を行った結果、当研究センターにて微生物二次代謝産物として単離された擬似ニ糖類IM-1および2、AG-401、-402、-501、-502についてゲンタマイシンと同程度の活性を持つことが確認された。

当研究センターにて利用できるすべての被検菌を用い50種の化合物の抗菌活性評価を行った。VS32およびVS29が顕著な抗菌活性を示した。

D. 考察

IM-1および2はその評価によってはすぐに臨床の場に送り込める候補化合物と成り得る。本結果を踏まえ、擬似三糖、四糖の選択的加水分解、半合成による擬似二糖ライブラリー構築を進め、最適な化合物を創製したい。

顕著な抗菌活性を示した化合物VS32およびVS29は筋ジストロフィーマウスを用いた評価を進める。また、VS32およびVS29の構造は毒性を回避する新しいネガマイシン誘導体創製の可能性を示した。

E. 結論

筋ジストロフィー治療薬を創製に向け、アミノグリコシド系抗生物質群からIM-1および2をネガマイシンのバーチャルスクリーニングによりVS32およびVS29を得た。VS32およびVS29の構造は新規ネガマイシン誘導体創製の可能性を示した。

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分担研究報告書

DMD患者の遺伝子解析と筋生検由来培養細胞を用いた評価

分担研究者　松尾雅文 神戸大学医学部小児科

研究要旨

Duchenne型筋ジストロフィー（DMD）は、進行性の筋萎縮を特徴とする伴性劣性遺伝病である。現在のところ、DMDに対する有効な治療はなく、DMD患者は心不全あるいは呼吸不全のために、20歳代に死亡する。DMDに関する遺伝子診断が進み、DMDはジストロフィン遺伝子のアウトオブフレーム変異あるいはナンセンス変異によって引き起こされることが判明した。本研究ではDMD症例でジストロフィン遺伝子のナンセンス変異を解析するとともに、ナンセンス変異に対するリードスルー誘導治療法に関する検討を行った。

A. 研究目的

Duchenne型筋ジストロフィー（DMD）は男児3500人に1人が発症する最も頻度の高い遺伝性進行性筋萎縮症である。DMDはジストロフィン遺伝子の異常に起因する筋肉のジストロフィン欠損を特徴とする。多くのDMDでは、このジストロフィン欠損はジストロフィン遺伝子のエクソン単位の欠失の異常によりジストロフィンmRNAのアミノ酸読み取り枠にずれを生じ（アウトオブフレーム）、mRNA上にストップコドンが新たに出現し、ジストロフィン合成が翻訳の途中で停止してしまうために生じる。また、1部のDMDではジストロフィン遺伝子の1塩基置換のためにナンセンス変異を生じ、ジストロフィンの合成が停止し、そのためにジストロフィンが欠損する。現在DMDの治療として、アウトオブフレーム異常に対しては、エクソンスキッピング誘導によりインフレームにかえる方法が、ナンセンス変異に対してはリボソーマルリードスルー誘導法がそれぞれ提唱されている。

本研究は、ジストロフィン遺伝子にナンセンス変異を持つDMDに対してmRNA上のナンセンス変異を読み飛ばすリードスルー現象を応用しようとするものである。この目的のためには従来抗生物質のゲンタマイシンが用いられていた。ゲンタマイシンの作用はリボゾームRNAに結合し、ナンセンスコドンと終結因子との結合を弱め、ナンセンスコドンとどれかのtRNAと

の結合を促し、新たなアミノ酸の伸長がおこりタンパクの合成が最後まで進行させるものである。しかしながら、ゲンタマイシンは高い毒性を有しており、臨床の現場で応用するには課題が残されている。
そのため、ゲンタマイシンにかわるリードスルー誘導薬の開発が活発におこなわれてきた。最近、ゲンタマイシンにかわる新たな化合物が発見されたので、そのリードスルー誘導によるジストロフィン発現について検討した。

B. 研究方法

神戸大学小児科を受診したDMD患者のジストロフィン遺伝子異常の解析を行った。まずMultiplex ligation probe amplification（MLPA）法にてエクソン単位の比較的大きな遺伝子の異常をスクリーニングし、欠失・重複変異の検出を図った。このMLPAで変異が発見できなかった症例について、ジストロフィンmRNAの解析を行った。

ジストロフィンmRNAの解析はリンパ球か筋生検で行った筋組織のRNAを抽出し、逆転写酵素を用いてcDNAに変換して行った。ジストロフィンcDNAの全長を10領域に分割し、それぞれをPCR増幅し、直接シークエンス法で塩基配列を解析した。

また、ナンセンス変異により２次的に生じたスプライシングの異常の解析は、ナンセンス変異を有するエクソンを中心に両側の2〜3個のエクソンを含んだ領域をPCR増幅し、その増幅産物の塩基配列を決定することにより解析した。

さらに、ナンセンス変異に対してリードスルー薬によるジストロフィン発現誘導効果を検討した。ナンセンス変異を有するDMD患者から同意を得て筋生検にて筋組織を得た。そして、培養筋細胞株を樹立した。株化した培養筋細胞の培養液中にリードスルー薬を添加した。添加後に筋細胞でのジストロフィン発現をモノクローナル抗体を用いた免疫染色で解析した。

C. 研究結果 と D. 考察

DMD患者でジストロフィン遺伝子異常の解析を施行し、ジストロフィン遺伝子のナンセンス変異を有する例をさらに同定することに成功した。

これまでに神戸大学小児科で同定したナンセンス変異は56件でジストロフィン遺伝子全体に分布していた。ナンセンス異常のホットスポットは見い出されなかった。ナンセンス変異になる塩基の変異をみるとCから Tへの変異によりTGAを形成する変異が最も高頻度にみられた。また、3種のス

トップコドンのうちTGAの頻度が最も高かった。

ジストロフィン遺伝子にナンセンス変異を有するすべての症例はDMDの表現型を示すものと考えられる。ところが、1部の例では表現型が重症なDMDではなく、軽症のBMDとなっている例もみられた。BMDへと表現型が軽症化する原因として、ナンセンスとなった1塩基の置換により二次的に誘導されるスプライシング様式の変化が考えられた。

このことはナンセンス変異に対するリードスルー薬の使用に際しては、その効果がストップコドンのリードスルーによるのかスプライシングの変異によるのかを厳格に区別しておく必要があることを示した。そのためには、一塩基置換によりスプライシングがどの様に変化するのかをあらかじめ知っておく必要がある。

そこで、ジストロフィン遺伝子のナンセンス変異例のスプライシング様式を解析した。ジストロフィン遺伝子のmRNAを解析した38例中7例では一塩基の置換によりスプライシング異常が誘発されることが確認できた。この様に、ナンセンス異常では高頻度でスプライシング異常も合併することが明らかとなった。また、スプライシングの異常によりジストロフィンの産生が可能となることが5例で確認された。

我が国で新たに開発されたリードスルー薬のナンセンス変異に及ぼす効果を患者細胞で解析した。コンパウンド2を患者由来培養細胞に導入したところ、ジストロフィンの発現が促進されることが確認された。しかし、その発現で治療効果が得られるレベルのものか不明である。今後、さらに検討を加えコンパウンド2の治療効果を明らかにする予定である。

また、新規に同定された化合物についても現在そのジストロフィン発現誘導効果を確認中であり、大きな効果が得られた時には、その効果を分子生物学的手法を用いて詳しく解析する予定である。そして、DMDの治療への応用をはかってゆく予定である。

E. 結論

DMD患者の解析から今後、リードスルー薬の開発あるいは使用に際して2次的スプライシング異常の併発に注意する必要があることが明らかとなった。また、現在検討中の新規の化合物によるリードスルー効果の著明な発現を期待したい。

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分担研究報告書

DMD患者由来培養筋細胞を用いた評価

分担研究者　斎藤加代子 東京女子医科大学附属遺伝子医療センター 所長

研究要旨

DMD患者の骨格筋からTGA型停止コドンを示す初代筋細胞（125M）を用い、RD1の最適濃度、細胞接着と増殖、dystrophinの発現と筋分化について観察し、蛍光抗体法により細胞を免疫学的に検討した。RD1による細胞毒性は観察されず、50, 100μg/ ml投与においてdystrophin, myosin陽性細胞が観察された。筋分化の指標であるfusion index ならびにdystrophin:myosin ratioの比較においても、RD1による筋分化、dystrophin発現ともに増加を認めた。

A. 研究目的

Duchenne muscular dystrophy (DMD)は原因遺伝子が解明されて２０年経過し、その遺伝子変異の詳細が分析可能となった。DMD患児の約２０％に点変異が認められ、特に、停止コドン型ノンセンス変異を有する患児に対して、リードスルーによるnonsense コドン抑制が根本治療として有用であると考えられ[1]、基礎的研究が積み重ねられてきた。 PTC124によるDMD nonsense コドンをリードスルーにより抑制しdystrophinが発現することが示され、現在米国においてDMDの治験が進められている。

リードスルーによるDMD治療効果が示唆されている薬剤にはgentamicinなどアミノグリコシド系薬剤があるが副作用が懸念される。主任研究者らは、これらの構造類似体であるRD1をin silico探索により得た。この薬剤を用いたDMDの根本治療の開発に寄与することを目的として、RD1のリードスルーによるnonsenseコドン抑制効果を、患者由来の培養骨格筋細胞を用いて検討した。

B. 研究方法

DMDの確定診断のための筋生検の施行に当たって、インフォームド・コンセントを得て、DMD患者の骨格筋の培養系を樹立し、TGA型停止コドンを示す初代筋細胞（１２５Ｍ）を用いた。正常対照は、human skeletal muscle myoblast (HSMM : Lonza社)を用いた。

RD1の最適濃度、細胞接着と増

殖、dystrophinの発現と筋分化について観察し、蛍光抗体法により細胞を免疫学的に検討した。増殖培地としてSkGM-2（GM: Lonza社）を用いて培養を行った。細胞はそれぞれ1×10^4 cells/ cm^2になるようにMatri-gel (BD Biosciences社) を薄層コートしたcollagen I-coated dish (IWAKI社) に播種した。播種後1日目にGM培地交換を行い、細胞が70-80%confluentになり使用した。分化培地(differentiation medium (DM) ; DMEM F12 + 2% horse serum + penicillin, streptomycin, amphotericin B)にRD1 を0, 50, 100, 250, 500 μg/ mlとなるよう調製し投与した。分化12日目におけるdystrophin (Abcam社)とmyosin (MF20)の発現を蛍光抗体染色にて検出した。

なお、本研究は東京女子医科大学の倫理委員会の審査承認を受けて実施された。

C. 研究結果

1）細胞培養系において、細胞増殖は良好で、対照細胞(HSMM)，患者由来培養骨格筋細胞(125M)ともに、骨格筋の分化の特徴である筋管細胞が観察された。HSMMでは分化による筋線維への形態学的変化ならびに蛍光抗体によるdystrophin，myosinの発現を確認した。dystrophinの発現については、対照細胞では細胞膜辺縁、膜直下に局在を認めたが、125Mでは細胞質全体に局在した。

2）125MではRD1による細胞毒性は観察されなかった。形態学的観察では筋管様の形態を認めるものの小さく細胞体は不完全であった。細胞接着については播種から分化12日目における経時的観察によって、collagen I-coated 単独と比べMatri-gelを薄層コートした細胞群において安定した接着ならびに増殖分化が確認された。

3）125Mにおける蛍光抗体法では、RD1の未投与においてdystrophin陽性細胞は認められなかった。RD1の50, 100 μg/ ml投与細胞において、dystrophin, myosin陽性細胞が観察された(図1)。

4）筋分化の指標であるfusion index ならびにdystrophin:myosin ratioの比較においても、RD1による筋分化、dystrophin発現ともに増加を認めた（図2）。

D. 考察

RD1を投与した125Mにおいてdystrophinが細胞質全体に局在したことはRD1の適正量投与を行うことでdystrophinのnon sense コドンのリードスルーによる抑制効果によりdystrophinが発現し細胞質中に留まったと考えられる。発現したdystrophinが機能するには

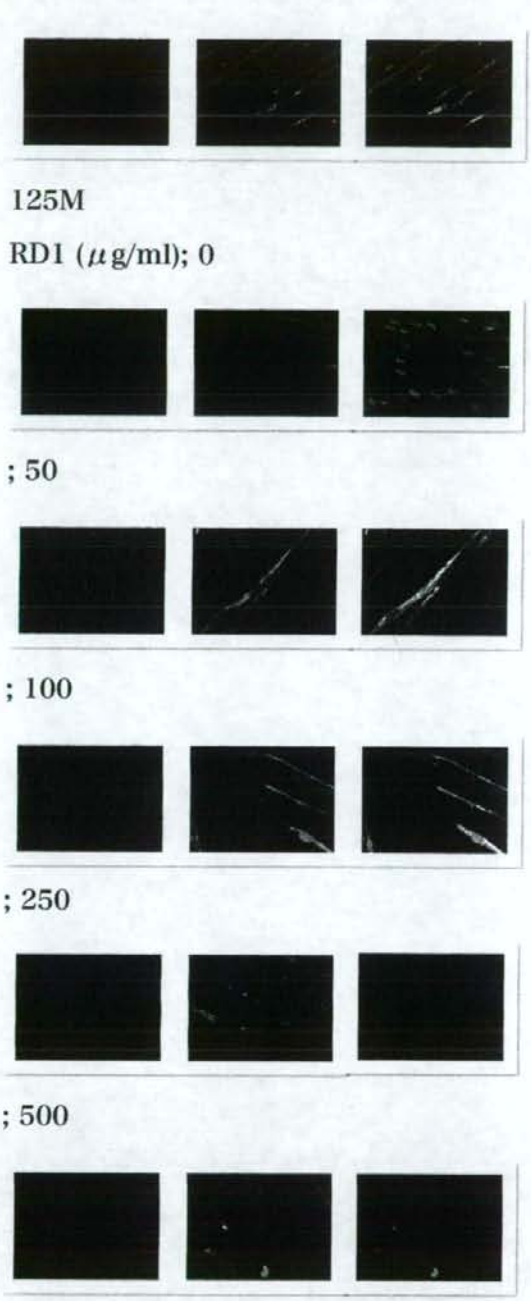
細胞内の適切な場所での局在の安定化が必要と考えられた。

細胞接着にはcollagen I-coated dish単独と比してMatri-gelをコートすることが有用と考えられた。しかしMatri-gelにはgentamicinを含むことから、125M においてＲＤ１を含まないＤＭのみでのdystrophin発現がバックグラウンドレベルではあるが観察された。　そこでMatri-gelのほかに筋細胞増殖分化への効果が示されているcollagen I以外collagen IV, laminin, fibronectin等の基質を用いた患者筋細胞培養の最適な細胞外基質の検討を行うことが必要であると考える。

筋分化の指標であるfusion index ならびにdystrophin:myosin ratioの比較を重ねて検討し、今後はリードスルーによる抑制効果で発現するｄｙｓｔｒｏｐｈｉｎについてWestern blottingの確認ならびに筋の機能についてCK値測定を課題とする。

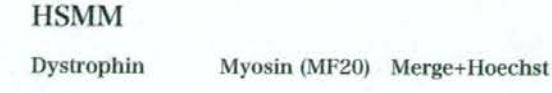
E. 結論

DMD において、nonsense コドンをもつ患者筋由来初代細胞においてRD1投与によりdystrophinが発現した。



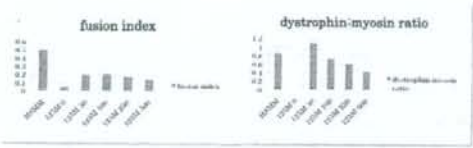
（図1）RD1投与による培養筋管細胞におけるdystrophinの発現。

筋生検より得られた初代骨格筋培養細胞由来の細胞免疫染色。正常対照(HSMM)とdystrophin　遺伝子exon60におけるstop



コドンを持つDMD患者(125M)。

RD1投与量を変えた効果により検出できたdystrophin (赤色)、myosin (緑色)を示す。核(青色)はHoechst 33258により染色された。



(図2) 筋分化の程度と筋繊維膜におけるdystrophinの存在。

細胞免疫染色像から計測した全核数とmyosin陽性細胞中の核数の比(fusion index)とdystrophin陽性細胞とmyosin陽性細胞の比(dystrophin:myosin ratio)を棒グラフに示す。

G. 研究発表

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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 α2-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 α2-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 β-galactosidase activities were measured from the same crude extract as described [43]. Stop codon readthrough was calculated by dividing the ratio of luciferase to β-galactosidase activity obtained with a given test construct by the ratio obtained from the control

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 + 2.5×10^{-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

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.

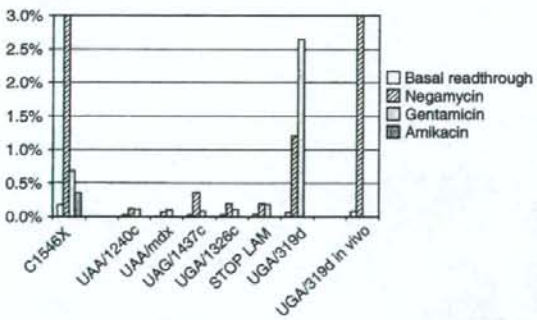

Figure 1. Induction of readthrough in NIH3T3 cells. Sequences corresponding to the C1546X stop mutation as well as other stop targets corresponding to DMC (c) or DMD (d) stop mutations or to the natural termination codon of the *LAMA2* gene (UGA/STOP LAM) were cloned in a dual gene reporter system between 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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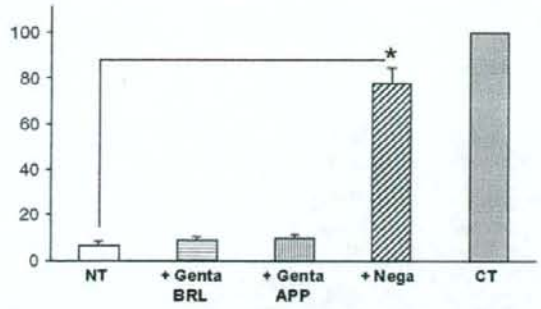

Figure 2. *LAMA2* **mRNA levels in cultured MDC1A myotubes treated with gentamicin and negamycin. Quantitative PCR were carried out on equal amounts of cDNAs, using the LightCycler real-time PCR machine (Roche Diagnostics, Germany).** *LAMA2* **mRNA levels were quantified relative to the mRNA of the 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

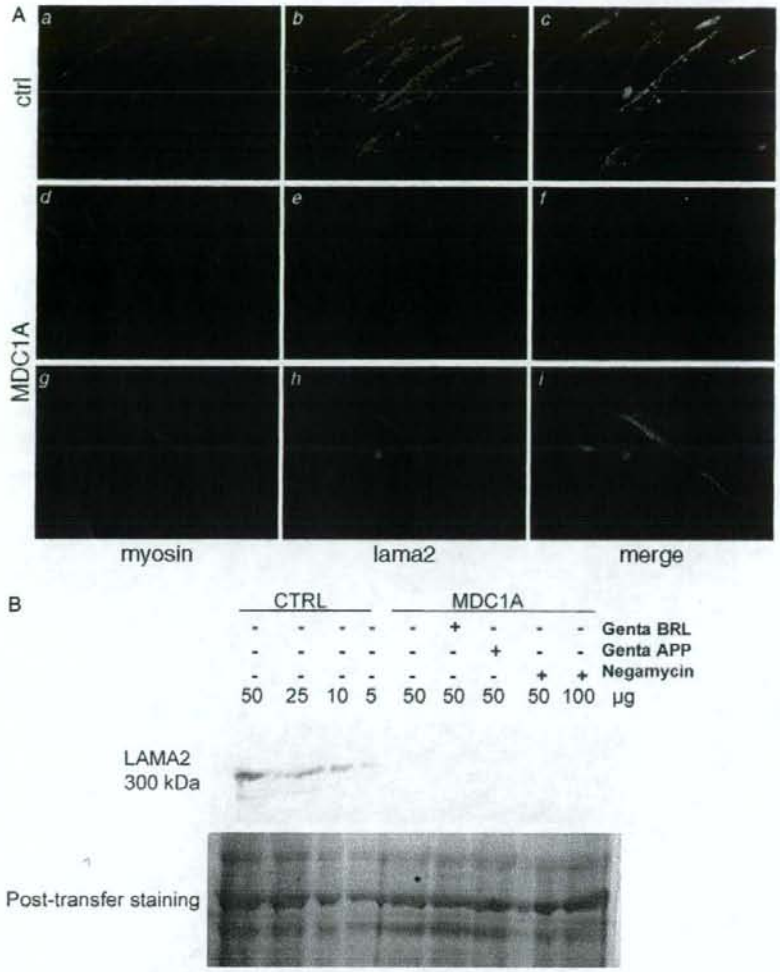

Figure 3. (A) Myosin and laminin α2-chain expression in control and MDC1A cultured myotubes after a 7-day negamycin treatment. Immunohistochemical analyses of primary myotubes from a control foetus (a–c) and a merosin-deficient CMD foetus (d–i) presenting a premature termination codon (C1546X) in the 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 $\alpha 2$ chain of laminin-211 assembles with the $\beta 1$ and $\gamma 1$ 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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