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Article

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Fatty Acid Cysteamine Conjugates as Novel and Potent Autophagy Activators that Enhance the Correction of Misfolded F508del-Cystic Fibrosis Transmembrane Conductance Regulator (CFTR)

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ABSTRACT

A depressed autophagy has previously been reported in cystic fibrosis patients with the common F508del-CFTR mutation. This report describes the synthesis and preliminary biological

characterization of a novel series of autophagy activators involving fatty acid cysteamine conjugates. These molecular entities were synthesized by first covalently linking cysteamine to docosahexaenoic acid. The resulting conjugate **1** synergistically activated autophagy in primary homozygous F508del-CFTR human bronchial epithelial (hBE) cells at submicromolar concentrations. When conjugate **1** was used in combination with the corrector lumacaftor and the potentiator ivacaftor, it showed an additive effect, as measured by the increase in the chloride current in a functional assay. In order to obtain a more stable form for oral dosing, the sulfhydryl group in conjugate **1** was converted into a functionalized disulfide moiety. The resulting conjugate **5** is orally bioavailable in the mouse, rat and dog and allows a sustained delivery of the biologically active conjugate **1**.

INTRODUCTION

Network pharmacology is a versatile and important tool for a medicinal chemist when faced with a challenging therapeutic target. This drug design technique, sometimes referred to as pathway pharmacology or polypharmacology,[1-4] involves the modulation of multiple therapeutic pathways in order to achieve a desired pharmacological response. We have recently described a variation of this technique which allowed network pharmacology to take place when two bioactives are delivered intracellularly at the same time and in equimolar concentrations.[5] In our approach, a plasma stable linker was used to first covalently join the two bioactives. Once delivered inside cells, intracellular enzymes could readily cleave the linker to simultaneously release the two bioactives. Since multiple biological pathways were simultaneously impacted by this type of intracellular delivery, the resulting pharmacology that could be produced was unique and could not be replicated by giving the two bioactives either individually or in combination. We have

demonstrated the utility of our methodology with two examples: the first involves the use of a fatty acid niacin conjugate in the inhibition of sterol regulatory element binding protein (SREBP) and the second involves the use of a fatty acid salicylate conjugate in the inhibition of NF-κB. Both of these therapeutic targets have historically been known to be difficult to attenuate by traditional medicinal chemistry approaches. In this communication, we will demonstrate how this approach could be used again to address another challenging therapeutic target; this time involving the activation of autophagy. Even though a depressed autophagy has been implicated in numerous diseases,[6] this report focuses primarily on its impact in cystic fibrosis (CF).

CF is a deadly orphan disease that affects over 70,000 patients worldwide, predominantly in the Caucasian population. It is caused by a defective cystic fibrosis transmembrane conductance regulator (CFTR),[7] a cAMP-dependent ion channel that regulates the flow of chloride ions across epithelial cell membranes. A malfunctioning CFTR ion channel will cause an imbalance in ion and fluid transport; and over time, this can have a debilitating effect on the pancreas, liver, kidneys, intestine and especially the lungs. Even though there are more than 1900 reported mutations in CF, more than 80% of CF patients worldwide carry the F508del-CFTR mutation, in either the homozygous or heterozygous form. With this particular mutation, the misfolded F508del-CFTR cannot be transported out of the endoplasmic reticulum (ER), and instead is degraded by the proteasome before it can rise to the cell surface. A restoration of F508del-CFTR function therefore requires the use of two agents: 1) a CFTR corrector to help transport the misfolded CFTR to the cell surface; 2) a CFTR potentiator to help keep this ion channel open at the cell surface. Intense research efforts in this field have recently resulted in the approval of the combination consisting of the corrector lumacaftor (VX-809)[8] and the potentiator ivacaftor (VX-770)[9] in CF patients with the homozygous F508del-CFTR mutation (Figure 1). However, the

clinical efficacy for this particular combination of drugs is modest, with an average improvement of 3% in lung function.[10] Therefore, numerous efforts are currently being taken to identify more effective CFTR correctors or potentiators,[11] as well as agents that can potentially improve the efficacy of the current combination of lumacaftor and ivacaftor.[12] In this report, we will demonstrate that the autophagy pathway is intricately linked to the trafficking of the CFTR; and an effective autophagy activator can enhance the transport of the misfolded CFTR to the cell surface and be useful as part of a triple drug combination to treat CF patients with the F508del-CFTR mutation.

RESULTS AND DISCUSSION

Autophagy and the trafficking of the F508del-CFTR

Normal cells typically use autophagy as a means for removing sources of reactive oxygen species, damaged mitochondria, misfolded proteins, and foreign pathogens. The autophagy process involves the initial formation and elongation of a membrane sac called the phagophore, which eventually develops into an autophagosome.[13] This is a double-membrane vesicle that is used to sequester the dysfunctional cellular components into the cytoplasmic cargo. The autophagosomes themselves will eventually fuse with the lysosomes to enable the degradation of the cargo. This process is used to essentially convert damaged cellular constituents into amino acids, lipids, sugars, and nucleotides, all basic building blocks for the regeneration of new proteins and organelles. In CF patients with the common F508del-CFTR mutation, a depressed autophagy could be traced back to decreased levels of the Beclin-1, a key protein that is needed for the autophagosome formation.[14] The defective F508del-CFTR is believed to induce an up-regulation of reactive oxygen species (ROS) and a corresponding increase in transglutaminase

(TG2) activity. The increased TG2 activity, in turn, drives the crosslinking and the subsequent disabling of Beclin-1. Once autophagy is depressed, there is a corresponding accumulation of p62. Since p62 has been shown to regulate aggresome formation, its accumulation effectively traps the misfolded CFTR and prevents its trafficking to the cell surface. The sequestered CFTR is then targeted for rapid degradation by the proteasome (Figure 2). A successful activation of autophagy could potentially allow this sequence of events to be reversed, and thereby allow more of the F508del-CFTR to rise to the cell membrane. Previous proof-of-concept studies using human epithelial cell lines (CFBE41o- and IB3-1 cells) that were transfected with the F508del-CFTR have shown that over expression of Beclin-1 or knock down of p62 via siRNA did indeed result in more of the misfolded CFTR being transported to the cell surface.[15] Even though this autophagy-based approach has the potential to address one of the underlying causes of CF, it has not been investigated extensively in the clinic. This is perhaps due in part to the lack of safe and effective agents that could selectively activate this particular pathway. Among the agents that have been reported to date (i.e., brefeldin A, rapamycin, valproic acid, trehalose, tunicamycin, carbamazepine, penitrem A) some could only activate autophagy at high concentrations; while others displayed many off-target side effects or non-drug like properties, which precluded their evaluation in the clinic.[16] Cysteamine and its disulfide form, namely cystamine, appear to be promising leads for autophagy activation, based on their safe and long term use in the clinic for patients with nephropathic cystinosis.[17] Administration of cysteamine to CFTR^{F508del} mice over a 5 week period showed a reduction in inflammatory markers (TNF, Cxcl2) and an increase in body weight, CFTR protein and the autophagy marker Beclin-1. Maiuri and coworkers have shown that cystamine (at 250 μM) could activate autophagy in brushed nasal epithelial cells from CF patients (homozygous F508del-CFTR) to allow the trafficking of the misfolded CFTR to the

cell membrane.[18] Cysteamine has since been evaluated in a pilot clinical trial involving homozygous F508del-CFTR CF patients, in combination with epigallocatechin gallate.[19, 20] Cysteamine administration, however, comes with many challenges because of its poor PK profile (i.e., short half-life) and the associated GI side effects (i.e., nausea, emesis). Because of its low potency, it is not clear if cysteamine could be administered at doses high enough to allow a restoration of autophagy without resulting in significant off-target effects. Furthermore, cysteamine itself has an unpleasant thiol odor and taste which can present significant patient compliance issues upon chronic dosing.

As outlined below, we have identified a more effective way of activating autophagy via the use of fatty acid cysteamine conjugates. These covalent conjugates have the potential of filling the current gap in the availability of more potent, yet safe and effective autophagy activators suitable for definitive proof-of-concept studies. These NCEs were prepared by covalently linking cysteamine to the omega-3 fatty acid (4Z,7Z,10Z,13Z,16Z,19Z)-docosa-4,7,10,13,16,19-hexaenoic acid (DHA). This process allows the simultaneous delivery of the two bioactives without having to address the individual PK and tissue distribution of the individual components. Our fatty acid cysteamine conjugates synergistically activated autophagy in cultured primary homozygous F508del-CFTR hBE cells at concentrations that were significantly lower than what had previously been reported with cysteamine.

Synergistic activation of autophagy with a fatty acid cysteamine conjugate

Cysteamine has been reported by Maiuri and co-workers to activate autophagy in certain human epithelial cell lines (CFBE41o- and IB3-1 cells) by restoring Beclin-1 via the inhibition of TG2.[14] However, the effect was modest and a high concentration of cysteamine (250 μM) was

required. We therefore hypothesized that if we could simultaneously deliver cysteamine along with a second bioactive, another pathway could perhaps work in concert with the TG2 inhibition to amplify the autophagy activation. We selected DHA as this second bioactive. This omega-3 fatty acid, by itself, has previously been shown to increase autophagosome formation via the AMPK pathway.[21, 22] In order to achieve a simultaneous intracellular delivery, we employed the same methodology that we described earlier[5] by first covalently linking cysteamine to DHA to form the fatty acid cysteamine conjugate **1** (Figure 3). The amide bond that is present in the covalent conjugate **1** is susceptible to cleavage by the intracellular enzyme, fatty acid amide hydrolase (FAAH). We have evaluated this hydrolysis process using the purified recombinant enzyme FAAH-1.[5] As shown in Figure 4, recombinant FAAH-1 readily hydrolyzed the amide bond to release DHA in a time-dependent fashion. Interestingly, FAAH is localized inside cells at the ER, the same compartment where autophagy is initiated. The covalent conjugate **1** allows cysteamine and DHA to be delivered at the same time, in equimolar concentrations, to the same subcellular compartment in which the mutant F508del-CFTR is trapped by the p62 aggresomes. This simultaneous intracellular delivery enables an unprecedented level of synergy to occur in terms of autophagy activation. Figure 5 summarizes an experiment where cultured primary homozygous F508del-CFTR hBE cells were incubated with the following treatment groups: 1) vehicle; 2) DHA (250 μM); 3) cysteamine (250 μM); 4) combination of cysteamine (250 μM) + DHA (250 μM); 5) fatty acid cysteamine conjugate **1** (3 μM). It should be noted that the fatty acid cysteamine conjugates have limited water solubility and need to be solubilized along with a protein carrier, such as FBS, prior to the serial dilution with the assay media in order to obtain the optimal effect. Compound **1**, at a concentration of 3 μM, increased Beclin-1; and this, in turn, activated autophagy, as noted by the increase in the ratio of LC3-II to LC3-I, a commonly used

marker for this catabolic process.[23] Autophagy activation was further accompanied by a corresponding decrease in p62, a key protein that participates in the sequestration of the F508del-CFTR. This synergistic activation in autophagy was observed only with the covalent fatty acid cysteamine conjugate **1**; and not with the individual components (i.e., cysteamine, DHA) or a combination with the individual components.[24] In cultured primary CF hBE cells obtained from this particular donor, cysteamine, by itself, did not activate autophagy even at a concentration of 250 μM. The omega-3 fatty acid DHA, as well as the combination consisting of cysteamine and DHA, did not activate autophagy in primary CF hBE cells at a concentration of 250 μM.

We next assessed how restoration of autophagy would affect the overall correction of the F508del-CFTR upon chronic exposure to the CFTR corrector lumacaftor and the potentiator ivacaftor. Figure 6 summarizes an experiment where cultured primary homozygous F508del-CFTR hBE cells (patient code KKCFFT006F) were incubated for 24 hr with **1** (3 μM) along with lumacaftor (3 μM) and ivacaftor (0.10 μM) in differentiation media.[25] Compared to the vehicle-treated group, treatment with lumacaftor/ivacaftor resulted in the expected increase in the CFTR band B (the immature, core-glycosylated isoform) and band C (the mature, complex-glycosylated isoform). When lumacaftor and ivacaftor were used in combination with the fatty acid cysteamine conjugate **1** (3 μM), a restoration of autophagy was observed, as noted by the increase in the level of Beclin-1 and the ratio of LC3-II to LC3-I (Figure 6C). Consistent with the previously proposed mechanism, an increase in autophagy was accompanied by a corresponding decrease in the level of p62, which, in turn, allowed some of F508del-CFTR to rise to the cell surface. As shown in Figures 6A and 6B, the combination consisting of **1** (3 μM) and lumacaftor/ivacaftor clearly produced an increase in both the CFTR band B and band C. With cultured primary homozygous F508del-CFTR hBE cells from this particular donor

(KKCFFT006F), we have observed autophagy activation even when **1** was used at the lower concentrations of 0.018-0.60 μM (this data is shown in the Supporting Information). Maiuri and coworkers[14] have previously reported that once autophagy is activated, a reduction in the turnover of plasma membrane-located F508del-CFTR in human CF airway epithelial cells is observed. This could potentially translate to an increase in the chloride current across the F508del-CFTR when the proper CFTR corrector and potentiator are available. The next stage of our validation involves measurement of the chloride current across the F508del-CFTR hBE cells.

A triple combination to correct F508del-CFTR

A successful correction of the F508del-CFTR needs to translate to an actual increase in the chloride current to be of benefit to CF patients. This important functional activity was assessed using the TECC-24 semi-automated format with cultured primary homozygous F508del-CFTR hBE cells. Unlike the traditional Ussing chamber assays, where the short circuit current (I_{SC}) is measured as an index of CFTR correction, the TECC-24 assay measures the equivalent current (I_{EQ}). The TECC-24 assay format offers some advantages over the Ussing chamber assay, one of which is the higher throughput; and the other involves the ability to measure the I_{EQ} with the test compounds continuously present in the recording medium. However, since the I_{EQ} was recorded in 5 min intervals, one disadvantage with the TECC-24 in the present format is the lack of sensitivity of the assay during the forskolin activation phase. Nevertheless, once steady state has been achieved, the bumetanide-inhibited CFTR chloride current obtained from a TECC-24 assay represents a reliable measure of functional efficacy. There are two basic protocols that are used to assess functional activity: 1) the acute ivacaftor protocol; and 2) the chronic ivacaftor pre-incubation protocol. In the acute ivacaftor protocol, cultured primary homozygous F508del-CFTR hBE cells were incubated with the fatty acid cysteamine conjugate **1** along with the CFTR

corrector lumacaftor for 24 hr. Forskolin and the CFTR potentiator ivacaftor were then added acutely just after benzamil was added to the apical side to inhibit sodium transport prior to the I_{EQ} measurements. This is the standard set of assay conditions that were used originally to assess the functional activity of the corrector lumacaftor.[8] In the chronic ivacaftor pre-incubation protocol, cultured primary homozygous F508del-CFTR hBE cells were incubated with the fatty acid cysteamine conjugate **1** along with both the corrector lumacaftor and the potentiator ivacaftor for 24 hr prior to the I_{EQ} measurements. This protocol was introduced more recently based on the findings from Cholon[26] and Veit[27] that the potentiator ivacaftor actually had a destabilizing effect on the corrected CFTR upon chronic pre-incubation with lumacaftor; and the chloride current measured under this protocol was significantly less than what was previously reported with the acute addition of ivacaftor. When a triple combination is used in a clinical setting, this chronic pre-incubation protocol might be more representative of what cells would be exposed to at a given time period. We have assessed the functional activity of **1** using this chronic ivacaftor pre-incubation protocol and have found a significant additive effect on top of lumacaftor/ivacaftor.[28] Figure 7 summarizes an experiment using the chronic ivacaftor pre-incubation protocol. Here, primary homozygous F508del-CFTR hBE cells (patient code CFFT0018I) was incubated in differentiation media for 24 hr at 37 ^{o}C with the following treatment groups: 1) vehicle + ivacaftor (0.10 μM); 2) lumacaftor (3 μM) + ivacaftor (0.10 μM); 3) **1** (0.075 μM) + lumacaftor (3 μM) + ivacaftor (0.10 μM). The next day, the differentiation media was replaced with Coon's F12 (without serum or bicarbonate) media; with the same concentrations of the test compounds added back to this new media.[29] To initiate the run, benzamil was added first to the apical side to block currents through the epithelial sodium channel (ENaC). Forskolin was added and the I_{EQ} was recorded over a 27 min period. The

antagonist bumetanide was then added to the basolateral side to inhibit the Na-K-2Cl cotransporter and block chloride secretion from the CFTR. The resulting bumetanide-inhibited CFTR chloride current (ΔBumetanide, μA/cm^2) is a reflection of the functional activity of the now corrected F508del-CFTR. As shown in Figures 7A and 7B, the triple combination consisting of **1** (0.075 μM) along with lumacaftor/ivacaftor showed a statistically significant increase in the bumetanide-inhibited CFTR chloride current (ΔBumetanide = 3.08 ± 0.24 μA/cm^2) over the lumacaftor/ivacaftor treatment group (ΔBumetanide = 1.91 ± 0.22 μA/cm^2). This increase in the CFTR chloride current could also be expressed as a % of the lumacaftor/ivacaftor control group, with the appropriate subtraction of the ΔBumetanide value corresponding to the vehicle group from both the triple combination and the lumacaftor/ivacaftor treatment group, in order to allow for a more direct comparison across the different assay runs. For this particular run, the triple combination allowed a 184% increase in the bumetanide-inhibited CFTR chloride current relative to the lumacaftor/ivacaftor treatment group; a nearly two-fold increase in the response (Figure 7B, with the appropriate subtraction of the ΔBumetanide value for the vehicle group of 0.52 ± 0.14 μA/cm^2). In this assay format using primary cells from this particular patient donor, we did not observe a noticeable change in the forskolin-stimulated ΔI_{EQ} for the triple combination. However, there was a statistically significant increase in the AUC for the triple combination over the lumacaftor/ivacaftor treatment group (Figure 7C, triple combination AUC = 173.3 ± 18.5 μA/(27 min * cm^2) vs lumacaftor/ivacaftor control group AUC = 134.2 ± 5.6 μA/(27 min * cm^2); vehicle group AUC = 22.3 ± 4.9 μA/(27 min * cm^2)). With this particular assay setup, the solubility of **1** in the Coon's F12 media was a limiting factor. At concentrations of > 0.075 μM, some of the precipitated, oily

fatty acid conjugates began to interfere with the current recording. The I_{EQ} readings became much more variable and the results were more difficult to interpret.[30]

Triple combinations using primary CF hBE cells from different donors

We have also evaluated the functional activity of **1** in a similar TECC-24 assay format using primary CF hBE cells from different donors. Figure 8 summarizes a TECC-24 assay that was carried out at a different laboratory, using cultured primary homozygous F508del-CFTR hBE cells from patient code KKCFFT006F. These primary hBE cells are of the same patient code as the one used in the band B/C western blots shown in Figure 6. Using the chronic ivacaftor pre-incubation protocol, cells were treated with the following groups: 1) vehicle + ivacaftor (0.10 μM); 2) lumacaftor (3 μM) + ivacaftor (0.10 μM); 3) **1** (0.15 μM) + lumacaftor (3 μM) + ivacaftor (0.10 μM). As detailed in the experimental section, there are some minor differences between the two TECC-24 assay formats. However, the basic protocol remains essentially the same. One major difference is in the antagonist that is used to block the chloride secretion from the CFTR. With this set up, $CFTR_{172}$-inh was used instead of bumetanide; and the antagonist-inhibited CFTR chloride current, ΔI_{EQ} ($CFTR_{inh}$-172), represents essentially the same functional readout as the ΔBumetanide described in Figure 7. With this particular TECC-24 set up, using cells from this particular patient donor, a significant increase in the peak forskolin response was observed with the triple combination (Figure 8A). As shown in Figure 8B, the peak forskolin ΔI_{EQ} for the triple combination was 24.47 ± 0.62 μA/cm^2. This increase in the peak forskolin response was statistically significant over the lumacaftor/ivacaftor treatment group (ΔI_{EQ} = 16.61 ± 2.16 μA/cm^2). When expressed as a % of the lumacaftor/ivacaftor control group, with the appropriate subtraction of the ΔI_{EQ} value of 7.50 ± 0.44 μA/cm^2 for the vehicle group, the triple combination with **1** afforded a 186% increase in the peak forskolin response; a nearly two-fold

increase in the response. In terms of the $CFTR_{inh}$-172 inhibited chloride current, the triple combination (ΔI_{EQ} = 7.47 ± 0.16 μA/cm^2) still showed a statistically significant increase over the lumacaftor/ivacaftor treatment group (ΔI_{EQ} = 5.45 ± 0.48 μA/cm^2). When expressed as a % of the lumacaftor/ivacaftor control group, this represents a 149% increase in the antagonist-inhibited chloride current (with the appropriate subtraction of the vehicle group ΔI_{EQ} = 1.37 ± 0.23 μA/cm^2). There was also a statistically significant increase in the AUC for the triple combination (triple combination AUC = 607.3 μA/(60 min * cm^2) vs lumacaftor/ivacaftor control AUC = 430.6 ± 40.5 μA/(60 min * cm^2); vehicle group AUC = 137.2 ± 20.3 μA/(60 min * cm^2). Even though the solubility of **1** was still limited in this TECC-24 assay set up, it did not appear to interfere with the I_{EQ} measurements as much as in the previous set up. We have evaluated **1** in concentrations of up to 0.6 μM and observed an additive effect on top of lumacaftor/ivacaftor (the entire data set is shown in the Supporting Information, along with the corresponding band B/band C data for this lower concentration of 0.15 μM of conjugate **1**). In this particular assay setup, at concentrations of ≥ 1.2 μM of **1**, solubility became an issue in the Coon's F12 media; and we began to experience an inhibition in the chloride current.[31]

A statistically significant increase in the peak forskolin response was also observed when **1** was evaluated in the same TECC-24 setup using primary cells from a different patient donor. Figure 9 summarizes an experiment where primary homozygous F508del-CFTR hBE cells (patient code KKCFFT004I) were treated with the following groups for 24 hr at 37 ^oC prior to I_{EQ} measurements: 1) vehicle + ivacaftor (0.10 μM); 2) lumacaftor (3 μM) + ivacaftor (0.10 μM); 3) **1** (0.30 μM) + lumacaftor (3 μM) + ivacaftor (0.10 μM). Under this chronic ivacaftor pre-incubation protocol, there was a statistically significant increase in the peak forskolin-activated CFTR chloride current with the triple combination. Even though the magnitude of the increase

was smaller than what was observed with the previous patient code, the ΔI_{EQ} (peak forskolin) for the triple combination still represent a 167% increase over the lumacaftor/ivacaftor treatment group (triple combination, $\Delta I_{EQ} = 8.24 \pm 1.18$ μA/cm^2 vs lumacaftor/ivacaftor control group $\Delta I_{EQ} = 6.250 \pm 0.36$ μA/cm^2; vehicle group, $\Delta I_{EQ} = 3.90 \pm 0.88$ μA/cm^2). With primary cells from this particular donor, we began to see an inhibition in the chloride current at concentrations ≥ 0.60 μM of **1** (additional data is presented in the Supporting Information). Having evaluated **1** in TECC-24 setups from different laboratories, using primary CF cells from different patient donors, we have noticed a wide range of responses. With some primary hBE cells, we did not experience any chloride current inhibition until we reached a concentration of ≥ 1.2 μM of **1**. In others, we began to experience significant variability and some chloride current inhibition at much lower concentrations. There are many contributing factors to the observed variations, including: a) different laboratories cultured and maintained primary hBE cells from different donors on different TECC-24 filter inserts; b) different protocols were used to remove the mucus film on the apical side of the hBE monolayers prior to I_{EQ} measurements; c) different sources of FBS were used to solubilize **1** prior to the serial dilution. However, one observation that was consistent across the different setups was the additive effect of **1** on top of lumacaftor/ivacaftor when used at lower concentrations, where solubility of **1** in the recording media was less of a factor. Thus far, because of the low solubility of **1** in the Coon's F12 media using this TECC-24 assay format, we have not been able to assess its functional activity at the higher concentration of 3 μM (i.e, the concentration where we observed a significant increase in the CFTR band C, Figure 6). In order to evaluate **1** at the higher concentrations, we have looked into different functional assay formats, including the forskolin-induced swelling of organoids.[32] The results from those studies will be disclosed in a separate communication.

Design of a plasma-stable and orally bioavailable fatty acid cysteamine conjugate

Even though the covalent conjugate **1** showed promising functional activity, when used in combination with lumacaftor/ivacaftor, it is rather unstable when exposed to air, presumably because of the sulfhydryl moiety. A more stable form is therefore needed for the appropriate *in vivo* studies. Converting the sulfhydryl group to the disulfide form, as shown with the cystamine derivative **2**, should enhance the overall stability of the covalent conjugate (Figure 3). In theory, the bis-fatty acid cystamine conjugate **3** should allow the intracellular delivery of the same covalent conjugate **1**. In practice, however, this type of bis-fatty acid was rather difficult to formulate for oral dosing, as well as for standard *in vitro* work. We, therefore, sought to replace one of the two fatty acids with a smaller substituent. Based on our earlier work with fatty acid niacin conjugates,[5] similar to the one shown with structure **6**, we selected nicotinic acid as the capping group for our first proof-of-concept compound. What we found was that the cystamine linker in **4** was still unstable in the plasma. To improve the plasma stability of our fatty acid cysteamine conjugate, a bis-geminal methyl group was installed adjacent to the disulfide moiety.[33] As shown in Figure 10, compound **5** was stable in rat, mouse, dog, and human plasma. Unlike the related compound **6**, which had significant inhibitory activity against SREBP,[5] the fatty acid cysteamine conjugate **5**, with this type of disulfide linker, did not show any SREBP activity at the highest tested concentration of 50 μM. Compound **5**, in turn, was prepared according to the synthetic sequence outlined in Scheme 1. Cysteamine was first reacted with 1,2-di(pyridin-2-yl)disulfane (**7**) to form the mixed disulfide derivative **8**. Treatment of **8** with DHA afforded the fatty acid derivative **9**. This was subsequently treated with 1-amino-2-methylpropane-2-thiol to afford the bis-geminal methyl derivative **9**. Standard amide coupling with nicotinic acid afforded the fatty acid cysteamine conjugate **5**. Unlike compound **1**, the fatty

acid cysteamine conjugate **5** is now sufficiently stable at room temperature and can be conveniently stored in the freezer over an extended period (initial stability > 1 year). Compound **5** can be conveniently formulated as a self-emulsifying dispersion (SED) using an aqueous mixture consisting of Tween, PEG400, and Peceol (glyceryl monoleate Type 40) for oral gavage administration. In a typical PK experiment, Sprague Dawley rats were dosed orally with 30 mg/kg of compound **5** (Figure 11). Serial blood collection was carried out at 0.125, 0.25, 0.5, 1, 2, 4, 5, 8 and 12 hrs. In order to quantify the unstable thiol metabolite **1**, the corresponding Ellman's adduct **15** (Figure 12) was prepared and used to prepare the necessary standard curve. A small quantity of the parent compound **5** was detected in the plasma upon oral dosing (C_{max} = 24.6 ± 4.39 ng/mL; AUC_{last} = 37.7 ± 16.2 h*ng/mL). There was a significantly higher and more sustained level of the biologically active metabolite **1** (C_{max} = 777 ± 435 ng/mL, AUC_{last} = 2929 ± 1282 h*ng/mL) and this was detected in the plasma even at the 8 and 12 hr time point.[34] This plasma level would support the feasibility of either a BID or QD dosing and is sufficient to support the functional activity displayed by **1** in the various assays discussed earlier.[35]

Even as the disulfide form, when **5** was administered to primary hBE cells, the disulfide bond should undergo the appropriate intracellular reduction to afford the biologically active metabolite **1**. The functional activity of the disulfide derivative **5** was evaluated in the TECC-24 assay format, and some of the results are described herein. Figure 13 summarizes an experiment where **5** was evaluated along with the corrector lumacaftor, upon acute exposure to the potentiator ivacaftor (additional TECC-24 assay data for **5** under the chronic ivacaftor pre-incubation protocol, as well as other functional data, can also be found in the Supporting Information). With this setup using primary hBE cells from this particular patient code (homozygous F508del-CFTR, CFFT028H), there was no significant change in the forskolin response. However, a two-

fold increase in the bumetanide-inhibited CFTR chloride current was observed when **5** (0.0375 μM) was used in combination with lumacaftor and ivacaftor (triple combination, ΔBumetanide = 3.73 ± 0.41 μA/cm^2 vs lumacaftor/ivacaftor control, ΔBumetanide = 1.54 ± 0.04 μA/cm^2). Figure 14 summarizes an experiment where **5** (0.018 μM) was evaluated in a different laboratory using primary homozygous F508del-CFTR hBE cells from another patient code (KKCFFT0012I) along with lumacaftor and ivacaftor. With this setup, although there was no change in the peak forskolin response, there was a significant additive effect for the triple combination when we looked at other parameters such as the CFTR$_{inh}$172-inhibited CFTR chloride current (triple combination, ΔI_{EQ} = 3.05 ± 0.18 μA/cm^2 vs lumacaftor/ivacaftor control, ΔI_{EQ} = 1.74 ± 0.29 μA/cm^2; vehicle group, ΔI_{EQ} = 0.18 ± 0.11 μA/cm^2), and the AUC (triple combination, AUC = 326.36 ± 17.16 μA/(60 min * cm^2) vs lumacaftor/ivacaftor control, AUC = 209.24 ± 40.42 μA/(60 min * cm^2); vehicle group, AUC = 63.83 ± 5.44 μA/(60 min * cm^2)). When expressed as a % of the lumacaftor/ivacaftor control group, both of these responses showed a ≥ 180% increase for the triple combination, a nearly two-fold increase over the lumacaftor/ivacaftor treatment group. In total, cultured primary homozygous F508del-CFTR hBE cells from five different patients were used to demonstrate the functional activity of conjugate **1** and the disulfide derivative **5** in the TECC-24 assay format, using either the chronic ivacaftor pre-incubation protocol or the standard acute ivacaftor protocol.

CONCLUSION

In summary, we have demonstrated the use of fatty acid cysteamine conjugates as novel autophagy activators that could enhance the correction of misfolded F508del-CFTR, when used

in combination with established CFTR modulators. Previous work by Maiuri and co-workers has demonstrated that autophagy could play a role in the trafficking of the mutant F508del-CFTR.[14, 15] However, the reagent employed in their studies, cysteamine, was a weak autophagy activator; and the effect was observed only at 250 μM in certain cell lines. This high concentration of cysteamine would be difficult to achieve in the various tissues because of its poor PK properties. By employing our covalent linking methodology, we have demonstrated that the fatty acid cysteamine conjugate **1** could synergistically activate autophagy in primary homozygous F508del-CFTR hBE cells at much lower concentrations. This level of activity could not be replicated by using the individual components (i.e., cysteamine, DHA) or a combination of the individual components, even at much higher concentrations. Immunoblotting and chloride conductance assays using cultured primary homozygous F508del-CFTR hBE cells were used to verify the functional activity of the fatty acid cysteamine conjugate **1**. Depending on the primary CF cells used, we have observed an improvement of nearly two-fold in either the peak forskolin response or in the bumetanide-inhibited CFTR chloride current when the conjugate **1** was used in combination with lumacaftor/ivacaftor. In order to obtain a more stable form for oral dosing, as well as to improve patient compliance, we converted the sulfhydryl group present in **1** to the corresponding odorless disulfide derivative. The resulting conjugate **5** is orally bioavailable in the mouse, rat and dog when formulated as a self-emulsifying dispersion. This disulfide form allowed a sustained delivery of the biologically active metabolite **1**, with plasma concentrations that were significantly higher than the concentrations at which functional activity was observed in primary homozygous F508del-CFTR hBE cells (0.075-0.3 μM). Since the biologically active metabolite **1** is so effective in activating autophagy, we are anticipating that a much lower dose of it would be needed in an *in vivo* setting. This would minimize the unpleasant smell, taste,

body odor and off-target side effects that are associated with the chronic administration of high doses of cysteamine.

This communication focuses primarily on the effect of autophagy on the correction of the misfolded F508del-CFTR. However, it is not the only significant biological activity that is associated with an autophagy activator such as **1** and its disulfide form **5**. The autophagy mechanism has previously been reported to have an important role in the innate and adaptive immunity and this could have a significant impact on the intracellular clearance of bacteria.[36, 37] While an autophagy activator can be helpful in enhancing CFTR correction, we believe that this unique anti-bacterial effect can potentially be just as useful in CF. It is widely known that the lack of a proper functioning CFTR in the lungs of CF patients causes a chronic bacterial infection which further deteriorates their lung function.[38-40] *Pseudomonas aeruginosa* is a particularly difficult pathogen to treat because of its ability to internalize within cells.[41] Airway epithelial cells thus become a reservoir of intracellular bacteria during chronic *P. aeruginosa* infection. Autophagy activation enables an alternative mechanism to clear the bacterial infection out of the bronchial epithelial cells;[42] and therefore, could potentially be useful when used in combination with anti-infective agents. We have evaluated the ability of compound **5** (CAT-5571) to activate autophagy and induce an intracellular clearance of *P. aeruginosa* in a number of *in vitro* and *in vivo* studies.[43] The results from those studies will be discussed in more detail in a separate communication.

EXPERIMENTAL SECTION

General Information: All chemical reagents and solvents were commercially available and used as received. (4Z,7Z,10Z,13Z,16Z,19Z)-Docosa-4,7,10,13,16,19-hexaenoic acid (DHA) was purchased from Nu-Chek Prep. Lumacaftor was purchased from Astatech; and ivacaftor was purchased from D-L-Chiral Chemicals. Reactions were generally run under argon or nitrogen. ^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz) spectra were recorded on a Varian 400MHz Unity Inova system in $CDCl_3$, unless otherwise noted. Chemical shifts are expressed in parts per million (ppm, δ); coupling constants are in hertz (Hz). Splitting patterns describe multiplicities s (singlet), d (double), t (triplet), q (quartet), m (multiplet), br (broad). High resolution mass spectroscopy data was analyzed by direct flow injection, utilizing electrospray ionization (ESI) on a Waters Qtof API US instrument in the positive mode. Analytical and other mass spectra were collected on an Agilent Technologies 1200 series system with an Agilent Technologies 6120 Quadruple LC-MS detector in positive mode. A SiliCycle C18 XDB, 3 × 100 mm column was used with a gradient of H_2O and acetonitrile each with 0.1% formic acid; UV detection at 254 nm and 210 nm. Normal phase flash chromatography was accomplished on Teledyne Isco systems using pre-packed silica gel columns. Sample purity was determined by LC-MS; all compounds were of >95% purity, as determined by at least two different HPLC methods. For the hydrolysis experiments and PK study, the samples were analyzed on the Agilent 6410 Triple Quad LC/MS mass spectrometer. Separation was achieved using a Gemini 3 μM C6 phenyl column with a gradient using water containing 0.1% formic acid (solvent A) and methanol containing 0.1% formic acid (solvent B).

(4Z,7Z,10Z,13Z,16Z,19Z)-N-(2-Mercaptoethyl)docosa-4,7,10,13,16,19-hexaenamide (1): The fatty acid cysteamine conjugate **1** was generated fresh from the well-characterized disulfide form prior to each assay, and was not isolated due to its unstable nature. In a typical run, the bis-

fatty acid derivative (4Z,4'Z,7Z,7'Z,10Z,10'Z,13Z,13'Z,16Z,16'Z,19Z,19'Z)-N,N'-(disulfanediylbis(ethane-2,1-diyl))bis(docosa-4,7,10,13,16,19-hexaenamide)[44] (125 mg, 0.162 mmol) was taken up in absolute EtOH (1.75 mL). Racemic dithiothreitol (DTT, 30 mg, 0.194 mmol) was added at rt, followed by aqueous NaOH (1 N solution, 250 μL). The resulting reaction mixture was stirred at rt for 30 minutes to allow for a complete reduction of the disulfide group. It was then diluted with DMSO (4.5 mL) to form a 50 mM DMSO stock solution of **1**. This freshly prepared stock solution was purged with inert gas and used directly for the assay. This stock solution should not be kept at rt for more than 3 hr. If necessary, the stock solution could be stored at -80 ^{o}C up to 5 days or at -20 ^{o}C for up to 3 days. Upon thawing to rt, it should be used (without refreezing) within 3 hr and then discarded appropriately.

N-(2-((2-((4Z,7Z,10Z,13Z,16Z,19Z)-docosa-4,7,10,13,16,19-hexaenamido)ethyl)disulfanyl)-2-methylpropyl)nicotinamide (5): (4Z,7Z,10Z,13Z,16Z,19Z)-N-(2-((1-Amino-2-methylpropan-2-yl)disulfanyl)ethyl)docosa-4,7,10,13,16,19-hexaenamide (**10**, 3.2 g, 6.5 mmol) and nicotinoyl chloride (1.8 g, 13 mmol) were taken up in CH_2Cl_2 (30 mL). Triethylamine (3 mL, 19.5 mmol) was added dropwise at 0 ^{o}C. The resulting mixture was stirred at rt for 18 hr. It was then diluted with water and extracted with CH_2Cl_2. The combined organic layers were washed with water (2 ×50 mL), brine (50 mL), dried over Na_2SO_4, and concentrated under reduced pressure. The resulting residue was purified by silica gel chromatography (pentanes/EtOAc) to afford N-(2-((2-((4Z,7Z,10Z,13Z,16Z,19Z)-docosa-4,7,10,13,16,19-hexaenamido)ethyl)disulfanyl)-2-methylpropyl)nicotinamide (2.2 g, 57% yield) as a light brown oil. ^{1}H NMR (400 MHz, DMSO-d6): δ 9.01 (s, 1H), 8.71-8.65 (m, 2H), 8.21-8.17 (m, 1H), 8.04-8.00 (m, 1H), 7.52-7.47 (m, 1H), 5.39-5.25 (m, 12H), 3.49-3.47 (m, 2H), 3.35-3.28 (m, 3H), 2.82-2.78 (m, 11H), 2.29-2.22 (m, 2H), 2.13-1.76 (m, 4H), 1.29 (s, 6H), 0.94-0.88 (m, 3H); ^{13}C

NMR (100 MHz, DMSO-d6, 5 overlapping peaks corresponding to the CH group present in the omega-3 fatty acid portion): δ 172.08, 165.92, 152.26, 148.98, 135.59, 131.99, 130.54, 129.27, 128.58, 128.52, 128.36, 128.31, 128.21, 128.15, 127.39, 123.81, 51.95, 48.35, 39.18, 38.69, 35.66, 25.79, 25.69, 25.65, 25.60, 23.56, 20.53, 14.59; MS calcd for $C_{34}H_{49}N_3O_2S_2$: 595.3; found, 596.3 $[M+H]^+$. High resolution MS (ES^+) calcd for $C_{34}H_{49}N_3O_2S_2$ ($M + H^+$) m/z, 596.3270; found, 596.3345.

2-(Pyridin-2-yldisulfaneyl)ethan-1-amine (8): A solution containing 1,2-di(pyridin-2-yl)disulfane (**7**, 26 g, 0.227 mmol) in MeOH (200 mL) was added dropwise at rt to a solution containing cysteamine (50 g, 0.227 mmol) in MeOH (200 mL). The resulting reaction mixture was stirred at rt for 2 h under an inert atmosphere of nitrogen and then concentrated under reduced pressure. The resulting residue was purified by silica gel chromatography (CH_2Cl_2/MeOH = 10/1) to afford 2-(pyridin-2-yldisulfanyl)ethan-1-amine (39 g, 92% yield). ^1H NMR (400 MHz, DMSO-d6): δ 8.50 (m, 1 H), 7.78-7.92 (m, 2 H), 7.28 (m, 1 H), 4.58 (br s, 2 H), 2.80-3.00 (m, 4 H). ^{13}C NMR (100 MHz, DMSO-d6): δ 159.37, 150.19, 138.65, 122.28, 119.96, 49.04, 38.49. R_f = 0.22 (CH_2Cl_2/MeOH/AcOH=85:15:5). MS calcd for $C_7H_{10}N_2S_2$: 186.03; found, 187 $[M+H]^+$.

(4Z,7Z,10Z,13Z,16Z,19Z)-N-(2-(Pyridin-2-yldisulfaneyl)ethyl)docosa-4,7,10,13,16,19-hexaenamide (9): A mixture containing 2-(pyridin-2-yldisulfanyl)ethan-1-amine (**8**, 5 g, 26.8 mmol), DHA (9.2 g, 26.8 mmol), and HATU (10.2 g, 26.8 mmol) were taken up in CH_2Cl_2 (100 mL) and stirred at rt. Triethylamine (18 mL, 40.3 mmol) was then added dropwise at rt. The resulting reaction mixture was stirred at rt for 18 hr. It was then diluted with water and extracted with CH_2Cl_2. The combined organic layers were washed with water (3 × 100 mL), brine (100

mL), dried over anhydrous Na_2SO_4, and concentrated under reduced pressure. The resulting residue was purified by silica gel chromatography (pentanes/EtOAc) to afford (4Z,7Z,10Z,13Z,16Z,19Z)-N-(2-(pyridin-2-yldisulfanyl)ethyl)docosa-4,7,10,13,16,19-hexaenamide (10 g, 75% yield) as a light brown oil. ^{1}H NMR (400 MHz, $CDCl_3$):): δ 8.49 (d, *J* = 4.4 Hz, 1 H), 7.65 (m, 1 H), 7.53 (d, *J* = 8.0 Hz, 1 H), 7.10-7.20 (m, 2 H), 5.22-5.40 (m, 12 H), 3.55 (m, 2 H), 2.95 (m, 2 H), 2.80-2.95 (m, 8 H), 2.42 (m, 2 H), 2.05 (m, 2 H), 0.97 (t, *J* = 7.2 Hz, 3 H). ^{13}C NMR (100 MHz, $CDCl_3$, 3 overlapping peaks due to the CH group of the omega-3 fatty acid) δ 172.46, 165.75, 159.17, 149.73, 137.01, 132.04, 129.25, 128.56, 128.11, 128.08, 127.87, 127.01, 121.31, 121.06, 38.87, 38.62, 37.23, 36.60, 31.43, 29.38, 25.84, 25.64, 25,54, 23.48, 20.57, 14.3. R_f = 0.50 (EtOAc/Hexane=1:2). MS calcd for $C_{29}H_{40}N_2OS_2$: 496.3; found, 497.5 $[M+H]^+$.

(4Z,7Z,10Z,13Z,16Z,19Z)-N-(2-((1-Amino-2-methylpropan-2-yl)disulfaneyl)ethyl)docosa-4,7,10,13,16,19-hexaenamide (10): 1-Amino-2-methylpropane-2-thiol (1.14 g, 8 mmol) was added dropwise at rt to a solution containing (4Z,7Z,10Z,13Z,16Z,19Z)-N-(2-(pyridin-2-yldisulfanyl)ethyl)docosa-4,7,10,13,16,19-hexaenamide **9** (4 g, 8 mmol) in a 1:1 mixture of MeOH/DMF (10 mL). The resulting reaction mixture was stirred at rt for 18 hr. It was then diluted with water and extracted with EtOAc. The combined organic layers were washed with water (2 ×50 mL), brine (50 mL), dried over Na_2SO_4, and concentrated under reduced pressure. The resulting residue was purified by silica gel chromatography (pentanes/EtOAc) to afford (4Z,7Z,10Z,13Z,16Z,19Z)-N-(2-((1-amino-2-methylpropan-2-yl)disulfanyl)ethyl)docosa-4,7,10,13,16,19-hexaenamide (3.2 g, 81% yield) as a light brown oil. ^{1}H NMR (400 MHz, $CDCl_3$): 6.37 (m, 1 H), 5.25-5.40 (m, 12 H), 4.30-4.50 (m, 2 H), 3.55 (m, 2 H), 2.95 (s, 2 H), 2.75-2.90 (m, 12 H), 2.40 (m, 2 H), 2.25 (m, 2 H), 2.05 (m, 2 H), 1.36 (s, 6 H), 0.97 (t, *J* = 7.2

Hz, 3 H). ^{13}C NMR (100 MHz, $CDCl_3$, 4 overlapping peaks due to CH group of the omega-3 fatty acid): 172.88, 132.05, 129.33, 128.58, 128.30, 128.29, 128.27, 128.21, 128.11, 128.07, 127.87, 127.01, 50.90, 49.23, 38.99, 38.49, 36.35, 29.71, 25.64, 25.54, 25.46, 23.36, 20.58, 14.32. R_f= 0.25 (CH_2Cl_2/MeOH=10:1). MS calcd for $C_{28}H_{46}2_3OS_2$: 490.31; found, 491.30 $[M+H]^+$.

5-((2-((4Z,7Z,10Z,13Z,16Z,19Z)-Docosa-4,7,10,13,16,19-hexaenamido)ethyl)disulfaneyl)-2-nitrobenzoic acid (15): In a typical run, (4Z,7Z,10Z,13Z,16Z,19Z)-N-(2-(pyridin-2-yldisulfanyl)ethyl)docosa-4,7,10,13,16,19-hexaenamide (compound 9, 1.0 g, 2.02 mmol) was taken up in 20 mL of MeOH/DMF (1:1) along with 5,5'-disulfanediylbis(2-nitrobenzoic acid) (Ellman's reagent, 0.8 g, 2.02 mmol). The resulting reaction mixture was stirred at rt for 4 h. Then it was concentrated under reduced pressure and purified by preparative-HPLC using an aqueous mixture of CH_3CN, buffered with 0.1% TFA, to provide 5-((2-((4Z,7Z,10Z,13Z,16Z,19Z)-docosa-4,7,10,13,16,19-hexaenamido)ethyl)disulfanyl)-2-nitrobenzoic acid (0.337 g, 28.6% yield) as a brown oil. This Ellman's adduct was then used to prepare the standard curves for the quantification of the fatty acid cysteamine conjugate **1** in the various mouse, rat and dog PK studies. This Ellman's adduct was stable in the -80 freezer for approximately 2 months. After this period, a fresh batch of the Ellman's adduct would need to be remade. ^{1}H NMR (400 MHz, DMSO-d6): δ 8.02-8.05 (m, 2 H), 7.88-7.91 (m, 2 H), 5.28-5.36 (m, 12 H), 3.28-3.33 (m, 2 H), 2.75-2.90 (m, 12 H), 2.23-2.25 (m, 2 H), 2.00-2.11 (m, 4 H), 0.89-0.93 (t, 3 H). ^{13}C NMR (100 MHz, DMSO-d6, 3 overlapping peaks corresponding to the CH group present in the omega-3 fatty acid portion): δ 174.52, 167.63, 145.78, 145.04, 132.17, 129.87, 129.49, 128.71, 128.52, 128.42, 128.19, 128.07, 127.99, 127.89, 127.13, 126.55, 124.78, 53.62, 38.57, 37.85, 36.27, 25.77, 25.76, 25.75, 25.68, 23.51, 20.70, 14.42. MS (ES^+) calcd for

$C_{31}H_{46}N_2O_5S_2$ 584.24; found 585 $[M+H]^+$.This basic procedure could be repeated to form the corresponding Ellman's adduct for the thiol metabolites shown in Figure 12.

Immunoblotting: Primary CF cells (homozygous F508del-CFTR hBE, source: ChanTest, KKCFFT006F) were prepared and grown on Snapwell filter inserts as previously described. The test compounds, solubilized in FBS according to the procedure outlined above and diluted to the desired concentration, were then added to the individual Snapwell filter inserts in differentiation media at 37 ^oC. Twenty-four hours after the drug addition, cells were snap frozen and later lysed in RIPA buffer. The amounts of proteins were determined by Bio-Rad protein assay. Fifty μg of total cell lysates were analyzed by immunoblotting with anti-Beclin-1, anti-p62 and anti-LC3 antibodies. The immuno-reactivity was normalized with actin as the loading control. Data were presented as Beclin-1/actin, p62/actin and LC3-II/LC3-I ratio compared to vehicle treated samples. Antibodies against LC3A/B antibodies (Cell signaling, 12741), Beclin-1 (Cell Signaling Technology, 3495), p62 (Cell Signaling Technology, 5114) and β-actin (Cell Signaling Technology, 4970) were used as primary antibodies.

Determination of CFTR band B/band C: *Materials and reagents*: 1) Lysis Buffer (Thermo Scienfic Pierce IP Lysis Buffer, PI-87788); 2) Protease Inhibitors: Complete Ultra Tablets, Mini, EDTA-Free (ThermoFisher Scientific 88666); 3) Sample Loading Buffer: NuPAGE LDS Sample Buffer, 4x (Life Technologies NP0007) NuPAGE Reducing Agent, 10x (Life Technologies NP0004); 4) Running Buffer: NuPAGE Tris-Acetate SDS Running Buffer, 20x (ThermoFisher Scientific LA0041); 5) Gel: NuPAGE 3-8% Tris-Acetate Gel, 1.5mm x 15 wells (EA03785BOX); 6) Transfer System: Xcell II Blot Module (ThermoFisher Scientific EI0002); 7) Transfer Buffer: NuPAGE Transfer Buffer, 20x (Life Technologies NP0006-1); 8) Blocking Buffer: Blotting Grade Blocker (Bio-Rad 170-6404, 5% in TBS containing 0.2% Tween-20); 9)

PVDF: Amersham HyBond (GE Healthcare Life Sciences 10600023); 10) Chemiluminescent Detection Reagent: SuperSignal West Pico OR SuperSignal West Femto (ThermoFisher Scientific, 34080 and 34095, respectively).

The test compounds, solubilized in FBS according to the procedure outlined below and diluted to the desired concentration, were added to primary homozygous F508del-CFTR hBE cells, grown on individual Snapwell filter inserts, in differentiation media at 37 ^{o}C. Twenty four hours after the drug addition, cells were lysed on ice, using ice-cooled buffers, as follows: 1) The growth medium was removed and cells were rinsed with 2 × 500µL ice cold DPBS. 2) The second wash was removed and 150 µL of DPBS was added to each well. 3) Cells were gently removed from Snapwell inserts by directly rinsing them with DPBS. 4) Cells were collected in suspension and placed into microcentrifuge tube on ice. 5) An additional 200 µL of ice cold DPBS was added to each well and steps 3-4 were repeated. 6) An additional 700 µL of ice cold DPBS was added to each microcentrifuge tube. 7) Tubes were centrifuged at 1,000 x *g* for 5 minutes at 4 °C. 8) The supernatant was removed as much as possible without disturbing the cell pellet (some mixing of the pellet with mucus may occur). 9) Each cell pellet was gently re-suspended in 30 µL of ice cold lysis buffer with protease inhibitor and incubated on ice for 20 min. 10) Samples were centrifuged at 12,500 x *g* for 10 min at 4 °C to pellet cellular debris. The supernatant was removed to a fresh tube.

For the SDS-PAGE and electrophoretic transfer: Each sample was prepared by adding: a) 15µL of the lysate; b) 5µL 4X LDS Sample Buffer; c) 2.5µL 10x Reducing Agent; d) 2.5µL Milli-Q water (*Note: samples are not to be heated before loading gel*). 2) The gel and running buffer were prepared according to manufacturer's protocol. Gel was run at 150V constant for 1 hr and proteins were transferred to PVDF overnight at 10V constant in Xcell II Blot Module.

For the Western blot, the PVDF membrane was removed from transfer sandwich and placed directly into 5% Blocking buffer. This was allowed to incubate for 1 hr at rt with shaking. The primary antibody was prepared by adding 5μL UNC-596 primary antibody to 10mL Blocking Buffer (per membrane). Membranes were incubated with primary antibody overnight at 4 °C with gentle shaking. Membranes were removed from 4°C and rinsed 3 × 10 minutes in TBST at rt. The secondary antibody was prepared by adding 1 μL goat anti-Mouse IgG-HRP in 10mL Blocking Buffer (per membrane). Secondary antibody was applied to the membrane and incubated for 1 hr at rt with shaking. Membranes were rinsed 3 × 10 minutes in TBST, and 2 × 10 minutes in TBS. SuperSignal West Femto was applied to membranes and then allowed to incubate for 5 minutes in low light. Membranes were exposed to X-ray film at regular intervals for up to 1 hour. Membrane was washed with 3 × 5 minutes to remove substrate. The subsequent primary antibody was prepared by adding 0.5μL Mouse anti-Na/K-ATPase antibody in 10mL blocking buffer. Membranes were incubated with primary antibody for 1 hour at rt with shaking and then washed 3 × 10 minutes in TBST. SuperSignal West Pico was applied to membranes and incubated for 5 min in low light. Membranes were exposed to X-ray film at regular intervals for up to 1 hour.

In order to increase sensitivity at the lower concentrations (e.g. 0.15 μM of conjugate **1**), the following modified protocols were used: Cells were rinsed in DPBS two times, followed by direct lysis in IP Lysis buffer (Thermo) for 20 minutes. Cellular debris was pelleted, and supernatants were used directly in samples without determination of protein concentration. Unheated samples were run on a 3-8% Tris-Acetate gel (Life Technologies) at 150V for 2.5 hours. Separated proteins were transferred to PVDF using the Xcell Blot Module (Life Technologies) for 1 hour at 25V constant. After overnight transfer, blots were blocked in 5%

Blotting Grade Blocker (Bio-Rad) for 1 hour at room temperature (RT) and followed by primary antibody treatment.

Hydrolysis experiment using FAAH-1: FAAH-1-32-579 (the N-term transmembrane region truncated) was cloned into pFastBacI (Invitrogen) with N-terminal FLAG and C-terminal His tags and then expressed in SF9 insect cells. Compound **1** was tested in a hydrolysis experiment using this recombinant FAAH-1 at an enzyme concentration of 1 nM and substrate concentration of 25 μM.[5] For the hydrolysis portion, compound **1** and enzyme were incubated for 2 hours at 37 °C. For the quantification portion, the samples were analyzed on the Agilent 6410 Triple Quad LC/MS mass spectrometer using the appropriate standard curves.

Automated equivalent current (I_{EQ}) assay for compound evaluation on F508del-CFTR chloride channel function in primary cultures of CF hBE cells:

(TECC-24 assay setup # 1, Rosalind Franklin University, primary homozygous F508del-CFTR hBE cells, patient code CFFT0018I or CFFT0028H). Primary CF hBE cells from patients homozygous for F508del-CFTR were grown on Costar 24-well HTS filter plates at an air liquid interface for four to six weeks. Cells were fed three times a week with differentiation media and maintained in an incubator at 37 ^{o}C and 90% humidity. The mucus film was removed three days before the experiment by incubating the apical surface of the cells with 70 μL of differentiation media plus 3 mM DTT for approximately 30 min followed by aspiration of the mucus and media. The apical surface was washed again with 70 μL of PBS for 30 min 24 hrs before the cells were treated with test compounds. For the experiment, the test and control compounds were applied to cells as prepared dilutions of DMSO stock solutions into FBS and differentiation media to the basolateral side only. The fatty acid cysteamine conjugates are highly lipophilic and

need to be solubilized with FBS in order to obtain the optimal effect. This was done by diluting the DMSO stock solution with FBS prior to the serial dilution: 100 μL of a 25 mM DMSO stock solution of the test compound was diluted in 10.0 mL of FBS in a centrifuge tube to prepare an intermediate 250 μM intermediate dilution 10× stock (1% DMSO, 99% FBS). To prepare the 0.3 μM solution of the fatty acid conjugate in 10 mL of differentiation media, 12 μL of the 10× stock solution was added to 9000 μL of differentiation media and 988 μL of 1% DMSO, 99% FBS solution. Other concentrations of the test compound could be prepared by adding the appropriate volume of the 10× stock solution to the differentiation media and 1% DMSO, 99% FBS solution. Both the vehicle and the positive control group were similarly solubilized with FBS and diluted with differentiation media to give a final concentration of 10 % FBS. Cells were incubated with either the control or test compounds for 24 hr. For the chronic ivacaftor pre-incubation protocol, all treatment groups (vehicle, positive control and fatty acid cysteamine conjugate-treated groups) were incubated along with 0.10 μM of ivacaftor for 24 hr. For the electrophysiological measurements, the differentiation media was changed to an experimental media that have been supplemented with either the control or test compounds (in the same concentration used during the 24 hr incubation period). HEPES-buffered Coon's F12 media without serum or bicarbonate was used as the experimental assay solution. The fatty acid cysteamine conjugates were solubilized in this experimental assay solution using the same FBS solubilization protocol described above (substituting the differentiation media with the HEPES-buffered Coon's F12 media without serum and bicarbonate). Cells were incubated for 4 hr in a CO_2 free incubator at 37 ^{o}C. Cells were then placed in pre-warmed heating blocks at 36 ^{o} C ± 0.5 for an additional 15 min before initiating the electrophysiological measurements.

In order to obtain the I_{EQ}, the transepithelial voltage (V_T) and conductance (G_T) were measured under current clamp conditions using a custom designed 24 channel current clamp (TECC-24) and a 24-well electrode manifold (EP Design BVBA, Bertem, Belgium). Measurements were made at approximately 5 min intervals on a robotic work station with custom software. Electrodes were washed after each test plate. To initiate the run, baseline V_T and G_T were measured for approximately 20 min and benzamil (3 μM final concentration) was added to the apical solution as a 25 μL volume. The apical solution was mixed gently by pipetting up and down several times. After an additional 15 min, forskolin (10 μM final concentration) was added to the apical (25 μL) and basolateral (75 μL) solutions. Forskolin, along with the potentiator ivacaftor, will maximally activate any CFTR that is expressed in the apical membrane. For the acute ivacaftor protocol, ivacaftor (0.1 μM) was added 10 min after the addition of forskolin. After an additional 27 min, bumetanide (20 μM final concentration) was added to the basolateral solution (75 μL) to terminate the run. Bumetanide inhibits the basolateral membrane Na-K-2Cl cotransporter and thereby blocks chloride secretion. For the chronic ivacaftor pre-incubation protocol, ivacaftor (0.10 μM) was incubated along with the test article and lumacaftor (3 μM) for 24 hr. With the chronic ivacaftor pre-incubation protocol, only forskolin was needed to initiate the run. For the data analysis portion, the measured V_T values were corrected for the electrode offset potentials and G_T were corrected for the series resistance (R_S) of the solution and blank filter at each time point. These corrected V_T and G_T values were used to calculate the I_{EQ} at each time point by using Ohm's Law where $I_{EQ} = V_T \cdot G_T$. Responses to reagents (benzamil, forskolin and bumetanide) were calculated as the change (Δ) in the I_{EQ} using the I_{EQ} values before and after the reagent addition. In addition, the peak response to forskolin and the area under the curve (AUC) were calculated. The time period included in the AUC calculation spanned the I_{EQ}

value at the time of forskolin addition up to the I_{EQ} value at the time of the bumetanide addition; a duration of 27 min. A one-thirds trapezoid method was used to calculate the AUC. For the acute ivacaftor protocol, cells were incubated with the test compounds with lumacaftor in differentiation media for 24 hr at 37 ^{o}C. The next day, the media was replaced with HEPES-buffered Coon's F12 media, with the test compounds added back. The plate was incubated for 30 min in a CO_2 free incubator before the sequential addition of forskolin and ivacaftor. With this set up, the I_{EQ} was recorded over a 45 min period.

(TECC-24 assay setup # 2, Charles River Laboratories, Cleveland, OH; primary homozygous F508del-CFTR hBE cells, patient code KKCFFT006F, KKCFFT004I or KKCFFT0012I). The chloride transport function of primary homozygous F508-del-CFTR hBE monolayers grown on Corning HTS Transwell-24 filter inserts (permeable support) can be monitored as the CFTR agonist evoked equivalent current (I_{EQ}) calculated from the output of a TECC-24 current clamp system as described above.

Primary homozygous F508del-CFTR hBE cells were prepared and grown on Corning HTS Transwell-24 filter inserts according to established procedures. Primary CF cells were kept in differentiation media. Prior to the assay, the mucus film was removed by adding 100 μL of the blank media to the apical side of the filter inserts. This was allowed to stand for 30-60 min, and then removed prior to the incubation with the test articles. For the assay, the fatty acid cysteamine conjugates were solubilized in FBS prior to the serial dilution in differentiation media in the same manner as described above. With the chronic ivacaftor pre-incubation protocol, cells were incubated in differentiation media for 24 hr in a CO_2 free incubator at 37 ^{o}C with the indicated treatment groups. For the I_{EQ} measurements, the differentiation media was first replaced with HEPES buffered Coon's F12 media (without serum or bicarbonate) with the

test compounds added back to this new media (in the same concentration used during the 24 hr incubation period, and with the prior solubilization in FBS before the serial dilution with Coon's F-12 media). The plate was then allowed to incubate at 37 ^{o}C for 4 hr (CO_2 free incubator) prior to the I_{EQ} measurements at ~35 ^{o}C. To initiate the run, benzamil (10 μM) was added to the apical side of the Transwell-24 filter inserts to block any currents deriving from ENaC. Ten minutes later, Forskolin (10 μM) was added and the I_{EQ} was then recorded over a 60 min period, with measurements made at approximately 5 minute intervals on a robotic workstation using custom software. The antagonist $CFTR_{inh}$-172 (20 μM) was then added to inhibit the CFTR chloride current. With the acute ivacaftor protocol, cells were incubated with the indicated treatment groups in differentiation media for 24 hr in a CO_2 free incubator at 37 ^{o}C. The differentiation media was then first replaced with HEPES buffered Coon's F12 media (without serum or bicarbonate) with the test compounds added back to this new media. The plate was allowed to incubate at 37 ^{o}C for 30 min in a CO_2 free incubator. Forskolin and ivacaftor were added simultaneously; and the I_{EQ} was recorded over a 60 min period. Data analyses were performed using Microsoft Excel software. Comparison of agonist evoked I_{EQ} among both the corrector positive control, negative control and test compound-treated epithelia were obtained with one-way ANOVA followed by Dunnett's multiple comparison test and/or Student's *t*-test when appropriate. Significant correction was defined at the level of $p < 0.05$.

Plasma stability studies: The *in vitro* stability of compound **5** was studied in human, mouse, beagle and rat plasma using detailed protocols have been previously been described.[5]

Rat PK study: The fatty acid cysteamine conjugate **5** was solubilized in a mixture of excipients consisting 40% Tween, 50% Peceol (glyceryl monoleate Type 40), 10% PEG400 and diluted with water to form a self-emulsifying aqueous mixture for oral administration to

animals.[5] For this study, Sprague Dawley rats that had been surgically implanted with indwelling jugular vein cannulae (JVC) were used (Agilux, Worcester, MA). Serial blood collection was carried out at the following time points: 0.125, 0.25, 0.5, 1, 2, 4, 5, 8 and 12 hrs post dose. The bioanalytical portion of the PK study was carried out using an LC/MS/MS system (Agilent Model No: HPLC: 1200, MS: 6410) and analyzed with the appropriate software (WinNonlin Phoenix 64 6.3.0 395). The various thiol metabolites were quantitated by using the corresponding Ellman's adduct to prepare the standard curves.[45]

ASSOCIATED CONTENT

The Supporting Information contains additional functional assay data. This material is available free of charge via the Internet at http://pubs.acs.org.

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NOTES: The authors declare no competing financial interest.

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ABBREVIATIONS

Cystic fibrosis (CF); cystic fibrosis transmembrane conductance regulatory protein (CFTR); epithelial sodium channel (ENaC); fatty acid amide hydrolase (FAAH); human bronchial epithelial (hBE); reactive oxygen species (ROS); self-emulsifying dispersion (SED); transglutaminase 2 (TG2).

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28. We have also evaluated the fatty acid cysteamine conjugate **1** by itself (without the presence of the CFTR corrector lumacaftor) and have observed no significant change in the CFTR chloride current (data not shown).

29. Upon changing the differentiation media to Coon's F12 media, the plate was incubated for 4 hr in a CO_2 free incubator prior to I_{EQ} measurements. With the chronic ivacaftor pre-incubation protocol, we have found that the longer 4 hr incubation period in Coon's F12 media was helpful since it allowed primary hBE cells to process and hydrolyze the lipophilic fatty acid conjugate **1** more completely. Without this longer incubation time,

some of the precipitated, oily fatty acid conjugates could interfere with the current recording; the results became more variable and harder to interpret.

30. Additional TECC-24 assay data for this run, as well as for other runs, are provided in the Supporting Information.

31. In this TECC-24 assay setup using cells from the same donor (KKCFFT006F), we have also evaluated **1** at concentrations as low as 0.075 μM and still observed a statistically significant increase in the AUC. This data set is shown in the Supporting Information.

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of these 2 metabolites were detectable in the peripheral plasma. Niacin, nicotinuric acid **14** and the thiol-nicotinamide metabolite **11** could not be detected in the peripheral plasma. A significant first pass metabolism of **11** had presumably taken place since a significant quantity of it was detected in the portal circulation (metabolite **11**; portal C_{max} = 3129 ± 564 ng/mL, portal AUC_{last} = 5888 ± 1297 Hr*ng/mL).

35. Compound **5** has also been dosed orally (100 mg/kg BID) in a mouse PK/tissue distribution study over a 3.5 day period in order to assess autophagy activation in the various tissues. Both the parent compound and the biologically active metabolite **1** were detected in plasma and lung tissues. Compound **5** has also been administered the Beagle dog orally with multiple doses over a 14 day period. The results from these studies will be discussed in more detail in a separate communication.

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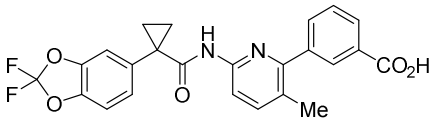
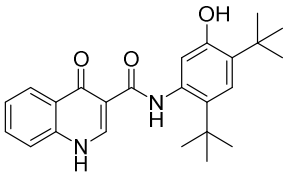
FIGURES AND SCHEMES.



Lumacaftor (VX-809)



Ivacaftor (VX-770)

Figure 1. Representative CFTR modulators

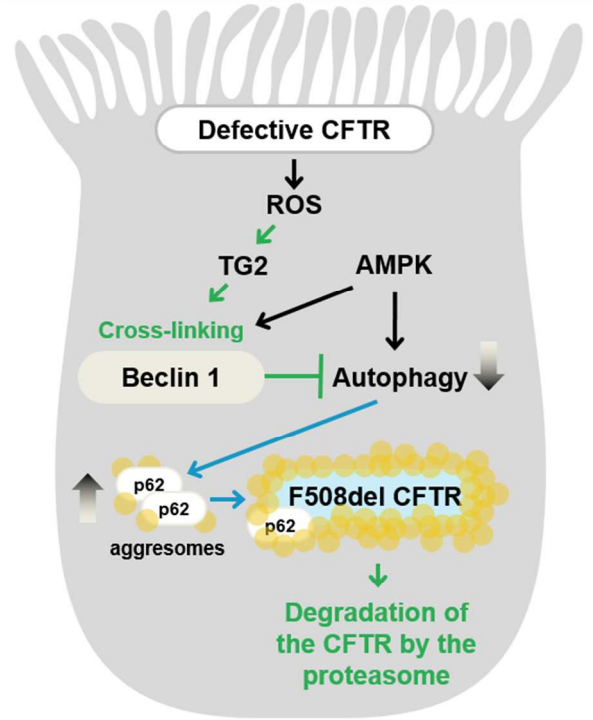


Figure 2. In CF, the defective CFTR induces an upregulation of TG2, which causes the cross-linking of Beclin-1 and the subsequent impairment of autophagy. This results in an increase in the p62 aggresomes, which can further trap the F508del-CFTR for the eventual degradation by the proteasome. A successful activation of autophagy would reverse these steps to allow more of the F508del-CFTR to reach the cell membrane. The fatty acid cysteamine conjugate **1** allows the simultaneous inhibition of TG2 and upregulation of AMPK to take place in order to activate autophagy more effectively.

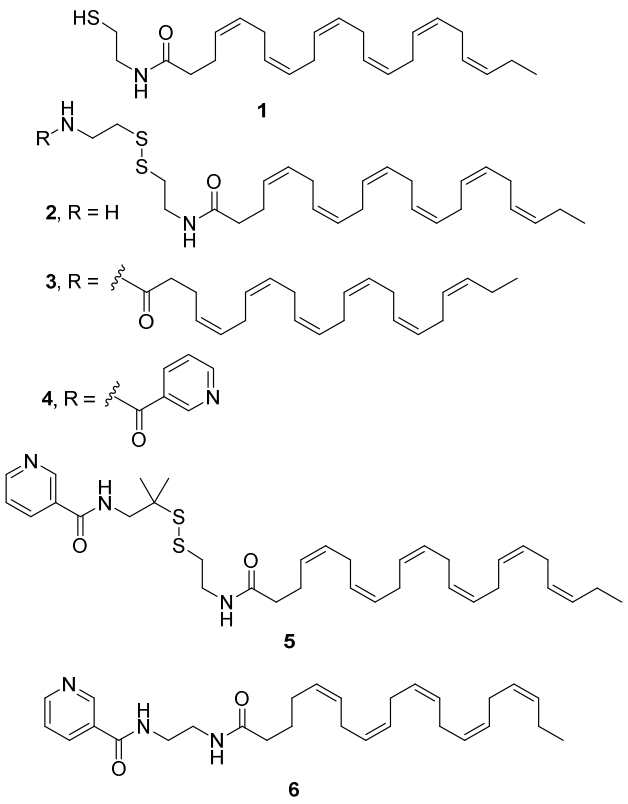


Figure 3. List of covalent fatty acid conjugates.

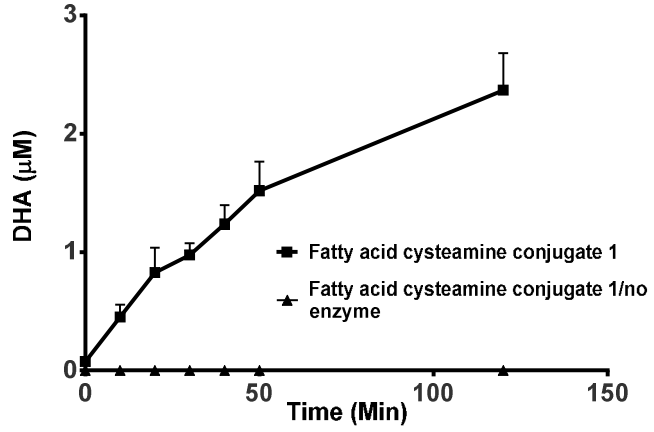


Figure 4. Hydrolysis of the fatty acid cysteamine conjugate **1** using purified recombinant FAAH-1. Measurements of DHA that was released upon hydrolysis of **1** using recombinant FAAH-1. Measurements were taken in the presence or absence of the recombinant enzyme FAAH-1.

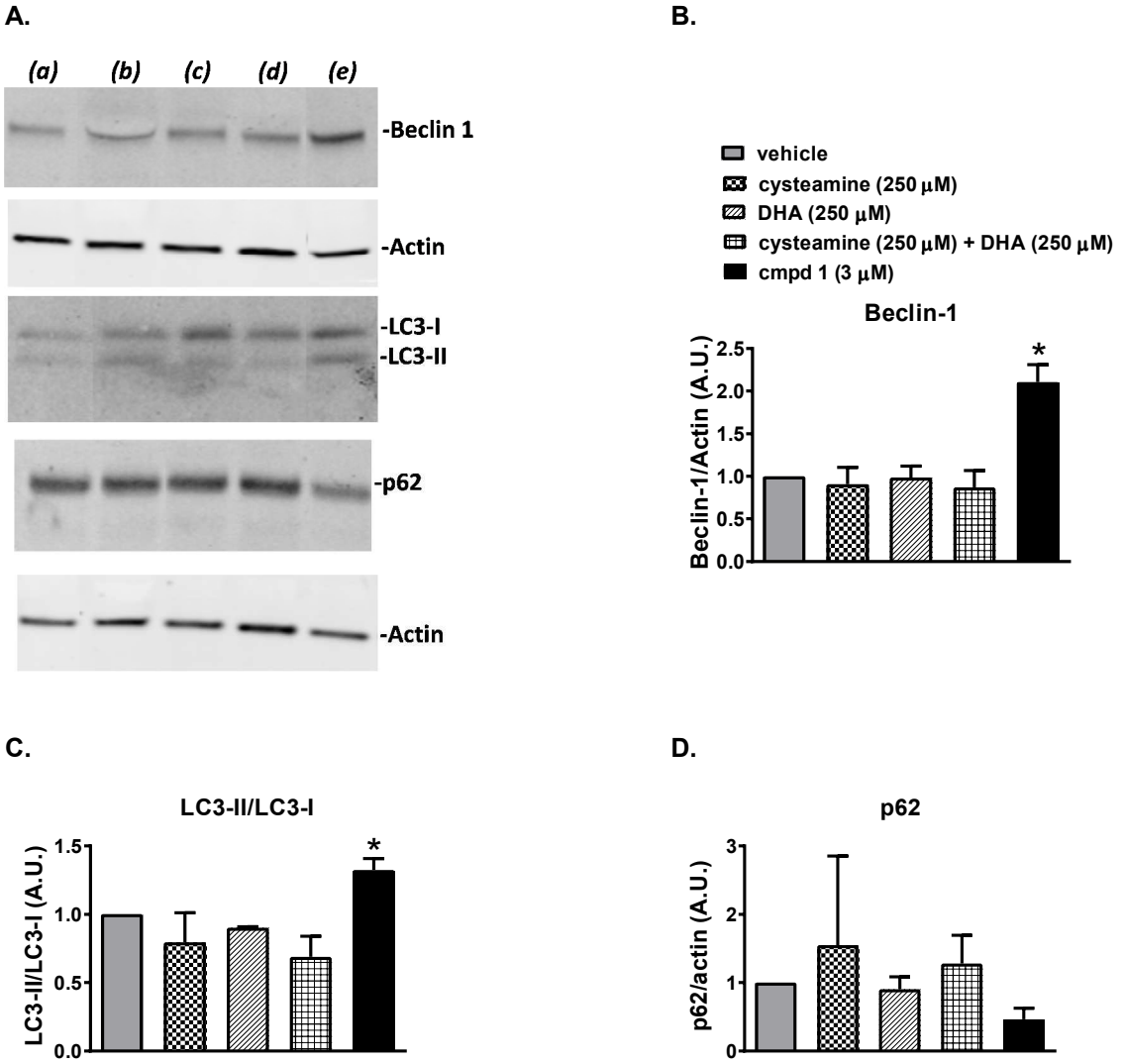


Figure 5. (A) Representative immunoblots of primary homozygous F508del-CFTR hBE cells (patient code KKCFFT004I) that were treated for 24 hrs at 37 ^{o}C with: *(lane a)* vehicle; *(lane b)* cysteamine (250 μM); *(lane c)* DHA (250 μM); *(lane d)* combination of cysteamine (250 μM) + DHA (250 μM); *(lane e)* fatty acid cysteamine conjugate **1** (3 μM). (B) Quantification of the immunoblots shown in (A) for Beclin-1 levels. (C) Quantification of the immunoblots shown in (A) for the ratio of LC3-II to LC3-I. (D) Quantification of the immunoblots shown in (A) for the p62 levels. Error bars represent standard error mean (SEM, n = 3). *p < 0.05, compared to the vehicle with ANOVA followed by Dunnett's multiple comparison test.

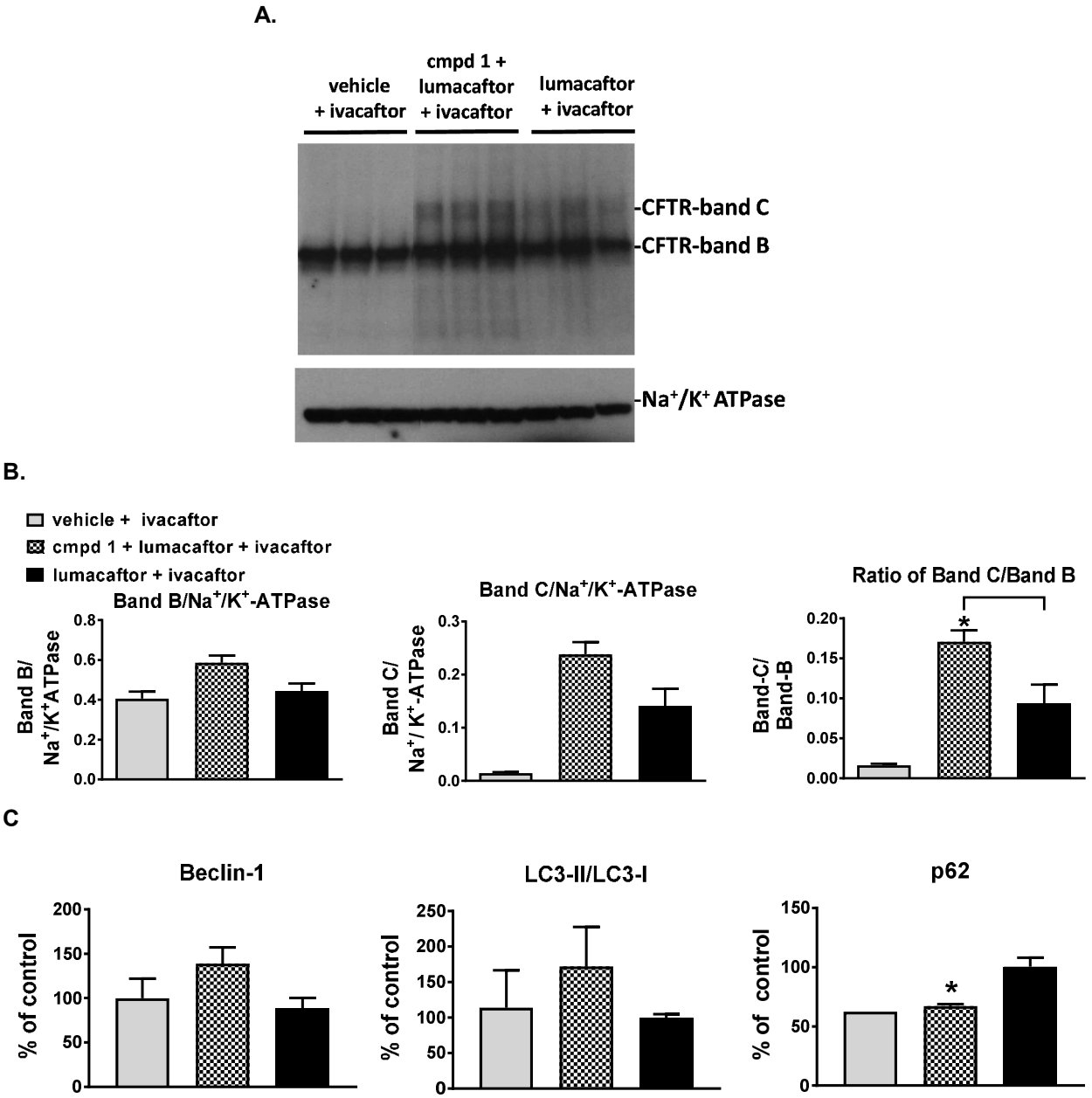


Figure 6. Determination of CFTR Band B and Band C. (A) Immunoblots of primary homozygous F508del-CFTR hBE cells (patient code KKCFFT006F) when treated for 24 hrs with: 1) vehicle + ivacaftor (0.10 μM); 2) lumacaftor (3 μM) + ivacaftor (0.10 μM); 3) **1** (3 μM) + lumacaftor (3 μM) + ivacaftor (0.10 μM). (B) Quantification of the immunoblots shown in (A) for the corresponding CFTR Band B, the CFTR Band C, and the ratio of Band C to Band B. (C) Quantification of the corresponding immunoblots for Beclin-1, the ratio of LC3-II/LC3-I and p62, expressed as a % of the lumacaftor/ivacaftor control group. Error bars represent standard deviation (SD). * $p < 0.05$, SD, n = 3. Statistically significant relative to the lumacaftor/ivacaftor treatment group with ANOVA followed by Dunnett's multiple comparison test.

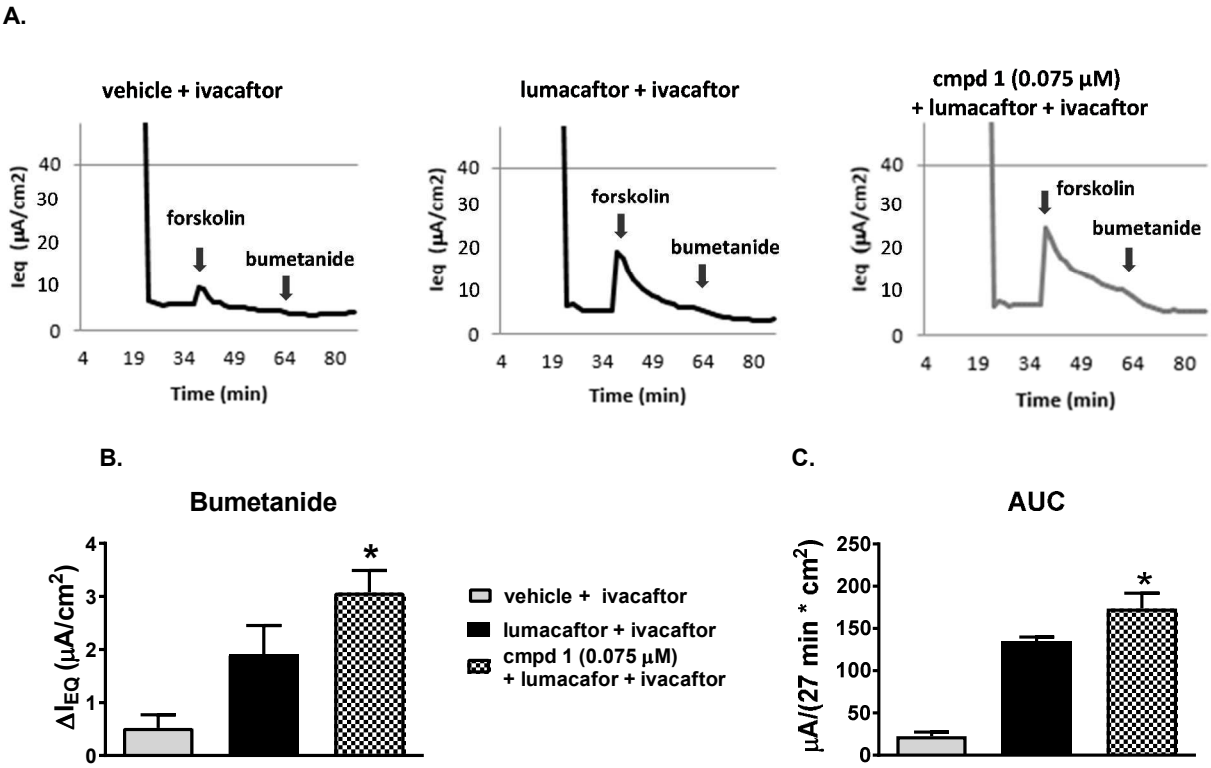


Figure 7. *(TECC-24 assay, Rosalind Franklin University, patient code CFFT018I)*: Chronic ivacaftor pre-incubation protocol. Primary homozygous F508del-CFTR hBE cells were incubated in differentiation media at 37 ^{o}C for 24 hr with: 1) vehicle + ivacaftor (0.10 µM); 2) lumacaftor (3 µM) + ivacaftor (0.10 µM); 3) Cmpd **1** (0.075 µM) + lumacaftor (3 µM) + ivacaftor (0.10 µM). The media was then switched to Coon's F12, with test compounds added back. Plate was incubated at 37 ^{o}C for 4 hr prior to I_{EQ} measurements. (A) Representative I_{EQ} traces following the addition of benzamil (3 µM) to first block currents deriving from ENaC. Forskolin (10 µM) was added and the I_{EQ} was recorded over a 27 min period at 37 ^{o}C. Bumetanide (20 µM) was added to block the chloride secretion from the CFTR. (B) Quantification of the bumetanide-inhibited CFTR chloride current for the three indicated treatment groups, ΔI_{EQ} (µA/cm^2). (C) Quantification of the AUC (computed for the time period that spans the I_{EQ} value at the time of forskolin addition up to the I_{EQ} value at the time of bumetanide addition). * $p < 0.05$, SEM, n = 3. Statistically significant relative to the lumacaftor/ivacaftor treatment group with ANOVA followed by Dunnett's multiple comparison test.

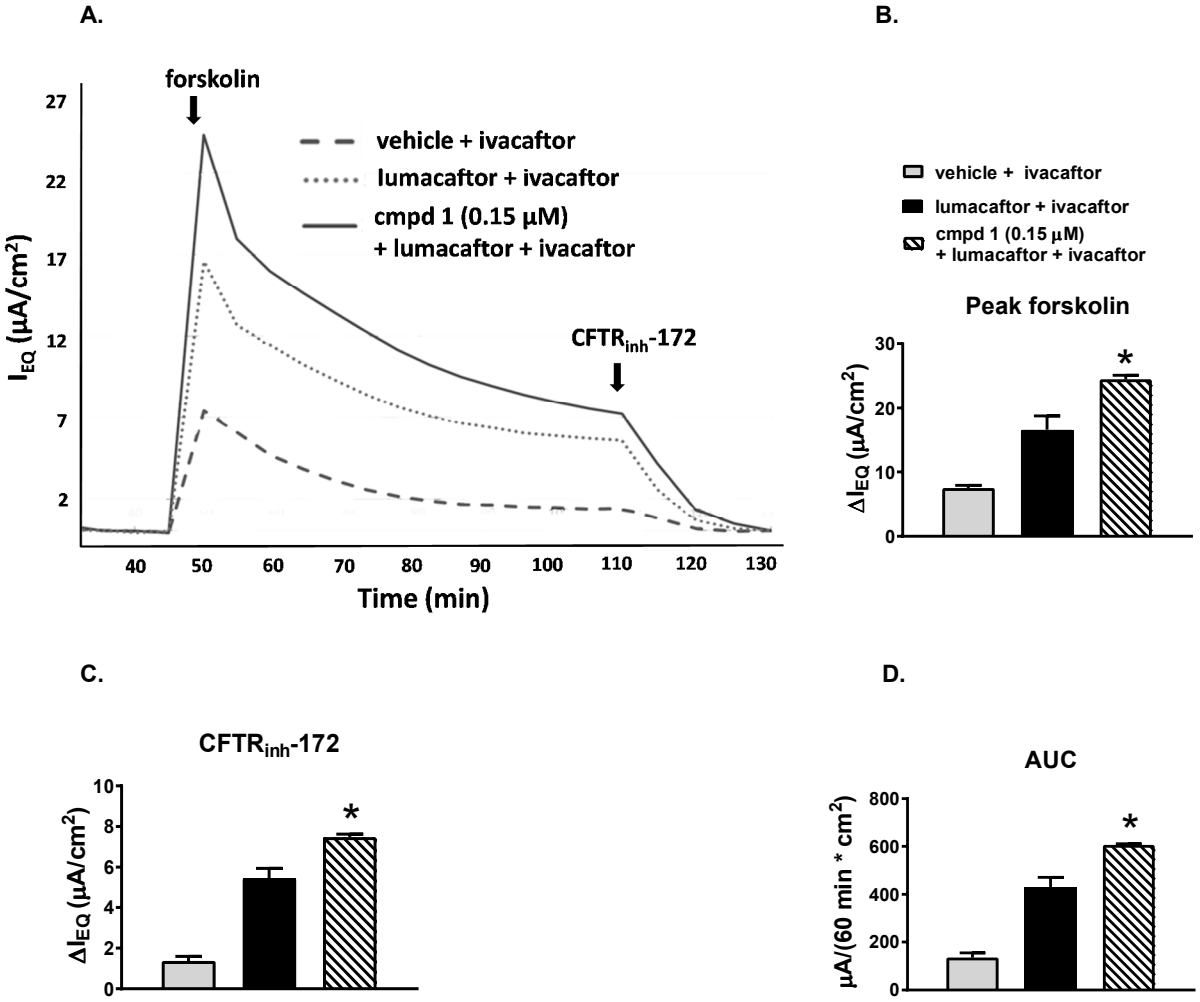
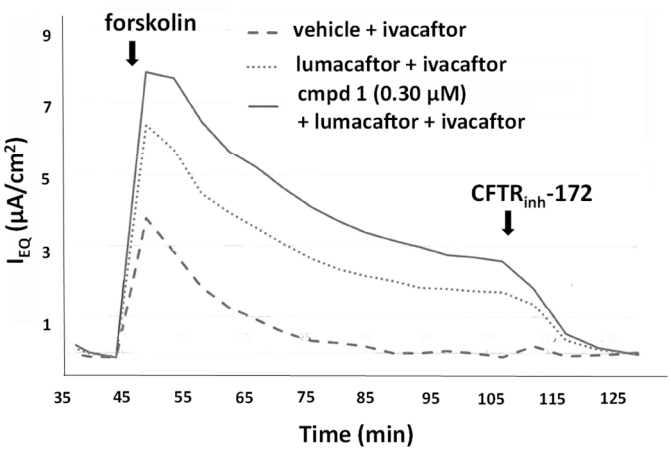


Figure 8. *(TECC-24 assay, Charles River Labs, patient code KKCFFT006F):* Chronic ivacaftor pre-incubation protocol. Primary homozygous F508del-CFTR hBE cells were incubated in differentiation media at 37 ^{o}C for 24 hr with: 1) vehicle + ivacaftor (0.10 µM); 2) lumacaftor (3 µM) + ivacaftor (0.10 µM); 3) cmpd **1** (0.15 µM) + lumacaftor (3 µM) + ivacaftor (0.10 µM). The media was then switched to Coon's F12, with the test compounds added back. Plate was incubated at 37 ^{o}C for 4 hr prior to I_{EQ} measurements. (A) Average I_{EQ} traces for the 3 treatment groups following the addition of benzamil (10 µM) to first block currents deriving from ENaC. Forskolin (10 µM) was then added, and the I_{EQ} was recorded over a 60 min period at 37 ^{o}C. $CFTR_{inh}$-172 (20 µM) was added to block the chloride secretion from the CFTR. (B) Quantification of the peak forskolin-activated chloride current, ΔI_{EQ} (µA/cm^2). (C) Quantification of the $CFTR_{inh}$-172 inhibited chloride current, ΔI_{EQ} (µA/cm^2). (D) Quantification of the AUC (computed for the time period that spans the I_{EQ} value at the time of forskolin addition up to the I_{EQ} value at the time of $CFTR_{inh}$-172 addition). * $p <$ 0.05, SEM, n = 4. Statistically significant relative to the lumacaftor/ivacaftor treatment group with ANOVA followed by Dunnett's multiple comparison test.

A.



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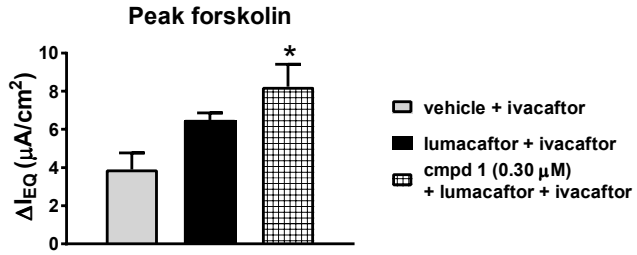
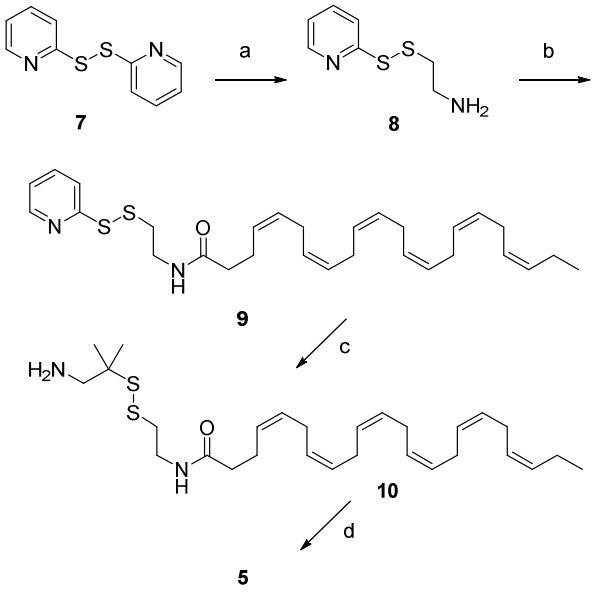


Figure 9. *(TECC-24 assay, Charles River Labs, patient code KKCFFT004I):* Chronic ivacaftor pre-incubation protocol. Primary homozygous F508del-CFTR hBE cells were incubated in differentiation media at 37 ^{o}C for 24 hr with: 1) vehicle + ivacaftor (0.10 µM); 2) lumacaftor (3 µM) + ivacaftor (0.10 µM); 3) cmpd **1** (0.30 µM) + lumacaftor (3 µM) + ivacaftor (0.10 µM). Same protocols as detailed in Figure 8. (A) Average I_{EQ} traces for the 3 treatment groups. (B) Quantification of the peak forskolin-activated chloride current, ΔI_{EQ} (µA/cm^2). * $p < 0.05$, SEM, n = 6. Statistically significant relative to the lumacaftor/ivacaftor treatment group with ANOVA followed by Dunnett's multiple comparison test.



Scheme 1. *Reagents and conditions:* (a) cysteamine, MeOH, rt; (b) DHA, HATU, Et_3N, CH_2Cl_2, rt; (c) 1-amino-2-methylpropane-2-thiol, MeOH/DMF (1:1), rt; (d) nicotinoyl chloride, Et_3N, CH_2Cl_2, 0 ^oC.

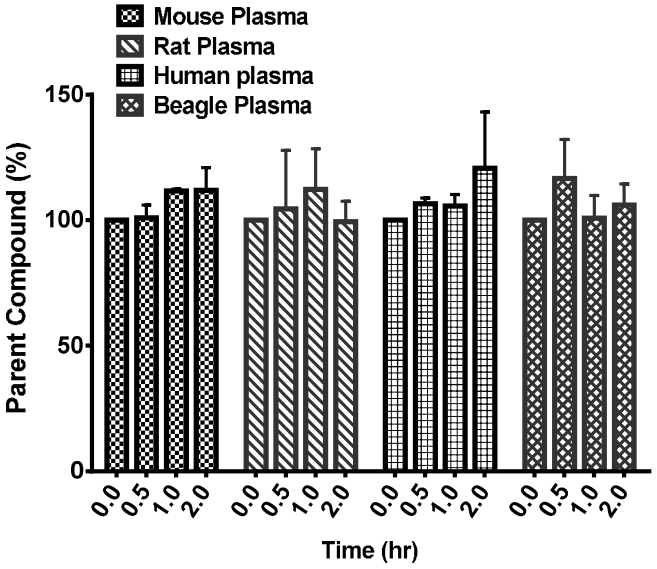


Figure 10. Plasma stability for the fatty acid cysteamine conjugate **5**.

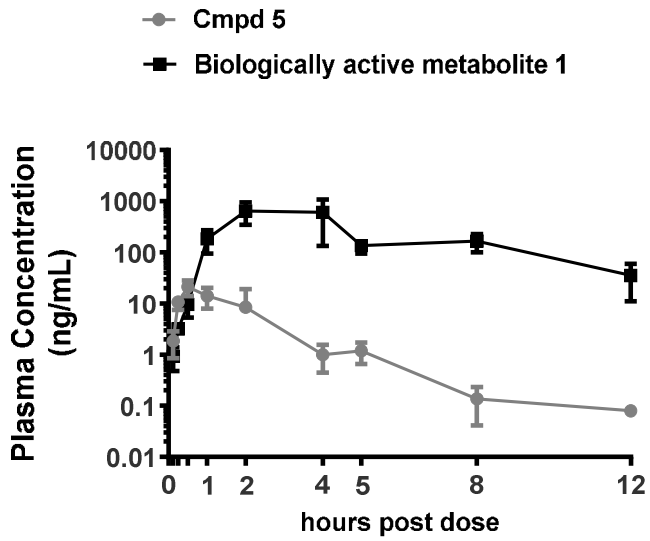


Figure 11. Plasma concentrations of the parent compound **5** and the biologically active metabolite **1**, upon oral dosing to Sprague Dawley rats (30 mg/kg).

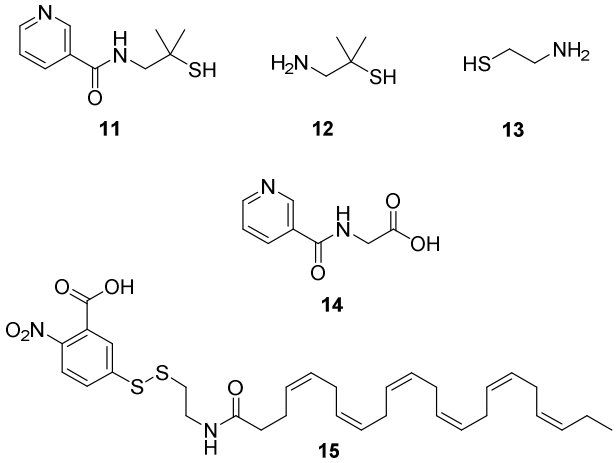


Figure 12. Potential metabolites obtained from oral dosing with **5** to Sprague Dawley rats (compound **15** represents the corresponding Ellman's adduct of conjugate **1**).

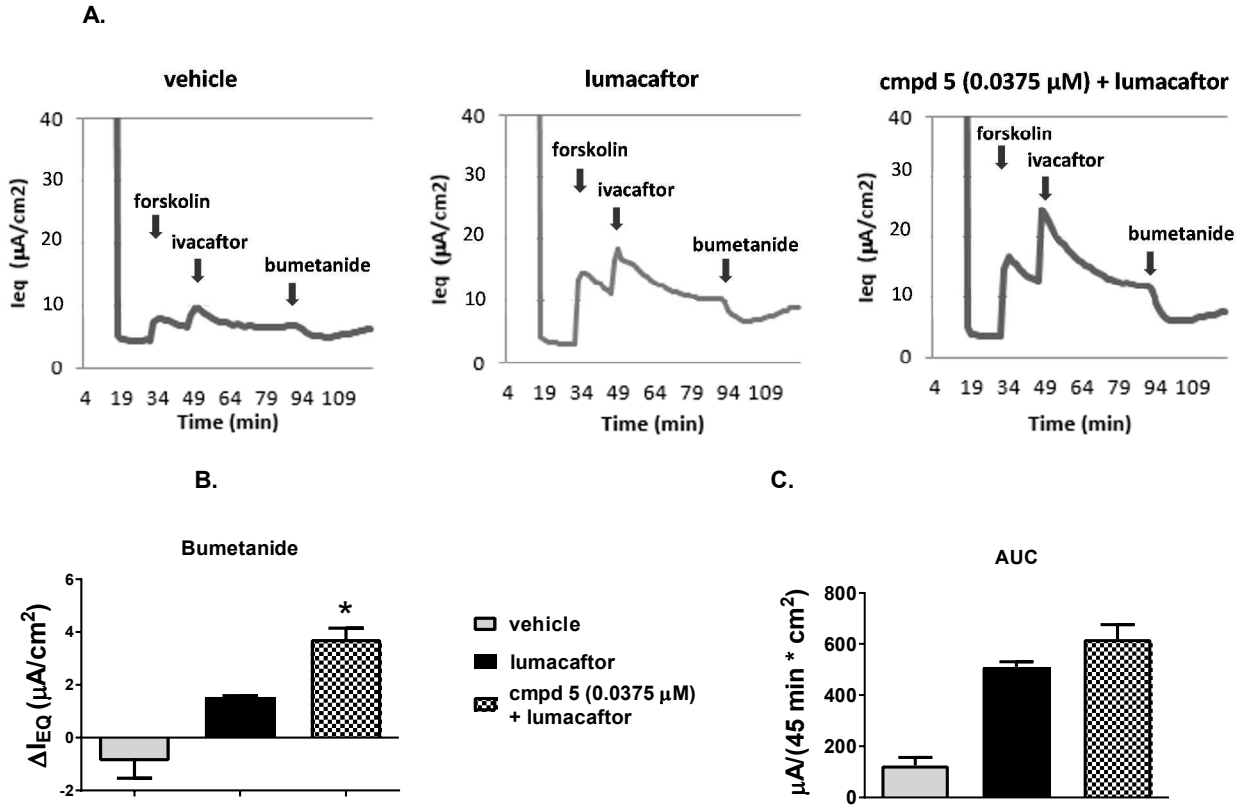


Figure 13. *(TECC-24 assay, Rosalind Franklin University, patient code CFFT028H)*: Acute ivacaftor protocol. Primary homozygous F508del-CFTR hBE cells were incubated in differentiation media at 37 ^{o}C for 24 hr with:1) vehicle; 2) lumacaftor (3 µM); 3) Cmpd **5** (0.0375 µM) + lumacaftor (3 µM). The media was then switched to Coon's F12, with test compounds added back. Plate was incubated at 37 ^{o}C for 30 min prior to I_{EQ} measurements. (A) Representative I_{EQ} traces following the addition of benzamil (3 µM) to first block the epithelial Na Channel. Forskolin (10 µM) was then added, followed by ivacaftor (0.10 µM). I_{EQ} was recorded over a 45 min period at 37 ^{o}C. Bumetanide (20 µM) was added to block the chloride secretion from the CFTR. (B) Quantification of the bumetanide-inhibited CFTR chloride current, ΔI_{EQ} (µA/cm^2). (C) Quantification of the AUC (computed for the time period that spans the I_{EQ} value at the time of forskolin addition up to the I_{EQ} value at the time of bumetanide addition). * $p < 0.05$, SEM, n = 3. Statistically significant relative to the lumacaftor/ivacaftor treatment group with ANOVA followed by Dunnett's multiple comparison test.

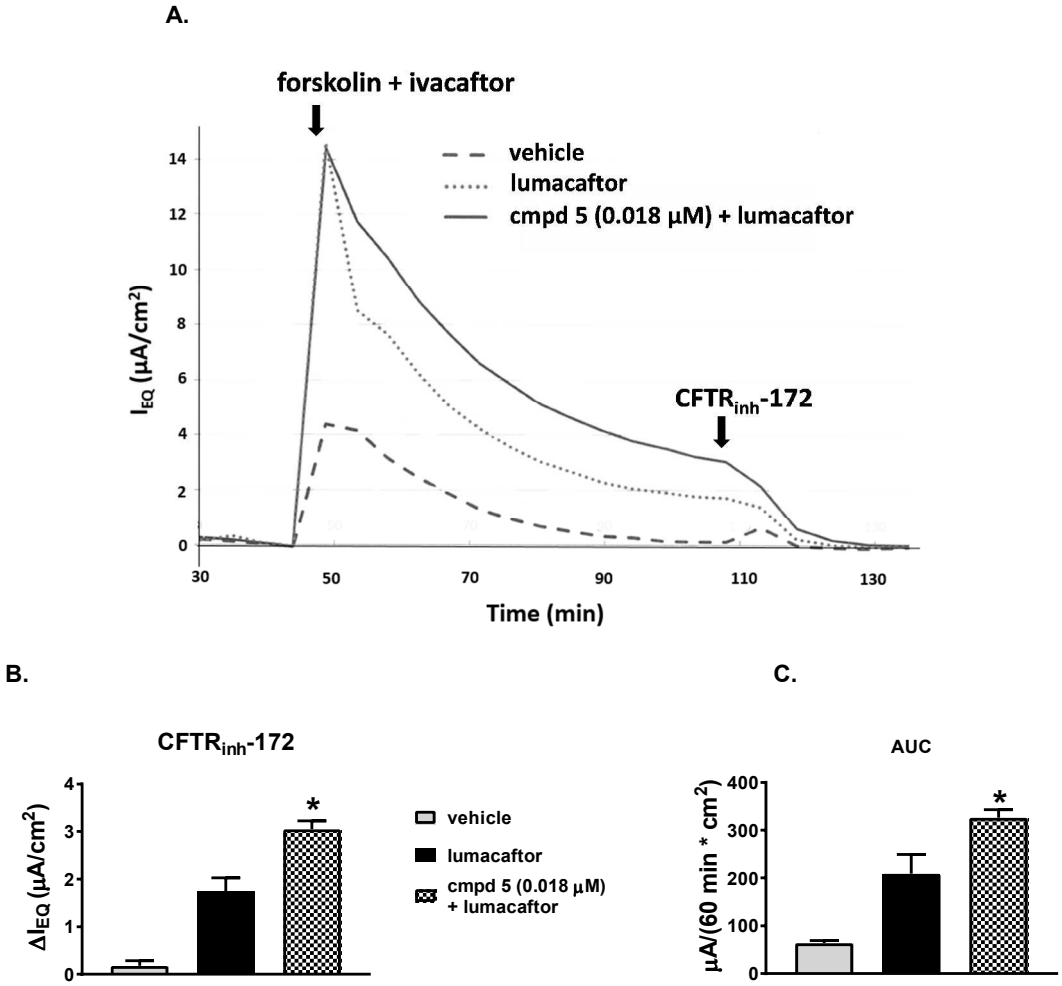
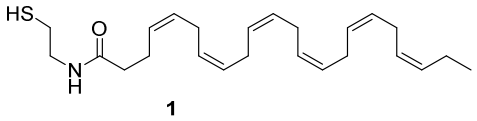


Figure 14. *(TECC-24 assay, Charles River Labs, patient code KKCFFT0012I):* Acute ivacaftor protocol. Primary homozygous F508del-CFTR hBE cells (n = 4) were incubated in differentiation media at 37 ^{o}C for 24 hr with: 1) vehicle; 2) lumacaftor (3 µM); 3) cmpd **5** (0.018 µM) + lumacaftor (3 µM). The media was then switched to Coon's F12, with the test compounds added back. Plate was incubated at 37 ^{o}C for 30 min prior to I_{EQ} measurements. (A) Average I_{EQ} traces for the indicated treatment groups following the addition of benzamil (10 µM) to first block currents deriving from ENaC. Forskolin (10 µM) and ivacaftor (0.10 µM) were then added, and the I_{EQ} was recorded over a 60 min period at 37 ^{o}C. $CFTR_{inh}$-172 (20 µM) was added to block the chloride secretion from the CFTR. (B) Quantification of the $CFTR_{inh}$-172 inhibited chloride current, ΔI_{EQ} (µA/cm^2). (C) Quantification of the AUC (computed for the time period that spans the I_{EQ} value at the time of forskolin addition up to the I_{EQ} value at the time of $CFTR_{inh}$-172 addition). * $p < 0.05$, SEM, n = 4. Statistically significant relative to the lumacaftor treatment group with ANOVA followed by Dunnett's multiple comparison test.

Table of Contents Graphic



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