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Original Research Article

Toxicological evaluation of Ultrapure and Potent Tannic Acid (UPPTA) by inhalation exposure

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ABSTRACT: The therapeutic options for coronal virus infections are limited. As SARS-CoV-2 directly targets the lungs and causes lung damage, treatment of COVID-19 with inhalants may offer more advantages over oral administration. Inhaled drug delivery provides a higher drug concentration in the target organ, where SARS-CoV-2 proliferates. In this study, we evaluated the potential systemic toxicity, relevant target organ toxicity, and toxicokinetics of Airnecflu®, Ultrapure, and Potent Tannic Acid (UPPTA) by metered-dose inhaler (MDI) inhalation to rodent and canine species once a day for 2 consecutive weeks. We further investigated the reversibility of the toxicity following a 3-week recovery period. No mortality related to the test article was observed in all the dose groups. Neither abnormalities related to the test article nor toxicologically significant changes were observed in both rodent and canine studies. In pathological examination, alveolar macrophage aggregation, perivascular/interstitial/alveolar inflammatory cell infiltration, and alveolar/bronchial epithelium hyperplasia were noted in the lung with bronchi involvement. However, after a 3-week recovery period, a substantial recovery was observed. There is limited systemic exposure to the inhalation administration. Therefore, inhalation of Airnecflu® UPPTA is safe to administer for respiratory disorders like COVID-19.

KEYWORDS: COVID-19; SARS-CoV-2; metered-dose inhaler; inhaled toxicity; pulmonary pathology

1. Introduction

Tannic acids, a group of tannin enriched in several species of sumac and Aleppo oak, are naturally occurring hydrolysable polyphenol compounds with galloyl moieties expanding from a central glucose moiety. The structures of tannic acids are composed of 1 to 10 units of galloyl moieties per molecule. Throughout human history, tannic acids have been used as (part of) herbal medicine worldwide for thousands of years. For example, tannic acids have been used to treat diarrhea, bleeding, coughing, and detoxification in China[1].

The study of the anti-viral activity of tannic acids can be traced back more than 70 years. Green first reported that tannic acids can inhibit influenza A virus multiplication and hemagglutination[2]. More

recently, tannic acids extracted from tea showed inhibitory activity against SARS-CoV with an IC_{50} of 3 μM[3]. The main protease of coronaviruses is essential for the processing of the replicase complex nonstructural proteins. In addition, the nasal epithelium is critical in the processing of viral spike protein priming for viral infection, and the cell surface protease transmembrane protease serine 2 (TMPRSS2) is highly expressed in the small airway epithelium[4]. Since the outbreak of novel coronavirus, causing severe pneumonia and sequelae in late 2019, drug candidates for SARS-CoV have been re-tested for SARS-CoV-2. Wang et al. reported fruits tannic acids are dual inhibitors targeting viral main protease ($3CL^{pro}$) and host TMPRSS2 with IC_{50} of 13.4 μM and 2.31 μM respectively[5]. However, the potency is weak, and it appears that crude tannic acids cannot reach the therapeutic level without administration in large quantities.

Shih et al. further examined the antiviral activities of proprietary processed tannic acid from Chinese gallnuts, Ultrapure and Potent Tannic Acid (UPPTA). The EC_{50} against SARS-CoV-2 is much improved to 0.5 μM in 3CLpro-overexpressed cellular system. Moreover, for the first documented human coronavirus infection, HCoV-OC43, the EC_{50} is 1.3 μM. The selectivity index (EC_{50}/CC_{50}) declared its effectiveness and safety in vitro. They also evaluated the pharmacokinetic and toxicology of UPPTA to prove its feasibility in drug development[6]. Also, a preliminary report reveals that patients infected by SARS-CoV-2 have a better clinical improvement[7].

As SARS-CoV-2 directly targets the lungs and causes lung damage, treatment of COVID-19 with inhalants may offer more advantages over oral administration. Because the viruses incubate deep in the respiratory tract, the systemically administered drug could not reach them easily. Therefore, high doses of drugs are required through oral or parenteral routes and may cause systemic over-exposure and unwanted side effects. The inhaled route of drug administration could provide a much higher concentration in the pulmonary system without much systemic exposure. Moreover, the peripheral metabolism of the drug could be great reduced when the drug is directly delivered to the lungs, minimizing systemic drug exposure[8].

Since the toxicity of UPPTA by inhalation has never been investigated, a thorough evaluation is needed before human use and investigational new drug applications. In this study, we evaluated the potential systemic and pulmonary toxicity of UPPTA by inhalation in both Sprague-Dawley (SD) rats and Beagle dogs once a day for 2 consecutive weeks. We also investigated the reversibility of toxicity following a 3-week recovery period after the exposure.

2. Materials and methods

The purification and manufacture of UPPTA active pharmaceutical ingredient (API) was described in the previous research[6]; the purity of cGMP-grade APIwas determined to be 98.4%.

2.1. Two-week toxicity study in SD rats following a 3-week recovery period

2.1.1. Aerosol generation and analysis

Inhalation exposure system and automatic metered-dose inhaler (MDI) nebulizer system (Beijing Huironghe Technology) were applied. Two bottles of control or test article were triggered to deliver the aerosol at the same time at the frequency of 12 times/min in single-cavity mode. The aerosol flow rate was set at 16 L/min, the dilution flow rate was 0 L/min, and the inner chamber exhaust flow rate is set at 14 L/min.

T_{99} of the exposure system was calculated by 4.6 × V/a, V is the volume of the chamber (0.00321 m^3), "a" (m^3/min) is the total aerosol in flow. During the experiment, the temperature was set at 20–26 °C, the humidity was set at 30–80%, the oxygen concentration was set at ≥19%, and the carbon dioxide concentration was set at <1%.

For analysis of the concentration of UPPTA, samples were collected with 3-stage wave plate sampler (5 mL of 30% acetonitrile water was added to each 1-stage wave tube) about 1 min after the beginning of administration. The sampling flow was set at 0.5 L/min. For analysis of the aerosol particle size parameters, including median mass aerodynamic diameter (MMAD), geometric standard deviation (GSD), and fine particle fraction, NGI laryngeal tube (NGI-1342 or NGI-1872, Copley Science, UK) and Copley Inhaler Testing Data Analysis Software, version 3.10 WIBU were applied. Sample conditions were set at pumping flow rate: 15 L/min; collecting time: 3 min.

2.1.2. Animals and treatment

The study procedures were approved by the Institutional Animal Care and Use Committee. 8–9 weeks old specific pathogen-free SD rats were purchased from the Zhejiang Vital River Laboratory Animal Technology Co. Ltd., China (license No. SCXK (Zhe) 2019-0001), housed at a maximum of 5 rats per cage and maintained on a 12/12-hrs light/dark cycle at 23 ± 4 °C and 55 ± 15% humidity. Animals had free access to SPF Rat Growth Breeding Feed diet (Jiangsu Xietong Pharmaceutical Bio-Engineering) and filtered water except during designated procedures. After the initial health examination, all animals were placed in a fixator for about 60 minutes once daily for 5 consecutive days for acclimation. Animals without abnormal behaviors during fixation acclimation were grouped and acclimated for an additional 5 days. All experiments were conducted in JOINN Laboratories (Suzhou) Co., Ltd. in accordance with the Good Laboratory Practice (GLP) guideline.

2.1.3. Experimental design

The objectives of this study are to evaluate toxicity and toxicokinetic properties of UPPTA administered by MDI followed by a 3-week recovery period.

In toxicity study, a total of 120 rats (60 rats/sex) were randomly assigned into 4 groups (15 animals/sex/group). Animals in Group 1 were given a 60-minute placebo by MDI as excipient control. Animals in Group 2 to 4 were given 15-minute, 30-minute, and 60-minute inhalation of UPPTA aerosol by MDI as low, medium, and high dose groups respectively. The dosing frequency was once daily for 14 consecutive days for a total of 14 doses. The first dosing day was defined as Day 1. The actual delivered dose was calculated with the formula: dose (mg/kg) = ventilation volume per minute (RMV) × Concentration × Duration/BW/1000, whereas the RMV = 0.608 × BW $(kg)^{0.852}$.

Clinical observation (including daily clinical observation, detailed clinical observation, and local observation), body weight, food consumption, body temperature, ophthalmoscopic examinations, respiratory function (tidal volume, respiratory frequency, and minute respiratory volume), and clinical pathology (hematology, coagulation, clinical chemistry, and urinalysis) were evaluated during the study. Ten animals per sex per group were euthanized at the end of the dosing period (Day15) and five animals per sex were euthanized at the end of the recovery period (Day 36) for macroscopic examination and complete necropsy examination in each group. Major organs were weighed, and histopathological examination was performed.

In toxicokinetic study, a total of 56 rats (8 rats/sex of dosing groups; 4 rats/sex of control group) were randomly assigned into 4 groups. Animals in Group 1 were given placebo by MDI for 60-minute

as excipient control. Animals in Group 2 to 4 were given 15-minute, 30-minute, and 60-minute UPPTA aerosol by MDI as low, medium, and high dose groups respectively. The dosing frequency was once daily for 14 consecutive days for a total of 14 doses. The first dosing day was defined as Day 1. The actual delivered dose was calculated with the formula: dose (mg/kg) = ventilation volume per minute (RMV) × Concentration × Duration/BW/1000, whereas the RMV = 0.608 × BW $(kg)^{0.852}$. Blood samples (approximately 0.3 mL, with EDTA-K_2 anticoagulation) were collected via jugular veins from animals of Group 1 to 4 for toxicokinetic analysis.

2.1.4. Clinical observation

All rats were observed twice daily (a.m. and p.m.) during the study for signs of, but not limited to, mortality, moribundity, mental/behavior status, respiration, secretion, feces, and water and food intake.

Detailed clinical observations included, but were not limited to, mental status, behavior, skin, fur, eyes, ears, nose, abdomen, external genitalia, anus, limbs, feet, and respiration were conducted prior to first dosing and once weekly after dosing.

Local observation of irritation at the snout and other contact sites with aerosol and the reaction symptoms (such as swelling, asthma, cough, asphyxia, and other symptoms) of animals after dosing were conducted as well.

2.1.5. Clinical pathology

Blood samples for hematology, coagulation and clinical chemistry determinations were collected via abdominal aorta prior to scheduled euthanasia. Rats were fasted overnight, then blood samples were collected into tubes containing EDTA-K2, sodium citrate, or separation gel and coagulation promoting for hematology, coagulation, or clinical chemistry, respectively. For analysis, Sysmex® XN Hematology system, Sysmex® CS-2000i and Sysmex CS-5100, and TBA-120FR were used for hematology, coagulation, and clinical chemistry analysis, respectively.Urine was collected using metabolism cages and analyzed by Cobas®6500 automatic urine analyzer.

2.1.6. Toxicokinetic study

Blood samples were collected from animals of control group at pre-dose time point and 4 h after the end of dosing on Day 1 and Day 14. Blood samples were collected from animals of the dosing groups at pre-dose time point, the end of dosing immediately (±2 min), and at post-dosing at 30 min, 1 h, 2 h, 4 h, 8 h and 24 h on Day 1 and Day 14. After blood collection, the blood samples were mixed by inverting at least 5 times and stored in an ice box. Plasma was separated by centrifugation at 2000 g for 10 min at 4 °C within 1 hr after blood collection. The plasma was dispensed in duplicate into labeled centrifuge tubes and stored below –60 °C. The analysis of the biological sample was performed by a validated LC-MS/MS method for the determination of plasma levels.

2.1.7. Animal euthanasia and pathology examination

Animals were euthanized with isoflurane inhalation followed by abdominal aorta exsanguination on Day 15 and Day 36. For histopathological evaluation, tissues/organs were fixed in 10% neutral buffered formalin followed by paraffin sectioning and hematoxylin and eosin (H&E) staining. A five-step grading system (minimal, slight, moderate, marked, or severe) was applied to rate the severity of microscopic lesions.

2.2. Two-week toxicity study in Beagle dogs following a 3-week recovery period

2.2.1. Aerosol generation and analysis

If not specified, the aerosol and its analysis were the same as those in the rodent study. For dog experiment, two bottles of control or test article were triggered at the same time at the frequency of 8 times/min in single-cavity mode. The aerosol flow rate was set at 20 L/min, the dilution flow rate was 8 L/min, and exhaust flow of the inner chamber was set at 26 L/min.

2.2.2. Animals and treatment

6 to 7-month-old conventional grade Beagle dogs were purchased from Jiangsu Marshall Biotechnology (license No. SCXK (Zhe) 2019-0012), housed individually in stainless steel cages and maintained on an approximate 12/12-hrs light/dark cycle at 22 ± 4 ℃ and 55 ± 15% humidity. Animals had free access to certified canine diet (Beijing Keaoxieli Feed) and tap water. After initial health examination, all animals underwent simulated domestication of inhalation for at least 5 days, about 135 minutes per day. Animals without abnormal behaviors during acclimation were grouped and acclimated for an additional 5 days. The study procedures were approved by the Institutional Animal Care and Use Committee.

2.2.3. Experimental design

To evaluate the potential toxicity and toxicokinetic properties, UPPTA was administered by MDI in Beagle dogs once a day for 2 consecutive weeks and the reversibility of toxicity following a 3-week recovery period was also investigated.

A total of 40 Beagle dogs (20/sex) were randomly assigned into 4 groups (5/sex/group). Animals in Group 1 were given a 90-minute inhalation of placebo by MDI. Animals in Group 2 to 4 were given 10-minute, 30-minute, and 90-minute UPPTA by MDI as low, medium, and high dose respectively. Dosing frequency was once daily for 2 consecutive weeks for a total of 14 doses. The day of the first dosing was defined as Day 1.

Placebo group received excipient control. The actual delivered dose was calculated with the same formula as in the rodent study. Animals were evaluated for clinical observation, body weight, food consumption, body temperature, electrocardiogram, blood pressure, ophthalmological examination, and clinical pathology, which are similar to the rodent study. Blood samples were collected on Day 1 and Day 14 for toxicokinetic analysis. At the end of the dosing period, the first 3 animals/sex in Group 1 to 4 were euthanized 2 weeks after dosing (Day 15). The remaining animals were euthanized at the end of the 3-week recovery period (Day 36). Systematic necropsy was performed for animals in Group 1 to 4. Also, organ weight, gross anatomy, and histopathology were examined.

2.2.4. Clinical observation and clinical pathology

The clinical observation processes are the same as in rat study. For clinical pathology, the equipment and procedures in the canine study are the same as those used in rodent study except for ADVIA 2120 Hematology system was used for hematology and the blood samples were collected via forelimb vein.

2.2.5. Toxicokinetic study

Blood samples (approximately 1 mL) were collected via forelimb subcutaneous vein for bioanalysis and toxicokinetic analysis. Blood samples were collected from animals of control group at pre-dose time point and at post-dosing 2 h on Day 1 and Day 14. Blood samples were collected from each animal of dosing groups at pre-dose time point, after the end of dosing immediately, and at post-dosing 2, 4, 8, 12

and 23 h on Day 1. On Day 14, blood samples from 3/sex animals of the dosing groups were collected at pre-dose time point, at the end of dosing immediately, and at post-dosing 2, 4, 8, 12 and 23 h. Blood samples from other 2/sex animals of dosing groups were collected at pre-dose time point, at the end of dosing immediately, and at post-dosing 2, 4, 8, 12, 23, 48, 72, 96, 120, 144, 264, 384 and 504 h.

2.2.6. Animal euthanasia and pathology examinations

Animals were euthanized after intramuscular injection of Xoletil 50 (12 mg/kg, 50 mg/mL) and xylazine hydrochloride (1 mg/kg, 20 mg/mL) followed by femoral artery bloodletting on Day 15 and Day 36. Histopathological evaluations were the same as in the rodent study.

2.3. Test article concentration in lung tissue

UPPTA was administered by MDI in SD rats and Beagle dogs once a day for 1 consecutive weeks. Frozen rat and dog lung samples were completely thawed in a cooler filled with ice and homogenized well. 20 μL rat lung matrix, 50 μL of internal standard (IS) (200 ng/mL), and 300 μL of tannase reaction solution (1 mg/mL) were added into an Eppendorf tube and mixed well by vortex. The mixtures were incubated at 30 ℃ for 4 h to complete the hydrolysis reaction. The hydrolysate was extracted with 3300 μL of extraction reagent (1.5% (w/w) formic acid in ACN), vortexed for about 2 min and then centrifuged at 12,000 rpm for 10 min at 2–8 ℃. The supernatant was collected and followed by evaporating to dryness under N_2. The dried extract was re-constituted in 200 μL reconstitution solution and filtered through a 0.22 μm membrane filter. The analysis of the biological sample was performed by a validated LC-MS/MS method for the determination of tissue levels.

2.4. Statistical analysis

Data from male and female animals were analyzed separately. All statistical tests were conducted as 2-sided tests, and the level of significance was set at 0.05 or $p \leq 0.05$. Group means and standard deviations (mean ± SD) were calculated using the Provantis system (SAS 9.2). Data were analyzed with the Levene's test to assess variance homogeneity. Data with homogeneous variance ($p > 0.05$ in Levene's test) were analyzed with the one-way ANOVA followed by the Dunnett's test for multiple comparisons. Data with variance heterogeneity ($p \leq 0.05$ in Levene's test) were logarithmically transformed using the natural logarithm (ln transformation). The Kruskal-Wallis test (non-parametric method) followed by the two-independent-sample test (Mann-Whitney U) for multiple comparisons was performed on the original data when transformed data showed variance heterogeneity.

When a dataset contained negative values, the logarithmical transformation was not applied, and either the original data or the rank transformed data were used for statistical analysis. When a dataset had zero values, the zero values were regarded as 1/10 of the smallest positive value in the dataset when the logarithmical transformation was performed.

3. Results

3.1. Analysis findings of the test article

In the SD rodent study, aerosol concentrations were analyzed from Day 1 to 14, and the aerosol particle size distribution was analyzed after the dosing on Days 1, 7 and 14. The actual delivered dose was 2.88 ± 0.96 mg/kg for the low-dose group, 5.35 ± 1.28 mg/kg for the medium-dose group and 11.70 ± 2.20 mg/kg for the high-dose group. The actual delivered dose and aerosol particle size distribution parameters are presented in **Table 1**.

Table 1. The actual delivered dose and aerosol particle size distribution parameters.

Dose	Low	Medium	High
SD rat			
Target dose (mg/kg)	3	6	12
Actual delivered dose (mg/kg)	2.88 ± 0.96	5.35 ± 1.28	11.70 ± 2.20
Average MMAD (μm)	3.11	3.07	3.26
Average GSD	1.55	1.56	1.57
Average FPF (%)	85.49	85.33	81.1
T_{99} (min)	0.92		
Beagle dog			
Target dose(mg/kg)	0.3	1.0	3.0
Actual delivered dose(mg/kg)	0.28 ± 0.09	0.98 ± 0.18	3.05 ± 0.48
Average MMAD(μm)	3.37	3.37	3.37
Average GSD	1.49	1.49	1.49
Average FPF (%)	82.81%	82.81%	82.81%
T_{99} (min)		0.49	

Note: MMAD: Mass Median Aerodynamic Diameter, GSD: Geometric Standard Deviation, FPF: Fine Particle Fraction (Fine Particle Fraction, MMAD < 5 μm). The T_{99} calculation formula of the exposure system is $T_{99} = 4.6 \times V/a$, V is the exposure tower volume (0.00321 m^3), a is the set total aerosol generation and dilution flow (m^3/min), a = 0.016 m^3/min for this test.

In the Beagle dog study, aerosol concentrations were analyzed from Day 1 to 14, and the aerosol particle size distribution was analyzed after the dosing on Days 1, 7 and 14. The actual delivered dose was 0.28 ± 0.09 mg/kg in low-dose group, 0.98 ± 0.18 mg/kg in medium-dose group and 3.05 ± 0.48 mg/kg in high-dose group respectively. The T_{99} (min) of the exposure system was 0.49 min (T99 = 4.6 × V/a (V = 0.003 m^3, a = 0.028 m^3/min)). The actual delivered dose and aerosol particle size distribution parameters are in **Table 1**.

3.2. Clinical observation and clinical pathology

In the rodent study, there is no test article-related abnormal changes (including daily clinical observation, detailed clinical observation, and local observation, body weight, food consumption, body temperature, ophthalmoscopic examinations, respiratory function, and clinical pathology (hematology, coagulation, clinical chemistry, and urinalysis) in all the dose groups during the study.

In Beagle dog study, no mortality related to test article was observed in all three dose groups during the whole study. There are no abnormalities related to the test article and no toxicologically significant changes were observed in body weight, food consumption, body temperature, electrocardiogram (limb II ECG), blood pressure, ophthalmic examination, coagulation, clinical chemistry, urinalysis, and gross anatomy in all the animals.

3.3. Toxicokinetic study

In the SD rodent study, Airnecflu®, UPPTA was not detected after 15 min, 30 min, and 60 min inhalation in most of the plasma samples.

In the Beagle dog study, after the first (Day 1) and last dosing (Day 14), Airnecflu®, UPPTA was not detected in all the plasma samples of excipient control group. The key TK parameters of Airnecflu®, UPPTA in the low, middle, and high dose groups are shown in the **Table 2**.

Table 2. The key toxicokinetic parameters of Airnecflu®, UPPTA in the Beagle dog study.

Analyte	Study day	Dose level (mg/kg)	Gender	T_{max} (hr)	C_{max} (ng/mL)	AUC_{0-23} (hr·ng/mL)
Airnecflu® UPPTA	D1 #	0.28	Male	-	-	-
			Female	-	-	-
		0.98	Male	15.67 ± 6.35	103.02 ± 49.40	1453.46 ± 719.50
			Female	12.00 ± 0.00	81.93 ± 36.90	1233.66 ± 739.20
		3.05	Male	14.20 ± 4.92	230.93 ± 98.45	3920.88 ± 1593.09
			Female	12.00 ± 0.00	356.43 ± 129.60	6237.47 ± 1984.03
	D14 #	0.28	Male	0.40 ± 0.89	90.10 ± 21.52	1773.31 ± 338.18
			Female	0.80 ± 1.79	126.14 ± 63.62	2785.10 ± 1219.51
		0.98	Male	2.40 ± 3.57	432.62 ± 121.85	8621.89 ± 2491.84
			Female	1.61 ± 3.57	738.87 ± 475.47	15077.32 ± 10791.66
		3.05	Male	2.01 ± 1.99	1162.13 ± 613.63	24206.36 ± 12767.67
			Female	7.21 ± 6.56	1684.23 ± 841.22	34553.42 ± 16559.60

-: Data under detectable limits; #, $n = 5$ for each group.

The analysis of the two one-sided *t*-test was applied in comparing the sexual difference of toxicokinetic parameters. The result showed that the exposure (AUC_{0-23}) of Airnecflu®, UPPTA had no statistical difference ($p > 0.05$) in both genders after inhalation of Airnecflu®, UPPTA at doses of 0.28, 0.98, and 3.05 mg/kg. The ratios (female/male) of mean C_{max} ranged from 0.80 to 1.71 and the ratios (female/male) of mean AUC_{0-23} ranged from 0.85 to 1.75 on the first dosing day (D1) and after repeated administration (D14). No gender differences were observed in the exposures of Airnecflu®, UPPTA.

In the Beagle dogs, after inhalation of Airnecflu®, UPPTA aerosol at doses of 0.98 and 3.05 mg/kg on Day 1, the mean exposure (AUC_{0-23}) in plasma increased dose-dependently in both genders. On Day 14, after inhalation of Airnecflu®, UPPTA aerosol at doses of 0.28, 0.98 and 3.05 mg/kg for 2 consecutive weeks, the mean exposure (AUC_{0-23}) increased dose-dependently in both genders. The ratio of mean exposure was slightly greater than the ratio of dose when comparing low dose to medium or high dose.

After consecutive 14-day administration of Airnecflu®, UPPTA at 0.98 and 3.05 mg/kg levels to Beagle dogs, the mean of Airnecflu®, UPPTA C_{max} ratios (Day14/Day1) ranged from 5.53 to 9.84 and the mean of AUC_{0-23} ratios (Day14/Day1) ranged from 6.78 to 13.87 (**Table 2**).

3.4. Organ weight change and pathology

In the SD rat study, test article-related increases in the absolute organ weight, organ-to-body weight ratio, and organ-to-brain weight ratio of lung with bronchi were observed in all dosing groups at the terminal necropsy (Day 15) (**Table 3**). These organ weight changes were probably correlated to the following pathology changes.

In the SD rats, test article-related minimal to slight alveolar macrophage aggregation and minimal to moderate perivascular/interstitial inflammatory cells infiltration were noted in the lung with bronchi of both sexes. Minimal alveolar epithelium hyperplasia was noted only in high dose group in the lung with bronchi (**Figure 1** and **Table 4**). After a 3-week recovery period (Day 36), there is substantial recovery of the test article-related microscopic findings in the lung with bronchi.

Table 3. Weight changes in terminal necropsy and their recover on Day 36 of the SD rats.

Sex		**Male**			**Female**	
Dose	Low	Medium	High	Low	Medium	High
Inhalation duration (min)	15	30	60	15	30	60
Terminal necropsy (Day 15) #						
Lung with bronchi						
Organ weight	1.61 g*	1.56 g*	1.69 g*	1.29 g	1.32 g*	1.34 g*
Organ-to-Body	0.46%*	0.46%*	0.48%*	0.52%*	0.52%*	0.55%*
Organ-to-Brain	78.47%*	78.28%	85.76%*	67.35%	67.46%	71.00%
Recovery necropsy (Day 36) ^						
Lung with bronchi						
Organ weight	1.53 g	1.59 g	1.67 g	1.27 g	1.23 g	1.24 g
Organ-to-Body	0.35%	0.35%	0.38%	0.47%	0.47%	0.47%
Organ-to-Brain	74.42%	78.70%	80.65%	64.00%	62.88%	65.06%

Data Format: Control data were presented in absolute (actual) values for the control group only; the data for dosed animal were presented as a percentage of change relative to the control group data. *$p \leq 0.05$; #, $n = 10$ for each group; ^, $n = 5$ for each group.

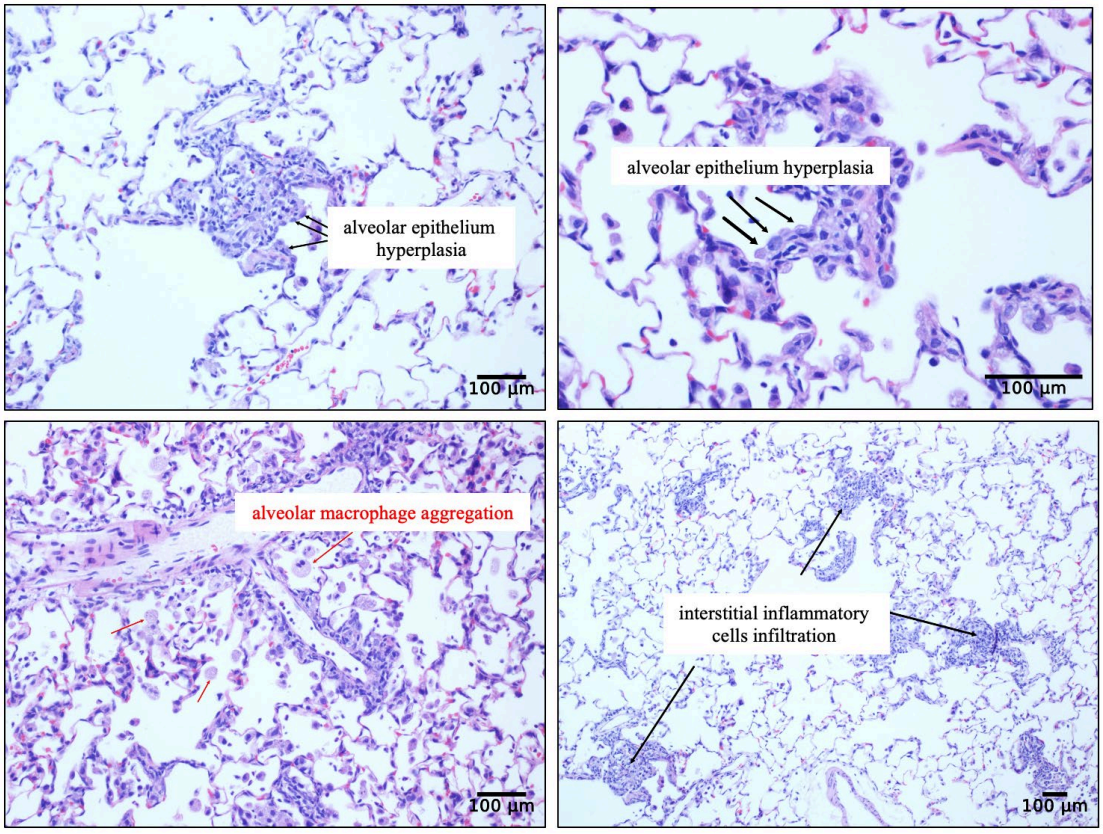

Figure 1. Examples of test article-related moderate alveolar macrophage aggregation, moderate interstitial/alveolar inflammatory cells infiltration, and minimal alveolar epithelium hyperplasia were noted in the lung with bronchi in SD rat. Representative lung H&E staining sections of rats with various treatments were displayed. Scale bar = 100 μm.

Table 4. UPPTA-related microscopic findings at the terminal and recovery necropsy in SD rat study.

Sex	**Male**				**Female**			
Dose	Control	Low	Medium	High	Control	Low	Medium	High
Inhalation duration/time (min)	60	15	30	60	60	15	30	60
Terminal necropsy (Day 15) #								
Lung with bronchi								
Alveolar macrophage aggregation								
Minimal	0	8	6	3	0	1	6	3
Slight	0	0	0	6	0	0	0	6
Infiltration, inflammatory cells perivascular; interstitial								
Minimal	0	5	3	1	0	10	6	1
Slight	0	4	5	2	0	0	4	3
Moderate	0	0	0	7	0	0	0	6
Hyperplasia, alveolar epithelium								
Minimal	0	0	0	1	0	0	0	2
Recovery necropsy (Day 36) *								
Lung with bronchi								
Alveolar macrophage aggregation								
Minimal	0	2	0	2	0	0	1	0
Infiltration, inflammatory cells perivascular; interstitial								
Minimal	0	2	2	2	0	0	1	3

#, n = 10 for each group; *, n = 5 for each group.

In the Beagle dog study, varying degrees of test article-related increases in the absolute weight, organ-to-body weight ratio, and organ-to-brain weight ratio of the lung with bronchi were observed in all dosing groups at the terminal necropsy (Day 15) (**Table 5**). These organ weight changes were probably correlated to the following pathology changes.

Table 5. Weight changes in terminal necropsy and their recover on Day 36 of the Beagle dog.

Sex	**Male**			**Female**		
Dose	Low	Medium	High	Low	Medium	High
Inhalation duration (min)	10	30	90	10	30	90
Terminal necropsy (Day 15) #						
Lung with bronchi						
Organ weight	72.12 g	87.26 g*	87.69 g*	64.63 g	71.08 g	78.32 g
Organ-to-Body	0.86%	1.01%	1.05%*	0.98%	1.08%	1.18%
Organ-to-Brain	100.97%	118.09%	122.51%	90.24%	106.19%	112.93%
Recovery necropsy (Day 36) ^						
Lung with bronchi						
Organ weight	73.00 g	72.50 g	76.15 g	68.17 g	61. 60 g	79.88 g
Organ-to-Body	0.88%	0.88%	0.93%	0.96%	0.87%	1.05%
Organ-to-Brain	105.53%	93.72%	97.50%	96.33%	90.18%	111.99%

Note: Data Format: Control data is presented in absolute terms; the data for dosed animals is presented as a percentage of change relative to the concurrent control group data. *$p \leq 0.05$, #, n = 3 for each group; ^, n = 2 for each group.

There was test article-related minimal to moderate alveolar macrophage aggregation, minimal to moderate perivascular/interstitial inflammatory cells infiltration, and minimal alveolar epithelium hyperplasia in both sexes' lung with bronchi. In addition, test article-related minimal to slight increased cellularity of lymphocytes in cortex and minimal inflammatory cells infiltrated in medulla were noted in both sexes' bronchial lymph node (**Table 6**). After a 3-week recovery period (Day 36), although test article-related microscopic findings were still observed in the lung with bronchi, the severity and incidence of above lesions were significantly lower than those observed at the terminal necropsy (Day 15), indicating a substantial recovery.

Table 6. UPPTA-related microscopic findings at the terminal and recovery necropsy in the Beagle dog study.

Sex	**Male**				**Female**			
Dose	Control	Low	Medium	High	Control	Low	Medium	High
Inhalation duration (min)	90	10	30	90	90	10	30	90
Terminal necropsy (Day 15) #								
Lung with bronchi								
Alveolar macrophage aggregation								
Minimal	0	0	1	0	0	0	2	0
Slight	0	0	1	2	0	0	1	2
Moderate	0	0	0	1	0	0	0	1
Infiltration, Inflammatory cells, interstitium/perivascular/alveous								
Minimal	1	0	1	0	1	2	1	0
Slight	0	0	1	2	0	0	2	2
Moderate	0	0	0	1	0	0	0	1
Hyperplasia, alveolar epithelium, bronchial epithelium								
Minimal	0	0	1	3	0	0	0	1
Lymph node, bronchial								
Increased cellularity, lymphocytic, cortex								
Minimal	0	1	0	1	0	0	2	0
Slight	0	0	1	2	0	0	1	3
Infiltration, inflammatory cells, medulla								
Minimal	0	0	1	0	0	0	1	3
Recovery necropsy (Day 36) *								
Lung with bronchi								
Infiltration, inflammatory cells, interstitium/perivascular								
Minimal	0	1	2	0	0	1	0	1

#, n = 3 for each group; *, n = 2 for each group.

3.5. Test article concentration in lung tissues

Airnecflu® UPPTA aerosol was administered to SD rats and Beagle dogs by repeated inhalation at actual mean delivered doses of 26.87 mg/kg and 8.10 mg/kg respectively once daily for 7 consecutive days. Test article concentration was measured in lung tissues after animal euthanasia. 425.90 and 10.46 μg UPPTA per gram tissue were detected in 3 rat whole lung tissues and 2 canine accessory lobes of lung respectively.

4. Discussion

Tannic acids have been used in many regions worldwide for multiple therapeutical applications. In Eastern countries, they were applied in traditional treatment in the herbal recipe for a various diseases such as diarrhea, bleeding, toxification, dementia, and coughing[1,9,10]. In Western countries, tannic acids were used for toxification, burn injuries, and diarrhea[11–13].

Commercial crude tannic acids are derived from various origins such as sumac galls, Aleppo oak galls, or sumac leaves. Among these, Chinese gallnut (*Galla chinensis*) which is produced by the Chinese aphid (*Schlechtendalia chinensis)* through stimulation of sumac leaves (*Rhus chinensis*) is the main resource of tannic acids in East Asia for traditional Chinese medicine[14,15].

The coronavirus' lifecycle starts with binding of the envelope spike protein to its receptor, angiotensin-converting enzyme 2 (ACE2). Through member fusion, the RNA genome is released into the host cytosol, where it is translated into the replicase proteins. The polyproteins, pp1a and pp1ab, are cleaved by a virus-encoded protease ($3CL^{pro}$) into individual replicase complex nonstructural proteins. Replication begins in virus-induced double-membrane vesicles derived from the endoplasmic reticulum (ER). Positive-strand genome then serves as a template for full-length negative-strand RNA and sub-genomic RNA (sgRNA). sgRNA translation results in the synthesis of both structural proteins and accessory proteins that are inserted into the ER–Golgi intermediate compartment (ERGIC) for virion assembly. Finally, subsequent positive-sense RNA genomes are incorporated into newly synthesized virions, which are then secreted from the cell membrane[16–18].

Because of the key role of $3CL^{pro}$ in proteolytic procession during coronavirus replication, it has been considered as a critical molecular target for the development of anti-coronavirus drug. In 2005, Chen et al. screened 720 natural compounds for inhibitory activity against SARS-CoV. Tannic acids, extracted from tea, showed the highest inhibitory activity against $3CL^{pro}$ with IC_{50} of 3 μM[3]. In late 2019, a novel coronavirus causing severe pneumonia outbreak, which was lately identified and named as SARS-CoV-2. Drug candidates once tested for SARS-CoV were re-tested for being potential treatment of SARS-CoV-2.

Wang et al. reported a molecular analysis which demonstrated that tannic acids from fruit formed a thermodynamically stable complex with SARS-CoV-2 $3CL^{pro}$ at a KD of 1.1 μM[5]. They also tested the inhibitory activity of tannic acid against SARS-CoV-2 $3CL^{pro}$ with IC_{50} of 13.4 μM. $3CL^{pro}$ of SARS-CoV and SARS-CoV-2 are 96% identical at the amino acid level, and only one different residue is presented at the binding pocket[19,20]. It is therefore predicted that tannic acids are effective in inhibiting both SARS-CoV-2 activity in addition to SARS-CoV.

Shih et al. further tested activities of proprietary purified tannic acids from Chinese gallnut, named ultrapure and potent tannic acid (UPPTA), Pentarlandir®[6]. The EC_{50} against two coronaviral strains SARS-CoV-2 and HCoV-OC43, the earliest-known human coronavirus, are 0.5 μM and 1.3 μM respectively as tested in $3CL^{pro}$-overexpressed cellular system. Even though SARS-CoV-2 shows only 59% identity of $3CL^{pro}$ at the amino acid level with HCoV-OC43 in the binding pocket[20], the inhibitory activities of Pentarlandir® are very close in both strains. It declares the potential of proprietary tannic acids as broad-spectrum pancoronal antivirus drug to treat other human coronavirus with similar $3CL^{pro}$ amino acid identity such as HCoV-OC43.

The UPPTA is a tannic acid derived from Chinese gallnut (*Galla chinensis*) with high proportion of 5-12 galloyl moieties as compared to other sources of tannic acid from *Quercus infectoria* and *Rhus chinensis*.

In fact, the inhibitory activities of tannic acids are positively correlated with the number of galloyl groups[21]. In addition, the Selectivity Index (EC_{50}/CC_{50}) of the coronaviruses are above 100 when treating UPPTA, which declare its effectiveness and safety. The pharmacokinetic (PK) and toxicology of UPPTA were also investigated by oral administration. In the PK studies, proprietary processed tannic acids were distributable to the pulmonary system without accumulation[6]. A 14-day repeated dose toxicity study in rat demonstrated its safety with NOAEL (no-observed-adverse-effect level) value above 2000 mg/kg/day[6]. These findings support UPPTA for further drug development for coronaviruses.

Though the safety profile of UPPTA by oral administration has been examined thoroughly, the potential toxicity of Airnecflu® UPPTA by MDI administration has never been investigated. In this study we evaluated the potential toxicity and the target organ toxicity of Airnecflu® UPPTA by inhalation in both SD rats and Beagle dogs, once a day for 2 consecutive weeks. All the procedures were under GLP regulations.

In our previous studies, we evaluated the acute and 7-day repeated dose toxicity of Airnecflu® UPPTA by inhalation in SD rats and Beagle dogs. The actual mean delivered doses used in the single dose and 7-day repeated dose toxicity of the rat studies were up to 139.20 and 26.87 mg/kg/day respectively. In the Beagle dog studies, the actual mean delivered doses used in single dose and 7-day repeated dose toxicity were up to 12.40, and 8.10 mg/kg/day respectively. These doses were defined as maximum tolerated dose (MTD) as there was no death, mortality, and/or significant systemic toxicity were observed. Therefore, the expected delivered doses in the 14-day repeatedly toxicological evaluation were set at 12.00 and 3.00 mg/kg/day in SD rat and Beagle dog studies respectively.

In this study, our findings revealed no death, mortality, or significant systemic toxicity at the actual mean delivered dose of Airnecflu® UPPTA up to 11.70 ± 2.20 mg/kg and 3.05 ± 0.48 mg/kg in SD rats and Beagle dogs respectively. In the pathology study, test article-related changes including alveolar macrophage aggregation, perivascular/interstitial inflammatory cells infiltration, and alveolar epithelium hyperplasia were mostly minimal to slight in SD rat and Beagle dog. Furthermore, after a 3-week period, substantial recovery of the test article-related microscopic findings was observed in the lung with bronchi in both SD rats and Beagle dogs, which indicating a reversible pathology that can recover.

In the toxicokinetic study, Airnecflu® UPPTA was not detected in most of the plasma samples of rat, indicating that it rarely enters the peripheral circulation after inhalation. However, in the canine toxicokinetic study, Airnecflu® UPPTA was detected in plasma of Beagle dog at very low level (C_{max} ~1 μM; molecular weight of UPPTA is 1465 g/mol) and the mean exposure (AUC_{0-23}) increased dose-dependently. The ratio of mean exposure was slightly greater than the ratio of dose when comparing low dose to medium or high dose after 14 days of administration. We also measured the concentration of Airnecflu® UPPTA in lung tissues at the end of the 7-day dosing period from previous study. Substantial levels of UPPTA were detected in the lung tissues. Taken together, there is a significant gradient of distribution of UPPTA when administered by MDI; UPPTA can be administered successfully by MDI to pulmonary tissue and there is little UPPTA distributed systemically.

Janower et al. reported potential hepatotoxicity caused by tannic acid through enema administration in a few cases after barium enema examinations[22]. However, liver toxicity was considered to be secondary to tannic acid administration with the performance of barium enemas. Shih et al. conducted the 14-day repeated dose toxicity study by oral route under GLP regulations. They evaluated a full panel of toxicology endpoints and found that the purified tannic acid had no significant abnormalities in all organs, biochemical parameters, or other evaluations[6]. In our phase II human study of UPPTA by oral

administration, no serious adverse event (SAE) was noted, and the adverse events (AE) were minimal and negligible[7].

We have reported the virucidal activity of proprietary UPPTA against SARS-CoV-2. Moreover, we have also investigated the virucidal activity of UPPTA against influenza viruses. UPPTA also showed virucidal activity to inhibit the infection of H1N1 and H3N2 at sub-μM concentrations in vitro. In addition, UPPTA further improved influenza-induced body survival rate, weight loss, and subsequent lung damage in vivo (unpublished findings).

Overall, the administration of UPPTA by either oral administration or MDI inhalation was well tolerated. The minimal pathology observed by MDI administration is, to a large extent, reversible. The no observed adverse effect levels (NOAEL) for MDI administration of UPPTA are 11.70 ± 2.20 mg/kg in SD rats, and 3.05 ± 0.48 mg/kg in Beagle dog, respectively. Airnecflu® UPPTA is considered safety for future use against either influenza or coronavirus infection, as it can be effectively delivered to reach therapeutic concentration in the target organ and minimize systemic exposure.

Author contributions

Conceptualization, LPL, YWM and GET; methodology, LPL, YWM and GET; software, LPL and YWM; validation, LPL and YWM; formal analysis, LPL and GET; investigation, LPL and TT; resources, TYC and GET; data curation, LPL, YWM and TT; writing—original draft preparation, LPL and YAL; writing—review and editing, LPL, YAL, TT and GET; visualization, LPL and GET; supervision, TYC and GET; project administration, LPL and YWM; funding acquisition, GET. All authors have read and agreed to the published version of the manuscript.

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Conflict of interest

The authors declare no conflict of interest.

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