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Safety assessment of propylparaben in juvenile rats

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ABSTRACT

There are conflicting literature reports that parabens, useful antimicrobial additives in pharmaceuticals, may have estrogenic activity. We conducted a comprehensive study to determine whether propylparaben (PP) administration to juvenile rats is associated with adverse effects on reproductive development and function. PP was administered orally once daily to groups of Crl:CD(SD) rats at doses of 0 (vehicle), 10, 100, or 1,000 mg/kg on Postnatal Days (PNDs) 4–90. In-life observations, clinical pathology, reproductive organ weights and histopathology, landmarks of sexual maturation, estrous cyclicity and functional reproductive competence were assessed. A conventional uterotrophic assay was conducted separately using the same doses. Systemic exposures to PP and 3 metabolites were evaluated on PND 7, 21 and 83. These studies demonstrated that PP was well tolerated when administered from PND 4–90 at all doses (AUC[0-T] on PND 83 = 69.9 ng•h/mL). Para-hydroxybenzoic acid, a non-estrogenic compound, was the predominant metabolite contributing to 95% of the total exposure at 1,000 mg/kg/day on PND 7. There was no evidence of estrogenic activity at any dose, and no effects on reproductive organs or function. The No-Observed-Adverse-Effect-Level (NOAEL) was 1,000 mg/kg/day.

1. Introduction

Propylparaben (PP), an n-propyl ester of p-hydroxybenzoic acid (pHBA), is a naturally occurring substance that is also manufactured for use as preservative in foods, cosmetics, toiletries, and pharmaceutical formulations (Elder, 1984; Soni et al., 2005). Despite its widespread use, there are concerns that PP may have estrogenic activity based on in vitro and in vivo literature reports. This led to a review on the use of parabens as excipients in pharmaceuticals, especially in pediatric formulations (EMA, 2013). There are 4 lines of in vitro evidence that PP is weakly estrogenic:

- PP has structural similarities and binding affinities to the estrogen receptor as alkylphenols (Blair et al., 2000);
- PP binds to estrogen receptors (rat and/or human ERα and ERβ) (Blair et al., 2000; Vo et al., 2010);
- PP has been reported to promote human breast cancer cell proliferation (Okubo et al., 2001; Byford et al., 2002; Wrobel and Gregoraszczuk, 2013); and
- PP induces ER reporter gene expression (Routledge et al., 1998; Miller et al., 2001; Watanabe et al., 2013; Bazin et al., 2013).

These literature reports also demonstrate that the estrogenic activity of PP is extremely weak (approximately 20,000–700,000-fold lower at maximum concentrations) compared to 17β-estradiol (Routledge et al., 1998; Watanabe et al., 2013; Okubo et al., 2001), which raises questions on the biological relevance of these findings. Nonetheless, there are also reports of in vivo estrogenic effects of PP:

- PP increased induction of yolk protein (vitellogenin) in sexually immature rainbow trout (Pedersen et al., 2000);
- PP reduced testicular and epididymal sperm counts (average PP intake of approximately 12.4 ± 3 mg/kg/day), as well as serum testosterone levels (approximate intake 125 ± 30 mg/kg/day) when administered in the diet to post-weaning (PNDs 19–21) male Crj:Wistar rats for 4 weeks (Oishi, 2002);
- PP increased uterine weights in immature mice and rats, as well as overiectomized mice (≥20 mg/kg in mice and ≥65 mg/kg in rat) (Lemini et al., 2003, 2004);
- PP increased myometrial hypertrophy and uterine weights when administered orally to young (PNDs 21–40) female Sprague-Dawley (SD) rats (1,000 mg/kg/day) (Vo et al., 2010);
- PP administered subcutaneously inhibited folliculogenesis and steroidogenesis in the ovaries of neonatal (PNDs 1–7) SD rats (≥250 mg/kg/day) (Ahn et al., 2012); and
- pHBA increased cornification of vaginal epithelial cells, as well as

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uterine weights in both immature and adult ovariectomized CD1 mice (Lemini et al., 1997).

As with the in vitro reports, the in vivo estrogenic effects of PP appear to be very weak compared to estradiol (E2). For example, in an uterotrophic assay in immature rats, PP was 0.03 to 0.005-fold less potent than E2 (Lemini et al., 2003). Similarly, in the Ahn study, primordial follicles were increased and early primary follicles decreased with both E2 (40 μg/kg/day) and PP (1,000 mg/kg/day) but at a 25,000-fold higher dose. Finally, E2 produced a 102% increase in myometrial thickness (relative to vehicle control) at 1 mg/kg/day, while PP produced half the increase (57%) at a 1,000-fold higher dose (Vo et al., 2010). The route of administration, the estrous stage of the animal at the time of uterine examination, or the underpowered studies could have impacted the conclusions from these studies. Conversely, there are reports in which PP administration was not associated with estrogenic effects in vivo. PP (up to 1,000 mg/kg/day) had no impact on implantation sites when administered subcutaneously to inseminated CF-1 mice on GDs 1–4 and sacrificed on GD 6 (Shaw and deCatanzaro, 2009). In another study, oral administration of PP for 8 weeks, initiating on PND 21, to male Wistar rats, had no effects on reproductive organ weights, sperm count or motility, or hormone levels. Importantly, this study was robustly powered and GLP compliant, with confirmation of systemic PP exposures (Gazin et al., 2013). Similarly, pHBA was shown to be estrogen inactive with no effects on uterine weight in immature Wistar rats in an uterotrophic assay (Lemini et al., 2003).

Considered together, although PP may be weakly estrogenic, the toxicological relevance of existing literature reports for human safety is questionable. Recognizing the lingering controversy over the use of parabens in pharmaceuticals, the EMA Safety Working Party published a draft reflection paper in 2013 on the use of methylparaben and propylparaben in human oral medicinal products that summarized key and conflicting literature, recommending a permitted daily exposure (PDE) of 5 mg/kg/day in children over 2 years of age (EMA, 2013). However, this paper acknowledged that there is insufficient literature to support the safe use of PP in children under 2 years, due to uncertainty about metabolizing capacity at this very early age. Therefore, the studies described here were conducted in juvenile animals as young as Postnatal Days (PND) 4 to definitively address whether use of PP in pediatric formulations is safe in neonates and infants under 2 years of age. Specifically, the hypothesis tested was that PP administration to juvenile rats from the neonatal period (PND 4) through early adult life (PND 90) would not be associated with evidence of estrogenic effects. The study was conducted under US FDA Good Laboratory Practice (GLP) regulations to ensure compliance with quality standards; incorporated larger group sizes (N = 10–25) to increase statistical power to identify a response; a longer dosing interval (PNDs 4–90); conduct of male necropsies after PND 90 in order to reduce inter-individual variability associated with the onset of spermatogenesis; and functional reproductive assessments after PP treatment for 86 days. Full toxicokinetic profiles were generated on PND 7, PND 21, and PND 83 and systemic exposures to PP and its metabolites confirmed in neonates, adolescent age and adult animals. Additionally in a separate study, the estrogenic activity of PP was also evaluated in an immature rat uterotrophic assay.

2. Experimental materials and methods

2.1. Ethics statements

The juvenile toxicology study was conducted in Charles River Laboratories Montreal ULC (CR MTL), Senneville, Quebec, Canada and sponsored by Bristol-Myers Squibb Company, New Brunswick, New Jersey, USA. The animal care and experimental procedures of the study were conducted in compliance with the guidelines of the USA National Research Council and the Canadian Council on Animal Care (CCAC). The study protocol and procedures involving the care and use of animals were reviewed and approved by CR MTL Institutional Animal Care and Use Committee (IACUC).

This study was designed to meet the requirements of the European Medicines Agency (EMA), Committee for Human Medicinal Products (CHMP), “Guideline on the need for Nonclinical Testing in Juvenile Animals on Human Pharmaceuticals for Paediatric Indications”; the United States Food and Drug Administration (US FDA) Guidance Document, “Nonclinical Safety Evaluation of Pediatric Drug Products”; and the ICH S3a guidelines, “Toxicokinetics: The Assessment of Systemic Exposure in Toxicity Studies”. The studies were also conducted in compliance with the OECD Principles of Good Laboratory Practice and the US Food and Drug Administration Good Laboratory Practice for Nonclinical Laboratory Studies, 21 CFR Part 58.

The rat uterotrophic assay study was conducted at Bristol-Myers Squibb (BMS) Company at its facility in New Brunswick, New Jersey, USA that is accredited by the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC), and under a protocol approved by the Institutional Animal Care and Use Committee (IACUC). The uterotrophic study was conducted in accordance with applicable Standard Operating Procedures.

2.2. Test materials

Propylparaben (ester of para-hydroxybenzoic acid; molecular formula $C_{10}H_{12}O_3$; molecular weight 180.20; Purity 99.7%) was obtained from Spectrum Chemicals and stored at ambient temperature. The control article, hydroxyethylcellulose (viscosity 80–125 cP at 2% in water), was obtained from Sigma Aldrich and stored at ambient temperature. The vehicle, 1% (w/v) hydroxyethylcellulose, was prepared in reagent grade water and also stored at ambient temperature.

2.3. Study animal assignments

Two separate studies with PP were conducted to assess the potential estrogen-mimetic effects on: (1) reproductive development and function in male and female rats when administered on PNDs 4 to 90; and (2) uterine weights in immature female rats when administered on PNDs 21 to 23.

For the first study, a total of 34 time-mated Crl:CD(SD) Sprague-Dawley females rats (*Rattus norvegicus*; F_0 generation) were received from Charles River Canada Inc. (St. Constant, QC, Canada) on Gestation Day (GDs) 18 (GD 0 = the day a vaginal plug was observed). These dams were used to produce the F_1-generation litters and for the cross-fostering/nursing of litters (see below) for Phase 1. An additional 60 dams were bred to produce sufficient pups to permit satellite toxicokinetic evaluation on PND 7 and PND 21 for Phase 2. A period of 3–4 days was allowed between arrival of rat dams and scheduled parturition in order to acclimate the animals to the laboratory environment.

The F_1 generation was the experimental population. On PND 3 (PND 0 = the day all pups in a litter were delivered by the dam), the litters of the dams were culled to 8 pups each (4/sex/litter, where possible), then assigned to cross-fostered litters (4/sex/litter; where possible) using a randomization procedure such that no more than one sibling from a given sex was assigned to a cross-fostered litter and no pup was assigned to its biological mother. On the same day (PND 3), each newly formed cross-fostered litter was randomly assigned to 4 groups (7–8 litters/group and 25 pups/sex/group; spares were available until PND 21 as replacements to maintain group size) using a computer-generated random series of numbers. The 25 animals were divided into 2 subsets of 10 rats/sex/group (End-of Dose Necropsy subset) and 15 rats/sex/group (Reproduction/Recovery subset).

Similarly, litters from the 60 Phase 2 dams were culled to form a minimum of 43 litters of up to 8 pups each (4/sex/litter), then assigned to cross-fostered litters (4/sex/litter; no more than 1 male and 1 female