

Inhibiting an Epoxide Hydrolase Virulence Factor from *Pseudomonas aeruginosa* 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, the mechanism of action of Cif has remained unclear. It catalyzes epoxide hydrolysis, but there is no known role for natural epoxides in CFTR regulation. It was demonstrated that the hydrolase activity of Cif is strictly required for its effects on CFTR. A small-molecule inhibitor that protects this key component of the mucociliary defense system was also uncovered. These results provide a basis for targeting the distinctive virulence chemistry of Cif and suggest an unanticipated role of physiological epoxides in intracellular protein trafficking.

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. We investigated 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 CFTR inhibitory factor (Cif), the founding member of a distinct class of epoxide hydrolase (EH) enzymes with homologues 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] thereby 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 the catalytic machinery of Cif 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 the 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

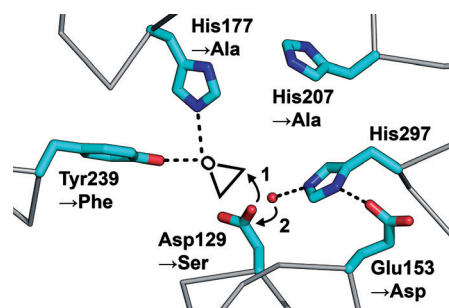


Figure 1. The active site of WT Cif is shown (stick model; PDB entry 3KD2). Based on analogy to other EH enzymes, a candidate mechanism is shown (curved arrows). As described in the text, active-site residues with a variety of proposed catalytic functions were mutated as indicated (straight arrows).

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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 the 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 wild-type (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 $d \leq 2.12$ Å, with stringent coordinate errors ≤ 0.2 Å. Each can be directly superimposed on the WT structure with a C_α RMSD value ≤ 0.13 Å (Figure 2, inset; Tables S1–S3 in the Supporting Information). Active-site changes are confined to the vicinity of the altered side chain (Figure S1 in the Supporting Information). Thermal denaturation experiments confirmed the stability of the mutants (Table S4). Collectively, the effects of these mutations support our model of Cif catalysis (Figure 1). The data also suggest that His207 plays a role in substrate binding.

With these mutants in hand, we determined whether the ability of Cif to reduce apical CFTR levels is dependent on its

EH activity. Wild-type or mutant Cif was 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, thus making it unlikely that Cif–protein interactions drive the 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, that is, 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 by using a radiolabeled reporter substrate (Table S5, Figure S3). However, this screen identified only weak inhibition (maximum $25\% \pm 3\%$ with $100 \mu\text{M}$ *N,N'*-di-(3,4-dichlorophenyl) urea (DCPU, **1**)), which was limited in part by compound solubility. Given the stereochemical deviation 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), which is consistent with the steric constraints of the Cif 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 monosubstituted 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) when using a 30% inhibition cut-off (Figure 3b and Table S7). Secondary assays (Tables S8 and S9) confirmed one of the two top hits, the thyroid hormone

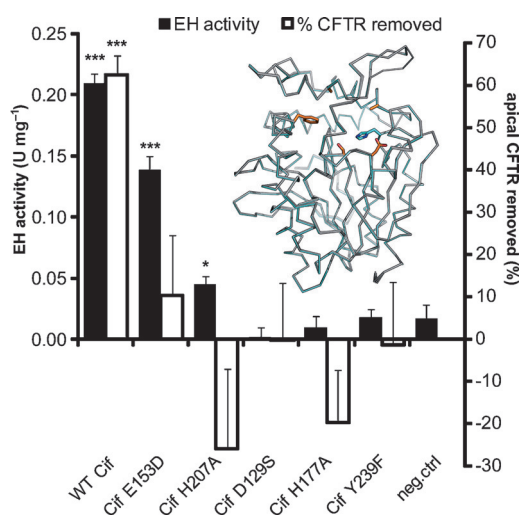


Figure 2. Epoxide hydrolysis is necessary but not sufficient to induce CFTR degradation. The EH activity for WT Cif and each mutant was determined using the adrenochrome assay (closed bars, left-hand axis). The activities of Cif mutants D129S, Y239F, and H177A were indistinguishable from control (buffer), while Cif mutants H207A and E153D retained residual activity ($n=3$, standard deviation (SD)). Apical WT CFTR levels were detected through cell-surface biotinylation of monolayers of polarized bronchial epithelial cells following treatment with purified Cif proteins, and the percentage reduction in CFTR was calculated compared to control cells (open bars, right-hand axis). WT Cif, but none of the mutants, triggered significant loss of CFTR ($n \geq 3$, standard error of the mean (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 models (C = orange). ***, $P < 0.001$; *, $P < 0.05$, based on individual comparisons to control.

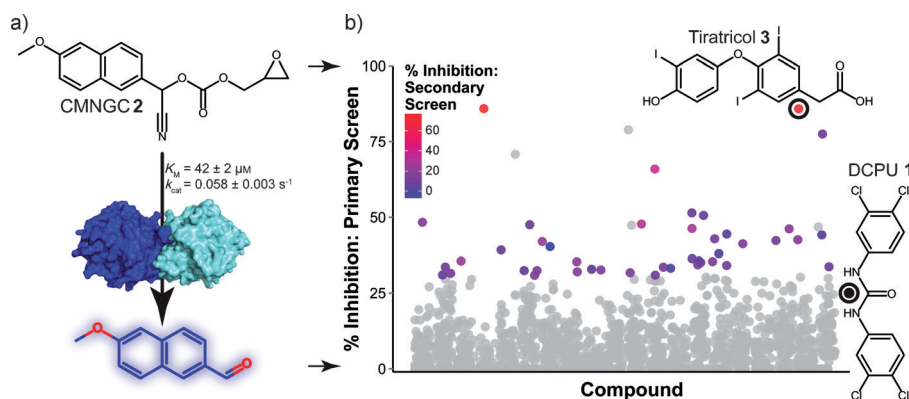


Figure 3. Inhibition of Cif activity. a) Hydrolysis of CMNGC by Cif converts the epoxide into a vicinal diol, which undergoes a chemical rearrangement to release a fluorescent product.^[13] b) A library of 1,600 molecules was tested for the ability to inhibit Cif-mediated hydrolysis of CMNGC. Results of the primary screen are displayed (gray and colored points), with the average value for the positive control (the inhibitor DCPU) displayed to the right for reference (black, circled). Molecules above a 30% inhibition cut-off were retested in a secondary screen, with results encoded by color. The point corresponding to tiratricol is circled. For details, see Tables S7 and S8.

analogue 3,3',5-triiodothyroacetic acid (tiratricol, **3**; Figure 3b), as an 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 (Figure S8 and S9), and has no significant effect on the 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 the 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 to be 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} value (**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 (Figure 4b and Table S10). 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 the 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 the structure of tiratricol 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 chronic obstructive pulmonary disease (COPD) and pneumonia, where *P. aeruginosa* infections can lead to high patient morbidity and mortality,^[16] Cif could potentially phenocopy CF in patients with WT CFTR, suppressing its apical-membrane levels^[8] and 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] thereby trapping patients in a downward spiral of infection and inflammation. While tiratricol itself causes metabolic complications,^[20] the HTS protocol and structural information presented herein provide a strong foundation for identifying and elaborating additional inhibitor chemistries to avoid these complications. Furthermore, broad-spectrum antibiotics can impact important commensal flora.^[21] However, Cif has a unique active-site geometry shared among homologues 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

Table 1: Cif inhibition by tiratricol derivatives.

	#	R^1	R^2	R^3	R^4	$\text{IC}_{50} [\mu\text{M}]^{\text{a}}$
	3	I	H	I	CH_2COOH	4.7 ± 0.6
	4	H	H	I	CH_2COOH	100 ± 2
	5	I	I	I	CH_2COOH	20 ± 2
	6	I	H	I	$\text{CH}_2\text{CH}_2\text{COOH}$	4.4 ± 0.5
	7	I	H	H	$\text{CH}_2\text{CH}(\text{NH}_2)\text{COOH}$	80 ± 10
	8	I	H	I	$\text{CH}_2\text{CH}(\text{NH}_2)\text{COOH}$	21 ± 2
	9	I	I	I	$\text{CH}_2\text{CH}(\text{NH}_2)\text{COOH}$	> 100

[a] IC_{50} = half maximal inhibitory concentration

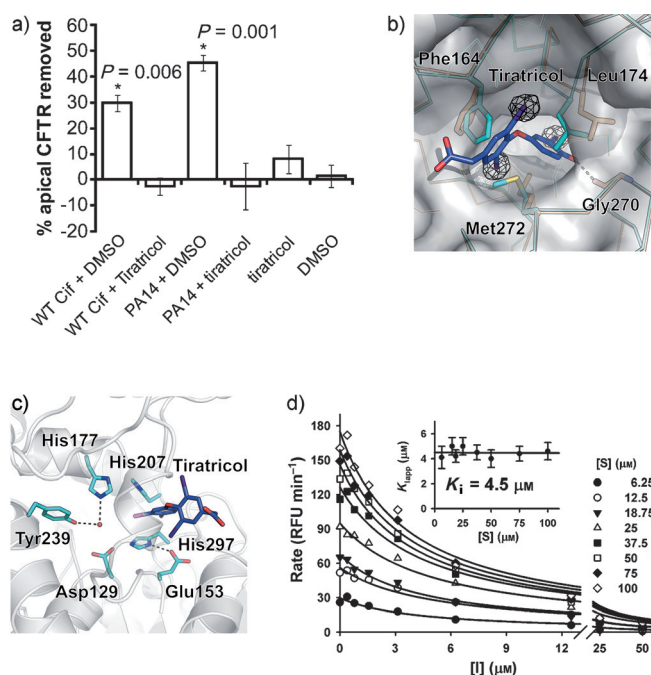


Figure 4. Stereochemistry of a micromolar Cif inhibitor that protects apical CFTR. **a)** Co-addition of tiratricol with purified Cif protein or *P. aeruginosa* PA14 inhibits the loss of CFTR from polarized human airway epithelial cells ($n=3$, SEM *, $P<0.01$ compared to buffer-treated cells). **b)** Tiratricol binds at the active-site tunnel. The tiratricol-bound model of Cif is shown (orange stick model, surface in gray), superimposed with the apo-model (cyan stick model). Bound tiratricol (blue stick model, oxygen red, iodine purple) participates in π -stacking with Phe164 and hydrogen bonding with the main-chain carbonyl group 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 densities for the iodine atoms of tiratricol were calculated by 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 molecule coordinated by His177 and Tyr239). Active-site side chains are shown for the tiratricol-bound model of Cif. **d)** Kinetic determination of the equilibrium inhibition constant (K_i) for tiratricol with Cif by using the fluorogenic substrate CMNGC. S = substrate, I = inhibitor, RFU = relative fluorescence units.

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 effect of Cif can be induced by highly purified protein, our data strongly suggest that at least one epithelial-derived epoxide/diol pair is responsible for the Cif-mediated loss of

CFTR. Finally, since this process has been shown to involve the 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 the Supporting Information for detailed methods.

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

Epoxide hydrolase enzyme assay: The radioactivity, 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 made using a fluorescent plate reader with $\lambda_{\text{ex}} = 330$ nm and $\lambda_{\text{em}} = 465$ nm.

High-throughput screening: The primary screen surveyed the Pharmakon 1600 (Microsource Discovery Systems) library^[14] at room temperature (ca. 25°C) in 96-well plates, with 25 μM CMNGC as the fluorogenic reporter substrate, 1 μM Cif protein, and test compounds at 10 μM or 10 μM DCPU as a positive control. The fluorescence signal was measured after 60 min. The secondary screen was performed at 37°C and with fluorescence readings made 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 (see Tables S2 and S10 for PDB IDs).

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 h 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.

Keywords: epoxide hydrolase · CFTR · inhibitors · *Pseudomonas aeruginosa* · virulence factors

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