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Article

Keywords:

Posted Date: July 3rd, 2026

DOI: <https://doi.org/10.21203/rs.3.rs-10093540/v1>

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Additional Declarations: There is **NO** Competing Interest.

Variant-selective RNA-targeting small molecules partially restore CFTR W1282X function in Cystic Fibrosis

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Keywords: Cystic Fibrosis, mRNA, small molecules, CFTR, splicing

ABSTRACT

RNA is an emerging therapeutic target enabling modulation of gene expression upstream of protein synthesis. Small molecules offer a powerful approach to targeting RNA by exploiting the dynamic structural landscape of the molecule. Here we show that the p.W1282X nonsense variant in CFTR, a common Class I variant causing cystic fibrosis, induces a distinct localized RNA conformation in exon 23 that persists within the cellular environment and differs significantly from the wild-type transcript. Using structure-guided virtual screening and biophysical validation, we identified small molecules that selectively engage this mutant RNA conformation over wild-type. ΔSHAPE-MaP analysis confirmed that the lead compound CTC20 and its SAR-guided analog CTC24 induce localized structural changes centered on the primary binding site at variant site, with CTC24 demonstrating greater potency and variant selectivity. In CFF-16HBEg-W1282X cells, both compounds produced directional increases in CFTR mRNA abundance, CFTR protein expression, and CFTR-mediated chloride transport, consistent with partial stabilization of the W1282X transcript against NMD-mediated degradation. These findings establish proof-of-concept for a variant-selective, small molecule RNA-targeting strategy for Class I cystic fibrosis and introduce a generalizable paradigm for structure-guided targeting of disease-associated RNA conformations in monogenic disorders.

INTRODUCTION

For decades, drug discovery has centered on protein targets, reflecting their pivotal roles in disease pathogenesis and cellular signaling. This protein-centric paradigm has yielded many therapeutic breakthroughs; however, currently approved drugs collectively engage only ~3% of the human proteome [1, 2]. In contrast, though approximately 75-80% of the human genome is transcribed into RNA, only ~1.5% is translated into proteins [3, 4], leaving the vast transcriptome, both non-coding and coding, largely untapped as a therapeutic resource. Beyond simply expanding the number of druggable targets, however, RNA targeting offers a

distinct mechanistic advantage even for coding transcripts: disease-causing mutations can alter RNA secondary structure in ways that create unique structural elements absent in the wild-type (WT) sequence, enabling allele-specific targeting with a level of selectivity that is difficult to achieve through protein-directed approaches. Together, these considerations, the breadth of the RNA as a target class and the potential for mutation-specific structural selectivity, underscore the need to re-evaluate traditional drug discovery strategies and position RNA as a compelling therapeutic target,

particularly for diseases previously considered undruggable by protein-directed approaches [5].

Recent clinical successes have validated RNA as a druggable target class for both small molecules and oligonucleotide-based therapeutics. The FDA approval of risdiplam, a small molecule that binds a specific site in SMN2 pre-mRNA to modulate splicing and treat spinal muscular atrophy, demonstrated that small molecules can selectively target RNA structures with therapeutic precision [6-8]. Oligonucleotide-based approaches, including antisense oligonucleotides (ASOs) and splice-switching strategies, have further established the feasibility of modulating RNA function with high sequence specificity [9-12]. This success reflects a broader recognition that RNA secondary structures, including hairpins, internal loops, and bulge motifs, form well-defined three-dimensional binding pockets amenable to small molecule recognition [13], and that the sequence-dependence of these structures can enable selective targeting of specific transcripts, including those harboring disease-causing mutations. Dysregulated RNA expression, aberrant splicing, and pathogenic variants underlie a wide spectrum of human diseases, including genetic disorders, cancers, and viral infections. Therapeutic modulation of RNA can correct these defects upstream of protein synthesis, restoring cellular homeostasis through novel mechanisms of action [14-18].

Beyond oligonucleotide-based approaches, small molecules targeting RNA may offer some advantages: they can be optimized using established medicinal chemistry frameworks, they possess favorable pharmacokinetic profiles, and may be amenable to oral administration [19, 20]. Importantly, in contrast to many gene therapy approaches, RNA-targeted therapies do not entail permanent genomic alterations, offering reversible and tunable control over gene expression [21, 22].

Realizing the full potential of RNA-targeted therapeutics requires a deeper understanding of RNA biology, particularly the structural and

dynamic principles governing RNA folding, recognition, and function [19, 23, 24]. These structural insights are essential for rational drug design, enabling the identification of mutation-specific binding pockets and the development of selective small molecules that target disease-relevant RNA conformations [19, 24, 25].

Cystic fibrosis (CF) is one disease with unmet therapeutic needs that could be a good candidate for RNA-targeted approaches. CF is a life-limiting autosomal recessive disorder caused by variants in the CF transmembrane conductance regulator (CFTR) gene, which encodes an epithelial ion channel critical for chloride and bicarbonate transport [26-28]. CFTR dysfunction disrupts ion and fluid homeostasis across epithelial surfaces, leading to multisystem disease, most notably in the lungs, pancreas, liver, and intestines [26, 27, 29]. In the respiratory tract, impaired CFTR activity compromises mucociliary clearance, resulting in viscous mucus, chronic infection, and progressive airway damage [30-33].

Current CF therapies target many but not all CF-causing variants, variant affecting protein folding or channel gating [34, 35]. However, people with CF (pwCF) harboring non-responsive variants do not have good therapeutic options [29, 36]. Among these, variants, nonsense variants such as CFTR p.W1282X (c.3846G>A) pose a significant therapeutic challenge. This variant introduces a premature termination codon (PTC), triggering nonsense-mediated mRNA decay (NMD) and drastically reducing CFTR mRNA levels [37-39]. Although the truncated CFTR W1282X protein retains partial function, its expression is limited by efficient NMD, impeding the efficacy of protein-correcting therapies [37, 40].

ASOs have emerged as a promising strategy to address this unmet need. Targeting exon 23, which harbors the W1282X variants, by ASO-induced exon skipping can stabilize CFTR-W1282X mRNA and promote production of a partially functional CFTR- Δ 23 isoform [41-44]. This

approach exemplifies the therapeutic potential of RNA modulation in CF and highlights the broader applicability of RNA-targeted strategies for genetic diseases driven by transcript-level defects [37, 38]. However, ASO-based approaches for CF face translational barriers, including delivery to the airways, motivating the parallel development of small molecules capable of engaging the W1282X RNA target directly with the pharmacokinetic advantages inherent to small molecule therapeutics.

Here, we demonstrate that the p.W1282X variant induces a distinct localized hairpin conformation in CFTR exon 23 *in vitro*, that is absent in the WT transcript, and that local conformational differences between CFTR WT and W1282X RNA persist within the cellular environment despite similar global secondary structure topologies. Using structure-guided screening, we identified small molecules that selectively bind the W1282X-induced conformation. To translate these virtual screening findings into testable therapeutics, we executed the targeted chemical synthesis of the lead compound, CTC20, and its structurally related analog, CTC24. These RNA-targeting small molecules produce a directional dose-dependent increase in CFTR mRNA abundance, directional improvements in CFTR protein expression, and partial restoration of CFTR-mediated chloride transport in W1282X-expressing cells. This work provides proof-of-concept for a variant-specific, small-molecule approach to Class I CF variants previously inaccessible to current therapies and establishes a foundation for further optimization toward clinically meaningful efficacy.

METHODS

DNA Templates

DNA templates for short RNAs (<200 nt) were generated by PCR amplification using primers designed with Primerize [45, 46] and synthesized by Thermo Fisher Scientific. Templates for longer RNAs (>200 nt) were obtained through the GeneArt gene synthesis service (Thermo Fisher Scientific). Each DNA template contained a T7

promoter sequence, and those intended for SHAPE assays additionally included 5' and 3' SHAPE cassettes. These cassettes serve as structured reference elements that enhance transcript stability during reverse transcription, enable accurate normalization and alignment of reactivity profiles, and provide internal controls for primer extension efficiency [47-52]. The sequences of all DNA templates used in this study are provided in **Supplementary Table 1**.

PCR Using Primerize Primers

Primerize was used to design four to eight primers for each DNA template [45, 46]. PCR reactions (50 μ L total volume) were prepared containing 2 μ L each of 100 μ M outer forward and reverse primers, 2 μ L of 1 μ M inner primers, 10 μ L of 5 $\times$ HF buffer, 1 μ L of 10 mM dNTP mix, 1 μ L of Phusion High-Fidelity DNA Polymerase (Thermo Fisher Scientific), and nuclease-free water to a final volume of 50 μ L. Thermal cycling was performed with the following parameters: initial denaturation at 98 $^{\circ}$ C for 30 s; 30 cycles of denaturation at 98 $^{\circ}$ C for 10 s, annealing at 60 $^{\circ}$ C for 30 s, and extension at 70 $^{\circ}$ C for 30 s; followed by a final extension at 72 $^{\circ}$ C for 10 min.

PCR products were analyzed on 2–4% agarose gels prepared in 1 $\times$ TBE buffer (Tris–Borate–EDTA) and stained with SYBR Safe DNA Gel Stain (Thermo Fisher Scientific). Electrophoresis was performed at 100 V for 30 min, and the bands were visualized under UV illumination.

PCR Using DNA Fragments

PCR reactions (50 μ L total volume) were prepared by combining two 25 μ L mixtures. Mixture 1 contained 0.1–0.25 μ g of DNA template, 1 μ L of 10 mM dNTPs, 1 μ L each of 10 μ M forward and reverse primers, and nuclease-free water to a final volume of 25 μ L. Mixture 2 contained 5 μ L of 10 $\times$ PCR reaction buffer, 0.25 μ L of Taq DNA Polymerase (5 U/ μ L), and 19.75 μ L of nuclease-free water. The two mixtures were combined immediately prior to thermal cycling. Thermal cycling conditions were as follows: initial

denaturation at 94 °C for 2 min; 30 cycles of denaturation at 94 °C for 30 s, annealing at the primer-specific melting temperature for 60 s, and extension at 72 °C for 90 s; followed by a final extension at 72 °C for 7 min. PCR products were analyzed as described above.

***In Vitro* Transcription and Purification of RNA**

In vitro transcription (IVT) was carried out using standard T7 RNA polymerase reactions[53] in a total volume of 50 µL. Each reaction contained 1–5 µg of linearized DNA template, 2.5 µL of 20× transcription buffer (100 mM Tris-HCl, pH 8.0; 100 mM spermidine; 200 mM DTT), the optimal concentration of MgCl₂, 5 µL of 50% PEG8000, 4 µL of each 100 mM NTP, an appropriate amount of T7 RNA polymerase, and nuclease-free water to bring the volume to 50 µL. Reactions were incubated at 37 °C for 30 min to 3 h, depending on RNA length.

Following transcription, RNAs were purified by electroelution from denaturing polyacrylamide gels prepared in 1× TBE buffer, followed by ethanol precipitation in the presence of sodium acetate overnight at –20 °C. RNA pellets were collected by centrifugation at 15,000 rpm for 15 min at 4°C, and the supernatant was carefully removed. The pellets were washed with 70% ethanol, air-dried, and resuspended in nuclease-free water for downstream applications. Purified RNAs were stored at -20°C or -80°C until use.

Selective 2' Hydroxyl Acylation analyzed by Primer Extension (SHAPE)

RNA samples (1 µM) were denatured in 2× folding buffer (100 mM HEPES, pH 8.0; 200 mM NaCl; 0.2 mM EDTA) by heating at 95 °C for 5 min, followed by rapid cooling on ice for 5 min. Refolding was carried out by incubating the RNA in refolding buffer (50 mM HEPES, pH 8.0; 100 mM NaCl; 5 mM MgCl₂) for 30 min at 37 °C. Chemical probing was performed using 1-methyl-7-nitroisatoic anhydride (1M7), 2-methylnicotinic acid imidazolide (NAI), 5-nitroisatoic anhydride (5NIA), or dimethyl sulfate (DMS). Stock solutions

of 1M7, NAI, and 5NIA were prepared in dimethyl sulfoxide (DMSO), while DMS was diluted 1:10 in ethanol. Final working concentrations ranged from 16 mM to 125 mM. Folded RNA samples were incubated with each reagent at 37 °C for 5–15 min, depending on the reagent, in a 96-well plate format. Control reactions were performed using the corresponding solvent (DMSO or ethanol) in place of the probing reagent. Reactions with 1M7 were quenched by adding quench buffer containing 0.5 M MES (pH 6.0), 5 M NaCl, Poly(A)Purist™ Magnetic Beads (Invitrogen), 0.25 µM fluorescein amidite-labeled primer, and nuclease-free water. For 5NIA, NAI, and DMS reactions, the same buffer was used with the addition of 10% (v/v) β-mercaptoethanol in place of MES. Quenching was performed at room temperature for 10 min, followed by magnetic separation for 7 min. Supernatants were discarded, and beads were washed twice with 70% ethanol, then air-dried under a fume hood for 30 min.

Beads containing immobilized RNA were resuspended in nuclease-free water, and reverse transcription was performed using SuperScript III Reverse Transcriptase (Thermo Fisher Scientific) according to the manufacturer's protocol, in a reaction containing 5× first-strand buffer, 0.1 M DTT, 10 mM dNTPs, and 200 U/µL enzyme. Reactions were incubated at 48 °C for 45 min, followed by addition of 0.4 M NaOH and incubation at 90 °C for 3 min to hydrolyze the RNA. Samples were cooled on ice for 3 min and neutralized with quench acid solution (1 vol 5 M NaCl, 1 vol 2 M HCl, 1.5 vol 3 M sodium acetate), followed by 1 min incubation at room temperature. Beads were again magnetically separated, washed twice with 70% ethanol, air-dried, and resuspended in formamide containing 350 ROX dye standard (1000:1). Samples were incubated at room temperature for 15 min, magnetically separated for 7 min, and supernatants transferred to a new 96-well plate. Each well was diluted 1:6.7 (3:20) with formamide/350 ROX dye mixture, and samples were submitted to the Cornell University

Biotechnology Resource Center for capillary electrophoresis [47, 48].

SHAPE Data Processing

The resulting data undergoes rigorous processing and normalization using HiTRACE (High-Throughput RNA Structure Analysis by Chemical Probing and Capillary Electrophoresis) in the MATLAB programming environment [54-57]. This process quantifies the extent of chemical modification at each nucleotide position and extracts meaningful structural insights, as each modification depicts accessibility and flexibility of the corresponding RNA region.

Cell Culture and in Cell SHAPE-Mutational Profiling (MaP)

CFF-16HBE CFTR W1282X and 16HBE14o- (CFTR WT) cells were cultured in T25 flasks using Minimum Essential Medium (MEM) supplemented with 10% fetal bovine serum (FBS) and 1% penicillin–streptomycin (100×). Upon reaching 80–90% confluency ($\sim 2 \times 10^6$ cells), cells were washed once with Dulbecco's phosphate-buffered saline (DPBS) and detached using TrypLE™ Express Enzyme (Thermo Fisher Scientific). The cell suspension was pelleted by centrifugation at 1200 rpm for 4 min, washed once in 1× PBS, pelleted again, and resuspended in 465 μ L of 1× PBS.

To each sample, 10 μ L of SUPERase-In™ RNase Inhibitor (Thermo Fisher Scientific) was added, followed by either 25 μ L of 2 M NAI (for SHAPE modification) or 25 μ L of DMSO (for control reactions). All experiments were performed in biological triplicates. Reactions were incubated at 37 °C for 15 min to allow in-cell RNA modification. To quench the reaction, cells were pelleted at 1200 rpm for 4 min, and the supernatant was discarded. Each pellet was lysed by addition of 1 mL of TRIzol™ Reagent (Thermo Fisher Scientific) and incubated at room temperature for 3–5 min. Total RNA was then extracted using the Direct-zol™ RNA Miniprep Plus Kit (Zymo Research) according to the manufacturer's instructions, including on-column DNase

treatment. Eluted RNA (88 μ L) was further treated with 10 μ L of 10× TURBO™ DNase buffer and 2 μ L of TURBO™ DNase enzyme at 37 °C for 30 min to ensure complete removal of genomic DNA. Samples were subsequently purified using the RNeasy Mini Kit (Qiagen). Purified RNA was stored at –80 °C until use.

To identify regions of RNA–protein interaction, we performed cell-free SHAPE-MaP experiments. Total RNA was extracted from cells as described previously [48, 58, 59] and subjected to the same SHAPE-MaP protocol under cell-free conditions. By directly comparing in-cell and cell-free SHAPE reactivity profiles, regions exhibiting reduced reactivity in the cellular context were identified as potential RNA–protein interaction sites, reflecting protection of nucleotides involved in protein binding.

SHAPE-MaP Library Preparation

Ten microliters of purified RNA were mixed with 1 μ L of 2 μ M reverse transcription primer targeting the region of interest, heated at 65 °C for 5 min, and immediately cooled on ice for 3 min. Subsequently, 8 μ L of freshly prepared 2.5× MaP buffer (125 mM Tris-HCl, pH 8.0; 187.5 mM KCl; 15 mM MnCl₂; 25 mM DTT; and 1.25 mM dNTPs) was added, and the reaction was incubated at 42 °C for 2 min. Reverse transcription was initiated by adding 1 μ L of SuperScript II Reverse Transcriptase (Invitrogen) to each reaction, followed by incubation at 42 °C for 3 h. The enzyme was then inactivated by heating at 70 °C for 15 min, and the resulting cDNA products were purified using G-25 spin columns (GE Healthcare) according to the manufacturer's protocol.

Sequencing libraries were prepared following the Small RNA workflow for RNA <500 nt [48, 59]. In brief, cDNA obtained from reverse transcription was used as a template for a two-step PCR amplification strategy to generate sequencing-ready libraries containing adapters. The sequences of all primers used in this assay are provided in **Supplementary Table 2**.

In the first PCR, cDNAs were amplified using sequence-specific primers flanking the region of interest. This initial amplification step introduced partial platform-specific adapter sequences, and the number of PCR cycles was minimized to prevent overamplification. The resulting double-stranded DNA (dsDNA) products were purified using the PureLink™ PCR Micro Kit (Life Technologies, Cat. No. K310250). In the second PCR, purified dsDNA was reamplified using primers containing Illumina barcodes and complete adapter sequences to generate fully compatible libraries. Amplified products were purified using Agencourt AMPure XP beads (Beckman Coulter, Cat. No. A63880). The resulting sequencing libraries were of uniform size and contained complete adapters, ensuring full coverage of the target region for downstream sequencing. Libraries were sequenced on MiSeq i100, 2X300 bp format, using 25M Reagent Kit (600 cycles), targeting approximately 700,000 reads per sample. (Illumina). All SHAPE-MaP sequencing libraries were processed and analyzed using ShapeMapper2[48, 59], following the developer's recommended workflow for alignment, mutation counting, and calculation of normalized SHAPE reactivity profiles[48, 59]. Two independent biological replicates were performed for each condition. Both replicates demonstrated high data quality as assessed by ShapeMapper2 quality metrics (**Fig. S1**). SHAPE reactivity profiles were averaged across replicates for all downstream structural and delta-SHAPE analyses. In-cell SHAPE-MaP was performed in two biological replicates for CFTR W1282X RNA, which showed high inter-replicate concordance (Pearson $r = 0.97$, $R^2 = 0.93$, $p < 10^{-250}$, $n = 429$ nucleotides) and were averaged prior to analysis. CFTR WT RNA in-cell data represent a single biological replicate with high sequencing coverage across the entire amplicon.

In Silico Virtual Screening

For in silico docking, the two hairpin structures flanking exon 23 that correspond to the SHAPE cassette regions that were included for experimental probing and data normalization were

removed to avoid introducing artificial structural constraints. Consequently, the base-pair numbering in the computational models differs from that shown in the SHAPE-derived structures.

Three-dimensional (3D) RNA structures were generated based on experimental SHAPE reactivity data using the RNAComposer, MC-Sym, Vfold web server [60-68]. The resulting RNA models were then subjected to virtual screening using the Schrödinger molecular modeling suite. RNA structures were preprocessed using the Protein Preparation Wizard to optimize hydrogen bonding networks and minimize potential steric clashes. Putative druggable binding pockets were identified and ranked using the SiteMap tool based on predicted druggability scores [69, 70]. The highest-ranking pocket located adjacent to the variant site was selected for virtual screening. A grid box of (20×20×20) Å was generated around this region, and docking simulations were performed against the ChemBridge Core Library (San Diego, CA. Available at: www.chembridge.com), consisting of approximately 211,000 drug-like compounds. The top 10% of compounds with the most favorable docking scores were selected for further analysis and validation[69, 71-73].

Surface Plasmon Resonance

Surface plasmon resonance (SPR) experiments were performed using a Biacore instrument (BIOCORE T100, T200 Sensivity Enhanced) [74]. *In vitro*-transcribed (IVT) RNAs were biotinylated using T4 RNA ligase (New England Biolabs). Briefly, 1 μM CFTR-W1282X RNA was denatured at 90 °C for 5 min, snap-cooled on ice for 3 min, and incubated at 4 °C for 16 h in a 20 μL reaction containing 1× T4 RNA ligase buffer, 1 mM ATP, 10 μM pCp-Biotin, 50% (w/v) PEG8000, and T4 RNA ligase. The reaction was terminated by heating at 70 °C for 10 min, and biotinylated RNA was purified using Sephadex G-50 resin (GE Healthcare). Purified RNA was refolded in RNA folding buffer (50 mM HEPES, pH 8.0; 100 mM NaCl; 5 mM MgCl₂) and immobilized on a streptavidin (SA) sensor chip (GE Healthcare, UK)

according to the manufacturer's protocol. Approximately 3000 resonance units (RU) of RNA were immobilized on the chip surface. Small molecules from the virtual screening library were prepared at a 200 μ M concentration and injected at a flow rate of 30 μ L/min. The association and dissociation phases were monitored for 180 s and 240 s, respectively. Biotinylated CFTR WT RNA and a blank reference channel were used as negative controls. All sensorgrams were processed and analyzed using Biacore Data Analysis Software (GE Healthcare) to determine hitting candidates [73, 75-78].

Microscale Thermophoresis

Microscale thermophoresis (MST) experiments were performed in triplicate using a Monolith X MST instrument (NanoTemper Technologies)[79, 80]. CFTR W1282X RNA was biotinylated as described above and refolded in RNA folding buffer (50 mM HEPES, pH 8.0; 100 mM NaCl; 5 mM $MgCl_2$). The folded RNA was then fluorescently labeled using NT-L020 Biotinylated Target Labeling Kit (NanoTemper Technologies) according to the manufacturer's instructions.

Following labeling, a serial dilution of the small-molecule compound CTC20 (2500 μ M to 0.0076 μ M) was prepared and incubated with a fixed concentration (20nM) of fluorescently labeled RNA in folding buffer at room temperature for 5 min to allow complex formation. Samples were loaded into MST Premium Capillaries (NanoTemper Technologies), and thermophoretic measurements were recorded using 100% LED/Excitation power (on the Monolith X system. Data were analyzed using MO.control 2 software and dissociation constants were determined by fitting the binding curves using a standard 1:1 binding model [81, 82].

Chemical Synthesis of CTC20 and CTC24

The target compounds CTC20 and CTC24 were synthesized through a four-step linear sequence starting from *N*-Boc- γ -aminobutyric acid and 4-chloro-1,2-phenylenediamine. Initial amide

coupling followed by cyclodehydration afforded the 5-chlorobenzimidazole intermediate A2, which was subsequently deprotected to provide the key amine intermediate, 3-(5-chloro-1*H*-benzo[*d*]imidazol-2-yl) propan-1-amine (A3). Final HATU-mediated coupling of A3 with the corresponding carboxylic acid precursors furnished the desired amide derivatives CTC20 and CTC24 (Fig. S2) (see Supplementary Note).

Cells and Culture Conditions

Immortalized human bronchial epithelial cells (CFF-16HBEge-W1282X, Cystic Fibrosis Foundation [CFF]) edited to express CFTR W1282X and cloned for high resistance [39, 44] were maintained in MEM (Gibco 11095-072) with 10% FBS (Gibco 26140-079) and 1% penicillin-streptomycin at 37 °C, 5% CO₂. For functional analysis, cells were plated on Costar 24-well high-throughput screening filter plates (0.4 μ M pore size, Polyester, Corning, catalog no. 3378) coated in collagen IV (Millipore Sigma, C7521). Cells were grown in a liquid/liquid interface (180 μ L apical/700 μ L basolateral) in a 37 °C incubator with 90% humidity and 5% CO₂ for 1 week until high resistance membranes were formed.

Small-Molecule Cell Treatment

CTC20 and CTC24 were synthesized by ChemBridge Corporation and dissolved in anhydrous DMSO at 50 mM. Working dilutions were prepared fresh in MEM to final concentrations of 0.5, 5, 25, 50 and 100 μ M (final DMSO $\leq$ 0.05%). Apical and basolateral treatment was initiated 3 d prior to running the functional assay. For combination therapy, VX-661 (3 μ M) + VX-445 (1 μ M) (Selleckchem) or vehicle (0.02% DMSO) were administered 24 h prior to CFTR functional assessment.

Positive control: phosphorodiamidate morpholino oligomer (PMO) ASOs targeting exon 23 splicing (ASO-23AB[44], Gene-Tools) were delivered via Endo-Porter (Gene Tools) at 40 μ M each, 3 d prior to functional assessment. Nonsense mediated decay inhibitor, SMG1i (0.3 μ M; CFF), was added

24 h prior to the functional assay.

RNA Isolation and RT-PCR

Total RNA was extracted using TRIzol, according to manufacturer instructions (Thermo Fisher Scientific). Reverse transcription was performed on total RNA using the GoScript Reverse Transcription System (Promega, cat # A501D), with an oligo-dT primer (Integrated DNA Technologies [IDT]).

Splicing of CFTR exons 22–24 was assessed by radiolabeled endpoint PCR of the resulting complementary DNA (cDNA) using GoTaq Green (Promega) spiked with α -³²P-deoxycytidine triphosphate. The CFTR primers used for amplification are:

- Forward (exon 22): 5'-CCAAACCATACAAGAAT-3'
- Reverse (exon 24): 5'-GATCACTCCACTGTTCAT-3'

Products were separated on a 6% nondenaturing polyacrylamide gel and quantified using a Typhoon 7000 phosphorimager (GE Healthcare). Exon 23 skipping = [Δ 23 band intensity / (WT + Δ 23)] $\times$ 100 (ImageJ 1.53).

Real-Time qPCR

Real-time qPCR was performed with PrimeTime Gene Expression Master Mix and PrimeTime qPCR probe assay kit targeting human non-F508del-CFTR (Integrated DNA Technologies [IDT]; qhCFTR-ex11WTF, qhCFTR-ex12WTR), and normalized to human HPRT1 (IDT; Hs.PT.58v.45621572). All reactions were analyzed in triplicate on 96-well plates and averaged together to comprise one replicate. Real-time PCR was performed on an Applied Biosystems ViiA 7 Real-Time PCR System. Results were analyzed by the $\Delta\Delta$ CT method to assess fold changes.

Automated Conductance Assay

CFTR function was measured using the TECC-24 robotic system (EP Design, Belgium) in a 24-well format. Cells were switched from culture media to

HEPES-buffered (pH 7.4) F12 Coon's modification running media (Sigma, F6636) apically and basolaterally and allowed to equilibrate for 1 h at 37°C without CO₂. For functional measurements, the resistance (R_t) measurements were recorded at 37°C. Conductance (G_t) was calculated by the reciprocal of the recorded R_t ($G_t = 1/R_t$) and plotted as conductance traces. AUC measurements the test periods were done using trapezoidal rule for each test period using Microsoft Excel. Independent experiments (n) included at least two technical replicates (wells) that were averaged and used as one biological replicate. Protocol:

1. Baseline (20 min)
2. Forskolin (10 μ M, apical + basolateral) to activate CFTR
3. VX-770 (10 μ M, apical + basolateral) to potentiate
4. Inh-172 (20 μ M, apical + basolateral) to inhibit CFTR Conductance

Protein isolation and immunoblot analysis

Cell lysates were prepared using Nonidet P-40 lysis buffer (1.0% Igepal, 150 mM NaCl, 50 mM Tris-HCl, pH 7.6), supplemented with protease inhibitor (Sigma-Aldrich, 11836170001). Protein concentration was determined using a Bradford protein assay (ThermoFisher Scientific, 23200). Proteins were mixed with sample buffer (BioRad, 1610791) and reducing agent (BioRad, 1610792) with final concentrations equalized to 2 mg/mL. The samples (30 μ g per lane) were loaded into a Criterion 3-8% Tris-Acetate gel (BioRad, 3450130). The separated samples were transferred onto 0.45 μ m polyvinylidene difluoride, then blocked in 5% nonfat dry milk solution. The membranes were incubated with antibodies against CFTR 596 (1:2000; Riordan Lab, UNC, CFF), 4°C overnight or Na⁺/K⁺ ATPase (1:20000; Santa Cruz sc-48345) for 2 h at room temperature. Horseradish peroxidase-conjugated secondary antibody (ThermoFisher, 32230) was incubated with membranes at room temperature for 1 h. Protein was detected via chemiluminescence.

Signal was quantified with ImageJ software.

RESULTS

Single-Nucleotide Substitution Induces Local Structural Rearrangement in CFTR mRNA

The W1282X variant in the CFTR gene, located within exon 23, arises from a single nucleotide substitution (G>A) that introduces a premature termination codon, triggering NMD and severely limiting CFTR protein expression [27, 38, 40, 44, 83-86].

To assess whether the W1282X variant alters the local secondary structure of the CFTR transcript, selective 2'-hydroxyl acylation analyzed by primer extension with capillary electrophoresis (SHAPE-CE) was performed on a 30-nucleotide region flanking the variant site (**Fig. 1A**). This targeted strategy enabled the detection of distinct conformational changes induced by W1282X (**Supplementary Table 1. #1-2**).

In CFTR WT RNA, SHAPE reactivity analysis revealed structured hairpins corresponding to the 5' and 3' SHAPE cassettes (see Methods), while the 30-nucleotide region of interest remained largely unstructured (**Fig. 1B**). In contrast, the W1282X variant displayed an additional distinct hairpin forming within the region of interest at the variant site, reflecting a localized conformational rearrangement directly induced by the G>A substitution (**Fig. 1C**).

To confirm these findings, we analyzed both CFTR WT and W1282X RNA using 1D ¹H-NMR experiments, focusing on the imino region [87-89]. Imino protons indicate complementary base-pairing, including AU and GC base complementarity, and stable interaction within loop regions such as tetraloops [90-95]. We observed an increase in the number of imino proton peaks in the W1282X RNA relative to WT (**Fig. S3**), consistent with greater overall base-pairing and structural stability in the W1282X construct compared to WT.

Structural persistence of the W1282X-induced folding hairpin within the full CFTR exon 23 and its splicing junction

To determine whether the local structural alteration observed in the *in vitro*-transcribed RNA fragment persists in a broader sequence context, we analyzed extended RNA constructs encompassing CFTR exon 23, enabling evaluation of long-range interactions and contextual influences on RNA folding near the W1282X variant (**Supplementary Table 1. #3-4**).

As expected for longer RNA sequences, both WT and W1282X RNAs displayed SHAPE reactivity profiles consistent with the adoption of multiple conformations in solution, reflecting their intrinsic structural dynamics. Despite this heterogeneity, distinct dominant structures emerged for each variant. In representative models, the WT region adopted a regular helix without hairpin formation (**Fig. 2A-B, Fig. S4A**), whereas the W1282X RNA consistently formed a prominent hairpin near the variant site (**Fig. 2C-D, Fig. S4B**). Notably, the predicted 3D structures of the CFTR RNA constructs, modeled using SHAPE-derived secondary structure constraints, also reveal conformational differences between the WT and W1282X variants (**Fig. 2**).

To validate the SHAPE-CE results, we performed SHAPE with mutational profiling (SHAPE-MaP), a higher-throughput sequencing-based approach, as an independent method to confirm the structural differences observed near the W1282X site. For these experiments, we used an extended RNA construct encompassing exon 23 and its splice junctions (**Supplementary Table 1. #5-6, Fig. 3, Fig. S5**). We focused on the splice junctions, as our previous study showed that the combination of ASO A and ASO B most effectively induces complete exon 23 skipping[44]. Structural characterization of these regions may clarify the interplay between RNA folding, splicing, and NMD-mediated transcript degradation. We therefore extended our structural analysis to these functionally relevant splice junction regions.

Consistent with our shorter-construct findings, this single-nucleotide substitution alters local folding near the variant site and induces conformational changes in neighboring regions, including the exon–intron junctions. (**Fig. S5**). SHAPE-MaP replicates showed high reproducibility for both WT ($R^2=0.9931$, $r=0.9965$) and W1282X ($R^2=0.9936$, $r=0.9968$) constructs (**Fig. S6**). Quality control metrics for SHAPE-MaP experiments is represented in (**Fig. S1**)

Together, these findings confirm that the G>A substitution in W1282X induces a stable, localized conformational rearrangement that persists in the extended exon context and propagates to the functionally relevant splice junction regions.

Identification of Potential Druggable Sites and Virtual Screening of the CFTR W1282X RNA Region of Interest

To extend our structural analysis beyond secondary structure, we used the SHAPE-constrained 2D model of the CFTR W1282X exon 23 construct (**Supplementary Table 1. #4**) to predict its three-dimensional (3D) architecture. Note that base-pair numbering in the computational models reflects a 29-nucleotide offset relative to the SHAPE-derived structures, due to removal of the cassette sequences for docking analysis (see Methods). This offset does not affect the structural or biological interpretation of the results. The experimentally derived secondary structure was input into RNAComposer [60-68], which generated a predicted atomic-level 3D model consistent with the observed folding patterns. Structured RNAs can adopt complex tertiary folds that create defined binding pockets suitable for small-molecule recognition. We therefore assessed the druggability of the modeled RNA.

The 3D structure was imported into the Schrödinger molecular modeling suite, where the SiteMap algorithm identified and characterized potential ligand-binding cavities based on pocket volume, geometry, hydrophobicity, hydrogen-bonding potential, and solvent accessibility[69,

70]. Multiple candidate pockets were predicted across the RNA surface (**Fig. 4A**), with a prominent site located adjacent to the W1282X variant (**Fig. 4B**). This region exhibited favorable SiteScore (1.01) and SiteMap hydrophilicity metric (0.9, indicating high potential for small-molecule binding.

To enable virtual screening, a 20×20×20 Å grid box was centered on the identified pocket using the Receptor Grid Generation module in Schrödinger Maestro. The ChemBridge Core Library (~211,000 drug-like compounds) was screened against this site using a tiered docking workflow comprising High-Throughput Virtual Screening (HTVS), Standard Precision (SP), and Extra Precision (XP) stages to progressively refine ligand selection[69, 71-73]. Compounds were ranked by GlideScore, reflecting predicted binding affinity and interaction quality. The top 20 candidates were further filtered based on QikProp-predicted ADME properties and removal of ligands with excessive net charge or multiple property violations. The resulting set of molecules, designated CFTR-Targeting Compounds (CTC1–CTC20), was prioritized for downstream biophysical validation.

To assess whether the identified binding site was maintained in the broader sequence context used for biophysical validation, all top-ranked compounds from the virtual screening were additionally docked against the extended exon 23 + splice junction construct (**Supplementary Table 1. #6**). Despite a lower SiteScore of 0.70 in this construct, docking predicted consistent binding poses proximal to the variant site for the top candidates, supporting the relevance of the identified pocket across both constructs and its compatibility with the extended RNA used for SPR, MST, and ΔSHAPE-MaP validation.

Biophysical Validation of Small-Molecule Interactions with CFTR RNA

To experimentally validate the predicted RNA-ligand interactions, we used Surface Plasmon resonance (SPR) to screen small-molecule

candidates against CFTR W1282X RNA. Screening was performed on both the exon 23 construct and the extended exon 23 + splice junction construct (**Supplementary Table 1. #4 and #6**), with refolded biotinylated RNA immobilized on a streptavidin sensor chip [73–78].

CTC20 showed a binding response to W1282X CFTR RNA on both constructs (**Fig. 5A–B**), confirming that compound engagement is mediated by the exon 23 region containing the variant site and is independent of the splice junction sequences. Parallel screening against an unrelated transcript, human nerve injury-induced long non-coding RNA (NIS-lncRNA), confirmed preferential binding to the mutant CFTR RNA. (**Fig. 5C; Fig. S7**). To quantify binding affinity, we performed microscale thermophoresis (MST) using the SPR-positive hits [79–82]. CTC20 emerged as a lead candidate, with a dissociation constant of 19.81 μM (**Fig. 5D**). Structure-activity relationship similarity analysis of CTC20 identified CTC24 as a structurally related analog (**Fig. S7A**). We next used ΔSHAPE -MaP to confirm that compound binding selectivity perturbs the W1282X conformation (**Supplementary Table 1. #5–6; Fig. 6**).

CTC20 produced no detectable ΔSHAPE signals at 25 μM , indicating insufficient structural perturbation at this concentration. At 50 μM , CTC20 induced three significant clusters: enhanced flexibility at positions 75–78 (mean $\Delta\text{SHAPE} = -0.16$), and protection at positions 157–159 (mean $\Delta\text{SHAPE} = +0.16$) and 174–176 (mean $\Delta\text{SHAPE} = +0.14$) (**Fig. 6A**). The protected region at 157–159 lies immediately downstream of the W1282X variant site within a helical stem consistent with local stabilization. The distant enhancement at positions 75–78, suggest allosteric propagation.

At 25 μM , CTC24 produced four clusters: protection at positions 57–59 (mean $\Delta\text{SHAPE} = +0.24$) and 117–119 (mean $\Delta\text{SHAPE} = +0.12$), and enhanced flexibility at positions 142–144 (mean $\Delta\text{SHAPE} = -0.13$) and 163–166 (mean $\Delta\text{SHAPE} = -0.15$). The flanking enhancement around the

W1282X, positions 142–144 lie 11–13 nucleotides upstream and positions 163–166 lie 8–11 nucleotides downstream, producing a pattern of increased flexibility bracketing the variant site (**Fig. 6B**). The flanking enhancements around the variant site indicate that CTC24 remodels or destabilizes the local structure surrounding W1282X.

Both compounds induced distal ΔSHAPE signals. Such long-range effects are commonly observed in small-molecule–RNA interactions and can arise from allosteric conformational propagation or direct contact with spatially proximal but sequence-distant nucleotides. Given that the enhanced sites occur in predicted loop/flexible regions, these changes most likely reflect allosteric redistribution of RNA dynamics upon binding at the variant site. Both compounds produced ΔSHAPE signals in WT RNA (**Fig. 6D–E**); however, neither affected the variant-site region (nucleotides 140–170) in WT context. This region selectivity supports preferential engagement of the disease-specific W1282X conformation over the corresponding wild-type structure.

In-cell SHAPE-MaP Analysis of CFTR RNA Structure

To validate the conformational differences observed in vitro and assess their physiological relevance, we performed in-cell SHAPE-MaP on CFTR RNA in 16HBE cells expressing either CFTR W1282X or WT, focusing on exons 22–24, which encompasses the W1282X variant site (**Fig. S8**). Nucleotide numbering in the in-cell amplicon differs from the in vitro-transcribed (IVT) construct due to the extended sequence context (see **Supplementary Table 1**). Parallel in-cell and cell-free SHAPE-MaP experiments on the same RNA species were used to distinguish intrinsic RNA structure from cellular influences (see Methods) [59].

Comparison of in-cell and cell-free ΔSHAPE profiles revealed that CFTR WT RNA undergoes substantially greater structural remodeling in the

cellular environment than W1282X RNA, with a larger number and higher amplitude of significant Δ SHAPE changes distributed across the amplicon (**Fig. 7A–B**). WT RNA exhibited 24 protected versus 5 enhanced nucleotides (ratio 4.8:1), consistent with extensive RNA-protein interactions that stabilize the WT transcript in cells. In contrast, W1282X RNA showed a near-balanced distribution (13 protected and 12 enhanced nucleotides; ratio ~1:1), indicating that the variant rewires the cellular RNA-protein interaction landscape rather than simply reducing protein engagement. Six protected sites were shared between constructs (positions 146–148 and 189–191) while 19. W1282X-specific enhancements particularly near the variant site (position 374 and positions 398–402), highlight variant-induced changes in local conformation and protein accessibility in cells. These patterns align with the stable hairpin observed *in vitro*, which renders the mutant RNA less accessible to endogenous remodeling factors.

Focused Δ SHAPE analysis around the variant site (positions 320–390, **Fig. 7C**) revealed opposing remodeling patterns. WT RNA displayed strong protection at position 338, (upstream of the variant), whereas W1282X exhibited significant enhancement at nucleotides 376 and 379 \ (downstream of the variant; strongest at 379, z-score=3.59)., These nucleotide-resolution differences demonstrate that the G→A substitution disrupts \ normal pattern of cellular structural remodeling and redistributes flexibility to a downstream region. Although minimum free energy predictions constrained by in-cell SHAPE data yielded similar global secondary structures for both transcripts, the pronounced local Δ SHAPE differences indicate that W1282X and WT RNAs populate distinct conformational ensembles in cells[59, 96, 97]. Such dynamic distinctions are functionally relevant and can be selectively recognized by small molecules[7, 98].

Despite these local differences, minimum free energy structure prediction constrained by in-cell

SHAPE reactivities yielded similar global secondary structure topologies for both constructs [59, 96, 97]. This is not uncommon in RNA biology and most likely reflects the sensitivity of Δ SHAPE analysis to subtle but real differences in conformational dynamics that are insufficient to redirect the global fold but are nonetheless functionally meaningful. RNA structure is not a single static conformation but an ensemble of states, and the local flexibility differences captured by Δ SHAPE indicate that W1282X and WT RNAs sample different conformational ensembles in cells even when their minimum free energy structures appear similar; precisely the kind of dynamic differences that small molecule ligands can selectively recognize [7, 98].

The W1282X-specific loss of protective protein contacts and increased flexibility near the variant create an accessible, unoccupied region that is less competitive with endogenous RNA-binding protein. This structural feature provides a mechanistic basis for the variant-selective binding observed with CTC20 and CTC24 *in vitro*. Moreover, the reduced structural plasticity of W1282X RNA in cells suggests decreased engagement by endogenous regulatory machinery, which may exacerbate pathology beyond the premature termination codon. Collectively, these data confirm that the disease-associated W1282X conformation persists in the cellular environment and strengthen the rationale for small-molecule targeting of this unique structural vulnerability.

CTC20 and CTC24 Provide Proof-of-Concept Rescue of CFTR W1282X Function CF-derived Cell Model

The W1282X variant is largely unresponsive to conventional CFTR modulators, which act on full-length or near full-length CFTR protein [37, 38, 83, 86, 99]. Having identified CTC20 and CTC24 as selective binders of the mutant RNA conformation, we tested whether this engagement could translate into functional CFTR rescue in 16HBEge CFTR W1282X cells the automated TECC assay to measure transepithelial conductance (Gt). Cells

were treated with compounds, CTC20 and CTC24 (0.5–100 μ M), alone or in combination with CFTR correctors VX-661 and VX-445. CFTR-mediated chloride conductance was measured after sequential addition of forskolin, VX-770, and the inhibitor Inh-172, with ASO-23AB and SMG1i as positive controls and DMSO as vehicle.

CTC20 produced directional increases in CFTR-mediated chloride conductance above vehicle cells across multiple concentrations, both alone and in combination with CFTR correctors (**Fig. 8A**). However, individual dose comparisons were not statistically significant (two-way ANOVA with Dunnett's post-hoc test, all $p > 0.05$; **Fig. 8C-D**). The drop in conductance following Inh-172 addition confirmed CFTR specificity of the observed signals (**Fig. 8A**). ASO-23AB and SMG1i produced substantially greater CFTR activity, confirming assay sensitivity (**Fig. 8C**). Notably, SMG1i-mediated rescue was most pronounced upon potentiator co-administration, consistent with the known gating dysfunction of the truncated W1282X protein, wherein NMD inhibition increases mRNA and protein abundance, but pharmacological potentiation is required to restore channel opening. The modulator-dependent response pattern of CTC20 is consistent with a similar RNA-level rescue mechanism.

CTC24 demonstrated a consistent directional increase in CFTR-mediated conductance across all modulator backgrounds (**Fig. 8B, 8E-F**). A statistically significant increase was observed at 100 μ M in combination with the triple modulator background (**Fig. 8F**, $p < 0.05$, two-way ANOVA with Dunnett's post-hoc test), with a trend toward greater efficacy at higher concentrations consistent with its greater potency demonstrated in Δ SHAPE analysis. The overall modulator background effect was robust ($p < 0.0001$), confirming that CFTR modulators substantially enhance the cellular response. ASO-23AB and SMG1i rescued CFTR as expected validating assay performance (**Fig. 8E**).

RT-PCR confirmed that CTC20 did not induce

exon 23 skipping at any concentration tested, in contrast to ASO-23AB (**Fig. 8G**), establishing a splicing-independent mechanism. Although equivalent splicing data for CTC24 were not obtained, its structural similarity to CTC20, SAR-guided origin, and comparable functional response pattern strongly suggest a conserved mechanism.

Collectively, these findings establish CTC20 and CTC24 as first-in-class small molecules capable of conferring some recovery of CFTR function by targeting the mutant RNA conformation through a splicing-independent mechanism, providing a validated starting point for further optimization.

CTC20 and CTC24 Increase CFTR Protein Expression and Maturation in W1282X Cells

To determine whether the functional improvements were reflected at the protein level, western blot analysis was performed on 16HBEge CFTR W1282X cells treated with 0.5–100 μ M of CTC20 or CTC24, in the absence and presence of VX-661 and VX-445 (**Fig. 9A, E**). Analyses of fully glycosylated CFTR band C and core glycosylated band B using three complementary metrics were assessed: total CFTR protein, normalized to housekeeping, Na⁺/K⁺-ATPase (Band C+B/Na⁺/K⁺-ATPase), mature surface CFTR (Band C/Na⁺/K⁺-ATPase), and maturation efficiency (Band C/Band B ratio).

CTC20 produced dose-dependent increases across all three metrics in the absence of correctors: total CFTR protein rose ~2.2-fold at 100 μ M (**Fig. 9B**), mature Band C/Na⁺/K⁺-ATPase reached ~2.7-fold above untreated (**Fig. 9C**), and Band C/B maturation efficiency increased from 1.0 to 1.7 (**Fig. 9D**). CTC24 showed a comparable profile, with total CFTR protein increasing ~2.8-fold (**Fig. 9F**), mature Band C/Na⁺/K⁺-ATPase reaching ~3.0-fold (**Fig. 9G**), and Band C/B ratio reaching ~1.35 (**Fig. 10H**). The modestly higher surface CFTR levels with CTC24 relative to CTC20 are consistent with its greater potency demonstrated in Δ SHAPE analysis and enhanced functional activity observed by TECC-24.

Co-treatment with VX-661 and VX-445 further increased total and mature CFTR protein levels for both compounds. Band C/Na⁺/K⁺-ATPase reached ~4.3-fold above untreated at 100 μ M + VX2 for CTC20 (**Fig. 9C**) and ~4.6-fold for CTC24 (**Fig. 9G**), consistent with additive effects on CFTR stability and trafficking. While correctors such as VX-809 are well established to increase the Band C/B ratio in Δ F508-CFTR by promoting maturation of the immature form, the W1282X truncation presents a distinct biogenic behavior. In this context, corrector treatment has been shown to increase both core-glycosylated (Band B) and complex-glycosylated (Band C) forms of W1282X-CFTR simultaneously, rather than selectively driving Band B to Band C conversion [100]. Consequently, the overall C/B ratio may remain unchanged or decrease even as total CFTR protein levels rise, reflecting enhanced overall biogenesis rather than impaired maturation efficiency [101]. Therefore, in the W1282X context, total Band B and C levels combined represent a more meaningful measure of corrector efficacy than the C/B ratio alone.

Although individual dose comparisons were not statistically significant for either compound (two-way ANOVA with Dunnett's post-hoc test, all $p > 0.05$), the consistent directional increase across independent metrics support and upstream effect on CFTR expression. This was directly confirmed by RT-qPCR (see below). ASO-23AB and SMG1i served as positive controls and benchmarks for maximal RNA-level rescue (**Fig. S9**). Direct quantitative comparison with CTC20 and CTC24 should be interpreted with caution. Antisense oligonucleotides engage RNA targets through Watson-Crick base pairing across 20–25 nucleotides with near-stoichiometric precision, achieving potency fundamentally distinct from small molecule interactions. The ability of these first-generation small molecules to produce directional improvements in CFTR protein levels and maturation, despite their distinct mechanism from antisense oligonucleotides, validates the therapeutic potential of targeting the mutant RNA

conformation and provides a foundation for further optimization.

CTC20 and CTC24 Increase CFTR W1282X mRNA Levels Consistent with Partial NMD Suppression

To determine whether the observed increases in CFTR protein and function were driven by changes in transcript abundance, we performed RT-qPCR on 16HBE_{cl}-CFTR W1282X cells treated with CTC20 or CTC24 (0.5–100 μ M), using ASO-23AB and SMG1i as positive controls.

Both compounds produced dose-dependent increases in CFTR mRNA levels relative to untreated cells. CTC20 increased CFTR transcript abundance ~1.8-fold at 50–100 μ M (**Fig. 10A**), while CTC24 produced a comparable increase of approximately 1.5-fold across the same concentration range (**Fig. 10B**), consistent with partial stabilization of the W1282X transcript against NMD-mediated degradation and mirroring the directional protein-level changes observed by western blot.

Taken together with the TECC-24 functional measurements (**Fig. 8A-F**), along with the CFTR mRNA (**Fig. 8G**), and protein quantification (**Fig. 9**), the RT-qPCR data support a model in which CTC20 and CTC24 partially stabilize the W1282X transcript against NMD-mediated degradation, producing more total CFTR protein available for downstream maturation and trafficking. While the W1282X premature termination codon is the primary trigger for NMD activation, small molecule engagement at the variant site may partially reduce the efficiency of transcript degradation, allowing a greater fraction of CFTR W1282X mRNA to escape NMD, consistent with the modest but reproducible increases in transcript abundance observed by RT-qPCR.

The consistent directional effects observed across mRNA, protein, and functional endpoints provide strong mechanistic support for targeting the

W1282X RNA structure and a clear rationale for continued optimization of this scaffold.

CONCLUSION

We investigated the structural consequences of the CFTR nonsense variant W1282X, which introduces a premature termination codon that triggers nonsense-mediated decay (NMD), and limits expression of a truncated yet partially functional CFTR protein. Using SHAPE-MaP (capillary electrophoresis and sequencing-based), we demonstrated that this single-nucleotide G→A substitution induces distinct and localized conformational rearrangements in the CFTR RNA, resulting in a distinct secondary structure relative to the WT transcript both *in vitro* and within the cellular environment. This altered conformation may modulate the folding dynamics and accessibility of adjacent sequences, with potential implications for interactions with RNA-binding proteins or components of the NMD pathway. In-cell SHAPE-MaP revealed that significant local nucleotide flexibility differences persist at and around the variant site despite similar global secondary structure topologies between W1282X and WT CFTR RNA, and that W1282X RNA is substantially less responsive to cellular structural remodeling than WT, consistent with stable localized hairpin identified *in vitro*. These findings underscore the sensitivity of RNA folding to point variants and demonstrate that W1282X the structural alterations persist in a physiological context, with potential effects on RNA-protein interactions and NMD efficiency.

By combining computational modeling, virtual screening, and biophysical validation (MST and SPR), we identified small molecules that selectively engage the mutant RNA conformation. ΔSHAPE-MaP confirmed that CTC20 and its SAR-derived analog CTC24 induce localized structural changes centered on the variant site (nucleotides 149–159), at concentrations where WT RNA is largely unaffected. CTC24, identified through 2D and 3D SAR similarity analysis of the CTC20 scaffold, demonstrated greater potency than

CTC20, producing focused variant-selective structural changes at 25 μM, a concentration at which CTC20 showed no detectable effect, suggesting greater potency and selectivity for the W1282X conformation. In cell-based functional assays, both compounds produced directional increases in CFTR-mediated chloride conductance in W1282X-expressing 16HBE cells, with CTC20 reaching statistical significance at 100 μM in combination with the triple modulator background. Western blot analysis demonstrated directional concentration-dependent increases in mature CFTR Band C protein and total CFTR protein for both compounds, consistent with improved CFTR biogenesis. RT-qPCR confirmed directional dose-dependent increases in W1282X CFTR mRNA levels for both compounds, reaching ~1.8-fold and 1.5-fold above untreated cells for CTC20 and CTC24 respectively, consistent with partial stabilization of the W1282X transcript against NMD-mediated degradation. These effects were splicing-independent and additive with CFTR correctors.

Although the magnitude of rescue was more modest than that achieved with ASO-23AB and SMG1i, direct quantitative comparison must be interpreted with caution and must account for fundamental mechanistic differences. Antisense oligonucleotides engage RNA targets through complementary base pairing across 20–25 nucleotides with near-stoichiometric precision, achieving potency that is fundamentally distinct from small molecule interactions. [102]. Similarly, SMG1i broadly inhibits the core NMD machinery [103, 104]. Recent advances in genome editing have similarly demonstrated proof-of-concept CFTR rescue for variants lacking pharmacological treatments [105]; however, such approaches require electroporation or viral delivery systems and involve permanent genomic modification, presenting significant challenges for *in vivo* therapeutic translation [106–108]. The ability of these first-generation small molecules to produce directional improvements in CFTR mRNA levels, protein expression, and chloride conductance,

despite their mechanistic distinction from oligonucleotide-based approaches, validates the therapeutic potential of targeting the mutant RNA conformation directly.

Furthermore, ASOs, kinase inhibitors, and genome editing approaches all face significant translational barriers. ASO delivery to target tissues and efficient cellular uptake remain major unresolved challenges [109], and SMG1 inhibition broadly suppresses a pathway that also regulates hundreds of physiological transcripts, raising concerns about global transcriptomic disruption [110, 111]. Additionally, the complex manufacturing processes associated with oligonucleotide-based therapeutics and gene editing tools contribute substantially to production costs, limiting patient access [112].

Small molecules as a class offer distinct advantages that make them particularly attractive for further development. Their low molecular weight enables passive diffusion across cell membranes and favorable oral bioavailability without the need for transfection agents or specialized delivery systems [112, 113]. Critically, small molecules are amenable to rapid iterative medicinal chemistry optimization, allowing systematic improvements in potency, selectivity, and pharmacokinetic properties through structure–activity relationship studies [83, 102]. The improvement in potency from CTC20 to CTC24 through SAR-guided design underscores the tractability of this scaffold. Future efforts will focus on expanding this SAR through the synthesis of next-generation analogs aimed at enhancing potency, variant selectivity, and *in vivo* pharmacokinetic properties.

Collectively, this work establishes a structure-guided small-molecule strategy for targeting the unique RNA conformation created by the W1282X variant. By demonstrating variant-selective engagement, partial NMD suppression, and coherent rescue across mRNA, protein, and functional readouts, we provide proof-of-concept for a new class of precision RNA-targeted therapeutics for CF patients unresponsive to current

modulators. Future optimization of potency, selectivity, and *in vivo* efficacy, together with deeper mechanistic studies, will further advance this approach and support its broader application to other variant-driven diseases.

ACKNOWLEDGMENTS

This work was supported by McKnight Brain Institute accelerator fund awarded to M. Khanna and funding from the Cystic Fibrosis Foundation to J. Centa and M. Hastings. We gratefully acknowledge access to instrumentation provided by the AMRIS Facility at the McKnight Brain Institute, University of Florida, including the NMR facility. We thank Amelia A. Bannell for her assistance with NMR data collection. We thank Dr. Yousong Ding for generously providing access to the Monolith X instrument in his laboratory for MST data acquisition. We are grateful to Dr. Alesha N. Jones for her guidance in establishing the SHAPE assay protocol in the early stages of this project, and to Dr. Jodi Lynne Bubenik for her invaluable assistance in the design and troubleshooting of SHAPE-MaP assays. Sequencing services were provided by the Interdisciplinary Center for Biotechnology Research (ICBR) NextGen DNA Sequencing Core at the University of Florida (RRID:SCR_019152).

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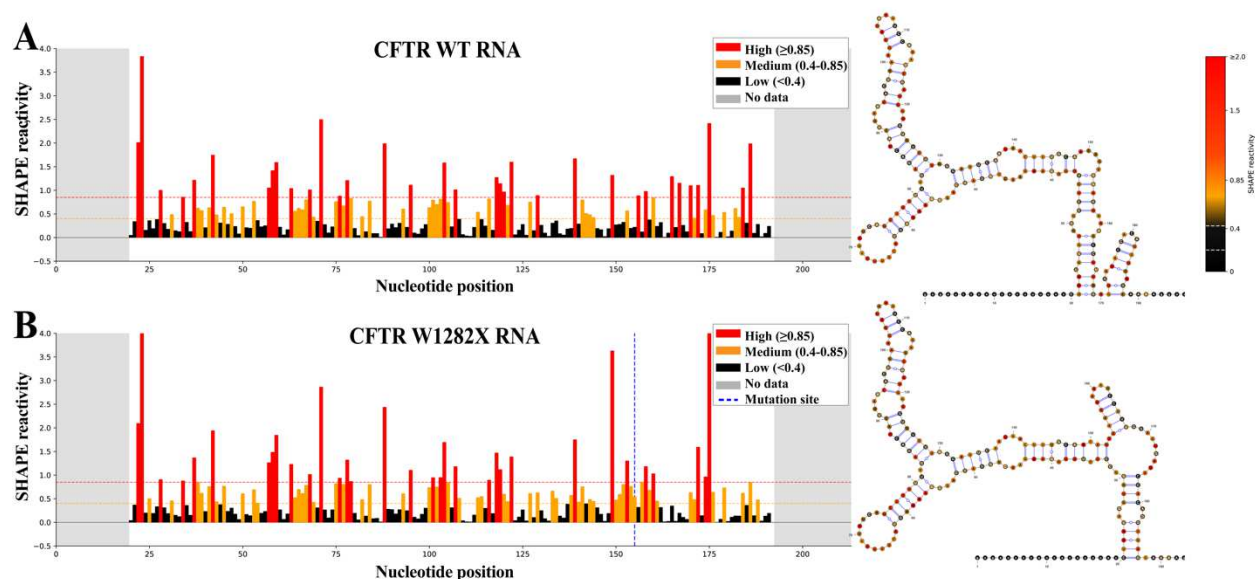


Figure 3. SHAPE-MaP reactivity profiles and predicted secondary structures of CFTR exon 23 RNA constructs. **A.** Averaged SHAPE-MaP reactivity profile of the CFTR WT RNA with the corresponding SHAPE-guided secondary structure shown to the right. **B.** Averaged SHAPE-MaP reactivity profile of the CFTR W1282X RNA with the corresponding SHAPE-guided secondary structure shown to the right. Nucleotides are colored according to SHAPE reactivity: red, high (≥ 0.85); orange, medium (0.4–0.85); black, low (< 0.4); gray, no data.

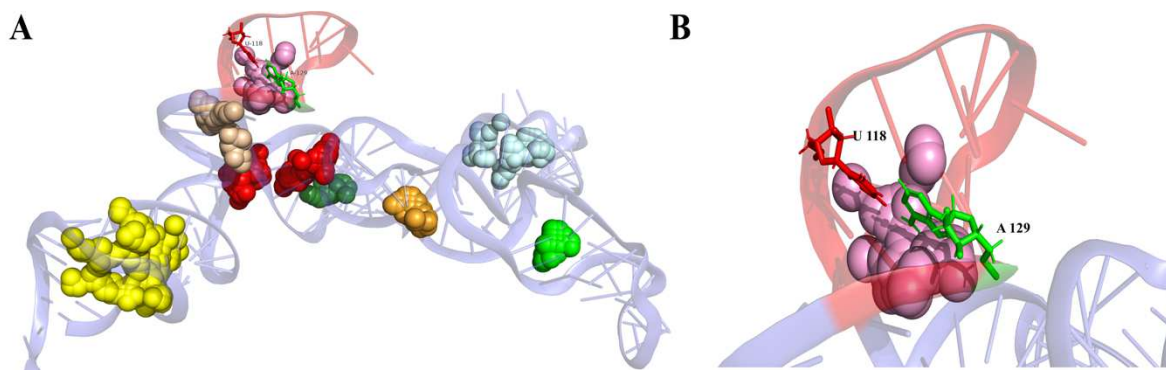


Figure 4. Molecular docking of RNA. **A.** Potential druggable sites predicted employing Schrödinger's Site Map tool on CFTR W1282X exon23. **B.** Binding mode of drug-like molecules from ChemBridge library.

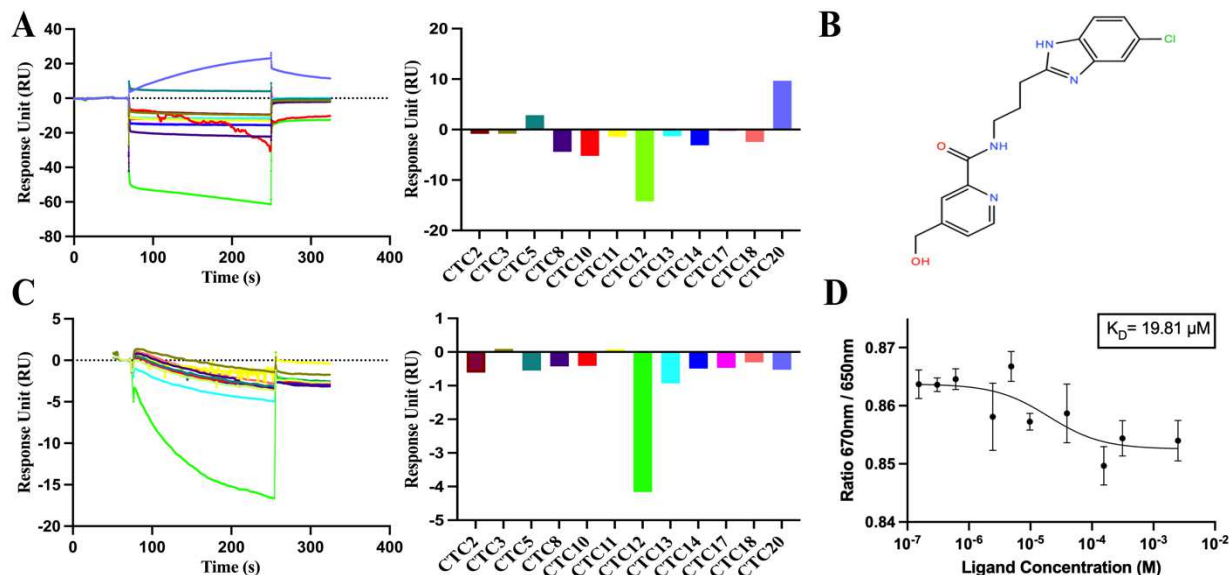


Figure 5. Physical Binding Assay. **A.** Sensorgram traces of the top 20 compounds (200 μ M) over immobilized CFTR W1282X RNA (12 traces are shown; noisy traces were excluded) (left panel), and the response signal of each compound (right panel). **B.** Sensorgram traces of the top 20 compounds (200 μ M) over Human NIS-lncRNA (left panel), and the response signal of each compound (right panel). **C.** Chemical structure of the lead compound CTC20 (O=C(NCCCC1=NC2C=CC(Cl)=CC2=N1)c1). **D.** MST results and K_D value for W1282X and CTC20.

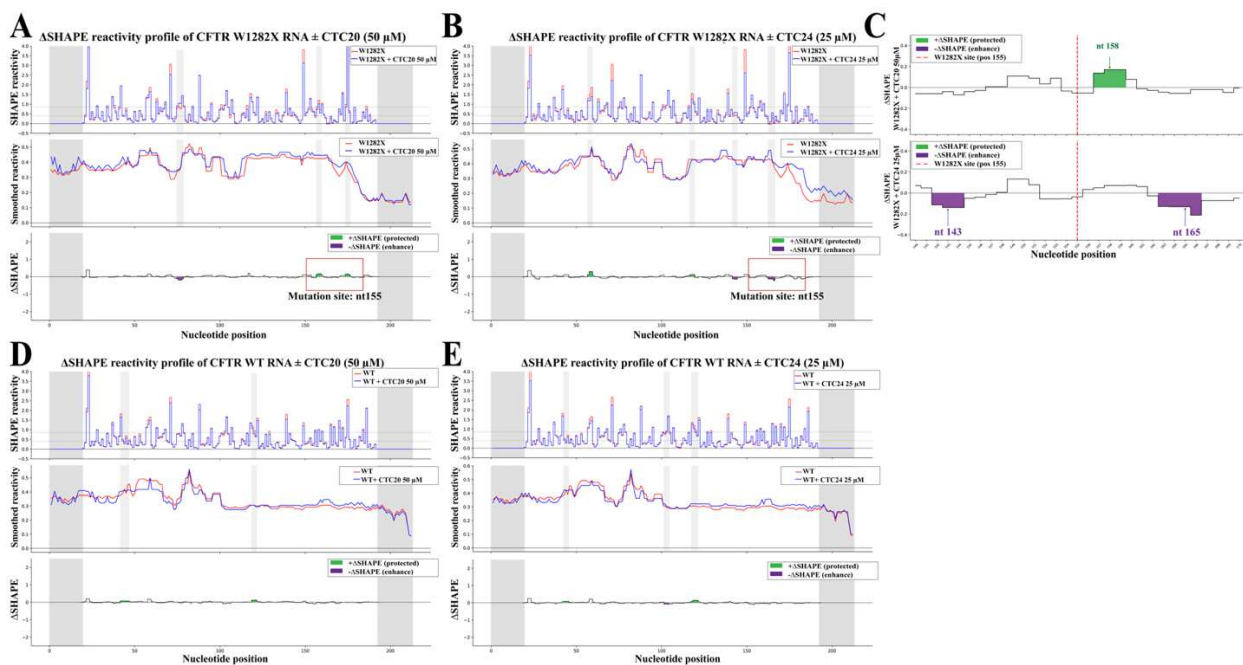


Figure 6. ASHAPE-MaP analysis of CTC20 and CTC24 binding to W1282X and CFTR WT IVT RNA. ASHAPE-MaP profiles for W1282X-CFTR RNA treated with CTC20 (50 μ M, **A**) or CTC24 (25 μ M, **B**), and CFTR WT RNA treated with CTC20 (50 μ M, **D**) or CTC24 (25 μ M, **E**). Each panel shows per-nucleotide SHAPE reactivity (top), 50-nucleotide sliding median (middle), and Δ SHAPE values (bottom); significant protection (+ Δ SHAPE, green) and enhancement ($-\Delta$ SHAPE, purple) are indicated. (**C**) Zoomed view of the boxed region in **A** and **B** (nt140-170), showing Δ SHAPE signals at the W1282X mutation site (dashed red line, nt155). Neither compound produced detectable effects in the equivalent WT region, confirming selective engagement of the W1282X RNA conformation. Grey shading indicates primer-binding sites excluded from analysis (nt 1-19 and nt 193-212).

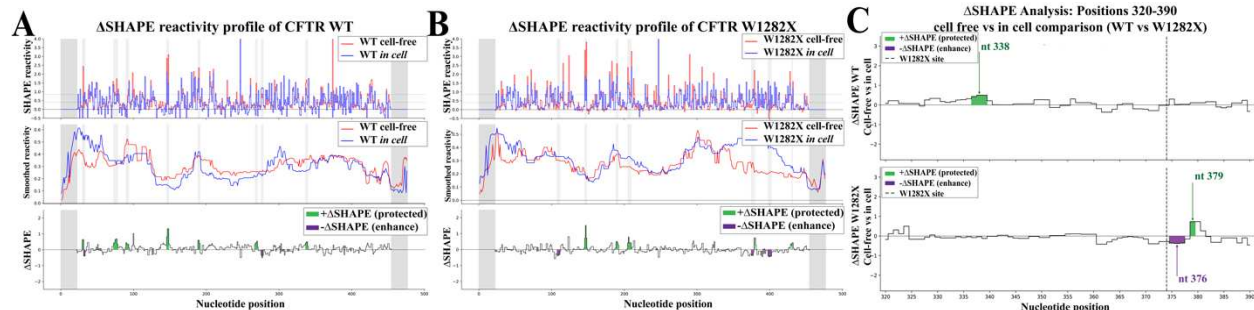


Figure 7. In-cell SHAPE-MaP analysis of CFTR WT and W1282X RNA. ΔSHAPE-MaP comparing cell-free (red) and in-cell (blue) SHAPE reactivity profiles for CFTR WT (A) and CFTR W1282X (B) RNA in 16HBE14o⁻ cells. Each panel shows per-nucleotide SHAPE reactivity (top), 50-nucleotide sliding median smoothed reactivity (middle), and ΔSHAPE values (bottom), with significant protection (+ΔSHAPE, green) and enhancement (-ΔSHAPE, purple) indicated. Grey shaded regions denote primer-binding sites excluded from analysis. CFTR WT RNA exhibited greater structural remodeling in the cellular environment compared to W1282X, which adopted a comparatively rigid conformation with fewer significant ΔSHAPE changes. C. Zoomed ΔSHAPE analysis of the region surrounding the W1282X mutation site (positions 320–390). The dashed vertical line indicates the mutation position (nt374 in this construct). W1282X RNA shows significant enhancement at nt376 and protection at nt379 downstream of the mutation, while WT RNA shows significant protection at nt338 upstream, demonstrating opposing patterns of cellular structural remodeling between the two constructs.

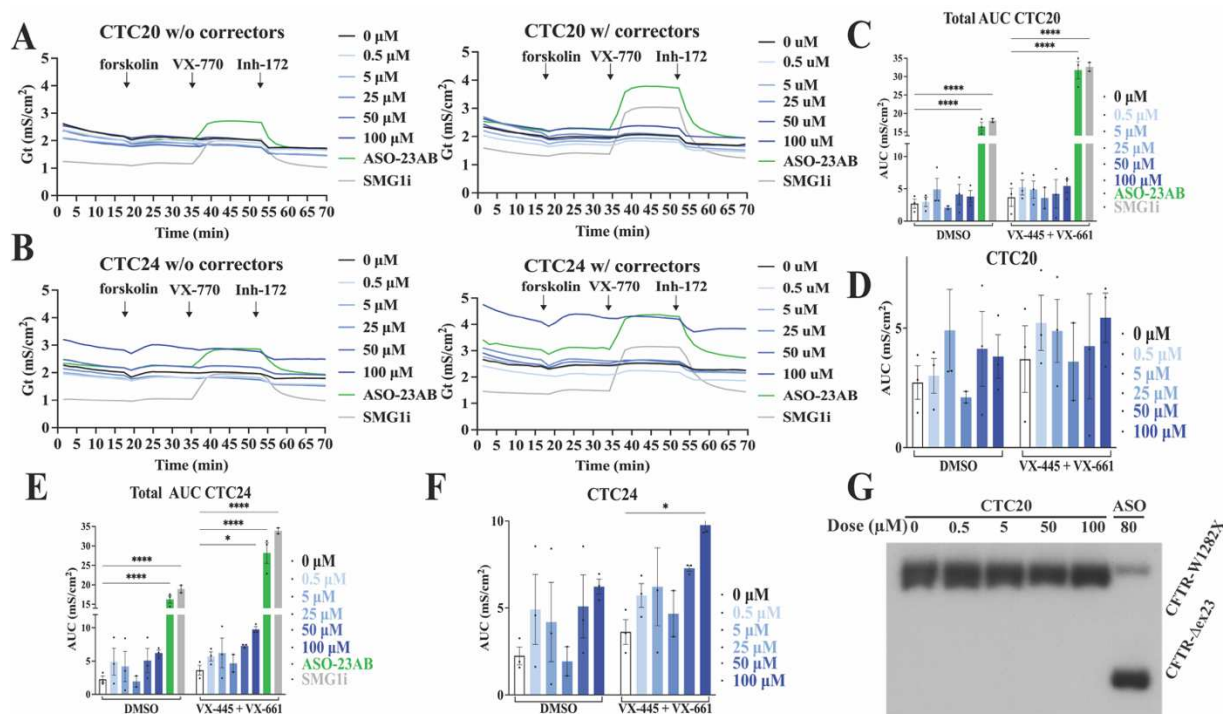


Figure 8. CTC20 and CTC24 partially restore CFTR-mediated chloride transport in W1282X cells via an exon 23 splicing-independent mechanism. A-B. Conductance (Gt) traces of 16HBE W1282X cells treated with CTC20 (A) or CTC24 (B) at 0.5–100 μ M, in the absence (left) or presence (right) of CFTR correctors VX-661 and VX-445 (VX2). Arrows indicate sequential addition of forskolin, CFTR potentiator VX-770, and CFTR inhibitor Inh-172. ASO-23AB and SMG1i are shown as positive controls. C-F. Total average area under the curve (AUC) quantification for CTC20 (C-D) and CTC24 (E-F). D and F excluded positive controls to better visualize compound dose-response. Data represent mean $\pm$ SEM, $n=3$ except $n=2$ for 25 μ M and SMG1i. (* two-way ANOVA with Dunnett's post-hoc test); * $P<0.05$ and **** $P<0.001$. G. RT-PCR analysis demonstrating that CTC20 does not alter CFTR exon 23 splicing at any concentration tested, unlike ASO-23AB (80 μ M) which induced robust exon 23 skipping. Upper band: CFTR-W1282X; lower band: CFTR- Δ ex23.

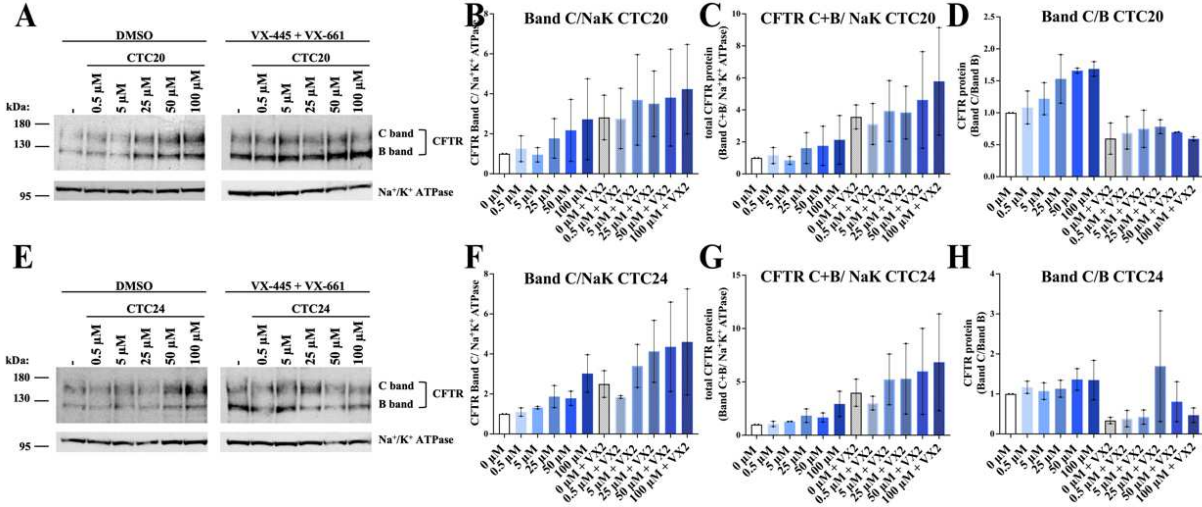


Figure 9. CTC20 and CTC24 induces directional increases in CFTR protein expression and maturation in W1282X cells. **A.** Representative western blot of CFTR protein isolated from 16HBEge-W1282X cells treated with CTC20 (0.5–100 μ M) $\pm$ VX-445+VX-661 (VX2). CFTR Band C (~180kDa) and Band B (~130kDa), and Na⁺/K⁺-ATPase loading control (~95kDa) are indicated. **B.** Quantification of total CFTR protein (Band C+B/Na⁺/K⁺-ATPase). **C.** Mature CFTR (Band C/Na⁺/K⁺-ATPase). **D.** Maturation efficiency (Band C/Band B), normalized to untreated cells. Full quantifications including ASO-23AB and SMG1i controls in Fig. S8. **E.** Representative western blot of CFTR in 16HBE-geW1282X cells treated with CTC24 (0.5–100 μ M) $\pm$ VX2, as in A. **F.** Total CFTR protein (Band C+B/Na⁺/K⁺-ATPase). **G.** Mature CFTR (Band C/Na⁺/K⁺-ATPase). **H.** Maturation efficiency (Band C/Band B), normalized to untreated cells. Data represent mean $\pm$ SEM, n=2.

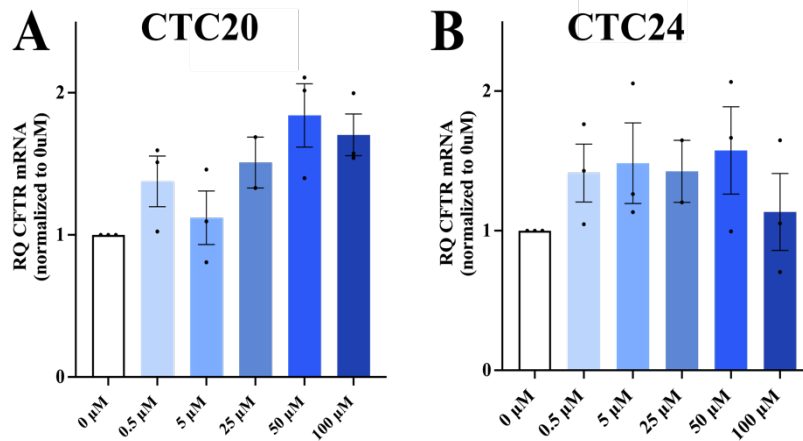


Figure 10. RT-qPCR analysis of CFTR mRNA levels in W1282X cells treated with CTC20 or CTC24. **A-B.** RT-qPCR quantification of total CFTR mRNA in 16HBEge-W1282X cells treated with CTC20 (A) and CTC24 (B) at 0.5–100 μ M. CFTR mRNA levels were quantified by RT-qPCR, normalized to the housekeeping gene HPRT1, and expressed as relative quantity (RQ) normalized to untreated cells (0 μ M). Data represent mean $\pm$ SEM, n=3 except n=2 for 25 μ M. Individual data points are shown.

SUPPLEMENTARY INFORMATION

Variant-selective RNA-targeting small molecules partially restore CFTR W1282X function in Cystic Fibrosis

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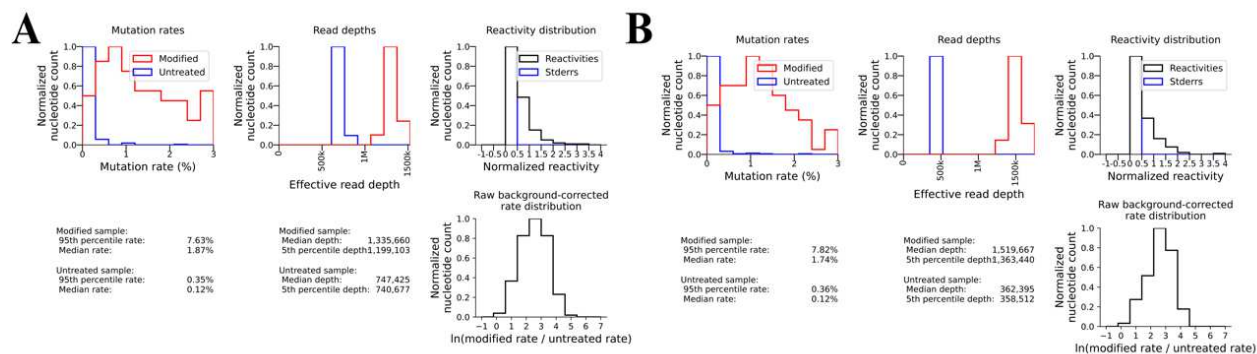


Figure S1. Quality control metrics for SHAPE-MaP experiments on IVT-transcribed WT (A) and W1282X RNA constructs (B). (**Top left**) Distribution of mutation rates in NAI-modified (red) and DMSO untreated (blue) samples, with the 95th percentile and median indicated. (**Top middle**) Effective sequencing read depth across the amplicon for modified and untreated samples. (**Top right**) Normalized SHAPE reactivity distribution. (**Bottom right**) Raw background-corrected mutation rate distribution. Both shows high modification rates (median > 1.5%), low background (untreated median < 0.15%), and high read depth (> 600,000 reads), confirming overall data quality.

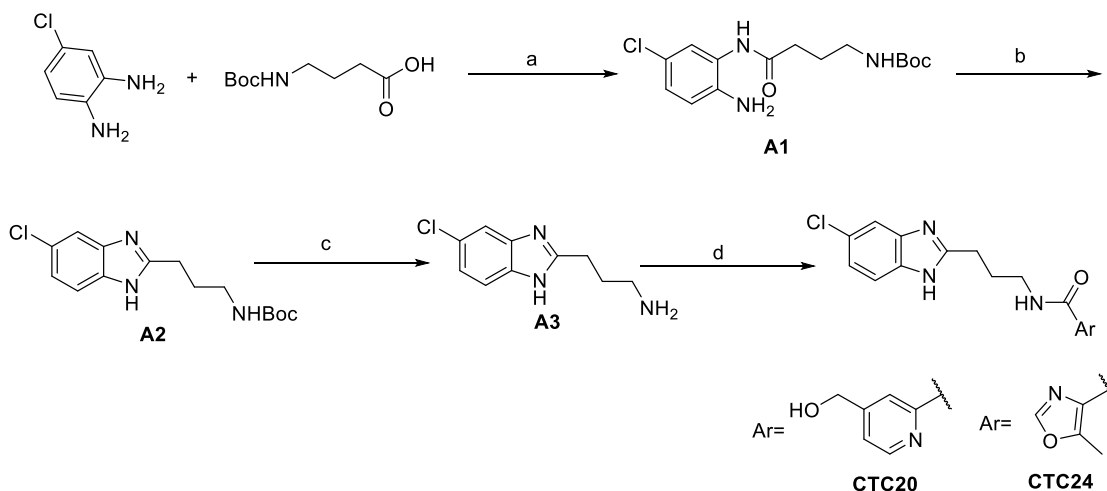


Figure S2. Synthetic Route of Compounds CTC20 & CTC24. Reagents and Conditions: (a) HOBt, EDC.HCl, Et₃N, DMF, rt, 2 h; (b) AcOH, 65 °C, 2 h; (c) TFA, DCM, 0 °C-rt, 4 h; (d) Ar-COOH, HATU, DIPEA, DMF, rt, 16 h.

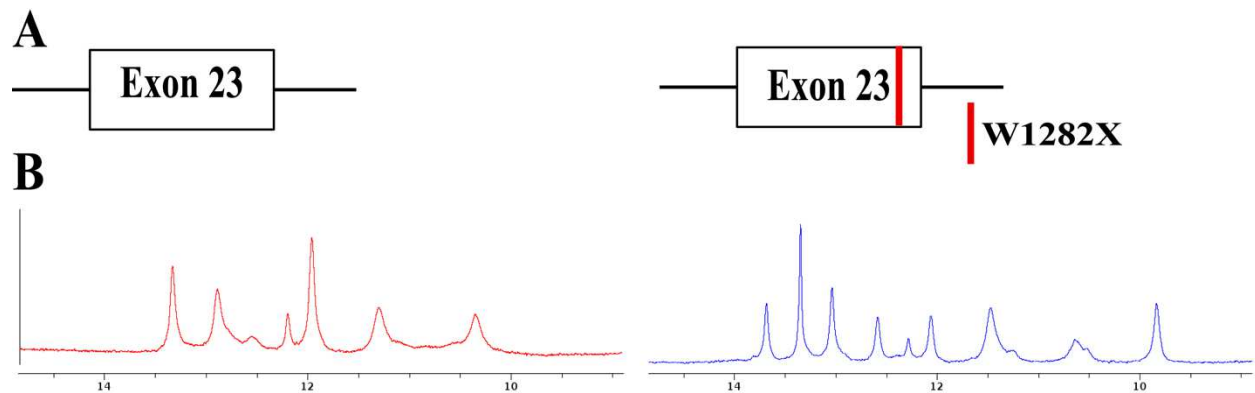


Figure S3. A. Schematic of exon 23 region, with W1282X location indicated in red. **B.** 1D ^1H NMR spectra showing the imino region of the RNA hairpin. The imino region of the RNA was shown to have fewer peaks in the absence of mutation (left panel, red) when compared to peaks in the presence of the mutation (right panel, blue).

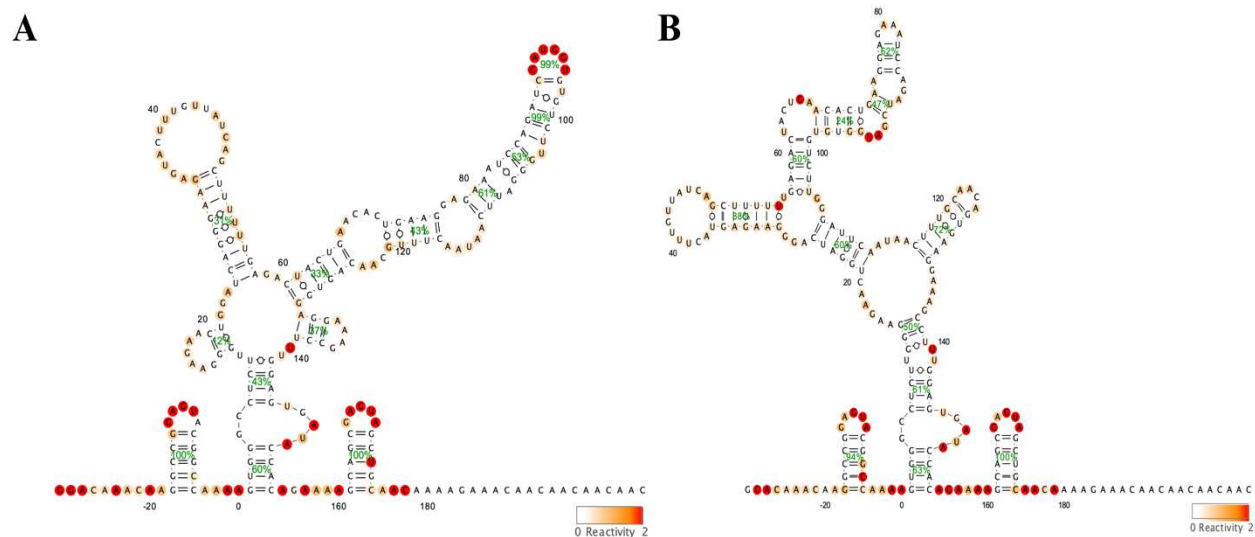


Figure S4. A. SHAPE reactivity profile of CFTR-WT Exon23 RNA. **B.** SHAPE reactivity profile of the CFTR-W1282X Exon23 RNA.

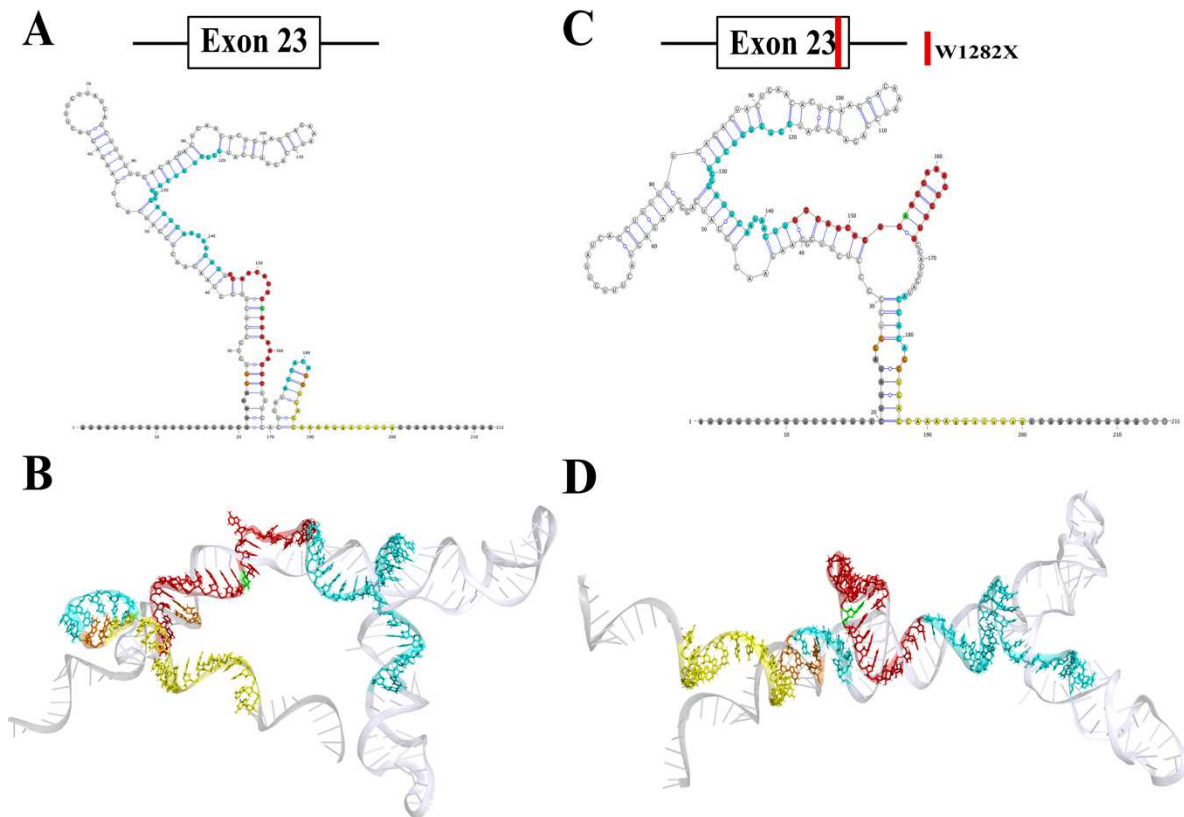


Figure S5. A. CFTR-WT Exon23 RNA and its splice junction. Colors (adjacent to mutation point (green)) cyan (exonic regions targeted by ASO-B), yellow (intronic regions targeted by ASO-A), orange (3' splice site), magenta (5' splice site), blue (cryptic 5' splice site) **B.** Predicted 3D RNA structure based on the SHAPE analysis in panel A. **C.** SHAPE profile of the CFTR-W1282X Exon23 RNA and its splice junction. **D.** Predicted 3D RNA structure based on SHAPE analysis in panel B.

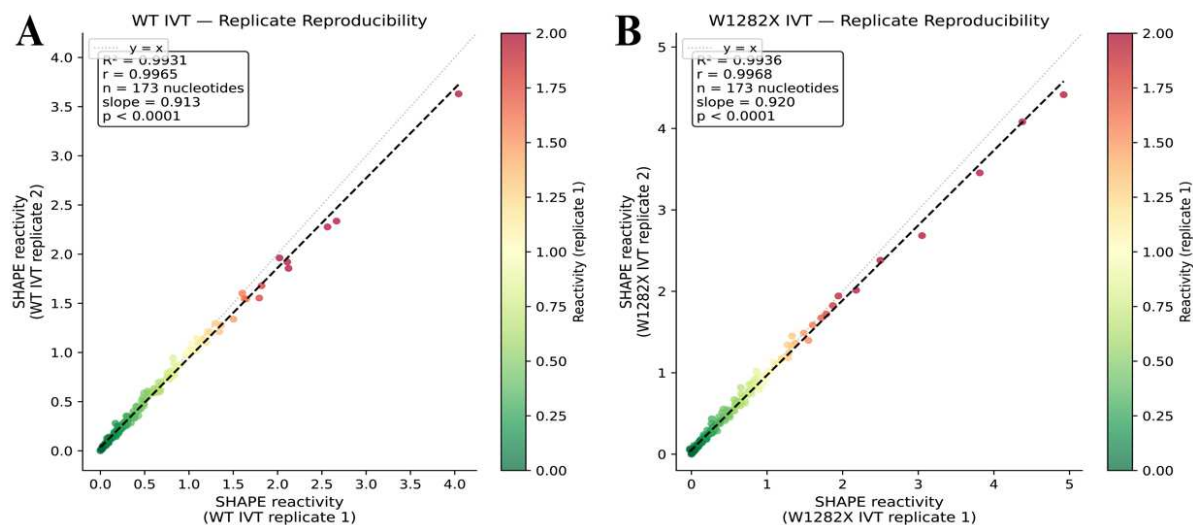


Figure S6. Reproducibility of SHAPE-MaP experiments. Scatter plots show SHAPE reactivity values for replicate 1 versus replicate 2 for **(A)** WT-CFTR and **(B)** W1282X-CFTR RNA constructs. Each point represents one nucleotide ($n = 173$). The dashed line indicates the linear regression fit, and the dotted line represents the ideal correlation ($y = x$). Pearson correlation coefficients (r) and coefficients of determination (R^2) are indicated in each panel.

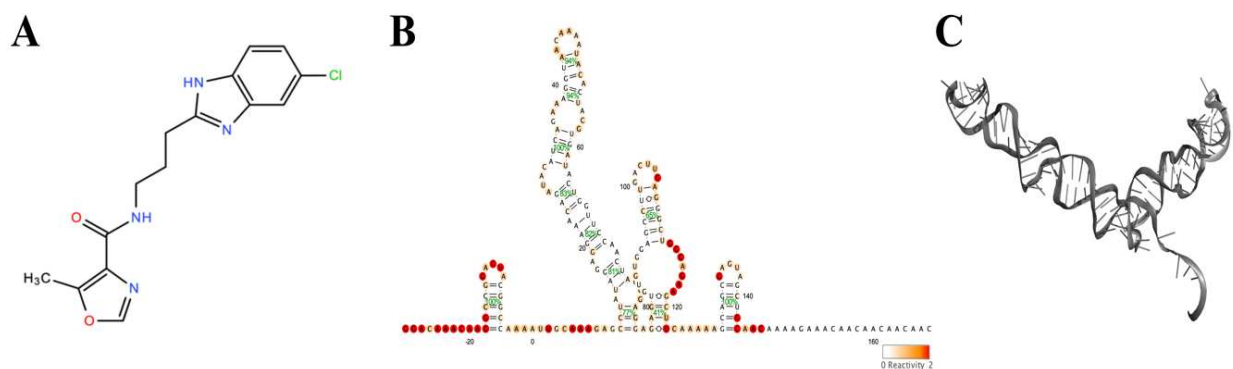


Figure S7. A. Chemical structure of the related analog CTC24. **B.** SHAPE reactivity profile of Human NIS-lncRNA. **C.** 3D model of the RNA in (B), obtained based on the 2D structure derived from the SHAPE assay.

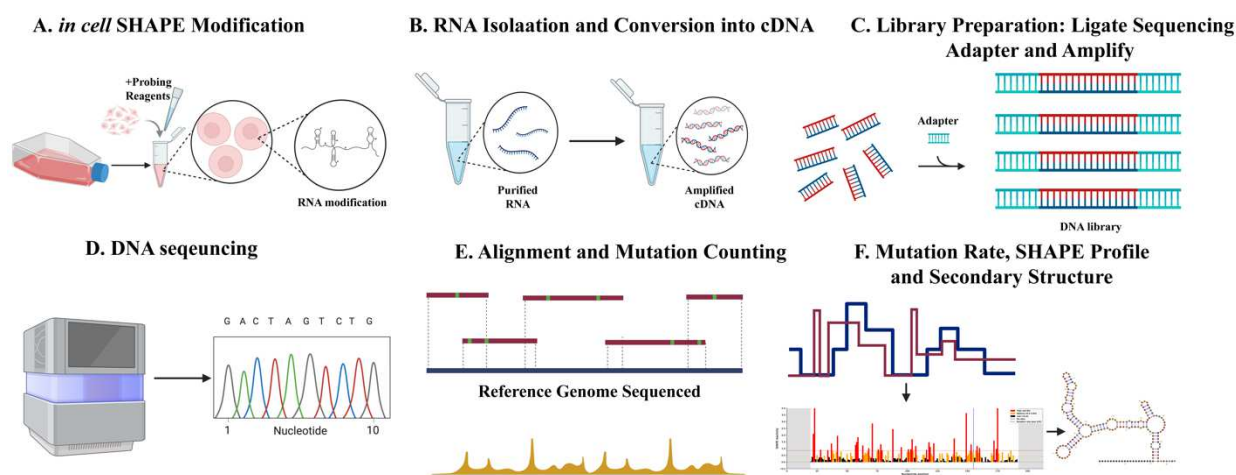


Figure S8. Schematic overview of the *in-cell* SHAPE-MaP workflow. **A.** Cells are treated with SHAPE reagents that selectively modify flexible RNA nucleotides. **B.** Modified RNA is purified, and reverse transcribed into cDNA, encoding the chemical adducts as mutations. **C.** Sequencing adapters are ligated, and libraries are amplified for next-generation sequencing. **D.** High-throughput sequencing data are aligned to reference transcripts, and **E.** mutation frequencies are quantified to generate SHAPE reactivity profiles. **F.** These profiles are used to model RNA secondary and tertiary structures with nucleotide-level resolution.

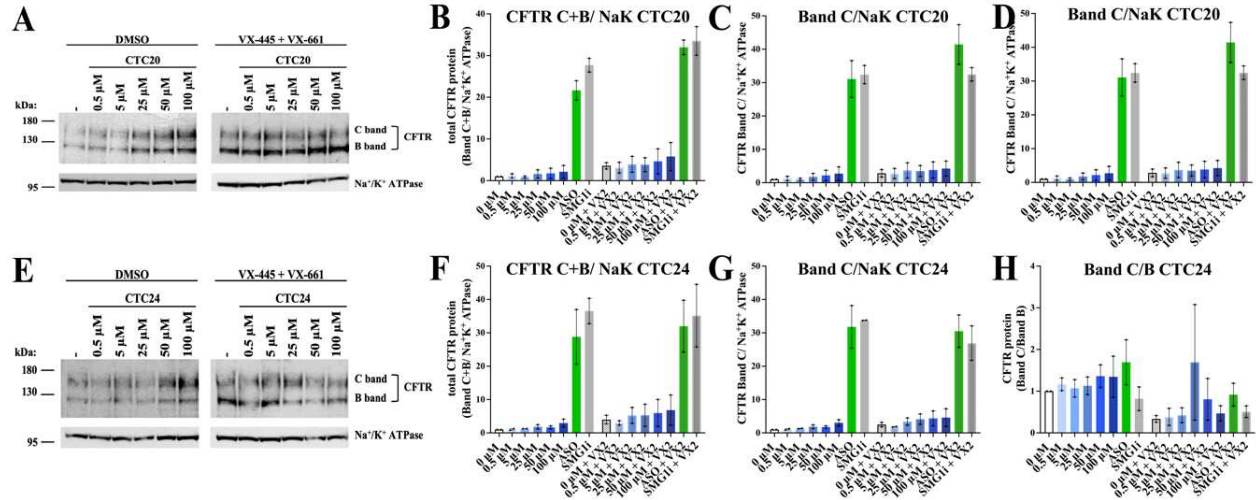


Figure S9. Supplementary quantification of CFTR functional and protein expression data for CTC20 and CTC24. **A.** Representative western blot of CFTR protein in 16HBEge-W1282X cells treated with CTC20 at the indicated concentrations, in the absence and presence of VX-445+VX-661. Band C (~180kDa), Band B (~130kDa), and Na⁺/K⁺-ATPase loading control (~95kDa) are indicated. ASO-23AB and SMG1i are included as positive controls. **B.** Total CFTR protein (Band C+B/Na⁺/K⁺-ATPase). **C.** Mature CFTR (Band C/Na⁺/K⁺-ATPase). **D.** Maturation efficiency (Band C/Band B), normalized to untreated cells. **E-H.** As in panels A-D, but for CTC24-treated cells at the indicated concentrations.

#	Name	DNA sequence	Primerize primer	Notes
1	CFTR WT exon 23-nt 109 to 137 with SHAPE Cassette	TTCTAATACGACTCACTATAGGACAAACA AGGCCGGAGTACGGCCAAAA CAATAACT TTGCAACAGTG G AGGAAAGC AAAAGCAGC GAGTAGCTGCAACAAAAGAAACAACAAC AACAAC	1F: TTCTAATACGACTCACTATAGG ACAAACAAGGCCGGA	DNA templates were designed for <i>in vitro</i> transcription to generate RNA constructs containing SHAPE cassettes for analysis of conformational changes near the mutation site. SHAPE cassette sequences are highlighted in orange and blue . The T7 promoter sequence is indicated in red , and the mutation site is marked in green .
			2R: CCTCCACTGTTGCAAAGTTATT GTTTTGGCCGTACTCCGGCCTT GTTTGTCTTATAGTGA	
			3F: AACTTTGCAACAGTGGAGGAA AGCAAAAGCAGCGAGTAGCTG CAACAAAAGA	
			4R: GTTGTTGTTGTTGTTTCTTTTGT TGCAGCTACTCGC	
2	CFTR W1282X exon 23-nt 109 to 137 with SHAPE Cassette	TTCTAATACGACTCACTATAGGACAAAC AAGGCCGGAGTACGGCCAAAA CAATAAC TTTGCAACAGTG A AGGAAAGC AAAAGCAG CGAGTAGCTGCAACAAAAGAAACAACAA CAACAAC	1F: TTCTAATACGACTCACTATAGG ACAAACAAGGCCGGAGTACGG CCA	
			2R: GCTTTCCTTCACTGTTGCAAAG TTATTGTTTTGGCCGTACTCCG GCCTTG	
			3F: TGCAACAGTGAAGGAAAGCAA AAGCAGCGAGTAGCTGCAACA AAAGA	
			4R: GTTGTTGTTGTTGTTTCTTTTGT TGCAGCTACTCGC	

#	Name	DNA sequence	Forward and Reverse primers	Annealing temperature (°C)	Notes
3	CFTR WT Exon23-FL with SHAPE Cassette	TAATACGACTCACTATAGGACAAACAAG GCCGGAGTACGGCCAAAAG TGGGCCTCTT GGGAAGAAGTGGATCAGGGAAGAGTACTT TGTTATCAGCTTTTTTGAGACTACTGAACA CTGAAGGAGAAATCCAGATCGATGGTGTGT CTTGGGATTCAATAACTTTGCAACAGTGGA GGAAAGCCTTTGGAGTGATACCACAGAAA AGCAGCGAGTAGCTGCAACAAAAGAAAC AACAACAACAAC	Forward primer: TAATACGACTCA CTATAGGACA	53.6	DNA templates were designed for <i>in vitro</i> transcription to generate RNA constructs corresponding to the full-length (FL) CFTR exon 23 , incorporating SHAPE cassettes for structural probing.
4	CFTR W1282X Exon23-FL with SHAPE Cassette	TAATACGACTCACTATAGGACAAACAAG GCCGGAGTACGGCCAAAAG TGGGCCTCTT GGGAAGAAGTGGATCAGGGAAGAGTACTT TGTTATCAGCTTTTTTGAGACTACTGAACA CTGAAGGAGAAATCCAGATCGATGGTGTGT CTTGGGATTCAATAACTTTGCAACAGTGGA GGAAAGCCTTTGGAGTGATACCACAGAAA AGCAGCGAGTAGCTGCAACAAAAGAAAC AACAACAACAAC	Reverse primer: GTTGTTGTTGTT GTT TCTTTTG		
5	CFTR WT Exon23 and its splice junction with linear 3'end	TAATACGACTCACTATA gtgatcccatcacttttacctt atagGTGGGCCTCTTGGGAAGAACTGGATCA GGGAAGAGTACTTTGTTATCAGCTTTTTTG AGACTACTGAACACTGAAGGAGAAATCCA GATCGATGGTGTGTCTTGGGATTCAATAAC TTTGCAACAGTGAGGAAAGCCTTTGGAGT GATACCACAGgtgagcaaaaggacttagccagaaaAAA AGAAACAACAACA	Forward primer: TAATACGACTCA CTATAGTGATCC	52	DNA templates were designed for <i>in vitro</i> transcription to generate RNA constructs corresponding to the full-length (FL) CFTR exon 23 , including flanking splicing junctions (portions of introns 22 and 23), to investigate RNA structure in a near-native context. Intron-derived sequences are indicated in

6	CFTR W1282X Exon23 and its splice junction with linear 3'end	TAATACGACTCACTATA gtgatcccatcacttttacctt atagGTGGGCCTCTTGGGAAGAACTGGATCA GGGAAGAGTACTTTGTTATCAGCTTTTTTG AGACTACTGAACACTGAAGGAGAAATCCA GATCGATGGTGTGTCTTGGGATTCAATAAC TTTGCAACAGTGAGGAAAGCCTTTGGAGT GATACCACAGgtgagcaaaaggacttagccagaaaAAA AGAAACAACAACAA	Reverse primer: TTGTTGTTGTTTC TTTTTTC		lowercase letters. These constructs do not contain SHAPE cassettes and instead include a 3'-end linearization sequence (colored in grey) to enhance the efficiency of biotin labeling. The resulting RNAs were used for microscale thermophoresis (MST) , and surface plasmon resonance (SPR) .
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Supplementary Table 1. DNA templates and primers used for *in vitro* transcription of RNA constructs in this study.

Primer name	Sequence 5'-3'	Notes
Reverse transcription primer	CTTTTTTCTGGCTAAGTCC	For <i>in vitro</i> transcribed RNA
Step 1 forward primer ^I	GACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNGTGATCCCATCACTTTTAC	
Step 1 reverse primer ^{II}	CCCTACACGACGCTCTTCCGATCTNNNNNCTTTTTTCTGGCTAAGTCC	
Reverse transcription primer	CCATATTTCTTGATCACTCCAC	<i>In Cell</i> and Cell free RNAs
Step 1 forward primer	GACTGGAGTTCAGACGTGTGCTCTTCCGATCTNNNNNGATCTGTGAGCCGAGTCTTTAA	
Step 1 reverse primer	CCCTACACGACGCTCTTCCGATCTNNNNNCCATATTTCTTGATCACTCCAC	

Step 2 forward primer ^{III}	CAAGCAGAAGACGGCATACTGAGAT [Barcode] GTGACTGGAGTTCAGAC	The barcode consists of a unique 6-nt sequence incorporated to enable sample multiplexing during Illumina sequencing.
Step 2 reverse primer ^{IV}	AATGATACGGCGACCACCGAGATCTACACTCTTTCCCTACACGACGCTCTTCCG	Identical for all samples; used as the universal reverse primer in Step 2 of library preparation.

Supplementary Table 2. Primer sequences used for library preparation in SHAPE-MaP experiments.

I Introduces a partial Illumina adapter sequence at the 5' end of the amplicon to enable downstream library preparation and sequencing.

II Introduces a partial Illumina adapter sequence at the 3' end of the amplicon to enable downstream library preparation and sequencing.

III and IV Completes the Illumina adapter sequence at the 5' end of the amplicon.

SUPPLEMENTARY NOTE

Variant-selective RNA-targeting small molecules partially restore CFTR W1282X function in Cystic Fibrosis

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Contents

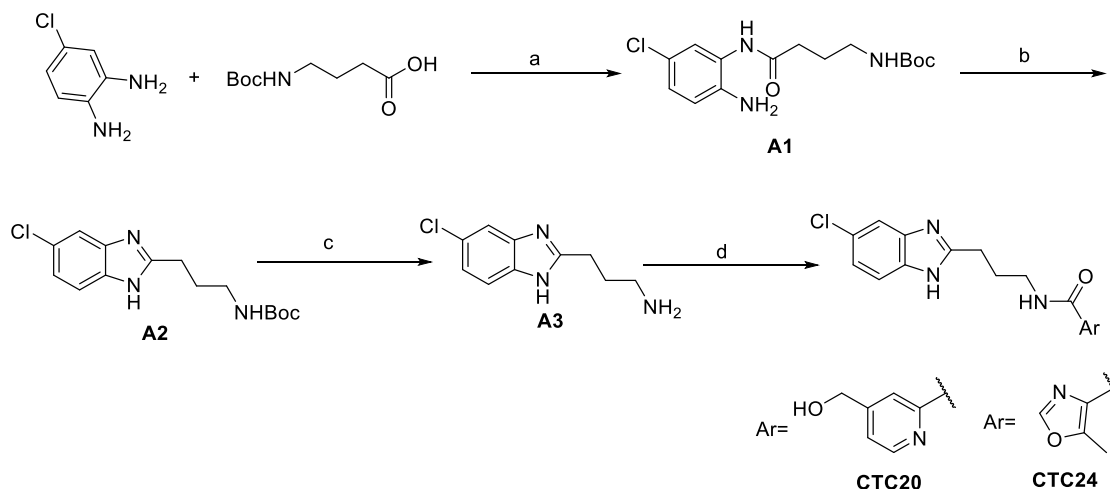
1.	General information	S3
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3.	^1H and ^{13}C NMR spectra of compounds	S8-S11
4.	HPLC data of CTC20 and CTC24	S12-S13

1. General Information

All materials were purchased from commercial suppliers and used without further purification. The purities of the final compounds were characterized by high-performance liquid chromatography (LC/MS) using a gradient elution program (Waters, Sunfire, C18 column, acetonitrile/water 5/95/95/5, 5 min, 0.05% TFA) and UV detection (254 nm). The purities of the final compounds were 95% or greater. Microwave experiments were performed using Biotage Initiator+. These reactions were performed in standard Biotage Microwave Reaction Vials (10–20 mL), and the reaction mixture was stirred by a magnetic stirring bar inside the microwave reaction vial. The desired temperature was set and monitored from the instrument's 10.4" touch screen. Temperature cannot exceed 250 °C as higher temperatures cannot be used with 10–20 mL vials in solvent-free reactions as recommended by the manufacturer. All microwave reactions were performed in sealed vials, and there was no pressure build-up during the reaction. After processing, the reaction mixture is immediately cooled with pressurized air. The reaction is heated through high-frequency microwaves (2.45 GHz), generated by magnetron. Melting points of the compounds were recorded with Reach Devices (RD-MP) digital melting point determination apparatus. NMR spectra were recorded on a Bruker NMR 400 MHz Avance III spectrometer operating at 400 MHz for ^1H NMR and 100 MHz for ^{13}C NMR. Chemical shifts are given in part per million (ppm) relative to tetramethyl silane (TMS) and coupling constants J are given in Hertz. HPLC-HRMS analyses were performed on reverse phase gradient using Agilent (Santa Clara, CA) 1200 series binary pump (G1312B), waters XTerra MS C₁₈ (3.5 μm ; 2.1 x 150 mm) + Phenomenex C18 security guard column (2 x 4 mm) using 0.2% acetic acid in H₂O/methanol as mobile phases; wavelength = 254 nm; and mass spectrometry was done with 6220 Agilent (Santa Clara, CA) TOF in electrospray ionization (ESI) mode with positive and negative method in both Profile and Centroid mode.

2. Experimental Details:

Supplementary Scheme 1. Synthetic Route of Compounds CTC20 & CTC24^a



^aReagents and Conditions: (a) HOBT, EDC.HCl, Et₃N, DMF, rt, 2 h; (b) AcOH, 65 °C, 2 h; (c) TFA, DCM, 0 °C-rt, 4 h; (d) RCOOH, HATU, DIPEA, DMF, rt, 16 h.

Procedure for the synthesis of *tert*-butyl (4-((2-amino-5-chlorophenyl)amino)-4-oxobutyl)carbamate (**A1**):

To a stirred solution of 4-((*tert*-butoxycarbonyl)amino)butanoic acid (5.0 g, 24.6 mmol, 1.0 equiv) in DMF (30 mL) were added triethylamine (8.64 mL, 61.5 mmol, 2.5 equiv) and HOBT (3.98 g, 29.5 mmol, 1.2 equiv) at room temperature (rt). The reaction mixture was stirred for 10 min, followed by the addition of 4-chlorobenzene-1,2-diamine (3.85 g, 27.1 mmol, 1.1 equiv). The resulting mixture was stirred at room temperature (rt) for 2 h. After completion of the reaction as monitored by TLC, the reaction mixture was poured into ice-cold water (2 × 500 mL) and extracted with ethyl acetate (2 × 500 mL). The combined organic extracts were washed with brine (50 mL), dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure to afford a brown gummy residue. The crude product was purified by flash column chromatography using 2% methanol in chloroform as the eluent to give compound **A1** (6.0 g, 74%) as a light brown solid.

Procedure for the synthesis of *tert*-butyl 3-(5-chloro-1*H*-benzo[*d*]imidazol-2-yl)propylcarbamate (A2):

tert-Butyl 4-((2-amino-5-chlorophenyl)amino)-4-oxobutylcarbamate (**A1**) (5.80 g, 17.69 mmol, 1.0 equiv) was dissolved in acetic acid (AcOH) (60 mL), and the resulting solution was heated at 65 °C for 2 h. After completion of the reaction, the mixture was cooled to room temperature and concentrated under reduced pressure to remove the solvent. The residue was dissolved in dichloromethane and carefully neutralized with saturated aqueous sodium bicarbonate solution. The aqueous layer was extracted with dichloromethane (250 mL), and the combined organic extracts were dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure to afford compound **A2** (5.46 g, 99.6%) as a pale brown solid.

Procedure for the synthesis of 3-(5-chloro-1*H*-benzo[*d*]imidazol-2-yl)propan-1-amine (A3):

To a stirred solution of *tert*-butyl 3-(5-chloro-1*H*-benzo[*d*]imidazol-2-yl)propylcarbamate (**A2**) (1.50 g, 4.84 mmol, 1.0 equiv) in dichloromethane (15 mL) at 0 °C was added trifluoroacetic acid (TFA) (3.70 mL, 48.4 mmol, 10 equiv) dropwise. The resulting reaction mixture was allowed to warm to room temperature and stirred for 4 h. After completion of the reaction as monitored by TLC, the mixture was concentrated under reduced pressure to afford crude compound (**A3**) (1.0 g, 99%) as a brown gummy. The crude product was used directly in the next step without further purification.

General Procedure for the syntheses of CTC20 and CTC24:

A carboxylic acid precursor (2.5 mmol, 1.0 equiv) was dissolved in DMF (10 mL). To the resulting solution were added 3-(5-chloro-1*H*-benzo[*d*]imidazol-2-yl)propan-1-amine (**A3**) (629 mg, 3.0 mmol, 1.2 equiv) and HATU (1.42 g, 3.75 mmol, 1.5 equiv), followed by DIPEA (1.30 mL, 7.5 mmol, 3.0 equiv). The reaction mixture was stirred at room temperature (rt) for 16 h. Upon completion of the reaction, as monitored by TLC, the mixture was diluted with ice-cold water (100 mL) and extracted with dichloromethane (2 × 100 mL). The combined organic extracts were washed with brine (15 mL), dried over anhydrous sodium sulfate, filtered, and concentrated under reduced pressure. The resulting crude product was purified by flash column chromatography using 2–3% methanol in chloroform as the eluent to afford the desired amide derivatives.

Analytical Data:

***Tert*-butyl (4-((2-amino-5-chlorophenyl)amino)-4-oxobutyl)carbamate (A1):** The reaction was carried out with 4-((*tert*-butoxycarbonyl)amino)butanoic acid (5 g, 24.6 mmol) and 4-chlorobenzene-1,2-diamine (3.85 g, 27.06 mmol); Purified by automated flash silica gel column chromatography using methanol in chloroform (2:98); Yield: 6 g (74%), light brown solid; mp: 80.2-92.4 °C; ¹H NMR (400 MHz, DMSO-*d*₆): δ 9.05 (d, *J* = 13.0 Hz, 1H), 7.15 (d, *J* = 8.4 Hz, 1H), 6.84 (t, *J* = 5.9 Hz, 1H), 6.72 (d, *J* = 2.4 Hz, 1H), 6.51 (dd, *J* = 8.4, 2.4 Hz, 1H), 5.16 (s, 2H), 2.94 (q, *J* = 6.7 Hz, 2H), 2.29 (q, *J* = 7.0 Hz, 2H), 1.65 (p, *J* = 7.1 Hz, 2H), 1.36 (s, 9H); ¹³C {¹H} NMR (100 MHz, DMSO-*d*₆): δ 171.03, 155.64, 143.55, 129.55, 126.76, 122.17, 115.26, 114.49, 77.47, 39.42, 33.07, 28.28, 25.64; LCMS(ESI) *m/z*: 350.1 [M+Na]⁺ for C₁₅H₂₂ClN₃O₃Na.

***Tert*-butyl (3-(5-chloro-1*H*-benzo[*d*]imidazol-2-yl)propyl)carbamate (A2):** The reaction was carried out with *tert*-butyl (4-((2-amino-5-chlorophenyl)amino)-4-oxobutyl)carbamate (A1) (5.8 g, 17.69 mmol); Yield: 5.46 g (99.6%), pale brown solid; mp: 147.5-149.1 °C; ¹H NMR (400 MHz, DMSO-*d*₆): δ 12.33 (brs, 1H), 7.54-7.40 (m, 2H), 7.11 (dd, *J* = 8.5, 2.1 Hz, 1H), 6.88 (t, *J* = 5.6 Hz, 1H), 2.98 (q, *J* = 6.6 Hz, 2H), 2.77 (t, *J* = 7.7 Hz, 2H), 1.84 (p, *J* = 7.3 Hz, 2H), 1.35 (s, 9H); ¹³C {¹H} NMR (100 MHz, DMSO-*d*₆): δ 156.36, 155.65, 125.52, 121.29, 77.48, 28.25, 27.83, 26.03; LCMS(ESI) *m/z*: 332.2 [M+Na]⁺ for C₁₅H₂₀ClN₃O₂Na.

3-(5-chloro-1*H*-benzo[*d*]imidazol-2-yl)propan-1-amine (A3): The reaction was carried out with *tert*-butyl (3-(5-chloro-1*H*-benzo[*d*]imidazol-2-yl)propyl)carbamate (A2) (1.5 g, 4.84 mmol); Yield: 1.2 g (99%), brown gummy; LCMS(ESI) *m/z*: 210.1 [M+H]⁺ for C₁₀H₁₃ClN₃.

***N*-(3-(5-Chloro-1*H*-benzo[*d*]imidazol-2-yl)propyl)-4-(hydroxymethyl)picolinamide (CTC20):** The reaction was carried out with 4-(hydroxymethyl)picolinic acid (382 mg, 2.5 mmol) and 3-(5-chloro-1*H*-benzo[*d*]imidazol-2-yl)propan-1-amine (A3) (629 mg, 3 mmol); Purified by automated flash silica gel column chromatography using methanol in chloroform (2:98); Yield: 258 mg (30%), white solid; mp: 65-73.5 °C; ¹H NMR (400 MHz, DMSO-*d*₆): δ 8.92 (t, *J* = 6.2 Hz,

1H), 8.52 (d, $J = 5.0$ Hz, 1H), 7.99 (s, 1H), 7.53-7.41 (m, 3H), 7.12 (dd, $J = 8.5, 2.1$ Hz, 1H), 5.55 (brs, 1H), 4.60 (s, 2H), 3.42-3.37 (m, 2H), 2.84 (t, $J = 7.6$ Hz, 2H), 2.02 (p, $J = 7.2$ Hz, 2H); ^{13}C { ^1H } NMR (100 MHz, DMSO- d_6): δ 164.09, 156.38, 153.55, 149.95, 148.12, 125.60, 123.44, 121.41, 119.07, 61.43, 38.51, 27.49, 26.18; LCMS(ESI) m/z : 345.200 $[\text{M}+\text{H}]^+$ for $\text{C}_{17}\text{H}_{18}\text{ClN}_4\text{O}_2$.

***N*-(3-(5-Chloro-1*H*-benzo[d]imidazol-2-yl)propyl)-5-methyloxazole-4-carboxamide**

(CTC24): The reaction was carried out with 5-methyloxazole-4-carboxylic acid (317 mg, 2.5 mmol) and 3-(5-chloro-1*H*-benzo[d]imidazol-2-yl)propan-1-amine (**A3**) (629 mg, 3 mmol); Purified by automated flash silica gel column chromatography using methanol in chloroform (2:98); Yield: 215 mg (27%), pale brown solid; mp: 186-193.5 °C; ^1H NMR (400 MHz, DMSO- d_6): δ 12.41 (brs, 1H), 8.32 (s, 1H), 8.30 (s, 1H), 7.50 (d, $J = 2.0$ Hz, 1H), 7.44 (d, $J = 8.5$ Hz, 1H), 7.11 (dd, $J = 8.5, 2.0$ Hz, 1H), 3.30-3.25 (m, 2H), 2.81 (t, $J = 7.6$ Hz, 2H), 2.54 (s, 3H), 1.96 (p, $J = 7.2$ Hz, 2H); ^{13}C { ^1H } NMR (100 MHz, DMSO- d_6): δ 161.18, 156.39, 152.08, 149.57, 128.48, 125.53, 121.34, 37.94, 27.54, 26.17, 11.20; LCMS(ESI) m/z : 319.100 $[\text{M}+\text{H}]^+$ for $\text{C}_{15}\text{H}_{16}\text{ClN}_4\text{O}_2$.

3. Spectral Data (^1H and ^{13}C NMR spectra of compounds):

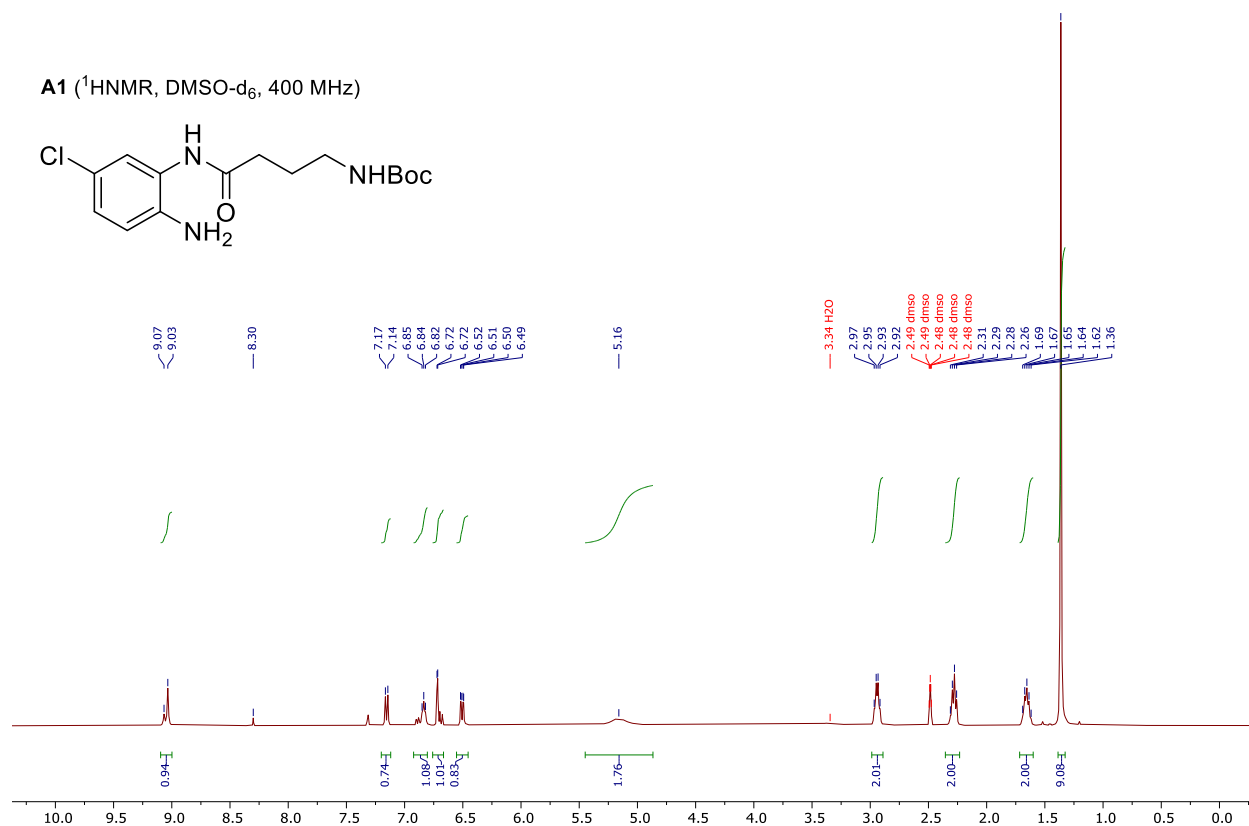


Figure SN1. ^1H NMR spectra (400MHz, DMSO- d_6) of A1

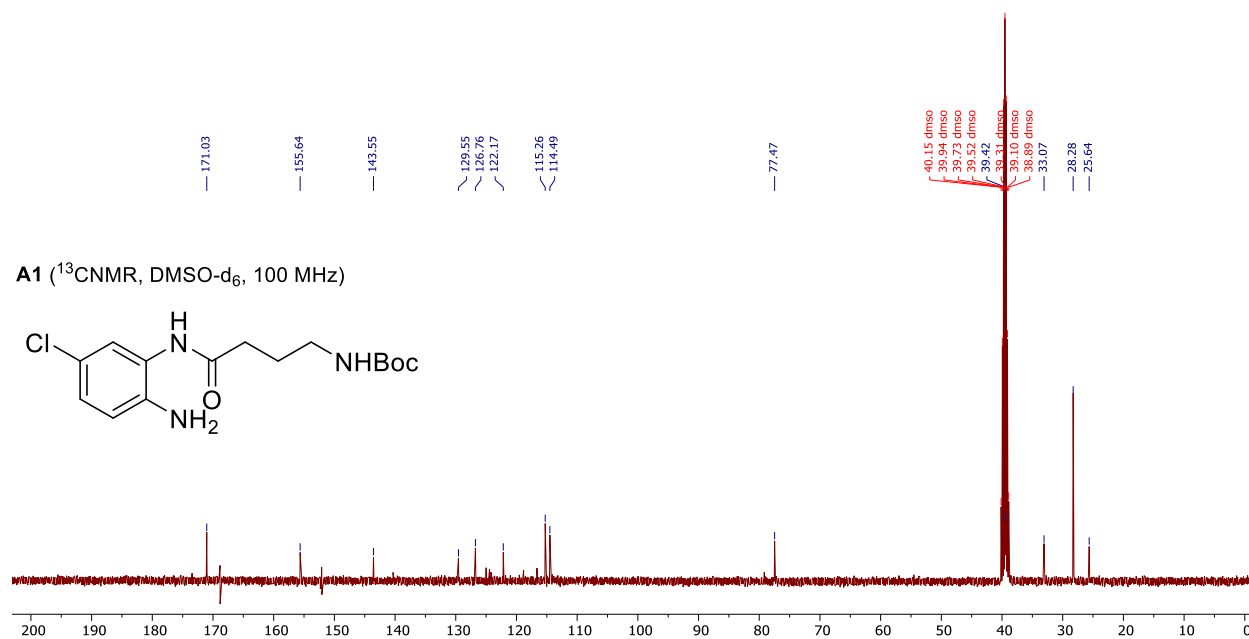


Figure SN2. $^{13}\text{C}\{^1\text{H}\}$ NMR spectra (100MHz, DMSO- d_6) of A1

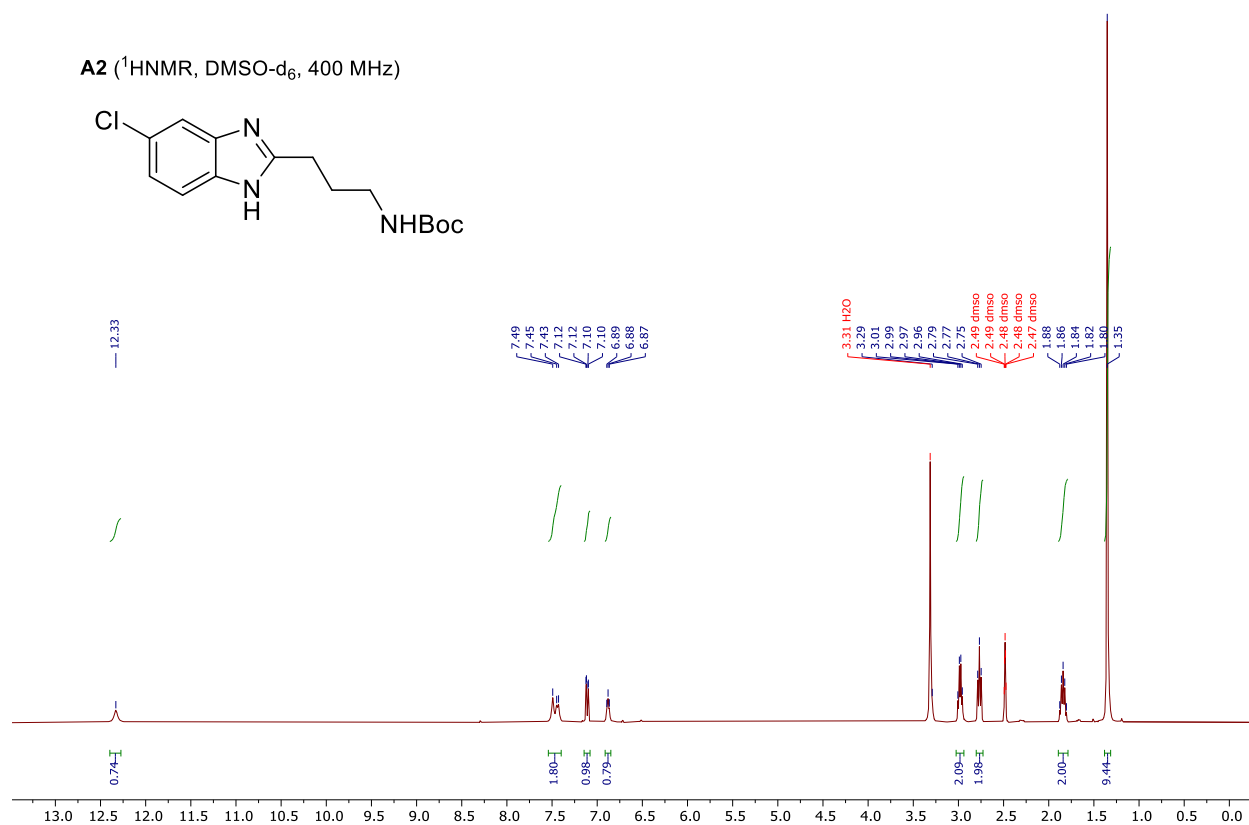


Figure SN3. ^1H NMR spectra (400MHz, DMSO- d_6) of A2

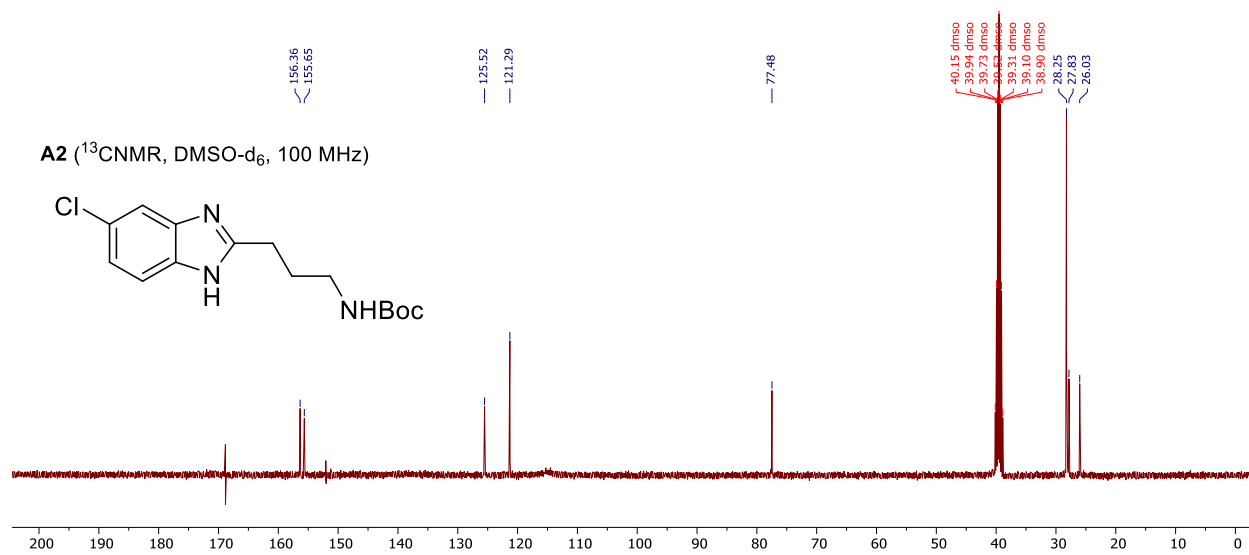


Figure SN4. $^{13}\text{C}\{^1\text{H}\}$ NMR spectra (100MHz, DMSO- d_6) of A2

CTC20 (^1H NMR, DMSO- d_6 , 400 MHz)

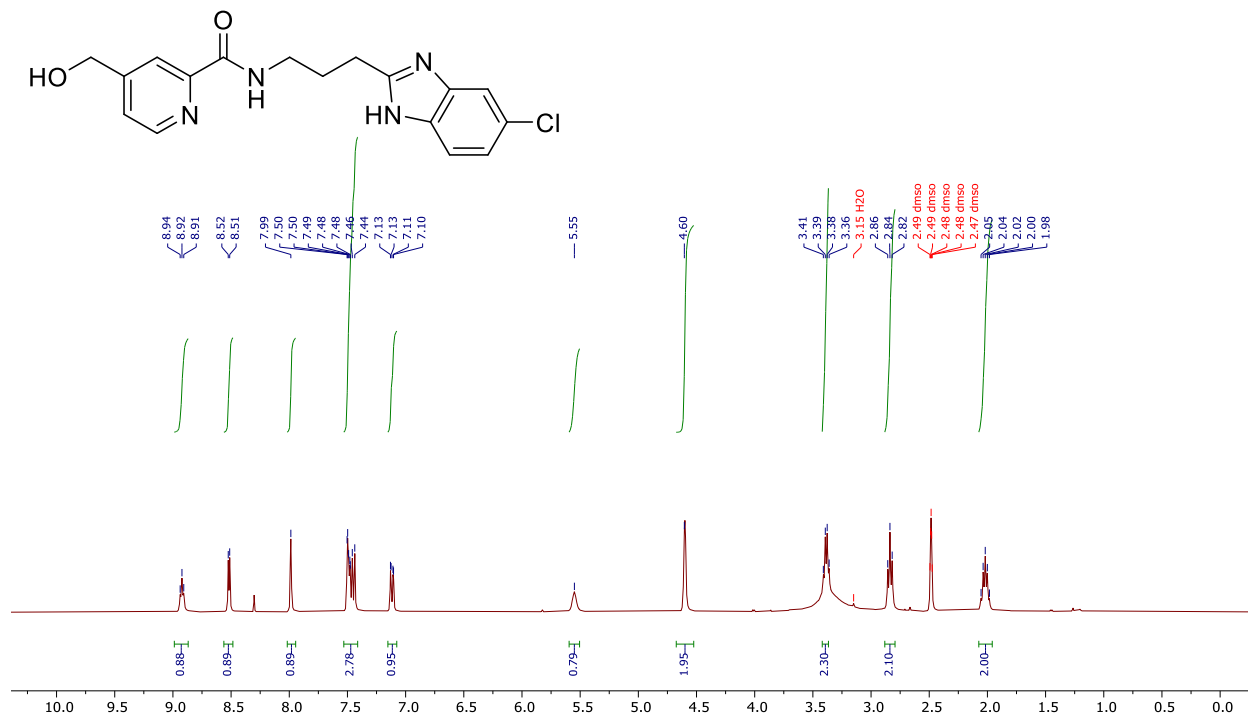


Figure SN5. ^1H NMR spectra (400MHz, DMSO- d_6) of CTC20

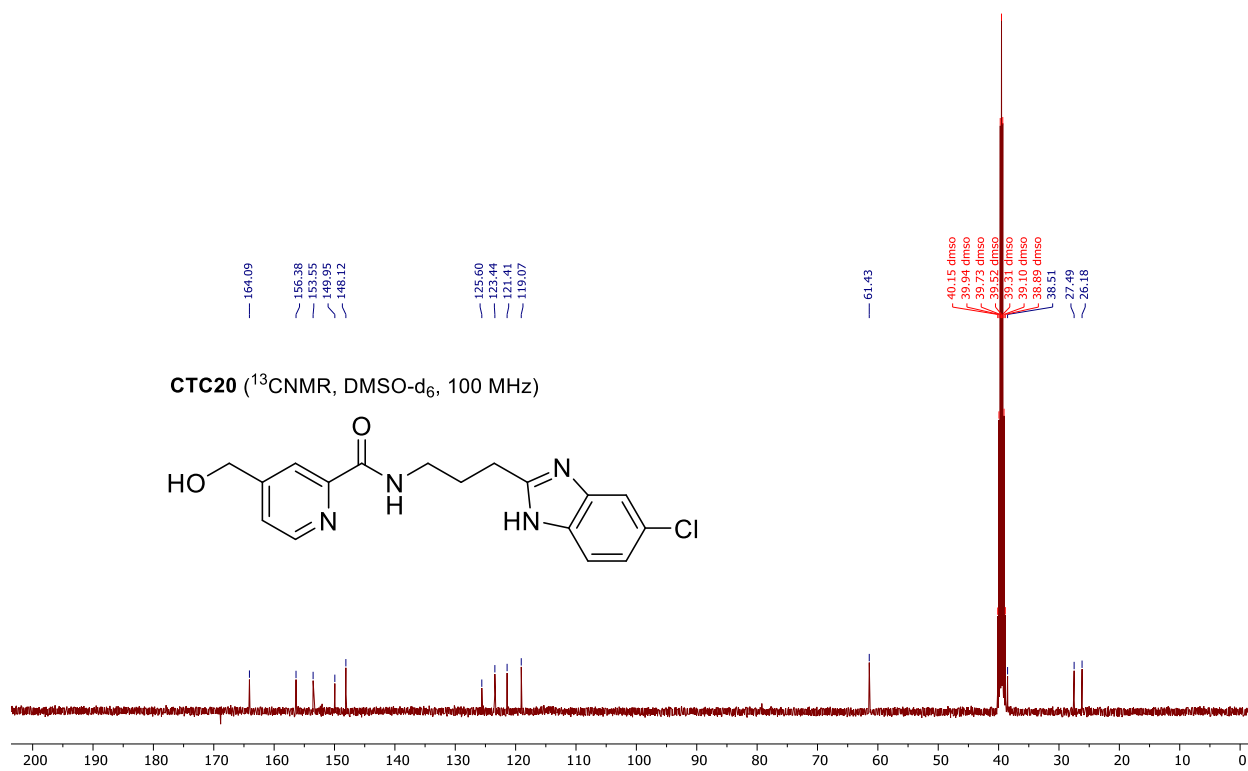


Figure SN6. $^{13}\text{C}\{^1\text{H}\}$ NMR spectra (100MHz, DMSO- d_6) of CTC20

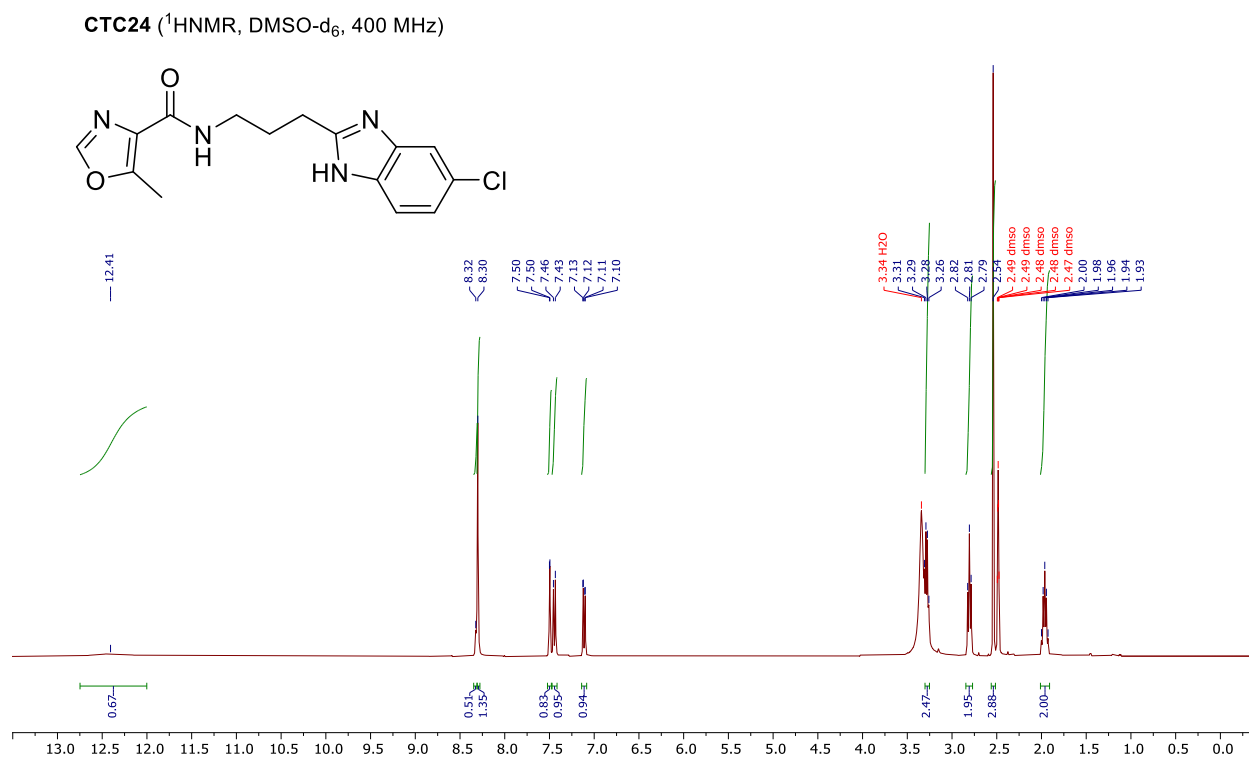


Figure SN7. ^1H NMR spectra (400MHz, DMSO- d_6) of CTC24

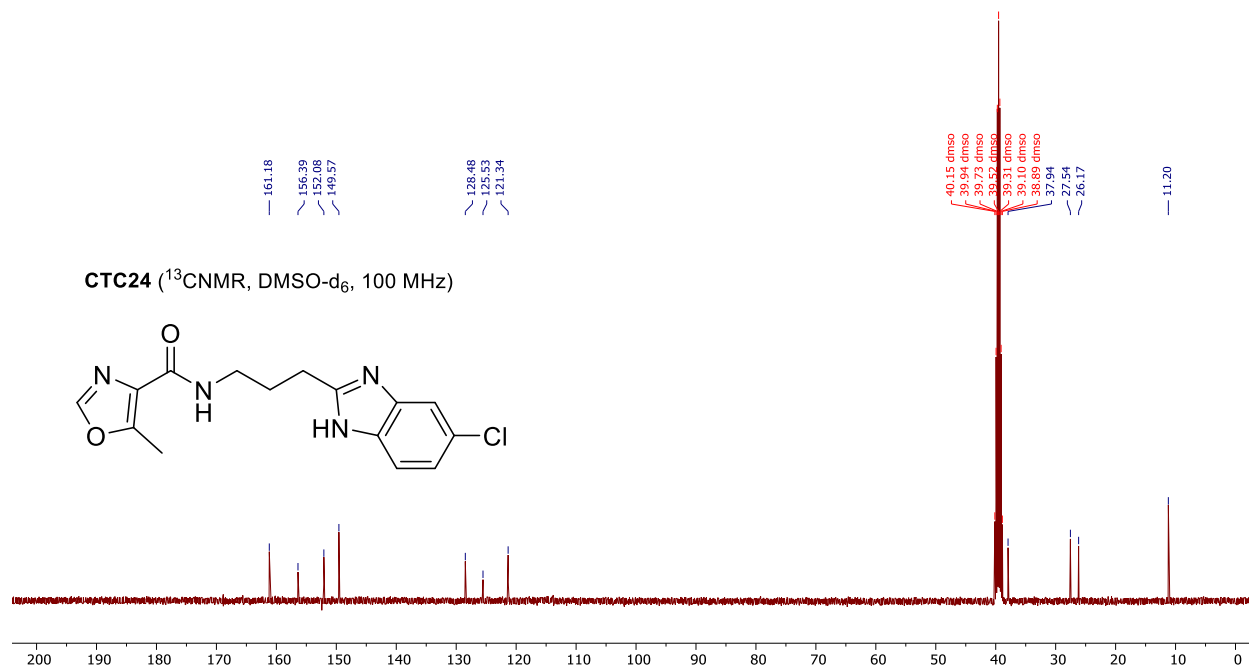
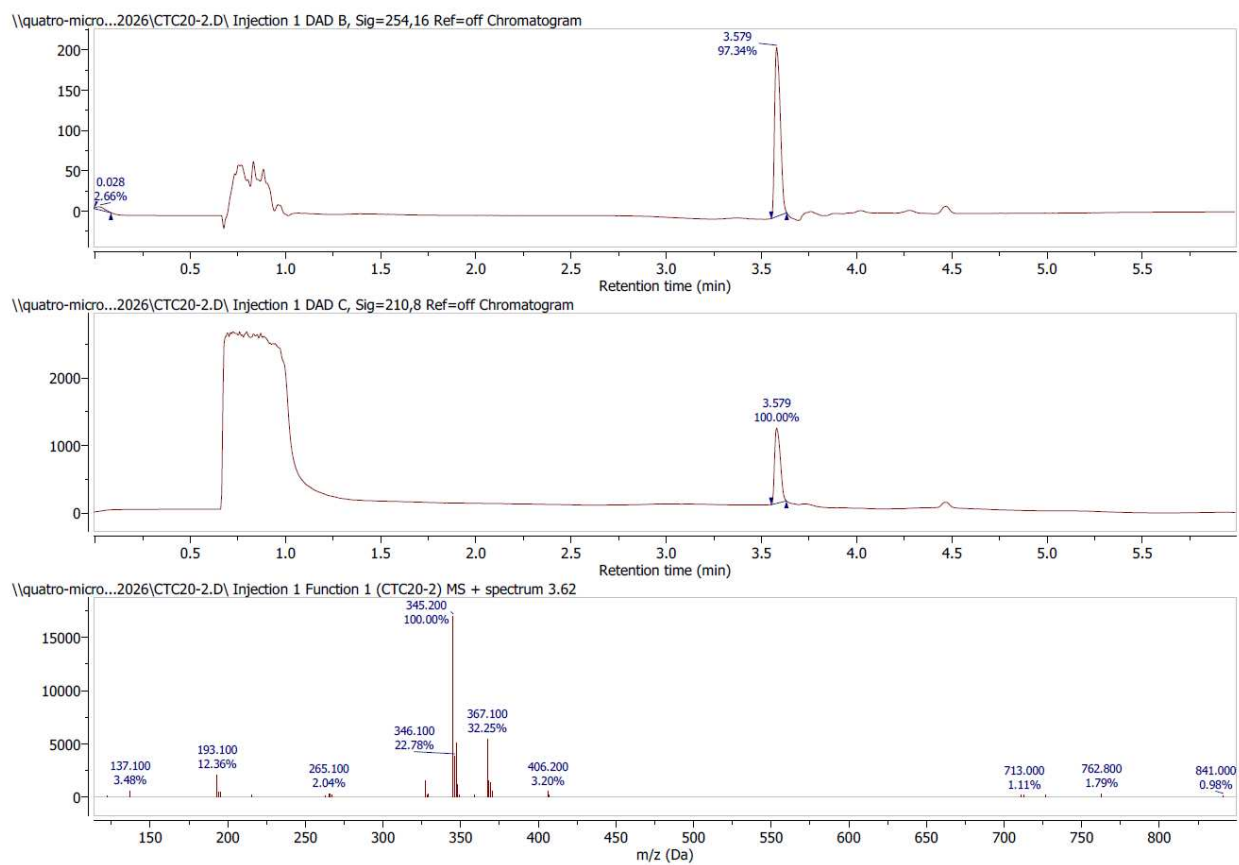


Figure SN8. $^{13}\text{C}\{^1\text{H}\}$ NMR spectra (100MHz, DMSO- d_6) of CTC24

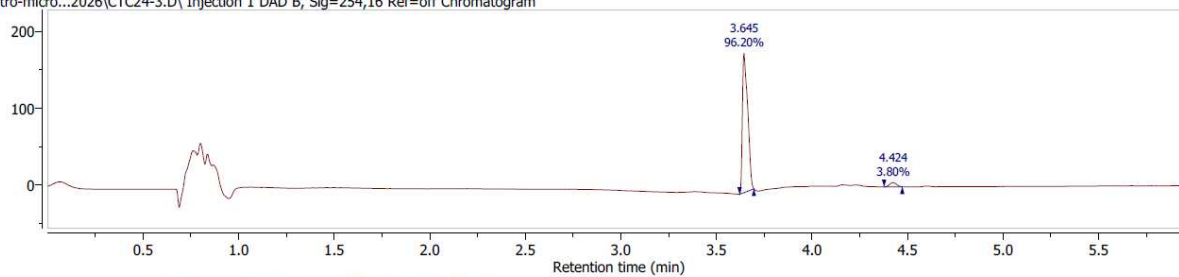
4. HPLC data of CTC20 and CTC24:

HPLC analysis of CTC20:

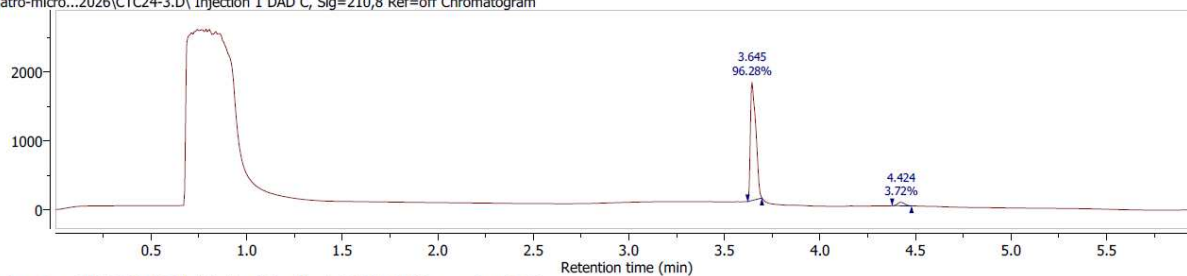


HPLC analysis of CTC24:

\\quatro-micro...2026\CTC24-3.D\ Injection 1 DAD B, Sig=254,16 Ref=off Chromatogram



\\quatro-micro...2026\CTC24-3.D\ Injection 1 DAD C, Sig=210,8 Ref=off Chromatogram



\\quatro-micro...2026\CTC24-3.D\ Injection 1 Function 1 (CTC24-3) MS + spectrum 3.69

