Regulatory Toxicology and Pharmacology 69 (2014) 398–407

Contents lists available at ScienceDirect

Regulatory Toxicology and Pharmacology

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

Safety evaluation of a whey protein fraction containing a concentrated amount of naturally occurring TGF-β2

Roy Forster [a,*], Michel Bourtourault [b], Yong Joo Chung [c], Jérémy Silvano [a], Guillaume Sire [a], François Spezia [a], Caroline Puel [b], Jacques Descotes [d], Takashi Mikogami [b]

[a] CiToxLAB France, 27005 Evreux Cedex, France
[b] Armor Protéines, Le Pont, 35460 Saint-Brice-en-Coglès, France
[c] Nestlé Research Center, Nestec, Vers-chez-les-Blanc, 1000 Lausanne, 26, Switzerland
[d] Lyon Poison Center, 162, Avenue Lacassagne, 69424 Lyon Cedex 03, France

ARTICLE INFO

Article history:
Received 23 January 2014
Available online 16 May 2014

Keywords:
TM0601p
Whey protein isolate
TGF-β2
Transforming growth factor

ABSTRACT

TM0601p is a whey protein isolate derived from cow milk, containing a concentrated amount of transforming growth factor β2 (TGF-β2), and is intended for nutritional use in infants and adults. *In vivo* and *in vitro* studies have been performed to evaluate the safety of this product. In a 13-week toxicity study, treatment of adult Sprague–Dawley rats by gavage at up to 2000 mg/kg/day did not result in any significant findings other than minor non-adverse changes in urinary parameters in females. The no-observed-adverse-effect level (NOAEL) was established as 2000 mg/kg/day. In a juvenile toxicity study, rat pups received 600 mg/kg/day by gavage from postnatal day (PND) 7 to PND 49. Transient lower bodyweight gain in the pre-weaning period was attributed to gastrointestinal effects of the viscous test material; following weaning, bodyweight gain was comparable to the vehicle controls. Reduced eosinophil counts and changes in urinary parameters (females) were recorded in treated pups at PND 49, and higher thymus weights were recorded in males only at the end of the recovery period (Day 77). None of the findings were considered adverse. There were no other significant findings and the NOAEL was established as 600 mg/kg/day. No evidence of genotoxicity was seen in the bacterial reverse mutation test or the *in vitro* micronucleus test. Overall the results obtained present a reassuring safety profile for TM0601p.

1. Introduction

Mammalian milk is rich in nutritional components, as well as biologically active molecules such as immunoglobulins, antimicrobial proteins, hormones and enzymes (e.g. lactoferrin, lactoperoxidase and lysozyme) (Séverin and Wenshui, 2005). Small amounts of growth factors, which are peptides involved in the regulation of cellular functions and the stimulation or inhibition of cell growth, can also be found in milk, including insulin-like growth factor 1 (IGF-1), transforming growth factor beta (TGF-β), platelet-derived growth factor (PDGF) and fibroblast growth factor (FGF).

TGF-β is a multifunctional protein expressed by a number of cell types, including epithelial cells, fibroblasts, platelets, osteoblasts, macrophages and lymphocytes. TGF-β plays an important role in embryonic development, the differentiation and control of cellular multiplication, tissue repair and the formation of bone and cartilage. Depending on the cell type under consideration, it can inhibit or stimulate proliferation, and control the renewal of extracellular matrix (Moses, 1992). It is also involved in the modulation of immune responses via effects on lymphocytes and macrophages, and plays a role in the mechanisms of immunological tolerance and in the modulation of inflammatory processes (Ando et al., 2007; Li et al., 2006; Oddy and McMahon, 2011).

Abbreviations: NOAEL, no-observed-adverse-effect level; FOB, functional observational battery; PND, postnatal day; TGF-β, transforming growth factor beta; IGF-1, insulin-like growth factor 1; PDGF, platelet-derived growth factor; FGF, fibroblast growth factor.

* Corresponding author. Address: CiToxLAB France, PO Box 563, 27005 Evreux Cedex, France. Fax: +33 2 3262 8705.

E-mail addresses: roy.forster@fr.citoxlab.com (R. Forster), michel.bourtourault@armor-proteines.fr (M. Bourtourault), YongJoo.Chung@rd.nestle.com (Y.J. Chung), jeremy.silvano@fr.citoxlab.com (J. Silvano), guillaume.sire@fr.citoxlab.com (G. Sire), francois.spezia@fr.citoxlab.com (F. Spezia), caroline.puel@soredab.org (C. Puel), descotes@me.com (J. Descotes), takashi.mikogami@armor-proteines.fr (T. Mikogami).

http://dx.doi.org/10.1016/j.yrtph.2014.05.003
0273-2300/

The importance of TGF-β in milk, where it participates in the prevention of allergy by inducing immunological tolerance via the oral route in the first weeks of an infant's life, has been repeatedly demonstrated (Ando et al., 2007; Nakao, 2010; Oddy and Rosales, 2010; Rautava et al., 2011).

At the molecular level, TGF-β is a dimeric molecule of 25 kD, with three homologous isoforms (TGF-β1, TGF-β2, TGF-β3). It is synthesized as an inactive precursor called the latent TGF-β complex, which is stored in the extra-cellular matrix. The extracellular activation of this complex leads to the release of TGF-β, which triggers cellular responses via binding to specific serine/threonine kinase receptors. TGF-β signaling is translocated to the nucleus following the phosphorylation of cytoplasmic proteins (Gleizes et al., 1997; Koli et al., 2001).

The concentration of TGF-β is normally in the range of 0.8–5.3 μg/L in human milk, and 13–71 μg/L in bovine milk, where the predominant form is TGF-β2 (Gauthier et al., 2006). The amino acid sequence of mature TGF-β2 is identical in humans and cattle, with 100% homology (determined by BLAST analysis).

Owing to their specific nutritional properties, cow milk protein fractions concentrated in TGF-β have been tested for potential beneficial actions in the area of infant nutrition, tissue repair and inflammatory diseases (Chen et al., 2012; de Medina et al., 2010; Ozawa et al., 2009). The nutritional efficacy of orally administered protein fractions concentrated in TGF-β (1–12 μg/g) has been demonstrated in human subjects with Crohn's disease (Fell, 2005) and psoriasis (Drouin et al., 2007; Poulin et al., 2006).

The extraction of fractions, consisting of basic proteins such as lactoferrin and lactoperoxidase, from cow milk by ion exchange chromatography permits the concentration of naturally occurring growth factors at an industrial level. TM0601p is a whey protein isolate mainly consisting of basic proteins, including TGF-β2 (*ca.* 150 μg/g of powder) at a higher concentration than in unprocessed cow milk on a dry matter or protein basis.

Although the efficacy of TGF-β2-rich protein fractions has been previously examined in human trials as described above, the safety of TM0601p has not been assessed under standard toxicological test conditions. The aim of the present article is to describe the results of a standard 13-week oral rat toxicity study and short-term *in vitro* genotoxicity assays conducted with TM0601p. Furthermore, since the proposed applications for TM0601p may involve use as an ingredient in infant formulas, safety assessment in a 6-week juvenile rat study is also presented.

2. Materials and methods

2.1. Test material

TM0601p (commercial name: Vitalarmor® GF-100) is a yellowish-gray powdered dietary ingredient produced with commonly used technology in the dairy industry: selective extraction of soluble proteins from fresh skimmed cow milk with strong cation-exchange chromatography, followed by ultrafiltration, microfiltration, pasteurization and spray drying. The protein content of TM0601p is *ca.* 95% in dry matter. Its major protein components are lactoferrin and lactoperoxidase, and it contains other minor protein components such as secretory component (a part of polymeric immunoglobulin receptor), complement factor C3 and lactophorin (glyCAM-1). This whey protein isolate is characterized by a concentrated content of TGF-β2, *ca.* 150 μg/g in powder (measured by ELISA: Quantikine DB250, R & D Systems); TGF-β in whey protein isolates is biologically active in both *in vitro* and *in vivo* assay systems (data not shown). TM0601p is a proprietary protein fraction and was supplied for these studies by Armor Proteins.

Determination of the TM0601p content in formulations for these studies was based on the determination of the TGF-β2 content in analytical samples using a sandwich ELISA method.

2.2. Experimental design: 13-week rat toxicity study by gavage

Groups of adult Sprague–Dawley rats (16M+16F controls and high dose; 10M+10F mid and low dose groups) received TM0601p (Batch No. 111217), at 0, 600 1200 or 2000 mg/kg/day for 13 weeks by daily oral gavage. TM0601p was administered as a suspension in 0.9% NaCl at a dosage volume of 10 ml/kg. The high dose-level was the maximum feasible dose, taking into account the dosage volume and viscosity of the formulations. At the end of the treatment period, 6 animals/sex in the high-dose and control groups were kept for a 4-week treatment-free period.

The rats were obtained from Charles River, France and were approximately 6 weeks old on the first day of administration; they were group housed and had free access to diet (SSNIFF Spezialdiäten GmbH, Soest, Germany) and water. Clinical signs were recorded daily. Body weight and food consumption were recorded weekly. Ophthalmological examinations were performed before and after the treatment period. A Functional Observation Battery (FOB) was performed on all animals in week 12. Hematology, clinical chemistry and urinary parameters were investigated at the end of the treatment period.

At scheduled sacrifice a full macroscopic post-mortem examination was performed. The body weight of each animal was recorded just before sacrifice and the ratios of organ weights to body weight were calculated. A microscopic examination was performed on a full range of tissues from high-dose and control animals. Peer review was performed for at least 20% of the slides.

Statistical analysis of body weight, food consumption, hematology, blood biochemistry and urinalysis data was performed using in-house software. PathData software (v.6.2d2, PDS Ltd., Switzerland) was used to perform the statistical analysis of organ weights.

2.3. Experimental design: 6-week juvenile toxicity study in rats

Mated female Sprague–Dawley rats (Charles River, France) were received in the test facility on gestation day GD 14. Following parturition, on PND 4 the litters were culled to standardized litters of 2M+2F or 3M+3F. The pups were then allocated to group 1 (vehicle control) or 2 (TM0601p). For each group:

- subset I was composed of 5 standardized litters (of 2M+2F)
- subset II was composed of 2 standardized litters (of 3M+3F)

Group 1 rat pups received the vehicle (0.9% NaCl) and group 2 rat pups received 600 mg/kg/day TM0601p (Batch No. 111217), daily by oral gavage from PND 7 to PND 49. Subset I pups were sacrificed on completion of the treatment period (PND 49) and subset II pups were sacrificed on completion of the 4 week recovery period (PND 77).

The dosing suspension of TM0601p was very viscous and in order to limit the amount of viscous material administered to pre-weaning rat pups, a dosage volume of 2 mL/kg/day was selected. This allowed us to achieve a dose-level of 600 mg/kg/day, providing a large multiple over human exposure.

Pups remained housed with their dams until weaning on PND 21. All animals had free access to SSNIFF R/M-H pelleted maintenance diet (SSNIFF Spezialdiäten GmbH, Soest, Germany) and tap water.

Clinical signs were recorded daily. Body weight was recorded on PNDs 4 and 7, then twice weekly. Food consumption was recorded twice weekly. Long bone growth (tibia length) was measured every 2 days from PND 7 to PND 21, then weekly.