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Pharmacokinetics, toxicity of nasal cilia and immunomodulating effects in Sprague–Dawley rats following intranasal delivery of thymopentin with or without absorption enhancers

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ABSTRACT

Thymopentin (TP 5), a synthetic pentapeptide, has been used in clinic as a modulator for immnuodeficiencies through intramuscular administration. The objectives of this study was to investigate the pharmacokinetics using normal rats and toxicity of nasal cilia as well as immunomodulating effects using immunosuppression rats after intranasal delivery of thymopentin with or without an absorption enhancer. The absorption extent of fluorescein isothiocyanate (FITC) labeled TP 5 via nasal delivery at a single dose is significantly improved by incorporating sodium deoxycholate, Brij 35 and chitosan, respectively. FITC-TP 5 can also be absorbed to such an extent ranging from 15 to 28% after intranasal administration of FITC-TP 5 alone, FITC-TP 5 with sodium caprylate, or with bacitracin, respectively. After seven consecutive days multiple dosing, TP 5 formulation with sodium deoxycholate or Brij 35 caused apparently injury to nasal cilia, indicating these two enhancers would not be appropriate for nasal delivery. Results from superoxide dismutase activity, maleic dialdehyde, T-lymphocyte subsets (CD3+, CD4+, CD8+ and CD4+/CD8+ ratio) analyses suggest that all the selected enhancers improve the modulating effects of TP 5 in the immunosuppression rats. On an overall evaluation, intranasal TP 5 alone, TP 5 with chitosan, or TP 5 with bacitracin formulation may be suitable for the future clinical application.

1. Introduction

Peptides and polypeptides play a critical role in the immune system and have shown promising immunomodulating properties [17]. Thymopentin (TP 5) is a synthetic pentapeptide (Arg-Lys-Asp-Val-Tyr), which represents the 32–36 fragment of the naturally occurring the 49 amino acid thymopoietins (TMPOs). It can reproduce the biological activity of thymopoietins and influences the immune system by promoting the differentiation of thymocytes and affecting the function of mature T-cells. It has been used as an immunomodulating agent for treatment of rheumatoid arthritis [1,33], acquired immunodeficiency syndrome (AIDS) [10,23], severe acute respiratory syndrome (SARS) [42], cutaneous T-cell lymphoma [5]/cancer immunodeficiency [7], and other primary immunodeficiencies.

Thymopentin is mainly administered intramuscularly in the present clinical therapy. As an alternative to parenteral

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application, the patient-friendly delivery of active peptides by mucosal routes of entry is of major interest. Peroral dosing, the most common route of application for the systemic delivery of drugs, is limited for most peptides due to extensive metabolism and poor mucosal permeability [20]. Nasal drug delivery has been proposed as the most feasible alternative to parenteral injections due to the high permeability of the nasal epithelium, the avoidance of first-pass metabolism and a convenient administration route. A number of peptide and protein drugs restricted to oral administrations have been intensively investigated for systemic medication through nasal administrations [39]. However, nasal TP 5 delivery, as a potentially useful administration route, has not been reported according to our knowledge.

In general, polypeptides are unable to overcome the mucosal barriers and/or are degraded before reaching the blood stream [26]. Nasal delivery TP 5 may encounter the same situation although the molecular weight of TP 5 (M_w = 679.8) is relatively low. Among the approaches explored so far in order to optimize the transport of the peptide molecules across mucosal barriers, the use of penetration enhancers or mucosal enzyme inhibitor represents a challenging but promising strategy. Therefore, the several TP 5 preparations for intranasal administrations were formulated by incorporating the five absorption enhancers, including sodium deoxycholate (SDch), sodium carprylate (SCa), polyoxyethylene lauryl ether (Brij 35), chitosan and bacitracin, respectively. Furthermore, TP 5 solution was administered intravenously and intranasally for evaluation of the effect of nasal delivery.

The objectives of this study were to investigate the pharmacokinetics, toxicity of nasal cilia and immunomodulating effects in Sprague–Dawley rats after intranasal administration of thymopentin with or without an absorption enhancer.

2. Materials and methods

2.1. Materials and animals

Thymopentin (TP 5) and fluorescein isothiocyanate labeled TP 5 (FITC-TP 5) were purchased from Chinese Peptide Company (Zhejiang, China); sodium deoxycholate (SDch), sodium caprylate (SCa), and bacitracin were purchased from Sigma–Aldrich (Shanghai, China); polyoxyethylene lauryl ether (Brij 35), and urethane were from Beijing Chemical Reagent Plant (Beijing, China); chitosan was from Qingdao Haihui Corporation (Shandong, China); cyclophosphamide was obtained from Nanjing Tianzun-Zezhong Chemicals, Co. Ltd. (Jiangsu, China); paraformaldehyde was from Tianjin Kemiou Chemical Reagent Corporation (Tianjin, China). All other chemicals and reagents were of analytical grade.

Superoxide dismutase (SOD) kit (catalogue no. 050520) and maleic dialdehyde (MDA) kit (catalogue no. 050520) were obtained from Nanjing Jiancheng Bioengineering Institute (Jiangsu, China); fluorescein isothiocyanate (FITC)-conjugated mouse anti-rat CD3 monoclonal antibody (CD3-FITC, catalogue no. 559975), phycoerythrin (PE)-conjugated mouse anti-rat CD4 monoclonal antibody (CD4-PE, catalogue no. 551397), peridinin chlorophyll-a-protein (PerCP)-conjugated mouse anti-rat CD8a monoclonal antibody (CD8-PerCP, catalogue no. 558824), IgG2a-PE isotype control antibody (catalogue no. 349053), IgG1-PerCP antibody (catalogue no. 349044), and hemolysin were supplied by BD Pharmingen (San Diego, CA, USA).

Sprague–Dawley rats weighing 200–220 g were obtained from Experimental Animal Center of Peking University, and maintained on a light/dark cycle. All animals were allowed free access to standard rat chow and water. Temperature and relative humidity were maintained at 25 °C and 50%, respectively. Rats were acclimatized for 7 days prior to experiment. All care and handling of animals were performed with the approval of Institutional Authority for Laboratory Animal Care of Peking University.

2.2. TP 5 formulations

Chitosan was dispersed in deionized water and 1% phosphoric acid was added into the above system under agitation until chitosan was dissolved completely. The final concentration of chitosan was 1% (w/v, pH 4.0). SDch (5%, w/v), SCa (5%, w/v), and Brij 35 (5%, w/v), or bacitracin (0.5%, w/v) was dissolved in pH 7.4 phosphate buffer solution (PBS, consisting of 10 mmol/l potassium dihydrogen phosphate and 40 mmol/l sodium hydroxide), respectively. The above solutions were used as the stock solutions of absorption enhancers.

FITC-TP 5 was dissolved in the stock solutions to prepare samples for nasal administration in pharmacokinetic study, respectively. In addition, FITC-TP 5 was directly dissolved in PBS (pH 7.4) solution for intravenous injection, or for nasal administration as the controls, respectively. Similarly, the unlabeled TP 5 for pharmacodynamic study was prepared with the same procedures as above.

2.3. Pharmacokinetics

2.3.1. Administrations

Nasal administration of TP 5 was performed as earlier reported [9]. Briefly, 40 normal male Sprague–Dawley rats were equally divided into eight groups (five each) and fasted for 12–16 h prior to the experiment, respectively. The rats were anesthetized by intraperitoneal injection of urethane (1.5 g/kg), and restrained in a supine position. The rats were tracheotomised to divert the airflow from the nasal passages and aid breathing. The esophagus was closed by ligation onto the tracheal cannula.

The rats in the Group I were given with 1 ml of FITC-TP 5 PBS solution by tail vein injection at a single dose of 0.5 mg/kg and those in the Group II were with 25 μl of FITC-TP 5 PBS solution by intranasal administration at a single dose of 10 mg/kg. The rats in the Group III through Group VIII were administered intranasally with 25 μl of FITC-TP 5 solution containing 5% SDch (w/v), 5% SCa (w/v), 5% Brij 35 (w/v), 1% chitosan (w/v), 0.5% bacitracin (w/v), or 1% chitosan (w/v) in combination with 0.5% bacitracin (w/v) at a single dose of 10 mg/kg, respectively. For the intranasal administration, FITC-TP 5 solution was carefully injected into nasal cavity using a microliter syringe with a blunt needle. In all above experiments, no visible leak of solution was observed from the administration sites.

2.3.2. Sampling and measurements

After administration, a volume of 0.5 ml blood sample was drawn at 0, 15, 30, 45, 60, 90, 120, 180, 240, 300 and 360 min from the orbit venous plexus of the rats, respectively. The blood samples were immediately put in the heparinized tubes, stored at 4 °C for 30 min and then centrifuged at 5000 revolutions per minute for 10 min at room temperature. The plasmas were collected and stored at −70 °C until analysis.

A volume of 100 μl plasma was diluted with PBS solution and FITC-TP 5 plasma concentrations were measured using a Varian Cary Eclipse fluorescent spectrophotometer (Palo Alto, CA, USA), and the determination conditions were as follows: excitation wavelength, 494 nm; emission wavelength, 515 nm; slit for excitation, 2.5 nm; slit for emission, 5 nm. The lower limit of quantitation (LOQ) of the assay was 10 ng/ml and linearity was obtained for FITC-TP 5 concentrations between 0.05 and 2.0 μg/ml ($r^2 = 0.9992$). The coefficient of variation of the inter-day and intra-day precision of the quality control samples ranged from 0.71 to 7.76% and accuracy ranged from 95.6 to 102.9%.

2.4. Toxicity of nasal cilia and pharmacodynamics

2.4.1. Immunosuppression model and administrations

The rat immunosuppression model was modified according to the previous report [32]. Briefly, 45 normal female Sprague–Dawley rats were equally divided into nine groups (five each) and fasted for 12–16 h prior to the experiment. The rats in Group 1 were given intranasally with 25 μl of physiological saline once daily for 10 days as a normal control. Cyclophosphamide solution was administered intraperitoneally to each rat in Group 2 through Group 9 at a dose of 35 mg/kg once daily for three consecutive days for construction of immunosuppression rats. From the fourth day, the rats in the Group 2 were given intranasally with 25 μl of physiological saline once daily for 7 days as an immunosuppression control. The rats in Group 3 was given with 1 ml of TP 5 solution by tail vein injection at a dose of 0.5 mg/kg once daily for seven consecutive days and those in the Group 4 through Group 9 were administered intranasally with 25 μl of TP 5 solution alone or TP 5 solution containing 5% SDch (w/v), 5% SCa (w/v), 5% Brij 35 (w/v), 1% chitosan (w/v), or 0.5% bacitracin (w/v) at a dose of 10 mg/kg once daily for seven consecutive days, respectively.

2.4.2. Sampling

On the 11th day, the body weight of the rat was weighed. A volume of 1 ml blood sample was taken from the orbit venous plexus of each rat and put into anticoagulant tube for T-lymphocyte subsets analysis using flow cytometry, as described below. Another 1.5 ml blood was taken by the same way and immediately centrifuged at 4000 revolutions per minute for 10 min. The plasma was collected and stored at −20 °C until assays for SOD activity and MDA content.

After blood sampling, the rats were sacrificed. The nasal septum mucosa was removed for morphological examination according to the reports [6,43]. The spleen and thymus of each rat were then removed immediately, washed using physiological saline, wiped off using filter paper and weighed for assessing changes of the immune organs.

2.4.3. Scanning electron microscope

The nasal septum mucosa was washed with cold saline, fixed with 2.5% glutaraldehyde solution and then with 1% osmic acid. The sample was dehydrated by a series of concentration ethanol, replaced by *n*-amyl acetate, dried at critical pointer of carbon dioxide and coated with gold by an ion coater. The processed nasal mucosa was examined with the scanning electron microscope (JSM-5600 LV, JEOL, Japan).

2.4.4. SOD activity and MDA assay

Superoxide dismutase (SOD) activity in plasma was measured according to the SOD kit instruction. The test principle is as follows: superoxide anions (O_2^-) are generated by a xanthine oxidase (XOD) system and oxidize hydroxylamine forming the product of nitrite. This product is stained with the kit reagent, thereby showing purple color. Absorbance of the stained solution is assayed at 550 nm using a spectrophotometer (752-type, Shanghai, China). When SOD is present in the sample, superoxide anion concentration is lowered through specific inhibition, thus lowering the colorimetric signal. Consequently, SOD activity is obtained after calculation. For each assay, a volume of 15 μl plasma sample was used. All the processing and assay validation met the requirements of the kit instruction. One unit of SOD activity is defined as that amount of enzyme required to inhibit the reduction of SOD by 50% under the specified conditions.

Plasma maleic dialdehyde (MDA) level was measured according to the MDA kit instruction. The assay principle is as follows: maleic dialdehyde, a secondary product of lipid peroxidation, reacts with thiobarbituric acid (TBA) in acidic medium to give a pink colored pigment at 95 °C. The absorbance of pink color is read at 532 nm using the spectrophotometer as above. For each assay, a volume of 100 μl plasma sample was used. Values were expressed as nanomoles of MDA formed per milliliter plasma.

2.4.5. Flow cytometry

The blood specimens were processed as follows: briefly, a volume of 100 μl anticoagulant blood, 2 μl CD3-FITC, 20 μl IgG2a-PE and 20 μl IgG1-PerCP, were added to one tube, mixed by vortex for 30 s and incubated at room temperature for 20 min as a control (isotype-specific antibody staining); a volume of 100 μl anticoagulant blood, 2 μl CD3-FITC, 5 μl CD4-PE and 5 μl CD8-PerCP, were added to another test tube, mixed by vortex for 30 s and incubated at room temperature for 20 min as a sample. During the incubation, the light was avoided. A volume of 2 ml hemolysin was added to the control and sample tubes, respectively. The control and sample tubes were further incubated at room temperature for 10 min. After red cells were lysed completely, the two specimens were centrifuged at a speed of 2000 revolutions per minute for 5 min. The cells in sediment for each tube were separately collected, washed with 2 ml PBS twice, fixed in 0.5 ml of 1% paraformaldehyde and the CD3+, CD4+ and CD8+ T-lymphocyte subsets were measured within 4 h using Becton Dickinson FACSCalibur flow cytometer (Englewood NJ, USA) with CELLQuest software for acquisition and analysis. Forward light scatter (FSC), side-light scatter (SSC), and log fluorescence parameters were utilized in the analysis. The lymphocyte gate was set by adjusting forward and side scatter so that the

population of lymphocytes was clearly defined from the monocytes and the dead cell populations and then drawing a gate encircling the lymphocyte population. A total of 15,000 total events per sample tube were collected.

2.5. Calculation and statistical analysis

Non-compartmental pharmacokinetics was used for calculating the parameters using the 3p87 software, a practical pharmacokinetic program compiled by Chinese Association of Mathematic Pharmacology (Beijing, China). The area under the plasma concentration-time curve (AUC) was calculated according to the trapezoidal rule. The absolute bioavailability (F) was calculated with the formula: $F = (AUC_{0-6\ h,\ i.n.} \times Dose_{iv})/(AUC_{0-6\ h,\ iv} \times Dose_{i.n.})$. The 'i.n.' and 'iv' represent 'intranasal' and 'intravenous', respectively. T_{max}, C_{max}, $t_{1/2}$ and MRT were obtained from the non-compartmental analyses and denote the time to reach maximal concentration, maximal concentration, terminal half-life and mean residence time of FITC-TP 5, respectively.

Net increment of body weight is the difference value of rat body weight between the first day and the end day of the experiment for pharmacodynamic study. The thymus index and spleen index were calculated using the following formulas: thymus index of rat = $(W_{thymus}/W_{rat}) \times 100\%$; spleen index of rat = $(W_{spleen}/W_{rat}) \times 100\%$. Where W_{thymus} is the thymus weight of rat, W_{spleen} is the spleen weight of rat and W_{rat} is the body weight of rat on the end day.

Data are presented as the mean ± standard error (S.E.). One-way analysis of variance (ANOVA) was used to determine significance among groups, after which post hoc tests with the Bonferroni correction were used for comparison between individual groups. A value of $p < 0.05$ was considered to be significant.

3. Results

3.1. Pharmacokinetics

The mean plasma FITC-TP 5 concentration versus time profiles following intravenous (iv) and intranasal (i.n.) administrations of FITC-TP 5 are illustrated in Fig. 1. (1) After iv administration at a dose of 0.5 mg/kg to rats, the mean maximal FITC-TP 5 concentration was 0.667 ± 0.067 μg/ml and the concentration dropped to 0.022 ± 0.061 μg/ml at 120 min. (2) As a control in absence of any enhancer, after i.n. administration of FITC-TP 5 solution at a dose of 10 mg/kg, the mean maximal concentration was 0.340 ± 0.068 μg/ml and the mean concentration of FITC-TP 5 at 360 min still remained at the level of 0.136 ± 0.020 μg/ml. The time to reach the peak concentration was 90 min, suggesting that FITC-TP 5 was slowly absorbed through nasal cavity. (3) After i.n. administration of FITC-TP 5 at a dose of 10 mg/kg in the presence of Brij 35, the mean maximal concentration was evidently increased to 1.692 ± 0.045 μg/ml and time to reach the peak concentration was 120 min. The mean concentration of FITC-TP 5 at 360 min was 0.219 ± 0.055 μg/ml. (4) For other i.n. administrations at the same dose of FITC-TP 5 in the presence of an enhancer, the mean maximal concentrations were as follows: 1.146 ± 0.099 μg/ml for SDch > 1.091 ± 0.185 μg/ml for chitosan ≥ 0.956 ± 0.100 μg/ml for chitosan plus bacitratcin > 0.791 ± 0.084 μg/ml for SCa > 0.417 ± 0.031 μg/ml for bacitracin, respectively. The times to reach peak concentrations varied from 30 to 90 min.

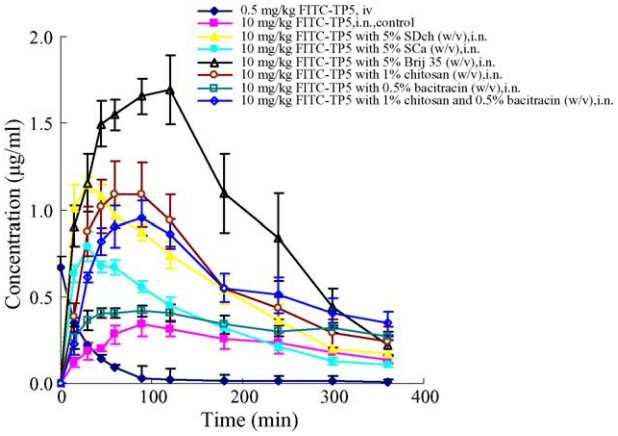

Fig. 1 – The mean plasma FITC-TP 5 concentration vs. time profiles following intravenous (iv) injection at a dose of 0.5 mg/kg or intranasal (i.n.) administrations at a dose of 10 mg/kg FITC-TP 5 with various formulations, respectively.

The pharmacokinetic parameters using non-compartmental analysis are presented in Table 1. In the presence of SDch, Brij 35, chitosan, or chitosan plus bacitracin, the C_{max} and $AUC_{0-6\ h}$, after i.n. administrations were significantly increased as compared with those of i.n. control group, respectively. For the i.n. administration in the presence of SCa, or bacitracin, these parameters were slightly increased, respectively. The rank order for the $AUC_{0-6\ h}$ values among groups was as follows: 6.06 ± 1.72 μg h/ml for Brij 35 > 3.52 ± 0.94 μg h/ml for chitosan plus bacitracin ≥ 3.50 ± 1.14 μg h/ml for chitosan > 3.38 ± 0.66 μg h/ml for SDch > 2.12 ± 0.30 μg h/ml for SCa > 1.87 ± 0.34 μg h/ml for bacitracin > 1.14 ± 0.31 for FITC-TP 5 alone (i.n. control). The values of T_{max} ranged from 0.65 ± 0.22 h for SCa to 1.55 ± 0.51 for the i.n. control. When compared with the iv administration group, the values of MRT after i.n. administrations with various formulations were significantly extended while those among groups after i.n. administrations were similar. The absolute bioavailability values (F) after i.n. administration of various formulations were as follows: 79.74 ± 0.17% for Brij 35 > 46.32 ± 0.09% for chitosan plus bacitracin ≥ 46.05 ± 0.11% for chitosan > 44.47 ± 0.07 for SDch > 27.89 ± 0.03 for SCa > 24.61 ± 0.03 for bacitracin > 15.0 ± 0.03% for i.n. control. The values of plasma $t_{1/2}$ for FITC-TP 5 varied ranging from 1.09 ± 0.42 h for the i.n. administration with Brij 35 group to 4.28 ± 1.39 h for the iv injection group.

3.2. Toxicity of nasal cilia

After intranasal administration of TP 5 with various formulations to immunosuppression rats at a dose of 10 mg/kg once daily for seven consecutive days, the scanning electron micrographs of rat nasal mucosal cilia are shown in Fig. 2A through H. As a negative control, the cilia were all in orderly

Table 1 – The pharmacokinetic parameters following intravenous (iv) injection at a dose of 0.5 mg/kg or intranasal (i.n.) administrations at a dose of 10 mg/kg FITC-TP 5 with various formulations, respectively

Parameters	T_{max} (h)	C_{max} (μg/ml)	$AUC_{0-6\ h}$ (μg h/ml)	$t_{1/2}$ (h)	MRT (h)	F (%)
0.5 mg/kg FITC-TP 5, iv		0.70 ± 0.17	0.38 ± 0.07	4.28 ± 1.39	0.96 ± 0.06	–
10 mg/kg FITC-TP 5, i.n., control	1.55 ± 0.51	0.36 ± 0.15	1.14 ± 0.31	4.06 ± 1.10	2.42 ± 0.45[a]	15.0 ± 0.03
10 mg/kg FITC-TP 5 with 5% SDch (w/v), i.n.	0.85 ± 0.68[a]	1.18 ± 0.09[a,b,c]	3.38 ± 0.66[a,b,c]	1.62 ± 0.35	2.08 ± 0.40[a]	44.47 ± 0.07[b,c]
10 mg/kg FITC-TP 5 with 5% SCa (w/v), i.n.	0.65 ± 0.22	0.82 ± 0.17[c]	2.12 ± 0.30[a,c]	1.88 ± 0.35	2.08 ± 0.06[a]	27.89 ± 0.03[c]
10 mg/kg FITC-TP 5 with 5% Brij 35 (w/v), i.n.	1.55 ± 0.51	1.93 ± 0.30[a,b,d]	6.06 ± 1.72[a,b,d]	1.09 ± 0.42	2.31 ± 0.29[a]	79.74 ± 0.17[b,d]
10 mg/kg FITC-TP 5 with 1% chitosan (w/v), i.n.	1.00 ± 0.31	1.13 ± 0.41[b]	3.50 ± 1.14[a,b]	1.75 ± 0.85	2.30 ± 0.22[a]	46.05 ± 0.11[b]
10 mg/kg FITC-TP 5 with 0.5% bacitracin (w/v), i.n.	1.10 ± 0.84	0.47 ± 0.06[c,d]	1.87 ± 0.34[a,c]	3.03 ± 1.04	2.68 ± 0.05[a]	24.61 ± 0.03[c]
10 mg/kg FITC-TP 5 with 1% Chitosan & 0.5% bacitracin (w/v), i.n.	1.15 ± 0.34	1.04 ± 0.17[b,c]	3.52 ± 0.94[a,b,c]	3.52 ± 0.83	2.61 ± 0.29[a]	46.32 ± 0.09[b,c]

Data are present as the mean ± S.E. ($n = 5$).
[a] $p < 0.05$ vs. 0.5 mg/kg TP 5, iv.
[b] $p < 0.05$ vs. 10 mg/kg TP 5, i.n., control.
[c] $p < 0.05$ vs. 10 mg/kg TP 5 with 5% Brij 35 (w/v), i.n.
[d] $p < 0.05$ vs. 10 mg/kg TP 5 with 1% chitosan (w/v), i.n.

arranged on the surface of the mucosa after i.n. administration of physiological saline (Fig. 2A). Similar phenomena were observed in the groups after i.n. administration of 10 mg/kg TP 5 with 1% chitosan (Fig. 2E), 10 mg/kg TP 5 with 0.5% bacitracin (Fig. 2F), 10 mg/kg TP 5 with 1% chitosan plus 0.5% bacitracin (Fig. 2G), or 10 mg/kg TP 5 alone (Fig. 2H), respectively. These results indicated that TP 5 alone, TP 5 with chitosan or with bacitracin did not affect the mucosal cilia.

On the contrary, the cilia shedding and obvious erosion were observed on the nasal mucosa of rats after i.n. administrations of 10 mg/kg TP 5 with 5% SDch (Fig. 2B), or 10 mg/kg TP 5 with 5% Brij 35 (Fig. 2D), respectively. The results suggested that these two enhancers exhibited a strong toxicity to nasal mucosal cilia of the rats, respectively.

In addition, the slight cilia erosion was found on the nasal mucosa after i.n. administration of 10 mg/kg TP 5 with 5% SCa (Fig. 2C), depicting that SCa may slightly be toxic to the cilia after the consecutive administrations with a formulation containing this adjuvant.

3.3. *Body weight and immune organs*

In contrasting to the normal rats, the induced immunosuppression rats showed an upright hair, reduced appetite, slight diarrhea and diminished activity. After treatment with TP 5, the situations were gradually improved.

After intravenous injection at a dose of 0.5 mg/kg or intranasal administrations at a dose of 10 mg/kg TP 5 with various formulations for seven consecutive days, the net increment of the rat body weight, thymus index and spleen index of immunosuppression rats are summarized in Table 2. The results showed that the net increment of body weight values of the immunosuppression rats were significantly lowered as compared with those of the normal control rats.

Table 2 – The net increment of the rat body weight, thymus index and spleen index of immunosuppression rats following intravenous (iv) injection at a dose of 0.5 mg/kg or intranasal (i.n.) administrations at a dose of 10 mg/kg TP 5 with various formulations for seven consecutive days, respectively

Treatment	Net increment of body weight (g)	Thymus index (mg/g)	Spleen index (mg/g)
Saline, i.n., as a normal control	88.20 ± 1.33	1.04 ± 0.15	1.23 ± 0.06
Saline, i.n., as an immunosuppression control	73.72 ± 3.96[a]	0.75 ± 0.08	1.11 ± 0.08
0.5 mg/kg TP 5, iv	77.66 ± 3.15	0.79 ± 0.07	1.16 ± 0.13
10 mg/kg TP 5, i.n.	83.0 ± 4.48	0.74 ± 0.12	1.05 ± 0.18
10 mg/kg TP 5 with 5% SDch (w/v), i.n.	87.16 ± 4.83	0.91 ± 0.06	1.02 ± 0.08
10 mg/kg TP 5 with 5% SCa (w/v), i.n.	84.04 ± 1.21	0.79 ± 0.06	1.29 ± 0.07
10 mg/kg TP 5 with 5% Brij 35 (w/v), i.n.	91.14 ± 4.34[b]	0.68 ± 0.10	0.93 ± 0.07
10 mg/kg TP 5 with 1% chitosan (w/v), i.n.	84.48 ± 3.20	0.88 ± 0.07	0.89 ± 0.05
10 mg/kg TP 5 with 0.5% bacitracin (w/v), i.n.	91.90 ± 3.41[b]	0.86 ± 0.13	1.03 ± 0.08

Each value represents the mean ± S.E. ($n = 5$); iv, intravenous injection; i.n., intranasal administration.
[a] $p < 0.05$ vs. saline solution as a control.
[b] $p < 0.05$ vs. model control.

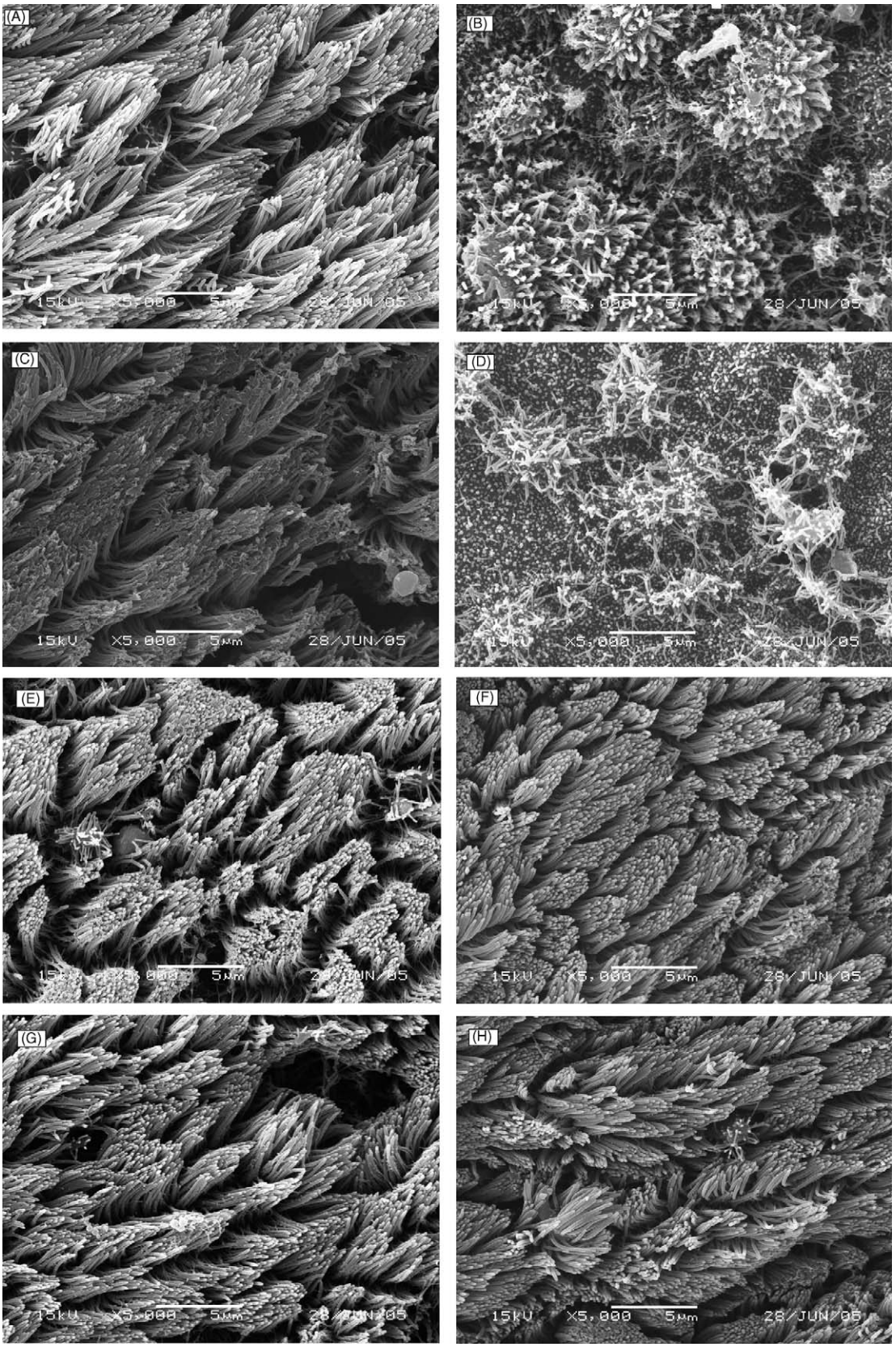

Fig. 2 – The scanning electron micrographs (5000×) of rat nasal cilia after intranasal administration of thymopentin (TP 5) with various formulations to immunosuppression rats at a dose of 10 mg/kg once daily for seven consecutive days, respectively. Keys: (A) physiological saline as a negative control; (B) 10 mg/kg TP 5 with 5% SDch (w/v); (C) 10 mg/kg TP 5 with 5% SCa (w/v); (D) 10 mg/kg TP 5 with 5% Brij 35 (w/v); (E) 10 mg/kg TP 5 with 1% chitosan (w/v); (F) 10 mg/kg TP 5 with 0.5% bacitracin (w/v); (G) 10 mg/kg TP 5 with 1% chitosan plus 0.5% bacitracin (w/v); (H) 10 mg/kg TP 5 alone (w/v).

Table 3 – The SOD and MDA values in immunosuppression rats following intravenous injection at a dose of 0.5 mg/kg or intranasal administrations at a dose of 10 mg/kg TP 5 with various formulations for seven consecutive days, respectively

Treatment	SOD (U/ml)	MDA (nmol/ml)
Saline, i.n., as a normal control	133.4 ± 3.2	4.4 ± 0.2
Saline, i.n., as an immunosuppression control	77.5 ± 16.5[a]	5.3 ± 0.2[a]
0.5 mg/kg TP 5, iv	118.6 ± 10.4[b]	2.8 ± 0.4[b]
10 mg/kg TP 5, i.n.	80.3 ± 7.1[a,c]	n
10 mg/kg TP 5 with 5% SDch (w/v), i.n.	101.6 ± 4.6	3.2 ± 0.2[b]
10 mg/kg TP 5 with 5% SCa (w/v), i.n.	97.5 ± 16.0	4.2 ± 0.5
10 mg/kg TP 5 with 5% Brij 35 (w/v), i.n.	112.7 ± 7.7[b,c]	3.6 ± 0.2[b]
10 mg/kg TP 5 with 1% chitosan (w/v), i.n.	145.9 ± 6.6[b,c]	4.2 ± 0.4
10 mg/kg TP 5 with 0.5% bacitracin (w/v), i.n.	142.2 ± 7.1[b,c]	4.4 ± 0.2

Each value represents the mean ± S.E. ($n = 5$); iv, intravenous injection; i.n., intranasal administration; n, not measured.
[a] $p < 0.05$ vs. normal control.
[b] $p < 0.05$ vs. immunosuppression control.
[c] $p < 0.05$ vs. 10 mg/kg TP 5, i.n.

Table 4 – The CD3+, CD4+ and CD8+ lymphocyte subpopulation changes in immunosuppression rats following intravenous injection at a dose of 0.5 mg/kg or intranasal administrations at a dose of 10 mg/kg TP 5 with various formulations for seven consecutive days, respectively

Treatment	CD3+ (%)	CD4+ (%)	CD8+ (%)	CD4+/CD8+
Saline, i.n., as a normal control	80.80 ± 2.33	43.36 ± 4.74	38.87 ± 2.89	1.17 ± 0.20
Saline, i.n., as a pathological model control	53.19 ± 1.36[a]	35.10 ± 0.85	18.01 ± 0.60[a]	1.95 ± 0.09[a]
0.5 mg/kg TP 5, iv	83.12 ± 2.65[b]	42.88 ± 2.38	41.82 ± 1.37[b]	1.03 ± 0.06[b]
10 mg/kg TP 5, i.n.	79.15 ± 2.02[b]	41.57 ± 1.88	39.62 ± 2.08[b]	1.06 ± 0.07[b]
10 mg/kg TP 5 with 5% SDch (w/v), i.n.	89.65 ± 1.61[b,c]	51.92 ± 2.83[b]	39.99 ± 2.08[b]	1.33 ± 0.14
10 mg/kg TP 5 with 5% SCa (w/v), i.n.	82.94 ± 2.05[b]	47.42 ± 5.23	35.85 ± 2.30[b]	1.38 ± 0.23
10 mg/kg TP 5 with 5% Brij 35 (w/v), i.n.	90.53 ± 2.19[b,c]	50.82 ± 1.13[b]	42.45 ± 3.07[b]	1.23 ± 0.13[b]
10 mg/kg TP 5 with 1% chitosan (w/v), i.n.	90.09 ± 1.25[b,c]	47.52 ± 2.15	44.44 ± 1.86[b]	1.08 ± 0.09[b]
10 mg/kg TP 5 with 0.5% bacitracin (w/v), i.n.	84.68 ± 2.10[b]	48.34 ± 3.61	37.90 ± 2.13[b]	1.31 ± 0.17

Each value represents the mean ± S.E. ($n = 5$); iv, intravenous injection; i.n., intranasal administration.
[a] $p < 0.05$ vs. normal control.
[b] $p < 0.05$ vs. pathological model control.
[c] $p < 0.01$ vs. 10 mg/kg TP 5, i.n.

Also, the thymus and spleen indexes were slightly reduced, respectively.

After iv or i.n. administration of TP 5 with various formulations for 7 days to the immunosuppression rats, the net increment values were improved at various extents. As compared with the normal control rats, the net increment values in the treatment groups were not significantly different, indicating that the body weight gain of pathological rats were gradually reversed to normal after treatment with TP 5. However, the reduced thymus index and spleen index were not obviously improved.

3.4. SOD activity and MDA content

The SOD and MDA values in immunosuppression rats following intravenous (iv) injection at a dose of 0.5 mg/kg or intranasal (i.n.) administrations at a dose of 10 mg/kg TP 5 with various formulations for seven consecutive days are described in Table 3. The results showed that the SOD values of the immunosuppression rats were significantly lowered and the MDA values markedly increased as compared with those of the normal control rats, respectively, indicating that the immunosuppression model was stably established.

The SOD values of the immunosuppresion rats after i.n. administration of TP 5 with Brij 35, chitosan, or bacitracin were significantly increased when compared with those of the immunosppression control rats. Those after i.n. administration of TP 5 alone, TP 5 with SDch, or TP 5 with SCa, were slightly increased. The MDA levels of the immunosuppresion rats after i.n. administration of TP 5 with various formulations were reduced when compared with those of the immunosuppresion model rats and were not significantly distinct from those of the normal control rats.

3.5. Peripheral blood T-lymphocyte subsets

The CD3+, CD4+ and CD8+ T-lymphocyte subsets changes in immunosuppression rats following administrations with various formulations are shown in Table 4. The results showed that, in the peripheral blood of immunosuppression rats, the T-lymphocyte percentages of CD3+ and CD8+ were significantly lowered and the percentage of CD4+ was slight reduced as compared with those of the normal control rats,

respectively. On the contrary, the CD4+/CD8+ ratio in the immunosuppression rats was markedly increased, indicating that the T-lymphocytes of the pathological rats were significantly affected by the immunosuppression.

After intravenous administration of TP 5 at a dose of 0.5 mg/k once daily for seven consecutive days to the immunosuppression rats, all the lowered CD3+ and CD8+ values were significantly increased as compared with untreated immunosuppression rats and the CD4+ percentage was reversed to the value of normal control rats. Correspondingly, the increased CD4+/CD8+ ratio in the immunosuppression rats was evidently reduced.

Also, all lowered CD3+ and CD8+ percentages after i.n. administration of TP 5 alone or TP 5 with various formulations were significantly increased when compared with those of the immunosuppression control rats, respectively. The CD4+ value was significantly increased after i.n. administration of TP 5 with SDch or with Brij 35 and obviously increased to normal values or above normal values after i.n. administration of TP 5 alone, TP 5 with SCa, with chitosan, or with bacitracin, respectively. The CD4+/CD8+ ratio was lowered after after i.n. administration of TP 5 with SDch, with SCa, or with bacitracin and significantly reduced after i.n. administration of TP 5 alone, TP 5 with Brij 35, or with chitosan, respectively.

4. Discussion

Thymopentin is a small molecule pentapeptide and stable during the formulating process in vitro [18]. Previous study showed that the pentapeptide was rapidly degraded in plasma [36], and degraded in vitro by human lymphocytes into two main fragments; the tetrapeptide (TP 4, thymocartin) Lys-Asp-Val-Tyr and the tripeptide (TP 3, thymotrinan) Asp-Val-Tyr [2]. The degradation products, both TP4 and TP3, are shown to exert similar immunomodulatory activities to TP5 affecting both humoral and cellular responses [13,27]. The pharmacokinetics of thymopentin has not been characterized due to the difficulty for measuring the blood concentrations using TP 5 itself. Consequently, FITC labeled TP 5 was used for pharmacokinetic analysis in the present investigation.

Absolute bioavailability represents the absorption extent of a drug. Our pharmacokinetic analysis indicates that three penetration enhancers including sodium deoxycholate (SDch), polyoxyethylene lauryl ether (Brij 35), and chitosan significantly improve the absorption extent of FITC-TP 5 via nasal mucosa. Sodium deoxycholate was thought as a potential absorption enhancer for drug delivery [21], and the enhanced absorption of FITC-TP 5 by sodium deoxycholate may be caused by the interaction mechanism that bile sodium was reported to able to disrupt lipid bilayers by intercalating between the phospholipids and forming mixed micelles [40,41]. The nasal promoting effect of Brij 35 may be related to the changed mucosal permeability [15]. Chitosan is a cationic polysaccharide widely employed as an absorption enhancer [19,12]. The ability of chitosan to work as an absorption enhancer was proven on Caco-2 cells, which serve as a model of intestinal epithelium [4,8,14,31], as well as in 'in vitro' experiments on nasal and buccal mucosae of animals [25,30]. The enhanced drug absorption by chitosan may be derived from the effect of calcium on adherent junctions rather than direct effect on tight junctions [19] while calcium ions are essential for the cells to maintain intercellular contacts. The removal of extracellular calcium in cultured epithelial cell lines or in vivo mucosae may result in the opening of tight junctions and prevent the formation of new tight junctions. This mechanism may be also true for the FITC-TP 5.

In comparison, other two enhancers, namely, sodium caprylate and bacitracin, increase the absorption of FITC-TP 5 to some extents as well. Previous studies demonstrated that the absorption promoting effect of sodium caprylate on insulin was in part associated with the chelating ability for calcium ions and the inhibitory action on leucine aminopeptidase activity [24]. In the present study, the immunomodulating activity of FITC labeled TP 5 was remained as the same as that of TP 5 (data from Chinese Peptide Company). Therefore, the increased absorption of FITC-TP 5 by sodium caprylate may be associated with the similar mechanism. Bacitracin is a protease inhibitor, which was proven to be more compatible with nasal cilia [28]. Incorporating bacitracin into a nasal formulation may be beneficial for the absorption of FITC-TP 5 due to the inhibition of TP 5 degradation by the proteases. The present results indicate that the absorption of FITC-TP 5 is slight increased as compared with the blank control (in absence of any enhancer) although it does not reach such an extent as expected. Among the five enhancers, Brij 35 seems to be the most significant factor for enhancing the absorption.

As compared with the iv administration route, the present results indicate that the mean residence time (MRT) of FITC-TP 5 after administration via nasal cavity is significantly extended and blood concentration maintains at a higher level for each formulation, suggesting that nasal TP 5 delivery may be suitable for enhancing the efficacy and thereby suitable for the future clinical therapy, as a more convenient administration route.

After intranasal delivery, peptides may be targeted to the nasal-associated lymphoid tissue (NALT) and to the deep cervical lymph nodes into which the NALT drains. The lymphatic drainage from the local nasal mucosa and NALT drains into the cervical lymph nodes and then to the systemic lymphatic system. Furthermore, peptides administered intranasally rapidly reach the brain through multiple pathways, consisting of olfactory neuronal, extraneuronal olfactory epithelial, trigeminal nerve and neuronal pathways [22,35]. Previous investigation showed that the intranasal administration of interferon (IFN) beta-1b led to a significant delivery throughout the rat central nervous system (CNS) and cervical lymph nodes, but a relatively low delivery to peripheral organs [29]. Therefore, intranasally administered peptides such as thymopentin are very likely to end up in the central nervous system. This can be good or bad. For treating multiple sclerosis (MS), intranasal administration of IFN beta-1b may offer a non-invasive approach and produces beneficial effects of tyrosine phosphorylation of IFN receptor in the CNS. For exerting immunomodulating effect, however, TP 5 targeted to the nasal-associated lymphoid tissue may be more beneficial because the nasal-associated lymphoid tissue (NALT), a mucosal inductive site for the upper respiratory tract, is important for the development of mucosal immunity. In the present study, the measured FITC-TP 5 after administration

via nasal cavity is most likely absorbed through the pathway of the nasal-associated lymphoid tissue (NALT). However, it is unclear that how much amount of TP 5 was delivered to the brain and what will happen after the TP 5 reaching the CNS. Thus these issues deserve further investigations.

In addition, the enhancer or additive may likely damage the nasal micro-surroundings, especially for a longer application case. For evaluating the possible influences, nasal mucosal cilia were assessed following the exposure of the TP 5 formulations to the nasal cavity of immunosuppression rats for 7 days, respectively. The present results indicate that sodium deoxycholate (SDch), and polyoxyethylene lauryl ether (Brij 35) lead to the irreversible injury of nasal mucosal cilia, respectively. For the potential application, chitosan, sodium caprylate (SCa), and bacitracin seem to be safer.

The improved net increments of body weight of rats indicate that the efficacy of TP 5 is shown in all treated groups including the i.n. administration of TP 5 alone via nasal cavity. This result suggests that the nasal delivery of TP 5 alone may also be effective in spite of a lower bioavailability. Results from assessments on immune organs shows that the reduced thymus index and spleen index were not obviously improved during the 7 days therapy, indicating that, for the immunosuppression caused by cyclophosphamide, the duration for recovery may need a longer time therapy using the immune modulator.

Superoxide Dismutase (SOD) catalyzes the dismutation of the superoxide radical (O^{2-}) into hydrogen peroxide (H_2O_2) and elemental oxygen (O_2), and as such, provides an important defense against the toxicity of superoxide radical. Another biomarker which provides an indication of lipid peroxidation level is the plasma concentration of maleic dialdehyde (MDA), one of several by-products of lipid peroxidation processes. In the plasmas of immunodeficiency patients, the SOD activity is significantly diminished [38], and the MDA levels pronouncedly increased [16]. Similarly, the present study shows that the SOD activity is significantly increased and the MDA level reduced after therapy, indicating that the nasal delivery is effective using either TP 5 alone or TP 5 formulation containing an enhancer. Interestingly, after multiple dosing TP 5 formulation with bacitracin, the efficacy for improving SOD or reducing MDA is the most significant although the absolute bioavailability is relative lower after a single dosing. These may suggest that the reducing protease degradation of TP 5 would be of clinical significance for longer administration.

According to the phenotype and function, T-lymphocytes are at least divided into four subsets, including cytotoxic T cells (T_c), suppressor T cells (T_s), delayed type hypersensitivity T cells (T_{dth}), inducer–helper T cells (T_i/T_h) and contrasuppressor T cells (T_{cs}). CD2 and CD3 cells are the common surface markers and express in all T-lymphocytes. CD4 express on the surface of T_i/T_h cells and CD8, the surface of T_s/T_c cells. The CD4+ and CD8+ represent the maturation of T-lymphocytes. In the immunological suppression or deficient patients with severe acute respiratory syndrome (SARS) [34], acquired immunodeficiency syndrome (AIDS) [37,3], and infections [11], etc., the peripheral blood CD3+, CD4+ and CD8+ values were diminished and CD4+/CD8+ ratio was irregularly changed. Similar results were obtained in our study using the immunosuppression rats induced by clyclophoshamide. The present results indicate that the nasal TP 5 delivery with various formulations either TP 5 alone or TP 5 with an enhancer is effective for improving the diminished CD3+, CD4+ and CD8+ counts, thereby reversing the irregular CD4+/CD8+ ratio to the normal values. In view of the efficacy for modulating the irregular CD4+/CD8+ ratio, i.n. administration of TP 5 alone, TP 5 with Brij 35, or TP 5 with chitosan seems to be more significant.

5. Conclusion

In conclusion, absorption extent of FITC labeled TP 5 via nasal delivery is significantly improved by incorporating sodium deoxycholate (SDch), Brij 35 and chitosan, respectively. FITC-TP 5 can also be absorbed to such an extent ranging from 15 to 28% after intranasal administration of FITC-TP 5 alone, FITC-TP 5 with sodium caprylate, or with bacitracin, respectively. Sodium deoxycholate and Brij 35 cause apparently injury to nasal mucosal cilia, indicating these two enhancers would not be suitable for nasal delivery. The pharmacodynamic investigations suggest that all the TP 5 formulations with or without an enhancer improve the immnuomudulating efficacy in the immunosuppression rats. On an overall evaluation, intranasal TP 5 alone, TP 5 with chitosan, or TP 5 with bacitracin formulation may be suitable for the future clinical application.

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