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Nephrotoxic and hepatotoxic potential of imidazolidinedione-, oxazolidinedione- and thiazolidinedione-containing analogues of *N*-(3,5-dichlorophenyl)succinimide (NDPS) in Fischer 344 rats☆

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Abstract

Nephrotoxicity of the agricultural fungicide *N*-(3,5-dichlorophenyl)succinimide (NDPS) in rats is believed to involve metabolism on the succinimide ring. To further investigate this hypothesis, we synthesized and tested the following NDPS analogues, which contain other cyclic imide rings and may therefore be metabolized differently than NDPS: 3-(3,5-dichlorophenyl)-2,4-oxazolidinedione (DCPO), 3-(3,5-dichlorophenyl)-2,4-imidazolidinedione (DCPI), 3-(3,5-dichlorophenyl)-1-methyl-2,4-imidazolidinedione (DCPM) and 3-(3,5-dichlorophenyl)-2,4-thiazolidinedione (DCPT). Male Fischer 344 rats were administered DCPO, DCPI, DCPM, DCPT (0.6 or 1.0 mmol/kg, i.p. in corn oil), NDPS (0.6 mmol/kg, i.p. in corn oil) or corn oil (4 ml/kg). As evidenced by diuresis, proteinuria, elevated blood urea nitrogen levels, increased kidney weights and proximal tubular damage, NDPS produced severe nephrotoxicity in the rats. In contrast, DCPO, DCPI, DCPM and DCPT were mild nephrotoxicants. None of the compounds elevated serum alanine transferase activity or liver weights in the rats, however DCPT produced centrilobular necrosis. These experiments confirm that NDPS-induced nephrotoxicity is critically dependent on the presence of the succinimide ring. Furthermore, replacement of the succinimide ring with a thiazolidinedione ring produced a more pronounced effect on the liver than on the kidney. Liver damage has been reported in type II diabetic patients taking troglitazone, rosiglitazone and pioglitazone. Since these compounds also contain a thiazolidinedione ring, DCPT may be useful for investigating the role of this structural feature in hepatotoxicity.

Keywords: Nephrotoxicity; Hepatotoxicity; *N*-(3,5-Dichlorophenyl)succinimide; 3-(3,5-Dichlorophenyl)-2,4-oxazolidinedione; 3-(3,5-Dichlorophenyl)-2,4-imidazolidinedione; 3-(3,5-Dichlorophenyl)-1-methyl-2,4-imidazolidinedione; 3-(3,5-Dichlorophenyl)-2,4-thiazolidinedione

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1. Introduction

N-(3,5-Dichlorophenyl)succinimide (NDPS, Fig. 1) was originally synthesized as an agricultural antifungal and antimicrobial agent and exhibited activity against a wide range of plant pathogenic microorganisms (Fujinami et al., 1972a). In spite of its promising activity, NDPS is not currently used in the US due to health concerns (Rankin et al., 1992). Characteristic signs of kidney damage caused by acute exposure to NDPS include diuresis, proteinuria, glucosuria, hematuria and elevations in blood urea nitrogen (BUN) levels and kidney weight (Rankin, 1982; Rankin et al., 1984, 1985a; Yang et al., 1985a). Clinical chemistry analyses and morphological evidence have indicated that NDPS is a selective nephrotoxicant in rats. For example, there were no reported effects on the liver (i.e. no elevations in serum alanine aminotransferase levels or liver weights) or other organs in rats when NDPS was administered chronically at a dose of 5000 ppm (Ito et al., 1974; Sugihara et al., 1975) or acutely at a dose of 0.8 mmol/kg (Rankin et al., 1995). Due to the selective nature of its nephrotoxicity, NDPS has been used as a model compound to investigate chemically induced kidney damage (Rankin et al., 1985b; Yang et al., 1985a; Kellner-Weibel et al., 1997).

Although the mechanism of NDPS toxicity is unknown, it has been demonstrated that there is an essential bioactivation step necessary for the production of the nephrotoxic metabolite (Rankin et al., 1987; Nyarko et al., 1997). This occurs via initial hydroxylation of the succinimide ring of NDPS by hepatic cytochromes P-450 to yield *N*-(3,5-dichlorophenyl)-2-hydroxysuccinimide (NDHS) (Rankin et al., 1987). Subsequent sulfation or glucuronidation of NDHS, followed by elimination, could yield the highly electrophilic species *N*-(3,5-dichlorophenyl)maleimide (NDPM) (Aleo et al., 1991). In fact, NDPM and a sulfate conjugate of NDHS were shown to be cytotoxic to isolated rat renal cortical cells (Rankin et al., 2001). Furthermore, covalent binding of ^{14}C-NDPS-derived radioactivity to rat renal and hepatic proteins in vivo was partly metabolism-dependent, which suggests that a reactive intermediate was generated (Henesey and Harvison, 2002).

Studies investigating the nephrotoxicity of NDPS have shown that it is important to have specific substituents on the phenyl ring of the parent compound, *N*-phenylsuccinimide, for nephrotoxicity to occur (Rankin et al., 1985b, 1992; Yang et al., 1985b, 1987). For example, *N*-(3,5-diiodophenyl)succinimide was equally as toxic as the 3,5-dichloro compound, whereas *N*-(3,5-difluorophenyl)succinimide was non-nephrotoxic (Yang et al., 1987; Kellner-Weibel et al., 1997). Replacement of the 3,5-dichloro substituents with other functional groups eliminated toxicity (Rankin et al., 1992). Another important structural feature in NDPS-induced nephrotoxicity is the presence of an intact, unsubstituted succinimide ring. Hydrolysis or methylation of the succinimide ring, or replacement of one or both carbonyl groups in the succinimide ring with methylene groups significantly attenuated NDPS nephrotoxicity (Yang et al., 1985a). NDPS analogues containing other cyclic imides were also investigated. Iprodione and vinclozolin, which contain substituted imidazolidinedione and oxazolidinedione rings, respectively, were non-nephrotoxic (Rankin et al., 1989). However, the presence of bulky substituents on the cyclic imide rings in iprodione

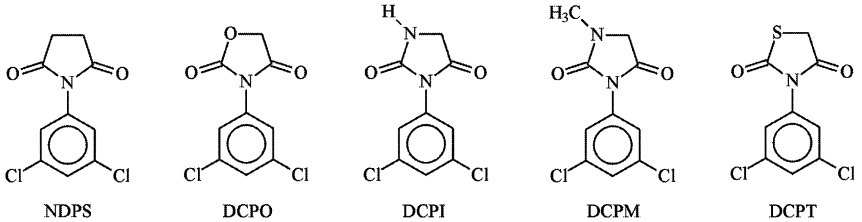

Fig. 1. Structures of NDPS and analogues.

and vinclozolin could conceivably interfere with the essential bioactivation step necessary to produce renal damage. Furthermore, replacing the succinimide ring of NDPS with an unsubstituted, six-membered glutarimide ring also abolished nephrotoxicity (Kellner-Weibel et al., 1995).

The present work was undertaken to study the effect of replacing the succinimide ring with other five-membered cyclic imide ring systems on NDPS-induced toxicity. Therefore, the following NDPS analogues were synthesized and evaluated for their nephrotoxic and hepatotoxic potential in male Fischer 344 rats (Fig. 1): 3-(3,5-dichlorophenyl)-2,4-oxazolidinedione (DCPO), 3-(3,5-dichlorophenyl)-2,4-imidazolidinedione (DCPI), 3-(3,5-dichlorophenyl)-1-methyl-2,4-imidazolidinedione (DCPM) and 3-(3,5-dichlorophenyl)-2,4-thiazolidinedione (DCPT). These compounds retain the 3,5-dichloro-substitution pattern in the phenyl ring, but their unsymmetrical cyclic imide rings could potentially be metabolized differently than a succinimide ring. For example, the presence of the oxygen, nitrogen or sulfur atoms in the cyclic imide rings of the NDPS analogues could affect cytochrome P450-dependent hydroxylation at the adjacent methylene group, which is a critical step in NDPS metabolic activation. Furthermore, the nitrogen and sulfur atoms of DCPI and DCPT, respectively or the methyl group of DCPM, represent alternative sites where competing oxidative metabolic pathways may occur. Determining the nephrotoxic potential of DCPO, DCPI, DCPM and DCPT may indicate if cyclic imides other than a succinimide ring can also induce kidney damage. These studies are relevant to human health since NDPS and the other compounds have structural features in common with drugs that are used to treat epilepsy and diabetes.

2. Methods

2.1. Chemicals/reagents

3,5-Dichlorophenyl isocyanate and ethyl glycolate were obtained from Alfa Aesar (Ward Hill, MA). Sarcosine, alanine aminotransferase assay kit (ALT, No. 505-P), blood urea nitrogen assay kit (BUN, No. 640-5) and urine/blood glucose assay kit (No. 510-DA) were all products of Sigma Chemical Co. (St. Louis, MO). Ethyl 2-mercaptoacetate and glycine were obtained from Aldrich Chemical Co. (Milwaukee, WI). Bio-Rad protein assay kit was obtained from Bio-Rad Laboratories (Hercules, CA). Labstix were purchased from Bayer Corporation (Elkhart, IN).

2.2. Animals

Male Fischer 344 rats (210–225 g) from Charles River Laboratories (Wilmington, MA) were used in all experiments. The animals were housed in standard stainless steel hanging cages under a 12:12 h light/dark cycle at ≈ 22 °C and 45–50% relative humidity. Rats were given a 1-week acclimation period prior to use in any experiments. Food (laboratory rodent diet No. 5001, PMI Foods, Inc., St. Louis, MO) and water were freely available during this period. The Institutional Animal Care and Use Committee of the University of the Sciences in Philadelphia approved all experiments involving rats.

2.3. Syntheses

Melting points were determined with a Thomas–Hoover capillary melting point apparatus. TLC (Silica Gel GF plates, Analtech, Inc., Newark, DE) was used to monitor progress of the reactions. ^{1}H-NMR and ^{13}C-NMR spectra were recorded at 90 and 22 MHz, respectively, on an Anasazi Eft-90 spectrometer. Chemical shifts (δ) are reported in ppm relative to the internal standard tetramethylsilane (TMS). The abbreviations used in reporting the data are as follows: s (singlet), d (doublet), t (triplet), q (quartet). Coupling constants (J) are reported in Hertz. Galbraith Laboratories (Knoxville, TN) or Micro-Analysis, Inc (Wilmington, DE) performed the elemental analyses.

3-(3,5-Dichlorophenyl)-2,4-oxazolidinedione (DCPO) was prepared from 3,5-dichlorophenyl isocyanate and ethyl glycolate using a method similar to that of Fujinami et al. (1971). DCPO was recrystallized from absolute ethanol until pure, m.p. 169–170 °C (lit. m.p. 165.5–166.5 °C

(Fujinami et al., 1971)). ^{1}H-NMR (DMSO-d_6): δ 7.79 (t, 1H, $J = 1.8$, Ar-H_4), 7.58 (d, 2H, $J = 1.8$, Ar-$H_{2,6}$) and 5.01 (s, 2H, CH_2). ^{13}C-NMR (DMSO-d_6): δ 170.5 (C = O), 154.9 (C = 0), 134.6 (Ar-$C_{3,5}$), 133.9 (Ar-C_1), 128.8 (Ar-C_4), 125.6 (Ar-$C_{2,6}$) and 68.7 (CH_2). *Anal*. Calc. for $C_9H_5Cl_2NO_3$ (246.04): C, 43.93; H, 2.05; N, 5.69; Cl, 28.82. Found: C, 44.04; H, 2.07; N, 5.64; Cl, 29.20.

3-(3,5-Dichlorophenyl)-2,4-imidazolidinedione (DCPI) was synthesized from 3,5-dichlorophenyl isocyanate and glycine using a variation on the method of Fujinami et al. (1972b). DCPI was recrystallized twice from absolute ethanol until pure, m.p. 194–196 °C (lit. m.p. 196.5–197 °C (Fujinami et al., 1972b)). ^{1}H-NMR (DMSO-d_6): δ 8.48 (s, 1H, NH), 7.68 (t, 1H, $J = 1.9$, Ar-H_4), 7.54 (d, 2H, $J = 1.9$, Ar-$H_{2,6}$), 4.08 (s, 2H, CH_2). ^{13}C-NMR (DMSO-d_6): δ 171.4 (C = O), 156.3 (C = O), 135.2 (Ar-$C_{3,5}$), 134.4 (Ar-C_1), 127.7 (Ar-C_4), 125.6 (Ar-$C_{2,6}$), 46.2 (CH_2). *Anal*. Calc. for $C_9H_6Cl_2N_2O_2$ (245.06): C, 44.11; H, 2.47; N, 11.43; Cl, 28.93. Found: C, 43.93; H, 2.30; N, 11.20; Cl, 29.57.

3-(3,5-Dichlorophenyl)-1-methyl-2,4-imidazolidinedione (DCPM) was prepared similarly to DCPI using sarcosine instead of glycine. DCPM was recrystallized from absolute ethanol until pure, m.p. 202–203.5 °C (lit. m.p. 157.5–158.5 °C (Fujinami et al., 1972b)). ^{1}H-NMR: δ 7.62 (t, 1H, $J = 2.0$, Ar-H_4), 7.46 (d, 2H, $J = 2.0$, Ar-$H_{2,6}$), 4.05 (s, 2H, CH_2), 2.88 (s, 3H, CH_3). Due to poor solubility, a ^{13}C-NMR spectrum could not be obtained for this compound. *Anal.* Calc. for $C_{10}H_8Cl_2N_2O_2$ (259.09): C, 46.36; H, 3.11; N, 10.81; Cl, 27.37. Found: C, 45.81; H, 3.07; N, 10.65; Cl, 27.74.

Ethoxycarbonylmethyl *N*-(3,5-dichlorophenyl)-thiolcarbamate was prepared from 3,5-dichlorophenyl isocyanate and ethyl 2-mercaptoacetate using a method similar to that of Kolbezen et al. (1954). The product was then filtered and dried in a vacuum oven at 50 °C for 4 h, m.p. 134–136 °C (lit. m.p. 134.5–135.5 °C (Kolbezen et al., 1954)). ^{1}H-NMR (acetone-d_6): δ 9.82 (s, 1H, NH), 7.63 (d, 2H, $J = 1.9$, Ar-$H_{2,6}$), 7.21 (t, 2H, $J = 1.9$, Ar-H_4), 4.19 (q, 2H, J = 7.2, CH_2), 3.82 (s, 2H, CH_2), 1.25 (t, 3H, $J = 7.2$, CH_3). ^{13}C-NMR (DMSO-d_6): 169.4 (R′CH_2C̲O_2R), 165.2 (R'NHCOSR), 147.3 (Ar-C_1), 134.6 (Ar-$C_{3,5}$), 123.2 (Ar-C_4), 117.2 (Ar-$C_{2,6}$), 61.3 (RCO_2C̲H_2CH_3), 31.6 (R′SCH_2R), 13.9 (CH_3).

3-(3,5-Dichlorophenyl)-2,4-thiazolidinedione (DCPT) was prepared from crude ethoxycarbonylmethyl *N*-(3,5-dichlorophenyl)thiolcarbamate using a method similar to that of Fujinami et al. (1971). DCPT was recrystallized from absolute ethanol until pure, m.p. 169.5–170.5 °C (lit. m.p. 164.5–165.5 °C (Fujinami et al., 1971)). ^{1}H-NMR (DMSO-d_6): δ 7.79 (t, 1H, $J = 1.9$, Ar-H_4), 7.54 (d, 2H, $J = 1.9$, Ar-$H_{2,6}$), 4.31 (s, 2H, CH_2). ^{13}C-NMR (DMSO-d_6): δ 170.1 (C = O), 134.5 (Ar-$C_{3,5}$), 133.2 (Ar-C_1), 127.9 (Ar-C_4), 126.2 (Ar-$C_{2,6}$), 30.1 (CH_2). *Anal.* Calc. for $C_9H_5Cl_2NO_2S$ (262.11): C, 41.24; H, 1.92; N, 5.34; Cl, 27.05; S, 12.23. Found: C, 41.16; H, 1.80; N, 5.16; Cl, 27.42; S, 12.33.

2.4. In vivo toxicity

The experimental design is a modification of a literature method (Rankin, 1982). Before any treatments began, a blood sample was obtained by amputating the tip of the tail. Toxicity was assessed at 24 and 48 h after each animal received a single dose of the appropriate test compound (0.6 or 1.0 mmol/kg, i.p. in corn oil), NDPS (0.6 mmol/kg) or corn oil only (4 ml/kg). The dose of 0.6 mmol/kg NDPS was chosen for comparison, since this is reproducibly nephrotoxic in rats (Griffin and Harvison, 1998). Dosages >1.0 mmol/kg are impractical due to solubility issues. On all days of the experiment, urine was collected for 6 h to measure baseline urine contents. Food and water were removed during this period to avoid dilution and contamination of the urine sample. Labstix were used to semi-quantitatively analyze urine for pH and the presence of protein, glucose, ketones and blood. After dosing (48 h), a blood sample was obtained by cardiac puncture under general anesthesia (methoxyflurane) for assessment of post-treatment BUN and blood glucose levels. ALT levels were analyzed on fresh serum. While still anesthetized, the rats were euthanized by cervical dislocation. After euthanasia, the kidneys and liver were removed, weighed

and the right kidney and a section of liver were fixed in formalin for histological analysis. Tissue sections were prepared and stained with hematoxylin and eosin by American Histolabs, Inc. (Gaithersburg, MD). Slides were coded and read by a person blind to the code. Urine protein concentrations were measured using the method of Bradford (1976).

2.5. Statistical analyses

Results are expressed as means ± S.E. ($N = 3–8$). The data were analyzed by a t-test or one-way analysis of variance (ANOVA) followed by a Student–Newman–Keuls post hoc test. When the normality and/or equal variance tests failed, the data were analyzed by the Kruskal–Wallis one-way analysis of variance on ranks (ANOVA on Ranks) test. Differences between means were considered significant when $P < 0.05$.

3. Results

3.1. Effect of NDPS analogues on rat morbidity

Rats treated with DCPI (1.0 mmol/kg only) appeared to go into circulatory shock within 24 h of dosing, showing signs of cyanosis and low body temperature. The animals were euthanized a day early; therefore Day 2 values are not included in the figures and tables. All other experiments were conducted for the full 48 h.

3.2. Nephrotoxicity/hepatotoxicity measurements

Table 1 indicates that diuresis occurred within 6 h (Day 1) following administration of NDPS (0.6 mmol/kg) and DCPT (0.6 and 1.0 mmol/kg) to the rats. Diuresis persisted in the DCPT-treated rats (both doses) for the duration of the experiment. Urine volumes in the NDPS-treated rats on Day 2 or the other treatment groups on either day were not significantly elevated from corn oil controls. Animals treated with NDPS (0.6 mmol/kg) exhibited proteinuria on Day 1 of the experiment (Fig. 2), but values returned to normal by Day 2. No other treatment groups showed signs of elevated urine protein levels at any time during the experimental period. Fig. 3 indicates that glucosuria was seen on Day 2 of the experiment, following administration of DCPT (0.6 and 1.0 mmol/kg). However, animals treated with DCPT (1.0 mmol/kg) showed a reduction in glucose levels on Day 1 before any signs of glucosuria on Day 2 of the experiment. The NDPS (0.6 mmol/kg) treatment group exhibited an elevation in urine glucose values, but these were not significantly different from controls. Urine glucose values were not significantly elevated by treatment with DCPO, DCPI or DCPM at any time point. DCPT (0.6 and 1.0 mmol/kg) produced persistent ketonuria (small to moderate, semi-quantitative analysis) throughout the experiment (data not shown). There was no significant evidence of ketonuria in any of the other treatment groups. Kidney weights were significantly increased in animals treated with NDPS (0.6 mmol/kg) and DCPT (1.0 mmol/kg only) (Table 2). Compared to rats treated with corn oil, kidney weights in the DCPO, DCPI and DCPM treatment groups were not significantly increased. Only animals treated with NDPS (0.6 mmol/kg) exhibited a toxicologically significant increase in BUN levels (Table 2).

None of the treatment groups showed any significant change in liver weights compared to the corn oil controls (data not shown). ALT values were also not significantly different from the control animals (data not shown).

Most treatment groups, except DCPI (0.6 mmol/kg) and DCPM (0.6 and 1.0 mmol/kg), exhibited a significant decrease in food intake on Day 1, but only NDPS (0.6 mmol/kg) treated animals showed a persistent decrease in food intake throughout the experiment (data not shown). By Day 2, most treatment groups returned to normal feeding patterns. Treatment with DCPM (0.6 mmol/kg) caused a significant decrease in water consumption on Day 1, but values returned to normal by 48 h. However, DCPT (0.6 mmol/kg) caused a significant increase on Day 2 (data not shown). NDPS (0.6 mmol/kg) and DCPT (0.6 and 1.0 mmol/kg) caused a persistent loss of body weight throughout the experiment (data not shown).

Table 1
Effect of NDPS analogues on urine volume

Compound	Dose (mmol/kg)	Urine volume (ml)[a]		
		Control Day	Day 1	Day 2
Corn oil		10.6±2.1	7.8±0.5	7.1±0.5
NDPS	0.6	13.4±1.4	18.6±2.1[b]	14.1±3.0
DCPO	0.6	10.2±0.7	12.1±1.1	8.6±1.2
	1.0	12.9±2.1	13.1±2.0	11.0±5.0
DCPI	0.6	9.5±1.3	7.5±1.7	6.9±1.2
	1.0	11.8±1.6	3.2±1.1[c]	n.d.[d]
DCPM	0.6	13.0±3.9	5.0±1.3	4.8±0.7
	1.0	10.4±0.8	6.4±1.4	7.6±1.7
DCPT	0.6	11.3±0.6	17.6±1.0[b,c]	25.6±3.3[b,c]
	1.0	13.8±1.9	20.6±1.0[b]	26.0±3.1[b,c]

[a] Values are reported as means±S.E. ($N = 4$–8 rats).
[b] Significantly different from corn oil control value.
[c] Significantly different from the Control Day within that treatment group.
[d] Not determined (n.d.) because animals were sacrificed a day early due to complications.

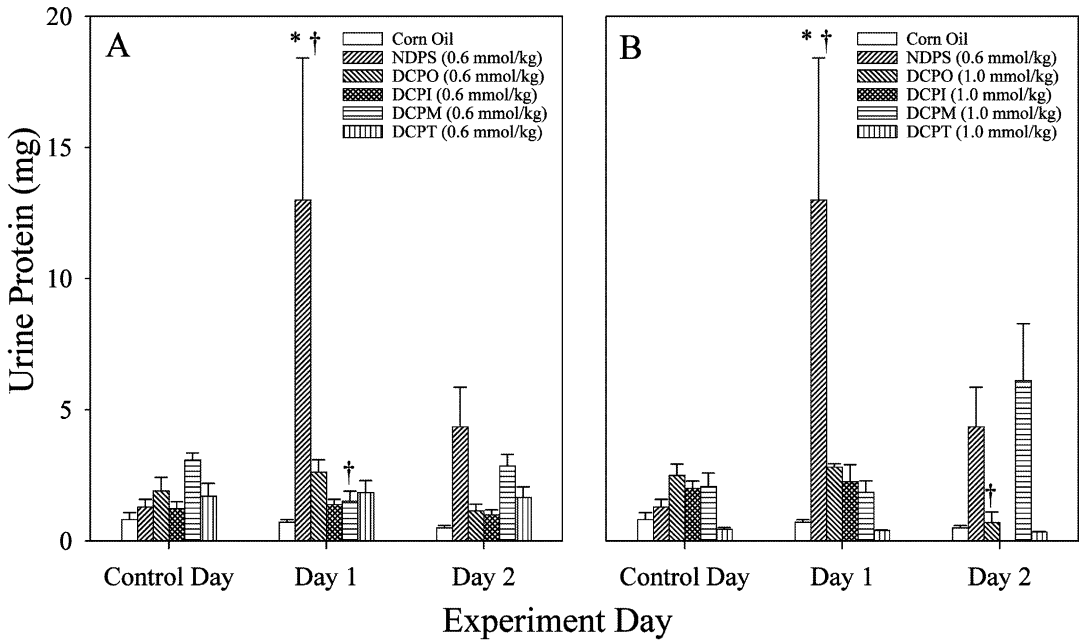

Fig. 2. Effect of NDPS analogues (0.6 mmol/kg, A; 1.0 mmol/kg, B) on urine protein levels ($N = 3$–8). Asterisks indicate values that are significantly different ($P < 0.05$) from corn oil controls; daggers indicate values that are significantly different ($P < 0.05$) from the Control Day within that treatment group. The Day 2 values for animals treated with DCPI (1.0 mmol/kg) are not included because they were sacrificed a day early due to complications.

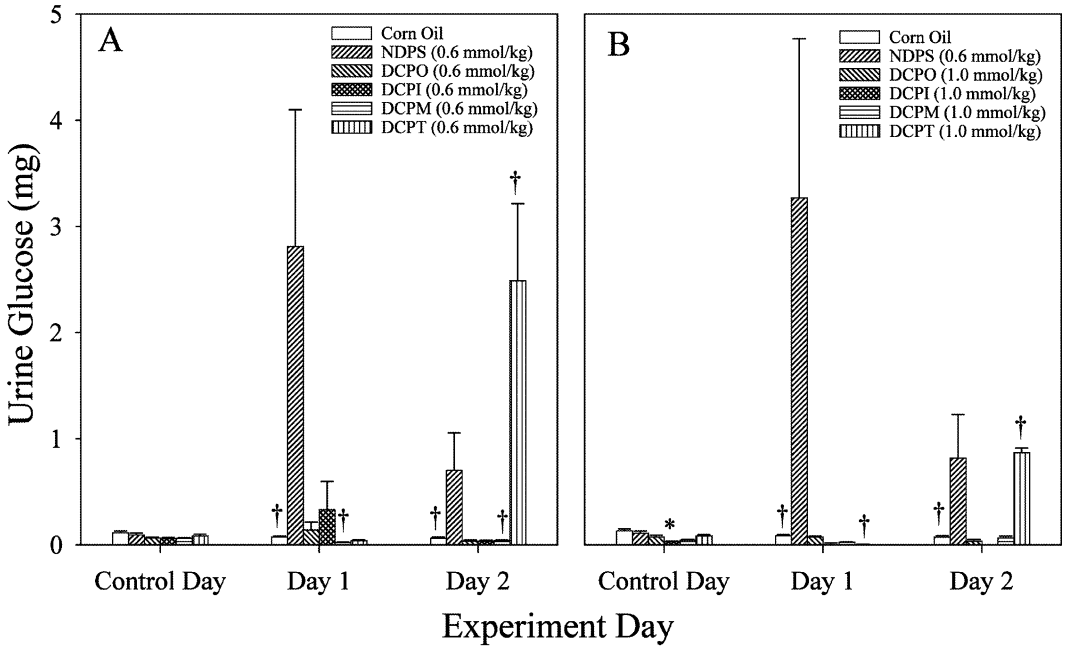

Fig. 3. Effect of NDPS analogues (0.6 mmol/kg, A; 1.0 mmol/kg, B) on urine glucose levels ($N = 3–8$). Asterisks indicate values that are significantly different ($P < 0.05$) from corn oil controls; daggers indicate values that are significantly different ($P < 0.05$) from the Control Day within that treatment group. The Day 2 values for animals treated with DCPI (1.0 mmol/kg) are not included because they were sacrificed a day early due to complications.

Table 2
Effect of NDPS analogues on kidney weights and BUN levels

Compound	Dose (mmol/kg)	Kidney weight[a] (g/100% BW)	BUN concentration[a] (mg/dl)	
			Pre-dose value	48 h post-dosing
Corn oil		0.350±0.002	19.2±1.6	19.7±0.7
NDPS	0.6	0.46±0.03[b]	21.4±2.0	118.4±42.9[c]
DCPO	0.6	0.37±0.01	15.9±1.2	16.2±1.1
	1.0	0.37±0.02	20.0±2.4	19.9±1.2
DCPI	0.6	0.38±0.01	18.0±1.1	19.7±1.7
	1.0	n.d.[d]	37.3±2.9[b]	n.d.[d]
DCPM	0.6	0.38±0.01	19.1±0.3	20.1±2.0
	1.0	0.38±0.01	17.0±0.7	20.6±1.8
DCPT	0.6	0.40±0.01	18.1±1.5	21.5±1.3
	1.0	0.46±0.01[b]	17.6±0.3	24.5±2.7[c]

[a] Values are reported as means±S.E. ($N = 3–8$ rats).
[b] Significantly different from corn oil control value.
[c] Significantly different from the pre-dose value within that treatment group.
[d] Not determined (n.d.) because animals were sacrificed a day early due to complications.

3.3. Kidney and liver histology

Slight swelling of some proximal convoluted tubules was noted in the rats that received corn oil (Fig. 4A). NDPS treatment (0.6 mmol/kg) caused distention in some of the distal tubules, with damage to the cells lining the tubules (Fig. 4B).

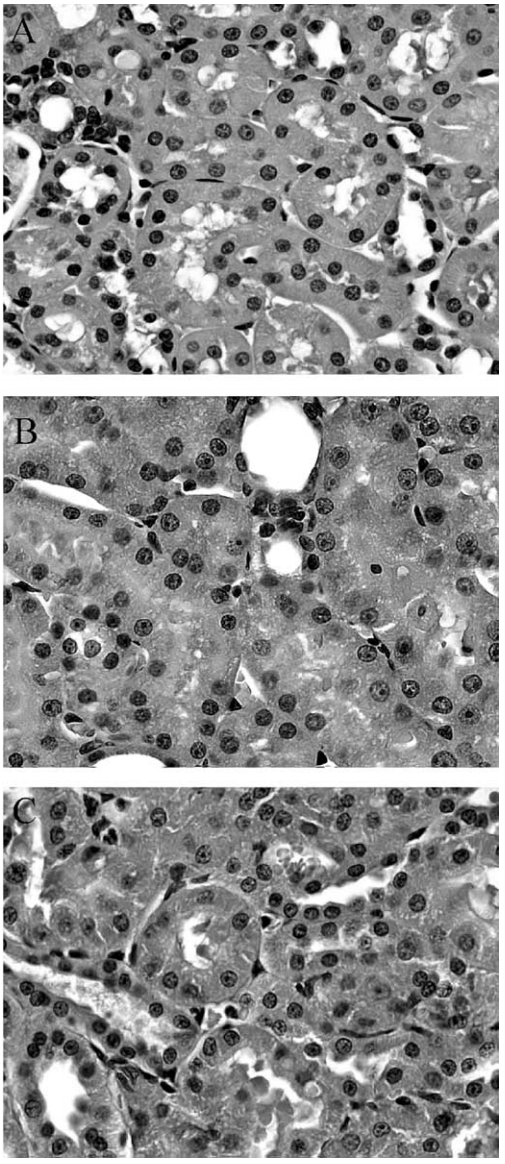

Fig. 4. Effect of corn oil (4 ml/kg, A), NDPS (0.6 mmol/kg, B) or DCPT (0.6 mmol/kg, C) on kidney morphology. Tissue sections were stained with H&E. Magnification is 400 ×.

Cells lining the proximal tubules exhibited severe swelling with occlusion of many proximal tubular lumina. Edema in the interstitium was also observed. Swelling of some vasculature caused accumulation of blood in the interstitial space. Cell casts and debris were seen scattered throughout the proximal tubules indicating that cell death had occurred and that proteinaceous material could appear in the urine. Occasional mitotic figures were observed. The glomeruli appeared normal. Treatment with DCPO, DCPI and DCPM produced minor renal morphological changes comparable to those observed in corn oil-treated animals (data not shown). DCPT (0.6 and 1.0 mmol/kg) treatment caused swelling of the proximal and distal tubules with evidence of minor cell damage and death (Fig. 4C). There were signs of cells breaking down, such as loss of the cell brush border. Proteinaceous debris also appeared in the proximal and collecting tubules. Occasional mitotic figures appeared in the lower part of the convoluted tubules, distal tubules and collecting ducts.

Treatment with corn oil (Fig. 5A) did not cause any noticeable injury to the liver. NDPS (Fig. 5B), DCPO, DCPI and DCPM (data not shown) produced minimal damage to the hepatocytes. There were signs of occasional swelling of the nuclei and loss of proteinaceous material in the cytoplasm. The damaged cells appeared to radiate out from the central veins. DCPT treatment (0.6 and 1.0 mmol/kg) caused swelling of the hepatocytes and the appearance of large areas of cell death around the centrilobular veins (Fig. 5C). The swelling forced vacuolization to occur in the cytoplasm. Some cells contained condensed nuclei. In many instances, the nuclear membrane had dissolved and the nuclear material had fragmented, causing irregular staining of the cytoplasm. The bile canaliculi appeared normal. DCPT treatment produced mostly nuclear, rather than cytoplasmic changes.

4. Discussion

In an attempt to further understand the importance of the succinimide ring towards the

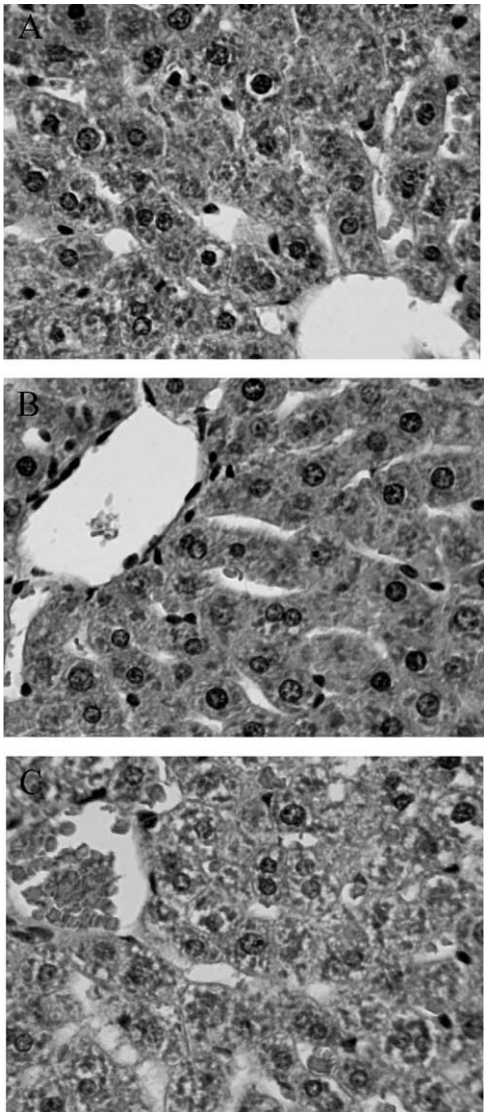

Fig. 5. Effect of corn oil (4 ml/kg, A), NDPS (0.6 mmol/kg, B) or DCPT (0.6 mmol/kg, C) on liver morphology. Tissue sections were stained with H&E. Magnification is 400 × .

mechanism of NDPS toxicity, we conducted a structure–activity relationship study. A series of NDPS analogues (Fig. 1) were synthesized and evaluated for their nephrotoxic and hepatotoxic potential in male Fischer 344 rats. The selection of these compounds was based on the fact that they each contain an oxygen, nitrogen, substituted nitrogen or sulfur atom in place of one of the methylene groups in the NDPS succinimide ring.

The analogues were tested in vivo at dosages of 0.6 and 1.0 mmol/kg, which are known nephrotoxic doses of NDPS (Rankin et al., 1984; Griffin and Harvison, 1998). NDPS (0.6 mmol/kg) caused diuresis, proteinuria, persistent loss of body weight due to reductions in food consumption, and increases in kidney weight and blood urea nitrogen (BUN) levels. Although urine glucose values were elevated in these animals, they were not significantly different from the control group. These data are consistent with previous studies and indicate that kidney damage had occurred (Rankin, 1982; Rankin et al., 1984, 1985a). The histology data supported the toxicological findings in that NDPS (0.6 mmol/kg) treatment caused severe damage to the proximal tubular cells. In contrast to NDPS treatment, corn oil produced a very slight effect on the kidneys. The glomeruli, proximal, distal and collecting tubules were essentially normal in these animals. DCPO, DCPI, DCPM and DCPT also did not produce the same severity of damage to the kidneys as occurred with NDPS. The histology data was generally consistent with the minor toxicological changes and alterations in kidney function that were seen. Mitotic figures were present in the kidneys of rats that received NDPS or DCPT, but not the other NDPS analogues. Since the mitotic figures were not observed in the corn oil-treated animals either, they are probably a consequence of NDPS or DCPT treatment. One possible explanation for the appearance of the mitotic figures in the kidneys of these animals could be localized recovery of some damaged tubular cells.

The glucosuria seen on Day 2 following treatment with NDPS (0.6 mmol/kg) and DCPT (0.6 and 1.0 mmol/kg) may be attributed to slight swelling and minor damage to the cells of the proximal tubules. Since glucose is absorbed along the proximal tubule (Vander, 1985), damage to this area may result in glucosuria. There may also be more subtle damage that cannot be observed by light microscopy to account for the decrease in glucose reabsorption. DCPM (1.0 mmol/kg) treatment also caused hyperglycemia on Day 2. This increase in blood glucose could be a result of the minor damage caused to the liver, as seen in the histology data (see below). This minor, focal

damage may cause a breakdown of glycogen, resulting in hyperglycemia.

DCPT-treated (0.6 and 1.0 mmol/kg) animals consumed a large amount of water on Day 2, which was not seen with any of the other treatment groups. This could also be a factor in the diuresis that was observed in these rats. The histology data of animals treated with DCPT (0.6 and 1.0 mmol/kg) showed some swelling of the proximal and distal tubules, which is also consistent with the observed diuresis.

Treatment with DCPT (0.6 and 1.0 mmol/kg) caused persistent, mild to moderate ketonuria. Similar to NDPS-treated rats, food consumption in the animals treated with DCPT was reduced on Day 1. Ketonuria could be a result of fasting or abnormalities of carbohydrate and lipid metabolism (Wilson et al., 1987). Under conditions of fasting or a diabetic environment, oxaloacetate is unavailable for condensation with acetyl CoA into the citric acid cycle and is therefore consumed and converted to glucose. Therefore, acetyl CoA is converted to acetoacetate. Acetoacetate can also be converted to acetone and β-hydroxybutyric acid, but the ketone test on the Labstix only reacts with the acetoacetic acid in urine, which is produced by the metabolism of fatty acids. However, since ketonuria was not seen in rats that received NDPS, decreased food consumption is unlikely to be a reason for the increase in urine ketone levels in the DCPT-treated animals. Another possibility could be a reaction between component(s) in the urine with the Labstix. In fact, false–positive ketonuria has been reported in patients taking thiol-containing drugs, such as mesna or captopril (Cantwell et al., 1986; Hinberg and Poon, 1987) and is due to a chemical reaction between the thiol group on the drug and the nitroprusside reagent on the dipstick (Csako, 1987). Although DCPT does not contain a free thiol group, it is conceivable that one might be generated by hydrolysis or metabolism of the thiazolidinedione ring in rats. These possibilities will require further investigation.

Collectively, the data suggest that DCPO, DCPI, DCPM and DCPT are mild nephrotoxicants in male Fischer 344 rats when compared to NDPS, which is a potent, nephrotoxic agent. These results are consistent with previous findings that NDPS-induced kidney damage is critically dependent on the presence of an intact, unsubstituted succinimide ring (Rankin et al., 1985b, 1992; Yang et al., 1985a,b, 1987; Kellner-Weibel et al., 1995, 1997).

In the present work, we have shown that replacement of the succinimide ring with other five-membered cyclic imides (oxazolidinedione, imidazolidinedione or thiazolidinedione) significantly attenuates nephrotoxicity. Iprodione and vinclozolin, that also contain imidazolidinedione and oxazolidinedione rings, respectively, were previously found to be non-nephrotoxic (Rankin et al., 1989). However, unlike the analogues described here, iprodione and vinclozolin have relatively bulky substituents present on their cyclic imide rings, which could interfere with metabolism. NDPS-induced nephrotoxicity is critically dependent on an initial hydroxylation step on the succinimide ring (Rankin et al., 1987; Nyarko et al., 1997). Furthermore, metabolism could yield a reactive intermediate, such as NDPM (Aleo et al., 1991; Rankin et al., 2001; Henesey and Harvison, 2002). In fact, each of the NDPS analogues contains an unsubstituted methylene group, where hydroxylation could still occur. However, the presence of a heteroatom adjacent to the methylene group could affect biotransformation at this critical site. Furthermore, DCPI, DCPM and DCPT each contain an additional site (nitrogen atom, methyl group or sulfur atom, respectively) for oxidation, which is not present in the succinimide ring of NDPS. Therefore, alternative metabolic pathways that do not result in the formation of a reactive intermediate may occur with the NDPS analogues or they may not be metabolized at all.

The potential hepatotoxicity of DCPO, DCPI, DCPM and DCPT was also evaluated. Liver weights and alanine aminotransferase (ALT) levels were normal in all treatment groups. The histological findings show that NDPS and the analogues, except for DCPT, produced only minor effects on liver morphology and that these effects were concentrated around some of the centrilobular veins. The minor damage consisted of swelling of some hepatocytes, condensation of some nuclei

and the appearance of vacuolization in the cytoplasm. These toxicological findings suggest that DCPO, DCPI, and DCPM are mild hepatotoxicants.

Treatment of animals with DCPT (0.6 and 1.0 mmol/kg) produced more widespread liver damage (that primarily radiated out from the centrilobular veins) than was observed with NDPS or the other analogues. The severe lesions consisted of swelling of the hepatocytes, which in many cases caused vacuolization to appear in the cytoplasm and karyolysis to occur. In spite of this damage, ALT levels and liver weights were normal at 48 h post-administration of DCPT. Severe cell death occurred at this time point, but was not accompanied by an inflammatory response. The DCPT-induced hepatotoxicity that we observed in rats is interesting since severe liver damage has been reported in diabetic patients treated with troglitazone, which, like DCPT, also contains a thiazolidinedione ring (Kohlroser et al., 2000; Graham et al., 2001). Furthermore, there have been several cases of hepatic injury reported in patients taking other thiazolidinedione-containing drugs, such as rosiglitazone (Forman et al., 2000; Gouda et al., 2001) and pioglitazone (Maeda, 2001; May et al., 2002).

Rats treated with DCPI (1.0 mmol/kg only) appeared to go into circulatory shock, however this compound did not produce any serious kidney or liver damage. One possible explanation for this finding could be the presence of an unsubstituted nitrogen atom, a potential site for *N*-oxidation, in the imidazolidinedione ring. *N*-Hydroxylation reactions have been associated with the induction of methemoglobinemia (Nohl and Stolze, 1998), which would be consistent with the symptoms observed in the DCPI-treated rats. We did not observe this effect with DCPM, the *N*-methylated analogue of DCPI, where direct oxidation of the nitrogen atom could not occur. Similarly, methemoglobinemia was not reported in rats that received iprodione, which like DCPM also contains an *N*-substituted imidazolidinedione ring (Rankin et al., 1989). Furthermore, phenacetin, but not its *N*-methylated derivative, produced methemoglobinemia in rats (Nelson et al., 1978). Thus, it is possible that the presence of an unsubstituted nitrogen atom in DCPI could be a factor in the toxicity that was observed in these animals. Whether or not DCPI actually produced methemoglobinemia in the rats would require further investigation.

In conclusion, DCPO, DCPI, DCPM and DCPT were mild nephrotoxicants in rats. This indicates that replacement of one of the methylene groups in the succinimide ring of NDPS with a heteroatom significantly reduces the adverse renal effects that are associated with this compound. Our findings extend previous results that NDPS-induced nephrotoxicity is critically dependent upon the presence of the succinimide ring. DCPO, DCPI and DCPM were mild hepatotoxicants. However, DCPT produced severe liver damage in rats. These results are interesting because thiazolinedione-containing drugs, such as troglitazone, rosiglitazone and pioglitazone, are associated with hepatotoxicity in humans. Although the mechanism of troglitazone-induced liver damage is controversial, metabolites derived from bioactivation of the thiazolidinedione ring have been detected in rats (Kassahun et al., 2001; Tettey et al., 2001). DCPT may therefore be a useful model compound for further investigating the role of the thiazolidinedione ring in hepatotoxicity.

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References

Aleo, M.D., Rankin, G.O., Cross, T.J., Schnellmann, R.G., 1991. Toxicity of *N*-(3,5-dichlorophenyl)succinimide and metabolites to rat renal proximal tubules and mitochondria. Chem.-Biol. Interact. 78, 109–121.

Bradford, M., 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Chem. 72, 248–254.

Cantwell, B.M.J., Pooley, J., Harris, A.L., 1986. False–positive ketonuria during ifosfamide and mesna therapy. Eur. J. Cancer Clin. Oncol. 22, 229–230.

Csako, G., 1987. False–positive results for ketone with the drug mesna and other free-sulfhydryl compounds. Clin. Chem. 33, 289–292.

Forman, L.M., Simmons, D.A., Diamond, R.H., 2000. Hepatic failure in a patient taking rosiglitazone. Ann. Intern. Med. 132, 118–121.

Fujinami, A., Ozaki, T., Yamamoto, T., 1971. Antimicrobial activity of 3-phenyloxazolidine-2,4-diones and related compounds. Agric. Biol. Chem. 35, 1707–1719.

Fujinami, A., Ozaki, T., Nodera, K., Tanaka, K., 1972a. Studies on biological activity of cyclic imide compounds. Part II. Antimicrobial activity of 1-phenylpyrrolidine-2,5-diones and related compounds. Agric. Biol. Chem. 36, 318–323.

Fujinami, A., Tottori, N., Kato, T., Kameda, N., 1972b. Antimicrobial activity of 3-phenylimidazolidine-2,4-diones and related compounds. Agric. Biol. Chem. 36, 1623–1630.

Gouda, H.E., Khan, A., Schwartz, J., Cohen, R.I., 2001. Liver failure in a patient treated with long-term rosiglitazone therapy. Am. J. Med. 111, 584–585.

Graham, D.J., Drinkard, C.R., Shatin, D., Tsong, Y., Burgess, M.J., 2001. Liver enzyme monitoring in patients treated with troglitazone. J. Am. Med. Assoc. 286, 831–833.

Griffin, R.J., Harvison, P.J., 1998. In vivo metabolism and disposition of the nephrotoxicant *N*-(3,5-dichlorophenyl)succinimide in Fischer 344 rats. Drug Metab. Dispos. 26, 907–913.

Henesey, C.M., Harvison, P.J., 2002. Renal damage, metabolism and covalent binding following administration of the nephrotoxicant *N*-(3,5-dichlorophenyl)succinimide (NDPS) to male Fischer 344 rats. Toxicology 170, 187–200.

Hinberg, I., Poon, R., 1987. Effect of captopril on reagent strip tests for urinary ketones. Clin. Chem. 33, 981.

Ito, N., Sugihara, S., Makiura, S., Arai, M., Hirao, K., Denda, A., Nishio, O., 1974. Effect of *N*-(3,5-dichlorophenyl)succinimide on the histological pattern and incidence of kidney tumors in rats induced by dimethylnitrosamine. Gann 65, 131–138.

Kassahun, K., Pearson, P.G., Tang, W., McIntosh, I., Leung, K., Elmore, C., Dean, D., Wang, R., Doss, G., Baillie, T.A., 2001. Studies on the metabolism of troglitazone to reactive intermediates in vitro and in vivo. Evidence for novel biotransformation pathways involving quinone methide formation and thiazolidinedione ring scission. Chem. Res. Toxicol. 14, 62–70.

Kellner-Weibel, G.L., Tchao, R., Harvison, P.J., 1995. Nephrotoxic potential of *N*-(3,5-dichlorophenyl)glutarimide and *N*-(3,5-dichlorophenyl)glutaramic acid in Fischer 344 rats. Toxicol. Lett. 80, 123–129.

Kellner-Weibel, G.L., Nyarko, A.K., Tchao, R., Henesey, C.M., Harvison, P.J., 1997. The effect of aromatic fluorine substitution on the nephrotoxicity and metabolism of *N*-(3,5-dichlorophenyl)succinimide in Fischer 344 rats. Toxicology 117, 73–83.

Kolbezen, M.J., Metcalf, R.L., Fukuto, T.R., 1954. Insecticidal activity of carbamate cholinesterase inhibitors. J. Agric. Food Chem. 2, 865–871.

Kohlroser, J., Mathai, J., Reichheld, J., Banner, B.F., Bonkovsky, H.L., 2000. Hepatotoxicity due to troglitazone: report of two cases and review of adverse events reported to the United States Food and Drug Administration. Am. J. Gastroenterol. 95, 272–276.

Maeda, K., 2001. Hepatocellular injury in a patient receiving pioglitazone. Ann. Intern. Med. 135, 306.

May, L.D., Lefkowitch, J.H., Kam, M.T., Rubin, D.E., 2002. Mixed hepatocellular-cholestatic liver injury after pioglitazone therapy. Ann Intern. Med. 136, 449–452.

Nelson, S.D., Forte, A.J., McMurtry, R.J., 1978. Increased toxicity of the *N*-methyl analogs of acetaminophen and phenacetin. Res. Commun. Chem. Pathol. Pharmacol. 22, 61–71.

Nohl, H., Stolze, K., 1998. The effect of xenobiotics on erythrocytes. Gen. Pharmacol. 31, 343–347.

Nyarko, A.K., Kellner-Weibel, G.L., Harvison, P.J., 1997. Cytochrome P450-mediated metabolism and nephrotoxicity of *N*-(3,5-dichlorophenyl)succinimide in Fischer 344 rats. Fundam. Appl. Toxicol. 37, 117–124.

Rankin, G.O., 1982. Nephrotoxicity following acute administration of *N*-(3,5-dichlorophenyl)succinimide in Sprague–Dawley rats. Toxicology 23, 21–31.

Rankin, G.O., Cressey-Veneziano, K., Brown, P.I., 1984. Onset of and recovery from acute *N*-(3,5-dichlorophenyl)succinimide-induced nephrotoxicity in Sprague–Dawley rats. Toxicology 30, 205–216.

Rankin, G.O., Yang, D.J., Cressey-Veneziano, K., Brown, P.I., 1985a. *N*-(3,5-Dichlorophenyl)succinimide nephrotoxicity in the Fischer 344 rat. Toxicol. Lett. 24, 99–105.

Rankin, G.O., Yang, D.J., Lahoda, E.P., Cressey-Veneziano, K., Bailey, M, Brown, P.I., 1985b. Acute nephrotoxicity of *N*-phenyl and *N*-(monochlorophenyl)succinimides in Fischer 344 and Sprague–Dawley rats. Toxicology 34, 299–308.

Rankin, G.O., Yang, D.J., Richmond, C.D., Teets, V.J., Wang, R.T., Brown, P.I., 1987. Effect of microsomal enzyme activity modulation on *N*-(3,5-dichlorophenyl)succinimide-induced nephrotoxicity. Toxicology 45, 269–289.

Rankin, G.O., Teets, V.J., Nicoll, D.W., Brown, P.I., 1989. Comparative acute renal effects of three *N*-(3,5-dichlorophenyl)carboximide fungicides: *N*-(3,5-dichlorophenyl)succinimide, vinclozolin, and iprodione. Toxicology 56, 263–272.

Rankin, G.O., Teets, V.J., Shih, H.C., Beers, K.W., Nicoll, D.W., Anestis, D.K., Brown, P.I., Hubbard, J.L., 1992. Renal effects of *N*-(3,5-disubstitutedphenyl)succinimides in the Fischer 344 rat. J. Appl. Toxicol. 12, 211–216.

Rankin, G.O., Beers, K.W., Nicoll, D.W., Anestis, D.K., Ball, J.G., Valentovic, M.A., Brown, P.I., 1995. Effect of dimethyl sulfoxide on *N*-(3,5-dichlorophenyl)succinimide (NDPS) and NDPS metabolite nephrotoxicity. Toxicology 100, 79–88.

Rankin, G.O., Hong, S.K., Anestis, D.K., Lash, L.H., Miles, S.L., 2001. In vitro nephrotoxicity induced by *N*-(3,5-dichlorophenyl)succinimide (NDPS) metabolites in isolated renal cortical cells from male and female Fischer 344 rats:

evidence for a nephrotoxic sulfate conjugate metabolite. Toxicology 163, 73–82.

Sugihara, S., Shinohara, Y., Miyata, Y., Inoue, K., Ito, N., 1975. Pathologic analysis of chemical nephritis in rats induced by *N*-(3,5-dichlorophenyl)succinimide. Lab. Invest. 33, 219–230.

Tettey, J.N., Maggs, J.L., Rapeport, W.G., Pirmohamed, M., Park, B.K., 2001. Enzyme-induction dependent bioactivation of troglitazone and troglitazone quinone in vivo. Chem. Res. Toxicol. 14, 965–974.

Vander, A.J., 1985. Renal physiology, 3rd ed. McGraw-Hill, New York, pp. 59–60.

Wilson, D.E., Zeikus, R., Chan, I.-F., 1987. Relationship of organ lipoprotein lipase activity and ketonuria to hypertriglyceridemia in starved and streptozocin-induced diabetic rats. Diabetes 36, 485–490.

Yang, D.J., Richmond, C.D., Teets, V.J., Brown, P.I., Rankin, G.O., 1985a. Effect of succinimide ring modification on *N*-(3,5-dichlorophenyl)succinimide-induced nephrotoxicity in Sprague–Dawley and Fischer 344 rats. Toxicology 37, 65–77.

Yang, D.J., Lahoda, E.P., Brown, P.I., Rankin, G.O., 1985b. Acute nephrotoxicity of isomeric *N*-(dichlorophenyl)succinimides in Sprague–Dawley and Fischer 344 rats. Fundam. Appl. Toxicol. 5, 1119–1127.

Yang, D.J., Lo, H.H, Teets, V.J., Brown, P.I., Rankin, G.O., 1987. Nephrotoxicity of *N*-(3,5-dihalophenyl)succinimides in Fischer 344 rats. J. Toxicol. Environ. Health 20, 333–346.