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Toxicological evaluation of ammonium 4,8-dioxa-3H-perfluorononanoate, a new emulsifier to replace ammonium perfluorooctanoate in fluoropolymer manufacturing

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ABSTRACT

Ammonium 4,8-dioxa-3H-perfluorononanoate (ADONA) was developed to replace ammonium perfluorooctanoate (APFO) as an emulsifier in the manufacture of fluoropolymers. The toxicity of ADONA was evaluated in acute and repeat-dose studies of up to 90-days duration, and in eye and skin irritation, dermal sensitization, genotoxicity, and developmental toxicity studies. ADONA was also evaluated as a peroxisome proliferator-activated receptor alpha (PPARα) agonist in rats. ADONA was moderately toxic orally and practically non-toxic dermally in acute studies in rats. It was a mild skin irritant and a moderate to severe eye irritant in rabbits. It was a weak dermal sensitizer in local lymph node assays in mice. ADONA was not genotoxic based on the weight of evidence from five assays. It was not developmentally toxic in rats except at maternally toxic doses. ADONA was a possible PPARα agonist in male rats. The liver was the primary target organ in male rats and the kidney was the primary target organ in female rats. NOAELs in 28- and 90-day oral studies in rats were 10 mg/kg/day for males and 100 mg/kg/day for females. These findings demonstrate that the toxicity profile for ADONA is acceptable for its intended use and is superior to that of APFO.

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1. Introduction

Ammonium 4,8-dioxa-3H-perfluorononanoate ($CF_3OCF_2CF_2CF_2$-$OCHFCF_2COO^-$ NH_4^+, ADONA) was developed to replace ammonium perfluorooctanoate (APFO) as an emulsifier used in the aqueous emulsion polymerization of fluoropolymers made from tetrafluoroethylene (TFE). Use of a fluorinated emulsifier in this process is essential, both to stabilize the final fluoropolymer dispersion and as a process safety measure to control the exothermic polymerization kinetics of TFE. APFO has been used since the 1950s for this purpose and as an intentionally added component in a variety of industrial and consumer products. However, evidence of widespread distribution and persistence of perfluorooctanoic acid (the aqueous dissociation product of APFO) in the environment, its biopersistence in humans, and its toxicity in laboratory animal studies [reviewed by Kennedy et al. (2004) and Lau et al. (2004, 2007)] have raised serious concerns about its continued use. In 2006, the US Environmental Protection Agency announced a Stewardship Program challenging fluoropolymer manufacturers to eliminate perfluorooctanoic acid and related chemicals from emissions and products by the year 2015 (USEPA, 2006). As a result, considerable efforts have been made to reduce the use of these compounds and to develop safer alternatives.

ADONA is a white to off-white crystalline solid at room temperature but it is generally made and used as a 30% aqueous solution. ADONA readily dissolves in aqueous media and dissociates to the corresponding carboxylic acid in equilibrium with its anion. The p*K*a of the carboxylic acid is less than 3; therefore, the anion is expected to predominate at physiological pH. ADONA is chemically highly stable and non-reactive. Additional properties of ADONA are listed in Table 1.

ADONA is not intentionally added to products. Most of the ADONA used in fluoropolymer manufacturing is either recaptured from waste streams and products for reuse or is thermally destroyed during processing. Certain fluoropolymers manufactured using ADONA as the emulsifier, such as polytetrafluoroethylene (PTFE), are used as non-stick coatings on cookware and other food contact surfaces. These fluoropolymers are sintered onto the substrate at temperatures exceeding 280 °C. ADONA decomposes at approximately 125–175 °C. Fluoropolymers and fluoroelastomers made using ADONA as the emulsifier are functionally indistinguishable from those made using APFO.

This article summarizes studies which have been performed to evaluate the toxicity of ADONA. Key treatment-related findings in these studies are presented and compared with those for APFO. A separate article summarizing the pharmacokinetics of ADONA is planned.

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Table 1
Properties of ADONA.

Physical state	Solid at 20 °C
Appearance	White to off-white crystalline solid
Molecular weight	395.1
Purity	>98.5%
Melting point	38 °C
Boiling point	100–105 °C
Decomposition temperature	125–175 °C
Vapor pressure	<0.0001 mm Hg at 20 °C
Water solubility	>700 mg/ml
Specific gravity	1.16 g/ml (30% aqueous solution)
pH	6.5 ± 1.0 (30% aqueous solution)
log P_{ow}	1.3
Stability	Stable, not readily biodegradable
Reactivity	Non-reactive

2. Materials and methods

2.1. Test material

ADONA was provided as a clear, colorless, 30 ± 0.3% (w/w) aqueous stock solution, pH 6.5, by Dyneon LLC, Gendorf, Germany. This stock solution was used either undiluted or diluted in an appropriate vehicle for the studies described in the following sections. ADONA doses and concentrations used in these studies are expressed as the ammonium salt and adjusted for the strength of the stock solution. The purity of ADONA in the stock solution was greater than 98.5% as determined by ^{19}F NMR spectroscopy. An alpha-branched isomer was present in the stock solution at approximately 0.4–0.7% (w/w) and several other structurally related impurities were present at less than 0.1% (w/w). The stock solution was demonstrated to be stable for at least 22 months at ambient room temperature in the dark. Dose formulations were generally prepared on the day of use and analyzed for ADONA content by ^{19}F NMR spectroscopy or LC-MS/MS.

2.2. Testing facilities and regulatory compliance

The studies described in the following sections were performed by NOTOX Safety and Environmental Research B.V. ('s-Hertogenbosch, The Netherlands), RCC Cytotest Cell Research (Rossdorf, Germany), MB Research Laboratories (Spinnerstown, PA), Charles River Laboratories (Spencerville, OH and Horsham, PA), and 3M Company (St. Paul, MN). Hepatic mRNA transcript analyses were performed at the Department of Biochemistry and Molecular Biology, University of Minnesota Medical School, Duluth, MN. Toxicokinetic (TK) samples from the 5-day oral toxicity study were analyzed by LCMS Limited (Raleigh, NC) and serum TK calculations were performed by the CIIT Center for Health Research (Research Triangle Park, NC). With the exceptions of the developmental toxicity screening study in rats and the hepatic mRNA transcript analyses, all of the studies were performed according to OECD Guidelines for the Testing of Chemicals and Good Laboratory Practices. Study protocols were approved by the testing facilities' Institutional Animal Care and Use Committees. Animal husbandry and use were in accordance with applicable local and international regulations and guidelines.

2.3. Acute toxicity studies

The acute oral toxicity of ADONA was evaluated in rats according to OECD Guideline 423 using the up-down toxic class method. ADONA (30% stock solution) was administered by oral gavage at doses of 300 and 2000 mg/kg to groups of three female Wistar rats. Dose volumes were 0.9 and 5.8 ml/kg, respectively. Clinical signs were recorded daily, body weights were recorded weekly, and necropsies were performed on day 15.

The acute dermal toxicity of ADONA was evaluated in rats according to OECD Guideline 402. ADONA (30% stock solution) was applied at a dose of 2000 mg/kg to sites representing approximately 10% of the total body surface area on the shaved backs of five male and five female Wistar rats. The dose volume was 5.8 ml/kg. Application sites were covered with semi-occlusive dressings for 24 h. Clinical signs were recorded daily, body weights were recorded weekly, and necropsies were performed on day 15.

2.4. Primary eye and skin irritation studies

The primary ocular irritancy of ADONA was evaluated in rabbits according to OECD Guideline 405. ADONA (0.1 ml of the 30% stock solution) was instilled into one eye of each of three male New Zealand White rabbits. The contralateral eye of each animal served as the control. Ocular examinations were performed at 1, 24, 48, and 72 h and 7, 14, and 21 days following instillation. Fluorescein staining was performed following the 24 h, 72 h and 7, 14, and 21 day examinations to quantify corneal epithelial damage. Ocular effects were scored using a standard scoring system.

The primary dermal irritancy of ADONA was evaluated in rabbits according to OECD Guideline 404. ADONA (0.5 ml of the 30% stock solution) was applied to intact sites on the backs of three male New Zealand White rabbits and the dose sites were covered with semi-occlusive dressings for 4 h. Animals were observed daily for signs of toxicity. Treatment sites were examined and scored for erythema and edema using a standard 4-point scoring system at 1, 24, 48, and 72 h after removal of the dressings. Body weights were recorded prior to dosing and following the last examination.

2.5. Dermal sensitization studies

The dermal sensitizing potential of ADONA was evaluated in two murine local lymph node assays (LLNA) performed according to OECD Guideline 429. In the first assay, ADONA (30% stock solution) was diluted with dimethylformamide (DMF) and applied to the dorsal surfaces of both ears (25 μl/ear) of female CBA mice (5 per group) once daily for three days at concentrations of 7.5%, 15%, and 30% (undiluted). The 15% and 30% concentrations caused no significant irritation in a preliminary rangefinding experiment. A vehicle control group was similarly treated with DMF. All animals were observed daily for signs of toxicity. Treatment sites were scored for irritation on day 3. Body weights were recorded on days 1 (pre-dose) and 6. Three days following the last treatment, animals were administered ^{3}H-methyl thymidine (20 μCi, iv). After 5 h, the animals were euthanized and the auricular lymph nodes were excised. Pooled lymph node cell suspensions from each animal were prepared, washed with phosphate-buffered saline (PBS), and treated overnight with 5% trichloroacetic acid (TCA) at 4 °C to precipitate DNA. DNA was recovered by centrifugation, resuspended in TCA, transferred to scintillation fluid, and measured for radioactivity (Packard 2800TR scintillation counter). Stimulation indices were calculated by dividing the mean dpm for each ADONA treatment group by the mean dpm for the vehicle control group. A stimulation index of 3 or greater was interpreted as evidence that the test article was a dermal sensitizer.

A second LLNA was performed to further investigate the findings of the first study. ADONA (30% stock solution) was diluted with dimethylsulfoxide (DMSO) and applied to the dorsal surfaces of both ears (25 μl/ear) of female CBA/J mice (5 per group) once daily for 3 days at concentrations of 7.5%, 15%, and 30% (undiluted). These concentrations caused no significant local irritation in a preliminary rangefinding experiment. A vehicle control group was similarly treated with DMSO, a positive control group was treated

with α-hexylcinnamaldehyde (25% in DMSO), and a naïve control group received no treatment. All animals were observed daily for signs of toxicity. Body weights were recorded on days 1 (pre-dose) and 6. Ear swelling measurements were performed on days 1 (pre-dose), 3, and 6. Three days following the last treatment, animals were administered 5-bromo-2′-deoxyuridine (BrdU, 150 mg/kg, ip). After 5 h, the animals were euthanized and the auricular lymph nodes were excised. Pooled lymph node cell suspensions from each animal were prepared in fetal bovine serum and fixed in 85% ethanol. One aliquot of fixed cells was stained with propidium iodide for analysis of total cells. A second aliquot, for analysis of proliferating (BrdU-positive) cells, was washed, denatured for 1 h in 1 N HCl containing 0.5% Triton X-100, neutralized with sodium tetraborate (pH 8.5), washed with a staining buffer, stained with BrdU-specific fluorescein-conjugated antibody (Becton Dickinson Immunocytometry Systems, San Jose, CA), washed again with staining buffer, and resuspended in Dulbecco's PBS containing propidium iodide. Total cells and BrdU-positive cells were counted using a FACScan flow cytometer (Becton Dickinson) equipped with a 15 mW Omnichrome argon laser emitting at 488 nm and analyzed using CellQuest version 3.3 software (Beckton Dickinson). A stimulation index was calculated for each ADONA treatment group by dividing the mean number of BrdU-positive cells by that for the DMSO control group and, in the case of the 30% ADONA group, by that for the naïve control group. Stimulation indices were statistically evaluated using Student's *t*-test. An EC3, the ADONA concentration estimated to give a stimulation index of 3, was calculated. Additional aliquots of lymph node cell suspensions (approximately 5×10^5 cells per mouse) were stained with fluorescently labeled anti-mouse B220 and CD3 antibodies (BD Biosciences) in PBS-based buffer, washed, and resuspended in PBS containing the viability dye 7AAD. Live cells were analyzed by flow cytometry to determine the %B (B220-positive) cells, %T (CD3-positive) cells, and the B:T cell ratio. Means for each treatment group were compared to those for the vehicle or naïve control groups. A stimulation index of 3 or greater or a 1.25-fold or greater increase over controls in either the %B cells or the B:T cell ratio was interpreted as evidence that the test article was a dermal sensitizer.

2.6. Genotoxicity studies

2.6.1. Bacterial reverse mutation assays

The mutagenic activity of ADONA was evaluated in reverse mutation assays in *Salmonella typhimurium* (strains TA1535, TA1537, TA98, and TA100) and *Escherichia coli* (WP2uvrA) performed according to OECD Guideline 471 using the plate incorporation method. ADONA (30% stock solution) was diluted with deionized water and tested in two independent assays at concentrations ranging from 3 to 5000 μg/plate in the presence and the absence of metabolic activation (rat liver S9). Strain-specific positive controls and vehicle controls were tested in parallel. The test substance was considered to be mutagenic if it induced a reproducible 2-fold or greater increase in the number of revertants in tester strain TA100 or a 3-fold or greater increase in revertants in any of the other tester strains.

2.6.2. In vitro mammalian gene mutation assay

The potential for ADONA to induce gene mutations in mammalian cells in vitro was evaluated in a Chinese hamster V79 cell HPRT assay performed according to OECD Guideline 476. ADONA (30% stock solution) was diluted with deionized water and tested in a rangefinder at concentrations up to 6000 μg/ml and in two independent assays at concentrations ranging from 93.8 to 1650 μg/ml in the presence of metabolic activation (rat liver S9) and from 93.8 to 1500 μg/ml in the absence of metabolic activation. In the first assay, cells were exposed for 4 h in the presence and the absence of S9 and, in the second assay, for 4 h in the presence of S9 and 24 h in the absence of S9. Cultures treated with positive controls (ethylmethane sulfonate with S9 and 7,12-dimethylbenz(a)anthracene without S9) and vehicle control (deionized water) were tested in parallel. All cultures were tested in duplicate. Cloning efficiency was used to monitor cytotoxicity. The test substance was considered to be mutagenic if it induced either a statistically significant, concentration-related ($p < 0.05$, least squares regression analysis) increase in mutant frequency that was outside the laboratory historical control range, or a reproducible 3-fold or greater increase in mutant frequency at any concentration.

2.6.3. In vitro chromosome aberration assay in human lymphocytes

The clastogenic potential of ADONA was evaluated in cultured human lymphocytes according to OECD Guideline 473. Human peripheral lymphocytes were obtained from healthy, non-smoking, male volunteers. ADONA (30% stock solution diluted with RMPI 1640 cell culture medium) was tested in a preliminary dose rangefinding experiment at concentrations ranging from 100 to 3951 μg/ml with and without metabolic activation (rat liver S9) and in two independent cytogenetic assays. Mitotic index was used to monitor cytotoxicity. In the first cytogenetic assay, cells were exposed to ADONA at concentrations ranging from 333 to 3000 μg/ml for 3 h with a 24-h fixation time in the presence and absence of S9. Based on the observed cytotoxicity, cultures exposed to 2100, 2200, and 2300 μg/ml in the absence of S9 and 1800, 2000, and 2200 μg/ml in the presence of S9 were scored for chromosome aberrations. In the second assay, cells were exposed to ADONA at concentrations ranging from 50 to 750 μg/ml for either 24 or 48 h with a 48-h fixation time in the absence of S9 and from 1000 to 2500 μg/ml for 3 h with a 48-h fixation time in the presence of S9. The following cultures were scored in the second assay based on cytotoxicity: 50, 200, and 400 μg/ml without S9 (24-h exposure, 24-h fixation time); 100, 200, and 400 μg/ml without S9 (48-h exposure, 48-h fixation time); and 1700, 1900, and 2300 μg/ml with S9 (3-h exposure, 48-h fixation time). Positive controls (mitomycin C without S9 and cyclophosphamide with S9) and negative control (culture medium) were tested in parallel. The test substance was considered to be clastogenic if it induced either a statistically significant ($p < 0.05$, one-sided Chi-square test), concentration-related increase in the number of cells with chromosome aberrations or, in the absence of a clear dose–response, a statistically significant increase in the frequency of cells with chromosome aberrations that was outside the laboratory historical control range.

2.6.4. Micronucleus assay in mice

The clastogenic potential of ADONA was evaluated in a micronucleus assay in mice performed according to OECD Guideline 474. ADONA (30% stock solution) was diluted with deionized water and administered by single oral gavage to groups of NMRI BR mice (5/sex/group with extra animals in the high-dose groups) at doses of 200, 400, and 800 mg/kg (males) or 180, 375, and 750 mg/kg (females). The highest doses for males and females were the maximum tolerated doses identified in a preliminary rangefinding experiment. A vehicle control group received water and a positive control group received cyclophosphamide (40 mg/kg, po) in physiological saline. Dose volumes were 10 ml/kg. Animals were observed twice daily for clinical signs. Animals were euthanized and bone marrow was harvested from the femurs of all animals at either 24 or 48 h after dosing. Bone marrow cells were flushed from the femurs with fetal calf serum, pelleted at approximately $1000 \times g$, resuspended in serum, spread on glass slides, fixed in methanol, Wright-Giemsa stained, cleared with xylene, embedded,

and examined microscopically to determine the ratio of polychromatic erythrocytes (PCE) to normochromatic erythrocytes (NCE) and the number of micronucleated PCE per 2000 PCE. The test substance was considered to be clastogenic if it induced a statistically significant ($p < 0.05$, one-sided Wilcoxon Rank Sum Test) increase in the frequency of micronucleated PCE that was outside the laboratory historical control range in any treatment group.

2.6.5. Bone marrow cytogenetic study in rats

The clastogenic potential of ADONA was evaluated in a bone marrow cytogenetic study in rats performed according to OECD Guideline 475. ADONA (30% stock solution) was diluted with deionized water and administered by single oral gavage to groups of male and female Wistar rats (5/sex/group, 8/sex in the high-dose groups) at doses of 280, 560, and 1120 mg/kg (males) or 235, 470, and 940 mg/kg (females). A second set of high-dose males and females was similarly dosed and designated for sacrifice at approximately 24 h after the other groups to account for potential delays in the uptake of the test substance or effects on cell cycle kinetics which could affect the optimum time for chromosome aberration detection. The highest doses for males and females were the maximum tolerated doses identified in a preliminary rangefinding experiment. A vehicle control group received water. A positive control group received cyclophosphamide (20 mg/kg, po) in water. Dose volumes were 10 ml/kg. Animals were observed daily for clinical signs and were weighed just prior to dosing and just prior to colchicine administration. At approximately 3 h prior to sacrifice, animals were administered colchicine (4 mg, ip) to arrest cells in metaphase. Animals were euthanized and bone marrow was harvested from the femurs of animals in all groups at 12–18 h after dosing and at 36–44 h after dosing in the additional male and female high-dose groups. Bone marrow cells were flushed from the femurs with Hank's Balanced Salt Solution, pelleted at approximately $1000 \times g$, swollen with hypotonic KCl, fixed in methanol:acetic acid (3:1), spread on glass slides, and Giemsa stained. Mitotic index (metaphases per 1000 cells) was determined. Chromosomes (approximately 100 metaphase spreads per animal) were examined microscopically for aberrations. The test substance was considered to be clastogenic if it induced a statistically significant ($p < 0.05$, one-sided Chi-squared test) increase in the number of cells with chromosome aberrations that was outside the laboratory historical control range in any dose group.

2.7. Repeat-dose toxicity studies

2.7.1. 5-Day oral toxicity study in rats

ADONA was evaluated in a 5-day oral toxicity study in rats performed according to OECD Guideline 401. ADONA (30% stock solution) was diluted with sterile water and administered by oral gavage once daily for five consecutive days to groups of male and female Sprague–Dawley rats (6/sex/group) at doses of 28, 104, and 298 mg/kg/day. A vehicle control group received sterile water. Dose volumes were 5 ml/kg. Three rats/sex/group were sacrificed at the end of the treatment period and 3/sex/group were sacrificed at the end of a 7-day post-treatment recovery period. Serum and liver specimens were collected from surviving animals at specified intervals following the last dose and analyzed for ADONA by LC/MS. Serum Cmax and AUC were calculated using WinNonlin Professional, Ver. 5.0.1 (Pharsight, Mountain View, CA). Clinical observations and food consumption measurements were performed daily and body weights were recorded on days 1 (pre-dose), 5, and 12. The following evaluations were performed on surviving animals at the end of the treatment and recovery periods: serum chemistry (alanine aminotransferase, aspartate aminotransferase, alkaline phosphatase, gamma glutamyl transpeptidase, total bilirubin, urea nitrogen, creatinine, calcium, phosphorus, total protein, albumin, globulin, albumin/globulin ratio, glucose, cholesterol, triglycerides, sodium, potassium, and chloride), hematology (red blood cell count, red blood cell morphology, hemoglobin, hematocrit, mean corpuscular volume, mean corpuscular hemoglobin, mean corpuscular hemoglobin concentration, reticulocytes, platelets, white blood cell count, and white blood cell differential), urinalysis (color, appearance, volume, specific gravity, pH, protein, glucose, bilirubin, ketones, nitrite, urobilinogen, leukocytes, blood, and sediment), gross necropsy, and organ weights (adrenal gland, brain, heart, kidneys, liver, testes, ovaries, pituitary, spleen, and thyroid gland). Histopathologic examinations were performed on the weighed organs plus stomach, duodenum, mesenteric lymph nodes, skeletal muscle, and spinal cord from all animals in the control and high-dose groups, all early deaths, and gross lesions from all groups. Kidneys from all mid-dose animals were also examined microscopically. Body weight, body weight change, food consumption, serum chemistry, hematology, urinalysis (specific gravity, pH, and total volume), and organ weight data were analyzed by Levene's test (Levene, 1960) followed by the Shapiro–Wilk test (Royston, 1995). Homogeneous, normally distributed data were further analyzed by single-factor ANOVA (Gad and Weil, 1994) followed by Dunnett's test (Dunnett, 1955, 1964). Non-homogeneous or non-normally distributed data were analyzed by Kruskal–Wallis nonparametric ANOVA (Siegel, 1956) followed by Dunn's test (Glantz, 1997). A significance level of $p < 0.05$ was used for all of the statistical tests except Levene's test for which $p < 0.01$ was considered significant.

2.7.2. 28-Day oral toxicity study in rats

ADONA was evaluated in a 28-day oral toxicity study in rats performed according to OECD Guideline 407. ADONA (30% stock solution) was diluted with deionized water and administered by oral gavage once daily for 28 consecutive days to groups of male and female Wistar rats (5/sex/group) at doses of 10, 30, and 100 mg/kg/day. A vehicle control group received deionized water. Dose volumes were 10 ml/kg. Clinical observations were performed daily. Body weights and food consumption were recorded weekly. A functional observational battery to assess hearing ability, pupillary reflex, static righting reflex, grip strength, and motor activity was performed on all animals during week 4. The following evaluations were performed on all surviving animals at the end of the treatment period: serum chemistry (the same parameters as in the 5-day study except that gamma glutamyl transpeptidase, triglycerides, and globulin were not measured), hematology (the same parameters as in the 5-day study except that red blood cell morphology was not evaluated and red blood cell distribution width, prothrombin time, and activated partial thromboplastin time were measured), gross necropsy, organ weights (the same organs as in the 5-day study except that pituitary and thyroid/parathyroid glands were not weighed and thymus, uterus, and epididymides were weighed). Histopathologic examinations were performed on approximately 34 tissues from all animals in the control and high-dose groups, all early deaths, gross lesions from all groups, and liver and thymus from all animals in the low- and mid-dose groups. Statistical analyses were performed using Dunnett's test for normally distributed continuous data, Steel's rank test (Miller, 1981) for non-normally distributed continuous data, and Fisher's exact test (Fisher, 1950) for frequency data. Armitage's test (Armitage, 1971) was performed on selected histopathology data to evaluate trends. All analyses were two-sided, with $p < 0.05$ considered significant.

2.7.3. 90-Day oral toxicity study in rats

The subchronic toxicity of ADONA was evaluated in a 90-day oral study in rats performed according to OECD Guideline 408. ADONA (30% stock solution) was diluted with deionized water

Table 2
Primer sequences used in mRNA transcript analyses.

Transcript	Upper primer	Lower primer	Length (bp)	GenBank reference
18s	CGC CGC TAG AGG TGA AAT TCT T	CAG TCG GCA TCG TTT ATG GTC	149	M11188
Ddit3	AGG TCC TGT CCT CAG ATG AAA TTG GG	TGC AGG GTC AAG AGT AGT GAA GGT	95	NM_024134
Acox	TGG AGA GCC CTC AGC TAT GG	CGT TTC ACC GCC TCG TAA G	338	NM_017340.1
Ehhadh	AGC ACT CAG GCT TGG AAT TCT GGA	GGC AAG CTT GGA ACT GGC TTG TTA	135	NM_133606.1
Cyp4a1	ACC TCT TTC ACT CCC GTG TG	GTG TGT GGC CAG AGC AGA GA	344	M57718

and administered by oral gavage once daily for 90 consecutive days to groups of Sprague–Dawley rats (10/sex/group) at doses of 1, 3, and 10 mg/kg/day (males) and 10, 30, and 100 mg/kg/day (females). These doses were based on the NOAELs for male and female rats in the 28-day study. A vehicle control group received water. Dose volumes were 5 ml/kg. Clinical observations were performed daily. Body weights and food consumption were recorded weekly. Plasma and liver specimens were collected from groups of satellite animals during weeks 1, 4, and 13 and analyzed by LC-MS/MS to monitor ADONA concentrations. Ophthalmoscopy was performed on all animals prior to dosing and on all control and high-dose animals during week 13. The following evaluations were performed on all surviving animals at the end of the treatment period: serum chemistry and hematology (same parameters as in the 28-day study), urinalysis (same parameters as in the 5-day study plus sodium, potassium, and calcium), gross necropsy, and organ weights (same organs as in the 28-day study). Histopathologic examinations were performed on approximately 35 tissues from all control and high-dose animals, all early deaths, gross lesions from all groups, livers from all animals, and thyroid glands from all females. Statistical analyses were the same as those used in the 28-day study.

2.8. Developmental toxicity screening study in rats

ADONA was evaluated in a developmental toxicity screening study in Sprague–Dawley rats. ADONA (30% stock solution) was diluted in deionized water and administered once daily by oral gavage to groups of 10 presumed-pregnant female rats at doses of 10, 30, 90, 270, and 500 mg/kg/day beginning on the first day of presumed gestation (GD 0) and continuing through the day of delivery or GD 24 for rats that did not deliver. A control group was administered deionized water. The dose volume was 5 ml/kg. The following maternal evaluations were performed: clinical observations (at least once daily during gestation, throughout parturition, and on postnatal days 1, 4, and 6), body weight and feed consumption (daily), duration of gestation, length of parturition, and necropsy. Pregnancy status and uterine contents were recorded. Uteri of apparently non-pregnant rats were stained with 10% ammonium sulfide to aid in the examination of implantation sites. Litter and pup (F_1) evaluations were performed through postnatal day (PND) 6 and included: litter size, pup viability at birth, pup survival and body weight on PND 1, 4, and 6, clinical observations, and necropsy. Necropsies of pups included a single cross-section of the head at the level of the frontal–parietal suture and examination of the cross-sectioned brain for apparent hydrocephaly. Homogeneous, continuous data were analyzed by ANOVA followed by Dunnett's test. Non-homogeneous data were ranked and ANOVA and Dunnett's test, if appropriate, were performed on the ranks. Frequency data were evaluated using Fisher's exact test.

2.9. Evaluation of PPARα activation in rats

ADONA (30% stock solution) was diluted in sterile 0.9% saline for injection (Baxter Healthcare Corp., Deerfield, IL) and administered to groups of male and female Sprague–Dawley rats (3/sex/group) at a dose of 30 mg/kg either by single oral gavage or by single intravenous injection. Control animals received no treatment. Dose volumes were 2.0 ml/kg. Approximately 48 h after dosing, all animals were euthanized and liver specimens (approximately 300 mg) were collected, placed in RNALater® (Applied Biosystems, Carlsbad, CA), and shipped on ice packs to the analytical facility where they were stored at approximately 4 °C prior to analysis. The liver specimens were analyzed by quantitative real-time PCR as previously described (Chang et al., 2009) for the following mRNA transcripts: peroxisomal bifunctional enzyme (*Ehhadh*), cytochrome P450-4a1 (*Cyp4a1*), acyl coenzyme A oxidase (*Acox*), and DNA damage inducible transcript (*Ddit3*). *Ehhadh*, *Cyp4a1*, and *Acox* are enzyme markers of PPARα-mediated gene transcription and *Ddit3* is a marker of general tissue injury. mRNA sequences for target genes were obtained from the GenBank database (http://www.ncbi.nih.gov) and gene-specific primers were designed using IDT PrimerQuest℠ (www.idtdna.com). Primer sequences used in this study are listed in Table 2. Target-specific mRNA expression was normalized against 18s rRNA and the results were reported as transcript-specific mRNA copies per 18s rRNA copy. Statistical significance was assessed using ANOVA followed by Dunnett's test.

3. Results

3.1. Acute toxicity studies

All three rats administered ADONA at an oral dose of 2000 mg/kg exhibited hunched posture, uncoordinated movements, and piloerection and died within 2 days of dosing. At necropsy, these animals exhibited dark red foci of the mucosa of the glandular stomach. All three rats administered ADONA at a dose of 300 mg/kg exhibited hunched posture for 1–2 days after dosing and survived to scheduled termination on day 15. There were no effects on body weight and no abnormal necropsy findings at 300 mg/kg.

All rats administered ADONA dermally at a dose of 2000 mg/kg for 24 h under semi-occluded conditions survived to scheduled termination on day 15. Clinical signs included mild erythema and scales at the test site, hunched posture, chromodacryorrhea, and piloerection. All clinical signs had fully resolved in all animals by day 9. There were no effects on body weight and no abnormal necropsy findings.

3.2. Primary eye and skin irritation studies

ADONA (0.1 ml of the 30% stock solution) caused moderate to severe ocular irritation when instilled into the eyes of rabbits. Effects included (values are combined mean scores for the 24, 48, and 72 h examinations over the maximum possible score): mild corneal opacity (1.0/4.0) with epithelial damage affecting 20–75% of the corneal surface, neovascularization of the cornea, iridial irritation (1.0/2.0), moderate to severe conjunctival redness (2.6/3.0), chemosis (1.3/4.0), and discharge (1.3/3.0). These effects had fully resolved in all animals by day 21.

Dermal exposure to ADONA (0.5 ml of the 30% stock solution) for 4 h under semi-occluded conditions caused slight to well-de-

Table 3
Murine local lymph node assays.[a]

Treatment group	First assay	Second assay					
	Stimulation index	Stimulation index	B cells		T cells	B:T cell ratio	
			%	Fold-increase vs. control	%	Ratio	Fold-increase vs. control
Naïve control	–[b]	1.0 ± 0.3 (5)	13.9 ± 2.1 (5)	(1.0)	70.5 ± 2.5 (5)	0.20 ± 0.04 (5)	(1.0)
Vehicle control	1.0 ± 0.3 (5)	1.0 ± 0.2 (5)	11.8 ± 1.2 (5)	(1.0)	73.0 ± 2.2 (5)	0.16 ± 0.02 (5)	(1.0)
Positive control	–	15.3 ± 1.1 (5)*	27.1 ± 1.3 (5)	2.30*	58.3 ± 0.7 (5)	0.46 ± 0.02 (5)	2.84*
ADONA 7.5%	1.8 ± 0.6 (5)	0.8 ± 0.0 (5)	12.3 ± 0.7 (5)	1.05	73.2 ± 1.7 (5)	0.17 ± 0.01 (5)	1.04
ADONA 15%	2.7 ± 0.8 (5)	2.4 ± 0.6 (5)	14.3 ± 0.7 (5)	1.22	72.9 ± 1.3 (5)	0.20 ± 0.01 (5)	1.20
ADONA 30%	4.9 ± 1.6 (5)*	3.8 ± 1.2 (4)*	22.4 ± 1.5 (4)	1.91*	62.6 ± 2.2 (4)	0.36 ± 0.04 (4)	2.21*
ADONA 30%[c]	–	8.9 ± 2.8 (4)*	22.4 ± 1.5 (4)	1.62*	62.6 ± 2.2 (4)	0.36 ± 0.04 (4)	1.79*

[a] Values, except fold-increases, are means ± SEM (*N*). In the first assay, dimethyl formamide (DMF) was used as the vehicle and there were no naïve or concurrent positive control groups. In the second assay, dimethylsulfoxide (DMSO) was used as the vehicle and α-hexylcinnamaldehyde (25% in DMSO) was the positive control.
[b] Not applicable.
[c] Values in this row are relative to the naïve control group. All other values are relative to the vehicle control group.
* Value meets a criterion for a dermal sensitizer (i.e., stimulation index ⩾3- or ⩾1.25-fold increase in %B cells or B:T cell ratio).

fined (grade 1–2) erythema at the treatment sites of all three rabbits. This erythema resolved within 48 h in two animals and within 72 h in the third animal. No edema or other treatment-related dermal signs were observed in any animal. Overall mean erythema and edema scores were 0.43 and 0.00, respectively. All animals gained weight and none exhibited clinical signs of toxicity.

3.3. Dermal sensitization studies

Key findings from the two local lymph node assays in mice are summarized in Table 3. In the first assay, ADONA caused no clinical signs of toxicity, no effects on body weight, and no signs of irritation at the test sites. The auricular lymph nodes of 4 of 5 animals in the 30% ADONA group were slightly enlarged. No macroscopic abnormalities of the surrounding area were noted. ADONA induced concentration-related increases in the stimulation index. The simulation index for the 30% ADONA treatment group was 4.9.

In the second assay, all animals appeared normal and gained weight except one animal in the 30% ADONA group which appeared emaciated, exhibited a wet anogenital area, and showed a small decrease in body weight on day 6. This animal was not administered BrdU and its lymph nodes were not harvested. The effects noted in this animal were not considered to be treatment-related since none of the other four animals in this dose group

Table 4
In vitro HPRT gene mutation assay in Chinese hamster V79 cells.[a]

Treatment group	Conc. (μg/ml)	S9	Cloning efficiency (%)		Mutant colonies (per 10^6 cells)		Fold-increase vs. vehicle control	
			A	B	A	B	A	B
First assay								
Vehicle control	0	–	(100.0)	(100.0)	19.2	9.5	(1.0)	(1.0)
Positive control	150.0	–	63.9	67.2	154.2	168.3	8.0[b]	17.8[b]
ADONA	103.1	–	70.3	98.8	11.9	7.2*	0.6	0.8
ADONA	206.3	–	66.7	86.9	33.7	19.6*	1.8	2.1
ADONA	412.5	–	50.1	30.3	24.1	17.7*	1.3	1.9
ADONA	618.8	–	0.0	5.4	19.7	22.2*	1.0	2.3
ADONA	825.0	–	0.0	0.1	14.6	29.4*	0.8	3.1[b]
Vehicle control	0	+	(100.0)	(100.0)	13.5	16.4	(1.0)	(1.0)
Positive control	1.3	+	39.5	47.9	615.3	661.8	45.5[b]	40.3[b]
ADONA	206.3	+	119.1	92.9	30.3	41.8	2.2	2.5
ADONA	412.5	+	100.9	82.1	17.3	25.3	1.3	1.5
ADONA	825.0	+	93.0	79.2	23.2	14.0	1.7	0.9
ADONA	1650.0	+	0.5	1.5	19.0	21.4	1.4	1.3
Second assay								
Vehicle control	0	–	(100.0)	(100.0)	16.0	11.1	(1.0)	(1.0)
Positive control	150.0	–	82.7	91.2	371.9	308.6	23.2[b]	27.7[b]
ADONA	93.8	–	101.6	107.4	11.5	13.3	0.7	1.2
ADONA	187.5	–	103.0	102.3	19.2	20.3	1.2	1.8
ADONA	375.0	–	108.8	103.8	10.5	18.0	0.7	1.6
ADONA	750.0	–	67.0	95.6	13.6	17.6	0.8	1.6
ADONA	1500.0	–	22.5	21.1	17.5	15.9	1.1	1.4
Vehicle control	0	+	(100.0)	(100.0)	10.4	7.8	(1.0)	(1.0)
Positive control	1.3	+	87.1	35.9	578.3	440.5	55.8[b]	56.1[b]
ADONA	93.8	+	103.4	99.2	12.2*	14.0	1.2	1.8
ADONA	187.5	+	105.4	99.5	8.1*	15.0	0.8	1.9
ADONA	375.0	+	104.4	100.8	14.8*	32.7	1.4	4.2[b]
ADONA	750.0	+	16.0	96.5	11.9*	18.8	1.1	2.4
ADONA	1500.0	+	2.5	12.5	21.0*	9.9	2.0	1.3

[a] Only cultures for which mutant colonies were counted are shown. Columns A and B are results for duplicate cultures. Treatment periods were 4 h with and without S9 in the first assay and 4 h with S9 and 24 h without S9 in the second assay. Positive controls were ethylmethane sulfonate in cultures with S9 and 7,12-dimethylbenz(a)anthracene in cultures without S9.
[b] Value exceeded the 3-fold criterion for a positive response; however, the increases for the ADONA-treated groups were not reproducible (were not observed in the duplicate cultures) and were therefore judged not to be toxicologically significant.
* Statistically significant dose response ($p < 0.05$, least squares regression analysis); however, mutant frequencies for individual groups were all within the laboratory historical control range for 4-h exposures (1.7–31.1 per 10^6 cells without S9, 1.0–29.1 per 10^6 cells with S9) and were therefore judged not to be toxicologically significant.

showed similar effects. Ear swelling data and examinations of the treatment sites indicated that none of the ADONA formulations was irritating. ADONA induced concentration-related increases in the stimulation index. The stimulation index for the 30% ADONA treatment group was 3.8 relative to the vehicle control group and 8.9 relative to the naïve control group. ADONA also induced concentration-related increases in the percentage of B lymphocytes (%B cells) and the ratio of B to T lymphocytes (B:T cell ratio) in the auricular lymph nodes of treated mice. The mean %B cells and B:T cell ratios for the 30% ADONA treatment group were more than 1.25-fold greater than the mean values for the vehicle and naïve controls. The calculated EC3 for ADONA was 16.4%. Based on the findings in both assays, ADONA was judged to be a weak dermal sensitizer (ECETOC, 2003).

3.4. Genotoxicity studies

3.4.1. Bacterial reverse mutation assays

ADONA, at concentrations up to 5000 μg/plate, did not induce significant increases in the number of revertant colonies in any of the *Salmonella* or *E. coli* strains tested, either in the presence or the absence of metabolic activation. No evidence of toxicity (reduction in the bacterial lawn or significant decrease in the number of revertants) was detected at any ADONA concentration tested. All criteria for a valid study were met. Under the conditions of the study, ADONA was not mutagenic.

3.4.2. In vitro mammalian gene mutation assay

Results of the in vitro HPRT gene mutation assay in Chinese hamster V79 cells are summarized in Table 4. ADONA concentrations from 3000 to 6000 μg/ml were excessively toxic and these cultures were not continued (data not shown). Statistically significant, concentration-related trends in mutant frequency were observed in culture B of the first assay without metabolic activation and in culture A of the second assay with metabolic activation. These trends were judged not to be toxicologically significant, however, because the mutant frequencies for these cultures were all within the laboratory historical control range and the results were not reproducible in the duplicate cultures. Greater than 3-fold increases in mutant frequency were seen for the positive control article in each of the duplicate cultures (8.0- to 56.1-fold) and for two cultures treated with ADONA (3.1-fold at 825 μg/ml without metabolic activation in culture B of the first assay and 4.2-fold at 375 μg/ml with metabolic activation in culture B of the second assay). Neither of these increases in the ADONA-treated cultures was reproducible and the mutant frequency for the first culture was within the historical control range. The mutant frequency for the second culture was slightly above the historical control range but was not concentration-related. Therefore, the increases in mutant frequency in the two ADONA-treated cultures were judged not to be toxicologically significant. Appropriate toxicity (cloning efficiency less than 50%) was attained at the highest ADONA concentrations tested in each assay. All criteria for a valid study were met. Under the conditions of the study, ADONA was not mutagenic.

3.4.3. In vitro chromosome aberration assay in human lymphocytes

Results of the chromosome aberration assays in cultured human lymphocytes are summarized in Table 5. In the first assay, ADONA induced statistically significant increases in the number of cells with chromosome aberrations at concentrations between 2100 and 2300 μg/ml (3-h exposure) in the absence of metabolic activation and at 2000 and 2200 μg/ml (3-h exposure) in the

Table 5
In vitro chromosome aberration assay in human lymphocytes.[a]

Treatment group	Conc. (μg/ml)	S9	Exposure time/fixation time (h)	Mitotic index (%)	Cells with aberrations (%)	Cells with aberrations other than caps (%)
First assay						
Vehicle control	0	–	3/24	(100)	1.0	1.0
Positive control	0.5	–	3/24	85	30.5***	27.5***
ADONA	2100	–	3/24	100	10.5***	9.0***
ADONA	2200	–	3/24	75	9.0***	8.0***
ADONA	2300	–	3/24	48	12.0***	10.5***
Vehicle control	0	+	3/24	(100)	1.5	1.0
Positive control	10	+	3/24	60	32.0***	28.5***
ADONA	1800	+	3/24	99	3.5	2.5
ADONA	2000	+	3/24	78	12.0***	11.5***
ADONA	2200	+	3/24	49	7.0**	7.0**
Second assay						
Vehicle control	0	–	24/24	(100)	0.0	0.0
Positive control	0.2	–	24/24	69	15.0***	13.5***
ADONA	50	–	24/24	98	0.0	0.0
ADONA	200	–	24/24	71	0.0	0.0
ADONA	400	–	24/24	46	0.0	0.0
Vehicle control	0	–	48/48	(100)	0.0	0.0
Positive control	0.1	–	48/48	73	18.0***	17.0***
ADONA	100	–	48/48	102	0.5	0.5
ADONA	200	–	48/48	65	0.0	0.0
ADONA	400	–	48/48	43	0.0	0.0
Vehicle control	0	+	3/48	(100)	1.5	1.5
Positive control	10	+	3/48	–[b]	38.7***	38.0***
ADONA	1700	+	3/48	148	1.5	1.5
ADONA	1900	+	3/48	68	7.5**	6.5**
ADONA	2100	+	3/48	92	5.0*	5.0*
ADONA	2300	+	3/48	35	10.5*	10.5*

[a] Only treatment groups in which aberrations were evaluated are shown. Values for mitotic index, cells with aberrations, and cells with aberrations other than gaps are means of duplicate cultures. Positive controls were cyclophosphamide in cultures with S9 and mitomycin C in cultures without S9.
[b] Culture was fixed after 24 h, therefore the mitotic index could not be calculated as a percentage of control.
* $p < 0.05$ (Chi-square test).
** $p < 0.01$ (Chi-square test).
*** $p < 0.001$ (Chi-square test).

Table 6
Micronucleus assay in mice.

Treatment group	Dose (mg/kg)	Sampling time (h)	Mortality (%)	Micronucleated PCE (per 2000 PCE)[a]	Ratio of PCE to NCE[a]
Males					
Vehicle control	0	24	0	1.0 ± 0.7 (5)	0.91 ± 0.06 (5)
Positive control[b]	40	48	0	33.0 ± 4.3 (5)**	0.55 ± 0.16 (5)
ADONA	200	24	0	1.4 ± 1.5 (5)	0.88 ± 0.08 (5)
ADONA	400	24	0	1.8 ± 1.3 (5)	0.90 ± 0.10 (5)
ADONA	800	24	28.6[c]	3.2 ± 1.3 (5)*,[d]	0.89 ± 0.10 (5)
ADONA	800	48		1.6 ± 0.9 (5)	0.89 ± 0.07 (5)
Females					
Vehicle control	0	24	0	1.8 ± 0.8 (5)	0.93 ± 0.04 (5)
Positive control[b]	40	48	0	22.0 ± 4.1 (5)**	0.60 ± 0.13 (5)
ADONA	180	24	0	2.0 ± 1.2 (5)	0.92 ± 0.06 (5)
ADONA	375	24	0	1.6 ± 1.1 (5)	0.92 ± 0.06 (5)
ADONA	750	24	35.7[c]	1.0 ± 1.0 (5)	0.87 ± 0.14 (5)
ADONA	750	48		1.8 ± 1.7 (4)	0.94 ± 0.07 (4)

[a] Values are means ± SD (*N*).
[b] Cyclophosphamide in physiological saline.
[c] Value is for the 24- and 48-h sampling times combined.
[d] Value is within the laboratory historical control range (0–5 micronucleated PCE per 2000 PCE).
* $p < 0.05$ (Wilcoxon Rank Sum Test).
** $p < 0.01$ (Wilcoxon Rank Sum Test).

presence of metabolic activation. In the second assay, ADONA induced chromosome aberrations at concentrations between 1900 and 2300 μg/ml (3-h exposure) in the presence of metabolic activation but did not induce aberrations at concentrations between 50 and 400 μg/ml (24- and 48-h exposures) in the absence of metabolic activation. The most common aberrations were chromatid and chromosome gaps. There were no increases in the number of cells exhibiting endoreduplication or polyploidy. Appropriate toxicity (mitotic index less than 50%) was attained at the highest ADONA concentrations tested in each assay. All criteria for a valid study were met. Under the conditions of the study, ADONA induced chromosome aberrations in cultured human lymphocytes with and without metabolic activation. However, as explained in Section 4, certain aspects of this study suggest that this may have been a false positive finding.

3.4.4. Micronucleus assay in mice

Results of the micronucleus assay in mice are summarized in Table 6. Four of 14 males administered ADONA at 800 mg/kg and 5 of 14 females administered ADONA at 750 mg/kg died. Clinical signs among animals in these dose groups included lethargy, hunched posture, ataxia, and rough coat. There were no deaths and no clinical signs of toxicity in the other groups. None of the ADONA treatment groups showed a significant decrease in the ratio of PCE to NCE, indicating the lack of a toxic effect on erythropoiesis. A small but statistically significant increase in the frequency of micronucleated PCE was observed in males in the 800 mg/kg treatment group at the 24-h sampling time. However, the frequency of micronucleated PCE was well within the laboratory historical control range and was therefore not considered toxicologically significant. The positive control article induced significant increases in the frequency of micronucleated PCE and caused the expected decreases in the ratio of PCE to NCE. All criteria for a valid study were met. Under the conditions of the study, ADONA was not clastogenic or aneugenic in male or female mice at oral doses up to and including the maximum tolerated dose.

3.4.5. Bone marrow cytogenetic study in rats

Results of the bone marrow cytogenetic study in rats are summarized in Table 7. Two of 8 high-dose males (1120 mg/kg) and 3 of 8 high-dose females (940 mg/kg) died. Clinical signs observed in high-dose animals included lethargy, hunched posture, ataxia, rough coat, and rales. No deaths or abnormal clinical signs were observed in the other groups. Body weight losses occurred in males and females in the high- and mid-dose groups. Mitotic indices for the ADONA treatment groups ranged from 65% to 105% in males and from 27% to 72% in females. ADONA did not induce statistically or toxicologically (outside the historical control range) significant increases in the number of cells with chromosome aberrations at any dose tested and did not induce polyploidy or endoreduplication. The positive control article induced significant increases in the number of cells with chromosome aberrations. All criteria for a valid study were met. Under the conditions of the study, ADONA was not clastogenic in male or female rats at oral doses up to and including the maximum tolerated dose.

3.5. Repeat-dose toxicity studies

3.5.1. 5-Day oral toxicity study in rats

Key findings in the 5-day oral toxicity study in rats are summarized in Table 8. Serum ADONA concentrations (Cmax and AUC) for surviving males and females following the last dose were dose-related and were higher in males than in females. Mean liver concentrations were much higher in males than in females on day 6. Liver concentrations at the end of the recovery period (day 13) had declined to approximately 2–4% (males) and 5–9% (females) of the day 6 levels. Liver concentrations were approximately equal to or less than the corresponding serum concentrations for both males and females.

All females in the 298 mg/kg/day dose group died between days 3 and 5. These females exhibited decreased activity, dramatically reduced food consumption, and had dark material on their fur at necropsy. Histopathologic examination of these animals revealed minimal to mild renal congestion, tubular dilation, and tubular degeneration/regeneration.

All males survived to scheduled termination and none exhibited clinical signs of toxicity. Statistically significant findings in high-dose (298 mg/kg/day) males during or at the end of the treatment period included: decreases in food consumption (approximately 19%),[1] body weight (9%), body weight gain (50%), and red blood cell count (13%), and increases in platelets (68%), total bilirubin (41%), glucose (50%), urea nitrogen (71%), calcium (5%), chloride (4%), absolute liver weight (47%), and relative kidney weight (23%). Relative liver weight was also increased (57%) in this group and,

[1] Percentages shown here and for the studies that follow are increases or decreases in the ADONA group mean relative to the corresponding control group mean rounded to the nearest whole number.

Table 7
Bone marrow cytogenetic study in rats.[a]

Treatment group	Dose (mg/kg)	Fixation period (h)	Mortality (%)	Body wt. change[b] (g)	Mitotic index (%)	Cells with aberrations (%)	Cells with aberrations other than gaps (%)
Males							
Vehicle control	0	12–18	0	11.6	(100)	0.8	0.8
Positive control[c]	20	12–18	0	5.2	47	48.0*	48.0*
ADONA	280	12–18	0	7.4	105	1.4	1.4
ADONA	560	12–18	0	−6.0	81	1.0	1.0
ADONA	1120	12–18	25[d]	−25.6	65	0.8	0.8
ADONA	1120	36–44		−28.4	80	1.0	1.0
Females							
Vehicle control	0	12–18	0	6.0	(100)	0.6	0.6
Positive control[c]	20	12–18	0	5.4	37	33.0*	33.0*
ADONA	235	12–18	0	3.6	62	1.2	1.2
ADONA	470	12–18	0	−4.6	52	0.5	0.5
ADONA	940	12–18	37.5[d]	−11.8	72	0.8	0.8
ADONA	940	36–44		−30.6[e]	27[e]	1.0[e]	1.0[e]

[a] Values for body weight change, mitotic index, cells with aberrations, and cells with aberrations other than gaps are means, $N = 5$ except where otherwise indicated.
[b] Mean body weight change from immediately prior to dosing to administration of colchicine.
[c] Cyclophosphamide in physiological saline.
[d] Value is for the 12–18 h and 36–44 h fixation periods combined.
[e] $N = 4$.
* $p < 0.001$ (one-sided Chi-square test).

although not statistically significant, this increase was considered to be toxicologically significant. Cholesterol was significantly decreased (59%) at the end of the recovery period. Group mean values for several of the clinical chemistry parameters in high-dose males (i.e., total bilirubin, glucose, calcium, chloride, and cholesterol) were within laboratory historical control ranges and are therefore of doubtful toxicological significance. Minimal to mild renal tubular dilation was observed in 3 of 3 high-dose males and minimal to mild renal tubular degeneration/regeneration was observed in 2 of 3 high-dose males.

At the mid-dose, 104 mg/kg/day, the only treatment-related, toxicologically significant findings were minimal to mild renal tubular dilation in 1 of 3 males and 2 of 3 females and minimal renal tubular degeneration in 1 of 3 males. The renal changes observed in high- and mid-dose animals were characterized by tubular cell degeneration with cellular flattening and dilation of tubular lumens and focal tubular cell regeneration with basophilic cytoplasm and infrequent small clusters of dense (hyperchromatic) nuclei and mitosis. A statistically significant decrease in red blood cell count (11%) was observed in males at this dose but the mean value was within the laboratory historical control range.

At the low dose, 28 mg/kg/day, no treatment-related effects were observed in females. In males at this dose, serum total bilirubin was significantly increased (41%) at the end of the treatment period and cholesterol was significantly decreased both at the end of the treatment period (46%) and the end of the recovery period (43%). The mean values for total bilirubin at the end of the treatment period and for cholesterol at the end of the recovery period were within historical control ranges and no significant decreases in cholesterol were observed in mid- and high-dose males at the end of the treatment period. The toxicological significance of these serum chemistry findings in males is therefore doubtful.

None of the treatment-related findings observed during or at the end of the treatment period among mid- and high-dose males or among mid-dose females were observed among recovery animals at the end of the 7-day recovery period.

3.5.2. 28-Day oral toxicity study in rats

Key findings in the 28-day oral toxicity study in rats are summarized in Table 9. No treatment-related deaths and no abnormal clinical signs were observed in any ADONA treatment group. There were no significant treatment-related effects on body weight, food consumption, or any functional observational parameters (i.e., hearing ability, pupillary reflex, static righting reflex, grip strength, and motor activity).

The only statistically significant findings in females were a slight decrease in serum calcium (3%), a small increase in serum creatinine (7%), and increases in absolute and relative adrenal weights (23% and 30%, respectively) at 100 mg/kg/day. Group mean values for each of these endpoints were within laboratory historical control ranges and no corresponding microscopic changes were noted in the adrenal glands. These changes in females are therefore of questionable toxicological significance.

In males, statistically significant clinical pathology findings included: decreases in hemoglobin (10%), hematocrit (7%), and total bilirubin (39%), and increases in alkaline phosphatase (39%), glucose (49%), inorganic phosphate (14%), potassium (9%), and urea (31%) at 100 mg/kg/day; decreased total bilirubin (30%) and increased glucose (40%) and potassium (8%) at 30 mg/kg/day; and decreased total bilirubin (30%) at 10 mg/kg/day. With the exception of decreased total bilirubin in males at 100 mg/kg/day, mean values for each of these parameters were within laboratory historical control ranges for male Wistar rats and are therefore of doubtful toxicological significance. The lower serum total bilirubin and elevated serum glucose values in males may reflect adaptive changes in the liver.

The livers of two high-dose males appeared enlarged at necropsy. Absolute liver weights were significantly increased in males at 30 and 100 mg/kg/day (34% and 64%, respectively) and relative liver weights were significantly increased in males in all groups (18%, 29%, and 62% at 10, 30, and 100 mg/kg/day, respectively). The mean relative liver weight for the male control group was slightly below the historical control range which may partially account for the statistically significant increases in relative liver weights for males in the 10 and 30 mg/kg/day groups.

The only significant histopathologic change was diffuse midzonal/centrilobular hepatocellular hypertrophy at minimal to moderate (dose-related) severity in the livers of all males in all ADONA treatment groups. No evidence of hepatocellular hypertrophy or other liver changes were observed in high-dose females (livers of mid- and low-dose females were not examined microscopically). There was no evidence of hepatocellular degeneration, necrosis, or other pathologic changes in males at any dose. The

Table 8
5-Day oral toxicity study in Sprague–Dawley rats – key findings.[a]

	Males				Females			
	Control	28 mg/kg/day	104 mg/kg/day	298 mg/kg/day	Control	28 mg/kg/day	104 mg/kg/day	298 mg/kg/day
Mortality, %	0	0	0	0	0	0	0	100
Body weight, g (day 6)	304 ± 7.7 (6)	307 ± 1.5 (3)	295 ± 15.5 (3)	283 ± 11.2 (3)*	203 ± 11.0 (6)	205 ± 11.0 (3)	205 ± 9.2 (3)	–[b]
Body weight gain, g (days 1–5)	34 ± 5.5 (12)	32 ± 5.3 (6)	36 ± 12.7 (6)	17 ± 7.7 (6)**	18 ± 8.1 (12)	18 ± 5.7 (6)	16 ± 5.4 (6)	–[b]
Food consumption, g/day (days 1–5)	31 ± 2.4 (12)	30 ± 1.3 (6)	31 ± 3.6 (6)	25 ± 2.3 (6)**	22 ± 1.8 (12)	22 ± 1.3 (6)	22 ± 1.9 (6)	4 ± 2.2 (6)[c],**
Toxicokinetics[d]								
Serum Cmax, μg/ml	–[e]	21.7 ± 10.3 (3)	55.5 ± 35.9 (3)	116.1 ± 76.4 (3)	–[e]	13.3 ± 10.3 (3)	46.6 ± 35.8 (3)	–[b]
Serum AUC, μg h/ml	–[e]	1232 ± 419 (3)	2370 ± 1081 (3)	4879 ± 2271 (3)	–[e]	441 ± 260 (3)	1545 ± 909 (3)	–[b]
Liver conc., μg/g (day 6)	<0.01 (3)	27.9 ± 33.8 (3)	12.9 ± 5.7 (3)	29.2 ± 22.5 (3)	<0.01 (3)	0.08 ± 0.04 (3)	0.31 ± 0.17 (3)	–[b]
Liver conc., μg/g (day 13)	<0.01 (3)	0.65 ± 0.10 (3)	0.35 ± 0.40 (3)	1.67 ± 1.2 (3)	<0.01 (3)	<0.01 (3)	0.02 ± 0.01 (3)	–[b]
Hematology								
Red blood cells, 10^6/μl (day 6)	7.57 ± 0.43 (6)	7.10 ± 0.14 (3)	6.76 ± 0.22 (3)[g],*	6.61 ± 0.46 (3)**	7.16 ± 0.42 (6)	6.92 ± 0.10 (3)	7.05 ± 0.10 (3)	–[b]
Platelets, 10^3/μl (day 6)	1020 ± 142 (6)	1246 ± 142 (3)	1145 ± 103 (3)	1712 ± 144 (3)**	1270 ± 114 (6)	1162 ± 89 (3)	1290 ± 225 (3)	–[b]
Serum chemistry								
Total bilirubin, mg/dl (day 6)	0.51 ± 0.06 (6)	0.72 ± 0.04 (3)[g],**	0.60 ± 0.03 (3)	0.72 ± 0.08 (3)[g],**	0.55 ± 0.06 (6)	0.56 ± 0.14 (3)	0.61 ± 0.14 (3)	–[b]
Glucose, mg/dl (day 6)	93 ± 10.1 (6)	119 ± 9.4 (3)	113 ± 18.9 (3)	140 ± 14.6 (3)[g],**	100 ± 9.6 (6)	114 ± 8.1 (3)	94 ± 14.5 (3)	–[b]
Urea nitrogen, mg/dl (day 6)	14 ± 2.0 (6)	18 ± 2.4 (3)	18 ± 1.7 (3)	24 ± 6.0 (3)**	18 ± 2.2 (6)	18 ± 1.9 (3)	18 ± 2.1 (3)	–[b]
Calcium, mg/dl (day 6)	10.12 ± 0.20 (6)	10.11 ± 0.18 (3)	10.27 ± 0.17 (3)	10.63 ± 0.27 (3)[g],*	10.49 ± 0.37 (6)	10.45 ± 0.21 (3)	10.21 ± 0.34 (3)	–[b]
Chloride, mmol/l (day 13)	102 ± 0.8 (6)	103 ± 0.7 (3)	104 ± 0.9 (3)	106 ± 0.5 (3)[g],**	102 ± 0.7 (6)	102 ± 1.5 (3)	103 ± 1.3 (3)	–[b]
Cholesterol, mmol/l (day 6)[f]	0.67 ± 0.13 (6)	0.36 ± 0.12 (3)**	0.52 ± 0.16 (3)	0.52 ± 0.15 (3)	0.70 ± 0.12 (6)	0.65 ± 0.11 (3)	0.70 ± 0.23 (3)	–[b]
Cholesterol, mmol/l (day 13)[f]	0.91 ± 0.12 (6)	0.52 ± 0.13 (3)[g],**	0.72 ± 0.20 (3)	0.54 ± 0.14 (3)[g],**	0.85 ± 0.13 (6)	0.72 ± 0.02 (3)	0.62 ± 0.05 (3)	–[b]
Triglyceride, mg/dl (day 6)	24 ± 8.1 (6)	16 ± 7.0 (2)	21 ± 5.2 (3)	18 ± 5.6 (3)	23 ± 5.3 (6)	21 ± 5.1 (3)	20 ± 5.1 (3)	–[b]
Organ weights								
Liver, g (day 6)	9.78 ± 1.01 (6)	11.06 ± 1.41 (3)	10.32 ± 1.32 (3)	14.38 ± 0.67 (3)**	6.53 ± 0.50 (6)	6.37 ± 0.21 (3)	6.97 ± 0.24 (3)	–[b]
Liver, % body weight (day 6)	3.22 ± 0.30 (6)	3.61 ± 0.44 (3)	3.50 ± 0.29 (3)	5.07 ± 0.07 (3)	3.22 ± 0.15 (6)	3.11 ± 0.10 (3)	3.40 ± 0.12 (3)	–[b]
Kidney, % body weight (day 6)	0.90 ± 0.092 (6)	0.96 ± 0.059 (3)	0.90 ± 0.016 (3)	1.10 ± 0.109 (3)**	0.85 ± 0.029 (6)	0.90 ± 0.042 (3)	0.89 ± 0.034 (3)	–[b]
Histopathology[h]								
Renal tubular degeneration	0/6	0/1	1/3 (1.0)	2/3 (1.5)	0/6	–[e]	0/3	6/6 (2.0)
Renal tubular regeneration	0/6	0/1	0/3	2/3 (1.0)	0/6	–[e]	0/3	4/6 (1.0)
Renal tubular dilation	0/6	0/1	1/3 (1.0)	3/3 (1.7)	0/6	–[e]	2/3 (1.5)	6/6 (1.7)
Renal congestion	0/6	0/1	0/3	0/3	0/6	–[e]	0/3	6/6 (2.0)

[a] Except for serum toxicokinetic, mortality and histopathology findings, all values are means ± SD (*N*).
[b] No data, all animals died.
[c] Food consumption for surviving animals on days 1–3.
[d] Serum samples were collected at 1, 24, 96, and 168 h after the final (5th) dose. Liver specimens were collected at necropsy on days 6 (approx. 24 h after the final dose) and 13 (recovery). Specimens were analyzed by LC/MS. TK assessment was performed using WinNonlin Professional, Ver. 5.0.1 (Pharsight, Mountain View, CA). Cmax and AUC are means ± SEM (*N*).
[e] Not evaluated or not calculated.
[f] Cholesterol values have been converted from mg/dl to mmol/l to facilitate comparison across studies.
[g] Mean value is within the laboratory historical control range.
[h] Histopathology findings are for early deaths (high-dose females) and animals euthanized at the end of the treatment period (day 6). There were no treatment-related histopathology findings at the end of the recovery period (day 13). Values are the number of animals with the finding/number examined. The numbers in parentheses are the average severity for animals with the finding (1 = minimal, 2 = mild/slight, 3 = moderate, 4 = severe).
* $p < 0.05$ (Dunnett's test).
** $p < 0.01$ (Dunnett's test).

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Table 9
28-Day oral toxicity study in Wistar rats – key findings.[a]

	Males				Females			
	Control	10 mg/kg/day	30 mg/kg/day	100 mg/kg/day	Control	10 mg/kg/day	30 mg/kg/day	100 mg/kg/day
Hematology								
Hemoglobin, mmol/l	9.3 ± 0.3	9.3 ± 0.3	9.0 ± 0.3	8.4 ± 0.2[b,**]	8.9 ± 0.7	8.7 ± 0.4	8.7 ± 0.2	8.7 ± 0.3
Hematocrit, l/l	0.447 ± 0.012	0.446 ± 0.019	0.439 ± 0.017	0.416 ± 0.015[b,*]	0.417 ± 0.033	0.414 ± 0.023	0.415 ± 0.014	0.413 ± 0.012
Serum chemistry								
Alkaline phosphatase, U/l	131 ± 17	144 ± 27	148 ± 23	182 ± 34[b,*]	71 ± 15	68 ± 5	78 ± 8	78 ± 11
Calcium, mmol/l	2.81 ± 0.04	2.72 ± 0.11	2.73 ± 0.05	2.75 ± 0.08	2.78 ± 0.05	2.73 ± 0.03	2.72 ± 0.05	2.69 ± 0.05[b,*]
Creatinine, μmol/l	38.9 ± 1.8	37.5 ± 2.2	39.3 ± 0.9	40.8 ± 0.5	42.3 ± 0.8	41.6 ± 1.5	44.3 ± 2.2	45.4 ± 1.7[b,*]
Glucose, mmol/l	6.57 ± 0.72	8.05 ± 1.43	9.23 ± 0.80[b,*]	9.81 ± 2.07[b,**]	6.58 ± 0.62	6.85 ± 0.81	6.88 ± 0.77	6.89 ± 0.62
Inorganic PO_4, mmol/l	2.56 ± 0.03	2.47 ± 0.14	2.74 ± 0.13	2.91 ± 0.25[b,**]	2.20 ± 0.12	2.14 ± 0.21	2.11 ± 0.30	2.10 ± 0.22
Potassium, mmol/l	3.79 ± 0.07	3.89 ± 0.11	4.11 ± 0.10[b,**]	4.15 ± 0.20[b,**]	3.85 ± 0.26	3.65 ± 0.09	3.68 ± 0.26	3.65 ± 0.11
Total bilirubin, μmol/l	2.3 ± 0.3	1.6 ± 0.4[b,**]	1.6 ± 0.2[b,**]	1.4 ± 0.1[**]	2.6 ± 0.3	2.6 ± 0.3	2.3 ± 0.2	2.5 ± 0.4
Urea, mmol/l	6.1 ± 0.9	6.0 ± 1.3	6.0 ± 0.7	8.0 ± 1.3[b,*]	6.0 ± 0.5	6.2 ± 0.5	7.0 ± 1.3	6.5 ± 0.9
Cholesterol, mmol/l	1.74 ± 0.25	1.40 ± 0.29	1.36 ± 0.32	1.38 ± 0.29	1.67 ± 0.43	1.67 ± 0.23	1.61 ± 0.32	1.34 ± 0.17
Necropsy[c]								
Liver enlarged	0/5	0/5	0/5	2/5	0/5	0/5	0/5	0/5
Organ weights								
Liver, g	9.46 ± 1.24	11.11 ± 1.44	12.70 ± 2.67[b,*]	15.49 ± 1.95[*]	6.46 ± 0.75	6.88 ± 0.82	6.15 ± 0.75	6.20 ± 0.49
Liver, % body weight	2.64 ± 0.17	3.12 ± 0.17[b,*]	3.41 ± 0.33[b,**]	4.27 ± 0.33[**]	2.91 ± 0.20	2.99 ± 0.20	2.81 ± 0.29	2.92 ± 0.13
Adrenals, g	0.059 ± 0.009	0.070 ± 0.016	0.064 ± 0.010	0.059 ± 0.011	0.073 ± 0.008	0.087 ± 0.010	0.081 ± 0.011	0.090 ± 0.006[b,*]
Adrenals, % body weight	0.017 ± 0.003	0.020 ± 0.005	0.017 ± 0.003	0.016 ± 0.002	0.033 ± 0.003	0.038 ± 0.007	0.037 ± 0.006	0.043 ± 0.005[b,*]
Histopathology[c]								
Hepatocellular hypertrophy	0/5	5/5[§] (1.2)	5/5[§] (1.6)	5/5[§] (2.8)	0/5	–[d]	–[d]	0/5
Thyroid follicular hypertrophy/hyperplasia	1/5 (1.0)	2/5 (1.0)	1/5 (1.0)	4/5[‡] (1.8)	0/5	–[d]	–[d]	0/5

[a] Except for necropsy and histopathology findings, all values are means ± SD, $N = 5$.
[b] Mean value is within the laboratory historical control range.
[c] Values are the number of animals with the finding/number examined. Numbers in parentheses are the average severity for animals with the finding (1 = minimal, 2 = mild-slight, 3 = moderate, 4 = severe).
[d] Tissue not examined.
[*] $p < 0.05$ (Dunnett's test).
[**] $p < 0.01$ (Dunnett's test).
[§] $p < 0.01$ (Fisher's exact test).
[‡] Incidence showed a positive dose-related trend ($p < 0.05$, Armitage's test) but was not statistically significant for any group (Fisher's exact test).

incidence of minimal to slight thyroid follicular hypertrophy/hyperplasia was not statistically significant for any group but did show a significant dose-related trend in males.

The high-dose, 100 mg/kg/day, was the NOAEL for females in this study. The minimal liver changes observed in male rats at 10 mg/kg/day were not deemed to be toxicologically significant and this dose was considered to be the NOAEL for males in this study.

3.5.3. 90-Day oral toxicity study in rats

Key findings in the 90-day oral toxicity study in rats are summarized in Table 10. Plasma and liver ADONA concentrations were dose-related in males and females. At 10 mg/kg/day, plasma Cmax values were approximately 2-times higher and liver concentrations were about 14-times higher in males than in females. Plasma Cmax values in males and females did not show any clear trends over the course of the study. Mean liver concentrations were slightly increased at weeks 4 and 13 for males in the 3 and 10 mg/kg/day dose groups compared with week 1; however, the increases were not statistically significant (Dunnett's test, $p = 0.05$).

One high-dose female was accidentally killed. Another high-dose female was euthanized in moribund condition on day 85. This animal's condition was attributed to acute renal tubular necrosis which, because it was not observed among any other animals, was considered to be an incidental finding unrelated to treatment. No other deaths occurred and no abnormal clinical signs were observed in any ADONA treatment group. There were no significant effects on body weight or food consumption. Opthalmoscopic examinations of high-dose animals during the last week of the study were normal. There were no treatment-related gross lesions noted at necropsy.

The only statistically significant findings in females were an increase in white blood cell count (35%) and a decrease in urinary calcium excretion (60%) at 100 mg/kg/day. Urinary calcium concentration was also decreased (52%) in females at 100 mg/kg/day but the decrease was not statistically significant. The increased white blood cell count at 100 mg/kg/day was within the historical control range for 13- to 22-week-old female Sprague–Dawley rats (Giknis and Clifford, 2006). Historical control data for the urinary calcium parameters were not available. Serum calcium levels in high-dose females were similar to controls (data not shown) and there was no microscopic evidence of treatment-related renal injury to support the lower urinary calcium concentration and excretion findings.

In males, statistically significant clinical pathology findings included: small decreases in red blood cell count (6%), hemoglobin (4%), and hematocrit (5%), and increases in prothrombin time (4%) and serum aspartate aminotransferase (12%) at 10 mg/kg/

Table 10
90-Day oral toxicity study in Sprague–Dawley rats – key findings.[a]

	Males				Females			
	Control	1 mg/kg/day	3 mg/kg/day	10 mg/kg/day	Control	10 mg/kg/day	30 mg/kg/day	100 mg/kg/day
Plasma Cmax, μg/ml[b]								
Week 1	<0.005 (3)	6.0 ± 0.8 (3)	22.3 ± 2.6 (3)	44.3 ± 6.7 (3)	<0.005 (3)	18.6 ± 5.2 (3)	50.7 ± 1.7 (3)	202 ± 93 (3)
Week 4	0.011 ± 0.001 (3)	8.3 ± 0.2 (3)	15.6 ± 3.8 (3)	22.2 ± 7.7 (3)	<0.005 (3)	11.2 ± 1.1 (3)	26.4 ± 5.3 (3)	71.8 ± 16.9 (3)
Week 13	0.005 ± 0.001 (4)	7.6 ± 1.6 (4)	21.2 ± 4.8 (3)	32.9 ± 7.0 (4)	<0.005 (4)	20.5 ± 7.8 (4)	59.1 ± 15.0 (4)	142 ± 35.9 (4)
Liver conc., μg/g[c]								
Week 1	<0.005 (3)	3.3 ± 0.8 (3)	6.0 ± 1.3 (3)	15.1 ± 1.6 (3)	<0.005 (3)	1.3 ± 0.5 (3)	3.5 ± 0.4 (3)	21.9 ± 12.0 (3)
Week 4	0.006 ± 0.002 (3)	4.5 ± 1.3 (3)	10.9 ± 3.7 (3)	20.8 ± 6.7 (3)	<0.005 (3)	1.5 ± 0.3 (3)	4.0 ± 1.8 (3)	29.5 ± 13.5 (3)
Week 13	<0.005 (4)	3.6 ± 0.8 (4)	12.7 ± 5.0 (4)	21.3 ± 4.6 (4)	<0.005 (4)	1.4 ± 0.3 (4)	4.3 ± 3.2 (4)	11.7 ± 7.1 (4)
Hematology								
Red blood cells, 10^6/μl	8.70 ± 0.49	8.42 ± 0.44	8.27 ± 0.39	8.21 ± 0.37[e,*]	7.92 ± 0.33 (9)	7.90 ± 0.38	7.83 ± 0.27	7.79 ± 0.47 (8)
Hemoglobin, mmol/l	10.1 ± 0.4	9.7 ± 0.2	9.6 ± 0.3[e,*]	9.7 ± 0.3[e,*]	9.6 ± 0.3 (9)	9.4 ± 0.4	9.4 ± 0.3	9.6 ± 0.4 (8)
Hematocrit, l/l	0.45 ± 0.02	0.44 ± 0.01	0.43 ± 0.02[e,*]	0.43 ± 0.02[e,*]	0.43 ± 0.02 (9)	0.43 ± 0.02	0.42 ± 0.01	0.42 ± 0.02 (8)
White blood cells, 10^3/μl	11.1 ± 2.2	7.6 ± 1.9 (9)[**]	8.7 ± 1.7[*]	11.0 ± 1.7	6.5 ± 1.4 (9)	6.5 ± 1.9	5.9 ± 0.9	8.8 ± 2.7 (8)[e,*]
Prothrombin time, s	16.6 ± 0.4	17.0 ± 0.4	16.9 ± 0.6	17.5 ± 0.5[e,**]	15.7 ± 0.4 (9)	16.0 ± 0.4	15.6 ± 0.7	16.0 ± 0.5 (7)
Serum chemistry								
Aspartate aminotransferase, U/l	75.6 ± 4.8	81.1 ± 10.2	84.5 ± 10.0	91.1 ± 18.6[e,*]	79.6 ± 15.2 (9)	86.1 ± 17.4	94.8 ± 59.6	83.8 ± 18.0 (8)
Cholesterol, mmol/l	1.61 ± 0.41	1.18 ± 0.21[*]	1.03 ± 0.32[**]	1.32 ± 0.29	1.97 ± 0.30 (9)	1.85 ± 0.44	2.08 ± 0.34	1.96 ± 0.54 (8)
Urinalysis								
Calcium, mmol/l	0.84 ± 0.55	0.70 ± 0.20	0.75 ± 0.28	0.69 ± 0.17	2.51 ± 1.50	2.81 ± 1.17	2.70 ± 1.91	1.20 ± 0.41 (8)
Calcium, mmol/TPV	0.013 ± 0.007	0.010 ± 0.002	0.012 ± 0.004	0.013 ± 0.003	0.027 ± 0.015	0.030 ± 0.018	0.027 ± 0.018	0.012 ± 0.005 (8)[§]
Organ weights								
Liver, g	11.66 ± 0.87	11.34 ± 1.35	10.97 ± 0.91	12.48 ± 1.23	6.54 ± 0.56	6.89 ± 0.67	6.68 ± 0.59	7.04 ± 0.76 (8)
Liver, % body weight	2.29 ± 0.09	2.27 ± 0.11	2.26 ± 0.14	2.40 ± 0.23	2.49 ± 0.14	2.42 ± 0.09	2.44 ± 0.14	2.65 ± 0.19 (8)
Histopathology[d]								
Hepatocellular hypertrophy	0/10	0/10	0/10	9/10 (1.7)	0/10	0/10	0/10	0/10

[a] Except for histopathology findings, all values are means ± SD; $N = 10$ except where otherwise indicated in parentheses.

[b] Blood specimens were collected from satellite animals at 0.5, 2, and 6 h post-dose during weeks 1, 4, and 13, processed to plasma, and analyzed by LC-MS/MS. The values shown are the highest mean ADONA concentrations during each week, means ± SD (N). The mean plasma Cmax for males administered 10 mg/kg/day was significantly different from that of females (two sample t-test, $p < 0.01$).

[c] Livers were collected from satellite animals at approximately 6 h post-dose during weeks 1, 4, and 13, perfused with normal saline to remove residual blood, and analyzed by LC-MS/MS. Values are μg ADONA per g wet tissue, means ± SD (N). ADONA concentrations at weeks 4 and 13 were not significantly different from those at week 1 for any male or female dose group (Dunnett's test, $p = 0.05$).

[d] Values are the number of animals with the finding/number examined. Numbers in parentheses are the average severity for animals with the finding (1 = minimal, 2 = mild-slight, 3 = moderate, 4 = severe).

[e] Mean value is within the historical control range for 13–22 week old Crl:CD Sprague–Dawley rats (Giknis and Clifford, 2006).

[*] $p < 0.05$ (Dunnett's test).

[**] $p < 0.01$ (Dunnett's test).

[§] $p < 0.01$ (Steel's test).

day; decreases in hematocrit (4%), hemoglobin (5%), white blood cell count (22%), and cholesterol (36%) at 3 mg/kg/day; and decreases in white blood cell count (32%) and cholesterol (27%) at 1 mg/kg/day. With the exception of the decreases in cholesterol and white blood cell counts in the low- and mid-dose groups, the mean values for each of these clinical pathology parameters were within the historical control ranges for 13- to 22-week-old male Sprague–Dawley rats (Giknis and Clifford, 2006). Cholesterol and white blood cell count were not significantly decreased at the high-dose and there was no microscopic evidence of altered red blood cell turnover (e.g., extramedullary hematopoiesis) to support the decreases in red blood cell parameters in males. Therefore, none of the clinical pathology findings in males were considered to be toxicologically significant.

Absolute and relative liver weights were slightly increased (5–8%) in high-dose males and females, but the increases were not statistically significant. Minimal to slight centrilobular hepatocellular hypertrophy was observed in 9 of 10 males in the 10 mg/kg/day dose group. No other treatment-related histopathologic changes were noted in any group.

The minor findings in males at 10 mg/kg/day and in females at 100 mg/kg/day were judged not to be toxicologically significant and these doses were considered to be NOAELs in this study.

3.6. Developmental toxicity screening study in rats

Key findings in the developmental toxicity screening study in rats are summarized in Table 11. The 500 mg/kg/day dose group was terminated on GD 2 due to two deaths, significant body weight loss (mean 26.1 g), reduced food consumption, and clinical signs that included decreased activity, dehydration, cold to touch, pale extremities, rales, ungroomed coat, urine-stained fur, and ptosis. Necropsy findings for these animals were unremarkable. Pregnancy status could not be confirmed in this group due to the early stage of gestation.

At 270 mg/kg/day, 4 of 10 females were found dead between GD 3 and 5 and another was euthanized in moribund condition on GD 21. Clinical signs were similar to those observed in the 500 mg/kg/day dose group but subsided in surviving animals after approximately 4–7 doses. Maternal food consumption was significantly reduced during gestation (17%). Food consumption was also reduced during the postnatal period (24%) but the reduction was not statistically significant. Absolute maternal body weights declined (mean 17.5 g) during GD 0–3 and mean body weight gain during GD 0–20 was significantly reduced (38%). Maternal weight gain during GD 3–6 was also reduced (33%), but not significantly so. Necropsy findings were unremarkable except for red areas in various tissues of two of the animals found dead. The animal euthanized on GD 21 had 12 dead fetuses and one early resorption *in utero*. The pregnancy status of the four early deaths in this group could not be confirmed due to the early stage of gestation.

All females in the 90 and 30 mg/kg/day dose groups survived to scheduled termination. One female in the 10 mg/kg/day dose group was euthanized on GD 23 due to problems during delivery resulting in adverse clinical signs (e.g., cold to touch and pale extremities). This event was not considered to be treatment-related since it did not occur at higher doses and because delivery problems such as this are known to occasionally occur in rats (Alan Hoberman, Charles River Laboratories, personal communication). Other than urine-stained abdominal fur in two females in the 90 mg/kg/day dose group, no significant treatment-related clinical signs were observed at 10, 30, and 90 mg/kg/day. Maternal weight gains were reduced during GD 0–3 (40%), GD 6–9 (34%), and GD 0–20 (12%) in the 90 mg/kg/day dose group but these reductions were not statistically significant. There were no unusual necropsy findings among dams in these groups except for the presence of

Table 11
Developmental toxicity screen in Sprague–Dawley rats – key findings.[a]

	Control	10 mg/kg/day	30 mg/kg/day	90 mg/kg/day	270 mg/kg/day
Maternal (F_0)[b]					
Number dosed	10	10	10	10	10
Deaths	0	1	0	0	5[§]
Number pregnant	9	9	10	10	6
Food consumption, g/day (GD 0–20)	23.4 ± 1.5 (9)	23.2 ± 1.8 (9)	23.4 ± 1.7 (10)	21.9 ± 2.5 (10)	19.4 ± 1.2 (6)[*]
Food consumption, g/day (PND 1–6)	35.2 ± 3.5 (8)	32.4 ± 5.4 (8)	32.6 ± 4.1 (9)	32.4 ± 4.8 (10)	26.8 ± 3.5 (3)
Weight gain, g (GD 0–3)	13.9 ± 5.7 (9)	16.7 ± 6.8 (9)	14.2 ± 6.6 (10)	8.4 ± 7.0 (10)	−17.5 ± 14.5 (6)[*]
Weight gain, g (GD 3–6)	13.9 ± 4.6 (9)	12.4 ± 4.5 (9)	16.1 ± 5.3 (10)	13.3 ± 5.9 (10)	9.3 ± 6.6 (6)
Weight gain, g (GD 6–9)	15.3 ± 3.9 (9)	11.2 ± 4.2 (9)	14.5 ± 2.0 (10)	10.1 ± 5.3 (10)	18.8 ± 8.5 (6)
Weight gain, g (GD 0–20)	152.9 ± 15.4 (9)	144.2 ± 17.0 (9)	147.8 ± 25.5 (10)	134.8 ± 23.3 (10)	95.0 ± 20.6 (6)[*]
Weight gain, g (PND 1–6)	27.5 ± 14.4 (8)	23.8 ± 13.4 (8)	26.0 ± 9.4 (10)	32.2 ± 9.8 (10)	33.0 ± 10.5 (3)
Duration of gestation, days	22.4 ± 0.5 (9)	22.6 ± 0.5 (8)	22.3 ± 0.5 (10)	22.2 ± 0.4 (10)	22.6 ± 0.5 (5)
Length of parturition, min	7.57 ± 2.57 (2)	13.00 ± 6.55 (5)	9.98 ± 1.24 (4)	9.27 ± 2.86 (5)	5.04 ± 1.83 (2)
Litters and pups (F_1)					
Litters delivered normally	9	8	10	10	5
Pups per litter	15.1 ± 1.4 (9)	13.8 ± 2.6 (8)	14.2 ± 3.8 (10)	14.6 ± 2.3 (10)	13.6 ± 2.8 (5)
Liveborn per litter	14.9 ± 1.3 (9)	13.2 ± 2.4 (8)	14.0 ± 3.8 (10)	14.5 ± 2.2 (10)	11.0 ± 5.7 (5)
Stillbirths per litter	0.2 ± 0.4 (9)	0.5 ± 0.8 (8)	0.2 ± 0.4 (10)	0.1 ± 0.3 (10)	2.4 ± 5.4 (5)
Dams with all pups dying PND 1–4, No. (%)	1 (11.1)	0	0	0	2 (40.0)
Pup survival, % (PND 1)	96.3	100	100	100	61.8[§]
Pup survival, % (PND 4)	87.3	99.1	97.9	98.6	47.3[§]
Pup survival, % (PND 6)	87.3	99.1	97.9	98.6	47.3[§]
Pup weight per litter, g (PND 1)	6.3 ± 0.5 (8)	6.1 ± 0.5 (8)	5.9 ± 0.6 (10)	5.5 ± 0.3 (10)[*]	4.6 ± 0.3 (4)[*]
Pup weight per litter, g (PND 4)	8.7 ± 0.9 (8)	8.7 ± 0.6 (8)	8.6 ± 1.3 (10)	7.7 ± 0.8 (10)	6.2 ± 0.5 (3)[*]
Pup weight per litter, g (PND 6)	11.1 ± 1.3 (8)	11.2 ± 0.8 (8)	11.0 ± 1.7 (10)	10.0 ± 1.0 (10)	8.3 ± 0.7 (3)[*]
Pups with gross malformations[c]	0/120	0/109	0/141	0/145	0/64

[a] Except for frequency and percentage data, all values are means ± SD (*N*). All maternal animals in the 500 mg/kg/day group were found dead or were euthanized in moribund condition on GD 2; no data are shown for this group.
[b] Maternal data during gestation are restricted to pregnant animals. Maternal data during the postnatal period are restricted to animals that delivered normally.
[c] Number with malformations/number examined, including stillbirths and pups found dead for which autolysis or cannibalization did not preclude evaluation.
[*] $p < 0.05$ (Dunnett's test).
[§] $p < 0.01$ (Fisher's exact test).

Table 12
Hepatic mRNA transcript levels in male and female Sprague–Dawley rats administered ADONA (30 mg/kg) by single oral gavage or intravenous injection.

Treatment group	Transcript[a]			
	Ddit3	*Cyp4a1*	*Ehhadh*	*Acox*
Males				
Control (untreated)	1.72 ± 0.22	8.30 ± 1.82	7.81 ± 1.23	40.3 ± 7.61
ADONA (oral)	1.51 ± 0.08	78.2 ± 45.4	53.0 ± 29.8	70.7 ± 17.6
ADONA (iv)	1.64 ± 0.14	93.8 ± 20.7	46.7 ± 12.2	65.4 ± 7.94
Females				
Control (untreated)	1.69 ± 0.15	22.1 ± 4.49	13.1 ± 1.80	47.7 ± 6.55
ADONA (oral)	1.48 ± 0.26	27.2 ± 2.29	18.7 ± 1.16	51.0 ± 4.96
ADONA (iv)	1.56 ± 0.10	19.0 ± 2.47	10.7 ± 2.00	45.3 ± 5.29

[a] Values are the mean number of mRNA copies per 18s rRNA ($\times 10^{-5}$) ± SEM, $N = 3$. None of the values for males or females administered ADONA were statistically significant compared with controls (ANOVA and Dunnett's, $p = 0.05$).

red gelatinous material in the right uterine horn of the animal in the 10 mg/kg/day dose group that was euthanized on GD 23. This animal had 2 live and 14 dead fetuses *in utero*.

With the exception of the dam in the 10 mg/kg/day dose group that was euthanized on GD 23, all surviving pregnant dams in the 10, 30, 90, and 270 mg/kg/day dose groups delivered normally. Duration of gestation and length of parturition were not significantly different from controls for any dose group. One dam in the control group and two dams in the 270 mg/kg/day dose group had all pups die between postnatal days 1 and 4. The mean number of pups per litter, percentage of liveborn pups per litter, and percentage of stillborn pups per litter were not significantly different from controls for any dose group. Pup survival on postnatal days 1, 4 and 6 (61.8%, 47.3%, and 47.3%, respectively) was significantly reduced in the 270 mg/kg/day group. Mean pup weight per litter was significantly reduced on postnatal days 1, 4 and 6 (25–29%) in the 270 mg/kg/day group and on postnatal day 1 (13%) in the 90 mg/kg/day group. Two pups in the 270 mg/kg/day dose group had body regions that were discolored purple and 10 pups from this group that were found dead had no milk present in their stomachs. Other clinical signs and necropsy findings in pups from treated groups were considered to be incidental and unrelated to treatment. There were no gross malformations noted at necropsy among pups from any group.

Maternal and developmental NOAELs for ADONA in this study were both 30 mg/kg/day.

3.7. Evaluation of PPARα activation in rats

Hepatic mRNA transcript levels in male and female Sprague–Dawley rats administered ADONA (30 mg/kg) by single oral gavage or intravenous injection are shown in Table 12. Mean *Ddit3* transcript levels for males and females in the ADONA dose groups were not significantly different from controls. In males, mean transcript levels of *Cyp4a1*, *Ehhadh*, and *Acox* were induced 9.4-, 6.8-, and 1.8-fold, respectively, in the oral ADONA dose group and 11.3-, 6.0-, and 1.6-fold, respectively, in the intravenous ADONA dose group; however, due to the small number of animals used in the study and the high inter-animal variation, none of these increases was statistically significant, even when data for the oral and intravenous dose groups were combined. Mean transcript levels of *Cyp4a1*, *Ehhadh*, and *Acox* in females administered ADONA were similar to controls. Since *Ddit3* was not induced by ADONA, the apparent induction of the other genes in males was not an indirect consequence of generalized hepatic injury. Although not statistically significant, the large fold-increases of *Cyp4a1* and *Ehhadh* in males are consistent with ADONA being a PPARα agonist in male Sprague–Dawley rats.

4. Discussion

ADONA was developed as a replacement for APFO as an emulsifier used in the manufacture of fluoropolymers. The studies described in this article were performed to evaluate the toxicity of ADONA. We were also interested in comparing the toxicological properties of ADONA with those of APFO which have been summarized in several recent reviews (Kennedy et al., 2004; Lau et al., 2004, 2007).

ADONA was moderately toxic (lethal dose between 300 and 2000 mg/kg) following single oral administration to female Wistar rats. The highest oral dose tested, 2000 mg/kg, caused dark red foci of the mucosa of the glandular stomach which may reflect local irritation/corrosion resulting from ADONA's surfactant properties. Only female rats were included in the acute oral study; however, in the bone marrow cytogenetic study, male Wistar rats tolerated acute oral (gavage) doses up to 560 mg/kg while doses ⩾1120 mg/kg caused deaths. Female rats in the same study tolerated oral doses up to 470 mg/kg while doses ⩾940 mg/kg caused deaths. Thus, the acute oral lethal dose of ADONA was similar for male and female rats. Acute oral lethal doses of ADONA in rats are similar to those reported for APFO (Kennedy et al., 2004).

ADONA was practically non-toxic (lethal dose greater than 2000 mg/kg) following single, 24-h, dermal administration to male and female rats. Transient clinical signs at this dose (e.g., hunched posture, chromodacryorrhea, and piloerection) suggest that ADONA was absorbed percutaneously to some extent. The acute dermal lethal dose for ADONA is consistent with reported dermal LD50 values for APFO in rats and rabbits (Kennedy et al., 2004).

As a 30% aqueous solution, ADONA was mildly irritating to the skin and moderately to severely irritating to the eyes of rabbits. All of the observed dermal and ocular effects in these studies were fully reversible. These findings for ADONA are consistent with those reported for APFO in rats and rabbits (Kennedy et al., 2004) although the studies with APFO were performed with powders and aqueous pastes so direct comparisons are not possible.

ADONA was a weak dermal sensitizer in two local lymph node assays in mice. These results were unexpected since ADONA is stable, non-reactive, and is not metabolized (3M unpublished data). It is unclear how ADONA could react with skin proteins to form a stable, immunogenic hapten-protein complex. APFO, which is also stable, non-reactive, and not metabolized (Kennedy et al., 2004), did not cause dermal contact sensitization in a Buehler study in guinea pigs (Moore, 2001). It is possible that the divergent findings for ADONA and APFO are simply due to differences in the two test systems. Another possible explanation is that an impurity in the ADONA formulation may have been responsible for the findings in the LLNA studies. This seems unlikely, however, because the concentrations of individual impurities in the test formulation were very low (less than 0.7%) and the structures of the impurities suggest that they would also be stable and non-reactive.

The weight of evidence from a battery of five genotoxicity studies indicates that ADONA is not directly genotoxic. ADONA did not induce mutations in reverse mutation assays in bacteria (*S. typhimurium* and *E. coli*) or in cultured mammalian (Chinese hamster V79) cells in either the presence or absence of metabolic activation. ADONA was not clastogenic at maximum tolerated oral doses in a micronucleus assay in mice or in a bone marrow cytogenetic study in rats. These findings for ADONA are consistent with those for APFO in similar studies (Kennedy et al., 2004).

ADONA did induce chromosome aberrations in an in vitro assay in human lymphocytes. However, several features of the study suggest that this may have been a false positive result. First, ADONA induced chromosome aberrations only over a relatively high and narrow range of concentrations (1900–2300 μg/ml). Second, the

percentage of cells with aberrations was not concentration-related in either of the two independent assays. Third, the percentage of cells with aberrations was increased both with and without metabolic activation in the first assay but only with metabolic activation in the second assay. These inconsistent findings in the two assays are difficult to explain since ADONA is not metabolized. Fourth, while mitotic indices ranged from 35% to 100% at ADONA concentrations that induced chromosome aberrations in the main study, mitotic indices as low as 8% were observed at similar ADONA concentrations in preliminary rangefinding experiments. Mitotic index often lacks reproducibility and often shows a poor correlation with other measures of cytotoxicity such as cell count, population doubling, confluence, or dye exclusion (Galloway, 2000; Kirkland et al., 2007). ADONA is a surfactant and it is plausible that high in vitro concentrations could disturb the integrity or functionality of lipid membranes, structural proteins, or enzymes, thus interfering with normal cell mitotic processes and leading indirectly to chromosome aberrations. These non-genotoxic mechanisms would be expected to exhibit dose thresholds and have been shown to be responsible for false positive findings in chromosome aberration assays with other compounds (Kirkland et al., 2007; Müller and Kasper, 2000; Kirkland and Müller, 2000; Galloway, 2000). Finally, although APFO is regarded as being non-genotoxic (Kennedy et al., 2004; Lau et al., 2007), it did induce chromosome aberrations in one of three in vitro chromosome aberration assays at concentrations similar to those at which ADONA induced aberrations (Murli, 1996). Regardless of the reason for the positive in vitro chromosome aberration findings with ADONA, the negative findings at maximum tolerated oral doses in the micronucleus study in mice and the bone marrow cytogenetic study in rats indicate that ADONA does not pose a clastogenic hazard under in vivo exposure conditions.

The 100% mortality in female rats administered ADONA at 298 mg/kg/day in the 5-day oral study was in marked contrast to the complete absence of mortality among males at the same dose. High mortality was also observed among pregnant female rats at doses ⩾270 mg/kg/day in the developmental toxicity study. No appreciable gender difference in mortality was seen following single oral doses in the bone marrow cytogenetic assay in rats. The deaths among high-dose females in the 5-day study would not appear to be due to gender-related toxicokinetic differences since serum and liver ADONA concentrations following the last dose were lower in females than in males at the mid- and low-doses. It is possible that a gender-related threshold phenomenon (e.g., saturation of an elimination route or tissue depot) may have resulted in an unexpectedly high internal dose in high-dose females; however, no data are available at the present time to support this theory. Although a somewhat higher incidence and severity of microscopic renal lesions was observed among high-dose females in the 5-day study compared with high-dose males, the differences do not appear to be sufficient to explain the difference in mortality. Additional studies are needed to determine the underlying basis for the mortality seen among female rats at high oral doses in repeat-dose studies.

Other important treatment-related findings in adult rats administered ADONA in oral repeat-dose and developmental toxicity studies included: reduced food consumption in males and females at 298 mg/kg/day in the 5-day study and in pregnant females at 270 mg/kg/day in the developmental study; reduced body weight gain in males at 298 mg/kg/day in the 5-day study and in pregnant females at ⩾90 mg/kg/day in the developmental study; increased liver weights in males at 298 mg/kg/day in the 5-day study and at ⩾30 mg/kg/day in the 28-day study; mild renal tubular injury in males and females at ⩾104 mg/kg/day in the 5-day study; and minimal to moderate hepatocellular hypertrophy in males at ⩾10 mg/kg/day in the 28- and 90-day studies. There was no correlation between treatment duration and the incidence or severity of hepatic effects observed in male rats in the 28- and 90-day studies. If anything, the minor hepatic changes observed in males at the end of the 90-day study at 10 mg/kg/day were less than those observed at the end of the 28-day study at the same dose.

The incidence of thyroid follicular hypertrophy/hyperplasia in male rats in the 28-day study showed a positive dose-related trend. While a direct effect of ADONA on the thyroid gland cannot be ruled out, the liver weight increases, hepatocellular hypertrophy, and increases in gene transcripts for hepatic *Cyp4a1* observed in male, but not female, rats treated with ADONA, are consistent with the idea that the thyroid follicular hypertrophy in male rats was an indirect effect of the hepatic changes. Activation of the thyroid gland secondary to increased thyroxine catabolism by substances that induce hepatic microsomal enzymes is a well-known phenomenon that has been observed in long-term studies in rats exposed to a wide variety of chemicals (Curran and DeGroot, 1991; Capen, 1997). Measurement of circulating thyroid hormones, especially TSH, in future studies with ADONA could provide support for this mechanism.

By comparison, for APFO, the main treatment-related findings in 28- and 90-day oral (dietary) toxicity studies in rats were: mortality in males and females at ⩾500 mg/kg/day; reduced food consumption in males and females at ⩾50 mg/kg/day; body weight loss and/or reduced body weight gain in males at ⩾6.5 mg/kg/day and in females at approximately 150 mg/kg/day; increased liver weights in males at ⩾0.64 mg/kg/day and in females at ⩾15 mg/kg/day; minimal to mild hepatocellular hypertrophy in males at ⩾0.64 mg/kg/day and in females at ⩾1.5 mg/kg/day; and minimal to moderate hepatocellular degeneration and necrosis in males and females at ⩾1.5 mg/kg/day (Metrick and Marias, 1977; Goldenthal, 1978; Perkins, 1992; Palazzolo, 1993; Kennedy et al., 2004).

The liver was the primary target organ in repeat-dose studies in rats with ADONA and with APFO. However, ADONA induced hepatic changes only in male rats whereas APFO induced hepatic changes in both males and females. Based on the lowest observed effect levels for liver weight increases and hepatocellular hypertrophy in male rats in 28- and 90-day studies, ADONA appears to be at least 15-times less potent than APFO in inducing these hepatic changes in male rats. Unlike APFO, ADONA did not cause hepatocellular degeneration or necrosis in rats, even at doses up to 100 mg/kg/day in 28- and 90-day studies. Finally, the incidence and severity of hepatic effects induced by APFO showed a positive correlation with treatment duration whereas the incidence and severity of hepatic effects induced by ADONA in male rats were similar in 28- and 90-day studies.

ADONA caused mild renal tubular injury in male and female rats at oral doses ⩾104 mg/kg/day in a 5-day oral toxicity study. These microscopic lesions were fully reversible by the end of a 7-day recovery period. Interestingly, no evidence of renal injury was seen in 28- and 90-day studies with ADONA despite the fact that the high-dose (100 mg/kg/day) for males and females in the 28-day study and for females in the 90-day study was virtually identical to a dose that caused renal lesions in the 5-day study. To the author's knowledge, histopathologic renal lesions have not been reported in rats administered APFO in repeat-dose studies although increases in relative kidney weight (Goldenthal, 1978) and occasional gross kidney lesions (Metrick and Marias, 1977) have been reported.

ADONA was not teratogenic or developmentally toxic in an oral screening study in Sprague–Dawley rats. Pup and litter parameters were unaffected except at relatively high, maternally toxic doses (⩾90 mg/kg/day) and gross malformations were not observed at any dose tested. Neonatal survival through postnatal day 6 was similar to that for controls except at a dose (270 mg/kg/day) that

caused significant maternal weight loss and 50% mortality. Maternal and developmental NOAELs for ADONA in this study were both 30 mg/kg/day.

The developmental toxicity of APFO has been recently reviewed (Lau et al., 2004, 2007). There were no significant findings in teratology studies with APFO in rats at oral doses up to 100–150 mg/kg/day or in rabbits at doses up to 50 mg/kg/day (Gortner, 1981, 1982; Staples et al., 1984). In a 2-generation study in rats, APFO caused small declines in postnatal weight gain, slight delays in sexual maturation, and increased post-weaning mortality only in F_1 generation animals at the highest dose, 30 mg/kg/day (Butenhoff et al., 2004). In mice, APFO caused significant increases in early full-litter resorptions and neonatal mortality at oral doses $\geqslant$5 mg/kg/day and developmental delays at $\geqslant$1 mg/kg/day (Lau et al., 2006). These strikingly different findings for APFO in rats and mice have been attributed to the slow elimination and resulting accumulation of APFO in female mice (Lau et al., 2005, 2006) as compared to the rapid urinary elimination of APFO by female rats (Kemper, 2003). Pharmacokinetic data in mice, rats, Cynomolgus monkeys, and occupationally exposed human subjects indicate that ADONA is eliminated much more rapidly than APFO and that gender and species differences in the pharmacokinetics of ADONA are less pronounced than those of APFO (3M unpublished data).

Relatively large fold increases were observed in hepatic *Cyp4a1* (9.4- and 11.3-fold) and *Ehhadh* (6.8- and 6.0-fold) gene transcripts in male (but not female) rats administered single oral and intravenous doses (30 mg/kg) of ADONA. Although not statistically significant, these increases are consistent with ADONA being a PPARα agonist in male Sprague–Dawley rats. The reason for the apparent lack of response in females is unknown but is consistent with the absence of other hepatic changes in females in repeat-dose studies with ADONA. Additional studies are needed to support these preliminary findings and further investigate this apparent gender difference.

PFOA is a PPARα agonist in both male and female rats (Sohlenius et al., 1992). Because ADONA and PFOA were evaluated under different experimental conditions, the available data do not permit a direct comparison of their relative potencies as PPARα agonists. Consequences of PPARα activation in rodents typically include: peroxisome proliferation; hepatocellular hypertrophy, proliferation and decreased apoptosis; enhanced activity of peroxisomal enzymes such as catalase and acyl coenzyme A oxidase; increased fatty acid β-oxidation; and reductions in serum cholesterol and triglycerides (Klaunig et al., 2003). Hepatocellular hypertrophy and occasional non-dose-related reductions in serum cholesterol were observed in male rats administered ADONA in repeat-dose studies.

More recent studies with PPARα-knockout mice suggest that PPARα signaling is involved in PFOA-induced neonatal mortality (Abbott et al., 2007). It is possible that the increased postnatal mortality observed at 270 mg/kg/day in the developmental toxicity study with ADONA was at least partially due to PPARα activation; however, the severe maternal toxicity observed at this dose would appear to be a more plausible explanation for this finding. PPARα-responsive genes were not induced in female rats following single 30 mg/kg doses of ADONA.

A number of PPARα agonists, including APFO, have been shown to induce liver, Leydig-cell, and pancreatic acinar-cell tumors in male rats in chronic studies (reviewed in Klaunig et al., 2003). Humans and non-human primates appear to be refractory to PPARα agonists due primarily to their low constitutive expression of the PPARα receptor. Bjork and Wallace (2009) have shown, for example, that APFO activates PPARα in cultured rat hepatocytes but not in primary human hepatocytes. Therefore, many of the effects caused by PPARα agonists in rodents, including tumor induction, are considered unlikely in humans although other possible modes of action by which APFO may induce Leydig-cell and pancreatic acinar-cell tumors are being investigated.

To summarize, ADONA was moderately toxic by single oral administration and practically non-toxic by acute dermal exposure in rats. It was a mild primary skin irritant and a moderate to severe primary eye irritant in rabbits. It was a weak dermal sensitizer in local lymph node assays in mice. ADONA was not genotoxic based on the weight of the evidence from five in vitro and in vivo assays. It was not developmentally toxic in rats except at relatively high, maternally toxic doses. ADONA was a possible PPARα agonist in male rats. The liver was the primary target organ in male rats and the kidney was the primary target organ in female rats in oral repeat-dose studies. NOAELs in 28- and 90-day oral studies in rats were 10 mg/kg/day for males and 100 mg/kg/day for females. Based on these findings, it is concluded that the toxicity profile for ADONA is acceptable for its intended use and is superior to that for APFO.

Conflict of interest

The author is employed by 3M Company. ADONA is manufactured by Dyneon LLC which is a wholly owned subsidiary of 3M.

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