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The urinary bladder carcinogen propoxur does not produce genotoxic effects in the urinary bladder of Wistar male rats

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ABSTRACT

Propoxur (PPX) is a carbamate insecticide which induced urinary bladder cancer in Wistar rats when fed at 5000 ppm in Altromin 1321 diet (1321). In the present investigation, PPX was studied for induction of several key events related to modes of action (MOA) of carcinogenicity in urinary bladders (UBs). Wistar rats were administered the compound for 28 days at 8000 ppm in Provini Liba SA 3883 diet, which is similar to the 1321 diet. *o*-Anisidine HCl (AH) was used as a genotoxic UB carcinogenic comparator, and trisodium nitrilotriacetate (NTA) as an epigenetic UB carcinogen comparator. Along with the non-dosed control and three test substance groups (PPX, AH, NTA), four more groups were additionally fed 2% ammonium chloride (AC) in the diet to acidify the urine, since 1321 was reported to increase urinary pH. AC did acidify the urine, as expected, although the 3883 diet itself did not increase pH values above 8. In the alkaline comet assay, AH produced DNA single strand breaks (SSBs) in the UB urothelium (UBU) irrespective of AC administration, whereas PPX and NTA did not. In the nucleotide ^{32}P-postlabeling assay (NPL), AH produced DNA adducts irrespective of AC administration, whereas PPX and NTA did not. Routine (H&E) histopathology evaluation of the UBU did not reveal any hyperplasia or evidence of luminal microprecipitates or calculi in any of the groups. Assessment of UBU proliferation as measured by immunohistochemistry of proliferating cell nuclear antigen, revealed that NTA and NTA plus AC increased the replicating fraction (RF). Also AH plus AC, but not AH alone, increased the RF of UBU, whereas PPX groups were not significantly different from controls. Thus, the results reveal no evidence for DNA SSBs, binding, or alteration of DNA synthesis in the UBU by PPX, while demonstrating UBU DNA damage by AH and showing that NTA does not damage DNA, but causes increased UBU proliferation. The findings are in accord with a genotoxic MOA for AH, and an epigenetic MOA for NTA. The MOA of PPX does not involve genotoxicity and may be specific to the 1321 diet.

1. Introduction

Propoxur (PPX) is a non-systemic carbamate insecticide introduced in 1959 for use in outdoor areas, agricultural settings, on pets and other similar uses. In multiple (oral diet) 2-year studies in male (M) and female (F) Wistar and S-D rats, using Altromin 1321 diet, PPX at greater than 1000 ppm (about 45 mg/kg/d in M and 60 mg/kg/d in F rats) induced urinary bladder (UB) neoplasia, which was dose and time related (FAO, 1989). The diet was associated with an increase in urine pH (unpublished Bayer AG company data 1984, 1988, 1989, 1993). In a study of PPX mode of action (MOA), Cohen et al. (1994) reported that the UB neoplasia was preceded by mild simple UB hyperplasia and increased cell proliferation, resulting from a direct mitogenic effect of PPX, rather than from cytotoxicity and consequent regeneration. PPX was inactive in standard *in vitro* and *in vivo* genotoxicity assays (FAO, 1989).

To examine the MOA of PPX in inducing UB neoplasia, several key events were monitored in the UB of M Wistar rats administered the test substance at 8000 ppm (about 600 mg/kg bw/day) for 28 days in Provimi Kliba SA 3883 diet, a diet chosen as virtually identical to the Altromin 1321 diet used in earlier studies, but which was no longer available (Supplemental Data I). To assess possible *in vivo* genotoxic UB urothelial effects, DNA single (SSBs) or double strand breaks (DSBs), and DNA adducts were measured. *o*-Anisidine (AH) was used as a genotoxic UB carcinogen. Also, to assess rates of UBU cell proliferation, which might be affected by either genotoxic or epigenetic effects, conventional hematoxylin and eosin (H&E) staining and immunohistochemistry of proliferating cell nuclear

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antigen (PCNA) were employed. Trisodium nitrilotriacetate (NTA) was used as an epigenetic UB carcinogen comparator.

For DNA adduct assessments, the nucleotide ^{32}P-postlabeling (NPL) assay (Randerath and Randerath, 1994) was used, and for SSBs or DSBs assessment, the single-cell gel electrophoresis or alkaline "Comet" assay was employed (Hartmann et al., 2003; Singh, 2000; Tice, 1995). These assays complement one another in that the Comet assay measures DNA breaks occurring as a result of DNA damage or its repair, which can occur without replication (and often before). NPL measures DNA adducts not repaired and thus complements the Comet assay. PCNA measures cell replication which can be stimulated without DNA damage. We report here on the application of these assays to investigate the effects of PPX in the rat urinary bladder.

2. Material and methods

2.1. Test animals and dosing

The study outline is given in Table 1. Wistar male rats, 5–6 weeks old at the start of the study were obtained from Taconic Farms. Rats were housed in the Department of Comparative Medicine's AALAC certified facility of NYMC under the supervision of a veterinarian. The rats were housed two per cage in solid bottom polycarbonate cages. Irradiated corn cob bedding was used in a temperature and humidity controlled room. Room temperature was maintained at 72 ± 10 °F and relative humidity at 55 ± 20%. Adequate fresh air was supplied to the animal room. Twelve hours of continuous low-level fluorescent lighting (5 ft candles) was provided daily (7 pm to 7 am) followed by 12 h of dark light cycle. Water was available *ad libitum* throughout the study and was supplied by an automatic watering system. Monitoring of the drinking water for microbiological and chemical contaminants was routinely conducted. Upon receipt the animals were maintained under observation for a minimum of 1 week during which they were fed NIH-07 diet. The diets were prepared and stored at 4 °C. Samples were retained and stored at −20 °C for analysis. Body weights of rats selected for inclusion in the study did not vary by more than 20% from the mean body weight. Identification was done by indelible tail marks and recorded in the raw data.

The dietary concentration of PPX (8000 ppm) in the 3883 diet was the highest tolerated dose level and was associated with induction of UB hyperplasia and tumors when given chronically (FAO, 1989). Diet samples were retained and stored at −20 °C. These samples were sent to Bayer Schering Pharma AG (Wuppertal, Germany) for PPX analysis. The means and standard deviations of the percent of nominal dietary concentration values of PPX in groups 3 and 4 were 100 ± 16 and 102 ± 13, respectively, indicating that the animals of these groups were exposed to the target dose of PPX.

Table 1
Experimental design.

Group identification	Test compound in diet (ppm, %) or (mg/kg bw) at 5 ml/kg for 4 weeks
1	Control diet (C)
2	C and ammonium chloride (AC) 2% in diet
3	Propoxur (PPX) 8000 ppm
4	PPX and AC
5	*o*-Anisidine hydrochloride (AH) 17 mg/kg bw 3 d per wk (M W F)[a]
6	AH 17 mg/kg bw and AC 2% in diet
7	Trisodium nitrilotriacetate (NTA) 20,000 ppm in diet
8	NTA and AC

Total number of male Wistar rats was 48 or 6 per group, non-fasted rats were euthanized at 4 weeks; rats were gavaged with AH three times/week dissolved in 0.5% CMC at 5 ml/kg bw.

[a] Mon, Wed, Fri.

Weekly and at termination the urine pH, color and appearance was measured. For pH measurement, the thymol blue spectroscopic method and pH meter were used.

2.2. Test materials

The sources of chemicals were as follows: 2-(1-methylethoxy)phenyl methylcarbamate, Propoxur, CAS 114-26-1 (PPX) was 99.0% pure and was provided by Bayer Schering Pharma AG (Wuppertal, Germany). *o*-Anisidine hydrochloride CAS 134-29-2 (AH) was 99.7% pure and was obtained from TCI America (Portland, OR, USA). Trisodium 2-(bis(carboxymethyl)amino)-acetate, trisodium nitrilotriacetate, CAS 5064-31-3(NTA) was 100% pure and was provided by Sigma–Aldrich Co. (St. Louis, MO, USA). Ammonium chloride (AC) 99.6% pure, was obtained from Fisher Scientific/EDM Chemicals Inc. (Giblestown, NJ, USA). Carboxymethylcellulose, Na salt (high viscosity) CAS 9004-32-4 (CMC) was 100% pure and was obtained from Sigma–Aldrich Co. The 3883 G4.S25 diet (GLP Batch 03/09) was obtained from Provimi Kliba SA (Kaiseraugust, CH), and the NIH-07 diet 5018 NIH meal was obtained from Purina Mills, LLC (Gray Summit, MD, USA).

The dose of 8000 ppm PPX was selected based in part on the doses of the 2-year rat bioassay. The PCNA sacrifice times were chosen based on known mechanistic data with PPX, which, at 8000 ppm, was reported to induce mild hyperplasia by 4 weeks (Cohen et al., 1994). NTA was chosen to be a negative control since it induces hyperplasia but was considered to be non-genotoxic. AC was used to lower the pH of the urine and reduce the UB hyperplasia and associated carcinogenicity of PPX (Cohen et al., 1994, 2007). PPX, AC and NTA were given in the diet and AH was given by gavage in 0.5% CMC.

2.3. Measurements and evaluations

Morbidity, mortality and clinical observations were conducted twice daily. Body weight, urine pH, urine color and appearance were performed the day prior to study initiation, thereafter weekly and before necropsy.

Non-fasted rats were euthanized by exsanguination under isofluorane anesthesia. The holding room lighting cycles were adjusted so that sacrifices occurred 6 h after dosing (optimal timing for the Comet assay monitoring) and after the onset of the dark cycle and prior to eating. Immediately after death the UB of each animal was removed, and, sampled for Comet, NPL and histopathology (UB only). The study entailed measurement of three endpoints: DNA single strand breaks (SSB) measured by the Comet assay, DNA adducts (NPL), and UB urothelial cell proliferation by immunohistochemistry (PCNA). These procedures (Comet, NPL and PCNA) were performed according to internal Standard Operating Procedures.

At necropsy the UB was removed, rinsed in cold isotonic saline and placed on a cold metal block for detailed sampling. The UB was removed and a sample taken for histopathology. The mucosal cells were scraped from the remainder and a portion was used immediately for Comet assays (Sasaki et al., 1998) and the remaining portion for NPL. Six rats of each group were killed and UB DNA samples prepared using two rats/DNA sample owing to the limited amount of tissue available.

2.4. Alkaline comet analysis

The UB of six rats of each group was scraped according to standard operating procedures for the alkaline version of the COMET assay (Hartmann et al., 2003; Singh, 2000; Tice, 1995). As a positive control, AH (groups 5 and 6) was used and compared to the test article-treated (groups 3 and 4) and negative control (groups 1 and