The development of the dual peroxisome proliferator–activated receptor (PPAR) α/γ agonist tesaglitazar as an oral antidiabetic was recently discontinued. Here we present tumor data from a 2-year carcinogenicity study in rats given 0.3, 1, 3, and 10 μmol/kg tesaglitazar and present with focus on the findings of subcutaneous fibrosarcomas. To investigate the mechanism for induction of fibrosarcomas, replicative DNA synthesis (immunohistochemical detection of BrdU-labeled cells) and expression of PPARγ (immunohistochemistry and reverse transcription–polymerase chain reaction) in subcutaneous adipose tissues was assessed in rats administered 1 or 10 μmol/kg for 2 weeks or 3 months. Poorly differentiated subcutaneous mesenchymal sarcomas with a predominant spindle cell appearance occurred at the highest dose level of 10 μmol/kg in both sexes, and these tumors were diagnosed as fibrosarcomas. The 10-μmol/kg dose was at or above the maximum tolerated dose and caused considerable cardiovascular mortality. Tesaglitazar stimulated DNA synthesis mainly in subcutaneous interstitial mesenchymal cells. The percentage of BrdU-labeled interstitial cells was increased at 1 and 10 μmol/kg after 2 weeks. The increase in DNA synthesis was still significant at the end of the 12-week treatment at 10 μmol/kg, the dose producing fibrosarcoma. However, at 1 μmol/kg, a dose below the no-observed-effect level for fibrosarcoma, the level of DNA synthesis was similar to control levels at 12 weeks. Immunohistochemical analyses showed no detectable PPARγ protein in the majority of BrdU-labeled interstitial mesenchymal cells in white and brown fat. This indicates that stimulation of DNA synthesis is not mediated via direct activation of PPARγ in these cells. The results suggest that the induction of rat fibrosarcoma by tesaglitazar, at exposures 100-fold above the human therapeutic exposure, may involve proliferation of undifferentiated mesenchymal cells in subcutaneous tissues.

Key Words: PPAR; tesaglitazar; fibrosarcoma; cell proliferation; DNA synthesis.

Peroxisome proliferator–activated receptors (PPARs) are a group of three homologous transcription factors within the nuclear receptor superfamily (Feige et al., 2006). The different subtypes, PPARα, PPARγ and PPARδ, have important physiological and dissimilar functions that are partly based on different tissue distributions. PPARα is highly expressed in liver, skeletal muscle, heart, and brown adipose tissue and coordinates expression of many genes important for fatty acid catabolism (Peters et al., 1997). There are two isoforms of PPARγ that are created by differential promoter usage and splicing (γ1 and γ2). PPARγ2 has an additional 30 amino acids at its N-terminal end and is expressed specifically in adipocytes and fatty livers. PPARγ1 is not only present in adipocytes but also in macrophages and at low levels in other cell types. PPARγ regulates genes important for adipocyte differentiation, lipid storage, and glucose homeostasis (Lehrke and Lazar, 2005). PPARδ is ubiquitously expressed and has been implicated in diverse functions, such as embryo implantation, lipid metabolism, and keratinocyte differentiation (Barak et al., 2001; Kim et al., 2006). Agonists to PPARα, the fibrates, have well-documented therapeutic effects in dyslipidemias, and agonists to PPARγ are effective in the treatment of insulin resistance, diabetes, and concurring dyslipidemia (Lee et al., 2006).

The toxicological effects of PPARα agonists (peroxisome proliferators) have been extensively investigated. PPARα agonists induce not only liver tumors in rats and mice but also frequently thyroid, pancreatic acinar cell, and testicular Leydig cell tumors. The modes of action for these tumors have been identified and are generally considered not to be relevant to humans (Cattley et al., 1998; Klaassen and Hood, 2001; Klaunig et al., 2003; McClain, 1989; Morimura et al., 2006).

Several PPARγ and dual PPARα/γ agonists induce other types of tumors in rodents. These include fibrosarcomas, liposarcomas, and bladder tumors in rats and hemangiosarcomas in mice (Cohen, 2005). Fibrosarcomas or liposarcomas are induced in rats by 8 of 11 recorded PPARγ and dual PPARα/γ
agonists (El-Hage, 2005). The commonality in tumor findings across this class of compounds has caused concerns regarding human risk and investigative studies to elucidate the mode of action are needed. A large number of pharmaceutical companies are currently collaborating in the International Life Science Institute PPAR initiative with the aim to produce mode of action frameworks for fibro-, lipo-, and hemangiosarcomas and bladder tumors (Cohen et al., 2006; Meek et al., 2003).

Tesaglitazar is a dual PPARα/γ agonist that is more potent on PPARγ than on PPARα (Cronet et al., 2001). The EC50 value for tesaglitazar in in vitro transactivation assays is 13.4 μM on rat PPARα, 3.6 μM on human PPARα, and approximately 0.2 μM on both rat and human PPARγ (unpublished data). Tesaglitazar has been shown to lower circulating triglyceride, glucose, and insulin levels in animal models of type 2 diabetes and insulin resistance with an estimated ED50 of 0.07 μmol/kg (Ljung et al., 2002; Oakes et al., 2005). Clinical evaluation has shown that tesaglitazar reduces abnormalities of glucose and lipid metabolism associated with insulin resistance and improves the atherogenic lipoprotein profile in patients with dyslipidemia of insulin resistance (Fagerberg et al., 2005; Goldstein et al., 2006; Hamren et al., 2005). Tesaglitazar was discontinued from further development in May 2006 as data from phase III clinical studies showed that its benefit-risk profile was unlikely to give an advantage over currently available therapies.

The carcinogenic potential of tesaglitazar has been assessed in a 2-year rat carcinogenicity study. At the highest dose level of 10 μmol/kg, tesaglitazar induced subcutaneous mesenchymal tumors that were classified as fibrosarcomas based on their predominant histological feature. As tesaglitazar has no genotoxic potential, we postulate that the mechanism for the induction of fibrosarcoma may be due to a trophic effect on connective tissues, similar to the proposed mechanism for a number of well-described nongenotoxic carcinogens (Shaw and Jones, 1994; Klaunig et al., 2003). In this article, we describe the findings of fibrosarcoma in rats and present the results of initial investigative studies, in which replicative DNA synthesis and PPARγ expression were assessed in subcutaneous adipose tissues. The results show long-term stimulation of DNA replication and potentially proliferation of interstitial mesenchymal cells, putative target cells for tumor formation, which do not appear to express PPARγ.

MATERIALS AND METHODS

Test substance. Tesaglitazar ([S]-2-ethoxy-3-[4-[4-(methylsulphonyloxy)phenethyl]oxy]phenyl)propanoic acid) was synthesized by AstraZeneca. The test substance was dissolved in vehicle, consisting of 5 mM sodium hydrogen carbonate buffer solution, pH 8.5.

Protein binding and calculation of safety margins. Tesaglitazar shows species differences in plasma protein binding. The fraction of nonprotein-bound tesaglitazar is 0.8% in rats and 0.1% in man. The free fraction of tesaglitazar has been used in the calculation of safety margins since the free concentration is considered responsible for both pharmacological and toxicological effects.

Animals. Male and female Wistar Hannover Galas rats were obtained from M&B AS, Møllegård, Denmark. The animals were allowed to acclimatize for 2 weeks before study start and were 6–8 weeks of age at the start of dosing. The animals were housed five rats per cage by sex and dose. Each cage was supplied with sterilized whitewood shavings. Water and food (rat and mouse no. 1 expanded SQC diet, supplied by Special Diets Services Limited, Stepfield, Witham, Essex, UK) was supplied ad libitum. There was automatic control of light cycle, temperature, and humidity; light hours were 0700–1900 h and target ranges for temperature and humidity were 20°C ± 2°C and 50 ± 15% respectively.

Carcinogenicity study. The carcinogenicity study was performed at Inveresk Research, Tranent, Scotland. Fifty male and 50 female rats per group were administered tesaglitazar at 0.3, 1, 3, and 10 μmol/kg (0.12, 0.41, 1.2, and 4.1 mg/kg) by daily oral gavage for 104 weeks. Due to high mortality, dosing of males at 10 μmol/kg tesaglitazar was stopped at week 76. Surviving animals in this group remained on the study. In the vehicle groups, 100 rats of each sex were used. The daily dose volume was 5 ml/kg. The doses administered in the study were based on previous 14-day and 3-month studies conducted by AstraZeneca. The low dose of 0.3 μmol/kg was chosen to give a threefold multiple of the therapeutic systemic exposure in humans to the nonprotein-bound fraction of tesaglitazar in plasma. The safety margins to therapeutic exposure for the highest dose of 10 μmol/kg were approximately 100.

Animals were observed daily for clinical signs of toxicity. Individual body weights were recorded daily up to week 61 and from then once weekly. Food consumption was recorded weekly up to week 13 and then every 4 weeks throughout the study.

Toxicokinetic analyses were performed on satellite animals. Blood samples were obtained from four male and female rats at weeks 13 and 53 at the following time points: 1, 3, 5, 8, and 24 h postdose. Satellite animals were subjected to a limited necropsy at which brain, heart, and liver were weighed.

Tailing blood samples of blood were taken from all surviving main study animals during weeks 53 and weeks 103 for hematology and clinical chemistry analyses. The animals were given free access to food and water before sampling.

All animals in the main study (including preterminally killed animals and animals found dead) were subjected to a detailed necropsy at which brain, heart, and liver were weighed. A full range of tissues from all animals was evaluated microscopically on hematoxylin and eosin (HE)–stained slides at AstraZeneca, and the diagnoses were subject to internal peer review.

Investigative study. Sixteen male and 16 female rats per group were administered vehicle or tesaglitazar at 1 and 10 μmol/kg (0.41 and 4.1 mg/kg) by daily oral gavage for 2 or 12 weeks. The daily dose volume was 10 ml/kg.

The doses of tesaglitazar were based on the rat carcinogenicity study, in which the high dose of 10 μmol/kg produced a statistical increase in fibrosarcomas, whereas the low dose of 1 μmol/kg did not (Table 4).

Body weights were recorded twice weekly up to week 4 and once weekly thereafter. Food consumption was recorded weekly throughout the study.

Samples for determination of plasma levels of tesaglitazar were taken at the end of the dosing periods (2 and 12 weeks) from four male and four female rats in all groups at 1 h after dosing (approximately Cmax).

Blood samples for clinical chemistry analyses were taken from all animals after about 2 and 12 weeks of dosing, respectively. The animals were given free access to food and water before sampling. Blood samples were taken from the orbital venous plexus under light enfuran (Efrane, Abbott Laboratories) and N2O anesthesia.

BrdU was administered to all animals during the last week before necropsy by a single subcutaneous injection of 100 mg/kg BrdU in physiological saline 7 days before sacrifice. On the same day, the drinking water was replaced by water containing 0.8 mg/ml BrdU, which was given throughout the following 7 days. BrdU is mutagenic, teratogenic, and a probable human carcinogen, and necessary safety precautions were taken to minimize human exposure. All animals were subjected to necropsy, and organ weights were recorded for heart...
and brain. Skin with subcutaneous layers from dorsal (interscapular brown fat) and ventral location (white fat) and jejunum was taken from all animals, fixed in neutral buffered formalin, and processed to paraffin wax for microscopic evaluation. HE-stained slides were prepared for histopathological examination and immunohistochemical stainings were prepared as detailed below. Frozen skin samples were taken for explorative work and were used for laser capture microdissection (LCM) and mRNA analysis.

**Immunohistochemical staining for BrdU and PPARγ.** Samples of skin were stained immunohistochemically for the presence of BrdU and PPARγ. All immunohistochemical staining was performed on the staining module Discovery XT (Ventana Medical Systems, Tucson, AZ). Solutions for deparaffinization, pretreatment, counterstaining, detection, and rinsing were supplied by Ventana Medical Systems. Both heat (40 min at 96°C in pH 8.0) and enzyme digestion (protease 0.02 U/ml 4-8 min) was used as pretreatment for BrdU. Heat (60 min at 96°C in pH 8.0) was used as pretreatment for PPARγ. The primary antibodies (mouse anti-BrdU, M0744, Dako, Glostrup, Denmark, and rabbit anti-PPARγ, H1000, sc-7196, Santa Cruz Biotechnology) were added manually at a dilution of 1/50 in PBS. The secondary antibodies (biotinylated goat anti-mouse IgG, E0433, Dako, and biotinylated goat anti-rabbit IgG, E0432, Dako) were automatically dispensed at a dilution of 1/250 (E0433) and 1/200 (E0432) in antibody diluent (760-108, Ventana Medical Systems) with 5% normal goat serum (X0907, Dako). The immunological reaction was visualized with diaminobenzidine (DAB) chromogen (DABMapKit 760-124, Ventana Medical Systems). After the staining, the slides were dehydrated in rising concentration of ethanol and mounted in Cytoseal XYL (760-2021, Ventana Medical Systems). After the staining, the slides were visualized with diaminobenzidine (DAB) chromogen (DABMapKit 760-124, Ventana Medical Systems). The immunological reaction was visualized with diaminobenzidine (DAB) chromogen (DABMapKit 760-124, Ventana Medical Systems) and counterstaining was performed with hematoxylin (760-2021, Ventana Medical Systems). After the staining, the slides were dehydrated in rising concentration of ethanol and mounted in Cytoseal XYL (760-2021, Ventana Medical Systems).

**Total RNA extraction, reverse transcription, and real-time RT-PCR.** Total RNA was extracted using RNAeasy Micro Kit (Qiagen, VWR International) according to manufacturer’s protocol. The extracted RNA was eluted in 14-μl RNase-free water, 2 μl was used for RNA analysis using the Pico LabChip (Agilent 2100 Bioanalyzer, Agilent Technologies, Santa Clara, CA), and the rest was stored at −80°C for later reverse transcription–polymerase chain reaction (RT-PCR) analysis. The amount of total RNA was also measured with NanoDrop ND-1000 Spectrophotometer (NanoDrop Technologies, Rockland, DE).

**Statistical analyses.** In the carcinogenicity study, body weight, food consumption, hematology, clinical chemistry, and organ weight data were analyzed for homogeneity of variance using the “F-Max” test. If the group variances appeared homogeneous, a parametric ANOVA was used, and pairwise comparisons were made using Fisher’s protected least significant difference method via Student t-test, i.e., pairwise comparisons were made only if the overall F-test was significant. If the variances were heterogeneous, log or square root transformations were used in an attempt to stabilize the variances. Expression levels were presented as fold changes compared to the vehicle group. No statistical analysis was performed since RNA was only obtained from two animals in each group.

<table>
<thead>
<tr>
<th>Primer and Probe Sequences for Real-Time RT-PCR</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Gene</strong></td>
</tr>
<tr>
<td>PPARγ forward</td>
</tr>
<tr>
<td>PPARγ probe</td>
</tr>
<tr>
<td>PPARγ reverse</td>
</tr>
<tr>
<td>PPARδx forward</td>
</tr>
<tr>
<td>PPARδx probe</td>
</tr>
<tr>
<td>PPARδx reverse</td>
</tr>
<tr>
<td>FABP4 probe</td>
</tr>
<tr>
<td>FABP4 reverse</td>
</tr>
</tbody>
</table>
RESULTS

Carcinogenicity Study

Toxicokinetics. The maximal plasma concentrations of tesaglitazar, $C_{\text{max}}$, and the area under the plasma concentration-time curves, $AUC_{(0-24h)}$, were equal at weeks 13 and 53 and only data for week 13 are presented in Table 2. $C_{\text{max}}$ of tesaglitazar appeared generally within 3 h after administration (data not shown). The exposure expressed as $AUC_{(0-24h)}$ and $C_{\text{max}}$ increased in fair proportion to the increase in dose. The exposure was higher in males compared to females.

Mortality and clinical observations. There was an increase in mortality in males given 3 and 10 μmol/kg and in females at 10 μmol/kg tesaglitazar (Fig. 1). A substantial increase in mortality was evident from week 50 in the males treated at 10 μmol/kg. By week 76, with loss of 28 males, dosing was stopped in this group and the mortality rate declined thereafter resulting in a terminal survival of 10 animals. The most probable cause of the demise of many of these animals was heart failure associated with myocardial hypertrophy. In males given 3 μmol/kg and in females given 10 μmol/kg tesaglitazar, there was a slight increase in mortality after about 70 weeks. Other findings noted clinically for the premature decedent animals treated with 10 μmol/kg tesaglitazar included irregular respiration, hunched posture, and subdued appearance.

Mean body weight gain was generally higher in the group administered 1 μmol/kg but lower at 3 and 10 μmol/kg tesaglitazar as compared to the vehicles group (data not shown). A slight increase in food consumption was noted throughout the study for both sexes in all dose groups, except for males given 10 μmol/kg at week 84 (data not shown).

Clinical chemistry and hematology. Due to the high mortality by 103 weeks, hematology and clinical chemistry data are only discussed for the 53-week time point. The most prominent findings were decreases in several red cell parameters (red blood cell count, hemoglobin, and hematocrit) and a decreased platelet count in both sexes at most dose levels (data not shown). An increase was observed for white blood cells and lymphocytes in both sexes given 1, 3, and 10 μmol/kg tesaglitazar (data not shown).

At week 53, males administered 0.3, 1, and 3 μmol/kg tesaglitazar had increased levels of aspartate aminotransferase and alanine aminotransferase levels in the plasma, whereas there was no clear effect in females (data not shown). Plasma cholesterol and triglycerides were reduced in all male and female groups treated with tesaglitazar (Table 3), and this is indicative of the pharmacological effect of tesaglitazar. Decreases in total protein were observed in males given 1, 3, and 10 μmol/kg and in females given 3 and 10 μmol/kg tesaglitazar (data not shown).

### Table 2

**Exposure to Tesaglitazar (total drug) at Week 13 in the Carcinogenicity Study**

<table>
<thead>
<tr>
<th>Dose (μmol/kg)</th>
<th>$C_{1h}$ (μmol/l)</th>
<th>$C_{\text{max}}$ (μmol/l)</th>
<th>$AUC_{(0-24h)}$ (μmol-h/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Male</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.3</td>
<td>0.88 ± 0.40</td>
<td>0.88 ± 0.20</td>
<td>10.2 ± 2.6</td>
</tr>
<tr>
<td>1</td>
<td>3.39 ± 1.31</td>
<td>3.78 ± 0.50</td>
<td>41.9 ± 7.1</td>
</tr>
<tr>
<td>3</td>
<td>12.0 ± 2.7</td>
<td>12.0 ± 1.4</td>
<td>126 ± 22</td>
</tr>
<tr>
<td>10</td>
<td>32.2 ± 5.7</td>
<td>32.2 ± 2.9</td>
<td>352 ± 38</td>
</tr>
<tr>
<td>Female</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.3</td>
<td>0.75 ± 0.30</td>
<td>0.81 ± 0.14</td>
<td>7.91 ± 1.3</td>
</tr>
<tr>
<td>1</td>
<td>2.23 ± 0.71</td>
<td>3.42 ± 1.19</td>
<td>35.8 ± 12.6</td>
</tr>
<tr>
<td>3</td>
<td>6.89 ± 1.10</td>
<td>7.26 ± 0.35</td>
<td>94.7 ± 7.9</td>
</tr>
</tbody>
</table>

Note. $C_{1h}$, plasma concentration at 1 h after dose; $C_{\text{max}}$, maximum plasma concentration; $AUC_{(0-24h)}$, area under the plasma concentration-time curve from 0 to 24 h.
Gross necropsy findings and organ weights. Macroscopic changes associated with the administration of tesaglitazar were generally confined to animals given 3 and 10 μmol/kg. There was a dose-dependent increase in the amount of body fat that was firmer in consistency compared to the body fat seen in control animals. There was an increase in the incidence of palpable subcutaneous masses and masses in the liver. Liver and heart were enlarged, which was reflected by an increase in organ weight. The increase in heart weight in the satellite animals at 13 weeks is shown in Table 3 in comparison to the increase in heart weight in the investigative study at 12 weeks. In addition, an increase in the quantity of fluid was found in body cavities.

Microscopic findings. There was a range of nonneoplastic microscopic changes in many organ systems, and only the most prominent changes will be described. There was a dose-dependent increase in the amount of subcutaneous fat and the fat displayed a polymorphic appearance. This was characterized by larger fat droplets in brown fat and by multiple small fat droplets in white fat. Polymorphic fat occurred in all treated groups in both sexes, including the males given 10 μmol/kg tesaglitazar. Areas of fibroplasia were seen in the skin of five males given 0.3 μmol/kg tesaglitazar. The liver displayed several changes characteristic of PPARγ activation, including hepatocellular hypertrophy, fatty change, necrosis, and an increased incidence of basophilic foci. These changes occurred in both sexes and at all doses. Hypertrophy of the zona glomerulosa of the adrenal glands and an increase in follicular cell hyperplasia of the thyroid gland was seen in both sexes given 1, 3, and 10 μmol/kg tesaglitazar.

Fat infiltration of the bone marrow (all treated groups) and of the parenchyma of the pancreas (in both sexes given 3 and 10 μmol/kg tesaglitazar) was also observed.

Neoplastic findings. Drug-related neoplastic findings are summarized in Table 4. There was an increase in hepatocellular adenomas in both sexes and an increase in hepatocellular carcinomas in female rats. There was an increased incidence of follicular cell adenomas of the thyroids in both sexes.

Poorly differentiated subcutaneous mesenchymal sarcomas were found in both sexes given 10 μmol/kg tesaglitazar. The increase in the females given 10 μmol/kg was lower compared to that in the male rats. A small number of subcutaneous mesenchymal tumors were present in the groups given 0.3, 1, and 3 μmol/kg tesaglitazar. These tumors showed a predominant spindle cell appearance that was consistent with the accepted morphological criteria for fibrosarcomas (Fig. 2A). Compared with the tumors seen at lower doses, those seen in rats given 10 μmol/kg tesaglitazar were more variable in appearance and displayed a range of additional morphological features. These included features that are characteristic of tumors of adipocyte and histiocyte origin. Selected tumors are shown in Figure 2. A number of immunocytochemical methods were used to further characterize the tumors. The tumors were negative for histiocytic markers, for muscle markers, and for S100 (data not shown). All tumors were classified as fibrosarcomas based on their predominant histological features, although a number of the tumors contained areas that were consistent with liposarcoma (Fig. 2B) or histiocytic sarcoma (Fig. 2C). The majority of the subcutaneous fibrosarcomas in animals given 10 μmol/kg tesaglitazar were found in the abdominal region (both dorsal and ventral), and the rest were found on the ventral or dorsal thorax, in the axillae, and on limbs but not in the interscapular region. These tumors were locally very invasive,
and the exact subcutaneous tissue of origin could not be determined. Animals bearing the tumors commonly died within a short period after detection of the tumor, the first death occurring at week 60, but most of them after week 76. A table of all tumors in the study is available as supplementary data.

**Investigative Study**

**Exposure, clinical observations, and clinical chemistry.** The plasma concentrations of tesaglitazar at 2 and 12 weeks are shown in Table 5. The levels were slightly lower at 2 weeks, and the exposure in the females was lower than in the males. In comparison to the plasma concentration at 1 h in the carcinogenicity study (Table 2), the plasma levels measured 1 h after dose in the investigative study were slightly lower.

The only clinical sign observed during the study was the occurrence of palpable subcutaneous masses. This was observed for all males and four females given 10 μmol/kg tesaglitazar. There was an indication of higher body weights in the groups administered 10 μmol/kg tesaglitazar for 12 weeks, but this was not statistically significant. The food consumption increased in all treated female groups and in all treated male groups sacrificed after 2 weeks. In the male rats sacrificed after 12 weeks, increased food consumption was only observed in the group administered 10 μmol/kg tesaglitazar.

Clinical chemistry showed decreases in plasma levels of total cholesterol and triglycerides. Similar effects were observed at both time points, and only data from 12 weeks are shown in Table 3. Plasma total protein was slightly decreased in both sexes given 10 μmol/kg tesaglitazar at 2 and 12 weeks (data not shown).

**Gross necropsy findings and organ weights.** There were no gross findings noted at necropsy at 2 weeks. At week 12, there were increased subcutaneous fat deposits in animals given

---

**TABLE 4**

Incidences of Treatment-Related Neoplastic Findings in the Rat Carcinogenicity Study

<table>
<thead>
<tr>
<th>Dose (μmol/kg)</th>
<th>Male</th>
<th>Female</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Group size</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Premature decedents</td>
<td>31</td>
</tr>
</tbody>
</table>

**Liver tumors**
- Hepatocellular adenoma: 2 2 2 5 1 0 0 1 4 5<sup>*</sup>
- Hepatocellular carcinoma: 0 0 0 2 2 0 0 1 3 9<sup>*</sup>

**Thyroid tumors**
- Follicular cell adenoma: 4 7 4 6 4 3 4 3 5 9<sup>*</sup>

**Fibrosarcoma**
- Subcutaneous: 0 0 1 2 13<sup>*</sup>
- Abdominal cavity: 1 0 0 0 1 0 0 0 0 0
- Sternum: 0 0 0 0 1 0 0 0 0 0

Note. All animals were subjected to necropsy when found dead, preterminally killed, or at scheduled termination at 104 weeks.
<sup>a</sup>Treatment was stopped at week 76 due to mortality, surviving animals remained on the study.
<sup>b</sup>Twenty-eight animals died during the dosing period and 12 animals died after week 76 in the nondosing period.
<sup>*</sup>Significant at <i>p</i> < 0.01 using Peto trend test.

---

**TABLE 5**

Exposure to Tesaglitazar (total drug) in the Investigative 12-Week Study

<table>
<thead>
<tr>
<th>Dose (μmol/kg)</th>
<th>Plasma concentration of tesaglitazar 1 h after dose (μmol/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 weeks</td>
</tr>
<tr>
<td>Male</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.29 ± 0.34</td>
</tr>
<tr>
<td>10</td>
<td>13.0 ± 1.3</td>
</tr>
<tr>
<td>Female</td>
<td></td>
</tr>
<tr>
<td>1</td>
<td>1.17 ± 0.14</td>
</tr>
<tr>
<td>10</td>
<td>10.2 ± 1.6</td>
</tr>
</tbody>
</table>

---

*Fig. 2.* Representative photographs of subcutaneous fibrosarcomas with spindle cell–shaped morphology in the control and low-dose tumors or areas with additional morphological features in the high-dose tumors. Spindle cell–shaped tumor from a male rat given 1 μmol/kg (A), tumor-containing adipocytes features from a female rat given 10 μmol/kg (B), and tumor-containing histiocyte features from a male rat given 10 μmol/kg (C). The scale bar represents 100 μm.
10 μmol/kg tesaglitazar, the males being more affected than the females.

At both time points, there was an increase in heart weight in both sexes in animals given 10 μmol/kg tesaglitazar (Table 3) and an increase in liver weight for all treated groups (data not shown).

**BrdU labeling.** BrdU-labeled cells were detected in brown and white subcutaneous adipose tissue. In the white fat, labeling was predominantly confined to cells with spindle-shaped nuclei, with occasional labeling of adipocytes (Fig. 3A). Thus, the majority of labeled cells were identified as interstitial mesenchymal cells. Similarly, in brown fat, the majority of labeled cells were interstitial mesenchymal cells with only a minor fraction of brown fat nuclei stained for BrdU (Fig. 3B).

The ranking method showed a statistically significant increase in BrdU labeling in white and brown adipose tissue in both treated groups at 2 weeks. By 12 weeks, increased labeling was only detected in animals given 10 μmol/kg tesaglitazar. The magnitude of this difference was similar in males and females, and therefore, sexes were combined in the subsequent assessment using image analysis to obtain an average labeling index. The image analysis results confirmed the rank data, showing a sustained stimulation of DNA synthesis in rats given 10 μmol/kg tesaglitazar for 12 weeks (Fig. 4). The statistical analysis of the image analysis was less powerful than the rank analysis because it was based on fewer animals. However, the results are in-line with those from the ranked tissues evaluation.

**PPARγ immunohistochemistry.** Immunohistochemical staining for PPARγ in subcutaneous tissues from control animals demonstrated that the protein was primarily detected in the nuclei of white adipocytes and brown adipocytes, with occasional staining of interstitial cells in the adipose tissues. PPARγ was also detected in sebocytes and pericytes and smooth muscle cells surrounding vessels, but no staining was detected in hair follicles, endothelial, or epithelial cells.

Treatment with tesaglitazar at 1 or 10 μmol/kg for 2 weeks or 1 μmol/kg for 12 weeks did not cause any significant changes in the PPARγ staining pattern in brown fat (Figs. 5A–E). In animals administered 10 μmol/kg for 12 weeks, a weaker staining was observed in the brown fat nuclei in combination with increased cytoplasmic staining (Fig. 5F).

![FIG. 3. Immunohistochemistry of BrdU-labeled cells in white (A) and brown (B) subcutaneous adipose tissue from a rat administered 1 μmol/kg for 2 weeks. The BrdU-staining spindle-shaped cells represent interstitial mesenchymal cells. The scale bar represents 50 μm.](image)

![FIG. 4. Mean levels of the percentage of BrdU-labeled cells in subcutaneous brown and white fat obtained by image analysis on eight animals per group. Asterisks indicate statistical significance obtained in the ranking method on all animals in the groups: *p < 0.05; **p < 0.001; ***p < 0.0001.](image)

![FIG. 5. Immunohistochemistry for PPARγ in control and treated animals in brown fat at 2 weeks (A, vehicle; B, 1 μmol/kg; C, 10 μmol/kg), in brown fat at 12 weeks (D, vehicle; E, 1 μmol/kg; F, 10 μmol/kg), in white fat at 2 weeks (G, vehicle; H, 1 μmol/kg; I, 10 μmol/kg), and in white fat at 12 weeks (K, vehicle; L, 1 μmol/kg; M, 10 μmol/kg). The scale bars represent 50 μm.](image)
In white fat, treatment with tesaglitazar at 1 µmol/kg for 2 or 12 weeks caused no changes in the staining pattern of adipocytes and interstitial cells (Figs. 5G, 5H, 5K, and 5L). Treatment with 10 µmol/kg of tesaglitazar caused polymorphic changes with increased number of smaller fat droplets, increased cytoplasm, and rounded adipocyte nuclei. These changes started to appear after 2 weeks but were more prominent after 12 weeks of treatment. The intensity of the staining at 12 weeks was more variable between cells in adipose tissue, and there was clearly an increase in cytoplasmic staining in many adipocytes in white fat in animals treated with 10 µmol/kg (Figs. 5I and 5M).

Expression of PPARγ, FABP4, and PPARα mRNA in subcutaneous fat. PPARγ mRNAs were clearly detected at a similar level in both brown and white fat by analysis of samples obtained by LCM from subcutaneous tissue. As expected, the expression of PPARα mRNA was more than 10-fold higher in brown fat as compared to white fat (data not shown). There were no evident treatment-related effects on PPARγ or PPARα mRNA levels in either tissue type (Figs. 6A and 6C). However, the FABP4 mRNA expression level was increased in tesaglitazar-treated animals in both brown and white adipose tissue in a dose-dependent manner (Fig. 6B).

Colocalization of BrdU and PPARγ. Staining for BrdU and PPARγ was performed on adjacent sections in white and brown fat from animals treated with 1 or 10 µmol/kg tesaglitazar. The majority of BrdU-labeled cells in white and brown fat did not stain for PPARγ (Fig. 7). Double staining for PPARγ and BrdU on the same section in brown adipose tissue confirmed that the nuclei of BrdU-positive spindle-shaped interstitial mesenchymal cells did not stain for PPARγ (Fig. 8A). Brown adipocytes stained positively for PPARγ and a small minority of these also stained for BrdU (Fig. 8B). It was noted that the BrdU-labeled interstitial mesenchymal cells appeared as single cells, while BrdU-labeled brown adipocytes often appeared in a cluster. Double staining in white fat was not satisfactory due to technical difficulties.

**DISCUSSION**

The fibrosarcomas produced by tesaglitazar in the carcinogenicity study were confined to the dose level of 10 µmol/kg in both sexes, and the no-observed-effect level (NOEL) was defined as 3 µmol/kg with an incidence of 4% (Table 4). The NOEL was based on historical background incidences of 0.8–6% (mean 3.2%) for male Wistar rats (Poteracki et al., 1998) and the fact that the tumors at 10 µmol/kg were histologically different from those in the lower doses and in the controls. The exposure to tesaglitazar (Table 2) was lower in the females, which may explain why the incidence of fibrosarcomas was lower in the females.

The exposure of 10 µmol/kg was at, or above, the maximally tolerated dose (MTD). This was evidenced by high cardiovascular mortality in the males to the extent that dosing at 10 µmol/kg was stopped in this gender at week 76. The cardiovascular effects were also indicated by the substantial increase in heart weight at 3 months, 44% in males and 25% in females (Table 3). The United States Food and Drug Administration recommends that the high dose in cancer studies with PPARγ
agonists should not increase heart weights by more than 25% at 3 months in order not to risk long-term survival (El-Hage, 2005). The remaining males in the 10-μmol/kg dose group were kept untreated for the remaining time of the study and most fibrosarcomas appeared after week 76. Notably, the safety margins to human therapeutic exposure, at a daily dose of 1 mg in the clinical studies, was 57 and 33 in male and female rats, respectively, at the NOEL (3 μmol/kg) calculated on the nonprotein–bound fraction of tesaglitazar. Plasma protein binding of tesaglitazar is higher in humans than in rats and the nonprotein–bound fraction is most relevant for calculation of safety margins.

The fibrosarcomas were associated with a dose-dependent increase in the amount of subcutaneous fat and fatty masses in animals given 3 and 10 μmol/kg tesaglitazar. The same type of fatty masses was also observed at 12 weeks in the investigative study and it is of interest to investigate these findings further with regard to their potential involvement in the formation of fibrosarcomas. In addition, a small number of animals given 10 μmol/kg showed fibroplasia in the skin.

Clinical chemistry and hematology findings of decreased red blood cell parameters and total protein is a general finding with PPARγ agonists due to increased plasma volume and hemodilution (Nesto et al., 2003). The increased plasma volume leads to cardiac hypertrophy, which also is a general finding with PPARγ agonists (Arakawa et al., 2004; El-Hage, 2005). The decrease in plasma triglycerides and cholesterol in both studies are attributed to the dual PPARγ/α agonism.

The plasma levels of tesaglitazar were slightly lower in the investigative study as compared to the carcinogenicity study (Tables 2 and 5). However, comparable exposure to tesaglitazar was supported by a similar increase in heart weight and a similar decrease in triglyceride levels in both studies at 12 weeks in the animals administered 10 μmol/kg tesaglitazar (Table 3).

Tesaglitazar, as many other PPAR agonists, was negative in a battery of standard genotoxicity tests (data not shown), and thus, the formation of fibrosarcomas is likely to involve an epigenetic mechanism. Assessment of BrdU incorporation in subcutaneous adipose tissues in the rat demonstrated that 10 μmol/kg tesaglitazar, a dose which produced fibrosarcomas in the rat carcinogenicity study, stimulated DNA replication in a manner that was different from a lower dose of 1 μmol/kg, which was below NOEL for fibrosarcoma in rats (Fig. 4). A comparable increase in BrdU-labeled cells was observed at 2 weeks in white and brown adipose tissue at both doses. However, the BrdU-labeling index at 12 weeks was completely normalized in animals given 1 μmol/kg, whereas the increase in BrdU-labeled cells was sustained over the investigated 12-week period in animals given 10 μmol/kg tesaglitazar.

The majority of labeled cells were identified as interstitial mesenchymal cells, and these undifferentiated cells are possible target cells for the development of fibrosarcoma.

Activation of PPARγ in adipose tissue will cause adipogenesis involving differentiation of preadipocytes into mature adipocytes (Rosen et al., 2000). Treatment with PPARγ agonists increases the cellularity of adipose tissue (deSouza et al., 2001; Okuno et al., 1998; Toseland et al., 2001), most likely due to proliferation and differentiation of preadipocytes and/or other interstitial stem cells. The proliferative effect in adipose tissue may be the consequence of a feedback mechanism to repopulate the preadipocyte pool, but little information is available on clonal expansion of preadipocytes and/or mesenchymal stem cells in vivo. It is difficult to define the identity of the undifferentiated mesenchymal cells, and it is
possible that these cells represent stem cells that are recruited to the tissue. Rosiglitazone, a potent PPARγ agonist, or high-fat feeding was recently found to induce recruitment of bone marrow progenitor cells into adipose tissue in mice (Crossno et al., 2006).

Increased BrdU incorporation in endothelial cells following 1-week exposure to a PPARγ ligand (troglitazone) has been detected in brown adipose tissue in mice (Breider et al., 1999). We found no endothelial cell labeling. This discrepancy may be related to important species differences. Another differential species-related response could be the occurrence of subcutaneous haemangiosarcomas in mice treated with troglitazone and our findings of fibrosarcomas in rats (Herman et al., 2002).

Cellular expression of PPARγ was evaluated to assess the role of this receptor in the observed proliferative effect. Immunohistochemical analysis of PPARγ showed that the receptor was expressed in the nuclei of white and brown adipocytes and in a small fraction of interstitial mesenchymal cells in control or treated animals (Fig. 5). The high staining intensity for PPARγ in normal mature lipid-containing cells is in agreement with the central role of PPARγ in adipocyte differentiation and lipid storage (Lehrke et al., 2005). However, treatment with 1 μmol/kg for 2 or 12 weeks did not significantly change the PPARγ protein staining (Fig. 5). After treatment with 10 μmol/kg tesaglitazar for 12 weeks, increased cytoplasmatic staining for PPARγ protein was noticed in white and brown adipocytes. This effect may be due to the polymorphic changes in adipocytes that occur after treatment with PPARγ agonists. It was recently shown that mitogenic stimulation can cause nuclear export and downregulation of PPARγ in cell cultures (Burgermeister et al., 2007). Treatment with tesaglitazar may thus have stimulated shuttling of PPARγ from the nucleus to the cytoplasm.

Immunohistochemical analysis of protein expression is not a quantitative method, and therefore, quantitative mRNA analyses on LCM samples were performed (Fig. 6). Although, the use of only two animals in these analyses precludes definite conclusions, the results indicate that there are no major treatment-related changes in PPARγ expression. Analyses of mRNA levels of PPARα and PPARγ in whole tissue samples from white and brown fat from a 6-month investigative study, using the same doses of tesaglitazar and with interim necropsies at 2 and 12 weeks, confirm the lack of treatment effects (manuscript in preparation). The mRNA encoding FABP4, aP2, was slightly increased in both brown and white adipocytes after treatment with tesaglitazar. FABP4 is one of the target genes of PPARγ and is directly activated by the ligand-activated receptor on its promoter (Tontonoz et al., 1994). The increase of FABP4 mRNA shows that the PPARγ protein in the tissue was functional and responded to tesaglitazar treatment.

Staining of adjacent sections in white and brown fat (Fig. 7) and double staining for BrdU and PPARγ on the same section in brown fat (Fig. 8) showed that the majority of the proliferating interstitial mesenchymal cells did not express PPARγ. Thus, the proliferative effect is unlikely to be directly mediated by PPARγ in the target cells. A small fraction of brown adipocytes was found to stain positive for BrdU. These cells most likely represent preadipocytes that have undergone replication and differentiation into brown adipocytes within the week that BrdU was administered to the animals.

Clearly, PPARγ-induced adipogenesis and tissue remodeling involves synthesis and release of growth factors from adipocytes, and thus, paracrine signaling via adipocytes is a possible mechanism. It appears that the tissue can adapt to moderate levels of chronic PPARγ activation without adverse effects since there was only a transient increase in proliferation at 2 weeks at the lower dose of 1 μmol/kg. In contrast, the tissue seems to be unable to adapt to sustained PPARγ activation within 12 weeks at doses above MTD. It cannot be assumed that the same type of signaling occurs at both the low and the high dose. It is conceivable that tesaglitazar may exert additional PPAR-independent effects at the high dose, for which evidence is available for other PPAR agonists (Feinstein et al., 2005; Peraza et al., 2005). Furthermore, the role of PPARα in the development of the fibrosarcomas is not clear. This type of tumor is produced by PPARγ and dual PPARα/γ agonists (El-Hage, 2005), and therefore, PPARγ is assumed to be the key player, but contributing effects of PPARα agonism cannot be excluded and requires further studies. This is important because all doses of tesaglitazar in the carcinogenicity study achieved activation of PPARα, which was evidenced by the liver enlargement and the occurrence of liver tumors (Klaunig et al., 2003).

The role of PPARγ agonism in carcinogenicity is controversial. Activation of PPARγ has been shown to result in cell cycle inhibition and promotion of differentiation of normal and tumor cells (Panigraphy et al., 2003). Despite antineoplastic effects in many tumor models, activation of PPARγ has also been shown to result in protumorigenic effects under certain circumstances (Panigraphy et al., 2003; Peraza et al., 2006). The protumorigenic effects may be concentration dependent, and it is not clear to what extent PPAR-independent mechanisms may play a role.

In summary, the commonality in the findings of lipomatous and fibrosarcomas in the class of PPARγ and α/γ agonists has caused concerns regarding risk assessment for chronic treatment. Mechanistic studies are needed to assess the relevance of these findings in humans. In this study, we present initial mechanistic data that suggest dose-dependent effects on proliferation of a putative target cell involved in tumor development. We hypothesize that the induction of subcutaneous fibrosarcoma by tesaglitazar, at exposures approximately 100-fold above the human therapeutic exposure, may involve proliferation of undifferentiated mesenchymal cells in subcutaneous tissues. A prolonged stimulation of proliferation can lead to enhanced malignant transformation of the affected cells.
or a growth stimulus for already transformed cells. Tesaglitazar could affect these processes either indirectly via PPAR-expressing neighboring cells and paracrine mitogenic signaling or directly in the target cells via PPARγ-independent mechanisms. Further studies will aim at identifying the nature and origin of the proliferating cells and the signaling pathways involved in the stimulation of proliferation.

**SUPPLEMENTARY DATA**

A table of all tumors in the carcinogenicity study is available as supplementary data. The list contains information on diagnosis, tissue, and incidence in the different dose groups. Supplementary data are available online at http://toxsci.oxfordjournals.org/.

**ACKNOWLEDGMENTS**

We thank Elisa Basmaci, Anna-Karin Lindström, and Kenneth Stockling at AstraZeneca Safety Assessment for excellent technical contribution to the studies. The authors acknowledge the AstraZeneca publication team for editorial assistance. In addition, Tom Martin (Inveresk Research) and Alli Manninen (AstraZeneca) are acknowledged as study director and study monitor for the carcinogenicity study and Karin Svens (AstraZeneca) as study director for the investigative study.

**REFERENCES**


