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Aminoglycoside-stimulated readthrough of premature termination codons in selected genes involved in primary ciliary dyskinesia

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ABSTRACT

Translational readthrough of premature termination codons (PTCs) induced by pharmacological compounds has proven to be an effective way of restoring functional protein expression and reducing symptoms in several genetic disorders. We tested the potential of different concentrations of several aminoglycosides (AAGs) for promoting PTC-readthrough in 5 genes involved in the pathogenesis of primary ciliary dyskinesia, an inherited disorder caused by the dysfunction of motile cilia and flagella. The efficiency of readthrough stimulation of PTCs cloned in dual reporter vectors was examined in 2 experimental settings: *in vitro* (transcription/translation system) and *ex vivo* (transiently transfected epithelial cell line). PTC-readthrough was observed in 5 of the 16 mutations analyzed. UGA codons were more susceptible to AAG-stimulated readthrough than UAG; no suppression of UAA was observed. The efficiency of PTC-readthrough *in vitro* (from less than 1% to ~28% of the translation from the corresponding wild-type constructs) differed with the AAG type and concentration, and depended on the combination of AAG and PTC, indicating that each PTC has to be individually tested with a range of stimulating compounds. The maximal values of PTC suppression observed in the *ex vivo* experiments were, depending on AAG used, 3–5 times lower than the corresponding values *in vitro*, despite using AAG concentrations that were 2 orders of magnitude higher. This indicates that, while the *in vitro* system is sufficient to examine the readthrough-susceptibility of PTCs, it is not sufficient to test the compounds potential to stimulate PTC-readthrough in the living cells. Most of the tested compounds (except for G418) at their highest concentrations did not disturb ciliogenesis in the cultures of primary respiratory epithelial cells from healthy donors.

Abbreviations: PTC, premature termination codon; AAG, aminoglycoside; PCD, primary ciliary dyskinesia; TNT, transcription/translation reaction; RT, readthrough

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Introduction

Nonsense mutations, which give rise to the in-frame STOP codons (UAA, UAG or UGA) in coding regions of mRNAs, lead to the premature termination of translation and generation of non-functional, truncated proteins.[1,2] It is estimated that premature termination codons (PTCs) constitute 12% of all deleterious alterations to the DNA sequence,[3] and are involved in the pathogenesis of ~1800 human genetic diseases.[2,4]

One of the ways to suppress an in-frame PTC and allow translation to proceed to the true end of a mutated transcript relies on the natural mechanism of decoding termination codons by near-cognate tRNAs; it is known as translational readthrough of PTC (PTC-readthrough). The resulting protein is often functional, even when the incorporated amino acid differs from the wild type.[5] PTC-readthrough can be stimulated by a number of compounds, including aminoglycoside antibiotics (AAGs), which influence the fidelity of a STOP codon recognition by changing the conformation of rRNA.[6] In genetic diseases caused by nonsense mutations, selective promotion of PTC-readthrough is an alternative to difficult and frequently unsuccessful gene therapy.[7] Over past few years, the therapeutic potential of a number of AAGs or their mimetics has been documented, the evidence ranging from a partial restoration of functional proteins[8-15] to the ongoing clinical trials involving patients with cystic fibrosis and Duchenne muscular dystrophy.[16-21] It has been shown that the efficiency of PTC-readthrough depends on the stimulating compounds; it also varies with the STOP codon type and its sequence context,[22-24] which makes each disease-causing PTC a highly individual case.

Primary ciliary dyskinesia (PCD), a rare recessive disease with the prevalence of 1/2,200 to 1/40,000 individuals, belongs to the class of ciliopathies and is caused by molecular defects of motile cilia and flagella.[25] The major symptoms include recurrent respiratory infections of the upper and lower airways, male infertility and randomization of the visceral organs symmetry.[26] Genetically, PCD is highly heterogeneous, with over 30 causative genes have

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Table 1. Characteristics of the analyzed PTC mutations in PCD-related genes.

Gene	Exon	Mutation	Frequency	Nucleotide substitution	Premature STOP codon	Nucleotide at +4 position
DNAH5	20	Q1031*	0.7	C/T	UAG	C
	32	R1711*	0.7	C/T	UGA	C
	34(a)	E1843*	0.3	G/T	UAA	G
	34(b)	R1883*	0.3	C/T	UGA	G
	37	Q2024*	0.3	C/T	UAG	T
	47	Q2592*	0.3	C/T	UAA	G
	49	R2677*	1.4	C/T	UGA	C
	62	R3481*	0.3	C/T	UGA	T
DNAH11	70	R3809*	1.0	C/T	UGA	T
SPAG1	16	Q672*	12.5	C/T	UAG	T
RSPH4A	1	Q109*	1.0	C/T	UAG	T
	3(a)	W356*	0.3	G/A	UGA	G
	3(b)	R490*	0.7	C/T	UGA	A
CCDC40	15	K841*	0.3	A/T	UAG	A
	20(a)	Y1118*	3.1	C/A	UAA	C
	20(b)	Q1120*	0.3	C/T	UAG	T

Insert sequences cloned into the pDluc vector are available from the authors upon request. The frequency column describes the proportion of unrelated Polish patients with the PTC in question (both homozygotes and compound heterozygotes are included; based on the data from 295 PCD families examined in our lab). Each of the letters a and b following some of the exon numbers indicates one of the 2 separate mutations in the same exon.

identified so far.[27,28] Almost a quarter of PCD patients have PTC mutations in one or both alleles (our unpublished data). However, no studies on the PTC-readthrough stimulation in PCD-causing genes have been published so far and no information about the therapeutic potential of this approach in PCD patients is available.

The aim of our study was to assess the efficacy of several AAG compounds (G418, paromomycin, gentamicin and amikacin) with known STOP codon suppression potential[29] in the context of 16 PTC mutations found among Polish PCD patients. The relevant AAG concentrations were also tested for their potential cyto- and ciliotoxicity in primary cultures of the ciliated respiratory epithelium from healthy donors.

Results

Construction of the mutation-specific recombinant pDluc-PTC reporter vectors

PTC mutations involved in PCD pathogenesis were selected based on the mutation occurrence in Polish patients (based on our previous studies in the collection of DNA from over 200 unrelated Polish PCD patients). Sixteen PTCs, localized in 5 genes: *DNAH5, DNAH11, SPAG1, RSPH4A* and *CCDC40*, were selected (Table 1). Throughout the text, mutations are referred to by the name of the gene and exon number (followed by "a" and "b," if 2 different mutations were in the same exon).

The selected PTCs in their natural context (minimum 8 nucleotides at either side) and the corresponding wild-type sequences (Table 1), were cloned in pDluc, the dual-luciferase reporter vector.[30] The sequences were inserted into the polylinker region located between 2 reporter luciferase genes, *rluc* from *Renilla* and *fluc* from firefly (Fig. 1A). Expression of the recombined pDluc can yield 2 types of product (Fig. 1B). The shorter product, containing only Rluc protein activity, is expressed when PTC in the insert causes premature termination of the protein synthesis. The longer product (Rluc-insert-Fluc), containing the activities of both reporter proteins, Rluc and Fluc, is observed in the control constructs (inserts with a wild-type sequence), or in the case when PTC is suppressed (PTC-readthrough). Since the Rluc signal served as an internal

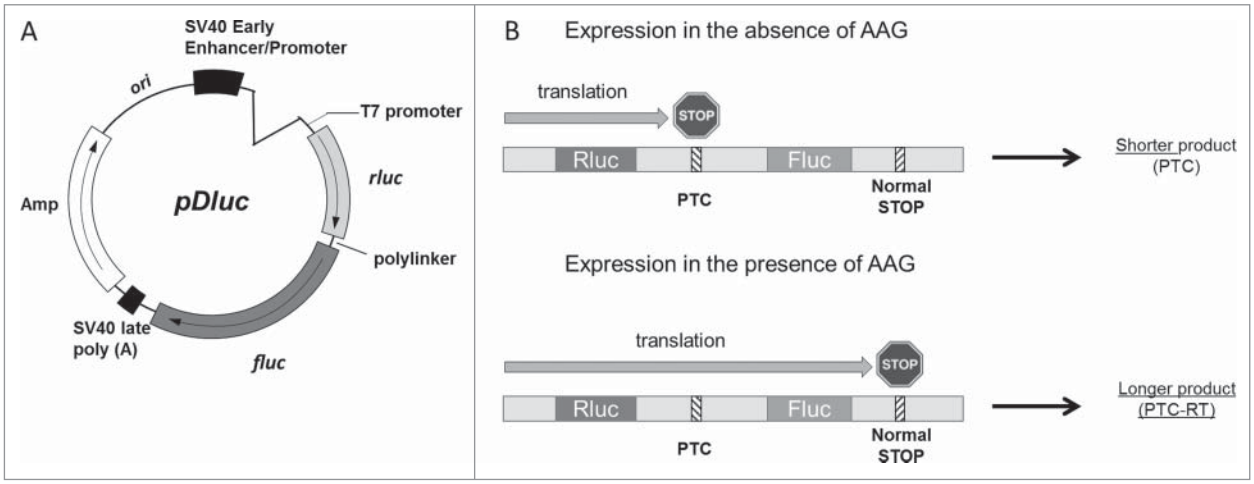

Figure 1. The principle of pDluc dual-luciferase reporter assay. (A) Structure of the pDluc plasmid. *rluc*– *Renilla* luciferase gene; *fluc*– firefly luciferase gene; *ori* – bacterial replication start; *Amp* – ampicillin resistance gene; polylinker – the site of the analyzed sequence insertion. (B) Expression of the pDluc vector with the insert containing an in-frame PTC, in the absence (upper panel) and presence (lower panel) of PTC-readthrough process in the presence of AAG.

calibrator, measuring the relative amounts/activities of both luciferase proteins allows comparison of the readthrough efficiency across different experiments.[30]

Selection of AAG concentrations for the in vitro *and* ex vivo *experiments*

While AAGs are best known as translation inhibitors in Prokaryotes, when used at high doses they also interfere with normal protein translation in Eukaryotes. To establish the range of tolerable AAG concentrations for the *in vitro* experiments, pDluc vectors with wild-type inserts were analyzed in the transcription/translation (TNT) system (rabbit reticulocytes). The observed reduction in the amount of protein product compared to the untreated controls was proportional to the concentration of the examined AAG. G418 exerted the strongest negative effect: at the highest tested concentration (5 μg/ml), it reduced the amount of the translated protein by more than 60%. The maximal AAG concentrations for the *in vitro* experiments were set up, so to prevent reduction of the translation efficiency by more than 50%: 15 μg/ml for gentamicin, paromomycin, amikacin and 1.5 μg/ml for G418.

The AAG concentrations used in the *ex vivo* experiments in the transfected HEK293FT cell lines did not exceed the LD_{50} doses reported in the literature (paromomycin, gentamicin: 1000 and 2000 μg/ml; G418: 200 and 400 μg/ml).[23,31]

Efficiency of the AAG-stimulated PTC-readthrough in the in vitro *TNT system*

The *in vitro* TNT system was used to examine the susceptibility of 16 PTCs cloned in pDluc to become suppressed by AAGs: gentamicin, paromomycin and amikacin at 5, 10, 15 μg/ml; G418 at 0.5, 1, 1.5 μg/ml. Parallel control reactions with pDluc constructs containing the corresponding wild-type insert were performed. The measurable readthrough (seen as the presence of Rluc-insert-Fluc product[22]) was observed for some of the PTCs in the presence of gentamicin, paromomycin and G418; a representative example of SDS-PAGE analysis of the TNT products is shown in Fig. 2. No PTC-readthrough was observed in the presence of amikacin at any of the concentrations tested; therefore, amikacin was excluded from further experiments.

Out of the 16 PTCs, only 5 (from *DNAH5*_20; *DNAH5*_32; *DNAH5*_49; *DNAH11*_70; *RSPH4A*_3a) underwent suppression in the presence of the analyzed AAGs. PTC from *SPAG1*_16 exhibited a very low level of readthrough, only at the highest concentration of G418. Since the results were not consistent across the repeated experiments, this PTC was conservatively considered as non-responsive to the AAGs examined. The remaining 10 PTCs were not suppressed in the presence of any concentration of the AAGs analyzed. The summary of TNT results is shown in Table 2 and Fig. 3.

The efficiency of the readthrough differed depending on the analyzed PTC. The highest response to AAG stimulation was observed for PTCs from *DNAH5*_32, *DNAH11*_70 and *RSPH4A*_3a. The amount of Rluc-insert-Fluc product, translated from constructs containing these PTCs in the presence of the highest AAG concentrations, ranged from 7.8 to 27.9% of the protein products generated from the corresponding wild-type constructs in the absence of AAG. PTCs from *DNAH5*_49 and *DNAH5*_20 were less susceptible to the AAG-stimulated readthrough: the amount of the longer product was several times lower than for the 3 best- responding PTCs (Table 2).

The amount of the Rluc-insert-Fluc product in the TNT system also differed depending on the AAGs used, reflecting

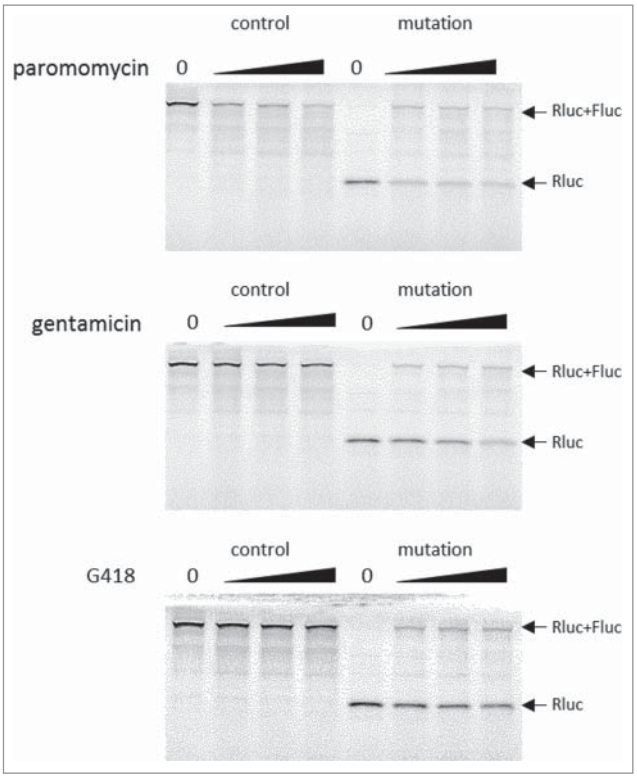

Figure 2. Representative example of SDS-PAGE separation of the radioactively-labeled TNT products obtained in the presence of increasing concentrations of AAGs. The results shown are for the pDluc construct containing *DNAH5_32* insert. Paromomycin and gentamicin concentrations were: 0, 5, 10, 15 μg/ml; G418 concentrations were: 0, 0.5, 1, 1.5 μg/ml. control: pDluc construct containing the wild-type sequence; mutation: PTC-containing template; Rluc+Fluc – the longer product (recombinant Rluc-insert-Fluc protein) translated from the control template and from the mutated template in the presence of PTC-readthrough activity; Rluc – the shorter product reflecting the presence of PTC in the template.

Table 2. Comparison of the PTC-readthrough efficiency for AAG/PTC combinations tested in the *in vitro* TNT system.

	Normalized PTC-readthrough efficiency (%) in the presence of AAG		
Location of PTC (gene_exon)	Paromomycin 15 μg/ml	Gentamicin 15 μg/ml	G418 1.5 μg/ml
*DNAH5*_32	27.9 ± 1.7	14.3 ± 2.3	26.5 ± 7.4
*DNAH5*_49	11.4 ± 1.6	2.7 ± 0.3	7.1 ± 0.6
*DNAH11*_70	7.8 ± 0.8	20.0 ± 3.8	24.9 ± 3.8
*RSPH4A*_3(a)	6.6 ± 0.3	12.5 ± 1.9	15.7 ± 0.4
*DNAH5*_20	2.7 ± 0.6	1.5 ± 0.0	5.1 ± 0.2
*SPAG1*_16	n/a	n/a	n/a

The PTC-readthrough efficiency is expressed as the proportion of the longer product (Rluc+Fluc) intensity in the AAG-treated reaction with PTC-containing pDluc construct, normalized by the respective value obtained in the untreated reaction with the corresponding wild-type construct (set as 100%). Only the data for the highest concentration of each AAG are shown. n/a – not analyzed due to the very low/inconsistent PTC-readthrough signal.

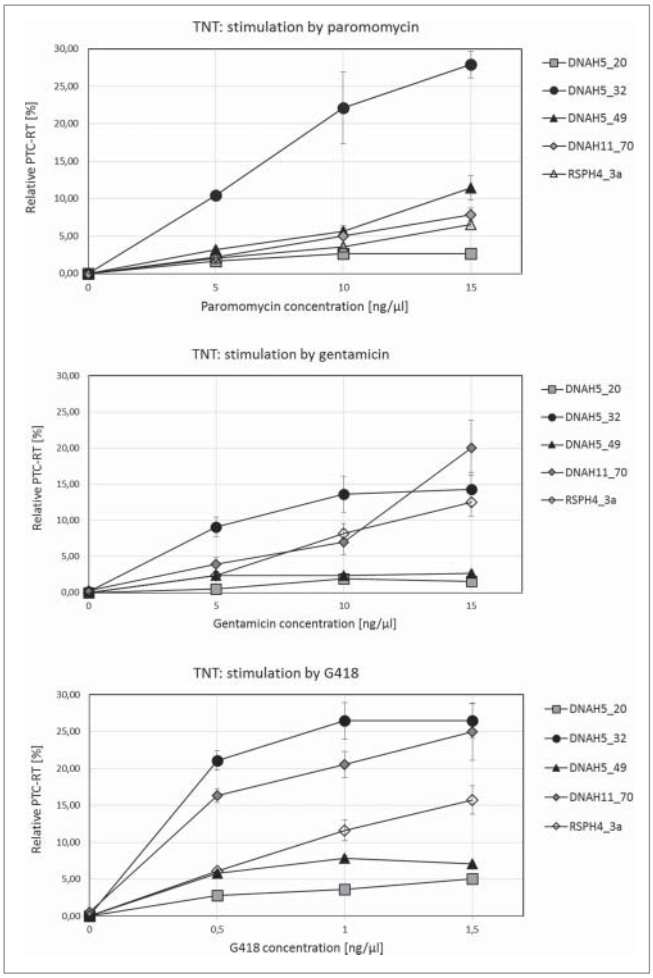

Figure 3. PTC-readthrough efficiency in 5 PTCs most effectively stimulated by AAGs in the *in vitro* TNT tests. The efficiency of PTC-readthrough for each pDluc-PTC construct in the presence of different concentrations of AAGs is expressed as the percentage of the longer protein product (Rluc-insert-Fluc) intensity normalized by the respective value for the untreated control (construct containing the wild-type insert). Upper panel: paromomycin; middle panel: gentamicin; lower panel: G418. Please note the different scale for G418 concentration.

varying ability of these compounds to stimulate the readthrough process. G418 and paromomycin at their highest concentrations appeared to be slightly more effective than gentamicin in stimulating readthrough of the responsive PTCs.

It has to be emphasized that the ability of each AAG to stimulate translational readthrough depended on the particular PTC. The highest stimulation was obtained for *DNAH5*_32 in the presence of either 15 μg/ml paromomycin or 1.5 μg/ml G418 (the amount of Rluc-insert-Fluc product: 27.9 $\pm$ 1.7% or 26.5$\pm$7 .4% of the untreated control constructs, respectively). The second most responsive PTC/AAG combination was *DNAH11*_70 in the presence of either 1.5 μg/ml G418 or 15 μg/ml gentamicin (24.9 $\pm$ 3.9% or 20.0 $\pm$ 3.8%, respectively).

Efficiency of the AAG-stimulated PTC-readthrough in the ex vivo *system*

To test the efficiency of AAG-stimulated PTC-readthrough in living cells, the expression of pDluc-PTC constructs was examined *ex vivo*, in the transiently transfected epithelial cells from the HEK293FT kidney cell line, incubated in the presence of gentamicin or paromomycin (1000 and 2000 μg/ml), or G418 (200 and 400 μg/ml). Five PTCs, best-responding in the *in vitro* experiments, were selected: *DNAH5*_20, *DNAH5*_32; *DNAH5*_49; *DNAH11*_70; *RSPH4A*_3a. For comparison, the construct containing poorly responding SPAG1_16 PTC was also included.

Table 3. Comparison of the PTC-readthrough efficiency for AAG/PTC combinations tested *ex vivo* in the HEK293FT cells transfected with pDluc constructs.

	Normalized PTC-readthrough efficiency (%) in the presence of AAG		
Location of PTC (gene_exon)	Paromomycin 2000 μg/ml	Gentamicin 2000 μg/ml	G418 400 μg/ml
DNAH5_32	2.3 $\pm$ 0.3	2.1 $\pm$ 0.4	2.5 $\pm$ 0.5
DNAH5_49	1.9 $\pm$ 0.6	1.9 $\pm$ 0.3	4.4 $\pm$ 1.1
DNAH11_70	2.6 $\pm$ 0.2	3.2 $\pm$ 0.1	6.1 $\pm$ 1.0
RSPH4A_3(a)	1.5 $\pm$ 0.0	2.0 $\pm$ 0.0	3.2 $\pm$ 0.5
DNAH5_20	1.2 $\pm$ 0.2	1.0 $\pm$ 0.3	2.3 $\pm$ 0.1
SPAG1_16	0.8 $\pm$ 0.0	1.1$\pm$ 0.0	1.3 $\pm$ 0.0

The PTC-readthrough efficiency is expressed as the ratio of Rluc to Fluc activities in the AAG-treated cells transfected with pDluc-PTC constructs, normalized by the ratio in the untreated cells transfected with the corresponding wild-type construct (set as 100%). Only the data for the highest concentration of each AAG are shown.

The readthrough efficiency in the transfected HEK293FT cells was expressed as the ratio of luminescence-based measurement of Fluc and Rluc activities in the whole cell lysates. The values obtained in the untreated cells (no AAG), transfected with pDluc constructs containing wild-type inserts, were assumed to represent 100% efficiency. Summary of the results is shown in Table 3 and Figs. 4 and 5.

The highest readthrough values in the presence of paromomycin, gentamicin and G418 did not exceed 2.6%, 3.3% and 6.1%, respectively. Thus, despite the higher AAGs concentrations used in the *ex vivo* experiment (up to 133-fold difference for paromomycin and gentamicin and 266-fold for G418), the PTC-readthrough was approximately 4 times lower than in the respective *in vitro* tests. Although G418 remained the most effective stimulator of PTC-readthrough, the best PTC/AAG combinations in the *ex vivo* experiments were different from those in the *in vitro* system: *DNAH11*_70 with 400 μg/ml G418 (6.1 $\pm$ 1.0%), and *DNAH5*_49 with 400 μg/ml G418 (4.4 $\pm$ 1.1%).

The ranking order of PTCs susceptibility to AAG-stimulated readthrough

The overall comparison of the results obtained in the *in vitro* and *ex vivo* tests (Table 4) revealed that, regardless of the AAG used and the testing system applied, PTCs in *DNAH11*_70 and *DNAH5*_32 remained the most, while in *DNAH5*_20 the least susceptible to suppression.

When the sequences of underlying codons were examined (Table 5), the significant readthrough was observed in 4 out of 7 UGA termination codons (*DNAH5*_32, *DNAH5*_49, *DNAH11*_70, *RSPH4A*_3a) and in one out of 6 UAG codons (*DNAH5*_20); no readthrough was observed in any of 3 UAA codons.

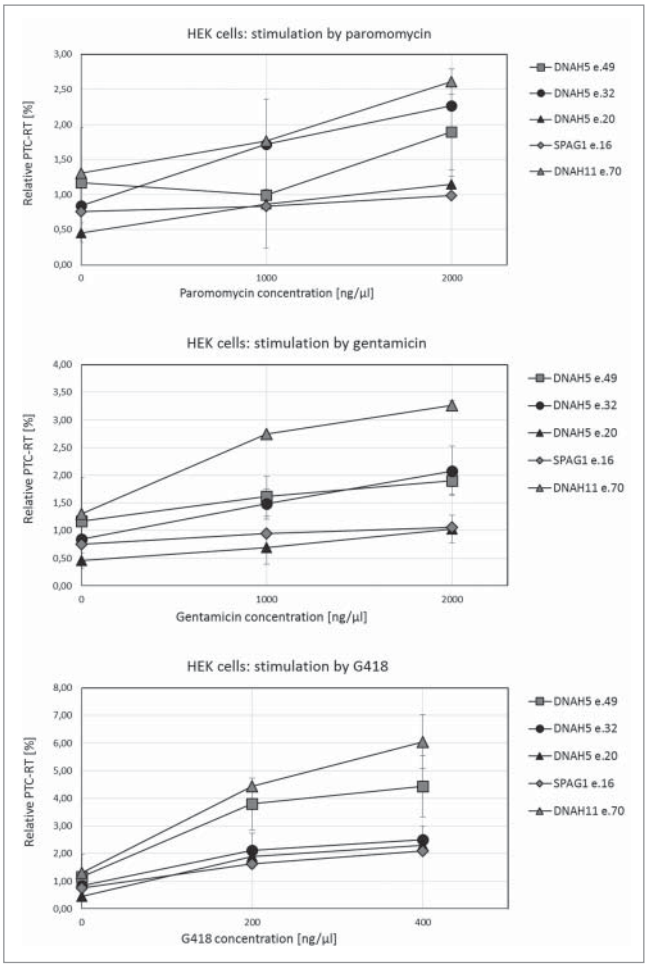

Figure 4. PTC-readthrough efficiency in 5 PTCs most effectively stimulated by AAGs in the *ex vivo* tests in pDluc- transfected HEK293FT cells. The efficiency of PTC-readthrough stimulated by different concentrations of AAGs in HEK293FT cells transfected with each pDluc-PTC construct is expressed as the ratio of Rluc to Fluc activities, normalized by the respective value for the untreated cells transfected with the control constructs (pDluc with the wild-type insert). Please note the different scale for G418 concentration; also note the difference in the y-scale between Figs. 1 and 2.

No clear correlation of the PTC-readthrough with the +4 position (the first base after STOP codon) was observed (Table 5). For example, of the 2 UGA codons followed by T, one was efficiently suppressed (*DNAH11_70*), while no readthrough was observed for the other one (*DNAH5_62*). Both UGA codons followed by C (*DNAH5_32* and *DNAH5_49*) were read through, but only one (*DNAH5_32*) with a high efficiency.

Biological effect of the AAGs in the primary respiratory epithelial cells

The primary respiratory epithelial cells from healthy individuals were cultured toward *in vitro* ciliogenesis.[32,33] The cells were exposed to AAGs during the adherent phase of the culture, when early steps of cell differentiation occur. The influence of different concentrations of AAGs on the viability of the cells and on their ability to grow cilia was analyzed during the suspension phase of the culture, when multi-cell spheroids are formed and cilia growth takes place.

The cytotoxic effect of AAGs was manifested by the increased amount of single dead cells floating in the medium; its direct estimation was not possible, since the dead cells were removed from the culture during each medium exchange. The cytotoxicity was therefore examined indirectly, by analyzing the number and size of viable spheroids visible in high speed videomicroscopy (HSVM).[34] The minimal number of spheroids required to consider AAG as non-cytotoxic was arbitrarily set at 15. Among all the tested AAG concentrations, only G418 at 400 μg/ml resulted in the reduction of that number below 15; the effect of this G418 concentration on the spheroids was therefore not analyzed any further.

Analysis of the spheroid size revealed that ~40% of all the spheroids in the untreated cultures were small (each occupying less than half of the field of view at 40x magnification). For the majority of AAG concentrations analyzed, the proportion of small spheroids in the treated culture was similar to that in the controls. A slight increase in the proportion of small spheroids (up to 21% compared to the untreated control) was observed only in cultures treated with gentamicin at 1000 or 2000 μg/ml and G418 at 200 μg/ml.

The process of ciliogenesis was examined using the HSVM analysis. As expected, spheroids in the untreated cultures differentiated asynchronously, randomly forming cilia on their outer surfaces.[35,36] The differentiation proceeded through several stages: I) the nude and immotile spheroids, II) the nude spheroids exhibiting motility (due to the presence of very short, microscopically invisible, cilia), and III) the motile spheroids covered with visible cilia. The proportion of spheroids at these 3 stages at day 16 of the suspension phase was used to assess the influence of AAGs on the ciliogenesis. Approximately 85% of the

Table 4. Ranking of the PTC-readthrough susceptibility of the analyzed PTCs depending on the AAG and the experimental system used.

In vitro TNT tests								
AAG	PTC-readthrough susceptibility							
Paromomycin	*DNAH5_32*	>	*DNAH5_49*	>	*DNAH11_70*	>	*RSPH4A_3a*	>	*DNAH5_20*
Gentamicin	*DNAH11_70*	>	*DNAH5_32*	>	*RSPH4A_3a*	>	*DNAH5_49*	>	*DNAH5_20*
G418	*DNAH5_32*	>	*DNAH11_70*	>	*RSPH4A_3a*	>	*DNAH5_49*	>	*DNAH5_20*
Ex vivo tests								
AAG	PTC-readthrough susceptibility							
Paromomycin	*DNAH11_70*	>	*DNAH5_32*	>	*DNAH5_49*	>	*RSPH4A_3a*	>	*DNAH5_20*
Gentamicin	*DNAH11_70*	>	*DNAH5_32*	>	*RSPH4A_3a*	>	*DNAH5_49*	>	*DNAH5_20*
G418	*DNAH11_70*	>	*DNAH5_49*	>	*RSPH4A_3a*	>	*DNAH5_32*	>	*DNAH5_20*

The ranking of the PTCs is based on the decreasing PTC-readthrough efficiency at the highest concentration of each AAG (from the left to the right).

Table 5. PTC-readthrough susceptibility depending on the termination codon context.

PTC location	PTC identity	Nucleotide at +4 position	PTC-readthrough (in *TNT*)	PTC-readthrough (in *HEK293FT*)
*DNAH5*_32	UGA	C	+++	+++
*DNAH5*_49	UGA	C	+	+
*RSPH4A*_3(a)	UGA	G	++	++
*DNAH5*_34(b)	UGA	G	−	n/a
*RSPH4A*_3(b)	UGA	A	−	n/a
*DNAH11*_70	UGA	T	+++	+++
*DNAH5*_62	UGA	T	−	n/a
*SPAG1*_16	UAG	T	+−	−
*RSPH4A*_1	UAG	T	−	n/a
*DNAH5*_37	UAG	T	−	n/a
*CCDC40*_20(b)	UAG	T	−	n/a
*DNAH5*_20	UAG	C	+	+
*CCDC40*_15	UAG	A	−	n/a
*CCDC40*_20(a)	UAA	C	−	n/a
*DNAH5*_47	UAA	G	−	n/a
*DNAH5*_34(a)	UAA	G	−	n/a

+: the presence of PTC-readthrough; the efficiency is proportional to the number of + signs; −: no PTC-readthrough; +− results not reproducible; **n/a**: not analyzed due to very low PTC-readthrough efficiency in TNT tests.

spheroids in the untreated cultures and in the majority of AAG-treated cultures were at stage II or III. A slight (~10%) decrease in the proportion of stage II or III spheroids was observed in cultures treated with gentamicin at 2000 μg/ml. In contrast, ~85% of the spheroids in cultures treated with G418 at 200 μg/ml represented stage I (i.e. were neither motile nor covered with visible cilia). This indicated a strong adverse effect of G418 on the cilia growth, although this effect was less pronounced than the direct cytotoxic effect of this AAG at 400 μg/ml.

Discussion

Among the PTCs examined in our study, the UGA termination codon was more susceptible to AAG-stimulated readthrough than UAG, and both were more responsive than UAA (UGA >UAG>>UAA). This was not surprising in light of the reported preference of these codons in the previous PTC-readthrough studies (for the recent review see[24]). On the other hand, 3 out of 7 constructs with the UGA codon did not yield any positive results at the examined range of the AAG concentrations. The UAG codon responded positively to AAG stimulation in only one out of 6 constructs containing this PTC. Consistent with the previous reports,[22,23,37,38] none of the constructs containing the UAA STOP codon showed any PTC-readthrough.

According to the literature, the efficiency of PTC-readthrough is also influenced by the identity of the +4 nucleotide, and cytosine in this position is known to increase the extent of PTC-readthrough.[39-43] In our experiments, no clear correlation of the AAG-stimulated PTC-readthrough with the cytosine at the +4 position was observed. Sequences other than the STOP codon and the +4 position (e.g. forming secondary structures downstream from the STOP codon) may be among additional factors involved in the susceptibility of the examined PTC to become suppressed.[24]

As expected, the efficiency of the suppression of the susceptible PTCs differed depending on the AAG type and concentration. However, neither the PTC type nor the nature of the stimulating compound alone were sufficient to predict the efficiency of the readthrough stimulation, indicating that all the possible combinations of AAG and responsive PTC should be examined.

Generally, the readthrough efficiency correlated positively with the stimulating agent's concentration. However, it has to be emphasized that the maximal AAG concentrations, which reflected the limits set to avoid cytotoxic effects of AAGs, differed in the *in vitro* and *ex vivo* experiments. The highest AAG concentrations used in the *ex vivo* system (2000 μg/ml for paromomycin and gentamicin; 400 μg/ml for G418) were over 2 orders of magnitude higher than those used in the *in vitro* TNT system (15 μg/ml and 1.5 μg/ml, respectively). Yet, the maximal efficiency of PTC-readthrough stimulation in the transfected cells was 2–12 times lower than *in vitro*.

We postulate that the observed difference reflected the limited permeability of the living cells to AAGs. While the formal proof for this scenario remains to be delivered, this explanation appears the most probable, as similar phenomena have been reported in other studies.[44,45]

Regardless of the mechanisms responsible for the lower efficiency of PTC-readthrough stimulation in the transfected cells, the most important question is, how to achieve more efficient PTC suppression in future *ex vivo* experiments? Increasing concentration of the tested AAGs in the cell medium cannot be considered, given that their upper limits were set to not exceed the LD_{50} doses reported in the literature.[23,31] In addition, G418 at 400 μg/ml appeared to be cytotoxic to the primary respiratory epithelial cells. In this context, it has to be noted that a number of new aminoglycoside derivatives and non-AAG compounds, with a higher PTC-readthrough stimulation potential and significantly lower oto- and nephrotoxicity, have been recently developed.[46-50] Thanks to their lower molecular mass, and considerably better membrane permeability, these compounds could be used as alternatives to AAGs in the context of the therapy of PTC-caused disorders.

In the *ex vivo* experimental system, there is a potential possibility of other readthrough-independent factors, which could affect the activity of luciferases in the presence of AAGs. The case of PTC124, a purported readthrough-stimulating agent, provides a good example of such confounding effects.[51] PTC124 is a specific Fluc inhibitor, which by stabilizing this

protein, transiently increases the Fluc luminescence in the transfected cells. In the literature, no spurious effect similar to that reported for PTC124/Fluc has ever been reported for any of the 4 AAGs examined in our study. Moreover, if the stimulation of Fluc activity by AAGs in the transfected cells was RT-independent, one would expect the increase in the ratio of Fluc to Rluc signals to be visible not only in constructs with PTC-containing inserts, but also in controls with the wild-type inserts. No such effect was observed in our study, indicating that the tested AAGs did not influence the activity of Fluc in an RT-independent manner.

The efficiency of the translational readthrough *in vivo* (i.e., in the primary cells with PTC in its full genomic context) may also depend on the process of nonsense-mediated decay (NMD), which lowers the quantity of some of the PTC-containing transcripts.[52,53] However, the transcripts from the recombinant pDluc vectors used in our experiments did not contain exon-intron junctions important for triggering NMD together with the poly-A tail signal. Therefore, the efficiency of PTC-readthrough stimulation in our experiments would not be affected by NMD, neither *in vitro* nor *ex vivo*.

The efficiency of chemical compound-stimulated PTC-readthrough in the *ex vivo* experiments may also depend on the type of the cells. In our study, it was analyzed in the transiently transfected HEK293FT cells of kidney origin, which represent the tissue vulnerable to the well-known nephrotoxic effects of AAGs.[54-56] However, the potential therapeutic use of AAG-stimulated PTC-readthrough in PCD patients would require targeting the respiratory epithelium, the tissue most severely affected tissue in the patients. Therefore, testing the susceptibility of PTCs to AAG-stimulated readthrough should be analyzed directly in these cells; moreover, the effect of AAGs on this tissue would have to be examined. The lack of an established epithelial cell line expressing cilia, and the extremely limited access to the epithelial cells from the patients with PTC mutations, renders the first task impossible at the present stage. We only succeeded to perform preliminary screening using the ciliated airway epithelium from healthy donors. These experiments showed that, although the majority of tested AAGs did not cause substantial negative effects, neither on the cells nor on the process of *ex vivo* ciliogenesis, G418 at 400 μg/ml exerted strong negative effects on these cells. Similar tests should also be performed for the new generation of PTC-readthrough stimulating non-AAG compounds.

Another reason, why the new compounds should be examined, is the possibility of a more effective readthrough of the examined PTCs – both in terms of the increase in the suppression of responsive PTCs, and of eliciting readthrough of those PTCs that did not positively respond to the tested AAGs. Considering the proportion of AAG-responsive PTCs (Table 1 and 5) among Polish PCD patients, we estimated that ~3% of Polish PCD patients might benefit from developing the paromomycin, gentamicin or G418-based treatment. Unfortunately, the PTC in *SPAG1*_16 (UAG_T) was not suppressed by the examined AAGs. Further search for the effective readthrough stimulator of this most frequent of all causative mutations in Polish PCD patients (alone accounting for 10% of the cases) is highly recommended.

Materials and methods

Insert synthesis and cloning

Oligonucleotides representing forward and reverse strands of PTC-containing and wild-type DNA sequences were synthesized (Genomed, Warsaw, Poland), with 4 bp overhangs (forward strand: 5′ TCGAG; reverse strand: 5′GATCT) added to enable ligation into the digested plasmid.

Phosphorylated and duplexed oligonucleotides were ligated into the *XhoI*- and *BglII*- digested polylinker region of pDluc, a dual-luciferase reporter vector.[30] 100 ng of each construct was electroporated into *Escherichia coli* strain DH5α (2.5 kV, 25 μF, 200 Ω). Plasmids were amplified in *E. coli* using carbenicillin selection (see Fig. 1A), and purified using Maxi- or Midi-prep Kit (Qiagen), according to the manufacturer's specifications. All insert sequences were confirmed by direct dideoxy-sequencing (Genomed).

In vitro *translation/transcription reactions and quantitation of RT*

Recombinant pDluc plasmids were used as templates for the *in vitro* protein synthesis in translation/transcription (TNT) Coupled Reticulocyte Lysate system (Promega, Madison, Wisconsin, USA) in the presence of radioactively labeled methionine and a range of AAG concentrations. Each reaction (12.5 μl total volume) contained: 6.25 μl reticulocyte lysate; 0.5 μl reaction buffer; 0.25 μl amino acid mix (minus methionine); 0.25 μl T7 polymerase; 0.5 μl radiolabeled [^{35}S]-methionine (Hartmann Analytic); 0.25 μl RNAsin (Promega, Madison, Wisconsin, USA); 200 ng DNA template (pDluc construct with a wild-type or PTC-containing insert) and 2.5 μl water or AAG dilution. The reaction was incubated at 30°C for 90 min, and stopped by adding 4 X SDS-PAGE Loading Buffer (BioRad, Hercules, California, USA) to the final concentration of 1X.

The reaction products were separated by 12% SDS-PAGE, and exposed to phosphor screens (Fujifilm Corporation, Tokyo, Japan). Phosphor screens were scanned using a Fujifilm FLA-500 Image Reader and analyzed using the ImageJ densitometry plug-in.[57] The extent of PTC-readthrough was quantified in several steps. First, the intensity of both protein products (longer Rluc-insert-Fluc and shorter Rluc) was normalized by the number of incorporated methionine residues. The proportion of a longer product (the intensity of Rluc-insert-Fluc protein divided by the intensity of both products) in the reaction with PTC-containing construct translated in the presence of AAGs was divided by the respective value for the wild-type construct translated in the absence of AAGs (representing 100% readthrough); this step accounted for the AAG-related decrease in the overall translation efficiency. The readthrough efficiency for each PTC/AAG combination was measured in at least 3 separate experiments, and the mean values were reported.

Ex vivo *translation in cultured cell lines*

For the use in *ex vivo* translation experiments, human kidney epithelial cells (HEK293FT) were cultured in the HEK growth medium: DMEM (Sigma-Aldrich Corporation, Missouri, USA) supplemented with 10% fetal calf serum (FCS), 1% GlutaMAX,

MEM Non-Essential Amino Acids (all 3 from ThermoFisher Scientific, Waltham, USA) and 1% pen-strep (Sigma-Aldrich Corporation, Missouri, USA), respectively. The cells were grown at 37°C, in a humidified atmosphere containing 5% CO_2; medium was changed 3 times a week.

On the day of transfection, the cells were detached from the culture vessels, and suspended in RPMI (ThermoFisher Scientific, Waltham, USA); 1×10^4 cells (10 μl of the suspension) were electroporated with 1 μg of the appropriate pDluc construct, using the Neon Transfection System (ThermoFisher Scientific, Waltham, USA). The transfected cells were seeded in HEK growth medium in a 24-well plate at a final density of $\sim 5.2 \times 10^4$ cells/cm^2 and incubated as above.

Twenty-four hours after transfection, a fresh growth medium, with or without gentamicin (0, 1000 or 2000 μg/ml), paromomycin (0, 1 or 2 μg/ml) or G418 (0, 200 or 400 μg/ml), was added and cells were incubated for 24 h at 37°C; 3 independent wells were used for each PTC/AAG concentration combination.

The cells were lysed using 100 μl of 1X Passive Lysis Buffer (Promega, Madison, Wisconsin, USA) and the PTC-readthrough efficiency was measured using the Dual-Luciferase Reporter Assay and dedicated Glomax Multi Detection System with injectors (Promega, Madison, Wisconsin, USA). Each lysate was measured in triplicate, using 20 μl of the lysate and 50 μl of Dual-Luciferase Reporter Assay reagents, at the Glomax acquisition settings recommended by the manufacturer.

The efficiency of PTC-readthrough was assessed in 2 steps. First, the ratio of Rluc to Fluc luminescence in the AAG-treated cells transfected with PTC-containing construct was calculated. Then, it was normalized with respect to the Rluc to Fluc ratio in the untreated cells transfected with the corresponding wild-type construct (set as 100%). Out of the 9 measurements of the luminescence for each AAG-construct combination in a single experiment, a maximum of 2 outlying measurements were rejected. All values were the average of at least 2 experiments.

Primary cell cultures

The cells from human respiratory epithelium were collected from routinely resected nasal polyps originating from healthy donors. The cells were preplated to remove contaminating fibroblasts and subjected to the *in vitro* ciliogenesis using a sequential 2-phase method, whereby cells are cultured in the adherent (ADH) phase, followed by the suspension (SUSP) phase.[32,33,58] ADH phase was initiated by seeding 5×10^5 cells/cm^2 into T75 culture flasks covered with a collagen gel. The cells were grown in ADH medium (DMEM-F12 + 2% Ultroser G (Pall Life Sciences) + 2% pen-strep (penicillin/streptomycin) + 0.2% nystatin (Sigma-Aldrich) at 37°C and 5% CO_2.); medium was changed 3 times a week. After 20 d in ADH, the cells were detached from the collagen using a 200 U/ml solution of collagenase IV (Worthington Biochemical Corp., in DMEM-F12 medium, 2% pen-strep, 0.2% nystatin), cell sheets were transferred to the SUSP medium (DMEM-F12, 10% Nu-Serum IV supplement (Beckton Dickinson), 2% pen-strep, 0.2% nystatin), and grown for 21 d at 37°C, with gentle rotation (80 rpm); medium was changed every 2 d.

The cells were treated with AAGs for 96 h during the ADH phase (16-19 of the culture), followed by one day of the ADH phase without AAG. The AAG concentrations were within the range used in previous studies:[23,31] paromomycin (1000 and 2000 μg/ml); gentamicin (1000 and 2000 μg/ml); amikacin (1000 and 2000 μg/ml) and G418 (200 μg/ml and 400 μg/ml).

Qualitative changes in the cell viability and in cilia differentiation were monitored during the SUSP phase, using high-speed video microscopy (HSVM, Sisson-Ammons Video Analysis software, Ammons Engineering, USA).[34]

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The use of control epithelial cells from polyps was approved by the local Ethical Committee.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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