Negamycin-therapy in Skeletal and Cardiac Muscles of mdx Mice

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Abstract

The ability of aminoglycoside antibiotics to promote read-through of nonsense mutations has attracted interest in these drugs as potential therapeutic agents in genetic diseases. However, the toxicity of aminoglycoside antibiotics may result in severe side effects during long-term treatment. In this study, we report that negamycin, a dipeptide antibiotic, also restores dystrophin expression in skeletal and cardiac muscles of mdx mice, a most widely accepted animal model for Duchenne muscular dystrophy (DMD) with a nonsense mutation in the dystrophin gene, and in cultured mdx myotubes. Dystrophin expression was detected up to 10% of normal levels, confirmed by immunoblotting. The toxicity of negamycin found to be less toxic than gentamicin. Furthermore, negamycin bound to a partial sequence of the eukaryotic rRNA-decoding A-site. We suggest that negamycin is a new therapeutic candidate for DMD and other genetic diseases caused by nonsense mutations.

Keywords : mdx mouse, dystrophin, Negamycin, nonsense mutation, muscular dystrophy


Introduction

Aminoglycoside antibiotics are known to decrease translational fidelity and cause read-through of termination signals in prokaryotic and eukaryotic cells [7, 19, 37]. Considerable attention has therefore been paid on aminoglycoside antibiotics as potential therapeutic agents for genetic diseases. Recently, gentamicin has been used in clinical trials for the treatment of cystic fibrosis, Hurler’s disease, infant neuronal coid and lipofuscinosis, diseases that are caused by nonsense mutations [4, 9, 22, 33].

The most common myopathy in children is Duchenne muscular dystrophy (DMD), which is a lethal X-linked recessive disorder affecting 1 in 3,500 live-born males characterized by mutations in the dystrophin gene. DMD patients and the mdx mice, are attempted treated pharmacologically with corticosteroids and corticosteroid-sparing agents, however, the use of corticosteroids is accompanied by significant side effects and is of limited and short-lived benefits [14, 16, 23]. On the other hand, 65% of patients with DMD the gene exhibit gross rearrangement (predominantly deletion or duplication), while the remaining 35% have a dystrophin gene with either nonsense or other point mutations.

Pre-clinical trials to develop drugs need reliable animal models of the disease. The mdx mouse, an animal model for DMD, carries a nonsense mutation (CAA to TAA) at nucleotide position 3,185 in exon 23 of the dystrophin gene [6, 34]. This causes an absence of dystrophin as part of the cytoskeleton of muscle and, thus, the dystrophin-associated glycoprotein complex in the sarcolemma. Identifying clinically useful methods to suppress the nonsense mutation within the dystrophin gene would be of benefit to a significant number of patients with DMD.

In 1999, Barton-Davis et al. reported that gentamicin restores dystrophin function to skeletal muscles of mdx mice [3]. Besides, gentamicin is currently in clinical
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trials for DMD patients. However, aminoglycoside antibiotics are associated with numerous side effects such as auditory and renal malfunctions, and excessive use can lead to the emergence of drug-resistant bacteria.

The serious side effects of gentamicin led us to search for other possible drug candidates to suppress nonsense mutations. Uehara et al. reported that negamycin (δ-hydroxy-β-lysine linked with methylhydrazinoacetic acid), a dipeptide antibiotic discovered in 1970, also induces the read-through of stop codons in a prokaryotic translation system [17, 35]. We therefore injected negamycin subcutaneously into mdx mice over period of 2 to 4 weeks. Immunofluorescence and immunoblotting analyses revealed that dystrophin protein expression was restored in skeletal and cardiac muscles of dystrophin-deficient mdx mice. The restoration of dystrophin was also found in immortalized mdx skeletal muscle cells (mdxSV40-T) prepared by introducing a temperature-sensitive SV40-T gene into mdx skeletal muscle cells using a retroviral vector.

It has been reported that aminoglycoside antibiotics bind to the A-site of rRNA and change its conformation [13, 24, 38]. In 1999, Griffey et al. showed the interaction between aminoglycoside antibiotics and the rRNA-decoding A-site of prokaryotes and eukaryotes using electrospray ionization mass spectroscopy (ESI-MS) [15]. To understand the mechanism of the read-through activity induced by negamycin during protein synthesis, we demonstrate here the interaction between a partial sequence of rRNA and negamycin, as estimated by time-of-flight mass spectrometry (TOF-MS).

Dystrophin deficiency in skeletal muscle of mdx mouse can be partially remedied by negamycin. These results predict that negamycin is a new therapeutic candidate for DMD and other genetic diseases caused by nonsense mutations.

This paper is a revision and expansion of two earlier studies, ‘Negamycin can restore dystrophin in mdx skeletal muscle’[1] and ‘Negamycin restores dystrophin expression in skeletal and cardiac muscles of mdx mice’[2].

Materials & Methods

Antibiotic treatment in mice

The amount of negamycin administered was adjusted to be equivalent to the number of molecules of gentamicin administered as described by Barton-Davis et al. [3]. Male mdx mice (7–8 weeks old, n = 45, or 4-week-old, n = 5) were injected subcutaneously with 0.2 ml of PBS daily. Animal experiments were carried out according to the manual of the University of Tokyo.

Vital staining with Evans Blue dye

Evans Blue dye was used as a marker of myofiber damage and histology was used to assess the muscle degeneration and regeneration, as described by Matsuda et al. [27]. All animals were given an intraperitoneal injection of Evans Blue dye (2% EB in PBS, 0.1 ml/10g, BW) 12 h prior to sacrifice.

Dystrophin staining of mouse tissues

Immunofluorescence staining was carried out on 7–10 μm transverse cryosections of the TA muscle and ventricular muscle from experimental mice. The cryosections were blocked for 15 min with 10%HS-PBS at room temperature and then washed in PBS. These specimens stained with a rabbit polyclonal anti-dystrophin (C-terminal region) antibody and detected with FITC conjugated anti-rabbit IgG antibody (Amarsham).

Negamycin treatment in mdx mouse skeletal muscle cells

Cell culture of mdx mouse skeletal muscle cells (mdxSV40-T) was carried out according to the procedure of Arakawa et al. [2]. Cells (2.0 x 10^5 cells/35-mm dish) were grown in growth medium (20% FBS in DMEM) at 32°C for 7 days in gelatin-coated culture dishes. Growth medium was replaced with differentiation medium (10% HS in MEM) at 38°C for next 5 days. After myotube formation, negamycin (4.0 x 10^-7 mol/ml, 100 μg/ml, was added daily for 7 days to the cultures.

Dystrophin staining of cultured skeletal muscle cells

The cultured cells were fixed in methanol at -20°C and then washed with PBS. Fixed cells were blocked for 30 min with 10% HS-PBS at room temperature, washed extensively with PBS and then stained with the monoclonal antibody MF20 against sarcomeric muscle myosin heavy chain or rat monoclonal anti-dystrophin antibody, and detected using a Alexa Fluor secondary antibodies.

Immunoblot analysis

Partial enrichment of the dystrophin/dystrophin-associated glycoprotein complex from muscle tissues of left hindlimb (600 mg wet weight) and heart (100 mg wet weight) isolated from mdx or B10 mice using the lectin wheat germ agglutinin-Sepharose CL-6B (Sigma). Immunoprecipitation from cultured cells was carried out using protein A-Sepharose CL-4B (Sigma) with rabbit polyclonal anti-dystrophin antibody as described previously [2].
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The protein concentrations of SDS-treated samples were estimated by the microbeuret method [21] and was adjusted to 20 or 100 µg/20µl, with SDS sample buffer. The samples were subjected to electrophoresis on 4%–8% gradient SDS-PAGE. Proteins were transferred to a PVDF membrane (Millipore) and treated with a blocking buffer. The membrane was treated with monoclonal anti-dystrophin C-terminal antibody (DYS2: Novocastra) followed by incubation with horseradish peroxidase (HRP)-conjugated anti-mouse IgG1 antibody (ZYMED) and then treated with an Enzyme Chemiluminescence (ECL) solution (Amersham Pharmacia Biotech). The pattern was visualized by exposing the membrane to X-ray film (Hyperfilm ECL, Amersham Pharmacia Biotech). For further details of these protocols, see Arakawa *et al.* [2].

Interaction between negamycin and rRNA (A-site)

HPLC-purified mammalian partial rRNA sequences (A-site: 5'-GGCGUCCGUACUUCGGUAAAAGUCGCC-3'; 27 mer) were synthesized by the TAKARA Biotechnology Co. A reaction mixture containing 5.0 x 10⁻⁸ mol of negamycin and 5.0 x 10⁻⁹ mol of the rRNA sequence was prepared in distilled water to a total volume of 1ml. The formation of a negamycin:rRNA complex was examined by the Orthogonal Acceleration TOF-MS (AccuTOF, JEOL). Measurements were performed at conditions produced by a 50 µl/min syringe pump at temperatures under 100°C. To prepare a modified A-site, bold nucleotides were changed in the partial, 1406-1494 loop region of the A-site (modified A-site: 5'-GGCGACUCUUCUUCCGAGAGUCGCC-3', 26 mer).

Changes in body weights of *mdx* mice treated with negamycin

Subcutaneous injection of male *mdx* mice (7-week-old, *n* = 4 per each dose) with negamycin was performed at various doses: 1.2 x 10⁻⁵ mol/kg (1x), 1.2 x 10⁻⁴ mol/kg (10x), 6.0 x 10⁻⁴ mol/kg (50x), and 1.2 x 10⁻³ mol/kg (100x) each day for 2 weeks. Gentamicin sulfate (1.2 x 10⁻³ mol/kg) was also injected into different male *mdx* mice (7-week-old, *n* = 2). As a control, PBS was injected into *mdx* mice every day. Body weights were measured every day.

Ototoxicity

Male *mdx* mice (7 weeks old, *n* = 2) were treated with 1.2 x 10⁻⁴ mol/kg (10x) negamycin, gentamicin or PBS(control) for 7 days. Hearing ability was determined by the auditory brainstem response, according to the method of Shapiro *et al.* [32].

![Figure 1. Dystrophin expression in skeletal muscles of negamycin-treated and untreated *mdx* mice. A, Immunofluorescent staining of dystrophin (a, c, and e) and EB staining (b, d, and f) of degenerating fibers. a and b, normal B10 mouse; c and d, untreated *mdx* mouse; e and f, negamycin-treated *mdx* mouse. Bar = 100 µm. B, Immunoblot analysis of full-length dystrophin proteins from the left hindlimb muscles. a, negamycin-treated *mdx* mouse; b, sample buffer; c, negamycin-treated *mdx* mouse (ten times the amount of protein used in a was loaded); d, untreated *mdx* mouse; e, B10 control mouse.](image-url)
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normal skeletal muscles. Dystrophin-positive fibers were not observed in cardiac muscle of *mdx* mice treated with negamycin for 2 weeks (data not shown). The restoration of dystrophin was observed in the cardiac muscles of *mdx* mice treated with negamycin after prolonged (4 weeks) negamycin treatment (Fig. 2A). There were no EB-positive fibers in the dystrophin-positive area. The full-length dystrophin protein was also detected by immunoblot analysis in the cardiac muscles of *mdx* mice (Fig. 2B). The dystrophin level in the cardiac muscle was also estimated to be less than 10%. Using WGA-conjugated affinity beads, the dystrophin/dystrophin-associated protein complex was successfully enriched from muscles of negamycin-treated *mdx* mouse. This study indicated that the accumulation of dystrophin-associated glycoprotein might also be recovered by the restoration of dystrophin in muscles of negamycin-treated animals. Moreover, with reference to Fig. 1, serum creatine kinase activity was decreased 35% in *mdx* mice treated with negamycin for 2 weeks (data not shown). Accordingly, it is reasonable to conclude that the physiologic function of the muscle cell membrane is present not only in the TA muscle but also in other muscles in the whole body by negamycin treatment. However, muscle contraction profiles in negamycin-treated and untreated *mdx* mice remained to be compared in detail.

*Dystrophin accumulates in negamycin-treated *mdx* skeletal muscle cells in culture*

In order to study the restoration of dystrophin in cultured *mdx* skeletal muscle cells, we first established *mdx*SV40-T cell line. Muscle cell differentiation of *mdx*SV40-T cells was confirmed morphologically as well as immunohistochemically by the expression of the sarcomeric myosin heavy chain (Fig. 3A). After 1 week of negamycin treatment (4.0 x 10^{-7} mol/ml, 100 µg/ml), dystrophin was detected immunohistochemically in an average of 6% of a total of 340 sarcomeric myosin heavy chain-positive myotubes from 4 different experiments, and dystrophin accumulation was confirmed by immunoblot analysis (Fig. 3B). Dystrophin was not detected in untreated *mdx*SV40-T cells.
Interaction between negamycin and the eukaryotic rRNA A-site

Analysis of its binding affinity should allow conclusions to be drawn on how negamycin affect rRNA. The mass measurement accuracy and resolution of MS easily provides this information in a rapid and sensitive manner. In the case of rRNA-aminoglycoside antibiotic and RNA-argininamidine complex analysis, changes in the chemical shift of the rRNA complex were caused by binding to other small molecules [8, 15, 24, 30]. In the present study, to examine the functional relationship between negamycin and its read-through activity, we investigated the interactions between negamycin and the eukaryotic rRNA-decoding A-site using TOF-MS. The ESI-MS was obtained from a mixture of 5 nmol partial eukaryotic rRNA A-site and 50 nmol negamycin in distilled water. A partial eukaryotic rRNA A-site was detected at a mass number (MN) of 8625.51 (M+H) by TOF-MS (Fig. 4A). The negamycin/eukaryotic rRNA A-site complex (MN: 9368.31), corresponding to a 3:1 ratio of negamycin to eukaryotic rRNA was detected (Fig. 4B). Under the same condition, binding of gentamicin to the eukaryotic rRNA A-site was detected (data not shown). To examine the affinity site more closely, binding of negamycin and a modified rRNA A-site, which was modified 5-nucleotids in the loop region as to block to form match pairs in the eukaryotic rRNA A-site sequence, was studied under these same conditions. The modified rRNA was often conjugated with Na⁺ or K⁺ ions, which appeared as complexes on mass spectra (Fig. 4C). However, no interaction between negamycin and the modified A-site was detected (Fig. 4D). The eukaryotic rRNA A-site showed stoichiometric and stereospecific affinities that were significantly higher than those of the modified rRNA A-site. Our results indicate that an interaction occurs involving three molecules of negamycin and one molecule of rRNA. Negamycin/eukaryotic rRNA complex might also induce a local conformational change in the eukaryotic rRNA and cause the read-through of the nonsense mutation.

Negamycin is less toxic than gentamicin in mdx mice

It is well known that aminoglycoside antibiotics are associated with severe side effects. Although these antibiotics are used routinely for the treatment of bacterial infections, they often cause nephrotoxicity and ototoxicity. We examined negamycin toxicity by measuring changes in body weight and hearing acuity in drug-treated mice. Male mdx mice (7-week-old) were injected subcutaneously with varying doses of negamycin for 2 weeks. The body weights of mice treated with lower doses of negamycin (1x and 10x the minimal effective dose) and untreated control increased gradually (Fig. 5); however, the body weights of mice treated with higher doses of negamycin (50x and 100x) decreased. In particular, it is noteworthy that the highest

Figure 4. Interaction between negamycin and a eukaryotic partial rRNA A-site. Deconvolution of a mass spectrum of the eukaryotic rRNA A-site (MN: 8625.51, A), the negamycin/rRNA A-site complex (MN: 9368.31, B), modified RNA (8459.72: RNA+3Na⁺+2K⁺, 8397.73: RNA+Na⁺+2K⁺, C), and negamycin/modified RNA (8397.91: RNA+Na⁺+2K⁺, D)

Figure 5. Changes in body weights of mdx mice during negamycin treatment. Mdx mice were treated with negamycin and weighed every day for 2 weeks. Mdx mice treated with PBS (open circles), or with negamycin at 1.2 x 10⁻⁵ mol/kg (closed circles), 1.2 x 10⁻⁴ mol/kg (crosses), 6.0 x 10⁻⁴ mol/kg (closed triangles), or 1.2 x 10⁻³ mol/kg (closed squares).
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A high dose of gentamicin (1.2 x 10^-3 mol/kg/day BW, 100x) killed all mice tested within 4 h (data not shown). Compared with gentamicin, negamycin treatment exhibited lower toxicity. While the antibacterial activity of negamycin resembles that of gentamicin, it is possible that negamycin may have different side effects.

Furthermore, to investigate the ototoxicity, we conducted otologic studies on untreated and antibiotic-treated mice. Auditory brainstem responses to sounds ranging from 95 to 25 decibels (dB) were recorded. In both untreated and negamycin-treated *mdx* mice, the normal P1, P2, P3, and P4 peaks of an auditory brainstem response were observed. On the other hand, gentamicin-treated mice lacked all waves of auditory brainstem response under 80 dB (Fig. 6). The number of revertant fibers increases as *mdx* mice age [25]. In the present study, the percentage of dystrophin-positive fibers in the skeletal muscle of *mdx* mice treated with negamycin was an average of 6%, which is significantly higher than the percentage of revertant fibers. In age-matched *mdx* skeletal and cardiac muscles, no truncated or shortened forms of dystrophin were detected in gentamicin-treated *mdx* mice.

Many human genetic diseases have a relatively high proportion of premature stop codon. Nonsense mutations are found in approximately 5%-15% of patients with DMD [23]. To overcome nonsense mutations, one can expect that the naturally occurring suppressor tRNA may play important roles. Especially in the case of selenoprotein synthesis, the selenocysteyl tRNA carries the anticodon for UGA, a stop codon [5, 39]. Unfortunately, however, the selenocysteyl tRNA function in for selenoprotein synthesis only in a presence of selenocysteine insertion sequence (SECIS) in the 3' untranslated region (UTR) of the mRNA, and SECIS is found only in the mRNAs encoding for patients of DMD. Barton-Davis *et al.* reported that gentamicin could restore functional dystrophin in *mdx* mice [3]. Although, Dunant *et al.* reported that gentamicin treatment failed to lead to a significant increase in full-length dystrophin protein expression in *mdx* mice [12]. In human trials, Politano *et al.* reported that one of four gentamicin-treated DMD patients exhibited increased dystrophin re-expression by immunohistochemistry and Western blot analysis [29]. In contrast, Wagner *et al.* and Serrano *et al.* reported that the dystrophin protein was not detectable by immunohistochemistry or immunoblot analysis in clinical trials of DMD/BMD patients treated with gentamicin [31, 36]. These discrepancies of gentamicin clinical trials can probably be explained by the likely variations in the purity of commercially available gentamicin sulfate and in the rations of its three distinct isomers.

Therapeutic significance of negamycin treatment

The mild Becker-type muscular dystrophy (BMD) is also caused by mutations in various portions of the dystrophin gene [10]. Hoffman *et al.* reported that in several cases of BMD, the phenotype was improved by dystrophin expressed at a level greater than or equal to 20% of normal [18]. Although we have no direct evidence as to whether 10% of the normal dystrophin level observed in this study would be sufficient to improve muscle performance in *vivo*, it is likely to be far better than a complete lack of dystrophin.

Danko *et al.* reported that dystrophin-positive fibers, called revertant fibers, were observed in 1% or fewer of the skeletal muscle fibers of 8-week-old *mdx* mice [11]. These dystrophin-positive *mdx* muscle fibers presumably arose from alternative splicing and/or a second mutation that skipped the primary mutation in exon 23. The number of revertant fibers increases as *mdx* mice age [25]. In the present study, the percentage of dystrophin-positive fibers in the skeletal muscle of *mdx* mice treated with negamycin was an average of 6%, which is significantly higher than the percentage of revertant fibers. In age-matched *mdx* skeletal and cardiac muscles, no truncated or shortened forms of dystrophin were detected in negamycin-treated *mdx* mice.

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selenoproteins. Except for selenocysteyl-tRNA, the level of other suppressor tRNAs is not high enough to overcome common nonsense mutations. Therefore, it is reasonable to search new drugs which induce read-through of premature stop codons.

The read-through of termination codons during normal protein synthesis may also be considered a source of side effects of negamycin. However, according to the cDNA databases that include the dystrophin gene, two or more stop codons usually exist near the termination codon in the UTR (data not shown). Extensive read-through of termination signals during protein synthesis may not occur. Both the type of stop codon and the nucleotide sequence surrounding the nonsense mutation have been reported to be critical for the read-through activity of gentamicin and other aminoglycoside antibiotics [20, 26, 28]. Therefore, the type of nonsense mutation and surrounding nucleotide sequence affected by the read-through activity induced by negamycin should be investigated.

We emphasize, however, that negamycin has not yet been approved for use in humans.

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