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HUMAN aP2 GENE PROMOTER-DRIVEN REPORTER ASSAY DISCRIMINATES NON-LIPOGENIC PPARγ LIGANDS

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HUMAN aP2 PROMOTER FUNCTIONALITY AND PPAR MODULATORS

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Non-standard abbreviations:

ABCA1: ATP-binding cassette transporter A1

aP2: adipocyte fatty acid binding protein

DMSO: dimethyl sulfoxyde

$EC_{50:}$ effective concentration 50% of maximum response

E_{max}: maximum efficacy

FABP4: fatty acid binding protein-4

HDL: high density lipoprotein

IBMX: isobutylmethylxanthine

LBD: ligand binding domain

PPARs: peroxisome proliferator-activated receptors

PPRE: peroxisome proliferator responsive element

SPPARMs: selective PPAR modulators

TZD: thiazolidinediones

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ABSTRACT

Peroxisome proliferator-activated receptors (PPARs) regulate storage and catabolism of fats and carbohydrates. PPARγ activity increases insulin sensitivity and adipocyte differentiation at the expense of adipogenesis and weight gain. The goal of this study was (i) to clone the promoter of the human aP2 gene, namely FABP4, (ii) to characterize its pharmacological regulation and (iii) determine its putative predictability for adipogenesis. Among the selected PPAR agonists, rosiglitazone and pioglitazone displayed the highest maximal efficacy (E_{max}) on reporter-gene assays in COS-7 cells co-transfected by either a GAL4-response element-based or a human aP2 promoter-based Luc reporter vector along with either chimaeric or full length hPPAR expression plasmids. The non-subtype selective GW-2331, the compounds L-165041, GW-0072 and indomethacin behaved as partial agonists relative to pioglitazone in full length human aP2-PPARγ2. Beyond their partial PPARγ agonist properties, these compounds elicited a lower maximal up-regulation of mouse aP2 mRNA in 3T3-L1 adipocytes as compared to pioglitazone; these properties paralleled a time-dependent increase in neutral lipids. By contrast, the selective PPARα agonist BM-17.0744 neither stimulated the human aP2-PPARα promoter reporter-gene assay thus demonstrating a specific interaction between PPARγ and the aP2 promoter, nor affected lipogenesis in 3T3-L1 cells. Altogether, these data characterized a functional promoter of the human aP2 gene; its *in vitro* pharmacological regulation in PPARγ-mediated reporter-gene assay may represent an interesting complement or an alternative to time-consuming procedures aiming at discriminating PPAR ligands with low lipogenic properties.

INTRODUCTION

Metabolic syndrome is characterized by the clustering of at least three risk factors among hypertension, certain types of dyslipidemia, impaired glucose tolerance and type II diabetes, and obesity. These metabolic abnormalities lead to atherosclerosis and related complications (Haffner and Taegtmeyer, 2003). The control of lipid and carbohydrate metabolism, including physiologic and pharmacological treatments, represents a valid rationale to reduce cardiovascular diseases in patients with metabolic syndrome (Wilson and Grundy, 2003; Beckman et al., 2003).

PPARs are a subclass of the nuclear receptor superfamily acting as ligand-dependent transcription factors (Kersten et al., 2000). Three subtypes were identified; the α isoform is the primary subtype expressed in liver, but also in heart and kidney, and acts as a major regulator of metabolism of fats, catabolism of fatty acids, synthesis and catabolism of lipoproteins (Barbier et al., 2002). PPARα is also involved in cholesterol efflux from peripheral tissues, and HDL reverse cholesterol-transport pathway (Chinetti et al., 2001).

The PPARγ isoform is essentially expressed in adipocytes where it stimulates lipoprotein lipase and promotes fatty acid uptake and storage in mature adipocytes. These effects, along with increased insulin sensitivity, decrease circulating free fatty acids and triglycerides (Reginato and Lazar, 1999). Like PPARα, PPARγ also influences lipid metabolism in macrophages from the arterial wall by up-regulating the expression of ABCA1 transporter which results in an increased cholesterol efflux (Chawla et al., 2001).

The functions of the ubiquitous PPARδ isoform remain quite mysterious so far, despite (i) a role in epidermal maturation and skin wound healing (Wahli, 2002), (ii) properties related to lipid metabolism as well as (iii) a putative role in fat metabolism recently reported (Oliver et al., 2001 ; Wang et al., 2003).

Thiazolidinediones (TZD) are efficacious insulin-sensitizing agents used in the treatment of type II diabetes and work through the binding and activation of PPARγ. However, both in

pharmacological and clinical use TZDs increase adiposity and body weight as well as elicit clinical side effects including edema, plasma volume expansion, hemodilution (Aleo et al., 2003; Yang et al., 2003; Schöfl and Lübben, 2003). These drawbacks stimulate the search for PPARγ modulators with modified pharmacological profiles that regulate fatty acids and glucose metabolism with a reduced increase in adiposity since this undesirable effect may favour the development of obesity, a risk factor often associated with insulin resistance (Oberfield et al., 1999). The adipocyte-specific PPARγ2 isoform contains additionally 30 amino acids at the amino terminus DNA-binding domain as compared to the PPARγ1 isoform, and is characterized by a greater constitutive transcription activation function than PPARγ1 (Werman et al., 1997). It has been hypothesized that PPARγ2 may regulate adipogenesis specifically while in the absence of ligand PPARγ1 does not (Ren et al., 2002); nevertheless the selective modulation of PPARγ1 *vs*. PPARγ2 has yet to be established and may represent a complex pharmacological challenge.

The identification of partial agonists for PPARγ represents an attractive pharmacological rationale which needs further validation to fulfil the aforementioned criteria. Regarding nuclear receptors, partial agonism can be achieved (i) either by modulating co-factor interactions (i.e., activator recruitment, repressor releasing, stoechiometry), (ii) by reducing the heterodimerization with retinoid-X-receptor, (iii) or by controlling the interaction between the PPAR DNA-binding domain and response elements in the promoter region of target genes. In the present study, this latter hypothesis was addressed and the partial efficacy for PPARγ modulators monitored in reporter gene and lipogenesis assays was related to lower neutral lipid accumulation *in vitro*. This paper also describes the cloning and functional characterization of the full-length promoter of the human homologue for aP2, namely fatty acid binding protein-4 (FABP4), which has not been described so far. Its *in vitro* regulation

may represent either an interesting complement or an alternative approach to adipocyte differentiation assays.

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METHODS

Plasmids

Chimaeric receptors containing the yeast GAL4 DNA binding domain fused to either human PPARα or PPARγ ligand binding domain were generated. A pFA-CMV plasmid (Stratagene, La Jolla, USA) containing a yeast GAL4 DNA binding domain (BD) downstream of a multiple cloning site was used as backbone vector. Human PPARα (Genbank ID: S74349) and PPARγ (Genbank ID: U63415) ligand binding domain (LBD position 619 to 1530 for PPARα, position 607 to 1518 for PPAR γ) was PCR-amplified and a *BamHI* restriction site was inserted at the 5' end of the LDB sequence and in frame with the GAL4 BD reading frame to generate chimaeric receptor genes. Each construct was controlled by automated DNA sequencing to validate the GAL4 BD-PPAR LBD chimaeric gene nucleotide sequence.

The corresponding reporter plasmid for these GAL4 chimaeric receptors (pFR-Luc, Stratagene, La Jolla, USA) contained five upstream activation sequences (UAS) of the yeast GAL4 gene promoter upstream of a canonical TATA box and adjacent to a luciferase reporter gene (pFR-Luc).

Cloning of the human full length PPARα and PPARγ2 plasmids as well as the construction of the corresponding reporter plasmid containing 3 copies of the consensus PPAR response element (PPRE3-HSV-luc) were reported previously (Wurch et al., 2002). The human PPARγ1 subtype (Genbank ID: L 40904) was PCR-amplified and cloned according to the same strategy.

Construction of the human fatty acid binding protein-4 gene promoter reporter plasmid

The promoter-reporter plasmid containing the luciferase coding sequence under the transcriptional control of a 5400 bp-long fragment having both the enhancer and core promoter elements of the human FABP4 gene promoter was obtained according to the

following strategy. A sequence coding for human FABP4 (accession number : J02874) was identified on BAC clone RP11-157I4 (accession number AC018616) of the Whitehead Institute for Genome research, this BACmid encompassed about 166,000 bp of human chromosome 8. The start codon of the FABP4 was located at position 24025 of AC018616 and the 5' end of its mRNA was mapped to 23978. By analogy to the mouse aP2 promoter (Graves et al., 1992), a 5400 bp-long genomic sequence upstream of the putative transcription start point was amplified by PCR on human genomic DNA and sense (5'-CATTCAGAAAGGAACTTTGTTTCAAATAAAAGGAGAG-3') and reverse (5'-ATTATT CTTCAAGGAGAGAAGGAAGCTGCA-3') primers and a long range polymerase mixture (Expand Long Template PCR system, Roche Applied Science). PCR products were cloned into pCR4.1 and fully sequenced on an automated DNA sequencer (ABI310 Genetic Analyzer, Applied Biosystems). Clones from independent amplifications were compared to rule out PCR errors. The 5403 bp-long promoter was further subcloned into a promoter-less pGL3Basic vector (Promega) upstream of a luciferase reporter gene.

Reporter gene assays

COS-7 cells (ATCC, CRL-1651) were seeded (12-15 x 10^3 cells par well in 96-well plates) in high glucose (4.5 g/l) Dulbecco's Modified Eagle's Medium (DMEM) containing 50 µg/ml gentamycin, 2.5 µg/ml fungizone and supplemented with 10% foetal calf serum. After 24 hours in a humidified incubator (37°C, 5% CO_2 in air), they were up to 60-80% confluence before transfection in serum-free and antibiotic-free medium by Lipofectamine plus reagent™ according to the instructions of the manufacturer. Cells were incubated for 4 hours with transfection mixtures containing 9 ng of PPAR (either wild type or chimaeric) and 37 ng of the corresponding reporter plasmid, then washed before the addition for 24 hours of fresh medium containing delipidated charcoal-stripped foetal calf serum and antibiobics. Then cells

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were treated with either compound or vehicle (0.1% dimethyl sulfoxide, DMSO) for a further 24 hours and luciferase activity was measured as previously described (Wurch et al., 2002). Cells treated with vehicle corresponded to the basal transcription level of luciferase gene which was subtracted from the ligand responses. Experimental points were best-fitted using the SigmaPlot v.4.0.1 software (four-parameter logistic equation) and EC_{50} values were deduced as the concentration of ligand which yielded 50% of its own maximal response. In case a plateau phase could not be reached due to insolubility or toxicity of the compound at high concentrations, an apparent EC_{50} value was calculated as the concentration of ligand yielding 50% of the maximal response at the highest investigated concentration.

Adipocyte differentiation assays

Murine 3T3-L1 cells (ATCC, CCL 92.1 passage 5-12) were seeded at 8-8.5 x 10^3 cells/well of 96-well plates and were grown to confluence at 37°C in 5% CO_2 in high glucose DMEM containing pyruvate, fetal calf serum 10%, gentamycin (50 µg/ml) and fungizone (2.5 µg/ml). Two days after reaching confluence, cells were incubated in the same medium containing a so-called differentiation cocktail (dexamethasone 1 µM + insulin 5 nM + isobutylmethylxanthine IBMX 0.5 mM) along with various concentrations of PPAR modulators or their vehicle (DMSO 0.1%).

For measurement of murine aP2 (maP2) mRNA, 3T3-L1 cells were maintained in the presence of the differentiation cocktail and drug treatment for 3 days. Then they were washed with PBS without Ca^{2+} and Mg^{2+}, dried and frozen at -80°C. Total RNA was isolated from murine 3T3-L1 cells using Rneasy Mini Kit according to manufacturer specification (QIAGEN, Valencia, CA, USA). Contaminating DNA in RNA preparation was removed by DNAse I treatment on-column at room temperature for 15 minutes. For reverse transcription, 500 ng of total RNA was used to generate cDNA in total volume of 20 µl. iScript (BIO-RAD, Hercules, CA, USA) was used for first-strand cDNA synthesis during 40 minutes at 42°C.

Reaction was stopped by a 5 minutes step at 85°C. Real-Time PCR was carried out on the iCycler iQ Real Time PCR Detection System (BIO-RAD) using gene specific primers and iQ SYBR green Supermix (BIO-RAD). The sequences of the primers are as follows: maP2 (NM_024406) forward 5'-GGG CGT GGA ATT CGA TGA AAT CA-3'; maP2 (NM_024406) reverse 5'-CCC GCC ATC TAG GGT TAT GAT-3'; m36B4 (NM_007475) forward 5'-GGA CCC GAG AAG ACC TCC TT-3' and m36B4 (NM_007475) reverse 5'-AAT GGT GCC TCT GGA GAT TTT CG-3'. The PCR reactions were performed in a final volume of 25 µl as follows: 5 minutes at 95°C to activate "hot start" enzyme and 40 cycles at 94°C for 30 seconds, 60°C for 30 seconds and 72°C for 30 seconds; followed by a melt curve from 65°C to 95°C (0.5°C every 10 seconds) to determinate specific temperature melting of each amplicon. m36B4 was used as internal reference for normalization of maP2 relative quantifications.

To examine lipid accumulation during adipogenesis, 3T3-L1 cells were maintained in the presence of the differentiation cocktail and drug treatment for 7 days without medium exchange. Then, cells were washed gently with PBS in the presence of Ca^{2+} and Mg^{2+}, before the addition of 5 µl Adipored reagent (Bio Whittaker Inc, Walkersville, USA) per well.

Adipored is a solution of Nile red whose maximum emission of the fluorescent signal (λ_{ex} = 485 nm, λ_{em} = 572 nm) depends on the hydrophobic environment, especially when the stain was partitioned in triglyceride droplets.

Statistical analysis

All data were expressed as mean ± S.E.M. Statistical significance (for results in Table 2 and 3) was determined with Dunnett multiple comparison procedure applied after a one-way analysis of variance (ANOVA) conducted on data (*P* value of less than 0.05 was considered significant).

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Materials

All molecular biology reagents were either from Invitrogen (Carlsbad, USA), Clontech (Palo Alto, USA), Roche Applied Science (Indianapolis, USA), Stratagene (La Jolla, USA), Promega (Charbonnieres, France) or Applied Biosystems (Forster City, USA). Cells were obtained from ATCC (Rockville, USA). Dulbecco's modified Eagle's medium, phosphate-buffered saline, gentamycin, fungizone and Lipofectamine plus reagent were purchased from Invitrogen–Life Technology (Cergy, France); delipidated and charcoal-stripped foetal calf serum was from Hyclone (Erembodegem, Belgium). Luminescence was measured by using a luciferase assay system from Promega (Charbonnieres, France). Triglyceride accumulation was quantified by using Adipored assay reagent (Biowhittaker, Walkersville, USA). Rosiglitazone, 2,2-dichloro-12-(4-chlorophenyl)dodecanoic acid (BM-17.0744), 2-(4-[2-(3-[2,4-difluorophenyl]-1-heptylureido)ethyl]phenoxy)-2-methyl-butyric acid (GW-2331), 4-(4-((2S, 5S)-5-(2-(bis(phenylmethyl)amino)-2-oxoethyl)-2-heptyl-4-oxo-3-thiazolidinyl)butyl)-benzoic acid (GW-0072), 5-(2,4-dioxothiazolidin-5-ylmethyl)-2-methoxy-N-(4-trifluoromethyl-benzyl)-benzamide (KRP-297) and [4-[3-(4-acetyl-3-hydroxy-2-propylphenoxy)-propoxyl]phenoxy]-acetic acid (L-165041) were synthesized by a Medicinal Chemistry Division of Pierre Fabre Research Center. Pioglitazone was purified from the medicine Actos (Takeda Pharmaceuticals, Japan). Fenofibrate, indomethacin and diclofenac sodium were obtained from Sigma (St. Quentin, France). All compounds were dissolved in DMSO and successive dilutions were made in order to obtain a final maximal DMSO concentration of 0.1%.

RESULTS

Homologous cloning of the human fatty acid binding protein 4 gene promoter

The promoter of the mouse aP2 gene has been identified as containing several several cis and trans acting elements (Graves et al., 1992) as well as PPAR response elements (Elbrecht et al., 1996). The human ortholog was cloned by homologous cloning: a gene encoding human acidic fatty acid-binding protein (hFABP4, or aP2) has been retrieved from the public databases (accession number J02874, Baxa et al., 1989), and it contained, besides the entire coding sequence, 63-bp of the 5' un-translated region. The entire sequence J02874 was used in a BLAST search against all publicly available human sequences. A high BLAST score (398 bits, E-value: $1.0\ 10^{-108}$) was obtained for entry AC018616, a BACmid of human chromosome 8 containing clone RP11-157I4 (Whitehead Center for human genome research). This location is in accordance with the chromosomal mapping of hFABP4 to position 8q21 (Prinsen et al., 1997). Four exons of haP2 were localized within the AC018616 sequence (Table 1). The exon/intron junctions corresponded to those previously identified for the mouse aP2 gene (Phillips et al., 1986). By analogy to the 5400 bp long aP2 promoter identified in mice (Graves et al., 1992), an equivalent human sequence was PCR-amplified from human genomic DNA and its promoter activity was evaluated in a luciferase reporter gene assay. Four nucleotide modifications were observed in the herein presented promoter sequence (deposited to the EMBL database as AJ627200) as compared to the AC018616 entry (Figure 1) : an AA doublet (position 3808 in AJ627200) within a poly-A stretch of 13 adenosine residues and a CA doublet (position 5030 in AJ627200) located within a CA-di-nucleotide stretch were missing.

Characterization of PPAR modulators in GAL4-reporter gene assays

In order to compare the efficacy of PPAR agonists, a selection of ligands or their vehicle (DMSO 0.1%) was incubated with COS-7 cells co-expressing chimaeric receptors formed by

the GAL4 DNA-binding domain and a human PPAR-ligand binding domain along with a GAL4-responsive reporter plasmid. Rosiglitazone transactivated PPARγ with a 8-fold higher potency than pioglitazone and both behaved as rather selective PPARγ full agonists in spite of a significant transactivation of GAL4:PPARα by pioglitazone at the highest concentration tested (100 μM). The well-known fenofibric acid and the novel compound BM-17.7044 induced a selective transactivation mediated by GAL4:PPARα with apparent potencies in the high micromolar range for both agents and a slightly lower efficacy for BM-17.0744 relative to fenofibric acid (Table 2). While the non-subtype selective PPARαγ ligands GW-2331 and KRP-297 elicited a maximal PPARα transactivation comparable to that of fenofibric acid, they displayed partial agonist properties in GAL4:PPARγ reporter-gene assays regarding both TZDs. The EC_{50} for the compound L-165041 were 211±66 (obtained in a GAL4:PPARδ specific set of experiments) and 8507±2836 nM for GAL4:PPARδ and GAL4:PPARγ chimeric proteins, respectively; it displayed a partial efficacy (65±11%) for GAL4:PPARγ, and remained inactive for GAL4:PPARα (Table 2). The three last compounds tested, namely GW-0072, indomethacin and diclofenac, behaved as a subtype-selective PPARγ agonist with a partial efficacy between 35-50% relative to pioglitazone. The evaluation of the nonsteroidal anti-inflammatory drugs (NSAIDs) indomethacin and diclofenac on GAL4:PPAR reporter gene assays was based on their previously reported low affinity for the γ isoform (Lehmann et al., 1997; Adamson et al., 2002).

Pharmacological characterization of the human aP2 promoter

COS-7 cells were transiently co-transfected with mammalian expression vectors containing either full-length human PPARα, PPARγ1 or PPARγ2 along with the human aP2-Luc plasmid. All selected compounds which displayed substantial PPARγ activating properties in the GAL4 reporter-gene assay elicited a concentration-dependant increase in luciferase

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expression/activity under the control of the aP2 promoter, whatever PPARγ1 (Figure 2, A) or PPARγ2 (Figure 2, B) co-expressed. In accordance with data obtained in GAL4 reporter gene assays GW-0072 and indomethacin displayed also a low efficacy in this human aP2 gene promoter driven reporter assay whereas diclofenac remained ineffective. The PPARα ligands fenofibric acid and BM-17.0744 remained inefficient not only in the full length PPARγ-aP2 reporter gene assays which was in accordance with their PPAR-subtype binding selectivity, but were inactive also in the PPARα-aP2 reporter gene assay thus demonstrating the specificity of the interaction between the PPARγ DNA-binding domains and this functional promoter aP2 (Figure 2 and Table 3). In contrast to previous results describing the activation of a common 'consensus' PPRE by all full length PPAR subtypes (Wurch et al., 2002), the present data suggest that the 5400 bp fragment upstream of the putative transcription start of the human FABP4 mRNA can be used in a reporter gene assay selective for both PPARγ1 and PPARγ2 but not PPARα. Moreover, besides the TZD rosiglitazone, pioglitazone and KRP-297 which induced a maximal receptor activation, the maximal efficacy achieved by the non-subtype selective agent GW-2331 relative to rosiglitazone was about 85% and 99% respectively for PPARγ1 and PPARγ2. L-165041 elicited also a concentration-dependent increase in luciferase activity with a maximal activity comparable to that of rosiglitazone, but with a lower potency (Table 3). No significant difference between PPARγ1 and PPARγ2 was detected with L-165041.

In order to further define the putative relationship between the activation profiles of the selected compounds on chimaeric *vs*. full length PPARγ proteins, aP2-dependent reporter gene assays were performed in COS-7 cells which were treated for 24 hours simultaneously with a constant and maximally effective concentration of rosiglitazone (100 nM) and increasing concentrations of L-165041 up to 30 μM. This agent reduced rosiglitazone-stimulated PPARγ transcriptional activity in a concentration-dependent manner, and to a

larger extent in PPARγ2 cells (41±4% reduction in PPARγ1; 48±9% reduction in PPARγ2); nevertheless the efficacy between rosiglitazone and L-165041 was almost comparable during this head-to-head comparison. Hence, the well-known PPARγ partial agonist GW-0072 was investigated (PPARγ1, Figure 3A) or (PPARγ2, Figure 3C) and it elicited a concentration-dependent inhibition of rosiglitazone-mediated luciferase expression in this aP2 promoter controlled cell-based assay (Figure 3B and 3D for respectively PPARγ1 and PPARγ2).

Beyond PPARγ reporter gene assays, so-called 'physiological experiments' on adipocyte differentiation were carried out to determine whether a relationship between these *in vitro* models may exist, at least for this PPAR modulator selection.

Effects of PPARs on 3T3-L1 adipocytes

Confluent murine 3T3-L1 preadipocytes differentiated into adipocytes upon treatment with dexamethasone, insulin and IBMX. Adipogenesis was stimulated in a concentration-dependant manner by rosiglitazone, pioglitazone, GW-2331 and L-165041, with regard to the up-regulation of maP2 mRNA after a 3-day treatment period; the threshold concentration for these compounds were 0.1, and 1 μM respectively for TZD and non-TZD agents**.** By contrast the selective PPARα activator BM-17.0744 remained ineffective as well as indomethacin and diclofenac. A full range of apparent intrinsic activities was observed with the highest concentration tested of ligands which displayed some PPARγ agonist properties (Figure 4, A). The level of maP2 mRNA appeared to be related with the development of the adipocyte phenotype and it was used as a rather early and sensitive marker of adipogenesis in the present study.

Nevertheless since many other genes and regulations may play key roles in this process, the accumulation of neutral lipid content was monitored in longer experiments (7 days) using Adipored reagent (Figure 4, B). Rosiglitazone and pioglitazone elicited the largest-dependent

lipid accumulation. All other compounds except BM-17.0744 were able to induce a concentration-dependent lipid accumulation, albeit with a different efficiency because of less adipogenic potential than TZDs (Figure 4, B). During the course of this assay, the highest concentration of BM-17.0744 (10 μM) did not appear to inhibit differentiation but lipid accumulation, if any, was marginally increased (Figure 4, B).

Photomicrographs illustrated the rosiglitazone-dependent accumulation of intracellular lipids (Figure 5, C and D), as monitored by Adipored staining. By contrast, BM-17.0744-treated 3T3-L1 cells (Figure 5, B) remained comparable to differentiated control cells (Figure 5, A).

DISCUSSION

This report presents the cloning strategy of the human adipocyte fatty-acid-binding protein (or aP2) gene promoter and its functional characterization based on human PPAR reporter-gene pharmacological assays. A key result of this study appears to be the selective activation of this promoter by liganded PPARγ of both PPARγ1 or PPARγ2 isoforms, since PPARα remained unable to mediate any aP2-driven reporter gene expression upon stimulation by either a selective PPARα agonist (BM-17.0744) or a dual PPARαγ agonist (KRP-297). These data are in accordance with the properties of PPARγ agonists on adipocyte differentiation and the up-regulation of murine aP2 both *in vitro* and *in vivo* (Lehmann et al., 1995; Henke et al., 1998). The lack of effect PPARα was not related to technical issues since a positive transactivation signal was obtained with both BM-17.0744 and KRP-297 when COS-7 cells were transiently co-transfected with full length PPARα and consensus $PPRE_{3x}$-HSV-Luc expression vectors (data not shown); positive non-subtype selective interactions between ligand-bound PPAR (α for instance) were also previously shown in reporter gene assays conducted with the same expression vectors but in HepG2 recipient cells (Wurch et al., 2002). Hence, besides (i) the tissue distribution of the various PPAR isoforms, (ii) the specific physiological programs relative to a tissue, (iii) the availability of cofactors required for ligand-dependent transcription factor activity and (iv) the affinity of ligands for PPAR ligand binding domains, one can speculate that the specific interaction between PPAR-DNA binding domain and responsive element(s) is of key relevance for the regulation of certain signalling pathways.

The selection of various PPAR modulators was made according to their reported potency and efficacy on the different human PPAR subtypes. Nevertheless their respective pharmacological properties were established under our experimental conditions with chimaeric GAL4:PPARs reporter-gene assays. A rather wide range for GAL4:PPARγ activation was observed with rosiglitazone and pioglitazone (full agonists), KRP-297, GW-

2331 and L-165041 (non-subtype selective agonists), GW-0072, indomethacin and diclofenac (partial PPARγ agonists), BM-17.0744 and fenofibric acid (almost inactive), in accordance with previous results (Willson et al., 2000; Lehmann et al., 1997; Adamson et al., 2002; Oberfield et al., 1999). Although being artificial, GAL4:PPAR ligand binding domain-related assays are often used to further characterize the agonist efficacy, but their putative relationship in a comparable model with the wild-type receptors and full promoters activities is sometimes lacking (Berger et al., 2003) or not related to the corresponding physiological function, namely adipogenesis (Camp et al., 2000; Reginato et al., 1998). Hence, the cloning of the human adipocyte fatty-acid-binding protein gene promoter was performed by homologous cloning, starting from the coding sequence of the human aP2 gene and retrieving a sequence with similar genomic organization as that described for the mouse aP2 gene (Phillips et al., 1986). Genomic location on human chromosome 8, the homologous of mouse chromosome 3, containing the aP2 gene (Heuckeroth et al., 1987), a 64 nt-long 5' un-translated region and exon/intron junctions similar to those described for the mouse aP2 gene (Phillips et al., 1986) were identified. The functionality of this putative promoter sequence was assayed upon fusion upstream to a luciferase reporter gene.

Data obtained from both chimaeric and full-length protein reporter gene assays suggest that a discrepancy may exist regarding the efficacy of so-called partial agonists for PPARγ (i.e., L-165041 and diclofenac for example). The former elicited a maximal response comparable to that of rosiglitazone in wild-type/human aP2 reporter gene assays although it is partial with PPARγ-ligand binding domain fused to an heterologous GAL4 DNA binding domain, as it was also reported for another compound MCC-555 (Reginato et al., 1998); the latter behaved as a partial agonist in the chimaeric assay whereas it was silent in the PPARγ full length/human aP2 assay. These data fit with those reported by Adamson et al. (2002) showing that diclofenac antagonizes PPARγ full-length reporter gene assay and suggest that apparent efficacy differs for one pharmacological model to another. As a consequence, since inter-

domain relationship in PPAR is likely crucial for its own activation, it appears quite difficult to characterize the so-called 'intrinsic efficacy' of a PPAR ligand which can also vary upon co-factor recruitment. Nevertheless data related to other PPARγ agonists (TZDs, GW-0072 and indomethacin) suggest a coherence between GAL4 and wild type/human aP2 procedure. The relevance of this model (reporter gene assay controlled by the human aP2 promoter) for characterizing the putative antagonistic properties of a PPARγ partial agonist is strengthened by the results obtained with GW-0072 added simultaneously with rosiglitazone even if an antagonistic effect down to its partial intrinsic efficacy could not be achieved in our experimental conditions. GW-0072 was previously characterized with chimaeric PPAR proteins as a partial agonist/antagonist for rosiglitazone (Oberfield et al., 1999).

In spite of slight differences in the efficacy of GW-2331 for instance (85±5% and 99±5% for PPARγ1 and PPARγ2, respectively), one cannot speculate upon the putative selectivity of a ligand toward PPARγ1 relative to PPARγ2, and no clear-cut interpretation can be drawn regarding the adipogenic potential of a compound relying only on the presence of additional 30 amino acids at the amino terminus DNA-binding domain of PPARγ2 which are moreover located outside the ligand binding domain. Even if PPARγ2 activation was proposed to play a unique role in adipogenesis (Ren et al., 2002), it was further demonstrated that PPARγ1 can also drive the differentiation of fat cells albeit with a lesser sensitivity, depending on both isoform expression level and ligand concentration (Mueller et al., 2002).

Not only a peculiar distribution of a compound but also a specific interaction profile with cofactors may modulate PPAR activation and putatively its adipogenic effect (Berger et al., 2003). The *in vitro* physiological significance of data from reporter gene assays was addressed by studying the adipogenic potential of some PPAR modulators in 3T3-L1 adipocytes, a well-documented albeit murine model. GW-2331 and L-165041 showed a less potent and efficacious up-regulation of maP2 mRNA than rosiglitazone and pioglitazone after

3 days of treatment which led to a lower accumulation of intracellular neutral lipids after 7 days. GW-0072 and the NSAIDs indomethacin and diclofenac behaved as low lipogenic compounds as demonstrated in aP2 mRNA and lipid accumulation experiments. The adipogenic properties of these compounds were only observed at the highest concentration (efficacy) and remained quite marginal at lower concentrations. As foreseeable, a treatment by the PPARα agonist BM-17.0744 resulted in a low increase in maP2 mRNA as compared to the control situation which was associated to a very low level of neutral lipids.

Altogether these data suggest that a positive relationship can exist between these *in vitro* pharmacological and physiological assays in spite of some specific compound behaviours; even if not totally parallel, reporter gene assays constitute a more rapid and sensitive procedure than adipocyte differentiation to identify agent with low adipogenic properties. These results on the functionality and specificity of the human aP2 promoter represent an interesting issue for addressing part of the lipogenesis signalling pathway and warrants future investigations, at least to determine the nature of DNA binding domain/promoter interactions for instance. Efficacy but also potency in full length PPAR/aP2 reporter gene assays constitute 'key' predictive parameters and subtle differences between compounds within a common chemical series should be tested to further strengthen the validity of the model. Other target genes for PPARγ, such as resistin (Way et al., 2001) adiponectin (Maeda et al., 2001) or aquaporin (Kishida et al., 2001), could also be evaluated following a comparable strategy to characterize, at least *in vitro* and with a larger predictability, attractive selective PPAR modulators (SPPARMs).

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LEGENDS FOR FIGURES

Figure 1: Sequence divergences between AJ627200 (haP2 promoter) and AC018616 (chromosome 8 clone RP11-157I4) sequences. The initiator codon ATG of the aP2 coding sequence is located at position 24025 in AC018616, the putative transcription start is at position 23978 in AC018616. The cloned haP2 promoter corresponds to position 18571 to 23977 (respectively 1 to 5403 of AJ627200).

Figure 2: Modulation of transcriptional activity of full length human PPARγ1 (A) and PPARγ2 (B) by various PPAR agonists. The transcription of the human aP2-driven reporter luciferase activity was measured. Results are expressed in percentage of stimulation *vs.* control (DMSO 0.1%) and are mean ± SEM values of at least three separate experiments, each experimental point performed in triplicate.

Figure 3: Effects of rosiglitazone and GW-0072 on full length human PPARγ1 (A, B) and PPARγ2 (C, D) transcriptional activity. Modulation of rosiglitazone (100 nM)-mediated PPAR activation by increasing concentrations of GW-0072 (B, D). The transcription of the human aP2-driven reporter luciferase activity was measured. Results are expressed in percentage of stimulation *vs.* control (DMSO 0.1%) and are mean ± SEM values of at least three separate experiments, each experimental point performed in triplicate.

Figure 4: Effects of PPAR modulators on markers of adipogenesis in murine 3T3-L1 adipocytes. (A) Measurement of aP2 mRNA after a 3-day treatment period by various agents at 10 µM (except for NSAIDs indomethacin and diclofenac which were added at 100 µM). Results from one representative experiment among three separate assays are expressed by the ratio of target gene mRNA over housekeeping gene (36B4) mRNA, each experimental point performed in triplicate. (B) Evaluation of intracellular triglycerides after a treatment for 7 days by various agents at 10 µM (except for NSAIDs indomethacin and diclofenac which were added at 100 µM). Results are expressed in fold induction *vs.* vehicle (DMSO 0.1%) and are mean $\pm$ SEM values of at least three separate experiments, each experimental point performed in triplicate.

Figure 5: Adipored staining of 3T3-L1 cells incubated for 7 days with DMSO 0.1% (A), BM-17.0744 (10 µM, B) or rosiglitazone (0.1 µM, C and 10 µM, D).

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Table 1: Computer-based localization of exon sequences within the human aP2 coding sequence as deduced from the AC018616 sequence. Nucleotide positions are based on the numbering of the AC018616 sequence. Amino acid (AA) sequence is deduced from the full-length human aP2 coding sequence (accession number J02874)

Exon		**Beginning**	**End**
1	Position AA	< 24025 MCD...	240096 ...KEN
2	Position AA	26593 GVG...	26766 ...KVK
3	Position AA	27677 STI...	27778 ...LVV
4	Position AA	28277 ECV...	> 28327 ...RA*

Table 2: Apparent EC_{50} and E_{max} values of transactivation responses for selected PPAR modulators at GAL4:PPAR chimaeric proteins. COS-7 cells were transiently transfected with either GAL4:PPARα or GAL4:PPARγ vectors along with pFR-Luc reporter plasmid as described in Materials and Methods. E_{max} (%) values were calculated for each independent experiment and expressed relative to the apparent maximal luciferase value activity obtained with fenofibric acid and pioglitazone respectively for α and γ isoforms. nd: not determined; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (*vs*. fenofibric acid for PPARα; *vs*. pioglitazone for PPARγ)

	GAL4:PPAR / pFR-Luc			
GAL4:PPAR	**α**		**γ**	
Ligand	**EC_{50} (nM)**	**E_{max} (%)**	**EC_{50} (nM)**	**E_{max} (%)**
PIOGLITAZONE	**nd**	**104 ± 3**	***9000 ± 1770***	***100***
ROSIGLITAZONE	**nd**	**inactive**	**1115 ± 242**	**95 ± 23**
KRP-297	**3070 ± 1030*****	**107 ± 14**	**1180 ± 400**	**54 ± 13**
GW-2331	**117 ± 37*****	**93 ± 8**	**1816 ± 342**	**81 ± 16**
BM-17.0744	**4550 ± 1670*****	**87 ± 8**	**> 100000**	**27 ± 2****
FENOFIBRIC A.	***16900 ± 1000***	***100***	**65200 ± 4800*****	**29 ± 1***
L-165041	**> 10000**	**inactive**	**8507 ± 2836**	**65 ± 11**
GW-0072	**nd**	**inactive**	**690 ± 11**	**35 ± 1***
INDOMETHACIN	**nd**	**inactive**	**15000 ± 2200**	**50 ± 7**
DICLOFENAC	**nd**	**inactive**	**13200 ± 5500**	**39 ± 4***

Table 3: Apparent EC_{50} and E_{max} values of transactivation responses for selected PPAR modulators at human full length PPARs. COS-7 cells were transiently transfected with either PPARα- or PPARγ1- or PPARγ2- vectors along with the reporter plasmid containing the luciferase sequence under the control of human aP2 promoter as described in Materials and Methods. E_{max} (%) values were calculated for each independent experiment and expressed relative to the apparent maximal luciferase value activity obtained with rosiglitazone. nd: not determined; *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$ (*vs*. fenofibric acid for PPARα; *vs.* rosiglitazone for PPARγ)

	h PPAR / h aP2-Luc					
Full length hPPAR	**α**		**γ1**		**γ2**	
Ligand	**EC_{50} (nM)**	**E_{max} (%)**	**EC_{50} (nM)**	**E_{max} (%)**	**EC_{50} (nM)**	**E_{max} (%)**
ROSIGLITAZONE	**> 10000**	**inactive**	***21 ± 3***	***100***	***32 ± 2***	***100***
PIOGLITAZONE	**> 10000**	**inactive**	**171 ± 15**	**107 ± 7**	**173 ± 29**	**125 ± 9***
KRP-297	**> 10000**	**inactive**	**168 ± 15**	**96 ± 5**	**183 ± 8**	**111 ± 10**
GW-2331	**nd**	**inactive**	**153 ± 22**	**85 ± 5**	**132 ± 28**	**99 ± 5**
BM-17.0744	**> 10000**	**inactive**	**> 10000**	**inactive**	**> 10000**	**inactive**
FENOFIBRIC A.	**nd**	**inactive**	**> 1000000**	**inactive**	**> 1000000**	**inactive**
L-165041	**nd**	**inactive**	**2500 ± 240****	**85 ± 12**	**2650 ± 220*****	**88 ± 2**
GW-0072	**nd**	**inactive**	**839 ± 361**	**25 ± 1*****	**215 ± 1**	**27 ± 1*****
INDOMETHACIN	**nd**	**inactive**	**18500 ± 600*****	**45 ± 2***	**12100 ± 200*****	**49 ± 7****
DICLOFENAC	**nd**	**inactive**	**nd**	**inactive**	**nd**	**inactive**

Figure 1:

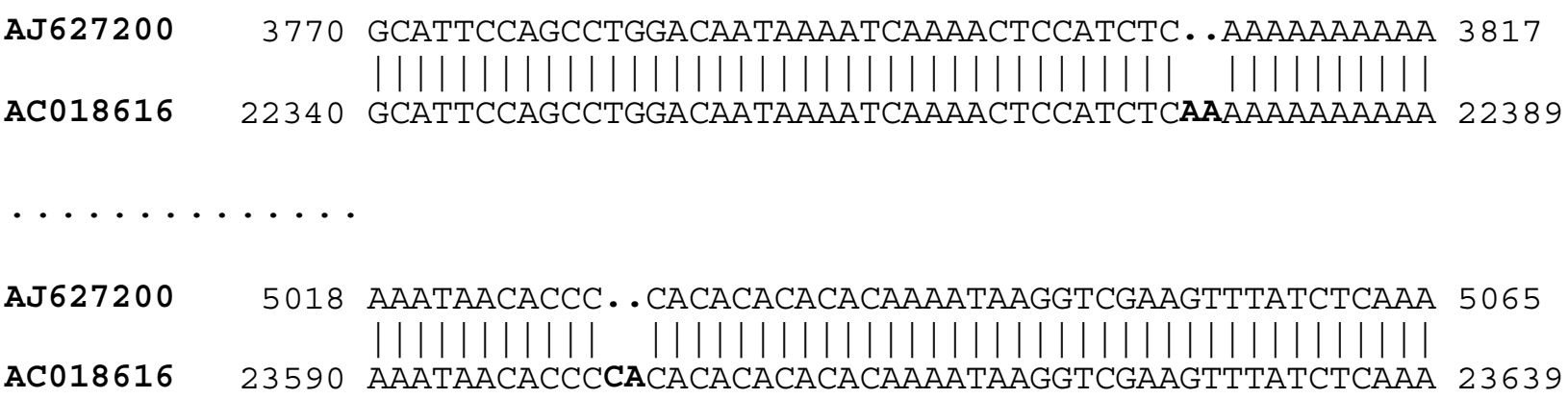


Figure 2:

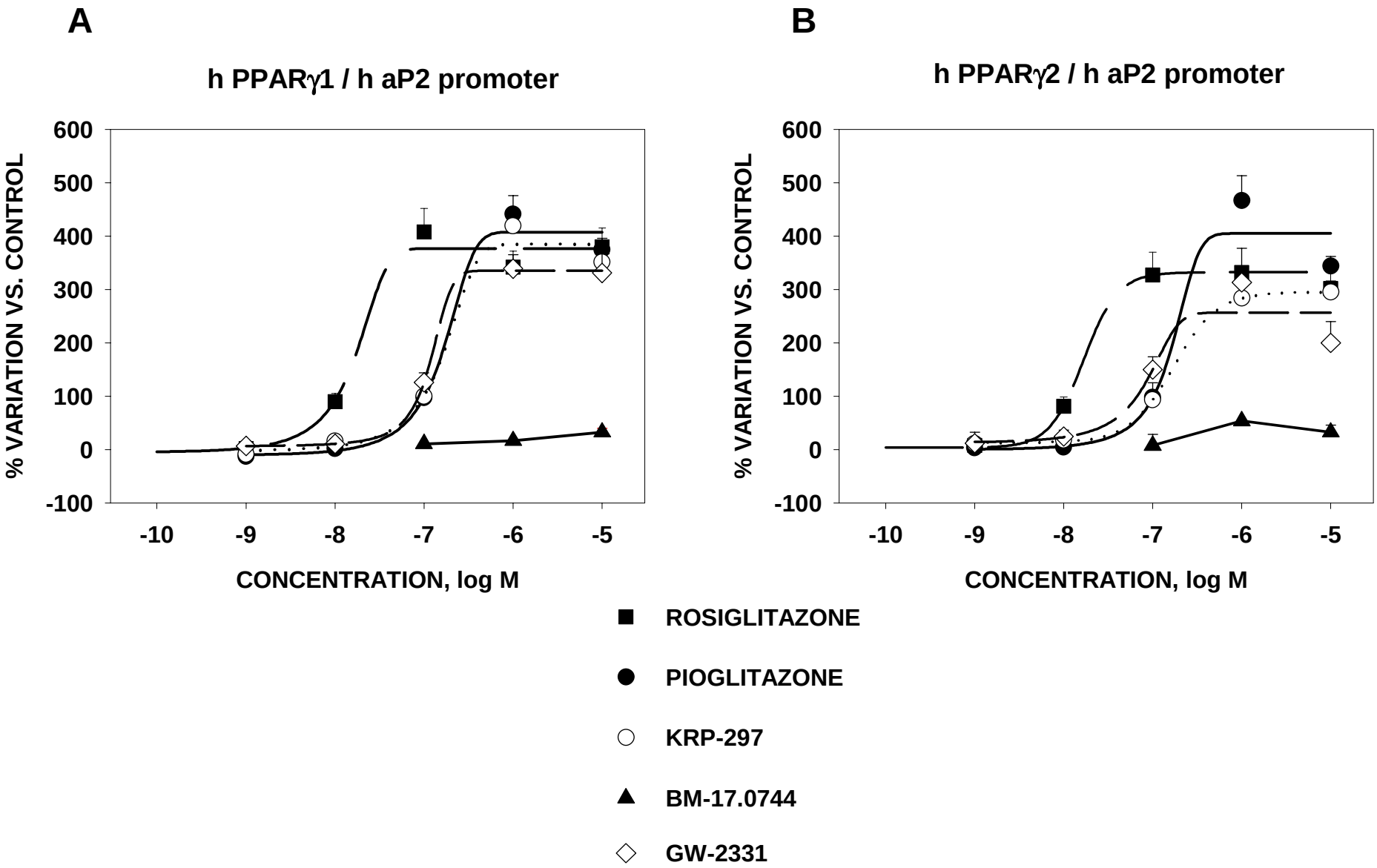


Figure 3:

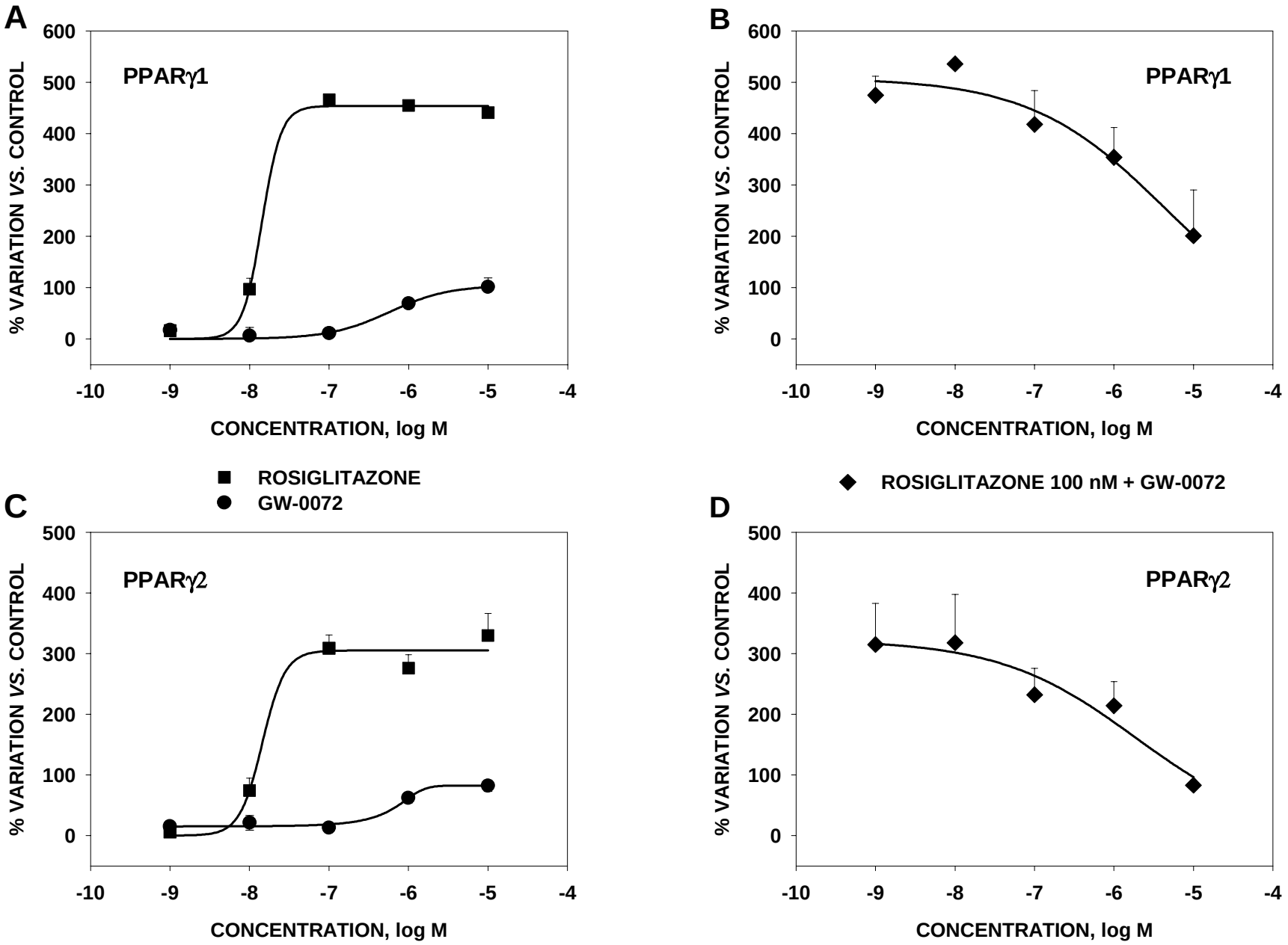


Figure 4:

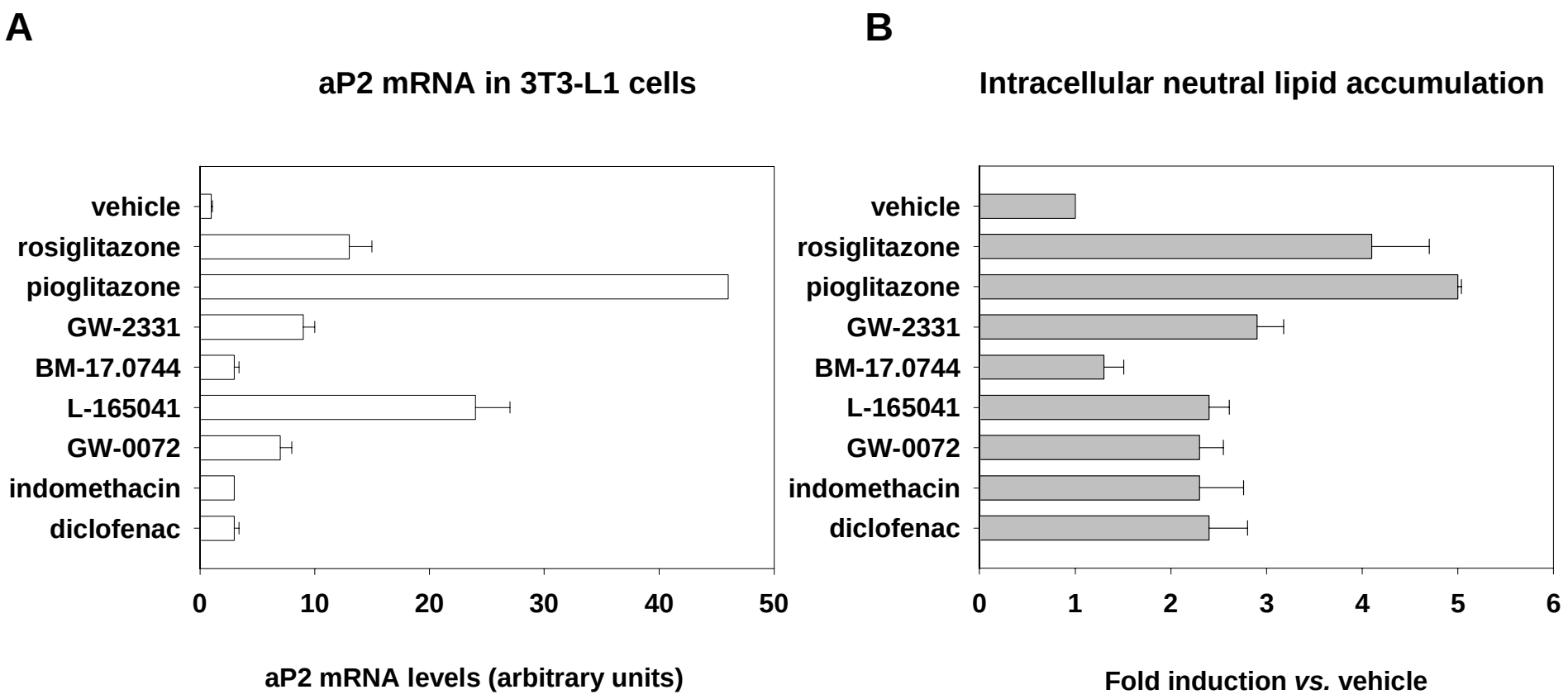
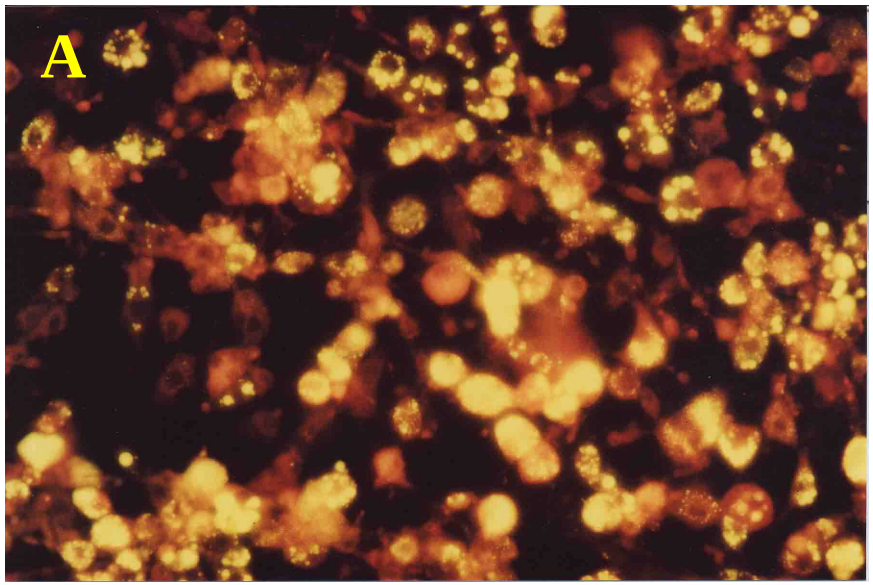
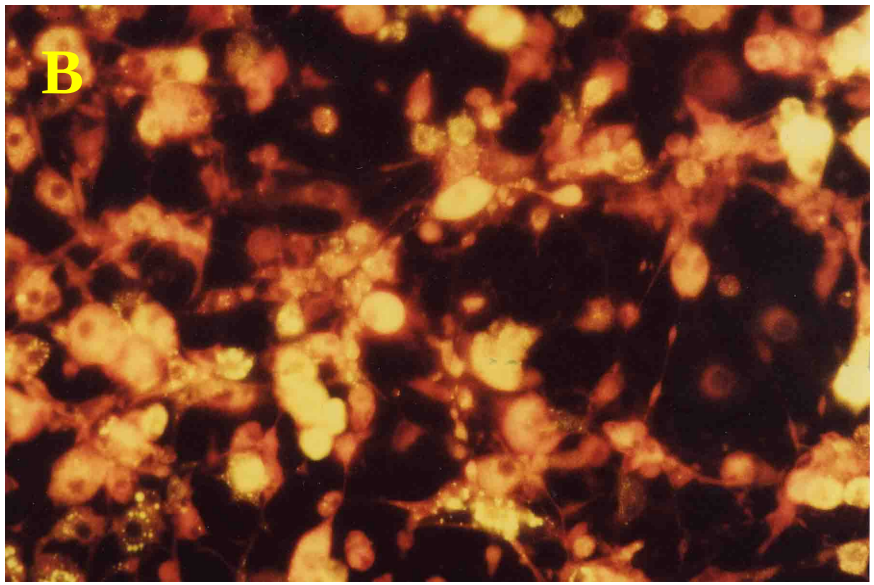


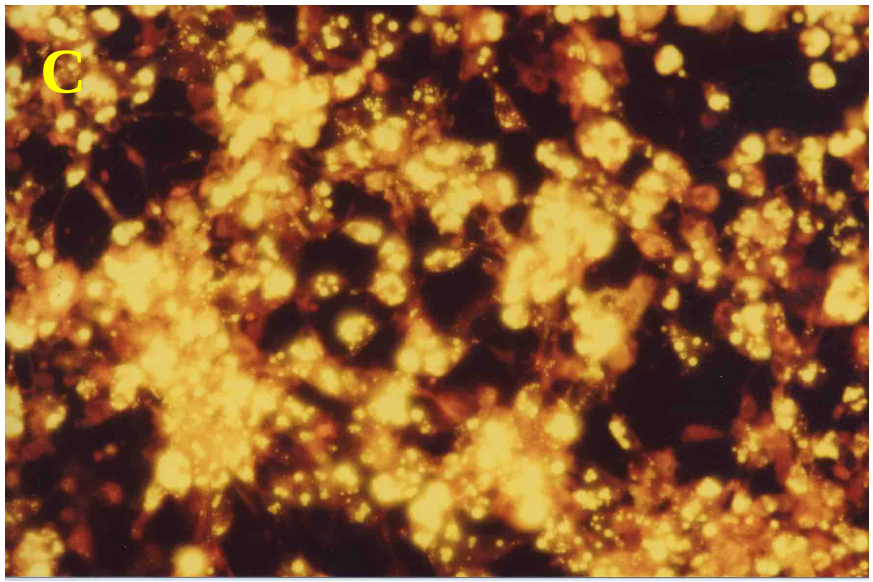
Figure 5:



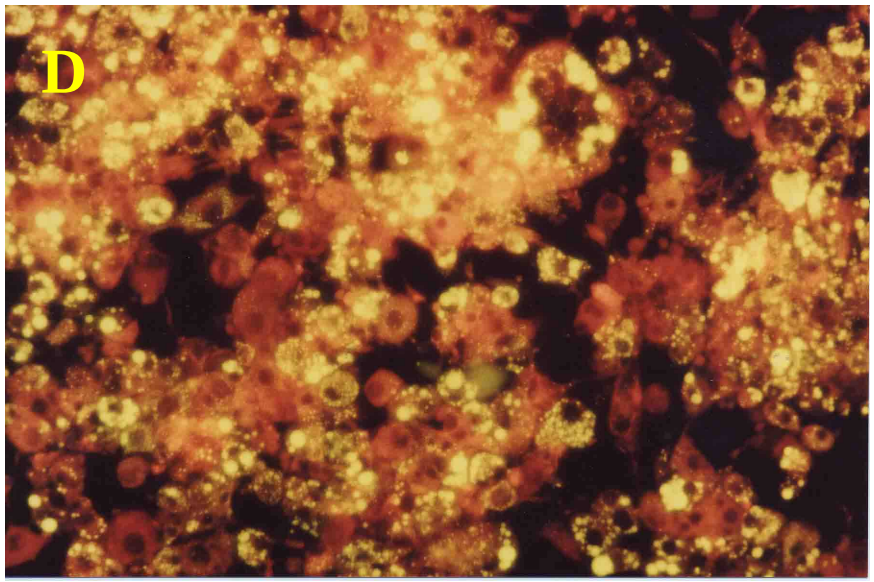
Differentiated cells : DMSO 0.1%



BM-17.0744 : 10 µM



Rosiglitazone : 0.1 µM



Rosiglitazone : 10 µM