Additive effect of dornase alfa and Nacystelyn on transportability and viscoelasticity of cystic fibrosis sputum

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OBJECTIVE: To investigate the effect of dornase alfa (DA), Nacystelyn (NAL) and their combination on mucociliary transportability and mucus viscoelasticity of cystic fibrosis (CF) sputum, and to assess whether the combination possesses an additive effect.
DESIGN: Determination of transportability in frog palate and viscoelasticity in vitro.
SETTING: Research laboratory at a medical centre.
PATIENTS: Sputa from 15 patients with CF, chronically infected with *Pseudomonas aeruginosa*, were studied.
INTERVENTIONS: Sputum samples were incubated without any drug solution as a control, and with normal saline, DA, NAL and a mixture of DA and NAL in concentrations approximating those achieved in clinical practice.
RESULTS: Normal saline (10% volume) by itself had a small effect on CF sputum transportability with a mean increase of 9%, and on viscoelasticity with a mean of decrease of 0.22 log units, respectively, compared with control (incubation without saline). DA (200 nM) further increased the transportability by a mean of 35% versus saline and decreased viscoelasticity by a mean of 0.30 log units. NAL (100 µM) increased the transportability by a mean of 32% and decreased viscoelasticity by a mean of 0.22 log units from the levels achieved with saline. The mixture of DA plus NAL at one-half of the above concentration of each agent produced an additional increase in the transportability, by a mean of 18%, and a further decrease in viscoelasticity, by a mean of 0.25 log units, compared with DA or NAL as a single treatment.
CONCLUSIONS: The combination of DA and NAL exhibits an additive effect for both the viscoelasticity and transportability of CF sputum samples. The two agents appear to act well together in breaking down the bonding due to extracellular DNA and mucins. Clinical studies should be undertaken to see whether the additive combination at lower concentration produces the anticipated benefits of improved airway clearance and fewer side effects.

Key Words: *Cystic fibrosis; Mucociliary transport; Mucolytics; Sputum viscoelasticity*

Résumé à la page suivante

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Effet additif de la dornase alfa et du nacystélyn sur la mobilisation et la viscoélasticité des expectorations dans le contexte de la mucoviscidose

OBJECTIF : Étudier l'effet de la dornase alfa (DA), du Nacystélyn (NAL) et de leur association sur la mobilisation des sécrétions mucociliaires et sur la viscoélasticité du mucus dans le contexte de la mucoviscidose (MV) et vérifier si leur association produit un effet additif.

PLAN D'ÉTUDE : Détermination de la mobilisation des sécrétions dans des palais de grenouille et de la viscoélasticité *in vitro*.

LIEU : Laboratoire de recherche dans un centre médical.

PATIENTS : Ont participé à l'étude 15 patients atteints de MV et présentant des infections chroniques à *Pseudomonas aeruginosa*.

INTERVENTIONS : Des prélèvements d'expectoration ont été mis en incubation, certains sans solution médicamenteuse servant de témoins, d'autres avec du sérum physiologique (SS), de la DA, du NAL et un mélange de DA et de NAL en des concentrations voisines de celles utilisées en clinique.

RÉSULTATS : Le SS (10 % en volume) seul a eu très peu d'effet sur la mobilisation des expectorations, soit une augmentation moyenne de 9 %, et sur la viscoélasticité, soit une diminution moyenne de 0,22 unité logarithmique par rapport aux témoins (incubation sans SS). La DA (20 nM), de son côté, a augmenté la mobilisation de 35 % en moyenne par rapport au SS et a diminué la viscoélasticité de 0,30 unité logarithmique en moyenne. Quant au NAL (100 µM), il a permis une augmentation moyenne de 32 % de la mobilisation et une diminution moyenne de 0,22 unité logarithmique de la viscoélasticité par rapport au SS. Enfin, le mélange de DA et de NAL à la moitié des concentrations indiquées précédemment pour chacune des substances a produit une augmentation moyenne de la mobilisation de 18 % et une diminution moyenne de la viscoélasticité de 0,25 unité logarithmique comparativement à la DA et au NAL seuls.

CONCLUSION : L'association de la DA et du NAL a eu un effet additif tant sur la viscoélasticité que sur la mobilisation des expectorations produites dans le contexte de la MV. Les deux agents semblent bien se compléter pour cliver les liaisons dues à l'ADN extracellulaire et aux mucines. Il faudrait mener des essais cliniques pour vérifier si l'effet additif de l'association à des concentrations plus faibles améliore toujours le dégagement des voies aériennes tout en produisant moins d'effets indésirables.

Mucociliary transport (MT) is a physiological response of the respiratory tract to clear both normal and excessive airway secretions (1). Impairment of MT, which may finally result in obstructive and damaged airways, is a characteristic feature of cystic fibrosis (CF). The decreased mucociliary transportability is primarily attributed to overproduction, accumulation and persistence of macromolecules (eg, DNA, albumin, etc) in the patient's airways (2,3). The direct approach to increase mucociliary transportability of the CF gel mucus in airways is mucolysis, ie, the disruption of the gel network by altering the degree of crosslinking or the interactions between the macromolecules that form it (4). This disruption may be achieved through mucolytic agents such as dornase alfa (DA) (Pulmozyme, Genetech, USA) and Nacystelyn (NAL) (SMB & Galephar, Belgium) (4,5).

Because substantial amounts of DNA of high molecular weight are the leading cause of the tenacious and viscous properties of CF sputum (2,3), DA has been applied to treat CF sputum in vitro to hydrolyze the excess DNA in CF airway mucus and, thus, reduce the viscosity, changing it from rigid, poorly deformable material to a more fluid gel, thereby facilitating MT (2). Clinical studies have reported improved lung function in CF patients after DA treatment, and DA is widely used as a treatment for CF lung disease (6,7).

NAL is a compound of *N*-acetylcysteine (NAC) and L-lysine (8). It has been found in vitro to reduce the mucous gel disulfide bonds to sulfhydryl bonds, thereby reducing the mucoprotein viscosity and enhancing transportability (9). Preliminary clinical trials with NAL have shown it to be effective in reducing the viscoelasticity of sputum in CF patients (10).

Despite the many different mechanisms by which the rheological properties of mucus can be altered (7), very few studies have been carried out regarding the additive effects of mucolytics on CF sputum. Our previous experiments demonstrated that the combination of DA and NAL at one-half the concentration of each agent significantly decreased the spinnability (one of the rheological parameters) of CF sputum more than either treatment by itself (4). We hypothesized that their combination would also improve viscoelasticity (the most important rheological factor) and mucociliary transportability more than either of them on their own.

To investigate this hypothesis, the researchers measured and compared in vitro the viscoelasticity (log G*, the major parameter of viscoelasticity) of CF sputum before and after treatment with DA, NAL and their combination. In the present study, the authors also employed the mucus depleted frog palate as the animal model to determine mucociliary transport velocity (MTV), a direct index of mucociliary transportability of CF patient sputum.

MATERIALS AND METHODS

Subjects

Sputum samples were collected from 15 patients with CF by voluntary expectoration during a routine clinical visit before a clinical trial of azithromycin. The patients, aged 10 to 19 years, were all infected with *Pseudomonas aeruginosa*. Eight of the patients were receiving oral NAC, usually 600 mg/day. Four of these eight patients were also receiving a daily inhalation of 2.5 mg DA. In most cases, the patient sputum was collected in the morning, before the administration of any mucolytic medication. Collection and use of sputum for this experiment were

approved by the Hannover Medical School Research Ethics Board.

Study design

The sputum samples, which had been stored at –80°C, were allowed to reach room temperature before analysis. Aliquots of sputum (approximately 5 mg to 18 mg) were subjected to the following treatment protocols: 1) treatment with 50 µg/mL DA (Pulmozyme) in 0.9% saline to achieve a final concentration of 5 µg DA/g of sputum (approximately 200 nM); 2) treatment with 309 µg/mL NAL in 0.9% saline to achieve a final concentration of 30.9 µg NAL/g of sputum (100 µM); 3) no drug treatment (DA or NAL), as a negative control; and 4) treatment with 0.9% saline, as a positive control to test the dilution effect. The samples in each protocol were incubated at 37°C for 30 min.

The concentrations of DA and NAL were chosen on the basis of previous experience (4) and preliminary experiments, which indicated a decrease in spinnability to about one-half the control value, but no significant effect on viscoelasticity by DA (100 nM). The threshold for viscoelasticity effect is higher than for spinnability (4), but at double the concentration of DA (5 µg/g), there is now an appreciable effect on log G* (11). The authors' previous study also indicated that a 30 min incubation period for the experimental drug was appropriate to show significant changes in mucus rheological properties (4).

To observe the combined effects of DA and NAL, protocols 1 and 2 were combined at one-half the concentration of each drug, and incubation was carried at 37°C for 30 min. One-half the concentration of each drug was applied to test for the possible interaction between them to avoid reducing log G* to too low a value. This could potentially approach that of uncrosslinked mucus and, as a consequence, reduce MTV to below an optimal value. An additional decrease in log G* or increase in MTV compared with that achieved by either treatment by itself would serve as evidence for supra-additivity of effect.

Viscoelasticity measurements

The viscoelasticity of microlitre quantities of sputum was measured by means of a magnetic microrheometer (12). An 80 to 120 µm steel sphere is placed within a 5 to 20 mg sample of sputum. An electromagnet oscillates this sphere, whose image is projected onto a pair of photocells via a microscope. The motion of the sphere is plotted against the driving force of the magnet on an oscilloscope, forming an ellipse, from which G* is measured. G* is the mechanical impedance or vector sum of viscosity and elasticity, reported here on a log scale.

For each treatment protocol, log G* at 10 rad/s (an intermediate frequency) was measured before any treatment (baseline), and then after 30 min of application of the treatment, leaving the steel microsphere in place to remove the major source of imprecision, namely the intra-aliquot inhomogeneity of sputum. The viscoelasticity measurement was performed once for each aliquot of the sample.

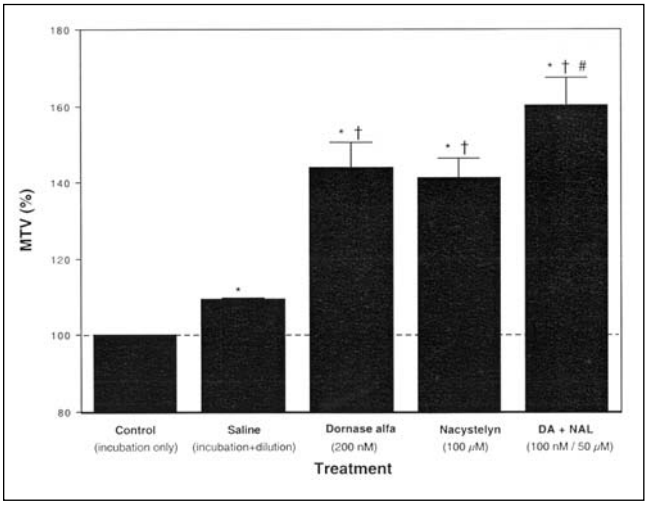

Figure 1) *Additive effect of dornase alfa (DA) (Pulmozyme, Genentech, USA) and Nacystelyn (NAL) (SMB & Galephar, Belgium) on transportability (mucociliary transport velocity [MTV]) of cystic fibrosis (CF) sputum in frog palates. Values are expressed as percentages of the respective values of the control sputum samples incubated without drugs. Each vertical bar represents the mean value ± SD for seven samples. *Statistically significant difference from the control value; †Statistically significant difference from the saline value; #Statistically significant difference from the DA or NAL value.*

MTV determination using frog palates

Mature leopard frogs (*Rana pipiens*) were sacrificed using the double pithing method, as introduced previously (13). Immediately after sacrificing the frog, the palate was excised by disarticulation of the jaw and removal of the upper part of head by cutting with scissors through the junction of the posterior pharynx and esophagus out to the skin of the back. The excised palate was then placed in the middle of a 20 L acrylic chamber (20 cm height, 40 cm width, 25 cm depth) at 100% humidity, measured with an aneroid hygrometer (Bacharach 15238, Bacharach Instruments Inc, USA), and at room temperature (20°C to 25°C).

Determination of MTV was made by measuring the rate of displacement of the treated sputum sample (approximately 10 mg), placed on the frog epithelial surface, using a stereomicroscope provided with a reticulated eyepiece. MTV was calculated by dividing the distance traveled (9 mm) by the time elapsed (min). Six measurements were made for each sample to minimize measurement variability, and the arithmetic mean of the six readings was calculated. The samples were applied to the frog palates in random order to account for temporal variations in transport rate.

Statistical analysis

The significance of the results was assessed by analysis of Fisher Protected Least Significant Difference (ANOVA). All results are presented as mean ± SD unless otherwise stated. The level of significance was set at 5 %.

TABLE 1
Viscoelasticity data (log G* at 10 rad/s) of treated and untreated cystic fibrosis (CF) sputum (n=15)

Treatment	Control	Saline	DA	NAL	DA+NAL
Before	2.98±0.08	3.05±0.07	3.08±0.09	3.06±0.07	3.17±0.04
After	2.91±0.09	2.75±0.06	2.48±0.08	2.54±0.07	2.36±0.06

Aliquots of cystitc fibrosis sputum samples were incubated without treatment (control), or with saline, dornase alfa (DA, 200 nM), Nacystelyn (SMB & Galephar, Belgium) (NAL, 100 µM), or DA+NAL (100 nM/50 µM) at 37°C for 30 min. All data are presented as log G (means plus or minus standard error) for 15 samples*

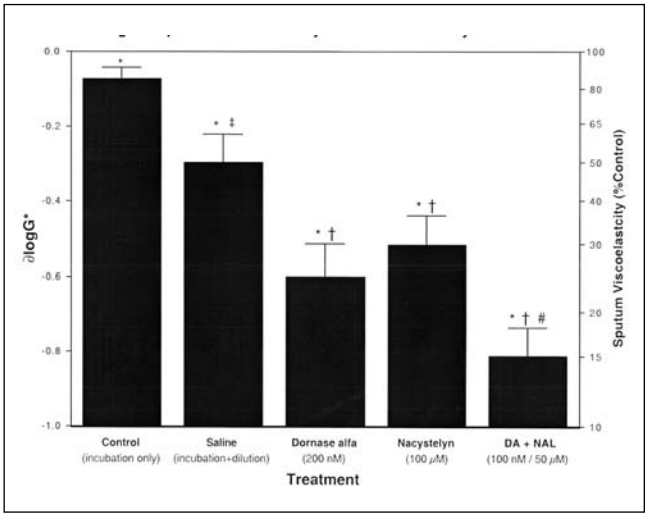

Figure 2) *Additive effect of dornase alfa (DA) (Pulmozyme, Genentech, USA) and Nacystelyn (NAL) (SMB & Galephar, Belgium) on viscoelasticity (log G*) of cystic fibrosis (CF) sputum in vitro. Values are expressed as the change in log G* from the pretreatment control values (rt ordinate: % control linear scale). Each vertical bar represents the mean value ± standard error for 15 samples. *Statistically significant difference from the pretreatment value; ‡Statistically significant difference from the control value; †Statistically significant difference from the saline value. #Statistically significant difference from the NAL value*

RESULTS

Ciliary transportability

Saline treatment produced a small but statistically significant increase in MTV from the untreated control (17.87±0.98 mm/min versus 16.35±0.91 mm/min) (Figure 1). The application of DA to the sputum (5 µg/g final concentration) produced a significant increase in MTV compared with incubation with normal saline (23.42±1.41 versus 17.87±0.98 mm/min). The application of 100 µM of NAL also increased MTV significantly more than normal saline (23.02±1.37 versus 17.87±0.98). There was no significant difference in MTV between the application of DA and NAL at the indicated concentrations.

Compared with treatment with normal saline, the combined treatment of DA and NAL (one-half the above mentioned concentration of each mucolytic agent) produced a significant increase in MTV (26.27±2.07 versus 17.87±0.98) (Figure 1). The combined treatment also significantly increased MTV compared with the application of either DA or NAL alone.

Sputum viscoelasticity

Table 1 shows the viscoelastic data, presented as log G* (mean ± SE) for 15 samples before and after different treatments. Figure 2 shows the values expressed as the change in log G* value (% control linear scale) from the pretreatment control values and the statistical significance between the different groups. Incubation only (untreated control) resulted in a slight decrease (0.073 log units, P=0.0395) from the pretreatment viscoelasticity. Saline treatment (incubation plus dilution) resulted in a further modest decrease in log G* (0.296 units, P=0.0105). DA treatment at 5 µg/g final concentration (approximately 200 nM) decreased the viscoelasticity by a mean of 0.60 log units (to approximately 25% control, P=0.0127). NAL treatment at 100 µM decreased the viscoelasticity by a similar amount (0.518 log units, P=0.0522 with respect to saline).

The combination DA plus NAL at one-half the concentration of each agent resulted in the greatest decrease in viscoelasticity (0.813 log units, approximately 15% control, P=0.0001). The decrease in viscoelasticity due to DA plus NAL was significantly greater than for NAL alone (P=0.012) but not for DA alone (P=0.0735).

Both components of G* (G′ – elasticity and G″ – viscosity) decreased with mucolytic treatment. There was no significant change in tan δ (G′/G″).

The response to in vitro mucolytic treatment (decrease in viscoelasticity compared with vehicle) was not different in those patients who had been receiving mucolytic drugs (NAC or DA) versus those who did not (Table 2). This was also the case in terms of in vitro ciliary transportability.

DISCUSSION

CF sputum treated with either DA (200 nM) or NAL (100 µM) demonstrated a significant increase in transportability and a significant decrease in viscoelasticity compared with CF sputum treated with saline. However, in comparison with singular treatments, the combined treatment of DA and NAL, at one-half the concentration of each mucotropic agent, showed an even larger, statistically significant increase in transportability and decrease in viscoelasticity of CF sputum. This finding is important because it indicates that the two agents clearly complement one another and, importantly, do not interfere with each other's action.

The enzyme DA has the ability to break down large concentrations of DNA, thus improving the rheological properties of CF sputum. In this study, CF sputum treated with DA at a final concentration of 5 µg/g demonstrated an

TABLE 2
Decrease in viscoelasticity (δlog G* at 10 rad/s) and increase in mucociliary transport velocity (MTV) of cystic fibrosis sputum of patients who had received mucolytic drugs versus those who had not

dlog G* at 10 rad/s	Control	Saline	DA	NAL	DA+NAL
No mucolytics (n=7)	0.140±0.08	0.246±0.07	0.554±0.09	0.424±0.07	0.823±0.04
Mucolytics (n=8)	0.014±0.06	0.340±0.06	0.640±0.08	0.600±0.07	0.804±0.06

MTV (% control)	Saline	DA	NAL	DA+NAL
No mucolytics (n=3)	109.3±0.7	141.3±15.7	140.4±12.3	152.5±14.5
Mucolytics (n=4)	109.5±0.6	145.8± 5.7	141.7± 4.6	165.9± 7.0

Aliquots of CF sputum samples were incubated without treatment (control), or with saline, dornase alfa (DA, 200 nM), Nacystelyn (NAL, 100 µM) (SMB & Galephar, Belgium) or DA+NAL (100 nM / 50 µM) at 37°C for 30 min. δlogG and MTV data are presented as means plus or minus standard errors for the number of samples indicated*

increase in transportability. This result is similar to that reported by Zahm et al (14). They observed improvements in sputum transportability (by frog palate assay) with DA at concentrations ranging from 0.2 to 20 µg/mL. In addition, DA at a concentration of 200 nM was found to decrease the sputum viscoelasticity in vitro. These data may explain our previous experimental results that DA at a concentration of 100 nM (lower than the present one) succeeded in decreasing the spinnability of CF sputum but failed to decrease the viscoelasticity (4).

NAL is a derivative of NAC, a thiol-reducing agent that breaks disulfide bonds. NAC has been widely used to treat MT disorders (15). Previous studies have shown that NAL has greater mucolytic activity than NAC (16, 17) and preliminary clinical trials with NAL have shown it to be effective in reducing the viscoelasticity of sputum in CF patients (10). Marriott et al (16), using porcine gastric mucus, found significant mucolytic activity with NAL starting at 8 µM concentration, while App et al (17) also found a significant activity at 10 µM in sputum, which is lower than the concentration used in the present study. Other thiol-reducing agents, such as dithiothreitol or mercaptoethane sulfonate, may overliquefy mucus (17), thereby making it unsuitable for clearance by ciliary action (18).

In the present study, two different doses of NAL and DA manifested significant effects on the viscoelasticity of CF sputum. The dose of NAL at 31 µg/g has equivalence (in terms of rheological and transport effects) to 5 µg/g of DA. The DA dose pertains to 2 to 4 µg/mL of DA in CF sputum, which is achieved by aerosol of DA at a dose of 2.5 mg in the clinic (19). The current dose of NAL in clinical development is 16 mg by dry powder inhaler (SMB & Galephar, Belgium, private communication). This dose is nominally 6.4-fold greater than DA, and thus, likely relevant to the mucolytic concentration used in our study, which was approximately six times as great as that of DA at the point of equipotency. This tendency to greater rheological effect of CF sputum with DA concurs with the results presented by Shah et al (20), who reported that CF sputum samples were more responsive to DA than to NAL; although in the latter study, the concentration of both drugs were much higher than those used in the present investigation.

The combined treatment of sputum with DA and NAL (at one-half the concentration of each mucolytic agent) increased transportability and decreased viscoelasticity significantly more than the singular treatments with either DA or NAL. Although synergism was not formally tested by studying the concentration dependence of the mucolytic behaviour, it is clear from previous studies (4,17) that reducing the concentration of either DA or NAL would have resulted in a decrease in viscoelastic effect. Thus, it is reasonable to describe the supra-additivity of mucolytic activity as an additive effect.

This additive behaviour of DA and NAL in increasing transportability and decreasing viscoelasticity of CF sputum may be due, in part, to cooperative rearrangements of the bonding and intermolecular interactions between neighbouring molecules. Reduction of the mucin disulfide bonds to sulfhydryl bonds by NAL may make DNA more accessible for action by DA. At the same time, cleavage of high molecular weight DNA by DA may assist in increasing the sputum transportability by NAL. These favourable alterations in sputum rheological properties would predict improved expectoration (18) and, ultimately, an improvement in lung function of CF patients, provided that overliquefication of sputum does not occur. Although reduction of the disulfide bonds in DA by NAL, and thus interference with its DA action, is a theoretical possibility, there is no suggestion from our findings that this might have occurred. DA should have little effect on normal mucin networks, as suggested by rheological experiments using tracheal mucus from healthy dogs (21).

CONCLUSIONS

The results of the present studies both in frog palate and in vitro suggest that the combined treatment of CF sputum with DA and NAL may lessen the respiratory burden caused by the impaired clearance of airway secretions with abnormal rheological properties in these patients. By combining DA and NAL, mucolysis may not only be more appropriate for rheological change, but also more cost effective in the treating disorders of impaired MT of sputum with abnormal rheological properties. NAL has not yet been tested clinically in North America. Because NAL appears to work in vitro at lower concentration than its par-

ent compound NAC, and its pH is more neutral, the potential for side effects is reduced. Furthermore, combining it with another mucolytic approach, as the results of the present study suggest, could minimize such undesirable effects. It is possible, however, that NAL interacts with DA molecules, leading to either a reduction in its hydrolytic activity or alteration in its conformation. Thus, data on drug interaction are needed before conducting further studies on patients. Additional studies with experiments in a large patient population are required to confirm these findings and investigate issues regarding the safety and efficacy of long term administration of DA and NAL in combination.

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