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Liver and kidney damage induced by 4-aminopyridine in a repeated dose (28 days) oral toxicity study in rats: Gene expression profile of hybrid cell death

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HIGHLIGHTS

- The present work shows for the first time *in vivo* cell death on liver and kidney.
- The hematologic and biochemical results are indicative of liver and kidney damage.
- The real-time PCR array analysis on liver tissue expressed a gene expression profile of necrotic and apoptotic induced cell death.
- KCNIP1 down regulation could mediate the necroptotic cell death through the Kv4.3 channel.

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ABSTRACT

4-Aminopyridine (4-AP) is an orphan drug indicated for the treatment of neuromuscular disorders. There is a great controversy around the use of this drug because of its narrow safety index and because a large number of adverse effects have been reported. Moreover, it was shown to induce cell death in different cell lines, being reported mainly apoptosis and necrosis as the principal pathways of cell death mediated by blockage of K channels or the Na, K-ATPase, but until now it was not described *in vivo* cell death induced by 4-aminipyridine. To provide new subchronic toxicity data and specifically, evaluate if 4-AP is able to induce *in vivo* cell death process and the main pathways related to it, a repeated dose (28 days) oral toxicity study, at therapeutic range of doses, was conducted in rats. The anatomical pathology, the biochemical and hematological parameters were analyzed and a real-time PCR array analysis was developed with an Ingenuity Pathway Analysis (IPA). The leucocytes number, the lactate dehydrogenase (LDH) and aspartate aminotransferase (AST) enzymatic activity were increased at all dose but the erythrocytes number, the hemoglobin concentration, the alkaline phosphatase (FAL) and alanine aminotransferase (ALT) enzymatic activity were increased only at highest dose studied. However, glucose levels decreased at all doses. The biochemical results are indicative of hepatic damage. The anatomy pathology studies showed cell death only on liver and kidney, and the real-time PCR array on liver tissue expressed a gene expression profile of necrotic and apoptotic induced cell death. The present work shows for the first time *in vivo* cell death on liver and kidney with features of apoptosis and necrosis induced by 4-AP and the gene expression profile shows that the cell death is mediated by necrotic and apoptotic pathways that support this finding.

1. Introduction

Aminopyridines, especially 4-aminopyrine (4-AP) have been used for many years on the pharmacological research to characterize different potassium channel subtypes, being 4-aminopyrine the first of aminopyridines used in clinical practice (Soni and Kam, 1982). 4-AP has also been registered by the Environmental Protection Agency (EPA) as a biocide to control birds (avicide), being developed by Phillips Petroleum Co. and marketed in 1963 under the name Avitrol® (Bischoff et al., 2001). Recently, it has been approved by the Food and Drug Administration (FDA) under the trade name of Dalfampridine, as an orphan drug indicated for the treatment of neuromuscular disorders such as multiple sclerosis, botulism, spinal cord injury, Alzheimer's disease, myasthenia gravis and Eaton-Lambert syndrome, as it improves interneuronal

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and neuromuscular synaptic transmission (Murray and Newsom-Davis, 1981). The current dose regimen approved by the FDA is 10 mg administered orally twice a day, observing in studies with doses higher than 25 mg adverse effects, such as seizures at doses of 30 mg administered twice a day, presenting a narrow safety index (Dunn and Blight, 2011; Goodman et al., 2007; Sedehizadeh et al., 2012). In Europe, the European Medicines Agency (EMA) has approved 4-AP on a temporary basis (July 2011) under the name Fampyra (10 mg/kg/12 h for 2 weeks) (EMA, 2011). The mechanism by which the 4-AP exerts its therapeutic effects has not been fully clarified, although it is known that it is a potassium channel blocker and through this inhibition it increases the conduction of action potentials in demyelinated axons (Bever and Judge, 2009; Sherratt et al., 1980; Smith et al., 2000). 4-AP also acts on other channels such as calcium channels, easing its conductance by blocking potassium channels which cause the depolarization and the opening of calcium channels, voltage-dependent (Dunn and Blight, 2011; Fiszer et al., 2007; Wu et al., 2009).

On the other hand, there is a great controversy around the use of this new drug because a large number of adverse effects have been observed in clinical studies, especially at neurological (confusion, seizures, involuntary movements and choreoathetoid) and cardiovascular level (hypertension and heart arrhythmias as a result of a possible prolongation of the QT interval) (EMA, 2011). Recent studies have shown that 4-AP, suppress cell proliferation and induce apoptosis in several types of cancer cell lines such as human hepatoblastoma HepG2 line (Kim et al., 2000), neuroblastoma cells (Rouzaire-Dubois et al., 1993), NF1 Schwann cells (Fieber et al., 2003), human melanoma cells (Nilius and Wohlrab, 1992), human prostate cancer LNCaP cell line (Rybalchenko et al., 2001), and astrocytoma cell lines (Chin et al., 1997). The mechanism by which apoptosis occurs is not clearly determined. Several authors suggest that 4-AP produces cell death by causing an increase in intracellular calcium (Wang et al., 2011), in various ways, being one of them through the Ca^{2+}/Calmodulin Protein Kinase II (CaMKII) (Li et al., 2012). However, the role of calcium in the induction of apoptosis is controversial, because it can occur without changes in intracellular calcium concentration (Iseki et al., 1993; Treves et al., 1994). Lately, the importance of the ionic mechanism linked to apoptosis has been emphasized, especially in the important role of potassium channels, associating this type of cell death with a loss of intracellular potassium and an excessive output of it, mediated through ion channels (Yu, 2003; Yu et al., 1999, 2001) or by inhibition of the ATPase Na+, K+ dependent. The latter produces a "hybrid death" originating necrosis and apoptosis in the same cells associated with a reduction of intracellular potassium and an increase of intracellular calcium and sodium (Xiao et al., 2002; Yu, 2003).

Although the induction of cell death by 4-AP in different culture cell lines has been described, there are no *in vivo* studies that confirms these effects, as well as there are no studies on the genetic pathways by which the 4-AP induces the cell death observed in the cell lines, therefore the objective of the present study was to provide new toxicity data and research the *in vivo* presence of cell death induced by 4-AP evaluating the possible genetic pathways that could mediate it. To reach this aim, a repeated 28 days oral dose toxicity study, in a therapeutic dose range similar to the one currently used in the treatment of neuronal disorders, was undertaken.

2. Methods

2.1. Animals and experimental design

The repeated dose (28 days) studies were conducted in accordance with European Union guidelines (EC Council Regulation No 440/2008, 2008).The studies were undertaken in accordance with the ethics requirements and authorized by the Official Ethical Committee of the Complutense University. Wistar male and female rats were acclimated for 7 days prior to study initiation with an evaluation of health status. The rats were individually housed in polycarbonate cages with sawdust bedding and maintained in environmentally controlled rooms (22 ± 2 °C and $50\% \pm 10\%$ relative humidity) with a 12 h light-dark cycle (light from 08:00 to 20:00 h). Food and water were available *ad libitum*.

The rats were 60-days old at initiation of treatment. The repeated dose (28 days) study was conducted in 48 rats (24 males, 24 females) divided in four groups of 6 males and 6 females each (control group and three treated group). Rats received a daily dose of either distilled water (control group) or 2, 4 and 10 mg/kg of 4-AP (Lab. Janssen Chemical) based on the individual animal body weights obtained on the day of dosing (treated groups). Doses of the control and treated groups were administered by esophageal gavage at a volume of 1 ml. These doses correspond to 1/2, 1/5 and 1/10 of lethal dose 50 (LD_{50}), using as reference a LD_{50} dose of 20 mg/kg in rats orally (Schafer et al., 1973), set by the FDA and EMA (EMA, 2011; USFDA, 2009)

Animals were dosed at approximately the same time each day (approximately 4–6 h into light cycle). Food but not water was withheld from 4 h before until 2 h after control and treatment administration. Animals were checked for clinical signs and mortality twice a day (a.m. and p.m.). All rats were deprived of food for 18 h, weighed, euthanized by CO_2 inhalation, exsanguinated, and necropsied on day 29.

2.2. General observations

All animals were observed twice daily for general appearance, behavior, signs of morbidity and mortality (once before treatment and once thereafter). Rats were observed for their general health condition and the condition of the skin and fur, eyes, nose, oral cavity, abdomen and external genitalia, evaluated for respiration rate and palpated for masses. Behavioral parameters tested were abnormal movements (tremor, convulsion and muscular contractions) reactions to handling and behavior in open field (excitability, responsiveness to touch and to sharp noise), changes in ordinary behavior (changes in grooming, head shaking and gyration), abnormal behavior (autophagy, backward motion) and aggression. Body weight, body weight gain and food and water consumption were measured daily, and at the end of the observation periods the rats were examined by necropsy, and the weights of the organs recorded.

2.3. Clinical parameters test

Blood samples for hematology and clinical chemistry evaluation were collected from animals after the last treatment. Ethylenediaminetetraacetic acid (EDTA) (Sigma, Madrid, Spain) was used as an anticoagulant for hematology samples and sodium citrate was used as an anticoagulant for clinical chemistry. Food was withheld for approximately 18 h before blood collection, and samples were collected early in the working day to reduce biological variation; water was provided *ad libitum*. Clinical pathology parameters (hematological and clinical biochemistry) were evaluated. Hematological parameters evaluated were: red blood cell count (RBC), hemoglobin, hematocrit, mean corpuscular volume (MCV), mean corpuscular hemoglobin (MCH), mean corpuscular hemoglobin concentration (MCHC), white blood cell count (WBC), neutrophils count, eosinophils count, lymphocytes count, monocytes count, basophils count, and platelet count. Clinical biochemical parameters evaluated were: glucose, urea, total protein, calcium, aspartate aminotransferase (AST), alanine aminotransferase (ALT), alkaline phosphatase and lactate dehydrogenase (LDH). Hematology variables were measured with a micro-cell counter CC 180 with autodilutor AD241 (Sysmex, Madrid, Spain). Clinical chemistry parameters were evaluated with a spectrophotometer UV/visibleBioMate 3S (ThermoFisher Scientific, Madrid, Spain) and special biochemistry parameters with CoulterLH 500 Hematology Analyzer (Beckman Coulter, Madrid, Spain).

2.4. Anatomical pathology analysis

The necropsy included a macroscopic examination of the external surface of the body, all orifices, the cranial cavity, the brain and spinal cord, the nasal cavity and paranasal sinuses, and the thoracic, abdominal, and pelvic cavities and viscera. Descriptions of all macroscopic abnormalities were recorded. Samples of the following tissues and organs were collected from all animals at necropsy and fixed in neutral phosphate-buffered 10% formaldehyde solution: adrenal glands, brain, heart, ileum, jejunum, caecum, colon, duodenum, rectum, stomach, esophagus, trachea, kidneys, liver, lungs, pancreas, spleen, skin, testicles with epididymes, ovaries with oviducts, bone marrow, thymus, thyroid and parathyroid glands, seminal vesicles, urinary bladder and uterus. All organ and tissue samples for histopathological examination were processed, embedded in paraffin (Merk, Madrid, Spain), cut at an approximate thickness of 2–5 μm, with microtome type Minot (Leitz, Madrid, Spain) and stained with hematoxylin and eosin. Slides of all organs and tissues listed above were collected from all animals of the control and treated groups. The cuts once mounted, were studied in an Orthoplan photomicroscope (Leitz, Madrid, Spain)

2.5. RNA extraction and purification

Being liver the most affected organ, as the anatomopathological and biochemical parameters studies showed; the liver from control and treated animals at the