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

Chi B. Vu, Robert J. Bridges, Cecilia Pena-Rasgado, Antonio E. Lacerda, Curtis Bordwell, Abby Sewell, Andrew J. Nichols, Sachin Chandran, Pallavi Lonkar, Dominic Picarella, Amal Ting, Allison Wensley, Maisy Yeager, and Feng Liu

†Catabasis Pharmaceuticals, One Kendall Square, Suite B14202, Cambridge, Massachusetts 02139, United States
‡Chicago Medical School, Rosalind Franklin University of Medicine and Science, 3333 Green Bay Road, North Chicago, Illinois 60064, United States
§Charles River Laboratories, 14656 Neo Parkway, Cleveland, Ohio 44128, United States

Supporting Information

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, involves the modulation of multiple therapeutic pathways in order to achieve a desired pharmacological response. We have recently described a variation of this technique that allowed network pharmacology to take place when two bioactives are delivered intracellularly at the same time and in equimolar concentrations. 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, 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), 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) and the potentiator ivacaftor (VX-770) 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. Therefore, numerous efforts are currently being taken to identify more effective CFTR correctors or potentiators, as well as agents that can potentially improve the efficacy of the current combination of lumacaftor and ivacaftor. 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. 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. 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 cross-linking 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 overexpression 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. 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 nondruglike properties, which precluded their evaluation in the clinic. 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. Administration of cysteamine to CFTRF508del 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. Mairui 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. Cysteamine has since been evaluated in a pilot clinical trial involving homozygous F508del-CFTR CF patients, in combination with epigallocatechin gallate. 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 ω-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. 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 ω-3 fatty acid, by itself, has previously been shown to increase autophagosome formation via the AMPK pathway. In order to achieve a simultaneous intracellular delivery, we employed the same methodology that we described earlier 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. 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. 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. 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 ω-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 h with 1 (3 μM) along with lumacaftor (3 μM) and ivacaftor (0.10 μM) in differentiation media. 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 Figure 6A and Figure 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 (these data are shown in the Supporting Information). Maiuri and co-workers 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 semiautomated 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; the other involves the ability to measure the I_EQ with the test compounds continuously present in the recording medium.
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 preincubation protocol. In the acute ivacaftor protocol, cultured primary homozygous F508del-CFTR hBE cells were incubated with the fatty acid cysteamine conjugate along with the CFTR corrector lumacaftor for 24 h. 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. In the chronic ivacaftor preincubation protocol, cultured primary homozygous F508del-CFTR hBE cells were incubated with the fatty acid cysteamine conjugate along with both the corrector lumacaftor and the potentiator ivacaftor for 24 h prior to the $I_{EQ}$ measurements. This protocol was introduced more recently based on the findings from Cholon and Veit that the potentiator ivacaftor actually had a destabilizing effect on the corrected CFTR upon chronic preincubation 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 preincubation protocol might be more representative of what cells would be exposed to at a given time period. We have assessed the functional activity of using this chronic ivacaftor preincubation protocol and have found a significant additive effect on top of lumacaftor/ivacaftor. Figure 7 summarizes an experiment using the chronic ivacaftor preincubation protocol. Here, primary homozygous F508del-CFTR hBE cells (patient code KC0018I) were incubated in differentiation medium for 24 h at 37 °C with the following treatment groups: (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): ($\ast$) $p < 0.05$, SD, $n = 3$, statistically significant relative to the lumacaftor/ivacaftor treatment group with ANOVA followed by Dunnett’s multiple comparison test.
The next day, the differentiation medium was replaced with Coon’s F12 (without serum or bicarbonate) medium; with the same concentrations of the test compounds added back to this new medium. To initiate the run, benzamil was added first to the apical side to block currents through the epithelial sodium channel (ENaC). Forskolin (10 μM) was added, and the I_{EQ} was recorded over a 27 min period at 37 °C. Bumetanide (20 μM) was added to block the chloride secretion from the CFTR. The resultingbumetanide-inhibited CFTR chloride current (Δbumetanide, μA/cm²) is a reflection of the functional activity of the now corrected F508del-CFTR. As shown in Figure 7A and Figure 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/cm²) over the lumacaftor/ivacaftor treatment group (Δbumetanide = 1.91 ± 0.22 μA/cm²). 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 2-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/cm²). 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·cm²) vs lumacaftor/ivacaftor control group AUC = 134.2 ± 5.6 μA/(27 min·cm²)); vehicle group AUC = 22.3 ± 4.9 μA/(27 min·cm²)). With this particular assay setup, the solubility of 1 in the Coon’s F12 medium 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.

**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. By use of the chronic ivacaftor preincubation 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

μM). In this assay format using primary cells from this

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in the antagonist that is used to block the chloride secretion from the CFTR. With this setup, CFTRinh-172 was used instead of bumetanide, and the antagonist-inhibited CFTR chloride current, $\Delta I_{EQ}$ (CFTRinh-172), represents essentially the same functional readout as the $\Delta$bumetanide described in Figure 7. With this particular TECC-24 setup, 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 $\Delta I_{EQ}$ for the triple combination was 24.47 ± 0.62 $\mu$A/cm$^2$. This increase in the peak forskolin response was statistically significant over the lumacaftor/ivacaftor treatment group ($\Delta I_{EQ} = 16.61 \pm 2.16 \mu$A/cm$^2$). When expressed as a % of the lumacaftor/ivacaftor control group, the appropriate subtraction of the vehicle group $\Delta I_{EQ} = 1.37 \pm 0.23 \mu$A/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 $\mu$A/(60 min·cm$^2$). Even though the solubility of I was still limited in this TECC-24 assay setup, it did not appear to interfere with the $I_{EQ}$ measurements as much as in the previous setup. We have evaluated I in concentrations of up to 0.6 $\mu$M 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 $\mu$M of conjugate I). In this particular assay setup, at concentrations of ≥1.2 $\mu$M I, solubility became an issue in the Coon’s F12 medium, 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 I was evaluated in the same

Figure 8. TECC-24 assay, Charles River Laboratories, patient code KKCFF006F: chronic ivacaftor preincubation protocol. Primary homozygous F508del-CFTR hBE cells were incubated in differentiation medium at 37 °C for 24 h with the following: (1) vehicle + ivacaftor (0.10 $\mu$M); (2) lumacaftor (3 $\mu$M) + ivacaftor (0.10 $\mu$M); (3) compound 1 (0.15 $\mu$M) + lumacaftor (3 $\mu$M) + ivacaftor (0.10 $\mu$M). The medium was then switched to Coon’s F12, with the test compounds added back. Plate was incubated at 37 °C for 4 h prior to $I_{EQ}$ measurements. (A) Average $I_{EQ}$ traces for the three treatment groups following the addition of benzenalm (10 $\mu$M) to first block currents deriving from ENaC. Forskolin (10 $\mu$M) was then added, and the $I_{EQ}$ was recorded over a 60 min period at 37 °C. CFTRinh-172 (20 $\mu$M) was added to block the chloride secretion from the CFTR. (B) Quantification of the peak forskolin-activated chloride current, $\Delta I_{EQ}$ ($\mu$A/cm$^2$). (C) Quantification of the CFTRinh-172 inhibited chloride current, $\Delta I_{EQ}$ ($\mu$A/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 CFTRinh-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.

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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 KKCFFT004H) were treated with the following groups for 24 h at 37 °C prior to $I_{FE}$ measurements: (1) vehicle + ivacaftor (0.10 μM); (2) lumacaftor (3 μM) + ivacaftor (0.10 μM); (3) compound 1 (0.30 μM) + lumacaftor (3 μM) + ivacaftor (0.10 μM). Under this chronic ivacaftor preincubation 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 $\Delta I_{FE}$ (peak forskolin) for the triple combination still represents a 167% increase over the lumacaftor/ivacaftor treatment group (triple combination, $\Delta I_{FE} = 8.24 \pm 1.18 \mu A/cm^2$ vs lumacaftor/ivacaftor control group $\Delta I_{FE} = 6.250 \pm 0.36 \mu A/cm^2$; vehicle group, $\Delta I_{FE} = 3.90 \pm 0.88 \mu A/cm^2$). With primary cells from this particular donor, we began to see an inhibition in the chloride current at concentrations of $\geq 0.60 \mu M$ (additional data are 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 $\geq 1.2 \mu M$. 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 the following: (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 medium was less of a factor. Thus, far, because of the low solubility of 1 in the Coon’s F12 medium 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 organeloids. 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 sulphydryl 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 linker 4. 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. On the basis of our earlier work with fatty acid niacin conjugates, 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. 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, 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 of >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 h. 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 ($C_{\text{max}} = 24.6 \pm 4.39$ ng/mL; $\text{AUC}_{\text{last}} = 37.7 \pm 16.2$ h·ng/mL). There was a significantly higher and more sustained level of the biologically active metabolite 1 ($C_{\text{max}} = 777 \pm 435$ ng/mL, $\text{AUC}_{\text{last}} = 2929 \pm 1282$ h·ng/mL), and this was detected in the plasma even at the 8 and 12 h time point. This plasma level would support the feasibility of either a b.i.d. or q.d. dosing and is sufficient to support the functional activity displayed by 1 in the various assays discussed earlier.

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 preincubation 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 2-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, $\Delta_{\text{bumetanide}} = 3.73 \pm 0.41$ μA/cm² vs lumacaftor/ivacaftor control, $\Delta_{\text{bumetanide}} = 1.54 \pm 0.04$ μA/cm²). 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<sub>inh</sub>-I<sub>72</sub>-inhibited CFTR chloride current (triple combination, \( \Delta I_{EQ} = 3.05 \pm 0.18 \mu A/cm^2 \)) vs lumacaftor/ivacaftor control, \( \Delta I_{EQ} = 1.74 \pm 0.29 \mu A/cm^2 \); vehicle group, \( \Delta I_{EQ} = 0.18 \pm 0.11 \mu A/cm^2 \) and the AUC (triple combination, AUC = 326.36 ± 17.16 \( \mu A/(60 \text{ min} \cdot \text{cm}^2) \)) vs lumacaftor/ivacaftor control, AUC = 209.24 ± 40.42 \( \mu A/(60 \text{ min} \cdot \text{cm}^2) \); vehicle group, AUC = 63.83 ± 5.44 \( \mu A/(60 \text{ min} \cdot \text{cm}^2) \)). When expressed as a % of the lumacaftor/ivacaftor control group, both of these responses showed a \( \geq 180\% \) increase for the triple combination, a nearly 2-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 preincubation 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. However, the reagent employed in their studies, cysteamine, was a weak autophagy activator, and the effect was observed only at 250 \( \mu M \) 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 2-fold in either the peak forskolin response or 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 \( \mu M \)). 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 antibacterial 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 that 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 cells42 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. 1H NMR (400 MHz) and 13C NMR (100 MHz) spectra...
were recorded on a Varian 400 MHz Unity Inova system in CDCl₃ 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 (doublet), t (triplet), q (quartet), m (multiplet), br (broad). High resolution mass spectroscopy data were analyzed by direct flow injection, utilizing electrospray ionization (ESI) on a Waters QqToF 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 quadrupole LC–MS detector in positive mode. A SiliCycle C18 XDB, 3 mm × 100 mm column was used with a gradient of H₂O and acetonitrile each with 0.1% formic acid; UV detection was at 254 and 210 nm. Normal phase flash chromatography was accomplished on Teledyne Isco systems using prepacked 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-hexaenamidoethyl]disulfanyl-2-methylpropyl nicotinamide (5).** (4Z,7Z,10Z,13Z,16Z,19Z)-N-[(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 mol) were taken up in CH₂Cl₂ (30 mL). Triethylamine (4.5 mL, 31.5 mmol) was added dropwise at 0°C. The resulting mixture was stirred at rt for 18 h. It was then diluted with water and extracted with CH₂Cl₂. The combined organic layers were washed with water (3 × 50 mL), brine (50 mL), dried over anhydrous Na₂SO₄, 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-yl)disulfanyl)ethyl]docosa-4,7,10,13,16,19-hexaenamide (9)**. A mixture containing 2-(pyridin-2-yl)sulfanylethanal-1-amine (8, 5 g, 26.8 mmol), DHA (9.2 g, 26.8 mmol), and HATU (10.2 g, 26.8 mmol) was taken up in CH₃Cl (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 h. It was then diluted with water and extracted with CH₂Cl₂. The combined organic layers were washed with water (3 × 100 mL), brine (100 mL), dried over anhydrous Na₂SO₄, 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-yl)sulfanylethanal)-ethyl]docosa-4,7,10,13,16,19-hexaenamide (10)**. 1-Amino-2-methylpropane-2-thiol (1.14 g, 8 mmol) was added to a solution containing **(4Z,7Z,10Z,13Z,16Z,19Z)-N-[(2-(Pyridin-2-yl)sulfanylethanal)-ethyl]docosa-4,7,10,13,16,19-hexaenamide (10)**. The resulting reaction mixture was stirred at rt for 30 min to allow for a complete reduction of the disulfide group. It was then diluted with water and extracted with CH₂Cl₂. The combined organic layers were washed with water (2 × 50 mL), brine (50 mL), dried over Na₂SO₄ 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-[(1-Amino-2-methylpropan-2-yl)sulfanylethanal]-ethyl]docosa-4,7,10,13,16,19-hexaenamide (8)**. A solution containing 1,2-di(pyridin-2-yl)sulfanylethanal (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 (CH₂Cl₂/MeOH = 1:1) to afford 2-(pyridin-2-yl)sulfanyl)ethanal-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). ¹³C NMR (100 MHz, DMSO-d₆): δ 159.37, 150.19, 138.65, 122.28, 119.86, 82.54, 48.39, 48.36, 48.13. R₁ = 0.22 (CH₂Cl₂/MeOH = 8:5:15 S:V). MS calcd for C₃₄H₄₉N₃O₂S₂: 595.3; found, 596.3270; m/z 596.3345. 2-(Pyridin-2-yl)sulfanyl)ethanal-1-amine (8). A solution containing 1,2-di(pyridin-2-yl)sulfanylethanal (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 (CH₂Cl₂/MeOH = 1:1) to afford 2-(pyridin-2-yl)sulfanyl)ethanal-1-amine (39 g, 92% yield). ¹H NMR (400 MHz, DMSO-d₆): δ 8.02–8.05 (m, 2 H). DOI: 10.1021/acs.jmedchem.6b01539 J. Med. Chem. 2017, 60, 488–473
immediately, cells were lysed on ice, using ice-cooled lysis buffer. The amounts of proteins were determined by Bio-Rad protein assay. An amount of 50 μg of total cell lysates was analyzed by SDS-page for protein concentration. Unheated samples were run on a 3–8% Tris-acetate gel (Life Technologies) at 150 V for 2.5 h. Separated proteins were transferred to PVDF using the Xcell blot module (Life Technologies) for 1 h at 25 V constant. After overnight transfer, blots were blocked in 5% blocking buffer (Bio-Rad) for 1 h at room temperature (rt) and followed by primary antibody treatment.

Hydrolysis Experiment Using FAAH-1. FAAH-1 was cloned into pFastBac (Invitrogen) with N-terminal FLAG and C-terminal His tags and then expressed in S. lividans at 37 °C with gentle shaking. Membranes were prepared according to manufacturer protocol. Gel was run at 150 V 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 h at rt with shaking. The primary antibody was prepared by adding 5 μL of UNC-596 primary antibody to 10 mL of 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 min in TBST at rt. The secondary antibody was prepared by adding 1 μL goat anti-mouse IgG–HRP in 10 mL of blocking buffer (per membrane). Secondary antibody was applied to the membrane and incubated for 1 h at rt with shaking. Membranes were rinsed 3 × 10 min in TBST and 2 × 10 min in TBS. SuperSignal West Femto was applied to membranes and then allowed to incubate for 5 min in low light. Membranes were exposed to X-ray film at regular intervals for up to 1 h. Membrane was washed with 3 × 5 min to remove substrate. The subsequent primary antibody was prepared by adding 0.5 μL of mouse anti-Na/K-ATPase antibody in 10 mL blocking buffer. Membranes were incubated with primary antibody for 1 h at rt with shaking and then washed 3 × 10 min 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 h.

In order to increase sensitivity at the lower concentrations (e.g., 0.15 μM 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 min. 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 150 V for 2.5 h. Separated proteins were transferred to PVDF using the Xcell blot module (Life Technologies) for 1 h at 25 V constant. After overnight transfer, blots were blocked in 5% blocking grade blocker (Bio-Rad) for 1 h at room temperature and then followed by primary antibody treatment.

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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 medium and 1% DMSO, 99% FBS solution. Both the vehicle and the positive control group were similarly solubilized with FBS and diluted with differentiation medium to give a final concentration of 10% FBS. Cells were incubated with either the control or test compounds for 24 h. For the chronic ivacaftor preincubation 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 h. For the electrophysiological measurements, the differentiation medium was changed to an experimental medium that has been supplemented with either the control or test compounds (in the same concentration used during the 24 h incubation period). HEPES-buffered Coon’s F12 medium 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 medium with the HEPES-buffered Coon’s F12 medium without serum and bicarbonate). Cells were incubated for 4 h in a CO2-free incubator at 37 °C. Cells were then placed in prewarmed heating blocks at 36 °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 (BP Design BVBA, Berchem, Belgium). Measurements were made at approximately 5 min intervals on a robotic workstation using 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 μM 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 preincubation protocol, ivacaftor (0.10 μM) was incubated along with the test article and bumetanide (3 μM) for 24 h. With the chronic ivacaftor preincubation protocol, only forskolin was needed to initiate the run. For the data analysis, portico chambers were measured V_{T} values were corrected for the electrode offset potentials and G_{T} values 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}/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-third tripezoid method was used to calculate the AUC. For the acute ivacaftor protocol, cells were incubated with the test compounds with bumetanide in differentiation medium for 24 h at 37 °C. The next day, the medium was replaced with HEPES-buffered Coon’s F12 medium, with the test compounds added back. The plate was incubated for 30 min in a CO2-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 KKCF7006F, KKCF70041, or KKCF70012L. The chloride transport function of primary homozygous F508del-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 medium. Prior to the assay, the mucus film was removed by adding 100 μL of the blank medium 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 medium in the same manner as described above. With the chronic ivacaftor preincubation protocol, cells were incubated in differentiation medium for 24 h in a CO2-free incubator at 37 °C with the indicated treatment groups. For the I_{EQ} measurements, the differentiation medium was first replaced with HEPES buffered Coon’s F12 medium (without serum or bicarbonate) with the test compounds added back to this new medium (in the same concentration used during the 24 h incubation period and with the prior solubilization in FBS before the serial dilution with Coon’s F-12 medium). The plate was then allowed to incubate at 37 °C for 4 h (CO2-free incubator) prior to the I_{EQ} measurements at ~35 °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 min intervals on a robotic workstation using custom software. The antagonist CFTRinh-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 medium for 24 h in a CO2-free incubator at 37 °C. The differentiation medium was then first replaced with HEPES buffered Coon’s F12 medium (without serum or bicarbonate) with the test compounds added back to this new medium. The plate was then allowed to incubate at 37 °C for 30 min in a CO2-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 was 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 that have been previously been described.5

**Rat PK Study.** The fatty acid cysteamine conjugate 5 was solubilized in a mixture of excipients consisting of 40% Tween, 50% Pecel (glyceryl monololate type 40), 10% PEG400 and diluted with water to form a self-emulsifying aqueous mixture for oral administration to animals. For this study, Sprague Dawley rats that had been surgically implanted with indwelling jugular vein cannulae (JVC) were used (Agulux, 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 h postdose. The bioanalytical portion of the PK study was carried out using an LC/MS/MS system (Agilent model: HPLC, 1200; MS, 6410) and analyzed with the appropriate software (WinNonlin Phoenix 64 6.3.0 395). The various thiol metabolites were quantified by using the corresponding Ellman’s adduct to prepare the standard curves.35

**ASSOCIATED CONTENT**

**Supporting Information**

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acs.jmedchem.6b01539.

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Autophagy activation, details of runs depicted in Figures 7–9 and 14, effect of FBS on chloride current, and evaluations of compounds 1 and 5 (PDF) Molecular formula strings (CSV)

**AUTHOR INFORMATION**

**Corresponding Author**


**ORCID**

Chi B. Vu: 0000-0002-6516-8846

**Notes**

The authors declare no competing financial interest.

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**ABBREVIATIONS USED**

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

**REFERENCES**


(24) We have evaluated the fatty acid cysteamine 1 in cultured primary homozygous F508del-CFTR hBE cells from different donors (KKCFFT006F and CFFT028H) and have observed a similar level of autophagy activation (data not shown).


(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 medium to Coon’s F12 medium, the plate was incubated for 4 h in a CO2 free incubator prior to iSO measurements. With the chronic ivacaftor preincubation protocol, we found that the longer 4 h incubation period in Coon’s F12 medium 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.


(34) In this rat PK experiment, the plasma levels of cysteamine 12 and bis-geminal cysteamine 13 (Figure 11) were not determined. However, in a separate PK experiment involving Sprague Dawley rats that have been surgically implanted with indwelling jugular vein cannula and portal vein cannula (similarly dosed orally at 30 mg/kg) only trace quantities of these two metabolites were detectable in the peripheral plasma. Niacin, nicotinamide 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 Cmax = 3129 ± 564 ng/mL, portal AUCt = 5888 ± 1297 h·ng/mL).

(35) Compound 5 has also been dosed orally (100 mg/kg b.i.d.) 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 tissue. Compound 5 has also been administered to 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.


