Enhanced Production of Functional Proteins from Defective Genes

Biao Xi, Fangxia Guan, and David S. Lawrence*

*Department of Biochemistry, The Albert Einstein College of Medicine,
1300 Morris Park Avenue, Bronx, New York 10461*

Received December 22, 2003; E-mail: dlawrenc@aecom.yu.edu

Nonsense mutations, which result in the truncation or absence of a key protein product, are associated with a host of genetic diseases.[1] Recently, gentamicin and other aminoglycoside antibiotics have been shown to suppress premature stop codon arrest by inducing the ribosome to read past the nonsense mutation via insertion of a random amino acid by a noncognate tRNA.[2] Although the precise mechanism of nonsense mutation suppression remains to be established, gentamicin exerts its antibiotic action by targeting the 30S ribosomal subunit, where it interferes with the initiation complex of protein formation.[3] Presumably, a related binding event in mammalian cells enables gentamicin to actively promote premature stop codon suppression. Aminoglycosides have now been used to suppress nonsense mutations in human cell lines and animal models of Hurler's syndrome, Duchenne muscular dystrophy, late infantile neuronal ceroid lipofuscinosis, and cystic fibrosis.[1] Unfortunately, gentamicin therapy has limitations. First and foremost, suppression rates tend to be low and attempts to enhance these rates could have devastating genome-wide consequences by repressing bona fide stop codon signals. Second, iototoxicity and nephrotoxicity are known and serious side effects of gentamicin therapy. Finally, aminoglycoside antibiotics suppress stop codons with dramatically different efficiencies (UGA > UAG > UAA) and the ability to read past these codons is further dependent upon the local sequence context.[2]

We have developed a strategy that could augment the efficacy of gentamicin-induced nonsense suppression by enhancing target protein formation and by reducing the amount of aminoglycoside required for activity. Figure 1a illustrates the gentamicin-induced suppression of a stop codon and the consequent synthesis of a protein with a hypothetical 25% suppression rate. We reasoned that larger amounts of protein could be produced, even at the same nonsense suppression rate, if suppression were conducted in the presence of a promoter-activating agent. The latter, in favorable cases, should enhance the quantity of message available for translation and thereby generate more target protein.

Our initial efforts focused on ataxia telangiectasia (A−T), a rare childhood disorder characterized by the eventual loss of motor control, moderate to severe immunodeficiency, premature aging, and a pronounced predisposition to cancer.[4] Like many rare childhood diseases, it is the absence, rather than the overabundance, of a key protein that is responsible for these devastating symptoms. Shiloh and his colleagues identified the gene (*atm*) that is defective in A−T.[4] A variety of *atm* deletion, nonsense, and missense mutations have been subsequently reported, the vast majority (>70%) of which result in protein truncation.[5] Truncated ATM protein is unstable and therefore not detected in most A−T cell lines.

We developed a cell-based screen for *atm* promoter enhancing agents by inserting the 700 bp *atm* promoter into pA3LUC, a promoterless plasmid containing a firefly luciferase cDNA reporter (LUC) (Figure 2). The human embryonic kidney 293T cell line
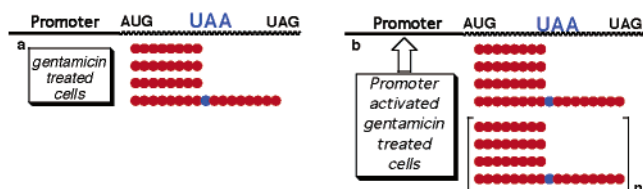

Figure 1. (a) Gentamicin-induced suppression of a premature stop codon results in the formation of protein product (highlighted in red; a hypothetical 25% suppression rate is shown in this example); (b) simultaneous treatment with gentamicin and a promoter-activating drug should generate more protein product, but at the same suppression rate.
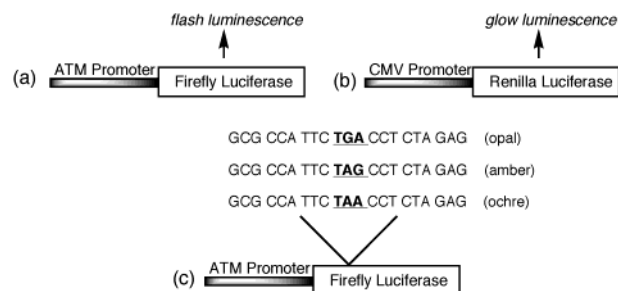

Figure 2. (a) Description of (a) the *atm*/pA3LUC plasmid, (b) the internal control plasmid containing the CMV promoter-driven renilla luciferase gene, and (c) the three constructs containing stop codons inserted 43 bp from the start site of the coding region of firefly luciferase (1650 bp).

was transiently cotransfected with the *atm*/pA3LUC plasmid and a plasmid containing the gene encoding renilla luciferase driven by the constitutive CMV promoter (a commonly employed internal control). The transfected cells were then plated into individual wells of 96-well plates. We assembled a library of nearly 400 FDA-approved drugs and individually screened these agents for their ability to activate the *atm* promoter. The obvious advantage associated with FDA-approved drugs is their established safety profile, which markedly reduces the expenses and lead-time associated with their application to disorders other than those for which they were originally intended. In addition, this structurally diverse family of chemicals is among the best understood of all biologically active compounds. Consequently, if a specific member of the FDA-approved family of drugs activates the expression of a specific gene, then there is a good likelihood that its mechanism of action can be deduced from the existing scientific literature.

Wells containing the transiently transfected 293T cells were treated with individual members at varied concentrations (0.25, 2.5, 25, and 250 μM) of our library of FDA-approved drugs. In addition, a series of controls were performed in each plate, including untreated (i.e., 5 mM glucose) and 25 mM glucose-exposed 293T cells. We previously found that high glucose levels upregulate ATM message and protein levels.[6] The amount of renilla luciferase activity is indicative of the number of cells present in a given well, whereas the quantity of firefly luciferase activity represents the influence

Table 1. Fold-Increase in *atm* Message Level as a Function of Time Following Treatment with Ofloxacin

| cell lines | fold-increase (*atm* message levels)[a] | | |
	2 h	6 h	24 h
GM13810 (A−T)[b]	1.2 ± 0.2	2.3 ± 0.3	5.4 ± 0.7
GM13860 (A−T)[b]	3.8 ± 0.9	2.3 ± 0.3	3.3 ± 0.3
GM05823 (A−T)[c]	2.2 ± 0.1	3.0 ± 0.2	5.8 ± 0.4

[a] As measured by RT-PCR, which were performed twice, each time in triplicate. Fold-increase relative to the corresponding untreated cell line. [b] Lymphoblastoid cell lines. [c] Fibroblast cell line.

Table 2. Fold-Increase in Stop Codon Read-through in 293T Cells Treated with Aminoglycoside Alone or Aminoglycoside in Combination with Promoter-Activating Agent

| protocol | fold-increase (read-through)[a] | | |
	opal	amber	ochre
geneticin	6.5 ± 0.8	14.9 ± 1.9	7.9 ± 3.0
gentamicin	8.7 ± 1.2	8.2 ± 1.8	6.7 ± 1.5
ofloxacin/gentamicin	14.2 ± 1.2	17.5 ± 1.9	9.6 ± 2.1
thioguanine/gentamicin	39.7 ± 2.0	29.2 ± 5.9	18.3 ± 2.3
ofloxacin/geneticin	14.6 ± 3.5	21.1 ± 1.1	7.1 ± 0.4

[a] As measured by the dual luciferase assay (in triplicate) and relative to untreated 293T cells transfected with the indicated stop codon-containing gene.

of the various drugs on *atm* promoter activity. In addition to 25 mM glucose, several FDA-approved drugs activate the *atm* promoter, including the fluorinated quinolone ofloxacin. Fluorinated quinolones are known to target eukaryotic topoisomerase II, an enzyme that catalyzes the interpenetration of DNA strands by introducing transient double strand breaks. Consequently, one possible mechanism by which ofloxacin activates the *atm* promoter is via the induction of double strand breaks, which is consistent with the role of ATM in DNA double strand break recognition and/ or repair.[7−9] If this mechanism is correct then the production of ATM protein would need to be sufficient to offset any drug-induced double strand breaks. However, several other drugs induce *atm* promoter activity, including the antimetabolite thioguanine.[10]

We subsequently examined the effect of ofloxacin on native *atm* message levels in 293T cells as well as in lymphoblastoid and primary fibroblast cell lines derived from A−T patients (Table 1). Message levels are enhanced 2.4 ± 0.1-fold in 293T cells treated with ofloxicin for 24 h. By contrast, an up to nearly 6-fold increase is observed in ATM-deficient (A−T) cell lines. The latter results offer the intriguing possibility that *atm* message levels are elevated more dramatically in an ATM-deficient environment. Most importantly, however, these results appear to validate the notion that promoter-activating agents identified in the luciferase screen upregulate the intracellular levels of the corresponding *atm* message.

We subsequently evaluated the ability of aminoglycosides, in combination with promoter-activating agents, to augment read-through of a stop codon inserted into the reading frame (Figure 2c) of firefly luciferase. Both gentamicin, and its structurally related congener geneticin, enhance stop codon read-through by 6- to 15-fold relative to untreated controls (Table 2). By contrast, the combined use of a nonsense-suppressing agent (i.e., gentamicin or geneticin) with a promoter-activating drug (i.e., ofloxacin or thioguanine) furnishes read-through levels that can reach 30-fold

greater than that observed with untreated cells. The latter, to a rough approximation, appears to reflect the enhanced message levels produced by the promoter-activating agent in 293T cells. In addition, the promoter-activation strategy provides enhanced read-through for all three stop codons, suggesting that the strategy outlined herein should prove applicable to nonsense mutations in general. Finally, in the most efficient case (gentamicin/thioguanine/ofloxacin; UGA stop codon), the level of functional luciferase induced from a defective gene is 10% of that generated from the corresponding native gene (i.e., no nonsense mutation present). This level of expression may prove noteworthy because it constitutes a significant fraction of the 50% level present in the vast majority of carriers of genetic disorders (i.e., one defective allele), individuals who generally do not display a disease phenotype.

In summary, we have examined the notion that a promoter-activating agent, in combination with an aminoglycoside antibiotic, can stimulate enhanced production of functional protein from nonsense codon-containing genes. We screened a library of FDA-approved drugs to identify agents that activate the *atm* promoter. FDA-approved drugs enjoy the obvious advantage that they have already been accepted for human use and thus can be rapidly applied to treat additional diseases or ailments. In addition, the biochemical mechanism of action of the overwhelming majority of these agents is extremely well understood. Consequently, it has not escaped our notice that the global response of a given promoter to a broad-spectrum of FDA-approved drugs may help to delineate the signaling pathways that influence the activity of that promoter.

Supporting Information Available: Library of FDA-approved drugs and the experimental protocols for reporter plasmid cloning, cell culture and transfections, and dual luciferase assay. This material is available free of charge via the Internet at http://pubs.acs.org.

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