Negamycin Analogue with Readthrough-Promoting Activity as a Potential Drug Candidate for Duchenne Muscular Dystrophy

Akihiro Taguchi,[†] Shigenobu Nishiguchi,[‡] Masataka Shiozuka,[§] Takao Nomoto,[†] Mayuko Ina,[†] Shouta Nojima,[†] Ryoichi Matsuda,[§] Yoshiaki Nonomura,[§,‖] Yoshiaki Kiso,[‡] Yuri Yamazaki,[†] Fumika Yakushiji,[†] and Yoshio Hayashi*,[†]

[†]Department of Medicinal Chemistry, Tokyo University of Pharmacy and Life Sciences, Hachioji, Tokyo 192-0392, Japan
[‡]Department of Medicinal Chemistry, Kyoto Pharmaceutical University, Yamashina-ku, Kyoto 607-8412, Japan
[§]Department of Life Sciences, Graduate School of Arts and Sciences, The University of Tokyo, Komaba, Tokyo 153-8902, Japan
[‖]Institute of Microbial Chemistry, Shinagawa-ku, Tokyo 141-0021, Japan

Ⓢ *Supporting Information*

ABSTRACT: A series of (+)-negamycin **1** analogues were synthesized, and their readthrough-promoting activity was evaluated for nonsense mutations in Duchenne muscular dystrophy (DMD). A structure−activity relationship study indicated that **11b** was the most potent drug candidate. Immunohistochemical analyses suggested that treatment with **11b** restored dystrophin expression in *mdx* mice, a DMD mouse model. Furthermore, **11b** decreased serum creatine kinase (CK) levels, an indicator of muscle fiber destruction. Most importantly, **11b** demonstrated lower toxicity than **1**, and thus, it could be a useful candidate for long-term treatment of DMD.

11b

KEYWORDS: *Negamycin, readthrough-promoting activity, Duchenne muscular dystrophy, nonsense mutations, hydrazino dipeptide, genetic disease*
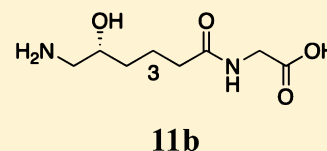
Duchenne muscular dystrophy (DMD), characterized by progressive muscle degeneration, is one of the most common hereditary disorders, affecting approximately 1 in 3500 live male births.[1] This disorder is caused by mutations in the DMD gene, located on the X-chromosome. The DMD gene encodes the protein dystrophin, which plays a crucial role linking the intracellular cytoskeleton and the extracellular matrix via the dystrophin-associated protein complex (DAPC). The loss of dystrophin function causes destabilization of the DAPC, which results in the breakdown of muscle fibers, loss of membrane integrity, and difficulty in walking and breathing, and it ultimately leads to death. Nonsense mutations, which lead to premature termination codons (PTCs) in the reading frame of the DMD gene, are responsible for up to 20% of DMD cases. The nonsense mutations yield truncated dystrophin proteins, which have no valuable biological function.[2] Presently, although the molecular basis for the disease is clear, there is no cure for DMD.[3] The only available treatment is glucocorticoid therapy, which can prolong ambulation and reduce the incidence of severe scoliosis, although it is limited to relatively short-term treatments due to severe side effects.[4−6]

Recently, a unique therapeutic strategy, so-called "readthrough drugs", was proposed to target genetic diseases caused by nonsense mutations.[7] These drugs promote a translational "skip" of PTCs, but not of normal termination codons, resulting in the production of full-length proteins. Specifically, gentamicin, an aminoglycoside antibiotic, was reported to promote the readthrough of disease-causing PTCs in mammalian cells. Furthermore, its treatment partially restored dystrophin expression in skeletal and cardiac muscles of *mdx*

mice, an animal model of DMD with a nonsense mutation in the dystrophin gene. In spite of these positive results, long-term administration of gentamicin is not recommended, due to its severe side-effects including ototoxicity[8] and nephrotoxicity.[9] Small molecules possessing readthrough-promoting activity have also been described for DMD treatment, including aminoglycosides,[10] RTC compounds,[11] and an oxadiazole derivative, ataluren (PTC-124, phase IIB).[12]

In the same vein, Arakawa et al.[13] reported that the dipeptidic antibiotic (+)-negamycin (**1**, [2-(3,6-diamino-5-hydroxyhexanoyl)-1-methylhydrazino]acetic acid, Figure 1)[14] also induced
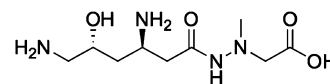

Figure 1. Structure of (+)-negamycin **1**.

the readthrough of PTCs in both a prokaryotic translational system[15] and *mdx* mice.[13] Therefore, **1** has been recognized as a potential therapeutic agent for diseases caused by nonsense mutations. Here, we designed and synthesized a series of negamycin analogues, and their biological activity was evaluated using a transgenic mouse strain, READ (readthrough evaluation and assessment by dual reporter),[16] which expresses a dual-reporter gene segmentalized with a PTC. Once the most potent

Table 1. Readthrough-Promoting and Antimicrobial Activities of Synthetic Negamycin Analogues

Compound	Structure	Yield (%)[a]	Readthrough activity[c]	Antimicrobial activity[d]
Gentamicin		NA[b]	1.00 ± 0.24	NT[e]
1 (+)-negamycin		NA[b]	1.00 ± 0.25	32/128/2/32/8
6		32	1.01 ± 0.16	>50/>50/25/>50/50
11a		27	0.83 ± 0.13	–
11b		27	1.36 ± 0.14	–
11c		43	0.81 ± 0.11	–
11d		44	0.92 ± 0.09	NT[e]
11e		24	< 0.8	–
11f		13	< 0.8	NT[e]
14		24	< 0.8	–

[a]Synthetic yields were calculated from intermediates **3** or **7** for analogues **6** or **11a−f** and **14**, respectively. [b]NA; not applicable, see ref 18. [c]Relative *in vivo* readthrough-promoting activity, which is expressed as a ratio compared to gentamicin. Samples were subcutaneously injected at the abdominal region of the READ mouse with a dosage of 0.1 mg/day/20 g body-weight for 7 days. Data are mean ± SD ($n = 4$). [d]The antimicrobial activity (MIC) against several microorganisms (*Staphylococcus aureus* FDA 209P/*Bacilus subtilis* NRRL B-558/*Escherichia coli* BEM11/*Shigella dysenteriae* J S11910/*Pseudomonas aeruginosa* A3, respectively). "− " denotes >128 μg/mL (MIC). See ref 24. [e]NT: not tested.

11b had been identified (Table 1), we used *mdx* mice to assess the effect on dystrophin expression, serum creatine kinase levels,[17] which are a clinical indicator of DMD, and general toxicity. We found that **11b** performed better than **1**, with markedly reduced toxicity, thus making it a promising therapeutic candidate.

(+)-Negamycin **1** was first isolated in 1970 from a microorganism closely related to *Streptomyces purpeofuscus*.[14] In an attempt to synthesize chiral **1**, we developed shortened, highly efficient synthetic routes.[18,19] Using one of these routes as a starting point,[19] here, we synthesized a series of analogues. Briefly, for the synthesis of analogue **6** (Scheme 1A), intermediate **3** was prepared from the commercially available ester **2** over 7 steps.[19] Then, **3** was converted to the *N*-protected *tert*-butyl ester **4** as a single diastereomer (diastereomeric excess (de) >99%) over 3 steps using Node's asymmetric Michael addition,[20] removal of the chiral auxiliary,[21] and protection of the inserted 3-amino group. The obtained intermediate **4** was then efficiently converted to the acid form by a microwave-assisted saponification, and it was subsequently coupled with a hydrazino ester using an EDC-HOBt (EDC, 1-ethyl-3-(3-dimethylaminopropyl)carbodiimide; HOBt, 1-hydroxybenzotriazole) method[22] to yield **5**. Deprotection of **5** with 4 M HCl/dioxane and purification by ion exchange chromatography afforded **6** (specific rotation: found $[\alpha]_D^{26.4}$ +14.0 (*c* 0.94, H$_2$O), literature data[23] $[\alpha]_D^{22.0}$ +8.5 (*c* 0.70, H$_2$O)).

Next, analogues **11a−f** were synthesized (Scheme 1B). Weinreb amide **7**[19] was prepared from **2** over six steps, and then it was reduced with diisobutylaluminium hydride (DIBAL-H) to the corresponding aldehyde, directly followed by treatment with (benzyloxycarbonylmethylene)triphenyl-phosphorane in THF under reflux conditions. After purification by flash chromatography on silica (Silica Gel 60N, KANTO CHEMICAL), we obtained **8** in 69% yield over two steps. After **8** was treated with Pd/C under a H$_2$ atmosphere, the resultant **9** was coupled with various amino acid *tert*-butyl esters or a hydrazinoacid *tert*-butyl ester[18] using an EDC-HOBt method to obtain **10a−f**. Deprotection of **10a−f** with 4 M HCl/dioxane and purification by reversed-phase HPLC afforded **11a−f** with 30−96% yield.

In the synthesis of analogue **14** (Scheme 1C), **7** was converted to the intermediate **12** by a procedure similar to that employed for **8** (Scheme 1B). Then, **12** was converted to the acid form by saponification and subsequently coupled with HCl·H-Gly-O*t*-Bu using an EDC-HOBt method to yield **13**. Deprotection of **13** with 4 M HCl/dioxane and purification by reversed-phase HPLC afforded **14** with 85% yield. The purity of each synthesized analogue for biological evaluation was over 95%.

To evaluate the readthrough-promoting activity, we adapted an *in vivo* dual-reporter gene expression system using READ mice.[16] This system encodes β-galactosidase and luciferase genes connected with a PTC (see the Supporting Information). β-Galactosidase activity is present constitutively, but luciferase

Scheme 1. Synthesis of Analogue 6[a]
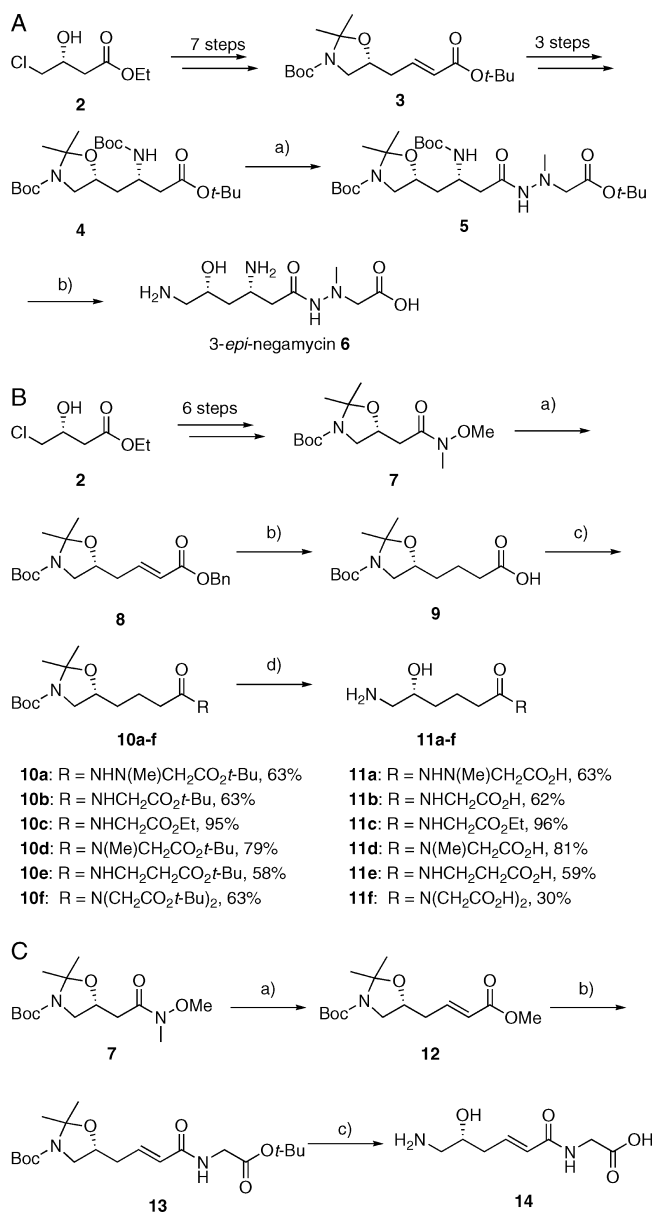
[a]Reagents and conditions: (A) Synthesis of **6**: (a) (i) KOH, MeOH, microwave (300 W), 100 °C, 10 min; (ii) PTSA·H₂N−N(Me)-CH₂CO₂t-Bu, HOBt·H₂O, Et₃N, EDC·HCl, CH₂Cl₂, rt, 4 h, 62% (2 steps); (b) (i) 4 M HCl/dioxane, rt, 1 h; (ii) ion exchange chromatography, 98%. (B) Synthesis of **11a−f**: (a) (i) DIBAL-H, toluene, −78 °C, 2 h; (ii) Ph₃P = CHCO₂Bn, THF, reflux, overnight, 69% (2 steps); (b) Pd/C, H₂, MeOH, rt, 1.5 h, quant; (c) amino acid t-Bu esters or hydrazinoacid t-Bu ester, HOBt·H₂O, Et₃N, EDC·HCl, DMF, rt, 3 h to overnight, 58−95%; (d) (i) 4 M HCl/dioxane, rt, 1 h; (ii) reversed-phase HPLC, 30−96%. (C) Synthesis of **14**: (a) (i) DIBAL-H, toluene, −78 °C, 2 h; (ii) Ph₃P = CHCO₂Me, THF, reflux, overnight, 63% (2 steps); (b) (i) KOH, MeOH/H₂O (2:1), rt, 4 h; (ii) HCl·H-Gly-Ot-Bu, HOBt·H₂O, Et₃N, EDC·HCl, DMF, rt, overnight, 44% (2 steps); (c) (i) 4 M HCl/dioxane, rt, 1 h; (ii) reversed-phase HPLC, 85%.

activity is only detected when readthrough occurs. Therefore, the activities of both enzymes in skeletal muscle were measured to calculate the activity ratio of luciferase to β-galactosidase after negamycin analogues (0.1 mg) were subcutaneously administered in the abdominal region of READ mice for 7 days.

The antimicrobial activity was also measured.[24] The results of these biological evaluations are shown in Table 1.

Since synthetic **1** showed similar levels of readthrough-promoting activity to the extracted native **1** (data not shown) and gentamicin, we first evaluated the importance of stereochemistry at the 3-amino group. The (+)-3-*epi*-negamycin **6** exhibited equipotent activity to **1**, suggesting that the stereochemistry of the 3-amino group might not be important for the activity. Next, analogue **11a** with no 3-amino group was prepared (Table 1). However, complete removal of the amino group led to a decrease and a loss of the readthrough-promoting and antimicrobial activities, respectively. Thus, the presence, but not the stereochemistry, of the 3-amino group was important for both biological activities.

In striking contrast, however, we observed that when both the *N*-methyl and amino groups were omitted from **11a**, the corresponding glycine analogue **11b** was a potent promoter of readthrough activity, demonstrating a 1.4-fold increase in functionality as compared to the case of **1**. Importantly, **11b** also did not display antimicrobial activity, making it a more selective readthrough-promoting analogue than **1**. In other words, it means that the readthrough-promoting activity can be distinguished from the antimicrobial activity.

Encouraged by these results, we synthesized additional analogues based on the chemical structure of **11b**. However, both the ethyl ester analogue **11c** and the *N*-methyl glycine analogue **11d** demonstrated decreased activities. From these results, we inferred that the glycine residue with a free carboxylic acid was functionally important, a hypothesis that we confirmed using **11e** and **11f**. Moreover, **14**, with the unsaturated amide structure, did not show any significant activity.

To understand the biological effects in detail, the most active **11b** was chosen for further *in vivo* immunohistochemical and biochemical evaluations. Regarding the immunohistochemical evaluation, **11b** was subcutaneously injected in the abdominal region of *mdx* mice at a dosage of 1 mg in phosphate-buffered saline (PBS, 0.2 mL)/day/20 g body-weight for 4 weeks. Dystrophin expression was clearly observed in the skeletal muscle of wild-type B10 mice (Figure 2A), while *mdx* mice
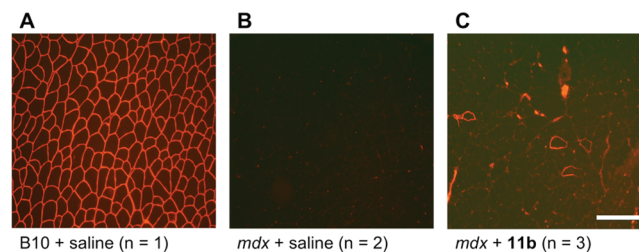

Figure 2. Dystrophin expression in skeletal muscles. Immunofluorescent staining of dystrophin in mouse muscle tissues was performed on 8 μm transverse cryosections.[12] (A) wild-type B10 mouse; (B) untreated *mdx* mouse; (C) **11b**-treated *mdx* mouse. Bar = 200 μm.

lacked this signal (Figure 2B). In contrast to these controls, dystrophin expression was only partially restored in the skeletal muscle of **11b**-treated *mdx* mice (Figure 2C). However, this result suggested that **11b** promotes PTC readthrough and is therefore a potential therapeutic candidate for DMD.

Regarding biochemical evaluation, we assessed levels of serum creatine kinase (CK)[17] in *mdx* mice treated subcutaneously with **11b** at a dosage of 1 mg in PBS (0.2 mL)/day/20

g body-weight for 4 weeks. As controls, the CK level in a wild-type B10 mouse was very low, while levels in *mdx* mice were very high. A statistically significant reduction of serum CK levels in **11b**-treated *mdx* mice was observed in comparison to the case of the untreated controls (Figure 3A). This result
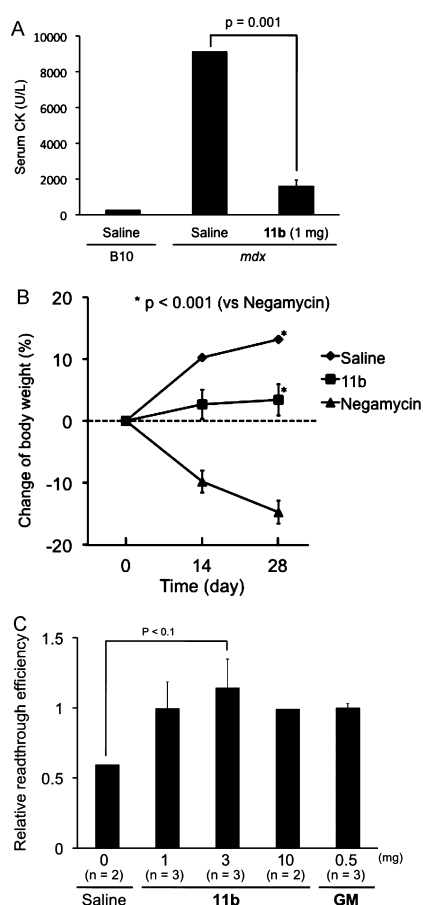

Figure 3. (A) Serum CK levels in *mdx* mice: wild-type B10 ($n = 1$); *mdx* ($n = 2$); **11b**-treated *mdx* ($n = 3$). (B) Effect of **11b** on the body-weight of *mdx* mice. The body-weight of **1**- and **11b**-treated mice ($n = 4$, 1 mg/day/20 g body-weight) over the course of 4 weeks was measured in comparison to that of saline-treated mice ($n = 2$) as a control. (C) Effects of the administration of high doses of **11b** on the readthrough-promoting activity in READ mice. P: Probability-value. Error bar indicates SD.

suggested that **11b** could enhance the strength of muscle fibers by increasing functional protein expression.

Next, we examined the acute *in vivo* toxicity of **11b** as compared to **1** by measuring the body-weight change of *mdx* mice for 4 weeks. Improving the *in vivo* toxicity profile of **1** was an important goal for the development of readthrough drugs based on the negamycin structure. As shown in Figure 3B, over the course of 4 weeks, the body-weight of saline-treated mice gradually increased, while that of **1**-treated mice (1 mg in 0.2 mL saline/day/20 g body-weight) markedly decreased. Conversely, the body-weight of **11b**-treated mice (1 mg in 0.2 mL saline/day/20 g body-weight) slowly increased during this time frame, indicating that **11b** exhibited a lower toxicity profile than **1**. We postulate that this lower toxicity is due to the absence of the hydrazine structure in **11b**. This improved toxicity profile strongly supports the potential of **11b** for the long-term treatment of DMD.

Finally, inspired by the low toxicity observed with **11b**, we tested the effects of high doses of **11b** on PTC readthrough-promoting activity. Accordingly, **11b**, or gentamicin or saline, as positive and negative controls, respectively, was administered subcutaneously in READ mice for 7 days. As shown in Figure 3C, the readthrough-promoting activity of **11b** was not dose-dependent at the levels tested. However, at a 3 mg dose, **11b** was more effective than gentamicin. For unknown reasons, there appeared to be a reduction in readthrough-promoting activity at the highest dosage of **11b**, an observation that we will pursue in the near future.

In summary, we have synthesized a series of (+)-negamycin analogues and evaluated their readthrough-promoting activity for DMD. On the basis of SAR studies, we identified **11b** as the most potent candidate. This analogue was then taken forward through immunohistochemical and biochemical studies, which demonstrated that treatment with **11b** restored some dystrophin expression in *mdx* mice and decreased their serum CK levels, indicating that the drug was protecting muscular tissues from collapse. Most importantly, **11b** was shown to have a lower toxicity profile than **1**, which might be useful for the long-term treatment of DMD. Further SAR studies to develop more efficient derivatives are under investigation.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information
Synthetic procedures, characterization of new products, biological assay protocols, and NMR data. This material is available free of charge via the Internet at http://pubs.acs.org.

■ AUTHOR INFORMATION

Corresponding Author
*E-mail: yhayashi@toyaku.ac.jp. Telephone: +81-42-676-3275. Fax: +81-42-676-3275.

■ ACKNOWLEDGMENTS

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