# Improved bioavailability of montelukast through a novel oral mucoadhesive film in humans and mice

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**Abstract**

The leukotriene receptor antagonist Montelukast (MTK) is an approved medication for the treatment of asthma and allergic rhinitis. The existing marketed tablet forms of MTK exhibit inconsistent uptake and bioavailability, which partially explains the presence of a significant proportion of MTK low- and non-responders in the population. Besides that, tablets are suboptimal formulations for patients suffering of dysphagia, for example seen in COVID-19 patients or in patients with neurodegenerative diseases such as Alzheimer's Disease. The increasing interest in repurposing of MTK for the treatment of such patients and the need for an improved bioavailability triggered us to reformulate MTK. The aim was to develop a mucoadhesive MTK film with a good safety and improved pharmacological, i.e. improved bioavailability, profile in humans as well as in a mouse model of Alzheimer´s Disease.

We tested dissolution of the mucoadhesive film containing MTK in saliva buffer and assessed pharmacoexposure and –kinetics after acute and chronic oral application in mice. Furthermore, we performed a Phase I safety / bioavailability / pharmacokinetic analysis in healthy volunteers. The latter included a comparison with the marketed tablet form as well as a quantitative analysis of the MTK levels in the cerebrospinal fluid.

The novel MTK film demonstrated significantly improved bioavailability compared to the marketed tablet in the clinical phase 1a study in healthy humans. Furthermore, there were measurable amounts of MTK present in the cerebrospinal fluid (CSF) at the 3.0 and 7.0 hour time points post drug administration in humans. In mice, MTK was detected in serum and CSF after acute and chronic exposure in a dose-dependent manner.

The developed mucoadhesive film of MTK represents a promising alternative for the tablet delivery. The oral film might lower the non-responder rate in patients with asthma and might be an interesting product for repurposing of MTK in other diseases. As we demonstrate BBB penetrance in a preclinical model as well as in a clinical study, the oral film of MTK might find its use as a therapeutic for acute and chronic neurodegenerative diseases such as dementias and stroke.

Keywords: Montelukast, drug delivery, COVID-19, Alzheimer' Disease, dementia

## Introduction

Montelukast (MTK) is a leukotriene receptor antagonist commonly used to treat patients suffering from chronic asthma as well as seasonal allergies and allergic rhinitis. MTK binds to the cysteinyl-leukotriene receptor 1 and to the leukotriene receptor GPR17 with high affinity and selectivity, thereby blocking the leukotriene signaling pathway[1,2]. It reduces leukotriene-mediated respiratory inflammation reaction such as vasoconstriction and relieves asthma symptoms[3]. Currently, MTK is marketed under the brand name Singulair® and in several generic products in oral tablet and other pharmaceutical forms such as oral granules. The available forms present a number of limitations such as inconsistent solubility, uptake, and bioavailability, for several reasons. Although MTK is freely soluble in water, its solubility increases significantly above pH 7.5 and drastically reduces under acidic conditions normally found in the gastrointestinal tract, in particular in the stomach[4]. This has led to relatively slow and inconsistent absorption into the blood stream, with maximum concentrations occurring between 2-4 hours following consumption, thereby limiting its use to chronic applications rather than for rapid acute treatment. Uptake and bioavailability of MTK is further determined by pharmacogenetics (for review see [5]). For example, more than 20% of the population is not responding to MTK with a clinical benefit [6]. Among the various genetic reasons are variations in the SLCO2B1 gene coding for the organic anion transporting OATP2B1, which has been associated with altered absorption of MTK[7]. Uptake of MTK was further modified by the intake of citrus juice[8]. Besides the physico-chemical and genetic basis for the insufficient uptake and bioavailability of MTK in its current tablet form, a further drawback is the inadequateness of the MTK tablets for patients suffering from dysphagia such as elderly patient, for patients with dementias, and for patients that require intubation or ventilation. In summary, the tablet form of MTK represent a number of limitations, and in consequence, there is an increasing interest in developing improved strategies for the delivery of MTK aiming for increased bioavailability[9].

Oral mucoadhesive application has several advantages, such as the relatively easy and convenient accessibility of the oral cavity, the limiting of first-pass effects and degradation during the gastro-intestinal transit and the rapid drug absorption due to high blood circulation in the mucosa (as reviewed in [10,11]). In general, buccal films are seen as a promising alternative application route for drugs as they combine several features, like better patient compliance (no swallowing and the possibility to modify the taste) and increased bioavailability (reviewed in [12]).

We developed a novel MTK oral mucoadhesive film to circumvent the limitations of MTK in its tablet form, tested its stability and dissolution, and performed a Phase I bioavailability and safety study in healthy volunteers, where we assessed MTK pharmacokinetics in serum and

cerebrospinal fluid. Furthermore, we wanted to investigate the possibility of repurposing MTK in form of the novel oral film for Alzheimer's Disease (AD). To test its suitability for a pre-clinical efficacy study of MTK in AD we assessed pharmacokinetics and tested the effects of acute and chronic application on pharmacoexposure in an animal model of AD, the 5xFAD mouse model.

## Materials and Methods

### *Film Preparation*

The mucoadhesive films were produced by a solvent-casting technique. For that, excipients and active pharmacological ingredient (API) in a range from 0 – 30.00 % (w/w) were mixed and dissolved/suspended in water. The resulting wet blend was spread to a thickness less than 1 mm onto a release liner. The wet film was dried in an oven for 60-75 minutes at 65°C. The dried film sheet was cut into film strips containing 10 mg of MTK. The composition of the film is depicted in table 1.

### *In vitro dissolution test*

Dissolution studies were carried out in a paddle type apparatus at 50 rpm and 37°C. Dissolution of MTK tablet, MTK mucoadhesive film and pre-solubilized MTK oral film was assessed in 900 mL of phosphate based saliva buffer (pH 6.8), For the dissolution test of pre-solubilized MTK film, a single film unit was mixed with 2 mL of saliva buffer pH 6.8 for 5 min and directly injected into the dissolution chamber. Aliquots of the release medium (8 mL) were collected at the following time points: 2.5, 5, 7.5, 10, 15, 20, 30, 45 minutes and analyzed by HPLC UV at 255 nm as specify in the USP.

### *Mechanical properties*

To determine potential impact of cold temperature during transport films containing 10 mg MTK were placed at -20°C for 15 and 30 days, respectively and were compared to a control product unexposed to cold temperature. The mechanical properties were evaluated by testing 3 films for folding endurance, and 8 films for elongation and tensile strength. A Mecmesin Multitest 1-d force testing system (SN 09-1004-11), including a Mecmesin Advanced Force Gauge 50N (SN 09-0071-11), was used to perform the tests. The flexibility of a film was assessed by repeatedly folding the film at the same place until cracking or breakage. The number of times the film was folded without breaking was recorded as the folding endurance value. A film strip was fixed at half-length on the bottom grip of the instrument, and then folded 10 times in both directions.

Elongation at break was measured by stretching the film to its maximum deformation until it torn apart. The test was carried out by affixing a film by its ends to the grips of a Mecmesin Multitest 1-d instrument and stretched at a constant speed of 10 mm/min until it cracked or broke. The length of the film section was measured before and after stretching. Elongation is defined as the ratio between the increase in film length as result of stretching, and the initial length of the film, expressed as a percentage of the initial length. Tensile strength was determined simultaneously

with the elongation test and is defined as the maximum force applied to the film area being stretched. All tests were performed in a room with controlled temperature and humidity.

*Clinical Study – Subjects and Study Design*

To determine the bioequivalence of MTK 10 mg mucoadhesive film (test drug - treatment A) and Singulair® 10 mg film-coated tablet (Merck Sharp &Dome) (reference drug – treatment B) a single-dose, randomized, Open-Label, Two Way Crossover comparative bioavailability pilot study was conducted in volunteers under fasting conditions (ethical approvement number MOD00167465). The clinic study was performed by BioPharma Services Inc (BPSI) (Toronto, Canada) and included 8 healthy non-smokers (for at least 6 months prior to first drug administration) male and non-pregnant female volunteers, with 18 years of age or older and a body mass index (BMI) within 18.5-29.9 kg/m2. To assess clinical health of the participants several laboratory tests were performed. Screenings included demographic data, medical and medication histories, physical examinations, body measurements, vital signs (seated blood pressure [BP], heart rate [HR], respiratory rate [RR] and temperature), electrocardiogram (ECG), hematology, biochemistry, serology, urinalysis, urine screening for drugs of abuse and cotinine, alcohol test and serum pregnancy test (for full list of clinical laboratory assessment see supplementary table 1). Clinical laboratory values needed to be within BPSI's acceptable range. A single dose of 10 mg MTK of either formulation was administered under fasting conditions. Blood sampling (4 mL) was performed 0.5, 1, 1.5, 2, 2.25, 2.5, 2.75, 3.25, 3.5, 3.75, 4, 4.25, 4.5, 5, 5.5, 6, 8,12 and 24 hours after dosing using a K2EDTA Vacutainer®. Additionally, CSF samples were taken 3.0 and 7.0 hours post-dose in study period 1. Participants were housed at least 10 hours prior to dosing and until 12 hours post-dose. The confinement period lasted in total at least 22 hours for each study period, with occurrence of adverse effects being closed monitored during this period. An investigator was present from approximately 30 minutes prior to dosing and until at least 12 hours (study period 1) or 4 hours (study period 2) after the last subject was dosed. The investigator remained on-call throughout the duration of the study. For safety, in each study period, vital signs (blood pressure [BP] and heart rate [HR]) were monitored before dosing and 3 and 24 hours post-dosing. The study lasted for at least 2 weeks from the study period 1 check-in, compromising a washout interval of minimum 7 days between the two clinical phases.

*Animals*

Acute exposure experiment: Two-month old C57BL6 female mice (n=10) were treated with a single dose of MTK mucoadhesive film. One group (n= 5) received a dose of 3,3 mg/kg/d of

MTK and another group (n=5) received 10 mg/kg/d of MTK. The respective films were punched out of an initial film into circular films using punch pliers with 3 mm diameter, which results in an area of 7 $mm^2$ per film. Based on the area of the film punch we had calculated the different concentrations of MTK in the initial film to reach 3.3 mg/kg/d and 10 mg/kg/d respectively in a 25 g mouse with one 7$mm^2$ film. For oral treatment mice were hold in the mouse grip and the film was placed on the oral mucosa using riffled forceps with a 0.5 diameter at the tip.

To assess serum levels of MTK blood samples were collected one, three and seven hours after the beginning of treatment (Fig 4A). For the first two time points blood was taken from the medial saphenous vein and for the last one by cardiac puncture. Mice were housed at the Paracelsus Medical University Salzburg in groups under standard conditions at a temperature of 22 °C and a 12 h light/dark cycle with ad libitum access to standard food and water. This study was approved by local Austrian ethical authorities (BMBWF-66.019/0019-V/3b/2018).

Chronic exposure experiment: Transgenic 5xFAD mice [13], which have three mutations in the gene for APP695 [APP K670N/M671L (Swedish), I716V (Florida), V717I (London)] and two mutations in the gene for PS1 [M146L, L286V] under the expression of the Thy1 promotor, were used for this experiment  Mice were housed at the animal facility of QPS Austria in groups under standard conditions with constant 12 h light/dark cycle, at a room temperature of 24°C , relative humidity of 40-70% and ad libitum access to standard rodent chow (altrumin) and water. The experiment was approved by local ethical committees (ABT13-14688/2018-4) and conducted at QPS. In this study, 5-months old animals (n=44) were treated daily with MTK mucoadhesive film for 13 weeks (Fig 4B). The film was placed on the mice buccal mucosa and the two different doses were used 3.3 mg/kg/d MTK (referred to as low dose) or 10 mg/kg/d MTK (referred to as high dose). As a control, animals received vehicle treatment (film without API).

*Quantitative assessment of MTK in –plasma and CSF human samples*

Serum and CSF was prepared as described in our previous work [14]. Samples were analyzed using an Agilent 1290 Infinity binary pump and autosampler with detection by an Agilent 6550 iFunnel QTof mass spectrometer (MS) (Agilent) using electrospray ionisation. The protonated molecules for MTK and tolbutamide (m/z 586.2177 and m/z 271.1116) were extracted with ±15 ppm mass windows to generate chromatograms with suitable combinations of specificity and signal/noise. Sample aliquots (5 µL) were injected into a mobile phase initially of 98% water / 0.1% formic acid (Channel A) and 2% acetonitrile / 0.1% formic acid (Channel B) delivered at 0.4 mL /min to an Acquity BEH C18 50 x 2.1mm, 1.7 µm column. The column was maintained at 50°C in an Agilent Infinity column oven. The mobile phase composition was programmed to

change linearly from 2% Channel B at 0.30 min post-injection up to 95% at 1.10 min, maintained at 95% until 1.75 min, and then returning to initial composition at 1.8 min. Column effluent was diverted to waste for the first 0.8 min post-injection to minimise source contamination. Data processing: MassHunter software (v B.05.01, Agilent UK). Calibration curves were fitted using the simplest regression model to minimize back-calculated calibration standard concentration residuals over the range of study sample concentrations.

*Quantitative assessment of MTK in –serum and CSF mouse samples (HPLC-MS/MS)*

The LC-MS/MS method for the quantification of montelukast in serum and CSF was described earlier[15] with a modified sample preparation as used in[16]. Briefly, sample preparation consisted of a simple protein precipitation protocol. Therefore, 12.5 µL 100% FA were added to 50 µL of serum or CSF, vortexed briefly before the addition of 150 µL 100% ACN containing MTK-d6 as internal standard. After vortexing for two minutes, all samples were centrifuged at 10 500 x g for 10 minutes at 4°C. For serum samples, 20 µL of the clear supernatant were added to 40 µL of mobile phase A (1 mM ammonium formate in water containing 0.1% FA). The supernatants (180 µL) of CSF extraction were dried under a constant nitrogen flow at 45°C. Completely dried samples were reconstituted with 60 µL 1 mM ammonium formate in 25/75 (vol/vol) acetonitrile/water containing 0.1% FA. Chromatographic separation was carried out on an Agilent 1200 series quaternary HPLC system using a Chromolith Performance RP18-e column (100x3 mm) from Merck operated a temperature of 45°C with 1 mM ammonium formate in water containing 0.1% FA as mobile phase A and 1 mM ammonium formate in 95/5 (vol/vol) acetonitrile/water containing 0.1% FA as mobile phase B. Gradient elution at a flow rate of 0.5 mL/min started from 25.0% to 95.0% B in 10.0 min, followed by a flushing step with 95.0% B for 0.8 min followed by an re-equilibration step with 25.0% B for 3.2 min. Total time for a single chromatographic run was 14.0 min. Injection volumes of 20 µL for serum and 30 µL for CSF samples were chosen. Selected reaction monitoring (SRM) measurements for MTK as well as for the d6-internal standard in obtained samples were performed on an API 4000 LC-MS/MS triple quadrupole system in positive ionisation mode. The quantifier ion transitions of MS/MS detection were m/z 586.2 to 568.2 for MTK and m/z 592.2 to 574.4 for MTK-d6. Calibration curves were derived from ratios of the peak areas of MTK and the internal standard using $1/\chi$-weighted linear least-squares regression of the area ratio versus the concentration of the corresponding internal standard MTK-d6. Analyst software 1.6.2 was used for detection, analysis and quantification of data.

*Statistical Analysis*

For statistical analysis of the human data, analysis of Variance (ANOVA) was used for ln-transformed AUCt, AUCinf and Cmax, and untransformed Tmax, λ and T1/2. Tmax was also analyzed using an additional nonparametric test (Wilcoxon test). The 90% confidence intervals (CI) for the Test/Reference ratios of geometric means for AUCt, AUCinf and Cmax were calculated based on the least square means (LSMEANS) and ESTIMATE of the ANOVA.

For statistical analysis of the animal data, Prism 8 software (GraphPad) was used. The data were tested for normal distribution with the Kolmogorov-Smirnov test. For comparison of two groups an unpaired t test was performed, whereas for comparison of more than two groups, one-way analysis of variance (ANOVA) was used with Tukey's or Bonferroni's multiple comparison test as a post-hoc test. The data were depicted as mean and standard error of the mean (SEM) or standard deviation (SD) with a 95% confidence interval as indicated in the respective figure legends. P values of $p < 0.0001$ and $p < 0.001$ were considered extremely significant (**** or ***), $p < 0.01$ very significant (**) and $p < 0.05$ significant (*).

## Results

*Composition, preparation, physical and mechanical properties of the MTK mucoadhesive film*

Besides the active pharmacological ingredient, the general composition of the film blend includes 60.0 – 85.0 %w/w wet of water as solvent and 5.0 -15.0 %w/w wet mucoadhesive polymer to form the film. Furthermore, it includes gum to modify viscosity, stabilizers to prevent degradation, plasticizers to tune mechanical properties, permeation enhancers to increase absorption and colorant and flavor for patient compliance (table 1).

The film was prepared using a solvent-casting technique, where excipients and active pharmacological ingredient (API) were mixed, dissolved in water and spread out to a thin layer that was dried and then cut into strips with a concentration of 10 mg of MTK per strip.

To assess the impact of exposure to freezing conditions on the mechanical properties of 10 mg MTK film, films were tested for folding endurance, elongation and tensile strength at $T_0$ as well as after 15 and 30 days at -20°C (table 2). Results of folding endurance testing showed that films from all three conditions can be folded at least 10 times without tearing and therefore meet the acceptance criteria of the folding test. A one-way ANOVA analysis was performed to compare the elongation mean values of the three conditions. At a $\alpha=0.05$ significance level the elongation data from films exposed to freezing conditions are not significantly different to the

initial product ($T_0$). A similar statistical analysis was applied to the tensile strength data and also revealed no significant differences between the three stability conditions.

These results show that the 10 mg MTK film has stable mechanical properties under freezing conditions for at least 30 days. Therefore, the integrity and quality of the film product are maintained in case to exposure to low temperatures during air transport of the films from the manufacturing site to the location where the clinical study would take place.

*Dissolution*

Release of MTK from films was compared to Singulair® tablets using a USP grade dissolution apparatus (Fig 1, table 3). In general, the dissolution experiments were conducted using a 10 mg dosage unit of either film or tablet. In these experiments, MTK release from coated tablets is compared to that from the mucoadhesive film and pre-solubilized film. The use of a "pre-solubilized" form of film simulated the condition in which the film, after applied in the oral mucosa, starts to slowly dissolve in saliva and is swallowed.

With MTK-film 80% of the API was released after approximately 6 minutes, while with the tablets an equivalent release was only achieved after 10 minutes. This highlights the rapid oral disintegration advantage of the film based platform. However, the most significant improvement using the film technology is observed when comparing the tablet to the pre-solubilized MTK-film. This experiment is particularly informative as it is a direct comparison of how API is released from swallowed MTK-tablets versus swallowed dissolved oral films under the same environmental conditions. This is important, as we cannot exclude that some patients, instead of placing the film on the mouth mucosa, might swallow the film. As a result, the pre-solubilized MTK-film reaches 80% released API in only approximately 1 minute, whereas the tablet reaches 80% released API after 10 minutes. This clearly demonstrates how the orally pre-solubilized MTK-film platform releases API more quickly than the MTK-tablet dosage in the stomach.

*Improved MTK bioavailability in human plasma and CSF in MTK mucoadhesive film*

We have performed a phase 1 clinical study in healthy human subjects to determine the pharmacokinetics of MTK administered in form of an oral film product compared to the marketed reference product Singulair® which both contained 10 mg MTK free base (Fig 2). Enrolled subjects had a mean age of 44 years and a mean BMI of 26 kg/m$^2$ (table 4). Pharmacokinetic parameters of MTK in plasma were collected for each subject individually and are summarized in table 5.

The area under the concentration-time curve (AUCt), measured from timepoint zero until the last sampling timepoint at 24 h, was significantly higher after the film (AUCt = 3673 ng*h/ml) than after the tablet (AUCt = 2409 ng*h/ml). The calculated film to tablet geometric mean ratio was 152.46% demonstrating a significantly higher extent of absorption after the film compared to the tablet. Similar results were obtained for the AUCinf, measured from timepoint zero to infinity, calculated as AUCt + $C_{last}/\lambda$, where $C_{last}$ is the last measurable concentration of MTK. The ratio of film to tablet was 153.15 %, again demonstrating significant higher extent of absorption of the film. Mean pharmacokinetic plasma profiles of MTK for both products over time are shown in figure 3. The geometric mean of Cmax was 338 ng/ml after treatment with Singulair® and 554 ng/ml after treatment with the film (Fig 3). Tmax was reached at 3.63 hrs after Singulair® and at 2.63 hrs after the film (Fig 3). These results indicate that the MTK film has approximately 1.5 times the Cmax and AUC (area under the curve) values compared to the Singulair® reference. This indicates that using equivalent API loading, the MTK mucoadhesive film exhibits significantly improved bioavailability. Furthermore, treatment with the film had a one hour earlier Tmax than the tablet.

Leukotriene blockers (i.e., leukotriene receptor antagonists and leukotriene synthesis inhibitors) can function to improve cognitive impairment by reducing the neuroinflammatory response within the brain (reviewed in [17-19]). Leukotriene blockers, such as MTK, must therefore cross the blood-brain barrier and accumulate in the CSF. To investigate the ability of MTK to cross of the blood-brain barrier in humans, CSF levels of MTK were measured 3 and 7 hours after dosing with the MTK mucoadhesive film. Measurable amounts, ranging from 3.2 – 4.7 ng/ml of MTK in the CSF were detected at both time points (table 6). A comparison of the mean concentrations of MTK in the CSF three hours (3.6 ng/ml) and seven hours (4.2 ng/ml) after application reveals a slower and different pharmacokinetic profile in the CSF than in the serum, where Cmax was reached after approximately 2.7 hours after application. Although, compared to serum levels the concentration of MTK in the CSF is lower, but could be more stable over time. As the mean concentration is slightly higher after seven hours than after three hours we might not have reached Cmax after seven hours. However, further studies with longer periods of observation are needed to monitor the temporal profile of MTK pharmacokinetics in the CSF in more detail.

*Clinical safety*

Six mild adverse effects (AEs), including somnolence, headache, pruritus, mechanical urticaria and back pain, were experienced by the subjects after taking MTK mucoadhesive film (table 7), four of which (pruritus, mechanical urticaria, headache and backpain) were classified as

unrelated or unlikely related to drug (table 8). The mild AEs that were classified as possibly related to the drug were somnolence reported by two subjects. No AEs were reported by the subjects after taking the reference product (table 7). No serious adverse events were reported during the conduct of this study (table 8).

*Pharmacoexposure and -kinetics of MTK in WT and in 5xFAD mice*

As outlined in the introduction, there is a current interest in repurposing of MTK for the treatment of patients with neurodegenerative diseases such as dementia patients [17,20,21], and of COVID-19 patients [22,23]. Both show dysphagia, and therefore, a mucoadhesive film might have certain advantages. Especially for repurposing of MTK in neurodegenerative diseases, preclinical evidence of efficacy in rodent models is typically required. Therefore, we tested the MTK buccal mucoadhesive film in the context of the present study for its pharmacoexposure and –kinetic profile in mice, healthy WT mice and 5-FAD mice, a mouse model of genetic AD. We first briefly assessed the serum pharmacokinetics of the buccal MTK film in 2 months old female C57bl6 wildtype (WT) mice. In a short-term pharmacokinetic experiment, WT animals received one dose of film (Fig 4A) with either 3.3 mg/kg/d (referred to as low dose) or 10 mg/kg/d (referred to as high dose) of MTK (Fig 4B). Regardless of the dose, the highest MTK concentration in serum was detected 1 hour post-dosing, whereas measurements 3 and 7 hours after the film application showed decreased MTK serum concentrations with time (Fig 5A). The animals receiving the high dose treatment showed, in general, higher MTK serum concentrations (Fig 5A). Next, we analyzed MTK serum concentration in 5 months old 5xFAD animals after chronic exposure. For that, animals were treated daily for 13 weeks with vehicle, low or high dose of MTK (Fig 4C), and serum MTK levels were assessed 7 hours after the last dose (Fig 5B). MTK serum levels were dose-dependent, with animals in the higher dose treatment group showing significantly higher serum concentrations compared to the animals in the lower dose treatment group (low dose: 103.7 ± 15.51 ng/ml, high dose 360.9± 67.5 ng/ml) (Fig 5B). As expected, in the vehicle treated group no MTK was detected in the serum (Fig 5B). Interestingly, MTK serum levels after chronic exposure were significantly higher in comparison with the serum concentrations determined 7-hours after a single dose administration (acute pharmacoexposure) (Fig 5C).

As a proof of concept we also analyzed MTK concentration in the CSF after chronic exposure and detected higher levels in the high-dose group than in the low dose group and no MTK in the vehicle group (Fig 5D), demonstrating again the ability of MTK to cross the BBB.

*Safety and general health in mice*

General health status of the animals was monitored during the chronic exposure study by weekly bodyweight assessment. Mean bodyweight was stable through the experiment (Fig 6A), with individual bodyweight fluctuating less than approximately 10 % of the initial bodyweight from week to week (Fig 6B). Mean bodyweight did not significantly differ between treatment groups (Fig 6C). Furthermore, the fecal motility of the animals was monitored for 20 minutes. There was no significant difference in the number of fecal boli between the groups (Fig 6D) showing no effect of the new formulation of MTK on fecal motility in 5xFAD mice. In summary, we conclude that prolonged treatment with low and high dose of MTK did not negatively affect general health of mice and no adverse events have occurred in mice.

## Discussion

Montelukast is an approved anti-allergic and anti-asthmatic drug which is commercialized only as solid dosage forms, in particular tablets. The limited number of MTK dosage forms available is associated to its physico-chemical properties and sensitivity to light, humidity, temperature and oxidation[9]. The commercialization of MTK in tablet forms might compromise patient adhesion to treatment among groups with swallowing difficulties such as elderly and children, or patients which require intubation / ventilation such as severe COVID-19 patients. Oral mucoadhesive films circumvent some of the limitations of solid tablets, like swallowing problems, and could be a promising alternative for MTK treatment with an improved patient compliance. In the present study we used for the first time a novel formulation of MTK, i.e. a mucoadhesive film, designed to be applied in the oral mucosa, in human and mice.

First, we compared the bioavailability and pharmacokinetic profile of MTK mucoadhesive relative to the reference formulation, Singulair® tablets. For equivalent drug loadings (10mg), MTK mucoadhesive film exhibits 50% better bioavailability compared to MTK tablets. The superior bioavailability of the MTK mucoadhesive film might be related to (1) sublingual MTK absorption, and/or (2) improved drug release. When applied in the oral mucosa, MTK mucoadhesive film releases the drug into the oral cavity, where it can be absorbed sublingually. By favoring pre-gastric absorption, MTK mucoadhesive film might limit first pass effects resulting in an increased bioavailability [9].Furthermore, as we show in vitro, the mucoadhesive film releases MTK twice as fast as the tablets, which could as well contribute to an increased MTK bioavailability. Alongside to its potential to improve patient compliance, this new formulation opens possibilities for the development of films containing a lower drug loading bioequivalent to the current reference formulation, Singulair® tablets. Additionally, as MTK mucoadhesive film shows earlier Tmax and greater Cmax compared to tablets, it might receive new indications, such as the treatment of acute allergic symptoms, in which a quick drug release and onset of action are desired.

As evidence arises from animal experiments and from case reports that MTK might be a valuable therapeutic in the field of neurodegenerative diseases[18-21,24], we found it relevant to investigate whether MTK can be detected in human CSF, as a proof of concept that MTK can pass the blood-brain barrier in humans. After a single dose treatment with MTK mucoadhesive film we detected MTK in the CSF of healthy subjects in a pharmacological relevant dose in the range of the IC50 (MTK IC50 < 5nM [25]). The initial results indicate that Tmax could in fact be delayed in comparison to the plasma level results. This may indicate that MTK accumulates in the CSF faster than its clearance. This is significant as it would allow a once a day dosing to maintain therapeutic CSF concentrations rather than multiple times in a single day, thereby improving patient compliance. Further clinical studies are needed to determine the pharmacokinetics of CSF accumulation and clearance of MTK, as, due to its invasiveness, CSF sampling was limited to two time points.

Montelukast was approved in 1998 for the treatment of chronic asthma and is generally well tolerated. In the safety meta-analysis of the 11 multicentre, randomized, controlled studies conducted by Storms et. al., (2001) a total of 3,386 adult patients (aged > 15 years) and 336 pediatric patients (6 to 14 years) were enrolled. From the total adult population 1,955 patients received montelukast and the remaining 1,431 were on placebo or an active comparator (inhaled beclomethasone). In the meta-analysis, discontinuation as a result of Adverse Events (AE) occurred in 3.7% (73 out of 1,955) adult patients treated with montelukast and 5.2% (61 out of 1,180) receiving placebo. It was also noted that there were no notable clinical differences in the frequency of reported AEs between patients receiving montelukast from those receiving placebo. The most common and frequently reported AE in the montelukast and placebo groups were upper respiratory infection, asthma, and headache. In total, the incidence of patients reporting an AE was 66.5% (1,300) with montelukast and 71.3% (841) with placebo. In 2 of 4 extension studies in the pooled analysis, no increase in treatment discontinuation or frequency of clinical or laboratory AEs were seen in patients receiving montelukast at substantially higher doses than the marketed 10-mg dose. In these 2 studies, patients received montelukast at 200 mg for 22 weeks, 100 mg for 12 weeks and 50 mg for 28 weeks with no observed change in the safety or tolerability profile. In all 4 extension studies, the total continuous exposure to montelukast for the adults was 698 patients for up to 6 months, 525 patients for at least 1 year, 152 patients for at least 2 years and 25 for up to 3 years, providing an overall exposure of 734 patient years[26]. In the study presented herein six adverse events were documented occurring after taking the MTK film. Two study subjects experienced somnolence, which was possibly related to MTK. The other adverse events were classified as unlikely related and unrelated to MTK. Sleep disturbances,

especially nightmares, have been reported after the use of MTK in asthma patients in children, but also in adults (reviewed in [27]), but they stop shortly after discontinuation of the treatment[28]. A retrospective study analyzing adverse reactions of MTK reported to the Netherlands Pharmacovigilance Center Lareb and the WHO Global database, VigiBase® identified aggression, nightmares and suicidal ideations as the most common reported adverse drug reactions and highlighted the importance of informing patients and parents of the possibility of neuropsychiatric side effects in the clinics [29]. Of course, these neuropsychiatric side effects need to be considered in future clinical studies, although they demonstrate possible effects of MTK on the brain.

Secondly, we analyzed pharmacokinetics of MTK in a transgenic mouse model of AD, because recently it was shown that pharmacological inhibition of leukotriene signaling had beneficial effects in several mouse models of AD[30-34]. The leukotriene receptor antagonist MTK has been proposed as an interesting candidate for drug repurposing in AD patients[20,21,24], due to its potential to modulate neuroinflammation and improve memory in animal models of stroke[35], epilepsy[36,37] and lewy body dementia[38]. However, the cellular and molecular mechanisms underlying MTK action on CNS remain yet poorly understood and pre-clinical experiments are needed to shade light onto its efficacy to modulate cognitive function. Here, we evaluated the pharmacokinetics of MTK mucoadhesive film in rodents, after acute and chronic treatment following different dosing and observed dose dependent MTK serum levels after acute and long-term treatment. Interestingly, MTK serum levels 7 hours post dosing were significantly lower after a single dose of 10 mg/kg/d compared to MTK serum levels after 3 months of daily treatment with the same dose. This suggest that MTK accumulates in the body during long-term treatment, which might stabilize and/or prolong effects of MTK. However, as acute pharmacoexposure experiments were performed in C57bl6 animals and chronic pharmacoexposure experiments were performed in transgenic 5xFAD mice, we cannot discharge the hypothesis, that the observed differences MTK pharmacokinetics are related to the different genetic background of the mouse models used.

Interestingly, we could detect dose dependent concentrations of MTK in the CSF of 5xFAD mice after chronic treatment. This constitutes an important prerequisite for future pre-clinical efficacy studies of MTK and shows the relevance of the 5xFAD mouse model as a tool to better understand the effects of MTK chronic treatment in AD. Overall, the results here presented, from both human and mice studies, pave the way for larger clinical phase 2 study to determine the efficacy of MTK to improve cognitive function in patients suffering from Alzheimer´s disease or to

alleviate symptoms seen in COVID-19 patients, which includes also neurological and cognitive deficits[39] (reviewed in [40]) .

## Conclusion

In summary we have demonstrated safety, convenience and improved bioavailability of MTK in form of the mucoadhesive film compared to the coated tablet in humans. Furthermore, we demonstrate BBB penetrance in a clinical study in human healthy subjects as well as in a preclinical mouse model for Alzheimer`s disease. This paves the way for potential therapeutic use of the oral mucoadhesive MTK film in acute and chronic neurodegenerative diseases such as Alzheimer´s disease, dementia with Lewy bodies, stroke, spinal cord injuries, and other diseases such as COVID-19.

## Availability of data and material

The datasets analyzed during the current study are available from the corresponding author on reasonable request.

## Conflict of interest

Justin Conway, Erick Gonzales-Labrada, Rodolphe Obeid, Horst Zerbe, Nadine Paiement are from IntelgenX, who has filed patent applications on the MTK film and its use. Ludwig Aigner is consultant at IntelgenX.

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## Figure Legends

**Figure 1**: Graphical representation comparing the in vitro dissolution rates of (Turquoise) the MTK oral film, (Blue) pre-solubilized MTK oral film, and (Red) MTL tablet Singulair®.

**Figure 2**: Study plan of the clinical Phase 1 study.

**Figure 3**: Pharmacokinetic profiles of MTK film and tablet.

MTK plasma concentration versus time profile after administration of 10 mg MTK film and tablet. Data are shown as mean +/- SEM.

**Figure 4**: Design of the preclinical testing of MTK.

Experimental setup of the pharmacoexposure and -kinetics experiment in WT and 5xFAD mice. (A) MTK Film for mice were prepared as punches suitable for placing the films on the toung of mice. (B) In a pre experiment C57Bl6 mice were treated with a single dose of MTK. Blood samples were taken 1, 3 and 7 hours after systemic application. (C) 5 months old 5xFAD transgenic mice were treated daily with vehicle or montelukast in two different doses for 89 days. On the last day of treatment mice were perfused and blood and CSF samples were collected approximately seven hours after the last application of MTK.

**Figure 5**: Serum levels of Montelukast after MTK film application.

(A) MTK concentration in the serum decreases over 7 hours. (B) Samples were taken approximately 7 hours after last oral application of MTK by cardiac puncture. Samples were analyzed for MTK concentration by HPLC-MS/MS. There is a dose dependent increase in serum levels of MTK. (C) After long term treatment the serum concentration of MTK after 7 hours is significantly higher than after single dose treatment with a dose of 10 mg/kg/d. (D) Pooled samples (n=14-15 per group) were analyzed for MTK concentration by HPLC-MS/MS. There is a dose-dependent increase in the MTK concentration in the CSF. Data are shown as mean +/-

SEM. One-way ANOVA with bonferroni post hoc test or students t-test were performed. P-value <0.05 were considered significant.

**Figure 6**: Safety profile of MTK film in mice.

Bodyweight of the mice was assessed on a weekly basis for 13 weeks as a parameter of health. (A) Mean bodyweight did not significantly differ between the weeks. (B) Individual bodyweigths did fluctuate less than +/- 10 % each week. (C) Bodyweights did not significantly differ between groups. (D) Fecal motility did not differ significantly between all groups. Data are shown as mean +/- SEM. One-way ANOVA with bonferroni post hoc test or students t-test were performed. P-value <0.05 were considered significant.

**Table 1**: General composition of the mucoadhesive film. API = active pharmacological ingredient.

| **Excipient** | **Function** | **Composition (%w/w wet)** |
|---|---|---|
| Water | Solvent | 60.0 – 85.0 |
| Mucoadhesive Polymer | Film forming polymer | 5.0 – 15.0 |
| Gum | Viscositiy modifier | 0.50 – 3.00 |
| Stabilizers | Degradation prevention | 0.01 – 0.05 |
| Colorant/Flavor | Patient compliance | 0.10 – 1.00 |
| Plasticizers | Tune mechanical properties | 0.10 – 4.00 |
| Permeation Enhancer | Increase oral absorption | 0.10 – 2.00 |
| API | therapeutic | 0 – 30.00 |

**Table 2**: Mechanical characterization of the mucoadhesive film: folding endurance, elongation and tensile strentgh of the 10 mg MTK film exposed to normal ($T_0$) and freezing conditions (T 15 days and 30 days). Results are shown as the average of three - eigth films (mean with SD).

| **Time** | **$T_0$** | **15 days** | **30 days** |
|---|---|---|---|
| Folding endurance | >10 | >10 | >10 |
| Elongation at break (%) | 62 ± 6 | 69 ± 7 | 65 ± 9 |
| Tensile strength (kPa) | 128 ± 10 | 115 ± 15 | 123 ± 13 |

**Table 3**: In vitro dissolution of 10 mg MTK film vs Singulair® tablet.

| **Sample** | **Time to 80% API released [min]** |
|---|---|
| Intelgenx MTK film | 6 |
| Pre-solubilized Intelgenx MTK film | <1 |
| Singulair® tablet | 10 |

**Table 4**: Study demographics of the clinical Phase 1 study

| | **Age** | **Height (cm)** | **Weight (kg)** | **BMI** |
|---|---|---|---|---|
| **Mean** | 44 ±6 | 174±10.9 | 79.2±14.1 | 26±3 |
| **Median** | 46 | 176.5 | 76.2 | 26.5 |
| **Range** | 31-50 | 154.8 - 186.8 | 55 - 98.7 | 22.4 - 29.4 |

**Table 5**: Comparative bioavailability analysis for plasma MTK buccal film versus Singulair tablet in humans.

| | Geometric means | | Ratio of geometric means (Test/Reference in [%]) | 90% confidence interval | Intra-subject CV (%) |
|---|---|---|---|---|---|
| Parameter | film | Singulair® | | | |
| AUCt (ng*h/mL) | 3673 | 2409 | 152.46 | 101.02-230.10 | 45.58 |
| AUCinf (ng*h/mL) | 3827 | 2499 | 153.15 | 99.77-235.1 | 45.88 |
| Cmax (ng/mL) | 554 | 338 | 163.89 | 99.12-270.99 | 57.05 |
| Tmax (hrs) | 2.63 | 3.63 | | | |

AUCt: Area under the concentration-time curve from time zero until the last measurable concentration or last sampling time t, whichever occurs first. AUCt is estimated using the trapezoidal method. AUCt/AUCinf: The proportion of AUCinf covered by the actual sample schedule. AUCinf: Area under the concentration-time curve from time zero to infinity, calculated as AUCt + Clast/λ, where Clast is the last measurable concentration. Cmax: The maximal observed plasma concentration. Tmax: Time when the maximal plasma concentration is observed.

**Table 6**: Descriptive statistics for MTK concentrations in cerebrospinal fluid at 3.0 and 7.0 hours post-dose after a single dose of 10 mg MTK oral mucoadhesive film in humans.

| **Descriptive Statistics** | **3.0 hours** | **7.0 hours** |
|---|---|---|
| N | 8 | 8 |
| Max (ng/mL) | 4.2 | 4.7 |
| Min (ng/mL) | 3.2 | 3.8 |
| Median (ng/mL) | 3.4 | 4.2 |
| Mean (ng/mL) | 3.6 | 4.2 |
| Std Dev (ng/mL) | 0.36 | 0.31 |
| CV (%) | 10.2 | 7.19 |

**Table 7**: Adverse events reported during the study.

| System Organ Classification/ Preferred Term (PT) | Reported Incidence by Treatment Group | |
|---|---|---|
| | MTK Film (N = 8) | MTK Tablet (N = 8) |
| **Nervous System Disorders** | | |
| Somnolence | 2 (25.0%)) | 0 (0.0%) |
| Headache | 1 (12.5%) | 0 (0.0%) |
| **Skin and Subcutaneous Tissue Disorders** | | |
| Pruritus | 1 (12.5%) | 0 (0.0%) |
| Mechanical urticatria | 1 (12.5%) | 0 (0.0%) |
| **Musculoskeletal and Connective Tissue Disorders** | | |
| Back pain | 1 (12.5%) | 0 (0.0%) |
| **Total** | 6 (75.0%) | 0 (0.0%) |

MTK Film: Montelukast 10 mg Oral Film; Lot No: 16E-MOT010-0001, by IntelGenx Corp.

MTK Tablet: Singulair® 10 mg Tablets; Lot No: L044452, by Merck Sharp & Dohme Ltd.,UK.

**Table 8**: Severity, relationship to study and action taken for adverse events.

| | Severity | | | Relationship to Drug | | | | Intervention | | |
|---|---|---|---|---|---|---|---|---|---|---|
| Treatment Group | Mild | Moderate | Severe | Unrelated | Unlikely | Possible | Probable | Pharmacologic | Other | None |
| A | 6 | 0 | 0 | 3 | 1 | 2 | 0 | 2* | 1* | 4 |
| B | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 | 0 |
| Total | 6 | 0 | 0 | 3 | 1 | 2 | 0 | 2 | 1 | 4 |

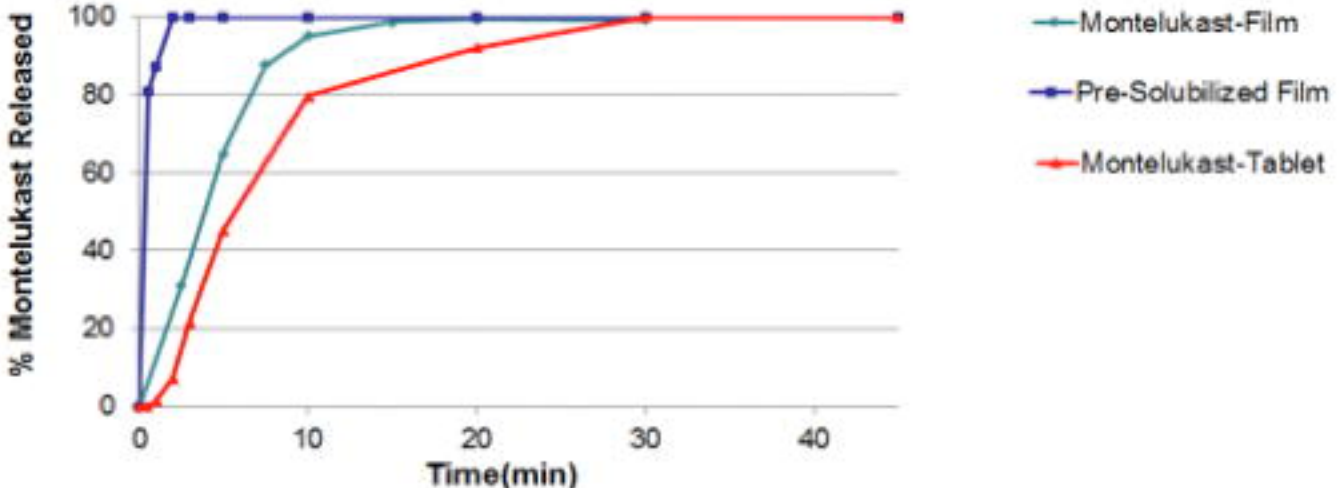
* One Subject was treated with both pharmacological and other interventions for AE Back pain in Period 1.

***Supplementary table 1***: Detailed clinical laboratory assessment of Phase 1 study subjects

| **TEST** | **COMPONENTS** | |
|---|---|---|
| Hematology | • Hemoglobin<br>• Platelet count<br>• WBC and differential | • Peripheral blood smear<br>• Hematocrit<br>• RBC |
| Serum Chemistry | • Glucose<br>• Calcium<br>• Sodium<br>• Chloride<br>• Albumin<br>• Protein<br>• Bilirubin<br>• Lactate Dehydrogenase | • AST<br>• ALT<br>• Potassium<br>• Alkaline Phosphatase<br>• Urea<br>• Uric Acid<br>• Creatinine<br>• Creatine Kinase |
| Urinalysis | • Bilirubin<br>• Blood<br>• Glucose<br>• pH<br>• Ketones | • Leukocytes<br>• Nitrites<br>• Protein<br>• Specific Gravity<br>• UBG |
| Additional Tests | Serology (HIV, Hepatitis B surface antigen, Hepatitis C antibody) | Alcohol test<br>Urine Cotinine<br>Serum hCG (females only at screening)<br>Urine hCG (females only at each period<br>check-in) |
| Urine Tests for Drugs of Abuse | Marijuana, Amphetamines, Phencyclidine, Barbiturates, Cocaine, Opiates, Benzodiazepines | |

***Supplementary table 2***: Detailed study demographics of Phase 1 study subjects

| Subject | Age | Height | | Weight | | | | |
|---|---|---|---|---|---|---|---|---|
| No. | (years) | (cm) | (in) | (kg) | (lb) | BMI | Gender | Race/Ethnicity |
| 1 | 41 | 172.3 | 67.8 | 84.6 | 186.5 | 28.5 | Male | Black |
| 2 | 45 | 154.8 | 60.9 | 55 | 121.3 | 23 | Female | Hispanic/Latino |
| 3 | 48 | 186.8 | 73.5 | 98.7 | 217.6 | 28.3 | Male | White |
| 4 | 47 | 173.5 | 68.3 | 74.1 | 163.4 | 24.6 | Female | Black |
| 5 | 46 | 161.5 | 63.6 | 76.8 | 169.3 | 29.4 | Male | Hispanic/Latino |
| 6 | 31 | 179.5 | 70.7 | 72.1 | 159 | 22.4 | Male | Black |
| 7 | 50 | 180.7 | 71.1 | 75.5 | 166.4 | 23.1 | Male | White |
| 8 | 46 | 182.5 | 71.9 | 96.5 | 212.7 | 29 | Male | White |


% Montelukast Released
100
80
60
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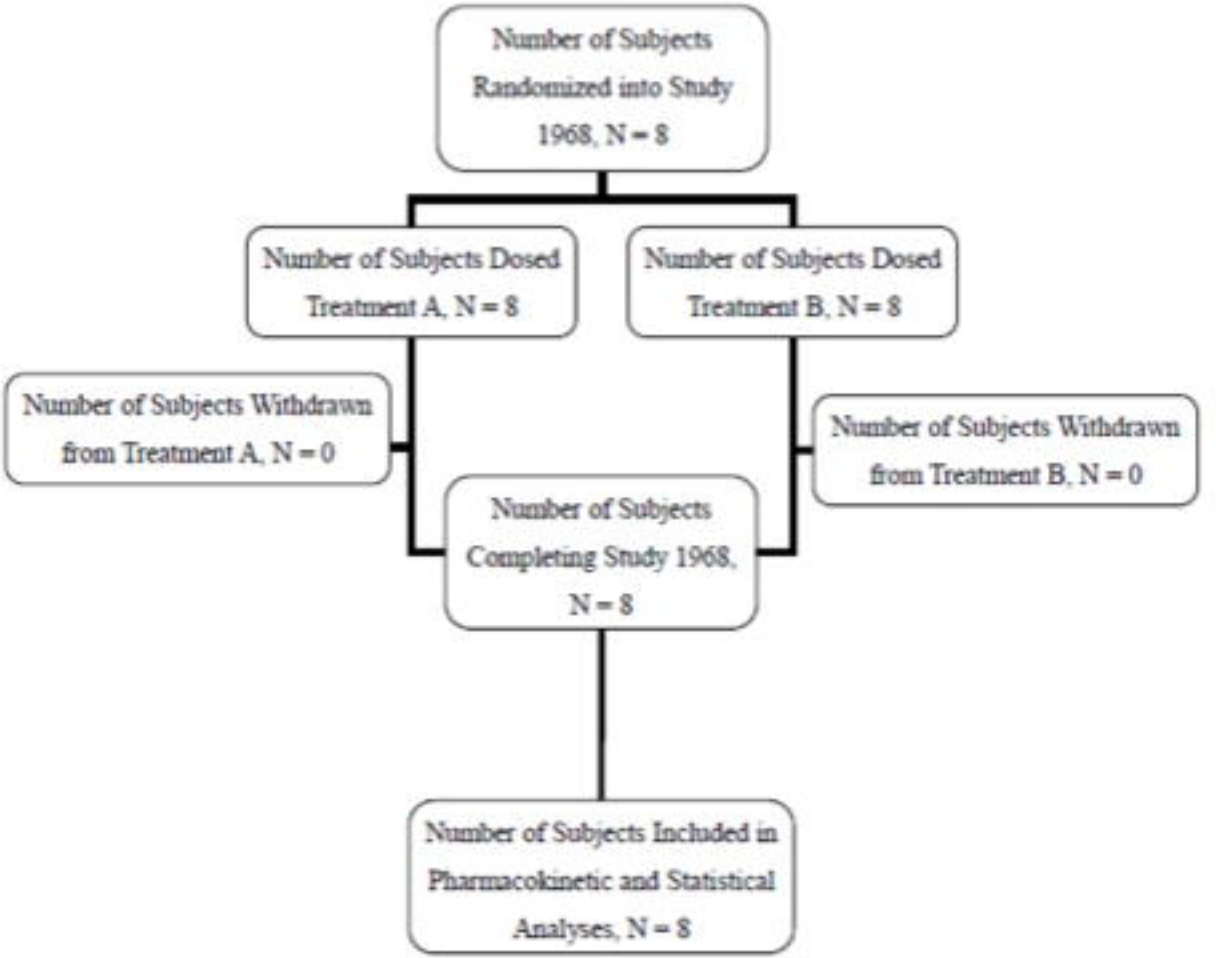
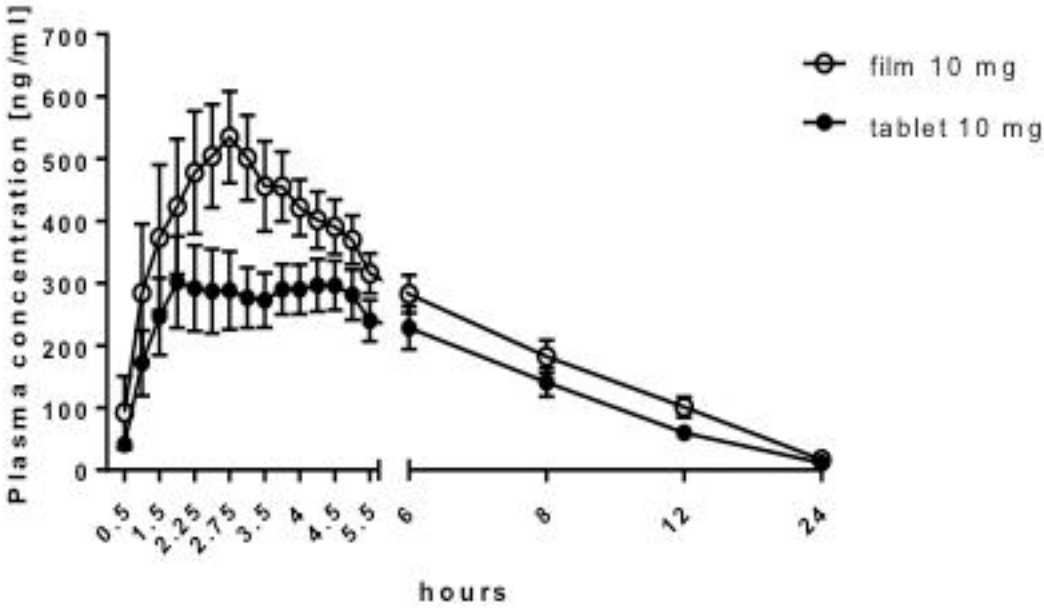
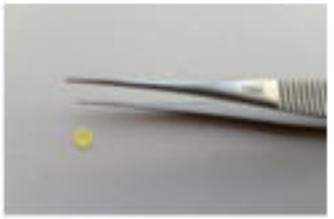
Time(min)
Montelukast-Film
Pre-Solubilized Film
Montelukast-Tablet


Number of Subjects Randomized into Study 1968, N = 8
Number of Subjects Dosed Treatment A, N = 8
Number of Subjects Dosed Treatment B, N = 8
Number of Subjects Withdrawn from Treatment A, N = 0
Number of Subjects Withdrawn from Treatment B, N = 0
Number of Subjects Completing Study 1968, N = 8
Number of Subjects Included in Pharmacokinetic and Statistical Analyses, N = 8

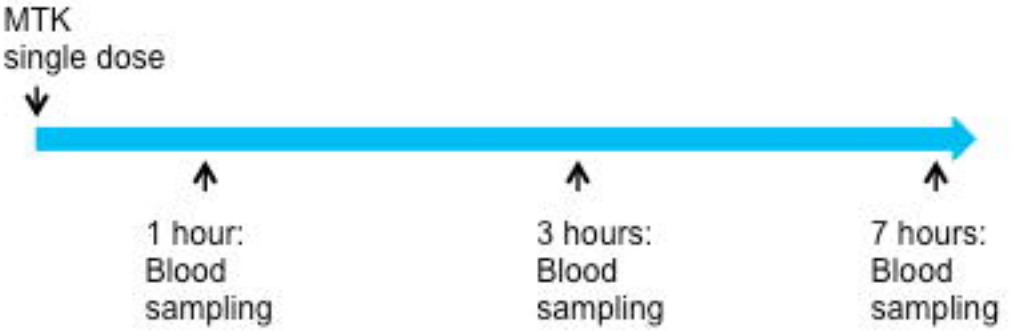



| Sample | Cmax (ng/ml) | Tmax (hrs) | AUC (AU) | Bioavailability |
|---|---|---|---|---|
| Intelgenx MTK film | 599 | 2.70 | 3910 | 1.5 |
| Singulair® tablet | 386 | 3.63 | 2617 | 1 |

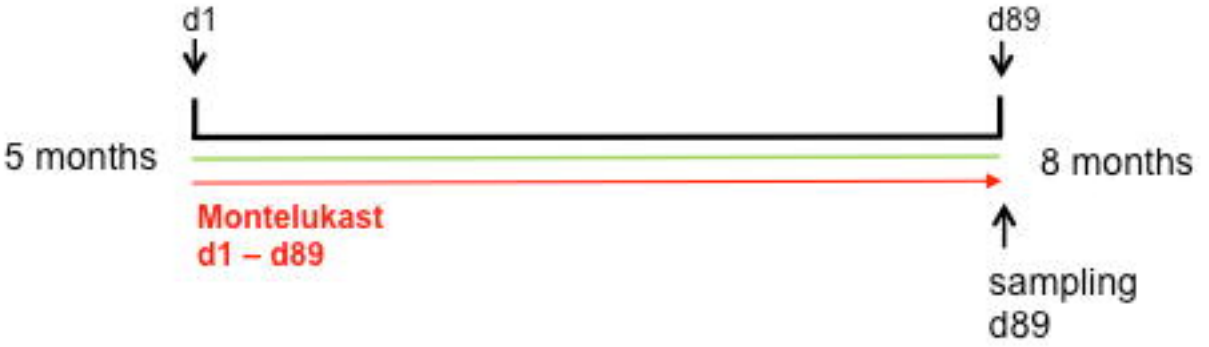
A

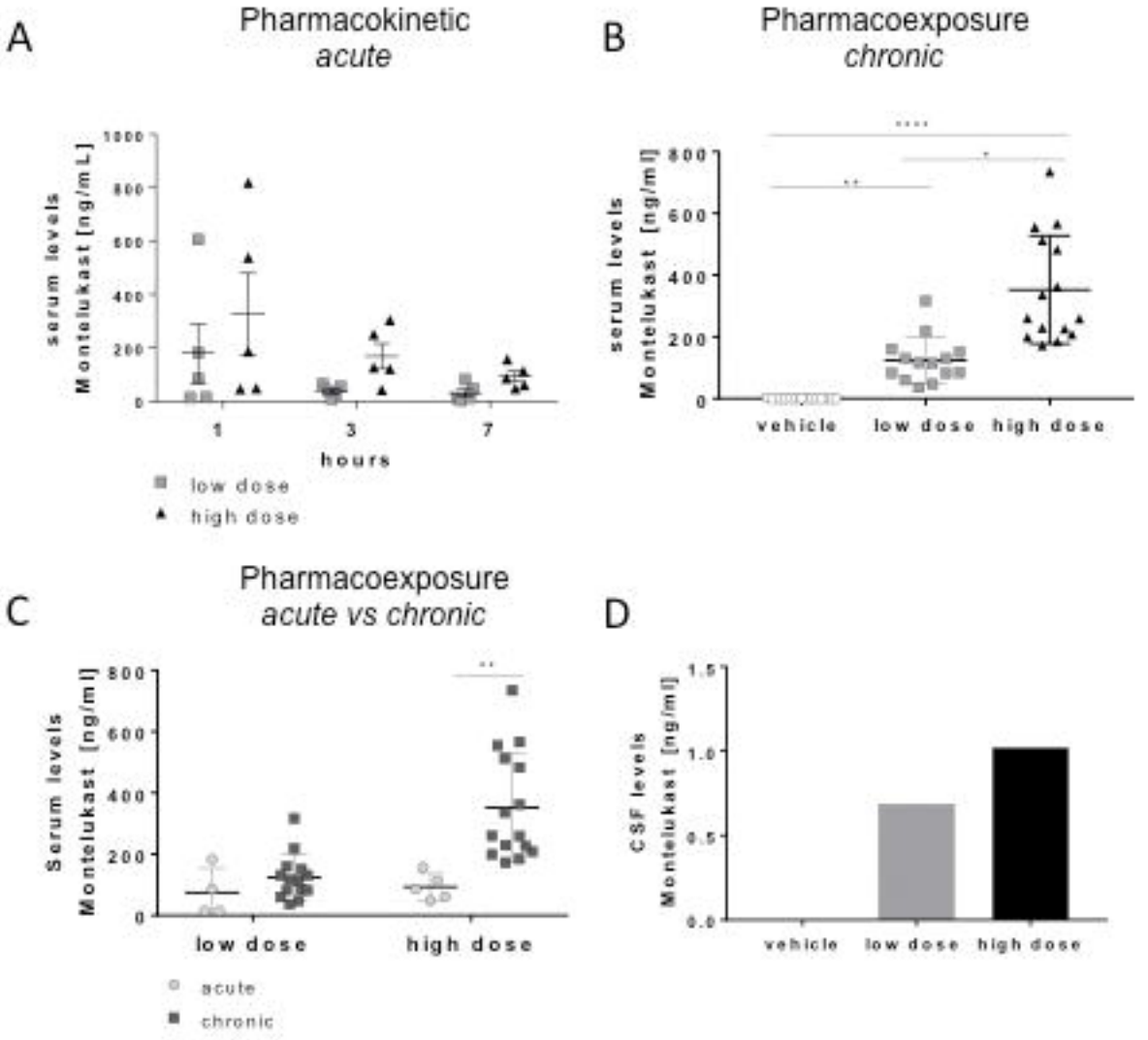


B

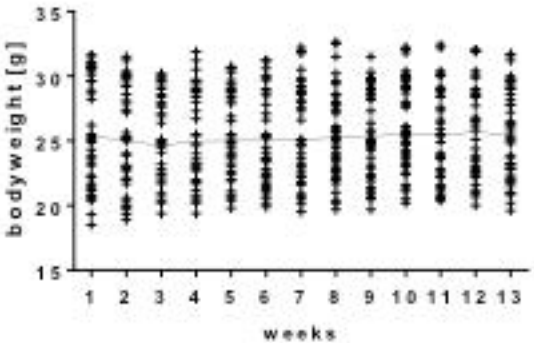

MTK
single dose
1 hour:
Blood
sampling
3 hours:
Blood
sampling
7 hours:
Blood
sampling

C

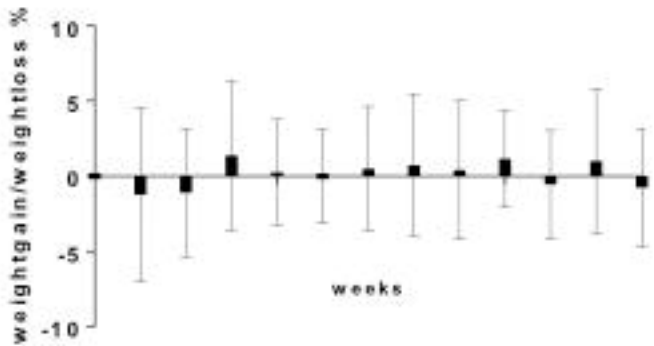

d1
d89
5 months
8 months
Montelukast
d1 – d89
sampling
d89


A
Pharmacokinetic
acute
serum levels
Montelukast [ng/mL]
1000
800
600
400
200
0
1
3
7
hours
low dose
high dose
B
Pharmacoexposure
chronic
serum levels
Montelukast [ng/ml]
800
600
400
200
0
****
**
*
vehicle
low dose
high dose
C
Pharmacoexposure
acute vs chronic
Serum levels
Montelukast [ng/ml]
800
600
400
200
0
**
low dose
high dose
acute
chronic
D
CSF levels
Montelukast [ng/ml]
1.5
1.0
0.5
0.0
vehicle
low dose
high dose

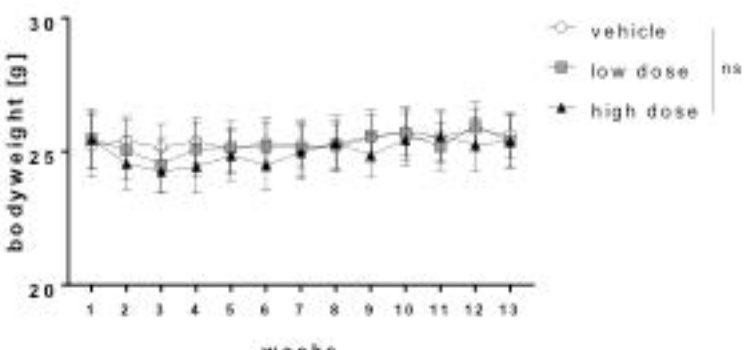
A Bodyweight over time




B




C Bodyweight between groups




D