Regulatory Toxicology and Pharmacology 80 (2016) 71–81

Contents lists available at ScienceDirect

Regulatory Toxicology and Pharmacology

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

# Genotoxicity and subchronic toxicity evaluation of dried *Euglena gracilis* ATCC PTA-123017

Ryan R. Simon [a], Trung D. Vo [a], Robert Levine [b, *]

[a] Intertek Scientific and Regulatory Consultancy, 2233 Argentia Road, Suite 201, Mississauga, ON L5N 2X7, Canada
[b] Algal Scientific Corporation, 14925 Galleon Court, Plymouth, MI 48170, USA

ARTICLE INFO

*Article history:*
Received 2 May 2016
Received in revised form
7 June 2016
Accepted 10 June 2016
Available online 14 June 2016

*Keywords:*
Rat
Genotoxicity
Toxicity
Safety
NOAEL

ABSTRACT

*Euglena gracilis* is a microalga capable of synthesizing various nutrients of interest in human and animal nutrition. When cultivated aerobically in the dark, *Euglena* synthesize paramylon, a storage polysaccharide comprised of high molecular weight *beta*-1,3-D-glucose polymers organized in cytoplasmic granules. *Beta*-glucans have been shown to have immune modulation effects, including anti-microbial, anti-tumor, and anti-oxidant properties, and metabolic effects, such as regulation of cholesterol and blood sugar levels. Preparations of *E. gracilis* and paramylon may therefore have potential utility as functional food ingredients for human and animal nutrition. A battery of toxicological studies was conducted on a dried preparation of *E. gracilis* and paramylon to support their safe food use. The dried alga was not genotoxic in a bacterial reverse mutation test and mammalian micronucleus test. In the subchronic toxicity study, rats were provided *E. gracilis* in the diet at levels of 0, 12,500, 25,000 or 50,000 ppm. Paramylon was provided at a concentration of 50,000 ppm. No effects that could be attributable to treatment were observed in clinical observations, body weight, food consumption, ophthalmology, hematology and clinical chemistry, urinalysis, and macroscopic and microscopic findings. A NOAEL of 50,000 ppm in the diet was determined for both ingredients.



## 1. Introduction

*Euglena gracilis* is a single-celled microalga belonging to the *Euglenaceae* family that occurs widely in nature. The species is primarily found in freshwater pools, ponds, and lakes. *E. gracilis* can adapt to diverse environments under conditions ranging from 1 to 38 °C and pH ranging from 2.3 to 11. As such, *E. gracilis* has been identified in a number of differing environments including vegetable and citrus waste-lagoons, raw sewage, water in tree holes, snow, bark of the honey locust tree, alkaline marshes, and in acid coal mine water (Buetow, 2011). Euglenozoa are classified taxonomically as members of the Protist kingdom and form paraphyletic supergroups that do not share evolutionarily lineages (Krajčovič et al., 2015). Protists are unicellular organisms that share the photosynthetic features of plants and the biosynthetic pathways found in animals; *Euglena* sp. can therefore be cultivated both like a plant by cultivation in water with carbon dioxide and sunlight or heterotrophically using a dissolved carbon and energy source like dextrose (O'Neill et al., 2015).

Historically, *E. gracilis* has been the subject of extensive research for use in large scale production of a variety of nutrients of importance to human and animal nutrition including vitamins A, C, and E, carotenoids, essential amino acids as well as the long-chain polyunsaturated fatty acids such as eicosapentaenoic acid (EPA), docosahexaenoic acid (DHA) and arachidonic acid (ARA) (Afiukwa and Ogbonna, 2007; Ogbonna et al., 1998, 2002; Krajčovič et al., 2015). Most of this work has been carried out with *Euglena* sp. grown photosynthetically and has not focused on the production of paramylon, the unique storage carbohydrate produced exclusively by Euglenoids. When cultivated aerobically in the dark with a carbon source, *Euglena* sp. synthesize large quantities of paramylon, often as high as 85% of the dry weight of the cell, compared to much lower levels of production when grown photosynthetically (Barsanti et al., 2001). Paramylon is an insoluble, non-digestible *beta*-glucan consisting of linear, high molecular weight *beta*-1,3-D-glucose polymers deposited in the cytoplasm as triple helical

*Abbreviations:* 2-AA, 2-aminoanthracene; ANOVA, analysis of variance; ARA, arachidonic acid; AST, aspartate aminotransferase; DHA, docosahexaenoic acid; EDTA, ethylenediaminetetraacetic acid; EPA, eicosapentaenoic acid; $LD_{50}$, median lethal dose; MCV, mean corpuscular volume; MF, Mutation Factor; MMS, methylmethanesulfonate; $NaN_3$, sodium azide; NOAEL, No-Observed-Adverse-Effect Level.

* Corresponding author.
*E-mail address:* robert.levine@algalscientific.com (R. Levine).

http://dx.doi.org/10.1016/j.yrtph.2016.06.007
0273-2300/

fibrils in a lamellar arrangement that produces highly crystalline discoid granules 1–11 μm in diameter (Monfils et al., 2011). Paramylon granules consist exclusively of glucose molecules linked in mostly *beta*-1,3 anomeric fashion, with trace amounts of other linkages present but none of *beta*-1,6-linkages that are characteristic of yeast-based *beta*-glucans (Krajčovič et al., 2015).

*Beta*-glucans are ubiquitous in nature. *Beta*-1,3-glucose polymers are found in fruits and vegetables as linear chains in *beta*-1,3/1,4 anomeric configuration, and various branched chain *beta*-1,3/1,6 glucans are constituents of highly conserved biopolymers that are integral to the structural integrity of the cell walls of yeast, bacteria, and fungi. Due to the extracellular location of *beta*-1,3-glucans within the cell wall of fungi, animals have developed pattern recognition systems (*e.g.*, Dectin-1) for insoluble *beta*-1,3-glucan complexes, and recognition of *beta*-glucans localized to the cell wall of invading pathogens forms an important part of the innate immune system that is managed by myeloid phagocytes (Qi et al., 2011). The ability of insoluble *beta*-glucan particulates to prime the immune system is the basis for development of a variety of fungal and yeast-based functional foods that are currently marketed for food use (GRN 239, 309, 413). For example, the consumption of baker's yeast *beta*-glucan has been reported to minimize post-exercise immunosuppression, and may decrease the severity and the duration of upper respiratory tract infections (Fuller et al., 2012; Carpenter et al., 2013). Similar to *beta*-1,3-glucans originating from fungal/yeast sources, paramylon also appears to have immune priming properties (Kankkunen et al., 2010). Paramylon consumption has been reported to have anti-tumor activity against pre-neoplastic colonic aberrant crypt foci in mice, inhibit the development of atopic dermatitis-like skin lesions in NC/Nga mice, and oral administration of paramylon granules had immunostimulatory effects in rainbow trout vaccinated against *Yersinia ruckeri* (Sugiyama et al., 2010; Skov et al., 2012; Watanabe et al., 2013). These findings suggest that paramylon and/or preparations of *E. gracilis* biomass may have potential utility as functional food ingredients or dietary supplements for human nutrition and/or for animal feed purposes.

Algal Scientific has developed a proprietary strain of *E. gracilis* (ATCC PTA-123017) that is being investigated for use as a dietary supplement, functional food ingredient, and animal/pet feed ingredient, as well as the source of high purity *beta*-1,3-glucan (paramylon). This manuscript details studies evaluating the genotoxicity and oral toxicity of two food ingredients, a dried cell preparation of *E. gracilis* and a high purity crystalline paramylon isolate from *E. gracilis*, both developed by Algal Scientific from a proprietary strain of *E. gracilis*. Genotoxicity was assessed using the bacterial reverse mutation assay and an *in vivo* mouse micronucleus test. A single dose study and a 90-day toxicity study were conducted in male and female Sprague-Dawley rats administered dried cells of *E. gracilis* and paramylon isolate administered in the diet.

## 2. Materials and methods[1,2]

### 2.1. Materials

The test article used in the genotoxicity, acute toxicity, and repeat-dose toxicity studies was dried cellular biomass of *E. gracilis* ATCC PTA-123017. The 90-day repeat-dose toxicity study was also conducted with a comparator substance, paramylon (CAS No. 9051-97-2). The dried alga is a yellow powder and consisted of 100% *E. gracilis*, whereas paramylon was supplied as a white powder and consisted of 100% paramylon. The test substances used in these studies were supplied by Algal Scientific Corporation (Plymouth, Michigan). Product specifications for these products are presented in Table 1 and Table 2. Two lots (040715-AM-1 and 052015-BG-1) of the dried algae and paramylon, respectively, meeting product specifications were used. The dried alga preparation is stable for over 24 months when stored at room temperature in a well-closed container to avoid moisture and direct sunlight. Paramylon is stable for at least 36 months under typical storage conditions.

### 2.2. Genotoxicity studies[3,4]

#### 2.2.1. Ames test

Two sets of experiments were conducted using the plate incorporation method with *Salmonella typhimurium* TA98, TA100, TA1535, and TA1537, and *Escherichia coli* WP2*uvr*A. All test strains were obtained from Molecular Toxicology Inc. (Boone, NC). The S9 microsomal faction (Molecular Toxicology Inc. Boone, NC) was freshly prepared on the day of use from liver homogenates from male Sprague-Dawley rats induced with phenobarbital and benzoflavone.

A total of eight concentrations (1.58, 5.0, 15.8, 50, 158, 500, 1,580 and 5,000 μg/plate) of the test substance were tested in the presence and absence of metabolic activation. The vehicle, sterile water, was used as the negative control. The following compounds were used as positive controls without metabolic activation: sodium azide ($NaN_3$); ICR 191 acridine; daunomycin; and methylmethanesulfonate (MMS). In the presence of metabolic activation, 2-aminoanthracene (2-AA) was used as a positive control. Each tester strain was tested in triplicate for the negative and positive controls, and the test compound.

The main test was conducted with the plate incorporation method, while the confirmatory test was conducted using the pre-incubation method. An additional test in the confirmatory test was conducted with TA1537 under the same conditions and concentrations.

After incubation, the number of colonies per plate was counted manually or with a plate counter (Colony Plate Reader, Model Colony-Doc-It™; Upland, CA). The mean and standard deviation were calculated for each set of triplicate plates.

#### 2.2.2. Micronucleus assay

Two sets of the *in vivo* mammalian erythrocyte micronucleus test were performed using male and female Swiss albino (ICR) mice (Charles River Laboratories, Inc.) housed in plastic solid bottom cages, and acclimatized for 5–18 days to the laboratory conditions (temperature: 19–23 °C; relative humidity: 44–55%; 12 air

[1] Studies were conducted at Product Safety Labs (Dayton, New Jersey) in compliance with Good Laboratory Practice standards as described under 40 CFR Part 160, 40 CFR Part 792, 21 CFR Part 58, and OECD Principles of Good Laboratory Practice (as revised in 1997) published in ENV/MC/CHEM (98)17 (OECD, 1998) (genotoxicity studies), and 21 CFR Part 58 and OECD Principles of Good Laboratory Practice (as revised in 1997) published in ENV/MC/CHEM (98)17 (OECD, 1998) (90-day repeat dose toxicity study).

[2] All animal investigations were conducted in accordance with the most recent "Guide for the Care and Use of Laboratory Animals" (NRC, 2011).

[3] The bacterial reverse mutation test was conducted based on the most recent test guidelines as described below: US EPA Health Effects Test Guidelines, OPPTS 870-5100; US FDA Toxicological Principles for the Safety Assessment of Food Ingredients, Redbook 2000, IV.C.1.a; OECD Guidelines for Testing of Chemicals, Section 4 (Test No. 471); ICH S2(R1) "Guidance on Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use"; and Commission Regulation (EU) No 440/2008 B.13/14.

[4] The micronucleus test was conducted based on the most recent test guidelines as described below: OECD Guidelines for the Testing of Chemicals, Test No. 474; US FDA Toxicological Principles for the Safety Assessment of Food Ingredients, Redbook 2000, IV.C.1.d; EPA Health Effects Test Guidelines, OPPTS 870-5395; and ICH S2(R1) "Guideline for Industry. Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use".