

Supplementary Appendix

This appendix has been provided by the authors to give readers additional information about their work.

Supplement to: Donaldson SH, Bennett WD, Zeman KL, et al. Mucus clearance and lung function in cystic fibrosis with hypertonic saline. *N Engl J Med* 2006;354:241-50.

APPENDIX MATERIAL

METHODS

Study Design

Complete inclusion and exclusion criteria are provided below.

Inclusion criteria:

1. Established diagnosis of CF
 - a. 2 gene mutations identified, or
 - b. Sweat chloride > 60 mmol/L, and
 - c. 1 or more typical CF clinical features
2. Age \geq 14 years
3. Able to perform spirometry and have post-bronchodilator FEV₁ \geq 50% of predicted at screening
4. Oxyhemoglobin saturation (by pulse oximetry) \geq 92% on room air
5. Able to provide informed consent

Exclusion criteria:

1. Unstable lung disease:
 - a. FEV₁ \geq 15% below best clinical measurement within 6 months
 - b. Requirement for IV antibiotics within 4 weeks of screening
 - c. Requirement for any change in pulmonary medication within 2 weeks of screening
2. Evidence of reactive airways
 - a. Clinical diagnosis of asthma
 - b. \geq 15% increase in FEV₁ after bronchodilator at screening
3. Hypertonic saline use within 2 weeks of screening
4. Unwilling or unable to either continue or discontinue cyclical therapies (e.g. inhaled tobramycin) for the 2 weeks prior to screening and the entire study period
5. Pregnancy, breast-feeding, or unwillingness to use barrier contraception during the entire study period
6. History of allergy or intolerance to amiloride, hypertonic saline, quinine, albuterol, or related compounds
7. Renal insufficiency (creatinine > 1.5 mg/dl)
8. Hyperkalemia (K⁺ \geq 5.0 meq/L)
9. Investigational drug use within 30 days of screening
10. Radiation exposure within the past year that would exceed Federal Regulations by participating in the study

Lung Function Assessments

Spirometry and plethysmographic lung volumes were expressed as a percentage of predicted norms from Knudson et al(1). The changes in FEV₁, FVC and FEF₂₅₋₇₅ during the baseline and treatment intervals were calculated as follows:

Baseline interval:

$$\frac{(\text{Average of values on Days 13,15}) - (\text{value on Day 1})}{\text{Value on Day 1}} \times 100$$

Treatment interval:

$$\frac{(\text{Average of value on Days 26, 28}) - (\text{value on Day 15})}{\text{Value on Day 15}} \times 100$$

Dose-Response of Amiloride on ASL Volume Response to HS

The effect of amiloride pretreatment on ASL height, measured 10 minutes after the addition of NaCl (0.8 mg) in perfluorocarbon, was determined over a range of amiloride concentrations (0.1 to 400 μM in the apical compartment) in CF epithelial cultures using XZ confocal microscopy.

Site of Amiloride's Block of Water Permeability

Osmotically driven water flow across the apical cell membrane was assessed by serially measuring cell height with XZ confocal microscopy before and after the addition of a hyperosmotic mannitol solution (PBS + 300 mM mannitol; 100 μl) to the apical surface of CF epithelia. Cell height measurements were facilitated by pre-labeling cells with the intracellular fluorophore, calcein-AM (Molecular Probes). The effect of amiloride (100 μM) on cell shrinkage was determined by comparing cell heights values measured before and 1-minute after PBS/mannitol addition.

To test whether cellular water permeability contributed to the HS-induced ASL volume response, CF epithelia were pretreated by adding HgCl_2 (1mM) to the apical surface(2). 10 minutes after HgCl_2 addition, NaCl (0.8 mg) was added in perfluorocarbon and the height of ASL serially monitored with XZ confocal microscopy.

Pharmacological Specificity of Amiloride on Transcellular Water Permeability

The effects of amiloride and two amiloride analogs (benzamil and dimethyl amiloride, each at 100 μM) on airway epithelial water permeability were tested by adding the test compound along with a hyperosmotic mannitol solution (PBS + 300 mM mannitol; 100 μl) to the apical surface of CF epithelial cultures. Cell height was serially measured with confocal microscopy and the rates of cell shrinkage through 60 seconds were compared.

RESULTS

Adverse Events

Adverse events occurring during the treatment interval are listed in Table 1.

Table 1: Adverse Events during Treatment Interval

Event	Placebo/HS N (% of group)	Amiloride/HS N (% of group)
Pulmonary exacerbation	1 (8.3%)	
Probable abdominal sepsis		1 (8.3%)
Acute FEV ₁ drop >15% after 1 st dose study meds.		1 (8.3%)
Fever (<24 hours)	1 (8.3%)	
Arthralgias	1 (8.3%)	
Diarrhea (<24 hours)		1 (8.3%)
Fatigue		1 (8.3%)
Rhinorrhea	1 (8.3%)	
Increased cough	1 (8.3%)	1 (8.3%)

Quality of Life

Individual domain scores measured during baseline and treatment intervals in each group are shown in Table 2. Baseline CFQ14+ domain scores were well matched in the two treatment groups, with the exception of a higher “eating disturbances” domain score in amiloride/HS group (p = 0.01). The respiratory symptom score during the treatment period was significantly higher in the placebo/HS group (p 0.01), which primarily reflected a significant improvement from baseline in this group (mean difference 7.6; p = 0.02). A significantly higher “treatment burden” during the treatment period was also detected in the placebo/HS group, as reflected by a mean difference of -20.2 (p = 0.03) in the placebo/HS group (Table 2).

Table 2: CFQ14+ (Quality of Life) Scores (Scores range from zero to 100 with higher scores indicating better quality of life)

		Amiloride/HS		Placebo/HS	
		Baseline Avg. (SE)	Treatment Avg. (SE)	Baseline Avg. (SE)	Treatment Avg.(SE)
Symptom Scores	Respiratory	73.3(4.5)	70.0(3.1)	74.7(4.1)	82.3(3.1)*#
	Weight	83.3(7.5)	73.3(9.7)	78.8(9.3)	87.9(8.1)
	Digestion	83.3(5.0)	88.9(2.9)	87.9(3.8)	89.9(3.5)
Quality of Life Domains	Treatment burden	64.2(5.5)	51.1(9.1)	79.8(5.4)	59.6(7.4)#
	Physical	85.4(4.9)	80.0(5.7)	75.0(7.9)	81.1(5.5)
	Role	88.9(8.2)	75.0(14.1)	72.9(8.3)	77.8(9.6)
	Vitality	65.0(4.3)	68.3(3.0)	59.8(6.0)	67.4(4.0)
	Emotional state	80.7(5.1)	84.7(4.7)	78.8(6.7)	86.1(5.0)
	Social/ Marginalization	73.3(6.2)	70.0(5.1)	77.0(4.1)	78.8(5.5)
	Body image	76.5(9.0)	80.0(8.7)	78.8(5.7)	85.9(5.0)
	Eating disturbances	100.0(0.0)	96.7(3.3)	85.9(5.0) [‡]	92.9(3.1)
Health Perception	Health	75.3(4.0)	71.1(5.3)	69.7(6.0)	75.8(4.4)

- * denotes $p = 0.01$ vs. amiloride/HS treatment period
- # denotes $p < 0.05$ vs. own group's baseline period
- Ψ denotes $p = 0.01$ vs. baseline period in amiloride/HS group

Laboratory Safety Data

No differences in bacterial density, serum chemistries, or hematological parameters were detected upon comparison of baseline and treatment interval data in either treatment group (Table 3).

Table 3: Laboratory Safety Data

	Amiloride/HS			Placebo/HS		
	Baseline (mean±SE)	Treatment (mean±SE)	Mean diff (95% CI)	Baseline (mean±SE)	Treatment (mean±SE)	Mean diff (95% CI)
Total bacteria, log ₁₀ cfu/ml	7.26 (0.19)	6.81 (0.40)	-0.45 (-1.63 – 0.73)	6.75 (0.31)	6.61 (0.36)	-0.14 (-0.82 – 0.54)
Pseudomonas, log ₁₀ cfu/ml	6.99 (0.24)	6.65 (0.21)	-0.34 (-1.20 – 0.51)	5.68 (0.60)	6.30 (0.38)	0.62 (-1.31 – 2.55)
K ⁺ , mmol/L	4.5 (0.1)	4.6 (0.0)	0.1 (-0.2 – 0.3)	4.4 (0.1)	4.4 (0.1)	0.0 (-0.3 – 0.3)
WBC, x10 ⁹ /L	10.8 (0.9)	10.4 (1.0)	-0.4 (-2.9 – 2.1)	9.4 (0.6)	8.9 (0.4)	-0.6 (-1.5 – 0.4)

Individual Lung Function and MC Responses to Study Medications

The absolute percent change in FEV₁ during the baseline and treatment periods are plotted for each individual subject (Figure 1), demonstrating that 10/11 subjects in the placebo/HS group improved during the treatment interval. In Figure 2, individual rates of MC (MCC_{1hr}) during baseline (Basal-MC) and treatment intervals (Durability-MC) are plotted, demonstrating that 9/11 subjects had sustained improvements in MC in response to placebo/HS.

Figure 1: Individual FEV₁ responses to amiloride/HS and placebo/HS during baseline and treatment intervals.

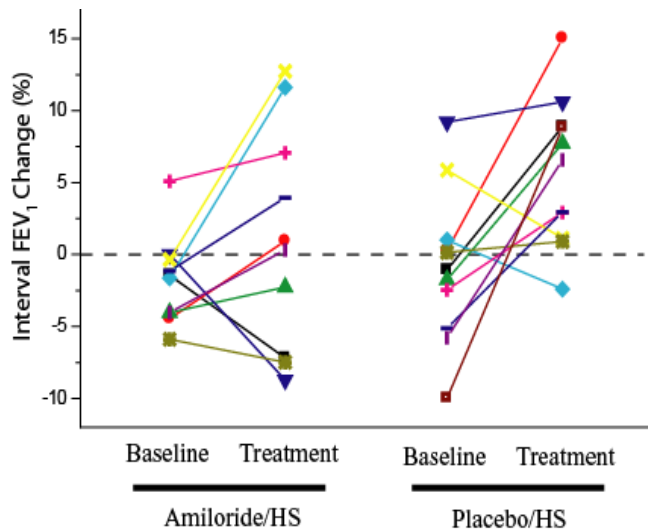
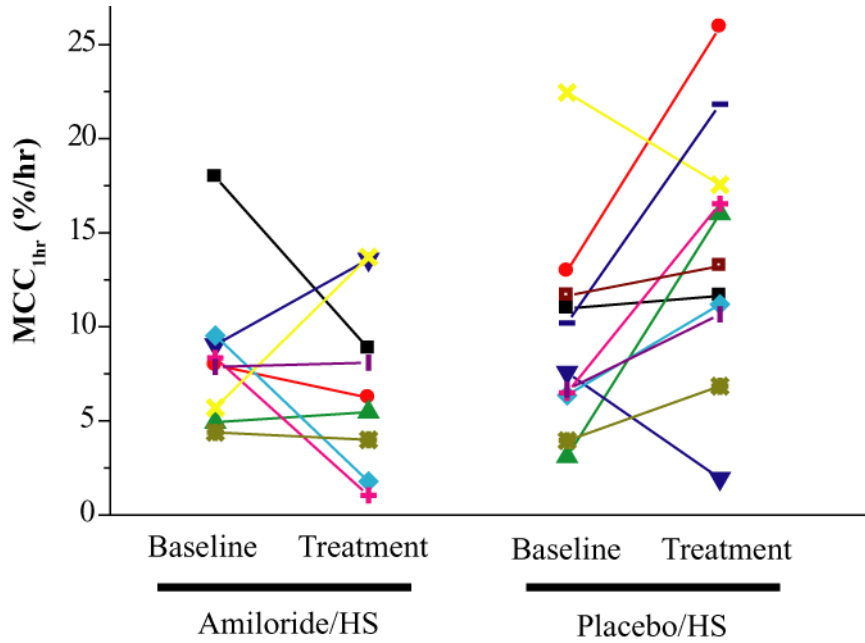


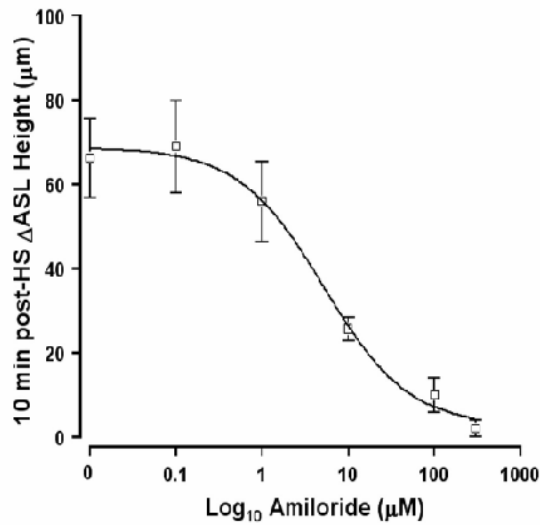
Figure 2: Individual MCC_{1hr} values before and during chronic treatment with amiloride/HS or placebo/HS (Basal-MC vs. Durability-MC).



Dose-Response of Amiloride on the ASL Volume Response

Increasing doses of amiloride blocked the ASL volume response to hypertonic NaCl in CF epithelia (N=3). From these data, an IC_{50} of $\sim 6 \mu M$ was calculated.

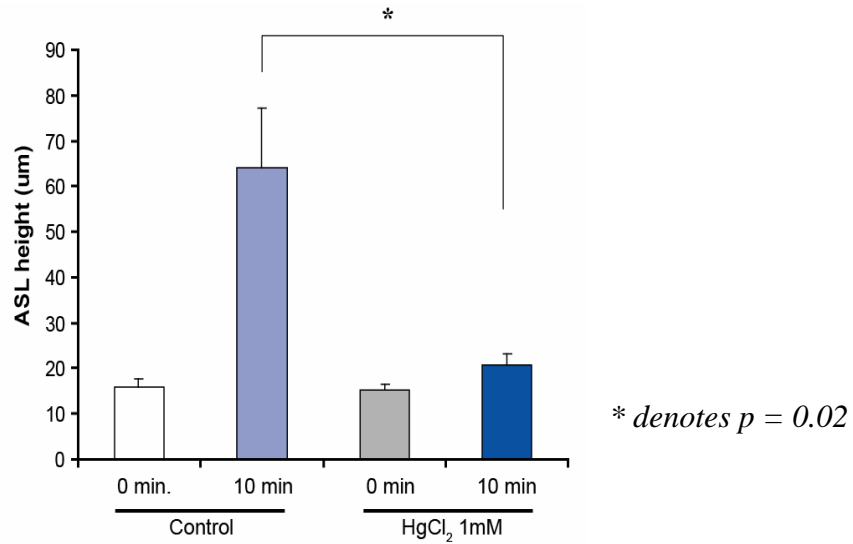
Figure 3: Amiloride dose-response relationship on ASL volume response



Cellular Path as Site of Amiloride's Block of Water Permeability

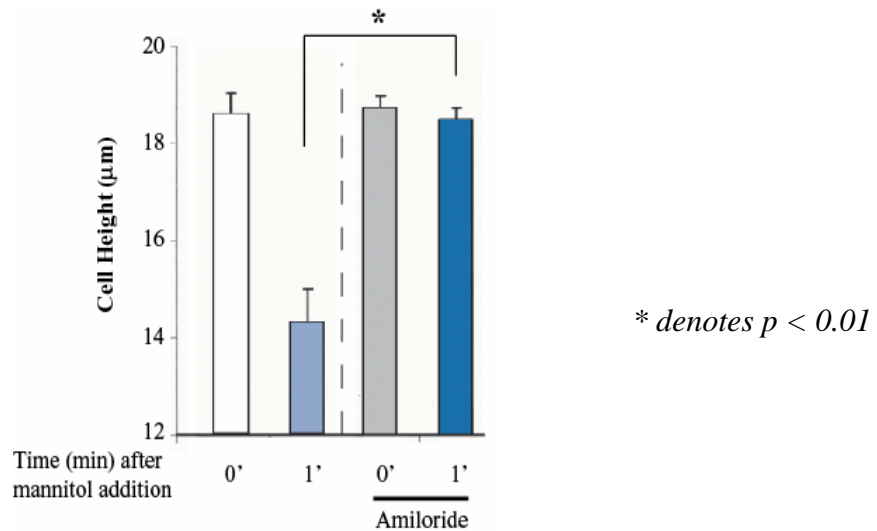
To test whether the cellular path was involved in the ASL volume response to hypertonic NaCl, CF epithelia (N=4) were treated with HgCl₂ (1 mM) prior to the addition of NaCl (0.8 mg) to the apical surface. HgCl₂, an inhibitor of cellular aquaporins, blocked the ASL volume response to hypertonic NaCl, mimicking the effects of amiloride and suggesting that transcellular water flow was critical to the ASL volume response to hyperosmotic stimuli (Figure 4).

Figure 4: Effect of HgCl₂ pretreatment on ASL height response to hypertonic saline



In a second series of experiments, cell height change in response to an apical hyperosmotic stimulus was monitored as an index of water flow across the apical cellular membrane in CF airway epithelia (N=4). Treatment with amiloride (100 µM) prevented cell shrinkage, suggesting that this agent blocked transcellular water flow at the apical cell membrane (Figure 5).

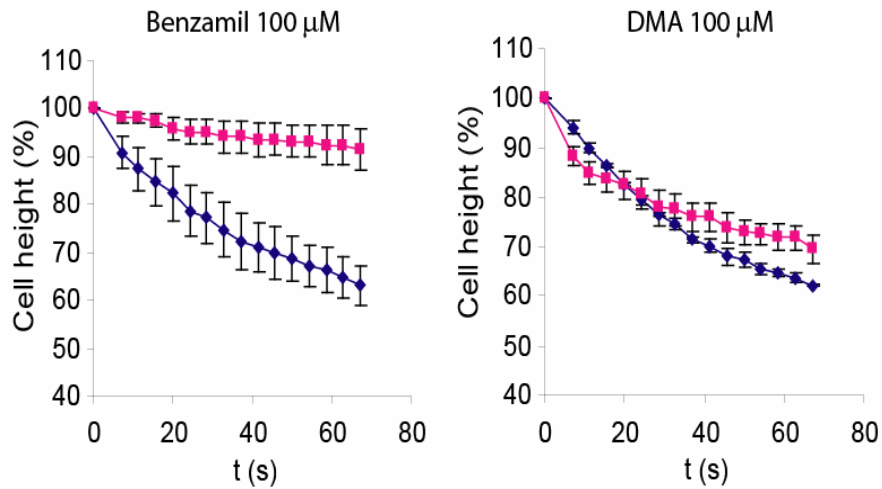
Figure 5: Cell height change in response to hyperosmotic stimulus in CF epithelia.



Pharmacological Specificity of Amiloride on Transcellular Water Permeability

Cell height change in response to the addition of an apical hyperosmotic stimulus was monitored \pm pretreatment with benzamil or dimethyl amiloride (each at 100 μ M). Whereas benzamil blocked osmotically driven cell shrinkage, as was observed with amiloride (see Figure 4 in Web Appendix), dimethyl amiloride (DMA) had no effect (Figure 6).

Figure 6: Effect of benzamil and dimethyl amiloride on transcellular water permeability



REFERENCES

- (1) Knudson RJ, Lebowitz MD, Holberg CJ, Burrows B. Changes in the normal maximal expiratory flow-volume curve with growth and aging. *Am Rev Respir Dis* 1983; 127(6):725-734.
- (2) Matsui H, Davis CW, Tarran R, Boucher RC. Osmotic water permeabilities of cultured, well-differentiated normal and cystic fibrosis airway epithelia [see comments]. *J Clin Invest* 2000; 105(10):1419-1427.