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Esketamine inhaled as dry powder: Pharmacokinetic, pharmacodynamic and safety assessment in a preclinical study

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ABSTRACT

Ketamine and its enantiomer esketamine have gained much attention in recent years as potent, fast-acting agents for the management of treatment-resistant depression. However, an alternative to oral ketamine administration is required to ensure adequate systemic exposure as the drug undergoes extensive first-pass metabolism. We propose dry powder inhalation as a new esketamine delivery route. Here, we examine the pharmacokinetics, pharmacodynamics, toxicology and safety of this novel esketamine administration method. Esketamine (10 mg/kg) and ketamine racemate (20 mg/kg) were administered to rats by dry powder inhalation, intravenous injection or intratracheal instillation and the pharmacokinetics of these treatments were compared. Analyte concentration of ketamine stereoisomers and their metabolites was assessed by LC-MS/MS method. Esketamine showed a clinically relevant pharmacokinetic profile, with high bioavailability (62%) and relatively low maximum concentration peaks. Esketamine exhibited high penetration of the blood-brain barrier, but pharmacodynamic examinations of brain homogenates showed no changes in selected protein phosphorylation or expression analyzed by the immunoblotting method. We conducted GLP-compliant 14-day and 28-day general toxicity studies in rats and dogs, respectively, subjected to dry esketamine powder inhalation. The maximum daily dosages were 46.5 mg/kg and 36.5 mg/kg, respectively. We also performed pharmacological safety studies. Esketamine inhaled as dry powder had an expected safety profile consistent with its known pharmacological action. None of its observed effects were considered toxicologically significant. The pharmacological safety studies confirmed that the observed effects were transient and that inhaled esketamine had a good safety profile. Hence, our preclinical studies demonstrated that dry powder inhalation is a highly efficacious and safe delivery route for esketamine and may be a viable alternative administration route meriting further clinical development.

1. Introduction

Ketamine is well-known in pharmacology and medicine as a non-competitive *N*-methyl-*D*-aspartate (NMDA) receptor antagonist. It occurs as a racemic mixture of esketamine (S-ketamine) and arketamine (R-ketamine). It was first synthesized in the 1960s [1], registered under the brand name Ketalar in 1969, and administered as an anesthetic and analgesic. However, it was later discovered that ketamine had another important mode of action. Forty years after the discovery of ketamine, it was observed that this drug has rapid antidepressant action [2]. Subsequent studies showed that a 40-min infusion of a subanesthetic ketamine dose significantly ameliorated the symptoms of depression [3–6]. Ketamine is generally well tolerated. The main concerns regarding its treatment-related use in psychiatry include psychoactive and abuse potential, sedation, and hypertension. Chronic use of ketamine may also lead to pronounced neuropsychiatric symptoms, urological and gastrointestinal adverse effects [7]. However, the psychotomimetic effects of ketamine are the main challenge and have restricted its medical applications. Ketamine has been widely misused as a recreational drug [8]. As a result of its abuse potential, ketamine has been classified as a scheduled drug. Esketamine has 3–4 × greater affinity for the NMDA receptor than arketamine [9,10]. Esketamine has similar efficacy to

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racemic ketamine, but the former has a relatively lower effective dose [11]. The aforementioned considerations and change in the administration route to intranasal delivery have led to the registration of esketamine (Spravato; Janssen) in 2019 by the FDA and the EMA as a therapeutic agent for treatment-resistant depression.

The ketamine administration route remains a challenge. Ketamine is extensively metabolized in the liver [12]. It undergoes strong first-pass metabolism which reduces its oral bioavailability to <20% [13–15]. Ketamine metabolites, such as norketamine and hydroxynorketamine, have demonstrated antidepressant efficacy *in vivo* [16,17]. However, oral ketamine delivery may have lower therapeutic efficacy than other administration routes [18]. To enhance bioavailability, other administration routes, such as intravenous and intramuscular injection or intranasal instillation, may be used. Nevertheless, they require either medical assistance or clear nasal airways [19–21]. Here, we propose dry powder inhalation as a novel administration route, as it is expected to be free of the deficiencies and limitations associated with other drug delivery methods. Hence, the objectives of this study were to 1) investigate the pharmacokinetics of inhaled dry esketamine powder versus inhaled dry ketamine racemate (esketamine plus arketamine) powder and their metabolites, 2) assess absolute esketamine bioavailability after dry powder inhalation and intratracheal instillation, 3) study the pharmacodynamics of dry esketamine and ketamine racemate powder in rat brain homogenate, and 4) assess the safety of inhaled dry esketamine powder via the GLP-compliant toxicology and safety pharmacology studies in rats and dogs.

2. Materials and methods

2.1. Pharmacokinetics and pharmacodynamics

2.1.1. Drugs (test items) and standards

Esketamine hydrochloride (S-Ketamine) and ketamine racemate hydrochloride were purchased from CU Chemise Uetikon GmbH (Lahr, Germany) and were micronized using Jet Mill J-50 (Tecnologia Meccanica, Italy), Esnorketamine (S-Norketamine), arnorketamine (R-Norketamine), eshydroxynorketamine (2S,6S-Hydroxynorketamine) and arhydroxynorketamine (2R,6R-Hydroxynrketamine) were obtained from Celon Pharma SA (Kiełpin, Poland). Racemic norketamine was prepared according to literature procedures starting from commercially available 2-chlorophenyl cyclopentyl ketone (Fluorochem, Hadfield, Glossop, UK) [22,23]. The obtained (R,S)-norketamine was separated into enantiomers via chiral resolution using the procedure described by Zanos [24]. He hydroxynorketamine derivatives (2R,6R and 2S,6S) were then prepared starting from corresponding obtained (S)-norketamine and (R)-norketamine using procedures described by Zanos [24]. Injectable 0.9% (w/v) sodium chloride USP was acquired from Baxter International (Deerfield, IL, USA).

2.1.2. Particle size examination

The particle size of the micronized actives (esketamine hydrochloride and ketamine hydrochloride) was measured by laser light scattering technique using dry dispersion particle size analyzer (RODOS/HELOS with Aspiros, Sympatec, Germany). Measured micronized powders respectively were weighed directly to a glass vial and transferred into the system. Then, mean particle sizes based on volume diameter and standard deviations were recorded. All the measurements were performed at least three times. The span calculation and its value were calculated through the following equation:

$$Span = \frac{d_{90} - d_{10}}{d_{50}} \quad (1)$$

The d_{50}, the median has been defined as the diameter where half of the population lies below this value. Similarly, 90% of the distribution lies below the d_{90}, and 10% of the population localises below the d_{10}.

2.1.3. Animals

Male Wistar rats (Charles River Laboratories, Senneville, QC, Canada), aged 8–9 weeks at treatment onset, were housed in groups of ≤3/cage at 21 ± 3 °C and 50 ± 20% relative humidity under a 12 h light/dark cycle, except during the designated experimental procedures. They were acclimatized for ≥9 days before the onset of the procedure. A standard certified commercial rodent chow (Envigo Global 18% Protein Rodent Diet #2018C; Envigo, Horst, The Netherlands) and water were provided *ad libitum* except during the designated procedures. Block randomization to 29 experimental groups (n = 3) was based on body weight. Each group was assigned to drug inhalation, intravenous injection, or intratracheal instillation treatment, as shown in Table 1.

2.1.4. Treatment

2.1.4.1. Dry powder inhalation. Animals were exposed to targeted doses of 10 mg/kg body weight and 20 mg/kg body weight for esketamine and ketamine racemate aerosol, respectively, or vehicle (air), for 30 min in small animal flow-past chambers. Aerosols were generated with a piston feed/rotating brush generator. The system provided ≥1.0 L/min atmosphere to each animal exposure port. It was balanced to ensure slight positive pressure at the animal exposure site. Equal aerosol delivery at each exposure position was achieved with a distribution network attached to the system. The distribution networks were identical for all exposure positions. For each inhalation exposure, multiple aerosol concentration filter samples were collected to determine concentrations gravimetrically. Achieved doses were calculated as follows:

$$\frac{Ec \times RMV \times T}{BW} \quad (2)$$

Table 1
Group assignment in pharmacokinetic study in rats.

Group no.	Treatment	Dosing route	PK timepoints (min) Post end of dosing
1	Air control		15
2	Air control		120
3	Esketamine	INH	-24, 0, 15
4	Esketamine	INH	-18, 5, 30
5	Esketamine	INH	-12, 5, 60
6	Esketamine	INH	-6, 20, 120
7	Esketamine	INH	0, 150, 240
8	Ketamine racemate	INH	-24, 0, 15
9	Ketamine racemate	INH	-18, 5, 30
10	Ketamine racemate	INH	-12, 5, 60
11	Ketamine racemate	INH	-6, 20, 120
12	Ketamine racemate	INH	0, 150, 240
13	Vehicle control	i.v.	30
14	Esketamine	i.v.	10
15	Esketamine	i.v.	30
16	Esketamine	i.v.	60
17	Esketamine	i.v.	5, 30, 120
18	Esketamine	i.v.	10, 60, 240
19	Ketamine racemate	i.v.	10
20	Ketamine racemate	i.v.	30
21	Ketamine racemate	i.v.	60
22	Ketamine racemate	i.v.	5, 30, 120
23	Ketamine racemate	i.v.	10, 60, 240
24	Vehicle control	i.t.	30
25	Esketamine	i.t.	10
26	Esketamine	i.t.	30
27	Esketamine	i.t.	60
28	Esketamine	i.t.	5, 30, 120
29	Esketamine	i.t.	10, 60, 240

Abbreviations: INH = dry powder inhalation, i.v. = intravenous administration, i.t. = intratracheal instillation.

where Ec = actual concentration delivered to animals (mg/L air), RMV = respiratory minute volume (L/min) = 0.499 x BW (kg)$^{0.809}$ [25], T = exposure duration (min), and BW = body weight. The achieved esketamine and ketamine racemate doses were 7.6 mg/kg body weight and 19.9 mg/kg body weight, respectively.

2.1.4.2. Intravenous and intratracheal administration. For intravenous and intratracheal administration, esketamine and ketamine racemate was dissolved in saline. Then 2 mL/kg body weight was intravenously injected into the tail vain to provide 10 mg/kg body weight or 20 mg/kg body weight, respectively. Saline was used as vehicle. Before pulmonary instillation of the vehicle or test item, each rat was anesthetized by inhalation of gaseous isoflurane in oxygen. The anesthetized animals were dosed by intratracheal instillation with a Penn-Century® microsprayer (Model IA-1B; Penn-Century, Bradenton, FL, USA) at a dose of 100 μL/animal. The opening of the trachea was observed with an otoscope inserted in the animal's mouth and the tip of the microsprayer will be inserted in the trachea. The administered dose was 100 μL/animal. The actual volume administered was calculated and adjusted according to the most recent body weight of each rat.

2.1.5. Sample collection

Blood was sampled by jugular venipuncture on three separate occasions per treatment group. Samples were collected 5 min, 10 min, 30 min, 60 min, 120 min, and 240 min post dosing. For the dry powder inhalation treatment, additional samples were drawn during administration at 24 min, 18 min, 12 min, and 6 min before the end of dosing (Table 1). Brain tissue was collected at 10 min, 30 min, 60 min, 120 min, and 240 min post dosing. Blood samples were placed in tubes containing EDTA and centrifuged at 2000 × *g* and ~4 °C for 15 min. The plasma was recovered and stored at ≤ -60 °C. After the final blood sample collection, the animals were sacrificed by exsanguination and their brains were excised. Each brain was split in half. For the pharmacokinetic analysis, one hemisphere was rinsed in ice-cold saline and 3 mL ice-cold pure water was added per gram tissue. The mixture was homogenized and left on ice for 10 min until the foam settled. For the pharmacodynamic analyses, the prefrontal cortices and hippocampi of the other hemisphere were dissected on ice and stored at ≤ -60 °C until analysis.

2.1.6. Bioanalysis

Biological samples were prepared by precipitating their proteins and extracting their analytes with acetonitrile in the presence of a ketamine-d4 internal standard (LGC Standards, Teddington, UK). One hundred and 50 μL of extraction solution were added to 50 μL biological material and the suspension was mixed for 1 min. The suspension was then centrifuged at 10,621g and ~20 °C for 4 min at and the supernatant was collected for analysis. Calibration standards and quality control samples were prepared by spiking 45 μL biological material with 5 μL standard working solution, adding the extraction solution, mixing, centrifuging, and collecting the supernatant. Analytes were determined as shown in Table 2 and Table 3.

2.1.7. Pharmacokinetic parameter calculation

Pharmacokinetic analyses of mean plasma and brain ketamine and metabolite concentrations as functions of time were performed using the ThothPro™ software (ver. 4.1, ThothPro, Gdańsk, Poland). The data were fitted to a one-compartment model based on the lower Akaike Information Criterion value [26].

In the PK analyses, the area under the concentration-time curve was calculated for the 0 to t concentration range ($AUC_{0\rightarrow t}$) according to the linear trapezoidal rule and the maximum plasma and brain concentrations (C_{max}) and times to C_{max} (t_{max}) were evaluated. For the IV group, the elimination rate constant (k_{el}) and elimination phase half-life ($t_{1/2kel}$) of the drug from the plasma or brain, the distribution volume of the first (central) compartment (V_c), and the total body clearance (Cl_B) were also determined.

Table 2
Parameters for determining ketamine in homogenized rat brain samples.

Apparatus	Agilent Technologies 1290 Infinity II UPLC system/Agilent Technologies 6460 QQQ MS/MS system	
Chromatographic column	Chiralpak AGP 150 mm × 3 mm; 5 μm	
Mobile phase	A: 10 mM ammonium acetate (pH 7.1) B: acetonitrile Isocratic elution	
MRM transitions (*m/z*)	R,S-Ketamine: 238.2 > 125.0; 238.2 > 207.0	
	Esketamine	Arketamine
Range (ng/mL)	28.3 ± 5.650	27.1 ± 5.425
Calibration curve	r = 0.999 Accuracy: 94.5–108%	r = 0.999 Accuracy: 93.6–107%
Quality control samples	Accuracy: 91.8–108%	Accuracy: 89.8–111%

Table 3
Parameters for determining ketamine metabolites in homogenized rat brain samples.

Apparatus	Agilent Technologies 1290 Infinity II UPLC system/Agilent Technologies 6460 QQQ MS/MS system	
Chromatographic column	Amylose-2 AGP 150 mm × 3 mm; 5 μm	
Mobile phase	A: 10 mM ammonium acetate (pH 9.0) B: acetonitrile C: isopropyl alcohol Gradient elution	
MRM transitions (*m/z*)	Hydroxynorketamine (R,S-HNK): 239.9 > 221.9, 239.9 > 209.0, 239.9 > 195.0 Norketamine (R,S-NOR): 224.0 > 206.9, 224.0 > 178.9, 224.0 > 124.9 Dehydroxynorketamine (R,S-DHNK): 221.9 > 205.0, 221.9 > 176.9, 221.9 > 141.9	
	eshydroxynorketamine	arhydroxynorketamine
Range (ng/mL)	12.5 ± 2.500	12.5 ± 2.500
Calibration curve	r = 0.997 Accuracy: 87.8–110%	r = 0.996 Accuracy: 85.6–107%
Quality control samples	Accuracy 152–207%[a]	Accuracy 153–182%[a]
	esnorketamine	arnorketamine
Range (ng/mL)	10.8–2150	10.8–2150
Calibration curve	r = 0.995 Accuracy: 89.6–115%	r = 0.995 Accuracy: 87.5–113%
Quality control samples	Accuracy: 90.5–114%	Accuracy: 101–113%

a = decomposition of R,S-Ketamine to R,S-HNK

Bioavailability (F) was calculated as follows:

$$F = \frac{AUC_{0\rightarrow tEV} \times Dose_{IV}}{AUC_{0\rightarrow tIV} \times Dose_{EV}} \times 100\% \quad (3)$$

where EV - extravascular, IV - intravascular.

2.1.8. Pharmacodynamics

2.1.8.1. Synaptic protein extract preparation. To purify synaptoneurosomes, rat prefrontal cortices and hippocampi were homogenized in 10 μL Syn-PER reagent (Thermo Fisher Scientific, Waltham, MA, USA) per milligram tissue with 1 × Halt protease inhibitor cocktail (Thermo Fisher Scientific, Waltham, MA, USA), 1x phosphatase inhibitor cocktail (PhosphoSTOP; Roche Diagnostics, Basel, Switzerland), and 0.1 M EDTA. The brain samples were homogenized on ice with a cordless motor (Kimble-Chase, Rochester, NY, USA). The homogenates were centrifuged at 1200 × *g* and 4 °C for 10 min and the tissue debris was discarded. They were then recentrifuged at 15,000 × *g* and 4 °C for 20 min and the pellets (synaptosomal fraction) were re-suspended in N-PER neuronal protein extraction reagent

(Thermo Fisher Scientific, Waltham, MA, USA) with protease and phosphatase inhibitors.

2.1.8.2. Western blotting. Protein concentration was determined with a bicinchoninic acid protein assay kit (Thermo Fisher Scientific, Waltham, MA, USA). Ten micrograms of protein mixture were loaded onto 7.5% and 12% stain-free gel (Bio-Rad Laboratories, Hercules, CA, USA) and transferred to nitrocellulose membranes for Western blotting. To establish sample preparation quality and transfer efficiency after the gel run, stain-free technology was used (CHEMI DOC XRS imaging system; Bio-Rad Laboratories, Hercules, CA, USA). The membranes were blocked with 5% skim milk and incubated overnight at 4 °C with primary antibody (Cell Signaling Technology (CST), Danvers, MA, USA, Santa Cruz Biotechnology, Dallas, TX, USA (SC) or Merck Millipore, Temecula, CA, USA (MM)) against: phospho-Akt (Ser473, CST 4060. 1:4000), phospho-ERK 1/2 (Thr202/Tyr204, CST 4370. 1:4000), phospho-4E-BP1 (Thr37/46, CST 2855, 1:2000), phospho-P70S6K (Ser235/236, CST 9205, 1:1000), phospho-eEF2 (Thr56, CST 2331, 1:1000), phospho-mTOR (Ser2448, CST 2971, 1:2000), pGSK3β (Ser9, CST 9315, 1:1000) with the corresponding total proteins Akt (CST 9372, 1:3000), ERK 1/2 (CST 9102, 1:4000), 4E-BP1 (CST 9644, 1:2000), P70S6K (CST 2708, 1:1000), eEF2 (CST 2332, 1:1000), mTOR (CST 2972, 1:2000), GSK3β (CST 9336, 1:2000) and synaptic protein expression PSD-95 (CST 2507, 1:1000), Synapsin-1 (CST 5297, 1:10,000), ARC (SC sc-17839, 1:500), and GluR1 (MM AB1504, 1:2,000). The following day, the membranes incubated with a secondary horseradish peroxidase anti-rabbit (CST 7074S, 1:2000-1:5000) or anti-mouse antibody (MM A9044, 1:2000) at ~20 °C for 2 h. At the end of incubation, the membranes were washed several times with TBS-Tween and the immunoreactive bands were detected with chemiluminescent substrate solution (Santa Cruz Biotechnology, Dallas, TX, USA). Chemiluminescence intensity was quantified with a CHEMI DOC XRS imaging system (Bio-Rad Laboratories, Hercules, CA, USA).

2.2. Toxicology and safety pharmacology

2.2.1. Drugs

Esketamine hydrochloride was purchased from CU Chemie Uetikon GmbH (Lahr, Germany). For the inhalation treatment, the esketamine was formulated with 79.2% (w/w) lactose (DFE Pharma, the Netherlands) and 0.8% (w/w) magnesium stearate (Peter Greven Nederland c.V., the Netherlands). The placebo consisted of 99.2% (w/w) lactose plus 0.8% (w/w) magnesium stearate. For the intravenous injection, the esketamine was dissolved in saline (Baxter International, Deerfield, IL, USA).

2.2.2. Solid-state flowability

Flowability of the dry inhalation powders was assessed by determining Carr's compressibility index and Hausner ratio. The tapped density measurement was performed using 50 g of the sample after mechanical taps in a measuring cylinder and Carr's index was computed from the tapped and bulk density (freely bulk density of powder) of inhalation powders by the following equation:

$$Carr's\ index = 100 \times \left(\frac{\rho_{tapped} - \rho_{bulk}}{\rho_{tapped}}\right) \quad (4)$$

Hausner ratio was computed from tapped density and bulk density from the following equation:

$$Hausner\ ratio = \frac{\rho_{tapped}}{\rho_{bulk}} \quad (5)$$

2.2.3. In vitro aerosolization study

The prepared inhalation powders were aerosolized through a dry powder inhalation device (Diskus type). The in vitro deposition of the aerosolized drug was studied using Next Generation Impactor (NGI, Copley, UK, Apparatus E; Ph. Eur). A total of 4 mg of micronized esketamine in dry inhalation powder was transferred into the NGI system. T To have a pressure of 4 kPa, an airstream of 80 L/min was produced throughout the system by attaching the outlet of the NGI to a vacuum pump for 4 s. The powder, which deposited in stages 1–8 (Micro-orifice collector, MOC), the mouthpiece and the pre-separator device were collected by rinsing with solvent. The drug content was then determined by HPLC. From drug deposition data the percentage of fine particle fraction (FPF), Mass Median Aerodynamic Diameter (MMAD), and geometric standard deviation (GSD) were calculated. CITDAS version 3.10, data processing software (Copley Scientific, Nottingham, UK) was used for data analysis.

2.2.4. Animals

Wistar Crl:CD (SD) rats of both sexes (Charles River Laboratories, Senneville, QC, Canada), aged 8–11 weeks at treatment onset, were housed in groups of ≤3/cage at 21 ± 3 °C, 50 ± 20% relative humidity, and 12 h light/dark cycle, except during designated experimental procedures. They were acclimatized for ≥2 weeks before the start of the procedure. Standard certified commercial rodent chow (Envigo Global 18% Protein Rodent Diet #2018C; Envigo, Horst, The Netherlands) and water were provided *ad libitum* except during designated procedures. Beagles (dogs) of both sexes (Marshall BioResources, North Rose, NY, USA), aged 7–8 months at treatment onset, were housed in groups of ≤2/cage at 21 ± 3 °C, 50 ± 20% relative humidity, and 12 h light/dark cycle, except during designated experimental procedures. They were acclimatized for ≥3 weeks before the start of the procedure. Four hundred grams of standard certified commercial rodent chow (Envigo Certified Global 25% protein Dog Diet #2025C; Envigo, Horst, The Netherlands) was provided daily. Water was available *ad libitum* except during designated procedures.

2.2.5. 14-day rat inhalation toxicity study

Block randomization to dedicated experimental groups was based on body weight. Animals were assigned as indicated in Table 4.

Animals were exposed to test items in small animal flow-past chambers for 120 min. Aerosols were produced with a piston feed/rotating brush generator. The system provided ≥1.0 L/min atmosphere to each animal exposure port and was balanced to ensure slight positive pressure at the animal exposure site.

Uniform aerosol delivery to each exposure position was achieved with a distribution network attached to the system and was identical for each individual exposure position.

For each inhalation exposure, multiple aerosol concentration filter samples were collected to determine concentrations gravimetrically. Achieved doses were calculated as follows:

$$\frac{Ec \times RMV \times T}{BW} \quad (6)$$

Table 4
Group assignment in 14-day rat inhalation toxicity study.

Group	Targeted dose (mg/kg/d)	Achieved average dose (mg/kg/d)	No. main animals	
			Male	Female
Vehicle	0	0	5	5
Low dose	4	4.3	5	5
Mid dose	12	13.8	5	5
High dose	40	46.5	5	5

where Ec = actual concentration delivered (mg/L air), RMV = respiratory minute volume (L/min) = 0.499 x BW (kg)$^{0.809}$ [25], T = exposure time (min), and BW = body weight.

Clinical signs were recorded immediately after exposure when the animal was returned to its cage and 1 h after each dose (±15 min). Food intake per cage and body weight were recorded daily. Fundoscopy and biomicroscopic ophthalmology were performed before and during the 2-week treatment.

Hematology, coagulation, clinical chemistry, and urinalysis were performed on all animals at termination. Animals were fasted and blood was collected from the abdominal aorta, and urine samples were collected over ~12–18 h.

Three males and three females per vehicle group and six males and six females per treatment group were used in the toxicokinetic study. Blood samples were collected on predose days 1 and 14, at 1 h post exposure onset, immediately post exposure, and 0.5 h, 1 h, 2 h, 4 h, and 24 h post exposure.

2.2.6. 28-day dog inhalation toxicity study with cardiovascular assessment

Block randomization to dedicated experimental groups was based on body weight. Animals were assigned as indicated in Table 5.

Dogs were exposed to the drugs by oronasal face mask inhalation for 120 min. Aerosols were produced with a piston feed/rotating brush generator and diluted with air, as required, to achieve target aerosol concentrations. The aerosols were discharged through a tube into a flow-past inhalation exposure system. The system provided ≥5.0 L/min aerosol to each animal. Equal aerosol delivery to each exposure position was achieved with a distribution network attached to the system that was identical for each individual exposure position.

For each inhalation exposure, multiple aerosol concentration filter samples were collected to determine concentrations gravimetrically. Achieved doses were calculated as follows:

$$\frac{Ec * RMV * T}{BW} \quad (7)$$

where Ec = actual concentration delivered to animals (mg/L air), RMV = respiratory minute volume (L/min) = 0.608 x BW (kg)$^{0.852}$ [25], T = exposure time (min), and BW = body weight.

Clinical signs were recorded immediately post exposure after the animal was returned to its cage and 1 h after each dose (±15 min). Food intake and body weight were recorded daily and weekly, respectively. Fundoscopy and biomicroscopic ophthalmology were performed before and after treatment. Electrocardiograms (heart rate (HR), PR interval (PR), QT interval (QT), corrected QT Interval (QTc), and QRS duration), blood pressure, and body temperature were obtained during pretreatment period, immediately post exposure, on day 2 post exposure, at 1 h, 4 h, and 24 h (±15 min) after dosing, and during week 4. Tracings were assessed for gross changes indicative of cardiac electrical dysfunction. Abnormalities of the heart rate, sinus, and atrioventricular rhythm or conductivity were determined.

Hematology, coagulation, clinical chemistry, and urinalysis were performed on all animals before treatment onset and at termination.

Table 5
Group assignment in 28-day dog inhalation toxicity study.

Group	Targeted dose (mg/kg/d)	Achieved average dose (mg/kg/d)	No. main animals		No. recovery animals	
			Male	Female	Male	Female
Vehicle	0	0	3	3	2	2
Low dose	5	5.7	3	3	2	2
Mid dose	15	18.0	3	3	2	2
High dose	35	36.5	3	3	2	2

Animals were fasted for ≥12 h and blood and urine samples were collected.

All animals were fasted overnight and euthanized upon completion of the treatment/recovery periods. All dogs were pre-anesthetized with acepromazine and euthanized by intravenous pentobarbital sodium overdose and exsanguination.

Necropsy consisted of an external examination with reference to all clinically recorded lesions and a detailed internal examination. Organs were then dissected, preserved, and histopathologically examined.

For the toxicokinetic study, blood samples were collected from each dog on treatment days 1 and 28 at pre-dose, immediately post exposure, and at 0.25 h, 0.5 h, 1 h, 2 h, 4 h, and 8 h post exposure.

2.2.7. Functional observational battery (FOB)

The FOB study was performed on male rats assigned by block randomization to the 0.5 mg/kg, 15 mg/kg, and 35 mg/kg body weight groups (n = 8/group). After ≥2 h of fasting, test and control/vehicle items were administered once by bolus intravenous injection into the tail vein. For ethical reasons and to avoid possible adverse pharmacological effects such as sedation, each dose was administered incrementally (25% of total volume; 2 min pause). In this manner, animals could be monitored, and dosing could be suspended if adverse effects were noted. The total dose volume was 1 mL/kg for all animals. FOB was recorded for each rat before dosing and at ~30 min, ~60 min, ~2 h, and ~24 h post dosing and comprised behavioral, neurological/neuromuscular, and autonomic domains assessment.

2.2.8. Respiratory safety pharmacology study

A respiratory safety study was performed on male rats assigned by block randomization to their respective dose groups (0.5 mg/kg, 15 mg/kg, or 35 mg/kg body weight; n = 8/group). After ≥2 h of fasting, test and control/vehicle items were administered once by bolus intravenous injection into the tail vein. For ethical reasons and to avoid possible adverse pharmacological effects such as sedation, each dose was administered incrementally (25% of total volume; 2 min pause). In this manner, animals could be monitored, and dosing could be suspended if adverse effects were noted. The total dose volume was 1 mL/kg for all animals. Respiratory measurements started from the last injection. Animals were set in 'head out' plethysmographs and their tidal volumes, respiratory rates, and minute volumes were measured for 15-min periods before dosing and at ~30 min, ~2 h, and ~24 h post dosing. Respiratory waveforms were recorded with Notocord HEM v. 4.2.

2.3. Ethics statement

The studies were reviewed and assessed by the Animal Care Committee (ACC) and each of the study (# 75723, 75749, 15705, 15706, 65585) was approved.

All animals used in the present study were maintained in accordance with the principles outlined in the current Guide to the Care and Use of Experimental Animals published by the Canadian Council on Animal Care and the Guide for the Care and Use of Laboratory Animals published by the National Institutes of Health.

2.4. Statistical analysis and data visualization

Statistical analysis and data visualization were performed in GraphPad Prism v. 7 (GraphPad Software, La Jolla, CA, USA). Pharmacokinetic data were reported as means ± range. Analyte concentrations were compared by two-way ANOVA (test item administered or analyte x time) followed by a post hoc Sidak's multiple comparisons test. Generalized ANOVA/ANCOVA was performed on the numerical toxicological data. An automatic transformation was used to assess the data for homogeneity of variance via Levene's test. Parametric and non-parametric trends were analyzed with the Williams and Shirley-Williams tests, re-

spectively. Homogeneous data were analyzed with ANCOVA/ANOVA and the significance of the differences between the control and treated groups was analyzed with Dunnett's test. Heterogeneous data were analyzed with Kruskal-Wallis test and the significance of the differences between the control and treatment groups was evaluated with non-parametric Dunnett's test.

For the safety pharmacology data and qualitative observations, χ^2 and Fisher's exact tests were used to identify significant differences between the control and treatments. Numerical data were analyzed for homogeneity of variance using the Levene median test and for normality using the Kolmogorov-Smirnov test. Homogeneous data were analyzed with ANOVA and the significance of the differences between the control and treatments was evaluated with Dunnett's test. Heterogeneous data were analyzed with Kruskal-Wallis test and the significance of the differences between the control and treatments was evaluated with Dunnett's test. Statistical significance was indicated at the $p < 0.05$, $p < 0.01$, and $p < 0.001$ acceptance levels.

3. Results

3.1. Esketamine and ketamine racemate aerodynamic characterization

Esketamine and ketamine racemate were characterized for particle size distribution (PSD) after micronization. Both substances have narrow particle size distribution (respectively span of 2.0 and 1.6) with a d_{50} below 2.3 μm for esketamine and d_{50} below 1.6 μm for ketamine racemate (Fig. 1, Table 6).

3.2. Pharmacokinetic properties of inhaled esketamine versus inhaled ketamine racemate

Blood esketamine concentration increased after esketamine and ketamine racemate exposure during the inhalation period and C_{max} was attained at the end of dosing (Fig. 2A, Table 7). Plasma esketamine concentration was significantly higher after racemate inhalation than it was after single enantiomer administration at 18 min ($p = 0.020$) and 24 min ($p = 0.031$) after the start of dosing, at the end of dosing ($p < 0.001$), and 5 min post end of dosing ($p = 0.044$). Esketamine C_{max} after ketamine racemate inhalation nearly doubled that after esketamine inhalation. Exposure calculated as the area under the curve (0-t) for esketamine was 1.5 × higher after ketamine racemate inhalation than esketamine inhalation.

After ketamine racemate inhalation, the esnorketamine concentration was significantly elevated 1 h after the end of dosing (Fig. 2C) while the eshydroxynorketamine concentration was significantly elevated 2.5 h after the end of dosing (Fig. 2E).

Esketamine reached higher concentrations in the brain homogenate than the plasma for all treatments (Fig. 2B, Table 7). The brain esketamine concentration was significantly higher after racemate administration than esketamine treatment at 15 min ($p < 0.0001$), 30 min ($p = 0.003$), and 60 min ($p = 0.011$) min post dosing.

There were no significant differences in blood or brain esketamine or arketamine concentration after ketamine racemate inhalation (Fig. 2A and B; Table 7). However, plasma esnorketamine concentration was significantly higher than plasma arnorketamine at 60 min post dosing (Fig. 2C). These discrepancies in concentration were evident for hydroxynorketamine enantiomers. Plasma eshydroxynorketamine concentration was substantially higher than plasma ardehydroxynorketamine concentration between 20 min and 4 h post dosing (Fig. 2E). Similar results were obtained for the brain homogenate and dramatic differences between eshydroxynorketamine and ardehydroxynorketamine concentrations were observed between 30 min and 4 h post dosing (Fig. 2F).

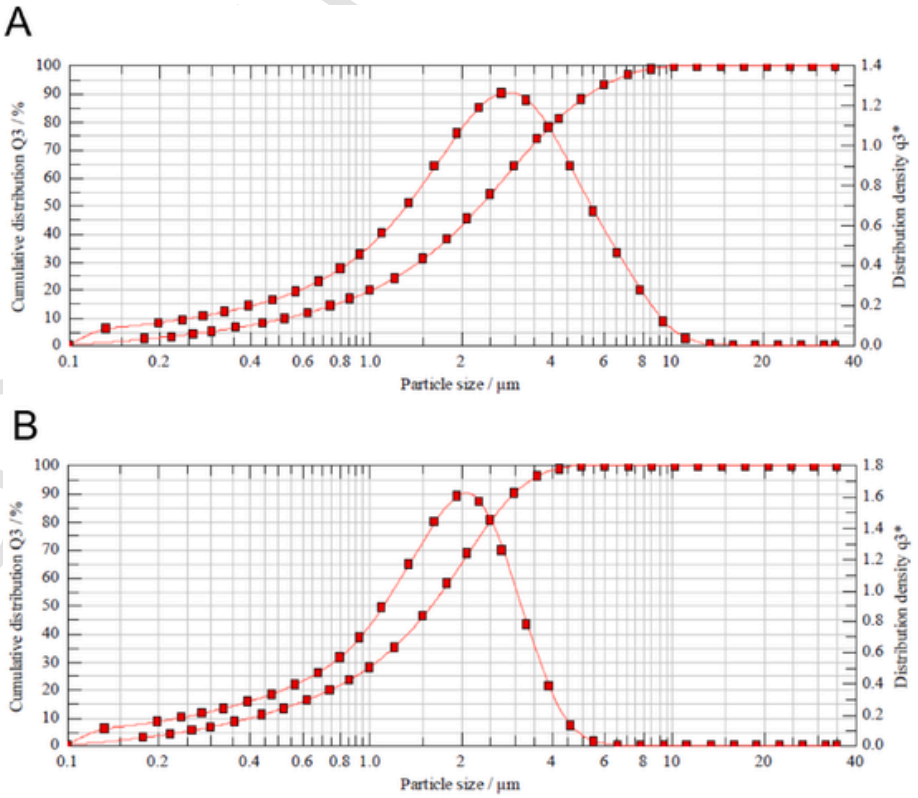

Fig. 1. Particle Size Distribution profile of esketamine (A) and ketamine racemate (B) used during the PK study presented as cumulative distribution and distribution density.

Table 6
Particle Size Distribution parameters of esketamine and ketamine racemate applied for the PK study.

	d_{10} (μm)	d_{50} (μm)	d_{90} (μm)	Span (μm)
Esketamine	0.6	2.3	5.2	2.0
Ketamine racemate	0.4	1.6	3.0	1.6

3.3. Bioavailability assessment of ketamine dry powder inhalation and intratracheal instillation

Esketamine and ketamine racemate was intravenously injected at 10 mg/kg and 20 mg/kg body weight, respectively, and to determine the bioavailability after inhalation (Fig. 3). Bioavailabilities of esketamine administered as a single enantiomer and as a racemate were 62% and 58%, respectively. Arketamine bioavailability was 73%.

To estimate administered drug losses in the airway passage, esketamine was applied by intratracheal instillation. The plasma and brain homogenate concentration-time curves showed rapid drug uptake (Fig.

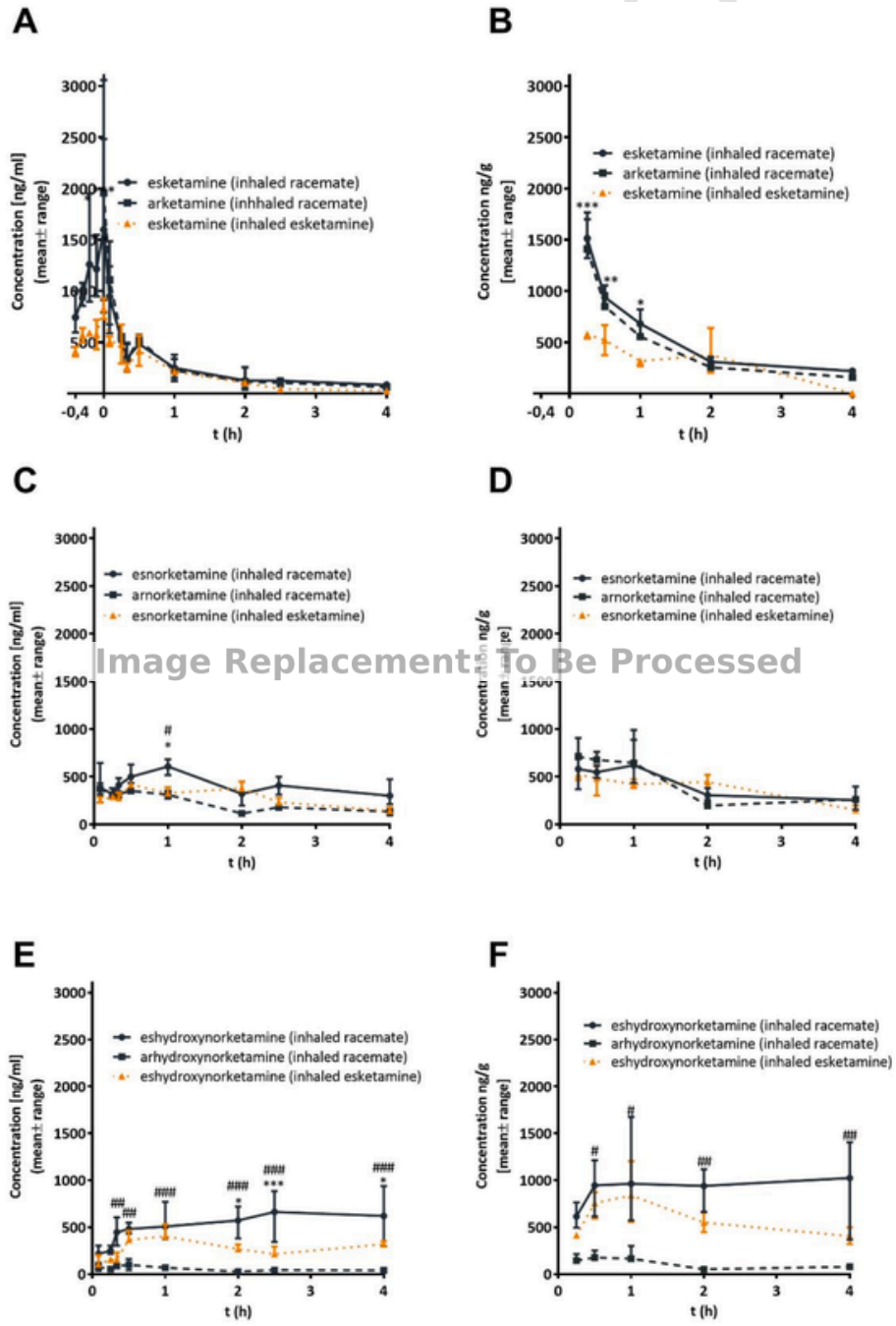

Fig. 2. Mean plasma concentrations of esketamine and arketamine in the plasma (A) and brain (B), esnorketamine and arnorketamine in the plasma (C) and brain (D), and eshydroxynorketamine and arhydroxynorketamine in the plasma (E) and brain (F) after dry powder inhalation of esketamine (10 mg/kg) or ketamine racemate (20 mg/kg). Data are presented as means ± range. Esketamine/esketamine metabolites of inhaled esketamine vs. esketamine/esketamine metabolites of inhaled ketamine racemate: *p < 0.05, **p < 0.01, ***p < 0.001; Esketamine/esketamine metabolites vs. arketamine/arketamine metabolites of inhaled ketamine racemate: #p < 0.05, ##p < 0.01, ###p < 0.001; #p < 0.05 for esketamine vs. arketamine after ketamine racemate inhalation.

Table 7
Pharmacokinetic parameters for plasma and brain esketamine and arketamine after ketamine racemate (19.9 mg/kg) and esketamine enantiomer (7.6 mg/kg) dry powder inhalation.

Parameter	Inhaled test item	Esketamine		Ketamine racemate			
	Analyte	Esketamine		Esketamine		Arketamine	
	Unit	Plasma	Brain	Plasma	Brain	Plasma	Brain
$AUC_{0\rightarrow t}$	µg*min/L	37,923	45,884	55,525	115,602	54,942	97,886
C_{max}	ng/mL	828	577	1598	1514	1958	1410
t_{max}	min	0.01	15	0.01	15	0.01	15
$AUC_{(n\rightarrow t)Brain}/AUC_{(n\rightarrow t)Plasma}$		2.17		2.49		2.24	

Abbreviations: $AUC_{0\rightarrow t}$ = area under concentration-time curve; $AUC_{(n\rightarrow t)}$ = area under concentration-time curve from first joint time point sampling to last sampling time point; C_{max} = maximum concentration; t_{max} = time to C_{max}.

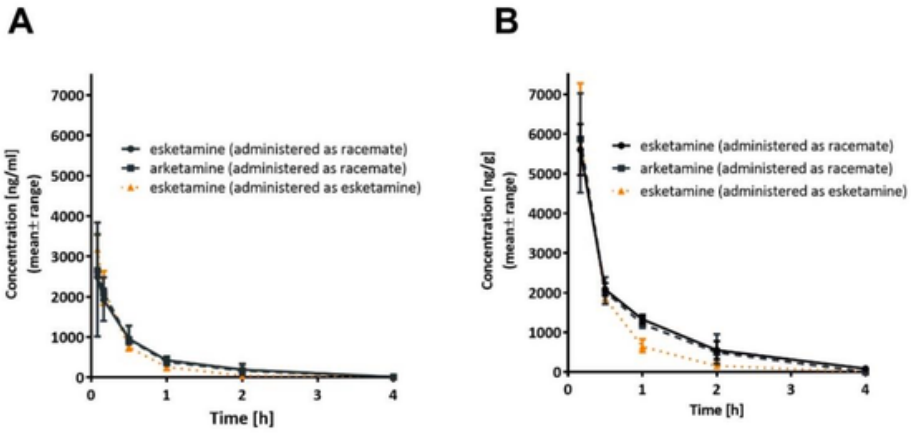

Fig. 3. Mean plasma concentration of esketamine and arketamine in the plasma (A) and brain (B) following intravenous injection of esketamine (10 mg/kg) or ketamine racemate (20 mg/kg). Data are presented as means ± range.

4, Table 9). Total esketamine bioavailability was 125%. Hence, drug absorption was complete.

3.4. Intracellular signaling activation induced by esketamine and ketamine racemate in animals

Brain tissue was collected during the pharmacokinetic study to assess molecular signaling in the neurons. No changes in protein phosphorylation or expression in the prefrontal cortices and hippocampi were detected regardless of administration route or drug (Supplementary Fig. A1–A4).

3.5. Toxicological study

3.5.1. Dry powder product containing esketamine

The composition for the esketamine dry powder was developed in-house. The physical properties were assessed by determining the bulk and tapped densities and the corresponding Carr's compressibility index and Hausner ratio (Table 10). Furthermore, the aerosol performance of the powders was studied in vitro by the use of an NGI impactor. Fine particle fraction (FPF) reached 50% of emitted dose on the system and MMAD of the particles was established at level 2.27 µm with GSD 1.94 (Table 11). The Aerodynamic Particle Size Distribution is shown in Fig. 5.

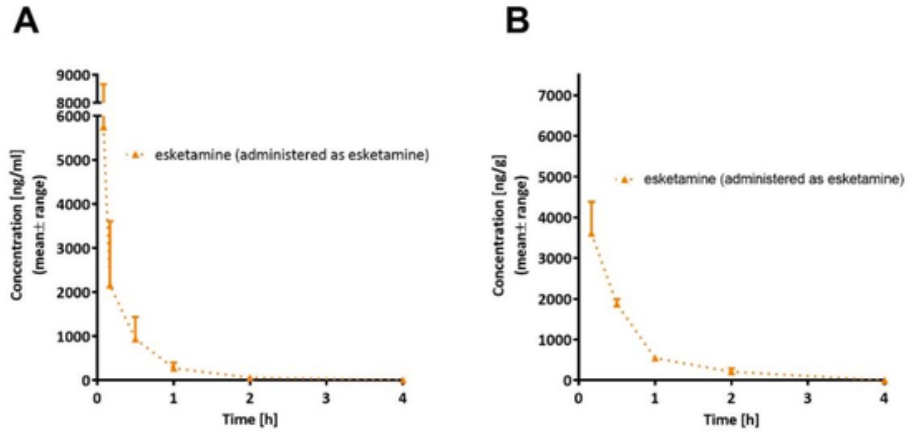

Fig. 4. Mean plasma concentration of esketamine and arketamine in the plasma (A) and brain (B) following intratracheal instillation of esketamine (10 mg/kg). Data are presented as means ± range.

Table 9
Pharmacokinetics parameters of plasma and brain esketamine after intratracheal esketamine enantiomer administration (10 mg/kg).

Parameter	Inhaled test item	Esketamine	
	Analyte	Esketamine	
	Unit	Plasma	Brain
$AUC_{0\rightarrow t}$	μg*min/L	97,624	131,899
C_{max}	ng/mL	5750	3612
t_{max}	1/min	0.04	10
$AUC_{(n\rightarrow t)Brain}/AUC_{(n\rightarrow t)Plasma}$		2.27	

Abbreviations: $AUC_{0\rightarrow t}$ = area under concentration-time curve; $AUC_{(n\rightarrow t)}$ = area under concentration-time curve from first joint time point sampling to last sampling time point; C_{max} = maximum concentration; t_{max} = time to C_{max}.

Table 10
The rheological properties of the esketamine dry inhalation powder.

Parameter	Value
Bulk density [g/mL]	0.623 ± 0.003
Tapped density [g/mL]	0.862 ± 0.006
Carr's Index	27.8 ± 1.0
Hausner Ratio	1.4 ± 0.0

Table 11
The inhalation properties of the esketamine dry inhalation powder.

Parameter	Value
FPF ≤ 5.0 μm (%)	50.0 ± 2.0
MMAD (μm)	2.27 ± 0.03
GSD	1.94 ± 0.01

Abbreviations: FPF – Fine Particle Fraction (here for particles below 5.0 μm), MMAD – mass median aerodynamic diameter, GSD – geometric standard deviation.

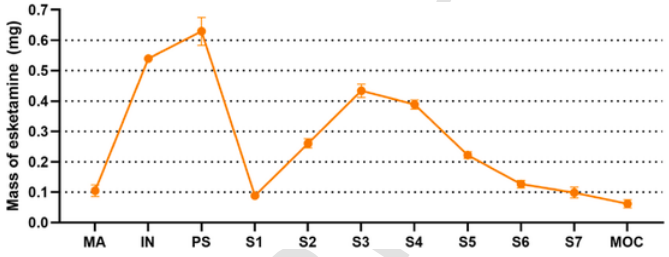

Fig. 5. Aerodynamic Particle Size Distribution of dry powder inhalation powder.
Abbreviations: MA – Mouthpiece adapter, IN – Induction port, PS – Preseparator, S1-7 – Size fractionation stages, MOC (S8) – Micro-orifice collector.

3.5.2. 14-day general toxicity in rats

No treatment-related deaths or adverse drug-related clinical signs were recorded at any point during the study. There were no esketamine-related effects on body weight, food consumption, ophthalmology, organ weight, hematology, clinical chemistry, coagulation, urinalysis, or macroscopic or microscopic examination. The no observed adverse effect level (NOAEL) in Wistar rats was 46.5 mg/kg after 14-day esketamine exposure. Exposure increased in a dose-dependent manner and the values were comparable between days 1 and 14 (Supplementary Table A.1).

The high dose was the maximum feasible dose as the generator was not able to produce a stable atmosphere with higher concentration of the aerosol.

3.5.3. 28-day inhalation toxicity study followed by 14-day recovery and cardiovascular safety assessment in dogs

There were no mortalities during the study. There were no significant esketamine-related changes in body weight, ophthalmoscopy, blood pressure, body temperature, hematology, clinical chemistry, coagulation, urinalysis, or organ weight. There were no esketamine-related gross pathological abnormalities at necropsy. The esketamine-related clinical signs in the treatment groups included slight to extreme incoordination, slight to extreme salivation, slightly decrease in activity, shaking, labored respiration, and slight increase in respiration. The aforementioned symptoms increased in severity and/or frequency in a dose-dependent manner. Most of the foregoing clinical signs were detected immediately post exposure and resolved 1 h post exposure. In a few rare cases, the animals returned to normal physiological condition only by the following morning. These clinical signs were not observed after every dose and did not manifest in all animals. All clinical signs were transient and resolved within 24 h. Exposure increased in a dose-dependent manner and the values were comparable between days 1 and 28 (Supplementary Table A.2).

Pre-treatment electrocardiographic morphology, rhythm, heart rate (HR), PR, QT, QTc interval, and QRS complex duration were normal in all dogs. Electrocardiograms recorded on the second day of treatment resembled those obtained from the examination before the onset of esketamine administration. No measurable effects on P-QRS-R complex morphology or PR, QT, QTc interval, or QRS complex duration were noted. Only the mean heart rate increased immediately post exposure, in a dose-dependent manner compared to that in pre-treatment and the control. Immediately post exposure, the mean heart rate increased by 7.8% in the control, 39.6% in the low-dose group, 48.1% in the mid-dose group, and 88.4% in the high-dose group. By treatment week 4, no further changes in heart rate were observed.

Microscopic examination revealed minimal to mild increases in the numbers of alveolar macrophages in two of three male and all the female vehicle controls and in all high-dose animals. However, there were no differences between the vehicle control and treated animals in terms of severity or incidence. After recovery, the numbers of alveolar macrophages had declined to normal levels. Hence, there was complete recovery.

The no observed adverse effect level (NOAEL) in Wistar rats was 36.5 mg/kg after 28-day esketamine exposure.

The high dose was the maximum feasible dose as the generator was not able to produce a stable atmosphere with higher concentration of the aerosol.

3.5.4. Functional observational battery (FOB)

Esketamine administration caused behavioral changes consistent with the pharmacological activity, in particular anesthetic, effect of this drug. Compared with the control animals and the data obtained before treatment onset, dose-related, statistically significant increases in the incidence of flattened posture were observed in the esketamine-treated animals at 30 min post dose. The number of animals with flattened postures gradually decreased in a dose-dependent manner. By 24 h post dose, the postures of all animals had returned to normal. At 30 min post dose, exploratory activity and arousal were at lower levels in the mid-dose and high-dose groups than they were in the control animals and before treatment onset. At 1 h post dose, exploratory activity returned to baseline levels and were comparable to that observed in the control animals. In contrast, arousal levels only returned to baseline levels by 24 h post dose. Other behavioral changes observed in the 35 mg/kg treatment group at 30 min and 60 min post dosing were circling, writhing, and/or retropulsion. Several esketamine-related clinical signs were observed in all treatment groups between administration of the first dose increment (25% of the total) and the start of the first scheduled FOB evaluation at 30 min post-dosing. These included slight to extreme decrease in activity, slight to moderate incoordination, slight to

moderate increase in respiration, slow to no righting reflex, and labored respiration. Clinical signs appeared earlier and were relatively more severe in the high-dose group. All changes detected during FOB were transient and vanished by 24 h post dosing.

3.5.5. Respiratory safety pharmacology study

At 15 mg/kg and 35 mg/kg and 30 min post-administration, tidal (37% and 41%, respectively) and minute (17% and 13%, respectively) volume increases, and respiratory rate decreases (12% for both dose levels) were observed, compared with the control. Nevertheless, none of these differences were statistically significant. Moreover, the aforementioned changes had resolved by 2 h and 24 h post dose. At 2 h post administration, no observed differences in respiratory rate, tidal volume, or minute volume in the treatment groups were associated with the drug itself, because all values remained within the normal/pre-dose ranges for this species (Fig. 6). Clinical signs observed soon after dose administration were generally more severe in the high-dose groups and were consistent with the pharmacological activity, in particular anesthetic, effect of ketamine.

4. Discussion

To the best of our knowledge, this study is the first to assess the pharmacokinetics, pharmacodynamics, and toxicology of inhaled dry powder esketamine.

During the first part of the study we compared the pharmacokinetics of esketamine inhaled as a single enantiomer and as a racemate in rats. Esketamine and ketamine racemate were micronized before administration and characterized for their particle size distribution. The particle size to reach the lung should be in the range of 1–5 μm [27]. The larger particles are expected to deposit in the upper airways not reaching the target organ. The smaller size may lead to very limited deposition and exhalation of the drug. The d_{50} value of the particle size distribution for both esketamine and ketamine racemate was 2.3 and 1.6 μm, respectively, confirming good properties for administration via inhalation.

The pharmacokinetic study aimed to compare the esketamine administered as an enantiomer or as a racemate. Therefore, equal esketamine doses were applied that corresponds to a ketamine racemate targeted dose of 20 mg/kg or 10 mg/kg for esketamine administered as an enantiomer. Inhaled ketamine caused relatively constant increases in blood esketamine and/or arketamine concentration during inhalation and maximum exposure occurred at the end of the dosing period (Fig. 3A).

The single time point esketamine concentrations were significantly higher after ketamine racemate administration than they were after single enantiomer esketamine administration. Similar trends were observed for C_{max} and $AUC_{0\rightarrow t}$ (Table 7). However, the achieved dose for esketamine that was ~25% lower than that for esketamine after ketamine racemate inhalation may explain the aforementioned higher concentrations. The enantiomers did not differ in terms of elimination constant and elimination half-time after intravenous esketamine and ketamine racemate injection (Table 8). The pharmacokinetic parameter measurements were consistent with those published elsewhere [28].

Intranasal delivery enables drug absorption through the richly vascularized nasal cavity mucosa [29–31]. Intranasal administration achieves >100% and 45–50% bioavailability in dogs and humans, respectively [32,33]. We calculated absolute esketamine bioavailabilities of 58% and 62% following single stereoisomer and ketamine racemate inhalation, respectively. No data were available for intranasal ketamine bioavailability in rats. Dry powder inhalation realizes the same ketamine bioavailability level as intranasal delivery in human. However, any further comparisons were hindered by differences in breathing

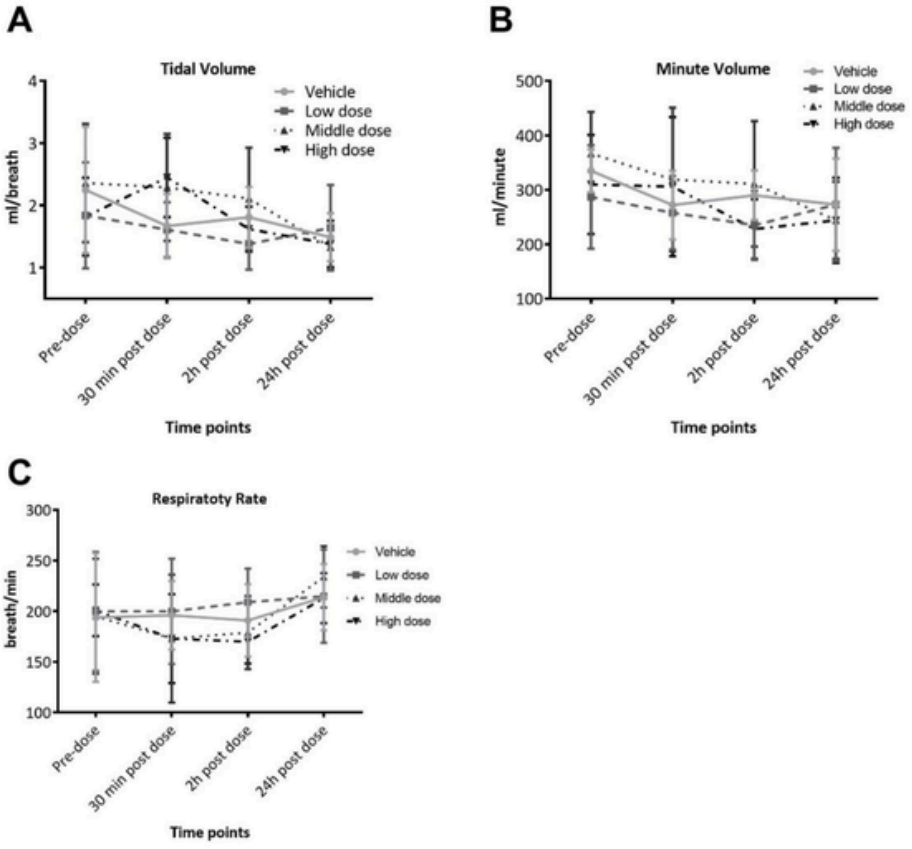

Fig. 6. Respiratory parameters of male Wistar rats following intravenous injection of esketamine (vehicle = 0 mg/kg, low dose = 5 mg/kg, mid dose = 15 mg/kg, high dose = 35 mg/kg); tidal volume (A), minute volume (B), respiratory rate (C). No significant changes were observed. Data are presented as means ± SD.

Table 8
Plasma pharmacokinetic parameters of esketamine and arketamine after intravenous administration of ketamine racemate (20 mg/kg) or esketamine (10 mg/kg).

Parameter	Test item	Esketamine		Ketamine racemate			
	Analyte	Esketamine		Esketamine		Arketamine	
	Unit	Plasma	Brain	Plasma	Brain	Plasma	Brain
$AUC_{0\rightarrow t}$	μg*min/L	78,030	168, 613	97,836	250, 696	96,282	208, 504
C_{max}	ng/mL	3179	5899	2494	5607	2653	5875
k_{el}	1/min	0.018	0.0268	0.020	0.0152	0.021	0.0155
$t_{½}k_{el}$	min	39.3	25.9	34.1	45.5	33.0	44.6
Cl_B	L/min	0.042	-	0.034	-	0.070	-
V_c	L	6.19	-	1.94	-	2.00	-
$AUC_{(n\rightarrow t)Brain}/AUC_{(n\rightarrow t)Plasma}$		2.63		2.76		2.69	

Abbreviations: $AUC_{0\rightarrow t}$ = area under concentration-time curve; $AUC_{(n\rightarrow t)}$ = area under concentration-time curve from first joint time point sampling to last sampling time point; C_{max} = maximum concentration; t_{max} = time to C_{max}; k_{el} = elimination rate constant; $t_{1/2kel}$ = half-life in elimination phase; V_c = distribution volume of first (central) compartment; Cl_B = total body clearance.

physiology and airway construction [34]. The calculated bioavailability values deem to be high, assuming that expected lung deposition in rats is as high as 10% [35]. However, taking into account the high potential of esketamine to be absorbed through epithelium, as it is the case for the intranasally delivered drug, it may not be excluded, that esketamine sediments on epithelium during inhalation is substantially absorbed there as well. Nevertheless, dry powder inhalation did not influence blood brain barrier crossing as the brain:plasma ratio was >1. Thus, the drug reached the target organ [36].

The main absorption organ for dry powder inhalation is the lung whence the drug may be immediately absorbed into circulation. We applied intratracheal esketamine instillation to determine whether lung absorption influences inhaled esketamine bioavailability. The calculated intratracheal bioavailability was 125% and the esketamine concentration curve for this administration resembled that for intravenous esketamine injection (Fig. 4A). This suggests that esketamine was rapidly and completely absorbed from the lungs, confirming that large surface area of the organ, thin epithelial membrane and high vascularization act highly enhancing for ketamine adsorption. The potential suprabioavailability after intratracheal instillation results rather from experimental setup and limitations of the study, and not the properties of esketamine. The relatively lower esketamine bioavailability after inhalation might be explained by esketamine particle ingestion, sedimentation and exhalation [37]. Nevertheless, this drug administration route regulates plasma levels and prevents high peak plasma drug levels associated with adverse effects such as psychotomimetic symptoms [38].

Ketamine is stereoselectively demethylated to norketamine which, in turn, is hydroxylated to hydroxynorketamine [39]. Norketamine (esnorketamine) and hydroxynorketamine may have antidepressant efficacy [17,40]. Therefore, we evaluated esnorketamine/arnorketamine and eshydroxynorketamine/arhydroxynorketamine (2S,6S and 2R,6R respectively) concentrations after dry ketamine powder inhalation (Fig. 2C–F). Our results corroborated reports indicating relatively faster *N*-demethylation of esketamine than arketamine and comparatively higher esnorketamine levels in rats [12,41]. There is even stronger stereoselectivity for eshydroxynorketamine metabolism. In fact, the relative differences in metabolite enantiomer concentration are even greater after ketamine racemate administration [24].

Earlier studies described the effects of ketamine on protein phosphorylation and expression in the brain and the involvement of the mTOR and pEF2 signaling pathways [42,43]. Here, we detected no relative differences in protein phosphorylation or expression regardless of the drug administered, the administration route, the time of analysis or the brain structure confirming results of other groups [24,44]. However, recent research suggests that esketamine may activate a distinct pathway by interaction with TrkB receptor enhancing BDNF signalling and hence exerting antidepressant action [45].

The aim of our toxicology study was to determine whether inhaled ketamine induces any toxic effects, particularly in the lungs. The toxicity assessment was performed according to OECD guidelines and the Scientific Advice procedure obtained from European Medicines for these studies. For this purpose, esketamine was formulated appropriately to correspond to the final product for clinical trials. The formulation included lactose as a carrier for inhalation and magnesium stearate as a lubricant to provide proper inhalation properties. Carr's index and Hausner Ratio for the formulated dry esketamine powder would suggest a rather poor flowability potential (Table 10), however, the FPF value reached a value of 50% and MMAD was 2.27 μm. Taking the rotating brush method for powder activation during the toxicology study into consideration, the powder properties provide an adequate characteristic for administration as dry powder inhalation [46–48], what was confirmed during the study by the gravimetric analysis. The maximum esketamine doses of 46.5 mg/kg and 36.5 mg/kg for rats and dogs, respectively, were also the maximum feasible doses because of the technical limitations of the device used to administer the drug to obtain stable concentration of the aerosol and maximal inhalation duration being applied.

In a repeated-dose toxicity studies performed on rats, ketamine racemate was intravenously injected at 2.5 mg/kg, 5 mg/kg, or 10 mg/kg body weight daily for 6 weeks [49]. In 3-month and 6-month repeat-dose toxicity studies, rats received intranasal administration of ≤27 mg/kg esketamine once daily [33]. Unlike the foregoing reports, our 14-day general toxicity study in rats presented no clinical signs such as changes in general activity, ataxia, food intake, or salivation. In our functional observational battery study, rats were administered ≤35 mg/kg esketamine in four equal increments. The assay confirmed that the anesthetic action of esketamine induced changes in activity, coordination, respiration, and righting reflex. Nevertheless, clinical signs were observed during our 28-day toxicology study in dogs. During 3-month and 9-month toxicological studies on once daily intranasal ≤10 mg/kg esketamine administration, appearance of these symptoms was independent of administration route [33]. Hence, formulated dry esketamine powder inhalation may induce adverse effects at higher doses compared to other administration routes. A possible explanation for this phenomenon is the fact that dry powder inhalation and intranasal administration have distinct esketamine exposure profiles. Compared to long-term intranasal studies in rats [50], our study showed at least 10 times lower C_{max} values for corresponding doses of 4.5, 15 and 45 mg/kg/day. Hence, peak plasma esketamine concentrations may be related to the observation of clinical signs.

As dry powder inhalation is a novel form of esketamine administration, we carefully examined the respiratory tract after treatment. Microscopic lung examinations after the 28-day toxicological study on dogs disclosed increases in the alveolar macrophages of all groups, including the controls, followed by complete recovery at 14 days post dosing. Particle clearance from the lung involves macrophage activation for insoluble particle phagocytosis and soluble compound diffusion or pinocytosis in the alveolar space [51]. Phagocytosis rapidly internalizes 100% of all particles within 24 h [52]. Increases in macrophage count followed by full recovery are natural processes associated with particle clearance and seem to be associated with unsoluble magnesium stearate particles. Hence, these effects resulted from inhalation of dry powder, not the active substance.

Cardiovascular assessment of dogs revealed transient, dose-dependent changes in heart rate that vanished by the end of the study. This pharmacological effect of esketamine was expected [53]. However, the observed increase in heart rate was very brief and transient.

Thus, it was not considered toxicologically relevant and the safety of esketamine doses ≤35 mg/kg was validated.

In conclusion, esketamine dry powder inhalation may provide several advantages over other administration routes. We showed that dry powder inhalation provided high bioavailability and rapid absorption of esketamine. The pharmacokinetic profile of dry powder inhaled esketamine ensures appropriate exposition with lower concentration maxima helping to limit adverse effects. We proved that dry esketamine powder inhalation was safe according to the results of our repeated-dose toxicology and safety pharmacology studies. Further enhancements of dry esketamine powder inhalation could culminate in a safe and efficacious drug delivery solution.

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CRediT authorship contribution statement

Mikołaj Matłoka: Conceptualization, Methodology, Formal analysis, Visualization, Data curation, Writing – original draft, Writing – review & editing, Funding acquisition, Project administration. **Sylwia Janowska:** Conceptualization, Methodology, Validation, Investigation, Writing – review & editing, Funding acquisition. **Anna Gajos-Draus:** Methodology, Formal analysis, Investigation, Data curation, Writing – original draft, Writing – review & editing, Visualization. **Hubert Ziółkowski:** Formal analysis, Writing – original draft, Writing – review & editing. **Monika Janicka:** Methodology, Investigation. **Przemysław Perko:** Methodology, Validation, Formal analysis, Investigation, Data curation, Writing – original draft, Visualization. **Kisło Kamil:** Methodology, Investigation, Supervision. **Piotr Pankiewicz:** Methodology, Validation. **Rafał Moszczyński-Pętkowski:** Resources. **Mateusz Mach:** Resources. **Paulina Dera:** Methodology. **Krzysztof Abramski:** Validation, Writing – original draft. **Małgorzata Teska-Kamińska:** Writing – original draft, Writing – review & editing, Visualization. **Ewa Tratkiewicz:** Conceptualization, Supervision, Project administration. **Maciej Wieczorek:** Conceptualization, Supervision, Funding acquisition. **Jerzy Pieczykolan:** Conceptualization, Writing – review & editing, Supervision, Project administration.

Declaration of competing interest

M. Matloka, S. Janowska, P. Perko, K. Kisło, P. Pankiewicz, R. Moszczyński-Pętkowski, M. Mach, K. Abramski, M. Teska-Kaminska, E. Traktiewicz, J. Pieczykolan are employees at Celon Pharma SA. A. Gajos-Draus, M. Janicka and P. Dera were employees at Celon Pharma SA, M. Wieczorek is the owner and CEO of Celon Pharma SA. S. Janowska, P. Perko, E. Traktiewicz and M. Wieczorek are authors of patent applications. H. Ziółkowski has no conflict of interest.

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Appendix A. Supplementary data

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