ALX-009 (OSCN-/bLF) EFFICACY AGAINST EMERGENT CYSTIC FIBROSIS PATHOGENS

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INTRODUCTION

The genetic causes of CF are linked to the mutation of a single gene, the *cftr* (cystic fibrosis transmembrane conductance regulator) gene. This gene codes for the CFTR membrane proteins involved in the ion exchange between the cell and the lumen. In the lung, ionic equilibrium is important to preserve the protective action of the Airway Surface Liquid (ASL) and mucus. However in CF patients, ASL and mucus volumes and compositions are modified. The ASL normally contains bactericidal/bacteriostatic products in solution that are less or not present in the ASL of CF patients. In addition, the volume of the ASL of CF patients is reduced with increased salt content; both factors dehydrate the mucus that becomes thick and accumulates at the surface of cells.

$OSCN^-$ and lactoferrin are among the bactericidal compounds that are deficient in the ASL of CF patients. $OSCN^-$ is a potent large spectrum antibiotic whereas lactoferrin is a multifunctional protein that inactivates/kills bacteria (Fig. 1).

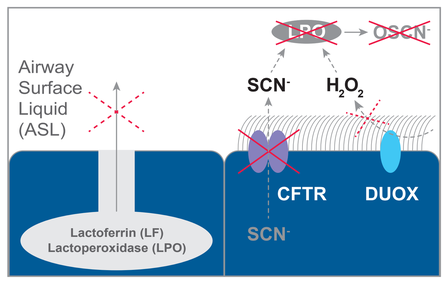

Figure 1. Impaired $OSCN^-$ and lactoferrin in CF patients lungs' defense

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.

$OSCN^-$ is a highly reactive compound that oxidizes free thiol radicals of proteins to create disulfide bonds that perturb the bacterial physiology. Lactoferrin may act by direct interaction with bacterial cell membranes or by depriving bacteria of iron due to its iron chelator activity. 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.

There is an extensive background describing the different antimicrobial properties of $OSCN^-$ and lactoferrin compounds but none of these works was performed according to standardized methods and used different product quality standards. These methodological differences hamper comparability and profiling of the antimicrobial properties of each compound. Here we report the efficacy of $OSCN^-$, bovine LF (bLF) and their combination over a large collection of multi-drug resistant clinical isolates from CF patients from several species.

METHODS

Bacterial isolates

The bacterial isolates obtained from CF patients were kindly provided by several laboratories and national CF repositories around the world. The tested isolates were *Achromobacter* spp. (n= 113), *Burkholderia* spp. (n=165), *Pseudomonas aeruginosa* (n=52), Multidrug Resistant (MDR) *Pseudomonas aeruginosa* (n=20) and *Stenotrophomonas maltophilia* (n=53). The MDR *Pseudomonas aeruginosa* isolates were selected according to the MDR criteria levels described by Magiorakos *et al.* (*Clin Microbiol Infect* 2012; 18:268–281).

Microbiological methods

1. Minimal Inhibitory Concentrations (MIC) for bLF, $OSCN^-$ and ALX-009 were obtained with the microdilution method described in guideline M07-A9 of CLSI with slight modifications. Results are reported as the average values of three independent experiments.
2. Fractional Inhibitory Concentration Index (FICI) were obtained with a microdilution checkerboard method. Microdilution experiments were done as reported above. FICI ≤0.5 indicated synergy for the combination; 0.5<FICI≤1.0: additive effect; 1.0< FICI ≤2.0: indifferent; and FICI >2.0 indicated antagonism between the components. Results are reported as the average values of three independent experiments.
3. Time kill curves for bLF, $OSCN^-$ and ALX-009 were obtained with the macrodilution method as described in guideline M26 of CLSI. From each bacterial group, isolates were selected according to their FICI to test an equal number of synergistic, additive and indifferent isolates. Results are reported as the average values of three independent experiments.
4. Minimal Biofilm Inhibitory Concentration experiments were performed with the MBEC™ assay (Innovotech, Vancouver, Ca) technology as described by the manufacturer. Results are reported as the average values of three independent experiments.

Test compounds

$OSCN^-$ solutions exempt of enzymes were produced by enzymatic reaction with Alaxia's proprietary technology. bLF is a pharma grade bovine Lactoferrin produced by Alaxia.

RESULTS

		OSCN-				bLF			
Bacteria	**n**	R	S	MIC_{Med}	MIC_{Ran}	R	S	MIC_{Med}	MIC_{Ran}
Achromobacter spp	113	0	113	58	12-132	99	14	24	0.25-64
Burkholderia spp	165	0	165	70	30-157	158	7	8	0.25-16
P. aeruginosa	52	0	52	48	28-107	47	5	32	1.0-64
P. aeruginosa MDR	20	0	20	38	27-56	15	5	64	0.5-64
S. maltophilia	53	0	53	45	20-96	27	26	32	0.5-96
All isolates	**403**	**0**	**403**	**58**	**12-157**	**346**	**57**	**16**	**0.25-96**

Table 1. Minimal Inhibitory Concentrations (MIC) values for $OSCN^-$, bLF. The MIC values are given in µg/ml for $OSCN^-$ and mg/ml for bLF. Med: Median. Ran: Range. MDR: Multi Drug Resistant. bLF: Bovine Lactoferrin. R: Resistant. S: Susceptible.

		Interaction			
Bacteria	**n**	Syn %	Add %	Ind %	Ant %
Achromobacter spp	113	17	69	14	0
Burkholderia spp	165	13	60	27	0
P. aeruginosa	52	4	34	62	0
P. aeruginosa MDR	20	10	65	25	0
S. maltophilia	53	27	73	0	0
All isolates	**403**	**14**	**60**	**26**	**0**

Table 2. $OSCN^-$ and bLF interaction by group measured by the fractional inhibitory concentration index (FICI). Add: Additive. Ant: Antagonist. Ind: Indifference. Syn: Synergy.

- All bacteria isolates were inhibited by $OSCN^-$ with a susceptibility ranking based on MIC_{median} as follows: *P. aeruginosa* MDR < *S. maltophilia* < *P. aeruginosa* < *Achromobacter* spp < *Burkholderia* spp.
- Under the CLSI conditions, bLF alone inhibited the growth of 14% of the total isolates with *Pseudomonas aeruginosa* and *Burkholderia* spp. being the most resistant to the bLF activity. Interestingly, 49% (26/53) of the *S. maltophilia* isolates were inhibited by bLF.
- A positive effect of the combination is observed on the MIC_{median} values for the whole collection. The addition of bLF allows to reduce the quantity of $OSCN^-$ required to inhibit the growth of bacteria of about 16% (average value) when compared to MIC values for $OSCN^-$ alone.
- The FICI data confirms the beneficial effect of the combination: for 76% of isolates, the combination presents either a synergistic (14%) or additive effect (60%). Finally, no antagonism between the two compounds has been detected so far.
- Interestingly, MIC data with *P. aeruginosa* and MDR *P. aeruginosa* demonstrate a higher susceptibility of the MDR phenotypes to $OSCN^-$ and to the combination. Indeed, $OSCN^-$ MIC_{median} value is about 16% lower and the MIC_{range} is narrower for the MDR phenotype compared to the antibiotic-sensible strains. Additionally, the FICI data also show striking differences between these two phenotypes: for 62% of the the antibiotic-sensible isolates, the combination is "Indifferent" with almost no synergistic activity. This situation is reverted in the MDR phenotype: for 65% of the isolates the combination is additive and the number of synergistic isolates doubled. Additional data are being obtained to confirm these observations.

ACKNOWLEDGMENTS

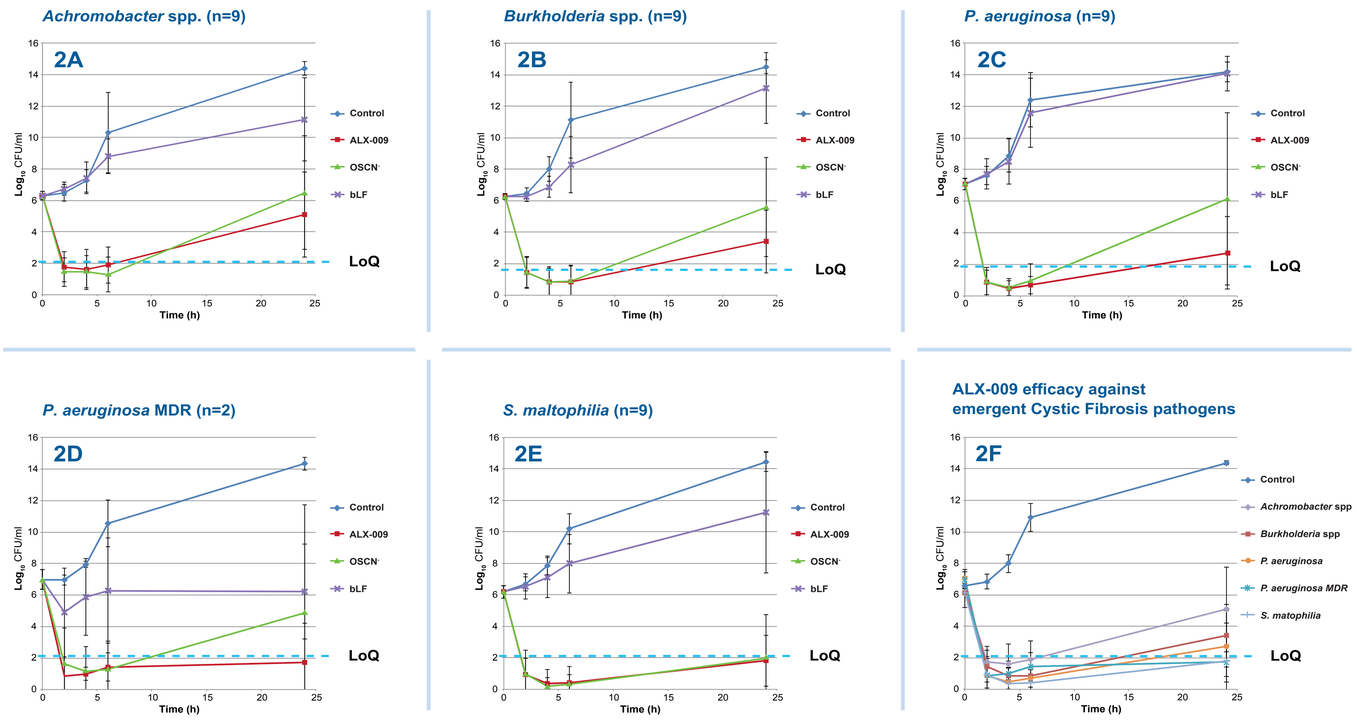

Figure 2. Time killing profile of a combination of 80µg/ml $OSCN^-$ and 4mg/ml bLF against emergent cystic fibrosis pathogens. 2A) *Achromobacter* spp.; 2B) *Burkholderia* spp., 2C) *P. aeruginosa*; 2D) MDR *P. aeruginosa*; 2E) *S. maltophilia*. F) Compiled average values of all the reported data in figures 2A to 2E. Each isolate was tested at least three times. Vertical bars show the standard deviation for the group at the given time point. CFU: Colony Forming Unit. LoQ, Lower level of quantification.

- The analysis of the FICI of the complete set of isolates let to define a dose of 80µg $OSCN^-$ and 4mg/ml bLF able to inhibit the growth of 90% of isolates. This combination was used to analyze the killing properties of the combination in planktonic cells.
- The killing profile of ALX-009 is similar for all the bacteria despite the MIC and FICI differences observed previously. For the whole set, an important CFU reduction is observed during the first 0-2h of the test, followed by a stabilization of the killing activity at times 2-6h with no apparent difference among the groups. However, at the end of the experiments (time 24h), a slow regrowth is observed for all bacterial groups being the most evident for *Achromobacter* spp.
- The *Burkholderia* spp., *Achromobacter* spp. and *S. maltophilia* species are characterized by their intrinsic multi-drug resistance. MDR *P. aeruginosa* evolved by continuous selection pressure of the antibiotic treatments. The time kill experiments with the tested ALX-009 formulation demonstrate that this investigational drug is able to overcome the natural or acquired resistance phenotypes of these bacteria.
- Taken together, this observation suggests that the activity of ALX-009 is independent of the resistance genetic determinants of these bacteria for which no or few pharmacological treatments are available.

	COMPOUNDS TESTED ALONE				COMPOUNDS TESTED IN COMBINATION (ALX-009)			
	OSCN- (±SD)		bLF (±SD)		OSCN- (±SD)		bLF (±SD)	
Strain	**MIC**	**MBIC**	**MIC**	**MBIC**	**MIC**	**MBIC**	**MIC**	**MBIC**
CIP 104116	61±3	103±5	>96000	>4000	71±14	77±4	125±0	250±0
LMG 27647	47±14	141±14	>96000	>4000	58±6	84±8	125±0	250±0

Table 2. Minimal Biofilm Inhibitory Concentration (MBIC) for $OSCN^-$ and bLF. Tested strains are *P. aeruginosa* isolates. Values correspond to the average concentration in µg/ml ± standard deviation. For the test with bLF alone, the reported values correspond to the maximal feasible doses tested for MIC or MBIC experiments.

- The inhibitory capacity of $OSCN^-$ is slightly reduced in bacteria embedded in biofilms when compared to the inhibitory values obtained against planktonic cells. bLF has no effect on the viability of cells when tested alone neither in planktonic cells nor bacterial biofilms.
- The presence of bLF in the ALX-009 investigational drug preserves the inhibitory capacity of $OSCN^-$ and probably its killing properties.
- Tests performed with sterilized (data not shown) or infected CF patients' sputa (see poster #368) revealed that ALX-009 efficacy is unaffected in presence of this biological matrix.

CONCLUSIONS

- ***ALX-009 demonstrates a positive bactericidal potential on a large panel of multiresistant and emergent CF bacterial strains for which few or no therapeutic options exist.***
- ***The antibacterial activity of ALX-009, measured by time kill experiments, suggests that the efficacy of the investigational drug is independent of the drug-resistance strategies developed/acquired by cystic fibrosis pathogens.***
- ***ALX-009 antibacterial activity is not blocked by biofilms or sputa from CF patients (see poster #368).***
- ***The data presented here confirm the potential therapeutic use of ALX-009 for the treatment of multiresistant bacterial infections in CF lung.***
- ***The pharmacodynamic profile of ALX-009 supports the enrolment of CF patients with lung infections involving bad bugs during the clinical development.***
- ***ALX-009 phase I clinical trial is ongoing (NCT02598999).***

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