Very High Sputum Cyanide Concentrations during Acute Pulmonary Exacerbations in Cystic Fibrosis Patients

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VERY HIGH SPUTUM CYANIDE CONCENTRATIONS DURING ACUTE PULMONARY EXACERBATIONS IN CYSTIC FIBROSIS PATIENTS

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ABSTRACT

Background: Cyanide (CN) has been shown to be chronically present in the airways of cystic fibrosis (CF) patients, as a byproduct Pseudomonas' metabolism. We sought to determine the concentrations of CN in the sputum of CF patients, who were admitted to the hospital with acute clinical exacerbations.

Methods: Sputum was collected within 1 day (median) of admission and immediately analyzed for the presence of total and free CN.

Results: We found 1) toxic levels of CN in the sputum of our patients, ranging from 27 to 401 µM, 2) the majority of the cyanide was in the form of free, thus diffusible, CN and 3) no cyanide in the blood.

Conclusions: While the chronic presence of CN in patients colonized with pseudomonas is not a new concept, the present data support the view that very high levels of free cyanide are present in the airways of CF patients during an acute clinical exacerbation, i.e. levels compatible with concentrations of CN able to virtually stop the mitochondrial activity. It becomes therefore urgent to investigate whether, in CF patients, cyanide is a significant factor affecting the short and long-term outcome and whether a specific treatment of CN intoxication of the airways is warranted during an exacerbation.

Introduction

Although the life expectancy of cystic fibrosis (CF) patients has steadily increased over the last 30 years, the current median survival of CF patients is still less than 40 years in the US [1, 2]. Current treatments aimed at restoring the function of the Cystic Fibrosis transmembrane conductance regulator (CFTR) are, unfortunately, not effective in every patient and must be used as early as possible to limit the development of irreversible structural lung damage. Therefore, the search for additional strategies aimed at impeding the development of airway remodeling and lung fibrosis must be pursued.

The pathophysiology of airway damage in CF is complex and multifarious. The lifelong persistence of microorganisms, e.g. staphylococcus pseudomonas, in the airways, which intensity worsens during acute episodes of clinical exacerbation, creates a pro-inflammatory microenvironment causing cellular dysfunction and destruction [3]. More specifically, the development of biofilms of pseudomonas aeruginosa, the most significant respiratory pathogen colonizing adult CF airways [4], is a significant contributor to the morbidity and mortality of this disease [5, 6]; this contention is supported by a clear benefit of the use of high dosages of antibiotics aimed at lowering pseudomonas bacterial load during episodes of acute clinical exacerbations.

Pseudomonas can exert its toxicity through various mechanisms, one of them, which role has been overlooked, is the production of cyanide (CN) [7, 8]. In a study published in 2008, Ryall et al [5] found CN in the sputum of CF patients colonized by pseudomonas at an average concentration of 72 µM. Hydrogen cyanide can also been identified in the expired gas of young CF patients colonized with pseudomonas [9]. Furthermore, other bacteria such as Burkholderia cepacia, a common colonizer of the CF airways, can also produce cyanide [10]. If in free form,

such concentrations of CN are well above levels capable of intoxicating cells [11] by inhibiting the mitochondrial respiratory chain [12-14]. For Instance, as reviewed by Cooper and Brown[15], cellular respiration is impeded in vitro by concentrations of free CN between 10-50 μM, concentrations that are much lower than local concentrations present in the airways of CF patients. This toxicity may in turn affect any cell in the vicinity of the biofilm, inhibiting critical cellular functions, i.e. from ciliary activity [16] to endothelial or smooth muscles, leading to cellular dysfunction, and eventually tissue necrosis [13], unless significant tolerance develops. In addition, CN intoxication is always associated with a deleterious increase in reactive oxygen species production [17]. Based on these findings, it has been hypothesized that cyanide generation could per se be a significant contributor to pseudomonas aeruginosa pathogenicity in CF patients [18]. It is not known, however, whether cyanide is independently associated with worsening lung function [5] and promotion of disease progression [12].

The aim of our study was to determine cyanide levels in the sputum of CF patients admitted in hospital with symptoms of acute clinical exacerbation. The latter was defined as at least one of the following symptoms: increase in cough and sputum production, shortness of breath, fever, or hemoptysis. Previous studies have looked at cyanide levels in an outpatient setting [19] or in patients whose criteria for recruitment was to be actively colonized by Pseudomonas [5]. Our rationale was that since pseudomonas aeruginosa can produce cyanide levels upwards of 300-500 μM in vitro [4, 20], much higher levels of CN than those previously reported may be well present in the airways during acute clinical exacerbations, wherein bacterial load is increased. Second, we wanted to determine whether the pool of cyanide present in the sputum of these patients was in a free/diffusible form. Indeed, as protein or iron-bound

cyanide would be virtually trapped in the sputum, their presence, even at very high concentrations, may not result in a noxious effect, per se. In contrast, free cyanide could diffuse outside the biofilm and cause significant cellular damage in the bacterium's vicinity. Finally, we determined the level of CN in the blood of few of these patients, although the very small amount of CN diffusing in the blood, if any, is expected to be rapidly metabolized [21]. Our objective was to expand our current knowledge on the presence of CN in CF patients during acute clinical exacerbations.

Methods

Human sputum and blood collection

Thirteen adult CF patients were recruited during their admission to the Penn State Milton S. Hershey Medical Center during an acute clinical exacerbation (Table 1). Subjects had a median age 35 years (range 23-48). Their FEV_1 averaged 39 ± 16.7% of expected values, with a mean admission days per year of 28, a mean BMI of 23 kg/m^2, and a mean CF-ABLE score of 3 [22].

Subjects were admitted to the hospital for acute exacerbations of their CF, commonly reporting increasing cough and fatigue (see Table 1). Subjects were approached typically within 1 day of admission, as to decrease the amount of time subjects were exposed to antibiotics as part of their standardized treatment algorithm. Informed consent was obtained from each patient and the study was approved by the Hershey Medical Center/Penn State University Institutional Review Board Committee. Culture and identification of bacteria found in patient's sputa was performed by the Clinical Microbiology laboratory at Penn State Milton S. Hershey Medical Center using a standardized protocol for Cystic Fibrosis culture [23]. All methods were carried out in accordance with relevant guidelines and regulations

Sputum was collected in the morning after administration of hypertonic saline nebulizers (as an aid for sputum expectoration), aliquoted into sterile containers for the clinical microbiology lab and the analytical lab, and transported to the laboratories for immediate analysis. Sputum was weighed and half was flash frozen using liquid nitrogen for further analysis. In addition, sputa specimens were processed, inoculated and incubated in CO_2 at 35°C for 4 days on the following media: Sheep Blood Agar, Chocolate Agar, Colistin-Nalidixic Acid Agar, MacConkey Agar, Mannitol Salt Agar, and Pseudomonas cepacia Agar. Bacterial identification

was performed by matrix-assisted laser desorption ionization time-of-flight (MALDI-TOF) mass spectrometry (Bruker Daltonics, Hamburg, DE).

The treatment strategy for the patients CF exacerbation included antibiotic therapy, chest physiotherapy, mucolytics (Dornase alpha), and airway surface liquid hydrators such as nebulized hypertonic saline [24].

Total CN measurement

Total cyanide was measured using the approach proposed by Lundquist and Sörbo [25]. Sputum was diluted with one ml saline and vortexed. 1 mL of this mixture was added to 3 mL of 0.33 mol/L perchloric acid and vortexed. After 2 minutes, the solution was centrifuged (Eppendorf, Hamburg, Germany) at 5000 rpm for 3 minutes and then filtered through glass wool in a Pasteur pipette. 2.5 mL of the filtrate was mixed with 500 µL of 1.0 mol/L sodium acetate before being mixed with 150 µL of 0.05 mol/L sodium hypochlorite. Within one minute, 500 µL of barbituric acid-pyridine reagent was added and mixed. After 5-10 minutes, a BioSpectrometer (Eppendorf, Hamburg, Germany) was used to determine spectrum of absorbance of a 2mL sample between 300 and 750 nm). The absorbance at 580 nm was proportional to CN concentration. Negative control and positive controls were made using the same methodology with 1 mL saline and 100 µL CN. Standardized curves were established for CN using concentrations of 0, 25, 50, 75, and 100 µM.

Blood samples were obtained on the same day as sputum collection with a K2-EDTA vacutainer. The same approach was used to determine the concentration of CN in the blood [25].

Cyanalyzer method for Free and Total CN in blood and sputum

The concentrations of free and total CN in blood and sputum from CF patients were measured based on method described previously [26] with slight modifications. Briefly, whole blood or diluted sputum (25 µL; sputum was diluted by weighing ≈1 g of sputum and adding 3 mL of deionized water) was pipetted into the sample chamber of a sampling device, to which 80 µL of deionized water was added. For free CN analysis, 200 µL each of NaOH (0.1 M), NDA (0.002M), and taurine (0.1 M) were added to the capture chamber of the sampling device. Free CN (as HCN) in the sample was then transferred from the sample chamber to the capture chamber using active microdiffusion with room air as the carrier gas. The free CN was captured in the basic solution of the capture chamber as CN- and reacted with NDA and taurine to form a CN-NDA-taurine complex. The fluorescence signal of the CN-NDA-taurine complex was measured using the Cyanalyzer (a CN measuring device based on the core concepts of Jackson et al. 2014) [26].

For total CN analysis, the same procedure was followed for the analysis of free CN, except H_2SO_4 (200 µL of 2 M) was added to the sample in the sample chamber to convert all cyanide in the sample to HCN. The HCN produced from the addition of H_2SO_4 was actively diffused to the capture chamber and reacted with NDA and taurine to form the CN-NDA-taurine complex. The fluorescence signal was then measured using the Cyanalyzer. The fluorescence signals obtained from the blood or sputum samples were converted to concentrations using a calibration curve from 10-200 µM CN.

Spirometry measurement

FEV_1 was determined on the day after admission (spirometer Vyaire, California, USA). All spirometry measurements were done and interpreted according the American Thoracic Society recommendations [27].

Data analysis

Data are expressed as mean ± SD, unless stated otherwise. Correlations were established between [CN] and patient characteristics (Age, BMI, admission days, FEV_1, and CF-ABLE) using Microsoft Excel software.

Results

Total CN measurement

Pseudomonas aeruginosa was isolated in twelve out of the thirteen patients. Total cyanide, analyzed by spectrophotometry, was however present in every sputum sample, with a concentration ranging from 27 to 401 µM (average of 153 ± 125 µM, Figure 1). We found no correlation between [CN] and age, FEV_1, admission days in previous year, BMI, or CF-ABLE scores. Of note, the seven patients with the highest [CN] (>100µM) had a similar FEV_1 and CF-able score than those with low CN.

Free vs total CN measurement

The concentration of free and total cyanide in sputum was determined in 4 patients (samples 10 to 13, Table 2). Virtually all the CN present in the sputum samples was in free form (95 ± 17% of total CN, Table 2) averaging 40.4 ± 7.3 µM. Of note, the concentration of total CN was in a similar range (44 ± 13.2 µM), although lower, than the concentration obtained with spectrophotometry (70 ± 24 µM), which is likely due to possible loss of CN through transportation of sputum samples. Finally, we found no measurable cyanide in the blood samples (10 to 13) by either of the 2 methods.

Discussion

This study shows that in the sputum of CF patients with an acute clinical exacerbation, cyanide can be present at very high concentrations, i.e. at levels that were clearly in the toxic range [14]. This study confirms and expands the findings from Ryall et al [5], who, using an ion-

sensing electrode, found up to 130 µM of CN (average 72 µM) in the sputum of 15 patients with bronchiectasis colonized with pseudomonas, but who had no evidence of clinical exacerbation. Of interest, just like in our present results, there was no correlation between the level of CN and the deterioration in lung function in that study. They also found that cyanide was undetectable in 10 patients with significant structural damage but no pseudomonas infection. Recently, Eiserich et al [19] found sputum CN concentrations ranging from 14.1– 98.1 µM in 12 cystic fibrosis patients who presented in an outpatient setting. Our patients displayed higher CN concentrations (averaging 153 µM), but were all presenting a clinical picture of CF exacerbation. Of note, in vitro, concentrations of free CN of less than 1 µM are sufficient to literally stop the cytochrome c activity [15]. Since acute clinical exacerbations are typically associated with an increased bacterial load, an exaggerated cyanogenesis in the airways is not unexpected. This contention is supported by the decrease by half of CN concentration in the lungs of a mouse model with pseudomonas infection after few hours into antibiotic treatment [28]. Finally, one patient displayed significant concentrations of CN in the sputum while no pseudomonas could be isolated. As Achromobacter, the main bacterium in this patient, does not produce cyanide [29], we speculated that the isolated sputum culture that was obtained in this patient may not be representative of the airway microbiome. Pseudomonas was nevertheless found in this patient's airway a few months prior to and after this acute exacerbation.

An important finding of this study is that the vast majority of CN in the sputum was in the form of free CN, which would be capable of diffusing into surrounding cells and blocking cytochrome C oxidase activity. This finding supports the view that CN found in the sputum of our patient is likely to be toxic for any cells in contact with the biofilm and the sputum but also in the

alveolar regions, wherein free CN can diffuse as HCN [9]. As rhodanese activity –the major enzymatic system inactivating CN into thiocyanate [21] – is lower in the lung parenchyma than in other tissues [30], the presence of such high levels of cyanide could be especially toxic to lung structures. Of note, cyanide was undetectable in the blood, indicating that although free cyanide is present in the sputum, both the quantity of CN capable of diffusing and the capacity of the body to metabolize CN made it unlikely for CN to be found in the blood or tissues. Therefore, despite the lack of measurable CN in the bloodstream, we cannot rule out that some systemic effects could have been produced by the diffusion of cyanide from the alveolar regions into the blood.

Whether CN could be used as a marker of an acute exacerbation in a given patient has already been discussed and debated [12]. The very large range of CN found among patients in our study and its lack of correlation with the respiratory function challenge the idea that CN could be used as a good surrogate to evaluate the respiratory status. More importantly, whether cyanide could play, by itself, a significant contribution to clinical exacerbation episodes and to the development of irreversible lung and airway damage has fundamental therapeutic implications. Indeed, various ways of sequestering CN are currently used to treat systemic CN intoxication [31]. Some of these antidotes could be offered to rapidly decrease free CN in the airways of patients presenting with acute clinical exacerbations, with the goal of bringing CN concentrations toward less concerning levels. Two outstanding questions remain to be clarified: first, the capacity of the airway epithelium to metabolize cyanide or to trap it in the proteins present the sputum following chronic exposure is not known and could render the presence of CN in the airway less toxic than in naïve subjects. In other words, we have no clear idea if the

chronic presence of cyanide in the airways should be a source of concern in these patients. secondly, the chronic presence of CN could also play a protective role by impeded fungal growth [32]. In the context of potential beneficial effects of CN, the treatment of the persistent presence of low levels of CN in the lungs is therefore open to debate.

During an acute exacerbation, the benefit of providing systemic or inhaled cyanide binding agents, in addition to the current standard treatment, should be seriously evaluated in CF patients.

Declarations

Ethics approval and consent to participate

Informed consent was obtained from each patient and the study was approved by the Hershey Medical Center/Penn State University Institutional Review Board Committee.

Consent for publication

NOT APPLICABLE

Availability of data and materials

All data generated or analysed during this study are included in this published article.

Competing interests

The authors have no conflict of interest or competing interests

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Authors' contributions

Daniel Guck, David Kim and Nicole Tubbs collected the sputum samples and performed the analysis. Daniel Guck, David Kim made the figure and wrote the first draft of result and method sections, reviewed and edited the final version.
Brian Logue, Nesta Bortey-Sam, David Craft performed some of the analysis and edited the paper
Philippe Haouzi conceived the study and wrote the first draft of the paper, he made the figure and reviewed and edited the final version of the manuscript

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N/A

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Figure legends

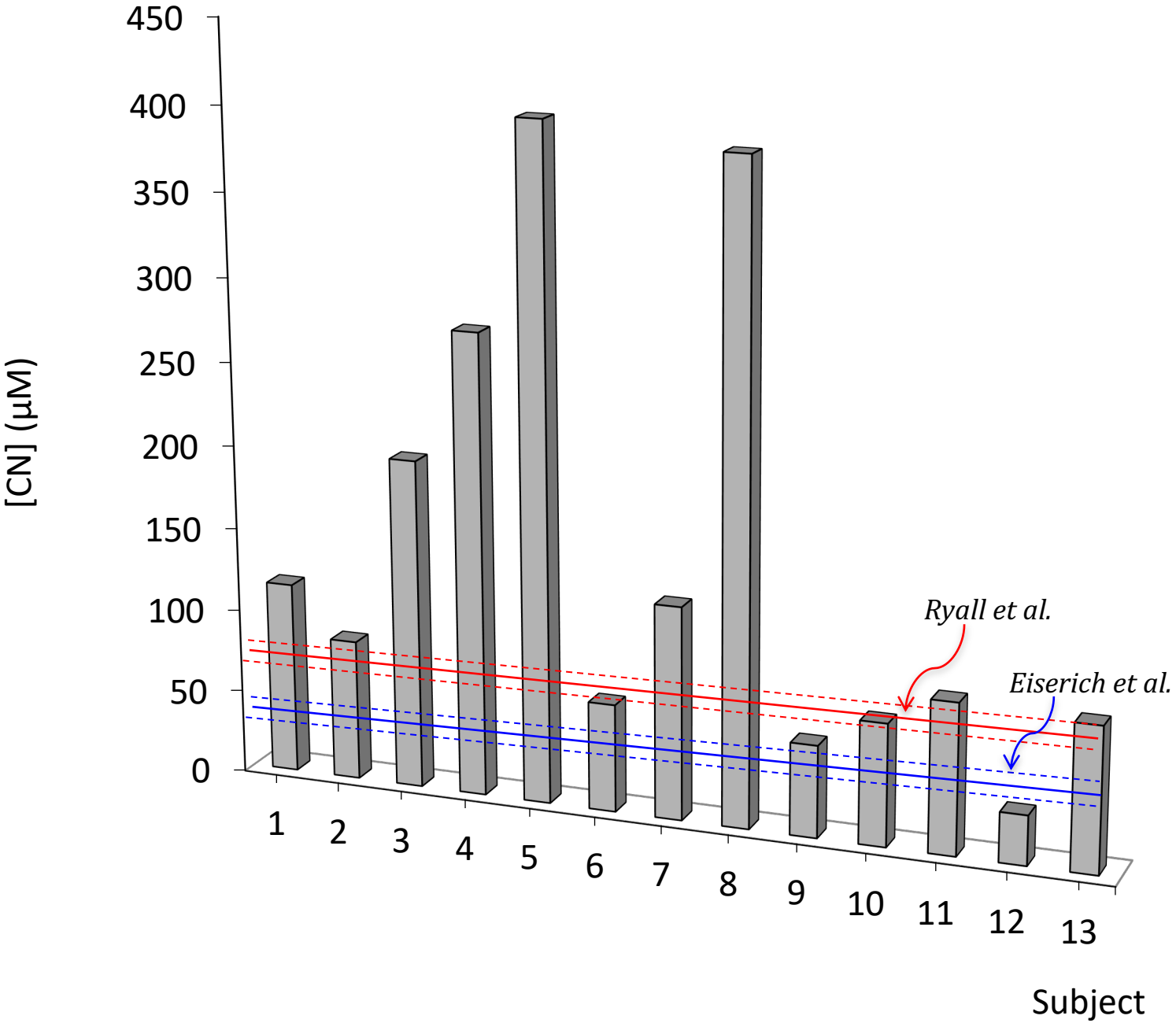
Figure 1. Total cyanide concentrations in the sputum samples of our 13 CF patients in acute clinical exacerbation. Mean CN concentration reached 153 ± 125 µM. Data from other studies reporting cyanide concentrations in the sputum of CF patients either colonized with pseudomonas (72 ± 6.6 µM) [5] or who presented in the outpatient setting but with no evidence for clinical exacerbation (37 ± 6.8 µM) [19], are also displayed.

Sample	Age	FEV_1	BMI	CF-ABLE	Admission Days in Previous Year	Reason for Admission	Days of Admission until Collection	Sputum Culture
1	34	30	22	3.5	9	A, B	1	Burkholderia, P. aeruginosa
2	26	47	27	3.5	10	A, B, D	1	P. aeruginosa , H. influenzae
3	35	30	24	3.5	19	A, B, D	1	P. aeruginosa, Achromobacter xylosoxidans
4	24	54	22	0	39	C	8	P. aeruginosa
5	35	62	25	0	9	B	1	S. aureus, P. aeruginosa
6	48	18	16	5	64	B, E	5	S. aureus, P. aeruginosa
7	36	66	23	3.5	10	A, B, D	1	P. aeruginosa
8	31	15	13	5	153	A, B	1	P. aeruginosa
9	36	31	24	3.5	15	A, B	1	P. aeruginosa
10	32	22	23	3.5	12	B, D, E	1	P. aeruginosa
11	29	40	26	3.5	5	B, D	2	P. aeruginosa
12	37	25	23	3.5	26	B	2	P. aeruginosa
13	23	54	22	1	14	B	1	S. aureus, Achromobacter xylosoxidans

Table 1. Sample and subject characteristic data at time of hospital admission. FEV_1 was calculated within 1 day of admission. Sputum cultures obtained at time of admission. *Reason for Admission Legend*: A - Shortness of breath, B - Increased sputum production, C - Hemoptysis, D -Weakness, E - Fever

Sample	[Free]	[Total]	%[Free]
10	48.8	58.3	83.7
11	38.4	49.2	78
12	43	41.6	103
13	31.4	27	116
Mean	**40.4**	**44**	**95**
SD	**7.3**	**13.2**	**17.5**

Table 2. Sputum Concentrations of free and total CN found in CF sputum using the Cyanalyzer in 4 subjects.


450
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[CN] (µM)
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Subject
Ryall et al.
Eiserich et al.

Figure 1