Published in final edited form as:

Angew Chem Int Ed Engl. 2015 August 17; 54(34): 9881–9885. doi:10.1002/anie.201503983.

Inhibiting an epoxide hydrolase virulence strategy protects CFTR**

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Abstract

Opportunistic pathogens exploit diverse strategies to sabotage host defenses. *Pseudomonas aeruginosa* secretes the CFTR inhibitory factor Cif and thus triggers loss of CFTR, an ion channel required for airway mucociliary defense. However, Cif's mechanism of action has remained unclear. It catalyzes epoxide hydrolysis, but there is no known role for natural epoxides in CFTR regulation. Here, we show that Cif's hydrolase activity is strictly required for its effects on CFTR. We also uncover a small-molecule inhibitor that protects this key component of the mucociliary

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**Funding support was provided by NIH grants R01-AI091699 (DRM), P30-GM106394 and R01-HL074175 (BAS), R00-HL098342 (JMB), R01-ES002710 (BDH), T32-AI007519 and T32-DK007301 (to CDB), NIEHS Superfund Research Program awards P42-ES004699 (BDH) and P42-ES007373 (BAS), as well as Cystic Fibrosis Foundation grants MADDEN08G0 and STANTO11R0.

defense system. Our results provide a basis for targeting Cif's distinctive virulence chemistry and suggest an unanticipated role of physiological epoxides in intracellular protein trafficking.

Keywords

epoxide hydrolase; *Pseudomonas aeruginosa* virulence factors; airway epithelial cells; X-ray crystallography; inhibitor design

In patients with underlying airway disease, infection by *Pseudomonas aeruginosa* is a major cause of morbidity and mortality.^[1] While this ubiquitous soil organism is routinely inhaled, the mucociliary elevator efficiently clears it from the airway under normal circumstances. However, pulmonary impairment enables the bacterium to colonize the lung, form biofilms,^[2] and trigger recurrent infections and inflammation. *P. aeruginosa* is often refractory to antibiotic treatment in patients with cystic fibrosis (CF) or other airway diseases, leading to chronic and debilitating pulmonary infections and, ultimately, to respiratory failure. As a result, there is a pressing need for new pathogen-specific therapeutic strategies. Here, we investigate a virulence factor that allows clinical isolates to sabotage an essential component of the mucociliary clearance machinery, the CF transmembrane conductance regulator (CFTR).^[3]

P. aeruginosa secretes an arsenal of toxins and virulence factors that aid the bacterium in airway colonization and persistence.^[4] Among them is the CFTR inhibitory factor (Cif), the founding member of a distinct class of epoxide hydrolases (EH) with homologs in several other airway pathogens, including *Acinetobacter* and *Burkholderia* species.^[5] Cif is delivered into the cytoplasm of human airway epithelial cells. There, it blocks the post-endocytic deubiquitination of CFTR,^[6] inducing a rapid drop in apical CFTR and chloride-secretion levels^[7] and thus phenocopying key aspects of CF^[8].

Cif is a member of the α/β hydrolase superfamily^[7,9] and catalyzes the hydrolysis of representative epoxides such as epibromohydrin (EBH). However, Cif has a non-canonical active site.^[5a,10] and neither EH enzymes nor epoxide/diol-based signals are known to regulate protein trafficking. Thus, to rigorously investigate Cif's catalytic machinery and to assess the possibility of a new physiological role for epoxides, we used our crystal structure^[9] to generate a panel of active-site mutations. According to our model of Cif mechanism (**Figure 1**), His¹⁷⁷ and Tyr²³⁹ position the substrate for hydrolysis and polarize the C-O bonds for epoxide ring-opening, attack by the Asp¹²⁹ nucleophile forms a covalent intermediate (**Figure 1**, arrow 1), and the Glu¹⁵³/His²⁹⁷ pair activates a water molecule for hydrolytic release of the product (**Figure 1**, arrow 2). His207 is located in the active-site tunnel. Site-directed Cif mutants were engineered to carry either disruptive (D129S, H177A, Y239F, or H207A) or conservative (E153D) substitutions in one of these key residues.

As predicted, each mutation reduces hydrolysis of EBH by Cif (**Figure 2**, closed bars). The D129S, H177A, and Y239F mutants lack detectable EBH hydrolysis, whereas the H207A and E153D mutants retain 22% and 66% of WT EH activity, respectively. None of these mutations significantly alter the overall structure of Cif. We determined the crystal structure of each mutant at a resolution $d = 2.12 \text{ \AA}$, with stringent coordinate errors $< 0.2 \text{ \AA}$. Each can

be directly superimposed on the WT structure with a C_α RMSD value 0.13 Å (**Figure 2, inset**; Tables S1-S3). Active-site changes are confined to the vicinity of the altered side chain (Figure S1). Thermal denaturation experiments confirmed the stability of the mutants (Table S4). Collectively, the effects of these mutations support the model of Cif catalysis (**Figure 1**). The data also suggest that His²⁰⁷ plays a role in substrate binding.

With these mutants in hand, we next determined whether Cif's ability to reduce apical CFTR is dependent on its EH activity. Wild-type or mutant Cif was individually applied to polarized human bronchial epithelial cells expressing WT-CFTR, and the amount of CFTR at the apical membrane was characterized by surface biotinylation and immunoblotting. Whereas WT Cif triggers a substantial loss of apical-membrane CFTR, none of the mutant Cif proteins mediate a statistically significant effect (**Figure 2**, open bars). The homologous protein from *A. nosocomialis* (aCif), which also hydrolyzes epoxides and reduces apical CFTR levels,^[5a] exhibits a similar EH dependence (Figure S2).

While Cif may participate in intracellular protein-protein interactions,^[6] the stability and external shape of the WT and mutant Cif proteins are essentially indistinguishable, making it unlikely that Cif:protein interactions drive loss of apical CFTR. Instead, the requirement for EH activity suggests the existence of an epoxide signal involved in the deubiquitination and post-endocytic recycling of CFTR – a previously unknown mammalian epoxide signaling mechanism. Furthermore, some mutations exhibit residual hydrolytic activity for epibromohydrin, but are unable to trigger robust loss of CFTR. Since our xenobiotic test substrate is much smaller and more hydrophilic than most cellular epoxides,^[11] these mutants have presumably selectively lost the ability to hydrolyze the authentic *in vivo* targets of Cif.

Our results also suggest that Cif-mediated loss of CFTR could be blocked by small-molecule EH inhibitors, several of which have been developed for other EH enzymes.^[12] We first surveyed 18 candidates for Cif inhibition using a radiolabeled reporter substrate (Table S5, Figure S3). However, this screen identified only weak inhibition (maximum 25% ± 3% with 100 μM N,N'-di-(3,4-dichlorophenyl) urea (DCPU, **1**)) limited in part by compound solubility. Given the stereochemical deviations of the Cif active site compared to previously characterized EHs,^[10] we suspected that a broader search of chemical space would be required to identify a potent Cif inhibitor. Neither of our previously described assays for Cif activity^[9] can be readily formatted for high-throughput screening (HTS). Furthermore, a screen of well-characterized 1,2-disubstituted fluorogenic soluble EH reporters showed no activity (Figure S4), consistent with the steric constraints of Cif's active site.^[9] However, a parallel survey of xenobiotic substrates revealed that Cif can hydrolyze glycidyl methacrylate (Figure S5), the hydrolytic trigger in a collection of mono-substituted fluorogenic epoxides that was being developed in parallel for mammalian microsomal EHs.^[13] Cif generated a robust hydrolysis signal with several members of the panel (Figure S6), and showed the highest activity against cyano(6-methoxynaphthalen-2-yl)methyl(oxiran-2-ylmethyl) (CMNGC, **2**) (**Figure 3a**).

Using CMNGC as a fluorescent reporter and DCPU as a positive control for inhibition, we next screened a library of 1,600 compounds,^[14] including FDA-approved and orphan drugs,

(Figure S7). The primary assay yielded excellent results, with an average Z' value of 0.82 ± 0.06 (Table S6), and a 2.9% hit rate (47 compounds) using a 30% inhibition cut-off (**Figure 3b**, Table S7). Secondary assays (Tables S8 and S9) confirmed one of the two top hits, the thyroid hormone analog 3,3',5-triiodothyroacetic acid (tiratricol, **3**; **Figure 3b**), as a *bona fide* inhibitor.

This chemical tool enabled us to test our hypothesis that a Cif inhibitor would protect CFTR in human airway epithelial cells. Control experiments confirmed that tiratricol by itself shows no significant cytotoxicity, does not affect bacterial growth rate (Figures S8 and S9), and has no significant effect on apical-membrane abundance of CFTR (**Figure 4a**). However, compared to dimethyl sulfoxide (DMSO; vehicle), tiratricol blocked the ability of either purified Cif protein or co-cultured *P. aeruginosa* to reduce levels of apical membrane CFTR (**Figure 4a**). These observations strongly support the importance of EH catalysis in triggering CFTR loss and demonstrate that an inhibitor could provide therapeutic benefit. Since tiratricol inhibits not only purified Cif, but also Cif encapsulated by *P. aeruginosa* within outer membrane vesicles,^[15] the inhibitor appears able to access compartments across a lipid bilayer.

To evaluate the stereochemistry of tiratricol-mediated inhibition, we also tested the inhibitory potential of six chemical variants. While a propionate replacement for the acetate moiety yields a similar IC_{50} (**6**, **Table 1**), all other substitutions individually and collectively reduced the potency of the scaffold. To explore the stereochemical basis of the observed structure-activity relationships, we co-crystallized Cif in complex with tiratricol and determined the structure to 1.75 Å resolution (Table S10; **Figure 4b**). Isomorphous difference and anomalous electron density maps clearly reveal the presence of the three tiratricol iodine atoms per Cif protomer (**Figure 4b**). Consistent with the substitution data, the R^1 , R^2 , and R^3 groups are in close contact with the protein surface, whereas the R^4 acetate moiety protrudes towards solvent. Thus, structural changes at the R^4 position could yield more selective compounds with equal or enhanced potency against Cif and improved pharmacological characteristics.

Tiratricol does not displace the active-site water molecule that occupies the putative site of the substrate epoxide oxygen (**Figure 4c**). Instead, it occludes the entrance, which is formed by a ring of hydrophobic residues. It also fills part of a vestibule immediately adjacent to the core active-site residues (**Figure 4c**). Together, these observations suggest that tiratricol may not be acting as a competitive inhibitor, and indeed, kinetic analysis of CMNGC hydrolysis reveals that the substrate concentration has little or no effect on the inhibitory equilibrium binding constant K_i (**Figure 4d**). Our results suggest that extending tiratricol's structure to reach the core active-site residues may generate competitive Cif inhibitors with greater potency and selectivity.

Cif inhibitors may contribute to successful therapy for the prevention and treatment of pulmonary infections caused by *P. aeruginosa*. In COPD and pneumonia, where *P. aeruginosa* infections can exhibit high patient morbidity and mortality,^[16] Cif can potentially phenocopy CF in patients with WT CFTR – suppressing its apical-membrane levels,^[8] thereby hampering mucociliary clearance and facilitating infection. In cystic

fibrosis, *P. aeruginosa* represents the dominant pulmonary pathogen and may be present in up to 97% of adult cases.^[17] Thus, although new modulator combinations can modestly restore CFTR function in some CF patients,^[18] Cif-mediated effects can antagonize their therapeutic efficacy,^[19] trapping patients in a downward spiral of infection and inflammation. And while tiratricol causes metabolic complications^[20] the HTS protocol and structural information presented here provide a strong foundation for identifying and elaborating additional inhibitor chemistries to avoid them. Furthermore, broad spectrum antibiotics can impact important commensal flora.^[21] However, Cif has a unique active-site geometry shared among homologs found in opportunistic pathogens.^[10] As a result, its EH activity likely represents a highly selective therapeutic target^[4] to bolster mucociliary defenses and combat airway infections in vulnerable patients.

Cif also provides important new clues to cellular physiology. Although epoxides derived from the arachidonic and linoleic acid pathways are potent signaling molecules, none have yet been implicated in protein trafficking or deubiquitination.^[22] Our results suggest that a bacterial virulence factor can hydrolyze mammalian signaling molecules containing an epoxide functionality, and that this activity is required to trigger the degradation of apical-membrane CFTR in human airway epithelial cells. To our knowledge, this represents the first example of a pathogenic EH that can subvert endogenous mammalian epoxide/diol signaling mechanisms to exert control over host physiological pathways. Furthermore, although epoxides are generated from polyunsaturated fatty acids in all cells, their impact on epithelial cells is poorly understood, particularly in comparison to the extensive literature on endothelial and immune responses.^[23] Because the Cif effect can be induced by highly purified protein, our data strongly suggest that at least one epithelial-derived epoxide/diol pair is responsible for Cif-mediated loss of CFTR. Finally, since this process has been shown to involve blockade of post-endocytic deubiquitination by USP10, our data also provide evidence for regulation of the peripheral protein trafficking machinery by a signaling epoxide.

Experimental Section

See Supporting Information for Detailed Methods.

Mutagenesis and protein purification

Cif mutations were generated using *in vivo* yeast recombineering or the QuikChange Mutagenesis Kit (Stratagene), and Cif proteins were expressed and purified as described previously.^[24]

Epoxide hydrolase enzyme assay

The radioactivity assay, adrenochrome, and fluorogenic assays were performed as described previously,^[9,13,25] using tritium labeled *cis*-stilbene oxide, epibromohydrin (Sigma), and CMNGC (**3**) as substrates, respectively. For assays with CMNGC, measurements were taken using a fluorescent plate reader with $\lambda_{\text{ex}} = 330 \text{ nm}$ and $\lambda_{\text{em}} = 465 \text{ nm}$.

High-throughput screening

The primary screen surveyed the Pharmakon 1600 (Microsource Discovery Systems) library^[14] at room temperature (~25 °C) in 96-well plates, using 25 µM CMNGC as the fluorogenic reporter substrate, 1 µM Cif protein, test compounds at 10 µM, or 10 µM DCPU as a positive control. The fluorescent signal was measured after 60 min. The secondary screen was performed at 37 °C and with fluorescent readings taken in kinetic mode.

Crystallization, data collection and processing, structure refinement, and analysis

Cif crystals were obtained by vapor diffusion (see Table S1 for composition) and flash cooled, data were collected and processed, and structures determined, refined, and visualized as described.^[9,24] For co-crystallization, solutions were supplemented with 200 µM tiratricol and 0.2% (v/v) DMSO. Structures have been deposited in the PDB (IDs, see Tables S2 and S10).

Cell culture

Parental human bronchial epithelial CFBE41o- cells stably transduced with WT-CFTR (CFBE cells)^[26] were cultured essentially as described,^[8] with modifications as described in the Supporting Information.

Determination of apical-membrane levels of CFTR

Polarized monolayers of CFBE cells were treated apically with 50 µg of purified Cif protein, *P. aeruginosa*, or buffer or medium control, and incubated for 1 hr at 37°C in a 5 % CO₂ incubator. The relative abundance of CFTR was then determined by surface biotinylation as described previously.^[27] Results are shown as an average of independent experiments performed a minimum of three times on separate days and with different passages of cells.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

We would like to thank Jessica St. Laurent for technical assistance, and Dr. Vivian Stojanoff and Dr. Jean Jakoncic at NSLS/Brookhaven, Abigail Fellows, Dr. William Winkelman, Dr. Alicia Ballok, Dr. George O'Toole, Dr. Joshua Weiner, Dr. Jeanine Amacher, Dr. Daniel MacEachran, and Dr. Henry Higgs for advice and assistance.

References

- [1]. Dudley MN, Loutit J, Griffith DC. Curr. Opin. Biotechnol. 2008; 19:637. [PubMed: 19036576]
- [2]. Davies JC, Bilton D. Respir. Care. 2009; 54:628. [PubMed: 19393107]
- [3]. Rogan MP, Stoltz DA, Hornick DB. Chest. 2011; 139:1480. [PubMed: 21652558]
- [4]. Bleves S, Viarre V, Salacha R, Michel GP, Filloux A, Voulhoux R. Int. J. Med. Microbiol. 2010; 300:534. [PubMed: 20947426]
- [5]. a) Bahl CD, Hvorecny KL, Bridges AA, Ballok AE, Bomberger JM, Cady KC, O'Toole GA Jr. Madden DR. J. Biol. Chem. 2014; 289:7460. [PubMed: 24474692] b) Ballok AE, Bahl CD, Dolben EL, Lindsay AK, St. Laurent JD, Hogan DA, Madden DR, O'Toole GA. J. Bacteriol. 2012; 194:5315. [PubMed: 22843844]

- [6]. Bomberger JM, Ye S, Maceachran DP, Koeppen K, Barnaby RL, O'Toole GA, Stanton BA. *PLoS Pathog.* 2011; 7:e1001325. [PubMed: 21455491]
- [7]. MacEachran DP, Ye S, Bomberger JM, Hogan DA, Swiatecka-Urban A, Stanton BA, O'Toole GA. *Infect. Immun.* 2007; 75:3902. [PubMed: 17502391]
- [8]. Swiatecka-Urban A, Moreau-Marquis S, Maceachran DP, Connolly JP, Stanton CR, Su JR, Barnaby R, O'Toole G A, Stanton BA. *Am. J. Physiol. Cell Physiol.* 2006; 290:C862. [PubMed: 16236828]
- [9]. Bahl CD, Morisseau C, Bomberger J, Stanton BA, Hammock BD, O'Toole GA, Madden DR. *J. Bacteriol.* 2010; 192:1785. [PubMed: 20118260]
- [10]. Bahl CD, Madden DR. *Protein Pept. Lett.* 2012; 19:186. [PubMed: 21933119]
- [11]. Spector AA, Norris AW. *Am. J. Physiol. Cell Physiol.* 2007; 292:C996. [PubMed: 16987999]
- [12]. Morisseau C, Hammock BD. *Annu. Rev. Pharmacol. Toxicol.* 2005; 45:311. [PubMed: 15822179]
- [13]. Morisseau C, Bernay M, Eschaich A, Sanborn JR, Lango J, Hammock BD. *Anal. Biochem.* 2011; 414:154. [PubMed: 21371418]
- [14]. Morisseau C, Sahdeo S, Cortopassi G, Hammock BD. *Anal. Biochem.* 2013; 434:105. [PubMed: 23219563]
- [15]. Bomberger JM, Maceachran DP, Coutermarsh BA, Ye S, O'Toole GA, Stanton BA. *PLoS Pathog.* 2009; 5:e1000382. [PubMed: 19360133]
- [16]. Murphy TF, Brauer AL, Eschberger K, Lobbins P, Grove L, Cai X, Sethi S. *Am. J. Respir. Crit. Care Med.* 2008; 177:853. [PubMed: 18202344]
- [17]. Filkins LM, Hampton TH, Gifford AH, Gross MJ, Hogan DA, Sogin ML, Morrison HG, Paster BJ, O'Toole GA. *J. Bacteriol.* 2012; 194:4709. [PubMed: 22753064]
- [18]. Boyle MP, Bell SC, Konstan MW, McColley SA, Rowe SM, Rietschel E, Huang X, Waltz D, Patel NR, Rodman D. *Lancet Respir. Med.* 2014; 2:527. [PubMed: 24973281]
- [19]. Stanton BA, Coutermarsh B, Barnaby R, Hogan DA. *PLoS One.* 2015 in press.
- [20]. Ma RC, Chan MH, Poon WT, So WY, Chow CC. *Intern. Med. J.* 2008; 38:611. [PubMed: 18715309]
- [21]. Mueller C, Macpherson AJ. *Gut.* 2006; 55:276. [PubMed: 16407387]
- [22]. Sudhahar V, Shaw S, Imig JD. *Curr. Med. Chem.* 2010; 17:1181. [PubMed: 20158473]
- [23]. Campbell WB, Fleming I. *Pflugers Arch.* 2010; 459:881. [PubMed: 20224870]
- [24]. Bahl CD, MacEachran DP, O'Toole GA, Madden DR. *Acta Crystallogr.* 2010; F66:26.
- [25]. a) Gill SS, Ota K, Hammock BD. *Anal. Biochem.* 1983; 131:273. [PubMed: 6614459] b) MacEachran DP, Stanton BA, O'Toole GA. *Infect. Immun.* 2008; 76:3197. [PubMed: 18458065] c) Cedrone F, Bhatnagar T, Baratti JC. *Biotechnol. Lett.* 2005; 27:1921. [PubMed: 16328991] d) Jones PD, Wolf NM, Morisseau C, Whetstone P, Hock B, Hammock BD. *Anal. Biochem.* 2005; 343:66. [PubMed: 15963942]
- [26]. Bebok Z, Collawn JF, Wakefield J, Parker W, Li Y, Varga K, Sorscher EJ, Clancy JP. *J. Physiol.* 2005; 569:601. [PubMed: 16210354]
- [27]. Bomberger JM, Guggino WB, Stanton BA. *Methods Mol. Biol.* 2011; 741:271. [PubMed: 21594791]

Figure 1.
The active-site of WT Cif is shown (stick figure; PDB entry 3KD2). Based on analogy to other EH enzymes, a candidate mechanism is shown (curved arrows). As described in the text, active-site residues with a variety of proposed catalytic functions were mutated as indicated (arrows).

Figure 2. Epoxide hydrolysis is necessary but not sufficient to induce CFTR degradation
 The EH activity for WT Cif and each mutant was determined using the adrenochrome assay (closed bars, left-hand axis). The activities of Cif-D129S, Y239F, and H177A were indistinguishable from control (buffer), while Cif-H207A and E153D retained residual activity (n=3, SD). Apical WT-CFTR levels were detected by cell-surface biotinylation of polarized bronchial epithelial cells monolayers following treatment with purified Cif proteins, and the percentage reduction in CFTR calculated compared to control cells (open bars, right-hand axis). WT Cif, but none of the mutants, triggered significant loss of CFTR (n=3, SEM). *inset* C α traces are shown for WT Cif (cyan) and all mutants (gray), following least-squares superposition. Mutant side chains are shown as stick figures (C=orange). ***, P < 0.001; *, P < 0.05, compared to controls.

Figure 3. Inhibition of Cif activity

a) Hydrolysis of CMNGC by Cif converts the epoxide into a vicinal diol, which undergoes a chemical rearrangement to release a fluorescent product.^[13] b) A library of 1,600 molecules was tested for the ability to inhibit Cif-mediated hydrolysis of CMNGC. Results of the primary screen are displayed (gray and colored points), with the average value for the positive-control inhibitor DCPU displayed to the right for reference (black circle). Molecules above a 30% inhibition cut-off were retested in a secondary screen, with results encoded by color (see inset scale). The point corresponding to tiratricol is circled. For details, see Tables S7 and S8.

Figure 4. Stereochemistry of a micromolar Cif inhibitor that protects apical CFTR

a) Co-addition of tiratricol with purified Cif protein or *P. aeruginosa* PA14 inhibits loss of CFTR in polarized human airway epithelial cells. $n=3$, SEM. *, $P<0.01$ compared to buffer-treated cells. b) Tiratricol binds at the active-site tunnel. The tiratricol bound model of Cif is shown (orange carbons, surface in gray), superimposed with the apo-model (cyan carbons). Bound tiratricol (carbon colored light purple) participates in π -stacking with Phe164 and hydrogen bonding with the main-chain carbonyl of Gly270. Residues Phe164, Leu174, and Met272 form a gasket controlling active site solvent accessibility and their side chains reorient upon tiratricol binding. Isomorphous difference density for iodine atoms of tiratricol (dark purple) were calculated using the apo-Cif structure factors from PDB entry 3KD2 and is shown at 10σ . c) Tiratricol does not directly occupy the epoxide oxygen binding site (indicated by a water coordinated by His177 and Tyr239). Cartoon representation and active site side chains are shown for the tiratricol-bound model of Cif. d) Kinetic determination of tiratricol equilibrium inhibition constant (K_i) of Cif using the fluorogenic substrate CMNGC.

Table 1

Cif inhibition by derivatives

	#	R ¹	R ²	R ³	R ⁴	IC ₅₀ (μM)
3	I	H	I	CH ₂ COOH	4.7 ± 0.6
	4	H	H	I	CH ₂ COOH	100 ± 2
	5	I	I	I	CH ₂ COOH	20 ± 2
	6	I	H	I	CH ₂ CH ₂ COOH	4.4 ± 0.5
	7	I	H	H	CH ₂ CH(NH ₂)COOH	80 ± 10
	8	I	H	I	CH ₂ CH(NH ₂)COOH	21 ± 2
	9	I	I	I	CH ₂ CH(NH ₂)COOH	>100