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Research Article

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HIGHLY EFFECTIVE LUNG DELIVERY OF A FULLY HUMAN MONOCLONAL ANTIBODY TARGETING PSEUDOMONAS AERUGINOSA FOLLOWING INTRA-NASAL ADMINISTRATION

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ABSTRACT

A time-course study was performed to assess lung concentrations and bioactivities of a fully human monoclonal antibody (mAb) targeting *Pseudomonas aeruginosa* (PA) flagella type b (LST-007) following intra-nasal administration. Intra-nasal administration of LST-007 (5 mg/kg) resulted in high mAb concentrations (11 μg/ml) within the bronchoalveolar lavage (BAL) fluid at 15 min post-administration, which decreased to 3 μg/ml at 4 hr. In marked contrast, LST-007 concentrations in blood were 110 and 11 fold lower at the same time points. Scrutinization of BAL fluid demonstrated the presence of both intact and immunoreactive LST-007 towards PAO1 bacteria, which impeded bacterial motility, underscoring the maintenance of biological activity. These compelling data lay credence that intra-nasal and thus potential inhalation modes of administration might represent *bona fide* routes for a targeted mAb delivery to the lung environment and establishment of therapeutically effective concentrations. Such delivery approaches could help combat life-threatening, pneumonia infections caused by PA.

KEY WORDS:Monoclonal antibody, intra-nasal, bronchoalveolar lavage; Pseudomonas aeruginosa

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INTRODUCTION

The ESKAPE[1] group of pathogens has been identified as an extremely worrisome set of bacteria which can stretch the clinician's drug armamentarium due to the capability of these bacteria to develop Multi-Drug Resistant (MDR) phenotypes. The "P" component of ESKAPE, namely *Pseudomonas Aeruginosa* (PA), a Gram-negative bacterium, is a major cause of a variety of nosocomial infections of which pneumonia (hospital and ventilator-associated) and urinary tract infections represent major components[2-4]. A major problem associated with infections caused by PA is due to the bacterium's agility to develop MDR phenotypes[5]. This underscores the urgent clinical need not only to develop innovative anti-PA therapeutic strategies which can retain activity towards MDR PA strains but also the need to develop novel and applicable routes of drug administration for new and even commonly used antibiotics[6]. Such all round research and development efforts could allow a rapid attainment of therapeutically active drug concentrations at target tissues thereby potentially interrupting the development of MDR phenotypes. We have recently reported[7] the characterization of a fully human mAb termed LST-007, which targets flagellin type b, a critically-important PA virulence factor. In a lethal mouse model of acute pneumonia driven by a PA isolate resistant to 19/21 antibiotics, i.v. administration of LST-007 afforded a significant improvement in survival, which outweighed the minimal improvement observed with a carbapenem antibiotic. In contrast with the anticipated PK profile of LST-007 in blood, analysis of the BAL fluid compartment following a single i.v. injection of LST-007 revealed that optimal mAb concentrations were achieved only at 24 hr post mAb administration[7]. Consequently, further beneficial effects of LST-007 might be harnessed if its bioactive concentration in lungs could be achieved more rapidly following administration. To that end, with our overall intent to embark on further *in-vivo* PA efficacy studies with LST-007 and additional anti-flagella mAbs, we wanted to address the question if intra-nasal administration of LST-007 might allow a more rapid targeted delivery of mAb to the lung compartment with concomitant establishment of therapeutic concentrations.

MATERIALS AND METHODS

All general chemicals were purchased from Sigma, Rehovot (Israel). Bacterial PA strains were obtained from ATCC (USA). NuPAGE gels, associated buffers, membranes and Colloidal Blue staining were obtained from In-Vitrogen (USA). Purified recombinant PA flagellin type b was expressed in pET28 bacterial system and purified as a histidine-tagged protein using Nickel agarose chromatography as previously described[7]. LST-007 was expressed in Chinese Hamster Ovary (CHO) cells and purified from serum-free supernatants using protein-G as previously described[7].

(i) *Binding of BAL and blood-containing LST-007 samples towards recombinant PA flagellin type b in ELISA*

Dilutions of BAL fluid (1:312.5 - 1:40,000) and blood (1:20 - 1: 2000) were made and added to wells of Maxisorp ELISA plates (Nunc, cat # 442404) pre-coated with 250 ng of recombinant PA flagellin type b. Following blocking overnight in PBS-10% fetal bovine serum, a secondary antibody consisting of a 1:10,000 dilution of goat anti-human IgG-Fc-HRP conjugate (Bethyl, cat # A80-104P) was added for 1 hr at room temperature and colorimetry performed following addition of TMB solution (Millipore, cat # ES001). A standard curve of exogenously added LST-007 (1 pg/ml - 1 μg/ml) was included in all assays.

(ii) *Binding of BAL fluid containing LST-007 samples towards immobilized, whole PA bacteria in ELISA*

PAK (flagellin type a), PAO1 (flagellin type b) or PA9721 (aflagellated strain) were grown overnight in 5 ml LB at 37^{o}C and irreversibly bound to poly-L-lysine-coated ELISA plates as previously described[7]. BAL samples from 15-240 min collection time points were diluted 1:312.5 - 1:40,000 and taken for fixed ELISA and developed colorimetrically following addition of goat anti-human IgG-Fc-HRP conjugate. In some experiments, samples at these time points were normalized to 0.5 μg/ml and taken for ELISA. Negative control of BAL fluid samples derived from mice injected with saline instead of LST-007 was included in all assays and subtracted from absorbances obtained with LST-007.

iii) *In-vitro PA motility assays*

Motility studies using BAL samples diluted to 0.1 μg/ml were performed as previously described[7].

iv) *Coomassie gel staining of BAL-containing LST-007 samples*

BAL fluid samples from the 15-240 min time points were reduced with DTT (final concentration 50 mM), adjusted with 4X LDS loading buffer and electrophoresed on 4-12% NuPAGE gels. Following removal of gel and washing with DDW, the gel was stained with Colloidal Blue according to the manufacturer's recommendations.

v) *Immunoblot with BAL fluid containing LST-007 samples*

200 μl samples of overnight growths of PAK or PAO1 were centrifuged, supernatant discarded and pellets resuspended in 1X LDS sample buffer containing 50 mM DTT. Following boiling for 10 min and a 10 min centrifugation at 10,000 g, clarified supernatants were electrophoresed as described above and electroblotted onto Nitrocellulose membrane. Membrane strips were prepared and incubated with BAL samples at 0.5 μg/ml and taken for Enhanced Chemiluminescence following incubation with a goat anti-human IgG-Fc-HRP conjugate.

vi) *In-vivo studies*

Female CD-1 mice, age 10-12 weeks (~ 25 g) were used. Animal handling was performed according to the National Institute of Health (NIH) and the Association for Assessment and Accreditation of Laboratory Animal Care (AAALAC). During acclimation (5 days) and following LST-007 dosing, mice were housed in a specific pathogen-free environment with 3 mice per cage, in polypropylene cages fitted with solid bottoms and filled with autoclaved sawdust as bedding material. Animals were provided *ad libitum* with a commercial rodent diet and had free access to autoclaved drinking water supplied to each cage. Automatically controlled environment conditions were set to maintain a temperature of 22-25ºC with a 12 hr light /12 hr dark cycle and air changes in the study room. LST-007 was prepared at a concentration of 2.5 mg/ml following dilution of stock antibody with sterile PBS. A total volume of 50 μl[8] was applied to both nares as follows: 25 μl for one naris, a minute delay and then application of the second 25 μl dose to the contralateral naris. Final dosing was therefore 2.5 mg/kg. A total of 15 mice were taken for simultaneous PK sampling from bleeds and Bronchoalveolar Lavage (BAL) fluid, with 3 mice dedicated each to the 15, 60 and 240 min time points and an additional 3 mice dedicated for sampling at 240 min following intra-nasal administration of equivalent volumes of sterile PBS. At the designated time points, mice were anesthetized by i.p. injection of 85 mg/kg Xylazine and 5 mg/kg Ketamine and bled, ~ 500 μl from the orbital sinus. The blood was collected into 1.5 ml eppendorf tubes, centrifuged and the upper sera layer aliquoted and stored at -80^{o}C until required. While the mice were still under anesthesia, they were placed on their backs and airway exposed for collection of BAL fluid by connection of veinflow to the airway attached to a 26G needle and 1 ml syringe. A total volume of 1.2

ml of saline was used to wash the lungs 2 times with a return BAL volume of ~ 2 ml per mouse. Following centrifugation, the clarified BAL supernatant was removed, aliquoted and stored at -80 ºC until assay.

RESULTS

Graph 1
Kinetics of LST-007 appearance in BAL fluid samples and blood.

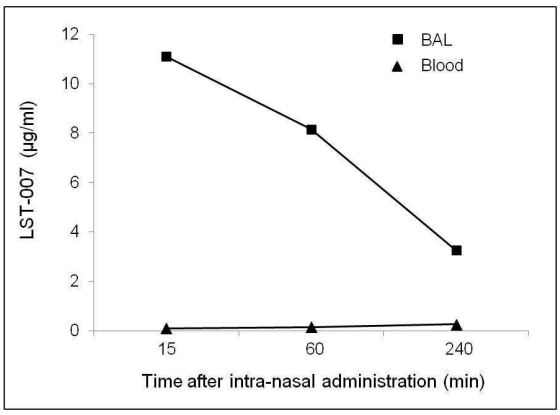

Figure 1
Time-course of LST-007 (µg/ml) concentrations in BAL fluid and plasma in mice following intra-nasal administration of LST-007 (5 mg/kg).

Samples of BAL fluid and blood were taken for quantification of LST-007 at 15-240 min following intra-nasal administration of the mAb (Fig 1). Concentrations in BAL fluid decreased from 66 µg/ml at 15 min post-administration to 3 µg/ml at 240 min. In marked contrast, concentrations in plasma were extremely low, increasing from 0.1 µg/ml at 15 min to 0.26 µg/ml at 240 min (Fig 1). Since 125 µg LST-007 was administered intra-nasally to each mouse, the total amount of mAb present in ~ 2 ml BAL fluid was quantified, enabling % bioavailability to be determined at each time point (Table 1, parantheses).To that end, at time points of 15, 60 and 240 min, the amount of LST-007 present in BAL fluid per mouse was calculated to be 22 µg, 16 µg and 6 µg (Table 1).

Table 1
Concentrations and amount of LST-007 in BAL fluid following intra-nasal administration of LST-007 (5 mg/kg) to mice

LST -007 (µg/ml) per mouse			
	15 min	60 min	240 min
BAL	11 (22 µg)	8 (16 µg)	3 (6 µg)
Sera	0.1	0.15	0.26

Coomassie gel staining of BAL fluid samples containing LST-007

Fifteen µl samples of LST-007 in BAL fluid from time points 15 min (Fig 2, lane A), 60 min (Fig 2, lane B) and 240 min (Fig 2, lane C) were electrophoresed on a protein gel and stained with Coomassie

Blue. A control lane D, consisting of 5 μg LST-007 was included as a positive control. All lanes demonstrated the presence of heavy and light mAb chains with no evidence of degraded products.

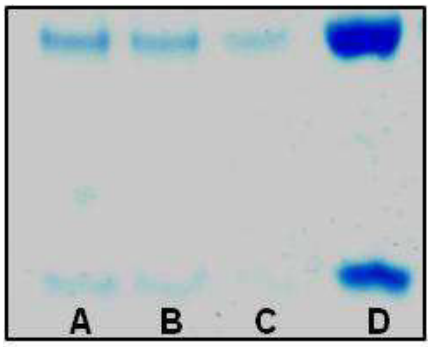

Figure 2

Coomassie-gel staining of reduced LST-007 in BAL fluid samples harvested at 15 min (lane A), 60 min (lane B) and 240 min (lane C). Lane D represents 5 μg of control LST-007. Upper band denotes 50 kD and lower band 25 kD, corresponding to V_H and V_L chains respectively.

Graph 2

Binding of BAL fluid containing LST-007 towards immobilized PAO1 in ELISA

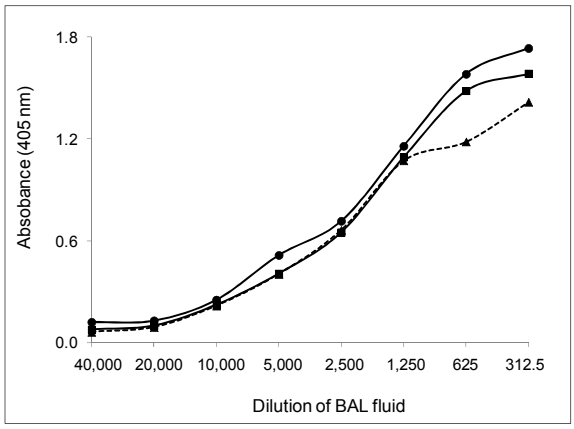

Figure 2

Binding of BAL fluid containing LST-007 towards immobilized PAO1 in ELISA. BAL fluid samples collected from time points 15 min (circles), 60 min (squares) and 240 min (triangles) were diluted 312.5 to 40,000 and taken for binding to PAO1 followed by colorimetric development via a secondary anti-human detecting antibody.

In addition to the standard ELISA employing recombinant PA flagellin type b to quantify binding of BAL-containing LST-007 samples (Fig 1), these same samples were taken for binding towards immobilized PAO1 bacteria[7]. In these studies, BAL samples harvested at time points 15-240 min following intra-nasal administration of LST-007, demonstrated specific binding towards PAO1 which decreased on dilution from 312.5 - 40,000 (Fig 2). Binding of all BAL samples to PAK (flagellin type a) or PA9721, a non-flagellated PA bacterium were negligible (data not shown).

Graph 3

Binding of normalized LST-007 (0.5 μg/ml) from BAL fluid samples harvested at 15 - 240 min following intra-nasal administration of LST-007 and immunoreactivities

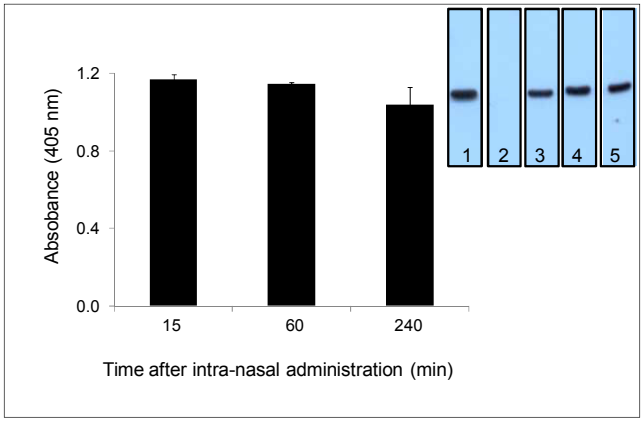

Figure 3

LST-007 in all BAL fluid samples was adjusted to 0.5 μg/ml and taken for binding towards PAO1 by ELISA. Inset Immunoreactive properties of LST-007 in BAL fluid samples towards PAO1 lysates. Lanes 1 and 2 demonstrate specificity of binding of control LST-007 (0.5 μg/ml) towards PAO1 (lane 1) but not PAK (lane 2). LST-007 (0.5 μg/ml) in BAL fluid samples from 15 min (lane 3), 60 min (lane 4) and 240 min (lane 5) all bound PAO1. (Mw of immunoreactive band = 52 kD).

Table 2

Bioactivities of BAL-fluid containing LST-007 as measured by inhibition of PAO1 motility in soft agar assays

BAL harvest time (min)	Halo diameter (mm)	% inhibition of PAO1 motility
15	2	83
60	3	75
240	4	67
Exogenous LST-007	2	83
Exogenous human isotype mAb	12	0

LST-007 in BAL fluid samples were added to soft agar at a final concentration of 0.1 μg/ml and their abilities to impede PAO1 motility measured as previously described[7]. All samples inhibited PAO1 motility by 67-83% (Table 2), an effect similarly observed with exogenously added LST-007 (0.1 μg/ml) but not a human isotype control mAb.

DISCUSSION

The development of anti-PA mAbs targeting virulence factors such as 011 LPS[9], PcrV[10] and flagella[7] represents a highly promising and innovative therapeutic approach to support antibiotic therapies and have already yielded highly promising clinical data[9,10]. Recently, we reported[7] the parenteral, pre-clinical therapeutic effect of a fully human anti-flagellin type b mAb termed LST-007, as demonstrated by its capability to improve animal survival (60-

75%) in a lethal mouse model of pneumonia driven by a MDR PA strain. LST-007's beneficial effect was purported to be, at least in part, due to its ability to impede bacterial motility at concentrations similar to LST-007's K_D towards PA flagellin type b[7]. In follow-up preliminary efficacy studies, prophylactic i.v. LST-007 (given 24 hr prior to PA infection) permitted 100% animal survival (Adawi *et al*, in preparation). This effect was presumably due to the 24 hr time period required for LST-007 to attain optimal concentrations in BAL fluid[7]. Thus, the present study aimed to evaluate if intra-nasal administration might permit a more rapid delivery of LST-007 into the lung compartment and achieve therapeutically-relevant concentrations. Interestingly, intra-nasal administration of LST-007 (5 mg/kg) resulted in a highly efficient delivery to the lung compartment within 15-60 min post-administration with calculated bioavailabilities of ~ 20% and 13% respectively. Examination of BAL fluid samples containing LST-007 demonstrated the presence of intact antibody by Coomassie gel staining, dilution-dependent recognition of recombinant PA flagellin type b (ELISA) as well as avid binding towards PAO1 bacteria using whole cell (ELISA) and lysate preparations (immunoblot). Additionally, normalization of LST-007 (0.1 μg/ml) in BAL fluid samples from all time points were highly effective in blunting PAO1 motility in soft agar assays, confirming our previous findings[7]. The minimal concentrations of LST-007 observed in blood throughout the 4 hr time course would confirm the capability of intra-nasal administration to essentially target delivery of the mAb to the lung compartment. MAbs[11] represent the fastest growing class of therapeutics with currently ~ 35 products approved and 100's in various stages of clinical development. The flexibility of therapeutic mAbs is underscored by accumulating data that their formulation and mode of administration can be fine-tuned in an effort to permit a more targeted delivery. For example, intra-nasal administration of cetuximab has been evaluated clinically in hereditary hemorrhagic telangiectasia with improved, short-term benefit[12]. Furthermore, promising pre-clinical reports have described inhalation delivery of cetuximab to treat lung tumors[13], nebulized anti-IL-13 FAb fragment to treat asthma[14], intra-nasal administration of nanobodies that effectively target RSV[15] and H5N1[16] influenza virus and similar administration of an IgM to prevent *Pneumocystis carnii* pneumonia[17]. In the case of hospital-associated pneumonias caused by PA, understanding the modified PK/PD "setting" is critical for successful anti-microbial therapy[18]. To that end, targeted delivery of innovative therapeutics (eg. anti-virulence mAbs) or even existing antibiotics to the lung compartment would clearly be advantageous since it would allow a rapid and much desired establishment of achievable therapeutics concentrations at presumed reduced dosing as compared to parenteral routes. The data herein describing the effective lung delivery of a novel anti-infective PA mAb following intra-nasal administration, support the rationale to investigate if this mode of delivery or follow-up inhalation approaches can combat lethal and chronic PA infections in pre-clinical models of pneumonia. Positive findings would undoubtedly further drive LST-007's clinical development and provide a basis for the development of additional monospecific anti-flagella mAbs (eg. targeting flagellin type a) or even bispecific mAbs harboring binding sites for flagellin type a and b.

CONCLUSION

Intra-nasal administration of LST-007, a highly novel anti-PA mAb targeting PA flagella type b, resulted in a targeted delivery to the lung compartment achieving concentrations in excess of those required to impede PA motility. Such a mode of administration or even inhalation technologies could be highly beneficial to patients suffering from life-threatening nosocomial infections caused by PA.

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