Regulatory Toxicology and Pharmacology 92 (2018) 220–225

Contents lists available at ScienceDirect

Regulatory Toxicology and Pharmacology

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

No treatment-related effects with aryloxyalkanoate dioxygenase-12 in three 28-day mouse toxicity studies

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ARTICLE INFO

Keywords:
AAD-12
28-Day
Mouse
Toxicology

ABSTRACT

The aryloxyalkanoate dioxygenase-12 (AAD-12) protein is expressed in genetically modified soybean events DAS-68416-4 and DAS-444Ø6-6. Expression of the AAD-12 protein in soybeans confers tolerance to the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) providing an additional herbicide choice to farmers. This enzyme acts by catalyzing the degradation of 2,4-D into herbicidally inactive metabolites. To meet evolving interpretation of regulations in the European Union, three separate 28-day repeat-dose oral mouse studies were conducted at increasing doses of up to 1100 mg AAD-12 protein/kg bw/day. No treatment-related effects were seen in any of these three studies.

1. Introduction

High-dose acute-oral rodent toxicity studies are required globally (with the exception of the European Union) for newly expressed proteins in genetically modified (GM) crops. This requirement is based on the knowledge that toxic proteins act acutely (Delaney et al., 2008; Sjoblad et al., 1992), and even proteins that can cause chronic effects at low doses, such as kidney bean lectin, are toxic acutely at high doses (Almeida et al., 1991; Noah et al., 1980). However, the European Commission uniquely requires a 28-day repeat-dose rodent study instead of an acute study to support the safety assessment of GM crops (EC, 2013). The AAD-12 (aryloxyalkanoate dioxygenase-12) enzyme is expressed in two GM soybean events (DAS-68416-4 and DAS-444Ø6-6) conferring tolerance to the herbicide 2,4-dichlorophenoxyacetic acid (2,4-D) (Griffin et al., 2013; Wright et al., 2010). AAD-12 degrades and inactivates 2,4-D and has good substrate specificity (Griffin et al., 2013). Three separate 28-day repeat-dose mouse studies were conducted with the AAD-12 protein to meet evolving EFSA requirements. An initial study was conducted at doses up to 47 mg AAD-12 protein/kg bw/day (mg/kg/day) and a second study was conducted at doses up to 142 mg/kg/day (based on slightly different AAD-12 expression levels in the DAS-68416-4 and DAS-444Ø6-6 soybean events and different estimates of soybean consumption by humans) to achieve a targeted high dose in both studies of 1000-fold over the worst-case daily dietary exposure in humans. A third study was conducted at a hazard, limit test dose of > 1000 mg/kg/day to meet the most current interpretation of the European regulations by EFSA. Here we report the results of these three 28-day repeat-dose mouse toxicity studies with the AAD-12 protein.

2. Methods and materials

2.1. Doses

In the first (#091024) and second (#151062) study, mice were dosed with the AAD-12 protein at 0, 0.47, 4.7, or 47 mg/kg/day and 0, 1.42, 14.2, or 142 mg/kg/day, respectively. In the first study, AAD-12 protein was administered through the diet, while in the second, it was administered *via* oral gavage. The high doses in both studies were chosen to achieve at least 1000-fold margin of safety over a worst-case human exposure to soybean grain based on different AAD-12 expression estimates in the two GM soybean events and different estimates of human consumption of soybeans [GEMS Cluster Diet data: http://www.who.int/foodsafety/chem/gems/en/index1.html for soybeans (+VD541: immature seeds, dry seeds, oil; and EFSA Comprehensive European Food Consumption Database, Food consumption data relevant to applications for GMOs, Product Category: Soybean (and derived products)]. Based on the available AAD-12 protein level observed in soybean grain, and 100% consumption of AAD-12 expressing grain in the diet, worst-case human exposure was estimated at 47 and 142 µg/kg/day in the first and second study, respectively (EFSA, 2017). It is noteworthy that the dose selection was based on expression levels in raw grain and did not consider that the AAD-12 protein is degraded by

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https://doi.org/10.1016/j.yrtph.2017.12.012
Received 25 October 2017; Received in revised form 13 December 2017; Accepted 15 December 2017
Available online 16 December 2017

Table 1
28-day repeat-dose oral mouse study design.

	Study 1 2010	Study 2 2015	Study 3 2016
Dose levels (mg AAD-12 protein/kg bw/day)*	• 0 (vehicle control) • 47 mg BSA/kg bw/day (protein control) • 0.47 • 4.7 • 47	• 0 (vehicle control) • 1.42 • 14.2 • 142	• 0 (vehicle control) • 1100 mg BSA/kg bw/day (protein control) • 1100
No. of mice/sex/dose	5 (total 50)	10 (total 80)	11 (total 66)
Route of administration	Diet formulated with AAD-12 protein	Oral gavage	
Parameters evaluated	• Daily cage-side observations • Weekly detailed clinical observations • Ophthalmic examinations • Body weights • Feed consumption • Hematology • Clinical chemistry • Selected organ weights • Gross and histopathologic examinations	• Daily cage-side observations • Daily clinical observations • Weekly detailed clinical observations (DCO's) • Ophthalmic examinations • Body weights • Body weight gains • Feed consumption • Hematology • Prothrombin time • Clinical chemistry • Selected organ weights • Gross and histopathological examinations	

* Dose levels were corrected for purity.

the processing of soybean to inactivate endogenous antinutrients so that it is safe for consumption by monogastric animals such as humans (Papineni et al., 2017). AAD-12 protein is below the detection limit in toasted soybean meal and oil. A third oral gavage study (#161005) was conducted at a hazard-limit test dose of 1100 mg/kg/day. Study designs used for these three studies are summarized in Table 1.

2.2. Dosing

Lyophilized purified AAD-12 protein produced heterologously in *Pseudomonas fluorescens* was used to dose the mice (Griffin et al., 2013). In the first study, a concentrated test-material feed mixture (i.e., the premix which contained the AAD-12 protein at a target concentration level) was formulated by adding necessary amounts of the AAD-12 protein to pre-chilled rodent diet (Certified Rodent LabDiet #5002 from PMI Nutrition International, St. Louis, MO). Dilutions of the premix to achieve targeted doses of AAD-12 protein were calculated based on known concentrations of AAD-12 protein in the premix, mean body weights, and feed consumption as previously described (Stagg et al., 2012). Control and test diets were stored at −20 °C. Daily aliquots of the diets were thawed and given to the mice. All dosing suspensions were prepared by mixing the test material (AAD-12 protein) in 0.5% aqueous methylcellulose at concentrations of 0.24, 2.37, or 23.67 mg/mL and administered at a dose volume of 6 mL/kg body weight to achieve the targeted dose levels in the second study and at a concentration of 110 mg/mL and administered at a dose volume of 10 mL/kg body weight in the third study. Dose suspensions were corrected for purity. In addition to the zero dose (vehicle control), bovine serum albumin (BSA), was included at a concentration that was equivalent to the high dose in the first and third study as a non-specific protein control group.

The concentrations of the AAD-12 Protein and BSA in 0.5% aqueous methylcellulose from the respective dose suspensions of the limit dose study (third study) were analyzed from the first mix, from a mix prepared near the middle, and from a mix prepared towards the end of the study. The analyses were conducted using amino acid analysis (AAA) method. The average recoveries for AAD-12 Protein and BSA in 0.5% aqueous methylcellulose were determined to be 71.7% and 73.2%, respectively based on nominal dosing suspensions at 110 mg/mL. The average recovery of these proteins (AAD-12 Protein and BSA) was within the acceptable experimental variation of 70–120% (Codex Alimentarius Commission, 2010).

2.3. Animals

Five, ten, and eleven, 7–8 week old, Crl CD1 (ICR) mice (Charles River Laboratories: Portage, MI or Raleigh, NC)/sex/dose were included in the first, second, and third study, respectively. During the one week acclimation period, each animal was evaluated by a laboratory veterinarian to determine the general health status and acceptability for study purposes. Before administration of test material began, animals were stratified by body weight and randomly assigned to a treatment group. Animals were caged individually at 20–26 °C, 30–70% RH, 10–15 air changes per hour, and 12:12 light/dark photoperiod. Feed (LabDiet Certified Rodent Diet #5002) and water were provided *ad libitum*. In accordance with the U.S. Department of Agriculture Animal Welfare Regulations, 9 CFR, Subchapter A, Parts 1–4, the Animal Care and Use Activities required for conduct of these studies were reviewed and approved by the Institutional Animal Care and Use Committee (IACUC).

2.4. In-life observations

Daily cage-side observations were made to monitor general health in all three studies. Clinical observations were conducted approximately 1 h post-dosing (gavage studies) each day in the second and third studies which included careful evaluation of abnormalities in the eyes, urine, feces, gastrointestinal tract, extremities, movement, posture, reproductive system, respiration, skin/hair-coat, and mucus membranes, as well as an assessment of general behavior, injuries, or palpable mass/swellings. Weekly detailed clinical observations, which included cage-side, hand held, and open field observations, were conducted on all animals in all three studies. The eyes of all animals were examined by a veterinarian pre-exposure and prior to the scheduled necropsy using indirect ophthalmoscopy. Eyes were also examined by a prosector during the necropsy using a moistened glass slide pressed to the cornea. All mice were weighed on test days 1, 2, 3, 4, 8, 15, 22, and 29. Body weight gains were calculated relative to test day 1. Feed consumption was determined at regular intervals in all three studies.

2.5. Clinical pathology

Blood was collected from the orbital sinus of non-fasted mice following anesthesia with Isoflurane/O_2 at the scheduled necropsy. Hematology and clinical chemistry analyses were conducted on five to