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Propiverine-induced accumulation of nuclear and cytosolic protein in F344 rat kidneys: Isolation and identification of the accumulating protein

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ABSTRACT

Male and female F344 rats but not B6C3F1 mice exposed for 104 weeks to propiverine hydrochloride (1-methylpiperid-4-yl 2,2-diphenyl-2-(1-propoxy)acetate hydrochloride), used for treatment of patients with neurogenic detrusor overactivity (NDO) and overactive bladder (OAB), presented with an accumulation of proteins in the cytosol and nuclei of renal proximal tubule epithelial cells, yet despite this, no increased renal tumor incidence was observed. In order to provide an improved interpretation of these findings and a better basis for human health risk assessment, male and female F344 rats were exposed for 16 weeks to 1000 ppm propiverine in the diet, the accumulating protein was isolated from the kidneys via cytosolic and nuclear preparations or laser-capture microdissection and analyzed using molecular weight determination and mass spectrometry. The accumulating protein was found to be D-amino acid oxidase (DAAO), an enzyme involved in amino and fatty acid metabolism. Subsequent reanalysis of kidney homogenate and nuclear samples as well as tissue sections using western blot and DAAO-immunohistochemistry, confirmed the presence and localization of DAAO in propiverine-treated male and female F344 rats. The accumulation of DAAO only in rats, and the limited similarity of rat DAAO with other species, including humans, suggests a rat-specific mechanism underlying the drug-induced renal DAAO accumulation with little relevance for patients chronically treated with propiverine.

Introduction

The benzilic acid derivative propiverine hydrochloride (1-methylpiperid-4-yl 2,2-diphenyl-2-(1-propoxy)acetate hydrochloride) was developed by Apogepha Arzneimittel GmbH, Dresden (Germany) and first registered as the immediate release drug formulation Mictonorm® in the German Democratic Republic (GDR) in 1981 (May et al., in press). Due to its neurotropic and musculotropic effects on the urinary smooth muscle, propiverine is used for treatment of patients with neurogenic detrusor overactivity (NDO) and overactive bladder (OAB, frequency and urge with/without urge incontinence)(Madersbacher, 2006). Upon oral administration, propiverine is rapidly and nearly completely absorbed and undergoes extensive and rapid first-pass metabolism in the liver to the main metabolite propiverine N-oxide (Fig. 1). Due to high plasma protein binding of both the parent compound (ca. 90%) and the N-oxide metabolite (ca. 60%), both demonstrate low renal clearance of<1 ml/min (Madersbacher and Mürtz, 2001). Despite this, drug accumulation does not occur following repeated and long-term treatment, but rather a steady state mean trough level of approx. 60 ng/ml plasma is observed when healthy volunteers are repeatedly given therapeutically effective doses of 15 mg t.i.d or 45 mg s.i.d (May et al., 2008).

Despite approval and marketing authorization in the GDR and subsequent treatment of patients, propiverine was subjected to pre-clinical and clinical testing as of 1989 in order to attain drug approval for marketing in Japan and later in additional European countries via the mutual recognition procedure (EMEA, 2003). A 52-week oral study with rats (Yamashita et al., 1990) and a 104-week oral carcinogenicity bioassay with rats and mice (Inoue et al., 1989a; Inoue et al., 1989b)[3] revealed interesting effects in that clear sex differences in the degree of pathology (non-neoplastic, pre-neoplastic and neoplastic lesions) were observed in male and female F344 rat kidneys while no renal

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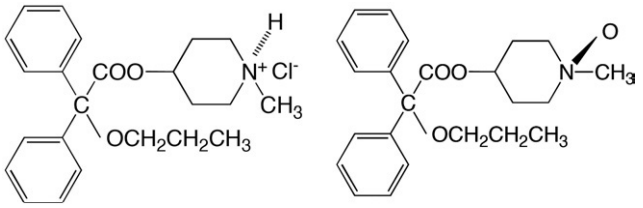

Fig. 1. Structures of propiverine and its major metabolite propiverine-N-oxide (Wuest et al., 2005).

pathological changes were observed in either sex of the B6C3F1 mice. F344 rat renal pathology consisted primarily of prominent hyaline droplets in the cytosol and nuclei of proximal tubule epithelial cells in both sexes. Pathologies in the renal cortex, medulla and papilla, were decisively more pronounced in males than in females. Similarly, increased incidences of simple hyperplasia were observed in the transitional epithelium of the papilla of male but not female rats with the highest incidences noted at the highest dose (1000 ppm) of propiverine applied (Inoue et al., 1989a).

In contrast, a one-year study with beagle dogs did not reveal any pathological changes in the kidneys (Kohda et al., 1989) thus raising the question whether the more apparent renal pathology observed in male than in female F344 rats is species-specific with a sex-specific component. Similar hyaline droplets in the nuclei of rat proximal tubule epithelial cells have previously been described (Gopinath et al., 1988). However, neither the treatment regimen nor the compound used in this study was disclosed and the accumulating protein(s) was not isolated, characterized or identified.

One of the best-characterized pathologies associated with renal hyaline droplet accumulation pathology is the male rat-specific α2u-globulin nephropathy and carcinogenicity (Swenberg, 1991; Dietrich, 1995). As α2u-globulin nephropathy is observed as cytosolic but not nuclear protein accumulation in proximal tubule epithelial cells of male rats only (females do not synthesize the hepatic isoform of α2u-globulin) (Dietrich and Rasonyi, 1995), the propiverine-induced accumulation of hyaline droplets in the renal proximal tubules of both male and female rats following propiverine exposure is unlikely to consist of α2u-globulin. More likely is that these hyaline droplets represent several proteins accumulating concurrently, with a contribution of α2u-globulin to the hyaline droplet pathology in male but not in female rats, thus possibly explaining the sex-specific component in the observed more prominent renal pathology in male F344 rats. As a first step towards providing a better understanding of this hyaline droplet phenomenon and its importance for human cancer risk assessment, and in order to distinguish it from the known α2u-globulin nephropathy, the protein(s) involved in the propiverine-induced hyaline droplet formation was isolated and identified.

Materials and methods

Chemicals and reagents. Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich GmbH, Germany. Rat diet pellets, placebo and 1000 ppm propiverine-supplemented, were prepared by Ssniff Spezialdiäten GmbH, Germany.

Animals, exposure regimen, and sample collection. Forty male and 40 female F344 rats (Charles River Laboratories GmbH, Germany) of 120–140 g body weight were housed at the University of Konstanz animal research facility (TFA). They were randomly marked by ear piercing, and allocated to cages in groups of five for acclimatization 26 days prior to study start. Animal housing and experimentation were approved by the animal care and use committee of the university of Konstanz and subject to permission by the responsible authority (Regierungspräsidium Freiburg, Germany).

Thirty male and 30 female F344 rats were placed on a 1000 ppm propiverine-supplemented diet for 16 weeks. The control group (10 males and 10 females) received placebo feed (see supplemental information, Figure S1). Feeding was *ad libitum* and food and water consumption per cage were monitored weekly. Animals were examined daily and weighed weekly.

At termination of exposure, animals were anaesthetized with pentobarbital sodium (50 mg/ml, 2.5 ml/kg i.p.). A total of five animals per treatment group and sex were retrograde perfused (Dietrich and Swenberg, 1991a; Dietrich and Swenberg, 1991b; Dietrich and Swenberg, 1991c). Right kidneys were sliced sagitally (three sections) and placed in histology fixative (2% (w/v) paraformaldehyde and 1% glutaraldehyde in 82.4 mM NaH2PO4 adjusted with 66.2 mM NaOH to pH 7.6 (864 mOsM)), embedded in paraffin, sectioned (2–3 μM) and stained with Mayer's hematoxylin and eosin (H&E, RCC Ltd., Switzerland). Left kidneys and livers were removed, snap-frozen in liquid nitrogen and stored at −80 °C for subsequent analyses. Organs (non-perfused) from the remaining animals per treatment group were taken for protein analysis (see supplemental information, Figure S1).

Pathological reading of the kidney sections. Hematoxylin and eosin (H&E) stained kidney sections of five animals per treatment group and sex were randomized for subsequent double-blind pathological assessment. Selected pathological lesions were photographed using a Nikon CoolPix950 digital camera mounted on a Zeiss microscope.

Renal cortex and liver protein extraction, concentration determination, SDS-PAGE and silver staining. Nuclear and cytosolic proteins were extracted from kidney (pooled cortices of five animals) and liver samples according to published protocols (Blobel and Potter, 1966). Protein concentrations of extracts were determined at λ=595 nm using a bovine γ-globulin standard calibration curve according to Bradford (Bradford, 1976). Samples were diluted with electrophoresis sample buffer to a final protein concentration of 2 μg/μl (Laemmli, 1970) and a total of 20 μg protein per lane was applied to 12% polyacrylamide gels. Analytical gel silver staining and molecular weight was estimated according to published methods (Heukeshoven and Dernik, 1985). For samples destined for mass spectrometric analyses, a modified silver staining method (Shevchenko et al., 1996) including reduction with dithiothreitol and reaction with iodoacetamide, avoiding glutaraldehyde and minimizing formaldehyde contact, was used.

Sample preparation for mass spectrometry. Protein bands of interest were excised from the silver-stained gel, as was a region of the gel without visible protein, which served as a concurrent control. Samples were destained and subjected to in-gel digestion with trypsin overnight at 37 °C (UCSF, 2002). The resulting digest solutions were transferred to fresh Eppendorf tubes and 5 μl of 1:1 water–acetonitrile containing 5% formic acid was added. The remaining gel pieces were treated with 50 μl of 1:1 water–acetonitrile containing 5% formic acid, mixed for 10 min, centrifuged at 12,000 ×g, sonicated for a further 5 min and the resulting supernatants were transferred to fresh Eppendorf tubes. The samples were vortexed, centrifuged at 12,000 ×g and dried completely under vacuum.

Sample preparation for MALDI-TOF-MS was carried out as previously described (Sze et al., 1998). Briefly, samples were suspended in 30 μl of 0.1% aqueous trifluoroacetic acid (TFA). C18 zip-tips were used to enrich the samples and remove salts and other contaminants. Samples were then eluted twice in 3 μl of 9:1 acetonitrile–water containing 0.1% (v/v) TFA directly onto Au-plated or stainless steel MALDI targets. A 1:1 acetonitrile–water containing 0.1% (v/v) TFA solution saturated with α-cyano-4-hydroxycinnamic acid (CHCA, 1 μl) was mixed with the eluted samples on the target and the spots were air-dried. MALDI-TOF-MS and MALDI-TOF/TOF-MS/MS spectra were collected between m/z 800 and 5000 using Ciphergen