3K inhibitor, completely reversed the insulin's suppressive effect. Further investigations indicated that treatment of rats with SULP increased plasma insulin levels, followed by activation of the PI3K/Akt signaling pathway, an event associated with the phosphorylation of the Forkhead box protein O1 (FOXO1) in the nucleus, the subsequent translocation of FOXO1 into cytoplasm and the termination of *CYP* transcription (Kodama et al., 2004; Kim and Novak, 2007). The Akt/p70S6K pathway does not seem to contribute to the SULP-induced suppressive effect on the aforementioned *CYPs*, as activation of p70S6K is connected with increased gene transcription (Kim and Novak, 2007). Notably, the activation of c-Jun N-terminal kinase (JNK) was markedly weaker in the liver of SULP-treated rats. Taking into account the accumulating evidence that Akt- and JNK-linked pathways have opposing effects on FOXO: Akt prevents FOXO nuclear localization and inhibits its activity, whereas JNK increases FOXO activity by promoting its import in the nucleus (Brunet et al., 1999; Essers et al., 2004; Wang et al., 2005), we

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hypothesize that the SULP-induced down-regulating effect on *CYPs* involving FOXO1 is the outcome of the drug's combined effect on both, Akt and JNK signaling pathways.

FOXO1 is a part of a complex cross-talking mechanism that also includes the nuclear receptors constitutive androstane receptor (CAR) and the pregnane X receptor (PXR) (Kodama et al., 2004). These nuclear receptors, along with the retinoic X receptor (RXR), the hepatocyte nuclear factor 1α (HNF1α) and 4α (HNF4α) (Liddle et al., 1998; Wiwi and Waxman, 2004) - are known to regulate the most important hepatic *CYP* genes, including members of the CYP3A and CYP2C subfamilies (Dvorak and Pavek, 2010). Importantly, SULP has also down-regulated the expression of CAR, PXR, RXRα and HNF1α, indicating that the drug's suppressive effect on *CYP3A1/2* and *CYP2C11* is potentially mediated by these nuclear factors.

Moreover, SULP up-regulated both *eNOS* and *iNOS* in the liver. It is well established that both of these enzymes possess a negative regulatory control on several *CYPs* (Hara and Adachi, 2002). The possible contribution of the NOS enzymes in the down-regulation of *CYP3A*, *CYP2C* and *CYP2D* should therefore, not be excluded. Finally, the SULP-induced suppression in hepatic CREB phosphorylation may contribute to the down-regulation of the afore-mentioned *CYPs*, as it is well defined that the cAMP/PKA/CREB signaling pathway controls the expression of *PXR* and *HNF1α* (Soutoglou et al., 2000; Kodama et al., 2007).

It is also possible that both GH and PRL, play a role in the down-regulation of these *CYPs* by SULP. SULP reduced plasma GH levels, followed by reduced phosphorylation of STAT5b: the major GH pulse-activated transcription factor, which is involved in the regulation of various *CYPs* in the liver (Waxman and O'Connor, 2006; Waxman and Holloway, 2009). In contrast, PRL was increased by SULP and it

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is well established that PRL possesses a down-regulating control on several *CYP* isoforms (Yamazoe et al., 1987; Fitzgerald and Dinan, 2008).

The SULP-induced perturbation of the above mentioned signaling pathways could be also a result of off-target drug effects, including the interaction of SULP with G protein coupled receptors (GPCRs), a hypothesis that likely merits further investigation (Kristiansen, 2004; Theodoropoulou et al, 2008). In conclusion, the present data indicate several possible mechanisms that could play a role in the down-regulation of the CYP3A, CYP2C and CYP2D enzymes by SULP. It is apparent that the *in vivo* SULP effect on *CYPs* is the outcome of the drug's combined effect on mainly the GH/STAT5b-, PRL-, cAMP/PKA/CREB-, PI3K/Akt/FOXO1-, JNK- and eNOS/iNOS signaling pathways. The use of the highly selective D_2-dopamine receptor antagonist L-741,626 confirmed the involvement of D_2-receptor-linked pathways in the modulation of the above mentioned signaling pathways, which mediate the down-regulation of *CYP3A*, *CYP2C* and *CYP2D*. However, more detailed studies are required to find out about their respective roles in the down-regulation.

Based on the above data and previous reports (Konstandi et al., 2008) a question arises as to what extent pharmacotherapy with D_2-dopamine receptor antagonists or agonists may influence the expression of *CYP3A, CYP2C* and *CYP2D,* and in turn, the metabolism of the majority of the prescribed drugs. The possibility that drugs acting as D_2-antagonists or agonists could modify the function of major signal transduction pathways involved in the regulation of *CYPs* with crucial importance in the metabolism of numerous prescribed drugs, toxicants and carcinogens, thus drastically affecting the outcome of pharmacotherapy, drug toxicity and carcinogenicity, leads to some very interesting prospects. For instance, several previous studies have reported a lower relative risk of cancer incidence amongst

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psychotic patients receiving drug therapy, usually including D_2-antagonists (Rassidakis et al., 1973; Rice, 1979; Craig and Lin, 1981; Mortensen, 1989). Could this fact be attributed to reduced expression of *CYP3A*, *CYP2C* and *CYP2D*? Or, should the strong SULP-mediated down-regulation of *CYP2D* be taken into account in assessing the effectiveness of pharmacotherapy and possible drug-drug interactions appearing in patients, who follow anti-depressant or anti-psychotic therapy, given the fact that the majority of these drugs are metabolized by CYP2D (Wojcikowski and Daniel, 2009)? Furthermore, it is worth noting that the present findings shade more light in the regulation of *CYP2D*, which has been considered as resistant to a direct hormonal regulation and it is known as “un-inducible” CYP.

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AUTHORSHIP CONTRIBUTIONS

Participated in research design: Konstandi.

Conducted experiments: Daskalopoulos, Konstandi, Malliou.

Contributed new reagents or analytic tools: Konstandi, Lang, Marselos.

Performed data analysis: Konstandi, Daskalopoulos.

Wrote or contributed to the writing of the manuscript: Konstandi, Lang, Daskalopoulos, Marselos.

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FOOTNOTES

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LEGENDS

Scheme 1. Insulin release from pancreatic beta-cells in response to increased plasma glucose levels is under dopaminergic control. Dopamine by stimulating D_2-dopaminergic receptors, expressed in beta-cells, restricts the release of insulin. The sulpiride-induced blockade of D_2-receptors prevents the dopamine's restricting effect on insulin release thus resulting in repression of the expression of various genes including CYPs.

Figure 1: D_2-dopaminergic mediated regulation of hepatic *CYP3A1/2*.

Assessment of the effect of the D_2-dopaminergic receptor antagonists, sulpiride (SULP) and L-741,626 on a) the enzyme activity: CYP3A1/2-catalyzed 6β-testosterone hydroxylation (pmoles/min/mg of protein) using HPLC, b) the CYP3A1/2 apoprotein level using western blot and c) the relative CYP3A1 and CYP3A2 mRNA expressions using qPCR. Comparisons were between controls (C) and drug-treated rats. Values are expressed as mean ± SE. ** $p<0.01$, *** $p<0.001$.

Figure 2: *In vitro* assessment of the role of D_2-dopaminergic and insulin/PI3K/Akt pathways in the regulation of hepatic *CYP3A1/2, CYP2C* and *CYP2D1/2*.

A. Assessment of the SULP effect on CYP3A1, CYP3A2, CYP2C11, CYP2D1 and CYP2D2 mRNA levels in primary hepatocytes. The role of the JNK-, cAMP/PKA- and PI3K-related signaling pathways in the SULP-induced *CYP* regulation was assessed using the following inhibitors before SULP: SP600125 (10 μM, 24 hours),

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H89 (10 µM, 24 hours) and wortmannin (WORT, 1µM, 24hours), respectively. **B.** Assessment of the effect of insulin (INS) on CYP3A1, CYP3A2, CYP2C11, CYP2D1 and CYP2D2 mRNA levels in primary hepatocytes [Treatment of primary hepatocytes with 1 µM INS for 24 hours, alone or in combination with the PI3K inhibitor, WORT (1 µM, 24 hours)]. Comparisons were between DMSO-(C)- and drug-treated cells. Values are expressed as mean ± SE.* $p<0.05$, ** $p<0.01$, *** $p<0.001$.

Figure 3: D_2-dopaminergic mediated regulation of hepatic *CYP2C11*.

Assessment of the effect of the D_2-dopaminergic receptor antagonists, sulpiride (SULP) and L-741,626 on a) the enzyme activity: CYP2C11-catalyzed 2α- and 16α-testosterone hydroxylation (pmoles/min/mg of protein) using HPLC b) the CYP2C11 apoprotein level using western blot and c) the relative CYP2C11 mRNA expression using qPCR. Comparisons were between controls (C) and drug-treated rats. Values are expressed as mean ± SE. *** $p<0.001$.

Figure 4: D_2-dopaminergic mediated regulation of hepatic *CYP2D1/2*.

Assessment of the effect of the D_2-dopaminergic receptor antagonists, sulpiride (SULP) and L-741,626 on a) the enzyme activity: CYP2D1/2-catalyzed 1′-bufuralol hydroxylation (Fluorescence $x10^{-6}$/min/mg protein) using HPLC b) the CYP2D1 apoprotein levels using western blot and c) the relative CYP2D1 and CYP2D2 mRNA expressions using qPCR. Comparisons were between controls (C) and drug-treated rats. Values are expressed as mean ± SE. ** $p<0.01$, *** $p<0.001$.

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Figure 5: ***In vivo*** **assessment of the effect of SULPon signal transduction.**

Western blot showing the sulpiride (SULP)-induced activation of Akt, p70S6K and inactivation of CREB and JNK in total cellular proteins (**A**). Western blot showing the nuclear (**B**) / cytosolic (**C**) STAT5b and FOXO1 activation following treatment with SULP. C, Control.

Figure 6: ***In vivo*** **assessment of the effect of L-741,626 on signal transduction.**

Western blot showing the L-741,626-induced activation of Akt and inactivation of CREB and JNK in total cellular proteins (**A**). Western blot showing the nuclear (**B**) / cytosolic (**C**) STAT5b and FOXO1 phosphorylation following treatment with L-741,626. C, Control.

Figure 7: ***In vivo*** **and** ***in vitro a*****ssessment of the role of D_2-dopaminergic receptor-linked pathways in the regulation of critical nuclear transcription factors in** ***CYP*** **regulation.**

Sulpiride (SULP) and L-741,626 (selective D_2-dopaminergic receptor antagonist) effect on CAR (**A**), PXR (**B**), RXRα (**C**), HNF1α (**D**) and HNF4α (**E**) expressions at relative mRNA level using qPCR. Comparisons were between controls (C) and drug-treated rats. For the *in vitro* assessment, primary hepatocytes were treated with DMSO (controls) or with either SULP alone or in combination with SP600125, H89 or wortmannin (WORT), inhibitors of JNK, PKA and PI3K signaling pathways, respectively. Values are expressed as mean ± SE. ** $p<0.01$, *** $p<0.001$.

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TABLES

Table 1: List of the oligonucleotide sequences used as primers for the quantitation of the gene mRNA levels using qPCR.

Gene	Primer sequence
CYP3A1	F: 5'-G G A AA T T C G A T G T G G A G T G C-3'
	R: 5'-A G G T TT G C C T TT C T C T T G C C-3'
CYP3A2	F: 5'-G T C A AA C G C C T G T G T TT G C C-3'
	R: 5'-A T C A G GG T G A G T G G C C A G G A-3'
CYP2D1	F: 5'-T G G A C C T C A G T A A C A T G C C A-3'
	R: 5'-G A T G C A A G G A T C A C A C C T T G-3'
CYP2D2	F: 5'-G G T G G A C T TT G A G A A C A T G C-3'
	R: 5'-T T G C A T C T C T G C T A G G A A G G-3'
CYP2C11	F: 5'-A G G A C A T C G G C C A A T C A A-3'
	R: 5'-G G G T A A A C T C A G A C T G C G G A-3'
CAR	F: 5'-C A G G C C T C C G G C C T A C C T G T-3'
	R: 5'-C C C T A C CC A C T C CC T G C CC C-3'
PXR	F: 5'-GAGCTCTGGGCAGAAACATC-3'
	R: 5'-A C A C G G C A G A T T T G A A G A C C-3'
RXR	F: 5´-T C A A T G G C G T C C T C A A G G T T C-3´
	R: 5´-T G T C A C G G C A G G T G T A G G T C A G-3´
HNF1α	F: 5'-G G G A A G A C T T C G C G C C A C C C-3'
	R: 5'-C C T C T C G C T G C T T G C G G A C G-3'
HNF4α	F: 5'-G C C C A A A A A C A T G C G C T G A G-3'
	R: 5'-G C A G A T G G T T G T C C T TT A G G-3'
eNOS	F: 5'-C T G C T G C CC G A G A T A T C T T C-3'
	R: 5'-C A G G T A C T G C A G T C CC T C C T-3'
iNOS	F: 5'-C G T G T G C C T G C T G C C T T C C T G C T G T-3'
	R: 5'-G T A A T C C T C A A C C T G C T C C T C A C T C-3'

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Table 2: Assessment of the SULP-induced alterations in plasma hormone and glucose levels.

	Control	Sulpiride
Insulin	0.4 ± 0.02	1.3 ± 0.18 ***
Glucose	8.0 ± 0.25	7.2 ± 0.10 *
GH	107.95 ± 2.99	29.76 ± 9.28***
T3	111.31 ± 5.80	66.86 ± 4.80***
T4	2.59 ± 0.10	1.40 ± 0.17***
TSH	1.66 ± 0.06	1.57 ± 0.08
CORT	166.3 ± 12.4	73.3 ± 9.5**
PRL	35.01 ± 5.80	177.78 ± 11.89***

*Plasma hormone levels :Insulin (pg/ml), glucose (mg/dl); GH: growth hormone (ng/ml), T3: triidothyronine (ng/dl), T4: thyroxin (µg/dl), TSH: thyroid stimulating hormone (ng/ml), CORT: corticosterone (mg/ml), PRL: prolactin (ng/ml); Values are expressed as mean ± SE (n=12), *p<0.05, **p<0.01, ***p<0.001.*

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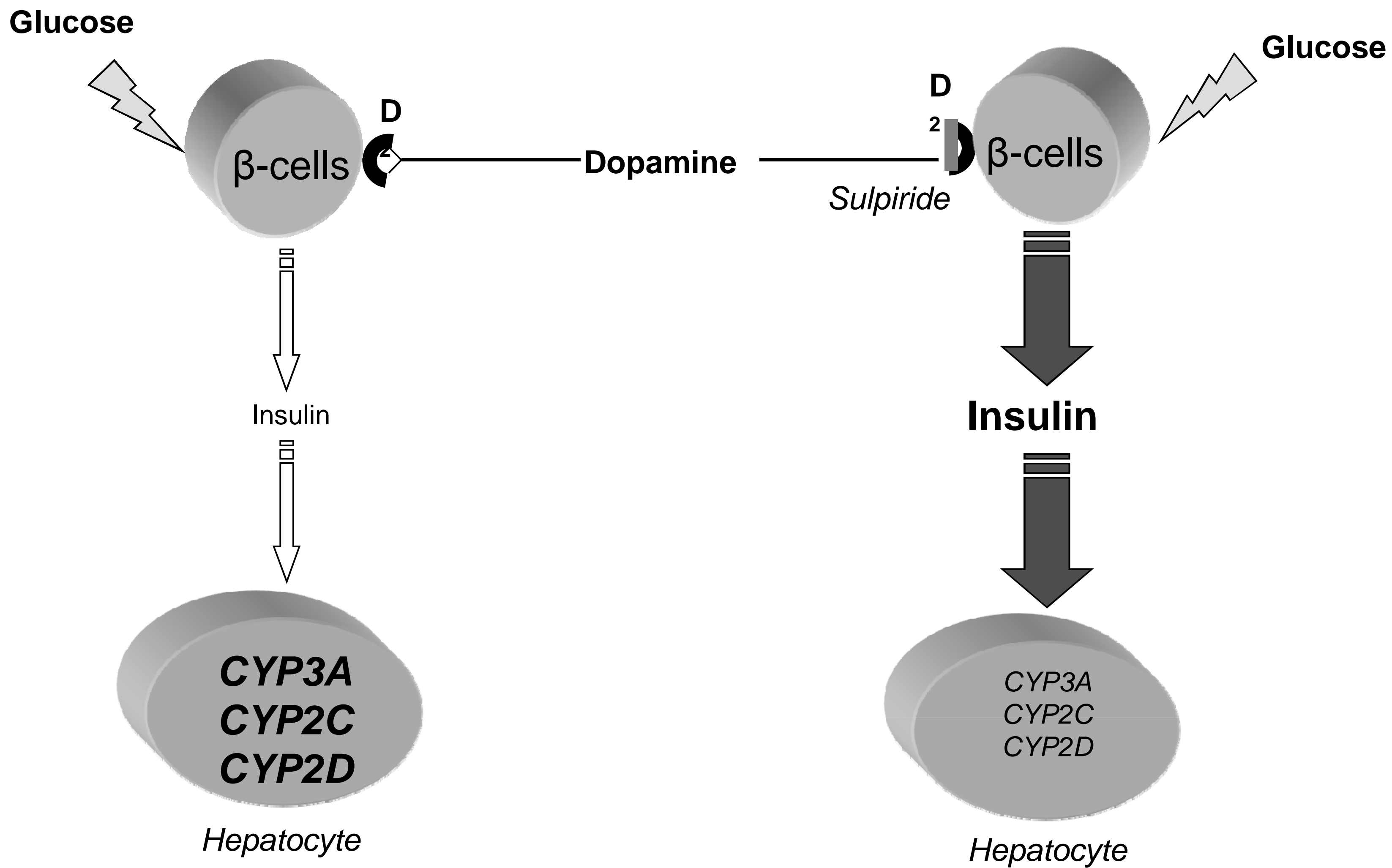

Scheme 1

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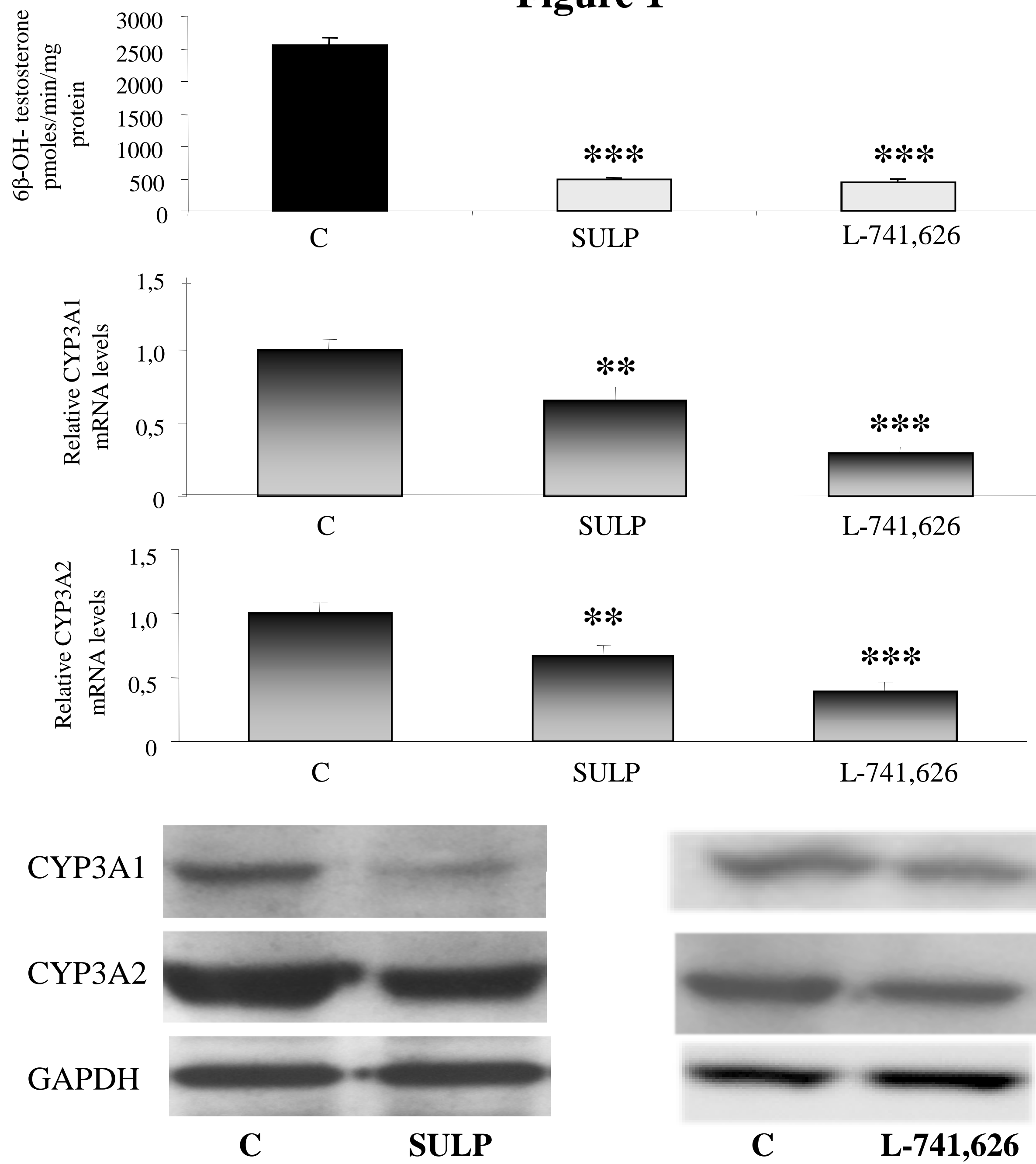

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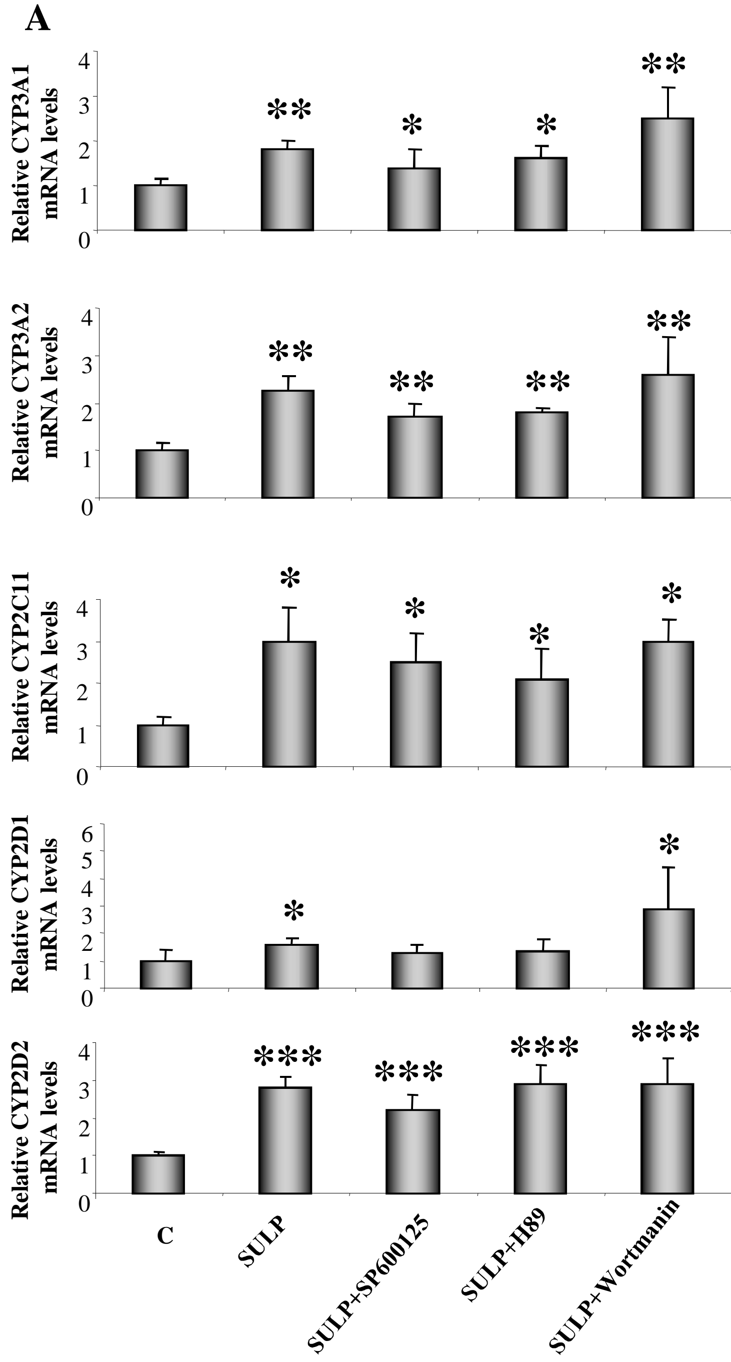
In vitro assessment

Figure 2



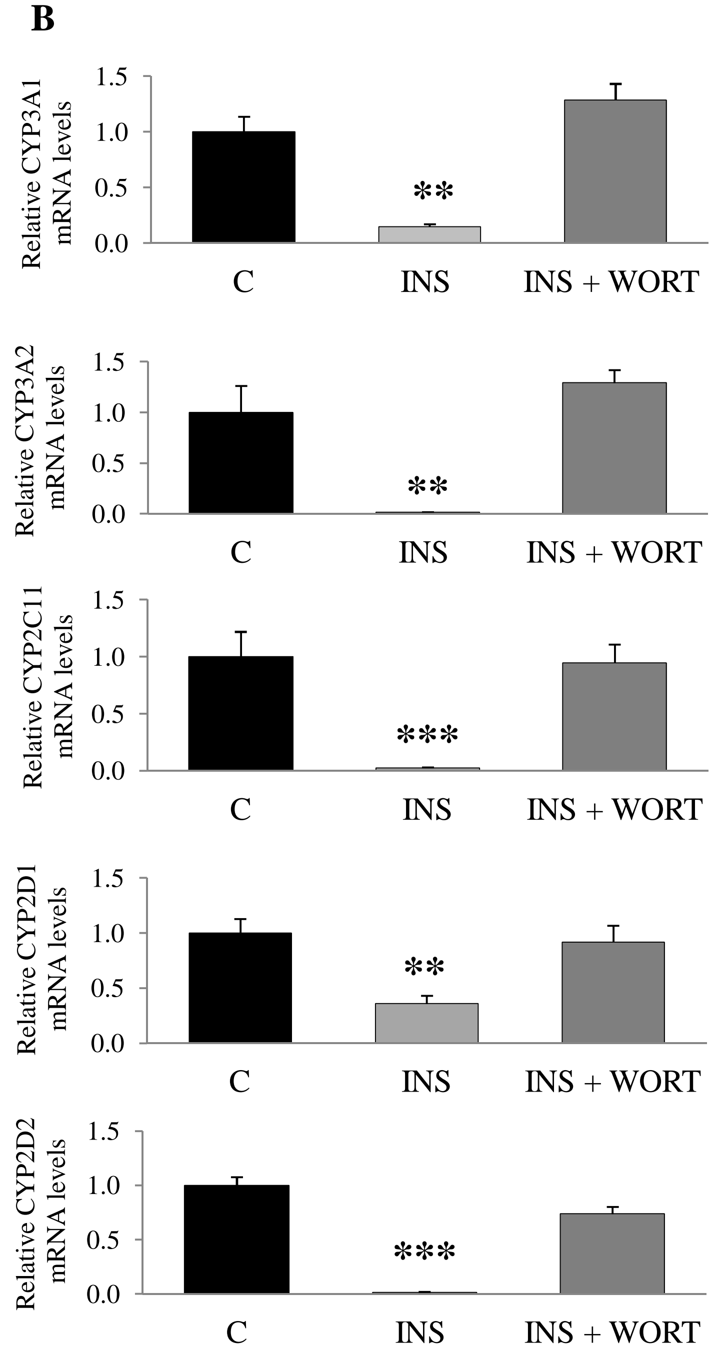


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Figure 3

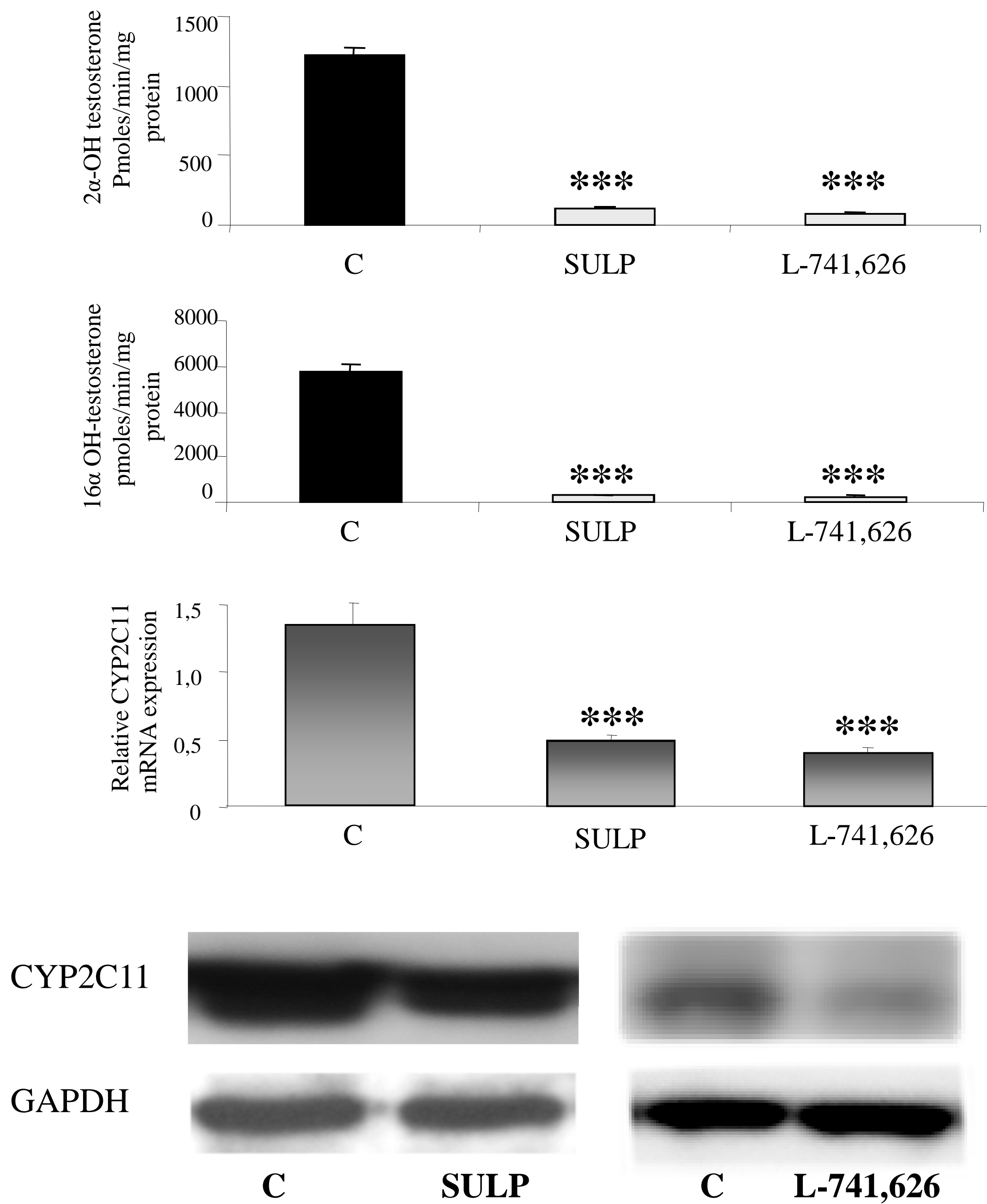

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Figure 4

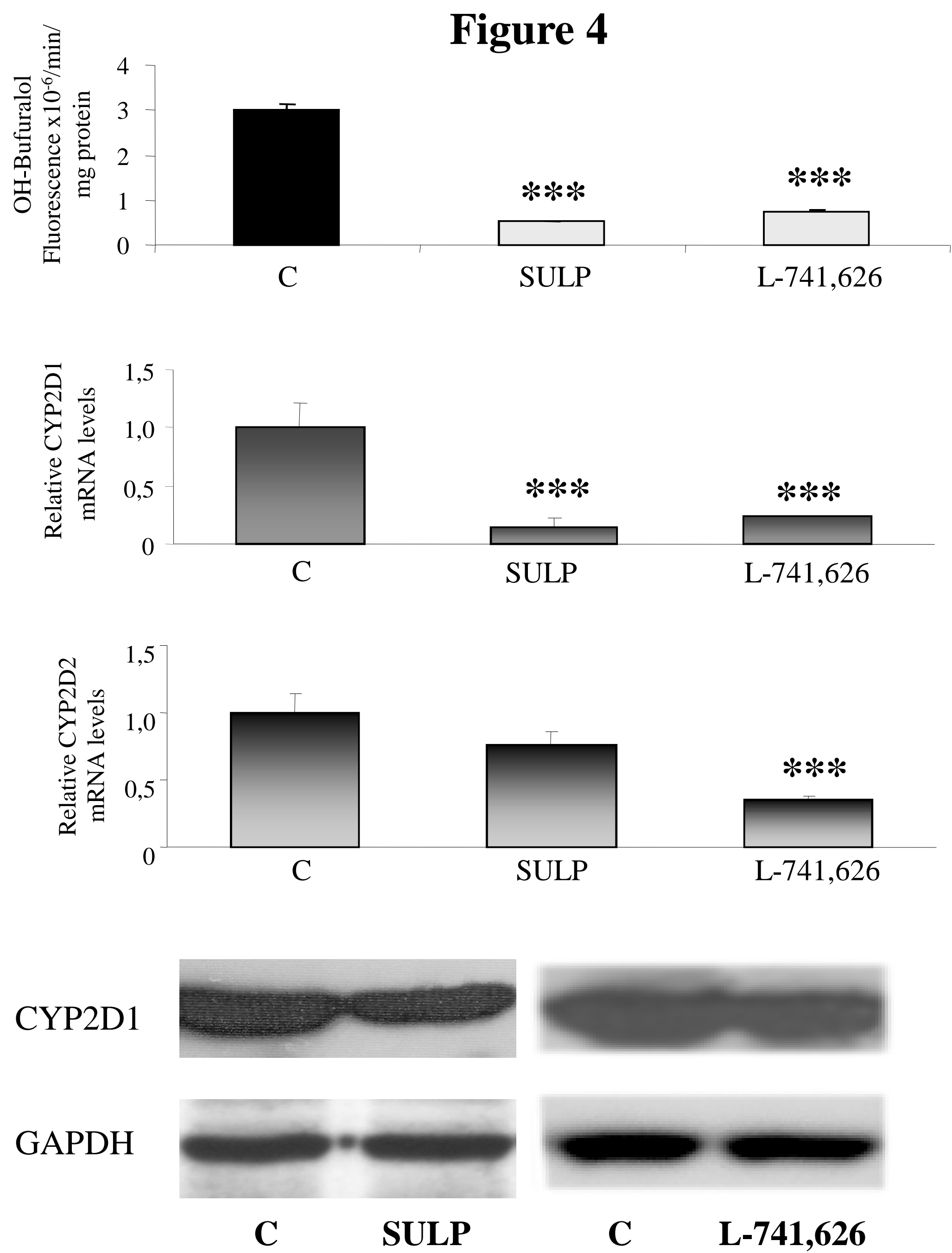

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Figure 5

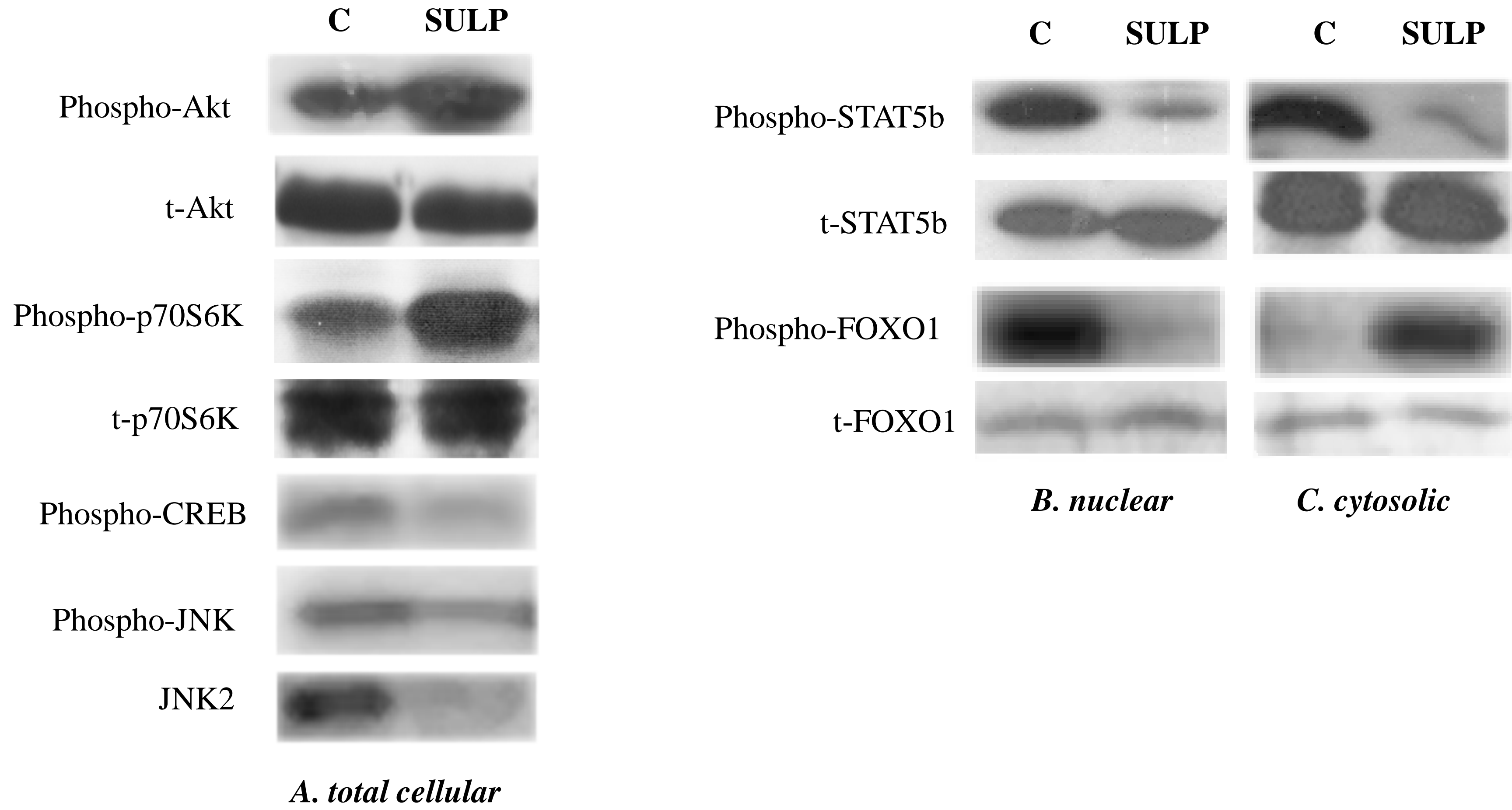

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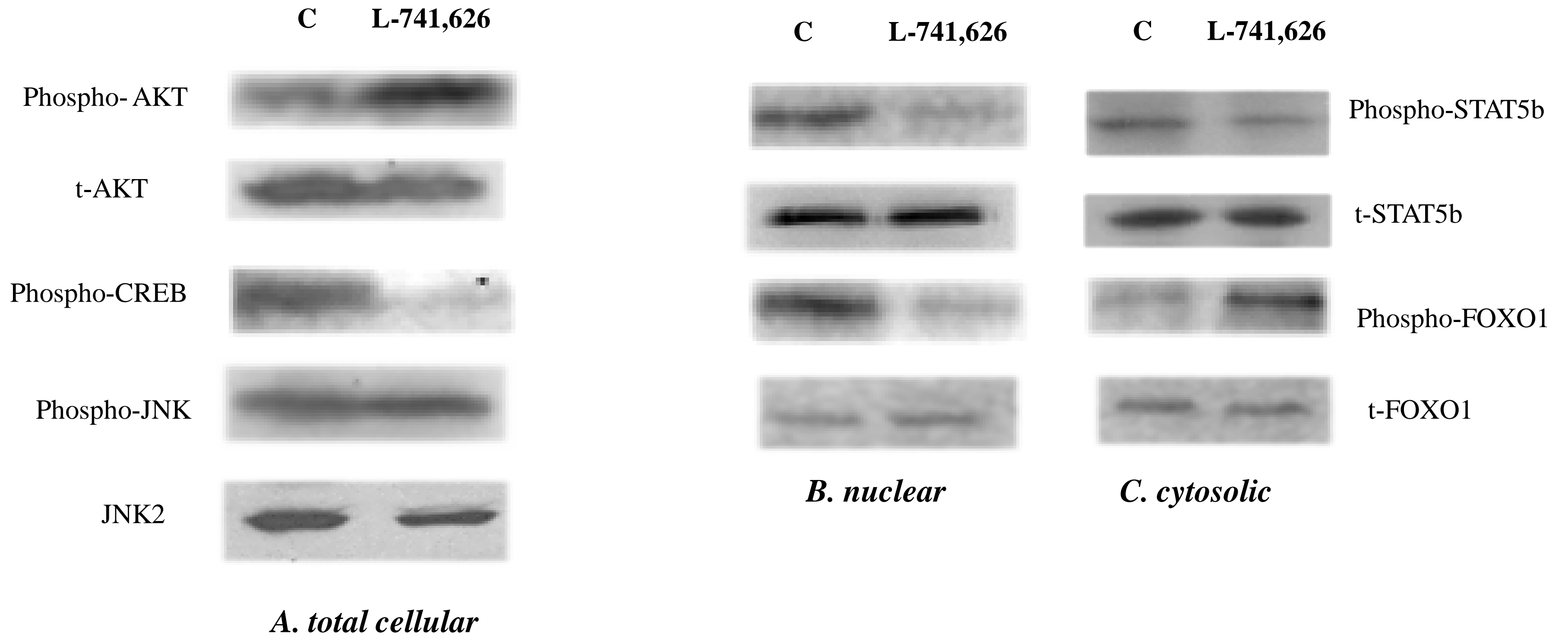
Figure 6



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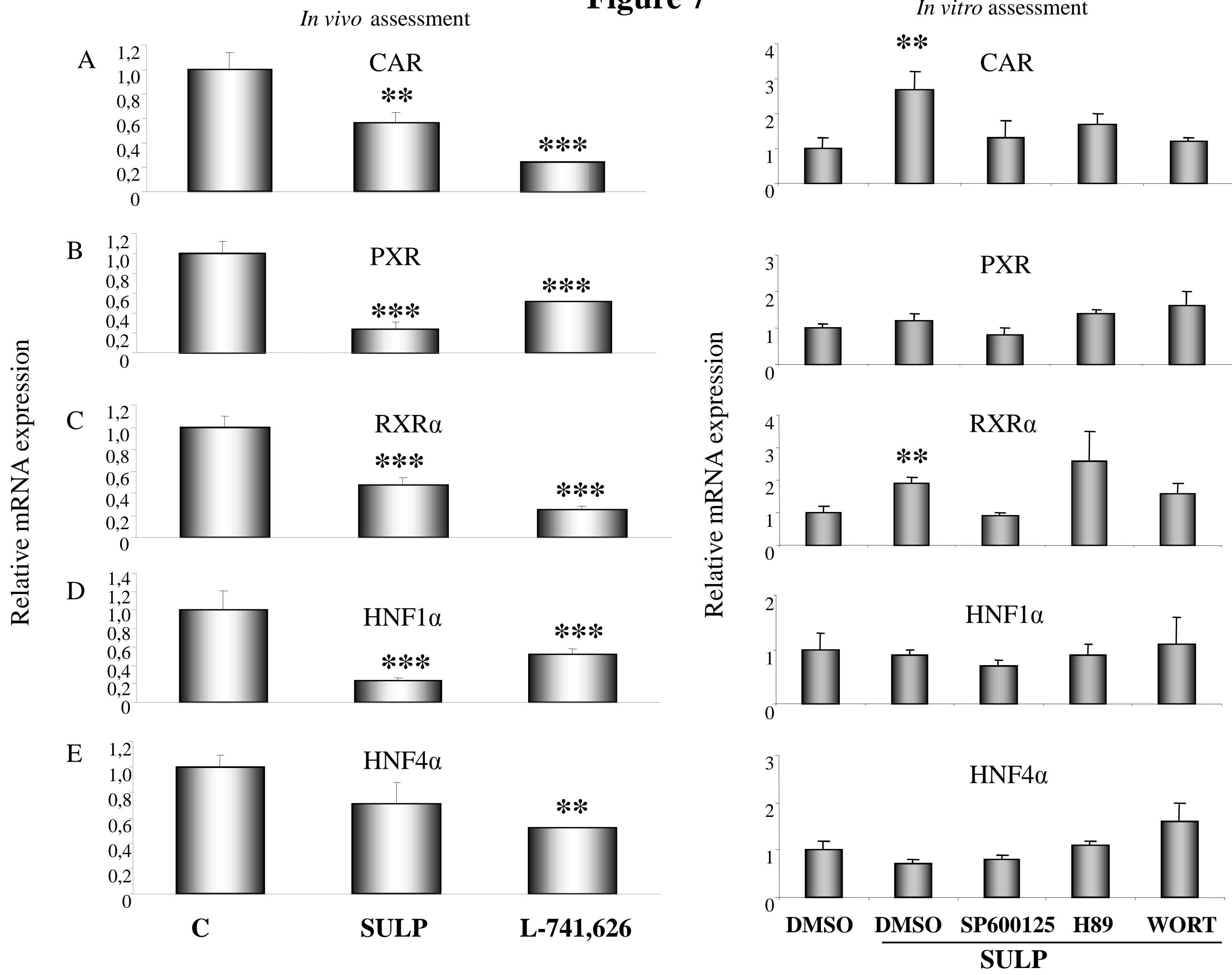

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