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Potential Agents for Treating Cystic Fibrosis: Cyclic Tetrapeptides That Restore Trafficking and Activity of ΔF508-CFTR

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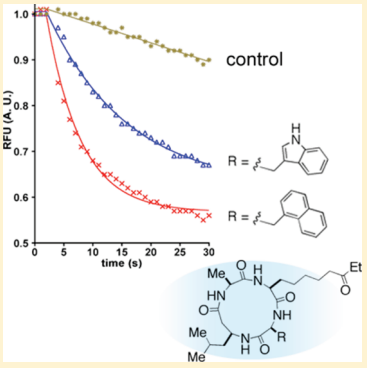
Supporting Information

ABSTRACT: Cystic fibrosis (CF) is a loss-of-function disease caused by mutations in the CF transmembrane conductance regulator (CFTR) protein, a chloride ion channel that localizes to the apical plasma membrane of epithelial cells. The most common form of the disease results from the deletion of phenylalanine-508 (ΔF508), leading to the accumulation of CFTR in the endoplasmic reticulum with a concomitant loss of chloride flux. We discovered that cyclic tetrapeptides, such as **11**, **14**, and **15**, are able to correct the trafficking defect and restore the cell surface activity of ΔF508-CFTR. Although this class of cyclic tetrapeptides is known to contain inhibitors of certain histone deacetylase (HDAC) isoforms, their HDAC inhibitory potencies did not directly correlate with their ability to rescue ΔF508-CFTR. In full HDAC profiling, **15** strongly inhibited HDACs 1, 2, 3, 10, and 11 but not HDACs 4−9. Although **15** had less potent IC_{50} values than reference agent vorinostat (**2**) in HDAC profiling, it was markedly more potent than **2** in rescuing ΔF508-CFTR. We suggest that specific HDACs can have a differential influence on correcting ΔF508-CFTR, which may reflect both deacetylase and protein scaffolding actions.



KEYWORDS: Cyclic peptides, CFTR, cystic fibrosis, HDAC inhibition

Cystic fibrosis (CF)[1] is a hereditary loss-of-function disease that affects various tissues of the respiratory and gastrointestinal systems, resulting in progressive disability and early death.[1] The disease is caused by defective trafficking and/or function of the CF transmembrane conductance regulator (CFTR) protein, a cAMP-activated channel that conducts chloride and bicarbonate ions across the apical plasma membrane, and controls trans-epithelial sodium ion transport.[2−4] About 90% of cases involve the deletion of phenylalanine-508 (ΔF508-CFTR).[5] This mutation results in a misfolded protein that is retained in the endoplasmic reticulum (ER), resulting in its degradation by the ubiquitin proteasomal system (UPS), such that little reaches the cell surface.[6−8]

Because current therapeutic options for CF focus on treating the symptoms rather than the underlying etiology, new drugs are needed to advance the treatment modalities. There has been recent interest in directly enhancing the function of ΔF508-CFTR using chemical agents.[9] One approach utilizes potentiators that increase the open probability of CFTR,[10] but these agents only address a minor cohort of CF patients. Thus, a crucial therapeutic goal is to first correct the folding and defective trafficking in the ER, which can be achieved with pharmacological chaperones, in the form of compounds that bind directly to ΔF508-CFTR. Along these lines, there have been reports on quinazolines,[11,12] quinolines,[13] bithiazoles,[14] and pyrazolylthiazoles[15] that modestly improve the trafficking defect of ΔF508-CFTR. In a recent phase 2a clinical study, VX-809, which improves the trafficking of ΔF508-CFTR to the cell surface, reduced sweat chloride levels (key biomarker) in CF patients.[9]

Alternatively, the folding of ΔF508-CFTR can be corrected using small molecule epigenetic regulators that alter proteostasis so that the mutation is tolerated by the epithelial cell.[16] Inhibitors of zinc-dependent HDACs can modulate CFTR function and have the potential to rescue ΔF508-CFTR.[17−19] Additionally, treatment of CF cells with the potent HDAC inhibitor trichostatin A (**1**; TSA) activated CFTR transcription, presumably by altering chromatin structure.[17] In mucosal epithelial cells, **1** gave rise to an acetylation pattern that was linked to CFTR expression and chromatin-associated transcription factors.[18] Potent HDAC inhibitors **1** and vorinostat (**2**) were found to increase ΔF508-CFTR mRNA levels by >15-fold and ΔF508-CFTR protein levels by 25-fold in a CF lung cell line that expresses ΔF508-CFTR, albeit from a viral promoter.[19] Under these conditions, Hutt et al.[19] reported restoration of ΔF508-CFTR trafficking and activity following chemical inhibition of HDACs. Moreover, RNAi-mediated silencing of HDAC1 and HDAC7 enhanced ΔF508-CFTR stability and

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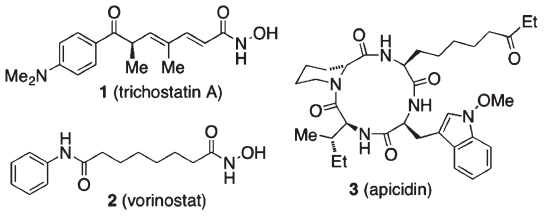


trafficking, while HDAC7 knockdown also improved function.[19]

Herein, we report on cyclic tetrapeptides, structurally related to the HDAC inhibitor natural product apicidin (**3**), that correct the maturation of ΔF508-CFTR from the ER, resulting in robust cell surface channel activity. Our data suggest potential involvement of both epigenetic and nonepigenetic processes related by protein acetylation pathways that adjust the proteostatic environment of the cell to promote favorable ΔF508-CFTR folding and function.

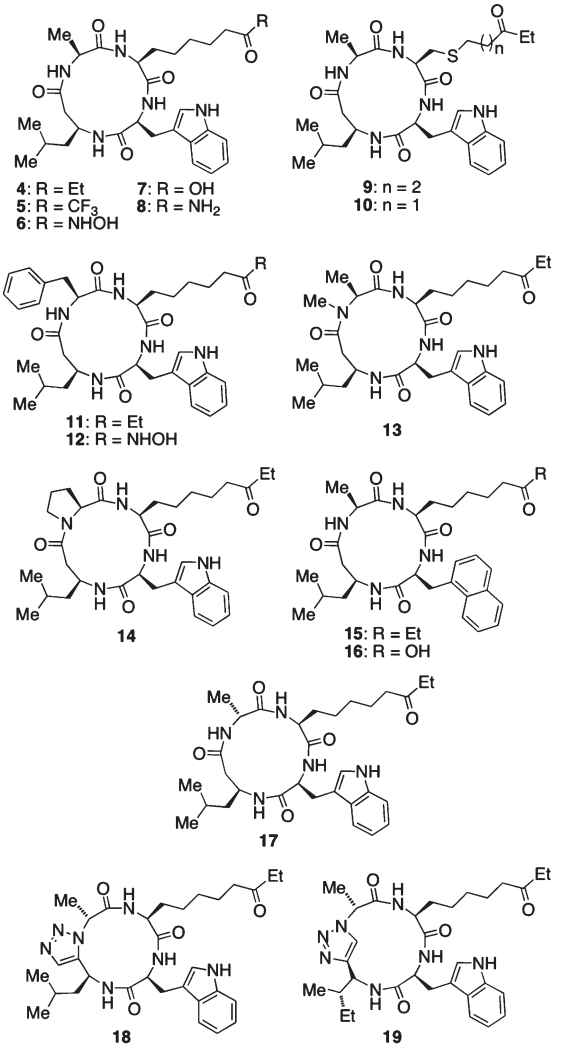
Conformationally biased cyclic tetrapeptides[20,21] and cyclic pseudotetrapeptides[22] related to apicidin (**3**) have potent HDAC inhibitory properties. Considering the connection between HDAC inhibition and the improvement of ΔF508-CFTR trafficking, we screened our collection of >40 derivatives (see Table S1 in the Supporting Information), many of which inhibited class I HDACs in the nanomolar range, for their ability to restore the function of ΔF508-CFTR. Our collection of cyclic tetrapeptides was designed to cover a broad range of pharmacophoric configurations related to the natural product apicidin (**3**). The inclusion of β-amino acids (**4**−**17**) or triazoles in the backbone (**18** and **19**) biased the macrocycles toward conformational homogeneity.[20−22] Amino acids were varied at each of the four positions to survey different stereochemistries, backbone amide alkylations, side chain identities, and zinc coordinating groups. The tetrapeptides of interest (Chart 1; **4**−**19**) were synthesized as described previously (Scheme S1 in the Supporting Information).[20−22]

Compounds were screened for their ability to restore ΔF508-CFTR function in CF bronchial epithelial cells expressing the halide-sensitive YFP-H148Q/I152L fluorescent reporter system (Table 1 and Figure 1).[23] Briefly, the fluorescence of this YFP mutant is quenched in the presence of halides, with iodide being the most potent. The rapid influx of extracellular iodide following activation with cAMP is dependent on the presence of a functional CFTR halide channel at the cell surface. The extent and rate of quenching indicate the level of CFTR and/or its activity at the cell surface.

The results of compound screening are reported in Table 1 as a fold increase in the rate of quenching relative to DMSO control. Several compounds were able to restore ΔF508-CFTR activity at concentrations of 1 μM, with **9**, **11**, and **15** being more potent than apicidin (**3**) (Table 1). Cyclic tetrapeptide **15**, our most potent corrector of ΔF508-CFTR function, showed greater effectiveness than the archetypal HDAC inhibitor vorinostat (**2**) in the iodide flux assay, at a 5-fold lower concentration (Figure 1a).[23] The level of ΔF508-CFTR correction activity for **15** was nearly 40%, which is a notable, clinically relevant value. The activity seen with **15** is due to restoration of ΔF508-CFTR since its effect was sensitive to the CFTR specific inhibitor CFInh-172 (Figure 1b).[24]

To further support the view that these cyclic tetrapeptides restore CFTR activity, we monitored the trafficking of the ΔF508-CFTR protein to the cell surface by Western blot analysis

Chart 1. Cyclic Tetrapeptides and Pseudotetrapeptides



(Figures 2 and S1 in the Supporting Information). During trafficking, wild-type CFTR and ΔF508-CFTR are glycosylated to give rise to proteins referred to as band B in an immunoblot analysis,[23] which are further processed in the Golgi to generate the slower migrating band C glycoform that indicates the protein has reached the cell surface (Figures 2 and S1 in the Supporting Information).[19,23] Band C was observed in the blots for most of the active cyclic tetrapeptides, which supports the restoration of cell surface CFTR activity by overcoming the trafficking defect associated with ΔF508-CFTR.

To gain additional insight into the relationship of HDAC inhibition to ΔF508-CFTR rescue, it is important to determine the HDAC isoform profile for the 11 known zinc-dependent isozymes according to the current state-of-the-art method. Unnatural trifluoroacetylated substrates have been developed to assay the class IIa HDACs (4, 5, 7, and 9),[25,26] which have markedly lower deacetylase activity against standard acetylated substrates than HDACs from class I (1, 2, 3, and 8), class IIb (6 and 10), and class IV (11).[27] Profiling by using these novel substrates revealed that the class IIa isoforms are rarely targeted effectively by known reference HDAC inhibitors at pharmacologically relevant concentrations. Accordingly, we had the HDAC

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Table 1. Results for ΔF508-CFTR Rescue and Selected HDAC Inhibition[a]

		IC$_{50}$ values (nM)			
compd	CFTR[b] (at 1 μM)	HDAC1	HDAC3[c]	HDAC6	HDAC8
4	60 ± 10	26	140	>10 μM	2200
5	IA	ND	62	3400	ND
6	60 ± 10	2	18	31	130
7	IA	120	610	2300	2300
8	IA	ND[d]	ND[d]	ND[d]	ND[d]
9	IA (140 ± 10 at 10 μM)	20	62	>10 μM	4900[e]
10	IA	>10 μM	>10 μM	>10 μM	>10 μM
11	120 ± 10	47	44	>10 μM	ND
12	70 ± 10	8	31	70	ND
13	50 ± 20	50	550	>10 μM	2000
14	100 ± 10	12	280	>10 μM	15
15	140 ± 30	8	32	>10 μM	ND
16	IA	200	990	4900	ND
17	IA	6500	4300	ND	ND
18	80 ± 10	7	9	6100	105
19	80 ± 10	25	16	>10 μM	ND
3	100	22	29	>10 μM	755
2[f]	30 (100 at 5 μM)	41	41	18	2000

[a] New compounds were purified by HPLC and characterized by MS and ^{1}H NMR (see the Supporting Information). HDACs 1, 3, and 8 are from class I; HDAC 6 is from class IIb. HDAC inhibition is given as IC$_{50}$ values, which are the means of at least two experiments performed in duplicate, unless otherwise noted; ND, not determined. Some HDAC inhibition data were previously published by us (refs 20−22). [b] The ΔF508-CFTR values are reported as fold increase over DMSO background (rate constant = 8.0 × 10^{-4}sec^{-1}) in rate of fluorescence decay based on iodide conductance with lung epithelial cells (CFBE41o-) expressing ΔF508-CFTR, at 1 μM test compound, unless otherwise noted. Data are means of two experiments performed in duplicate; IA, inactive at 1 μM. [c] In complex with nuclear receptor corepressor 2 (NCoR2). [d] Weak HDAC inhibition: IC$_{50}$ = 2700 nM in a HeLa cell nuclear extract assay. [e] Not performed in duplicate. [f] IC$_{50}$ values are from BPS Bioscience (ref 28).

inhibition profile for key compound **15** performed at BPS Bioscience (San Diego, CA), employing appropriate substrates for all of the HDAC isoforms (Table 2).[28] The IC$_{50}$ values for HDAC1 and HDAC3 from BPS for **15** were somewhat higher than those determined by us, probably due to their use of different enzyme substrates. Despite the broader, more potent HDAC inhibition profile of **2** as compared to **15** (Table 2), **15** was much more potent as a ΔF508-CFTR correcting agent. Likewise, **11** corrected CFTR channel function with greater potency than its analogue, **12** (Figure 3), even though **12** had broader, more potent HDAC inhibitory activity (Table 1). Additionally, several compounds with potent class I HDAC inhibition were not active in the CFTR assay (Table S1 in the Supporting Information). It is tempting to speculate that one reason for the improved ΔF508-CFTR rescue activity of **15** relative to **2** and **11** relative to **12** is that **15** and **11** lack potent inhibition of HDAC6, which is known to positively regulate the activity of Hsp90, a chaperone involved in CFTR processing.[29,30] From considering this data set, it would appear that potent inhibition of class I HDACs is necessary, but not sufficient, for correcting ΔF508-CFTR function.

Our findings draw attention to the question of how HDAC inhibition relates to the mechanism of ΔF508-CFTR rescue.

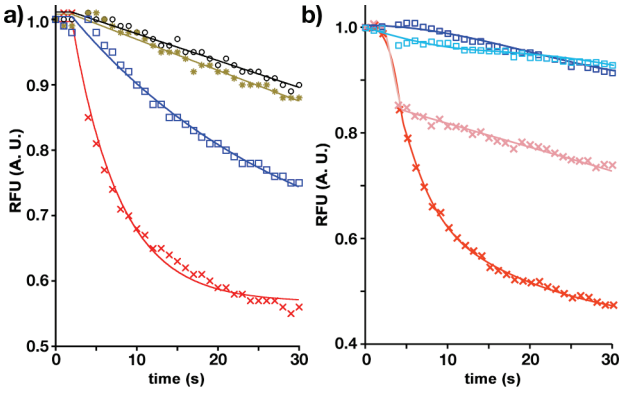

Figure 1. Cyclic tetrapeptides restore cell surface CFTR activity to ΔF508-CFTR. (a) Yellow fluorescent protein (YFP)-quenching curves for CFBE41o-YFP cells alone (black open circles) or treated for 24 h with DMSO (taupe stars), 1 μM **15** (red crosses), or 1 μM vorinostat (**2**; blue open squares) after stimulation with forskolin and genistein. (b) YFP-quenching curves for CFBE41o-(YFP) cells treated for 24 h with **15** and stimulated with forskolin and genistein in the presence (pink crosses) or absence (red crosses) of CFInh172. Control: DMSO in the absence (blue squares) or presence (light blue squares) of CFInh172. Abbreviations: RFU, relative fluorescence units; A.U., arbitrary units.

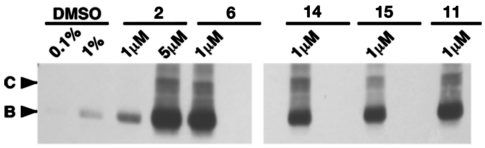

Figure 2. Cyclic tetrapeptides restore trafficking to ΔF508-CFTR. Western blot analysis of CFTR glycoforms following treatment with the indicated compounds.

Clearly, siRNA silencing experiments indicate that the knockdown of HDAC7 is a relevant molecular mechanism in ΔF508-CFTR rescue.[19] So, how might one explain the absence of HDAC7 inhibition for cyclic tetrapeptides that are active in CFTR rescue, such as **3** and **15**? It is known that expression of HDAC7 is selectively down-regulated in cells treated with vorinostat (**2**) or the depsipeptide HDAC inhibitor romidepsin.[31] Thus, we carried out a Western blot analysis to assess whether treatment of the lung epithelial cells with **15** would likewise suppress expression of HDAC7, secondary to the inhibition of class I HDACs. Indeed, this analysis indicated a reduction in expression of HDAC7 (see Figure S2 in the Supporting Information). Although the class IIa HDACs have much lower intrinsic deacetylase activity against known acetylated lysine substrates, they are able to bind ε-*N*-Ac-Lys residues on histone tails with affinities comparable to class I and class IIb HDACs.[26,31,32] HDAC7 could therefore function in cells as an ε-*N*-Ac-Lys-binding protein or could act on as yet undetermined substrates, to exert its influence in the process of ΔF508-CFTR rescue, rather than by functioning directly as a deacetylase.

Our results suggest avenues for further structure−activity optimization. First, CFTR activity varied considerably for a subset of compounds that differ by only a single amino acid substitution, for example, altering position four resulted in increased activity in the following order: **4** (Ala) < **14** (Pro) < **11** (Phe). Second, replacement of the naphthyl group in our most active compound, **15**, with indole, as in **4**, led to substantial

Table 2. IC_{50} Values (nM) in HDAC Profiling for Compounds 15 and 2[a]

compd	HDAC1	HDAC2	HDAC3[b]	HDAC4	HDAC5	HDAC6
15	120	290	180	>10000	>10000	>10000
2	41	66	41	inactive	15000	18

compd	HDAC7	HDAC8	HDAC9	HDAC10	HDAC11
15	>10000	>10000	>10000	150	300
2	21000	2000	inactive	58	133

[a] Determined by BPS Bioscience (ref 28). [b] In complex with nuclear receptor corepressor 2 (NCoR2).

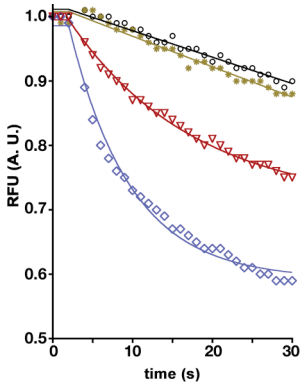

Figure 3. Iodide ion flux data for **11** (blue open squares) and **12** (red open triangles), which differ only in the Zn^{2+}-binding ketone (**11**) or hydroxamic acid (**12**) moiety, at 1 μM; results for 0.1% DMSO control (taupe stars) and absence of treatment (black open circles). Abbreviations: See Figure 1.

reduction of activity. Further variations at these two positions could yield more potent compounds. Finally, given that the collection of active compounds contains cyclic tetrapeptides with different stereochemistry and different backbone subunits, via β-amino acids or disubstituted 1,2,3-triazoles, our lead compounds offer a basis for future improvements.

In summary, we have discovered that certain cyclic tetrapeptides possess notable activity in correcting ΔF508-CFTR function. The relationship between HDAC inhibition and CFTR function at the cell surface is intriguing and leads to the suggestion that ΔF508-CFTR maturation could reflect distinct steps in folding, trafficking, and chloride channel function at the cell surface that may be differentially sensitive to distinct HDAC-based biological pathways. More generally, our findings provide further impetus for focused studies on the relationship between HDAC inhibition and CF biology. In this realm, the modulation of epigenetic or non-epigenetic processes, linked by protein acetylation/deacetylation cycles that impact the functional protein environment of the cell, could offer a useful approach for treating not only CF but also other protein-misfolding diseases.[33−35]

■ ASSOCIATED CONTENT

Supporting Information. Experimental methods, compound characterization data, full table of cyclic peptides tested in the CFTR flux assay (Table S1), and Scheme S1 and Figures S1 and S2. This material is available free of charge via the Internet at http://pubs.acs.org.

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Author Contributions

⊥These authors contributed equally to this work.

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