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Antidiabetic and hypolipidemic potential of DRF 2519—a dual activator of PPAR-α and PPAR-γ

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Abstract

We investigated the biological activity of Dr. Reddy's Research Foundation (DRF) 2519, a benzoxazinone analogue of the thiazolidinedione class of compounds. In the in vitro transactivation assay, DRF 2519 showed interesting dual activation of Peroxisome Proliferator Activated Receptor (PPAR) alpha and gamma. In insulin-resistant *ob/ob* mouse model, DRF 2519 showed significant alleviation of insulin resistance and dyslipidemia, which is better than rosiglitazone. Fatty Zucker rats treated with DRF 2519 showed better reduction of plasma insulin, triglyceride and free fatty acid levels than those treated with rosiglitazone. In addition, these rats were able to clear plasma lipids better when challenged with exogenous lipid (i.v.). DRF 2519 treatment resulted in improved plasma lipid profiles in high-fat-fed Sprague–Dawley rats. Treated rats showed better plasma lipid clearance and hepatic triglyceride secretion. When compared to DRF 2519, fenofibrate was comparatively less efficacious while rosigltiazone showed no activity in these models. In ex vivo studies, DRF 2519 showed induction of liver acyl CoA oxidase mRNA and increase in lipoprotein lipase (LPL) protein expression and activity in adipose tissue. In the in vitro studies, DRF 2519 inhibited the lipid biosynthesis and secretion of apolipoprotein B from human hepatoma (Hep)G2 cells. It also enhanced insulin-induced relaxation of rat aortic smooth muscle. These results indicate that DRF 2519, a dual activator of PPAR-α and γ, could be an interesting development candidate in the management of metabolic disorders and associated complications.
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Keywords: Antidiabetic; Hypolipidemic; Insulin sensitizer; Peroxisome proliferators-activated receptor (PPAR)

1. Introduction

Type 2 diabetes is characterised by defective insulin action (insulin resistance) (Defronzo et al., 1992). Insulin resistance is associated not only with hyperinsulinemia and hyperglycemia but also with other disorders such as atherosclerosis, hypertension and abnormal lipid profile collectively referred to as Syndrome X or Insulin Resistance Associated Disorders (Reaven, 1988; Haffner et al., 1992). A lipid profile consisting of low High Density Lipoprotein (HDL)-cholesterol and high Low Density Lipoprotein (LDL)-cholesterol (atherogenic lipid profile) is characteristic of type 2 diabetes. This 'atherogenic lipid profile' results from decreased Very Low Density Lipoprotein (VLDL) removal by lipoprotein lipase and increased hepatic VLDL synthesis (Reaven, 1988).

The most promising new class of agents targeting insulin resistance is the thiazolidinedione derivatives. They decrease glucose levels while simultaneously reducing circulating insulin and free fatty acids (Perry and Petrie, 1998). The thiazolidinediones are believed to be ligands for specific subtypes of peroxisome proliferator activated receptors (PPARs). The PPARs are the members of nuclear hormone receptor family of transcription factors, a diverse group of proteins that mediate ligand-dependent transcriptional activation and repression (Schoonjans et al., 1996a,b). They are involved in the regulation of lipid and glucose metabolism, adipocyte differentiation and also implicated in cancer and inflammatory disease (Loviscach and Henry, 1999). To date, three subtypes of PPAR—α, γ and δ—have so far been identified (Schoonjans et al., 1996a,b; Staels and Auwerx,

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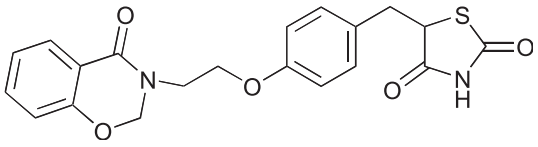

Fig. 1. Chemical structure of DRF 2519.

1997). PPAR-α is reported to be primarily involved in hepatic lipid metabolism, whereas PPAR-γ plays a central role in adipogenesis and glucose homeostasis (Schoonjans et al., 1996a,b; Loviscach and Henry, 1999; Staels and Auwerx, 1997). PPAR-δ is believed to be involved in basic lipid metabolism in brain (Basu-Modak et al., 1999). The hypotriglyceridemic activity of fibrate group of drugs is presumably due to their activation of PPAR-α, while the hypoglycemic and hypotriglyceridemic action of thiazolidinediones is through the activation of PPAR-γ leading to increased insulin sensitivity of peripheral tissues and lipoprotein lipase activity in the adipose tissue (Staels and Auwerx, 1997). These observations provide the impetus for exploring the possibility of developing dual PPAR-α and -γ activators, which will be useful for the treatment of dyslipidemia associated with atherosclerosis and type 2 diabetes. Recently, several other groups have also reported the efficacy of such coligands (Murami et al., 1996; Shinkai, 2001; Etegen et al., 2002).

Here, we report the biological activity of Dr. Reddy's Research Foundation (DRF) 2519, a benzoxazinone analogue of thiazolidinedione (Fig. 1) with dual PPAR-α and PPAR-γ activation property. In different animal models of diabetes, obesity and dyslipidemia, it shows interesting insulin-sensitizing and lipid-lowering activities through its dual activating property. The compound also showed lipid-lowering effect in normoglycemic hyperlipidemic animal models. We have compared the efficacy of DRF 2519 with PPAR-γ activator rosiglitazone, PPAR-α activator fenofibrate and another dual activator thiazolidinedione Kyorin Research Pharma (KRP)-297. In ex vivo studies, DRF 2519 showed significant effect on Acyl CoA oxidase and lipoprotein lipase. In in vitro cell-based assay, it showed significant reduction in lipid biosynthesis and apolipoprotein B secretion. DRF 2519 also showed significant increase in insulin-induced relaxation of isolated rat aortic ring.

2. Materials and methods

2.1. Animals

C57 BL/6J- *ob/ob* mice were obtained from Jackson Laboratory, Bar Harbour, ME, USA at 6 weeks of age. Zucker *fa/fa* rats were obtained from IFFA–CREDO, L'ARBRESLE CEDEX, France. Sprague–Dawley rats were bred at Dr. Reddy's Research Foundation (DRF) animal house. All animals were maintained under 12 h light and 12 h dark cycle at 25 ± 1 °C. All animals were given standard laboratory chow [National Institute of Nutrition, Hyderabad, India] and water ad libitum. Male Sprague–Dawley rats weighing 180–200 g were made hyperlipidemic by feeding a high-fat diet containing 2% cholesterol and 1% sodium cholate mixed with standard laboratory chow (National Institute of Nutrition). All animal experiments were carried out in accordance with internationally valid guidelines, and the experimental protocols were approved by DRF animal ethics committee.

2.2. Chemicals

DRF 2519 was synthesised by the Medicinal Chemistry department, Dr. Reddy's Research Foundation, Hyderabad (Lohray et al., 1991). Rosiglitazone was synthesised by the published procedure (Cantello et al., 1994) and was found to be 99% pure. Fenofibrate, insulin (from bovine pancreas) and Tyloxapol were purchased from Sigma (St. Louis, USA). Intralipid (20%) was purchased from Pharmacia (Stockholm, Sweden). Carboxy methyl cellulose was purchased from Loba Chemical Pvt. (Mumbai, India).

2.3. Drug treatment and blood sampling

Ob/ob mice were used at 10 weeks of age. DRF 2519, rosiglitazone and KRP-297 were administered orally for 14 days at doses mentioned. Animals in the control group received vehicle only (0.25% carboxy methyl cellulose, 10 ml/kg). For glucose tolerance test, animals were fasted for 5 h and then challenged with 3 g/kg glucose orally. The blood samples were collected at 0, 30, 60 and 120 min after glucose load, for plasma glucose estimation. Male Zucker *fa/fa* rats were used at 18 weeks. DRF 2519 and rosiglitazone were administerd at 3 mg/kg, p.o. dose for 10 days. For lipid clearance test, 20% intralipid was administered i.v. (5 ml/kg) and plasma triglyceride levels were measured at 0, 1, 10, 30, 60 and 120 min after injection.

Hyperlipidemic Sprague–Dawley rats were treated orally with DRF 2519, rosiglitazone, KRP-297 and fenofibrate for 6 days. Triton induced hepatic triglyceride output was measured by i.v. injection of Tyloxapol at 250 mg/kg (5 ml/kg in saline). Plasma triglyceride levels were measured at 0, 1, 2, 4 and 8 h after injection. Plasma lipid clearance test was performed to the control and treated animals after 5 ml/kg of i.v. injection of 20% intralipid. Plasma triglyceride levels were measured at 0, 1, 10, 30, 60 and 120 min after injection.

Blood samples were collected in fed state from the animals under mild ether anesthesia from retro-orbital sinus, 1 h after drug administration.

2.4. PPAR transactivation

The response element, upstream activated sequence of galactose DNA binding domain yeast transcription factor (UASGAL4 X 5) is present upstream of pFR-Luc reporter

(Promega, Madison, WI, USA) that contains Simian virus early promoter for luciferase assay. GAL4 fusions were made by fusing human PPAR-γ1 or PPAR-α ligand binding domain (amino acids: 174–475) to the C-terminal end of the yeast GAL4 DNA binding domain (amino acids: 1–147) of pM1 vector. pAdVantage (Promega) vector was used to enhance luciferase expression.

Human Embryonic Kidney (HEK) 293T cells were grown in Dulbecco's Modified Eagle's medium supplemented with 10% fetal bovine serum at 37 °C in 5% CO_2. One day prior to transfection, cells were plated to 50–60% confluence in Dulbecco's Modified Eagle's medium containing 10% delipidated fetal bovine serum. Cells were transfected by Superfect. Three hours after transfection, the reagent was removed and cells were maintained in Dulbecco's Modified Eagle's medium-delipidated fetal bovine serum. Forty-two hours after transfection, cells were placed in phenol red free Dulbecco's Modified Eagle's medium-delipidated fetal bovine serum and treated for 18 h with the test compounds or vehicle alone. The cells were lysed and assayed for luciferase activity. Luciferase activity was determined as fold activation relative to untreated cells by using Luclite kit (Packard, CT, USA) in a Packard Topcount.

2.5. Extraction of total RNA and semiquantitation of Acyl CoA oxidase mRNA

High-fat-fed rats were treated with DRF 2519, fenofibrate and rosiglitazone at 30 mg/kg dose for 6 days. Livers were excised under aseptic condition and total RNA was extracted by the Qiagen RNA midikit (Germany) following the manufacturer's instruction. First-strand cDNA was generated from 1 μg of RNA in a 20 μl volume by using the random primer in the first-strand cDNA synthesis kit (Boehringer–Manheim, 40724 Hilden, Germany). The reverse transcription reaction mixture (2.5 μl) was amplified in a 100 μl volume with primers specific for rat Acyl CoA oxidase and ribosomal protein L27 (RPL27) as control in presence of 5 μCi of [^{32}P]deoxycytidine 5′-triphosphate (4000 Ci/mmol, Jonaki, BRIT, India). All sequences of the primers, expected fragment length, T_m values of the primers are as shown in Table 1.

Linearity of the Polymerase Chain Reaction (PCR) was tested by amplification of 200 ng of total RNA per reaction from 15–40 cycles. The linearity range was found to be between 15–35 cycles. In no case did the amount of RNA used for PCR exceeded 200 ng per reaction. The samples were amplified for 33 cycles by using the following parameters: 95 °C/2 min/1 cycle, followed by 95 °C/1 min, 55 °C/1 min and 72 °C/1 min.

PCR products (2 μl) were electrophoresed on 8% polyacrylamide gel electrophoresis; gels were fixed, dried and autoradiographed. Bands were quantitated by gel documentation system using Gelwork 1D intermediate software (UVP, UK). Levels of mRNA were expressed as the ratio of signal intensity for the target gene relative to that of ribosomal protein L27.

2.6. Lipoprotein lipase activity

Lipoprotein lipase activity was measured in epididymal fat pad from *ob/ob* mice. Epididymal fat pads were excised from *ob/ob* mice treated with DRF 2519 at 10 mg/kg for 14 days. Lipoprotein lipase activity in tissue homogenate was measured as described (Iverius and Lindqvist, 1986). Out of 50% tissue homogenate, 200 μl was incubated for 1 h at 37 °C with 300 μl of substrate mixture consisting of 0.125 μCi glycerol tri[1-^{14}C]oleate, 0.3 mg phosphatidylcholine and 2.5 mg of triolein emulsified in 150 μl of 15% fatty acid free bovine serum albumin and 150 μl of 200 mM Tris hydrochloride buffer, pH 8.5. Pooled human serum (100 μl) was used as source of apolipoprotein CII. Incubation was terminated by the extraction of free oleate released by lipoprotein lipase from intact triolein by transferring an aliquot of incubation mixture (0.2 ml) to a tube containing 3 ml of methanol:chloroform:heptane (1.41:1.25:1) and agitated. To this 1 ml of 0.1 M carbonate borate buffer, pH 10.5 was added and agitated again. Phase separation was attained by centrifugation at 2500 rpm for 30 min at room temperature. Radioactivity in the aqueous layer was determined in a scintillation counter (Topcount, Packard).

Lipoprotein lipase protein levels in the epididymal fat tissue homogenate was measured using a lipoprotein lipase enzyme linked immunosorbent assay (ELISA) kit procured from Daiichi Pure Chemicals, Tokyo, Japan. Lipoprotein lipase level was measured in the tissue homogenate by following the manufacturer's instructions.

2.7. Incorporation of [^{14}C]acetate into total lipids in HepG2 cells

HepG2 cells were cultured at 37 °C in an atmosphere of 5% CO_2. Cells were maintained in Dulbecco's Modified

Table 1
Sequences of the primers used

Gene	Sequence of sense, primer and position starting from ATG	Sequence of antisense, primer and position starting from ATG	Expected fragment size (base pair)	T_m °C (5′/3′), sense/antisense
rACO	5′-CACAAGTAAACGCTCGGAATC-3′ (1830–1849)	5′-GGCTTCAAGTGCTTGTGGTA-3′ (2040–2021)	210	62/60
rRPL27	5′-AGCGATCCAAGATCAAGTCCTT-3′ (206–227)	5′-CCTGTCTTGTATCGCTCCTCAA-3′ (389–368)	184	64/66

ACO: Acyl CoA oxidase; RPL27: ribosomal protein L27.

Eagle's medium containing 10% fetal bovine serum, 100 U/ml penicillin and 100 μg/ml streptomycin. Cells were seeded on 6 well plates at 3.5×10^5 cells per well. After 24 h when the plate was 70% confluent, 5 μl of rosiglitazone or DRF 2519 in dimethyl sulphoxide (final concentration 0.25%) was added along with 2 ml of Dulbecco's Modified Eagle's medium + 10% fetal bovine serum. Eighteen hours later, cells were changed to a fresh medium containing 10% delipidated serum along with the compound. [1,2-^{14}C]acetate was added to a final concentration of 0.5 mmol/l for another 6 h at 37 °C. The cells were washed in phosphate buffered saline twice and lysed in minimum volume of 0.1 %. Igepal in 0.1 N NaOH per well. An aliquot of the lysate was used for the extraction of total lipids. To this, 4 ml of chloroform: methanol (2:1) was added and mixed. This was followed by the addition of 0.5 ml of 0.88% KCl. The tubes were vortexed and the layers separated by centrifugation at 2500 rpm for 30 min. The bottom chloroform layer was separated and dried under nitrogen. This residue was reconstituted in minimum volume of chloroform; methanol and radioactivity was measured in a scintillation counter (Topcount, Packard).

2.8. Apolipoprotein B secretion from HepG2 cells

HepG2 cells cultured in Dulbecco's Modified Eagle's medium + 10% fetal bovine serum were seeded at 1×10^5 cells/well in a 24 well plate prior to experiment. Experiment was initiated when cells were 70% confluent. The medium was replaced with fresh medium containing different concentrations of DRF 2519 in 100% dimethyl sulfoxide. The final concentration of dimethyl sulfoxide in the medium was 0.1%. An equivalent concentration of dimethyl sulfoxide was added in the control wells also. After 24 h incubation with the compound under standard tissue culture conditions, the medium was removed and used for measurement of apolipoprotein B. Apolipoprotein B level in media was measured by microwell enzyme immunoassay method using a commercial kit procured from AlerCHEK, Portland, USA.

2.9. In vitro vascular reactivity study

Male Wistar rats (250–359 g) were sacrificed by cervical dislocation. The thoracic aorta (aorta thoracic descendens) was dissected and transferred in to Krebs–Hansleit solution of the following composition: 118 mM NaCl, 25 mM $NaHCO_3$, 2.5 mM $CaCl_2$, 4.7 mM KCl, 1.2 mM KH_2PO_4, 1.2 mM $MgSO_4$, 11.1 mM glucose continuously gassed with carbogen (95% O_2 and 5% CO_2). Rat aorta were cleaned from connective tissue without damaging the endothelium and cut into rings of approximate length of 4 mm. Rings were individually mounted in organ bath (Hugo Sachs, Germany) containing Krebs–Hansleit solution and were allowed to equilibrate for 60 min under resting tension of approximately 1 g. After equilibration, rings were contracted with phenylephrine [1, 3, 10 and 30 μM, contact time 10 min] in a cumulative concentration dependent manner. The concentration, which produced maximum contraction, was used to study relaxation response. The presence of undamaged endothelium was checked by relaxation to acetylcholine [acetylcholine 1 μM; 10 min] after precontraction with phenylephrine (10 μM). Rings showing less than 70% contraction of the maximal phenylephrine effects were discarded. A cumulative dose-dependent relaxation was studied with insulin in presence and absence of test compound.

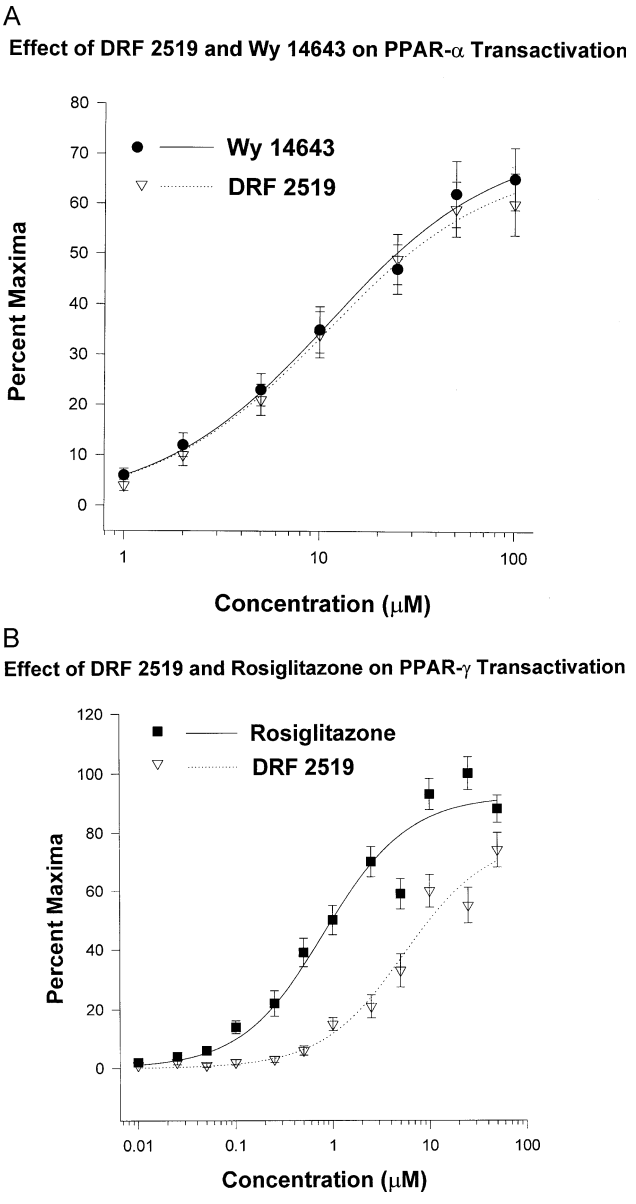

Fig. 2. Activation of (A) PPAR-α and (B) PPAR-γ by DRF 2519. HEK-293 cells were transfected with Gal4-PPAR-α or PPAR-γ1-LBD, pGL2 (Gal4X5)-SV 40-Luc reporter construct and pAdvantage. Luciferase activity was calculated as fold activation relative to untreated cells. Fourfold activation by WY 14,643 at 25 μM and 87-fold activation by rosiglitazone at 25 μM has been considered as maximum effect for PPAR-α and PPAR-γ, respectively. Values are an average of three experiments conducted in triplicates.

To study the effect of compound, the rings were pre-incubated with DRF 2519 (100 μM in dimethyl sulfoxide) for 30 min and contraction with phenylephrine was carried out as described above. At the peak contraction, insulin was added at 0.01, 0.1, 0.3, 1, 3 and 10 μM for every 5 min in a cumulative manner and the relaxation response with insulin was observed. Percent relaxation with insulin was calculated and compared with vehicle treated rings.

2.10. Analytical methods

Plasma glucose, triglyceride and free fatty acid were measured spectrophotometrically using commercially available kits (Pointe Scientific, USA and Boehringer Mannheim, Germany). Plasma insulin was measured using Radio Immuno Assay (RIA) kit from Linco Research, USA.

2.11. Data analysis and statistics

The percent reduction was calculated according to the formula

$$1 - \frac{(TT/OT)}{(TC/OC)} \times 100$$

TT: Test day treated; OT: zero day treated; TC: test day control; OC: zero day control. The statistical analyses were performed using One-Way Analysis of Variance (ANOVA) or *t*-test. $P < 0.05$ was considered significant.

3. Results

3.1. PPAR transactivation assay

Comparative dose–response study was performed with DRF 2519 and Wyeith (WY) 14,643 {2-[4-Chloro-6-(2,3-dimethylphenylamino)pyrimidin-2-ylsulfanyl]acetic acid} for PPAR-α and DRF 2519 and rosiglitazone for PPAR-γ. DRF 2519 showed dual activation of PPAR-α and PPAR-γ. DRF 2519 showed similar activation of PPAR-α as compared to WY 14,643 but less potency for PPAR-γ as compared to rosiglitazone (Fig. 2A, B). Neither WY 14,643 showed any significant PPAR-γ activation nor rosiglitazone showed any significant PPAR-α activation. Fenofibrate at 300 μM concentration showed only 1.5-fold activation with PPAR-α. DRF 2519 did not show any significant PPAR-δ activation even at 50 μM concentration.

Table 2
Effect of DRF 2519 in *ob/ob* mice

Group	Glucose (mg/dl)	Triglyceride (mg/dl)	FFA (mmol/l)	Insulin (ng/ml)
Control	385.93 ± 52.04	129.49 ± 18.76	1.4 ± 0.18	43.07 ± 11.8
0.3 mg/kg	232.85 ± 40.94	81.65 ± 5.4	1.2 ± 0.15	30.15 ± 4.5
1.0 mg/kg	212.05 ± 14.65[a]	77.52 ± 1.12[a]	0.78 ± 0.1[a]	23.69 ± 1.6[a]
3.0 mg/kg	126.03 ± 6.33[a]	57.44 ± 5.6[a]	0.55 ± 0.12[a]	12.89 ± 1.2[a]
10.0 mg/kg	102.65 ± 8.98[a]	33.33 ± 4.8[a]	0.46 ± 0.09[a]	1.02 ± 0.25[a]

Male *ob/ob* mice were treated with DRF 2519 for 14 days. End day values are expressed as mean ± S.E. No. of animals (n) = 5 per group.
FFA: free fatty acid.
[a] $P < 0.05$ as compared to control (ANOVA).

Table 3
Comparative effect of DRF 2519 and rosiglitazone in *ob/ob* mice

Plasma parameters	DRF 2519		Rosiglitazone	
	ED_{50} (mg/kg)	E_{max} (mg/kg)	ED_{50} (mg/kg)	E_{max} (mg/kg)
Glucose	1.2	75% at 10	3.6	63% at 10
Triglyceride	1.5	74% at 10	10.0	53% at 10
Free fatty acid	2.0	67% at 10	>10	48% at 10
Insulin	1.1	98% at 10	5.0	72% at 10

ED_{50} was calculated according to the regression analysis of the dose–response curve.

3.2. Effect in ob/ob mice

DRF 2519 showed a dose-dependent reduction in plasma glucose, triglyceride, free fatty acid and insulin levels in *ob/ob* mice after 14 days of treatment (Table 2). DRF 2519 showed better efficacy than rosiglitazone (Table 3). The compound showed dose-dependent improvement in oral glucose tolerance test (maximum reduction of 73% in area under curve for glucose; Fig. 3). KRP-297, another dual activator showed ED_{50} values of 3, 10, 8 and 10 mg/kg for plasma glucose, triglyceride, insulin and free fatty acid. DRF 2519 and rosiglitazone, in these models, ameliorated polydipsia but did not affect food consumption. No signif-

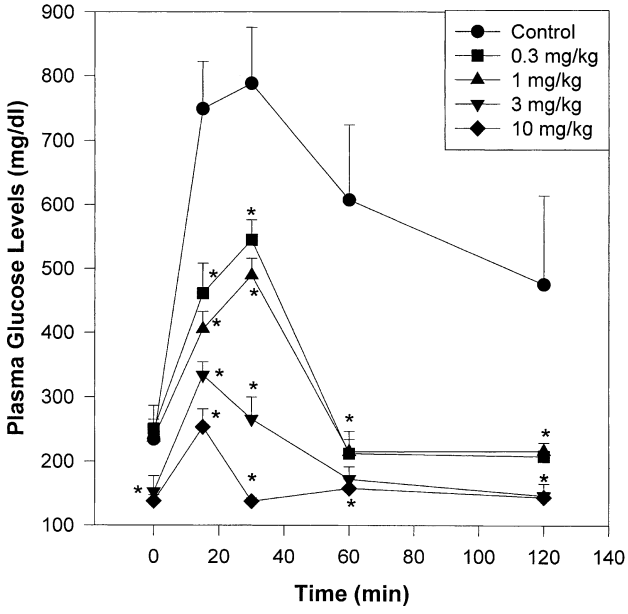

Fig. 3. Effect of DRF 2519 on glucose tolerance test in *ob/ob* mice. Animals were treated for 14 days at different doses and then subjected to oral glucose load (3 g/kg) after 5 h fast. Values are expressed as mean ± S.E. No. of animals (n) = 5 per group. *$P < 0.05$ as compared to control (ANOVA).

Table 4
Effect of DRF 2519 and rosiglitazone in Zucker *fa/fa* rats

Group	Triglyceride (mg/dl)	FFA (mmol/l)	Insulin (μU/ml)
Control	251.64 ± 29.46	0.281 ± 0.07	145.44 ± 29.52
DRF 2519	104.06 ± 7.90[a]	0.097 ± 0.04[a]	48.24 ± 7.68[a]
Rosiglitazone	167.44 ± 17.16[a]	0.216 ± 0.04 (NS)	68.40 ± 12.0[a]

Male Zucker *fa/fa* rats were treated with DRF 2519 and rosiglitazone at 3 mg/kg dose for 10 days. All values are expressed as mean ± S.E. No. of animals (n) = 5 per group.
FFA: free fatty acid; NS: not significant.
[a] $P < 0.05$ as compared to control (ANOVA).

icant difference in body weight change was observed among the treated groups.

3.3. Effect in Zucker fa/fa rats

In obese Zucker *fa/fa* rats, both DRF 2519 and rosiglitazone showed reduction in plasma triglyceride, free fatty acid and insulin levels at 3 mg/kg dose after 10 days of treatment (Table 4). DRF 2519 showed better reduction in plasma triglyceride (59% vs. 34%) and free fatty acid (65% vs. 23%) levels than rosiglitazone. KRP 297 at similar dose showed 60% and 50% reduction in triglyceride and free fatty acid. When challenged with i.v. intralipid, DRF 2519 treatment showed significant improvement (64% decrease in area under the triglyceride concentration vs. time graph) in plasma triglyceride clearance in these animals (Fig. 4). No significant difference in body weight was observed between DRF 2519 treated group and the vehicle treated group.

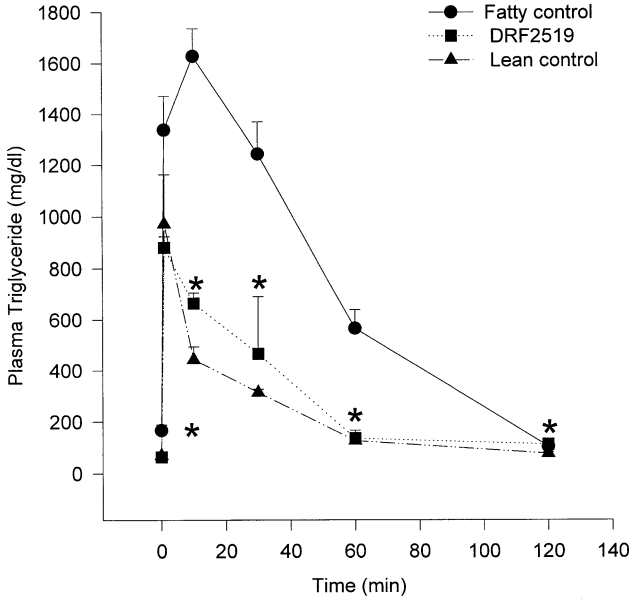

Fig. 4. Effect of DRF 2519 on plasma triglyceride clearance in Zucker *fa/fa* rats. Animals were treated with DRF 2519 for 10 days and loaded with 20% intralipid (5 ml/kg; i.v.). Values are expressed as mean ± S.E. No. of animals (n) = 5 per group. *$P < 0.05$ as compared to fatty control (ANOVA).

Table 5
Effect of DRF 2519 on high-fat-fed Sprague–Dawley rats

Group	TG (mg/dl)	TC (mg/dl)	HDL (mg/dl)	LDL (mg/dl)
Normal fed	36.82±13.10	113.19±4.18	64.34±1.50	41.48±3.73
High-fat-fed	116.05±18.08	342.53±59.34	21.89±2.12	297.43±58.44
DRF 2519 (3 mg/kg)	58.2±2.72[a]	225.42±10.2[a]	25.21±4.51	180.22±15.54
DRF 2519 (10 mg/kg)	37.60±3.80[a]	167.36±27.91[a]	31.61±1.19[a]	131.23±10.58[a]
DRF 2519 (30 mg/kg)	23.45±3.70[a]	126.31±12.37[a]	47.37±4.06[a]	84.25±6.93[a]

Male Sprague–Dawley rats weighing 180–200 g were made hyperlipidemic by feeding high-fat diet containing 2% cholesterol and 1% sodium cholate for 6 days. Compounds were given for 3 days at the respective doses. The high-fat diet was continued during the treatment period. Values are expressed as mean±S.E. No. of animals (n)=5 per group.
TG: triglyceride; TC: total cholesterol; HDL: high density lipoprotein; LDL: low density lipoprotein.
[a] $P < 0.05$ as compared to high-fat-fed group (ANOVA).

3.4. Effect in high-fat-fed rats

DRF 2519 was tested in a hyperlipidemic but normoglycemic rat model. These animals, when fed with high-fat-fed containing diet, showed significant increase in plasma triglyceride, total and LDL-cholesterol levels and concomitant decrease in plasma HDL level. DRF 2519 treatment significantly reduced plasma triglyceride, total and LDL-cholesterol and increased HDL-cholesterol levels (Table 5). DRF 2519 showed significantly better activity than fenofibrate (Table 6). KRP 297, at 100 mg/kg dose, showed 52% and 49% reduction in total cholesterol and triglyceride and 98% increase in HDL-cholesterol. The compound treatment did not show any significant change in body weight compared to control rats after 6 days of treatment. Fat-fed rats showed a significant increase in plasma apolipoprotein B levels as compared to control rats (50.39 ± 22.52 vs. 5 ± 1 mg/dl). DRF 2519 treatment reduced significantly this elevated apolipoprotein B levels to 7.76 ± 3.64 mg/dl. Tyloxapol blocks all clearance of VLDL particles, hence, the rate of accumulation of these particles in the plasma reflect their secretion rates. Hepatic triglyceride secretion

Table 6
Comparative effect of DRF 2519 and fenofibrate in high-fat-fed rats

Plasma parameters	DRF 2519		Fenofibrate	
	ED_{50} (mg/kg)	E_{max} (mg/kg)	ED_{50} (mg/kg)	E_{max} (mg/kg)
Triglyceride	2.8	80% at 30	12	48% at 60
Total cholesterol	10.2	63% at 30	45	65% at 60
HDL-cholesterol	7.4	119% at 30	30	114% at 60
LDL-cholesterol	6.5	72% at 30	6	73% at 60

ED_{50} was calculated according to the regression analysis of the dose–response curve.

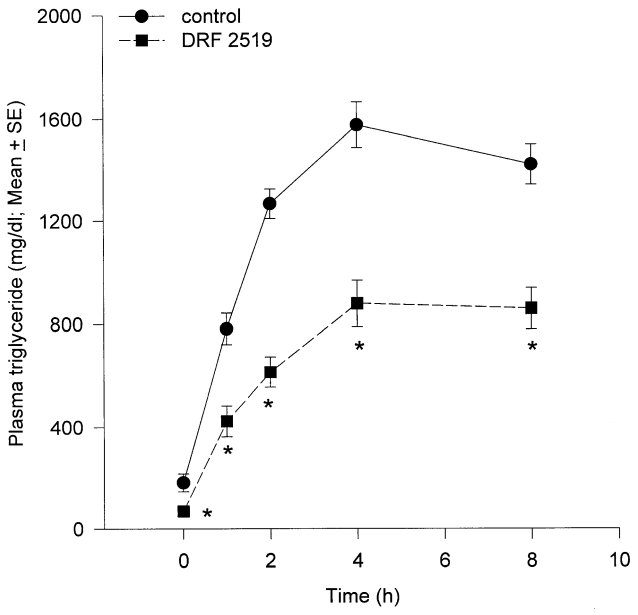

Fig. 5. Effect of DRF 2519 on hepatic triglyceride secretion in high-fat-fed rats. Animals were treated with DRF 2519 (30 mg/kg) for 6 days and injected with tyloxapol (250 mg/kg, 5 ml/kg; i.v.). Values are expressed as mean ± S.E. No. of animals (n) = 5 per group. *P < 0.05 as compared to control (ANOVA).

rate was measured after i.v. tyloxapol injection in both control and DRF 2519 (30 mg/kg) treated rats. DRF 2519 significantly reduces hepatic triglyceride secretion rate (202 vs. 349 mg/dl/h for treated vs. control) in this model (Fig. 5). Rats fed with normal diet showed a triglyceride secretion rate of 222 mg/dl/h. In an i.v. lipid tolerance study (20% intralipid) in fat-fed rats, DRF 2519 treatment showed significant improvement of the triglyceride clearance as compared to untreated rats (Fig. 6).

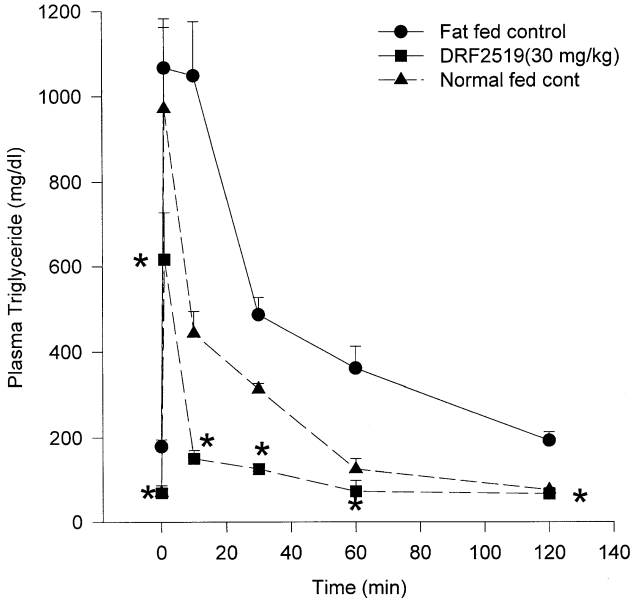

Fig. 6. Effect of DRF 2519 on plasma triglyceride clearance in high-fat-fed rats. Animals were treated with DRF 2519 (30 mg/kg) for 6 days and loaded with 20% intralipid (5 ml/kg, i.v.). Values are expressed as mean ± S.E. No. of animals (n) = 5 per group. *P < 0.05 as compared to high-fat-fed control (ANOVA).

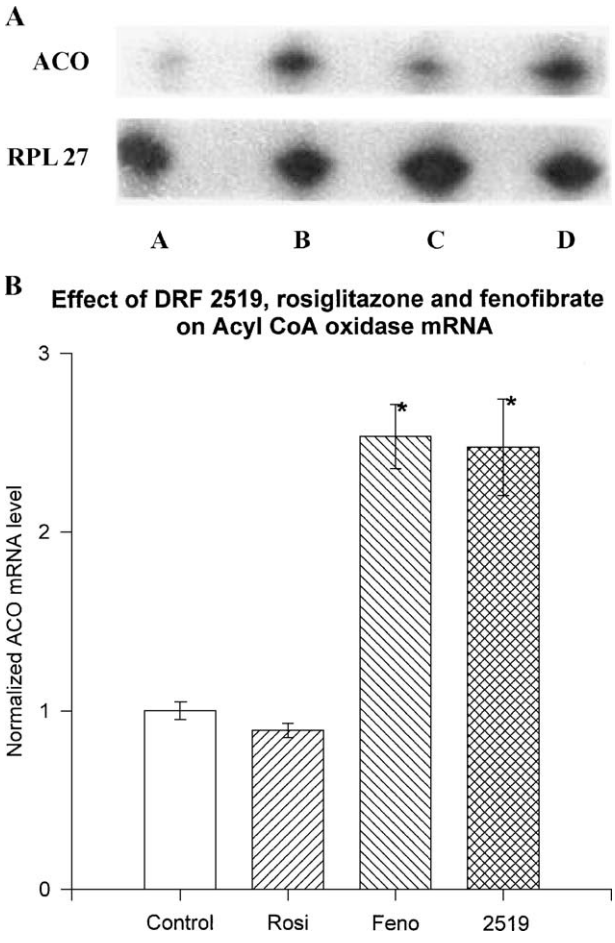

Fig. 7. Induction of Acyl CoA oxidase mRNA by DRF 2519. High-fat-fed rats were treated with DRF 2519, rosiglitazone and fenofibrate (30 mg/kg) for 6 days. Extraction of RNA and RT–PCR procedure are as described in Materials and methods. Panel (A) represents the autoradiogram. Lane A: control; lane B: fenofibrate; lane C: rosiglitazone; and lane D: DRF 2519. (B) Amount of Acyl CoA oxidase mRNA normalised by RPL27 mRNA. Feno: fenofibrate; Rosi: rosiglitazone; 2519: DRF 2519. Values are expressed as mean ± S.E. No. of animals (n) = 4 per group. *P < 0.05 as compared to vehicle treated fat-fed rats (Control).

Table 7
Effect of DRF 2519 treatment on lipoprotein lipase activity in *ob/ob* mice

Group	Mean CPM/g tissue	Percent increase	LPL protein (ng/ml)	Percent increase
Control	786,450 ± 48,261		0.398 ± 0.014	
DRF 2519	1,177,181 ± 59,747[a]	50	0.534 ± 0.046[a]	34

Ob/ob mice were treated with DRF 2519 at 10 mg/kg dose for 14 days. Epididymal fat pad was excised, processed and lipoprotein lipase assay was performed as mentioned in Materials and methods. All values are expressed as mean ± S.E. No. of animals (n) = 5 per group.

[a] P < 0.05 as compared to control (ANOVA).

Table 8
Effect of DRF 2519 on lipid biosynthesis in HepG2 cells

Concentration (μM)	Percent inhibition
10	53 ± 3[a]
30	58 ± 2[a]
100	94 ± 2[a]

HepG2 cells were preincubated with the compound for 18 h and then with (^{14}C) acetate and the compound for another 6 h. Values are expressed as mean ± S.E. No. of animals (n) = 4 per group.
[a] $P < 0.05$ as compared to the vehicle treated group.

3.5. Acyl CoA oxidase mRNA measurement

Acyl CoA oxidase mRNA was measured in the liver of high-fat-fed Sprague–Dawley rats treated with DRF 2519, rosiglitazone and fenofibrate (30 mg/kg dose for 6 days). Both fenofibrate and DRF 2519 (30 mg/kg) showed ~ 2.5-fold enhancement of Acyl CoA oxidase mRNA, whereas rosiglitazone at same dose failed to show any induction (Fig. 7A, B).

3.6. Lipoprotein lipase activity in epididymal fat pad

DRF 2519 treatment showed a 50% increase of lipoprotein lipase activity and 34% increase in the protein level in epididymal fat tissue of *ob/ob* mice after 14 days of treatment at 10 mg/kg dose (Table 7).

3.7. Lipid biosynthesis study in HepG2 cells

DRF 2519 treatment showed significant reduction in lipid biosynthesis as evidenced by [^{14}C]acetate incorporation. A 53% and 94% reduction was observed at 30 and 100 μM concentration (Table 8). Rosiglitazone failed to show any effect even at 100 μM.

3.8. Apolipoprotein B secretion from HepG2 cells

DRF 2519 showed a dose-dependent inhibition of apolipoprotein B secretion in HepG2 cells after overnight treatment (Table 9).

Table 9
Effect of DRF 2519 on apolipoprotein B secretion from HepG2 cells

Group	Apolipoprotein B concentration (mg/dl)	Percent reduction in secretion (%)
Control	31.13 ± 2.32	
DRF 2519 (10 μM)	23.28 ± 3.24	25
DRF 2519 (30 μM)	23.65 ± 3.09	24
DRF 2519 (100 μM)	13.91 ± 0.75[a]	55
DRF 2519 (300 μM)	7.05 ± 0.38[a]	77

HepG2 cells were incubated with DRF 2519 at different concentrations as mentioned for 24 h. Apolipoprotein B secreted in the media was measured by an ELISA kit. All values are mean ± S.E. No. of animals (n) = 8 per group.
[a] $P < 0.05$ as compared to control.

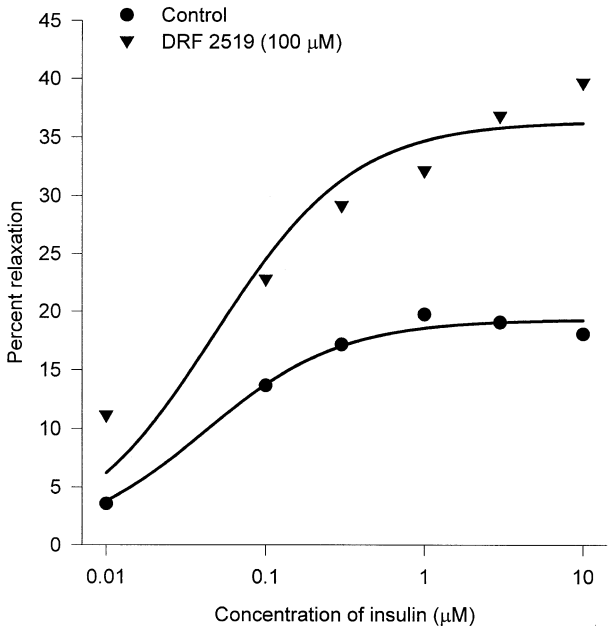

Fig. 8. Effect of DRF 2519 on insulin-induced relaxation of rat aortic ring. Thoracic aorta was excised from Wistar rats and the relaxation was measured on preconstricted aortic rings. Values are an average of three experiments.

3.9. Vascular reactivity study

Cumulative concentration–response curves for phenylephrine (1, 3, 10 and 30 μM, contact time 10 min) in endothelium-intact aorta were performed. In these studies, 10 μM phenylephrine induced approximately 70% of maximal contraction in a consistent manner, and hence, this dose was selected for studying the insulin-induced relaxation. In the precontracted state, insulin showed concentration (0.01–10 μM)-dependent relaxation. The preparation pretreated with 100 μM DRF 2519 for 10 min showed significant improvement in insulin-induced relaxation as compared to insulin alone (Fig. 8). Both vehicle (dimethyl sulfoxide) and compound per se did not alter the insulin and phenylephrine responses.

4. Discussion

Insulin resistance in type 2 diabetes is associated with both hyperglycemia and hyperlipidemia. Transcription factors PPARs offer subtype-dependent effect on different metabolic pathways. It is conceivable that this property of PPARs can be exploited to have a compound which will ameliorate both insulin resistance and hyperglycemia. DRF 2519, a novel thiazolidinedione analogue showed dual activation of α and γ isoforms of PPARs. This property of DRF 2519 is at variance from that of classic thiazolidinediones, which are known to be PPAR-γ selective ligands (Forman et al., 1995; Lehman et al., 1995). In this report, we present the antidiabetic and lipid-lowering potential of DRF

2519 in different genetic animal models of type 2 diabetes (Herberg and Coleman, 1997; Hunt, 1979) and hypolipidemic activity in normal and high-fat diet induced hyperlipidemic models.

In in vitro transactivation assay, DRF 2519 showed activation with both PPAR-α and PPAR-γ isoforms. We have used rosiglitazone, a potent and selective PPAR-γ activator (Lehman et al., 1995; Balfour and Plosker, 1999) for comparison. DRF 2519 showed slightly lower transactivation potential as compared to rosiglitazone. Fibrates which are used clinically are known to be low affinity ligands of PPAR-α. For this reason, we have used WY 14,643, a more potent PPAR-α specific ligand (Wilson et al., 2000), as a standard in our transactivation assay. DRF 2519 showed PPAR-α activation, and this activity was compared to WY 14,643. As reported previously (Lehman et al., 1995; Forman et al., 1997), our study also indicated that rosiglitazone is a PPAR-γ specific ligand while WY 14,643 is PPAR-α specific, which validates our assay procedure.

We studied the insulin-sensitizing and lipid-lowering potential of DRF 2519 in various animal models of insulin resistance and hyperlipidemia. Among the thiazolidinedione series of insulin sensitizers, rosiglitazone is claimed to be the most potent, efficacious and less toxic (Balfour and Plosker, 1999). We have compared the antidiabetic potential of DRF 2519 with rosiglitazone in our in vivo studies. In a preliminary study, we found that fenofibrate was the more potent hypolipidemic agent than clofibrate, bezafibrate and gemfibrozil (data not shown). Therefore, in vivo hypolipidemic potential of DRF 2519 was compared with fenofibrate. Among the dual PPAR activators, another thiazolidinedione analogue, KRP 297 has recently entered in Phase III clinical trial. We have also compared the efficacy of DRF 2519 with KRP 297 in our studies.

Ob/ob mice exhibit moderate hyperglycemia, marked hyperinsulinemia and impaired glucose tolerance (Genuth et al., 1971). DRF 2519 treated animals showed dose-dependent reduction in plasma glucose, triglyceride, insulin, free fatty acid levels and a marked improvement in glucose tolerance after 14 days of treatment. DRF 2519 showed better efficacy than both rosiglitazone and KRP 297.

Obese Zucker fatty rats (*fa/fa*) display hyperinsulinemia, hyperlipidemia but normoglycemia, and are widely used as an excellent model for insulin resistance associated with obesity (Hunt, 1979; Assimacopoulos-Jeannet and Jeanrenaud, 1976). DRF 2519 treatment showed significant reduction in plasma insulin and lipid levels in this model. This effect was better than rosiglitazone and comparable to KRP 297. Zucker *fa/fa* rats has defective clearance of plasma triglyceride levels as compared to control lean rats when challenged with intralipid. DRF 2519 treatment also normalised plasma triglyceride clearance in these rats.

The studies in different genetic insulin-resistant animal models clearly indicate that dual activator DRF 2519 treatment improves insulin sensitivity and its efficacy is better than the potent PPAR-γ agonist rosiglitazone. This is clearly in contrast to our in vitro study, which shows that rosiglitazone is a more potent PPAR-γ activator than DRF 2519. Pharmacokinetic studies did not show any significant difference in the pharmacokinetic profiles of DRF 2519 and rosiglitazone, which indicates the noninvolvement of pharmacokinetic-related issues in different pharmacodynamic effects of these two molecules. Although the detailed mechanism of both PPAR-α and PPAR-γ are not clear yet, several recent studies have indicated that glitazones induce lipoprotein lipase and fatty acid transporter protein (Schoonjans et al., 1996a,b; Martin et al., 1997) through PPAR-γ. This induction leads to increased fatty acid uptake in the adipose tissue and, subsequently, will lead to the utilization of glucose instead of fatty acid as the energy source by the muscle (Oakes et al., 1997). The role of PPAR-α activators, such as fibrates on insulin resistance, is not at all clear. Because fibrates increase hepatic oxidation of fatty acid and reduce synthesis and secretion of triglycerides (Staels et al., 1998), this will increase diversion of fatty acid from peripheral tissue (e.g., skeletal muscle and fat tissue) to the liver, decrease fatty acid synthesis and lower delivery of triglyceride to peripheral tissues. By this action on fatty acid pathway, fibrates can increase the insulin-stimulated glucose disposal in skeletal muscle (Randle et al., 1963; Boden, 1994), and thereby ameliorate insulin resistance. In addition, fibrates through their plasma triglyceride lowering activity, can lower skeletal muscle triglyceride content and thereby reduce insulin resistance (Goodpaster and Kelly, 1998). Interestingly, few recent publications have also shown insulin-sensitizing property of fibrate in insulin-resistant animal models (Boden, 1994; Guerre-Milo et al., 2000). We believe that contribution of PPAR-α to insulin sensitization could most probably be the reason for better efficacy of DRF 2519 over rosiglitazone.

The insulin-resistant state is commonly associated with lipoprotein abnormalities such as hypertriglyceridemia, high levels of VLDL, small dense LDL (Taskinen, 1995) and low levels of HDL-cholesterol (Reaven et al., 1993), which are risk factors for coronary heart disease. The metabolic abnormalities together with hypertension and type 2 diabetes may cluster in the same individual, constituting the metabolic syndrome X or insulin resistance-associated disorder (Reaven, 1988). Results from the Helsinki heart study and Veteran Affairs—HDL Cholesterol Intervention Trial study—demonstrate that fibrates significantly reduce the incidence of cardiovscular disease in patients with type 2 diabetes by raising HDL-C levels and lowering triglyceride levels without lowering the LDL-C levels. (Huttunen et al., 1991; Rubins et al., 1999). As described earlier, our main objective behind developing a dual activator was to improve the lipid-lowering property over the currently available thiazolidinediones. DRF 2519 showed better PPAR-α activation than the fibrates. Studies in different genetic models, e.g., *ob/ob* and Zucker *fa/fa* rats have shown the lipid lowering potential of DRF 2519. To study the lipid lowering

activity of DRF 2519 in further details, we used an animal model in which PPAR-γ ligands (thiazolidinediones) did not show any significant effect, whereas PPAR-α ligands showed significant lipid lowering activity.

High-fat-fed rat model had been used previously to study the efficacy of fibrates (Petit et al., 1988). These animals are hypercholesterolemic, hypertriglyceridemic but are nondiabetic. Normally, rodent plasma total cholesterol contains a very high proportion of HDL-cholesterol and very low LDL-cholesterol. This makes therapeutic interpretation of cholesterol lowering in normal rodents difficult. In case of hyperlipidemic rats, the plasma cholesterol is predominantly LDL-cholesterol, which reflects the clinical situation more closely. PPAR-γ ligands failed to show any activity in this model (data not shown). DRF 2519 showed better efficacy than fenofibrate as well as KRP 297 in this model. To confirm the PPAR-α activation by DRF 2519 and fenofibrate in this model, we measured the enhancement of Acyl CoA oxidase (a well-known marker of PPAR-α target gene) message level. DRF 2519 and fenofibrate at 30 mg/kg showed significant enhancement of Acyl CoA oxidase mRNA, whereas at similar dose, rosiglitazone showed no significant enhancement. To get further insight on the effect of DRF 2519 on lipid synthesis in liver, we have used human hepatoma cell lines (HepG2) and studied the effect on total lipid synthesis. DRF 2519 showed significant reduction in total lipid synthesis at 10 μM concentration, whereas rosiglitazone did not show any reduction even at 100 μM concentration (data not presented). Fenofibrate, on the other hand, shows similar effect but only at a concentration of 250 μM onwards. This data further substantiate the role of PPAR-α activation for the potent hypolipidemic activity of DRF 2519.

The mechanism responsible for hypertriglyceridemia associated with insulin resistance may be complex but a commonly proposed feature for most of the mechanisms involves overproduction of VLDL triglyceride and VLDL apolipoprotein B by the liver and decreased triglyceride uptake in peripheral tissues (Grundy et al., 1979; Howard, 1987; Reaven and Chen, 1998; Gliemann et al., 1972). DRF 2519 treatment in hyperlipidemic rats showed reduction in hepatic triglyceride secretion, increased clearance of plasma triglyceride and also reduction of elevated plasma apolipoprotein B levels. Although our animal models show similar lipoprotein profile as that of humans, in order to confirm the effect of DRF 2519 on VLDL apolipoprotein B metabolism, we have used HepG2 cell line and measured the apolipoprotein B secretion. This class of pharmacophore has high protein binding nature (data not shown); perhaps this could be one of the reasons for high concentrations requirements. No toxicity to the cells was noticed even at 300 μM concentration of the drug. It is known that lipoprotein lipase plays an important role in removal of plasma triglyceride by hydrolysing the triglycerides of VLDL and chylomicron particles (Goldberg, 1996). DRF 2519 increase the lipoprotein lipase activity associated with fat tissue, which might be the major mechanism for increased clearance of plasma triglyceride. Taken together, DRF 2519 treatment normalises increased hepatic triglyceride secretion and increased peripheral clearence of triglyceride, which leads to improvement of dyslipidemia in hyperlipidemic animal models. Recently, it has been shown that insulin resistance promotes the development of vasospastic angina (Shinozaki et al., 1995) as well as obstructive coronary artery disease in nondiabetic subjects (Shinozaki et al., 1996). In addition, abnormalities in endothelium-dependent arterial relaxation are also described in several insulin resistance states including hypertension and obesity (Baron et al., 1993; Steinberg et al., 1996). Several epidemiological studies indicate a close relationship between plasma insulin level and cardiovascular disease (Defronzo et al., 1992; Reaven, 1988; Haffner et al., 1992). In view of this, a drug which reduce insulin level by virtue of its insulin-sensitizing property was expected to be beneficial to cardiovascular disease. In isolated rat aorta experiment, the pretreatment with DRF 2519 significantly improved insulin-induced relaxation. This effect of DRF 2519 may be due to sensitization of vascular tissue to insulin action or other mechanism involving of calcium channel (Ali et al., 1999). United Kingdom Prospective Diabetes Study trial results indicate that any improvement in glycemic control across the diabetic range in type 2 diabetes patients is likely to reduce the cardiovascular complications, which is secondary to diabetes (United Kingdom Prospective Diabetes Study Group, 1998). In a nutshell, all these observations suggest that a molecule like DRF 2519 would also have beneficial effect in insulin resistance-induced hypertension and cardiovascular disease.

In summary, DRF 2519, by virtue of its dual PPAR-α and PPAR-γ ligand property, and thereby through its action on both liver and adipose tissue, shows better amelioration of hyperglycemia, hyperinsulinemia, abnormal lipid metabolism and hypertension than the PPAR-α or PPAR-γ selective ligands. Our data along with the KRP 297 data also prove that thiazolidonediones cannot be generalised as the selective ligand of PPAR-γ alone. We believe that DRF 2519 can be beneficial to a whole range of complications associated with type 2 diabetes.

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