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ORIGINAL RESEARCH

A Proof of Concept Study to Detect Urease Producing Bacteria in Lungs Using Aerosolized 13C Urea

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This is a ''proof of concept'' study to determine whether inhalation of 13C-urea can be safely used to detect the presence of urease producing bacteria in the airways of patients with cystic fibrosis (CF) by detecting $13CO_2$ in breath. This was a prospective, 2-part, open label, single-center, single-arm, single-administration, dose-escalation investigational device exemption trial. First, the safety of 20 and 50 mg inhaled 13C-urea was evaluated in 6 healthy adult participants. Then, 3 adult CF participants colonized with *Pseudomonas aeruginosa* were enrolled for each dose of inhaled 13C-urea. The safety of inhaled 13C urea was assessed by spirometry and physical examination. 13C-urea was administered using a jet nebulizer, followed by serial spirometry (10 min and 30 min post inhalation) and collection of exhaled breath at 5, 10, and 15 min post inhalation. There was no clinical significant change in any of the spirometry values compared to baseline in healthy participants and CF patients. Mean of $13CO_2/12CO_2$ delta over baseline (DOB) values in CF participants at 5, 10, and 15 min post inhalation was as follows: 20 mg dose 4‰ (2.2‰–4.9‰), 1‰ (1.0‰–1.4‰), and 1‰ (0.4‰–1.5‰); 50 mg dose: 10‰ (6.2‰–14.5‰), 3‰ (2.1‰–4.3‰), and 1.5‰ (0.6‰–2.3‰). Inhaled 13C-urea for detection of urease producing bacteria was safe, and preliminary data suggest that $13CO_2/12CO_2$ DOB values may be higher in CF patients with *P. aeruginosa* at 5–10 min after inhalation of 13C-urea. A future direction is to investigate use of inhaled 13C-urea in young children who have difficulty producing sputum for culturing.

Introduction

Cystic Fibrosis (CF) results in multi-organ dysfunction and 85% of mortality is a result of lung disease associated with decreased mucociliary clearance and chronic bacterial infection especially *Pseudomonas aeruginosa*. Chronic colonization with *P. aeruginosa* is strongly associated with more rapid decline in lung function. Therefore, the Cystic Fibrosis Foundation's Pulmonary Therapies Committee (CFFPTC) strongly recommends chronic administration of aerosolized tobramycin in all CF patients older than 6 years with moderate to severe disease who have *P. aeruginosa* present in airway sputum cultures.[1–3] The CFFPTC also recommends use of chronic aerosolized tobramycin in patients with mild disease (forced expiratory volume in one second [FEV1] > 90% predicted) colonized with *P. aeruginosa* to reduce exacerbation risk. Improved antipseudomonal treatments have increased both life expectancy and quality. However, there remains an unmet need for rapid, noninvasive diagnostic analysis of lung *P. aeruginosa* burden and mucoid status.[4] Such a test would enable real-time monitoring of infection, colonization, mucoid status, and response to antibiotic therapy of the entire lung. It would also significantly improve CF management that currently relies upon indirect measures such as lung function and culture of expectorated sputum. Sputum collection is highly variable, samples only a part of the lung, and is not applicable to younger patients. Thus, bronchoalveolar lavage that is invasive and unsuitable for repetitive use may be required.[4] A noninvasive breath test for detection of bacteria is an attractive diagnostic tool.

Urease enzyme is a widely expressed virulence factor of many bacterial and fungal pathogens, including *P. aeruginosa*, which hydrolyzes urea to produce ammonia and carbon dioxide (CO_2). The enzymology and general genetics of microbial ureases and their virulence roles in a range of infections have been extensively reviewed.[5–10] Urease activity is the basis of the breath test used to determine stomach colonization by *Helicobacter pylori* in which stable isotope-labeled ^{13}C-urea is orally administered, with *H. pylori* urease catalyzing formation of $^{13}CO_2$ that is then exhaled and

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measured. Two key features make it highly attractive to investigate whether alternate delivery routes of ^{13}C-urea may be useful to assess other infections: commercial availability of U.S. Food and Drug Administration (FDA)-approved analysis instrumentation for breath $^{13}CO_2$ and commercial availability of ^{13}C-urea at good manufacturing practice grade. In a parallel animal study, several of the current authors and colleagues used direct lung delivery of ^{13}C-urea to detect *Mycobacterium tuberculosis* and the dose–response of the bacterial load to treatment in infected rabbits as a result of *M. tuberculosis* urease production of $^{13}CO_2$.[11]

We hypothesized that we could detect urease producing bacteria in the lungs of patients with CF by delivering ^{13}C-urea using an inhaled dosage form. We selected CF patients colonized with *P. aeruginosa* to ensure the presence of at least one urease producing bacteria in the lungs. We performed a ''proof of concept'' study to determine whether inhalation of ^{13}C-urea could be safely administered, and then if it could be used to detect urease producing bacteria, including but not limited to *P. aeruginosa*, in the airways of patients with CF.

Methods

Ethical consideration and study design

This was a prospective, 2-part, open label, single-center, single-arm, single-administration, dose-escalation investigational device exemption trial approved by the FDA in accordance with Good Clinical Practice guidance. This study was also approved by the Institutional Review Board at the University of New Mexico, and patients provided written informed consent before any study-related procedures were being performed. The trial was prospectively registered at ClinicalTrials.gov (NCT01303068).

Study part I was designed to determine the safety of aerosolized ^{13}C-urea 20 mg and 50 mg in healthy participants (control group). Study part II would then proceed, contingent on favorable safety of aerosolized ^{13}C-urea in the control group. Study part II was designed to determine the safety and dose–response of aerosolized ^{13}C-urea 20 mg and 50 mg in detecting *urease producing bacteria* in the lungs of participants with CF with a confirmed *P. aeruginosa* colonization.

The ^{13}C-urea Breath Test Kit in this study contained ^{13}C-urea lyophilized in a 10 mL glass vial with an aluminum crimp closure containing 20 mg or 50 mg of active ingredient (Coldstream Laboratories, Lexington, KY) to be diluted with 3 mL of sterile water and nebulized by PARI LC Sprint nebulizer (PARI, Midlothian, VA).

The control group for part I met the following inclusion criteria: (1) at least 18 years old at the time of providing informed consent; (2) In good health with no chronic condition or acute respiratory illness or use of antibiotics within 2 weeks of screening visit; (3) No tobacco smoking within 6 months before the screening visit and agree to not smoke during the study; and (4) Forced expiratory volume in 1 s (FEV_1) of at least 80% of predicted value at the screening visit. The inclusion criteria for part II included the following: (1) at least 18 years old at the time of providing informed consent; (2) Diagnosed with CF at least 24 months before the screening visit; (3) Documented presence of *P. aeruginosa* in at least 3 sputum cultures within 2 years before the screening visit, one of which within 6 months before the screening visit; (4) $FEV_1 > 60\%$ or more than 1.5 L at screening visit; (5) No acute respiratory illness within 2 weeks of screening visit or use of antibiotics; (6)Any abnormal ECG; and (7) No tobacco smoking within 6 months before the screening visit and agreeing to not smoke during the study.

The exclusion criteria for participants in both parts included: (1) Positive *H. pylori* serology; (2) Intolerance of albuterol; (3) Females who were pregnant or nursing or of child-bearing potential who were not using a medically acceptable form of birth control; (4) Any condition or history that, in the judgment of the investigator, would compromise the ability of the subject to comply with the study protocol or to complete the study; and (5) Use of an investigational agent within 28 days before Day 1.

Part I and II studies consisted of 2 visits; a screening visit followed within 7 days by a study visit. A follow-up phone call was scheduled for 24-h after completing the study visit to discuss potential adverse events. Potential participants who were found to be eligible at screening visit proceeded to the study visit. At study visit, inclusion and exclusion criteria were reviewed and spirometry performed. Only for CF patients, 2 puffs of albuterol (Ventolin HFA 90 mcg/inhalation, GSK) were administered and spirometry was repeated in 10 min. The ^{13}C-urea solution was nebulized, and breath specimens were collected at 5, 10, and 15 min after completion of inhalation in an impermeable bag. Spirometry was repeated at 10 and 30 min post inhalation of ^{13}C-urea. All the adverse events were reported during the visit. Healthy participants completed part I with one participant at a time starting with inhaled ^{13}C-urea 20 mg followed by inhaled ^{13}C-urea 50 mg. When the safety of inhaled ^{13}C-urea doses in the healthy cohort was established, part II in CF participants started similar to part I.

Outcome Measures

The primary outcome of both studies was safety of inhaled ^{13}C-urea assessed by spirometry, physical examination, and any observed or reported adverse events. The secondary outcome was the isotopic ratio of $^{13}CO_2$ to $^{12}CO_2$ in exhaled air before study drug administration and at 5, 10, and 15 min after study drug administration. The kinetics of the production of $^{13}CO_2$ was characterized by determining the isotopic ratio of $^{13}CO_2$ to $^{12}CO_2$ in exhaled air of CF subjects before study drug administration and at 5, 10, and 15 min.

Analysis of breath specimen

After collection, all breath test specimens were picked up within 24 h by the central laboratory (Quest Diagnostics, San Juan Capistrano, CA) to be analyzed within 7 days of collection. The isotopic ratio of $^{13}CO_2$ to $^{12}CO_2$ was calculated from breath samples analyzed by a POCone® Infrared Spectrophotometer (Otsuka America Pharmaceutical, Rockville, MD). The relative increase in isotopic ratio, δ, is determined automatically by the spectrophotometer:

$$\delta = \left(\frac{R_x - R_{std}}{R_{std}}\right) \times 1000$$

where R_x is the ratio of the abundance of ^{13}C to ^{12}C in the postadministration sample and R_{std} is the corresponding ratio at baseline. The units of measurement of this ratio, δ, are per mille (‰), and the delta over baseline (DOB) is defined as

DOB = (δ at time *t* after nebulization) − (δ baseline before nebulization)

DOB thus reports on any increase in $^{13}CO_2$ in exhaled breath after ^{13}C-urea nebulization.

Sample size

The sample size was based upon a dose escalation model, not a calculation of statistical power. Three healthy participants received a single 20 mg dose followed by 3 participants who received a single 50 mg dose. The dose was not escalated until the first cohort completed the study at the 20 mg dose and completed the safety follow-up call/visit. After the safety of the 50 mg dose in healthy participants was evaluated, part II started enrolling 3 participants with CF who received a single 20 mg dose followed by 3 participants with CF who received a single 50 mg dose. If a participant developed an adverse event or new symptom that remained unresolved at 48 h, no new participant was to be enrolled until the relationship between the adverse event and symptom was evaluated. If any participant experienced an adverse event considered by the data safety monitor to be dose limiting, then 3 additional participants were to be enrolled at that current dose, and no more than 30 participants would be enrolled. Dose escalation was to stop if more than one additional participant experienced dose limiting toxicity.

A descriptive analysis of the data was performed using SAS (version 9.2). Modeling of DOB decay curves was performed using Origin 5.0 (Microcal Software, Northampton, MA).

Results

A total of 12 patients, 6 in the control group and 6 CF patients, were enrolled in the study; 3 control and 3 CF participants were enrolled and completed the study for each inhaled ^{13}C-urea 20 mg and 50 mg doses. Mean age of controls was 32 years (24–41 years old); mean values (range) of lung function at screening were as follows: FEV_1 3.8 L (3.2–4.4 L), forced vital capacity (FVC) 4.4 L (4.0–5.1 L), and FEV_1/FVC 87% (82%–93%). Mean age of CF participants was 26 years (23–31 years old); mean values (range) of spirometry at screening were as follows: FEV_1 3.7L (2.7–4.0 L), FVC 4.5 L (3.1–5.5), and FEV_1/FVC 84% (83%–84%).

There was no clinical significant change in any of the lung functions compared to baseline in healthy participants and CF patients (Table 1). No adverse event was reported at any doses by healthy or CF participants.

Mean DOB values (range) in controls at 5, 10, and 15 min post inhalation were as follows: 20 mg dose: 0.80‰ (0.4‰–1.2‰), 0.43‰ (0.1‰–1.0‰), and 0.13‰ (−0.5‰–0.6‰); and 50 mg dose: 2.13‰ (−0.1‰–5.5‰), 0.77‰ (−0.1‰–1.8‰), and 0.07‰ (−0.5‰–0.7‰). Mean DOB values in CF participants at 5, 10, and 15 min post inhalation were as follows: 20 mg dose: 3.93‰ (2.2‰–4.9‰), 1.2‰ (1.0‰–1.4‰), and 0.83‰ (0.4‰–1.5‰); and 50 mg dose: 9.53‰ (6.2‰–14.5‰), 3.0‰ (2.1‰–4.3‰), and 1.63‰ (0.6‰–2.3‰). Table 2 presents DOB values for each participant.

The decrease in mean DOB as a function of time was modeled using first order exponential decay, leading to half-lives of 2.7 and 3.3 min for CF patients at 20 and 50 mg doses, respectively, while for controls, these half-lives were 6.8 and 6.0 min, respectively. The mean DOB values for the 2 doses as a function of time after nebulization are plotted in Fig. 1. It can be seen in Fig. 1 and Table 2 that at early time points (5 and 10 min), the DOB values in CF patients are greater than in controls.

Discussion

This proof of concept study shows that inhaled ^{13}C-urea in controls and CF participants was safe as demonstrated by serial spirometry before and up to 30 min post inhalation of

Table 1. Mean (Range) Serial Spirometry Values at Each Study Visit Before and After Administration of Urea

	Baseline	*Post albuterol*	*10 min post urea inhalation*	*30 min post urea inhalation*
Control (*N* = 3), 20 mg		NA		
FEV_1 (L)	3.85 (3.32–4.41)		3.7 (3.09–4.33)	3.74 (3.16–4.38)
FVC (L)	4.4 (4.01–5.09)		4.29 (3.79–5.03)	4.35 (3.9–5.09)
FEV_1/FVC (%)	88 (83–93)		86 (82–91)	86 (81–91)
Control (*N* = 3), 50 mg		NA		
FEV_1 (L)	3.68 (3.24–4.0)		3.67 (3.22–3.99)	3.73 (3.25–4.08)
FVC (L)	4.25 (3.91–4.65)		4.22 (3.87–4.68)	4.23 (3.88–4.78)
FEV_1/FVC (%)	87 (83–91)		87 (83–52)	88 (84–96)
CF (*N* = 3), 20 mg				
FEV_1 (L)	3.35 (1.93–4.14)	3.4 (1.93–4.14)	3.40 (1.88_4.17)	3.36 (1.92–4.12)
FVC (L)	4.28 (2.33–5.45)	4.29 (2.31–5.58)	4.32 (2.21–5.66)	4.25 (2.29–5.57)
FEV_1/FVC (%)	79 (73–83)	80 (74–84)	80 (74–85)	80 (74–84)
CF (*N* = 3), 50 mg				
FEV_1 (L)	3.39 (1.9–4.18)	3.40 (1.95–4.21)	3.37 (1.91–4.01)	3.38 (1.87–4.18)
FVC (L)	4.26 (2.27–5.45)	4.34 (2.39–5.66)	4.24 (2.29–5.6)	4.29 (2.24–5.63)
FEV_1/FVC (%)	81 (75–84)	79 (74–82)	81 (75–84)	80 (74–84)

CF, cystic fibrosis; FVC, forced vital capacity; FEV1, forced expiratory volume in one second; NA, not applicable.

Table 2. All Delta Over Baseline Values for Healthy and Cystic Fibrosis Participants at Different Doses

	Healthy			*CF*		
	5 min post dose, DOB (‰)	*10 min post dose, DOB (‰)*	*15 min post dose, DOB (‰)*	*5 min post dose, DOB (‰)*	*10 min post dose, DOB (‰)*	*15 min post dose, DOB (‰)*
20 mg	0.4	0.1	–0.5	2.2	1.0	0.4
	1.2	1.0	0.3	4.6	1.2	0.6
	0.8	0.2	0.6	4.9	1.4	1.5
50 mg	–0.1	–0.01	–0.5	6.2	2.1	0.6
	1.0	0.6	0.0	7.9	2.6	2.0
	5.5	1.8	0.7	14.5	4.3	2.3

DOB, delta over baseline.

^{13}C-urea. No clinically significant adverse events were noted in healthy and CF participants.

There is a significant amount of endogenous urea already present in the airway surface fluid, also called epithelial lining fluid with concentrations typically close to those of plasma at between 2–4 mM;[12] therefore, it is likely that the lung is well adapted to such concentrations of urea. Some historical data upon the safety of inhaled urea solutions are available in the literature. In one study of 56 adults with asthma, a 4 M solution of urea was nebulized for 10 min using a Wright nebulizer.[13] They reported ''mild but variable impairment of ventilatory capacity,'' which was much lower than when methacholine was used. Waldron-Edward and Skoryna intermittently nebulized 2–14 g/day of urea over ''short intervals'' to patients with COPD and bronchiectasis for 14–30 days with an intermittent-positive pressure ventilator without adverse effects on lung function.[14] They also administered 2M concentration to 6 patients with CF for 10 min with a DeVilbiss nebulizer with an output of 6 mL/min without report of adverse effects.

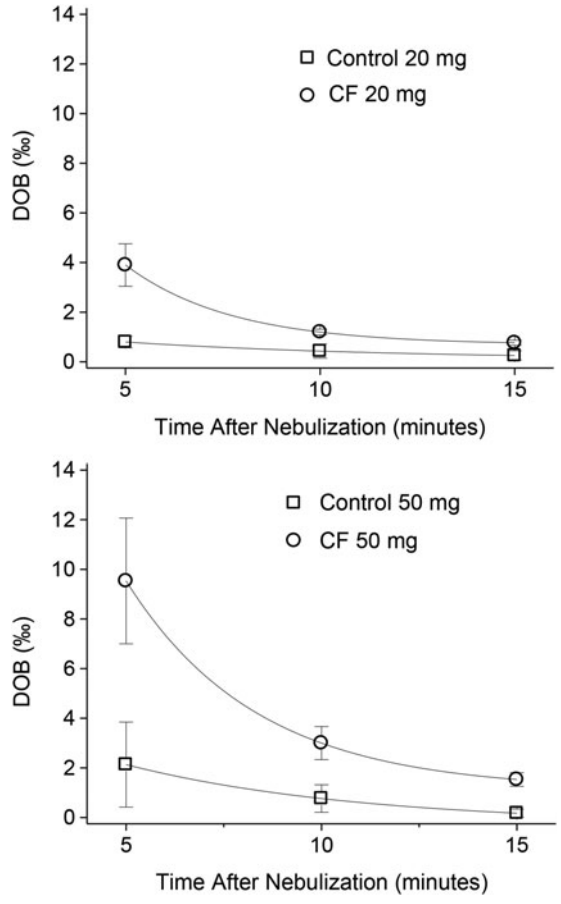

FIG. 1. $13CO_2$ Delta over Baseline (DOB) as a function of time after nebulization of 13C-urea in normal controls (*open squares*) and *Pseudomonas aeruginosa* colonized CF patients (*open circles*) using a dose of 20 mg (*upper Fig.*) and 50 mg (*lower Fig.*). $N = 3$, data are mean ± SE. CF, cystic fibrosis.

Recent data support the hypothesis that hypertonic or hypotonic nebulized solutions tend to cause the most symptoms and so in our study, isotonic solutions of urea were used at much lower molarities of urea than in the historical studies described above.[15–17] The higher dose of 50 mg in 3 mL for nebulization was designed to be close to isotonic (final urea molarity 0.27 M, total osmolality 310 mOsm) to lower the chance of causing cough or bronchospasm in CF patients.. The lower dose of 20 mg ^{13}C-urea (final urea molarity 0.11 M, total osmolality 310 mOsm) was also used to determine if the magnitude of the exhaled $^{13}CO_2$ signal was dependent upon urea dose. The molarities of urea in this study were significantly lower than in historical studies (2 and 4 M).

The DOB values in the CF patients 5 min post dose were higher than at 10 and 15 min post dose, and all the values in controls. The effect appeared to be dose related since the DOB values in CF patients at 5 min were higher at the 50 mg dose compared to 20 mg dose of inhaled ^{13}C-urea. Preliminary analysis showed that the decrease in DOB for individual participants could be approximated by first order exponential decay, with ½ lives in the range of 1.1–6.5 min. No statistically significant effect of dose upon half-life in either group was observed. We might expect to see even higher DOB values in CF patients at time points earlier than 5 min after nebulization.

This is the first human study using inhaled ^{13}C-urea for detection of urease producing bacteria in the lungs of the patients with confirmed colonization with *P. aeruginosa*. We selected CF patients with confirmed *P. aeruginosa* colonization since an *in vitro* study had confirmed measurements of urease activity of *P. aeruginosa* by Isotope Ratio Mass Spectrometry (direct communication with Dr. Timmins) using the same technique as prior studies of *M. tuberculosis*.[11] The headspace gas δ $^{13}CO_2$ values are

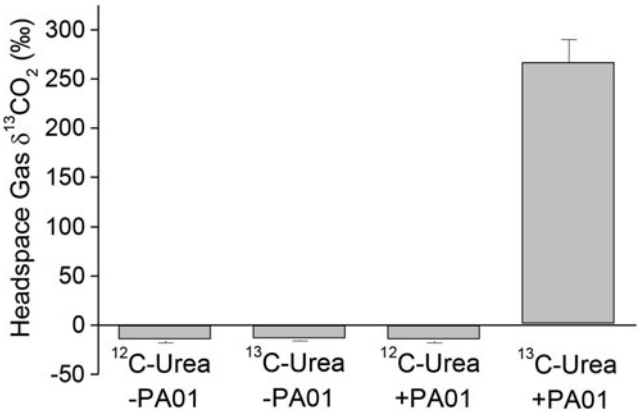

FIG. 2. Increase in headspace gas $13CO_2$ is dependent upon both 13C-urea and *P. aeruginosa*. Luria broth with and without *P. aeruginosa* PA01 (+PA01, –PA01, respectively, 108 CFU/mL), unlabeled and 13C-labeled urea (12C-urea, 13C-urea, respectively, final concentration 0.1 mg/mL) for 30 min, and headspace gas analyzed for presence of $13CO_2$ (as δ $13CO_2$), data are mean ± standard error, $n = 3$).

shown in Fig. 2, and it can be seen that a large increase in headspace δ $^{13}CO_2$ observed (from–16‰ to over 260‰) was dependent upon both the addition of ^{13}C-urea and the presence of *P. aeruginosa.* These data suggested that it could be possible to detect the urease activity of bacteria such as *P. aeruginosa* in the lungs of CF patients by administering nebulized ^{13}C-urea and analyzing exhaled breath for $^{13}CO_2$. It is important to note that CF patients who participated in this trial had confirmed *P. aeruginosa* and they may have been colonized with other urease producing bacteria.

Future research will evaluate DOB at earlier time points after nebulization, as it would appear that the differences between normal controls and CF patients colonized with urease producing bacteria (*P. aeruginosa)* may be even higher and that enhanced sensitivity may result. After appropriate development, the use of inhaled ^{13}C-urea as a diagnostic may offer an advantage for detecting early urease producing bacteria, including but not limited to *P. aeruginosa* infection. *P. aeruginosa* remains the predominant organism infecting lungs of patients with CF and has been associated with significant respiratory complications, poorer lung function, and increased morbidity and mortality.[3] It is well known that when lungs of CF patients are colonized with *P. aeruginosa*, organism cannot be eradicated. Furthermore, identifying initial *P. aeruginosa* infection in young children or other patients who have difficulty producing sputum for culturing *P. aeruginosa* is challenging. Future studies in use of inhaled ^{13}C-urea as a diagnostic tool may offer an early detection of *P. aeruginosa* so that antipseudomonal therapies might be used where appropriate to reduce colonization by *P. aeruginosa.* The approach may also enable monitoring of bacterial load as a result of antibacterial therapy to confirm a bacterial response.

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Author Disclosure Statement

Graham Timmins is the cofounder and currently Chief Science Advisor for Avisa Pharma, Inc., a clinical stage company that has licensed a number of patents (of which Timmins is inventor or coinventor) on $13CO_2$ breath test detection of lung diseases from the tech transfer arm of UNM, STC.UNM. Timmins has license revenue and stock interest managed through STC.UNM. Dr. Kelly was a paid consultant for Avisa in 2014–2015.

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