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Short Communication

Tobramycin is a suppressor of premature termination codons

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Abstract

Premature translation terminations (PTCs) constitute the molecular basis of many genetic diseases, including cystic fibrosis, as they lead to the synthesis of truncated non-functional or partially functional protein. Suppression of translation terminations at PTCs (read-through) has been developed as a therapeutic strategy to restore full-length protein in several genetic diseases. Phenotypic consequences of PTCs can be exacerbated by the nonsense-mediated mRNA decay (NMD) pathway that detects and degrades mRNA containing PTC. Modulation of NMD, therefore, is also of interest as a potential target for the suppression therapy. Tobramycin is an aminoglycoside antibiotic, normally used to treat *Pseudomonas aeruginosa* pulmonary infection in CF patients. In the present study, by using yeast as a genetic system, we have examined the ability of Tobramycin to suppress PTCs as a function of the presence or absence of NMD. Results demonstrate that Tobramycin exhibits read-through ability on PTCs and preferentially in absence of NMD.

Keywords: Tobramycin; Aminoglycoside antibiotics; Read-through; NMD; PTCs; Cystic fibrosis; Yeast

1. Introduction

In spite of the introduction of novel classes of antibiotics, aminoglycosides continue to be widely used for the treatment of cystic fibrosis (CF) lung disease. In fact they are highly efficient against *Pseudomonas aeruginosa*, act in synergism with β-lactam antibiotics [1] and penetrate the sputum of patients with CF [2]. In addition, aminoglycoside antibiotics have been investigated to suppress premature translation termination by inducing a ribosomal read-through of the premature, but not the natural termination codons [3].

Nonsense mutations generate premature termination codons (PTCs) that result in a premature translational termination and may involve the synthesis of truncated abnormal proteins potentially toxic to cells through dominant negative or gain-of-function effects [4].

Functional consequences of the nonsense mutations are minimised by the nonsense-mediated RNA decay pathway (NMD), a cellular mechanism aimed to detect and degrade PTC containing mRNA [5]. NMD, as part of an mRNA surveillance system, has therefore a crucial role in preventing accumulation of aberrant transcripts and their translation [5]. However, NMD can also eliminate transcripts that would possibly synthesize functional truncated products thus alleviating the phenotype of genetic diseases like CF. In this case modulation of NMD is an important therapeutic goal for achieving restoration of sufficient full-length protein expression levels.

Tobramycin is an aminoglycoside antibiotic used to treat various types of bacterial infections, particularly Gram-negative infections. Tobramycin inhalation solution was the first antibiotic

Abbreviations: CF, cystic fibrosis; CFTR, Cystic Fibrosis Transmembrane Conductance Regulator; NMD, nonsense-mediated mRNA decay; PTCs, premature termination codons; RLUs, relative light units; SD, standard deviation

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to be developed and approved (in 1998) for use as an aerosolized antibiotic in patients with CF [6,7]. Currently, aerosolized Tobramycin is used for the treatment of *P. aeruginosa* pulmonary infection in CF patients.

In the present study we have investigated the potential read-through ability of Tobramycin at each of the in frame PTCs (UAG, UGA, UAA) by using the yeast *Saccharomyces cerevisiae* as a genetic system for a functional or non-functional NMD context [8]. We have shown that Tobramycin has the ability to induce read-through at PTCs; this effect is particularly efficient preferentially in absence of NMD.

2. Material and methods

2.1. Yeast strains, media and transformation procedure

The yeast *S. cerevisiae* strains used in the present study are the wild type CW04 (*MATa, his3-11, his3-15, leu2-3, leu2-112, ade2-1, ura3-1, trp1-1, can1-100*) and its isogenic CWA1 (*MATa, his3-11, his3-15, leu2-3, leu2-112, upf1::LEU2, ade2-1, ura3-1, trp1-1, can1-100*) carrying the *UPF1* gene deletion [9].

Yeast cells were transformed using the LiCl procedure [10] and the $URA3^+$ transformants were selected on plate containing synthetic minimal medium (SD) w/o uracil (1,7 g/L yeast nitrogen base, 5 g/L ammonium sulphate, 2 g/L glucose, 2 μg/mL adenine, 1 μg/mL histidine, 2 μg/mL tryptophan and 6 μg/mL leucine). For the yeast cultures, 6000 cells/mL was seeded in SD medium and treated with increasing concentration of antibiotic. After incubation at 30 °C for 24 h, yeast cell number/mL was estimated by reading OD_{600} at a spectrophotometer (1 OD_{600} = 1 × 10^7 yeasts/mL) and the IC_{50} values were calculated.

2.2. Dual-luciferase reporter assay and real time PCR

Yeast strains were transformed with the Renilla/Firefly dual-luciferase reporter plasmids carrying either a nonsense (UAG, UGA, UAA) or a sense (CAG, CGA, CAA) codon between the luciferase genes [11]. Protein extracts were prepared from 10.000 exponentially growing yeast cells treated with increasing concentrations of Tobramycin, Gentamicin or G418 using 100 μl of passive lysis buffer 1× (dual luciferase reporter assay kit, Promega Corporation, Madison, WI, USA). Dual luciferase assay was performed by following manufacturer's instruction. Briefly, 10 μL of luciferase assay reagent II was added to 2 μL of cellular lysate. After the quantification of Firefly luciferase by reading for 10 s in Turner Biosystems luminometer 20/20n (Promega Corporation, Madison, WI, USA), this reaction was turned off and the Renilla luciferase activity was simultaneously measured after the addition of 10 μL of 1× Stop & Glo buffer tube. The read-through percentage was calculated as the nonsense Firefly/Renilla activity ratio normalized to the sense Firefly/Renilla activity ratio × 100 as described [11,12] and reported relative to untreated yeasts with NMD active (0 NMD+) or untreated yeasts with NMD abolished (0 NMD−) set arbitrarily to 1. Mean ± SD values were determined for each percentage activity for at least four independent experiments.

Total RNA from cultures yeasts was isolated using RiboPure™-Yeast Kit (Life Technologies-Ambion. Carlsbad. CA). Total RNA (300 ng) was reverse-transcribed to cDNA using the SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen) in a 20 μl reaction. The cDNA (1 μl) was then amplified for 40 PCR cycles using the iQ™ SYBR® Green Supermix (BioRad) in an iQ ™5 Multicolor Real-Time PCR Detection System (Bio-Rad). The SYBR Green real-time PCR reactions were performed in duplicates for both target and normalizer genes. Primer sequences for detection of Firefly luciferase mRNA were 5′-TAC AAA GGA TAT CAG GTG GCC C-3′ (forward primer) and 5′-TTT CGC GGT TGT TAC TTG ACT G-3′ (reverse primer); primer sequences for amplification of *CAN1-100* transcripts were 5′-AAG CTG CAA ACC CCA GAA AA-3′ and 5′-TGC GGC AGA AAT AAT GGT TG-3′. Primer sets were purchased from Sigma-Genosys (The Woodlands. TX). Results were collected with iQ5™ Optical System Software (version 2.0; Bio-Rad). Relative quantification of gene expression was performed using the comparative threshold method (ΔΔCt method). Changes in mRNA expression level were calculated following normalization with the PGK1 (forward primer: 5′-AAG GCT GGT GCT GAA ATC GT-3′; reverse primer: 5′-GGT GGA CCG TTC CAG ACA AT-3′) calibrator gene and expressed as fold change over untreated samples.

3. Results

In order to evaluate the potential ability of Tobramycin in suppressing nonsense mutations (read-through) we have used the read-through reporter system based on the Renilla and Firefly luciferase open reading frames separated by either a sense or a nonsense codon [9], adapted to the yeast *S. cerevisiae* by Keeling et al. [11]. We took advantage of the yeast system because it prompted us to monitor read-through activity in genetic contexts in which NMD is either functional (NMD+) or abolished (NMD−) upon the deletion of the *UPF1* gene from the chromosome (see Material and Methods). To validate the yeast system in monitoring translational read-through mediated by drugs, we have used as reference G418 (Geneticin) and Gentamicin, two aminoglycoside antibiotics with well-known read-through ability [13,14]. More recently, indeed, we have reported the read-through action of G418 and other aminoglycosides such as Gentamicin, Streptomycin, Amikacin, and Tobramycin on globin mRNA carrying beta-thalassemia nonsense mutations, by FACS analyses, demonstrating the G418-mediated de novo production of beta-globin in K562 cells carrying the beta-39 nonsense mutation [13].

First we have evaluated the effects of Tobramycin, Gentamicin and G418 on yeast proliferation, the relative IC_{50} values were calculated and found to be 800 μg/mL ± 6.2 for Tobramycin, 250 μg/mL ± 10.3 for Gentamicin and 5.27 μg/mL ± 0.05 for G418, respectively (data not shown). Then, the wild type (NMD+) or Δ*upf1* (NMD−) yeast strains of *S. cerevisiae*, were transformed with dual-luciferase reporter plasmids in which the two luciferase sequences were separated either by a UAG nonsense codon or a CAG sense codon. Transformants were grown in the absence or presence of increasing amounts of G418,