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The U UGA C sequence provides a favorable context to ELX-02 induced CFTR readthrough

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ARTICLE INFO

Introduction

Cystic fibrosis (CF) is an autosomal recessive disease caused by mutations in the gene encoding the cystic fibrosis transmembrane conductance regulator (CFTR) protein. CFTR modulators have proven to clinically benefit patients carrying the F508del mutation [1]. Such treatments are however ineffective for patients carrying nonsense mutations introducing a premature termination codon (PTC). Those patients represent around 12% of all CF patients in Europe and have a more severe phenotype [2].

PTCs are associated with two main defects, the degradation of the PTC-containing transcript by nonsense-mediated mRNA decay (NMD) and, in case of escape from NMD degradation, the production of a truncated nonfunctional polypeptide [3]. Both defects lead to the absence of available CFTR target for modulator therapy. A number of small molecules have been shown to decrease NMD mRNA degradation, reduce transcription fidelity, favoring the improper recognition of the PTC and the recruitment of near-cognate

tRNAs in place of the termination complex and finally the restoration of functional full-length protein [4]. The best-characterized drugs active against PTCs are aminoglycoside antibiotics, including gentamicin [5], and derivatives such as ELX-02 [6'-(R)-Methyl-5-O-(5-amino-5,6-dideoxy- α -L-talofuranosyl)-paromamine sulfate], previously referred to as NB-124 [6,7].

ELX-02 has already been evaluated *in vitro* for two frequent PTCs in CFTR: G542X [8], W1282X and three rare PTCs: W679X, Q250X and R1162X [9]. We aimed to identify other PTCs with high ELX-02 induced readthrough levels to target patients who would best benefit from this therapy.

Material and methods

Using a dual reporter gene assay, measuring luciferase activity and beta-galactosidase activity as internal system calibration, we determined the baseline readthrough level for 9 rare nonsense mutations, previously not studied, and their readthrough responses upon treatment with ELX-02 (Table 1). NIH3T3 cells were transfected with the reporter plasmid containing complementary oligonucleotides corresponding to nonsense mutations embedded in their natural context [10], 3 codons on each side.

CFTR activity was measured in Human Nasal Epithelial (HNE) cells obtained after nasal scraping as previously published [11,12].

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Table 1
List of mutations and corresponding sequences. The corresponding PTC is in bold.

Mutation	Sequence of mutation
S1196X	ATT GAG AAT TGA CAC GTG AAG
S466X	GCC AAG ACT TGA CTT CTA ATG
S434X	AGT AAT TTC TGA CTT CTT GGT
E1418X	TTG GTC ATA TAA GAG AAC AAA
E60X	TGG GAT AGA TAG CTG GCT TCA
Y275X	GTT AAG GCA TAG TGC TGG GAA
Q220X	GAG TTG TTA TAG GCG TCT GCC
E831X	GAC TTA AAG TAG TGC TTT TTT
Q290X	AAC TTA AGA TAA ACA GAA CTC
G542X	TTG CAA CAG TGA AGG AAA GCC
W1282X	ATA GTT CTT TGA GAA GGT GGA
W679X	CCT GTC TCC TAG ACA GAA ACA
Q250X	TAC AGA GAT TAG AGA GCT GGG
R1162X	TCT GTG AGC TGA GTC TTT AAG

The study was approved by the Ile-de France 2 Ethics Committee and written informed consent was obtained from each adult and parent. After conditional reprogramming with ROCK inhibitor [13] and amplification, cells were cultured in ALI conditions for 3–4 weeks to establish a differentiated epithelium [11]. Differentiated ALI cultures of HNE were incubated at the basal side for 48 h with ELX-02 1 mM (Eloxx Pharmaceuticals), SMG1i 5 μ M (NMDi hereafter) (kind gift from CF Foundation) [14], and the CFTR modulator triple combination VX-445 (3 μ M)/VX-661 (3 μ M)/VX-770 (100 nM) (ETI). The sum of the I_{sc} change after Forskolin/IBMX and VX-770 and its inhibition by Inh-172 (5 μ M) (ΔI_{sc} Inh172) served as an index of CFTR function. It was compared to the normal values obtained in 7 healthy controls of 11.6 (1.7) μ A/cm².

Total RNA was purified with TRIzol™ Reagent from ALI HNE cultures after the Ussing chamber studies. Reverse transcription (RT), followed by quantitative real time RT PCR (qPCR), was performed for mRNA quantification. Expression of mRNAs was determined using the 2(-ddCt) method.

Data are presented as mean (SEM) and were tested by non-parametric Mann-Whitney test.

Results

Out of the 9 tested mutations S466X (p.Ser466*, c.1397C>G) and S1196X (p.Ser1196*, c.3587C>G) were the most responsive mutations in the dual reporter gene assay experiments in NIH3T3 fibroblasts, with respectively, an ELX-02-induced readthrough of $6.5 \pm 0.1\%$ and $5 \pm 0.1\%$ of the WT level (Fig. 1A). We then investigated the response in HNE cells from 2 patients carrying respectively S1196X/S1196X and Y275X/S466X PTC mutations. At baseline, CFTR activity was null for both patients (Fig. 1B). ELX-02 induced a significant enhancement of the short circuit response, providing evidence of an increase in CFTR activity, as assessed by measuring ΔI_{sc} Inh172 currents (Fig. 1B). In the S1196X HNEs, ΔI_{sc} Inh172 was increased by 11 fold above vehicle, reaching 14.5% of the WT level ($p < 0.01$) and in the S466X/Y275X cells by 38.5 fold, corresponding to 16.3% of the WT level ($p < 0.001$). This was associated with an increase in CFTR transcript level for both mutations (Fig. 1C).

The combination of PTC readthrough by ELX-02 and inhibition of NMD by NMDi considerably increased CFTR transcripts (Fig. 1C) but this was not associated with an increase in CFTR activity (Fig. 1B). The combination of ETI with ELX-02 and NMDi maximized CFTR activity up to 27% of WT ($p = 0.03$) in S1196X-homozygous cells and to 46% of WT in S466X/Y275X cells ($p = 0.05$). As a whole, CFTR activity was significantly correlated to the transcript level ($R^2 = 0.97$; $p = 0.01$) (Fig. 1D).

Discussion

Our results show that the S1196X and S466X CFTR variants provide a favorable context for stabilization of CFTR transcript and their readthrough by ELX-02.

ELX-02 is a eukaryotic ribosomal selective glycoside whose readthrough properties were optimized and mitochondrial toxicity lowered through successive rounds of medicinal chemistry [15,16]. ELX-02 has been shown to induce efficient readthrough in FRT cells, patient-derived human bronchial cells and organoids carrying PTC mutations with the highest incidence rate, G542X and the W1282X alleles and three rare PTCs: W679X, Q250X and R1162X [7,8,9]. ELX-02 has since advanced into the clinic and ongoing evaluation in Phase 2 clinical trials in CF patients with at least one copy of the G542X allele is currently ongoing.

As reported for other mutations, our results show that ELX-02 by itself increases CFTR transcript [8]. This is consistent with stabilization of the PTC containing transcript by ELX-02 during the first round of translation, enabling to further enhance readthrough targets in the second round of translation [17,18]. This was already shown in cancer cells where NB124/ELX-02 counteracted p53 mRNA degradation by NMD [17].

Interestingly, we observed low basal mRNA level in primary cells carrying S1196X in contrast to Y275X/S466X cells. This was previously reported by Sharma et al. who showed decreased level of S1196X CFTR transcript in HEK and primary HNE cells, whereas CFTR mRNA bearing nonsense variants in the 5' exons did not elicit NMD even though mechanistic models predict that these variants should activate NMD [21]. This confirms that the level of mRNA target is different according to the PTC and its environment and might impact on readthrough efficiency.

Combining transcript stabilization by NMD inhibitors and readthrough is theoretically beneficial, as greater mRNA stability should make it possible to produce more protein as already reported both *in vitro* [19] and humans [20]. Our observation that addition of SMG1i increased CFTR transcript level but not CFTR activity, suggests that (i) ELX-02 leads to production of stable mRNA but (ii) that increasing transcript level by NMD inhibition is not beneficial in those 2 specific cases. Functional response maximization by ETI confirms that correctors enhance effect of translational readthrough of nonsense mutations [21]. Since we have previously shown that the correction level of Cl⁻ secretion in cultured HNE cells could predict the clinical efficacy of CFTR modulators in patients, the rescued CFTR activity upon ELX-02 can be considered relevant from a clinical point of view [11,22].

Remarkably, in the dual reporter system, the highest level of readthrough was obtained for S1196X and S466X both harboring a UGA PTC with a U nucleotide upstream and a C nucleotide downstream. This is consistent with previous data showing that aminoglycoside induced readthrough is dependent on both the nature of the PTC (UAA<UAG<UGA) and the identity of the up- and downstream nucleotides, with a positive influence of an uracil in position -1 and a cytidine in position +4 [17,23]. Y275X, which is combined with S466X in our patient carries the "A UAG U" sequence and yields a lower level of readthrough to ELX-02. As it was shown that the readthrough levels obtained with the dual reporter system are similar to those obtained *in vivo* [24], we hypothesize that a large part of the CFTR rescue is due to S466X readthrough. Among previously tested mutations responding to ELX-02 (G542X, W1282X, W679X, Q250X and R1162X) at least three of them (G542X, W1282X and R1162X) have the "TGA", the most favorable stop codon [8,9].

Collectively, our data show that the combination of UGA with U upstream and C downstream provides a responsive context for ELX-02 readthrough. Patients carrying mutations with this favourable context should be primarily targeted for clinical trials

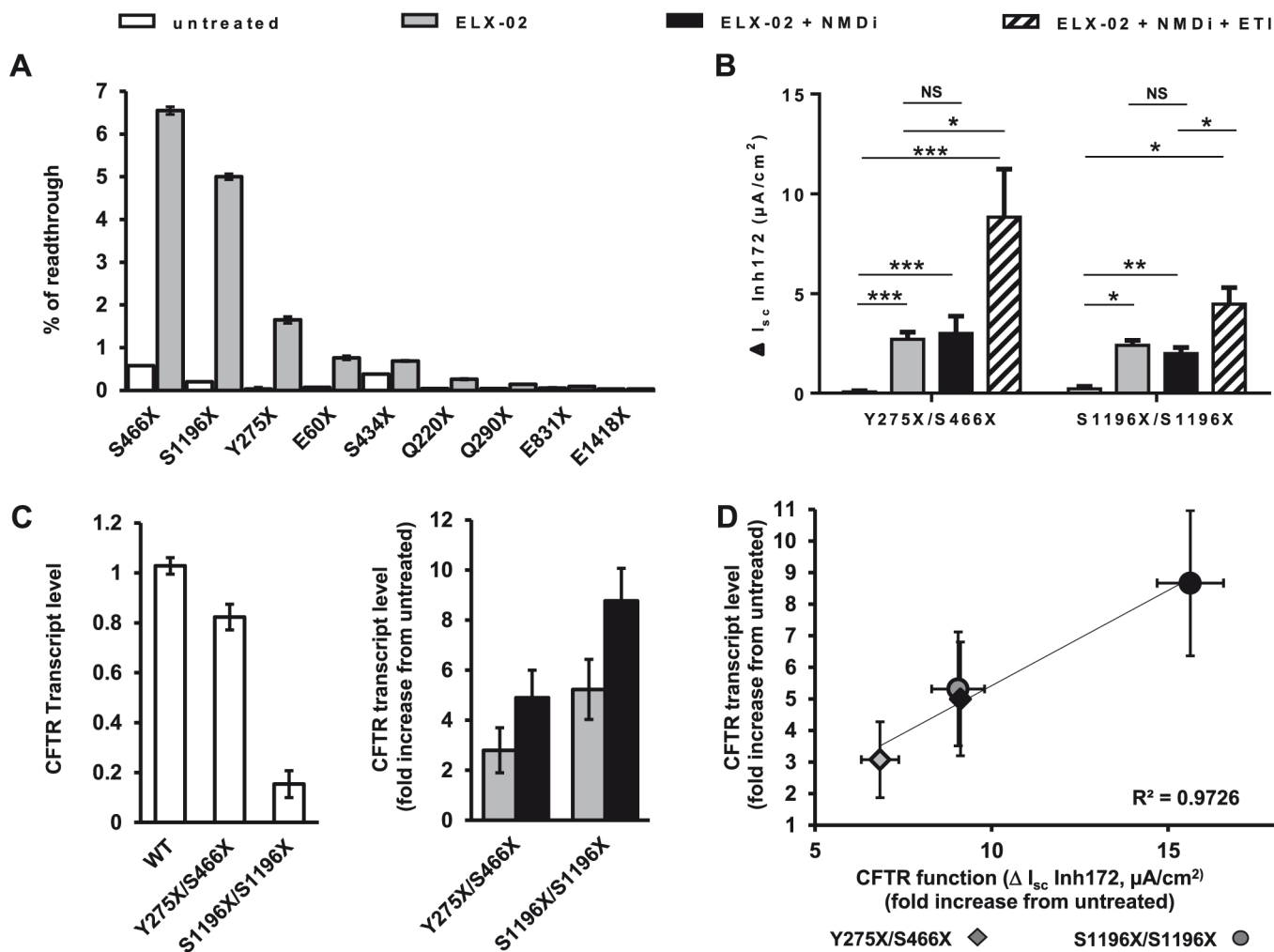


Fig. 1. legend. Effect of ELX-02 on rare CFTR nonsense mutations Basal and ELX-02-induced readthrough level in cell culture in 9 different NIHT3 Fibroblasts harboring rare nonsense CFTR mutations. Average CFTR activity of primary culture of human nasal epithelium carrying the S1196X/S1196X and Y275X/S466X genotype in control conditions and after treatment with ELX-02 (1 mM) or ELX-02 (1 mM) + NMDi (5 μ M), or ELX-02 (1 mM) + NMDi (5 μ M) + ETI (VX-661 3 μ M, VX-445 3 μ M, VX-770 100 nM). The I_{sc} response to CFTR-specific inhibitor Inh172 is indicative of CFTR function (μ A/cm²). Values of the I_{sc} responses to CFTR inhibitor Inh172 were presented as positive values. Left panel: Average basal CFTR transcript level (relative to WT CFTR control and normalized with GAPDH) in primary culture of human nasal epithelium carrying the S1196X/S1196X and Y275X/S466X genotype in control conditions. Right panel: Fold increase of the CFTR transcript level (CFTR/GAPDH) after treatment for 48 h with ELX-02 (1 mM; gray) alone or in combination with NMDi (5 μ M; black) from untreated. Presented values expressed as average \pm SEM. Correlation between CFTR function (fold increase from untreated of the I_{sc} response to Inh172), and CFTR transcript level (fold increase from untreated) in primary culture of human nasal epithelium after treatment with ELX-02 (1 mM, gray) or ELX-02 (1 mM) + NMDi (5 μ M), (black). Pearson correlation ($r = 0.97$; $p = 0.01$).

with this molecule. This may be also applied for other genetic diseases sharing this common defect.

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Iwona M. Pranke: analyzed and interpreted results, wrote the initial draft

Jessica Varilh: performed experiments

Aurélien Hatton: performed experiments

Caroline Faucon: wrote the initial draft and critically reviewed

Emmanuelle Girodon: wrote the initial draft and critically reviewed

Elise Dreano: performed experiments

Benoit Chevalier: interpreted results and wrote the initial draft

Sabrina Karri: performed experiments

Philippe Reix: wrote the initial draft and critically reviewed

Isabelle Durieu: wrote the initial draft and critically reviewed

Laure Bidou: wrote the initial draft and critically reviewed
Olivier Namy: wrote the initial draft and critically reviewed
Magali Taulan: interpreted results, wrote the initial draft and critically reviewed

Alexandre Hinzpeter: interpreted results, wrote the initial draft and critically reviewed

Isabelle Sermet-Gaudelus: wrote the initial draft, critically reviewed, and had the leadership responsibility for the research activity planning and execution

Conflict of Interest

Iwona M. Pranke, Jessica Varilh, Aurélien Hatton, Caroline Faucon, Emmanuelle Girodon, Elise Dreano, Benoit Chevalier, Sabrina Karri, Philippe Reix, Isabelle Durieu, Laure Bidou, Olivier Namy, Magali Taulan, Alexandre Hinzpeter have no conflict of interest. Isabelle Sermet-Gaudelus has received Vertex Innovation Award from Vertex therapeutics and participated to Scientific Advisory Boards (Vertex therapeutics, Eloxx Therapeutics).

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