Mechanism and Implications of Brown Adipose Tissue Proliferation in Rats and Monkeys Treated with the Thiazolidinedione Darglitazone, a Potent Peroxisome Proliferator-Activated Receptor-γ Agonist

MICHAEL D. ALEO, GREGG R. LUNDEEN, DAVID K. BLACKWELL, WARD M. SMITH, GERALD L. COLEMAN, STANLEY W. STADNICKI, and WILLIAM M. KLUWE


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ABSTRACT

Thiazolidinediones represent an established class of insulin sensitizing agents for treating noninsulin-dependent diabetes mellitus. Darglitazone, a thiazolidinedione approximately 200-fold more potent than ciglitazone, was evaluated in preclinical safety assessment studies using rats (1, 5, and 50 mg/kg/day) and cynomolgus monkeys (50, 75, and 100 mg/kg/day). Darglitazone was a potent adipogenic agent in rats, causing hyperplastic/hypertrophic changes and firmness of white and perirenal, dorsal thoracic, and interscapular brown adipose tissue (BAT). Progressive changes in BAT size, morphology, firmness, and fatty acid composition preceded clinical signs of impaired respiration and the subsequent development of a dose-dependent, life-threatening hydrothorax. The characteristics of the pleural effusate were consistent with lymphatic fluid. These adverse effects were ameliorated/reversed upon drug withdrawal and were insulin-dependent since rats rendered totally insulinopenic by streptozotocin pretreatment did not develop BAT changes or hydrothorax. Although the effects of darglitazone on BAT changes were consistent with enhanced sensitivity to endogenous glucocorticoids, adrenal-ectomy, and dietary dehydroepiandrosterone administration were without a protective effect. Treated monkeys also developed white and BAT hyperplasia/hypertrophy, peripheral edema, and hydrothorax-related morbidity/mortality. Both species developed reversible, dose-related reductions in red blood cell parameters and follicular atresia. Peripheral and pulmonary edema are purportedly a multifactorial process involving vasodilation, increased endothelial permeability, and/or plasma volume expansion due to reduced renal sodium excretion. Moreover, profound alterations in BAT hypertrophy/hyperplasia/firmness may lead to discrete hydrothorax by restricting normal thoracic lymphatic drainage. Similar effects on adipose tissue, hemodilution, and edema (peripheral and pulmonary) were observed clinically with darglitazone and/or several other structurally similar/dissimilar PPAR-γ agonists.

Ciglitazone, englitazone, and troglitazone represent early examples of thiazolidinediones, a new class of oral hypoglycemic agents, that are efficacious in several rodent models of noninsulin-dependent diabetes mellitus (NIDDM) (Hulin et al., 1996) and entered clinical development around the 1990s.

Like currently marketed thiazolidinediones such as pioglitazone (Hoffman et al., 1991) and rosiglitazone (Kramer et al., 2001), this class of compounds sensitizes skeletal muscle and adipose tissue to the effects of insulin (Hulin et al., 1996; Saltiel and Olefsky, 1996). Unlike sulfonylureas or biguanides, which stimulate insulin secretion directly from functioning pancreatic β-cell islet cells, this class of compounds increases the translocation of glucose transporters from intracellular storage pools to the plasma membrane of insulin-sensitive tissues (Hulin et al., 1996; Saltiel and Olefsky, 1996; Petrie et al., 1997). Although thiazolidinediones were initially developed without specific knowledge of a molecular mechanism of action, later work by researchers such as Leh-

ABBREVIATIONS: NIDDM, noninsulin-dependent diabetes mellitus; PPAR-γ, peroxisome proliferator-activated receptor-γ; BAT, brown adipose tissue; IBAT, interscapular brown adipose tissue; TBAT, thoracic brown adipose tissue; STZN, streptozotocin; CORT, corticosterone; DHEA, dehydroepiandrosterone; SHAM, sham-operated; ADX, adrenalectomized; VMH, ventromedial hypothalamus; AUC, area under the concentration curve; Cmax, concentration of drug at peak serum levels.
mann et al. (1995) subsequently defined the mode of action and receptor target as agonist activity at the peroxisome proliferator-activated receptor-γ (PPAR-γ). This nuclear hormone receptor is expressed to a greater extent in adipose tissue relative to skeletal muscle and liver tissue (Braissant et al., 1996; Vidal-Puig et al., 1996). Heterodimerization of this receptor with the retinoid X receptor regulates genes that control expression and translocation of glucose transporters. This effect lowers peripheral insulin resistance and restores glycemic control by enhancing glucose disposal in peripheral tissues.

Despite numerous articles publicizing the clinical efficacy and molecular mechanism of action of thiazolidinediones, information in the peer-reviewed literature regarding the safety profile of this class of compounds in multiple animal species is incomplete. For example, ciglitazone reportedly caused cata- racts in animals (Kraegen et al., 1989). Tanabe-174 (approximately 10× more orally efficacious than ciglitazone) caused a nonregenerative anemia and cardiac hypertrophy in rodents and dogs at drug levels near projected therapeutic levels in humans (Williams et al., 1993), whereas DRP-2189 caused he- modilation and cardiac hypertrophy in rats (Lohray and Bhus- han, 1999). Troglitazone, the first approved compound of this class for humans, was subsequently removed from the market- place after it was associated with a low incidence of liver injury in diabetics (Gale, 2001). The preclinical evaluation and develop- ment of newer more potent drug candidates of this pharma- cological and/or structural class would be facilitated if more were known regarding correlations between the safety profile of these agents in animals and humans. Understanding the spec- ificity and degree of concordance would enhance risk assess- ment and management strategies before initiation of clinical trials since this type of information can be used to set margins of safety based on experience and develop biomarkers used in the conduct of clinical trials. Still, complete disclosure of the preclinical safety assessment profile of earlier clinical candi- dates along with more potent and structurally diverse com- pounds compared with any of their adverse effects observed in human clinical trials are warranted in the peer-reviewed liter- ature.

The present report describes our experience with the safety profile of darglitazone in rats and cynomolgus monkeys. Dar- glitazone has an oral potency at least 10 to 200× greater than troglitazone and ciglitazone, respectively (Hulin et al., 1996). At the receptor level, darglitazone is approximately 20 to 150× more potent than resiglitazone and pioglitazone, respec- tively, at displacing [3H]darglitazone from PPAR-γ bind- ing (based on unpublished IC50 values from Donald E. Wild- er; Pfizer Global R&D). Compared with in-house experience with an earlier less potent thiazolidinedione (englitazone), darglitazone caused dramatic effects on white and brown adipose tissue and unexpected morbidity/mortality in rats and monkeys due to hydrothorax during the conduct of sub- acute safety studies at maximum tolerated daily doses. Be- cause of the similarity and severity of this finding in two preclinical toxicology species, additional studies were con- ducted to generate a perspective regarding the potential rel- evance of these preclinical findings to humans, at that time in the absence of any relevant clinical safety data regarding darglitazone or this class of compounds. The relationship between deaths due to hydrothorax to the profound hyper- trophic/hyperplastic changes observed in BAT was investi- gated in rats to determine a probable mechanism of action. The effects of darglitazone observed in treated rats and mon- keys (peripheral and pleural fluid retention) were consistent with some of the clinical effects observed with this compound during clinical trials. The similarities of these reversible findings in humans treated with darglitazone to what is now known about other structurally similar and diverse PPAR-γ agonists suggest that alterations in adipose tissue, hemoglo- bin and hematocrit levels (hemodilation), and fluid retention (edema) may represent a pharmacologic rather than a struc- tural class effect.

Materials and Methods

Animals. Male and female Long-Evans or female Sprague-Daw- ley rats (150–240 g) were obtained from Charles River Breeding Laboratories (Wilmington, MA) and allowed free access to water and Agway Probal RMH 3200 diet (Agway Country Foods, Inc., Syracuse, NY) unless noted otherwise. Rodents were housed individually in wire rack cages in an environmentally controlled room on a 12-h light/dark cycle. Male and female cynomolgus monkeys (Macaca fascicularis) were obtained from Charles River Research Primates Center (Port Washington, NY), housed in an environmentally con- trolled space with a dawn to dusk light cycle, and fed once daily with a complete nutritionally balanced diet (Agway Primate 18) enriched with seasonal fruits and vegetables. Animal care and use were con- ducted in accordance with all applicable state and federal regu- lations and guidelines. These activities complied with or exceeded the Animal Welfare Act Regulation, title 9 CFR parts 1 to 3, and the Association for Assessment and Accreditation of Laboratory Animal Care, International Standards as set forth by the Guide for the Care and Use of Laboratory Animals. Our facilities are registered as a research facility with the U.S. Department of Agriculture-Animal and Plant Health Inspection Service-Regulatory Enforcement of An- imal Care. An institutional Animal Care and Use Committee ap- proved all animal procedures used in the conduct of these studies.

Materials. Streptozotocin (STZ), corticosterone (CORT), and de- hydroepiandrosterone (DHEA) were obtained from Sigma-Aldrich (St. Louis, MO). STZ was dissolved in 0.01 M sodium citrate buffer (pH 7.4), and CORT was dissolved in peanut oil immediately before use. DHEA was administered in the diet to animals as a ground feed ad- mixture at a level of 0.6%. Darglitazone was administered orally as the sodium salt, and all daily doses were expressed as the free acid equiva- lent based on an activity factor of 94.5%. All dose levels stated in the text are based on the weight of the free acid. Darglitazone, chemically known as [±]-5-(4-(3-(5-methyl-2-phenyl-4-oxazolyl)-propionyl)phenyl- methyl)-thiazolidine-2,4-dione-sodium salt, was synthesized according to good manufacturing processes and contained <0.5% impurities based on various analytical techniques designed to determine the pre- sence of process related impurities. The compound was dissolved daily before use in distilled water and administered at a dosing volume of 10 ml/kg. Rats were dosed daily approximately 1 h after the beginning of the light cycle. Monkeys were dosed daily approximately 3 h before receiving their daily food ration at 11:00 AM.

General Experimental Design. Animals were assessed daily for clinical signs of toxicity. Body weight and food consumption were determined weekly. For toxicokinetic determinations, animals were periodically bled in the nonfasted state during the conduct of the study to quantitate serum drug levels. Potential changes in hema- tological parameters and serum chemistry were determined peri- odically during the course of study by collecting blood from rats (retroorbital sinus) or monkey (venopuncture) in the fasted state before receiving the daily dose of darglitazone. Urinalysis was per- formed on samples obtained from fasted animals after an overnight collection period. Samples were analyzed manually or using auto- mated analyzers (Technicon H-1E (Technicon, White Plains, NY), Hitachi 704/717 (Boehringer Mannheim Corp., Indianapolis, IN),
Experiment I: Standard 3-Month Subacute Toxicity Assessment in Rats. Male and female rats were treated daily for 3 months by oral gavage at doses of 0, 1, 5, and 50 mg/kg/day (15 animals/sex/group).

Experiment II: Chronology and Reversibility Study in Rats. In a separate study, female rats were treated daily for 5 to 36 days by oral gavage at doses of 0 and 50 mg/kg/day (6–20 animals/group). Nine treatment groups, along with corresponding controls, were treated as follows (days of treatment/days of reversal): 5/0, 10/0, 20/0, 20/20, 30/0, 30/30, 36/0, 36/19, and 36/40 to assess the onset and reversibility of the observed toxicity.

Experiment III: Role of Insulin or Insulin-Dependent/Regulated Processes. In another series of studies, four groups of female rats (12–18 animals/group) were treated by daily oral gavage at doses of 0 and 50 mg/kg/day for two months. Animals were assigned to either a control group (normal or STZN-pretreated) or a darglitazone treated group (normal or STZN-pretreated). Rats were rendered insulinopenic through the destruction of pancreatic islet cells by pretreating the animals with a single dose of STZN (75 mg/kg, i.v.) 8 days before initiation of darglitazone treatment. Fasting serum glucose levels before the start of treatment were: 103 ± 8 mg/dl in normal animals, 360 ± 154 mg/dl in STZN-pretreated animals administered vehicle, 99 ± 6 mg/dl in normal animals subsequently administered darglitazone for 2 months, and 345 ± 122 mg/dl (mean ± S.D.) in STZN-pretreated animals subsequently administered darglitazone for two months.

Experiment IV: Role of Endogenous Glucorticoids. In this study, female rats were treated daily for 2 weeks by oral gavage at doses of 0, 5, and 50 mg/kg/day (eight animals per group). Surgically altered rats were obtained from Charles River Breeding Laboratories (Releigh, NC). Rats were either sham-operated (SHAM) or adrenalectomized (ADX) 2 weeks before initiation of treatment. ADX animals had their drinking water replaced with normal saline to maintain proper salt balance. CORT was administered in peanut oil at a dose of 20 mg/kg/day s.c. (1 ml/kg) to half of the SHAM and ADX animals placed on treatment. In a separate series of studies, normal animals were supplemented with 0.6% DHEA in ground diet. Darglitazone-treated animals were pair-fed to their ADX or DHEA respective controls to account of hyperphagic episodes observed in previous studies.

Experiment V: Subchronic Toxicity/Reversibility Assessment in Monkeys. Male (3.4–5.4 kg) and female (2.7–3.6 kg) cynomolgus monkeys (Charles River Primate Co.) were treated daily by oral gavage at doses of 0, 50, 75, and 100 mg/kg/day (2/sex/dose) for 2.5 to 3.5 months. Because morbidity/mortality was observed during the in-life portion of the study, animals given darglitazone at 50 mg/kg/day were treated for 106 days, with an interim necropsy on days 71 to 72, followed by a reversibility period of 50 days with the remaining animals. Animals given 0, 75, and 100 mg/kg/day were treated for 71 to 72 days, at which time an interim necropsy was performed. The remaining animals were followed for 85 days to assess reversibility of the drug effects.

Analysis of Serum Darglitazone Levels. Whole blood was collected periodically during the rat (retro-orbital sinus) and monkey studies (venopuncture). Serum samples were deproteinized with 1 N HCl before extraction with ethyl acetate. The organic phase was then alkalinized with 0.01 M NaOH, dehydrated with sodium sulfate, and evaporated to dryness before being reconstituted in mobile phase (45:55 v/v acetonitrile/10 mM phosphate buffer, pH 3.0) for analysis. The extracted serum samples were analyzed under isocratic conditions by reverse phase high-performance liquid chromatography using a Nova-Pak C18 column (150 × 3.9 mm, 4-μm packing) from Millipore Corp. (Milford, MA). Detection was at 280 nm, and the flow rate was 1.2 ml/min.

Fatty Acid Analysis. Tissues were suspended in methanolic sodium hydroxide and heated in an airtight tube at 100°C for 30 min. Upon cooling to room temperature, acidified boron trichloride was added to the tissue. The acidified suspension was heated at 85°C for 10 min. Upon cooling to room temperature, fatty acid methyl esters were extracted by the addition of a 50:50 mixture of hexane/ether. Fatty acid methyl esters were analyzed by injection of the organic phase into an HP 5970A series gas chromatograph (Hewlett Packard, Palo Alto, CA) equipped with an HP-1 silicone gum capillary column. Ramp conditions were raised at a rate of 20°C/min from 50–250°C. Peaks were assigned based on comparisons with retention times with authentic standards.

Statistical Analysis. Comparisons between two treatment groups were made using an unpaired Student's t test. Comparisons among multiple treatments were made using a one-way analysis of variance followed by Fisher's protected least significant difference test. Statements of significance were based on a p value <0.05. All results are expressed as mean ± S.D.

Results

Experiment I: Three-Month Subacute Toxicity Assessment in Rats. During the conduct of the subacute toxicity assessment, there were no discernible changes in clinical pathologic parameters or histologic changes suggestive of major organ damage (i.e., liver, heart, and kidneys) to either male or female rats during 3 months of continuous daily dosing (data not shown). Nevertheless, there was evidence of a mild dose-related alteration in erythrocyte parameters in both male and female rats that were observed within 1 month of treatment. These changes were evident during the first analysis that was determined 30 days after consecutive daily treatment. Broad 10 to 15% reductions in circulating red blood cells, hemoglobin, and hematocrit along with a 57 to 100% increase in circulating reticulocytes (Table 1) were observed in the absence of any bone marrow changes that would be consistent with hematotoxicity. Monthly assessment of clinical pathology parameters showed that the specific effects and magnitude of these changes in erythrocyte parameters were stable and persisted during the 3-month treatment period (data not shown). These findings are consistent with the now well known hemodilution effect of thiazolidinediones.

Darglitazone administration to rats was associated with a time- and dose-dependent increase in morbidity/mortality that became evident after the first month of consecutive daily exposure. The total incidence of morbidity/mortality, which occurred during the 3-month study, can be found in Table 1. The 2-fold higher incidence of mortality in the high-dose female rats corresponded with a 2-fold higher serum darglitazone level compared with high-dose males (22.4 ± 2.9 vs. 10.5 ± 1.7 μg of darglitazone/ml of serum at Cmax, respectively, p < 0.05). Death was preceded by clinical signs of impaired respiration (cyanosis and dyspnea) and was ultimately attributed to the accumulation of a clear acellular nonclotting fluid in the thoracic cavity (hydrothorax) of all involved rats during post-mortem examination.

Progressive changes in the gross appearance, size, and palpable firmness of IBAT were observed to varying degrees in all treated male and female rats that survived the 3-month study and in all animals that died before the end of study. At the time of necropsy, there was a pronounced discoloration and increased size and firmness of IBAT in treated animals. IBAT was transformed in treated animals from a small soft, dark-rust brown colored fat mass to a large firm, off-white colored IBAT mass in treated animals (Fig. 1, A and B). In
addition, the profound adipogenic activity of darglitazone was clearly evident in the TBAT areas that are found along the descending aorta and major lymphatic trunks and within the thoracic cavity and the perirenal area (Fig. 1, C and D). Examination of BAT by light microscopy revealed morphological transformation of adipocytes from small multiloculated to large uniloculated lipid droplet containing cells (photomicrographs not shown). There was also evidence of an increase in white adipose tissue mass. Although there were no pathological changes found in major organ systems (heart, lung, kidney, brain, bone marrow, and testes), there were significant changes in ovarian morphology of treated female rats. These changes consisted of a decrease in the number of corpusa lutea as well as an increase number of atretic follicles (Table 2). The incidence and severity of these ovarian changes were dose-related. Subsequent analyses showed a treatment-related decrease (2-fold) in serum progesterone levels at daily doses of 5 and 50 mg/kg (data not shown).

**Experiment II: Chronology and Reversibility Study in Rats.** Because of the profound effects observed in the first

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose mg/kg/day</th>
<th>Mortality Incidence</th>
<th>Serum Drug Levels</th>
<th>RBC</th>
<th>Hgb</th>
<th>HCT</th>
<th>Reticulocytes % vol</th>
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<tbody>
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<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>0/15</td>
<td>0.0 ± 0.0a</td>
<td>8.09 ± 0.25a</td>
<td>15.85 ± 0.43a</td>
<td>50.01 ± 1.06a</td>
<td>2.64 ± 1.02a</td>
</tr>
<tr>
<td>Low</td>
<td>1</td>
<td>0/15</td>
<td>0.4 ± 0.1b</td>
<td>7.97 ± 0.22a</td>
<td>15.59 ± 0.46a</td>
<td>48.89 ± 1.59a</td>
<td>2.25 ± 0.96a</td>
</tr>
<tr>
<td>Mid</td>
<td>5</td>
<td>1/15</td>
<td>1.7 ± 0.4c</td>
<td>7.63 ± 0.29b</td>
<td>15.11 ± 0.38b</td>
<td>47.85 ± 1.17b</td>
<td>2.53 ± 0.94a</td>
</tr>
<tr>
<td>High</td>
<td>50</td>
<td>7/15</td>
<td>10.5 ± 1.7d</td>
<td>6.64 ± 0.55c</td>
<td>13.57 ± 0.75c</td>
<td>42.91 ± 2.41c</td>
<td>4.14 ± 2.27c</td>
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<tr>
<td>Female</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
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<td>0/15</td>
<td>0.0 ± 0.0a</td>
<td>8.16 ± 0.36a</td>
<td>16.46 ± 0.80a</td>
<td>51.75 ± 2.04a</td>
<td>2.26 ± 0.46a</td>
</tr>
<tr>
<td>Low</td>
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<td>0.5 ± 0.2b</td>
<td>7.75 ± 0.47b</td>
<td>15.45 ± 0.62b</td>
<td>48.61 ± 1.99b</td>
<td>2.99 ± 1.26b</td>
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<tr>
<td>Mid</td>
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<td>1/15</td>
<td>2.3 ± 0.7c</td>
<td>7.45 ± 0.38b</td>
<td>15.17 ± 0.67b</td>
<td>47.49 ± 1.66b</td>
<td>3.27 ± 1.50b</td>
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<tr>
<td>High</td>
<td>50</td>
<td>15/15</td>
<td>22.4 ± 2.9d</td>
<td>6.76 ± 0.73c</td>
<td>14.29 ± 1.29c</td>
<td>44.89 ± 4.05c</td>
<td>4.56 ± 1.72c</td>
</tr>
</tbody>
</table>

RBC, red blood cells; HCT, hematocrit; Hgb, hemoglobin.

**Fig. 1.** Representative photographs of control (A and C) and darglitazone-treated (B and D) rats from experiment I. Dorsal view of interscapular brown adipose tissue (IBAT) in control (A) and a rat treated with 50 mg/kg/day darglitazone for 36 days (B). Note increased size and off-white coloration if IBAT (arrow) compared with the smaller normal rust-like coloration of control IBAT (arrow). Deep ventral view of thoracic brown adipose tissue (TBAT) of the same control (C) and treated animal (D). Note proliferation of TBAT around the descending aorta, paravertebral lymphatic trunks, and perirenal area (arrows), and infiltration of TBAT in the dorsal chest wall. This animal had a fat body firmness of 3 mm Hg, showed some clinical signs of dyspnea and cyanosis, but no appreciable amount of fluid in the thoracic cavity.
TABLE 2
Experiment I: Severity and incidence of morphological changes in ovaries after three month subchronic toxicity assessment of darglitazone in female rats

<table>
<thead>
<tr>
<th>Grade/Incidence</th>
<th>Control (1 mg/kg/day)</th>
<th>Low (5 mg/kg/day)</th>
<th>Mid (10 mg/kg/day)</th>
<th>High (50 mg/kg/day)</th>
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<tr>
<td>Absent</td>
<td>15</td>
<td>12</td>
<td>4</td>
<td>0</td>
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<td>+</td>
<td>0</td>
<td>2</td>
<td>5</td>
<td>6</td>
</tr>
<tr>
<td>++</td>
<td>0</td>
<td>1</td>
<td>6</td>
<td>6</td>
</tr>
<tr>
<td>+++</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>3</td>
</tr>
<tr>
<td>Incidence</td>
<td>0/15</td>
<td>3/15</td>
<td>11/15</td>
<td>15/15</td>
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</table>

*Grade/Incidence key: 0, no change from control; 1, small increase in size; 2, large increase in size; 3, large increase in size and firmness.*

3-month rat study, female rats were used in subsequent studies to investigate 1) the chronology and reversibility of events associated with prolonged treatment with darglitazone (based on animal survival, hyperplastic/hypertrophic changes in BAT, red blood cell parameters, and ovarian morphology), 2) the probable relationship between thoracic fluid volume and the proliferation/firmness of BAT, and 3) hypothesized changes in the fatty acid composition of BAT. At 50 mg/kg/day (where serum darglitazone levels were 21.2 ± 8.9 µg/ml at Cmax), palpable increases in IBAT size and firmness were noted within 5 days of drug administration (Fig. 2). IBAT size and firmness (based on palpation) progressed as a function of time during treatment and was not fully reversed even after drug withdrawal (Fig. 2). Animal survival was not adversely affected until IBAT size and firmness were at a maximum. Within 21, 28, and 36 days of treatment 1/46 (2%), 3/46 (7%), and 8/46 (18%) of all treated animals expired or were euthanized in moribund condition (Fig. 2). Mortality increased with increasing length of treatment in the presence of hydrothorax. The incidence of morbidity/mortality paralleled the incidence of hydrothorax found at necropsy. Two additional animals died of hydrothorax during the 1-month recovery period.

The presence of hydrothorax was difficult to detect in rats until potentially life-threatening and obvious signs of pulmonary dysfunction were present. Therefore, we also determined whether there was a relationship between the volume of fluid found in the pleural cavity and the firmness of dorsal TBAT (determined at necropsy) with the size and firmness of IBAT (a palpable clinical assessment). Trying to develop a clinically assessable and relevant marker of this effect was necessary since the amount of fluid in the thoracic cavity and the mass and firmness of dorsal TBAT and aortic BAT cannot be followed during the in-life portion of the study. In general, thoracic fluid volume increased with increasing TBAT toxicity such that the greatest accumulation of thoracic fluid occurred in rats with TBAT toxicity ≥10 mm Hg (Fig. 3). The severity of dyspnea and cyanosis observed before death also correlated well with the presence and volume of pleural fluid found in the thoracic cavity at necropsy (data not shown). By clinical assessment, there was a good correlation between palpable IBAT size and firmness in animals with the greatest accumulation of fluid. This suggested that in the rat IBAT size and firmness could be used as surrogate markers of TBAT firmness and thoracic fluid accumulation when present.

Fluid that was found in the pleural cavity at necropsy was further characterized to determine its etiology. Based on its appearance (clear), color (pale yellow), specific gravity (range 1.020–1.027), cellularity (mostly acellular), and glucose (range 120–182 mg/dl), protein (2.8–4.1 g/dl) and lactate dehydrogenase (30–200 U/l) content, the characteristics of the fluid were consistent with a modified transudate derived from plasma. These results suggest that the pleural fluid was lymphatic in origin. From this, we deduced that the accumulation of fluid in the thoracic cavity may be due to impaired lymphatic drainage caused by the increased firmness and size of TBAT in the rat.

Although the composition of dietary fat intake remained constant during the study, there were distinct biochemical changes in the fatty acid composition of TBAT from treated animals. Compared with controls, TBAT from treated animals (50 mg/kg/day for 36 days) had a 274% increase in the unsaturated fatty acid of palmitoleic acid (C16:1) combined with a 36% reduction in saturated fatty acid content of stearic acid (C18:0) (Table 3). There was a 16% reduction in the ratio of unsaturated to saturated fatty acids in TBAT.
The two STZN-pretreated animals that died of hydrothorax (dyspnea and cyanosis) and had pleural fluid volumes ranging between 1 to 15.5 ml found in the thoracic cavity at the time of necropsy. Therefore, only rats that were marginally insulinopenic and had no histologic evidence of sustained pancreatic damage from the STZN pretreatment succumbed to hydrothorax.

All normoglycemic rats treated with darglitazone developed the characteristic profound increase in prominence and firmness of IBAT mass, dorsal thoracic and perirenal BAT hypertrophy/hyperplasia, and follicular atresia. In contrast, half of the STZN-pretreated animals that received darglitazone (9/18 animals) had only small to moderate increases in BAT mass (dorsal thoracic, interscapular, and perirenal). Follicular atresia was also dependent upon endogenous insulin since STZN pretreatment reduced the incidence and severity of this effect compared with normal animals treated with darglitazone (data not shown).

**Experiment IV: Role of Endogenous Glucocorticoids.**

Darglitazone-treated animals share similar features to genetically obese or VMH-lesioned animals (e.g., BAT enlargement, discoloration, and morphological changes in the relative content of certain BAT fatty acids, hyperphagia, and changes in ovarian function). Since these genetically obese or VMH-lesioned animals are overly sensitive to endogenous glucocorticoids, we investigated whether the adverse effects of darglitazone on BAT proliferation were related to enhanced sensitivity to endogenous glucocorticoids. Animals were either ADX or SHAM before study. Half of all ADX and SHAM animals received a replacement dose of corticosterone daily, as described under Materials and Methods.

After 2 weeks of study, untreated ADX and SHAM rats had an equivalent IBAT mass of 0.26 ± 0.06 g and 0.34 ± 0.07 g (p > 0.05), respectively (Table 4). SHAM animals treated with darglitazone developed an IBAT mass approximately twice as large but were exposed to twice the serum levels of darglitazone (C_{max}) compared with ADX animals treated at the same dose. Daily replacement of corticosterone resulted in the development of similarly sized IBAT mass and serum darglitazone levels (C_{max}) in both treated SHAM and ADX animals. This effect was observed at both doses of darglitazone (5 and 50 mg/kg/day). These results were identical whether the effect on IBAT size was expressed as C_{max} or as parent drug exposure over the course of 8 h (AUC_{0-8 h}). The 50% reduction in IBAT size in darglitazone-treated ADX animals corresponded to the 50% reduction in serum drug levels rather than the nearly 100% loss of serum corticosterone.

DHEA supplementation in the diet of darglitazone-treated animals was ineffective in preventing BAT proliferation. There were no differences in BAT weight, serum darglitazone exposure (C_{max} or AUC_{0-8 h}), and baseline corticosterone levels between darglitazone-treated animals given a normal or DHEA-supplemented diet (data not shown). Although dietary supplementation with 0.6% DHEA effectively antagonizes the enhanced sensitivity to endogenous levels of corticosterone in genetically obese animals, the lack of an effect further illustrates that darglitazone does not enhance sensitivity to endogenous glucocorticoids.
TABLE II: Fatty acid composition of thoracic brown adipose tissue obtained from darglitazone treated female rats

Rats were treated daily by oral gavage at 50 mg/kg/day for either 1 month (darglitazone-treated) or 1 month followed by a reversal period of 1 month (darglitazone-treated reversal) before assessment of fatty acid content. Values shown are the relative percentages of total fatty acids expressed as means ± S.D. (n = 6).

<table>
<thead>
<tr>
<th>Fatty Acid Type</th>
<th>Control</th>
<th>Darglitazone-Treated</th>
<th>Control</th>
<th>Darglitazone-Treated Reversal</th>
<th>Control</th>
<th>Darglitazone-Treated Reversal</th>
</tr>
</thead>
<tbody>
<tr>
<td>Palmitoleic (C16:1)</td>
<td>3.5 ± 0.8</td>
<td>9.6 ± 2.4*</td>
<td>2.7 ± 1.0</td>
<td>5.9 ± 1.7*</td>
<td>3.5 ± 0.8</td>
<td>9.6 ± 2.4*</td>
</tr>
<tr>
<td>Palmitic (C16:0)</td>
<td>19.2 ± 2.4</td>
<td>19.4 ± 0.9</td>
<td>17.9 ± 1.6</td>
<td>18.6 ± 1.0</td>
<td>19.2 ± 2.4</td>
<td>19.4 ± 0.9</td>
</tr>
<tr>
<td>Linoleic (C18:2)</td>
<td>24.0 ± 2.5</td>
<td>22.9 ± 2.3</td>
<td>23.0 ± 2.7</td>
<td>21.9 ± 1.2</td>
<td>24.0 ± 2.5</td>
<td>22.9 ± 2.3</td>
</tr>
<tr>
<td>Oleic (C18:1)</td>
<td>31.5 ± 3.3</td>
<td>32.1 ± 1.1</td>
<td>29.2 ± 2.4</td>
<td>35.0 ± 1.2*</td>
<td>31.5 ± 3.3</td>
<td>32.1 ± 1.1</td>
</tr>
<tr>
<td>Stearic (C18:0)</td>
<td>6.9 ± 0.6</td>
<td>4.4 ± 1.3*</td>
<td>7.0 ± 1.0</td>
<td>4.9 ± 1.1*</td>
<td>6.9 ± 0.6</td>
<td>4.4 ± 1.3*</td>
</tr>
<tr>
<td>Total saturated</td>
<td>26.1 ± 2.9</td>
<td>23.8 ± 0.9</td>
<td>24.9 ± 1.5</td>
<td>23.4 ± 1.4</td>
<td>26.1 ± 2.9</td>
<td>23.8 ± 0.9</td>
</tr>
<tr>
<td>Total unsaturated</td>
<td>59.0 ± 1.6</td>
<td>64.6 ± 1.5*</td>
<td>54.9 ± 3.6</td>
<td>62.8 ± 1.6*</td>
<td>59.0 ± 1.6</td>
<td>64.6 ± 1.5*</td>
</tr>
<tr>
<td>Ratio (sat./unsat.)</td>
<td>0.44 ± 0.04</td>
<td>0.37 ± 0.02*</td>
<td>0.45 ± 0.02</td>
<td>0.37 ± 0.03*</td>
<td>0.44 ± 0.04</td>
<td>0.37 ± 0.02*</td>
</tr>
</tbody>
</table>

sat., saturated; unsat., unsaturated.

* Results are significantly different from corresponding controls (P < 0.05).

TABLE IV

Experiment IV: Summary of ADX and CORT replacement therapy in darglitazone-treated female rats

SHAM rats were pair-fed to their corresponding ADX rats for 14 days. Corticosterone (CORT) was administered daily (20 mg/kg s.c.) in peanut oil. Darglitazone was administered daily by oral gavage for 14 days. ADX animals were given free access to normal saline. Values with different superscripts within the same column were significantly different from each other by one-way analysis of variance followed by Fisher’s least significant difference test (means ± S.D., n = 3–4, P < 0.05).

<table>
<thead>
<tr>
<th>Treatment Dose</th>
<th>Serum CORT</th>
<th>IBAT Size</th>
<th>Cmax</th>
<th>Serum CORT</th>
<th>IBAT Size</th>
<th>Cmax</th>
</tr>
</thead>
<tbody>
<tr>
<td>mg/kg/day</td>
<td>ng/ml</td>
<td>g</td>
<td>µg/ml</td>
<td>ng/ml</td>
<td>g</td>
<td>µg/ml</td>
</tr>
<tr>
<td>Sham</td>
<td>271 ± 145</td>
<td>0.34 ± 0.07bc</td>
<td>N.D.</td>
<td>373 ± 114</td>
<td>0.41 ± 0.08a</td>
<td>N.D.</td>
</tr>
<tr>
<td>0</td>
<td>159 ± 66</td>
<td>1.06 ± 0.45a</td>
<td>3.0 ± 1.8</td>
<td>287 ± 222</td>
<td>1.07 ± 0.12a</td>
<td>2.8 ± 0.5</td>
</tr>
<tr>
<td>5</td>
<td>263 ± 246</td>
<td>2.34 ± 0.70bc</td>
<td>20.9 ± 3.0</td>
<td>243 ± 157</td>
<td>2.34 ± 0.60bc</td>
<td>31.8 ± 22.3</td>
</tr>
<tr>
<td>50</td>
<td>6 ± 9</td>
<td>0.26 ± 0.06bc</td>
<td>N.D.</td>
<td>46 ± 44</td>
<td>0.39 ± 0.06bc</td>
<td>N.D.</td>
</tr>
<tr>
<td>ADX</td>
<td>&lt;2</td>
<td>0.66 ± 0.15ab</td>
<td>0.4 ± 0.1</td>
<td>147 ± 131</td>
<td>1.05 ± 0.36bc</td>
<td>1.0 ± 0.2</td>
</tr>
<tr>
<td>5</td>
<td>&lt;2</td>
<td>1.36 ± 0.32bc</td>
<td>9.3 ± 4.1</td>
<td>174 ± 181</td>
<td>3.13 ± 1.31bc</td>
<td>19.1 ± 16.9</td>
</tr>
</tbody>
</table>

N.D., not determined.

TABLE V

Experiment V: Major hematological findings during the three month subchronic toxicity assessment of darglitazone in cynomolgus monkeys

Serum drug levels were determined during day 17 of daily oral gavage dosing. Erythrocyte parameters were determined 14 days after continuous daily dosing. This depressed trend in erythrocyte parameters persisted during the course of drug treatment. Mortality incidence was based on results at the end of the complete study (treatment plus reversibility period). Data are expressed as means ± S.D. (n = 4; two animals per sex). Values with different superscripts within the same parameter and sex distribution are significantly different from each other by one-way analysis of variance followed by Fisher’s least significant difference test (P < 0.05).

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Dose</th>
<th>Mortality Incidence</th>
<th>Cmax</th>
<th>AUC 0–24 h</th>
<th>RBC</th>
<th>Hgb</th>
<th>HCT</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>mg/kg/day</td>
<td>µg/ml</td>
<td>µg/hr/ml</td>
<td>10^6/mm³</td>
<td>g/dl</td>
<td>% vol</td>
<td></td>
</tr>
<tr>
<td>Male/Female</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0</td>
<td>0/4</td>
<td>0.0 ± 0.0a</td>
<td>0.0 ± 0.0a</td>
<td>5.92 ± 0.50a</td>
<td>11.58 ± 0.80a</td>
<td>40.72 ± 3.15a</td>
</tr>
<tr>
<td>Low</td>
<td>50</td>
<td>1/4</td>
<td>43 ± 6b</td>
<td>147 ± 22b</td>
<td>4.93 ± 0.28b</td>
<td>10.02 ± 0.54b</td>
<td>34.82 ± 1.76b</td>
</tr>
<tr>
<td>Mid</td>
<td>75</td>
<td>1/4</td>
<td>37 ± 3b</td>
<td>154 ± 12b</td>
<td>5.12 ± 0.32b</td>
<td>10.40 ± 0.57b</td>
<td>35.98 ± 1.98b</td>
</tr>
<tr>
<td>High</td>
<td>100</td>
<td>0/4</td>
<td>42 ± 8b</td>
<td>167 ± 46b</td>
<td>4.79 ± 0.24b</td>
<td>9.80 ± 0.48b</td>
<td>33.52 ± 1.27b</td>
</tr>
</tbody>
</table>

RBC, red blood cell counts; Hgb, hemoglobin; Hct, hematocrit.
hypertrophy of periaortic and perirenal adipose tissue (all treated animals) and scrotum (males only), mild erythroid hyperplasia of sternal marrow (all treated animals), and ectasia of the mammary ducts (one intermediate dosed male and female). These effects were observed at initial serum drug levels and exposures (C_{max} AUC_{0–24 h}) of 60 ± 23 μg/ml:228 ± 54 μg·h/ml, 82 ± 21 μg/ml:400 ± 147 μg·h/ml, and 68 ± 33 μg/ml:410 ± 225 μg·h/ml in the low-, intermediate-, and high-dose group (day 1), respectively. Serum drug levels and exposure declined during the first 17 days of treatment, the results of which are also presented in Table 5.

During the 50- to 85-day post-treatment recovery period, monkeys in the intermediate- and high-dose groups showed complete reversibility of depressions in erythrocyte parameters within 2 weeks after discontinuing treatment. Menstrual cycle and postovulatory progesterone surges returned in all previously treated females within 4 weeks post-treatment. There was no evidence of pericardial effusion or depressed QRS amplitudes during the reversal period. Hypertrophy of subcutaneous adipocytes surrounding the eye and/or edema (low- and high-dose animals), erythroid hyperplasia of sternal marrow (low- and high-dose animals), and ectasia of mammary ducts (one intermediate- and all high-dose animals) were present but less pronounced at final necropsy after completion of the reversal period detailed above (data not shown).

### Discussion

Safety assessment studies in at least two animal species (rodent and nonrodent) are typically conducted before entering clinical trials with experimental therapeutics. Building safety margins based on experience, as well as a perspective on how safety findings in animal studies eventually translate into clinical outcomes, is of paramount importance. Although thiazolidinediones are effective as a class in treating NIDDM in animals and humans, the concordance (degree and specificity) between the safety profile of specific thiazolidinediones in animals and humans is generally lacking in the peer-reviewed literature. This article reports the relevance of preclinical safety assessment findings in rats and nonhuman primates to humans of a potent thiazolidinedione, darglitazone.

The major adverse event identified in darglitazone-treated rats and monkeys was the development of a life-threatening hydrothorax. The high incidence of morbidity/mortality occurred at doses ≥5 mg/kg/day (serum drug exposure ≥2 μg/ml at C_{max}) in the rat and ≥50 mg/kg/day (serum drug exposure ≥37 μg/ml at C_{max}) in the monkey. Increased IBAT and TBAT size, changes in fatty acid composition, and morphological changes in the appearance of BAT cells were observed in rats within 5 days of treatment. As treatment progressed, IBAT and TBAT became larger and firmer (based on touch and tonicity measurements) and were followed by the accumulation of fluid (∼15.5 ml in rats and ∼200 ml in monkeys) within the thoracic cavity with clinical signs of peripheral edema, dyspnea, and hemodilution. Severely affected animals eventually succumbed to hydrothorax.

Mechanistically these effects appear to represent multiple aspects of an exaggerated pharmacologic response to insulin sensitization coupled to receptor target expression in select tissues. For example, fluid extravasation into tissue, in the form of peripheral and pleural edema, can be caused by several mechanisms. Troglitazone and rosiglitazone increase endothelial permeability (see review by Wang et al., 2002), whereas troglitazone, but not rosiglitazone, has vasodilatory properties by itself (Walker et al., 1998). Insulin has both vasodilatory (McNally et al., 1995) and hemodynamic (Baron, 1994) properties. Hemodilution has been observed with other thiazolidinediones due to plasma volume expansion, an effect thought to be due to reduced renal sodium excretion (see review by Niemeyer and Janney, 2002). Finally, hypertrophic/hyperplastic changes in BAT, the development of hydrothorax and mortality in rats were insulin-dependent since animals that were rendered totally insulinopenic with STZN were devoid of these effects. Since STZTN also profoundly reduces expression of PPAR-γ in adipose tissue (Vidal-Puig et al., 1996), it appears that the protective effect of STZTN is directly linked to significant reductions in both endogenous insulin levels and PPAR-γ expression in sensitive tissues.

Although many of the effects listed above appear to be common to other thiazolidinediones, to our knowledge no one has reported life-threatening hydrothorax in animals treated with thiazolidinediones. While BAT proliferation (eiglitazone and troglitazone) and firmness of dorsal thorax in rats (troglitazone) occurs with less potent thiazolidinediones (Chang and Wyse, 1988; Herman et al., 1997), only nonlife-threatening signs of labored breathing/rales were reported in troglitazone-treated rats (de la Iglesia et al., 1998). Differences in the physical characteristics of BAT may have some bearing on this manifestation. Darglitazone has opposing effects on the content of certain saturated/unsaturated fatty acids in BAT compared with cultured adipocytes treated with troglitazone or pioglitazone (Kurebayashi et al., 1997). Moreover, manifestation of a life-threatening hydrothorax may also have a mechanical component as well. Obstructive compression or narrowing of lymphatic vessels due to the presence and increased firmness (tonicity) of extensive BAT hypertrophy/hyperplasia around the descending aorta and major thoracic lymphatic trunks (Fig. 1) may be implicated in the pathogenesis of hydrothorax. Ohtani and Ohtani (1997) have shown that obstruction or narrowing of the lymphatic trunks draining the diaphragmatic lymph alone is sufficient to cause hydrothorax in rats. Since the majority of pleural fluid (87%) is drained through the lymphatic system in the dog (Nakamura et al., 1988), it is conceivable that abnormal fat accumulation around the major lymphatics may contribute to the development of hydrothorax in rats and monkeys. Based on these findings and limited in-house experience with another less potent thiazolidinedione (englitazone), the severity of these PPAR-γ-insulin-dependent responses (BAT proliferation and firmness, edema, and hydrothorax) would appear to be potency driven.

The possibility that these adipogenic effects were linked to enhanced sensitivity to endogenous glucocorticoids was quickly disproved. Despite some similarities in the clinical profile of darglitazone-treated rats to genetically obese or VMH-lesioned animals (i.e., adipogenic activity in the form of BAT proliferation, discoloration, morphological changes, and increases in palmitoleic and oleic acid content, hyperphagic episodes, and changes in ovarian function and histology) (Haessler and Crawford, 1965; Saito et al., 1985), we proved darglitazone did not enhance sensitivity to endogenous glucocorticoids. Even though ADX or DHEA dietary supplemen-
agitation effectively corrects these abnormalities in obese and VMH-lesioned animals, these same surgical and dietary manipulations were without substantial effect in darglitazone-treated animals. The partial protective effect observed in ADX animals was due to differences in drug exposure rather than changes in the glucocorticoid status or sensitivity of the animal (Table 4). Therefore, the profound adipogenic activity of darglitazone observed here in vivo and by others in vitro (Rabelo et al., 1997) can be explained by its high affinity binding to the PPAR-γ (approximately 50 nM; unpublished information by D. E. Wilder, Pfizer Global R&D), a receptor implicated in the differentiation of white or brown adipocyte precursors to mature adipocytes (Brun et al., 1996). Binding to this receptor by darglitazone (Camirand et al., 1998) and other thiazolidinediones (Foellmi-Adams et al., 1996; Sears et al., 1996) also induces terminal differentiation (i.e., expression of uncoupling protein) of brown adipocytes in culture. Therefore, the adipogenic activity of thiazolidinediones is strongly related to PPAR-γ binding and potency rather than enhanced glucocorticoid sensitivity.

The clinical significance of these effects on adipose tissue proliferation, hemodilution, abnormal fluid retention/distribution, and hydrothorax could not be discounted as a rodent specific-phenomenon. These effects were also observed in darglitazone-treated monkeys. In humans, troglitazone promotes subcutaneous fat proliferation (Mori et al., 1999). Peripheral edema has also been reported with this class of compounds (Niemeyer and Janney, 2002). Pleural edema has rarely been associated with troglitazone or rosiglitazone treatment but was reversible upon discontinuation of drug treatment (Koshida et al., 1998; Inoue and Sano, 2000; Wang et al., 2002). Peripheral edema and pleural effusion (one case) was also observed during early clinical trials with darglitazone (information on file, Pfizer Global R&D) and were also both reversible. This generalized phenomenon is not linked to the chemical class since recent clinical trials with a highly selective and more potent PPAR-γ agonist, farglitazar (a t-tyrosine-based nonthiazolidinedione), showed a dose-dependent weight gain, peripheral edema and decreases in hemoglobin (Fiedorek et al., 2000; Raz et al., 2000; Wilson et al., 2000). These clinical findings suggest some degree of concordance of this phenomenon (adipose hypertrophy/hyperplasia, peripheral and pulmonary edema, and hemodilution) in rats and monkeys with humans. Given the clinical effects of GI262570, the phenomenon itself is most probably linked to the pharmacologic target itself as a function of potency and administered dose rather than the thiazolidinedione structure.

Other effects observed in rats and monkeys range from reversible changes in erythrocyte parameters to follicular atresia. Reversible depressions in erythrocyte parameters have also been reported in troglitazone-treated rodents (Herman et al., 1997). In both cases, bone marrow depression was not evident, suggesting that the effect was not the result of hematotoxicity. Unlike Tanabe-174 where a nonregenerative anemia occurred in rats and dogs (Williams et al., 1993), the present findings were consistent with the hemodilution effect of this pharmacologic class of compounds.

Alterations in female ovulatory cycle and fertility may be caused by multiple mechanisms. Blackwell et al. (1998) proposed that darglitazone enhanced insulin action in reproductive tissues within the body based on work by Poretsky and Kalin (1987). Additional work by Barbieri et al. (1983) implicates insulin in causing an androgen excess that would in turn cause follicular atresia with the loss of estrogen producing cells. Recently, Gasic et al. (2001) have shown that troglitazone inhibits 3β-hydroxysteroid dehydrogenase, an effect that would directly reduce ovarian progesterone production. Therefore, we postulate that the hormonal alterations observed with darglitazone might be the result of enhanced sensitivity to insulin or inhibition of this enzyme. Further work would be needed to confirm either proposed mechanism. The clinical relevance of this effect was not determined during clinical trials.

In summary, darglitazone, an insulin-sensitizing agent for the treatment of NIDDM, caused BAT proliferation, hemodilution, and abnormal estrous cycling in rodents and nonhuman primates. Prolonged treatment at high doses was associated with morbidity/mortality in rodents caused by the development of hydrothorax. The adverse effects of thiazolidinedione treatment in rats and monkeys manifest themselves clinically as subcutaneous fat accumulation, peripheral edema, hemodilution, and anecdotable cases of pulmonary edema. Many of these effects are fully reversible. The concordance between some of the effects observed in preclinical safety assessment studies and clinical studies should aid in setting appropriate margins of safety for future clinical trials with a new generation of potent thiazolidinediones or PPAR-γ agonists for the treatment of NIDDM.

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References


Address correspondence to: Dr. Michael D. Aleo, Pfizer Global R & D, Groton Laboratories, Drug Safety Evaluation, MS 8274-1229, Eastern Point Rd., Groton, CT 06340. E-mail: michael_d_aleo@groton.pfizer.com