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Evidence against Resveratrol as a viable therapy for the rescue of defective F508 CFTR

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Abstract

BACKGROUND—Resveratrol, a natural phenolic compound, has been reported to rescue mutant F508 CFTR in expression systems and primary epithelial cells. Although this implies a therapeutic benefit to patients with CF, investigations were performed using resveratrol concentrations greatly in excess of those achievable in plasma. We evaluated the efficacy of resveratrol as a CFTR corrector in relevant primary airway cells, using physiologically achievable resveratrol concentrations.

METHODS—Cells expressing wt or F508 CFTR were exposed to chronic or acute resveratrol. CFTR mRNA and protein expression were monitored. The effects of resveratrol on primary F508 human airway cells were evaluated by equivalent current analysis using modified Ussing chambers.

RESULTS—Consistent with previously published data in heterologous expression systems, high doses of resveratrol increased CFTR expression; however physiologically relevant concentrations were without effect. In contrast to heterologous expression systems, resveratrol was unable to increase mutant CFTR channel activity in primary airway cells. Elevated amiloride-sensitive currents, indicative of sodium transport and characteristically elevated in CF airway cells, were also unaffected by resveratrol

CONCLUSIONS—High concentrations of resveratrol can increase CFTR mRNA and protein in some cell types. In addition, acute resveratrol exposure can stimulate CFTR mediated chloride secretion, probably by increasing cellular cAMP levels. Resveratrol at physiologically achievable levels yielded no benefit in primary F508 airway cells, either in terms of amiloride-sensitive currents of CFTR currents.

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AUTHOR CONTRIBUTIONS

Y. Jai, K. Shah, N. Bradbury performed the experiments N. Bradbury and R. Bridges designed the research study Y. Jai, K. Shah, R. Bridges and N. Bradbury analysed the data N. Bradbury wrote the paper

COMPETING INTERESTS

None

Keywords

CFTR; cystic fibrosis; resveratrol; human airway cell culture; conductance

Introduction

Cystic fibrosis (CF) is caused by mutations in the cystic fibrosis transmembrane conductance regulator (*cftr*) gene, which encodes a cAMP/PKA activated anion conductance found in the apical membranes of epithelia lining the airways, pancreas, intestine and other epithelial tissues [1,2]. Clinically, CF patients display markedly reduced lung function, as well as pancreatic insufficiency resulting in intestinal malabsorption. The most common mutation in CFTR leading to cystic fibrosis is a deletion of phenylalanine at position 508 (F508), and is present in at least one allele in ~90% of CF subjects [3]. The F508-CFTR protein fails to achieve the correct stable conformation in the endoplasmic reticulum and is retained for proteosomal degradation [4,5], leading to an absence of CFTR protein in the apical membrane of epithelial cells. Interestingly, F508-CFTR can, at least partially, be rescued by incubation of cell cultures for 24 h at reduced temperature (<30°C) [6] or by incubation of cells with various small molecule correctors [7,8,9] leading to the appearance of F508-CFTR in the plasma membrane. Recent FDA approval of a potentiator/corrector combination (Ivacaftor/Lumacaftor, marketed as Orkambi; Vertex Pharmaceuticals) represents the first pharmacological approach to treat patients with the F508 mutation. As an initial approach, Orkambi is a welcome compound, yet whether it will be of benefit to all F508 patients' remains to be determined.

Recently, several publications have reported that resveratrol is efficacious in correcting defective F508-CFTR processing and trafficking in cell models [10,11,12,13], and few mouse models [11,13]. Resveratrol (3,4',5-trihydroxystilbene) is a naturally occurring polyphenolic flavonoid compound found in various fruits and vegetables, and is abundant in grapes, berries and peanuts [14]. In primary mouse nasal epithelial cells and human sinonasal epithelial cultures [11,12], 100 µM resveratrol has been reported to increase significantly CFTR-mediated chloride transport. Additionally, inclusion of 50 µM resveratrol for 18 hrs in the cell culture medium bathing F508-CFTR CFPAC-1 cells and wt-CFTR expressing MDCK1 cells resulted in a small increase in CFTR protein expression and cell chloride permeability [10]. While such data suggest a salutary effect of resveratrol in ameliorating the consequences of F508 CFTR, it should be noted that these observations were made using resveratrol concentrations ~50-fold greater than those pharmacologically achievable in plasma [15,16,17]. Studies in humans have indicated a very low bioavailability of orally administered resveratrol [16,18,19], with only low micromolar (~2 µM) levels detectable in the blood even after high dose administration [15,16,17]. Resveratrol preparations are becoming widely available as nutritional supplements, being cited as having potent anti-inflammatory and anti-oxidant properties [20,21], yet the utility of such supplements is still unclear. The aim of the present study was to evaluate reported effects of resveratrol on F508-CFTR biogenesis and transepithelial ion transport, and to determine if such effects could be recapitulated at physiologically relevant concentrations of resveratrol.

Materials and Methods

Reagents and antibodies

Resveratrol, forskolin and isobutyl-methyl-xanthine (IBMX) were obtained from Sigma (St. Louis, MO) at the highest grade available. Small molecule F508 CFTR corrector 106951 (1-(benzo[d][1,3]dioxol-5-yl)-N-(5-((S)-(2-chlorophenyl)((R)-3-hydroxypyrrolidin-1-yl)methyl)thiazol-2-yl)cyclopropanecarboxamide) also known as VRT-534 [22,23] was obtained through the CFFT Chemical Compound Distribution Programme at Rosalind Franklin University of Medicine and Science. CFTR antibody 596 was obtained from Dr J.R. Riordan from the University of North Carolina through the Cystic Fibrosis Foundation Therapeutics Network. Tubulin antibodies and IRDye® secondary antibodies were obtained from Licor (Lincoln, NE).

Cell Culture

Flp Hek293 cells constitutively expressing either wt or F508 CFTR [24,25] were cultured in Dulbecco's Modified Eagle Medium (DMEM) + GlutaMAX™ (Gibco/Invitrogen, Gaithersburg, MD), supplemented with 10% foetal calf serum (Hyclone). T84 cells [25] (ATTC, Gaithersburg MD) were grown in DMEM/Ham's F12 mix (1:1) supplemented with 5% foetal calf serum (Hyclone). For electrophysiologic measurements, T84 cells, a well described model for CFTR dependent chloride secretion [26,27], were seeded on permeable supports and grown until confluent. Primary human bronchial epithelial cells (from patients of undisclosed sex) were cultured as previously described [28,29]. Briefly, airway cells were obtained from the CF Center Tissue Procurement and Cell Culture Center (University of North Carolina) through an agreement with Cystic Fibrosis Foundation Therapeutics Inc, and were obtained from individuals with CF. Cells were seeded onto 0.4 µm SnapWell™ culture inserts (Corning Costar) that had been coated with NIH 3T3 conditioned media and bovine brain extract (Ultrosor G, Pall Life Sciences, France). After 4 days the apical media was removed at the cells grown at an air-liquid interface for >14 days before use. This resulted in a monolayer of fully differentiated ciliated columnar cells. Studies were performed using cells isolated from 3 non-CF patients and 3 CF patients homozygous for the F508 mutation.

Quantitative real-time (qRT)-PCR experiments

Total RNAs were isolated from cells using TRIzol® RNA isolation reagent following the manufacturer's recommendations (Invitrogen, Gaithersburg, MD). The purity and integrity of the extracted RNA was confirmed by optical density measurements (260/280 nm ratios). Complementary DNA synthesis reactions were performed using cDNA synthesis with SuperScript III Kit (Invitrogen) according to manufacturer instruction. Real-time PCR was performed using the Applied Biosystems 7500 Detection System. Briefly, 20 ng of cDNA and gene specific primers:

CFTR Forward Primer: 5'-TGG CTC CTT GGA AAC ACT CCT CTT-3'

CFTR Reverse Primer: 5'-TGT CGG CTA CTC CCA CGT AAA TGT-3'

GAPDH Forward Primer: 5' - GGA AGG TGA AGG TCG GAG TC-3'

GAPDH Reverse Primer: 5'-CTG GAA GAT GGT GAT GGG ATT TC-3')

were added to SYBR Green Master Mix (Applied Biosystems) and subjected to PCR amplification. All PCR reactions were run in duplicate. The amplified transcripts were quantified using the comparative C_t method.

Electrophoresis and Immunoblot Analysis

Cells were washed with ice-cold PBS and lysed in NP-40 buffer (1% vol/vol nonidet type P40, 10% vol/vol glycerol, 25 mM HEPES-Na [pH 7.4]), containing protease inhibitors (Complete EDTA-free protease inhibitor cocktail; Roche) for 20 mins on ice. Nuclei and unbroken cells were removed by centrifugation (15,000 g for 5 min at 4°C. Solubilized proteins were resolved using 4-12% NuPAGE™ gels (Invitrogen) and transferred to PVDF. After block, membranes were probed with the appropriate primary antibody and binding visualized with IR Dye 800-goat-anti-mouse IgG (1:15,000 dilution; Licor, Lincoln, NE) using an Odyssey SA Infrared Imaging System (Li-COR).

cAMP measurements

Cellular cAMP levels were measured using a commercially available direct cAMP enzyme immunoassay kit (Sigma). Cells grown on 35mm dishes were exposed to reagents for 10 mins. Cellular cAMP was extracted with ice-cold ethanol and levels determined according to the manufacturer's instructions.

Electrophysiological Assessment of CFTR activity

Short circuit current analysis was performed as previously described [30]. Briefly, T84 cells were grown in Snapwell filters (Costar), and used 14-20 days after seeding. Filters were mounted in a modified Ussing Chamber, and equilibrated for 15 min prior to addition of forskolin. T84 monolayers had resistances of $\sim 2,000 \Omega \text{cm}^{-2}$. Changes in I_{sc} were calculated as a difference current between the plateau phase of the response and the baseline value. For primary airway cells, equivalent currents (I_{eq}) were determined using a high throughput modified Ussing chamber. Changes in I_{eq} were calculated as a difference conductance between the plateau phase of the response and the baseline value. Amiloride was added at the start of experiments to inhibit ENaC mediated sodium currents.

Statistics

Results are expressed as means $\pm$ SD, with n = number of experiments, after checking normality of distribution using the Shapiro-Wilk test. Unpaired Student's t -tests were performed using Sigma Plot software version 10.0 (Systat Software Inc, San Jose, CA USA). All statistical tests were performed at a 5% significance level (i.e., $P < 0.05$).

Results

Effect of Resveratrol on wt and F508 CFTR expression

A series of experiments were performed using a model cell, Hek293, stably expressing wt of DF508 CFTR, as well as T84 cells, a human colonic epithelial cell line that expresses endogenous wt-CFTR. In dose-response experiments involving 24-h incubation of cells with different concentrations of resveratrol (0-100 μ M from DMSO stock), immunoblot analysis revealed a concentration dependent increase in both band b and band c of CFTR at resveratrol concentrations $> 30 \mu$ M. At 100 μ M resveratrol, CFTR levels were almost 2.5-fold greater than that observed in the absence of resveratrol (Fig 1a,b). Expression of the endogenous housekeeping protein β -tubulin were unaffected by resveratrol (Fig 1a). Previous studies have suggested that high levels of resveratrol are able to increase ER export of immature band b F508 CFTR and increase the level of mature band c F508 CFTR [10,11]. To see if we could reproduce such observations, we exposed Hek293 cells stably expressing F508 CFTR to increasing concentrations of resveratrol. The results were less dramatic for F508 CFTR, where only the maximally used resveratrol concentration (100 μ M) caused a small but variable increase in band c F508 CFTR expression (Fig 1c,d) that was greater than seen in the absence of resveratrol. No consistent changes were observed for band b F508 CFTR levels. T84 cells, a human colonic epithelial cell line that expresses large amounts of endogenous wt CFTR driven off the native promoter have been widely used as a model for CFTR mediated ion transport. When T84 cells were exposed to increasing concentrations of resveratrol for 24 hours, a small increase in CFTR expression was observed that followed a similar dose-response as for wt-CFTR expression in Hek293 cells, with increases in CFTR protein levels only being observed at resveratrol concentrations greater than 50 μ M (Fig 1e,f).

As resveratrol has been assigned gene transcriptional properties, we determined whether the observed effects of resveratrol on increasing CFTR expression could, at least in part, be attributed to enhanced gene expression. To determine if increases in CFTR in response to resveratrol were due to increased mRNA transcript levels, CFTR mRNA was quantified following 24 hour exposure to resveratrol. For heterologously expressed wt CFTR, resveratrol caused a dose-dependent increase in CFTR mRNA levels, paralleling that seen for protein, with mRNA levels increasing only at resveratrol concentrations greater than 50 μ M (Fig 2). For native wt CFTR expressing T84 cells, although resveratrol increased protein expression at high levels, no significant changes in CFTR mRNA were observed (Fig 2). This observation suggests that, at least in T84 cells, resveratrol does not act by altering mRNA levels.

Effect of Resveratrol on Cl⁻ secretion in T84 cells

CFTR dependent chloride secretion in T84 cells is mediated by increases in intracellular cAMP. Since resveratrol has been shown to increase cAMP levels, we determined if resveratrol would be able to initiate a cAMP-dependent secretory response through such a mechanism. Incubation of cells with increasing concentrations of resveratrol caused a small, but significant, dose-dependent increase in CFTR mediated chloride transport, as monitored by increases in short-circuit current (Isc) (Fig 3a). At maximal concentrations tested,

resveratrol (100 μ M) caused an increase in *I*_{sc} to approximately 40% of that observed for maximally efficacious forskolin concentrations. Application of basal bumetenide caused an inhibition of stimulated chloride transport due to blockade of the basolateral Na/K/2Cl cotransporter. Of note, resveratrol concentrations below 60 μ M (which had no long-term effect on CFTR protein expression levels) when applied acutely were still able to elicit small but significant increases in chloride secretion. To determine if resveratrol was stimulating chloride secretion through a cAMP dependent or independent pathway, we evaluated whether or not resveratrol would act synergistically or additively with sub-optimal levels of the adenylate cyclase activator, forskolin. When resveratrol was given concomitantly with low dose forskolin (20 nM; which elicits a minimal response alone), a marked leftward shift in the resveratrol dose-response curve was observed, such that there was a marked increase in resveratrol induced *I*_{sc} that was significantly greater than that observed for resveratrol alone (in the absence of forskolin) (Fig 3b). Indeed, the observed secretory response in the combined presence of resveratrol and forskolin was significantly greater than the arithmetic sum of the responses to resveratrol and forskolin alone, arguing for a synergistic interaction between the two compounds (Fig 3c).

Effect of Resveratrol on [cAMP]_i

Resveratrol has been reported to increase cellular cAMP by acting as a cyclic nucleotide phosphodiesterase inhibitor [31]. To determine if resveratrol could increase cAMP levels in Hek293 and T84 cells, we measured intracellular cAMP levels following acute exposure to cyclase activators, known phosphodiesterase inhibitors and resveratrol. As expected, cAMP levels were increased by the adenylate cyclase activator, forskolin (Table 1), and the non-selective phosphodiesterase inhibitor, isobutyl-1-methyl-xanthine (IBMX). Resveratrol too, at 60 μ M and 100 μ M caused a small but significant increase in intracellular cAMP (Table 1) in both Hek293 and T84 cells, which was dose-dependent. That resveratrol could augment cAMP levels in the presence of forskolin, was observed when the level of cAMP in the presence of both resveratrol (100 μ M) and forskolin (20 nM) was greater than the sum of cAMP levels when either was used alone (Table 1).

Effects of Resveratrol on Primary human airway cells

To determine if resveratrol would be able to increase mutant CFTR function in a relevant human tissue, we grew primary human airway cells from patients homozygous for the F508 mutation at an air liquid interface. Cells were exposed on the serosal surface to media containing resveratrol (10 μ M or 30 μ M), CFTR corrector 951 (6 μ M), a combination of resveratrol and corrector 951, or to DMSO vehicle alone. Cells were exposed to compounds for 24 hours prior to analysis of ion transport properties by equivalent current analysis in a modified Ussing chamber. Uncorrected airway epithelia from patients with CF display increased ENaC dependent sodium absorption and minimal CFTR mediated chloride secretion, and so initial studies were performed to determine if resveratrol abrogated enhanced sodium transport. Although sodium transport was not directly measured, changes in conductance in response to the application of amiloride were used as an indicator of ENaC activity. All CF HBE cells displayed a marked amiloride-sensitive current, consistent with previous published data (Fig 4a,b). No significant change in the size of the amiloride-sensitive conductance was observed for either resveratrol alone, or in combination with

corrector 951, compared to DMSO vehicle control (Fig 4a,b). For CFTR channel activity, CFTR mediated chloride secretion was determined as the forskolin stimulated conductance (following ablation of sodium currents with amiloride). F508-CFTR primary airway cells exposed to DMSO vehicle alone showed a minimal increase above baseline (absence of DMSO) in response to forskolin stimulation (Fig 4a,c). Prior incubation of cells with resveratrol caused no increase in forskolin stimulated conductance above that observed in the absence of resveratrol; indeed a small decrease in conductance compared to DMSO vehicle was observed (9.61 ± 1.21 Ieq for DMSO control vs 6.3 ± 0.55 Ieq for $30 \mu\text{M}$ resveratrol; mean $\pm$ SD $n=6$). That our cells could be responsive to correctors was shown by experiments where corrector 951 exposure caused a marked increase in F508-CFTR mediated conductance (~ 3 -fold above DMSO control), arguing that if resveratrol were efficacious, we would have detected such improvement. Resveratrol in combination with corrector 951 had no additive effect on ion conductance at $10 \mu\text{M}$ resveratrol above that observed for corrector 951 alone, but caused a reversal of the 951 correction at higher concentrations ($30 \mu\text{M}$ resveratrol (23.32 ± 0.11 Ieq for Corr 951 vs. 16.48 ± 2.1 Ieq for Corr 951 + $30 \mu\text{M}$ resveratrol; mean $\pm$ SD, $n=6$)).

Discussion

Cystic fibrosis is characterized by altered fluid and electrolyte transport, in most cases associated with the inability of F508 CFTR to reach the plasma membrane. The search for small molecular correctors to increase F508 CFTR trafficking from the ER to the plasma membrane is currently underway in several pharmaceutical and academic laboratories. Recently, the first FDA approved drug to treat F508 CFTR, Ivacaftor (Vertex Pharmaceuticals) has been released, although the long term efficacy still remains to be determined. Several natural phytoestrogenic, polyphenolic compounds such as curcumin, genistein and apigenin, have been previously reported to increase F508 CFTR expression at the plasma membrane [32,33,34]. For example, studies showing the efficacy of curcumin in increasing plasma membrane CFTR expression have been reported, where incubation of F508-CFTR transfected BHK cells with $5 \mu\text{M}$ curcumin for 18 hrs resulted in a small increase in cell surface F508-CFTR protein expression and cell chloride permeability [32]. However, subsequent studies by Verkman and colleagues found that the maximally achievable plasma curcumin level was 60 nM , well below that reported to be required for F508-CFTR correction [35]. Recently, resveratrol has received broad interest due to its antioxidant, antimutagenic, anti-inflammatory and chemoprotective properties [36,37]; and like curcumin is readily available in many Health Food stores. Resveratrol has also been reported to have beneficial effects on the activity of mutant CFTR [10,11,12]. Reported studies have used resveratrol at concentrations of $\sim 100 \mu\text{M}$ to correct F508-CFTR trafficking, however the maximum achievable plasma concentrations following high doses or oral resveratrol are only $\sim 2 \mu\text{M}$ [15,16,17]. The aim of the present study was to evaluate the reported effects of resveratrol on F508-CFTR biogenesis and to determine if such effects were recapitulated at physiologically relevant concentrations in human airway epithelial cells.

Resveratrol was able to increase wt CFTR expression in heterologous expression systems at concentrations $>60 \mu\text{M}$, but not at physiologically relevant concentrations. Such increases as

were observed for CFTR protein, were also reflected in increases in CFTR mRNA levels, arguing for a genomic component for resveratrol increasing CFTR expression rather than a “CFTR folding” effect. Interestingly, although resveratrol increased CFTR expression in native expressing T84 cells, it did so without an apparent increase in CFTR mRNA expression. Thus, the mechanism(s) by which resveratrol exerts its effects on CFTR may be specific for the tissue and/or expression system employed. Moreover, although resveratrol could increase CFTR mRNA and protein for wt CFTR in a heterologous expression system, the effects on F508-CFTR were singularly lacking. Our observations are in contrast to those of previous publications [10] showing a positive effect of 50 μ M resveratrol on

F508-CFTR expression in the pancreatic duct cell line CFPAC [38]. However, recent studies by Liu *et al* [39] have argued that resveratrol induces apoptosis in CFPAC cells by reducing miR-21 expression, which in turn causes a reduction in BCL-2 expression and a loss of apoptotic inhibition. Thus the effects of resveratrol in CFPAC cells may, at least in part, reflect general cellular apoptotic initiation rather than a specific effect on CFTR. Interestingly the related, but less drastic cellular degradative pathway of autophagy is also related to BCL-2 activity [40,41]. BCL-2 is reported to reduce the pro-autophagocytic actions of beclin-1, thus a resveratrol induced reduction in BCL-2 would lead to an increase in autophagocytic activity in cells [42]. Thus is it possible that an increase in cellular fragility or senescence following resveratrol exposure may allow the ER quality control to become more leaky allowing F508 CFTR to more easily exit the ER.

Similar to our observations in T84 cells, Zhang *et al* could not detect any change in CFTR mRNA levels in human nasal epithelial cells [12]; however we could detect changes in Hek293 cells. Why such differences should occur in different cell types is not clear, but may reflect a difference in mRNA stability rather than production, although this would need to be formally determined. The mechanism(s) of action of resveratrol on CFTR protein still remain unclear, although there are reports that resveratrol can act as a CFTR potentiator [12,43] to directly modulate CFTR channel activity. However, it is likely that multiple pathways will likely be responsible for the observed phenomenology. Certainly it is known that resveratrol modulates proteosomal activity [44], and since CFTR is degraded through a proteosomal pathway [45], it is not unreasonable to suppose that alterations in proteosomal degradation pathways could alter CFTR protein levels. Indeed, proteosomal inhibition has been shown to rescue F508 function in mouse intestine [46]. In addition to this classic degradative pathway, CFTR is also known to undergo quality control at the plasma membrane [47,48]. Whether resveratrol modulates these pathways is currently unknown.

Resveratrol is now known to increase cellular cAMP levels both through direct activation of adenylate cyclase [49] and through inhibition of phosphodiesterases [50]. Interestingly, resveratrol dependent cAMP increases are also associated with alterations in gene expression [51]. Indeed the observation that resveratrol increases cAMP may explain, at least in part, the ability of resveratrol to increase CFTR mRNA and subsequently, protein. In our heterologous expression system, wt CFTR is driven off a CMV promoter; CMV promoters have been shown to contain three cAMP response elements (CRE) that can be stimulated by forskolin induced cAMP elevations [51]. The endogenous CFTR promoter has also been shown to be mildly response to cAMP levels [52], although it shows weak

sensitivity to cAMP stimulation. Thus, it is likely that the observed effects of resveratrol on wt CFTR expression in heterologous expression systems may be an artifact of increased gene promoter activity. The low levels of cAMP generated by resveratrol alone may likely be insufficient to sustain a genomic response on the weakly sensitive endogenous CFTR promoter. It has been shown that F508 CFTR fails to fold productively and is rapidly eliminated from cells [53]. When overwhelmed by misfolded proteins, cells can dispose of such material by producing aggresomes [53]; although initially thought to be subject to ER degradation, CFTR is now known to be also degraded following its traffic through post-Golgi compartments [48,54]. Given the low level of increased CFTR production of wt CFTR expressing heterologous cells, it is unlikely that we would increase the production of

F508 CFTR beyond the capacity of the cell's quality control machinery. Why we see no increase in F508 CFTR in response to resveratrol is not immediately apparent to us, but may reflect nonsense mediated decay of F508 mRNA during co-translational processing and ERAD of F508-CFTR [55].

Further evidence of resveratrol's ability to increase cellular cAMP is derived from the ability of acute resveratrol exposure to increase CFTR mediated chloride transport across T84 cells. Alone, resveratrol was able to elicit a small dose-dependent increase in CFTR mediated ion transport. When coupled with low levels of forskolin (at levels which marginally stimulate chloride transport alone) resveratrol caused a marked increase in chloride secretion, above that predicted for the arithmetic sum of the individual forskolin and resveratrol responses, arguing for a synergistic effect of resveratrol and forskolin through the same pathway. Thus, such synergism is easily described by a model in which resveratrol acts as a PDE inhibitor, augmenting the effects of forskolin, leading to enhanced cAMP production. We have previously reported similar synergistic effects of forskolin and the non-selective PDE inhibitor, IMBX, on cAMP stimulated mucin secretion [56]. Certainly it is possible that the effects of resveratrol on transepithelial chloride transport are mediated, not by cAMP, but by a direct action on CFTR. Indeed, resveratrol has been shown to increase the open probability (P_o) of murine CFTR [12], and it is likely that some potentiator component exists in our observations; however it is unlikely that this is the sole mechanism of action as potentiators do not, *ab initio*, cause CFTR activity, but rather enhance activated CFTR activity. In contrast, resveratrol can initiate chloride secretion, consistent with its actions in raising cAMP levels. Although monomeric resveratrol can potentiate CFTR, oligomeric resveratrol blocks CFTR channels [57].

The gold standard for the verification and validation of small molecule correctors and potentiators to treat mutant CFTR has been, and continues to be, primary human airway epithelial cells. Our assay for CFTR function is a robust and sensitive assay, and we estimate that we have the ability to detect a level of correction for resveratrol equivalent to 10% of that observed for corrector 951. Thus, our inability to detect therapeutic effects of resveratrol on increasing CFTR mediated chloride transport in F508 airway cells is unlikely due to any deficiencies in our assay. Interestingly, it appears that resveratrol was slightly antagonistic to the corrective effects of corrector 951. The negative interaction between resveratrol, proposed to be a CFTR potentiator, and a CFTR corrector is reminiscent of the negative interactions proposed for other CFTR correctors (VX-809; Lumacafor, Vertex

Pharmaceuticals) and the CFTR potentiator VX-770 (Ivacaftor, Vertex Pharmaceuticals) [58]. Nevertheless, such a combination has recently received FDA approval for F508 patients, who show some improvement in some cases. The dichotomy between clinical data and experimental data for the combination of Ivacaftor and Lumacaftor is curious and yet to be resolved, though one possible interpretation is that there are sites of action for correctors, other than CFTR, which give rise to the clinical response. The clinical observations are indeed consistent with the proposal that both a corrector and a potentiator are likely required for efficient treatment of patients with CFTR, so it is still theoretically possible that resveratrol might have some role in the treatment of patients with CF.

In addition to reduced cAMP stimulated chloride transport, enhanced ENaC dependent sodium transport is a hallmark of CF airways [59,60]. We were unable to see any effects of resveratrol on amiloride sensitive conductance, consistent with a lack of effect on ENaC mediated sodium transport. In contrast, previously published data [12] has suggested that resveratrol might actually increase sodium absorption, a condition which would be expected to further exacerbate the problem, by further drying out the surface airways.

In summary, we were unable to document any salutary effects of resveratrol in the functional correction of F508 CFTR in native primary human airway cells using resveratrol concentrations that might be expected in human plasma. The low bioavailability of resveratrol suggests that maximal serum concentrations reported may be an order of magnitude lower than that required to correct F508 CFTR. Does this mean that resveratrol is of no benefit?, certainly it is unlikely at present. However, it is possible that different pharmaceutical preparations or medicinal chemistry on resveratrol could increase its bioavailability, either by increasing its absorption or reducing its breakdown. Thus understanding the metabolic pathways with which resveratrol interacts may be of utility. As a single therapy, resveratrol seems unlikely to be useful, but it is also true that a single drug regimen is unlikely to work anyway on patients with F508-CFTR. Whether adjunct compounds that enhance the actions of resveratrol will be discovered remains to be determined.

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ABBREVIATIONS

CF	cystic fibrosis
CFTR	cystic fibrosis transmembrane conductance regulator
DMEM	Dulbecco's modified Eagle Medium
ENaC	epithelial sodium channel
IBMX	isobutyl-methyl xanthine
SDS-PAGE	sodium dodecylsulphate polyacrylamide gel electrophoresis

wt

wild-type variant

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Highlights

- Resveratrol increases cAMP levels in epithelial cells
- Resveratrol at physiological concentrations can increase heterologous but not endogenous CFTR expression
- Resveratrol does not affect amiloride sensitive currents in primary airway cells
- Resveratrol does not increase F508 activity in human airway cells

GENERAL SIGNIFICANCE

Taken together, our results do not support the use of resveratrol supplements as a therapy for patients with cystic fibrosis. It is possible that further modifications of the resveratrol backbone would yield a more efficacious compound.

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Figure 1. Effects of Resveratrol on CFTR protein expression

Representative immunoblot analyses of CFTR and tubulin (used as a loading control) protein expression from wtCFTR expressing Hek293 total cell lysates (a), F508CFTR expressing Hek293 cells (c) and wtCFTR expressing T84 cells (e). Cells were pretreated for 24 hrs with increasing concentrations of resveratrol, or with DMSO vehicle alone. Quantitation was performed using analysis software from Licor (b,d,f). Data represents means $\pm$ SD for 3 separate experiments. * p <0.05 for difference from control.

Figure 2. Effect of resveratrol on CFTR mRNA

mRNA was extracted from cells exposed to varying concentrations of resveratrol for 24 hours. CFTR specific mRNA was amplified using primers described in *Methods*. Data are shown as fold increase over DMSO control (i.e., absence of resveratrol). Data represents means $\pm$ SD for 3 separate experiments. * $p < 0.05$ for difference from control.

Figure 3. Resveratrol-induced chloride secretion from T84 cells

(a) Representative current trace showing a time- and dose-dependent increase in resveratrol stimulated I_{sc} ($n=3$). Forskolin (10 μM) was applied to determine the maximal level of chloride secretion achievable. (b) Representative current trace for resveratrol stimulated I_{sc} in the presence of marginally efficacious forskolin levels (20 nM). (c) Synergism between resveratrol and forskolin is observed for chloride secretion; (●) for I_{sc} in the presence of resveratrol alone, (□) calculated I_{sc} for the combination of resveratrol and forskolin (20 nM), (◇) actual experimental values for I_{sc} in the combined presence of resveratrol and forskolin (20 nM). Data are mean $\pm$ S.D. ($n=3$). $*p < 0.05$

Figure 4. Effect of resveratrol on ion transport in F508 primary human airway cells

Primary human airway cells from a patient homozygous for the F508 mutation were subject to a conductance assay for chloride and sodium transport. Experiments were performed using compounds either alone or in combination as shown. All compounds were exposed to cells for 24 hours prior to assay. (a) Representative conductance traces for cells under the conditions shown. The first arrow marks the addition of amiloride and the second arrow the addition of forskolin. (b) and (c) Data represents means $\pm$ SD for 6 separate experiments. * $p < 0.05$ for difference from control (DMSO alone).

Table 1

Effect of Resveratrol on intracellular cAMP levels

Condition	Intracellular cAMP (pmol/mg protein) Mean $\pm$ SD (<i>n</i> = observations)	
	Hek293	T84
Control (unstimulated)	3.5 $\pm$ 0.4 (5)	4.4 $\pm$ 0.72 (4)
Forskolin (10 μ M)	370 $\pm$ 29 (4) *	204 $\pm$ 14 (4) *
Forskolin (20 nM)		8.4 $\pm$ 1.0 (4) *
IBMX (1 mM)	260 $\pm$ 31 (4) *	182 $\pm$ 21 (4) *
Resveratrol (60 μ M)	8.3 $\pm$ 0.8 (4) *	6.7 $\pm$ 0.7 (4) *
Resveratrol (100 μ M)	10.7 $\pm$ 0.9	8.9 $\pm$ 0.4 (4) *
Forskolin (20 nM) + Resveratrol (100 μ M)		83.4 $\pm$ 5.7 (4) *

T84 cells were exposed to compounds at the concentrations shown, for 10 mins at 37°C. Cells were quenched according to the manufacturers protocol. cAMP is shown as pmol/mg protein for the number of experiments shown in parentheses.

* $p < 0.05$ for difference from unstimulated control by Student's t-test.