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Capturing the direct binding of CFTR correctors to CFTR using click chemistry

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Abstract

Cystic fibrosis (CF) is a lethal genetic disease caused by the loss or dysfunction of the CF transmembrane conductance regulator (CFTR) channel. F508del is the most prevalent mutation of the *CFTR* gene and encodes a protein defective in folding and processing. VX-809 has been reported to facilitate the folding and trafficking of F508del-CFTR and augment its channel function. The mechanism of action of VX-809 previously has been poorly understood. In this study, we sought to answer a fundamental question underlying the mechanism of VX-809: Does it bind CFTR directly to exert its action? We synthesized two VX-809 derivatives, ALK-809 and SUL-809, which possess an alkyne group and retain the rescue capacity of VX-809. By using a Cu(I)-catalyzed click chemistry, we provide evidence that the VX-809 derivatives bind CFTR directly *in vitro* and in cells. Our findings will contribute to elucidation of the mechanism of action of CFTR correctors and design of more potent therapeutics to combat CF.

Keywords

CFTR correctors; click chemistry; cystic fibrosis; protein expression; VX-809

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Supporting information for this article is given via a link at the end of the document.

Cystic fibrosis (CF) is the most common autosomal recessive genetic disorder among Caucasians. CF is caused by mutations in the gene encoding an ion-channel protein, the CF transmembrane conductance regulator (CFTR).^[1] CFTR is predominantly expressed at the apical plasma membrane of epithelial cells lining several organs and functions as a cAMP-regulated chloride/bicarbonate channel.^[2] Mutations in the *CFTR* gene may cause defects in protein synthesis, processing, and trafficking and channel gating/conductance, consequently affecting CFTR-mediated transepithelial chloride and fluid homeostasis.^[3] Clinically, CFTR dysfunction causes CF lung disease, gastrointestinal disorders, pancreatic disease, hepatic disease, and reproductive abnormalities.^[1a, 4] Over 1900 *CFTR* mutations have been identified to date and can be categorized into different classes based on the nature of the resulting CFTR defects.^[4a, 5] The most-common mutation (c.1521-1523delCTT) encodes a mutant protein with a deletion of phenylalanine at position 508 (F508del-CFTR). F508del-CFTR has folding defect and is retained in the endoplasmic reticulum (ER), where it is targeted to ubiquitin-dependent proteosomal degradation.^[6] Several physical, genetic, and pharmacological approaches have been shown to partially rescue F508del-CFTR by facilitating its exit from the ER and allowing it to traffic to the plasma membrane, thereby restoring the channel function.^[7] Among pharmacologic agents, VX-809 showed promise as a CFTR corrector by modulating the folding and trafficking of F508del-CFTR, thus improving its expression, stability, and single-channel open probability at the plasma membrane.^[8] Effects of VX-809 seem to be specific for CFTR.^[5, 9] Recently, a Phase 3 combination study of VX-809 and ivacaftor (VX-770, a CFTR potentiator) showed positive results in CF patients homozygous for F508del and U.S. Food and Drug Administration (FDA) approved this combination therapy to treat CF patients ages 12 and older with two copies of F508del.^[10] However, the mechanism of action of VX-809 has been poorly understood. In this study, we sought to answer a fundamental question regarding the mechanism of action of VX-809: Does VX-809 bind CFTR directly to exert its effect?

To study the mechanism of action of VX-809 in rescuing F508del-CFTR, and especially to tackle the fundamental question of whether VX-809 binds directly to CFTR to exert its effect, one convenient approach is to generate derivatives of VX-809 that not only retain the rescuing capacity of VX-809, but also possess a tag for biochemical investigations (e.g., immunoprecipitation or microscopic visualization). In this study, we used click-chemistry-based approaches to determine whether there is direct binding between VX-809 derivatives and CFTR. A click chemistry is a bio-orthogonal reaction that can efficiently yield a specific product.^[11] High selectivity and the exquisite reliability of click chemistry render it broadly applicable and beneficial for chemical and biological studies.^[12] We first designed two VX-809 derivatives, ALK-809 and SUL-809, which possess an alkyne functional group at one end (Scheme 1). The rationale for generating these alkyne-containing VX-809 derivatives includes (1) the alkyne group can be specifically conjugated to biotin-azide molecules through Cu(I)-catalyzed cycloaddition, and by using the conjugated biotin moiety as a tag, it is possible to precipitate the corrector-associated CFTR and visualize their association in cells, thus gaining direct evidence whether these correctors bind directly to CFTR, and (2) complementary click chemistry approaches (Figure 1) can be explored *in vitro* and in cells, which will corroborate our findings. ALK-809 and SUL-809 were synthesized according to Scheme 2.

To determine whether ALK-809 and SUL-809 retain the rescuing capacity of VX-809, we treated HEK293-FLAG-F508del-CFTR cells (HEK293 cells expressing a FLAG-F508del-CFTR) with these compounds for 24 h and then used Western blotting to probe CFTR expressions. We found that both VX-809 derivatives had increased efficacy (statistically significant) in promoting F508del-CFTR maturation compared to VX-809 (Figure 2A) and that, similar to VX-809, ALK-809 and SUL-809 increased the expression of wild-type (WT)-CFTR in HEK293-FLAG-WT-CFTR cells (HEK293 cells expressing a FLAG-WT-CFTR) (Figure 2B). The differences in efficacies between VX-809 and SUL-809, VX-809 and ALK-809 are not statistically significant for WT-CFTR. Our data suggest that the introduction of an alkyne group did not compromise the efficacy of VX-809 in rescuing the maturation of F508del-CFTR.

We utilized two assays to investigate whether ALK-809 and SUL-809 augment the channel function of F508del-CFTR. The first assay monitored CFTR-mediated iodide efflux in HEK293-FLAG-F508del-CFTR cells pretreated with VX-809, ALK-809, or SUL-809. We found that both ALK-809 and SUL-809 significantly increased the rate of iodide efflux compared to DMSO-pretreated controls, with 5- to 10-fold increases in maximal efflux rate (Figure 3A). The differences in efficacies between VX-809 and SUL-809, VX-809 and ALK-809 are not statistically significant.

We also tested the effect of ALK-809 and SUL-809 on the channel function of F508del-CFTR in primary human bronchial epithelial (HBE) cells from CF patients homozygous for F508del. As shown in Figure 3C and Figure S1, ALK-809 and SUL-809 increased the F508del-CFTR-mediated short-circuit current (I_{sc}) with efficacies comparable to VX-809. The specificity of CFTR-mediated I_{sc} was verified by inhibition using a CFTR channel blocker, CFTR_{inh}-172 (Figure 3C and Figure S1). Taken together, our data demonstrate that ALK-809 and SUL-809 retain the F508del-CFTR-correcting capacity of VX-809. Considering that these derivatives were modified based on the structure of VX-809, they represent useful tools to study the mechanism of action of VX-809.

After confirming that ALK-809 and SUL-809 have similar efficiencies to that of VX-809 in augmenting the expression and function of F508del- and WT-CFTR, we performed *in vitro* click chemistry using cell lysates from HEK293-FLAG-WT-CFTR cells or HEK293-FLAG-F508del-CFTR cells pre-treated with ALK-809, SUL-809, or VX-809 (used as a negative control), Biotin-PEG3-Azide or Sulfo-Link-Biotin-Azide, and reagents from the Click-iT[®] protein-reaction buffer kits (Figure 1, Approach 1). HEK293 parental cells were used as negative controls. We precipitated the protein complexes using NeutrAvidin beads and immunoprobed for CFTR. Our data suggested that ALK-809 and SUL-809 bind WT-CFTR (Figure 4A), and that upon using Sulfo-Link-Biotin-Azide, the effect was more prominent (Figure 4B). Due to the low expression level of F508del-CFTR, we were unable to observe binding between F508del-CFTR and either ALK-809 or SUL-809 using this immunoblotting approach.

We therefore employed an alternative approach to perform the *in vitro* click chemistry. We treated HEK293-FLAG-WT-CFTR cells or HEK293-FLAG-F508del-CFTR cells with ALK-809 or VX-809 (used as a negative control). We then used anti-FLAG beads to

immunoprecipitate CFTR (please note that by doing so, we increased the CFTR concentration for the following click chemistry reaction), which would also have the bound corrector molecules if there is direct binding. The beads were used for solid-phase click chemistry to conjugate Sulfo-Link-Biotin-Azide, and the end-products were probed for biotin activity using streptavidin HRP (Figure 1, Approach 2). We found that WT- and F508del-CFTR, when treated with ALK-809, exhibited significantly higher HRP activity than VX-809-treated controls (Figure 4C), suggesting that ALK-809 was bound to both WT- and F508del-CFTR.

Since our *in vitro* data suggested that VX-809 derivatives bind directly to F508del-CFTR to exert their correcting effects, we continued to investigate whether such an association can be visualized in cells. We treated HEK293-FLAG-WT-CFTR cells and HEK293-FLAG-F508del-CFTR cells with ALK-809 or VX-809, performed in-cell click chemistry with Sulfo-Link-Biotin-Azide, and fluorescently labeled CFTR and biotin for visualization of their locations. HEK-293 parental cells were used as negative controls. Our data showed that the biotin moiety was localized intracellularly in ALK-809-treated HEK293-FLAG-WT-CFTR cells and HEK293-FLAG-F508del-CFTR cells, indicating the specificity of the click-chemistry-based biotin tagging of the alkyne derivative of VX-809 (Figure 5). We observed that 0.02% of total ALK-809 (green) is co-localized with WT-CFTR (red) and 0.023% of ALK-809 is co-localized with F508del-CFTR in selected region of interest (ROI). Importantly, the intracellular co-localizations were observed primarily in regions near the nuclei (Figure 5). As expected, no click chemistry reaction was observed in VX-809 treated cells or in parental HEK293 cells. Our results suggest that ALK-809 did enter the cells, bound F508del-CFTR, and facilitated the processing and trafficking of the misfolded protein to the plasma membrane.

A recent study by Ren and colleagues suggests that VX-809 modulates the conformation of membrane-spanning domain 1 (MSD1) of CFTR to enhance the interactions among MSD1, nucleotide-binding domain1 (NBD1), and MSD2, therefore increasing global protein folding and assembly and restoring CFTR function.^[13] The most significant and innovative feature of our study lies in the utilization of a bio-orthogonal click chemistry approach to demonstrate that two derivatives of VX-809, ALK-809 and SUL-809, bind directly to F508del-CFTR and WT-CFTR *in vitro* and associate with CFTR intracellularly, especially in regions near the nuclei, a finding consistent with the current thinking that VX-809 may induce F508del-CFTR conformational change during its synthesis and processing. To the best of our knowledge, our study is the first to use a novel click chemistry approach to provide direct evidence for direct binding between VX-809 derivatives and CFTR.

The click chemistry approach we used facilitates the capture of corrector-associated F508del-CFTR-containing macromolecular complexes (interactome). In combination with using modern proteomics technology, we might be able to analyze these macromolecular complexes and identify important proteins involved in the process of CFTR rescue. Such information will systematically advance our understanding of the mechanisms of action of CFTR correctors.

In conclusion, we designed and synthesized two alkyne-containing VX-809 derivatives that retain the rescuing capability of VX-809 on F508del-CFTR. By using a Cu(I)-catalyzed click chemistry approach, we conjugated these alkyne-containing CFTR correctors to biotin-azide compounds and provide *in vitro* and in cells evidence to support our hypothesis that CFTR correctors bind directly to CFTR to exert therapeutic action. Our findings will contribute to a better understanding of mechanisms underlying CFTR correction and facilitate the design of more-potent and specific correctors to combat CF.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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A) Approach 1

B) Approach 2

Figure 1.

Schematic representation of click chemistry approaches used in this study. Approach 1 and Approach 2 depicted here are complementary to each other and were used to test our hypothesis that VX-809 binds directly to CFTR to exert its rescue functionality. Please note that the depiction does not reflect the actual sizes of the molecules or moieties. IP: immunoprecipitation.

Figure 2.

ALK-809 and SUL-809 retained the efficacy of VX-809 in rescuing and promoting the maturation of F508del-CFTR. **A)** (Left panel) a representative blot showing that ALK-809 and SUL-809 rescued and promoted the maturation of FLAG-F508del-CFTR expressed in HEK293 cells (the cells are named as HEK293-FLAG-F508del-CFTR cells in this manuscript). DMSO was used as the solvent control. A purified FLAG-WT-CFTR protein was used to help characterize the maturation of F508del-CFTR. Band 'C' represents the mature CFTR, whereas band 'B' represents the immature CFTR. (Right panel) quantification of data from experiments as represented in the left panel ($n = 3$). The maturation levels of F508del-CFTR under different treatment conditions were quantified as the percentage expression of mature band C normalized to the immature band B. * $P < 0.05$ compared to DMSO control. **B)** (Left panel) a representative blot showing that ALK-809 and SUL-809 increased the expression of FLAG-WT-CFTR expressed in HEK293 cells (the cells are named as HEK293-FLAG-WT-CFTR cells in this manuscript). (Right panel) quantification of the data from experiments as represented in the left panel ($n = 3$). The WT-CFTR expression levels were normalized to β -actin, the loading control. * $P < 0.05$ compared to DMSO control. Parental: HEK293 cells.

Figure 3.

ALK-809 and SUL-809 augmented the channel function of F508del-CFTR. **A)** ALK-809 and SUL-809 augmented the channel function of FLAG-F508del-CFTR expressed in HEK293 cells. (Left panel) representative traces of CFTR-mediated iodide efflux under different treatment conditions. Parental: HEK293 cells. F508del: FLAG-F508del-CFTR channel. (Right panel) bar graph showing the mean maximal iodide efflux rate at 2 min after adding CFTR channel activating agents, forskolin and IBMX. * $P < 0.05$ compared to DMSO control. **B)** Representative CFTR-dependent short-circuit currents (I_{sc}) in polarized primary bronchial epithelial cells isolated from a non-CF subject (top panel) or F508del homozygous CF patient (bottom panel). VX-770 is a CFTR potentiator. **C)** (Left panel) ALK-809 and SUL-809 augmented the F508del-CFTR mediated I_{sc} in primary bronchial epithelial cells from F508del homozygous CF patients. (Right panel) a CFTR channel blocker, CFTRinh-172, was used to verify the effects shown in the left panel were CFTR mediated. All data represent the results from three independent experiments ($n = 3$). * $P < 0.05$ compared to DMSO control.

Figure 4.

ALK-809 and SUL-809 bind directly to CFTR. **A)** Immunoblots showing the binding of ALK-809 and SUL-809 to FLAG-WT-CFTR expressed in HEK293 cells. Biotin-PEG3-Azide was used for click chemistry (please see Approach 1, Figure 2). **B)** Immunoblots showing the binding of ALK-809 to FLAG-WT-CFTR expressed in HEK293 cells. Sulfo-Link-Biotin-Azide was used for click chemistry (please see Approach 1, Figure 1). **C)** ALK-809 binds to WT- and F508del-CFTR as evidenced by solid-phase click chemistry approach (please see Approach 2, Figure 1). The bar graph represents quantification of the streptavidin-HRP activity of click chemistry end-products, which reflects the binding of CFTR correctors to CFTR. The solid-phase click chemistry was carried out with ALK-809-bound CFTR (on FLAG beads), Sulfo-Link-Biotin-Azide, and other reagents from the Click-iT[®] protein reaction buffer kit according to the protocol provided by the manufacturer. The data represent the results from three independent experiments (n = 3). * $P < 0.05$. Parental: HEK293 cells. WT: FLAG-WT-CFTR expressed in HEK293 cells. F508del: FLAG-F508del-CFTR expressed in HEK293 cells. Please see the Experimental section for details.

Figure 5.

Localization and association of ALK-809 and CFTR can be visualized in cells using a click chemistry approach. Immunofluorescence data showing the localization of CFTR (red) and biotin (green) in VX-809- and ALK-809-treated parental, HEK293-FLAG-WT-CFTR and HEK293-FLAG-F508del-CFTR cells. Blue indicates the nuclei stained with DAPI. The in-cell click chemistry was performed using Sulfo-Link-Biotin-Azide and following the manufacturer's instructions. F508del-CFTR: HEK293-FLAG-F508del-CFTR cells. WT-CFTR: HEK293-FLAG-WT-CFTR cells. Parental: HEK293 cells.

Scheme 1.

The structures of VX-809 and its alkyne derivatives, ALK-809 and SUL-809.

Scheme 2. Synthesis of **A)** ALK-809 and **B)** SUL-809. rt: room temperature.