Modulation of $\Delta F508$ Cystic Fibrosis Transmembrane Regulator Trafficking and Function with 4-Phenylbutyrate and Flavonoids

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Over 70% of patients with cystic fibrosis have the $\Delta F508$ mutation. This protein is a partially functional chloride ($Cl^-$) channel that is prematurely degraded in the endoplasmic reticulum. Specific members of the flavonoid class of compounds have been shown to increase $Cl^-$ conductance of wild-type and $\Delta F508$ cystic fibrosis transmembrane regulator (CFTR). Although flavonoid effects on CFTR processing are unknown, evidence of effects on heat shock proteins, specifically those that have been shown to interact with CFTR, led us to believe that there would be an effect on CFTR processing through modulation of CFTR–chaperone interactions. We sought to determine (i) the effect of apigenin, genistein, kaempferol, and quercetin on CFTR processing and flavonoid to directly stimulate CFTR would increase $Cl^-$ conductance. Our results show no significant effect on CFTR processing as measured by immunoblotting with 1 μM or 5 μM of apigenin, genistein, kaempferol, or quercetin. However, despite no effect on CFTR processing as determined by immunoblot, immunofluorescence demonstrated a favorable change in the intracellular distribution of CFTR with 24 h treatments of apigenin, kaempferol, and genistein. Furthermore, we observed an increase in $Cl^-$ conductance as measured by $Cl^-$ efflux in cells that were treated for 24 h with 4-PBA and then assayed with forskolin and 1 μM or 5 μM genistein, and also with cells treated for 24 h with either 4-PBA, 5 μM apigenin, or 1 μM quercetin. Thus, a combination of chronic treatment with 4-PBA or select flavonoids, followed by acute flavonoid exposure, may be beneficial in cystic fibrosis.

Cystic fibrosis (CF) is an autosomal recessive disorder caused by mutations in the cystic fibrosis transmembrane regulator (CFTR) gene. CFTR is a cAMP-activated, voltage-gated chloride ($Cl^-$) channel. The most common mutation, $\Delta F508$, is observed in over 70% of persons with CF. The $\Delta F508$ CFTR protein is a temperature-sensitive processing mutant with a reduced open time for $Cl^-$ conductance. Despite this relative impairment in $Cl^-$ transport, the $\Delta F508$ CFTR has measurable conductance when it reaches the cell surface in artificial systems (1–3). In cell culture systems, most of the mutant protein is not able to undergo the normal folding pathway through the endoplasmic reticulum and Golgi to reach the cell surface. If $\Delta F508$ can be rescued from premature proteolysis and reach the plasma membrane, the degree to which $Cl^-$ conductance can be restored will be critically important to the success of pharmacologic therapies using the rescue approach.

Cellular chaperones play a key role in the intracellular processing of CFTR. Chemical (e.g., glycerol) or pharmacologic (e.g., phenylbutyrate [PBA]) treatments that restore $\Delta F508$ CFTR to the processing pathway also restore a portion of cAMP-mediated $Cl^-$ conductance to the cell surface. Retrieval of mutant CFTR from the proteolytic pathway likely involves modulation of CFTR–heat shock protein (Hsp) interactions. Hsp or heat shock cognate (Hsc) proteins are constitutive and stress-inducible proteins that can protect normal cells against protein damage by physical interaction during synthesis, folding, assembly, and degradation (4). Down-regulation of Hsc70 and upregulation of Hsp70 have been implicated in PBA-mediated regulation of $\Delta F508$ CFTR trafficking (5–8).

The flavonoids are a class of polyphenolic compounds that have been shown to induce $Cl^-$ transport through wild-type and mutant CFTR (9–12). It is unclear whether some part of this effect is due to manipulation of CFTR trafficking. Flavonoids are naturally occurring components in fruits, vegetables, tea, and legumes. In addition to stimulation of CFTR, some flavonoids exhibit antiinflammatory properties secondary to inhibition of lipooxygenase and cyclooxygenase (13). These compounds may be antitumorigenic (14), antibacterial against methicillin-resistant Staphylococcus aureus (15), and function as antioxidants in a structure-specific manner (16). This broad spectrum of activity could be useful in the treatment of the secondary complications of CF. More important, in terms of ameliorating the physiologic defect in CF, is the ability of genistein to interact directly with CFTR at the nuclear binding domain to increase $Cl^-$ channel open time and thereby increase $Cl^-$ conductance (12, 17, 18). Combinations of a chemical chaperone with a flavonoid have shown promise in vitro (18) and in vivo (19).

The first hypothesis of this article is that specific flavonoids (apigenin, genistein, kaempferol, and quercetin) facilitate $\Delta F508$ CFTR trafficking at low concentrations, possibly through a similar mechanism of favorable interactions with cellular chaperones as that seen with the butyrates. The second hypothesis is that these flavonoids stimulate CFTR-mediated $Cl^-$ conductance, potentiating pretreatment with 4-PBA to promote $\Delta F508$ CFTR trafficking to the cell surface.

Materials and Methods

Cell Culture

IB3–1 (ΔF508/W1282X) bronchial epithelial cells (20) were cultured on uncoated T-75 flasks in LHC-8 supplemented with glutamine (genticin-free formulation; Biofluids, Rockville, MD), 5% bovine serum albumin (BSA) (Biofluids), penicillin-streptomycin (Gibco, BRL, Gaithersburg, MD), 1% fungizone (Biofluids), and 1% tobramycin (Eli Lily, Indianapolis, IN). Pharmacologic treatments were initiated at 80% confluence. Flavonoids were solubilized in dimethyl sulfoxide (DMSO) and studied at 1 and 5 μM of apigenin (Sigma-Aldrich, St. Louis, MO), kaempferol (Sigma), genistein (Calbiochem, La Jolla, CA), and quercetin (Sigma). Higher concentrations led to loss of cells from the monolayers (see 3–4.5 dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide [MTT] Assay below). Final DMSO concentration was 0.05%. After 48 h, cells were washed twice in phosphate-buffered saline (PBS) and lysed with 500 μl/250 ml flask of RIPA buffer (50 mM Tris-HCl,
pH8, 150 mM NaCl, 1% Triton X-100, 1% SDS with 0.1 mM phenylmethylsulfonyl fluoride, 10 μM aprotinin, 1 mM Na orthovanadate, and 5 mM ethylenediamine tetraacetic acid) at 4°C.

MTT Assay
Chemosensitivity to each flavonoid and to 4-PBA was assessed with the MTT assay (21) to quantify cell viability. IB3-1 cells were plated at 5,000 cells/ml in 24-well tissue culture plates. At 70% confluency the test drugs were added at 0, 1, 5, 25, 50, and 100 μM for 0, 24, and 72 h. An empty well served as the blank. One milliliter MTT (5 mg/ml) was then added for 1.5 h at 37°C. The medium was discarded and the cells were resuspended in 1 ml DMSO. The optical density at 540 nm was determined and data were normalized to the value obtained in the absence of test compound.

Antibodies
Polyclonal anti-CFTR antisera 181 and 169 (CFTR181 and CFTR169) were generated in rabbits against human CFTR peptides in the region before nucleotide binding domain 1 (NBD1), and in the R domain, respectively (22). Anti-CFTR181 was used at a 1:1,000 dilution for immunoblotting and for immunocytochemistry, and Anti-CFTR169 was applied at a 1:500 dilution for immunoprecipitation. Donkey anti-rabbit immunoglobulin (Ig) G-horseradish peroxidase conjugate (Amersham Pharmacia Biotech, Arlington Heights IL) and donkey anti-rabbit Cy3 conjugate (Jackson Immunoresearch, West Grove, PA) were secondary antibodies for immunoblotting and immunofluorescence, respectively. Rat monoclonal anti-Hsc70 and mouse monoclonal anti-Hsp70 antisera (StressGen, Victoria, BC, Canada) were used for immunoblotting at a 1:3,000 dilution. Goat anti-rat IgG-horseradish peroxidase (Amersham Biosciences, Piscataway, NJ), and sheep anti-mouse IgG-horseradish peroxidase (Amersham Biosciences) were used as secondary antibodies at a 1:3,000 dilution, respectively.

Immunoblotting
Protein lysates were separated by 6% sodium dodecylsulfate (SDS)–polyacrylamide gel electrophoresis. The electrophoresed proteins were transferred to a nitrocellulose membrane in 1% Fairbanks solution. Both 1C–1 cells were grown as indicated above, washed with Dulbecco’s PBS, and then fixed with acetone at -20°C for 9 min. The acetone was neutralized with PBS and nonspecific binding was blocked by incubation in a solution of 10 mM NH4Cl and 5% BSA. The cells were then incubated with anti-CFTR181 diluted in 1% BSA (1:1,000) overnight at room temperature in a humidity chamber. Cells were then washed again with PBS and exposed to secondary antibody (1:1,000) for 40 min. After washing again in PBS, nuclear stain was applied for 5 min. Cells were washed a final time and SlowFade (Molecular Probes, Eugene, OR) applied to the coverslip before mounting on glass. Fluorescence was imaged using a Zeiss Axiovert (Thornwood, NY) microscope and images were captured with a digital charge-coupled device camera (Roper Scientific, Tucson, AZ) and IP Lab Spectrum Software (Scanco-lytics, Fairfax, VA).

Cl- Efflux Assay
Two separate efflux assays were performed: (i) cells grown in the absence of flavonoids and challenged with flavonoids to measure acute transport effects by efflux, and (ii) cells grown in flavonoids for 24 h to measure chronic effects on CFTR trafficking. In the first assay, cells were grown in the standard medium and efflux was then performed as described previously (25, 23) using the following solutions: lactated Ringer’s solution (LR), LR with 13 μM forskolin, and LR with 13 μM forskolin and 1 or 5 μM of flavonoid (apigenin, genistein, kaempferol or quercetin). In the second assay, cells were grown in the presence of 1 and 5 μM concentrations of flavonoid. Efflux was then performed using LR, or LR with 13 μM forskolin. Rate constants were then calculated by fitting the data to the equation Y = Ae-kt + B, where Y is the amount of radioactivity in the cells at time t, A is the initial amount of radioactivity in the cells, B is the small amount of radioactivity in the wash before collection of the initial time point, and k is the rate constant in min-1) using Origin software (MicrocSoft Software, Inc., Northampton, MA).

Results
\[ ΔF508 CFTR Protein Expression Is Unaffected by the Flavonoids \]

The MTT assay was performed to define the acceptable concentrations of each flavonoid for chronic exposure. Up to 5 μM flavonoid (Figure 1) led to preservation of cell numbers as measured by the optical density at 540 nm. Higher concentrations resulted in a dose-dependent loss of viable cells by 72 h of exposure.

The IB3-1 cell line contains one ΔF508 allele and one W1282X allele. The gentamicin that is a standard component of the LHC-8 medium can be eliminated from the formulation for these experiments to avoid read-through of the stop codon during translation. During growth at 37°C, band B is favored and little to no band C is detectable by immunoblotting (Figure 2A). Growth at the permissive temperature 27°C restores band C. Growth at 37°C in the presence of the DMSO vehicle also is partially effective at restoring some band C (Figure 2A). The data in the bar graph in Figure 2A were derived by densitometry of the single immunoblot. Data are expressed as a percent of band B at 37°C. Both bands B and C were increased with 27°C or PBA. DMSO is required to solubilize the isoflavonoids, and all estimates of ΔF508 processing from band B to band C in Figures 2A–2E were referenced to band B in cells at 37°C. Representative immunoblots are shown in Figures 2A–2E. There was no decrease in mature CFTR band C expression with the doses of flavonoid tested. The graphs in Figure 2A–2E represent an average of 3–4 independent experiments for each flavonoid at the specified concentrations. Student’s t test was then performed comparing band B and band C from the 37°C lane to each of the flavonoid treatments at 1 and 5 μM concentrations. As there was no statistically significant difference found for any treatment,
we conclude that there is no detrimental effect at low concentrations of the flavonoids tested on CFTR processing in IB3–1 cells. Although we were not able to show conclusive improvement in CFTR processing with flavonoid treatment alone, it is still noteworthy that the flavonoids we tested at the concentrations described did not appear to be detrimental in the production or processing of CFTR.

Apigenin, Genistein, Kaempferol, and Quercetin Do Not Interfere with CFTR-Hsp70 or CFTR-Hsc70 Interactions as Measured by Immunoprecipitation

We have focused on Hsc70 and Hsp70, two members of the 70 kDa Hsp family. Experiments were performed to measure total protein expression of Hsp70 and Hsc70, as well as quantity of Hsp70 complexed with CFTR and Hsc70 complexed with CFTR. There were no differences in total protein expression of Hsp70 or Hsc70 with flavonoid treatment. However, previous experience (5) has shown us that it may not be total cellular protein expression of these chaperone proteins that are a factor as much as the amount of chaperone associated with CFTR.

We therefore looked at Hsp70 and Hsc70 complexed with CFTR. As we have reported previously, there is a visible increase in Hsp70-CFTR complex and a decrease in Hsc70-CFTR complex with 4-PBA treatment, as seen in the representative immunoblots shown in Figure 3A. There was no significant change seen in Hsc70-CFTR complexes or Hsp70-CFTR complexes with apigenin, genistein, or kaempferol; however, there was a statistically significant ($P = 0.049$) decrease in the Hsc70-CFTR complex in cells treated with 5 μM quercetin compared with control cells. Based on the mechanism of action of 4-PBA, one might expect this decrease in Hsc70-CFTR complex to lead to improved CFTR processing, but we did not see this reflected in total protein expression by immunoblot (Figure 2E) or in CFTR distribution by immunofluorescence (Figure 4H). This supports our hypothesis that there are likely multiple chaperones that could be affected differently by butyrates or the flavonoids, and that the mechanism is quite complex.

We next examined the effects of the flavonoids on expression of two additional chaperones, calreticulin and calnexin (Figure 3B) in complex with CFTR. Neither chaperone was appreciably affected by these treatments.

Flavonoids Affect ΔF508 CFTR Trafficking

Despite the apparent lack of effects of flavonoids on CFTR bands B or C, we hypothesized that stimulation of CFTR at the

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**Figure 2.** (A) IB3–1 cells were exposed to the following control conditions: 37°C, 37°C DMSO, 27°C, and 27°C DMSO. The immunoblot confirms our previous finding that there is an increase in overall CFTR, including band B (open bars) by 27°C or 4-PBA at 37°C. DMSO in low concentrations also was associated with a slight favoring of band C (closed bars). The immunoblot was analyzed by densitometry as described in Materials and Methods and the results are displayed in the graph to the left of the immunoblot as a percentage of band B at 37°C. The effect of apigenin (B), genistein (C), kaempferol (D), and quercetin (E) on CFTR protein expression is shown in the next panels. IB3–1 cells were treated with 1 and 5 μM concentrations of flavonoid and compared with control cells grown at 37°C and 37°C or 27°C in the presence of DMSO. Western blots were performed with CFTR181 as described and CFTR band C and band B were quantitated by densitometry. Representative immunoblots are shown accompanying each graph. The value for CFTR band B at 37°C was set at 100 and band C and band B in all other conditions were normalized to that value. Each bar represents the average of three to four independent experiments. Error bars represent ± 1 SD. There is no statistically significant difference seen with any of the treatment groups. Note that a single immunoblot was selected for display in B through E and that the first three lanes were reproduced against the experimental conditions for this figure. (Continues)
cell surface could also result from a redistribution of CFTR without a detectable change in total protein expression. IB3–1 cells were treated with 1 μM of apigenin, genistein, kaempferol, or quercetin for 24 h and then probed for CFTR using immunofluorescence (Figures 4A–4H). In cells grown at 37°C, there is a scant amount of CFTR in a predominantly perinuclear distribution. With the addition of 1 mM 4-PBA and DMSO (a known chemical chaperone) there is a change in the distribution of CFTR from predominantly perinuclear to a dispersed pattern throughout the cytoplasm, including the cell periphery. With apigenin, genistein, and kaempferol we again see a more peripheral distribution of CFTR. Quercetin treatment gives a pattern similar to that seen in the 37°C control cells. These patterns of CFTR expression were not predictable by patterns seen on immunoblots or by immunoprecipitation experiments, because band C simply indicates passage through the Golgi and does not guarantee presence in the periphery or plasma membrane. Thus, the measurement of the processing of CFTR from band B to band C, is not of sufficient resolution to predict subcellular localization.

Acute Exposure to Genistein Stimulates Cl– Efflux in IB3–1 Cells that Have Been Treated with 4-PBA

4-PBA regulates chaperone-mediated ΔF508 processing but does not stimulate Cl– conductance (5, 24–26). We examined the combination of pretreatment with 4-PBA with acute exposure to flavonoids to see if the combination might augment cAMP-mediated Cl– transport. IB3–1 cells were cultured as described above and left untreated at 37°C or were treated with 1 mM 4-PBA. Efflux assays were then conducted in LR, 13 μM forskolin, and 13 μM forskolin with 1 and 5 μM flavonoid. The data were collected and analyzed to calculate the rate constant of Cl– efflux shown in Table 1.

Table 1 shows no significant improvement in the rate constant for cells grown at control conditions when stimulated with forskolin and 1 or 5 μM apigenin, genistein, kaempferol, or quercetin compared with cells stimulated with forskolin alone. In cells treated with 4-PBA, there was no statistically significant improvement in the rate constant when the cells were stimulated with forskolin and 1 or 5 μM apigenin, kaempferol, or quercetin; however, significant increases (P = 0.003) in the rate constant were seen with stimulation by forskolin and 1 μM genistein (k = 6.32 ± 0.80, n = 5) or 5 μM genistein (k = 6.46 ± 1.16, n = 5) (Table 1). The efflux curves for genistein stimulation compared with the DMSO control are shown in Figure 5A. Thus, genistein activates ΔF508 mediated Cl– secretion in cells pretreated with 4-PBA to augment the cell surface pool.

Chronic Exposure to Flavonoids in the Absence of 4-PBA Variably Promotes Forskolin-Stimulated Cl– Efflux

Cells were chronically exposed to 1 μM and 5 μM flavonoids at 37°C and then assayed by forskolin-mediated Cl– efflux. Only 1 mM 4-PBA, 5 μM apigenin, and 1 μM quercetin, each for 24 h, was associated with a slightly increased first-order rate constant for forskolin-mediated Cl– efflux (see Table 1). The efflux data for the active concentration of the two flavonoids is included in Figure 5B. Thus, putting together the findings by immunofluorescence with anti-CFTR antibodies and radioisotopic Cl– efflux assays, 4-PBA, apigenin, or quercetin chronically, or 4-PBA chronically with acute intermittent exposure to genistein, are the most favorable conditions for the restoration of ΔF508 function.

Discussion

Flavonoids exhibit features that make them ideal candidates for therapeutic agents. Their antimicrobial, antiinflammatory, and antioxidant properties, combined with the fact that they are naturally found in foods and appear to be well tolerated thus far in human clinical trials (27), make them an ideal candidate as a therapeutic agent. We have demonstrated that apigenin, genistein, kaempferol, and quercetin do not have any detrimental

Figure 2. (Continued)
Figure 3. (A) Effect of flavonoids on Hsp70/chaperone-CFTR complex formation. IB3–1 cells were treated with 1 and 5 μM concentrations of flavonoid and compared with control cells grown at 37°C and 27°C in the presence of DMSO as well as cells treated with 1 mM 4-PBA. Immunoprecipitation was performed with CFTR169 and the complexes were then probed by immunoblot with anti-Hsp70 and Hsc70 antibody. Representative blots are shown for Hsp70-CFTR (closed bars) and Hsc70-CFTR (open bars) complexes. Each complex was quantitated by densitometry and the values at 37°C for CFTR bands B and C were set at 100 and DMSO and flavonoid treatment values were normalized to the 37°C control. Each point on the graph represents an average of three independent experiments. Error bars represent ± 1 SD. There is a visible increase in Hsp70-CFTR complex and a relative decrease in Hsc70-CFTR complex with 1 mM 4-PBA treatment. *P < 0.05 decrease in Hsc70-CFTR complex with 5 μM quercetin treatment compared with control cells grown at 37°C. (B) Effect of flavonoids on calnexin (closed bars) or calreticulin (open bars)-CFTR complex formation. Experimental conditions were analogous to those of Figure 2A and analyzed in a similar fashion. There is no effect of experimental or control conditions on these complexes.

Figure 4. Change in CFTR distribution with DMSO, 4-PBA, and flavonoid treatments. CFTR was detected with CFTR181 and Cy3-labeled donkey anti-rabbit antibody as described in Materials and Methods. In the absence of CFTR181 (A), there is a scant amount of nonspecific staining seen. Cells grown at 37°C (B) show CFTR localized predominantly to the perinuclear region. DMSO treatment (C) leads to a vesicular pattern of CFTR with some spread from the perinuclear region. A 1 mM 4-PBA treatment (D) is associated with a more peripheral distribution of CFTR away from the nucleus. Apigenin (E), genistein (F), and kaempferol (G) at 1 μM also induce a pattern of peripheral CFTR distribution similar to that seen with 4-PBA treatment and the vesicular pattern noted with DMSO treatment alone. Cells treated with 1 μM quercetin (H) appear similar to control cells (B).

published studies also demonstrate that serum levels above 10 μM are difficult to achieve (28–31).

Treatments that restore ΔF508 CFTR to the processing pathway may involve modulation of Hsp-CFTR interactions as seen with the butyrates. Downregulation of Hsc70 and upregulation of Hsp70 have been implicated in PBA-mediated regulation of ΔF508 CFTR trafficking (5–8). Quercetin has also been shown to downregulate Hsp in tumor cells, specifically Hsp70 and Hsp27 (32–34). Hansen (33) showed that quercetin inhibited the synthesis of Hsp70 in various cell lines, but in a cell type–specific manner. This effect was not seen in our experiments using IB3–1 cells, which once again demonstrates that the effects of flavonoids are cell line–specific and may be modulated by the stress the cells are under as well. Thus, we may not have detected a substantial increase in CFTR-related efflux with quercetin in contrast to the
TABLE 1. Effects of acute and chronic exposure to flavonoids on radiotopic chloride efflux in IB3–1 cells

<table>
<thead>
<tr>
<th>Treatment</th>
<th>Acute Flavonoida (N)</th>
<th>P value</th>
<th>Chronic Flavonoida (N)</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>37°C</td>
<td>4.28 ± 1.19 (10)</td>
<td>0.625</td>
<td>6.60 ± 0.85 (6)</td>
<td>0.006</td>
</tr>
<tr>
<td>1 mM 4-PBA</td>
<td>4.14 ± 1.14 (9)</td>
<td>0.208</td>
<td>5.65 ± 0.88 (6)</td>
<td>0.360</td>
</tr>
<tr>
<td>1 μM apigenin</td>
<td>5.12 ± 2.3 (5)</td>
<td>0.304</td>
<td>5.05 ± 0.18 (6)</td>
<td>0.160</td>
</tr>
<tr>
<td>5 μM apigenin</td>
<td>4.73 ± 0.82 (5)</td>
<td>0.369</td>
<td>5.56 ± 0.65 (6)</td>
<td>0.030</td>
</tr>
<tr>
<td>1 μM genistein</td>
<td>6.32 ± 0.80 (5)</td>
<td>0.003⁰</td>
<td>7.14 ± 1.83 (6)</td>
<td>0.096</td>
</tr>
<tr>
<td>5 μM genistein</td>
<td>6.46 ± 1.16 (5)</td>
<td>0.000¹</td>
<td>5.28 ± 0.63 (6)</td>
<td>0.080</td>
</tr>
<tr>
<td>1 μM kaempferol</td>
<td>4.80 ± 0.71 (5)</td>
<td>0.262</td>
<td>4.95 ± 1.04 (6)</td>
<td>0.216</td>
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<tr>
<td>5 μM kaempferol</td>
<td>4.76 ± 0.65 (5)</td>
<td>0.169</td>
<td>5.29 ± 0.59 (6)</td>
<td>0.075</td>
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<tr>
<td>1 μM quercetin</td>
<td>4.55 ± 1.44 (5)</td>
<td>0.562</td>
<td>5.50 ± 0.54 (6)</td>
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<td>5 μM quercetin</td>
<td>4.78 ± 1.14 (5)</td>
<td>0.330</td>
<td>4.98 ± 1.74 (6)</td>
<td>0.299</td>
</tr>
</tbody>
</table>

Data are mean ± S.D. Units are mm⁻¹.

⁰ Cells were grown in 4-PBA for 24 h to promote ΔF508 trafficking. Efflux was performed with flavonoids supplementation of forskolin efflux solution.

¹ P < 0.05, unpaired t test.

² Cells were grown in flavonoids for 24 h and efflux was performed in forskolin efflux solution in the absence of flavonoids.

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