細胞はわずかにジストロフィン陽性となった。このことは、コンパウンド２がわずかながらジストロフィン発現を促進する作用を有することを可能性を示した。

考察

本研究結果は、コンパウンド2のリードスルー誘導効果を示唆するものであった。今後さらに検討を重ね、コンパウンド２の有効性の議論に結論を得る予定である。

G. 研究発表

1. 論文発表


1. Habara Y, Yagi M, Takeshima Y, Matsuo M: A strong exonic splicing enhancer in dystrophin exon 19 achieve proper splicing without an upstream polypyrimidine tract. J Biochem 2007, in press.
2. Nishiyama A, Takeshima Y, Saiki K, Narukage A, Oyazato Y, Yagi M, Matsuo M: Two novel missense mutations in the myostatin gene identified in Japanese patients with Duchenne muscular dystrophy. BMC Med Genet 2007, 8:1-9.
3. Tran VK, Takeshima Y, Zhang Z, Habara Y, Haginoya K, Nishiyama A, Yagi M, Matsuo M: A nonsense mutation-created intraexonic splice site is active in the lymphocytes, but not in the skeletal muscle of a DMD patient. Hum Genet 2007, 120(5):737-742.
4. Beroud C, Giraud TS, Matsuo M, Hamroun D, Humbertclaude V, Monnier N, Moizard MP, Voelckel MA, Calemard LM, Boisseau P et al: Multi-exon skipping leading to an artificial DMD protein lacking amino acids from exons 45 through 55 could rescue up to 63% of patients with Duchenne Muscular Dystrophy. Hum Mutat 2007, 28(2):196-202.
5. Sahashi K, Masuda A, Matsuura T, Shinmi J, Zhang Z, Takeshima Y, Matsuo M, Sobue G, Ohno K: In vitro and in silico analysis reveals an efficient algorithm to predict the splicing consequences of mutations at the 5' splice sites. Nucleic Acids Res 2007, 35(18):5995-6003.
6. Zhang Z, Habara Y, Nishiyama A, Oyazato Y, Yagi M, Takeshima Y, Matsuo M: Identification of seven novel cryptic exons embedded in the dystrophin gene and

characterization of 14 cryptic dystrophin exons. J Hum Genet 2007, 52(7):607-617.

## 研究成果の刊行に関する一覧表

雑誌

Allamand V, Bidou L, Arakawa M, Shiozuka M, Hatin I, Paturneau-Jouas M, Gartioux C, Butler-Browne GS, Mouly V, Rousset JP, Matsuda R, and Guicheney P Antibiotic-mediated readthrough of premature stop codons leads to the stabilization of laminin 2 chain mRNA in congenital muscular dystrophy myotubes. J. Gene Med. 10: 217-224 (2008)

Hayashi Y, Regnier T, Nishiguchi S, Sydnes MO, Hashimoto D, Hasegawa J, Katoh T, Kajimoto T, Shiozuka M, Matsuda R, Noda M, and Kiso Y. Efficient total synthesis of (+)-Negamycin, a potential chemotherapeutic agent for genetic diseases. Chem. Comm. in press

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## 研究成果による特許権等の知的財産権の出願状況

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RESEARCH ARTICLE

# Drug-induced readthrough of premature stop codons leads to the stabilization of laminin α2 chain mRNA in CMD myotubes

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## Abstract

**Background** The most common form of congenital muscular dystrophy is caused by a deficiency in the α2 chain of laminin-211, a protein of the extracellular matrix. A wide variety of mutations, including 20 to 30% of nonsense mutations, have been identified in the corresponding gene, *LAMA2*. A promising approach for the treatment of genetic disorders due to premature termination codons (PTCs) is the use of drugs to force stop codon readthrough.

**Methods** Here, we analyzed the effects of two compounds on a PTC in the *LAMA2* gene that targets the mRNA to nonsense-mediated RNA decay, *in vitro* using a dual reporter assay, as well as *ex vivo* in patient-derived myotubes.

**Results** We first showed that both gentamicin and negamycin promote significant readthrough of this PTC. We then demonstrated that the mutant mRNAs were strongly stabilized in patient-derived myotubes after administration of negamycin, but not gentamicin. Nevertheless, neither treatment allowed re-expression of the laminin α2-chain protein, pointing to problems that may have arisen at the translational or post-translational levels.

**Conclusions** Taken together, our results emphasize that achievement of a clinical benefit upon treatment with novel readthrough-inducing agents would require several favourable conditions including PTC nucleotide context, intrinsic and induced stability of mRNA and correct synthesis of a full-length active protein. Copyright © 2007 John Wiley & Sons, Ltd.

**Keywords** congenital muscular dystrophy; laminin α2 chain; premature termination codon; antibiotic-mediated readthrough; nonsense-mediated mRNA decay

## Introduction

Congenital muscular dystrophy (CMD) is a clinically and genetically heterogeneous group of neuromuscular disorders with autosomal recessive inheritance. The 'classic' (or occidental) form of CMD is characterized by clinical manifestations mainly affecting skeletal muscle [1–4]. A specific deficiency of the α2 chain of laminin-211 is responsible for about 30 to 40% of these cases (MDC1A, MIM#607855). Numerous mutations have now been identified in the *LAMA2* gene encoding the α2 chain of laminin, leading to either complete or partial protein deficiency [5–9]. Notably, 20 to 30% of the mutations identified in the *LAMA2* gene are nonsense mutations leading to a premature termination codon (PTC).

Thus, up to 15% of 'classical' CMD patients would potentially benefit from a pharmacological strategy designed to promote translation of the endogenous *LAMA2* mRNA interrupted by a PTC. From early work in *Escherichia coli*, it has been demonstrated that aminoglycoside antibiotics such as gentamicin can specifically act on ribosomal RNA to suppress codon-anticodon recognition by aminoacyl tRNAs, thereby interfering with the translation termination process and suppressing stop codons [10–14]. Aminoglycoside-mediated translational readthrough of PTCs involved in several human genetic disorders, including Duchenne muscular dystrophy, has now been reported with variable degrees of success [15–26]. It has also become increasingly evident that only a small subset of stop codon mutations would benefit from gentamicin treatment, depending on their nucleotide context [21,27–30]. In addition, strong side effects of gentamicin, the most commonly used aminoglycoside antibiotic, have been well documented [31,32] and hamper its utilization as a potential therapeutic agent especially if long-term treatment is required. Furthermore, gentamicin-induced readthrough is dependent on its chemical composition and its reproducibility is debatable [33].

To address these problems, we tested two other compounds with antibiotic activities, negamycin and amikacin [34], known to provide effective suppression of nonsense mutations in cellular and animal models and to present a lower toxicity than gentamicin [20,34–38]. Unlike gentamicin and amikacin, that belong to the aminoglycoside family, negamycin is a dipeptide antibiotic, which also interacts with the ribosomal A site to mediate readthrough [38].

Due to the absence of an adequate animal model for MDC1A, we chose an *ex vivo* approach based on muscle explants obtained from a patient presenting a complete merosin deficiency due to a homozygous nonsense mutation (c.C4687A) in exon 31 of the *LAMA2* gene causing a PTC (C1546X) [39]. We treated myotubes derived from this patient with either gentamicin or negamycin. Since readthrough efficiency depends on the nature of the PTC and its surrounding sequences [21,28,40–43], in parallel we measured termination readthrough for this specific *LAMA2* mutation, in its nucleotidic context, in a sensitive and reproducible dual reporter assay [40,43]. In addition, the presence of PTCs often results in the rapid degradation of the mutant mRNA by the nonsense-mediated mRNA decay (NMD) pathway (for a review, see [44]). It has been suggested that gentamicin, by allowing some translational readthrough, may result in reduced levels of NMD [14,45].

In this study, using a dual reporter assay, we first demonstrated, *ex vivo* as well as *in vivo*, that negamycin is as effective as gentamicin in inducing PTC readthrough. Interestingly, negamycin-induced readthrough of the *LAMA2* PTC studied here was among the highest levels reached using this assay. Moreover, by quantitative reverse-transcription polymerase chain reaction (RT-PCR) we demonstrated that negamycin treatment, in contrast to gentamicin, strongly stabilized the patient's laminin $\alpha$2-chain mRNA levels. Indeed, mutant mRNA levels were significantly decreased in the untreated patient's cells, likely due to the NMD pathway. However, re-expression of the laminin $\alpha$2-chain protein could not be detected in our experimental conditions.

In conclusion, our data demonstrate that for some mutations, stabilization of the mutant mRNA can be obtained through a sufficient level of readthrough, which, in some cases, could allow a synergistic effect on PTC suppression. Nonetheless, this may not be sufficient to allow re-expression of a functional full-length protein, suggesting that numerous steps need to be fulfilled to obtain a clinical benefit through PTC-induced suppression.

# Materials and methods

## Cell culture and transfection

NIH3T3 cells were cultivated in Dulbecco's modified Eagle's medium (DMEM) (Invitrogen-Gibco, Cergy, France) supplemented with 7% fetal calf serum (Gibco BRL), and incubated at 37 °C in humidity saturated 6.5% $CO_2$. Cells were electroporated with 18 µg of each pAC(lacZ-luc) plasmid construct, as described [21], and plated immediately in culture medium without antibiotic. After 20 h and 28 h the medium was replaced with fresh medium complemented or not with gentamicin (Invitrogen-Gibco, Cergy, France), negamycin (both used at 600 µg/ml) or amikacin (GIBCO BRL; at 2mg/ml). Two days after transfection, cells were harvested, spun for 5 min at 4 °C and lysed by repeated pipetting in 150 µl of cold luciferase assay buffer. For each construct, at least five independent transfection experiments were performed.

## Reporter plasmid cloning and stop mutation targets

Complementary oligonucleotides corresponding to the sequence of each stop mutation embedded in its natural nucleotide context were annealed and cloned in a dual gene reporter system between the lacZ and luc coding sequences as previously described [21]. The nucleotidic context around the C1546X stop mutation sequence is: 5′ GTC ACA GGA TTC TGA ACG TGC CGA CCT 3′. For the other stop targets oligonucleotides sequences were previously published [21].

## Enzyme assays

Luciferase and $\beta$-galactosidase activities were measured from the same crude extract as described [43]. Stop codon readthrough was calculated by dividing the ratio of luciferase to $\beta$-galactosidase activity obtained with a given test construct by the ratio obtained from the control



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construct where the stop codon was replaced by a sense codon. Each value of readthrough efficiency corresponds to the mean of five to six independent experiments. Standard deviation errors did not exceed 20%.

## Cell culture and immunohistochemistry

Primary myoblasts obtained from a control foetus (12 weeks of gestation) and a merosin-deficient CMD foetus (15 weeks of gestation), presenting a homozygous nonsense mutation in exon 31 of the *LAMA2* gene, were grown in F10 medium + 20% SVF + penicillin/streptomycin (10 U/ml and 10 μg/ml, respectively; Invitrogen-Gibco). Muscle cells were obtained in accordance with the French legislation on ethical rules. Myoblasts were allowed to fuse for 7 days on 0.5% gelatin-coated coverslips in antibiotic-free or negamycin-containing (300 μg/ml; Institute of Microbiology, Tokyo, Japan) differentiation medium (DMEM 4.5 g glucose + 2% horse serum + $10^{-6}$ M insulin + $2.5 \times 10^{-6}$ M dexamethasone). Immunohistochemical analyses were performed on methanol-fixed cells (10 min at 4 °C) as follows: non-specific sites were blocked for 30 min in 5% bovine serum albumin (BSA)/1X phosphate-buffered saline (PBS), and cells were then incubated overnight at 4 °C with primary antibodies against myosin (MF20, a gift from Dr. Denis Furling, Inserm-UMR S 787, Institut de Myologie, Paris, France) and laminin α2 chain (4H8-2, Alexis Biochemicals, Lausen, Switzerland) diluted into 1% BSA/1X PBS. Following three washes in 1% BSA/1X PBS, cells were incubated for 90 min in appropriate FITC or Cy3-conjugated secondary antibodies (Jackson ImmunoResearch, West Grove, PA, USA). After three additional washes, cells were mounted with Mowiol containing DAPI (0.05 μg/μl final) to stain all of the nuclei and observed under a Zeiss microscope. Images were captured under identical conditions using the Metaview software (Ropper Scientific GmbH, Germany).

## RNA extraction and quantitative RT-PCR

Total RNAs were extracted using conventional TRIzol™ reagent (Life Technologies, Inc.) extraction protocol as recommended by the manufacturer. RNA quality control and quantification was performed using the Bioanalyzer apparatus (Agilent). First strand cDNA was synthesized from 1 μg of total RNA using oligo$(dT)_{18}$ and Superscript reverse transcriptase II (Invitrogen-Gibco, Cergy, France) as recommended by the manufacturer. Quantitative PCR was then carried out on equal amounts of cDNAs, using the LightCycler real-time PCR machine (Roche Diagnostics, Germany), and *LAMA2* mRNA levels were quantified relative to the mRNA of the troponin T1 (*TPNT1*) gene. In each experiment, results were expressed relative to control myotubes normalized to 100. The oligonucleotide pairs used for the amplifications are as follows: *TPNT1* forward: 5′ TGGAGCTGCAGACACTCATC 3′ and reverse: 5′ GCTTCTGTTCTGCCTTGACC 3′; *LAMA2* forward: 5′ TGTGCTGCAGAATCAGAACC 3′ and reverse: 5′ ATTGATTTTGGTGGGGATCA 3′. Mean values of several quantitative RT-PCRs (n = 4) are presented + standard deviation to mean. Statistical significance was determined by a t-test (*$p < 0.001$).

## Protein extraction and immunodetection

Total proteins were extracted in SDS buffer (80 mM Tris-HCl, pH6.8, 10% SDS, 0.12 M sucrose, 10 mM EDTA, 1 mM PMSF, 1 mM benzamidine). Concentrations were determined using a BCA protein assay kit (Pierce, Rockford, IL, USA) with BSA as a standard. Proteins were subsequently resolved under reducing conditions by sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS-PAGE) on a 10% gel and transferred to PVDF membranes. Detection of the laminin α2 chain was performed by using an affinity purified polyclonal antibody (rabbit 180, a gift from Dr. Kevin P. Campbell, Molecular Physiology and Biophysics, Howard Hughes Medical Institute, Iowa City, USA). HRP-conjugated secondary antibody anti-rabbit IgG (DakoCytomation, Glostrup, Denmark) was used at a dilution of 1 : 2000. Immunoblots were developed using enhanced chemiluminescence (SuperSignal West Pico chemiluminescent substrate; Pierce, Rockford, IL). In order to control the amounts of proteins which had been loaded, the gel was stained with Coomassie blue following transfer.

## *In vivo* studies

All animal studies conformed to the guidelines on animal use procedures approved by Inserm. Mice were anesthetized by intraperitoneal injection of 10 mg/ml ketamine (Clorketam 1000; Vétoquinols SA, Lure, France) and 5 mg/ml xylazine (Rompun 2%; Bayer Pharma, Puteaux, France). Plasmid DNA (35 μg) was injected percutaneously into the tibialis anterior muscle. Two minutes after the injection, transcutaneous electric pulses were applied through external stainless steel plates (200 V/cm, 8 pulses of 20 ms each, 2 Hz) on a ECM 830 BTX electroporator (San Diego, USA).

Animals were subsequently treated with gentamicin (34 mg/kg; Invitrogen-Gibco) or PBS once daily for 3 days, by percutaneous injections. On the last day, mice were euthanized and the injected muscles were harvested.

# Results

## Negamycin promotes readthrough in a dual reporter assay in NIH3T3 cells

The C1546X mutation in the *LAMA2* gene leads to a UGA premature termination codon (PTC) which has been



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reported to be the most sensitive codon to aminoglycoside-induced readthrough [28,41,42]. Using a previously published dual reporter assay [40], we demonstrated that this PTC is responsive to amikacin and gentamicin (0.4% and 0.6%, respectively, which correspond to 2.5- and 3.8-fold increases in readthrough; Figure 1). Interestingly, readthrough was increased up to 3% (19-fold) following negamycin treatment which is among the highest levels we have found using this assay [21]. To examine more precisely the effect of negamycin on other stop targets, five other nonsense mutations leading to PTCs in either the human or murine dystrophin genes (UGA/319d and UAA/mdx, respectively) and the *LAMA2* gene (UGA/1326c, UAG/1437c, UAA/1240c), as well as the natural termination codon of the *LAMA2* gene (UGA/STOP LAM), were tested under the same conditions. These targets have previously been analyzed for gentamicin sensitivity [21]. For six of the seven stop targets presented in Figure 1, negamycin is equally or more effective than gentamicin in promoting readthrough. Nevertheless, in five of these targets the efficiency remained modest (0.4%; 2.5-fold). Only one stop mutation (UGA/319d) was more sensitive to gentamicin (2.6%, 44-fold) than to negamycin (1.2%, 20-fold). Thus, among six mutations only two can be highly bypassed by antibiotic treatment: C1546X by negamycin and UGA/319d by gentamicin; the latter also being moderately bypassed by negamycin.

## Negamycin allows readthrough *in vivo*

Since the availability and metabolism of negamycin might be modulated *in vivo*, the results in cultured cells might not reflect exactly the situation in muscles.

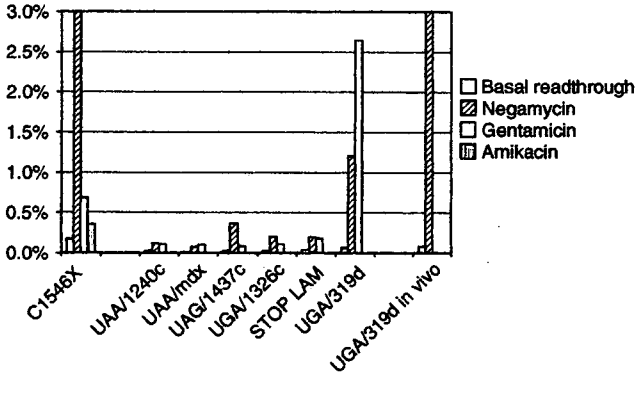


**Figure 1. Induction of readthrough in NIH3T3 cells. Sequences corresponding to the C1546X stop mutation as well as other stop targets corresponding to DMC (c) or DMD (d) stop mutations or to the natural termination codon of the *LAMA2* gene (UGA/STOP LAM) were cloned in a dual gene reporter system between lacZ and luc coding sequences. NIH3T3 cells were treated with gentamicin, negamycin or amikacin. Termination readthrough is expressed as the luciferase/β-galactosidase ratio of the test construct normalized to an in-frame control where stop codons have been replaced by a sense codon. Each value of readthrough efficiency corresponds to the mean of four to six independent experiments. Standard deviation errors did not exceed 20%**

As previously shown for gentamicin [21], we assessed the effect of negamycin *in vivo*, in C57BL/6 mice. The vector carrying the UGA/319d mutation was injected into skeletal muscle with subsequent electrotransfer and thereafter mice were treated intramuscularly once daily with negamycin (34 mg/kg) or PBS for the following 3 days. Results showed that both basal and negamycin-induced readthrough efficiency were similar in NIH3T3 cells and *in vivo*, increasing from 0.08% in the absence to 3% (38-fold) in the presence of the drug (Figure 1).

## Stabilization of *LAMA2* mRNA in negamycin-treated myotubes

The mutation in exon 31 of the *LAMA2* gene leads to the replacement of a cysteine residue by a premature UGA stop (C1546X) that is placed more than 50 nucleotides upstream of the 3′ most exon-exon junction and is therefore in a favourable condition for the degradation of the mutant mRNA by the NMD pathway [44]. Indeed, quantitative RT-PCR analysis demonstrated a significant decrease in the level of *LAMA2* transcripts which were reduced to 6.25% of control values (Figure 2). We hypothesized that, by promoting translational readthrough of the mutant mRNA, gentamicin and negamycin would prevent its rapid degradation by the NMD pathway. Treatment of the patient's myotubes by two different batches of gentamicin (GIBCO BRL or American Pharmaceutical Partners, Inc.) did not trigger a significant increase in the level of transcripts. However, negamycin treatment strongly sustained the stabilization of the *LAMA2* transcripts up to 77% of control levels (Figure 2). These results are in agreement with those obtained in *S. cerevisiae* where previous studies indicated an inverse relationship between readthrough level and NMD-dependent decrease in mRNA abundance [20,46]. Moreover, the mRNA quantification data can be correlated to those obtained in our cell culture expression system in which negamycin was more efficient than gentamicin in promoting translational readthrough of the *LAMA2* nonsense mutation (Figure 1).

## Neither gentamicin nor negamycin enabled re-expression of laminin α2 chain in MDC1A myotubes

In parallel to the mRNA studies, we investigated whether gentamicin or negamycin treatment induced re-expression of the laminin α2 chain in the patients' myotubes at the protein level. Control and MDC1A myoblasts were allowed to differentiate *ex vivo* for 7 days and subsequently myotubes were treated with gentamicin (GIBCO BRL or American Pharmaceutical Partners, Inc.) or negamycin for 7 or 15 days. Expression of myosin and the laminin α2 chain was then assessed on fixed myotubes by immunohistochemistry (Figure 3A). First, we observed that MDC1A myoblasts were indeed able to efficiently differentiate into myotubes as indicated by



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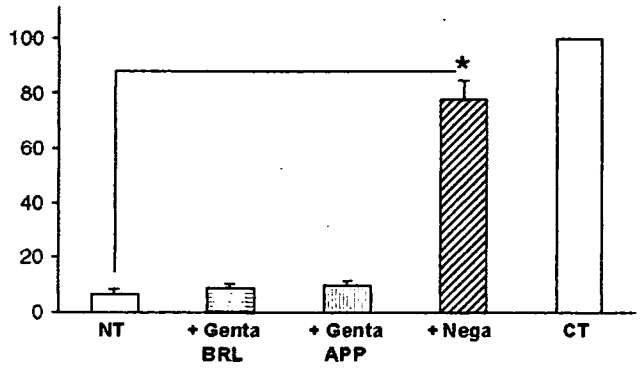


**Figure 2.** *LAMA2* **mRNA levels in cultured MDC1A myotubes treated with gentamicin and negamycin. Quantitative PCR were carried out on equal amounts of cDNAs, using the LightCycler real-time PCR machine (Roche Diagnostics, Germany).** *LAMA2* **mRNA levels were quantified relative to the mRNA of the troponin T1 (*TPNT1*) gene. In each experiment, results were expressed relative to control myotubes normalized to 100. Mean values are presented + standard deviation to mean. Statistical significance was determined by a t-test (*$p < 0.001$)**

the strong expression of myosin (panel d) which was comparable to that observed in the control cells (panel a). However, immunohistochemical analyses did not allow detection of the laminin $\alpha 2$ chain following either a 7- or 15-day-long treatment with doses as high as 300 μg/ml of gentamicin or negamycin (panels h, i and data not shown). Biochemical analyses confirmed these results for all three compounds tested (Figure 3B), and even though the detection threshold of our experiment was as low as 1% (Figure 3B and data not shown).

## Discussion

Here we present *in vitro* and *in vivo* data that address important questions regarding the potential use of drugs able to induce readthrough of stop codon mutations, to treat genetic disorders due to the presence of a PTC. Indeed, re-expression of a functional protein, which could provide a clinical benefit, is dependent on the correct realization of several steps. We first analyzed the efficacy of drug-mediated readthrough of premature termination codons by different compounds with antibiotic activities. We showed that negamycin, a dipeptide antibiotic, showed a higher suppression activity than gentamicin on several PTCs. In particular, the C1546X *LAMA2* mutation that we analyzed in more detail is especially responsive to negamycin as compared to gentamicin and amikacin. This illustrates the importance of testing the efficiency of treatments in sensitive, rapid, and reproducible systems such as the one presented here. Since there are no hot spots of mutation within the *LAMA2* gene, all mutations are 'private' and the consequences of the treatment may vary considerably between patients. The variable response to readthrough drugs has been recently illustrated in cystic fibrosis patients treated with gentamicin [30]. In that report, it was demonstrated, using the same dual reporter system as in the present work, that suppression of stop mutations in the *CFTR* gene with parenteral gentamicin can be predicted *in vitro*. The study established that in a small subgroup of patients with the Y122X mutation, gentamicin treatment was associated with clinical benefit and significant modification of the CFTR-mediated $Cl^-$ transport in nasal and sweat gland epithelium [30].

Although readthrough-inducing compounds primarily affect translation, it is becoming clear that mutant mRNA stability is a significant factor in the process of termination readthrough. Importantly, we demonstrated that negamycin has a dual action since it enables readthrough levels high enough to strongly counteract mRNA degradation by the NMD pathway. Indeed, it has been shown that termination readthrough above the threshold value of 0.5% antagonizes NMD, leading to mRNA stabilization [46]. This likely explains the striking stabilization of *LAMA2* mRNA observed following negamycin treatment, since we detected 3% of readthrough. On the other hand, the level of gentamicin-induced readthrough (0.6%) being just above the threshold value was probably not sufficient for mRNA stabilization. This would have important consequences for predicting the efficiency of pharmacological readthrough on patients' mutations before envisioning clinical trials, and again stresses the necessity to test *in vitro* the response of individual mutations to pharmacological treatment. Accordingly, gentamicin response was shown to depend on the efficiency of NMD in cystic fibrosis which varied greatly between patients and cell types [47]. In addition, in that study, the authors did not observe stabilization of the nonsense *CFTR* transcripts after gentamicin treatment in patients and cell lines [47], whereas it had been shown that G418, a more potent readthrough drug than gentamicin, could restore CFTR mRNA levels [16].

Recently, PTC124, a new molecule, has been shown to induce ribosomal readthrough of PTCs [48]. Interestingly, the levels of a nonsense-containing mRNA were unaffected in PTC124-treated cells. More generally, the synthesis and stability of few, if any, cellular mRNAs are altered in response to levels of PTC124. Although an off-target effect might be detrimental, the lack of stabilization of PTC-containing mRNAs by PTC124 may limit its use to mutations which do not lead to mRNA degradation by the NMD pathway.

In the present study, since gentamicin treatment did not allow stabilization of the mutant *LAMA2* mRNA, it seems coherent that no re-expression of the protein occurred. On the other hand, considering the extent of mRNA stabilization that we detected following treatment with negamycin, the lack of laminin $\alpha 2$-chain re-expression may appear surprising. Several hypotheses, which are not mutually exclusive, may explain our results: (1) a longer treatment may be needed in order to be able to detect this large extracellular matrix protein; however, it was not possible to treat the cells for longer periods of time since the myotubes started detaching from the gelatine-coated dishes after 16 to 17 days of differentiation. (2) It is thought that PTCs would be misread by near-cognate aminoacyl-tRNAs bearing anti-codons with one

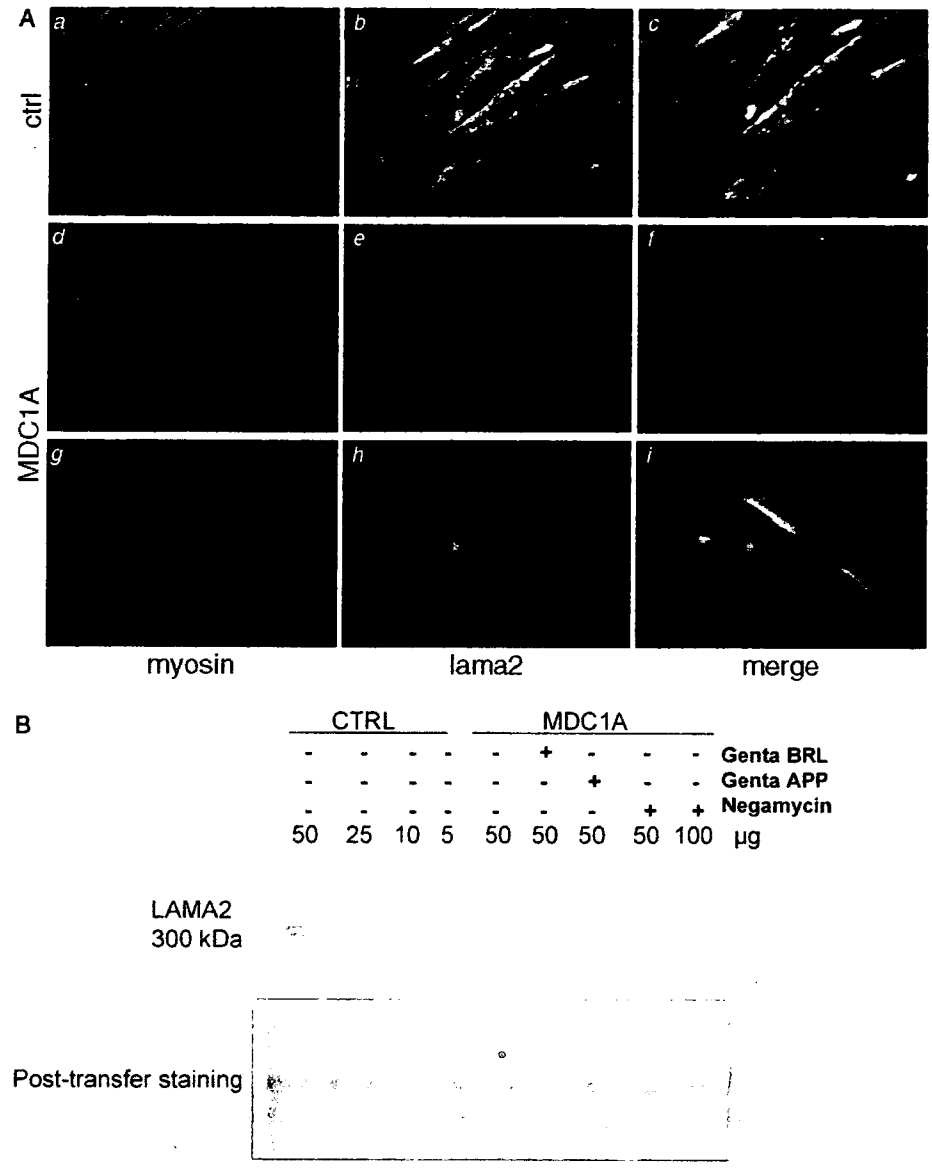


**Figure 3. (A) Myosin and laminin α2-chain expression in control and MDC1A cultured myotubes after a 7-day negamycin treatment. Immunohistochemical analyses of primary myotubes from a control foetus (a–c) and a merosin-deficient CMD foetus (d–i) presenting a premature termination codon (C1546X) in the laminin α2 chain with antibodies against myosin (a, d, g) and the laminin α2 chain (b, e, h). Magnification 60× (a–c, g–i). Magnification 25× (d–f). c, f and i are merged images. (B)Western blot analysis in negamycin-treated and untreated control and MDC1A samples. Detection of laminin α2 chain on total proteins using an affinity purified polyclonal antibody (rabbit 180, a gift from Dr. Kevin P. Campbell). In order to control the amounts of proteins which had been loaded, the gel was stained with Coomassie blue following transfer**

base difference. In *E. coli*, Nilsson and Ryden-Aulin [49] showed that stop codons UAG and UAA are replaced by glutamine whereas UGA would be substituted by tryptophan. Following this hypothesis, readthrough of the *LAMA2* UGA PTC would thus lead to the replacement of the normal cysteine by a tryptophan or an arginine residue. This residue is localized close to the junction site where the α2 chain of laminin-211 assembles with the β1 and γ1 chains to form the heterotrimeric molecule which will then be secreted. It seems likely that the insertion of a tryptophan residue would perturb this assembly and destabilize the protein which might thereafter be degraded. It should be noted that replacement of the cysteine residue by a tryptophan was predicted to be deleterious by using the Web server PolyPhen [50] for polymorphism phenotyping [51]. It should be emphasized that treatments aiming at re-expressing a full-length protein by suppression of a PTC will be faced with this general problem. Although some structural proteins, like dystrophin, might tolerate large modifications of their coding sequences, enzymatic proteins, which are involved in most genetic disorders, will be very sensitive to the specific residue inserted in place of the PTC. This would necessitate a precise analysis of the site of the mutation in the context of the three-dimensional and functional structure of the protein, and of the potential near-cognate tRNA involved in decoding the stop codon upon drug treatment.

In conclusion, our study demonstrates that negamycin, a compound less toxic and more efficient for premature termination readthrough than gentamicin, appears an attractive alternative for treatment of patients carrying a PTC mutation. Its striking effect on mRNA stabilization suggests that, even for patients with a low level of mutant mRNA, treatment might be effective through readthrough-induced mRNA stabilization. However, although sufficient levels of mRNA are likely to be a preliminary requirement, correct synthesis of the corresponding full-length protein also depends on the effect of the insertion of a novel amino acid in place of the premature stop codon, which should be carefully considered prior to treatment.

Overall, our results emphasize that numerous steps need to be fulfilled to achieve a clinical benefit by inducing suppression of nonsense mutations.

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## References

1. Fardeau M. Congenital myopathies. In *Skeletal Muscle Pathology*, Mastaglia FL, Detchant. LWo (eds). Churchill Livingstone: Edinburgh, 1992; 237–281.
2. Banker BQ. The congenital muscular dystrophies. In *Myology*, Engel AG, Franzini-Amstrong C (eds). McGraw-Hill: New York, 1994; 1275–1289.
3. Tomé FMS, Guicheney P, Fardeau M. Congenital muscular dystrophies. In *Neuromuscular Disorders, Clinical and Molecular Genetics*, Emery AEH (ed). John Wiley: Chichester, 1998; 21–57.
4. Tomé FM. The Peter Emil Becker Award lecture 1998. The saga of congenital muscular dystrophy. *Neuroped* 1999; **30**: 55–65.
5. Helbling-Leclerc A, Zhang X, Topaloglu H, *et al.* Mutations in the laminin α2-chain gene (LAMA2) cause merosin-deficient congenital muscular dystrophy. *Nat Genet* 1995; **11**: 216–218.
6. Nissinen M, Helbling-Leclerc A, Zhang X, *et al.* Substitution of a conserved cysteine-996 in a cysteine-rich motif of the laminin α2-chain in congenital muscular dystrophy with partial deficiency of the protein. *Am J Hum Genet* 1996; **58**: 1177–1184.
7. Allamand V, Guicheney P. Merosin-deficient congenital muscular dystrophy, autosomal recessive (MDC1A, MIM#156225, LAMA2 gene coding for α2 chain of laminin). *Eur J Hum Genet* 2002; **10**: 91–94.
8. Allamand V, Sunada Y, Salih MAM, *et al.* Mild congenital muscular dystrophy in two patients with an internally deleted laminin α2-chain. *Hum Mol Genet* 1997; **6**: 747–752.
9. Guicheney P, Vignier N, Helbling-Leclerc A, *et al.* Genetics of laminin alpha 2 chain (or merosin) deficient congenital muscular dystrophy: from identification of mutations to prenatal diagnosis. *Neuromuscul Disord* 1997; **7**: 180–186.
10. Palmer E, Wilhelm JM, Sherman F. Phenotypic suppression of nonsense mutants in yeast by aminoglycoside antibiotics. *Nature* 1979; **277**: 148–150.
11. Singh A, Ursic D, Davies J. Phenotypic suppression and misreading in Saccharomyces cerevisiae. *Nature* 1979; **277**: 146–148.
12. Burke JF, Mogg AE. Suppression of a nonsense mutation in mammalian cells in vivo by the aminoglycoside antibiotics G-418 and paromomycin. *Nucleic Acids Res* 1985; **13**: 6265–6272.
13. Martin R, Mogg AE, Heywood LA, Nitschke L, Burke JF. Aminoglycoside suppression at UAG, UAA and UGA codons in Escherichia coli and human tissue culture cells. *Mol Gen Genet* 1989; **217**: 411–418.
14. Kaufman RJ. Correction of genetic disease by making sense from nonsense. *J Clin Invest* 1999; **104**: 367–368.
15. Howard MT, Anderson CB, Fass U, *et al.* Readthrough of dystrophin stop codon mutations induced by aminoglycosides. *Ann Neurol* 2004; **55**: 422–426.
16. Bedwell DM, Kaenjak A, Benos DJ, *et al.* Suppression of a CFTR premature stop mutation in a bronchial epithelial cell line. *Nat Med* 1997; **3**: 1280–1284.
17. Barton-Davis ER, Cordier L, Shoturma DI, Leland SE, Sweeney HL. Aminoglycoside antibiotics restore dystrophin function to skeletal muscles of *mdx* mice. *J Clin Invest* 1999; **104**: 375–381.
18. Wilschanski M, Famini C, Blau H, *et al.* A pilot study of the effect of gentamicin on nasal potential difference measurements in cystic fibrosis patients carrying stop mutations. *Am J Respir Crit Care Med* 2000; **161**: 860–865.
19. Clancy JP, Bebök Z, Ruiz F, *et al.* Evidence that systemic gentamicin suppresses premature stop mutations in patients with cystic fibrosis. *Am J Respir Crit Care Med* 2001; **163**: 1683–1692.
20. Keeling KM, Bedwell DM. Clinically relevant aminoglycosides can suppress disease-associated premature stop mutations in the *IDUA* and *P53* cDNAs in a mammalian translation system. *J Mol Med* 2002; **80**: 367–376.
21. Bidou L, Hatin I, Perez N, Allamand V, Panthier J-J, Rousset J-P. Premature stop codons involved in muscular dystrophies show a broad spectrum of readthrough efficiencies in response to gentamicin treatment. *Gene Ther* 2004; **11**: 619–627.
22. Kellermayer R, Szigeti R, Keeling KM, Bedekovics T, Bedwell DM. Aminoglycosides as potential pharmacogenetic agents in the treatment of Hailey-Hailey disease. *J Invest Dermatol* 2006; **126**: 229–231.
23. Wagner KR, Hamed S, Hadley DW, *et al.* Gentamicin treatment of Duchenne and Becker muscular dystrophy due to nonsense mutations. *Ann Neurol* 2001; **49**: 706–711.
24. Politano L, Nigro G, Nigro V, *et al.* Gentamicin administration in Duchenne patients with premature stop codon. Preliminary results. *Acta Myologica* 2003; **22**: 15–21.
25. Wilschanski M, Yahav Y, Yaacov Y, *et al.* Gentamicin-induced correction of CFTR function in patients with cystic fibrosis and CFTR stop mutations. *N Engl J Med* 2003; **349**: 1433–1441.
26. James PD, Raut S, Rivard GE, *et al.* Aminoglycoside suppression of nonsense mutations in severe hemophilia. *Blood* 2005; **106**: 3043–3048.
27. Phillips-Jones M, Hill L, Atkinson J, Martin R. Context effects on misreading and suppression at UAG codons in human cells. *Mol Cell Biol* 1995; **15**: 6593–6600.
28. Howard MT, Shirts BH, Petros LM, Flanigan KM, Gesteland RF, Atkins JF. Sequence specificity of aminoglycoside-induced stop codon readthrough: potential implications for treatment of Duchenne muscular dystrophy. *Ann Neurol* 2000; **48**: 164–169.
29. Aurino S, Nigro V. Readthrough strategies for stop codons in Duchenne muscular dystrophy. *Acta Myologica* 2006; **25**: 5–12.
30. Sermet-Gaudelus I, Renouil M, Fajac A, *et al.* In vitro prediction of stop-codon suppression by intravenous gentamicin in patients with cystic fibrosis: a pilot study. *BMC Med* 2007; **5**: 5.
31. Swann SK. Aminoglycoside nephrotoxicity. *Semin Nephrol* 1997; **17**: 27–33.
32. Forge A, Schacht J. Aminoglycoside antibiotics. *Audiol Neurootol* 2000; **5**: 3–22.

33. Dunant P, Walter MC, Karpati G, Lochmuller H. Gentamicin fails to increase dystrophin expression in dystrophin- deficient muscle. *Muscle Nerve* 2003; **27**: 624–627.
34. Wolstencroft EC, Mattis V, Bajer AA, Young PJ, Lorson CL. A non-sequence-specific requirement for SMN protein activity: the role of aminoglycosides in inducing elevated SMN protein levels. *Hum Mol Genet* 2005; **14**: 1199–1210.
35. Hamada M, Tadeuchi T, Kondo S, *et al.* A new antibiotic, negamycin. *J Antibiot* 1970; **23**: 170–171.
36. Uehara Y, Hori M, Umezawa H. Negamycin inhibits termination of protein synthesis directed by phage f2 RNA in vitro. *Biochim Biophys Acta* 1974; **374**: 82–95.
37. Arakawa M, Nakayama Y, Hara T, *et al.* Negamycin can restore dystrophin in mdx skeletal muscle. *Acta Myologica* 2001; **20**: 154–158.
38. Arakawa M, Shiozuka M, Nakayama Y, *et al.* Negamycin restores dystrophin expression in skeletal and cardiac muscles of mdx mice. *J Biochem (Tokyo)* 2003; **134**: 751–758.
39. Guicheney P, Vignier N, Zhang X, *et al.* PCR based mutation screening of the laminin alpha2 chain gene (LAMA2): application to prenatal diagnosis and search for founder effects in congenital muscular dystrophy. *J Med Genet* 1998; **35**: 211–217.
40. Bidou L, Stahl G, Hatin I, Namy O, Rousset J-P, Farabaugh PJ. Nonsense-mediated decay mutants do not affect programmed -1 frameshifting. *RNA* 2000; **6**: 952–961.
41. Martin R, Philips-Jones MK, Watson FJ, Hills LS. Codon context effects on nonsense suppression in human cells. *Biochem Soc Trans* 1993; **21**: 846–851.
42. Manuvakhova M, Keeling K, Bedwell DM. Aminoglycoside antibiotics mediate context-dependent suppression of termination codons in a mammalian translation system. *RNA* 2000; **6**: 1044–1055.
43. Cassan M, Rousset JP. UAG readthrough in mammalian cells: Effect of upstream and downstream stop codon contexts reveal different signals. *BMC Mol Biol* 2001; **2**: 3.
44. Maquat LE. Nonsense-mediated mRNA decay in mammals. *J Cell Sci* 2005; **118**: 1773–1776.
45. Moriarty PM, Reddy CC, Maquat LE. Selenium deficiency reduces the abundance of mRNA for Se-dependent glutathione peroxidase 1 by a UGA-dependent mechanism likely to be nonsense codon-mediated decay of cytoplasmic mRNA. *Mol Cell Biol* 1998; **18**: 2932–2939.
46. Keeling KM, Lanier J, Du M, *et al.* Leaky termination at premature stop codons antagonize nonsense-mediated mRNA decay in *S. cerevisiae*. *RNA* 2004; **10**: 691–703.
47. Linde L, Boelz S, Nissim-Rafinia M, *et al.* Nonsense-mediated mRNA decay affects nonsense transcript levels and governs response of cystic fibrosis patients to gentamicin. *J Clin Invest* 2007; **117**: 683–692.
48. Welch EM, Barton ER, Zhuo J, *et al.* PTC124 targets genetic disorders caused by nonsense mutations. *Nature* 2007; **447**: 87–91.
49. Nilsson M, Ryden-Aulin M. Glutamine is incorporated at the nonsense codons UAG and UAA in a suppressor-free *Escherichia coli* strain. *Biochim Biophys Acta – Gene Structure and Expression* 2003; **1627**: 1–6.
50. Available: www.bork.embl-heidelberg.de/PolyPhen.
51. Sunyaev S, Ramensky V, Koch I, Lathe Wr, Kondrashov A, Bork P. Prediction of deleterious human alleles. *Hum Mol Genet* 2001; **10**: 591–597.

# Efficient Total Synthesis of (+)-Negamycin, a Potential Chemotherapeutic Agent for Genetic Diseases.

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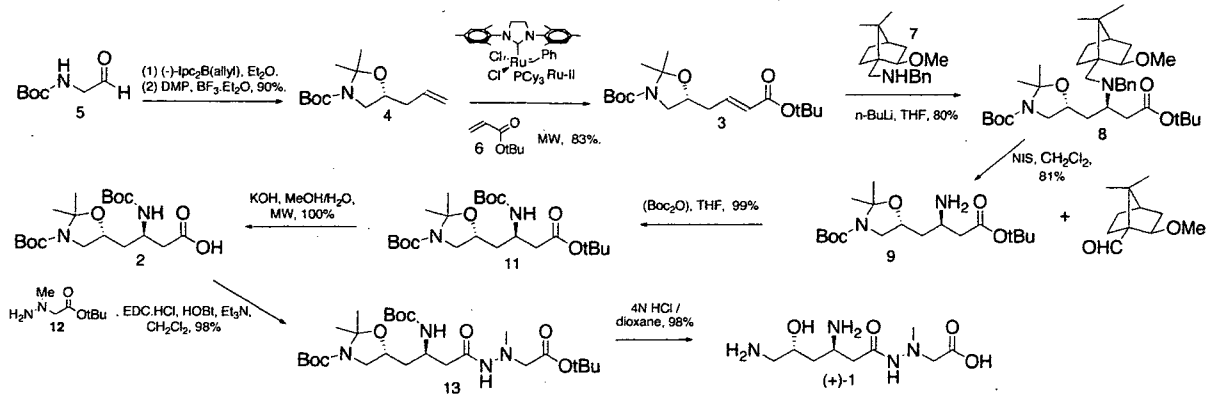
**Herein, we describe an efficient strategy for the total synthesis of (+)-Negamycin using commercially available achiral *N*-Boc-2-aminoacetaldehyde as starting material with 42% overall yield for a limited number of steps.**

(+)-Negamycin (**1**, Scheme 1), an unusual antibiotic containing a hydrazine peptide bond was isolated for the first time by Umezawa et al. in 1970 from culture filtrates of three strains related to Streptomyces purperfuscus. This natural product exhibits very low acute toxicity and strong inhibitory activity against multiple drug-resistant enteric Gram-negative bacteria including Pseudomonas aerginosa.[1] (+)-**1**'s antimicrobial activity is derived from a genetic miscoding on bacterial ribosomal systems, and thereby leading to a specific inhibition of protein biosynthesis.[2] Because this miscoding causes read-through of termination signals, considerable attention is focused on (+)-**1** as a potential therapeutic agent against genetic diseases. Indeed, the aminoglycoside antibiotic gentamicin and the less toxic negamycin both restore dystrophin expression in skeletal and cardiac muscles of mdx mices, an animal model of Duchenne muscular dystrophy (DMD) with a nonsense mutation in the dystrophin gene.[3] Therefore, an efficient shortened synthetic route of (+)-**1** and its derivatives appears significant to develop promissing new therapeutic candidates for DMD and other diseases caused by nonsense mutations. The first total synthesis of (+)-**1** from D-galacturonic acid was reported in 1972 and confirmed the assigned structure of the natural product.[4] Over three decades, numerous total syntheses have been reported on both racemic and optically active (+)-**1** but with moderate overall yield.[5]

Our fast and efficient route consists first on an asymmetric allylboration of *N*-Boc-glycinal **5** using the established Brown's procedure for preparation of chiral allylic alcohols[6] that led to a corresponding chiral intermediate (Scheme 1). This resulting chiral amino alcohol was directly engaged without further purification to form the target oxazoline **4** by treatment with *2,2*-dimethoxypropane (DMP) in the presence of boron trifluoride diethyl ether complex ($BF_3{\cdot}Et_2O$) in acetone. As a result, **4** was generated in high yield after purification by silica gel column chromatography (90%). To prepare the key intermediate **3**, a cross-metathesis (CM) reaction between **4** and *tert*-butyl acrylate **6** was investigated. Although the efficiency of ruthenium-based catalysts for ring-opening metathesis polymerization (ROMP) and ring-closing metathesis (RCM) is now well established, most alkene CM variants have fewer successful applications because of the multiple possible side reactions that cause relatively low synthetic yields.[7] Because our substrates are categorized as rapid and slow homodimerizable compounds according to Grubbs et al.'s empirical model for predicting the outcome of CM reactions,[8] we screened different reaction conditions to avoid forming unwanted dimers and selectively provide the target compound **3** by varying catalysts (Grubbs first [Ru-I] and second-generation [Ru-II] catalysts), amount of reactant (1 or 5 equiv of **6**), duration of reaction as well as heating method (conventional or microwave-assisted heating).




**Scheme 1** Total synthesis of (+)-negamycin.

As detailed in Supporting Informations, the conversion and chemoselectivity enhancements are definitely more pronounced for [Ru]-**II** than [Ru]-**I**. Furthermore, we observed that microwave irradiation drastically shortened the CM reaction time by 20 folds. As it pertains to microwave-assisted synthesis, this acceleration is commonly attributed to the very high local temperatures and the ease to which microwave irradiation reaches such conditions but the scientific community is still divided in opinion on the involvement of a specific non-thermal effect induced by the dielectric heating produced using microwave.[9] Interestingly, although our observations provide a new example for the thermal effect, the involvement of such "specific effect" is neither confirmed nor disproved in these reaction conditions. Bargiggia et al. arrived to similar conclusions while studying CM reactions[10] and Garbaccia et al. have described similar observations for RCM reactions in 2003.[11] As a result, the desired product **3** was isolated with 83% yield. NOE experiments revealed that the stereochemistry of the olefin moiety in **3** was an *E* configuration ($J_{\text{vinylic protons}}$ = 15.7 Hz). Thus, the desired chiral intermediate **3** was obtained with 75% yield after 2 steps from achiral *N*-Boc-glycinal **5**.

With intermediate **3** in hands, our focus shifted toward the asymmetric Michael addition reaction. Recently, Node et al. reported a highly stereoselective asymmetric Michael addition toward *tert*-butyl $\alpha,\beta$-unsaturated carbonyl compounds using chiral amine **7**, [12] This approach, when applied to the $\alpha,\beta$-unsaturated *tert*-butyl ester **3**, allowed the introduction of the amine moiety with an excellent enantiomeric excess.[13] The chiral reagent **7** was prepared from the corresponding ketopinic acid[12] and reacted with **3** in the presence of *n*-BuLi in THF at -78°C to afford compound **8** as a single diastereomer (*de* >99%) with 80% yield after purification. Removal of both benzyl and 2-methoxybornyl protecting groups located on the same amine moiety could be achieved efficiently using 4 equiv of *N*-iodosuccinimide (NIS) in dichloromethane to obtain free amino compound **9** with 81% yield. This deprotection proceeded by oxydation with NIS to imine and subsequent spontaneous hydrolysis to afford *tert*-butyl esters of $\beta$-amino acids and 2-methoxy-D-bornylaldehyde.[12] No epimerization was observed during this reaction. Furthermore, one of the advantages of the protocol is that the initial chiral inducer **7** can be easily regenerated from 2-methoxy-D-bornaldehyde, generated during the cleavage by reductive amination, using benzylamine in the presence of sodium cyanoborohydride (data not shown).

The last part of the synthesis of (+)-**1** consisted of introducing a hydrazine unit, prior to a final deprotection. A *Boc*-protection of **9** using standard procedures was first quantitatively performed to afford *N*-protected *tert*-butyl ester **11**, that was then efficiently converted to acid **2** by a microwave-assisted saponification with 2M KOH in MeOH, and coupling with hydrazine unit **12** was then performed using the classical EDC·HCl-HOBt method. The synthesis of hydrazine **12** was achieved by reacting *N*-methyl hydrazine with *t*-butyl bromoacetate with 40% yield after purification. Deprotection of compound **13** and purification by ion exchange chromatography on Amberlite CG50 ($NH_4^+$ form) afforded the target compound (+)-**1** with 98% yield, $[\alpha]_D^{25.2}$ +2.4° (c 0.36, $H_2O$), lit. $[\alpha]_D^{29.0}$ +2.5° (c 2.00, $H_2O$). The final compound was fully characterized and compared with the published data for the natural product to confirm the success of this new total synthesis of (+)-**1** (e.g. $^1$H NMR chart for natural and synthesized (+)-**1**, available in Supporting Information). Furthermore, the in vivo read-through activity of termination codons during protein biosynthesis[3] of the synthesized (+)-**1** in mice was almost similar to that of the native (+)-**1** (data not shown). Further derivatization of the **1** structure using the above synthetic methodology will contribute to a better understanding on the structure-activity relationship of **1** and the development of more potent compounds with efficient read-through activity. Studies in this regard are currently in progress and details pertaining to the biological activity will be soon published elsewhere.

In conclusion, the proposed synthetic route for the total synthesis of optically active (+)-negamycin starting from *N*-Boc-glycinal **5**, led to the desired product with a total yield of 42% from only 8 steps. To our knowledge, this study represents the most efficient strategy to prepare (+)-**1**. Current efforts with this new synthetic approach are now expanding into medicinal chemistry to discover new drug candidates with potent read-through activity for Duchenne Muscular Dystrophy. The chemical biology of Negamycin is also now investigated to better understand its read-through mechanism.

## Notes and references

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† Electronic Supplementary Information (ESI) available: Analytical data.
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1 Kondo, S.; Shibahara, S.; Takahashi, S.; Maeda, K.; Umezawa, H.; Ohno, M. *J. Am. Chem. Soc.* **1971**, *93*, 6305.
2 Mizuno, S.; Nitta, K.; Umezawa, H. *J. Antibiot.* **1970**, *23*, 581.
3 (a) Welch, E. M.; Barton, E. R.; Zhuo, J.; Tomizawa, Y.; Friesen, W. J.; Trifillis, P.; Paushkin, S.; Patel, M.; Trotta, C. R.; Hwang, S.; Wilde, R. G.; Karp, G.; Takasugi, J.; Chen, G.; Jones, S.; Ren, H.; Moon, Y.-C.; Corson, D.; Turpoff, A. A.; Campbell, J. A.; Conn, M. M.; Khan, A.; Almstead, N. G.; Hedrick, J.; Mollin, A.; Risher, N.; Weetall, M.; Yeh, S.; Branstrom, A. A.; Colacino, J. M.; Babiak, J.; Ju, W. D.; Hirawat, S.; Northcutt, V. J.; Miller, L. L.; Spatrick, P.; He, F.; Kawana, M.; Feng, H.; Jacobson, A.; Peltz S. W.; Lee S. H. *Nature* **2007**, *447*, 87. (b) Arakawa, M.; Shiozuka, M.; Nakayama, Y.; Hara, T.; Hamada, M.; Kondo, S.; Ikeda, D.; Takahashi, Y.; Sawa, R.; Nonomura, Y.; Sheykholeslami, K.; Kondo, K.; Kaga, K.; Kitamura, T.; Suzuki-Miyagoe, Y.; Takeda, S.; Matsuda, R. *J. Biochem.* **2003**, *134*, 751.
4 Shibahara, S.; Kondo, S.; Maeda, K.; Umezawa, H.; Ohno, M. *J. Am. Chem. Soc.* **1972**, *94*, 4353.
5 (a) Wang, Y.-F.; Izawa, T.; Kobayashi, S.; Ohno, M. *J. Am. Chem. Soc.* **1982**, *104*, 6465. (b) Iida, H.; Kasahara, K.; Kibayashi, C. *J. Am. Chem. Soc.* **1986**, *108*, 4647. (c) Tanner, D.; Somfai, P. *Tetrahedron Lett.* **1988**, *29*, 2373. (d) Kasahara, K.; Iida, H.; Kibayashi, C. *J. Org. Chem.*; **1989**; *54(9)*; 2225 (e) Schimdt, U.; Stabler, F.; Lieberknecht, A. *Synthesis* **1992**, 482. (f) Maycock, C. D.; Barros, M. T.; Santos, A.

G.; Godinho, L. S. *Tetrahedron Lett.* **1992**, *33*, 4633. (g) Masters, J. J.; Hegedus, L. S. *J. Org. Chem.* **1993**, *58*, 4547. (h) Socha, D.; Jurczak, M.; Chmielewski, M. *Tetrahedron Lett.* **1995**, *36*, 135. (i) Davies, S. G.; Ichihara, O. *Tetrahedron: Asymmetry* **1996**, *7*, 1919. (j) Jain, R. P.; Williams, R. M. *J. Org. Chem.* **2002**, *67*, 6361.

6 (a) Jadhav, P. K.; Bhat, K. S.; Perumaal, T.; Brown, H. C. *J. Org. Chem.* **1986**, *51*, 432; (b) Racgerlaa, U. S.; Brown, H. C. *J. Org. Chem.* **1991**, *56*, 401.

7 Hansen E. C.; Lee D. *Acc. Chem. Res.* **2006**, *39*, 509.

8 Chatterjee, A. K.; Choi, T.L.; Sanders, D. P.; Grubbs, R. H. *J. Am. Chem. Soc*, **2003**, *125*, 11360.

9 Strauss C. R. *Angew. Chem. Int. Ed.* **2002**, *41*, 3589.

10 Bargiggia, F. C.; Murray, W. V. *J. Org. Chem.* **2005**, *70*, 9636.

11 Garbaccia, S.; Desai, B.; Lavastre, O.; Kappe, O. C. *J. Org. Chem.* **2003**, *68*, 9136.

12 Katoh T., Watanabe T., Nishitani M., Ozeki M., Kajimoto T., Node M. *Tetrahedron Lett.* **2008**, *49*, 598.

13 (a) Nishide, K.; Ozeki, M.; Kunishige, H.; Shigeta, Y.; Patra, P. K.; Hagimoto, Y.; Node M. *Angew. Chem. Int. Ed.* **2003**, *42*, 4515. (b) Node M.; Hashimoto, D.; Hatoh, T.; Ochi, S.; Ozeki, M.; Watanabe, T.; Kajimoto, T. Manuscript in preparation.

TOPICS トピックス

# ナンセンスコドンを読み飛ばせ

★[*Nature*誌2007年5月3日掲載記事を読む]

点変異により1つの塩基が置換し，本来の翻訳終結部位より上流に未熟終止コドン（premature termination codon）を生じ，その結果，分子量が小さいタンパク質が作られて細胞機能に重大な支障をきたすことがある。このようなナンセンス変異による遺伝性疾患は単一遺伝性疾患の5～15%程度（多いものでは70%）を占めるといわれている。近年，このナンセンス変異型遺伝性疾患を克服するために未熟終止コドンを読み飛ばす治療法〔リードスルー療法（readthrough therapy）〕の研究が進んできた。

## 未熟終止コドンのリードスルー

原核細胞において，アミノグリコシドはrRNAの構造変化を引き起こし，mRNA上の未熟終止コドンとrRNAのA部位との結合を阻害する。その結果，未熟終止コドンをリードスルーして翻訳を続行させることが知られていた。これを真核細胞で起こさせ，遺伝性疾患の治療可能性を示したのは，米国のBedwellらである。嚢胞性線維症の原因遺伝子にナンセンス変異をもつHeLa細胞や気管上皮細胞に対し，ゲンタマイシンやG418といったアミノグリコシド系抗菌物質の投与により，正常遺伝子産物を発現させたという報告であった[1)2)]。未熟終止コドンのリードスルーによって機能的な全長タンパク質が合成されるのである（図1）。

これらの研究成果の後，1999年米国のLee Sweeneyらはデュシェンヌ型筋ジストロフィーのモデル動物である**mdxマウス**にゲンタマイシン（図2A）を投与し，リードスルーさせることにより，筋組織内に正常マウスの20%程度のジストロフィンの蓄積および筋力の上昇を認めた[3)]。しかし，ゲンタマイシンの血中濃度の安全域は狭く，聴覚毒性や腎毒性などの重篤な副作用を有するので，遺伝性疾患の治療薬として長期間ヒトに投与することは危険であるため，臨床的実用化には至っていない。

ジストロフィン遺伝子上にナンセンス変異を有し，機能的なジストロフィンを合成できず，筋変性を起こすマウス。

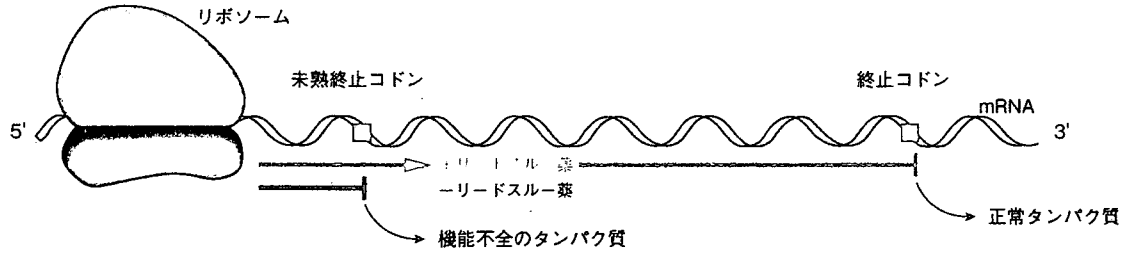
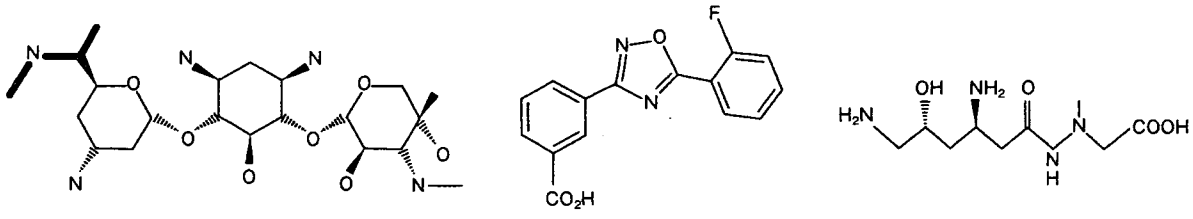



図1　リードスルーの概念



(A) ゲンタマイシン　　(B) PTC124　　(C) ネガマイシン

図2　ゲンタマイシン，PTC124，ネガマイシンの構造

## ナンセンス変異型遺伝性疾患の治療薬としての新規物質

2007年Lee Sweeneyらは，リードスルー活性が高くリードスルー療法薬として有望な新規物質PTC124を*Nature*誌に発表した。このPTC124（図2B）は，80万種の低分子化合物群から特定された分子で，毒性はほとんど認められず，しかも経口投与でも効果が認められる優れたリードスルー物質である。ナンセンス変異を含むレポーター細胞によって検出されたリードスルー活性は，濃度とコドンの種類（UGA，UAG，UAA）に依存し，処理後20時間での最大活性はUGAに対して12倍，UAGには4倍，UAAには2倍であった。またゲンタマイシン有効濃度の100～1,000倍薄い濃度においても有意なリードスルー効果を示した。デュシェンヌ型筋ジストロフィー患者およびmdxマウス由来の培養筋細胞をPTC124で処理したところ，それぞれ正常の40～60%，35%の回復をジストロフィン免疫染色から確認し，mdxマウスへの4週間の経口投与でも機能的な筋強度の修復，筋変性の保護，血清**クレアチンキナーゼ活性**の低下，筋組織内におけるジストロフィンの蓄積を認めた。さらに，彼らはレポーター細胞を用いた解析から，PTC124は未熟終止コドンを特異的に認識し，正常な終止コドンにおいてはリードスルーを誘起しないことを示唆する結果を報告した。

## リードスルー薬の可能性

今回発表されたPTC124は，米国において健常人を対象とした第I相臨床試験において安全性が確認され[5]，現在，ナンセンス変異を有するデュシェンヌ型筋ジストロフィーと遠位性線維症の患者を対象に，用量依存的な薬理効果を調べる第II相臨床試験が行われている。中間評価ではすでに薬理効果を見出しているが，確実な治療効果を得るために，より高用量での治験が行われている。

一方，筆者らはゲンタマイシンより毒性が低いジペプチド系抗菌物質ネガマイシン（図2C）が，デュシェンヌ型筋ジストロフィーや先天性筋ジストロフィーのモデル系においても高いリードスルー活性を有することを報告してきた[6][7]（図3）。さらにネガマイシンと類似した構造をもつ分子群からリードスルー惹起物質を探索したところ，リードスルー活性が高く，安全性の高い新規物質を特定している。この分子の構造はPTC124

**筋中に多量に存在する酵素。筋細胞の崩壊や筋細胞膜の透過性が亢進すると血中に漏出する。筋ジストロフィーの臨床的診断マーカーになっている。**

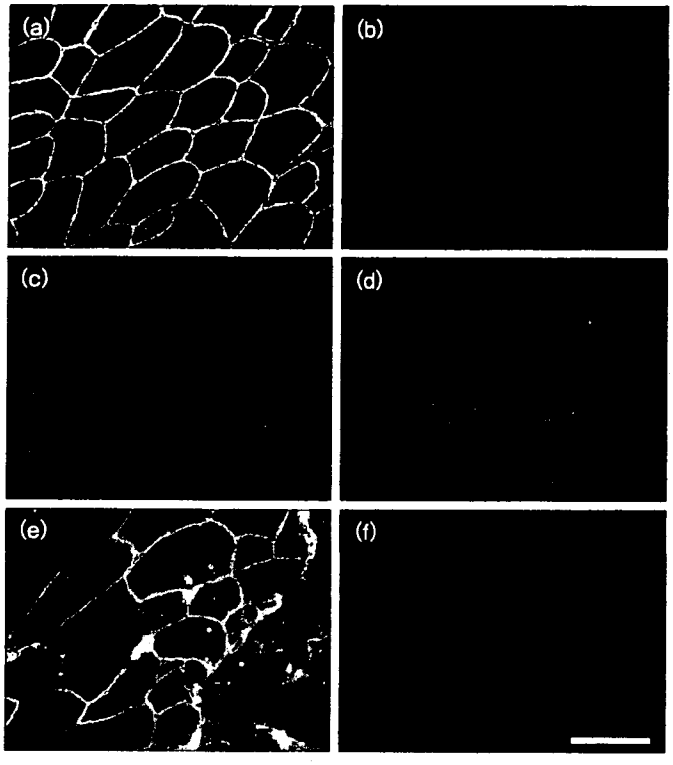



図3

とはまったく異なり，抗菌活性はなく，経口投与でも活性を示すことから，PTC124と同様に有望なリードスルー薬物候補であると考えている。

正常遺伝子の導入による遺伝子治療は，ベクターの安全性や導入効率などに問題があり，本格的な実用には至っていない。一方，ナンセンス変異症例は多くの遺伝性疾患に認められる。せめてナンセンス変異型遺伝性疾患やP53のナンセンス変異による癌だけでも薬物治療を可能にしたい。リードスルー活性は未熟終止コドンの種類や周辺塩基配列に影響を受け，薬物ごとに異なる傾向がある。リードスルー療法の確立のためには，リードスルー活性が高く，毒性が低い薬物分子をより多く特定するため，探索をさらに続けていく必要がある。遺伝性疾患が飲み薬で治せれば素晴らしいではないか！


[引用文献]

1) Howard, M. T., Frizzell, R. A. & Bedwell, D. M. *Nat. Med.*, **2**, 467–469 (1996).

2) Bedwell, D. M., Kaenjak, A., Benos, D. J., Bebok, Z., Bubien, J. K. *et al. Nat. Med.*, **3**, 1280–1284 (1997).

3) Barton, E. R., Cordiner, L., Shoturma, D. I., Leland, S. E. & Sweeney, H. L. *J. Clin. Invest.*, **104**, 375–381 (1999).

4) Welch, E. M., Barton, E. R., Zhuo, J., Tomizawa, Y., Friesen, W. J. *et al. Nature*, **447**, 87–91 (2007).

5) Hirawat, S., Welch, E. M., Elfring, G. L., Northcutt, V. J., Paushkin, S. *et al. J. Clin. Pharmacol.*, **47**, 430–444 (2007).

6) Arakawa, M., Shiozuka, M., Nakayama, Y., Hara, T., Matsuda, R. *et al. J. Biochem.*, **134**, 751–758 (2003).

7) Allamand, V., Bidou, L., Arakawa, M., Shiozuka, M., Matsuda, R. *et al. J. Gene Med.* (*In press*)



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## ― 編集後記 ―

◇めっきり寒くなりました。それでも私の子供の頃は，もっと寒かったような気がします。軒下には氷柱が下がり，池には厚い氷が張っていました。暖冬という言葉が頻繁に聞かれるようになったのはいつの頃からでしょうか。暖かい冬は個人的にはうれしいのですが，地球全体にとっては大問題です。温暖化による海面上昇で，水没の危機に見舞われている国や地域も多数あります。日本の沿岸域でさえ，その被害を受ける恐れがあり，他人事では済まされません。
今回の特集は，雪と氷の世界を生活の場に選んだ生き物たちを紹介しています。極地方に暮らす生物にとっても，温暖化は深刻です。寒さに適応した彼らの体は，気温の上昇に非常に敏感です。ほんの数℃気温や水温が上昇しただけでも，生態系を破壊するような事態になりかねません。彼らの特異な体のしくみや生態を眺めつつ，温暖化についても考える冬にしたいですね。　（K）

（お詫びと訂正）
2007年11月号トピックス「ナンセンスコドンを読み飛ばせ」p. 12図3の説明を追加いたしますとともに，関係各位に深くお詫びいたします。
正常マウスでは発現しているジストロフィン（a）が，未投与のmdxマウスでは発現していない（c）のに対し，mdxマウスにネガマイシンを投与するとジストロフィンの回復が認められた（e）。変性筋線維を可視化するエバンスブルー染色では，正常マウスでは変性が起きていない（b）のに対し，mdxマウスでは多く見られる脆弱な筋線維（d）が，投与によって減少している（f）のが確認できる。スケールバーは100 μm



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