2-Aminothiazole-4-carboxamides Enhance Readthrough of Premature Termination Codons by Aminoglycosides

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S Supporting Information

ABSTRACT: Nonsense mutations introduce a premature termination codon (PTC) and are the underlying cause of multiple rare genetic diseases and cancers. Although certain aminoglycosides bind to eukaryotic ribosomes enabling incorporation of an amino acid at the PTC and formation of full-length protein, they are inefficient and toxic at therapeutic doses. Library screening in assays that measure readthrough at a PTC in the TP53 gene in human HDQ-P1 cells identified six novel 2-aminothiazole-4-carboxamide derivatives that potentiate the PTC readthrough (PTCR) efficiency of G418 when used in combination. The two most potent compounds incorporated a 4-indazole motif on the 2-aminothiazole nitrogen and a hydrophobic aryl substituent on the carboxamide nitrogen. These compounds are valuable tools to further investigate the therapeutic potential of aminoglycoside-induced PTCR.

KEYWORDS: Premature termination codon (PTC) readthrough, 2-aminothiazole-4-carboxamides, library screening, cell-based assay, nonsense mutations

Nonsense mutations are single base substitutions in DNA that generate a premature termination codon (PTC), preventing the formation of full-length functional protein from mRNA. Nonsense mutations are the underlying cause of 10–12% of rare genetic disease cases, and they similarly inactivate tumor suppressor genes in about 10% of sporadic cancer patients.¹ It has been found that the binding of aminoglycosides, such as gentamicin and G418, to eukaryotic ribosomes alters the architecture of the ribosomal decoding center, thereby enabling pairing of a near-cognate aminoacyl-tRNA complex to a PTC and incorporation of an amino acid instead of premature translation termination.² This process, termed PTC readthrough (PTCR), enables formation of full-length protein from mRNAs bearing a PTC and functional rescue.

Building on this observation, the ability of aminoglycosides to promote PTCR has been intensively studied as a therapeutic strategy for treatment of cystic fibrosis, Duchenne muscular dystrophy, spinal muscular dystrophy, lysosomal storage disorders, and other genetic diseases.³ However, gentamicin and G418 are too toxic to be used in humans at the elevated doses required to induce sufficient readthrough to exert a therapeutic effect. Current research in this area has focused on developing safer and more potent aminoglycosides related to neomycin (TC series), kanamycin B (JL series), paromomycin (cf. NB30, NB54, NB74, NB84, and NB124),^{3–5} exploring the use of nonaminoglycoside antibiotics (tylosin, josamycin, spiramycin, azithromycin, and negamycin),^{6,7} and identifying

small molecules inducers through library screening. Active compounds found in screening programs include clitocine (a nucleoside analog),⁸ escin (a mixture of saponins),⁹ and the heterocycles amlexanox,¹⁰ PTC124 (used to treat Duchenne muscular dystrophy),¹¹ RTC13, 14, 204, and 219,^{12–14} and GJ071/072¹⁵ (Figure 1).

In this latter context, Baradaran-Heravi et al. recently developed an automated immunofluorescence assay for the high throughput screening of compound libraries to detect molecules that induce readthrough at a PTC in the TP53 gene in HDQ-P1 breast cancer cells.¹⁶ Importantly, although no activity was observed for the libraries evaluated when tested alone, it was discovered that certain molecules potentiate the PTCR efficiency of the aminoglycoside G418 when used in combination. This led to the discovery of the phthalimide compound **1** (CDX5–1) as the first enhancer of aminoglycoside-induced PTCR (Figure 2).

At present, it is not known whether this enhancement effect occurred through direct interaction with the ribosome and/or ribosome associated proteins, through interaction with upstream factors, or through some other mechanism. Indeed, a number of cellular mechanisms contribute to restricting PTCR,

Received: December 3, 2018

Accepted: April 9, 2019

Published: April 9, 2019

Figure 1. Structures of representative nonaminoglycoside small molecule inducers of PTCR.

Figure 2. Structures of CDX5-1 1, IDC16 2, 1C8 3, and general formula 4 for the subset of IDC16 mimics built using the 2-aminothiazole-4/5-carboxamide linker (in blue). “Het”Ar corresponds to aromatic/heteroaromatic diversity elements on the left and right sides of structure 4.

including nonsense-mediated RNA decay (NMD) and alternative RNA splicing.^{17–19} Consequently, it was of interest to evaluate a library of molecules for their PTCR activity that were purposely designed to mimic the structure of the indole compound 2 (IDC16),²⁰ which impacts HIV pre-mRNA alternative splicing. As illustrated in Figure 2, the library contains molecules wherein the central B/C-rings of IDC16 have been replaced by different linker motifs, and the A/D-ring surrogates correspond to different aromatic/heteroaromatic

motifs. In an anti-HIV screen and subsequent studies, the diheteroarylamide compound 3 (1C8) was shown to perturb the function of the splicing factor SRSF10.^{21,22} In contrast, as described in this study, the results of the PTCR screen of this same library has revealed that a subset of 2-aminothiazole-4/5-carboxamide-based IDC16 mimics (cf. structure 4), which had no effect on HIV replication, significantly enhances G418 promoted PTC readthrough. In particular, compounds 9a and 9f (Table 1) display greater potency than phthalimide 1.

Table 1. Structures of 2-Amino-4-carboxamides 5–9 and Their ER Values at 10 and 3 μ M Concentrations

10 μ M + G418	7.0	10 μ M + G418	10.7	10 μ M + G418	8.7
3 μ M + G418	3.8	3 μ M + G418	3.5	3 μ M + G418	1.6
10 μ M + G418	8.0	10 μ M + G418	11.2	10 μ M + G418	5.4
3 μ M + G418	1.4	3 μ M + G418	2.5	3 μ M + G418	1.2
10 μ M + G418	23.3	10 μ M + G418	23.3	10 μ M + G418	15.8
3 μ M + G418	13.1	3 μ M + G418	13.1	3 μ M + G418	3.4
10 μ M + G418	27.2	10 μ M + G418	27.2	10 μ M + G418	20.1
3 μ M + G418	1.4	3 μ M + G418	1.4	3 μ M + G418	8.2

To evaluate the 390 molecules comprising the library of IDC16 mimics, it was screened using the 96-well plate automated immunofluorescence-based PTCR assay.¹⁶ The compounds were dispensed using a 96-pin tool delivering a volume of 110 nL, resulting in a final concentration of ~ 20 μ M for most compounds. In the absence of any treatment, few cells expressed detectable p53, and exposure to a suboptimal concentration of the aminoglycoside G418 (50 μ M for 48 h) caused a small increase in the percentage of cells expressing

p53, indicating induction of a modest level of readthrough. In this preliminary screen, all compounds were tested in the presence of 50 μ M G418, and their biological activity was expressed as a readthrough ER (enhancement ratio) (ER = % cells expressing p53 when exposed to a library compound and G418/% cells expressing p53 when exposed to G418 alone). Using a modest ER of 1.4 as an initial cutoff, 33 of the 93 2-aminothiazole-4/5-carboxamides present in the library were identified as potentially active (see Table 1A in the [Supporting Information](#)).

The 33 initial hit molecules were subsequently tested in a quantitative capillary electrophoresis western analysis assay to formally demonstrate PTC readthrough by measuring formation of full-length p53, the readthrough product. Cells were initially exposed to the compounds alone at 10 μ M, to G418 alone at 25 μ M, or to the compounds at 10 μ M together with 25 μ M G418 for 72 h. The cells were then lysed, cellular proteins were separated by capillary electrophoresis, and the amount of full-length p53 protein was determined by quantitative immunoblotting. Vinculin was also measured in the same capillaries and served as an internal protein loading control. The amount of full-length p53 was normalized to that of vinculin to correct for any variations in loading of the capillaries. The amount of full-length p53 produced was then expressed as the ER, as above. In all experiments, full-length p53 was undetectable in untreated cells, and exposure to 25 μ M G418 elicited a small amount of readthrough, defined as 1 (see Figures 1A to 4A in the [Supporting Information](#)). From these experiments, the 16 compounds shown in [Tables 1 and 2](#)

Table 2. Structures of 2-Amino-5-carboxamides 10a–c and Their ER Values at 10 and 3 μ M Concentrations

10a R ¹ =		10b R ¹ =		10c R ¹ =
10mM + G418		7.8		4.7	
3 mM + G418		2.2		1.6	
				19.2	
				1.2	

were active with an ER > 4.5. Looking at this collection of hits in the secondary assay, it is noteworthy that, with the exception of the 2-pyridinone compound **5**, the two quinolines **6a** and **6b** and the indole compound **7**, 12 of the compounds possessed either a 5-indazole (**8a–c**, **10a–c**) or a 4-indazole (**9a–f**) motif as the “left side” component.

To further distinguish their activities, the 16 compounds were tested as above, but at a lower 3 μ M concentration. All compounds showed a lower PTC readthrough ER than at 10 μ M. However, the six molecules **5**, **6a**, **9a**, **9b**, **9d**, and **9f** stood out as maintaining an ability to enhance G418-mediated PTC readthrough, with compounds **9a** and **9f** being the most potent ([Tables 1](#), [Figure 3A](#); [Figures 1A–4A](#) in the [Supporting Information](#)). Interestingly, all three isomeric 2-amino-5-carboxamides **10a–c** were eliminated in the more stringent screen at 3 μ M concentration. Note, when tested side by side at 10 μ M in combination with G418, compounds **9a** and **9f** showed ERs of 25.7 and 8.8, respectively, higher than the previously reported phthalimide **1**, with an ER of 2.8 ([Figure 3B](#)). A concentration of 30 μ M was required for compound **1**

Figure 3. PTC readthrough activity of the six active compounds (A) and comparison of compounds **9a** and **9f** to CDX5-1 (**1**) (B). (A) Cells were exposed to the compounds alone at 10 μ M (top panel) or at 10 or 3 μ M in combination with 25 μ M G418 (middle and bottom panels, respectively), and full-length p53 (FL-p53), the readthrough product, was measured by automated capillary electrophoresis western analysis. Vinculin is the loading control. The numbers under the lanes indicate the readthrough ER. (B) Cells were exposed to the indicated compound concentrations without or with 25 μ M G418, and FL-p53 and ER were measured as in A.

to approach the activity of 10 μ M **9a** and **9f**, indicating they are at least 3-fold more potent ([Figure 3B](#)). Compound **9f** also enhanced readthrough by the other aminoglycoside gentamicin with an ER of 10.8 when used at 3 μ M together with 1 mg/mL gentamicin ([Figure 5A](#) in the [Supporting Information](#)).

Like the other 2-aminothiazole-4-carboxamides present in the library, the six advanced hits were prepared using the Hantzsch protocol illustrated in the central part of [Scheme 1](#). While 5-aminopyridinone **11** and 6-aminoquinoline **12** were both commercially available, the 4-aminoindazole **15** was prepared by reacting 2-methyl-3-nitroaniline **13** with NaNO₂ in HOAc, followed by hydrazine-Pd/C (cat) reduction of the nitro group in the derived indazole **14**.²³ Reaction of amine **11** with benzoylisothiocyanate in acetonitrile at room temperature for 6 h gave compound **16a**. Similarly, amines **12** and **15** were converted to intermediates **17a** and **18a** (63–75% range for the three amines).²⁴ Treatment of the derived *N*-acylthioureas with sodium hydroxide in methanol at 75 °C readily cleaved the benzoyl group to give the monosubstituted thioureas **16b–18b** in 54–77% yield.²⁵ Assembly of the aminothiazole-4-carboxylic acid substructure **16d–18d** involved reaction of thioureas **16b–18b** with ethyl bromopyruvate in hot EtOH (51–72%) and hydrolysis of the ester group in the derived intermediates **16c** (NaOH-H₂O) and **17c/18c** (LiOH, MeOH-H₂O) (44–78%). Subsequent reaction of acids **16d–18d** with the requisite amine (1,4-benzodioxan-6-amine for **5** and **9b**, 3,4-methylenedioxyaniline for **6a**, *p*-fluoroaniline for **9a**, 3,4,5-trimethoxyaniline for **9d**, and *p*-thiomethylaniline for **9f**) under peptide coupling conditions (HOAt/EDC/DIEA in DMF) completed the synthesis of the target compounds. As determined by ¹H and ¹³C NMR, these compounds (like all others in the compound library) were isolated pure by flash

Scheme 1^a

^aReagents and conditions: (i) NaNO₂, HOAc, 0 °C 1 h then RT 3 d, 89%; (ii) hydrazine, Pd/C (10%) (cat), MeOH, reflux, 3 h, 71–89%; (iii) PhCONCS, acetone, RT, 2 h, 63–75%; (iv) LiOH, MeOH–H₂O, 75 °C, 1 h, 54–77%; (v) BrCH₂COCO₂Et, EtOH, 90 °C, 3 h, 51–72%; (vi) LiOH, MeOH–H₂O, then 1 N HCl, 44–78%; vii (Het)Ar–NH₂, HOAc, EDC, DIEA, DMF, RT, 18 h.

column chromatography (absence of any extraneous peaks), and HPLC analysis established their purity to be >98%.

From the SAR perspective, although the original choice of the “left” and “right” side heterocycles used to construct the IDC16 mimic library had no logical connection to their ultimate ability to enhance aminoglycoside induced PTCR, the fact that a relatively large assortment of 2-aminothiazole-4/5-carboxamide derivatives was tested and only a few displayed potent activity provided pertinent insights into the nature of binding interactions these compounds have with a putative protein target.

Looking at the data in Tables 1 and 2 for the indazole-based compounds at 10 μM concentration, it was remarkable to observe that the attachment of the carboxamide motif to the thiazole ring could be varied from C-4 to C-5, and that for compounds 8 and 9, the 2-aminothiazole linker could be attached at either C-4 or C-5 of the indazole ring. Indeed, due to the different connectivities of the components in compounds 8, 9, and 10, their overall geometry is significantly different. This variation in structure due to modifications in the middle portion of the molecules proved to be acceptable for interaction with their putative target at the higher concentration. However, in the more stringent assay at 3 μM concentration where a more optimal binding to the target is required to out-compete with any endogenous ligand, it was observed that activity was maintained only for the 4-aminoindazole compounds 9.

That a 4-aminoindazole motif was found as the left-side component in the four advanced hits 9a, 9b, 9d, and 9f

strongly indicated that this system possibly interacts with the putative target through proximal H-donor/H-acceptor H-bonding interactions. This is in-line with the fact that an indazole scaffold is often used in the design of kinase inhibitors,²⁶ and this suggested that compounds 9 may be targeting an upstream kinase implicated in ribosome functioning, or alternatively some other ribosome associated protein that may similarly engage H-bonding to these agents via the NH/C=O functionality in an exposed backbone amide motif. In a similar way, the 2-pyridinone 5 and quinoline nitrogen in 6a could form H-bonds with this supposed binding pocket.

Noticeable also from the assay results was the finding that the active compounds frequently incorporated oxygen functionality in the right (carboxamide) side of their structures: a dioxane ring in 5, 6b, and 9b, a dioxolane system in compounds 6a, 8b, 10c, a mono-OMe group in compounds 9c, 9e, 10a, and tri-OMe substitution in 9d. Indeed, four of the advanced hits (5, 6a, 9b, and 9d) identified in this study possessed an oxygen bearing functionality in their right-side component. However, the ER values for these molecules (ER = 3.5–5.3) were significantly lower than that for the two most active compounds 9a (ER = 13.1) and 9f (ER = 8.2). Note also that the 2-methoxypyridine compound 9e and, in particular, the 4-methoxyphenyl compound 9c were considered inactive in the secondary assay at lower concentration. This points to the preference of having a less polar more hydrophobic motif on the right-side aromatic ring, such as the fluorine atom in 9a and the SMe substituent in 9f. Note further the absence of activity for the isomeric and geometrically distinct compound 10b, which also contains the 4-thiomethylphenyl motif.

Looking more closely at compound 9a, it is intriguing that the N-4-fluorophenyl carboxamide motif in its structure is also found in compound GJ072 and that the left side component in this nonaminoglycoside PTCR compound is a pyridine ring, which, like the indazole ring in 9a, can engage in H-bonding interactions with a putative protein target. At present, it is not understood how compounds 9a/9f have the ability to enhance the aminoglycoside induced PTCR, or how compound GJ072 and its congeners can directly affect PTCR. Further, although it is currently not known whether these compounds impact mRNA alternative splicing through interaction with splicing factors, compounds 9a and 9f did not increase the levels of TP53 R213X mRNA levels in HDQ-P1 cells (Figure 6A in the Supporting Information), indicating that they do not inhibit NMD. In addition, compounds 9a and 9f showed no cytotoxicity at 1 μM, but they reduced cell numbers to 42% and 60% of untreated controls during 72 h incubation at the PTCR enhancing concentration of 3 μM (Figure 7A in the Supporting Information). This cytotoxicity was intrinsic to the compounds as it was observed in the absence of G418 and coinubation with G418 did not further increase cytotoxicity. It is not currently not known whether their cytotoxicity is an on-target effect or whether it can be dissociated from their activity as PTCR enhancers.

In light of these observations, further development/SAR studies will focus on designing molecules that contain desirable characteristics of both the advanced hits identified in the present study and the GJ compounds. In particular, it will be of interest to use active molecules with these characteristics as probes to identify the putative targets of the two apparently distinct but, in some aspects, structurally similar PTCR

inducing/aminoglycoside enhancing agents. In conclusion, besides the potential for therapeutic application of these 2-aminothiazole-4-carboxamides in combination with subtoxic doses of aminoglycosides, these novel compounds are valuable tools to study the underlying mechanisms of PTCR.

■ ASSOCIATED CONTENT

■ Supporting Information

The Supporting Information is available free of charge on the ACS Publications website at DOI: 10.1021/acsmchemlett.8b00610.

Table 1A: Composition of the sublibrary of 2-aminothiazole-4/5-carboxamides and the preliminary screening results (pages S2 to S10). Figures 1A–7A: Structures of the thirty-three 2-aminothiazole-4/5-carboxamides identified in the primary screen; gels showing their PTC readthrough activity; and graphs showing their effect on TP53 mRNA levels and cytotoxicity (pages S11 to S15). Experimental Procedures: Biology and Chemistry (pages S16–S27). Spectral data (^1H NMR, ^{13}C NMR, and HRMS) for all final compounds and ^1H NMR for all intermediates prepared (pages S28 to S62). HPLC data on final compounds **5**, **6a**, **9a**, **9b**, **9d**, and **9f** (pages S63–S69) (PDF)

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The manuscript was written through contributions of all authors. All authors have given approval to the final version of the manuscript.

Funding

Authors A.B.H., A.D.B., A.K., S.H.F., and M.R. received funding from Canadian Cancer Society Grant 704700 and Genome BC Grant POC027. A.B.H. received funding from Mitacs Award IT03752.

Notes

The authors declare no competing financial interest.

■ ACKNOWLEDGMENTS

We thank M. Christian Buchwalder for invaluable assistance in obtaining the HPLC data for the active compounds identified in this study.

■ ABBREVIATIONS

DMAP, dimethylaminopyridine;; HOAt, 1-hydroxy-7-azabenzotriazole;; EDC, 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide;; DIEA, diisopropylethylamine

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