Toxicology and Applied Pharmacology 289 (2015) 515–524

Contents lists available at ScienceDirect

Toxicology and Applied Pharmacology

journal homepage: www.elsevier.com/locate/ytaap

Low dose evaluation of the antiandrogen flutamide following a Mode of Action approach

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ARTICLE INFO

Article history:
Received 31 July 2015
Revised 6 October 2015
Accepted 14 October 2015
Available online 17 October 2015

Keywords:
Flutamide
Low-dose
Leydig cell hyperplasia
Mode of action
Threshold

ABSTRACT

The dose–response characterization of endocrine mediated toxicity is an on-going debate which is controversial when exploring the nature of the dose–response curve and the effect at the low-end of the curve. To contribute to this debate we have assessed the effects of a wide range of dose levels of the antiandrogen flutamide (FLU) on 7-week male Wistar rats. FLU was administered by oral gavage at doses of 0, 0.001, 0.01, 0.1, 1 and 10 mg/kg/day for 28 days. To evaluate the reproducibility, the study was performed 3 times. The molecular initiating event (MIE; AR antagonism), the key events (LH increase, Leydig cell proliferation and hyperplasia increases) and associated events involved in the mode of action (MOA) of FLU induced testicular toxicity were characterized to address the dose response concordance. Results showed no effects at low doses (<0.1 mg/kg/day) for the different key events studied. The histopathological changes (Leydig cell hyperplasia) observed at 1 and 10 mg/kg/day were associated with an increase in steroidogenesis gene expression in the testis from 1 mg/kg/day, as well as an increase in testosterone blood level at 10 mg/kg/day. Each key event dose–response was in good concordance with the MOA of FLU on the testis. From the available results, only monotonic dose–response curves were observed for the MIE, the key events, associated events and in effects observed in other sex related tissues. All the results, so far, show that the reference endocrine disruptor FLU induces threshold effects in a standard 28-day toxicity study on adult male rats.

1. Introduction

One of the most discussed topics dealing with endocrine disruption concerns the existence or not of "low dose" effects and the concept of non-monotonic dose response curves. Low dose effects have been suggested to be different from those effects observed at doses typically evaluated in standard toxicity studies (Vandenberg, 2014; Vandenberg et al., 2012). Although it is generally accepted that such a phenomenon can occur *in vitro* (e.g. cell proliferation vs cytotoxicity), this concept is not so clear when considering *in vivo* toxicity studies. This uncertainty, coupled with published *in vivo* data in particular using bisphenol A (BPA) (Vandenberg et al., 2009; Wetherill et al., 2002), has led to the current toxicology testing paradigm being heavily challenged and suggested being inadequate to fully evaluate the toxicity profile of a given chemical (Myers et al., 2009). Specifically, the dose levels used as well as the parameters measured in standard regulatory toxicity studies are considered to be insufficient to identify low dose effects (Vandenberg et al., 2012).

In the current regulatory arena, there is an increasing demand for a greater knowledge of the toxicity profile of chemical substances to ensure adequate safety evaluation. Thus, additional or refined parameters are included in the revised guidelines for a number of standard toxicity studies. Furthermore, information concerning the mode of action (MOA)/adverse outcome pathway (AOP) for adverse effects induced by a chemical is actively encouraged by the regulatory authorities to aid in the decision making processes for chemical regulation. The European Chemicals Agency (ECHA) recently hosted a workshop to determine how MOA data should be presented and how they could contribute to the Classification and Labelling (CLH) and regulatory assessment of biocides and pesticides (European Chemicals Agency, 2014). Associated with this is the increasing effort towards identifying adverse outcome pathways (AOPs) for toxicity, which was launched by the OECD in 2012 (OECD, 2013).

Critical to the acceptance of a proposed MOA for a given adverse effect are the identification and characterization (according to dose and kinetic) of a sequence of biologically plausible events that are considered responsible for the adverse effect induced. Further confidence in the proposed MOA can be gained by exploring (and dismissing) alternative MOAs.

Consequently, when considering the debate surrounding EDs and low dose effects, it would seem logical to use an MOA/AOP approach to first determine if such effects occur. Fundamental to this approach is to specify the adverse effect to be investigated, in terms of changes

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http://dx.doi.org/10.1016/j.taap.2015.10.009
0041-008X/

in the key events, so that if low dose effects do occur they can be characterized to fully appreciate their contribution to the effect observed. Such an approach was recommended in a recent UK "Royal Society of Chemistry" workshop, where it was agreed that a better understanding of the dose response characteristics of EDs on the adverse effect and also on the underlying molecular and cellular events would help improve the risk assessment process for such chemicals. Furthermore the most appropriate way to address this would be to generate mechanistic data at human relevant exposure levels (Carmichael et al., 2011; Royal Society of Chemistry, 2014).

To contribute to this effort we have further characterized the MOA of the anti-androgen flutamide (FLU) in terms of the dose response curves for each key event leading to Leydig cell hyperplasia in the adult rat which eventually will result, with this chemical, in testicular tumors (Schering, 2000). FLU is a pure non-steroidal anti-androgen used in the treatment of androgen-dependent prostate cancer at doses around 10 mg/kg/day. FLU acts as a competitive antagonist leading to AR blockade (Labrie, 1993; Sufrin and Coffey, 1976). To our knowledge, this is the first time that each key event contributing to the MOA has been characterized at doses up to 10 000 fold lower than the dose known to induce clear testicular effects in rats (Rouquié et al., 2009). Young adult animals were exposed to FLU for 28 days at 0, 0.001, 0.01, 0.1, 1 and 10 mg/kg/d. The no observed effect level (NOEL) was assumed to be around 0.1 mg/kg/d also based on previous publications (Ludwig et al., 2011). A dose response curve was established for each key event leading to Leydig cell hyperplasia using a variety of standard and molecular methods.

2. Material and methods

2.1. Animals and housing

The studies were performed in accordance with "The Directive 2010/63/EU of the European Parliament and the Council of 22 September 2010 on the protection of animals used for scientific purposes, by the Official Journal of the European Union, L276/33-79, 2010". Male Wistar Rj:WI (IOPS HAN) rats (6 weeks of age on arrival) were purchased from R. Janvier (Le Genest St. Isle, France) and were acclimatized for 7 days. Each animal was individually housed in a stainless steel wire mesh cage under controlled environmental conditions (20 °C–24 °C, 40–70% relative humidity) with a 12-h light/dark cycle. Filtered tap water and pelleted rodent diet (Scientific Animal Food and Engineering, Epinay-sur-Orge, France) were available *ad libitum*. Prior to treatment, rats were assigned to a group by the stratified randomization method based upon the body weight on the day before the initiation of administration, so that there was no significant variation in mean body weights among the groups.

2.2. Dosing and experimental design

Three studies following the identical protocol were conducted (Table 1). Flutamide (FLU; CAS no. 13311-84-7; Sigma, St. Quentin Fallavier, France) was administered in suspension to rats (7 weeks old at start of treatment, 16 animals per group) by oral gavage at a daily dose of 0 (control), 0.001, 0.01, 0.1, 1 and 10 mg/kg body weight, for 28 consecutive days, using a dose volume of 5 ml/kg body weight. Methylcellulose in sterilized water (0.5% wt/vol) was used as the vehicle for all FLU dosing solutions. The dose levels were set after taking into account published toxicity data generated for FLU from 28-day rat toxicity studies (Toyoda et al., 2000; Andrews et al., 2001; Friry-Santini et al., 2007; Rouquié et al., 2009; Ludwig et al., 2011). The dose level of 10 mg/kg body weight/day was selected in order to observe phenotypic anchorage associated with testicular microscopic changes in all the treated animals. Four additional dose levels were selected: the NOAEL (no observed adverse effect level) determined based on standard toxicological parameters in toxicity studies; the NOAEL divided by 10, 100 and 1000 (Ludwig et al., 2011). Clinical observations were performed daily, and body weights and physical examinations were recorded weekly. Exactly 24 h after the last dose, body weights were recorded, then all animals were sacrificed by isoflurane (Baxter, France) inhalation followed by exsanguination under deep anesthesia.

In each studies, terminal blood samples were collected from each animal and placed into tubes containing lithium heparin (for plasma preparation, studies #1 and #2) or tubes containing clot activator (for serum preparation, study #3). All samples were stored at −80 °C until hormonal analysis.

2.3. Tissue collection

For all studies, at necropsy, the following organs were excised from each rat, trimmed free of fat and connective tissue, and weighed: liver, testes, epididymis, ventral prostate, seminal vesicles, adrenal glands and pituitary glands. All paired organs were weighed together with the exception of the testes and epididymis, which were weighed separately. The left testis was then decapsulated and cut into three equal parts. The left testis and pituitary in all studies, and ventral prostate in study #1, were flash-frozen in liquid nitrogen prior to storing at −80 °C until analyses.

2.4. Histopathology

At necropsy, each right testis was preserved in Bouin's fixative in study #1 and in 10% neutral buffered formalin in studies #2 and #3 (Carlo Erba Reagents, Italy). Bouin's fixative was used for histological examination whereas 10% neutral buffered formalin was used both for histological examination and immunohistochemistry purposes. Paraffin-

Table 1
Summary of the studies and parameters measured in this article. This publication relies on four 28 days of studies performed on young adult Wistar rats exposed to flutamide (FLU), in Bayer CropScience Sophia Antipolis facility. Studies #1 to #4 followed the same treatment protocol by oral gavage. In studies #1 to #3, doses range ran from 0.001 mg/kg/day to 10 mg/kg/day. In study #4, doses range ran from 0.2 to 30 mg/kg/day. For each study, the table shows the objectives, the duration of treatment, the FLU dose groups and the observed parameters. Study #4 has already been published by Ludwig et al. (2011).

	Objectives	Duration	Flutamide doses	Parameters
Study #1	Dose–response evaluation of key events, associated events and associated tissues	28 days	0, 0.001, 0.01, 0.1, 1 and 10 mg/kg/day	Histopathology Steroidogenesis and *Pdgfd* transcripts (testis) Testosterone
Study #2	Dose–response evaluation of key events, associated events and associated tissues	28 days	0, 0.001, 0.01, 0.1, 1 and 10 mg/kg/day	Histopathology Testosterone level
Study #3	Dose–response evaluation of key events, associated events and associated tissues	28 days	0, 0.001, 0.01, 0.1, 1 and 10 mg/kg/day	Histopathology Cell proliferation (testis) Hepatic phase 1 and 2 enzyme transcripts Testosterone, LH and FSH levels
Study#4 (Ludwig et al., 2011)	Dose–response evaluation	28 days	0, 0.2, 1, 6 and 30 mg/kg/day	Histopathology Whole genome rat microarrays Testosterone and hydroxyflutamide levels