

Introduction

Phenylketonuria (PKU): Inherited metabolic disorder primarily resulting from mutations in the phenylalanine hydroxylase gene (which encodes the enzyme responsible for the catabolism of phenylalanine, Phe).

Primary therapy: Dietary restriction of protein intake

Complementary treatments approved: Efficacy only in a limited subset of patients or frequently associated with immune-mediated adverse effects and allergic reactions. Survey data indicates that more than 75% of PKU patients in both the US and China are **untreated**.

Multiple investigational pharmacological agents are currently under development. **Genetically modified bacteria** have exhibited promising therapeutic efficacy in clinical investigations. **Orally administered engineered bacteria** capable of metabolizing Phe represent an optimal therapeutic modality for PKU, owing to the physiological process of enterorecirculation of Phe between the bloodstream and the small intestine.

We genetically engineered the probiotic *E. coli* Nissle 1917 (EcN) to include genes facilitating Phe degradation, **CBT102**, which demonstrated efficient Phe degradation under both aerobic and hypoxic conditions, as well as in PKU mouse models. CBT102 was nearly undetectable in fecal samples by 48 hours.

The purpose of this study is to identify and characterize potential toxicities of the genetically modified bacterial product CBT102, thereby supporting safety monitoring during clinical trials.

Methods

- Sprague-Dawley (SD) rats and Beagle dogs were selected for non-GLP toxicology studies.
- The designs included single-dose maximum tolerated dose (MTD) studies and 14-day dose range finding (DRF) studies to determine the potential toxicity in each species.
- Toxicity evaluations included mortality, clinical observations, body weights, food consumption, ophthalmology, clinical pathology, cytokines, organ weights, macroscopic and microscopic pathology. *In vivo* micronucleus assays, *in vivo* comet assays and biodistributions were also combined in the rat DRF study.

Conclusion

- Overall, the genetically modified bacterial product CBT102 was well tolerated in these studies.
- There was no evidence of genotoxic potential, and CBT102 does not meaningfully enter systemic circulation consistent with a safe, GI-restricted biodistribution.
- As no adverse findings were identified, the no observed adverse effect level (NOAEL) was established at the high dose level in each DRF study.
- These findings support further development of CBT102 and underscore its potential as a promising treatment for PKU.

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In the rat MTD study, up to 2.63×10^{12} live cells/kg/day was well tolerated. Abnormal findings were limited to brown staining around the anal area and a slight elevation in alkaline phosphatase.

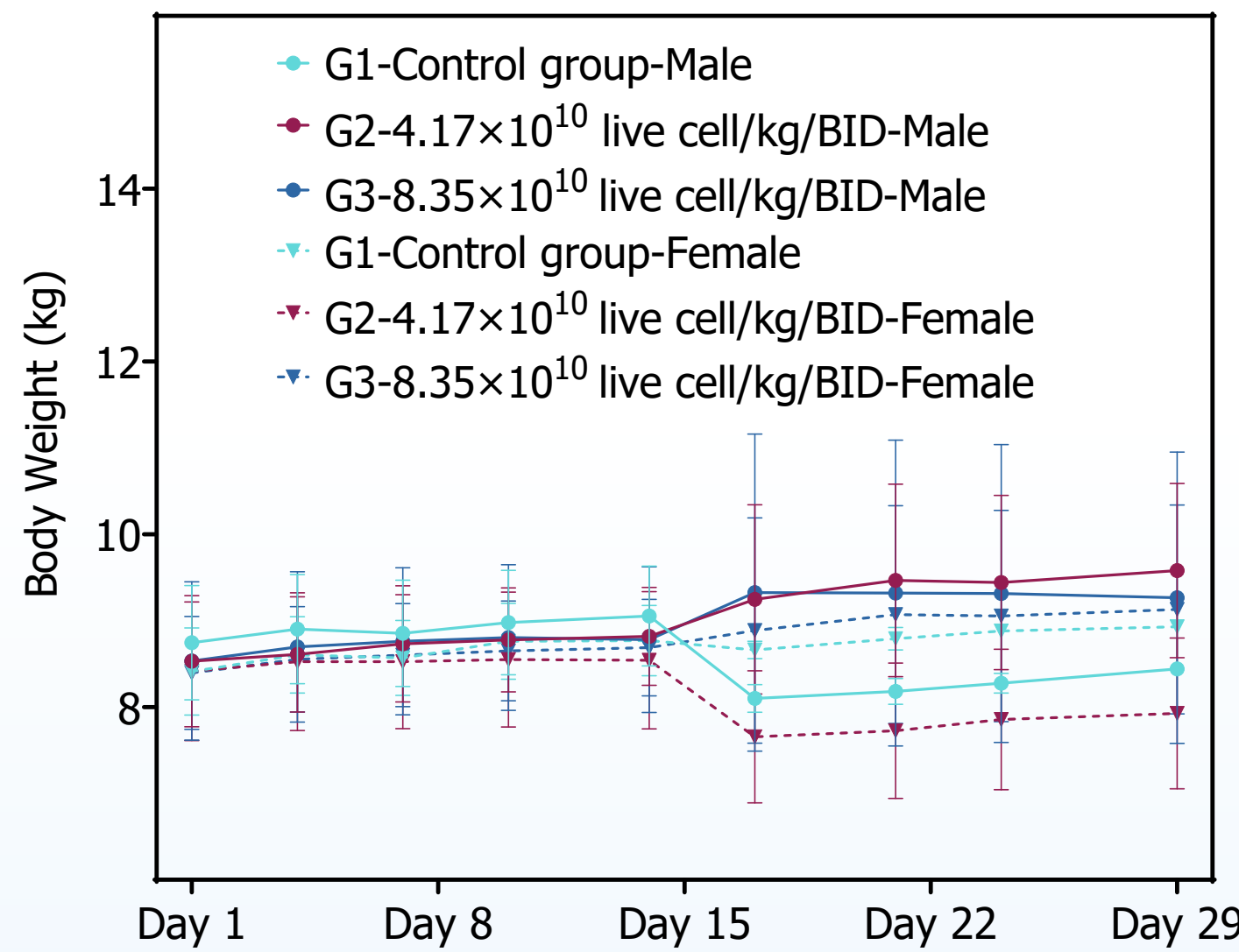
Clinical Observation-Male		Finding (No.)		
Dose Level (live cell/kg/day)		5.00×10 ¹¹	1.75×10 ¹²	2.63×10 ¹²
Day 2	anal stain (brown)	—	(1)	—
Clinical Chemistry-Male		Finding		
Dose Level (live cell/kg/day)		5.00×10 ¹¹	1.75×10 ¹²	2.63×10 ¹²
TS on Day 3		alkaline phosphatase	—	↑ 23.2% ↑ 38.98%

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In the dog MTD study, up to 4.17×10^{10} live cells/kg (BID) was well tolerated. Yellow ocular discharge was observed in CBT102-treated animals after dosing, and a relationship to the test article could not be excluded.

Detailed Clinical Observations From Day 1 to Day 7	Male		Female	
	G1	G2	G1	G2
Ocular discharge				
Days Observation Recorded	0	3	0	1
Animals Affected	0	1	0	1
Days First to Last Seen	—	2 - 4	—	2 - 2

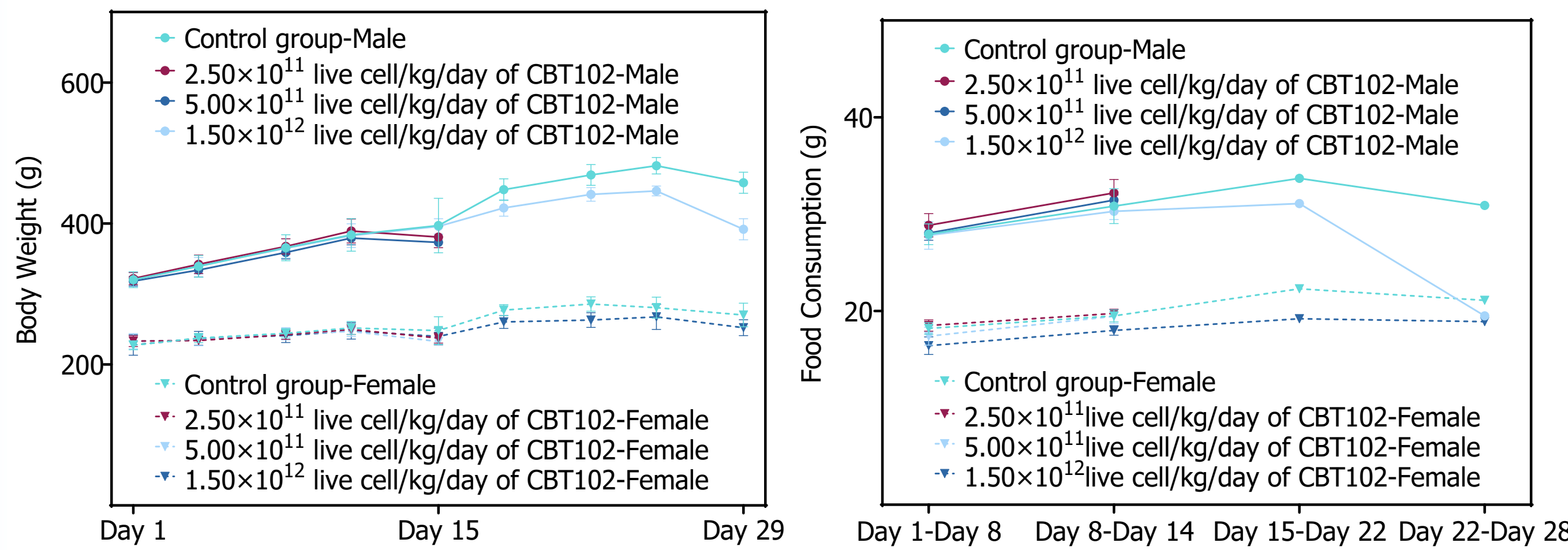
In the dog DRF study, up to 8.35×10^{10} live cells/kg (BID) was well tolerated. Abnormal findings were limited to soft stools across all CBT102-dosed groups.



Cageside Observations From Day 1 to Day 29	Male			Female		
	G1	G2	G3	G1	G2	G3
Soft stool						
Days Observation Recorded	4	9	11	3	10	4
Animals Affected	3	4	3	2	4	2
Days First to Last Seen	2 - 13	4 - 14	6 - 23	3 - 4	4 - 26	6 - 5

3

In the rat DRF study, up to 1.50×10^{12} live cells/kg/day was well tolerated. During the dosing period, abnormal findings included soft stool, anal staining (brown), and loose feces across dose groups of male animals.



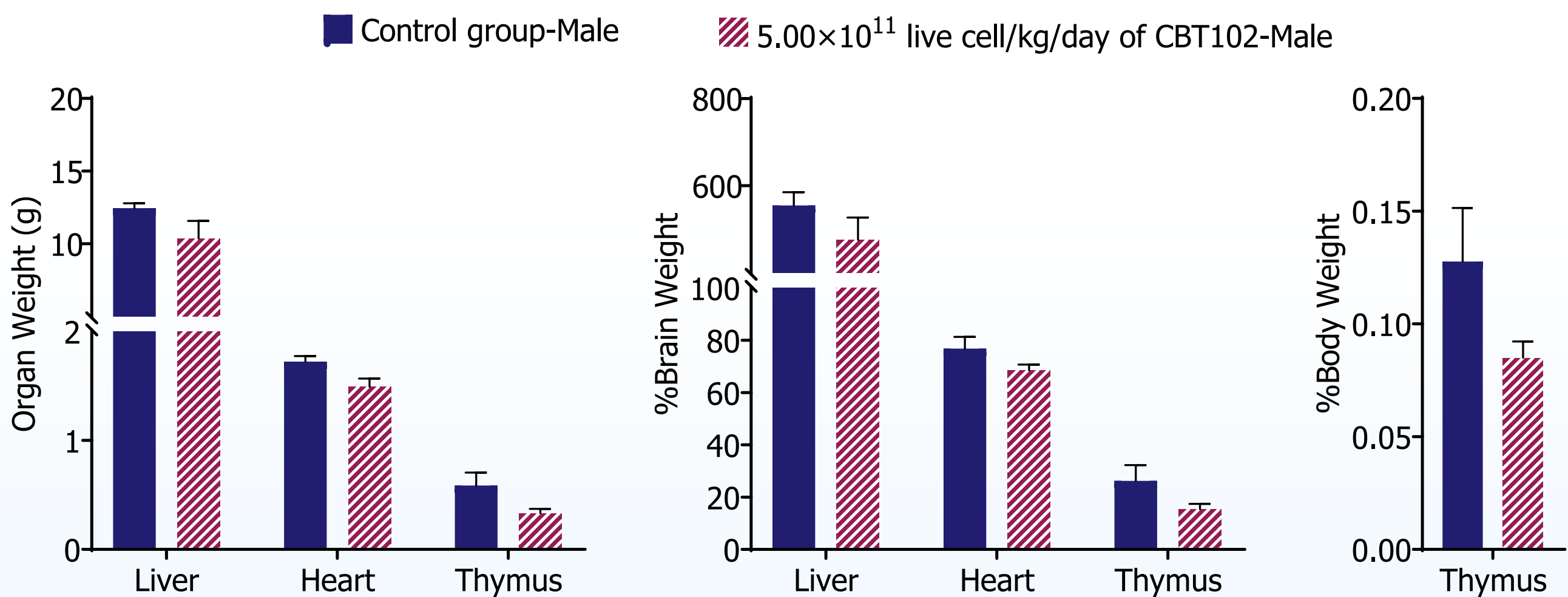
No abnormal **Body Weight** was noted in the dosing phase. During the recovery period, decrease in body weights of male animals and significant decrease in **Food Consumption** of all animals were noted in the 1.50×10^{12} live cells/kg/day group.

Clinical Chemistry-Male	Dose Level (live cell/kg/day)	Finding (No.)		
		2.50 ×10 ¹¹	5.00 ×10 ¹¹	1.50 ×10 ¹²
TS on Day 15	Total Protein	—	↓ (2)	—
	Globulin	—	↓ (2)	—
	IgG	—	↓ (2)	↓ (1)
	IgM	—	↓ (2)	↓ (3)
	C ₄	—	—	↑ (3)
RS on Day 29	Blood Urine Nitrogen	—	—	↑ (1)
	Creatine	—	—	↑ (2)
	Cholesterol Total	—	—	↓ (1)
	Triglyceride	—	—	↓ (2)
	Na	—	—	↓ (1)
	Cl	—	—	↓ (2)
	C ₄	—	—	↑ (1)

Hematology-Male		Finding (No.)		
Dose Level (live cell/kg/day)		2.50 ×10 ¹¹	5.00 ×10 ¹¹	1.50 ×10 ¹²
TS on Day 15	—	—	—	—
	WBC	—	—	↑ (2)
RS on Day 29	ABLYPM	—	—	↑ (2)
	ABRETTIC	—	—	↓ (2)

Hematology results showed a decrease in ABRETIC and increases in WBC and ABLYPM in the 1.50×10^{12} live cell/kg/day group of male animals.

Clinical chemistry revealed decreases in total protein, globulin, IgG, and IgM in the 5.00×10^{11} live cell/kg/day group of male animals, decreases in CHO, TG, Na, Cl, IgG and IgM along with increases in BU, CRE and C₄ in 1.50×10^{12} live cell/kg/day group of male animals.



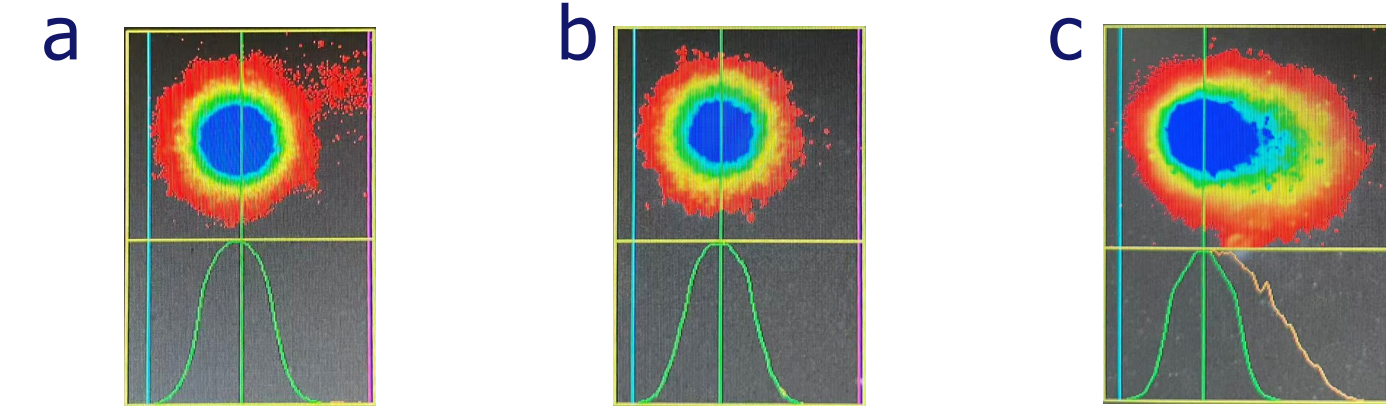
Organ Weight analysis revealed decreases in the absolute weights and the organ-to-brain weight ratios of liver and heart, and decreases in the absolute weights, the organ-to-body weight ratios and the organ-to-brain weight ratios of thymus in 1.50×10^{12} live cell/kg/day group of male animals. However, these organ weight changes lacked histopathological correlation and were considered secondary to reduced body weights.

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CBT102 showed no genotoxicity or cytotoxicity effects.

In the *in vivo* **Micronucleus Assay** of the rat DRF study, there were no statistically significant differences observed in the proportion of polychromatic erythrocytes (PCEs) among total erythrocytes across all dose levels in both genders when compared to the excipient control group, suggested that CBT102 did not induce bone marrow cytotoxicity.

In the *in vivo* **Comet Assays** of the rat DRF study, results of liver, stomach, and jejunum tissues showed that CBT102 did not induce DNA damages and genotoxic effects in the examined tissues.



Representative results of Comet Assays in the liver. a, Vehicle group; b, CBT102-dosed group; c, Positive control.

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The genomic DNA (gDNA) copy number of CBT102 was detected by a specific real-time absolute quantitative PCR assay. The results indicated **no systemic exposure** and only trace tissue presence of CBT102.

Times Points		Samples
SD Rat DRF Study	■ 1h, 4h, 24h and 48 hours post-dose on Day 14	■ Blood ■ Heart, Liver, Spleen, Lung, Kidney, Brain
	■ Day 29 (RS)	
Beagle Dog DRF Study	■ 24h and 48 hours post-dose on Day 14	
	■ Day 29 (RS)	

No gDNA was detected in blood samples in rat DRF study and in all male animal blood samples from dog DRF study, indicating rapid clearance or degradation.

For the biodistribution, the gDNA was also analyzed in the six organs in the DRF studies. The proportion of the administered dose detected was minimal in all dose levels, with the highest percentage being only 6.4‰ in one sample from the rat DRF study and 0.1‰ in one sample from the dog DRF study, respectively.

The distribution of gDNA in organs showed no notable differences between genders.