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A new antiallergic agent that binds to dimerized translationally controlled tumor protein and inhibits allergic symptoms is nontoxic

M Kim¹, YB Jin², K Lee¹ and YS Lee¹

Abstract

Dimerized translationally controlled tumor protein (dTCTP) plays a role in allergic diseases. A 7-mer peptide, dimerized translationally binding protein 2 (dTBP2), binds to dTCTP and inhibits dTCTP, suggesting that the 7-mer peptide may have therapeutic potential. We assessed the safety of dTBP2 by examining its cytotoxicity to both human bronchial epithelial cells and mice. dTBP2 did not cause cytotoxicity to the epithelial cells in concentrations up to 100 µg/ml. Also, dTBP2 caused no adverse effects upon repeated administration of 50 mg/kg over 24 h to mice. Hence, we conclude that dTBP2 is a safe candidate drug for use in the therapy of allergic diseases.

Keywords

Dimerized TCTP, dTBP2, cytotoxicity, repeated-dose toxicity, allergy, safety

Introduction

Translationally controlled tumor protein (TCTP), also known as immunoglobulin E (IgE)-dependent histamine-releasing factor (HRF) or fortilin, is a house-keeping protein^{1,2} found in culture supernatants of some cell lines,³ sera from atopic/asthmatic patients and bronchoalveolar lavage fluids from mice with airway inflammation.⁴ TCTP exhibits diverse functions by interacting with proteins such as tubulin,⁵ polo-like kinase,⁶ Na, K-ATPase,⁷ and Bcl-xL⁸ or with ions such as Ca²⁺.⁹ TCTP induces histamine and IL-4 secretion from basophils,¹⁰ causes chemotaxis of eosinophils, augments IL-8 production from eosinophils,¹¹ stimulates the secretion of IL-8 and granulocyte-macrophage colony-stimulating factor in human bronchial epithelial cells,¹² and increases the production of IL-4 in T-helper 2 (T_H2) cells.⁴ TCTP, therefore, seems to influence allergic disease through a variety of effects on the inflammatory cells.

In a previous study, we showed that the effects of TCTP are seen only when TCTP is in a dimerized form (dTCTP) but not when in its monomeric form.⁴ We identified a 7-mer peptide, called dTBP2, which binds to dTCTP and inhibits the cytokine-like effect of TCTP.¹³ dTBP2 exerted this inhibitory effect by reducing the binding of dTCTP to the cell surface

in BEAS-2B cells. Also, dTBP2 significantly decreased the allergic symptoms (frequency of sneeze and nasal rub, and the infiltration of eosinophils into the nasal mucosa) that result when ovalbumin (OVA)-sensitized mice are challenged with OVA, suggesting the potential of dTBP2 to be useful in the therapy of some allergic diseases.

The use of peptides as drugs has several advantages over the use of recombinant proteins and antibodies. They are less immunogenic, more stable, can be synthesized more cheaply, and are not expressed.^{14,15} Their metabolites are amino acids that are normally present in the body constituents and are therefore safer than more complex chemicals. Nevertheless, usage of these peptides as drugs has been limited by their low oral bioavailability, rapid degradation in blood plasma, and relatively

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high costs. This limitation of peptides as potential drug candidates has been somewhat mitigated by the development of new technologies for their production, alternative routes for their administration, and new strategies to increase their plasma residence times.¹⁶

Because dTBP2 seemed to have the potential to serve as a prodrug in the treatment of allergic inflammation, we evaluated its toxicity to both human bronchial epithelial cell line (BEAS-2B) and mice.

Materials and methods

dTBP2, used in this study, was synthesized and purified to >98% homogeneity (A & PEP Inc., Yeongi, Korea). The purity and identity of the synthetic peptide were confirmed by high-performance liquid chromatography and mass spectroscopy, respectively.

Assessment of toxicity

- (a) In human bronchial epithelial cells, BEAS-2B cells were maintained in bronchial epithelial growth medium (BEGM, Lonza, Maryland, USA). When the cells became 80% confluent, they were passaged in a 96-well culture plate and incubated with various concentrations of dTBP2 (0, 1, 10, 100, and 500 $\mu\text{g/ml}$) in 1% penicillin/streptomycin/BEBM (Lonza). After 20 h, cell counting kit-8 solution (Dojindo Laboratories, Kumamoto, Japan) was added to each well and incubated for 1 h. The absorbance at 450 nm of the resulting supernatant was measured using microplate reader (VersaMax, Molecular Devices, Sunnyvale, CA, USA).
- (b) Twenty-four-day repeated-dose toxicity study was conducted in ICR mice (20 males and 20 females; Orient Bio Inc., Seongnam, Korea), aged 6 weeks and weighing 19–24 g, after 1 week of adaptation. The animals were maintained at a controlled temperature ($22 \pm 2^\circ\text{C}$) and humidity ($50 \pm 10\%$) under a time-controlled system with a 12-h light/dark cycle. Standard diet (Cargill Agri Purina, Seongnam, Korea) and water were supplied ad libitum. Studies were conducted according to the Guidelines for the Use and Care of Laboratory Animals, approved by the Institutional Animal Care and Use Committee of the Korea Institute of Radiological and Medical Sciences (KIRAMS).

After acclimation to the animal room, the mice were assigned randomly to four treatment groups receiving, respectively, 5, 25, and 50 mg/kg of dTBP2 in

phosphate-buffered saline (PBS) or PBS alone (negative control group, NC), intraperitoneally (i.p.) (0.5 ml/mouse) every even numbered day for 24 days. All animals were examined for clinical signs twice a day. The body weights were measured prior to the start of study (D0) and then immediately prior to dTBP2 injection on every other day to the end of the study (D24). Hematology parameters were measured using a Hemavet 950 Multispecies Hematology Analyzer (DREW Scientific Inc., Connecticut, USA). These included white blood cells (WBCs), neutrophils, lymphocytes, monocytes, eosinophils, basophils, and red blood cells (RBCs). Serum biochemical analyses including triglyceride, total protein, total cholesterol, total bilirubin (TBIL), albumin, creatinine, glucose, blood urea nitrogen, aspartate aminotransferase (AST), alanine aminotransferase (ALT), and alkaline phosphatase (ALP) were performed using DRI-CHEM 4000 (FUJIFILM Co., Tokyo, Japan). During autopsy, brains, hearts, lungs, livers, kidneys, spleens, stomachs, and intestines were grossly evaluated, organ weights measured, and fixed in 10% neutral-buffered formalin. Livers were sectioned (thickness of 3–5 μm) and stained with hematoxylin and eosin for microscopy.

Data analysis

Data are presented as the mean $\pm$ standard deviation. Statistical comparisons were conducted using the statistics software, SPSS, version 14.0 (SPSS Inc., Illinois, USA). In case of body weight, the one-factor (time) analysis of variance (ANOVA) with post hoc testing was used for determining group differences. The residuals were analyzed for homogeneity of variance and normality of distributions. Statistical comparisons were made using the Student's *t* test (independent group) and Dunnett's test. *P* values of 0.05 or less were considered statistically significant.

Results and Discussion

Assessment of cytotoxicity

We first examined the cytotoxicity of dTBP2 to BEAS-2B cells. In a previous study, we showed 7.5 nM or 75 nM of dTBP2 inhibited the function of equimolar dTCTP in these cells.¹³ Also, 0.5 μM of dTBP2 could reduce binding of 5 μM of dTCTP to the cell surface in Jurkat T cells. In the present study, various concentrations of dTBP2 (0, 1, 10, 100, and 500 $\mu\text{g/ml}$) were tested for cytotoxicity (Figure 1). The viability of BEAS-2B cells was decreased to

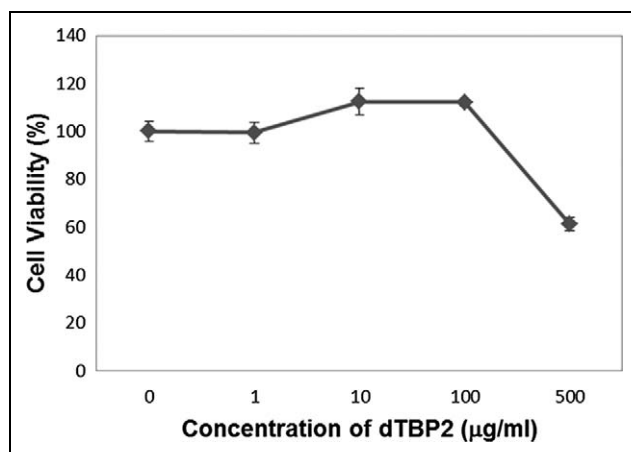


Figure 1. Cytotoxicity of dTBP2 in BEAS-2B cells. BEAS-2B cells were seeded in a 96-well culture plate and cultured overnight. Next day, the cells were treated with indicated concentrations of dTBP2 for 20 h, followed by incubation with cell counting kit-8 solution for 1 h. The resulting supernatants were measured at 450 nm using a microplate reader. Viability of the cells untreated with dTBP2 was calculated as 100%. dTBP2: dimerized translationally-binding protein2.

60% of NC by 500 µg/ml (529 µM) of dTBP2. At concentrations of 1, 10, and 100 µg/ml, dTBP2 showed no cytotoxicity. We conclude that dTBP2 is safe at about 200- to 14,000-fold of effective concentration at least in BEAS-2B cells.

Schoonen et al. tested the cytotoxicity of toxic reference compounds and pharmaceutical compounds in four different cell lines, human liver (HepG2), human cervix (HeLa), human endometrium (ECC-1) and Chinese hamster ovary cells (CHO).¹⁷ They found that the selected compounds have similar cytotoxicity on the tested cell lines, except only 3 of the 100 compounds. Cytotoxicity of dTBP2 was tested in just human bronchial epithelial cell line, BEAS-2B cells, but the half-maximal inhibitory concentration (IC₅₀) was estimated at 529 µM, suggesting that dTBP2 may be a nontoxic peptide.

24-Day repeated-dose toxicity study

dTBP2 at a concentration of 2.5–5 mg/kg has been shown to reduce allergic symptom score and eosinophil infiltration in a rhinitis model of BALB/c mice.¹³ To evaluate systemic toxicity of dTBP2 at higher doses, mice were injected with 12 doses of 5, 25, or 50 mg/kg of dTBP2 for 24 days. During the experimental period, all mice survived, and there were no gross signs of toxicity. At the beginning of experiment, the

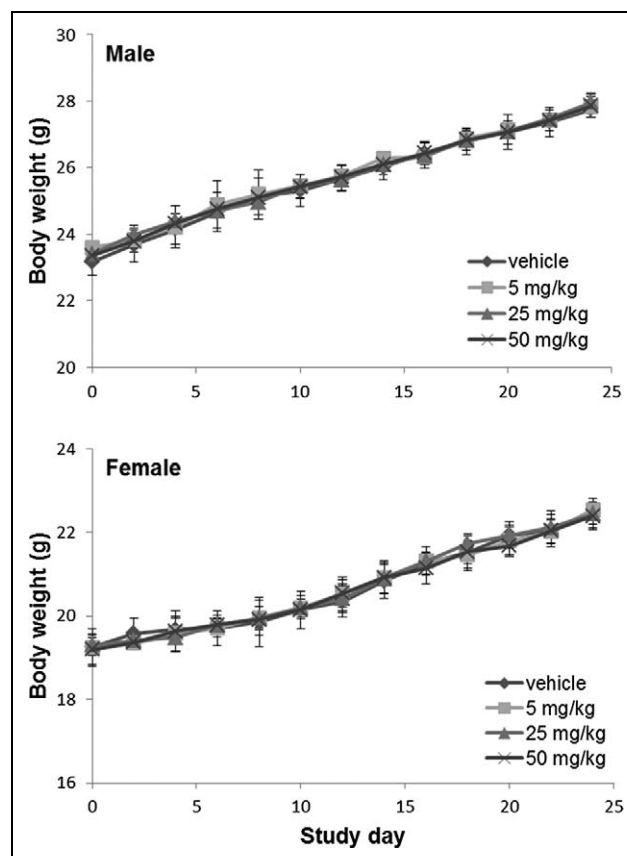


Figure 2. Changes in body weights of the mice. The body weights of all mice were measured every other day before administration. Negative control mice were injected the same volume of vehicle (PBS). Each value was expressed as the mean body weight $\pm$ SD ($n = 5$). PBS: phosphate-buffered saline; SD: standard deviation.

mean weights of mice were about 23 g in male and about 19 g in female, which increased to 27 g in males and about 22 g in females at the end of the experiment. There were no statistical differences in the mean body weights of male and female mice among each group (Figure 2 (a) and (b)). No significant difference in each organ weight was observed between the control and dTBP2-treated groups (Table 1).

Hematologic parameters were within the normal range for all animals, and there was no significant difference between the dTBP2-treated groups and the control group (Table 2). No significant differences were found also in serum biochemical parameters of the animals in all the groups (Table 3). However, significant increases in TBIL were found in all the groups. Abnormal increase in TBIL (1–1.3 mg/dl) was found in all groups when compared to a normal value of bilirubin in serum, which ranged 0.3–0.8 mg/dl in mice.¹⁸ We therefore conducted microscopic examination of

Table 1. Absolute organ weights of mice after 24 days of treatment with dTBP2.^a

Sex	dTBP2 (mg/kg)	Organ weight (g)							
		Brain	Heart	Lung	Liver	Kidney	Spleen	Stomach	Intestine
Male	0	0.44 ± 0.03	0.17 ± 0.02	0.40 ± 0.03	1.40 ± 0.05	0.48 ± 0.01	0.13 ± 0.02	0.27 ± 0.01	2.60 ± 0.12
	5	0.44 ± 0.01	0.17 ± 0.03	0.41 ± 0.07	1.47 ± 0.05	0.47 ± 0.02	0.14 ± 0.02	0.27 ± 0.04	2.76 ± 0.08
	25	0.43 ± 0.03	0.17 ± 0.01	0.40 ± 0.04	1.39 ± 0.09	0.48 ± 0.04	0.14 ± 0.03	0.24 ± 0.04	2.68 ± 0.11
	50	0.44 ± 0.02	0.16 ± 0.01	0.38 ± 0.01	1.37 ± 0.06	0.47 ± 0.02	0.15 ± 0.01	0.27 ± 0.01	2.65 ± 0.19
Female	0	0.44 ± 0.02	0.16 ± 0.04	0.34 ± 0.02	1.10 ± 0.04	0.33 ± 0.02	0.14 ± 0.02	0.18 ± 0.04	2.46 ± 0.09
	5	0.44 ± 0.01	0.14 ± 0.05	0.36 ± 0.03	1.15 ± 0.04	0.32 ± 0.02	0.15 ± 0.03	0.18 ± 0.03	2.53 ± 0.11
	25	0.44 ± 0.01	0.15 ± 0.04	0.34 ± 0.05	1.10 ± 0.07	0.32 ± 0.02	0.15 ± 0.02	0.19 ± 0.03	2.59 ± 0.13
	50	0.45 ± 0.02	0.14 ± 0.02	0.34 ± 0.05	1.12 ± 0.06	0.31 ± 0.02	0.16 ± 0.02	0.19 ± 0.04	2.54 ± 0.39

^aData are expressed as the mean organ weight ± SD (*n* = 5). Statistical differences are not observed.

Table 2. Hematological parameters of mice after 24 days of treatment with dTBP2^a

Sex	dTBP2 (mg/kg)	WBC (K/μl)	Neutrophil (K/μl)	Lymphocyte (K/μl)	Monocyte (K/μl)	Eosinophil (K/μl)	Basophil (K/μl)	RBC (M/μl)
Male	0	6.39 ± 0.62	1.44 ± 0.34	4.62 ± 1.33	0.32 ± 0.07	0.13 ± 0.01	0.03 ± 0.01	9.05 ± 1.49
	5	6.64 ± 1.84	1.41 ± 0.35	4.68 ± 1.24	0.34 ± 0.03	0.11 ± 0.07	0.02 ± 0.01	9.01 ± 0.50
	25	6.46 ± 1.40	1.42 ± 0.48	4.60 ± 1.06	0.36 ± 0.19	0.12 ± 0.09	0.03 ± 0.04	9.14 ± 0.44
	50	6.20 ± 1.12	1.45 ± 0.77	4.46 ± 0.52	0.30 ± 0.13	0.13 ± 0.08	0.03 ± 0.04	9.17 ± 0.54
Female	0	7.30 ± 1.49	1.51 ± 0.23	4.48 ± 0.71	0.50 ± 0.08	0.12 ± 0.07	0.02 ± 0.02	9.43 ± 0.55
	5	7.51 ± 1.02	1.51 ± 0.58	4.71 ± 1.22	0.50 ± 0.20	0.11 ± 0.11	0.03 ± 0.04	9.54 ± 1.69
	25	7.12 ± 0.42	1.48 ± 0.24	4.51 ± 0.48	0.49 ± 0.12	0.13 ± 0.04	0.04 ± 0.02	9.61 ± 0.95
	50	7.34 ± 1.53	1.42 ± 0.36	4.38 ± 0.72	0.52 ± 0.21	0.11 ± 0.10	0.03 ± 0.03	9.64 ± 1.19

^aData are expressed as the mean ± SD (*n* = 5). Statistical differences are not observed.

the livers of the control group and the group treated with 50 mg/kg of dTBP2 (Figure 3). The histological observation of liver sections showed no abnormal changes in any group, and other indices of liver function such as AST, ALT, and ALP were also in normal range, but we observed faint red color in mice serums probably caused by the breakdown of RBCs during serum preparation. Therefore, the abnormal increases of TBIL observed in the mice in all groups appear to be due to the contamination of bilirubin originating from RBCs.

Teshima et al. speculated that the amounts of TCTP secreted into the peritoneal cavity of OVA-sensitized/ macrophage colony-stimulating factor challenged mice to be about 2 μg.¹⁹ In our previous report, dTBP2 was found effective at 0.1:1 molar ratio with dTCTP in BEAS-2B cells.¹³ Therefore, when the weight of mouse is supposed as to be 20 g, it can be calculated that the working concentration of dTBP2, say about 1 kDa, may be about 0.005–0.05 mg/kg in the inflamed site. In addition, in a mouse rhinitis model, dTBP2 reduced allergic symptoms and eosinophils infiltration when injected i.p. at a dose of 2.5 mg/kg and showed augmented effect at a dose

of 5 mg/kg.¹³ The repeated administration of the maximum dose of 50 mg/kg for 24 days of the maximum dose of 50 mg/kg employed in this study clearly proved to be nontoxic in mice. It should be noted that 50 mg/kg used in this study is 10³- to 10⁴-fold higher than the calculated dose based on the report of by Teshima et al., and 10- to 20-fold higher than the effective dose in a mouse rhinitis model.

Though we confirmed the minimal toxicity both in vitro and in vivo, further studies are needed to confirm the toxicity and the pharmacokinetic profiles of this prodrug. For example, the IC_{50s} in various cell types need should to be measured along with whether and how its cellular toxicity might depend on incubation time. In addition to gross toxicity upon repeated treatment of dTBP2 in mice, which is reported in the present study, further toxicological monitoring including food and water intake urine analysis and histological analysis should also be conducted. Furthermore, determination of lethal dose and toxicokinetics of dTBP2 are necessary for drug development. As dTBP2 is an antiallergic drug candidate, its possible immunogenicity as well as local toxicity needs to be profiled.

Table 3. Biochemical parameters of mice after 24 days of treatment with dTBP2.^a

Sex	dTBP2 (mg/kg)	TG (mg/dl)	TP (g/dl)	TCH (mg/dl)	TBIL (mg/dl)	ALB (g/dl)	CRE (mg/dl)	GLU (mg/dl)	BUN (mg/dl)	AST (U/l)	ALT (U/l)	ALP (U/l)
Male	0	210.20 ± 59.08	5.36 ± 0.36	149.00 ± 4.06	1.20 ± 0.36	2.90 ± 0.26	0.14 ± 0.08	266.33 ± 10.66	18.43 ± 1.70	39.67 ± 4.92	26.00 ± 0.82	192.33 ± 9.74
	5	208.75 ± 25.96	5.48 ± 0.21	146.00 ± 5.72	1.06 ± 0.17	2.84 ± 0.14	0.13 ± 0.04	265.60 ± 15.21	18.87 ± 0.33	38.50 ± 3.20	25.60 ± 1.85	189.00 ± 9.06
	25	215.20 ± 29.89	5.72 ± 0.56	147.40 ± 6.09	1.10 ± 0.14	2.86 ± 0.33	0.15 ± 0.05	269.25 ± 17.88	17.93 ± 1.58	37.20 ± 2.71	25.25 ± 3.03	190.75 ± 9.65
	50	209.50 ± 33.48	5.62 ± 0.25	148.25 ± 5.89	1.08 ± 0.16	2.84 ± 0.12	0.16 ± 0.08	262.33 ± 6.94	18.80 ± 1.80	38.00 ± 2.12	24.50 ± 3.20	192.00 ± 5.39
Female	0	183.50 ± 14.40	5.60 ± 0.28	112.50 ± 13.35	1.25 ± 0.05	3.23 ± 0.19	0.15 ± 0.05	236.50 ± 17.23	18.73 ± 1.99	57.25 ± 3.49	26.25 ± 2.17	267.00 ± 11.77
	5	181.67 ± 9.67	5.95 ± 0.41	116.75 ± 14.65	1.28 ± 0.08	3.38 ± 0.23	0.18 ± 0.08	234.00 ± 13.14	18.50 ± 1.02	58.00 ± 9.20	26.33 ± 2.49	266.00 ± 13.75
	25	185.33 ± 10.62	5.93 ± 0.33	114.67 ± 4.50	1.25 ± 0.11	3.35 ± 0.11	0.13 ± 0.04	233.00 ± 5.48	18.48 ± 1.21	57.67 ± 1.25	27.25 ± 3.11	270.75 ± 10.13
	50	183.25 ± 6.87	5.80 ± 0.25	113.25 ± 14.92	1.23 ± 0.08	3.10 ± 0.08	0.15 ± 0.09	235.50 ± 10.26	18.67 ± 0.75	57.00 ± 1.41	26.75 ± 1.48	268.50 ± 9.71

TG: triglyceride; TP: total protein; TCH: total cholesterol; TBIL: total bilirubin; ALB: albumin; CRE: creatinine; GLU: glucose; BUN: blood urea nitrogen; AST: aspartate amino transferase; ALT: alanine amino transferase; ALP: alkaline phosphatase.

^aData are expressed as the mean ± SD (n = 5). Statistical differences are not observed.

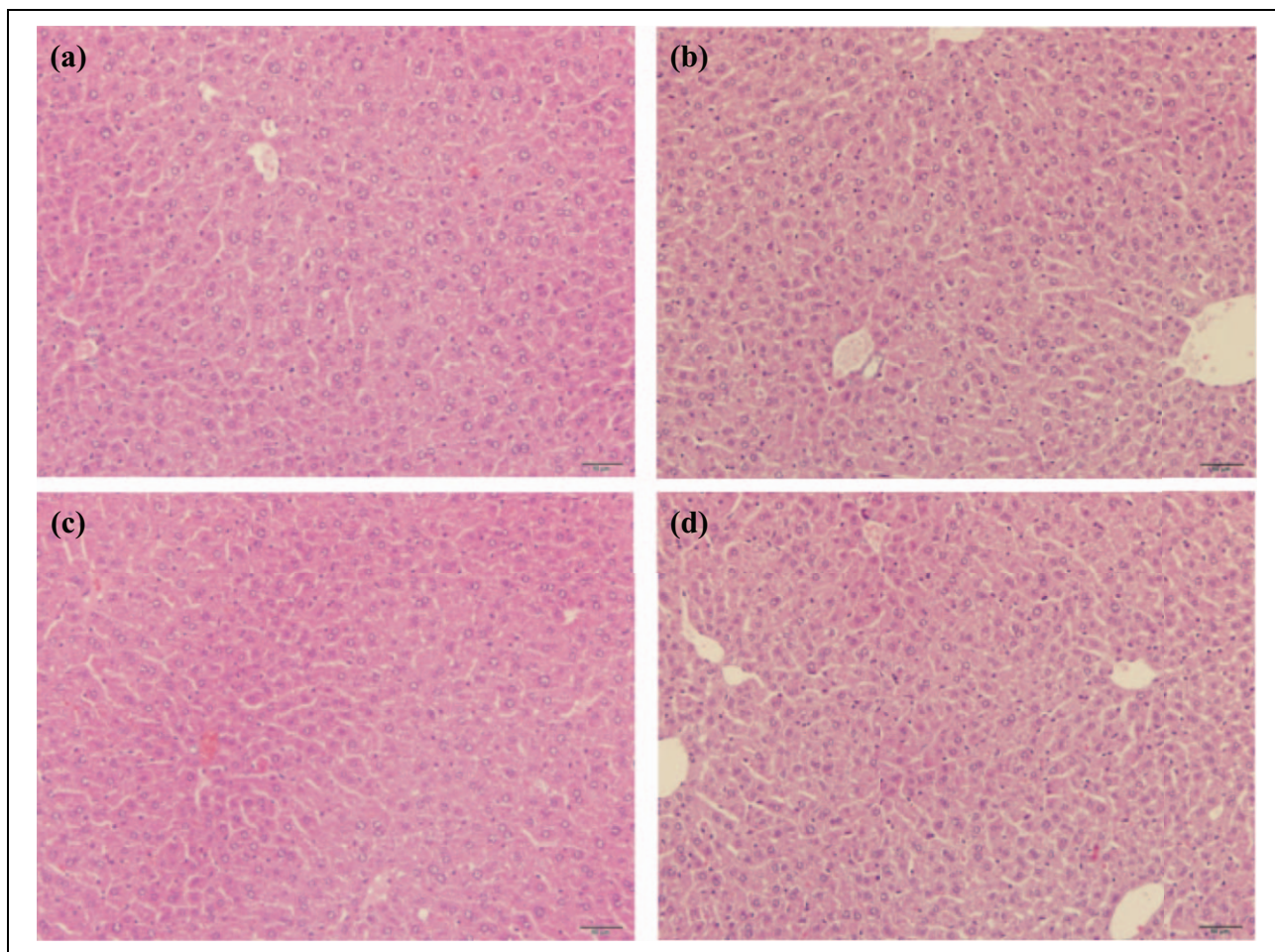


Figure 3. Histology of liver tissues. Liver tissues of mice treated with 50 mg/kg dTBP2 were fixed in 10% neutral-buffered formalin and then sectioned. The tissue sections were stained with hematoxylin and eosin and observed at magnification 200 $\times$. Scale bars indicate 50 μ m. Negative control male (a) and female (b) mice administered vehicle, and male (c) and female (d) mice administered 50 mg/kg of dTBP2.

We conclude therefore that dTBP2 is a safe candidate drug for the therapy of allergic diseases. As peptide drugs are relatively unstable physicochemically, they can usefully be modified into more stable drugs having enhanced bioavailability. This is an ongoing investigation in our laboratory, by applying the method of peptidomimetics. To extend this study into a new drug development via modification and formulation of dTBP2; and toxicological and pharmacokinetic studies in animals, it is necessary to administer the drug in its final dosage form. Also to be applied as a safe and an effective antiallergic drug, the general profiles of absorption, distribution, metabolism, and excretion of dTBP2 need to be assessed. Such assessments will be the goal of our succeeding studies.

Authors' Note

M Kim and YB Jin contributed equally to this work.

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Conflict of Interest

M. Kim and K. Lee have submitted an application for a patent on the use of dTBP2 for allergic diseases.

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