NONCLINICAL SAFETY OF ALX-009, AN ANTIMICROBIAL THERAPY FOR CYSTIC FIBROSIS LUNG INFECTIONS

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INTRODUCTION

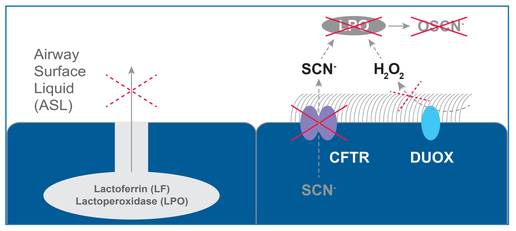
The physical entrance of microorganisms to the body may produce diseases symptoms by diverse microorganism-host interactions. In healthy people, the microbial infection is first managed by the innate defenses that pre-exist in all individuals. This innate immune defense system begins to act within minutes after the microbial invasion by eliminating the infection agents thanks to broadly non-specific and specific effector molecules but without recruitment of any cellular component.

In the lung, one of these innate defenses is the so called Lactoperoxidase system. This system produces, among others, the hypothiocyanite ($OSCN^-$) with proven broad antimicrobial properties. $OSCN^-$ is a highly reactive compound that oxidizes free thiol residues of bacterial proteins with consequent perturbation of the bacterial physiology and induction of bacterial death. In addition, the Airway Surface Liquid (ASL) is composed of a large number of antimicrobial proteins and peptides, including lactoferrin. Lactoferrin may act by direct interaction with bacterial cell membranes and/or by depriving bacteria of iron due to its iron chelator activity.

$OSCN^-$ and lactoferrin, two components of the innate immune system, are among the bactericidal compounds that are deficient in the ASL of Cystic Fibrosis (CF) patients (Fig. 1).

By providing both molecules, ALX-009, a fixed combination of $OSCN^-$ and lactoferrin, will contribute to restore the natural capacity of the lung to fight against infections. Previous *in vitro* studies have demonstrated the broad spectrum of action of ALX-009 that validates its potential to fight against chronic lung infections caused by naturally or acquired multidrug resistant bacteria such as *Achromobacter* spp., *Burkholderia* spp., *Pseudomonas aeruginosa* and *Stenotrophomonas maltophilia.*

Fig. 1



Presence of $OSCN^-$ is significantly influenced by the CFTR function. Indeed, the precursor molecule SCN^- transits by the CFTR channel to the ASL where it reacts with H_2O_2 via a lactoperoxidase enzymatic reaction to produce $OSCN^-$. However, in CF patients, the SCN^- traffic via the CFTR channel is inefficient and the secretion of lactoperoxidase seems also impaired by the obstruction of goblet cells by the thick mucus covering the CF lung epithelia. $OSCN^-$ production is then compromised. Lactoferrin secretion is also blocked by the mucus, reducing even more the natural ASL defense capacity.

TEST ITEMS

Test items, bovine lactoferrin (bLF) and $OSCN^-$, were pharma grade. Due to its high reactivity leading to limited half-life, $OSCN^-$ was produced just before use in these studies. $OSCN^-$ doses are reported as the average values of each production during the in-life study period. The high reactivity of $OSCN^-$ hampers also the production of highly concentrated solutions. For this reason, the dose increase required by guidelines, in classical study designs of toxicological and safety pharmacology studies, was achieved by an increase of dosing time across the treated groups. In addition, analytical detection of $OSCN^-$ in the pharmacokinetics (PK)/toxicokinetics (TK) studies was precluded by technical limitations inherent to its physicochemical properties. Therefore, SCN^-, the most abundant and stable metabolite of $OSCN^-$, was used as an exposure marker to $OSCN^-$. $OSCN^-$ exposure in animals included in the inhalation studies was expressed as SCN^- exposure in the PK/TK measurements.

STUDY DESIGNS

The studies were conducted under GLP conditions. All the studies were designed to fulfill the requirements of ICH M3(R2) to proceed with a First-In-Man study with ALX-009 and subsequent clinical phases II and III.

Study designs, dosing regimens and major findings are detailed in tables 1 and 2. Sub-acute and chronic safety profiles of ALX-009 were evaluated by administering ALX-009 by inhalation to rats, by snout-only device or with mask inhalator to dogs. The dose increase was done by applying different inhalation times for the low, mid and high dose groups. The dosing regimen for the sub-acute studies was based on the results of previous Maximal Tolerated Dose studies, not reported here. Control groups received air only and were submitted to the procedure at time identical to the high dose group of each study. Satellite animals were allocated to the sub-acute and chronic rat studies for pharmacokinetics examinations. In the sub-acute and chronic dog studies, pharmacokinetic samples were obtained from main study animals. All the studies were approved by the local Ethical Committee and were designed to minimize number of animals and animal suffering.

RESULTS AND DISCUSSION

GENOTOXICITY AND SAFETY PHARMACOLOGY FINDINGS (Table 1).

Under the experimental conditions of the studies, ALX-009 did not show any genotoxic potential in any of the *in vitro* and *in vivo* tests performed.

The analysis of undesirable pharmacological activity demonstrated that single or repeated dosing of ALX-009 in rats do not affect their behavior or physiological function as measured by a modified Irwin test.
The effects on the cardiovascular system were assessed by means of the administration of a single dose via intravenous injection and did not reveal any changes in telemeterized dogs. Additionally, effects on the electrocardiogram (ECG), blood pressure and pulse rate parameters were also analyzed in the dog chronic general toxicology study by inhalation; no treatment-related effects on the ECG, blood pressure and pulse rate parameters were observed.
Single administration of ALX-009 did not reveal effects on the rat's respiratory function after 6h of continuous inhalation. Under the experimental conditions tested, there were no statistically significant differences between the minute volumes (overall pulmonary ventilation), recorded for ALX-009 treated groups, and the air-treated control group. Minor changes reported as higher respiratory rates and lower tidal volume were recorded during administration in the mid dose group, and post exposure in low and mid dose groups. However, none of these changes reached statistical significance when compared with control group values and were thus considered not related to ALX-009 administration.

Table 1. ALX-009 Safety Pharmacology and Genotoxicity study designs and findings.

STUDY	Administration route	Test system	Dosing regimen*	Findings
SAFETY PHARMACOLOGY				
Central Nervous System	Inhalation	Crl:WI(Han) rats. 10 animals per dose and per sex	**Low:** 1.5h (2.07 SCN^- / 12.5 bLF) **Mid:** 3h (3.45 SCN^- / 20.2 bLF) **High:** 6h (6.68 SCN^- / 40.6 bLF) Doses in mg/kg/d	No treatment related effects
Respiratory system	Inhalation	Crl:WI(Han) rats. 8 male animals per dose	Single dose **Low:** 1.5h (1.29 SCN^- / 7.6 bLF) **Mid:** 3h (3.26 SCN^- / 18.0 bLF) **High:** 6h (5.36 SCN^- / 32.1 bLF) Doses in mg/kg/d	No treatment related effects
Cardiovascular system	Intravenous (bolus)	Beagle dogs 4 male animals	0.041 $OSCN^-$ / 3.34 bLF mg/kg on a crossover study design	No treatment related effects
GENOTOXICITY				
AMES	Direct contact	Bacteria	0.01 $OSCN^-$ / 4.4 bLF mg/ml then 6 serial dilutions at 1/3 each	No treatment related effects
***In vitro* micronucleus test**	Direct contact	Human lymphocytes	**Low:** 5.12 $OSCN^-$ / 0.25 bLF **Mid:** 10.24 $OSCN^-$ / 0.5 bLF **High:** 20.47 $OSCN^-$ /1.0 bLF Doses in µg/ml	No treatment related effects
***In vivo* micronucleus test**	Intravenous (bolus)	OF1 Mice 5 animals per dose and per sex	2 administrations at 24-hours interval **Low:** 1.2 $OSCN^-$ / 0.1 bLF **Mid:** 2.25 $OSCN^-$ / 0.2 bLF **High:** 4.3 $OSCN^-$ / 0.4 bLF Doses in mg/kg/d	No treatment related effects

TOXICOLOGICAL FINDINGS

The toxicological profile of ALX-009 is described in table 2. In both rats and dogs the most significant findings involved the respiratory organs. In rats, the organ weights and gross pathology changes were no longer visible after 4-weeks off treatment. Furthermore, the incidence and severity of these events did not increase with chronicity of treatment as revealed by the analysis of the 13-week interim and 26-week animals. At the histopathological level, the test animals showed irritant and inflammatory events, some of these being related to the inhalation procedure itself, as revealed by the lack of dose-dependency and similar incidence in the control groups. The findings attributable to ALX-009 were mostly of inflammatory nature (cell infiltrates) and superficial epithelial cell damage as concluded from the observations of the sub-acute 4-weeks study. These changes were partially reverted in the recovery groups. Chronicity of administration did not reveal an increased incidence or severity of the findings after analysis of the week 13 and week 26 test groups. Despite a dose-dependency, the total or partial recovery of changes after an off period, and the lack of increased severity or incidence of changes among the different groups tested suggest that the observed changes might be of adaptive nature. Finally, minimal hematology, blood chemistry and urinalysis changes were also reported in the sub-acute and acute studies. However, these changes either did not reach statistical significance or were not dose-dependent; for these reasons, they were considered as incidental and not related to the administration of ALX-009.

In the sub-acute study in dogs, the toxicity findings concerned the gross pathology and histopathology observations with events of similar nature as compared to rats: dose-dependent dark areas in the lungs, inflammatory cell infiltrates. In contrast, and compared to rats, no modifications of the lungs or bronchi weights were reported.
Additionally, all the reported events observed in dogs after the 4-week administration were no longer seen in the 4-week recovery group. Due to the lack of evidence of significant toxic changes in this study, the same dosing regimen was used for the chronic dog study.

During the course of the chronic study (at week 7), one female of the high dose group experienced some clinical signs, the most significant being a rapid and irregular breathing, coughing and gasping, all of them visible only during the administration period and lasting a few minutes after the end of the administration. Despite these clinical signs, the animal remained calm and did not try to remove the mask inhalator during administrations. The week after (week 8), similar signs were observed in the 3/6 females and 1/6 males of the high dose group and a first dose reduction was applied. Due to the increase in the incidence of the events, it was concluded that the dosing regime was no longer tolerated and the decision was taken to reduce the dosing to a single inhalation period of 10 min, 20 min and 40 min per day for the low, mid and high dose groups, respectively. After reduction of dosing, the frequency and severity of the clinical signs decreased as the study progressed. The analysis of the 13-week interim killing animals and the 39-week animals revealed only events in the respiratory organs, similar to those observed during the 4-week study. The chronicity of the administration did not show significant differences between the interim killing groups and the main study receiving ALX-009 for 39 weeks and were comparable to the adverse events observed in the sub-acute study.

Table 2. ALX-009 General toxicology study design and findings.

	SUB-ACUTE TOXICITY WITH RECOVERY - RATS	ACUTE TOXICITY WITH 13-WEEKS INTERIM KILLING - RATS	SUB-ACUTE TOXICITY WITH RECOVERY - DOGS	ACUTE TOXICITY WITH 13-WEEKS INTERIM KILLING - DOGS
STUDY DESIGN				
Administration route	Inhalation	Inhalation	Inhalation	Inhalation
Test system	Crl:WI(Han) rats. Main: 10 animals per dose and per sex. Recovery: 5 animals per sex for Control and High dose groups. Satellite animals: 12 per dose and per sex.	Crl:WI(Han) rats. Main: 12 animals per dose and per sex. Interim: 8 animals per dose et per sex. Satellite animals: 9 per per dose and per sex.	Beagle dogs Main: 3 animals per dose and per sex. Recovery: 2 animals per sex for control and High dose groups	Beagle dogs Main: 4 animals per dose and per sex. Interim killing: 2 animals per sex and per dose
Duration of treatment	**Main and satellite:** single daily dose for 4 consecutive weeks **Recovery:** single daily dose for 4 consecutive weeks plus 4 weeks off	**Main and satellite:** single daily dose for 26 consecutive weeks **Interim killing:** single daily dose for 13 consecutive weeks	**Main and satellite:** 2 daily doses with at least 2 hours off for 4 consecutive weeks **Recovery:** 2 daily doses with at least 2 hours off for 4 weeks plus 4 weeks off	**Main and interim killing:** 2 daily doses for 8 consecutive weeks then once a day till the end of study due to dose limiting toxicities
Dosing regime	**Low:** 1.5h (2.07 SCN^- / 12.5 bLF) **Mid:** 3h (3.45 SCN^- / 20.2 bLF) **High:** 6h (6.68 SCN^- / 40.6 bLF)	**Low:** 1.5h (1.07 SCN^- / 6.42 bLF) **Mid:** 3h (2.10 SCN^- / 12.7 bLF) **High:** 6h (4.39 SCN^- / 26.1 bLF)	**Low:** 15 min bid(0.46 SCN^- / 2.72 bLF) **Mid:** 30 min bid (0.92 SCN^- / 5.68 bLF) **High:** 60 min bid (1.59 SCN^- / 9.48 bLF)	**Initial schedule:** **Low:** 15 min bid (0.44 SCN^- / 2.55 bLF) **Mid:** 30 min (0.80 SCN^- / 4.66 bLF) **High:** 60 min (1.8 SCN^- / 10.4 bLF) **After dose limiting toxicities:** **Low:** 10 min bid (0.29 SCN^- / 1.25 bLF) **Mid:** 20 min bid (0.53 SCN^- / 2.53 bLF) **High:** 60 min bid (1.19 SCN^- / 5f.41 bLF) **After dose limiting toxicities** **Low:** 10 min (0.14 SCN^- / 0.80 bLF) **Mid:** 20 min (0.28 SCN^- / 1.52 bLF) **High:** 60 min (0.56 SCN^- / 3.09 bLF)
FINDINGS				
Mortality	No treatment related deaths	No treatment related deaths	None	None
General health observations	No treatment related signs	No treatment related signs	No treatment related signs	On week 7, 1/6 females exposed to the high dose experienced being cold to touch, rapid and irregular breathing, coughing and gasping. A first dose reduction was applied. The week after (week 8) similar signs were observed in 3/6 females and 1/6 males of the high dose group. Due to the severity of the observations, it was considered the dose was no longer tolerated and dosing duration was reduced for all groups to once a day. The frequency of these signs decreased as the study progressed to a total recovery.
Weight	No treatment related effects	No treatment related effects	Lower body weight gain in some animals of the middle and high dose groups during the in life phase. Due to the lack of dose-response, change was not attributed to ALX-009 administration	No evidence of test article-related effects on group mean bodyweight. Individual body weight losses were evident during Week 6 for animals exhibiting the greatest reaction to treatment. Recovery from any body weight loss was apparent following reduction to once daily dosing.
Food consumption	No treatment related effects	No treatment related effects	No treatment related effects	No evidence of test article-related effects on group mean food consumption. Individual reduced food consumption were evident during Week 6 for animals exhibiting the greatest reaction to treatment. Recovery from any body weight loss was apparent following reduction to once daily dosing.
Ophthalmology	No treatment related effects	No treatment related effects	No treatment related effects	No treatment related effects
Electrocardiography	Not evaluated	Not evaluated	No treatment related effects	No treatment related effects
Blood pressure	Not evaluated	Not evaluated	Not evaluated	No treatment related effects
Hematology	Minor changes with no statistical significance and/or no dose dependency	Minor changes with no statistical significance and/or no dose dependency	No treatment related effects	No treatment related effects
Blood	Minor changes with no statistical significance and/or no dose dependency	Minor changes with no statistical significance and/or no dose dependency	No treatment related effects	No treatment related effects
Urinalysis	Minor changes with no statistical significance and/or no dose dependency	Minor changes with no statistical significance and/or no dose dependency	No treatment related effects	No treatment related effects
Organ weights	Increased mean lung and bronchi weights in test groups compared to controls. Differences no longer apparent in the recovery group	Increased mean lung and bronchi weights in test groups compared to controls at weeks 13 and 26; however severity of changes did not increase with chronicity maybe indicative of an adaptive change	No treatment related effects	Increased mean lung and bronchi weights in the mid and high dose groups and kidneys weight in males of the high dose group. Lower spleen mean weights in all males treated groups and in the female mid and high groups
Gross pathology	Dose dependent increase of pale areas in the lungs and in the number of enlarged tracheobronchial lymph nodes. Differences no longer apparent in the recovery group	Dark areas in the lungs and enlarged mediastinal and tracheobronchial lymph nodes observed in all treated groups. No dose dependency and no increase with chronicity of dosing (13 weeks vs 26 weeks)	Dark areas on the lungs and enlarged tracheobronchial lymph nodes seen for all animals of all treated groups. Events no longer seen after the 4-weeks recovery period.	Dark areas on the lungs and enlarged tracheobronchial lymph nodes seen for all animals of all treated groups.
Histopathology	Irritant changes in the nose and larynx and inflammatory changes in the lungs, nasal passages and trachea. Laryngeal changes comprised epithelial ulceration, squamous metaplasia, squamous hyperplasia and submucosal inflammation. Findings considered adverse due to the presence of epithelial ulceration in association with moderate or marked submucosal inflammation. Partial to full recovery observed after 4-weeks off dose	Dose related increase incidence and/or severity of inflammatory cell infiltrates in lungs, larynx and trachea similar to these described in the 4-weeks study. Despite dose dependency, changes did not increase with chronicity of administration (13 weeks vs 26 weeks).	Increased incidence in alveolar macrophages/inflammatory cells scattered in the alveolar airways in treated groups. Also a mixed inflammatory cell infiltration (mononuclear cells, mainly macrophages) was seen at the perivascular / peribronchiolar / intraseptal areas of the lungs. Complete recovery of lung findings was apparent following 4-weeks off dose.	Inflammatory changes in the lungs and increased cellularity of the tracheobronchial lymph nodes; inflammatory changes also evident in the nasal turbinates after 39 weeks of treatment. Little or no difference in the severity of changes related to treatment in the lungs and tracheobronchial lymph nodes after 13 weeks or 39 weeks of dosing
NOAEL	2.07 SCN^- / 12.5 bLF mg/kg/d	2.10 SCN^- / 12.7 bLF mg/kg/d	1.59 SCN^- / 9.48 bLF mg/kg/d	0.77 SCN^- / 4.32 bLF mg/kg/d*

*values correspond to the average exposure doses for the whole study

TOXICOKINETICS

ALX-009 is a locally acting drug candidate and systemic exposure assessments were conducted to better characterize systemic safety.

bLF was detected in plasma of rats and dogs only occasionally with no dose-dependency or chronicity of administration. Due to the paucity of available data, bLF pharmacokinetic evaluation was not possible for any of the two tested species.

SCN^- was also quantified in control animals of both species, as expected for an endogenous product. The analysis of the pharmacokinetic parameters indicated a similar exposure pattern in both species:
- There is not sex difference on the exposure levels
- T_{max} occurred at 0.5-1h after the end of exposure
- The observed C_{max} is dose dependent but not proportionate to the dose as predicted for a linear relationship.

After repeated dosing by inhalation, the rate of systemic exposure increased without signs of accumulation in plasma. The rate (C_{max}) and extent (AUC_{ALL}) of systemic exposure to SCN^- was generally similar during weeks 4, 13 and 26 or 39, indicating that a steady-state was achieved by week 4.

CONCLUSIONS

- **The safety pharmacology, genotoxicity as well as sub-acute and chronic administration toxicology testing demonstrate that ALX-009 does not induce major safety signals. In dogs, and during chronic administration, ALX-009 induced irregular breathing, coughing and gasping in some animals at the higher dose tested. However, these events were reversible and disappeared either after some off-dosing periods or after dose reduction.**
- **bLF was occasionally detected in plasma, indicative of a low risk of systemic exposure. In contrast, dose-dependent but non-linear SCN^- systemic exposure was observed in both species.**
- **Taken together, all results demonstrate that ALX-009 exhibits a favorable safety profile to support administration by inhalation to healthy volunteers and cystic fibrosis patients.**
- **A first-in-man dose escalation study of ALX-009 and its components in healthy volunteers and cystic fibrosis suffering patients is ongoing (NCT02598999).**

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