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Toxicologic evaluation of a novel, highly soluble biotin salt, magnesium biotinate

Jason L. Blum [a], Melissa Ellis [a], Jayson X. Chen [a], Odete Mendes [a], Sarah Sylla [b], Sara Perez Ojalvo [b], James Komorowski [b,*]

[a] Product Safety Labs, 2394 US Highway 130, Dayton, NJ, 08810, United States
[b] JDS Therapeutics LLC, Harrison, NY, 10528, United States

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ABSTRACT

A novel, highly soluble biotin salt, magnesium biotinate (MgB), was assessed for general and genetic toxicity using several toxicologic tests. This battery of tests included *in vitro* bacterial reverse mutation test, *in vitro* mammalian micronucleus assay, and oral acute, 14-day, and 90-day repeat-dose toxicity in Sprague-Dawley (SD) rats. The results of the *in vitro* studies indicate that MgB is not mutagenic, clastogenic, or aneugenic. The acute oral toxicity study established an $LD_{50} \geq 5000$ mg MgB/kg. In the 14-day oral toxicity study, doses of MgB up to 2500 mg MgB/kg/day produced no clinical signs or mortality. In the 90-day oral toxicity study, administration of 600 mg MgB/kg/day resulted in no clinical signs and was determined to be the no-observed-adverse-effect-level (NOAEL), which equates to 39 g biotin/day for a 70 kg human. Since MgB is composed of 93% biotin, the 600 mg NOAEL equates to approximately 1.3 million times the current recommended daily allowance of 30 μg biotin/day and 3900 times supplement levels of 10 mg biotin/day. Based on the toxicologic profile and lack of findings in various *in vitro* and *in vivo* studies, MgB may be considered safe for long-term human use.

1. Introduction

Biotin is an essential B-complex vitamin that was initially discovered in 1927 (Institute of Medicine, 1998). Human cells cannot synthesize biotin so consequently it must be supplied from microbial and dietary sources (Zempleni et al., 2016a,b). This colorless, water-soluble nutrient plays a pivotal role in both metabolic and epigenetic pathways (Nur et al., 2020; Zempleni et al., 2012). It is an essential cofactor for five carboxylases involved in fatty acid, glucose, and amino acid metabolism, and is also a regulator of gene expression, including histone modification and DNA repair (Hosoya and Kubo, 2014; Institute of Medicine, 1998; Mock, 2017; Zempleni et al., 2012).

In humans, biotin is needed for normal development, growth, and health. Studies have shown that it may help treat or prevent specific disorders such as alopecia, biotin-responsive basal ganglia disease, congenital anomalies, multiple sclerosis, onychorrhexis (brittle fingernails), photoaging, and Type 2 diabetes (Alfadhel et al., 2013; Chessa et al., 2019; Famenini and Goh, 2014; Komorowski et al., 2019a,b; Maebashi et al., 1993; Mock, 2009; Sedel et al., 2015, 2016). Furthermore, a number of genetic disorders associated with secondary biotin deficiency including biotinidase deficiency, holocarboxylase synthetase deficiency, and biotin transport deficiency are treated with supplemental biotin (Wolf, 2012; Elrefai and Wolf, 2015; Mardach et al., 2002).

Foods rich in biotin include liver, egg yolks, and yeast, but relying on dietary sources alone is insufficient for some individuals (Staggs et al., 2004). Biotin deficiency frequently occurs with alcoholism, inflammatory bowel disorders, seborrheic dermatitis, Lenier's disease, pregnancy, long-term use of anticonvulsant drugs and parenteral nutrition, and the aforementioned genetic disorders (Dasgupta, 2019). Long-term consumption of raw egg whites can also contribute to biotin deficiency as the protein, avidin, has high affinity for biotin (Zempleni et al., 2016a, b). Signs and symptoms of biotin deficiency may include growth retardation and neurological and dermatological disorders (Coates et al., 2010).

Disorders related to biotin deficiency are often responsive to administration of oral biotin. Both the recommended dietary allowance and tolerable upper intake level have yet to be established; however, the adequate daily intake level is 30 μg for adults and pregnant women and 35 μg for lactating women (Dasgupta, 2019; Institute of Medicine, 1998). A daily dose of 0.3–10 mg of biotin is used in dietary

* Corresponding author.
E-mail address: jkomorowski@jdstherapeutics.com (J. Komorowski).

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Abbreviations

ABAS	absolute basophil
AEOS	absolute eosinophil
ALB	albumin
ALKP	alkaline phosphatase
ALT	alanine aminotransferase
ALUC	absolute large unstained cells
ALYM	absolute lymphocyte
AMON	absolute monocyte
ANOVA	analysis of variance
ARET	absolute reticulocytes
AST	aspartate aminotransferase
BILI	bilirubin
BUN	blood urea nitrogen
BW	body weight
BWG	body weight gain
CALC	calcium
CHOL	cholesterol
CL	chloride
CREA	creatinine
GLOB	globulin
GLUC	glucose
HCT	hematocrit
HGB	hemoglobin
IPHS	intraparenchymal hemorrhages
K	potassium
LD	lethal dose
MCHC	mean corpuscular hemoglobin concentration
MCV	mean corpuscular volume
MgB	magnesium biotinate
MEM	minimal essential medium
NA	sodium
NOAEL	no-observed-adverse-effect-level
OECD	Organization for Economic Cooperation and Development
PLT	platelet
RBC	red blood cell
RDW	red cell distribution width
SD	standard deviation
SDH	sorbitol dehydrogenase
S9	rat liver S9 mix
TP	total protein
TRIG	triglycerides
WBC	white blood cell
w/w	weight per weight
V79	Chinese hamster V79

supplements, a daily intake of 5–10 mg/kg body weight is used in severe and/or genetic disorders, and doses up to 300 mg have been clinically studied in progressive multiple sclerosis (Sedel et al., 2015, 2016, 2016; Tourbah et al., 2018).

Establishing reliable and consistent regimens for biotin administration can be challenging due to its low solubility (National Center for Biotechnology Information, 2020). A prior study found that a novel biotin salt, magnesium biotinate (MgB), has 40 times more solubility than D-biotin. Moreover, a preclinical model showed that administration of MgB to rats resulted in serum levels 47% higher than those administered the same amount of D-biotin (Ojalvo et al., 2019). As Ojalvo et al. (2019) did not assess toxicity, a series of tests (bacterial reverse mutation, *in vitro* micronucleus, and oral acute and repeat-dose testing in rats) were performed to assess the potential toxicity of MgB. All testing followed appropriate OECD and FDA guidelines (Table 1).

2. Materials and methods

2.1. Chemical and materials

The test substance, MgB, was supplied by JDS Therapeutics, LLC. (Harrison, NY, USA). The composition of MgB (by weight) is 93% D-biotin, 4.5% magnesium, and 2.5% sodium chloride with a molecular formula of $Mg(C_{10}H_{15}N_2O_3S)_2$. It is manufactured as a stable, non-hygroscopic, off-white powder.

Unless otherwise described, all reagents were obtained from Mediatech Inc (Manassas, VA, USA) or Sigma-Aldrich Corporation (St. Louis, MO, USA). For the bacterial reverse mutation test, chemically-induced rat liver S9 mix (S9), overlay agar, minimal glucose agar plates and oxoid nutrient broth #2 were obtained from Molecular Toxicology, Inc (Boone, NC, USA). For the mammalian *in vitro* micronucleus test, S9 fraction from liver homogenates from Sprague Dawley (SD) rats induced with phenobarbital and β-naphthoflavone was obtained from Eurofins BioPharma Product Testing (Eurofins; Planegg/Munich, Germany) for the pre-experiment and from Trinova Biochem GmbH (Giessen, Germany) for the main experiment.

Table 1
Guidelines followed for testing.

Test	Guidelines Followed
Bacterial reverse mutation test	• US FDA GLP: 21 CFR Part 58, 1987 • OECD[a] Principles of GLP (as revised in 1997): ENV/MC/CHEM(98)17, OECD, Paris, 1998 • OECD Guidelines for Testing of Chemicals, Section 4, Test No. 471: "Bacterial Reverse Mutation Test" (1997) • US FDA Toxicological Principles for the Safety Assessment of Food Ingredients, Redbook 2000, IV.C. 1. a. (2007) • ICH S2 (R1) Guidance on Genotoxicity Testing and Data Interpretation for Pharmaceuticals Intended for Human Use (2012)
Mammalian *in vitro* micronucleus test	• OECD Guidelines for Testing of Chemicals, Section 4 (Test No. 487): In Vitro Mammalian Micronucleus Test (2016). • OECD Principles of Good Laboratory Practice (as revised in 1997); ENV/MC/CHEM(98)17, OECD, Paris, 1998.
Rat oral dosing tests	• Acute oral testing • US FDA GLP: 21 CFR Part 58, 1987 • OECD Principles of GLP (as revised in 1997): ENV/MC/CHEM(98)17, OECD, Paris, 1998 • OECD Guidelines for Testing of Chemicals and Food Ingredients, Section 4 (Test No. 425): Acute Oral Toxicity: Up-and-Down Procedure (2008) • 14-day and 90-day repeat-dose oral testing • US FDA Toxicological Principles for the Safety Assessment of Food Ingredients, Redbook 2000, IV. C.3 a. Short-Term Toxicity Studies with Rodents (2007). • OECD Guidelines for Testing of Chemicals and Food Ingredients, Section 4 (Test No. 407): Health Effects, Repeated Dose 28-Day Oral Toxicity Study in Rodents (2008). • OECD Guidelines for Testing of Chemicals and Food Ingredients, Section 4 (Test No. 408): Health Effects, Repeated Dose 90-Day Oral Toxicity Study in Rodents (1998).

[a] OECD: Organization for Economic Cooperation and Development.

2.2. Animals

All *in vivo* studies were performed under protocols approved by the Product Safety Labs' IACUC committee in an American Association for Accreditation of Laboratory Animal Care accredited facility and the animals were housed individually in suspended, stainless steel cages, conforming to the size recommendations in the latest Guide for the Care and Use of Laboratory Animals (National Research Council, 2011). The animals received filtered water and rodent chow (Envigo Teklad Global 16% Protein Rodent Diet #2016; Envigo-Teklad) *ad libitum*. Animal rooms had photoperiods set to 12-hr light:dark cycles.

Female SD rats used for the acute oral dosing study were from SAGE Labs (St. Louis, MO, USA) and were 9–10 weeks at study initiation. Animals used for the 14-day range-finding and 90-day definitive repeat dose oral studies were SD rats (males and females) from Charles River Laboratories (Stone Ridge, NY, USA). The rats were 6–7 weeks of age upon arrival. At study initiation, animals were weighed and randomly assigned to treatment groups in such a way that intrasex variation in body weights was less than 20% between treatment groups.

2.3. Experimental design

2.3.1. Bacterial reverse mutation test

The five strains of bacteria used in this assay, purchased from Molecular Toxicology, Inc. (Boone, NC), included *Salmonella typhimurium* (TA1535, TA1537, TA98, TA100) and *Escherichia coli* (WP2 [uvrA]). MgB concentrations were as follows: 1.58, 5, 15.8, 50, 158, 500, 1580 and 5000 μg/plate. The main study used the plate incorporation method and the pre-incubation modification method was used to confirm the results. Both were performed in either the absence or presence of S9 mix. All experiments were performed in triplicate.

The plate incorporation method included mixing 100 μL of prepared MgB at the indicated concentrations, positive or negative control, 500 μL S9 (5% v/v S9 fraction, including sterile cofactors: 8 mM $MgCl_2$, 33 mM KCl, 100 mM sodium phosphate buffer pH 7.4, 5 mM glucose-6-phosphate, and 4 mM NADP) (Maron and Ames, 1983) or substitution buffer when S9 was not included (sodium phosphate), and 100 μL bacteria suspension (approximately 1×10^9 bacteria/mL) into 2000 μL overlay agar maintained at approximately 45 °C and pouring this over the surface of a minimal glucose agar plate. MgB was prepared in sterile water. Positive controls, sodium azide (15 μg/mL), ICR 191 acridine (10 μg/mL), daunomycin (60 μg/mL), and methylmethanesulfonate (MMS; 25 μL/mL), were prepared in sterile water, and 2-aminoanthracene (100 μg/mL) was prepared in dimethylsulfoxide. Fresh bacterial suspension cultures were prepared in oxoid nutrient broth #2 and incorporated during late exponential growth phase. After pouring, plates were placed inverted in an incubator at approximately 37 °C for 65 h. The pre-incubation reactions containing MgB or control, bacterial suspension, and S9/substitution buffer were incubated under agitation at approximately 37 °C for 30 min prior to mixing with the overlay agar and being poured onto the minimal agar plates.

The number of revertant colonies per plate was determined by counting manually and/or using a plate counter (Colony Plate Reader: Model Colony-Doc-It™). The means and standard deviations were calculated for each set of triplicate plates. Criteria for a valid assay were as previously described (Mortelmans and Zeiger, 2000; Gatehouse, 2012). A response was considered positive for mutagenic potential if the mutation factor (mean revertant colony count divided by mean revertant colony count for the corresponding negative control) was $\geq$2 for TA98, TA100, and WP2 or $\geq$ 3 for TA1535 and TA1537 strains, indicating a substantial increase in revertant colonies, and was dose-dependent and reproducible.

2.3.2. In vitro mammalian cell micronucleus test

The *in vitro* micronucleus test assay was conducted to assess clastogenicity and aneugenicity potential of MgB using Chinese hamster V79 (V79) cells during or after exposure to MgB by detecting both numerical and structural chromosomal aberrations. The V79 cells underwent routine testing and were free of mycoplasma contamination. Cells were maintained in minimal essential medium (MEM) supplemented with 10% fetal bovine serum, 100 μg/mL penicillin-streptomycin solution, 2 mM L-glutamate, 2.5 μg/mL amphotericin b, and 25 mM HEPES buffer solution, in 75 cm^2 cell culture plastic flasks at 37 °C and 5% CO_2 in air.

Experiment 1 tested a 4 h incubation of the V79 cells either with or without S9 activation. Plates without MgB were included for both tests. Experiment 2 was an incubation without S9 metabolic activation for 24 h. Cytochalasin B (1.5 μg/mL) was added to the culture media and cells were cultured for an additional 20 h (Experiments 1) or 23 h (Experiment 2) at 37 °C.

Metabolic activation was achieved using liver homogenate S9 fraction from SD rats. The S9 supernatant was mixed with S9 cofactor solution (8 mM magnesium chloride, 33 mM potassium chloride, 5 mM glucose-6-phosphate, and 5 mM nicotinamide adenine dinucleotide phosphate). The final concentration of S9 mix used in the media was 5%.

Magnesium biotinate was dissolved and diluted in cell culture medium within 1 h of addition. Ten concentrations were used in the pre-experiment assay ranging between 3.9 and 2000 μg/mL. Six concentrations were used in Experiment I ranging between 125 and 2000 μg/mL. Seven concentrations were used in Experiment II between 50 and 2000 μg/mL. MMS (20 and 25 μg/mL) served as the positive clastogenic control. Colchicine (0.16 and 1.5 μg/mL) served as the positive aneugenic control in plates without S9 activation. Cyclophosphamide (2.5 μg/mL) served as positive clastogenic control in plates with S9 activation. All positive controls were prepared in MEM and culture medium alone served as the negative control.

Cells were prepared on fixed slides and then analyzed for micronuclei and cytotoxicity. For each experimental point, at least 2000 binucleated cells per concentration (1000 binucleated cells per slide) were analyzed for micronuclei according to the Fenech criteria (Fenech, 2000). Mononucleated and multinucleated cells and cells with more than six micronuclei were not considered. A cytokinesis block proliferation index was determined from 500 cells as an assessment of cytotoxicity. This index was used to calculate the percent cytosis, an indicator of growth inhibition of treated cultures compared with control.

2.3.3. Acute oral toxicity study in rats

Prior to dosing, a group of female rats was fasted overnight, weighed, and examined for health and then three healthy individuals were selected. The rats were administered 5000 mg/kg, which was divided into two doses administered 2 h apart using a stainless steel ball-tipped needle attached to a syringe. Following administration, the animals were observed for mortality, signs of gross toxicity, and behavioral changes several times immediately after dosing and then daily for 14 days. Body weights were measured and recorded on days 7 and 14 following dosing. Gross necropsies were performed after the animals were sacrificed with CO_2 inhalation 14 days post-administration.

2.3.4. 14-Day repeat oral dose toxicity study in rats

Forty SD rats (20 males and 20 females) were used to determine the general toxicity of MgB. Each dose level consisted of five males and five females. Rats were administered vehicle or MgB (120, 600, or 2500 mg MgB/kg/day) in a volume of 10 mL/kg twice daily by oral gavage for 14 consecutive days. Following administration, the animals were observed for mortality, signs of gross toxicity, and behavioral changes approximately 30 min and several hours post-dose each day. Body weights were measured and recorded weekly and then prior to sacrifice. Food consumption was measured at the same time as body weight and food efficiency was calculated. Animals were euthanized on day 15 and gross necropsies were performed.

2.3.5. Subchronic 90-day repeat oral toxicity study in rats

Forty male and forty female SD rats were used to evaluate potential

subchronic toxicity of repeated exposure to MgB. Prior to the start of the study and near its conclusion, the eyes of all study animals were examined by focal illumination and indirect ophthalmoscopy.

Rats were administered vehicle or MgB (120, 600, or 2500 mg MgB/kg/day) in a volume of 10 mL/kg twice daily by oral gavage for at least 90 days. All animals were weighed on Day 1 (prior to study start), and weekly and then just prior to sacrifice in order to calculate organ-to-body weight ratios. Food consumption and body weights were measured and food efficiency was calculated.

During Week 12, samples were collected for hematology, clinical chemistry, and urinalysis and evaluated (Table 2). Animals were placed in metabolism cages and fasted for 15 h prior to blood sample collection. Blood samples for determining prothrombin and partial thromboplastin times (coagulation) were collected from the inferior vena cava under isoflurane anesthesia. Animals were euthanized by exsanguination followed by full necropsy. Gross evaluations were made to the external surface of the body, all orifices, the musculoskeletal system, and the cranial, thoracic, abdominal, and pelvic cavities (Table 3). Tissues were weighed wet and preserved in 10% neutral buffered formalin or modified Davidson's fixative, and stored in ethanol for histopathological examination.

2.4. Statistical analyses

Statistical comparisons were performed for in-life phase data (body weight, food consumption, and food efficiency), organ weights, and clinical pathology results using Provantis (ver 9; Staffordshire, UK). Treated and control groups were compared using a two-way analysis of variance (ANOVA), testing the effects of both time and treatment with methods accounting for repeated measures for one independent variable. If statistically significant results were obtained, Dunnett's test was performed to assess the differences between control and treated groups. Where homogenous variances and normal distribution were observed in single measurements of continuous data (e.g. organ weight and relative organ weight), inter-group comparisons were made using one-way ANOVA. If statistically significant results were obtained, Dunnett's test was performed to assess the inter-group differences. When assumptions for normal distributions were not met, a Kruskal-Wallis non-parametric analysis was performed and when found significant, inter-group comparisons were made using Dunn's test. Analysis of the micronucleus test was performed using a non-parametric χ^2 test.

Table 2
Hematology, coagulation, and clinical chemistry parameters evaluated in blood samples collected from male and female rats on designated days.

Parameter	Parameter
Hematology – Day 86	
RBC (10^6/μl)	ANEU (10^3/μl)
Hemoglobin (g/dl)	ALYM (10^3/μl)
Hematocrit (%)	AMON (10^3/μl)
MCV (fl)	AEOS (10^3/μl)
MCH (pg)	ABAS (10^3/μl)
MCHC (g/dl)	ALUC (10^3/μl)
RDW (%)	AHSN (10^3/μl)
Platelet count (10^3/μl)	AIL (10^3/μl)
WBC (10^3/μl)	AIM (10^3/μl)
ARET (10^3/μl)	
Coagulation – Day 86	
PT (sec)	APTT (sec)
Clinical Chemistry–Day 86	
AST (U/l)	Glucose, fasting (mg/dl)
ALT (U/l)	Total protein (g/dl)
SDH (U/l)	Albumin (g/dl)
ALKP (U/l)	Globulin (g/dl)
BILI (mg/dl)	Calcium (mg/dl)
BUN (mg/dl)	Inorganic Phosphorus (mg/dl)
Creatinine (mg/dl)	Sodium (mmol/l)
Total Cholesterol (mg/dl)	Potassium (mmol/l)
Triglycerides (mg/dl)	Chloride (mmol/l)
Urinalysis Day 86	
Urinary Volume (ml)	Urinary Specific Gravity
Urinary pH	Urobilinogen (EU/dL)

ABAS, absolute basophil; AEOS, absolute eosinophil; ALKP, alkaline phosphatase; ALT, alanine aminotransferase; ALUC, absolute large unstained cell; ALYM, absolute lymphocyte; AMON, absolute monocyte; ANEU, absolute neutrophil; APTT, activated partial thromboplastin time; ARET, absolute reticulocyte count; AST, aspartate aminotransferase; BILI, total bilirubin; BUN, blood urea nitrogen; MCH, mean corpuscular hemoglobin; MCHC, mean corpuscular hemoglobin concentration; MCV, mean corpuscular volume; PT, prothrombin time; RBC, erythrocyte count; RDW, red cell distribution width; SDH, sorbitol dehydrogenase; WBC, total white blood cell and differential leukocyte count.

Table 3
Organs and tissues removed for macroscopic and microscopic pathology.

Organ/tissue	Organ/tissue	Organ/tissue
Accessory genital organs (prostate and seminal vesicles)	Heart[a]	Rectum
Adrenals[a]	Ileum with Peyer's patches	Salivary glands (sublingual, submandibular, parotid)
All gross lesions	Jejunum	Skeletal muscle
Aorta	Kidneys[a]	Skin
Bone (femur)	Larynx	Spinal cord (cervical, mid-thoracic, lumbar)
Bone marrow (femur & sternum)	Liver[a]	Spleen[a]
Brain[a] (cerebral and cerebellar cortices, medulla/pons)	Lungs	Sternum
Cecum	Lymph nodes (mandibular and mesenteric)	Stomach
Cervix	Mammary gland	Testes[a]
Colon	Nose and nasal turbinates	Thymus[a]
Duodenum	Ovaries with oviducts[a]	Thyroid/parathyroid
Epididymides[a]	Pancreas	Trachea
Esophagus	Peripheral nerve (sciatic)	Urinary bladder
Eyes (with optic nerve)	Pharynx	Uterus[a]
Harderian gland	Pituitary gland	Vagina

[a] Weighed organs.

3. Results

3.1. Bacterial reverse mutation test

No concentration-related or substantial MgB-related increases in revertants were observed for any of the five bacterial strains tested in either the absence or presence of S9 using either the plate incorporation or the pre-incubation method. The mean revertant colony counts for each strain treated with MgB were close to or within the expected range of laboratory historical controls and published values (Mortelmans and Zeiger, 2000; Gatehouse, 2012). Treatment with MgB (1.58, 5.0, 15.8, 50, 158, 500, 1580, and 5000 μg/plate) showed no significant mutation increases compared to the negative control in the *Salmonella typhimurium* (TA 100) plate incorporation method, with 117 ± 5.7 (-S9) and 121 ± 6.5 (+S9) colonies at 1.58 μg/plate and 116 ± 8.5 (-S9) and 117 ± 8.5 (+S9) at 5000 μg/plate compared to 118 ± 3.1 (-S9) and 126 ± 5.5 (+S9) colonies with negative control (Fig. 1). Further, the results of the pre-incubation modification confirmed the results of the plate incorporation method. For all tests, treatment of the various cell types with the positive control substances (sodium azide, 2-aminoanthracene, ICR 191 acridine, daunomycin, and methylmethanesulfonate) resulted in the expected reversion mutation factor levels.

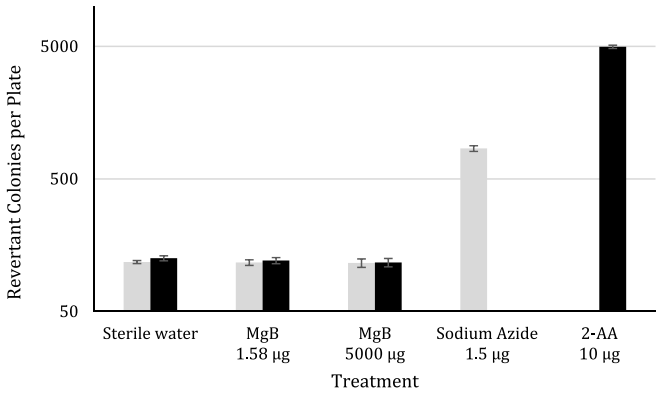

Fig. 1. Mean revertant colony counts of negative control (sterile water), lowest MgB concentration (1.58 µg), highest MgB concentration (5000 µg), and positive controls without and with S9 metabolic activator in one of five tested bacterial strains, *Salmonella typhimurium* (TA 100). The six additional MgB concentrations (5, 15.8, 50, 158, 500, and 1580 µg) not shown were all within the expected range, 107 ± 6.2–120 ± 7.4 µg/plate. Experiments were performed in triplicate and mean and standard deviation was calculated for each set of triplicate plates. 2-AA: 2-aminoanthracene. Grey bars are values from plates treated without S9 activation and black bars are values from plates with S9 activation.

3.2. In vitro mammalian cell micronucleus test

No increase above 30% cytostasis and no statistically significant increase in aberrant cells were noted in Experiment 1 (with or without S9 activation) or Experiment 2 at all MgB concentrations. Micronucleus frequency with MgB showed no significant increases compared to negative control in Experiment 1 (without S9) (Table 4–A). A significant decrease in micronucleus frequency was observed by 2000 mg/mL MgB compared to negative control in Experiment 1 (with S9 activation) (Table 4–B). A decrease in micronucleus frequency is not considered to be toxicologically important. The positive controls resulted in the expected significant increases in micronucleus frequency in both 0.80 (-S9) and 0.70 (+S9) frequency at 2000 µg/plate compared to 0.70 (-S9) and 1.35 (+S9) frequency with negative control.

Concentrations of 1500 and 2000 µg/mL of MgB in Experiment 2 produced statistically significant increases compared to control treated cells (1.2% each versus 0.55%) (Table 4–C). However, these observed increases were considered to not be biologically relevant since the numbers of micronucleated cells were within the historical control limits of the negative control of the lab and no dose-response relationship was observed. The clastogenic positive control, methylmethanesulfonate and the aneugenic positive control, colchicine, induced the expected significant ($p < 0.05$) increases in micronucleus frequency (5.5% and 7.6%, respectively) demonstrating that the assay provided valid results.

Table 4
Results of *In Vitro* micronucleus testing.

4-A. Experiment 1 without S9 activation			
Dose Group	Conc (mg/mL)	Micronucleus Frequency (%)	P-value
Negative control	0	0.70	N/A
4	1000	1.20	0.1031
5	1500	1.15	0.1372
6	2000	0.80	0.7140
MMS	20	4.00	<0.0001 *
Colchicine	1.5	2.67	<0.0001 *
4-B. Experiment 1 with S9 activation			
Dose Group	Conc (mg/mL)	Micronucleus Frequency (%)	P-value
Negative control	0	1.35	N/A
4	1000	1.25	0.7801
5	1500	1.05	0.3836
6	2000	0.70	0.0413 *
Cyclophosphamide	2.5	4.30	<0.0001 *
4-C. Experiment 2 without S9 activation			
Dose Group	Conc (mg/mL)	Micronucleus Frequency (%)	P-value
Negative control	0	0.55	N/A
5	1000	1.05	0.0759
6	1500	1.20	0.0273 *
7	2000	1.20	0.0273 *
MMS	25	5.50	<0.0001 *
Colchicine	0.16	7.60	<0.0001 *

3.3. Acute oral toxicity study

All animals survived a single oral dose of 5000 mg MgB/kg of body weight and gained weight during the study. Shortly following administration, all animals exhibited diarrhea, ano-genital staining, and/or soft feces. These signs cleared by 24 h following administration and animals remained active and healthy for the remainder of the study. There were no gross abnormalities when necropsied after the conclusion of the 14-day observation period. These data support an MgB oral LD50 ≥ 5000 mg/kg.

3.4. 14-Day repeat dose oral toxicity study

At doses up to 2500 mg MgB/kg/day for 14 days, no mortality or clinical signs were observed in any of the animals. Further, there were no significant changes in mean body weights, daily body weight gain, daily food consumption, and food efficiency (Tables 5 and 6).

Gross examination of the organs and tissues during necropsy showed no MgB-related macroscopic observations. Incidental macroscopic findings were limited to a small right testis and epididymis in one Group 3 (600 mg MgB/kg/day) male as well as a fluid-filled uterus in one Group 2 (120 mg MgB/kg MgB) female. As these findings were limited to individual animals and not observed in a dose dependent manner, they were considered not toxicologically relevant but attributed as incidental or congenital in the male and estrous cycle-related variability in the female.

3.5. Subchronic 90-day oral toxicity study in rats

MgB related mortality during the course of the 90-day study was limited to one female at 2500 mg MgB/kg/day and was associated with diarrhea, anogenital staining, decreased feed intake, and reduced fecal output. Macroscopic and microscopic evidence of gastric ulceration as well as depletion of lymphoid and bone marrow tissue were also observed. Through a combined assessment of the in-life, clinical, and anatomical pathology findings, it was determined that the 2500 mg MgB/kg/day dose contributed to the animal's moribundity. Additionally, one male in the 600 mg MgB/kg/day group had clinical signs of respiratory distress and esophageal trauma associated with gavage and unrelated to MgB. Otherwise there were no MgB-related macroscopic observations. Incidental gross findings included fluid-filled uteri in the control (n = 2), 120 mg MgB/kg/day (n = 3), and 600 mg MgB/kg/day (n = 2) female groups, which correlated with estrous cycle-related dilation upon histological evaluation and were considered normal and not related to MgB treatment. Ophthalmological exams at the end of the treatment period did not show any clinically relevant findings.

The mean weekly body weight of males in the 120 and 600 mg MgB/

Table 5
Mean body weights in male and female rats during a 14-day treatment with 2500 mg MgB/kg/day.

Parameter	Day 1	Day 5	Day 8
Body weights (g)			
Females	191.8 ± 7.9	222.8 ± 6.7	240.4 ± 7.8
Males	247.4 ± 12.4	306.8 ± 17.1	352.4 ± 26.1

Table 6
Mean daily body weight gain, daily food consumption, and food efficiency in male and female rats during a 14-day treatment with 2500 mg MgB/kg/day.

Parameter	Days 1–8	Days 8–15	Days 1–15
Daily body weight gain (g/day)			
Females	4.43 ± 1.61	2.51 ± 0.33	3.47 ± 0.88
Males	8.49 ± 1.05	6.51 ± 1.62	7.50 ± 1.28
Daily food consumption (g/day)			
Females	19.29 ± 0.81	20.77 ± 0.75	20.03 ± 0.67
Males	27.86 ± 2.55	29.89 ± 4.24	28.87 ± 3.33
Daily food efficiency			
Females	0.299 ± 0.082	0.121 ± 0.017	0.173 ± 0.042
Males	0.304 ± 0.015	0.216 ± 0.030	0.259 ± 0.017

kg/day groups and mean daily body weight gains in the 120 mg MgB/kg/day group were comparable to the control group. The 2500 mg MgB/kg/day males had a non-statistically significant decrease in both body weight and body weight gain that was interpreted as not adverse due to a lack of histopathological findings. Mean daily body weight gain of males in the 120 and 600 mg MgB/kg/day groups showed statistically significant increases on Study Days 29–36 ($p < 0.05$) and Study Days 78–85 ($p < 0.01$), respectively, compared with control. Further, the mean daily body weight gain in the 600 mg MgB/kg/day group also showed a statistically significant decrease on Study Days 50–57 ($p < 0.01$). However, without corresponding significant changes in body weights, all three of these statistically significant outcomes were considered not toxicologically relevant.

In females, average daily food consumption was similar in all groups throughout the study and mean food efficiencies were also similar. In males, mean food efficiencies were similar in all groups except for a statistically significant decrease between Study Days 50–57 ($p < 0.05$–0.01) for the 600 and 2500 mg MgB/kg/day groups and a statistically significant increase on Study Days 78–85 ($p < 0.01$) in the 600 mg MgB/kg/day group. Without corresponding effects on body weights in the males, all three statistically significant outcomes were considered to be incidental and not toxicologically relevant.

Clinical pathology and hematology results are presented in Tables 7 and 8 for males and females, respectively. Both hematocrit and absolute numbers of basophils were significantly decreased ($p < 0.05$) in males treated with 600 mg MgB/kg/day. In females, a statistically significant decrease was observed in serum alanine aminotransferase (ALT; $p < 0.05$) in the 2500 mg MgB/kg/day group. Treatment of male rats with MgB (all levels) and female rats in the 600 and 2500 mg MgB/kg/day groups showed non-significant increases in sodium, potassium, and chloride that were dose-dependent. These changes were within the laboratory's historical control limit ranges and not associated with any

Table 7
Hematology, coagulation, and clinical chemistry parameters in male rats following a 13-week dietary treatment with MgB.

Parameter	Group 1 Control	Group 2 120 mg/kg/day	Group3 600 mg/kg/day	Group 4 2500 mg/kg/day
Hematology – Day 86				
RBC (10^6/μl)	8.953 ± 0.511	9.237 ± 0.409	8.751 ± 0.453	8.945 ± 0.557
Hemoglobin (g/dl)	16.41 ± 0.76	16.61 ± 0.70	15.68 ± 0.80	16.40 ± 0.78
Hematocrit (%)	50.79 ± 2.24	52.03 ± 2.11	48.35 ± 1.94[a]	50.49 ± 2.29
MCV (fl)	56.78 ± 1.42	56.16 ± 1.67	55.27 ± 1.91	56.55 ± 2.68
MCH (pg)	18.35 ± 0.69	17.94 ± 0.59	17.93 ± 0.82	18.37 ± 0.65
MCHC (g/dl)	32.33 ± 0.53	31.96 ± 0.38	32.42 ± 0.60	32.49 ± 0.57
RDW (%)	13.03 ± 1.22	13.56 ± 097	13.61 ± 0.65	13.11 ± 1.17
Platelet count (10^3/μl)	1048.8 ± 118.2	1012.5 ± 178.6	941.0 ± 246.5	989.2 ± 158.0
WBC (10^3/μl)	11.645 ± 1.934	11.612 ± 2.804	11.189 ± 3.138	10.320 ± 2.509
ANEU (10^3/μl)	1.560 ± 0.468	1.480 ± 0.543	1.949 ± 0.918	1.640 ± 0.358
ALYM (10^3/μl)	9.471 ± 1.653	9.573 ± 2.462	8.835 ± 2.487	8.137 ± 2.263
AMON (10^3/μl)	0.279 ± 0.081	0.231 ± 0.149	0.326 ± 0.179	0.250 ± 0.038
AEOS (10^3/μl)	0.202 ± 0.056	0.180 ± 0.048	0.175 ± 0.052	0.210 ± 0.111
ABAS (10^3/μl)	0.087 ± 0.038	0.106 ± 0.051	0.051 ± 0.026[a]	0.052 ± 0.018
ALUC (10^3/μl)	0.046 ± 0.019	0.045 ± 0.033	0.059 ± 0.045	0.033 ± 0.016
ARET (10^3/μl)	192.47 ± 35.25	227.79 ± 47.53	216.96 ± 55.31	206.72 ± 48.71
Coagulation – Day 86				
PT (sec)	10.21 ± 0.23	10.08 ± 0.30	9.91 ± 0.39	9.89 ± 0.30
APTT (sec)	35.12 ± 4.63	31.10 ± 4.69	33.43 ± 8.04	31.31 ± 5.29
Clinical Chemistry–Day 86				
AST (U/l)	3.60 ± 0.28	3.60 ± 0.22	3.61 ± 0.22	3.79 ± 0.32
ALT (U/l)	80.50 ± 9.58	95.60 ± 16.46	89.40 ± 31.37	91.90 ± 14.15
SDH (U/l)	31.1 ± 3.9	29.6 ± 3.1	30.0 ± 4.4	27.8 ± 4.2
ALKP (U/l)	68.2 ± 12.3	67.2 ± 12.9	72.6 ± 17.6	70.3 ± 19.7
BILI (mg/dl)	11.9 ± 1.7	11.8 ± 1.5	11.8 ± 1.8	13.0 ± 1.6
BUN (mg/dl)	9.39 ± 0.52	9.19 ± 0.55	9.40 ± 0.39	9.64 ± 0.69
Creatinine (mg/dl)	50.6 ± 15.9	53.7 ± 15.0	59.4 ± 12.2	58.5 ± 10.6
Total Cholesterol (mg/dl)	0.1555 ± 0.022	0.148 ± 0.038	0.160 ± 0.040	0.172 ± 0.036
Triglycerides (mg/dl)	102.1 ± 21.1	102.1 ± 21.1	118.9 ± 22.0	118.1 ± 9.9
Glucose, fasting (mg/dl)	6.26 ± 0.57	6.28 ± 0.57	6.38 ± 0.48	6.88 ± 0.65
Total protein (g/dl)	5.81 ± 0.32	5.75 ± 0.42	5.78 ± 0.36	5.94 ± 0.45
Albumin (g/dl)	0.096 ± 0.016	0.087 ± 0.015	0.081 ± 0.014	0.095 ± 0.020
Globulin (g/dl)	63.4 ± 35.3	55.7 ± 19.2	54.9 ± 21.1	45.1 ± 9.0
Calcium (mg/dl)	3.989 ± 3.854	2.186 ± 1.850	2.753 ± 2.680	1.178 ± 1.133
Inorganic Phosphorus (mg/dl)	140.9 ± 5.0	142.9 ± 2.4	145.2 ± 1.4	147.3 ± 2.5
Sodium (mmol/l)	5.261 ± 0.266	5.474 ± 0.289	5.807 ± 0.492	5.789 ± 0.358
Potassium (mmol/l)	99.52 ± 3.78	101.41 ± 1.98	103.77 ± 1.31	103.93 ± 1.67
Chloride (mmol/l)	2.21 ± 0.17	2.19 ± 0.19	2.17 ± 0.26	2.17 ± 0.27
Urinalysis Day 86				
Urinary Volume (ml)	6.875 ± 4.581	8.300 ± 7.48	9.650 ± 10.462	8.050 ± 4.907
Urinary pH	6.65 ± 0.41	6.50 ± 0.53	6.60 ± 0.70	7.10 ± 1.22
Urinary Specific Gravity	1.0250 ± 0.0047	1.0240 ± 0.0077	1.0240 ± 0.0074	0.9206 ± 0.2202
Urobilinogen (EU/dL)	0.60 ± 0.42	0.44 ± 0.39	0.28 ± 0.25	0.82 ± 1.18

[a] Means are significantly different from Group 1 controls.

Table 8
Hematology, coagulation, and clinical chemistry parameters in female rats following a 13-week dietary treatment with MgB.

Parameter	Group 1 Control	Group 2 120 mg/kg/day	Group3 600 mg/kg/day	Group 4 2500 mg/kg/day
Hematology – Day 86				
RBC (10^6/μl)	8.363 ± 0.361	8.232 ± 0.245	8.565 ± 0.258	8.397 ± 0.407
Hemoglobin (g/dl)	15.54 ± 0.49	15.83 ± 0.68	15.86 ± 0.62	15.51 ± 0.45
Hematocrit (%)	47.81 ± 1.23	47.87 ± 1.83	48.45 ± 2.07	46.97 ± 1.84
MCV (fl)	57.22 ± 1.55	57.52 ± 1.97	56.57 ± 1.32	56.02 ± 2.35
MCH (pg)	18.72 ± 0.49	19.06 ± 0.79	18.50 ± 0.44	18.50 ± 0.83
MCHC (g/dl)	32.92 ± 0.35	33.22 ± 0.47	32.97 ± 0.37	33.04 ± 0.29
RDW (%)	11.81 ± 0.33	11.78 ± 0.33	11.59 ± 0.35	11.89 ± 0.37
Platelet count (10^3/μl)	1107.5 ± 96.3	1051.9 ± 124.4	1149.3 ± 90.4	1079.9 ± 119.9
WBC (10^3/μl)	7.762 ± 2.132	7.679 ± 1.243	7.178 ± 1.432	6.351 ± 1.990
ANEU (10^3/μl)	0.729 ± 0.357	0.835 ± 0.299	0.707 ± 0.258	0.987 ± 0.874
ALYM (10^3/μl)	6.637 ± 1.881	6.424 ± 1.980	6.100 ± 1.494	4.859 ± 2.427
AMON (10^3/μl)	0.169 ± 0.085	0.187 ± 0.081	0.172 ± 0.045	0.352 ± 0.698
AEOS (10^3/μl)	0.143 ± 0.053	0.147 ± 0.045	0.128 ± 0.045	0.120 ± 0.039
ABAS (10^3/μl)	0.040 ± 0.023	0.031 ± 0.014	0.030 ± 0.015	0.026 ± 0.017
ALUC (10^3/μl)	0.042 ± 0.021	0.050 ± 0.034	0.044 ± 0.016	0.027 ± 0.017
ARET (10^3/μl)	164.18 ± 27.47	86.77 ± 27.04	178.86 ± 25.62	171.20 ± 27.12
Coagulation – Day 86				
PT (sec)	9.50 ± 0.30	9.52 ± 0.29	9.50 ± 0.32	9.27 ± 0.27
APTT (sec)	30.72 ± 5.65	28.42 ± 4.64	31.51 ± 5.49	29.16 ± 4.38
Clinical Chemistry–Day 86				
AST (U/l)	81.90 ± 27.60	78.30 ± 16.64	91.00 ± 15.90	71.28 ± 29.24
ALT (U/l)	32.1 ± 12.5	27.3 ± 4.7	30.6 ± 7.4	20.4 ± 4.4[a]
SDH (U/l)	6.680 ± 5.038	4.940 ± 1.657	7.980 ± 4.692	3.638 ± 1.986
ALKP (U/l)	40.3 ± 9.3	35.0 ± 11.5	40.3 ± 6.8	51.4 ± 52.2
BILI (mg/dl)	0.096 ± 0.026	0.087 ± 0.019	0.094 ± 0.025	0.086 ± 0.024
BUN (mg/dl)	13.2 ± 1.5	13.1 ± 1.9	14.1 ± 2.6	13.7 ± 1.8
Creatinine (mg/dl)	0.219 ± 0.035	0.221 ± 0.61	0.234 ± 0.055	0.253 ± 0.042
Total Cholesterol (mg/dl)	90.4 ± 20.1	81.5 ± 18.8	84.3 ± 21.3	79.4 ± 11.2
Triglycerides (mg/dl)	62.7 ± 37.5	55.0 ± 24.4	55.0 ± 26.3	53.0 ± 11.7
Glucose, fasting (mg/dl)	118.2 ± 10.8	116.7 ± 9.5	129.7 ± 13.2	129.8 ± 20.1
Total protein (g/dl)	6.68 ± 0.71	6.78 ± 0.81	6.43 ± 0.50	6.54 ± 0.71
Albumin (g/dl)	4.61 ± 0.65	4.47 ± 0.53	4.44 ± 0.45	4.73 ± 0.71
Globulin (g/dl)	2.07 ± 0.20	2.29 ± 0.44	1.99 ± 0.41	1.86 ± 0.19
Calcium (mg/dl)	9.68 ± 0.53	9.61 ± 0.74	9.80 ± 0.73	10.37 ± 0.70
Inorganic Phosphorus (mg/dl)	5.22 ± 0.58	5.12 ± 0.54	5.23 ± 1.11	5.99 ± 0.91
Sodium (mmol/l)	140.2 ± 1.9	140.2 ± 5.7	144.1 ± 6.4	145.7 ± 1.9
Potassium (mmol/l)	4.853 ± 0.406	4.900 ± 0.332	4.854 ± 0.439	5.134 ± 0.214
Chloride (mmol/l)	99.40 ± 1.93	99.58 ± 4.11	102.78 ± 3.72	102.04 ± 3.48
Urinalysis Day 86				
Urinary Volume (ml)	7.000 ± 3.691	9.150 ± 5.548	5.550 ± 3.640	10.994 ± 11.182
cUrinary pH	6.83 ± 0.43	6.60 ± 0.97	6.45 ± 0.60	7.22 ± 1.09
Urinary Specific Gravity	1.0194 ± 0.0077	1.0185 ± 0.0085	1.0250 ± 0.0066	0.9609 ± 0.1719
Urobilinogen	0.56 ± 0.42	0.28 ± 0.25	0.36 ± 0.34	0.38 ± 0.35

[a] Means are significantly different from Group 1 controls.

histopathologic changes. Therefore, these changes were considered to be due to individual variability and unrelated to MgB administration. Measurements of coagulation and urinalysis showed no significant changes in either sex at the levels tested.

Tables 9 and 10 present data for the weights of various organs for males and females, respectively. Evaluation of the mean organ weights and organ weights relative to total body weight showed that males from the 2500 mg MgB/kg/day group had significantly decreased absolute ($p < 0.01$) and relative heart weight ($p < 0.05$) compared with control without microscopic correlates and were considered to be secondary to lower final body weight. Females from the 2500 mg MgB/kg/day group had significantly increased absolute and relative liver weights ($p < 0.05$). Differences in absolute and relative weights of the heart and liver at the 2500 mg MgB/kg/day level were observed without microscopic changes and were not considered adverse.

Significantly decreased absolute and relative thymus weights ($p < 0.05$), which like the thymic lymphocyte depletion, were deemed a secondary stress response and not associated with MgB. Additionally, microscopic observations showed minimal thymic lymphocyte depletion in male and female rats (two of each) treated with 2500 mg MgB/kg/day. This depletion was considered secondary to stress and not directly attributable to MgB administration (Everds et al., 2013). There were no microscopic findings directly attributable to MgB.

4. Discussion

Biotin is an essential nutrient that acts as a coenzyme for five biotin-dependent carboxylases involved in amino acid metabolism, fatty acid synthesis, and glucogenesis. Through these pathways, biotin plays a vital role in mediating nutrient metabolism, cell signaling, DNA repair, and immune function, among numerous other processes. Biotin is sold as an ingredient in both dietary supplements and functional foods to support these metabolic functions and also to help treat or prevent symptoms of biotin deficiency (Dasgupta, 2019; Zempleni et al., 2016a,b). However, the Recommended Dietary Allowance (RDA) and Tolerable Upper Intake Level has yet to be established due to lack of data on adverse events from high dose intake. Clinical studies show no toxicity in patients treated with up to 200 mg orally to treat inborn errors of biotin metabolism (Mock, 1996) and 300 mg orally to treat multiple sclerosis (Tourbah et al., 2016). Sawamura et al. (2007) reported the no observed adverse effect level of biotin as 38.4 mg/kg/day after feeding rats a 20% casein diet with biotin added at 0.04, 0.08, 0.10, 0.20, 0.50, 0.80 or 1.0% for 28 days. However, some limitations of that study include short duration, did not include histopathology, and involved a small number of animals. Therefore, while the no observed adverse effect level of biotin has been reported, further studies are needed to confirm that dose level and to report on how that value varies when

Table 9
Terminal body weights and absolute and relative organ weights of male rats on Day 93 following a 13-week dietary treatment with MgB.[a].

	Group 1 Control	Group 2 120 mg/kg/day	Group 3 600 mg/kg/day	Group 4 2500 mg/kg/day
Absolute weights				
Terminal body weight (g)	477.6 ± 37.3	489.2 ± 52.4	490.4 ± 48.3	448.5 ± 31.4
Adrenals (g)	0.0633 ± 0 .0090	0.0630 ± 0.0128	0.0690 ± 0.0106	0.0595 ± 0.0071
Brain (g)	2.246 ± 0.107	2.286 ± 0.096	2.263 ± 0.123	2.158 ± 0.103
Epididymides (g)	1.6633 ± 0.2587	1.6285 ± 0.1528	1.5853 ± 0.1851	1.5231 ± 0.1546
Heart (g)	1.451 ± 0.127	1.427 ± 0.126	1.523 ± 0.1851	1.271 ± 0.081**
Kidneys (g)	3.177 ± 0.448	3.177 ± 0.448	3.240 ± 0.369	2.982 ± 0.235
Liver (g)	11.801 ± 1.602	11.801 ± 1.602	12.087 ± 1.541	11.203 ± 0.869
Spleen (g)	0.854 ± 0.144	0.854 ± 0.144	0.884 ± 0.132	0.819 ± 0.108
Testes (g)	3.793 ± 0.329	3.793 ± 0.329	3.734 ± 0.228	3.663 ± 0.363
Thymus (g)	0.2332 ± 0.0645	0.2332 ± 0.0645	0.2759 ± 0.0823	0.1948 ± 0.0780
Relative Organ-to-body weight ratios[b]				
Adrenals/BW	0.1335 ± 0.0235	0.1295 ± 0.0261	0.1406 ± 0.0149	0.1334 ± 0.0201
Brain/BW	4.730 ± 0.442	4.713 ± 0.446	4.651 ± 0.482	4.835 ± 0.422
Epididymides/BW	3.5243 ± 0.7990	3.3520 ± 0.3681	3.2747 ± 0.5918	3.4001 ± 0.3054
Heart/BW	3.042 ± 0.202	2.929 ± 0.205	3.107 ± 0.138	2.839 ± 0.165[d]
Kidneys/BW	6.636 ± 0.629	6.627 ± 0.719	6.611 ± 0.460	6.671 ± 0.605
Liver/BW	24.635 ± 1.977	24.962 ± 2.484	24.610 ± 1.440	25.014 ± 1.551
Spleen/BW	1.786 ± 0.256	1.842 ± 0.250	1.809 ± 0.237	1.830 ± 0.235
Testes/BW	8.010 ± 1.188	7.493 ± 0.884	7.700 ± 1.077	8.196 ± 0.950
Thymus/BW	0.4845 ± 0.1098	0.5542 ± 0.1070	0.5614 ± 0.1460	0.4271 ± 0.1435
Relative Organ-to-brain weight ratios[c]				
Adrenals/BrW	0.0283 ± 0.0044	0.0276 ± 0.0058	0.0305 ± 0.0041	0.0276 ± 0.0035
Epididymides/BrW	0.7442 ± 0.1343	0.7126 ± 0.0630	0.7018 ± 0.0888	0.7061 ± 0.0671
Heart/BrW	0.647 ± 0.063	0.625 ± 0.055	0.674 ± 0.066	0.590 ± 0.046
Kidneys/BrW	1.417 ± 0.205	1.411 ± 0.136	1.433 ± 0.153	1.383 ± 0.110
Liver/BrW	5.272 ± 0.817	5.354 ± 0.878	5.343 ± 0.629	5.207 ± 0.541
Spleen/BrW	0.382 ± 0.075	0.396 ± 0.077	0.391 ± 0.056	0.380 ± 0.048
Testes/BrW	1.694 ± 0.184	1.591 ± 0.139	1.652 ± 0.095	1.698 ± 0.150

BW, body weight; BrW, brain weight.
[a] All data are presented as mean values ± standard deviations with N = 10.
[b] Relative organ-to-body weight ratios are calculated as organ weight (mg)/body weight (g).
[c] Relative organ-to-brain weight ratios are calculated as organ weight (mg)/brain weight (mg).
[d] Means are significantly different from Group 1 controls.

testing different biotin salts.

MgB is a novel biotin salt that provides a highly soluble and bioavailable form of biotin. An *in vitro* assay comparing the water solubility of MgB to D-biotin showed that MgB is 40 times more soluble than D-biotin. Several preclinical studies have demonstrated that MgB has superior absorption, tissue absorption, and activity compared to D-biotin (Komorowski et al., 2018, 2019; Ojalvo et al., 2019). Although the safety profiles of the individual molecular components of MgB, magnesium and biotin, are already established, the toxicology for the compound had not been fully reported on thus far. Therefore, the objective of these studies was to conduct a comprehensive toxicologic evaluation of this novel biotin salt to determine its safety for humans at chronic doses.

The results of five toxicological studies on MgB showed no evidence of mutagenicity or genotoxicity and no MgB-related toxic effects when given up to 2500 mg/kg for 14 days and 600 mg/kg for 90 days. MgB administration at 2500 mg/kg for 90 days however did cause adverse outcomes which led to one early sacrifice and stress-induced responses. Macroscopic and microscopic evidence of gastric ulceration, and depletion of lymphoid and bone marrow tissue were observed. These data are consistent with anecdotal reports of gastric upset in humans taking high doses of biotin. Although the level of magnesium is low in the compound, magnesium at high doses can have laxative or other effects leading to gastric distress, and therefore it is possible that the direct gavage dosing administration of magnesium and biotin led to the gastric issues observed. Dosing with 2500 mg MgB/kg was also associated with post-administration stress-related thymic depletion during microscopic evaluation in both males and females. It is recognized that deficiencies of both biotin and magnesium increase involution of the thymus (Báez-Saldaña and Ortega, 2004; Malpuech-Brugère et al., 1999), suggesting a role for both nutrients in supporting thymus function. While the thymic depletion observed in the present study was not considered to be a direct effect of MgB, the possible fine balance between deficiency and excess of biotin and magnesium is an effect needing further examination.

A MgB-related increase in absolute and relative liver weights was also observed in females at 2500 mg/kg/day. Previous studies have found effects of biotin on liver weight. Feeding rats a biotin-containing diet decreased liver weights and increased biotin accumulation in the liver in a dose-dependent manner (Sawamura et al., 2007). Low liver biotin levels have also been associated with increased liver weight in broiler chicks (Pearce and Balnave, 1978). Moreover, Ojalvo et al. (2019) found dose-dependent increases in liver biotin levels when supplementing with 0.1, 1, and 100 mg MgB/kg with no effects on liver toxicity markers. It is evident however that at 2500 mg/kg, MgB may have adverse effects on the liver, further supporting the use of MgB at or below the maximum tolerated dose level of 600 mg/kg. Finally, dosing at 2500 mg MgB/kg also decreased heart weight, however these results were not associated with cardiac histological changes. A previous report by Sawamura et al. (2007) showed that supplementation of biotin to weanling male Wister rats caused a significant decrease in heart weight when the level of biotin in the diet reached 0.08%. This was also observed with a significant decrease in food intake and body weight gain over the course of the 28 day study. However, Sawamura et al. (2007) indicated that further studies were necessary since that study used a sample size of four rats per group and no mechanism for these observations were provided. There are studies indicating false negative results in cardiac troponin-T assay for acute myocardial infarction (IFCC, 2019; Mumma et al., 2020). However, Mumma et al. (2020) concluded that this risk is very small compared to the benefits of supplementing biotin in cases of deficiency. While further investigation should be carried out to determine the toxicological significance of these findings, the current

Table 10
Terminal body weights and absolute and relative organ weights of female rats on Day 93 following a 13-week dietary treatment with MgB.[a].

	Group 1 Control	Group 2 120 mg/kg/day	Group 3 600 mg/kg/day	Group 4 2500 mg/kg/day
Absolute Weights				
Terminal body weight (g)	312.7 ± 29.1	314.5 ± 23.8	316.1 ± 15.0	310.3 ± 22.3
Adrenals (g)	0.0797 ± 0.0135	0.0696 ± 0.0133	0.0759 ± 0.0139	0.0792 ± 0.0140
Brain (g)	1.998 ± 0.064	2.055 ± 0.071	2.025 ± 0.093	1.957 ± 0.107
Heart (g)	1.021 ± 0.102	1.095 ± 0.073	1.031 ± 0.084	1.080 ± 0.135
Kidneys (g)	2.066 ± 0.311	2.112 ± 0.207	2.098 ± 0.121	2.247 ± 0.368
Liver (g)	8.148 ± 1.321	8.459 ± 0.713	8.309 ± 0.588	9.654 ± 1.905[d]
Ovaries w/ Oviducts (g)	0.1243 ± 0.0104	0.1218 ± 0.0326	0.1277 ± 0.0115	0.1149 ± 0.0098
Spleen (g)	0.617 ± 0.082	0.605 ± 0.093	0.638 ± 0.055	0.597 ± 0.048
Thymus (g)	0.269 ± 0.0512	0.2878 ± 0.0441	0.2747 ± 0.0448	0.1991 ± 0.0652
Uterus (g)	0.797 ± 0.264	0.918 ± 0.396	0.707 ± 0.192	0.641 ± 0.195
Relative Organ-to-body weight ratios[b]				
Adrenals/BW	0.2557 ± 0.0442	0.2202 ± 0.0312	0.2411 ± 0.0491	0.2542 ± 0.0321
Brain/BW	6.423 ± 0.420	6.398 ± 0.372	6.417 ± 0.377	6.322 ± 0.399
Heart/BW	3.268 ± 0.187	3.500 ± 0.356	3.264 ± 0.251	3.478 ± 0.309
Kidneys/BW	6.587 ± 0.484	6.723 ± 0.552	6.651 ± 0.509	7.210 ± 0.826
Liver/BW	25.958 ± 2.063	26.947 ± 1.988	26.310 ± 1.824	30.979 ± 4.518[d]
Ovaries w/ Oviducts/BW	0.4011 ± 0.0540	0.3850 ± 0.0891	0.4056 ± 0.0490	0.3720 ± 0.0403
Spleen/BW	1.989 ± 0.339	1.925 ± 0.259	2.022 ± 0.195	1.930 ± 0.200
Thymus/BW	0.6891 ± 0.1979	0.9174 ± 0.1380	0.8725 ± 0.1590	0.6444 ± 0.2810[d]
Uterus/BW	2.569 ± 0.908	2.892 ± 1.133	2.240 ± 0.614	2.074 ± 0.674
Relative Organ-to-brain weight ratios[c]				
Adrenals/BrW	0.0398 ± 0.0064	0.0346 ± 0.0057	0.0377 ± 0.0079	0.0405 ± 0.0067
Heart/BrW	0.511 ± 0.042	0.546 ± 0.034	0.511 ± 0.053	0.552 ± 0.065
Kidneys/BrW	1.032 ± 0.129	1.053 ± 0.092	1.037 ± 0.064	1.148 ± 0.177
Liver/BrW	4.070 ± 0.568	4.215 ± 0.253	4.111 ± 0.354	4.933 ± 0.912[d]
Ovaries w/ Oviducts/BrW	0.0623 ± 0.0059	0.0607 ± 0.0154	0.0632 ± 0.0067	0.0587 ± 0.0040
Spleen/BrW	0.309 ± 0.044	0.301 ± 0.042	0.315 ± 0.025	0.306 ± 0.030
Thymus/BrW	0.1348 ± 0.0266	0.1436 ± 0.0212	0.1359 ± 0.0226	0.1012 ± 0.0302[d]
Uterus/BrW	0.399 ± 0.133	0.456 ± 0.188	0.349 ± 0.091	0.329 ± 0.105

BW, body weight; BrW, brain weight.
[a] All data are presented as mean values ± standard deviations with N = 10.
[b] Relative organ-to-body weight ratios are calculated as organ weight (mg)/body weight (g).
[c] Relative organ-to-brain weight ratios are calculated as organ weight (mg)/brain weight (mg).
[d] Means are significantly different from Group 1 controls.

data suggest that supplementation with MgB at higher doses (2500 mg/kg) may alter liver, thymic, and gastric function and therefore lower levels (600 mg/kg or below) should be used chronically to avoid potential toxicity issues.

The safety of magnesium biotinate can be established based on the results of the toxicity data described in this report. Moreover, the safety of MgB is supported by other preclinical efficacy studies and the known safety of its components. The maximum tolerated dose level of 600 mg MgB/kg identified in this study is above typical, recommended intake. However, the lack of toxicity found in these studies and the fact that no upper intake level of biotin has been established based on the absence of toxic effects in other animal and clinical studies supports its use at that dosage.

5. Conclusion

In conclusion, the studies we present here constitute a comprehensive toxicologic evaluation of MgB, a novel biotin salt shown to have superior solubility and activity compared to D-biotin. MgB demonstrated no evidence of mutagenicity, clastogenicity, or aneugenicity in two *in vitro* genotoxicity studies. An acute oral toxicity study established a $LD_{50} > 5000$ mg/kg of body weight. No treatment-related mortality or clinical signs resulted from a 14-day repeat dose oral toxicity study using daily doses of 120, 600, and 2500 mg MgB/kg/day. In a 90-day subchronic oral toxicity study, MgB in daily doses up to 600 mg/kg were well-tolerated and did not cause ophthalmic or clinical signs in either male or female rats. Therefore, the NOAEL derived from the 90-day subchronic oral toxicity study was 600 mg/kg of MgB, equating to 39 g for an average 70 kg human. Since MgB is composed of 93% biotin, this NOAEL is about 1,300,000 times the daily value dose of 30 μg/day for biotin and 3900 times the 10 mg dose found in some higher dose dietary supplements. Based on the toxicologic profile and safety of various *in vitro* and *in vivo* studies, MgB may be considered safe for humans for long-term clinical use.

CRediT authorship contribution statement

Jason L. Blum: Conceptualization, Methodology, Investigation, Validation, Formal analysis, Resources, Data curation, Visualization, Supervision, Project administration, Writing – review & editing. **Melissa Ellis:** Conceptualization, Methodology, Investigation, Validation, Formal analysis, Resources, Data curation, Visualization, Supervision, Project administration, Writing – review & editing. **Jayson X. Chen:** Conceptualization, Methodology, Investigation, Validation, Formal analysis, Resources, Data curation, Visualization, Supervision, Project administration, Writing – review & editing. **Odete Mendes:** Conceptualization, Methodology, Investigation, Validation, Formal analysis, Resources, Data curation, Visualization, Supervision, Project administration, Writing – review & editing. **Sarah Sylla:** Conceptualization, Writing – original draft, Writing – review & editing, Visualization, Supervision. **Sara Perez Ojalvo:** Conceptualization, Writing – original draft, Writing – review & editing, Visualization, Supervision. **James Komorowski:** Conceptualization, Writing – original draft, Writing – review & editing, Visualization, Supervision.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: This research was financially supported by JDS Therapeutics, LLC, Harrison, NY, United States. James Komorowski, Sara Perez Ojalvo, and Sarah Sylla are all employed by JDS Therapeutics, LLC. All other authors declare no conflict of interest.

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