A Toxicological Assessment of Creatyl-L-Leucine

Abstract/Poster ID 1621/P861

Robin Reddeman,[1] Róbert Glávitis,[2] John R. Endres,[1] Timothy S. Murbach,[1] Gábor Hirka,[2] Adél Vértesi,[2] Erzsébet Béres,[2] and Ilona Pasics Szakonyiné[2]

[1]AIBMR Life Sciences, Inc., Seattle WA, USA; [2]Toxi-Coop Zrt., Budapest, Hungary

Abstract

Creatyl-L-leucine (CLL) is a synthetic derivative of creatine and L-leucine joined by an amide bond between the carboxyl group of creatine and the amino group of L-leucine for the purpose of protecting creatine from spontaneous degradation in solution. Because of interest in this novel compound for use in beverages, we investigated its toxicological potential, according to internationally accepted guidelines in [1] a bacterial reverse mutation test, [2] an in vitro mammalian chromosomal aberration test, [3] an in vivo mammalian micronucleus test, and [4] a 90-day repeated-dose oral toxicity study in rats. No evidence of mutagenicity, up to the recommended concentrations in the in vitro tests, or genotoxic activity, up to the limit dose in the in vivo study, was observed. No mortality, general toxicity, or toxic effects on organs or tissues was observed in Hsd.Han Wistar rats in the 90-day gavage study at doses of 1250, 2500, and 5000 mg/kg bw/d, and a NOAEL was determined as the highest dose level.

Introduction

- The studies were recently published in *the International Journal of Toxicology*.[1]
- Creatine and leucine occur naturally in foods such as meats, seafood and dairy products with leucine also being found in some grains, soybeans, nuts, beans, seaweed and sesame.[2,3]
- Creatine and leucine are not known to naturally form bonds with one another endogenously in humans or in nature.
- CLL is synthesized by subjecting L-leucine to a series of chemical processes, resulting in creatine and leucine connected by an amide bond, thus, creating a novel compound.

Bacterial Reverse Mutation Test

S. typhimurium strains: TA98, TA100, TA1535, and TA1537 and *E. coli* strain: WP2 *uvrA*.

OECD (471), EC (440/2008 B13/14), EPA (OPPTS 870.5100), ICH (S2(R1)) and GLP compliant.

- No concentration-related or biologically relevant increases in revertant colonies were observed in any tester strain up to the highest level tested with or without metabolic activation (5000 µg/plate).

In Vitro Mammalian Chromosomal Aberration Test

Cells: V79 (Chinese hamster lung) cells.

OECD (473), EPA (OPPTS 870.5375) and GLP compliant.

- No aberrations, abnormal metaphases, or dose-responses occurred up to the highest level tested (2000 µg/plate, with and without metabolic activation).

Test Article, creatyl-L-leucine

- Soluble in water
- Test article provided by the sponsor of the studies, VPX Sports Nutrition.
- Purity of >99%
- Formulated in water for the bacterial reverse mutation test, mouse micronucleus test, and 90-day toxicity study, and in Dulbecco's modified Eagle's medium for the chromosomal aberration test.

In Vivo Mammalian Erythrocyte Micronucleus Test

Male SPF Crl:NMRI BR mice.

OECD (474), EPA (OPPTS 870.5395) and GLP compliant.

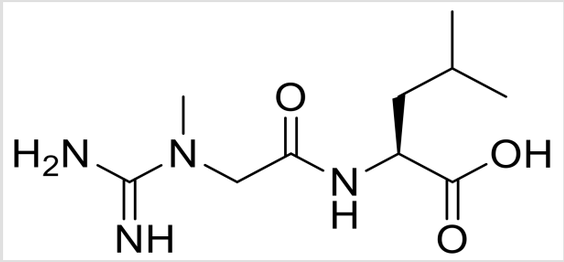
Performed with permission of the laboratory's Institutional Animal Care and Use committee, following principles and guidelines for Care and Use of Laboratory Animals.

- No statistically significant increases in the frequency of micronucleated polychromatic erythrocytes compared to control were detected up to the highest level tested (limit dose of 2000 mg/kg bw).

Ninety-Day Repeated Oral Toxicity Study

SPF Hsd.Han Wistar rats (38-46 days old), OECD (408) and GLP compliant. Performed with permission of the laboratory's Institutional Animal Care and Use committee, following principles and guidelines for Care and Use of Laboratory Animals.

- Ten animals per sex per group were utilized. Doses were chosen based on an unpublished OECD (407)-compliant 14-d repeated-dose oral toxicity study in Hsd.Han Wistar rats. A maximum tolerated dose could not be established; the NOAEL was estimated as the high dose of 5000 mg/kg bw/d. Thus, doses for the 90-day study were 0 (vehicle control), 1250, 2500 and 5000 mg/kg bw/d.
- Gavage dosing of the test article (at a dose volume of 20 mL/kg, prepared not longer than three days before administration) was performed daily for 90 consecutive days. The recovery and stability of the test article solution were analytically verified up front. Concentration and homogeneity of the dosing solutions were tested three times (weeks 1, 8, and 13) during the study.
- Evaluated: Mortality, behavior and clinical observations, body weight, food consumption, feed efficiency, ophthalmologic examination, functional observation battery, hematological and clinical chemistry analysis, gross and histopathology and organ weights (absolute and relative).
- Outcomes:
  - **Mortality:** There were no mortalities in the control, 1250, 2500, or 5000 mg/kg bw/d groups during the 90-day test period.
  - **Cage-side observations:** Slightly soft stool was observed in the intermediate-and high-dose groups which was likely caused by the relatively large concentration of the test article; the change was not considered toxicologically relevant due to the absence of related changes in body weight, food consumption or biochemical parameters in those test groups
  - **Weight/weight gain**: There were no toxicologically relevant differences in body weight, weight gain, food consumption, or feed efficiency in any of the test groups compared to controls.
  - **Blood/serum**: Slight sporadic differences in several laboratory parameters were statistically significantly different compared to controls; however, they were not considered toxicologically relevant. Findings were as follows:
    - Decreased % basophils (male low- and high-dose groups), effect not dose related, not consistent across sexes, were within historical control ranges and were without corresponding histopathological findings.
    - Decreased hemoglobin and hematocrit (females high-dose group) effect not consistent across sexes, results were within historical control ranges and were without corresponding histopathological findings.
    - Decreased platelet counts (males and females high-dose groups) were within historical control ranges and were without corresponding histopathological findings.
    - Decreased activated partial thromboplastin time (females high-dose) effect not consistent across sexes, results were within historical control ranges and were without corresponding histopathological findings.
    - Increased creatinine (females intermediate- and high-dose groups) effect not consistent across sexes, results were within historical control ranges and were without corresponding histopathological findings.
    - Increased urea (females high-dose group) effect not consistent across sexes, results were within historical control ranges and were without corresponding histopathological findings.
    - Increased glucose (males intermediate-dose group), effect not dose related, not consistent across sexes, results were within historical control ranges and were without corresponding histopathological findings.
    - Increased cholesterol (females high-dose group), effect not consistent across sexes, results were within historical control ranges and were without corresponding histopathological findings.
    - Increased calcium ion (males high-dose group and females low- and high-dose groups), results were within historical control ranges and were without corresponding histopathological findings.
    - Decreased sodium ion (all male test groups) and increased sodium (females low-dose group) effect not dose related, not consistent across sexes, were within historical control ranges and were without corresponding histopathological findings.
    - Decreased chloride ion (males high-dose group), effect not consistent across sexes, results were within historical control ranges and were without corresponding histopathological findings.
    - Increased total protein (males intermediate-group) effect not dose related, not consistent across sexes, results were within historical control ranges and were without corresponding histopathological findings.
  - **Organ gross/weights:** No test-article related gross pathological lesions or organ weight differences were observed.
  - **Histopathology:** No test article-related findings, were found during histopathological examinations. Findings not considered toxicologically relevant included:
    - Slight alveolar emphysema (two male and one female control, one high-dose male), alveolar histiocytosis (one control female), and hemorrhage of the thymus (four male controls, two high-dose males, two mid-dose females) are considered consequences of exsanguination.
    - Smaller than normal thymus glands were observed in two mid-dose males and the changes were consistent with accelerated involution of the thymus which is common in this age of experimental rats.
    - Pyelectasia was observed in a single control male and one-sided lymphocytic pyelitis was observed in a single high-dose female; thus, they were considered individual findings unrelated to test article administration.
    - Hyperplasia of the bronchus-associated lymphoid tissue is considered an immunomorphological phenomenon and is not considered to have toxicological significance.
    - Dilation of the uterine horns in five controls and two high-dose females is considered a a common neurohormonal phenomenon in connection with the proestrus phase of the sexual cycle.
  - **Conclusion:** The NOAEL for the 90-day repeated-dose oral toxicity study on Hsd.Han:Wistar rats is considered to be the highest dose tested, 5000 mg/kg bw/d.



Chemical Structure of Creatyl-L-Leucine

Conclusions

- No evidence of in vitro mutagenicity or clastogenicity or in vivo genotoxicity was detected in the bacterial reverse mutation, in vitro mammalian chromosomal aberration and in vivo mouse micronucleus tests.
- No target organs or treatment-related toxicological effects were identified. The NOAEL of the 90-day repeated dose oral toxicity study was 5000 mg/kg bw/d—the highest dose tested.

References: (1) Reddeman R, Glávits R, Endres J, et al. A Toxicological Assessment of Creatyl-L-Leucine. *Int J Toxicol*. 2018:1091581817751142. **(2)**USDA, ARS. USDA food composition databases. Nutrient lists. Leucine. 2016. **https://ndb.nal.usda.gov/ndb/foods.** . **(3)** Jager R, Purpura M, et al. Analysis of the efficacy, safety, and regulatory status of novel forms of creatine. Amino Acids. 2011;40(5):1369-1383.