Reproductive Toxicology 49 (2014) 33–42

Contents lists available at ScienceDirect

Reproductive Toxicology

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

# *In vivo* preliminary investigations of the effects of the benzimidazole anthelmintic drug flubendazole on rat embryos and fetuses

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ARTICLE INFO

*Article history:*
Received 29 April 2014
Received in revised form 19 June 2014
Accepted 23 June 2014
Available online 30 June 2014

*Keywords:*
Flubendazole
Filarial diseases
Benzimidazole anthelmintic
Embryolethality
Teratogenicity
Eye development

ABSTRACT

Flubendazole, in a new formulation with high systemic bioavailability, has been proposed as a macrofilaricide against filarial diseases.

To investigate embryotoxic activity, the new flubendazole formulation was administered orally to Sprague Dawley rats at 2, 3.46, 6.32 mg/kg/day on gestation day (GD) 9.5 and 10.5. Embryos/fetuses were evaluated on GD 11.5, 12.5 or 20.

At 6.32 mg/kg/day ($C_{max}$ = 0.801 μg/mL after single administration), flubendazole initially induced an arrest of embryonic development followed by a generalized cell death that led to 100% embryolethality by GD 12.5.

At 3.46 mg/kg/day ($C_{max}$ = 0.539 μg/mL after single administration), flubendazole markedly reduced embryonic development by GD 12.5 without causing cell death. On GD 20, 80% of fetuses showed malformations.

At 2 mg/kg/day ($C_{max}$ = 0.389 μg/mL after single administration), it did not interfere with rat embryo-fetal development.



## 1. Introduction

Lymphatic filariasis (commonly known as elephantiasis) and onchocerciasis (also known as river blindness) are tropical diseases caused by parasitic nematodes belonging to the filarioidea family. Epidemiological data from the World Health Organization (WHO) estimates that nearly 1.4 billion individuals in 73 countries worldwide are at risk for lymphatic filariasis [1]. With regard to onchocerciasis, approximately 26 million people in Africa are afflicted by the disease among which 746,000 are visually impaired and 265,000 are blinded [2].

For lymphatic filariasis, 99% of the cases are caused by *Wuchereria bancrofti*. Almost all remaining cases are due to *Brugia malayi*. *B. timori* is restricted to few islands of Indonesia and produces elephantiasis similar to that of *B. malayi*. With regard to onchocerciasis all cases are caused by *Onchocerca volvulus* [3].

The life cycles of these parasitic nematodes are very similar although the vectors differ. The infective stage larvae are transmitted to humans by the bite of an infected vector during a blood meal [4]. Parasitic nematodes causing lymphatic filariasis are transmitted by a wide range of mosquitos (*Culex* spp., *Anopheles* spp. and *Mansonia* spp.) [5]. In the case of onchocerciasis the vector is a black fly of genus *Simulium* spp. After entry into the host, the larvae molt twice before reaching the adult stage of macrofilariae. The adult stage then produced microfilariae. Following ingestion by a vector, the microfilariae develop into an infective stage larvae which migrate through the hemocoel to the proboscis. Other humans can then be infected when the vector takes a blood meal [6].

To tackle lymphatic filariasis and onchocerciasis the WHO relies on Preventative Chemotherapy (PCT) (also called Mass Drug Administration (MDA). The Global Program to Eliminate Lymphatic Filariasis (GPELF) currently distributes single annual dose of albendazole plus either diethylcarbamazine or ivermectin in endemic areas [7]. The African Program for Onchocerciasis control (APOC) relies on the active community participation for the distribution of single doses of ivermectin in high prevalent areas

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http://dx.doi.org/10.1016/j.reprotox.2014.06.009
0890-6238/

(Community-Directed Treatment with Ivermectin (CDTI)). The main strategy of these programs is to reduce morbidity and transmission of the parasite by treatment of all eligible members of the population in endemic areas regardless of whether individuals are infected or not [8].

All the current available drugs are predominantly directed against microfilariae.

In order to improve these control strategies, macrofilaricides for lymphatic filariasis and onchocerciasis need to be identified [9].

In this context, the Drugs for Neglected Diseases *initiative* (DND*i*), together with their partners, aim to develop a complementary tool targeting "the adult stage of the parasites" for the treatment of individuals.

It was suggested that flubendazole might be a selective macrofilaricide candidate for lymphatic filariasis and onchocerciasis which could be used in co-endemic areas [10]. Flubendazole belongs to the benzimidazole class of drugs, and is well known to be efficacious against a variety of worm infestations with several drugs in this class marketed as efficacious anthelmintics [11]. Flubendazole inhibits tubulin polymerization thus causing immobilization of the parasite and its eventually death. Flubendazole was developed by Janssen in the 1970s and is currently registered in Europe as an anthelmintic in humans for intestinal nematodes (5 mg/kg for 3 days) and for veterinary use [11]. The currently registered human product Fluvermal® is not contraindicated in pregnancy although the use in pregnant women or in those liable to become pregnant is not recommended.

The poor solubility and very low bioavailability provided by actual marketed formulations of flubendazole preclude its use for treatment of filarial infections, which require systemic exposure in order to reach adult filarial worms which live in the tissues and internal vessels. Therefore, the first step of the DND*i* program was the identification of a new formulation allowing flubendazole to be systemically available when administered orally. Among various new flubendazole formulations tested, the most promising one showing a very high oral bioavailability resulted to be an Amorphous Solid Dispersion of flubendazole (flubendazole-ASD). This formulation showed very encouraging results in murine efficacy models of filariasis [12].

Helminth infections during pregnancy in humans have been associated with adverse outcomes, including maternal anemia, low birth weight, and perinatal mortality [13–15]. Women of childbearing potential therefore, represent an important part of population in need of effective and safe therapies. Consequently, as part of the preclinical development program of a new formulation of flubendazole, its potential embryotoxicity required a re-evaluation. In fact, available information on the embryotoxicity and teratogenicity of old formulations of flubendazole in animals are scant and controversial, essentially summarized in document EMEA/267/97 [16] and are not supported by toxicokinetic data, thus preventing any extrapolation to humans.

In a first step in the re-evaluation of flubendazole's embryotoxicity, we have examined the direct effects of flubendazole itself (the raw material) and its metabolites, reduced or hydrolyzed flubendazole, on rat embryos cultured *in vitro* from gestational age 9.5 to 11.5 days, using the rat whole embryo culture (WEC) method [17]. Results demonstrated that flubendazole induced retardation of growth and dysmorphogenic effects at concentrations $\geq$0.5 μg/mL. The head, optic and otic systems, branchial arches and posterior body portion were affected. Diffuse areas of cell death were seen in various embryonic districts. The No Observed Effect Level (NOEL) was 0.25 μg/mL. Reduced and hydrolyzed flubendazole were less embryotoxic than the parent compound, with NOELs 4-fold and >40-fold higher than that of flubendazole, respectively.

As a second step of this re-evaluation, in the present study, we evaluate the possible embryotoxic activity of flubendazole new formulation (Flubendazole-ASD) and investigate its embryotoxic nature when administrated to pregnant rats by oral gavage on gestation day (GD) 9.5 and 10.5, the period recognized susceptible by the WEC model [17]. Systemic exposure evaluation was also conducted.

## 2. Materials and methods

### 2.1. Test article

Flubendazole was formulated by dispersion in a polymeric matrix. This formulation is called Amorphous Solid Dispersion (ASD) which consists in a two component system where the polymer acts as a solvent and drug as a solute. Flubendazole Amorphus Solid Dispersion (Flubendazole-ASD) was developed and provided by AbbVie (Abbott Park, IL, USA). The test article was suspended in a 5% Lutrol F127 (BASF) water solution and administered at 2, 3.46 and 6.32 mg/kg/day, expressed as flubendazole.

Concentrations of suspensions were 0.2, 0.346 and 0.632 mg/mL, as flubendazole, respectively. The volume of administration was 10 mL/kg. Suspensions of Flubendazole-ASD were prepared daily, immediately before treatment, in order to avoid crystallization and guarantee bioavailability of the test item.

### 2.2. Animals

Ten- to 12-week-old Crl:CD(SD)BR virgin male and female rats were supplied by Charles River, Calco, Lecco (Italy), and maintained under standard conditions (room temperature 21.5 ± 1.5 °C; humidity 55 ± 5% and artificial light 6.00 am to 6.00 pm) with food (4RF25 GLP pelletted rat feed supplied by Mucedola, Settimo Milanese, Milano) and tap water *ad libitum*. Nestlets® nesting (Datesand, UK) were given to animals as enrichments.

Female rats were mated overnight with male breeder rats 1:1. The paired animals were left in cohabitation from 4.00 pm to 9.00 am the next morning. Copulation was ascertained by vaginal smear. The day on which spermatozoa were found in the vaginal smear was considered as Day 0 of pregnancy.

All the above environmental conditions, as well as all the procedures adopted throughout the study for housing and handling the animals, were in strict compliance with EC and Italian Guidelines for Laboratory Animal Welfare.

The study was carried out at a certified AAALAC and GLP facility. The Principles of Good Laboratory Practice (GLP) are accepted by the worldwide Regulatory Authorities on the basis of intergovernmental agreements.

### 2.3. Experimental design

Flubendazole-ASD was administered orally once daily to pregnant rats on GD 9.5 and 10.5 (i.e. in the afternoon of GD 9 and 10) at 2, 3.46 and 6.32 mg/kg/day. Control animals received the vehicle alone (5% Lutrol F127 water solution). There were 10 presumed pregnant females in each dose-group. Animals were observed daily for clinical signs. Body weight was recorded for each animal on GD 0, 6, 9, 10, 11, 14, 18 and 20. Animals were Cesarean-sectioned at different time-points during gestation (GD 11.5, 24 h after the last dose; and GD 12.5, 48 h after the last dose) and at term on GD 20 (Table 1).

The uteri of dams C-sectioned pre-term were removed and placed in Tyrode's salt solution at 37.8 °C. Each uterus was cut along the antimesometrial borders and the individual implantation sites removed with their decidual material. Each embryo was divested of remaining decidua under a stereomicroscope and observed for viability. Live embryos were scored using the method of Brown and Fabro [18] and morphological abnormalities were also recorded.