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Fluorescence assay quantification of pre-terminal stop codon read through rate in Ataluren treated yeast

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1 Abstract

Specialized ribosomes are characterized by selective preferences in the translation initiation of certain mRNAs [1]. Modification of the corresponding ribosomal genes with genetic engineering tools like TALEN or CRISPR can permanently change the morphology of the transcribed proteins and thereby influence the translational behaviour. Off-target effects are very hard to control when using modifications on the DNA level. Small molecules are only temporarily modifying ribosomes but have less potential risks compared to DNA transforming techniques. Firefly luciferase (FL) is a potent ATP-dependent bioluminescent reporter enzyme which we used in a dual-reporter assay with Renilla [2]. We coupled them to LamB3-PTC constructs in order to detect translational readthrough. Interestingly Ataluren is a strong FL inhibitor and can also restore the activity of nonfunctional nonsense alleles via promotion of near-cognate tRNA insertion [2][3]. With the following procedure, the results were not significant between treated yeast and the control group.

2 Introduction

A sub-population of ribosomes, called specialised ribosomes, can favour the translation of subsets of mRNAs. It was shown by comparative protein synthesis assays that different heterologous mRNA reporters, which are based on Luciferase are preferentially translated by distinct populations of specialised ribosomes. These mRNAs include reporters carrying premature termination codons (PTCs). This allows the identification of specialised ribosomes that alter the efficiency of translation termination leading to enhanced synthesis of the wild-type protein. These strains can be used to identify novel therapeutic targets in the ribosome [1]. Firefly Luciferase (FL) is an ATP-dependent bioluminescent reporter enzyme. It is broadly used in chemical biology and drug discovery assays. Ataluren (previously known as PTC124; 3-[5-(2-fluorophenyl)-1,2,4-oxadiazol-3-yl]benzoic acid) is an unusually potent FL-inhibitor. Although it also increases cellular FL activity levels by posttranslational stabilisation. This inhibition and stabilisation is the result of an inhibitory product formed during the FL-catalysed reaction between its natural substrate (ATP and Ataluren). It was also demonstrated that the inhibitory activity is assisted by free coenzyme A which is present in high concentration in luciferase detection reagents. This might explain why Ataluren increases the FL activity instead of inhibiting in cell-based reporter gene assays [2]. Ataluren (PTC124) is a bioactive molecule thought to modulate the translation machinery in human cells as well as yeast cells. The substance allows a read-through of PTCs such as UAG, UAA and UGA during mRNA translation and helps with the production of full-length proteins by promoting the insertion of near-cognate tRNAs at the site of the nonsense codon without apparent effects on transcription. The resulting read-through proteins retain function and contain amino acid replacements like Gln, Lys, and Tyr at UAA and UAG codons, and Trp, Arg, and Cys at the UGA codon. It identifies the specific amino acids inserted during nonsense suppression when premature termination is bypassed. In conclusion, the tRNA selection is attributable to base mispairing at codon position 1 and 3 and not at the typical wobble position. It also seems to prefer the use of certain nonstandard base pairs, e.g.: U-G. Ataluren's retention of similar specificity of near-cognate tRNA insertion has important implications for its general use in therapeutic nonsense suppression. It is currently being evaluated for treatment of diseases such as cystic fibrosis, aniridia and mucopolysaccharidosis I [3] [4].

3 Materials and Methods

Two overnight yeast cultures were prepared in 3 mL of SC-ura-leu medium each and grown at 28 °C. The following day, the cultures were diluted to an OD600 of 0.9 and one of them (treated) was inoculated with an appropriate amount of Ataluren to reach a final concentration of 2μM (Calc.1) the other culture (untreated) served as control. The cultures were then incubated overnight at 28 °C, shaking at 180 rpm. When the cells had reached an OD600 of 0.8 (treated: 0.82, untreated: 0.83), the total cell number was calculated according to the OD value measured with the spectrophotometer (OD600= 0.1 corresponds to 1×10^6 $\frac{cells}{mL}$). 5 mL of each culture were transferred into 15 mL centrifugation tubes each, pelleted by centrifugation (4000g, 10min, 4 °C) and resuspended in 4,1 mL H_2O(Calc.2) to reach a cell count of 5×10^5 per well. 50 μL were transferred into six wells of a *black/white* 96 multiwell isoplate (Perkin Elmer Inc., Waltham, MA, USA) for each culture. This was followed by addition of 20 μL of 1x Passive Lysis Buffer (PLB) using a multi-channel pipette and incubation for 1 min at room temperature. After incubation, the measurements were started instantly and continued in a batch of six samples each. For the luminescence measurements we used the Dual-Luciferase Reporter (DLRTM) Assay System (Promega Inc., Madison, WI, USA) with 50 μL Firefly substrate (LAR II) and 50 μL Renilla substrate (Stop & Glo®), automatically injected by the dispenser of the GloMax Multi Microplate Detection System (Promega Inc., Madison, WI, USA). After 2 sec upon substrate addition, luminescence signals were detected with 10 sec integration time.

4 Results

Renilla: Read 1 untreated:	Renilla: Read 1 treated:
2459,91	5690,65
4331,86	7028,82
5310,64	4228,71
4712,03	4013,92
5155,56	3944,39
5512,75	3183,52
Firefly: Read 2 untreated:	Firefly: Read 2 treated:
1,17E+08	1,11E+08
1,07E+08	3,00E+30
1,11E+08	1,07E+08
1,22E+08	1,02E+08
1,18E+08	1,16E+08
1,17E+08	1,17E+08
Ratio ½ untreated:	Ratio ½ treated:
2,11E-05	5,11E-05
4,04E-05	2,34E-27
4,80E-05	3,95E-05
3,87E-05	3,92E-05
4,38E-05	3,41E-05
4,71E-05	2,73E-05

Figure 1: Luminescence data

Grubbs test: (https://www.graphpad.com/quickcalcs/grubbs2/)
Ratio $\frac{1}{2}$ untreated:

Descriptive Statistics

Mean: 0.00003985
SD: 0.00000988
# of values: 6
# of rows w/o data: 1
Outlier detected? Yes
Significance level: 0.05 (two-sided)
Critical value of Z: 1.8871466793

Your data

Row	Value	Z	Significant Outlier?
1	0.000021	1.89835096	Significant outlier. P < 0.05
2	0.000040	0.05568496	
3	0.000048	0.82514988	
4	0.000039	0.11643219	
5	0.000044	0.39991927	
6	0.000047	0.73402904	

Figure 2: Grubbs test (untreated)

Data point 1 will be omitted from further calculations.
Ratio $\frac{1}{2}$ treated:

Descriptive Statistics

Mean: 0.00003187
SD: 0.00001745
# of values: 6
# of rows w/o data: 1
Outlier detected? No
Significance level: 0.05 (two-sided)
Critical value of Z: 1.8871466793

Your data

Row	Value	Z	Significant Outlier?
1	0.000051	1.10191423	
2	0.000000	1.82570191	Furthest from the rest, but not a significant outlier (P > 0.05).
3	0.000040	0.43732818	
4	0.000039	0.42014061	
5	0.000034	0.12795191	
6	0.000027	0.26163301	

Figure 3: Grubbs test (treated)

Normality Test: Shapiro-Wilk:
(http://sdittami.altervista.org/shapirotest/ShapiroTest.htmL)
Ratio $\frac{1}{2}$ untreated:

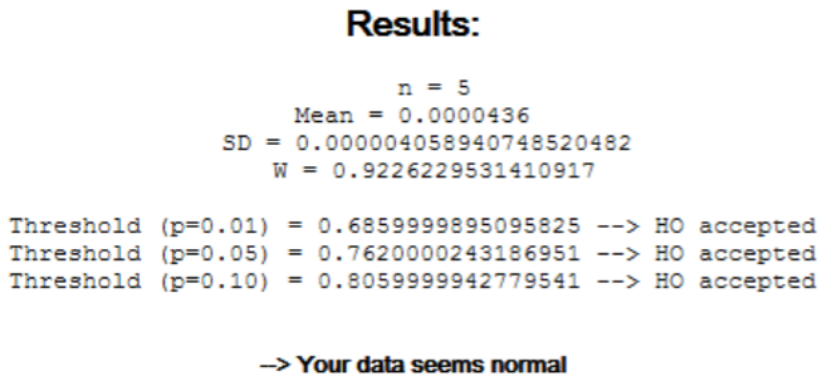

Figure 4: Shapiro-Wilk test (untreated)

Ratio $\frac{1}{2}$ treated:

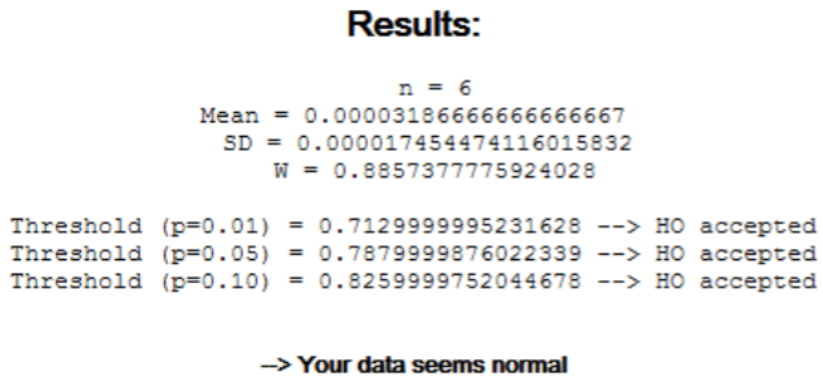

Figure 5: Shapiro-Wilk test (treated)

Hypothesis:
H0: $\mu 1 = \mu 2$ There is no difference in the LAMB3-PTC expression between treated and untreated cells
H1: $\mu\ 1 \neq \mu\ 2$ There is a difference in the LAMB3-PTC expression between treated and untreated cells

Test of Significance:
(https://www.socscistatistics.com/tests/anova/Default2.aspx)

Summary of Data

	Treatments					
	1	2	3	4	5	Total
N	5	6				11
ΣX	0.0002	0.0002				0.0004
Mean	0	0				0
ΣX^2	0	0				0
Std.Dev.	0	0				0

Result Details

Source	*SS*	*df*	*MS*	
Between-treatments	0	1	0	*F* = 2.12636
Within-treatments	0	9	0	
Total	0	10		

The *f*-ratio value is 2.12636. The *p*-value is .178779. The result is *not* significant at $p < .05$.

Figure 6: Test of Significance

The results are not significant, therefore H0 is accepted.

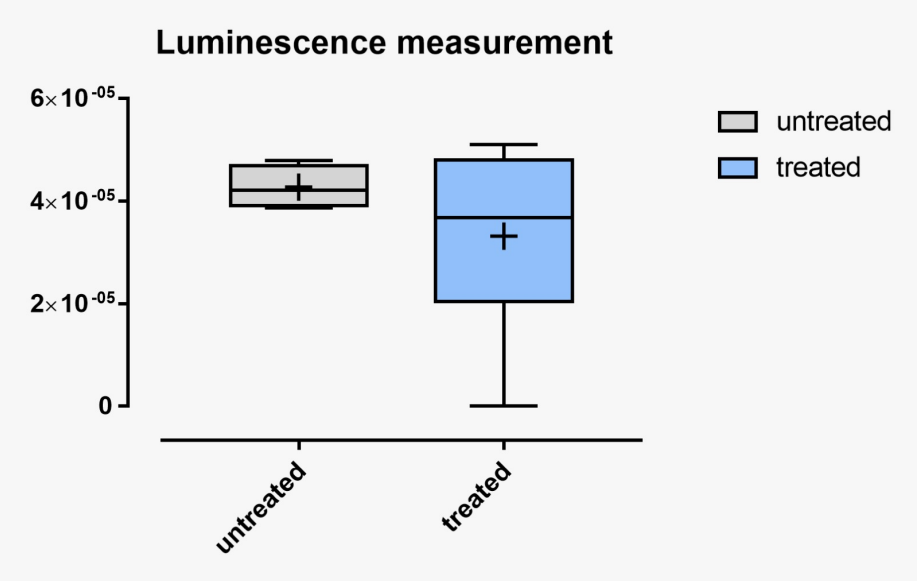

Figure 7: Box-whisker plot

There is a large spread in the data set of treated yeast, which translates to a low precision.

5 Discussion

Roy and colleagues have shown that Ataluren mediates nonsense suppression by incorporation of near-cognate tRNAs at pre-terminal stop codons. They demonstrated that GLn, Lys and Tyr are preferentially integrated at UAA/UAG codons while Trp, Arg and Cys are incorporated at UGA condons [3]. In our data no significant effect between treated and untreated yeast cells could be identified (Figure 6), although both sets were normally distributed (Figure 4, Figure 5). As expected FL luminescence levels are very high compared to Renilla luminescence due to the stabilization effect of Ataluren on FL [2]. Systematic errors have not been eliminated and might be present in the procedure. Therefore uncertainties in relation to the sequence context, cell count and reference value for the Ataluren concentration are present. The lack of a reference value also prevents a statement on trueness and accuracy of the data. As seen in Figure 7 the precision in the treated group is rather low. In order to improve the significance and possible errors, further tests with various concentration levels of reporter, Ataluren and cells have to be executed.

6 Appendix

Buffers and solutions:

1. Media for S. cerevisiae growth

SC (Synthetic complete)

0.17 % Yeast nitrogen base

0.5 % Ammonium sulfate

2 % Glucose

(2 % Agar)

Fill up with dH_2O

Adjust pH at 5.8 with NaOH and autoclave at 125 °C, 12-14 min, 1.5-2 bar

For 1L SC-media add following amino acids at hand-hot temperature:

10mL Complete dropout (-ade, -tyr, -ura, -leu)

1mL 1% Adenine

4mL 1% Uracil

5mL 1% L-Tyrosine

6mL 1% L-Leucine

Complete Dropout (-ade, -tyr, -ura, -leu)

0.2% (w/v) L-Arginine

0.1% (w/v) L-Histidine

0.6% (w/v) L-Isoleucine

0.4% (w/v) L-Lysine

0.1% (w/v) L-Methionine

0.6% (w/v) L-Phenylalanine

0.5% (w/v) L-Threonine

0.4% (w/v) L-Tryptophan

Dilute in 100 mL dH_2O and filtrate

2. Luminescence measurements

1x Passive lysis buffer (PLB) Dilute 5x PLB to 1x PLB, provided by Promega (Promega Inc., Madison, WI, USA).
LAR II, Stop & Glo® Solubilize lyophilized Luciferase Assay Substrate in 10 mL Luciferase Assay Buffer. Mix 200 μL Stop & Glo® Substrate (50x) with 10mL Stop & Glo® Buffer.

Calculations:

Calc.1:

$M_{ataluren} : 284,24 \frac{g}{mol}$
Ataluren stock solution: 1.4212 mg dissolved in 5 mL $H_2O = 1 \frac{\mu mol}{mL}$
Inoculated with 26 μL in 13 mL to reach a concentration of 2 μM

Calc.2:

$0,8 \frac{cells}{mL} \times 10^7 cells \times 5mL = \frac{5 \times 10^5 cells}{50 \mu L} \times V_2$

$V_2 = 4,1mL$

References

[1] Johann W Bauer, Clemens Brandl, Olaf Haubenreisser, Bjoern Wimmer, Manuela Weber, Thomas Karl, Alfred Klausegger, Michael Breitenbach, Helmut Hintner, Tobias von der Haar, et al. Specialized yeast ribosomes: a customized tool for selective mrna translation. *PLoS One*, 8(7):e67609, 2013.

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