(19) DANMARK

(10) DK/EP 2203173 T3

(12) Oversættelse af europæisk patentskrift

Patent- og Varemærkestyrelsen

(51) Int.Cl.: *A 61 K 31/56 (2006.01)* *A 61 K 31/57 (2006.01)* *A 61 K 38/17 (2006.01)* *A 61 P 21/00 (2006.01)* *C 07 J 1/00 (2006.01)* *C 07 J 41/00 (2006.01)*

(45) Oversættelsen bekendtgjort den: **2016-02-29**

(80) Dato for Den Europæiske Patentmyndigheds bekendtgørelse om meddelelse af patentet: **2015-12-23**

(86) Europæisk ansøgning nr.: **08842559.0**

(86) Europæisk indleveringsdag: **2008-10-27**

(87) Den europæiske ansøgnings publiceringsdag: **2010-07-07**

(86) International ansøgning nr.: **NL2008050673**

(87) Internationalt publikationsnr.: **WO2009054725**

(30) Prioritet: **2007-10-26 EP 07119351** **2007-10-26 US 670 P**

(84) Designerede stater: **AT BE BG CH CY CZ DE DK EE ES FI FR GB GR HR HU IE IS IT LI LT LU LV MC MT NL NO PL PT RO SE SI SK TR**

(73) Patenthaver: **Academisch Ziekenhuis Leiden, Albinusdreef 2, 2333 ZA Leiden, Holland**
BioMarin Technologies B.V., J.H. Oortweg 21, 2333 CH Leiden, Holland

(72) Opfinder: **VAN DEUTEKOM, Judith Christina Theodora, Abeelstraat 13, NL-3329 AA Dordrecht, Holland**
AARTSMA-RUS, Annemieke, Navona 42, NL-2134 BE Hoofddorp, Holland
VAN OMMEN, Garrit-Jan, Boudewijn, Westerstraat 73, NL-1015 LW Amsterdam, Holland
PLATENBURG, Gerardus Johannes, Wijngaardenlan 56, NL-Voorschoten 2252 XR, Holland
DE KIMPE, Josephus, Johannes, Veilingstraat 175, 3521 BE Utrecht, Holland

(74) Fuldmægtig i Danmark: **Zacco Denmark A/S, Arne Jacobsens Allé 15, 2300 København S, Danmark**

(54) Benævnelse: **MIDLER OG FREMGANGSMÅDER TIL MODVIRKNING AF MUSKELLIDELSER**

(56) Fremdragne publikationer:
WO-A-2006/007910
WO-A1-2007/004979
WO-A2-2004/083446
US-A1- 2007 021 360
CHAUBOURT E ET AL: "Muscular nitric oxide synthase (muNOS) and utrophin" JOURNAL OF PHYSIOLOGY - PARIS, vol. 96, no. 1-2, 2002, pages 43-52, XP002469418
RADLEY ET AL: "Duchenne muscular dystrophy: Focus on pharmaceutical and nutritional interventions" INTERNATIONAL JOURNAL OF BIOCHEMISTRY AND CELL BIOLOGY, EXETER, GB, vol. 39, no. 3, 10 October 2006 (2006-10-10), pages 469-477, XP005831292 ISSN: 1357-2725
SEGALAT L ET AL: "CAPON expression in skeletal muscle is regulated by position, repair, NOS activity, and dystrophy" EXPERIMENTAL CELL RESEARCH, SAN DIEGO, CA, US, vol. 302, no. 2, 15 January 2005 (2005-01-15), pages 170-179, XP004649912 ISSN: 0014-4827
ANDERSON J E ET AL: "Correlated NOS-I and myf5 expression by satelite cells in mdx mouse muscle

Fortsættes ...

regeneration during NOS manipulation and deflazacort treatment" NEUROMUSCULAR DISORDERS, vol. 13, no. 5, 2003, pages 388-396, XP002469419
ARRUDA V R: "The role of immunosuppression in gene- and cell based treatments for Duchenne Muscular Dystrophy" MOLECULAR THERAPY, vol. 15, no. 6, June 2007 (2007-06), pages 1040-1041, XP009096112
GRANCHELLI J A ET AL: "Pre-clinical screening of drugs using the mdx mouse" NEUROMUSCULAR DISORDERS, PERGAMON PRESS, GB, vol. 10, no. 4-5, 1 June 2000 (2000-06-01), pages 235-239, XP002305261 ISSN: 0960-8966
ROLLAND J F ET AL: "Overactivity of exercise-sensitive cation channels and their impaired modulation by IGF-1 in mdx native muscle fibers: Beneficial effect of pentoxifylline" NEUROBIOLOGY OF DISEASE, BLACKWELL SCIENTIFIC PUBLICATIONS, OXFORD, GB, vol. 24, no. 3, 1 December 2006 (2006-12-01), pages 466-474, XP024901469 ISSN: 0969-9961 [retrieved on 2006-12-01]
ZHOU GUANG-QIAN; XIE HUI-QI; ZHANG SU-ZHEN; YANG ZHI-MING: "Current understanding of dystrophin-related muscular dystrophy and therapeutic challenges ahead" CHINESE MEDICAL JOURNAL (ENGLISH EDITION), vol. 119, no. 16, 20 August 2006 (2006-08-20), pages 1381-1391, XP002542429

DESCRIPTION

[0001] A muscle disorder is a disease that usually has a significant impact on the life of an individual. A muscle disorder can either have a genetic cause or a non-genetic cause. An important group of muscle diseases with a genetic cause are Becker Muscular Dystrophy (BMD) and Duchenne Muscular Dystrophy (DMD). These disorders are caused by defects in a gene for a muscle protein.

[0002] Becker Muscular Dystrophy and Duchenne Muscular Dystrophy are genetic muscular dystrophies with a relatively high incidence. In both Duchenne and Becker muscular dystrophy the muscle protein dystrophin is affected. In Duchenne dystrophin is absent, whereas in Becker some dystrophin is present but its production is most often not sufficient and/or the dystrophin present is abnormally formed. Both diseases are associated with recessive X-linked inheritance. DMD results from a frameshift mutation in the DMD gene. The frameshift in the DMD gene results in the production of a truncated non-functional dystrophin protein, resulting in progressive muscle wasting and weakness. BMD occurs as a mutation does not cause a frame-shift in the DMD gene. As in BMD some dystrophin is present in contrast to DMD where dystrophin is absent, BMD has less severe symptoms then DMD. The onset of DMD is earlier than BMD. DMD usually manifests itself in early childhood, BMD in the teens or in early adulthood. The progression of BMD is slower and less predictable than DMD. Patients with BMD can survive into mid to late adulthood. Patients with DMD rarely survive beyond their thirties.

[0003] Dystrophin plays an important structural role in the muscle fiber, connecting the extracellular matrix and the cytoskeleton. The N-terminal region binds actin, whereas the C-terminal end is part of the dystrophin glycoprotein complex (DGC), which spans the sarcolemma. In the absence of dystrophin, mechanical stress leads to sarcolemmal ruptures, causing an uncontrolled influx of calcium into the muscle fiber interior, thereby triggering calcium-activated proteases and fiber necrosis.

[0004] For most genetic muscular dystrophies no clinically applicable and effective therapies are currently available. Exon skipping techniques are nowadays explored in order to combat genetic muscular dystrophies. Promising results have recently been reported by us and others on a genetic therapy aimed at restoring the reading frame of the dystrophin pre-mRNA in cells from the *mdx* mouse and DMD patients[1-11]. By the targeted skipping of a specific exon, a DMD phenotype (lacking dystrophin) is converted into a milder BMD phenotype (partly to largely functional dystrophin). The skipping of an exon is preferably induced by the binding of antisense oligoribonucleotides (AONs) targeting either one or both of the splice sites, or exon-internal sequences. Since an exon will only be included in the mRNA when both the splice sites are recognised by the spliceosome complex, splice sites are obvious targets for AONs. Alternatively, or additionally, one or more AONs are used which are specific for at least part of one or more exonic sequences. Using exon-internal AONs specific for an exon 46 sequence, we were previously able to modulate the splicing pattern in cultured myotubes from two different DMD patients with an exon 45 deletion[11]. Following AON treatment, exon 46 was skipped, which resulted in a restored reading frame and the induction of dystrophin synthesis in at least 75% of the cells. We have recently shown that exon skipping can also efficiently be induced in human control and patient muscle cells for 39 different DMD exons using exon-internal AONs[1, 2, 11-15].

[0005] Hence, exon skipping techniques applied on the dystrophin gene result in the generation of at least partially functional - albeit shorter - dystrophin protein in DMD patients. Since DMD is caused by a dysfunctional dystrophin protein, it would be expected that the symptoms of DMD are sufficiently alleviated once a DMD patient has been provided with functional dystrophin protein. However, the present invention provides the insight that, even though exon skipping techniques are capable of inducing dystrophin synthesis, DMD symptom(s) is/are still further alleviated by administering to a DMD patient an adjunct compound for reducing inflammation, preferably for reducing muscle tissue inflammation, and/or an adjunct compound for improving muscle fiber function, integrity and/or survival. According to the present invention, even when a dystrophin protein deficiency has been restored in a DMD patient, the presence of tissue inflammation and damaged muscle cells still continues to contribute to the symptoms of DMD. Hence, even though the cause of DMD - i.e. a dysfunctional dystrophin protein - is alleviated, treatment of DMD is still further improved by additionally using an adjunct therapy according to the present invention. Furthermore, the present invention provides the insight that a reduction of inflammation does not result in significant reduction of AON uptake by muscle cells. This is surprising because, in general, inflammation enhances the trafficking of cells, blood and other compounds. As a result, AON uptake/delivery is also enhanced during inflammation. Hence, before the present invention it would be expected that an adjunct therapy counteracting inflammation involves the risk of negatively influencing AON therapy. This, however, appears not to be the case.

[0006] The present disclosure therefore provides a method for alleviating one or more symptom(s) of Duchenne Muscular Dystrophy or Becker Muscular Dystrophy in an individual, the method comprising:

- administering to said individual a compound for providing said individual with a (at least partially) functional dystrophin protein, and
- administering to said individual an adjunct compound for reducing inflammation, preferably for reducing muscle tissue inflammation, and/or an adjunct compound for improving muscle fiber function, integrity and/or survival.

[0007] The method for alleviating one or more symptom(s) of Duchenne Muscular Dystrophy or Becker Muscular Dystrophy in an individual comprises administering to said individual an adjunct compound for reducing inflammation, preferably for reducing muscle tissue inflammation, and/or an adjunct compound for improving muscle fiber function, integrity and/or survival.

[0008] The present invention relates to a combination of:

- an antisense oligonucleotide comprising a sequence which is complementary to a part of human dystrophin pre-mRNA exon 51 and said oligonucleotide is represented by SEQ ID NO:204, and
- an adjunct compound for reducing inflammation, wherein this compound comprises a steroid, said combination being for use as a medicament, for alleviating one or more symptom(s) of Duchenne Muscular Dystrophy in said individual, according to claim 1.

[0009] It has surprisingly been found that the skipping frequency of a dystrophin exon from a pre-MRNA comprising said exon, when using an oligonucleotide directed toward the exon or to one or both splice sites of said exon, is enhanced if cells expressing said pre-mRNA are also provided with an adjunct compound for reducing inflammation, preferably for reducing muscle tissue inflammation, and/or an adjunct compound for improving muscle fiber function, integrity and/or survival. The enhanced skipping frequency also increases the level of functional dystrophin protein produced in a muscle cell of a DMD or BMD individual.

[0010] The present disclosure further provides a method for enhancing skipping of an exon from a dystrophin pre-mRNA in cells expressing said pre-mRNA, said method comprising

- contacting said pre-mRNA in said cells with an oligonucleotide for skipping said exon and,
- contacting said cells with an adjunct compound for reducing inflammation, preferably for reducing muscle tissue inflammation, and/or an adjunct compound for improving muscle fiber function, integrity and/or survival.

[0011] As Duchenne and Becker muscular dystrophy have a pronounced phenotype in muscle cells, it is preferred that said cells are muscle cells. Preferably said cells comprise a gene encoding a mutant dystrophin protein. Preferably said cells are cells of an individual suffering from DMD or BMD.

[0012] The present invention further provides a method for enhancing skipping of an exon from a dystrophin pre-mRNA in cells expressing said pre-mRNA in an individual suffering from Duchenne Muscular Dystrophy or Becker Muscular Dystrophy, the method comprising:

- administering to said individual a compound for providing said individual with a (at least partially) functional dystrophin protein, and
- administering to said individual an adjunct compound for reducing inflammation, preferably for reducing muscle tissue inflammation, and/or an adjunct compound for improving muscle fiber function, integrity and/or survival

[0013] An individual is provided with a functional dystrophin protein in various ways. In one embodiment an exon skipping technique is applied. However, alternative methods are available according to the disclosure, such as for instance stop codon suppression by gentamycin or PTC124[16, 17] (also known as 3-(5-(2-fluorophenyl)-1,2,4-oxadiazol-3-yl)benzoic acid), and/or adeno-associated virus (AAV)-mediated gene delivery of a functional mini- or micro-dystrophin gene[18-20]. PTC124 ™ is a registered trademark of PTC Therapeutics, Inc. South Plainfield, New Jersey.

[0014] As defined herein, a functional dystrophin is preferably a wild type dystrophin corresponding to a protein having the amino acid sequence as identified in SEQ ID NO: 1. A functional dystrophin is preferably a dystrophin, which has an actin binding

domain in its N terminal part (first 240 amino acids at the N terminus), a cystein-rich domain (amino acid 3361 till 3685) and a C terminal domain (last 325 amino acids at the C terminus) each of these domains being present in a wild type dystrophin as known to the skilled person. The amino acids indicated herein correspond to amino acids of the wild type dystrophin being represented by SEQ ID NO:1. In other words, a functional dystrophin is a dystrophin which exhibits at least to some extent an activity of a wild type dystrophin. "At least to some extent" preferably means at least 30%, 40%, 50%, 60%, 70%, 80%, 90%, 95% or 100% of a corresponding activity of a wild type functional dystrophin. In this context, an activity of a functional dystrophin is preferably binding to actin and to the dystrophin-associated glycoprotein complex (DGC)[56]. Binding of dystrophin to actin and to the DGC complex may be visualized by either co-immunoprecipitation using total protein extracts or immunofluorescence analysis of cross-sections, from a biopsy of a muscle suspected to be dystrophic, as known to the skilled person.

[0015] Individuals suffering from Duchenne muscular dystrophy typically have a mutation in the gene encoding dystrophin that prevent synthesis of the complete protein, i.e of a premature stop prevents the synthesis of the C-terminus. In Becker muscular dystrophy the dystrophin gene also comprises a mutation compared tot the wild type but the mutation does typically not include a premature stop and the C-terminus is typically synthesized. As a result a functional dystrophin protein is synthesized that has at least the same activity in kind as the wild type protein, not although not necessarily the same amount of activity. The genome of a BMD individual typically encodes a dystrophin protein comprising the N terminal part (first 240 amino acids at the N terminus), a cystein-rich domain (amino acid 3361 till 3685) and a C terminal domain (last 325 amino acids at the C terminus) but its central rod shaped domain may be shorter than the one of a wild type dystrophin[56]. Exon - skipping for the treatment of DMD is typically directed to overcome a premature stop in the pre-mRNA by skipping an exon in the rod-domain shaped domain to correct the reading frame and allow synthesis of remainder of the dystrophin protein including the C-terminus, albeit that the protein is somewhat smaller as a result of a smaller rod domain. In a preferred embodiment, an individual having DMD and being treated by a method as defined herein will be provided a dystrophin which exhibits at least to some extent an activity of a wild type dystrophin. More preferably, if said individual is a Duchennes patient or is suspected to be a Duchennes patient, a functional dystrophin is a dystrophin of an individual having BMD: typically said dystrophin is able to interact with both actin and the DGC, but its central rod shaped domain may be shorter than the one of a wild type dystrophin (Aartsma-Rus et al (2006, ref 56). The central rod domain of wild type dystrophin comprises 24 spectrin-like repeats[56].For example, a central rod shaped domain of a dystrophin as provided herein may comprise 5 to 23, 10 to 22 or 12 to 18 spectrin-like repeats as long as it can bind to actin and to DGC.

[0016] Alleviating one or more symptom(s) of Duchenne Muscular Dystrophy or Becker Muscular Dystrophy in an individual may be assessed by any of the following assays: prolongation of time to loss of walking, improvement of muscle strength, improvement of the ability to lift weight, improvement of the time taken to rise from the floor, improvement in the nine-meter walking time, improvement in the time taken for four-stairs climbing, improvement of the leg function grade, improvement of the pulmonary function, improvement of cardiac function, improvement of the quality of life. Each of these assays is known to the skilled person. As an example, the publication of Manzur at al (2008, ref 58) gives an extensive explanation of each of these assays. For each of these assays, as soon as a detectable improvement or prolongation of a parameter measured in an assay has been found, it will preferably mean that one or more symptoms of Duchenne Muscular Dystrophy or Becker Muscular Dystrophy has been alleviated in an individual.

[0017] Detectable improvement or prolongation is preferably a statistically significant improvement or prolongation as described in Hodgetts et al (2006, ref 57). Alternatively, the alleviation of one or more symptom(s) of Duchenne Muscular Dystrophy or Becker Muscular Dystrophy may be assessed by measuring an improvement of a muscle fiber function, integrity and/or survival as later defined herein.

[0018] An adjunct compound for reducing inflammation comprises any therapy which is capable of at least in part reducing inflammation, preferably inflammation caused by damaged muscle cells. Said adjunct compound is most preferably capable of reducing muscle tissue inflammation. Inflammation is preferably assessed by detecting an increase in the number of infiltrating immune cells such as neutrophils and/or mast cells and/or dendritic cells and/or lymphocytes in muscle tissue suspected to be dystrophic. This assessment is preferably carried out in cross-sections of a biopsy[57] of muscle tissue suspected to be dystrophic after having specifically stained immune cells as identified above. The quantification is preferably carried out under the microscope. Reducing inflammation is therefore preferably assessed by detecting a decrease in the number of immune cells in a cross-section of muscle tissue suspected to be dystrophic. Detecting a decrease preferably means that the number of at least one sort of immune cells as identified above is decreased of at least 1%, 2%, 3%, 5%, 7%, 10%, 12%, 15%, 17%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or more compared to the number of a corresponding immune cell in a same individual before treatment. Most preferably, no infiltrating immune cells are detected in cross-sections of said biopsy.

[0019] In the present disclosure, an adjunct compound for improving muscle fiber function, integrity and/or survival comprises

any therapy which is capable of measurably enhancing muscle fiber function, integrity and/or survival as compared to an otherwise similar situation wherein said adjunct compound is not present. The improvement of muscle fiber function, integrity and/or survival may be assessed using at least one of the following assays: a detectable decrease of creatine kinase in blood, a detectable decrease of necrosis of muscle fibers in a biopsy cross-section of a muscle suspected to be dystrophic, and/or a detectable increase of the homogeneity of the diameter of muscle fibers in a biopsy cross-section of a muscle suspected to be dystrophic. Each of these assays is known to the skilled person.

[0020] Creatine kinase may be detected in blood as described in 57. A detectable decrease in creatine kinase may mean a decrease of 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or more compared to the concentration of creatine kinase in a same individual before treatment.

[0021] A detectable decrease of necrosis of muscle fibers is preferably assessed in a muscle biopsy, more preferably as described in 57 using biopsy cross-sections. A detectable decrease of necrosis may be a decrease of 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or more of the area wherein necrosis has been identified using biopsy cross-sections. The decrease is measured by comparison to the necrosis as assessed in a same individual before treatment.

[0022] A detectable increase of the homogeneity of the diameter of a muscle fiber is preferably assessed in a muscle biopsy cross-section, more preferably as described in 57.

[0023] A treatment is about at least one week, about at least one month, about at least several months, about at least one year, about at least 2, 3, 4, 5, 6 years or more.

[0024] In one embodiment of the disclosure an adjunct compound for increasing turnover of damaged muscle cells is used. An adjunct compound for increasing turnover of damaged muscle cells comprises any therapy which is capable of at least in part inducing and/or increasing turnover of damaged muscle cells. Damaged muscle cells are muscle cells which have significantly less clinically measurable functionality than a healthy, intact muscle cell. In the absence of dystrophin, mechanical stress leads to sarcolemmal ruptures, causing an uncontrolled influx of calcium into the muscle fiber interior, thereby triggering calcium-activated proteases and fiber necrosis, resulting in damaged muscle cells. Increasing turnover of damaged muscle cells means that damaged muscle cells are more quickly broken down and/or removed as compared to a situation wherein turnover of damaged muscle cells is not increased. Turnover of damaged muscle cells is preferably assessed in a muscle biopsy, more preferably as described in 57 using a cross-section of a biopsy. A detectable increase of turnover may be an increase of 5%, 10%, 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or more of the area wherein turnover has been identified using a biopsy cross-section. The increase is measured by comparison to the turnover as assessed in a same individual before treatment.

[0025] Without wishing to be bound to theory, it is believed that increasing turnover of muscle cells is preferred because this reduces inflammatory responses.

[0026] According to the present disclosure, a combination of a therapy for providing an individual with a functional dystrophin protein, together with an adjunct therapy for reducing inflammation, preferably for reducing muscle tissue inflammation in an individual, is particularly suitable for use as a medicament. Such combination is even better capable of alleviating one or more symptom(s) of Duchenne Muscular Dystrophy or Becker Muscular Dystrophy as compared to a sole therapy for providing an individual with a functional dystrophin protein. This embodiment also enhances the skipping frequency of a dystrophin exon from a pre-MRNA comprising said exon, when using an oligonucleotide directed toward the exon or to one or both splice sites of said exon. The enhanced skipping frequency also increases the level of functional dystrophin protein produced in a muscle cell of a DMD or BMD individual.

[0027] Further provided is therefore a combination of a compound for providing an individual with a functional dystrophin protein, and an adjunct compound for reducing inflammation, preferably for reducing muscle tissue inflammation in said individual, for use as a medicament. Since said combination is particularly suitable for counteracting DMD, the present disclosure also provides a use of a compound for providing an individual with a functional dystrophin protein, and an adjunct compound for reducing inflammation, preferably for reducing muscle tissue inflammation in said individual, for the preparation of a medicament for alleviating one or more symptom(s) of Duchenne Muscular Dystrophy. Said combination may be used in order to alleviate one or more symptom(s) of a severe form of BMD wherein a very short dystrophin protein is formed which is not sufficiently functional.

[0028] In the disclosure, preferred adjunct compound for reducing inflammation include a steroid, a TNFαinhibitor, a source of mIGF-1 and/or an antioxidant. However, any other compound able to reduce inflammation as defined herein is also encompassed within the present disclosure. Each of these compounds is later on extensively presented. Each of the compounds extensively presented may be used separately or in combination with each other and/or in combination with one or more of the adjunct

compounds used for improving muscle fiber function, integrity and/or survival. An adjunct compound of the invention comprises a steroid.

[0029] Furthermore this disclosure provides that, a combination of a therapy for providing an individual with a functional dystrophin protein, together with an adjunct therapy for improving muscle fiber function, integrity and/or survival in an individual is particularly suitable for use as a medicament. Such combination is even better capable of alleviating one or more symptom(s) of Duchenne Muscular Dystrophy as compared to a sole therapy for providing an individual with a functional dystrophin protein.

[0030] The disclosure therefore further provides a combination of a compound for providing an individual with a functional dystrophin protein, and an adjunct compound for improving muscle fiber function, integrity and/or survival in said individual, for use as a medicament. This combination is also particularly suitable for counteracting DMD. A use of a compound for providing an individual with a functional dystrophin protein, and an adjunct compound for improving muscle fiber function, integrity and/or survival in said individual, for the preparation of a medicament for alleviating one or more symptom(s) of Duchenne Muscular Dystrophy is therefore also provided in the disclosure. In one embodiment of the disclosure, said combination is used in order to alleviate one or more symptom(s) of a severe form of BMD wherein a very short dystrophin protein is formed which is not sufficiently functional.

[0031] In the disclosure, preferred adjunct compounds for improving muscle fiber function, integrity and/or survival include a ion channel inhibitor, a protease inhibitor, L-arginine and/or an angiotensin II type I receptor blocker. However, any other compound able to improving muscle fiber function, integrity and/or survival as defined herein is also encompassed within the present disclosure. Each of these compounds is later on extensively presented. Each of the compounds extensively presented may be used separately or in combination with each other and/or in combination with one or more of the adjunct compounds used for reducing inflammation.

[0032] A pharmaceutical preparation may be made which comprises at least one of the above mentioned combinations comprising a compound for providing an individual with a functional dystrophin protein together with an adjunct compound according to the invention. Further provided is therefore a pharmaceutical preparation comprising:

- a compound for providing an individual with a functional dystrophin protein, and
- an adjunct compound for reducing inflammation, preferably for reducing muscle tissue inflammation in said individual, and/or an adjunct compound for improving muscle fiber function, integrity and/or survival in said individual, and
- a pharmaceutically acceptable carrier, adjuvant, diluent and/or excipient. Examples of suitable carriers and adjuvants are well known in the art and for instance comprise a saline solution. Dose ranges of compounds used in a pharmaceutical preparation are designed on the basis of rising dose studies in clinical trials for which rigorous protocol requirements exist.

[0033] A compound for providing an individual with a functional dystrophin protein is combined with a steroid. As shown in the Examples, such combination results in significant alleviation of DMD symptoms. The present disclosure therefore provides a method for alleviating one or more symptom(s) of Duchenne Muscular Dystrophy in an individual, the method comprising administering to said individual a steroid and a compound for providing said individual with a functional dystrophin protein. A combination of a steroid and a compound for providing an individual with a functional dystrophin protein for use as a medicament is also provided, as well as a use of a steroid and a compound for providing an individual with a functional dystrophin protein for the preparation of a medicament for alleviating one or more symptom(s) of DMD. This also enhances the skipping frequency of a dystrophin exon from a pre-MRNA comprising said exon, when using an oligonucleotide directed toward the exon or to one or both splice sites of said exon. The enhanced skipping frequency also increases the level of functional dystrophin protein produced in a muscle cell of a DMD or BMD individual.

[0034] Said combination may be used in order to alleviate one or more symptom(s) of a severe form of BMD wherein a very short dystrophin protein is formed which is not sufficiently functional.

[0035] A steroid is a terpenoid lipid characterized by a carbon skeleton with four fused rings, generally arranged in a 6-6-6-5 fashion. Steroids vary by the functional groups attached to these rings and the oxidation state of the rings. Steroids include hormones and drugs which are usually used to relieve swelling and inflammation, such as for instance prednisone, dexamethasone and vitamin D.

[0036] According to the present disclosure supplemental effects of adjunct steroid therapy in DMD patients include reduction of tissue inflammation, suppression of cytotoxic cells, and improved calcium homeostasis. Most positive results are obtained in

younger boys. Preferably the steroid is a corticosteroid (glucocorticosteroid). Preferably, prednisone steroids (such as prednisone, prednizolone or deflazacort) are used[21].

[0037] Dose ranges of (glucocortico)steroids to be used in the therapeutic applications as described herein are designed on the basis of rising dose studies in clinical trials for which rigorous protocol requirements exist. The usual doses are about 0.5 - 1.0 mg/kg/day, preferably about 0.75 mg/kg/day for prednisone and prednisolone, and about 0.4 - 1.4 mg/kg/day, preferably about 0.9 mg/kg/day for deflazacort.

[0038] A steroid may be administered to said individual prior to administering a compound for providing an individual with a functional dystrophin protein. It is preferred that said steroid is administered at least one day, more preferred at least one week, more preferred at least two weeks, more preferred at least three weeks prior to administering a compound for providing said individual with a functional dystrophin protein.

[0039] In another preferred embodiment of the disclosure, a compound for providing an individual with a functional dystrophin protein is combined with a tumour necrosis factor-alpha (TNFα) inhibitor. Tumour necrosis factor-alpha (TNFα) is a pro-inflammatory cytokine that stimulates the inflammatory response. Pharmacological blockade of TNFα activity with the neutralising antibody infliximab (Remicade) is highly effective clinically at reducing symptoms of inflammatory diseases. In *mdx* mice, both infliximab and etanercept delay and reduce the necrosis of dystrophic muscle[24, 25], with additional physiological benefits on muscle strength, chloride channel function and reduced CK levels being demonstrated in chronically treated exercised adult *mdx* mice[26]. Such highly specific anti-inflammatory drugs designed for use in other clinical conditions, are attractive alternatives to the use of steroids for DMD. In one embodiment, the use of a TNFα inhibitor is limited to periods of intensive muscle growth in boys when muscle damage and deterioration are especially pronounced.

[0040] One aspect of the present disclosure thus provides a method for alleviating one or more symptom(s) of Duchenne Muscular Dystrophy in an individual, the method comprising administering to said individual a TNFα inhibitor and a compound for providing said individual with a functional dystrophin protein. A combination of a TNFα inhibitor and a compound for providing an individual with a functional dystrophin protein for use as a medicament is also provided in the disclosure, as well as a use of a TNFα inhibitor and a compound for providing an individual with a functional dystrophin protein for the preparation of a medicament for alleviating one or more symptom(s) of DMD. In one embodiment of the disclosure, said combination is used in order to alleviate one or more symptom(s) of a severe form of BMD wherein a very short dystrophin protein is formed which is not sufficiently functional. A preferred TNFα inhibitor according to the disclosure is a dimeric fusion protein consisting of the extracellular ligand-binding domain of the human p75 receptor of TNFα linked to the Fc portion of human IgG1. A more preferred TNFα inhibitor is ethanercept (Amgen, America)[26]. The usual doses of ethanercept is about 0.2 mg/kg, preferably about 0.5 mg/kg twice a week. The administration is preferably subcutaneous.

[0041] In another preferred embodiment according to the disclosure, a compound for providing an individual with a functional dystrophin protein is combined with a source of mIGF-1. As defined herein, a source of IGF-1 preferably encompasses mIGF-1 itself, a compound able of enhancing mIGF-1 expression and/or activity. Enhancing is herein synonymous with increasing. Expression of mIGF-1 is synonymous with amount of mIGF-1. mIGF-1 promotes regeneration of muscles through increase in satellite cell activity, and reduces inflammation and fibrosis[27]. Local injury of muscle results in increased mIGF-1 expression. In transgenic mice with extra IGF-1 genes, muscle hypertrophy and enlarged muscle fibers are observed[27]. Similarly, transgenic *mdx* mice show reduced muscle fiber degeneration[28]. Upregulation of the mIGF-1 gene and/or administration of extra amounts of mIGF-1 protein or a functional equivalent thereof (especially the mIGF-1 Ea isoform [as described in 27, human homolog IGF-1 isoform 4: SEQ ID NO: 2]) thus promotes the effect of other, preferably genetic, therapies for DMD, including antisense-induced exon skipping. The additional mIGF-1 levels in the above mentioned transgenic mice do not induce cardiac problems nor promote cancer, and have no pathological side effects. One aspect of the present disclosure thus provides a method for alleviating one or more symptom(s) of Duchenne Muscular Dystrophy in an individual, the method comprising administering to said individual a compound for providing said individual with a functional dystrophin protein, and providing said individual with a source of mIGF-1, preferably mIGF-1 itself, a compound able of increasing mIGF-1 expression and/or activity. As stated before, the amount of mIGF-1 is for instance increased by enhancing expression of the mIGF-1 gene and/or by administration of mIGF-1 protein and/or a functional equivalent thereof (especially the mIGF-1 Ea isoform [as described in 27, human homolog IGF-1 isoform 4: SEQ ID NO: 2]). A combination of mIGF-1, or a compound capable of enhancing mIGF-1 expression or an mIGF-1 activity, and a compound for providing an individual with a functional dystrophin protein for use as a medicament is also provided in the disclosure, as well as a use of mIGF-1, or a compound capable of enhancing mIGF-1 expression or mIGF-1 activity, and a compound for providing an individual with a functional dystrophin protein for the preparation of a medicament for alleviating one or more symptom(s) of DMD. In one embodiment, such combination is used in order to alleviate one or more symptom(s) of a severe form of BMD

wherein a very short dystrophin protein is formed which is not sufficiently functional.

[0042] Within the context of the disclosure, an increased amount or activity of mIGF-1 may be reached by increasing the gene expression level of an IGF-1 gene, by increasing the amount of a corresponding IGF-1 protein and/or by increasing an activity of an IGF1-protein. A preferred mIGF-1 protein has been earlier defined herein. An increase of an activity of said protein is herein understood to mean any detectable change in a biological activity exerted by said protein or in the steady state level of said protein as compared to said activity or steady-state in a individual who has not been treated. Increased amount or activity of mIGF-1 is preferably assessed by detection of increased expression of muscle hypertrophy biomarker GATA-2 (as described in 27).

[0043] Gene expression level is preferably assessed using classical molecular biology techniques such as (real time) PCR, arrays or Northern analysis. A steady state level of a protein is determined directly by quantifying the amount of a protein. Quantifying a protein amount may be carried out by any known technique such as Western blotting or immunoassay using an antibody raised against a protein. The skilled person will understand that alternatively or in combination with the quantification of a gene expression level and/or a corresponding protein, the quantification of a substrate of a corresponding protein or of any compound known to be associated with a function or activity of a corresponding protein or the quantification of said function or activity of a corresponding protein using a specific assay may be used to assess the alteration of an activity or steady state level of a protein.

[0044] In a method of the disclosure, an activity or steady-state level of a said protein may be altered at the level of the protein itself, e.g. by providing a protein to a cell from an exogenous source.

[0045] Preferably in the disclosure, an increase or an upregulation of the expression level of a said gene means an increase of at least 5% of the expression level of said gene using arrays. More preferably, an increase of the expression level of said gene means an increase of at least 10%, even more preferably at least 20%, at least 30%, at least 40%, at least 50%, at least 70%, at least 90%, at least 150% or more. In another preferred embodiment of the disclosure, an increase of the expression level of said protein means an increase of at least 5% of the expression level of said protein using western blotting and/or using ELISA or a suitable assay. More preferably, an increase of the expression level of a protein means an increase of at least 10%, even more preferably at least 20%, at least 30%, at least 40%, at least 50%, at least 70%, at least 90%, at least 150% or more.

[0046] In another preferred embodiment of the disclosure, an increase of a polypeptide activity means an increase of at least 5% of a polypeptide activity using a suitable assay. More preferably, an increase of a polypeptide activity means an increase of at least 10%, even more preferably at least 20%, at least 30%, at least 40%, at least 50%, at least 70%, at least 90%, at least 150% or more. The increase is preferably assessed by comparison to corresponding activity in the individual before treatment.

[0047] A preferred way of providing a source of mIGF1 according to the disclosure is to introduce a transgene encoding mIGF1, preferably an mIGF-1 Ea isoform (as described in 27, human homolog IGF-1 isoform 4: SEQ ID NO: 2), more preferably in an AAV vector as later defined herein. Such source of mIGF1 is specifically expressed in muscle tissue as described in mice in 27.

[0048] In another preferred embodiment of the disclosure, a compound for providing an individual with a functional dystrophin protein is combined with an antioxidant. Oxidative stress is an important factor in the progression of DMD and promotes chronic inflammation and fibrosis[29]. The most prevalent products of oxidative stress, the peroxidized lipids, are increased by an average of 35% in Duchenne boys. Increased levels of the enzymes superoxide dismutase and catalase reduce the excessive amount of free radicals causing these effects. In fact, a dietary supplement *Protandim*® (LifeVantage) was clinically tested and found to increase levels of superoxide dismutase (up to 30%) and catalase (up to 54%), which indeed significantly inhibited the peroxidation of lipids in 29 healthy persons[30]. Such effective management of oxidative stress thus preserves muscle quality and so promotes the positive effect of DMD therapy. Idebenone is another potent antioxidant with a chemical structure derived from natural coenzyme Q10. It protects mitochondria where adenosine triphosphate, ATP, is generated by oxidative phosphorylation. The absence of dystrophin in DMD negatively affects this process in the heart, and probably also in skeletal muscle. Idebenone was recently applied in clinical trials in the US and Europe demonstrating efficacy on neurological aspects of Friedreich's Ataxia[31]. A phase-IIa double-blind, placebo-controlled randomized clinical trial with Idebenone has recently been started in Belgium, including 21 Duchenne boys at 8 to 16 years of age. The primary objective of this study is to determine the effect of Idebenone on heart muscle function. In addition several different tests will be performed to detect the possible functional benefit on muscle strength in the patients. When effective, Idebenone is a preferred adjunct compound for use in a method according to the present disclosure in order to enhance the therapeutic effect of DMD therapy, especially in the heart. One aspect of the present disclosure thus provides a method for alleviating one or more symptom(s) of Duchenne Muscular Dystrophy in an individual, the method comprising administering to said individual an antioxidant and a compound for providing said individual with a functional

dystrophin protein. A combination of an antioxidant and a compound for providing an individual with a functional dystrophin protein for use as a medicament is also provided in the disclosure, as well as a use of an antioxidant and a compound for providing an individual with a functional dystrophin protein for the preparation of a medicament for alleviating one or more symptom(s) of DMD. In one embodiment of the disclosure, said combination is used in order to alleviate one or more symptom(s) of a severe form of BMD wherein a very short dystrophin protein is formed which is not sufficiently functional. Depending on the identity of the antioxidant, the skilled person will know which quantities are preferably used. An antioxidant may include bacoside, silymarin, curcumin, a polyphenol, preferably epigallocatechin-3-gallate (EGCG). Preferably in the disclosure, an antioxidant is a mixture of antioxidants as the dietary supplement *Protandim*® (LifeVantage). A daily capsule of 675mg of *Protandim*® comprises 150 mg of B. monniera (45% bacosides), 225mg of S. marianum (70-80% silymarin), 150 mg of W. somnifera powder, 75mg green tea (98% polyphenols wherein 45% EGCG) and 75mg turmeric (95% curcumin).

[0049] In another preferred embodiment of the disclosure, a compound for providing an individual with a functional dystrophin protein is combined with an ion channel inhibitor. The presence of damaged muscle membranes in DMD disturbs the passage of calcium ions into the myofibers, and the consequently disrupted calcium homeostasis activates many enzymes, e.g. proteases, that cause additional damage and muscle necrosis. Ion channels that directly contribute to the pathological accumulation of calcium in dystrophic muscle are potential targets for adjunct compounds to treat DMD. There is evidence that some drugs, such as pentoxifylline, block exercise-sensitive calcium channels[32] and antibiotics that block stretch activated channels reduce myofibre necrosis in *mdx* mice and CK levels in DMD boys[33]. One embodiment of the disclosure thus provides a method for alleviating one or more symptom(s) of Duchenne Muscular Dystrophy in an individual, the method comprising administering to said individual an ion channel inhibitor and a compound for providing said individual with a functional dystrophin protein. A combination of an ion channel inhibitor and a compound for providing an individual with a functional dystrophin protein for use as a medicament is also provided in the disclosure, as well as a use of an ion channel inhibitor and a compound for providing an individual with a functional dystrophin protein for the preparation of a medicament for alleviating one or more symptom(s) of DMD. In one embodiment of the disclosure, said combination is used in order to alleviate one or more symptom(s) of a severe form of BMD wherein a very short dystrophin protein is formed which is not sufficiently functional.

[0050] Preferably in the disclosure, ion channel inhibitors of the class of xanthines are used. More preferably, said xanthines are derivatives of methylxanthines, and most preferably, said methylxanthine derivates are chosen from the group consisting of pentoxifylline, furafylline, lisofylline, propentofylline, pentifylline, theophylline, torbafylline, albifylline, enprofylline and derivatives thereof. Most preferred is the use of pentoxifylline. Ion channel inhibitors of the class of xanthines enhance the skipping frequency of a dystrophin exon from a pre-MRNA comprising said exon, when using an oligonucleotide directed toward the exon or to one or both splice sites of said exon. The enhanced skipping frequency also increases the level of functional dystrophin protein produced in a muscle cell of a DMD or BMD individual.

[0051] Depending on the identity of the ion channel inhibitor, the skilled person will know which quantities are preferably used. Suitable dosages of pentoxifylline are between about 1 mg/kg/day to about 100 mg/kg/day, preferred dosages are between about 10 mg/kg/day to 50 mg/kg/day. Typical dosages used in humans are 20 mg/kg/day.

[0052] In one embodiment of the disclosure, an ion channel inhibitor is administered to said individual prior to administering a compound for providing an individual with a functional dystrophin protein. In this embodiment, it is preferred that said ion channel inhibitor is administered at least one day, more preferred at least one week, more preferred at least two weeks, more preferred at least three weeks prior to administering a compound for providing said individual with a functional dystrophin protein.

[0053] In another preferred embodiment of the disclosure, a compound for providing an individual with a functional dystrophin protein is combined with a protease inhibitor. Calpains are calcium activated proteases that are increased in dystrophic muscle and account for myofiber degeneration. Calpain inhibitors such as calpastatin, leupeptin[34], calpeptin, calpain inhibitor III, or PD150606 are therefore applied to reduce the degeneration process. A new compound, BN 82270 (Ipsen) that has dual action as both a calpain inhibitor and an antioxidant increased muscle strength, decreased serum CK and reduced fibrosis of the *mdx* diaphragm, indicating a therapeutic effect with this new compound[35]. Another compound of Leupeptin/Carnitine (Myodur) has recently been proposed for clinical trials in DMD patients.

[0054] MG132 is another proteasomal inhibitor that has shown to reduce muscle membrane damage, and to ameliorate the histopathological signs of muscular dystrophy[36]. MG-132 (CBZ-leucyl-leucyl-leucinal) is a cell-permeable, proteasomal inhibitor (Ki=4nM), which inhibits NFkappaB activation by preventing IkappaB degradation (IC50 = 3 μM). In addition, it is a peptide aldehyde that inhibits ubiquitin-mediated proteolysis by binding to and inactivating 20S and 26S proteasomes. MG-132 has shown to inhibit the proteasomal degradation of dystrophin-associated proteins in the dystrophic mdx mouse model[36]. This compound is

thus also suitable for use as an adjunct pharmacological compound for DMD. Further provided in the disclosure is therefore a method for alleviating one or more symptom(s) of Duchenne Muscular Dystrophy in an individual, the method comprising administering to said individual a protease inhibitor and a compound for providing said individual with a functional dystrophin protein. A combination of a protease inhibitor and a compound for providing an individual with a functional dystrophin protein for use as a medicament is also provided in the disclosure, as well as a use of a protease inhibitor and a compound for providing an individual with a functional dystrophin protein for the preparation of a medicament for alleviating one or more symptom(s) of DMD. In one embodiment of the disclosure, said combination is used in order to alleviate one or more symptom(s) of a severe form of BMD wherein a very short dystrophin protein is formed which is not sufficiently functional. Depending on the identity of the protease inhibitor, the skilled person will know which quantities are preferably used.

[0055] In another preferred embodiment of the disclosure, a compound for providing an individual with a functional dystrophin protein is combined with L-arginine. Dystrophin-deficiency is associated with the loss of the DGC-complex at the fiber membranes, including neuronal nitric oxide synthase (nNOS). Expression of a nNOS transgene in *mdx* mice greatly reduced muscle membrane damage. Similarly, administration of L-arginine (the substrate for nitric oxide synthase) increased NO production and upregulated utrophin expression in *mdx* mice. Six weeks of L-arginine treatment improved muscle pathology and decreased serum CK in *mdx* mice[37]. The use of L-arginine as an adjunct therapy in combination with a compound for providing said individual with a functional dystrophin protein has not been disclosed.

[0056] Further provided in the disclosure is therefore a method for alleviating one or more symptom(s) of Duchenne Muscular Dystrophy in an individual, the method comprising administering to said individual L-arginine and a compound for providing said individual with a functional dystrophin protein. A combination of L-arginine and a compound for providing an individual with a functional dystrophin protein for use as a medicament is also provided in the disclosure, as well as a use of L-arginine and a compound for providing an individual with a functional dystrophin protein for the preparation of a medicament for alleviating one or more symptom(s) of DMD. In one embodiment of the disclosure, said combination is used in order to alleviate one or more symptom(s) of a severe form of BMD wherein a very short dystrophin protein is formed which is not sufficiently functional.

[0057] In another preferred embodiment of the disclosure, a compound for providing an individual with a functional dystrophin protein is combined with angiotensin II type 1 receptor blocker Losartan which normalizes muscle architecture, repair and function, as shown in the dystrophin-deficient *mdx* mouse model[23]. One aspect of the present disclosure thus provides a method for alleviating one or more symptom(s) of Duchenne Muscular Dystrophy in an individual, the method comprising administering to said individual angiotensin II type 1 receptor blocker Losartan, and a compound for providing said individual with a functional dystrophin protein. A combination of angiotensin II type 1 receptor blocker Losartan and a compound for providing an individual with a functional dystrophin protein for use as a medicament is also provided in the disclosure, as well as a use of angiotensin II type 1 receptor blocker Losartan and a compound for providing an individual with a functional dystrophin protein for the preparation of a medicament for alleviating one or more symptom(s) of DMD. In one embodiment of the disclosure, said combination is used in order to alleviate one or more symptom(s) of a severe form of BMD wherein a very short dystrophin protein is formed which is not sufficiently functional. Depending on the identity of the angiotensin II type 1 receptor blocker, the skilled person will know which quantities are preferably used.

[0058] In another preferred embodiment of the disclosure, a compound for providing an individual with a functional dystrophin protein is combined with an angiotensin-converting enzyme (ACE) inhibitor, preferably perindopril. ACE inhibitors are capable of lowering blood pressure. Early initiation of treatment with perindopril is associated with a lower mortality in DMD patients[22]. One aspect of the present disclosure thus provides a method for alleviating one or more symptom(s) of Duchenne Muscular Dystrophy in an individual, the method comprising administering to said individual an ACE inhibitor, preferably perindopril, and a compound for providing said individual with a functional dystrophin protein. A combination of an ACE inhibitor, preferably perindopril, and a compound for providing an individual with a functional dystrophin protein for use as a medicament is also provided in the disclosure, as well as a use of an ACE inhibitor, preferably perindopril, and a compound for providing an individual with a functional dystrophin protein for the preparation of a medicament for alleviating one or more symptom(s) of DMD. In one embodiment of the disclosure, said combination is used in order to alleviate one or more symptom(s) of a severe form of BMD wherein a very short dystrophin protein is formed which is not sufficiently functional. The usual doses of an ACE inhibitor, preferably perindopril are about 2 to 4 mg/day[22].

[0059] In a more preferred embodiment of the disclosure, an ACE inhibitor is combined with at least one of the previously identified adjunct compounds.

[0060] In another preferred embodiment of the disclosure, a compound for providing an individual with a functional dystrophin protein is combined with a compound which is capable of enhancing exon skipping and/or inhibiting spliceosome assembly and/or

splicing. Small chemical compounds, such as for instance specific indole derivatives, have been shown to selectively inhibit spliceosome assembly and splicing[38], for instance by interfering with the binding of serine- and arginine-rich (SR) proteins to their cognate splicing enhancers (ISEs or ESEs) and/or by interfering with the binding of splicing repressors to silencer sequences (ESSs or ISSs). These compounds are therefore suitable for applying as adjunct compounds that enhance exon skipping.

[0061] Further provided by the disclosure is therefore a method for alleviating one or more symptom(s) of Duchenne Muscular Dystrophy in an individual, the method comprising administering to said individual a compound for enhancing exon skipping and/or inhibiting spliceosome assembly and/or splicing, and a compound for providing said individual with a functional dystrophin protein. A combination of a compound for enhancing exon skipping and/or inhibiting spliceosome assembly and/or splicing and a compound for providing an individual with a functional dystrophin protein for use as a medicament is also provided in the disclosure, as well as a use of a compound for enhancing exon skipping and/or inhibiting spliceosome assembly and/or splicing and a compound for providing an individual with a functional dystrophin protein for the preparation of a medicament for alleviating one or more symptom(s) of DMD. In one embodiment of the disclosure, said combination is used in order to alleviate one or more symptom(s) of a severe form of BMD wherein a very short dystrophin protein is formed which is not sufficiently functional. Depending on the identity of the compound which is capable of enhancing exon skipping and/or inhibiting spliceosome assembly and/or splicing, the skilled person will know which quantities are preferably used. In a more preferred embodiment of the disclosure, a compound for enhancing exon skipping and/or inhibiting spliceosome assembly and/or splicing is combined with a ACE inhibitor and/or with any adjunct compounds as identified earlier herein.

[0062] A pharmaceutical preparation comprising a compound for providing an individual with a functional dystrophin protein, any of the above mentioned adjunct compounds, and a pharmaceutically acceptable carrier, filler, preservative, adjuvant, solubilizer, diluent and/or excipient is also provided. Such pharmaceutically acceptable carrier, filler, preservative, adjuvant, solubilizer, diluent and/or excipient may for instance be found in Remington: The Science and Practice of Pharmacy, 20th Edition. Baltimore, MD: Lippincott Williams & Wilkins, 2000.

[0063] Therefore, here disclosed is a method, combination, use or pharmaceutical preparation according to the invention, wherein said adjunct compound comprises a steroid, an ACE inhibitor (preferably perindopril), angiotensin II type 1 receptor blocker Losartan, a tumour necrosis factor-alpha (TNFα) inhibitor, a source of mIGF-1, preferably mIGF-1, a compound for enhancing mIGF-1 expression, a compound for enhancing mIGF-1 activity, an antioxidant, an ion channel inhibitor, a protease inhibitor, L-arginine and/or a compound for enhancing exon skipping and/or inhibiting spliceosome assembly and/or splicing.
As described in the disclosure before, an individual is provided with a functional dystrophin protein in various ways, for instance by stop codon suppression by gentamycin or PTC124[16, 17], or by adeno-associated virus (AAV)-mediated gene delivery of a functional mini- or micro-dystrophin gene[18-20].

[0064] Preferably, however, said compound for providing said individual with a functional dystrophin protein comprises an oligonucleotide, or a functional equivalent thereof, for at least in part decreasing the production of an aberrant dystrophin protein in said individual. Decreasing the production of an aberrant dystrophin mRNA, or aberrant dystrophin protein, preferably means that 90%, 80%, 70%, 60%, 50%, 40%, 30%, 20%, 10%, 5% or less of the initial amount of aberrant dystrophin mRNA, or aberrant dystrophin protein, is still detectable by RT PCR (mRNA) or immunofluorescence or western blot analysis (protein). An aberrant dystrophin mRNA or protein is also referred to herein as a non-functional dystrophin mRNA or protein. A non functional dystrophin protein is preferably a dystrophin protein which is not able to bind actin and/or members of the DGC protein complex. A non-functional dystrophin protein or dystrophin mRNA does typically not have, or does not encode a dystrophin protein with an intact C-terminus of the protein. Said oligonucleotide preferably comprises an antisense oligoribonucleotide. In a preferred embodiment an exon skipping technique is applied. Exon skipping interferes with the natural splicing processes occurring within a eukaryotic cell. In higher eukaryotes the genetic information for proteins in the DNA of the cell is encoded in exons which are separated from each other by intronic sequences. These introns are in some cases very long. The transcription machinery of eukaryotes generates a pre-mRNA which contains both exons and introns, while the splicing machinery, often already during the production of the pre-mRNA, generates the actual coding region for the protein by splicing together the exons present in the pre-mRNA.

[0065] Exon-skipping results in mature mRNA that lacks at least one skipped exon. Thus, when said exon codes for amino acids, exon skipping leads to the expression of an altered product. Technology for exon-skipping is currently directed towards the use of antisense oligonucleotides (AONs). Much of this work is done in the *mdx* mouse model for Duchenne muscular dystrophy. The *mdx* mouse, which carries a nonsense mutation in exon 23 of the dystrophin gene, has been used as an animal model of DMD. Despite the *mdx* mutation, which should preclude the synthesis of a functional dystrophin protein, rare, naturally occurring dystrophin positive fibers have been observed in *mdx* muscle tissue. These dystrophin-positive fibers are thought to have arisen from an apparently naturally occurring exon-skipping mechanism, either due to somatic mutations or through alternative splicing. AONs directed to, respectively, the 3' and/or 5' splice sites of introns 22 and 23 in dystrophin pre-mRNA, have been shown to

interfere with factors normally involved in removal of intron 23 so that also exon 23 was removed from the mRNA[3, 5, 6, 39, 40].

[0066] By the targeted skipping of a specific exon, a DMD phenotype is converted into a milder BMD phenotype. The skipping of an exon is preferably induced by the binding of AONs targeting either one or both of the splice sites, or exon-internal sequences. An oligonucleotide directed toward an exon internal sequence typically exhibits no overlap with non-exon sequences. It preferably does not overlap with the splice sites at least not insofar as these are present in the intron. An oligonucleotide directed toward an exon internal sequence preferably does not contain a sequence complementary to an adjacent intron. Further provided is thus a method, combination, use or pharmaceutical preparation according to the invention, wherein said compound for providing said individual with a functional dystrophin protein comprises an oligonucleotide, or a functional equivalent thereof, for inhibiting inclusion of an exon of a dystrophin pre-mRNA into mRNA produced from splicing of said pre-mRNA. An exon skipping technique is preferably applied such that the absence of an exon from mRNA produced from dystrophin pre-mRNA generates a coding region for a functional - albeit shorter - dystrophin protein. In this context, inhibiting inclusion of an exon preferably means that the detection of the original, aberrant dystrophin mRNA is decreased of at least about 10% as assessed by RT-PCR or that a corresponding aberrant dystrophin protein is decreased of at least about 10% as assessed by immunofluorescence or western blot analysis. The decrease is preferably of at least 20%, 30%, 40%, 50%, 60%, 70%, 80%, 90% or 100%.

[0067] Once a DMD patient is provided with a functional dystrophin protein, the cause of DMD is taken away. Hence, it would then be expected that the symptoms of DMD are sufficiently alleviated. However, as already described before, the present invention provides the insight that, even though exon skipping techniques are capable of providing a functional dystrophin protein, a symptom of DMD is still further alleviated by administering to a DMD patient an adjunct compound for reducing inflammation, preferably for reducing muscle tissue inflammation, and/or an adjunct compound for improving muscle fiber function, integrity and/or survival. Moreover, the present invention provides the insight that an adjunct therapy counteracting inflammation does not negatively influence AON therapy. The present invention further provides the insight that the skipping frequency of a dystrophin exon from a pre-MRNA comprising said exon is enhanced, when using an oligonucleotide directed toward the exon or to one or both splice sites of said exon. The enhanced skipping frequency also increases the level of functional dystrophin protein produced in a muscle cell of a DMD or BMD individual.

[0068] Since an exon of a dystrophin pre-mRNA will only be included into the resulting mRNA when both the splice sites are recognised by the spliceosome complex, splice sites are obvious targets for AONs. One embodiment of the present disclosure therefore provides a method, combination, use or pharmaceutical preparation, wherein said compound for providing said individual with a functional dystrophin protein comprises an oligonucleotide, or a functional equivalent thereof, comprising a sequence which is complementary to a non-exon region of a dystrophin pre mRNA. In one embodiment of the disclosure, an AON is used which is solely complementary to a non-exon region of a dystrophin pre mRNA. This is however not necessary: it is also possible to use an AON which comprises an intron-specific sequence as well as exon-specific sequence. Such AON comprises a sequence which is complementary to a non-exon region of a dystrophin pre mRNA, as well as a sequence which is complementary to an exon region of a dystrophin pre mRNA. Of course, an AON is not necessarily complementary to the entire sequence of a dystrophin exon or intron. AONs which are complementary to a part of such exon or intron are preferred. An AON is preferably complementary to at least part of a dystrohin exon and/or intron, said part having at least 13 nucleotides.

[0069] Splicing of a dystrophin pre-mRNA occurs via two sequential transesterification reactions. First, the 2'OH of a specific branch-point nucleotide within the intron that is defined during spliceosome assembly performs a nucleophilic attack on the first nucleotide of the intron at the 5' splice site forming the lariat intermediate. Second, the 3'OH of the released 5' exon then performs a nucleophilic attack at the last nucleotide of the intron at the 3' splice site thus joining the exons and releasing the intron lariat. The branch point and splice sites of an intron are thus involved in a splicing event. Hence, an oligonucleotide comprising a sequence which is complementary to such branch point and/or splice site is preferably used for exon skipping. Further provided is therefore a method, combination, use or pharmaceutical preparation according to the disclosure, wherein said compound for providing said individual with a functional dystrophin protein comprises an oligonucleotide, or a functional equivalent thereof, comprising a sequence which is complementary to a splice site and/or branch point of a dystrophin pre mRNA.

[0070] Since splice sites contain consensus sequences, the use of an oligonucleotide or a functional equivalent thereof (herein also called an AON) comprising a sequence which is complementary of a splice site involves the risk of promiscuous hybridization. Hybridization of AONs to other splice sites than the sites of the exon to be skipped could easily interfere with the accuracy of the splicing process. To overcome these and other potential problems related to the use of AONs which are complementary to an intron sequence, one preferred embodiment of the disclosure provides a method, combination, use or pharmaceutical preparation, wherein said compound for providing said individual with a functional dystrophin protein comprises an oligonucleotide, or a functional equivalent thereof, comprising a sequence which is complementary to a dystrophin pre-mRNA exon. Preferably, said AON is capable of specifically inhibiting an exon inclusion signal of at least one exon in said dystrophin pre-

mRNA. Interfering with an exon inclusion signal (EIS) has the advantage that such elements are located within the exon. By providing an AON for the interior of the exon to be skipped, it is possible to interfere with the exon inclusion signal thereby effectively masking the exon from the splicing apparatus. The failure of the splicing apparatus to recognize the exon to be skipped thus leads to exclusion of the exon from the final mRNA. This embodiment does not interfere directly with the enzymatic process of the splicing machinery (the joining of the exons). It is thought that this allows the method to be more specific and/or reliable. It is thought that an EIS is a particular structure of an exon that allows splice acceptor and donor to assume a particular spatial conformation. In this concept it is the particular spatial conformation that enables the splicing machinery to recognize the exon. However, the disclosure is certainly not limited to this model. It has been found that agents capable of binding to an exon are capable of inhibiting an EIS. An AON may specifically contact said exon at any point and still be able to specifically inhibit said EIS.

[0071] Using exon-internal AONs specific for an exon 46 sequence, we were previously able to modulate the splicing pattern in cultured myotubes from two different DMD patients with an exon 45 deletion[11]. Following AON treatment, exon 46 was skipped, which resulted in a restored reading frame and the induction of dystrophin synthesis in at least 75% of the cells. We have recently shown that exon skipping can also efficiently be induced in human control and series of patients with different mutations, including deletions, duplications and point mutations, for 39 different DMD exons using exon-internal AONs[1, 2, 11-15].

[0072] A functional equivalent of an oligonucleotide preferably means an oligonucleotide as defined herein wherein one or more nucleotides have been substituted and wherein an activity of said functional equivalent is retained to at least some extent. Preferably, an activity of said functional equivalent is providing a functional dystrophin protein. Said activity of said functional equivalent is therefore preferably assessed by quantifying the amount of a functional dystrophin protein. A functional dystrophin is herein preferably defined as being a dystrophin able to bind actin and members of the DGC protein complex. The assessment of said activity of an oligonucleotide is preferably done by RT-PCR or by immunofluorescence or Western blot analyses. Said activity is preferably retained to at least some extent when it represents at least 50%, or at least 60%, or at least 70% or at least 80% or at least 90% or at least 95% or more of corresponding activity of said oligonucleotide the functional equivalent derives from. Throughout this application, when the word oligonucleotide is used it may be replaced by a functional equivalent thereof as defined herein.

[0073] Hence, the use of an oligonucleotide, or a functional equivalent thereof, comprising or consisting of a sequence which is complementary to a dystrophin pre-mRNA exon provides good anti-DMD results. An oligonucleotide, or a functional equivalent thereof, is used which comprises or consists of a sequence which is complementary to at least part of dystrophin pre-mRNA exon 2, 8, 9, 17, 19, 29, 40-46, 48-53, 55 or 59, said part having at least 13 nucleotides. However, said part may also have at least 14, 15, 16, 17 , 18, 19, 20, 21, 22, 23, 24, 25, 26, 27 , 28 , 29, 30 nucleotides.

[0074] Most preferably in the disclosure an AON is used which comprises or consists of a sequence which is complementary to at least part of dystrophin pre-mRNA exon 51, 44, 45, 53, 46, 43, 2, 8, 50 and/or 52, said part having at least 13 nucleotides. However, said part may also have at least 14, 15, 16, 17 , 18, 19, 20, 21, 22, 23, 24, 25, 26, 27 , 28 , 29, 30 nucleotides. Most preferred oligonucleotides of the disclosure are identified by each of the following sequences SEQ ID NO: 3 to SEQ ID NO: 284. Accordingly, a most preferred oligonucleotide as used in the disclosure is represented by a sequence from SEQ ID NO:3 to SEQ ID NO:284. A most preferred oligonucleotide as used in the disclosure is selected from the group consisting of SEQ ID NO:3 to NO:284. The antisense oligonucleotide of the invention comprises a sequence which is complementary to a part of human dystrophin pre-mRNA exon 51 and said oligonucleotide is represented by SEQ ID NO:204.

[0075] Said exons are listed in decreasing order of patient population applicability. Hence, the use of an AON comprising a sequence which is complementary to at least part of dystrophin pre-mRNA exon 51 is suitable for use in a larger part of the DMD patient population as compared to an AON comprising a sequence which is complementary to dystrophin pre-mRNA exon 44, et cetera.

[0076] In a preferred embodiment, an oligonucleotide of the disclosure which comprises a sequence that is complementary to part of dystrophin pre-mRNA is such that the complementary part is at least 50% of the length of the oligonucleotide of the invention, more preferably at least 60%, even more preferably at least 70%, even more preferably at least 80%, even more preferably at least 90% or even more preferably at least 95%, or even more preferably 98% or more. In a most preferred embodiment of the disclosure, the oligonucleotide consists of a sequence that is complementary to part of dystrophin pre-mRNA as defined herein. For example, an oligonucleotide may comprise a sequence that is complementary to part of dystrophin pre-mRNA as defined herein and additional flanking sequences. In a more preferred embodiment of the disclosure, the length of said complementary part of said oligonucleotide is of at least 13, 14, 15, 16, 17 , 18, 19, 20, 21, 22, 23, 24, 25, 26, 27 , 28 , 29, 30 nucleotides. Preferably in the disclosure, additional flanking sequences are used to modify the binding of a protein to the oligonucleotide, or to modify a thermodynamic property of the oligonucleotide, more preferably to modify target RNA binding

affinity.

[0077] One preferred embodiment of the disclosure provides a method, combination, use or pharmaceutical preparation according to the invention, wherein said compound for providing said individual with a functional dystrophin protein comprises an oligonucleotide, or a functional equivalent thereof, which comprises:

- a sequence which is complementary to a region of a dystrophin pre-mRNA exon that is hybridized to another part of a dystrophin pre-mRNA exon (closed structure), and
- a sequence which is complementary to a region of a dystrophin pre-mRNA exon that is not hybridized in said dystrophin pre-mRNA (open structure).

[0078] For this embodiment, reference is made to our WO 2004/083432 patent application. RNA molecules exhibit strong secondary structures, mostly due to base pairing of complementary or partly complementary stretches within the same RNA. It has long since been thought that structures in the RNA play a role in the function of the RNA. Without being bound by theory, it is believed that the secondary structure of the RNA of an exon plays a role in structuring the splicing process. Through its structure, an exon is recognized as a part that needs to be included in the mRNA. Herein this signalling function is referred to as an exon inclusion signal. A complementary oligonucleotide of this embodiment is capable of interfering with the structure of the exon and thereby capable of interfering with the exon inclusion signal of the exon. It has been found that many complementary oligonucleotides indeed comprise this capacity, some more efficient than others. Oligonucleotides of this preferred embodiment, i.e. those with the said overlap directed towards open and closed structures in the native exon RNA, are a selection from all possible oligonucleotides. The selection encompasses oligonucleotides that can efficiently interfere with an exon inclusion signal. Without being bound by theory it is thought that the overlap with an open structure improves the invasion efficiency of the oligonucleotide (i.e. increases the efficiency with which the oligonucleotide can enter the structure), whereas the overlap with the closed structure subsequently increases the efficiency of interfering with the secondary structure of the RNA of the exon, and thereby interfere with the exon inclusion signal. It is found that the length of the partial complementarity to both the closed and the open structure is not extremely restricted. We have observed high efficiencies with oligonucleotides with variable lengths of complementarity in either structure. The term complementarity is used herein to refer to a stretch of nucleic acids that can hybridise to another stretch of nucleic acids under physiological conditions. It is thus not absolutely required that all the bases in the region of complementarity are capable of pairing with bases in the opposing strand. For instance, when designing the oligonucleotide one may want to incorporate for instance a residue that does not base pair with the base on the complementary strand. Mismatches may to some extent be allowed, if under the circumstances in the cell, the stretch of nucleotides is capable of hybridising to the complementary part. In a preferred embodiment of the disclosure a complementary part (either to said open or to said closed structure) comprises at least 3, and more preferably at least 4 consecutive nucleotides. The complementary regions are preferably designed such that, when combined, they are specific for the exon in the pre-mRNA. Such specificity may be created with various lengths of complementary regions as this depends on the actual sequences in other (pre-)mRNA in the system. The risk that also one or more other pre-mRNA will be able to hybridise to the oligonucleotide decreases with increasing size of the oligonucleotide. It is clear that oligonucleotides comprising mismatches in the region of complementarity but that retain the capacity to hybridise to the targeted region(s) in the pre-mRNA, can be used.
However, preferably at least the complementary parts do not comprise such mismatches as these typically have a higher efficiency and a higher specificity, than oligonucleotides having such mismatches in one or more complementary regions. It is thought that higher hybridisation strengths, (i.e. increasing number of interactions with the opposing strand) are favourable in increasing the efficiency of the process of interfering with the splicing machinery of the system. Preferably, the complementarity is between 90 and 100%. In general this allows for approximately 1 or 2 mismatch(es) in an oligonucleotide of around 20 nucleotides

[0079] The secondary structure is best analysed in the context of the pre-mRNA wherein the exon resides. Such structure may be analysed in the actual RNA. However, it is currently possible to predict the secondary structure of an RNA molecule (at lowest energy costs) quite well using structure-modelling programs. A non-limiting example of a suitable program is RNA mfold version 3.1 server[41]. A person skilled in the art will be able to predict, with suitable reproducibility, a likely structure of the exon, given the nucleotide sequence. Best predictions are obtained when providing such modelling programs with both the exon and flanking intron sequences. It is typically not necessary to model the structure of the entire pre-mRNA.

[0080] The open and closed structure to which the oligonucleotide is directed, are preferably adjacent to one another. It is thought that in this way the annealing of the oligonucleotide to the open structure induces opening of the closed structure whereupon annealing progresses into this closed structure. Through this action the previously closed structure assumes a different conformation. The different conformation results in the disruption of the exon inclusion signal. However, when potential (cryptic) splice acceptor and/or donor sequences are present within the targeted exon, occasionally a new exon inclusion signal is

generated defining a different (neo) exon, i.e. with a different 5' end, a different 3' end, or both. This type of activity is within the scope of the present disclosure as the targeted exon is excluded from the mRNA. The presence of a new exon, containing part of the targeted exon, in the mRNA does not alter the fact that the targeted exon, as such, is excluded.
The inclusion of a neo-exon can be seen as a side effect which occurs only occasionally. There are two possibilities when exon skipping is used to restore (part of) an open reading frame of dystrophin that is disrupted as a result of a mutation. One is that the neo-exon is functional in the restoration of the reading frame, whereas in the other case the reading frame is not restored. When selecting oligonucleotides for restoring dystrophin reading frames by means of exon-skipping it is of course clear that under these conditions only those oligonucleotides are selected that indeed result in exon-skipping that restores the dystrophin open reading frame, with or without a neo-exon.

[0081] Further provided is a method, combination, use or pharmaceutical preparation according to the disclosure, wherein said compound for providing said individual with a functional dystrophin protein comprises an oligonucleotide, or a functional equivalent thereof, which comprises a sequence that is complementary to a binding site for a serine-arginine (SR) protein in RNA of an exon of a dystrophin pre-mRNA. In our WO 2006/112705 patent application we have disclosed the presence of a correlation between the effectivity of an exon-internal antisense oligonucleotide (AON) in inducing exon skipping and the presence of a (for example by ESEfinder) predicted SR binding site in the target pre-mRNA site of said AON. Therefore, in one embodiment of the disclosure an oligonucleotide is generated comprising determining a (putative) binding site for an SR (Ser-Arg) protein in RNA of a dystrophin exon and producing an oligonucleotide that is complementary to said RNA and that at least partly overlaps said (putative) binding site. The term "at least partly overlaps" is defined herein as to comprise an overlap of only a single nucleotide of an SR binding site as well as multiple nucleotides of said binding site as well as a complete overlap of said binding site. This embodiment preferably further comprises determining from a secondary structure of said RNA, a region that is hybridised to another part of said RNA (closed structure) and a region that is not hybridised in said structure (open structure), and subsequently generating an oligonucleotide that at least partly overlaps said (putative) binding site and that overlaps at least part of said closed structure and overlaps at least part of said open structure. In this way we increase the chance of obtaining an oligonucleotide that is capable of interfering with the exon inclusion from the pre-mRNA into mRNA. It is possible that a first selected SR-binding region does not have the requested open-closed structure in which case another (second) SR protein binding site is selected which is then subsequently tested for the presence of an open-closed structure. This process is continued until a sequence is identified which contains an SR protein binding site as well as a(n) (partly overlapping) open-closed structure. This sequence is then used to design an oligonucleotide which is complementary to said sequence.

[0082] Such a method for generating an oligonucleotide is also performed by reversing the described order, i.e. first generating an oligonucleotide comprising determining, from a secondary structure of RNA from a dystrophin exon, a region that assumes a structure that is hybridised to another part of said RNA (closed structure) and a region that is not hybridised in said structure (open structure), and subsequently generating an oligonucleotide, of which at least a part of said oligonucleotide is complementary to said closed structure and of which at least another part of said oligonucleotide is complementary to said open structure. This is then followed by determining whether an SR protein binding site at least overlaps with said open/closed structure. In this way the method of WO 2004/083432 is improved. In yet another embodiment of the disclosure the selections are performed simultaneously.

[0083] Without wishing to be bound by any theory it is currently thought that use of an oligonucleotide directed to an SR protein binding site results in (at least partly) impairing the binding of an SR protein to the binding site of an SR protein which results in disrupted or impaired splicing.

[0084] Preferably, an open/closed structure and an SR protein binding site partly overlap and even more preferred an open/closed structure completely overlaps an SR protein binding site or an SR protein binding site completely overlaps an open/closed structure. This allows for an improved disruption of exon inclusion.

[0085] Besides consensus splice sites sequences, many (if not all) exons contain splicing regulatory sequences such as exonic splicing enhancer (ESE) sequences to facilitate the recognition of genuine splice sites by the spliceosome[42, 43]. A subgroup of splicing factors, called the SR proteins, can bind to these ESEs and recruit other splicing factors, such as U1 and U2AF to (weakly defined) splice sites. The binding sites of the four most abundant SR proteins (SF2/ASF, SC35, SRp40 and SRp55) have been analyzed in detail and these results are implemented in ESEfinder, a web source that predicts potential binding sites for these SR proteins[42, 43]. There is a correlation between the effectiveness of an AON and the presence/absence of an SF2/ASF, SC35 and SRp40 binding site. In a preferred embodiment, the disclosure, thus provides a method, combination, use or pharmaceutical preparation as described above, wherein said SR protein is SF2/ASF or SC35 or SRp40.

[0086] In one embodiment of the disclosure, a DMD patient is provided with a functional dystrophin protein by using an

oligonucleotide, or a functional equivalent thereof, which is capable of specifically binding a regulatory RNA sequence which is required for the correct splicing of a dystrophin exon in a transcript. Several cis-acting RNA sequences are required for the correct splicing of exons in a transcript. In particular, supplementary elements such as intronic or exonic splicing enhancers (ISEs and ESEs) or silencers (ISSs and ESEs) are identified to regulate specific and efficient splicing of constitutive and alternative exons. Using sequence-specific antisense oligonucleotides (AONs) that bind to the elements, their regulatory function is disturbed so that the exon is skipped, as shown for DMD. Hence, in one preferred embodiment of the disclosure, an oligonucleotide or functional equivalent thereof is used which is complementary to an intronic splicing enhancer (ISE), an exonic splicing enhancer (ESE), an intronic splicing silencer (ISS) and/or an exonic splicing silencer (ESS). As already described herein before, a dystrophin exon is in one preferred embodiment skipped by an agent capable of specifically inhibiting an exon inclusion signal of said exon, so that said exon is not recognized by the splicing machinery as a part that needs to be included in the mRNA. As a result, a mRNA without said exon is formed.

[0087] An AON used in a method of the present disclosure is preferably complementary to a consecutive part of between 13 and 50 nucleotides of dystrophin exon RNA or dystrophin intron RNA. In one embodiment an AON used in a method of the present disclosure is complementary to a consecutive part of between 16 and 50 nucleotides of a dystrophin exon RNA or dystrophin intron RNA. Preferably, said AON is complementary to a consecutive part of between 15 and 25 nucleotides of said exon RNA. More preferably, an AON is used which comprises a sequence which is complementary to a consecutive part of between 20 and 25 nucleotides of a dystrophin exon RNA or a dystrophin intron RNA.

[0088] Different types of nucleic acid may be used to generate the oligonucleotide. Preferably, said oligonucleotide comprises RNA, as RNA/RNA hybrids are very stable. Since one of the aims of the exon skipping technique is to direct splicing in subjects it is preferred that the oligonucleotide RNA comprises a modification providing the RNA with an additional property, for instance resistance to endonucleases and RNaseH, additional hybridisation strength, increased stability (for instance in a bodily fluid), increased or decreased flexibility, reduced toxicity, increased intracellular transport, tissue-specificity, etc. Preferably said modification comprises a 2'-*O*-methyl-phosphorothioate oligoribonucleotide modification. Preferably said modification comprises a 2'-*O*-methyl-phosphorothioate oligodeoxyribonucleotide modification. One embodiment thus provides a method, combination, use or pharmaceutical preparation according to the invention, wherein an oligonucleotide is used which comprises RNA which contains a modification, preferably a 2'-*O*-methyl modified ribose (RNA) or deoxyribose (DNA) modification.

[0089] In one embodiment the disclosure provides a hybrid oligonucleotide comprising an oligonucleotide comprising a 2'-*O*-methyl-phosphorothioate oligo(deoxy)ribonucleotide modification and locked nucleic acid. This particular combination comprises better sequence specificity compared to an equivalent consisting of locked nucleic acid, and comprises improved effectivity when compared with an oligonucleotide consisting of 2'-*O*-methyl-phosphorothioate oligo(deoxy)ribonucleotide modification.

[0090] With the advent of nucleic acid mimicking technology it has become possible to generate molecules that have a similar, preferably the same hybridisation characteristics in kind not necessarily in amount as nucleic acid itself. Such functional equivalents are of course also suitable for use in a method of the disclosure.

[0091] Preferred examples of functional equivalents of an oligonucleotide are peptide nucleic acid and/or locked nucleic acid. Most preferably, a morpholino phosphorodiamidate is used. Suitable but non-limiting examples of equivalents of oligonucleotides of the invention can be found in[44-50]. Hybrids between one or more of the equivalents among each other and/or together with nucleic acid are of course also suitable. In a preferred embodiment of the disclosure, locked nucleic acid is used as a functional equivalent of an oligonucleotide, as locked nucleic acid displays a higher target affinity and reduced toxicity and therefore shows a higher efficiency of exon skipping.

[0092] In one embodiment of the disclosure an oligonucleotide, or a functional equivalent thereof, which is capable of inhibiting inclusion of a dystrophin exon into dystrophin mRNA is combined with at least one other oligonucleotide, or functional equivalent thereof, that is capable of inhibiting inclusion of another dystrophin exon into dystrophin mRNA. This way, inclusion of two or more exons of a dystrophin pre-mRNA in mRNA produced from this pre-mRNA is prevented. This embodiment of the disclosure is further referred to as double- or multi-exon skipping[2,15]. In most cases double-exon skipping results in the exclusion of only the two targeted exons from the dystrophin pre-mRNA. However, in other cases it was found that the targeted exons and the entire region in between said exons in said pre-mRNA were not present in the produced mRNA even when other exons (intervening exons) were present in such region. This multi-skipping was notably so for the combination of oligonucleotides derived from the DMD gene, wherein one oligonucleotide for exon 45 and one oligonucleotide for exon 51 was added to a cell transcribing the DMD gene. Such a set-up resulted in mRNA being produced that did not contain exons 45 to 51. Apparently, the structure of the pre-mRNA in the presence of the mentioned oligonucleotides was such that the splicing machinery was stimulated to connect exons 44 and 52 to each other.

[0093] Further provided is therefore a method, combination, use or pharmaceutical preparation according to the disclosure, wherein a nucleotide sequence is used which comprises at least 8, preferably between 16 to 80, consecutive nucleotides that are complementary to a first exon of a dystrophin pre-mRNA and wherein a nucleotide sequence is used which comprises at least 8, preferably between 16 to 80, consecutive nucleotides that are complementary to a second exon of said dystrophin pre-mRNA.

[0094] In one preferred embodiment of the disclosure said first and said second exon are separated in said dystrophin pre-mRNA by at least one exon to which said oligonucleotide is not complementary.

[0095] According to the disclosure, it is possible to specifically promote the skipping of also the intervening exons by providing a linkage between the two complementary oligonucleotides. Hence, in one embodiment stretches of nucleotides complementary to at least two dystrophin exons are separated by a linking moiety. The at least two stretches of nucleotides are thus linked in this embodiment so as to form a single molecule. Further provided is therefore a method, combination, use or pharmaceutical preparation according to the disclosure wherein said oligonucleotide, or functional equivalent thereof, for providing said individual with a functional dystrophin protein is complementary to at least two exons in a dystrophin pre-mRNA, said oligonucleotide or functional equivalent comprising at least two parts wherein a first part comprises an oligonucleotide having at least 8, preferably between 16 to 80, consecutive nucleotides that are complementary to a first of said at least two exons and wherein a second part comprises an oligonucleotide having at least 8, preferably between 16 to 80, consecutive nucleotides that are complementary to a second exon in said dystrophin pre-mRNA. According to the disclosure, the linkage may be through any means but is preferably accomplished through a nucleotide linkage. In the latter case the number of nucleotides that do not contain an overlap between one or the other complementary exon can be zero, but is preferably between 4 to 40 nucleotides. The linking moiety can be any type of moiety capable of linking oligonucleotides. Preferably in the disclosure, said linking moiety comprises at least 4 uracil nucleotides. Currently, many different compounds are available that mimic hybridisation characteristics of oligonucleotides. Such a compound, called herein a functional equivalent of an oligonucleotide, is also suitable for the present disclosure if such equivalent comprises similar hybridisation characteristics in kind not necessarily in amount. Suitable functional equivalents are mentioned earlier in this description. As mentioned, oligonucleotides of the disclosure do not have to consist of only oligonucleotides that contribute to hybridisation to the targeted exon. There may be additional material and/or nucleotides added.

[0096] The DMD gene is a large gene, with many different exons. Considering that the gene is located on the X-chromosome, it is mostly boys that are affected, although girls can also be affected by the disease, as they may receive a bad copy of the gene from both parents, or are suffering from a particularly biased inactivation of the functional allele due to a particularly biased X chromosome inactivation in their muscle cells. The protein is encoded by a plurality of exons (79) over a range of at least 2,6 Mb. Defects may occur in any part of the DMD gene. Skipping of a particular exon or particular exons can, very often, result in a restructured mRNA that encodes a shorter than normal but at least partially functional dystrophin protein. A practical problem in the development of a medicament based on exon-skipping technology is the plurality of mutations that may result in a deficiency in functional dystrophin protein in the cell. Despite the fact that already multiple different mutations can be corrected for by the skipping of a single exon, this plurality of mutations, requires the generation of a large number of different pharmaceuticals as for different mutations different exons need to be skipped. An advantage of a compound capable of inducing skipping of two or more exons, is that more than one exon can be skipped with a single pharmaceutical. This property is not only practically very useful in that only a limited number of pharmaceuticals need to be generated for treating many different DMD or particular, severe BMD mutations. Another option now open to the person skilled in the art is to select particularly functional restructured dystrophin proteins and produce compounds capable of generating these preferred dystrophin proteins. Such preferred end results are further referred to as mild phenotype dystrophins.

[0097] Each compound, an oligonucleotide and/or an adjunct compound as defined herein for use according to the present disclosure may be suitable for direct administration to a cell, tissue and/or an organ *in vivo* of individuals affected by or at risk of developing DMD or BMD, and may be administered directly *in vivo*, *ex vivo* or *in vitro*.

[0098] Alternatively, suitable means for providing cells with an oligonucleotide or equivalent thereof are present in the art. An oligonucleotide or functional equivalent thereof may for example be provided to a cell in the form of an expression vector wherein the expression vector encodes a transcript comprising said oligonucleotide. The expression vector is preferably introduced into the cell via a gene delivery vehicle. A preferred delivery vehicle is a viral vector such as an adeno-associated virus vector (AAV) or a retroviral vector such as a lentivirus vector[4, 51, 52] and the like. Also plasmids, artificial chromosomes, plasmids suitable for targeted homologous recombination and integration in the human genome of cells may be suitably applied for delivery of an oligonucleotide as defined herein. Preferred for the current disclosure are those vectors wherein transcription is driven from PolIII promoters, and/or wherein transcripts are in the form fusions with U1 or U7 transcripts, which yield good results for delivering small transcripts. It is within the skill of the artisan to design suitable transcripts. Preferred are PolIII driven transcripts. Preferably

in the form of a fusion transcript with an Ulor U7 transcript[4, 51, 52]. Such fusions may be generated as described[53, 54]. The oligonucleotide may be delivered as is. However, the oligonucleotide may also be encoded by the viral vector. Typically this is in the form of an RNA transcript that comprises the sequence of the oligonucleotide in a part of the transcript.

[0099] Improvements in means for providing cells with an oligonucleotide or equivalent thereof, are anticipated considering the progress that has already thus far been achieved. Such future improvements may of course be incorporated to achieve the mentioned effect on restructuring of mRNA using a method of the disclosure.

[0100] The oligonucleotide or equivalent thereof can be delivered as is to the cells. When administering the oligonucleotide or equivalent thereof to an individual, it is preferred that the oligonucleotide is dissolved in a solution that is compatible with the delivery method. For intravenous, subcutaneous, intramuscular, intrathecal and/or intraventricular administration it is preferred that the solution is a physiological salt solution. Particularly preferred for a method of the disclosure is the use of an excipient that will aid in delivery of a compound as defined herein, preferably an oligonucleotide and optionally together with an adjunct compound to a cell and into a cell, preferably a muscle cell. Preferred are excipients capable of forming complexes, vesicles and/or liposomes that deliver such a compound as defined herein, preferably an oligonucleotide and optionally together with an adjunct compound complexed or trapped in a vesicle or liposome through a cell membrane. Many of these excipients are known in the art. Suitable excipients comprise polyethylenimine (PEI), or similar cationic polymers, including polypropyleneimine or polyethylenimine copolymers (PECs) and derivatives, ExGen 500, synthetic amphiphils (SAINT-18), lipofectinTM, DOTAP and/or viral capsid proteins that are capable of self assembly into particles that can deliver such compounds, preferably an oligonucleotide and optionally together with an adjunct compound as defined herein to a cell, preferably a muscle cell. Such excipients have been shown to efficiently deliver (oligonucleotide such as antisense) nucleic acids to a wide variety of cultured cells, including muscle cells. Their high transfection potential is combined with an excepted low to moderate toxicity in terms of overall cell survival. The ease of structural modification can be used to allow further modifications and the analysis of their further (*in vivo*) nucleic acid transfer characteristics and toxicity.

[0101] Lipofectin represents an example of a liposomal transfection agent. It consists of two lipid components, a cationic lipid N-[1-(2,3 dioleoyloxy)propyl]-N,N,N-trimethylammonium chloride (DOTMA) (cp. DOTAP which is the methylsulfate salt) and a neutral lipid dioleoylphosphatidylethanolamine (DOPE). The neutral component mediates the intracellular release. Another group of delivery systems are polymeric nanoparticles.

[0102] Polycations such like diethylaminoethylaminoethyl (DEAE)-dextran, which are well known as DNA transfection reagent can be combined with butylcyanoacrylate (PBCA) and hexylcyanoacrylate (PHCA) to formulate cationic nanoparticles that can deliver a compound as defined herein, preferably an oligonucleotide and optionally together with an adjunct compound across cell membranes into cells.

[0103] In addition to these common nanoparticle materials, the cationic peptide protamine offers an alternative approach to formulate a compound as defined herein, preferably an oligonucleotide and optionally together with an adjunct compound as colloids. This colloidal nanoparticle system can form so called proticles, which can be prepared by a simple self-assembly process to package and mediate intracellular release of a compound as defined herein, preferably an oligonucleotide and optionally together with an adjunct compound. The skilled person may select and adapt any of the above or other commercially available alternative excipients and delivery systems to package and deliver a compound as defined herein, preferably an oligonucleotide and optionally together with an adjunct compound for use to deliver said compound for the treatment of Duchenne Muscular Dystrophy or Becker Muscular Dystrophy in humans.

[0104] In addition, a compound as defined herein, preferably an oligonucleotide and optionally together with an adjunct compound could be covalently or non-covalently linked to a targeting ligand specifically designed to facilitate the uptake in to the cell, cytoplasm and/or its nucleus. Such ligand could comprise (i) a compound (including but not limited to peptide(-like) structures) recognising cell, tissue or organ specific elements facilitating cellular uptake and/or (ii) a chemical compound able to facilitate the uptake in to cells and/or the intracellular release of an a compound as defined herein, preferably an oligonucleotide and optionally together with an adjunct compound from vesicles, e.g. endosomes or lysosomes.

[0105] Therefore, in a preferred embodiment of the disclosure, a compound as defined herein, preferably an oligonucleotide and optionally together with an adjunct compound are formulated in a medicament which is provided with at least an excipient and/or a targeting ligand for delivery and/or a delivery device of said compound to a cell and/or enhancing its intracellular delivery. Accordingly, the present disclosure also encompasses a pharmaceutically acceptable composition comprising a compound as defined herein, preferably an oligonucleotide and optionally together with an adjunct compound and further comprising at least one excipient and/or a targeting ligand for delivery and/or a delivery device of said compound to a cell and/or enhancing its

intracellular delivery. It is to be understood that an oligonucleotide and an adjunct compound may not be formulated in one single composition or preparation. Depending on their identity, the skilled person will know which type of formulation is the most appropriate for each compound.

[0106] In a preferred embodiment the disclosure provides a kit of parts comprising a compound for providing an individual with a functional dystrophin protein and an adjunct compound for reducing inflammation, preferably for reducing muscle tissue inflammation, and/or an adjunct compound for improving muscle fiber function, integrity and/or survival.

[0107] In a preferred embodiment of the disclosure, a concentration of an oligonucleotide as defined herein, which is ranged between about 0.1 nM and about 1 μM is used. More preferably, the concentration used is ranged between about 0.3 to about 400 nM, even more preferably between about 1 to about 200 nM. If several oligonucleotides are used, this concentration may refer to the total concentration of oligonucleotides or the concentration of each oligonucleotide added. The ranges of concentration of oligonucleotide(s) as given above are preferred concentrations for *in vitro* or *ex vivo* uses. The skilled person will understand that depending on the oligonucleotide(s) used, the target cell to be treated, the gene target and its expression levels, the medium used and the transfection and incubation conditions, the concentration of oligonucleotide(s) used may further vary and may need to be optimised any further.

[0108] More preferably, a compound preferably an oligonucleotide and an adjunct compound to be used to prevent, treat DMD or BMD are synthetically produced and administered directly to a cell, a tissue, an organ and/or patients in formulated form in a pharmaceutically acceptable composition or preparation. The delivery of a pharmaceutical composition to the subject is preferably carried out by one or more parenteral injections, e.g. intravenous and/or subcutaneous and/or intramuscular and/or intrathecal and/or intraventricular administrations, preferably injections, at one or at multiple sites in the human body.

[0109] Besides exon skipping the present disclosure provides the insight, it is also possible to provide a DMD patient with a functional dystrophin protein with a therapy based on read-through of stopcodons. Compounds capable of suppressing stopcodons are particularly suitable for a subgroup of DMD patients which is affected by nonsense mutations (~7%) resulting in the formation of a stop codon within their dystrophin gene. In one embodiment of the disclosure said compound capable of suppressing stopcodons comprises the antibiotic gentamicin. In a recent study in *mdx* mice, gentamicin treatment induced novel dystrophin expression up to 20% of normal level, albeit with variability among animals. Human trials with gentamicin have however been inconclusive[55]. PTC124 belongs to a new class of small molecules that mimics at lower concentrations the readthrough activity of gentamicin. Administration of PTC124 resulted in the production of full-length and functionally active dystrophin both *in vitro* and in *mdx* mice[16]. Phase I/II trials with PTC124 are currently ongoing, not only for application in DMD but also for cystic fibrosis[16, 17]. The references 16 and 17 also describe preferred dosages of the PCT124 compound for use in the present disclosure. Further provided is therefore a method, combination, use or pharmaceutical preparation according to the disclosure, wherein said compound for providing said individual with a functional dystrophin protein comprises a compound for suppressing stop codons. Said compound for suppressing stop codons preferably comprises gentamicin, PTC124 or a functional equivalent thereof. Most preferably, said compound comprises PTC124.

[0110] In one embodiment of the disclosure, an individual is provided with a functional dystrophin protein using a vector, preferably a viral vector, comprising a micro-mini-dystrophin gene. Most preferably, a recombinant adeno-associated viral (rAAV) vector is used. AAV is a single-stranded DNA parvovirus that is non-pathogenic and shows a helper-dependent life cycle. In contrast to other viruses (adenovirus, retrovirus, and herpes simplex virus), rAAV vectors have demonstrated to be very efficient in transducing mature skeletal muscle. Application of rAAV in classical DMD "gene addition" studies has been hindered by its restricted packaging limits (< 5 kb). Therefore, rAAV is preferably applied for the efficient delivery of a much smaller micro- or mini-dystrophin gene. Administration of such micro- or mini-dystrophin gene results in the presence of a at least parially functional dystrophin protein. Reference is made to[18-20].

[0111] A compound for providing an individual with a functional dystrophin protein and at least one adjunct compound according to the invention can be administered to an individual in any order. In one embodiment, said compound for providing an individual with a functional dystrophin protein and said at least one adjunct compound are administered simultaneously (meaning that said compounds are administered within 10 hours, preferably within one hour). This is however not necessary. In one embodiment at least one adjunct compound is administered to an individual in need thereof before administration of a compound for providing an individual with a functional dystrophin protein. Further disclosed is therefore a method comprising:

- administering to an individual in need thereof an adjunct compound for reducing inflammation, preferably for reducing muscle tissue inflammation, and/or administering to said individual an adjunct compound for improving muscle fiber function, integrity and/or survival, and, subsequently,

- administering to said individual a compound for providing said individual with a functional dystrophin protein.

[0112] In yet another embodiment, said compound for providing an individual with a functional dystrophin protein is administered before administration of said at least one adjunct compound.

[0113] Further provided is a method for at least in part increasing the production of a functional dystrophin protein in a cell, said cell comprising pre-mRNA of a dystrophin gene encoding aberrant dystrophin protein, the method comprising:

providing said cell with a compound for inhibiting inclusion of an exon into mRNA produced from splicing of said dystrophin pre-mRNA, and

providing said cell with an adjunct compound for reducing inflammation, preferably for reducing muscle tissue inflammation, and/or providing said cell with an adjunct compound for improving muscle fiber function, integrity and/or survival,

the method further comprising allowing translation of mRNA produced from splicing of said pre-mRNA. In one embodiment of the disclosure said method is performed *in vitro,* for instance using a cell culture.

[0114] In this context, increasing the production of a functional dystrophin protein has been earlier defined herein.

[0115] Unless otherwise indicated each embodiment as described herein may be combined with another embodiment as described herein.

[0116] In this document and in its claims, the verb "to comprise" and its conjugations is used in its non-limiting sense to mean that items following the word are included, but items not specifically mentioned are not excluded. In addition the verb "to consist" may be replaced by "to consist essentially of" meaning that a compound or adjunct compound as defined herein may comprise additional component(s) than the ones specifically identified, said additional component(s) not altering the unique characteristic of the disclosure.

[0117] In addition, reference to an element by the indefinite article "a" or "an" does not exclude the possibility that more than one of the element is present, unless the context clearly requires that there be one and only one of the elements. The indefinite article "a" or "an" thus usually means "at least one".

[0118] The word "approximately" or "about" when used in association with a numerical value (approximately 10, about 10) preferably means that the value may be the given value of 10 more or less 1% of the value.

[0119] The invention is further explained in the following examples. These examples do not limit the scope of the invention, but merely serve to clarify the invention.

Brief description of the drawings

[0120]

Figure 1. Schematic Representation of Exon Skipping.
In a patient with Duchenne's muscular dystrophy who has a deletion of exon 50, an out-of-frame transcript is generated in which exon 49 is spliced to exon 51 (Panel A). As a result, a stop codon is generated in exon 51, which prematurely aborts dystrophin synthesis. The sequence-specific binding of the exon-internal antisense oligonucleotide PRO051 interferes with the correct inclusion of exon 51 during splicing so that the exon is actually skipped (Panel B). This restores the open reading frame of the transcript and allows the synthesis of a dystrophin similar to that in patients with Becker's muscular dystrophy (BMD).

Figure 2. Prescreening Studies of the Four Patients.
Magnetic resonance images of the lower legs of the four patients (the left leg of Patient 3 and right legs of the other three patients) show the adequate condition of the tibialis anterior muscle (less than 50% fat infiltration and fibrosis) (Panel A). The diagnosis of Duchenne's muscular dystrophy in these patients was confirmed by diaminobenzidine tetrahydrochloride staining of cross sections of biopsy specimens obtained previously from the quadriceps muscle (Panel B). No dystrophin expression was observed, with the exception of one dystrophin-positive, or revertant, fiber in Patient 2 (arrow). Reverse-transcriptase-

polymerasechain-reaction (RT-PCR) analysis of the transcript region flanking the patients' mutations and exon 51 confirmed both the individual mutations in nontreated myotubes (NT) and the positive response to PRO051 (i.e., exon 51 skipping) in treated myotubes (T) on the RNA level (Panel C). The efficiencies of exon skipping were 49% for Patient 1, 84% for Patient 2, 58% for Patient 3, and 90% for Patient 4. A cryptic splice site within exon 51 is sometimes activated by PRO051 in cell culture, resulting in an extra aberrant splicing product, as seen in the treated sample from Patient 4. Lane M shows a 100-bp size marker, and lane C RNA from healthy control muscle. Sequence analysis of the RT-PCR fragments from treated and untreated myotubes identified the precise skipping of exon 51 for each patient (Panel D). The new in-frame transcripts led to substantial dystrophin synthesis, as detected by immunofluorescence analysis of treated myotubes with the use of monoclonal antibody NCL-DYS2 (Panel E).
No dystrophin was detected before treatment

Figure 3. RT-PCR Analysis of RNA Isolated from Serial Sections of Biopsy Specimens from the Patients.
After treatment with PRO051, reverse-transcriptase-polymerase-chain-reaction (RT-PCR) analysis shows novel, shorter transcript fragments for each patient. Both the size and sequence of these fragments confirm the precise skipping of exon 51. No additional splice variants were observed. At 28 days, still significant in-frame RNA transcripts were detected, suggesting prolonged persistence of PRO051 in muscle. Owing to the small amount of section material, high-sensitivity PCR conditions were used; this process precluded the accurate quantification of skipping efficiencies and the meaningful correlation between levels of RNA and protein. M denotes size marker, and C control.

Figure 4. Dystrophin-Restoring Effect of a Single Intramuscular Dose of PRO051.
Immunofluorescence analysis with the use of the dystrophin antibody MANDYS106 clearly shows dystrophin expression at the membranes of the majority of fibers throughout the biopsy specimen obtained from each patient (Panel A). The areas indicated by the squares are shown in higher magnification in Panel B. For comparison, a sample from an untreated patient with Duchenne's muscular dystrophy (DMD) and a healthy control sample from gastrocnemius muscle (HC) are included with the samples from the patients. Putative revertant fibers are indicated by arrows. The total number of muscle fibers that contained dystrophin and laminin α2 were counted manually and the ratios of dystrophin to laminin α2 were plotted (Panel C). Western blot analysis of total protein extracts isolated from the patients' biopsy specimens with the use of NCL-DYS1 antibody show restored dystrophin expression in all patients (Panel E). For each patient, 30 µg (right lane) and 60 µg (left lane) were loaded; for comparison, 3 µg of total protein from a healthy gastrocnemius muscle sample was also loaded (to avoid overexposure). Because of the relatively small deletions in the DMD gene of these patients, no differences were observed in protein sizes. In Patient 1, a transfer irregularity disturbed signal detection in the 60-µg lane. To correct for the varying density of muscle fibers in the different cross sections, the total fluorescent dystrophin signal (area percentage) in each section was plotted as a ratio to the area percentage of laminin α2 (Panel D).

Figure 5 from reference example. Exon 23 skipping levels on RNA level in different muscle groups (Q: quadriceps muscle; TA: tibialis anterior muscle; DIA: diaphragm muscle) in mdx mice (two mice per group) treated with PS49 alone (group 3) or with PS49 and prednisolone (group4).

Figure 6A,B from reference example In muscle cells, DMD gene exon 44 (A) or exon 45 (B) skipping levels are enhanced with increasing concentrations of pentoxyfilline (from 0 to 0.5 mg/ml). **Figuur 6C** Exon 23 skipping levels on RNA level in different muscle groups (Q: quadriceps muscle; TA: tibialis anterior muscle; Tri: triceps muscle; HRT: heart muscle) in mdx mice (two mice per group) treated with PS49 alone (group 3) or with PS49 and pentoxyfilline (group4).

Figure 7. Dystrophin (DMD) gene amino acid sequence

Figure 8 from reference example. Human IGF-1 Isoform 4 amino acid sequence.

Figure 9 from reference example. Various oligonucleotides directed against the indicated exons of the dystrophin (DMD)-gene

Examples

Example 1

[0121] In a recent clinical study the local safety, tolerability, and dystrophin-restoring effect of antisense compound PRO051was assessed. The clinical study was recently published. The content of the publication is reproduced herein under example 1A. In brief, PRO051 is a synthetic, modified RNA molecule with sequence 5'-UCA AGG AAG AUG GCA UUU CU-3', and designed to

specifically induce exon 51 skipping[59]. It carries full-length 2'-O-methyl substituted ribose moieties and phosphorothioate internucleotide linkages. Four DMD patients with different specific DMD gene deletions correctible by exon 51 skipping were included. At day 0, a series of safety parameters was assessed. The patient's leg (i.e. tibialis anterior muscle) was fixed with a tailor-made plastic mould and its position was carefully recorded. A topical anesthetic (EMLA) was used to numb the skin. Four injections of PRO051 were given along a line of 1.5 cm between two small skin tattoos, using a 2.5 cm electromyographic needle (MyoJect Disposable Hypodermic Needle Electrode, TECA Accessories) to ensure intramuscular delivery. Each injection volume was 200 µl, containing 200 µg PRO051, dispersed in equal portions at angles of approximately 30 degrees. At day 28, the same series of safety parameters was assessed again. The leg was positioned using the patient's own mould, and a semi-open muscle biopsy was taken between the tattoos under local anesthesia using a forceps with two sharp-edged jaws (Blakesley Conchotoma, DK Instruments). The biopsy was snap-frozen in liquid nitrogen-cooled 2-methylbutane. Patients were treated sequentially. At the time of study, two patients (nr. 1 and 2) were also on corticosteroids (prednisone or deflazacort), one had just stopped steroid treatment (nr.4) and one patient never used steroids (nr.3) (see Table 1). This latter patient was also the one who lost ambulance at the youngest age when compared to the other three patients. The biopsy was analysed, for detection of specific exon skipping on RNA level (RT-PCR analysis, not shown) and novel expression of dystrophin on protein level (immunofluorescence and western blot analyses, summarized in Table 1). Assessment of the series of safety parameters (routine plasma and urine parameters for renal and liver function, electrolyte levels, blood cell counts, hemoglobin, aPTT, AP50 and CH50 values) before and after treatment, indicated that the PRO051 compound was locally safe and well tolerated. For immunofluorescence analysis, acetone-fixed cross-sections of the biopsy were incubated for 90 minutes with monoclonal antibodies against the central rod domain (MANDYS106, Dr. G. Morris, UK, 1:60), the C-terminal domain (NCL-DYS2, Novocastra Laboratories Ltd., 1:30) or, as reference, laminin- α2 (Chemicon International, Inc, 1:150), followed by Alexa Fluor 488 goat anti-mouse IgG (H+L) (Molecular Probes, Inc, 1:250) antibody for one hour. Sections were mounted with Vectashield Mounting Medium (Vector Laboratories Inc.).

For quantitative image analysis the ImageJ software (W. Rasband, NIH, USA; http://rsb.info.nih.gov/ij) was used as described[60,61]. Entire cross-sections were subdivided into series of 6-10 adjacent images, depending on section size. To ensure reliable measurements, staining of the sections and recording of all images was performed in one session, using fixed exposure settings, and avoiding pixel saturation. The lower intensity threshold was set at Duchenne muscular dystrophy background, and positive fluorescence was quantified for each section (area percentage), both for dystrophin and laminin-α2. Western blot analysis was performed as described[1], using pooled homogenates from sets of four serial 50 µm sections throughout the biopsy. For the patients 30 and 60 µg total protein was applied and for the control sample 3 µg. The blot was incubated overnight with dystrophin monoclonal antibody NCL-DYS1 (Novocastra Laboratories, 1:125), followed by goat anti-mouse IgG-HRP (Santa Cruz Biotechnology, 1:10.000) for one hour. Immuno-reactive bands were visualized using the ECL Plus Western Blotting Detection System (GE Healthcare) and Hyperfilm ECL (Amersham, Biosciences). Signal intensities were measured using ImageJ.

Novel dystrophin protein expression at the sarcolemma was detected in the majority of muscle fibers in the treated area in all four patients. The fibers in each section were manually counted after staining for laminin-α2, a basal lamina protein unaffected by dystrophin deficiency. The individual numbers varied, consistent with the biopsy size and the quality of the patients' muscles. In the largest sections, patient 2 had 726 fibers, of which 620 were dystrophin-positive, while patient 3 had 120 fibers, of which 117 were dystrophin-positive. The dystrophin intensities were typically lower than those in a healthy muscle biopsy. Western blot analysis confirmed the presence of dystrophin in varying amounts. The dystrophin signals were scanned and correlated to the control (per µg total protein). The amounts varied from 3% in patient 3 with the most dystrophic muscle, to 12% in patient 2 with the best preserved muscle. Since such comparison based on total protein does not correct for the varying amounts of fibrotic and adipose tissue in Duchenne muscular dystrophy patients, we also quantified the dystrophin fluorescence signal relative to that of the similarly-located laminin-α2 in each section, by ImageJ analysis. When this dystrophin/laminin-α2 ratio was set at 100% for the control section, the two patients that were co-treated with corticosteroids showed the highest percentages of dystrophin, 32% in patient 1 and 35% in patient 2 (Table 1). The lowest percentage of dystrophin was detected in patient 3, 17%. In patient 4 an intermediate percentage of 25% was observed. These percentages correlated to the relative quality of the target muscle, which was best in patients nr. 1 and 2, and worst in patient nr.3.

Table 1.

	Patient 1	Patient 2	Patient 3	Patient 4
Age (yrs)	10	13	13	11
Age at Loss of Ambulation (yrs)	9	11	7	10
Steroid Treatment	Yes	Yes	Never	Until Jan 2006
Ratio Dystrophinlaminin-alpha2	32%	35%	17%	25%

[0122] Conclusion: the effect of the PRO051 antisense compound was more prominent in those patients that were also subjected to corticosteroids.

Example 1A

[0123] Reproduced from Van Deutekom JC et al, (2007) Antisense Oligonucleotide PRO051 Restores Local Dystrophin in DMD Patients. N Engl J Med., 357(26): 2677-86.

Methods

Patients and Study Design

[0124] Patients with Duchenne's muscular dystrophy who were between the ages of 8 and 16 years were eligible to participate in the study. All patients had deletions that were correctable by exon-51 skipping and had no evidence of dystrophin on previous diagnostic muscle biopsy. Concurrent glucocorticoid treatment was allowed. Written informed consent was obtained from the patients or their parents, as appropriate. During the prescreening period (up to 60 days), each patient's mutational status and positive exon-skipping response to PRO051 in vitro were confirmed, and the condition of the tibialis anterior muscle was determined by T_1-weighted magnetic resonance imaging (MRI).[62] For patients to be included in the study, fibrotic and adipose tissue could make up no more than 50% of their target muscle.

[0125] During the baseline visit, safety measures were assessed. In each patient, the leg that was to be injected was fixed with a tailor-made plastic mold and its position was recorded. A topical eutectic mixture of local anesthetics (EMLA) was used to numb the skin. Four injections of PRO051 were given along a line measuring 1.5 cm running between two small skin tattoos with the use of a 2.5-cm electromyographic needle (MyoJect Disposable Hypodermic Needle Electrode, TECA Accessories) to ensure intramuscular delivery. The volume of each injection was 200 µl containing 200 µg of PRO051, which was dispersed in equal portions at angles of approximately 30 degrees.

[0126] At day 28, safety measures were assessed again. The leg that had been injected was positioned with the use of the patient's own mold, and a semiopen muscle biopsy was performed between the tattoos under local anesthesia with a forceps with two sharp-edged jaws (Blakesley Conchotoma, DK Instruments).[63] The biopsy specimen was snap-frozen in 2-methylbutane cooled in liquid nitrogen.

[0127] Patients were treated sequentially from May 2006 through March 2007 and in compliance with Good Clinical Practice guidelines and the provisions of the Declaration of Helsinki. The study was approved by the Dutch Central Committee on Research Involving Human Subjects and by the local institutional review board at Leiden University Medical Center. All authors contributed to the study design, participated in the collection and analysis of the data, had complete and free access to the data, jointly wrote the manuscript, and vouch for the completeness and accuracy of the data and analyses presented.

Description of PR0051

[0128] PRO051 is a synthetic, modified RNA molecule with sequence 5'-UCAAGGAAGAUGGCAUUUCU-3'.[12] It carries full-length 2'-O-methyl-substituted ribose molecules and phosphorothioate internucleotide linkages. The drug was provided by Prosensa B.V. in vials of 1 mg of freeze-dried material with no excipient. It was dissolved and administered in sterile, unpreserved saline (0.9% sodium chloride). PRO051 was not found to be mutagenic by bacterial Ames testing. In regulatory Good Laboratory Practice safety studies, rats that received a single administration of up to 8 mg per kilogram of body weight intramuscularly and 50 mg per kilogram intravenously showed no adverse effects; monkeys receiving PRO051 for 1 month appeared to tolerate doses up to 16 mg per kilogram per week when the drug was administered by intravenous 1-hour infusion or by subcutaneous injection, without clinically relevant adverse effects.

In Vitro Prescreening

[0129] A preexisting primary myoblast culture[1] was used for the prescreening of Patient 4. For the other three patients,

fibroblasts were converted into myogenic cells after infection with an adenoviral vector containing the gene for the myogenic transcription factor (MyoD) as described previously.[1, 64, 65] Myotube cultures were transfected with PRO051 (100 nM) and polyethylenimine (2 µl per microgram of PRO051), according to the manufacturer's instructions for ExGen500 (MBI Fermentas). RNA was isolated after 48 hours. Reverse transcriptase-polymerase chain reaction (RT-PCR), immunofluorescence, and Western blot analyses were performed as reported previously[1,12] PCR fragments were analyzed with the use of the 2100 Bioanalyzer (Agilent) and isolated for sequencing by the Leiden Genome Technology Center.

Safety Assessment

[0130] At baseline and at 2 hours, 1 day, and 28 days after injection, all patients received a full physical examination (including the measurement of vital signs) and underwent electrocardiography. In addition, plasma and urine were obtained to determine renal and liver function, electrolyte levels, complete cell counts, the activated partial-thromboplastin time, and complement activity values in the classical (CH50) and alternative (AP50) routes. The use of concomitant medications was recorded. At baseline and on day 28, the strength of the tibialis anterior muscle was assessed with the use of the Medical Research Council scale[66] to evaluate whether the procedures had affected muscle performance. (On this scale, a score of 0 indicates no movement and a score of 5 indicates normal muscle strength.) Since only a small area of the muscle was treated, clinical benefit in terms of increased muscle strength was not expected. At each visit, adverse events were recorded.

RNA Assessment

[0131] Serial sections (50 µm) of the frozen muscle-biopsy specimen were homogenized in RNA-Bee solution (Campro Scientific) and MagNA Lyser Green Beads (Roche Diagnostics). Total RNA was isolated and purified according to the manufacturer's instructions. For complementary DNA, synthesis was accomplished with Transcriptor reverse transcriptase (Roche Diagnostics) with the use of 500 ng of RNA in a 20-µl reaction at 55°C for 30 minutes with human exon 53 or 54 specific reverse primers. PCR analyses were performed as described previously.[1,12] Products were analyzed on 2% agarose gels and sequenced. In addition, RT-PCR with the use of a primer set for the protein-truncation test[67] was used to rapidly screen for aspecific aberrant splicing events throughout the *DMD* gene.

Assessment of Protein Level

[0132] For immunofluorescence analysis, acetone-fixed sections were incubated for 90 minutes with monoclonal antibodies against the central rod domain (MANDYS106, Dr. G. Morris, United Kingdom) at a dilution of 1:60, the C-terminal domain (NCL-DYS2, Novocastra Laboratories) at a dilution of 1:30, or (as a reference) laminin α2 (Chemicon International), a basal lamina protein that is unaffected by dystrophin deficiency, at a dilution of 1:150, followed by Alexa Fluor 488 goat antimouse IgG (H+L) antibody (Molecular Probes) at a dilution of 1:250 for 1 hour. Sections were mounted with Vectashield Mounting Medium (Vector Laboratories). ImageJ software (W. Rasband, National Institutes of Health, http://rsb.info.nih.gov/ij) was used for quantitative image analysis as described previously.[60,61] Entire cross sections were subdivided into series of 6 to 10 adjacent images, depending on the size of the section. To ensure reliable measurements, staining of the sections and recording of all images were performed during one session with the use of fixed exposure settings and the avoidance of pixel saturation. The lower-intensity threshold was set at background for Duchenne's muscular dystrophy, and positive fluorescence was quantified for each section (area percentage), both for dystrophin and laminin α2.

[0133] Western blot analysis was performed as described previously[1] with the use of pooled homogenates from sets of four serial 50-µm sections throughout the biopsy specimen. For each patient, two amounts of total protein - 30 µg and 60 µg - were applied, and for the control sample, 3 µg. The Western blot was incubated overnight with dystrophin monoclonal antibody NCL-DYS1 (Novocastra Laboratories) at a dilution of 1:125, followed by horseradish-peroxidase-labeled goat antimouse IgG (Santa Cruz Biotechnology) at a dilution of 1:10,000 for 1 hour. Immunoreactive bands were visualized with the use of the ECL Plus Western blotting detection system (GE Healthcare) and Hyperfilm ECL (Amersham Biosciences). Signal intensities were measured with the use of ImageJ software.

Results

Prescreening of Patients

[0134] The study was planned to include four to six patients. Six patients were invited to participate, and one declined. The remaining five patients were prescreened. First, the condition of the tibialis anterior muscle was evaluated on MRI. The muscle condition of four patients was deemed to be adequate for the study (Figure 2A), and the absence of dystrophin was confirmed in the patients' original biopsy specimens (Figure 2B). Second, the mutational status and positive exon-skipping response to PRO051 of these four patients were confirmed in fibroblast cultures. PRO051 treatment generated a novel, shorter fragment of messenger RNA for each patient, representing 46% (in Patient 4) to 90% (in Patient 1) of the total RT-PCR product (Figure 2C). Precise exon-51 skipping was confirmed by sequencing (Figure 2D). No other transcript regions were found to be altered. Immunofluorescence analyses showed a preponderance of dystrophin-positive myotubes (Figure 2E), a finding that was confirmed by Western blot analysis (not shown). Thus, the four patients were judged to be eligible for PRO051 treatment. Their baseline characteristics are shown in Table 2.

Safety and Adverse Events

[0135] All patients had one or more adverse events. However, only one patient reported mild local pain at the injection site, which was considered to be an adverse event related to the study drug. Other events included mild-to-moderate pain after the muscle biopsy. Two patients had blistering under the bandages used for wound closure. In the period between injection and biopsy, two patients reported a few days of flulike symptoms, and one patient had mild diarrhea for 1 day. At baseline, the muscle-strength scores of the treated tibialis anterior muscle in Patients 1, 2, 3, and 4 were 4, 2, 3, and 4, respectively, on the Medical Research Council scale. None of the patients showed changes in the strength of this muscle during the study or significant alterations in standard laboratory measures or increased measures of complement split products or activated partial-thromboplastin time. No local inflammatory or toxic response was detected in the muscle sections of the patients (data not shown). Patient 3 successfully underwent preplanned surgery for scoliosis in the month after the study was completed.

RNA and Protein Level

[0136] At day 28, a biopsy of the treated area was performed in each patient. Total muscle RNA was isolated from serial sections throughout the biopsy specimen. In all patients, RT-PCR identified a novel, shorter fragment caused by exon-51 skipping, as confirmed by sequencing (Figure 3). Further transcript analysis showed no other alterations (data not shown). Immunofluorescence analyses of sections throughout the biopsy specimen of each patient showed clear sarcolemmal dystrophin signals in the majority of muscle fibers (Figure 4A and 4B). Dystrophin antibodies proximal and distal to the deletions that were used included MANDYS106 (Figure 4A and 4B) and NCL-DYS2 (similar to MANDYS106, not shown). The fibers in each section were manually counted after staining for laminin α2.[68] The individual numbers varied, consistent with the size of the biopsy specimen and the quality of the muscle. In the largest sections, Patient 2 had 726 fibers, of which 620 were dystrophin-positive, whereas Patient 3 had 120 fibers, of which 117 were dystrophin-positive (Figure 4A and 4C). The dystrophin intensities were typically lower than those in a healthy muscle biopsy specimen (Figure 4B). The single fibers with a more intense dystrophin signal in Patients 2 and 3 could well be revertant fibers (Figure 4B).

[0137] Western blot analysis confirmed the presence of dystrophin in varying amounts (Figure 4E). The dystrophin signals were scanned and correlated to the control (per microgram of total protein). The amounts varied from 3% in Patient 3, who had the most-dystrophic muscle, to 12% in Patient 2, who had the best-preserved muscle. Since such comparison on the basis of total protein does not correct for the varying amounts of fibrotic and adipose tissue in patients with Duchenne's muscular dystrophy, we also quantified the dystrophin fluorescence signal (Figure 4A and 4B) relative to that of the similarly located laminin α2 in each section by ImageJ analysis. When the ratio of dystrophin to laminin α2 was set at 100 for the control section, Patients 1, 2, 3, and 4 had ratios of 33, 35, 17, and 25, respectively (Figure 4D).

Discussion

[0138] Our study showed that local intramuscular injection of PRO051, a 2OMePS antisense oligoribonucleotide complementary to a 20-nucleotide sequence within exon 51, induced exon-51 skipping, corrected the reading frame, and thus introduced

dystrophin in the muscle in all four patients with Duchenne's muscular dystrophy who received therapy. Dystrophin-positive fibers were found throughout the patients' biopsy specimens, indicating dispersion of the compound in the injected area. Since no delivery-enhancing excipient was used, PRO051 uptake did not seem to be a major potentially limiting factor. We cannot rule out that increased permeability of the dystrophic fiber membrane had a favorable effect. The patients produced levels of dystrophin that were 3 to 12% of the level in healthy control muscle, as shown on Western blot analysis of total protein. Since the presence of fibrosis and fat may lead to some underestimation of dystrophin in total protein extracts, we determined the ratio of dystrophin to laminin α2 in the cross sections, which ranged from 17 to 35, as compared with 100 in control muscle. The dystrophin-restoring effect of PRO051 was limited to the treated area, and no strength improvement of the entire muscle was observed. Future systemic treatment will require repeated administration to increase and maintain dystrophin expression at a higher level and to obtain clinical efficacy.

[0139] Because of medical-ethics regulations regarding interventions in minors, we could not obtain a biopsy specimen from the patients' contralateral muscles that had not been injected. However, the patients showed less than 1% of revertant fibers in the original diagnostic biopsy specimens obtained 5 to 9 years before the initiation of the study (Table 2 and Figure 2B). We consider it very likely that the effects we observed were related to the nature and sequence of the PRO051 reagent rather than to a marked increase in revertant fibers. Indeed, a single, possibly revertant fiber that had an increased dystrophin signal was observed in both Patient 2 and Patient 3 (Figure 4B).

[0140] In summary, our study showed that local administration of PRO051 to muscle in four patients with Duchenne's muscular dystrophy restored dystrophin to levels ranging from 3 to 12% or 17 to 35%, depending on quantification relative to total protein or myofiber content. Consistent with the distinctly localized nature of the treatment, functional improvement was not observed. The consistently poorer result in Patient 3, who had the most advanced disease, suggests the importance of performing clinical trials in patients at a relatively young age, when relatively little muscle tissue has been replaced by fibrotic and adipose tissue. Our findings provide an indication that antisense-mediated exon skipping may be a potential approach to restoring dystrophin synthesis in the muscles of patients with Duchenne's muscular dystrophy.

Example 2: Reference example

[0141] In a pre-clinical study in *mdx* mice (animal model for DMD) the effect of adjunct compound prednisone on AON-induced exon skipping was assessed. *Mdx* mice (C57Bl/10ScSn-mdx/J) were obtained from Charles River Laboratories (The Netherlands). These mice are dystrophin-deficient due to a nonsense mutation in exon 23. AON-induced exon 23 skipping is therapeutic in *mdx* mice by removing the nonsense mutation and correction of the open reading frame. Two *mdx* mice per group were injected subcutaneously with: Group 1) physiologic salt (wk 1-8), Group 2) prednisolone (1mg/kg, wk 1-8), Group 3) mouse-specific antisense oligonucleotide PS49 designed to specifically induce exon 23 skipping (100mg/kg, wk 4 (5 times),week 5-8 (2 times), Group 4) prednisolone (1mg/kg, wk 1-8) + PS49 (100mg/kg, wk 4 (5 times),week 5-8 (2 times). PS49 (5' GGCCAAACCUCGGCUUACCU 3') has a full-length phosphorothioate backbone and 2'O-methyl modified ribose molecules.

[0142] All mice were sacrificed at 1 week post-last-injection. Different muscles groups, including quadriceps, tibialis anterior, and diaphragm muscles were isolated and frozen in liquid nitrogen-cooled 2-methylbutane. For RT-PCR analysis, the muscle samples were homogenized in the RNA-Bee solution (Campro Scientific, The Netherlands). Total RNA was isolated and purified according to the manufacturer's instructions. For cDNA synthesis with reverse transcriptase (Roche Diagnostics, The Netherlands), 300 ng of RNA was used in a 20 µl reaction at 55°C for 30 min, reverse primed with mouse DMD gene-specific primers. First PCRs were performed with outer primer sets, for 20 cycles of 94°C (40 sec), 60°C (40 sec), and 72°C (60 sec). One µl of this reaction (diluted 1:10) was then re-amplified using nested primer combinations in the exons directly flanking exon 23, with 30 cycles of 94°C (40 sec), 60°C (40 sec), and 72°C (60 sec). PCR products were analysed on 2% agarose gels. Skipping efficiencies were determined by quantification of PCR products using the DNA 1000 LabChip® Kit and the Agilent 2100 bioanalyzer (Agilent Technologies, The Netherlands). No exon 23 skipping was observed in the muscles from mice treated with physiologic salt or prenisolone only (groups 1 and 2). Levels of exon 23 skipping were detected and per muscle group compared between mice treated with PS49 only (group 3) and mice treated with PS49 and adjunct compound prednisolone (group 4). In the quadriceps (Q), tibialis anterior (TA), and diaphragm (DIA) muscles, exon 23 skipping levels were typically higher in group 4 when compared to group 3 (Figure 5). This indicates that adjunct compound prednisolone indeed enhances exon 23 skipping levels in *mdx* mice treated with PS49.

Example 3: Reference example

[0143] **A**., **B**. Differentiated muscle cell cultures (myotubes) derived from a healthy control individual were transfected with 250 nM PS188 ([5' UCAGCUUCUGUUAGCCACUG 3'; SEQ ID NO:10] an AON optimized to specifically skip exon 44) or 250 nM PS221 ([5' AUUCAAUGUUCUGACAACAGUUUGC 3'; SEQ ID NO: 60] an AON optimized to specifically skip exon 45) in the presence of 0 to 0.5 mg/ml pentoxifylline, using the transfection reagent polymer UNIFectylin (2,0 µl UNIFectylin per µg AON in 0,15M NaCl). UNIFectylin interacts electrostatically with nucleic acids, provided that the nucleic acid is negatively charged (such as 2'-*O*-methyl phosphorothioate AONs). Pentoxyfillin (Sigma Aldrich) was dissolved in water. Total RNA was isolated 24 hrs after transfection in RNA-Bee solution (Campro Scientific, The Netherlands) according to the manufacturer's instructions. For cDNA synthesis with reverse transcriptase (Roche Diagnostics, The Netherlands), 500 ng of RNA was used in a 20 µl reaction at 55°C for 30 min, reverse primed with DMD gene-specific primers. First PCRs were performed with outer primer sets, for 20 cycles of 94°C (40 sec), 60°C (40 sec), and 72°C (60 sec). One µl of this reaction (diluted 1:10) was then re-amplified using nested primer combinations in the exons directly flanking exon 44 or 45, with 30 cycles of 94°C (40 sec), 60°C (40 sec), and 72°C (60 sec). PCR products were analysed on 2% agarose gels. Skipping efficiencies were determined by quantification of PCR products using the DNA 1000 LabChip® Kit and the Agilent 2100 bioanalyzer (Agilent Technologies, The Netherlands).

[0144] Both with PS188 and PS221, increasing levels of exon 44 or 45 skipping were obtained with increasing concentrations of the adjunct compound pentoxifylline when compared to those obtained in cells that were not co-treated with pentoxyfilline (see Figure 6). These results indicate that pentoxifylline enhances exon skipping levels in the muscle cells.

C.

[0145] In a pre-clinical study in *mdx* mice (animal model for DMD) the effect of adjunct compound pentoxyfilline on AON-induced exon skipping was assessed. *Mdx* mice (C57Bl/10ScSn-mdx/J) were obtained from Charles River Laboratories (The Netherlands). These mice are dystrophin-deficient due to a nonsense mutation in exon 23. AON-induced exon 23 skipping is therapeutic in *mdx* mice by removing the nonsense mutation and correction of the open reading frame. Two *mdx* mice per group were injected subcutaneously with: Group 1) pentoxyfilline (50 mg/kg, wk 1-2), Group 2) mouse-specific antisense oligonucleotide PS49 designed to specifically induce exon 23 skipping (100mg/kg, wk 2 (2 times), Group 3) pentoxyfilline (50 mg/kg, wk 1-2) + PS49 (100mg/kg, wk 2 (2 times). PS49 (5' GGCCAAACCUCGGCUUACCU 3') has a full-length phosphorothioate backbone and 2'*O*-methyl modified ribose molecules.

[0146] All mice were sacrificed at 1 week post-last-injection. Different muscles groups, including quadriceps, tibialis anterior, triceps and heart muscles were isolated and frozen in liquid nitrogen-cooled 2-methylbutane. For RT-PCR analysis, the muscle samples were homogenized in the RNA-Bee solution (Campro Scientific, The Netherlands). Total RNA was isolated and purified according to the manufacturer's instructions. For cDNA synthesis with reverse transcriptase (Roche Diagnostics, The Netherlands), 300 ng of RNA was used in a 20 µl reaction at 55°C for 30 min, reverse primed with mouse DMD gene-specific primers. First PCRs were performed with outer primer sets, for 20 cycles of 94°C (40 sec), 60°C (40 sec), and 72°C (60 sec). One µl of this reaction (diluted 1:10) was then re-amplified using nested primer combinations in the exons directly flanking exon 23, with 30 cycles of 94°C (40 sec), 60°C (40 sec), and 72°C (60 sec). PCR products were analysed on 2% agarose gels. Skipping efficiencies were determined by quantification of PCR products using the DNA 1000 LabChip® Kit and the Agilent 2100 bioanalyzer (Agilent Technologies, The Netherlands). No exon 23 skipping was observed in the muscles from mice treated with pentoxyfilline only (groups 1). Levels of exon 23 skipping were detected and per muscle group compared between mice treated with PS49 only (group 2) and mice treated with PS49 and adjunct compound pentoxyfilline (group 3). In the quadriceps (Q), tibialis anterior (TA), triceps (Tri) and heart (HRT) muscles, exon 23 skipping levels were typically higher in group 3 when compared to group 2 (Figure 6c). This indicates that adjunct compound pentoxyfilline indeed enhances exon 23 skipping levels in *mdx* mice treated with PS49.

Table 2. Baseline characteristics of the DMD patients

	Patient 1	Patient 2	Patient 3	Patient 4
Age (yrs)	10	13	13	11
Deletion	Exon 50	Exons 48-50	Exons 49-50	Exon 52
Age at Loss of Ambulation (yrs)	9	11	7	10
Scoliosis	No	No	Yes	Yes
Creatine Kinase Levels (U/l)[1]	5823	2531	717	4711
Steroid treatment	Yes	Yes	Never	Until Jan 2006
Strength TA muscle (MRC scale)	4	2	3	4

Table 2. Baseline characteristics of the DMD patients				
	Patient 1	**Patient 2**	**Patient 3**	**Patient 4**
MRI status TA muscle	Moderate[2]	Moderate[2]	Moderate[2]	Moderate[2]
% Revertant fibers	N.D.	<1%	N.D.	
1 normal level: <200 U/l 2 less than 50% fat infiltration and/or fibrosis [Mercuri et al., 2005)				

References

[0147]

1. 1. Aartsma-Rus A, Janson AA, Kaman WE, et al. Therapeutic antisense-induced exon skipping in cultured muscle cells from six different DMD patients. Hum Mol Genet 2003;12(8):907-14.
2. 2. Aartsma-Rus A, Janson AA, Kaman WE, et al. Antisense-induced multiexon skipping for Duchenne muscular dystrophy makes more sense. Am J Hum Genet 2004;74(1):83-92.
3. 3. Alter J, Lou F, Rabinowitz A, et al. Systemic delivery of morpholino oligonucleotide restores dystrophin expression bodywide and improves dystrophic pathology. Nat Med 2006;12(2):175-7.
4. 4. Goyenvalle A, Vulin A, Fougerousse F, et al. Rescue of dystrophic muscle through U7 snRNA-mediated exon skipping. Science 2004;306(5702):1796-9.
5. 5. Lu QL, Mann CJ, Lou F, et al. Functional amounts of dystrophin produced by skipping the mutated exon in the mdx dystrophic mouse. Nat Med 2003;6:6.
6. 6. Lu QL, Rabinowitz A, Chen YC, et al. Systemic delivery of antisense oligoribonucleotide restores dystrophin expression in body-wide skeletal muscles. Proc Natl Acad Sci U S A 2005;102(1):198-203.
7. 7. McClorey G, Fall AM, Moulton HM, et al. Induced dystrophin exon skipping in human muscle explants. Neuromuscul Disord 2006;16(9-10):583-90.
8. 8. McClorey G, Moulton HM, Iversen PL, et al. Antisense oligonucleotide-induced exon skipping restores dystrophin expression in vitro in a canine model of DMD. Gene Ther 2006;13(19):1373-81.
9. 9. Pramono ZA, Takeshima Y, Alimsardjono H, Ishii A, Takeda S, Matsuo M. Induction of exon skipping of the dystrophin transcript in lymphoblastoid cells by transfecting an antisense oligodeoxynucleotide complementary to an exon recognition sequence. Biochem Biophys Res Commun 1996;226(2):445-9.
10. 10. Takeshima Y, Yagi M, Wada H, et al. Intravenous infusion of an antisense oligonucleotide results in exon skipping in muscle dystrophin mRNA of Duchenne muscular dystrophy. Pediatr Res 2006;59(5):690-4.
11. 11. van Deutekom JC, Bremmer-Bout M, Janson AA, et al. Antisense-induced exon skipping restores dystrophin expression in DMD patient derived muscle cells. Hum Mol Genet 2001;10(15):1547-54.
12. 12. Aartsma-Rus A, Bremmer-Bout M, Janson A, den Dunnen J, van Ommen G, van Deutekom J. Targeted exon skipping as a potential gene correction therapy for Duchenne muscular dystrophy. Neuromuscul Disord 2002;12 Suppl:S71-S77.
13. 13. Aartsma-Rus A, De Winter CL, Janson AA, et al. Functional analysis of 114 exon-internal AONs for targeted DMD exon skipping: indication for steric hindrance of SR protein binding sites. Oligonucleotides 2005;15(4):284-97.
14. 14. Aartsma-Rus A, Janson AA, Heemskerk JA, CL de Winter, GJ Van Ommen, JC Van Deutekom. Therapeutic Modulation of DMD Splicing by Blocking Exonic Splicing Enhancer Sites with Antisense Oligonucleotides. Annals of the New York Academy of Sciences 2006;1082:74-6.
15. 15. Aartsma-Rus A, Kaman WE, Weij R, den Dunnen JT, van Ommen GJ, van Deutekom JC. Exploring the frontiers of therapeutic exon skipping for Duchenne muscular dystrophy by double targeting within one or multiple exons. Mol Ther 2006;14(3):401-7.
16. 16. Welch EM, Barton ER, Zhuo J, et al. PTC124 targets genetic disorders caused by nonsense mutations. Nature 2007;447(7140):87-91.
17. 17. Hirawat S, Welch EM, Elfring GL, et al. Safety, tolerability, and pharmacokinetics of PTC124, a nonaminoglycoside nonsense mutation suppressor, following single- and multiple-dose administration to healthy male and female adult volunteers. Journal of clinical pharmacology 2007;47(4):430-44.
18. 18. Wang B, Li J, Xiao X. Adeno-associated virus vector carrying human minidystrophin genes effectively ameliorates muscular dystrophy in mdx mouse model. Proc Natl Acad Sci U S A 2000;97(25):13714-9.
19. 19. Fabb SA, Wells DJ, Serpente P, Dickson G. Adeno-associated virus vector gene transfer and sarcolemmal expression of a 144 kDa micro-dystrophin effectively restores the dystrophin-associated protein complex and inhibits myofibre

degeneration in nude/mdx mice. Hum Mol Genet 2002;11(7):733-41.
20. 20. Wang Z, Kuhr CS, Allen JM, et al. Sustained AAV-mediated dystrophin expression in a canine model of Duchenne muscular dystrophy with a brief course of immunosuppression. Mol Ther 2007;15(6):1160-6.
21. 21. Manzur AY, Kuntzer T, Pike M, Swan A. Glucocorticoid corticosteroids for Duchenne muscular dystrophy. Cochrane Database Syst Rev 2004;2.
22. 22. Duboc D, Meune C, Pierre B, et al. Perindopril preventive treatment on mortality in Duchenne muscular dystrophy: 10 years' follow-up. American heart journal 2007;154(3):596-602.
23. 23. Cohn RD, van Erp C, Habashi JP, et al. Angiotensin II type 1 receptor blockade attenuates TGF-beta-induced failure of muscle regeneration in multiple myopathic states. Nat Med 2007;13(2):204-10.
24. 24. Grounds MD, Torrisi J. Anti-TNFalpha (Remicade) therapy protects dystrophic skeletal muscle from necrosis. Faseb J 2004;18(6):676-82.
25. 25. Hodgetts S, Radley H, Davies M, Grounds MD. Reduced necrosis of dystrophic muscle by depletion of host neutrophils, or blocking TNFalpha function with Etanercept in mdx mice. Neuromuscul Disord 2006;16(9-10):591-602.
26. 26. Pierno S, Nico B, Burdi R, et al. Role of tumour necrosis factor alpha, but not of cyclo-oxygenase-2-derived eicosanoids, on functional and morphological indices of dystrophic progression in mdx mice: a pharmacological approach. Neuropathology and applied neurobiology 2007;33(3):344-59.
27. 27. Musaro A, McCullagh K, Paul A, et al. Localized Igf-1 transgene expression sustains hypertrophy and regeneration in senescent skeletal muscle. Nat Genet 2001;27(2):195-200.
28. 28. Barton ER, Morris L, Musaro A, Rosenthal N, Sweeney HL. Muscle-specific expression of insulin-like growth factor I counters muscle decline in mdx mice. J Cell Biol 2002;157(1):137-48.
29. 29. Disatnik MH, Dhawan J, Yu Y, et al. Evidence of oxidative stress in mdx mouse muscle: studies of the pre-necrotic state. J Neurol Sci 1998;161(1):77-84.
30. 30. Nelson SK, Bose SK, Grunwald GK, Myhill P, McCord JM. The induction of human superoxide dismutase and catalase in vivo: a fundamentally new approach to antioxidant therapy. Free radical biology & medicine 2006;40(2):341-7.
31. 31. Hart PE, Lodi R, Rajagopalan B, et al. Antioxidant treatment of patients with Friedreich ataxia: four-year follow-up. Archives of neurology 2005;62(4):621-6.
32. 32. Rolland JF, De Luca A, Burdi R, Andreetta F, Confalonieri P, Conte Camerino D. Overactivity of exercise-sensitive cation channels and their impaired modulation by IGF-1 in mdx native muscle fibers: beneficial effect of pentoxifylline. Neurobiol Dis 2006;24(3):466-74.
33. 33. Whitehead NP, Streamer M, Lusambili LI, Sachs F, Allen DG. Streptomycin reduces stretch-induced membrane permeability in muscles from mdx mice. Neuromuscul Disord 2006;16(12):845-54.
34. 34. Badalamente MA, Stracher A. Delay of muscle degeneration and necrosis in mdx mice by calpain inhibition. Muscle Nerve 2000;23(1):106-11.
35. 35. Burdi R, Didonna MP, Pignol B, et al. First evaluation of the potential effectiveness in muscular dystrophy of a novel chimeric compound, BN 82270, acting as calpain-inhibitor and anti-oxidant. Neuromuscul Disord 2006;16(4):237-48.
36. 36. Bonuccelli G, Sotgia F, Schubert W, et al. Proteasome inhibitor (MG-132) treatment of mdx mice rescues the expression and membrane localization of dystrophin and dystrophin-associated proteins. Am J Pathol 2003;163(4):1663-75.
37. 37. Voisin V, Sebrie C, Matecki S, et al. L-arginine improves dystrophic phenotype in mdx mice. Neurobiol Dis 2005;20(1):123-30.
38. 38. Soret J, Bakkour N, Maire S, et al. Selective modification of alternative splicing by indole derivatives that target serine-arginine-rich protein splicing factors. Proc Natl Acad Sci U S A 2005;102(24):8764-9.
39. 39. Mann CJ, Honeyman K, McClorey G, Fletcher S, Wilton SD. Improved antisense oligonucleotide induced exon skipping in the mdx mouse model of muscular dystrophy. J Gene Med 2002;4(6):644-54.
40. 40. Graham IR, Hill VJ, Manoharan M, Inamati GB, Dickson G. Towards a therapeutic inhibition of dystrophin exon 23 splicing in mdx mouse muscle induced by antisense oligoribonucleotides (splicomers): target sequence optimisation using oligonucleotide arrays. J Gene Med 2004;6(10):1149-58.
41. 41. Mathews DH, Sabina J, Zuker M, Turner DH. Expanded sequence dependence of thermodynamic parameters improves prediction of RNA secondary structure. J Mol Biol 1999;288(5):911-40.
42. 42. Cartegni L, Chew SL, Krainer AR. Listening to silence and understanding nonsense: exonic mutations that affect splicing. Nat Rev Genet 2002;3(4):285-98.
43. 43. Cartegni L, Wang J, Zhu Z, Zhang MQ, Krainer AR. ESEfinder: A web resource to identify exonic splicing enhancers. Nucleic Acids Res 2003;31(13):3568-71.
44. 44. Braasch DA, Corey DR. Locked nucleic acid (LNA): fine-tuning the recognition of DNA and RNA. Chem Biol 2001;8(1):1-7.
45. 45. Braasch DA, Corey DR. Novel antisense and peptide nucleic acid strategies for controlling gene expression. Biochemistry 2002;41(14):4503-10.
46. 46. Elayadi AN, Corey DR. Application of PNA and LNA oligomers to chemotherapy. Curr Opin Investig Drugs 2001;2(4):558-61.

47. 47. Larsen HJ, Bentin T, Nielsen PE. Antisense properties of peptide nucleic acid. Biochim Biophys Acta 1999;1489(1):159-66.
48. 48. Summerton J. Morpholino antisense oligomers: the case for an RNase H-independent structural type. Biochim Biophys Acta 1999;1489(1):141-58.
49. 49. Summerton J, Weller D. Morpholino antisense oligomers: design, preparation, and properties. Antisense Nucleic Acid Drug Dev 1997;7(3):187-95.
50. 50. Wahlestedt C, Salmi P, Good L, et al. Potent and nontoxic antisense oligonucleotides containing locked nucleic acids. Proc Natl Acad Sci U S A 2000;97(10):5633-8.
51. 51. De Angelis FG, Sthandier 0, Berarducci B, et al. Chimeric snRNA molecules carrying antisense sequences against the splice junctions of exon 51 of the dystrophin pre-mRNA induce exon skipping and restoration of a dystrophin synthesis in Delta 48-50 DMD cells. Proc Natl Acad Sci U S A 2002;99(14):9456-61.
52. 52. Denti MA, Rosa A, D'Antona G, et al. Chimeric adeno-associated virus/antisense U1 small nuclear RNA effectively rescues dystrophin synthesis and muscle function by local treatment of mdx mice. Hum Gene Ther 2006;17(5):565-74.
53. 53. Gorman L, Suter D, Emerick V, Schumperli D, Kole R. Stable alteration of pre-mRNA splicing patterns by modified U7 small nuclear RNAs. Proc Natl Acad Sci U S A 1998;95(9):4929-34.
54. 54. Suter D, Tomasini R, Reber U, Gorman L, Kole R, Schumperli D. Double-target antisense U7 snRNAs promote efficient skipping of an aberrant exon in three human beta-thalassemic mutations. Hum Mol Genet 1999;8(13):2415-23.
55. 55. Wagner KR, Hamed S, Hadley DW, et al. Gentamicin treatment of Duchenne and Becker muscular dystrophy due to nonsense mutations. Ann Neurol 2001;49(6):706-11.
56. 56. Aartsma-Rus A et al, (2006), Entries in the leiden Duchenne Muscular Dystrophy mutation database: an overview of mutation types and paradoxical cases that confirm the reading-frame rule, Muscle Nerve, 34: 135-144.
57. 57. Hodgetts S., et al, (2006), Neuromuscular Disorders, 16: 591-602.
58. 58. Manzur AY et al, (2008), Glucocorticoid corticosteroids for Duchenne muscular dystrophy (review), Wiley publishers, The Cochrane collaboration.
59. 59. Van Deutekom JC et al, (2007) Antisense Oligonucleotide PRO051 Restores Local Dystrophin in DMD Patients. N Engl J Med., 357(26): 2677-86.
60. 60. Yuan H, Takeuchi E, Taylor GA, McLaughlin M, Brown D, Salant DJ. Nephrin dissociates from actin, and its expression is reduced in early experimental membranous nephropathy. J Am Soc Nephrol 2002;13:946-56.
61. 61. Koop K, Bakker RC, Eikmans M, et al. Differentiation between chronic rejection and chronic cyclosporine toxity by analysis of renal cortical mRNA. Kindney Int 2004;66:2038-46.
62. 62. Mercuri E, Bushby K, Ricci e., et al. Muscle MRI findings in patients with limb girdle muscular dystrophy with calpain 3 deficiency (LGMD2A) and early contractures. Neuromuscul Disord 2005;15:164-71.
63. 63. Dorph C, Nennesmo I, Lundberg IE. Percutaneous conchotome muscle biopsy: a useful diagnostic and assessment tool. J Rheumatol 2001;28:1591-9.
64. 64. Havenga MJ, Lemckert AA, Ophorst OJ, et al. Exploiting the natural diversity in adenovirus tropism for therapy and prevention of disease. J Virol 2002;76:4612-20.
65. 65. Roest PA, van der Tuijn AC, Ginjaar HB, et al. Application of in vitro Myo-differentation of non-muscle cells to enhance gene expression and facilitate analysis of muscle proteins. Neuromuscul Disord 1996;6:195-202.
66. 66. John J. Grading of muscular power: comparison of MRC and analogue scales by physiotherapists. Int J Rehabil Res 1984;7:173-81.
67. 67. Roest PA, Roberts RG, van der Tuijn AC, Heikoop JC, van Ommen GJ, den Dunnen JT. Protein truncation test (PTT) to rapidly screen the DMD gene for translation terminating mutations. Neuromuscul Disord 1993;3:391-4.
68. 68. Cullen MJ, Walsh J, Roberds SL, Campbell KP. Ultrastructural localization of adhalin, alpha-dystroglycan and merosin in normal and dystrophic muscle. Neuropathol Appl Neurobiol 1996;22:30-7.

REFERENCES CITED IN THE DESCRIPTION

This list of references cited by the applicant is for the reader's convenience only. It does not form part of the European patent document. Even though great care has been taken in compiling the references, errors or omissions cannot be excluded and the EPO disclaims all liability in this regard.

Patent documents cited in the description

- WO2004083432A [0078] [0082]
- WO2006112705A [0081]

Non-patent literature cited in the description

- The Science and Practice of PharmacyLippincott Williams & Wilkins20000000 [0062]
- **VAN DEUTEKOM JC et al.**Antisense Oligonucleotide PRO051 Restores Local Dystrophin in DMD Patients.N Engl J Med., 2007, vol. 357, 262677-86 [0123]
- **AARTSMA-RUS AJANSON AAKAMAN WE et al.**Therapeutic antisense-induced exon skipping in cultured muscle cells from six different DMD patients.Hum Mol Genet, 2003, vol. 12, 8907-14 [0147]
- **AARTSMA-RUS AJANSON AAKAMAN WE et al.**Antisense-induced multiexon skipping for Duchenne muscular dystrophy makes more senseAm J Hum Genet, 2004, vol. 74, 183-92 [0147]
- **ALTER J LOU FRABINOWITZ A et al.** Systemic delivery of morpholino oligonucleotide restores dystrophin expression bodywide and improves dystrophic pathologyNat Med, 2006, vol. 12, 2175-7 [0147]
- **GOYENVALLE AVULIN AFOUGEROUSSE F et al.**Rescue of dystrophic muscle through U7 snRNA-mediated exon skippingScience, 2004, vol. 306, 57021796-9 [0147]
- **LU QLMANN CJLOU F et al.**Functional amounts of dystrophin produced by skipping the mutated exon in the mdx dystrophic mouseNat Med, 2003, vol. 6, 6- [0147]
- **LU QLRABINOWITZ ACHEN YC et al.**Systemic delivery of antisense oligoribonucleotide restores dystrophin expression in body-wide skeletal muscles.Proc Natl Acad Sci U S A, 2005, vol. 102, 1198-203 [0147]
- **MCCLOREY GFALL AM MOULTON HM et al.**Induced dystrophin exon skipping in human muscle explants.Neuromuscul Disord, 2006, vol. 16, 9-10583-90 [0147]
- **MCCLOREY GMOULTON HMIVERSEN PL et al.**Antisense oligonucleotide-induced exon skipping restores dystrophin expression in vitro in a canine model of DMDGene Ther, 2006, vol. 13, 191373-81 [0147]
- **PRAMONO ZATAKESHIMA YALIMSARDJONO HISHII ATAKEDA SMATSUO M.**Induction of exon skipping of the dystrophin transcript in lymphoblastoid cells by transfecting an antisense oligodeoxynucleotide complementary to an exon recognition sequenceBiochem Biophys Res Commun, 1996, vol. 226, 2445-9 [0147]
- **TAKESHIMA YYAGI MWADA H et al.** Intravenous infusion of an antisense oligonucleotide results in exon skipping in muscle dystrophin mRNA of Duchenne muscular dystrophy.Pediatr Res, 2006, vol. 59, 5690-4 [0147]
- **VAN DEUTEKOM JCBREMMER-BOUT MJANSON AA et al.** Antisense-induced exon skipping restores dystrophin expression in DMD patient derived muscle cells.Hum Mol Genet, 2001, vol. 10, 151547-54 [0147]
- **AARTSMA-RUS ABREMMER-BOUT MJANSON ADEN DUNNEN JVAN OMMEN GVAN DEUTEKOM J.**Targeted exon skipping as a potential gene correction therapy for Duchenne muscular dystrophy.Neuromuscul Disord, 2002, vol. 12, S71-S77 [0147]
- **AARTSMA-RUS ADE WINTER CLJANSON AA et al.** Functional analysis of 114 exon-internal AONs for targeted DMD exon skipping: indication for steric hindrance of SR protein binding sites.Oligonucleotides, 2005, vol. 15, 4284-97 [0147]
- Therapeutic Modulation of DMD Splicing by Blocking Exonic Splicing Enhancer Sites with Antisense Oligonucleotides**AARTSMA-RUS AJANSON AAHEEMSKERK JACL DE WINTERGJ VAN OMMENJC VAN DEUTEKOM.**Annals of the New York Academy of Sciences20060000vol. 1082, 74-6 [0147]
- **AARTSMA-RUS AKAMAN WEWEIJ RDEN DUNNEN JTVAN OMMEN GJVAN DEUTEKOM JC.**Exploring the frontiers of therapeutic exon skipping for Duchenne muscular dystrophy by double targeting within one or multiple exons.Mol Ther, 2006, vol. 14, 3401-7 [0147]
- **WELCH EMBARTON ERZHUO J et al.**PTC124 targets genetic disorders caused by nonsense mutations.Nature, 2007, vol. 447, 714087-91 [0147]
- **HIRAWAT S WELCH EMELFRING GL et al.**Safety, tolerability, and pharmacokinetics of PTC124, a nonaminoglycoside nonsense mutation suppressor, following single- and multiple-dose administration to healthy male and female adult volunteers.Journal of clinical pharmacology, 2007, vol. 47, 4430-44 [0147]
- **WANG BLI JXIAO X.**Adeno-associated virus vector carrying human minidystrophin genes effectively ameliorates muscular dystrophy in mdx mouse modelProc Natl Acad Sci U S A, 2000, vol. 97, 2513714-9 [0147]
- **FABB SAWELLS DJSERPENTE PDICKSON G.**Adeno-associated virus vector gene transfer and sarcolemmal expression of a 144 kDa micro-dystrophin effectively restores the dystrophin-associated protein complex and inhibits myofibre degeneration in nude/mdx mice.Hum Mol Genet, 2002, vol. 11, 7733-41 [0147]
- **WANG ZKUHR CSALLEN JM et al.**Sustained AAV-mediated dystrophin expression in a canine model of Duchenne muscular dystrophy with a brief course of immunosuppression.Mol Ther, 2007, vol. 15, 61160-6 [0147]

- **MANZUR AY KUNTZER TPIKE MSWAN A.** Glucocorticoid corticosteroids for Duchenne muscular dystrophyCochrane Database Syst Rev, 2004, 2- [0147]
- **DUBOC DMEUNE CPIERRE B et al.**Perindopril preventive treatment on mortality in Duchenne muscular dystrophy: 10 years' follow-upAmerican heart journal, 2007, vol. 154, 3596-602 [0147]
- **COHN RDVAN ERP CHABASHI JP et al.**Angiotensin II type 1 receptor blockade attenuates TGF-beta-induced failure of muscle regeneration in multiple myopathic statesNat Med, 2007, vol. 13, 2204-10 [0147]
- **GROUNDS MDTORRISI J.**Anti-TNFalpha (Remicade) therapy protects dystrophic skeletal muscle from necrosisFaseb J, 2004, vol. 18, 6676-82 [0147]
- **HODGETTS SRADLEY HDAVIES MGROUNDS MD.**Reduced necrosis of dystrophic muscle by depletion of host neutrophils, or blocking TNFalpha function with Etanercept in mdx mice.Neuromuscul Disord, 2006, vol. 16, 9-10591-602 [0147]
- **PIERNO SNICO BBURDI R et al.**Role of tumour necrosis factor alpha, but not of cyclo-oxygenase-2-derived eicosanoids, on functional and morphological indices of dystrophic progression in mdx mice: a pharmacological approachNeuropathology and applied neurobiology, 2007, vol. 33, 3344-59 [0147]
- **MUSARO AMCCULLAGH KPAUL A et al.** Localized Igf-1 transgene expression sustains hypertrophy and regeneration in senescent skeletal muscleNat Genet, 2001, vol. 27, 2195-200 [0147]
- **BARTON ERMORRIS LMUSARO AROSENTHAL NSWEENEY HL**Muscle-specific expression of insulin-like growth factor I counters muscle decline in mdx miceJ Cell Biol, 2002, vol. 157, 1137-48 [0147]
- **DISATNIK MHDHAWAN JYU Y et al.**Evidence of oxidative stress in mdx mouse muscle: studies of the pre-necrotic stateJ Neurol Sci, 1998, vol. 161, 177-84 [0147]
- **NELSON SKBOSE SKGRUNWALD GKMYHILL PMCCORD JM.**The induction of human superoxide dismutase and catalase in vivo: a fundamentally new approach to antioxidant therapy.Free radical biology & medicine, 2006, vol. 40, 2341-7 [0147]
- **HART PELODI RRAJAGOPALAN B et al.**Antioxidant treatment of patients with Friedreich ataxia: four-year follow-up.Archives of neurology, 2005, vol. 62, 4621-6 [0147]
- **ROLLAND JFDE LUCA ABURDI RANDREETTA FCONFALONIERI PCONTE CAMERINO D.**Overactivity of exercise-sensitive cation channels and their impaired modulation by IGF-1 in mdx native muscle fibers: beneficial effect of pentoxifylline.Neurobiol Dis, 2006, vol. 24, 3466-74 [0147]
- **WHITEHEAD NPSTREAMER MLUSAMBILI LISACHS FALLEN DG.**Streptomycin reduces stretch-induced membrane permeability in muscles from mdx mice.Neuromuscul Disord, 2006, vol. 16, 12845-54 [0147]
- **BADALAMENTE MASTRACHER A.**Delay of muscle degeneration and necrosis in mdx mice by calpain inhibitionMuscle Nerve, 2000, vol. 23, 1106-11 [0147]
- **BURDI RDIDONNA MPPIGNOL B et al.**First evaluation of the potential effectiveness in muscular dystrophy of a novel chimeric compound, BN 82270, acting as calpain-inhibitor and anti-oxidant.Neuromuscul Disord, 2006, vol. 16, 4237-48 [0147]
- **BONUCCELLI GSOTGIA FSCHUBERT W et al.**Proteasome inhibitor (MG-132) treatment of mdx mice rescues the expression and membrane localization of dystrophin and dystrophin-associated proteinsAm J Pathol, 2003, vol. 163, 41663-75 [0147]
- **VOISIN VSEBRIE CMATECKI S et al.**L-arginine improves dystrophic phenotype in mdx mice.Neurobiol Dis, 2005, vol. 20, 1123-30 [0147]
- **SORET JBAKKOUR NMAIRE S et al.**Selective modification of alternative splicing by indole derivatives that target serine-arginine-rich protein splicing factorsProc Natl Acad Sci U S A, 2005, vol. 102, 248764-9 [0147]
- **MANN CJHONEYMAN KMCCLOREY GFLETCHER SWILTON SD.**Improved antisense oligonucleotide induced exon skipping in the mdx mouse model of muscular dystrophyJ Gene Med, 2002, vol. 4, 6644-54 [0147]
- **GRAHAM IRHILL VJMANOHARAN MINAMATI GBDICKSON G.**Towards a therapeutic inhibition of dystrophin exon 23 splicing in mdx mouse muscle induced by antisense oligoribonucleotides (splicomers): target sequence optimisation using oligonucleotide arraysJ Gene Med, 2004, vol. 6, 101149-58 [0147]
- **MATHEWS DHSABINA JZUKER MTURNER DH.**Expanded sequence dependence of thermodynamic parameters improves prediction of RNA secondary structure.J Mol Biol, 1999, vol. 288, 5911-40 [0147]
- **CARTEGNI LCHEW SLKRAINER AR.**Listening to silence and understanding nonsense: exonic mutations that affect splicingNat Rev Genet, 2002, vol. 3, 4285-98 [0147]
- **CARTEGNI LWANG JZHU ZZHANG MQKRAINER AR.**ESEfinder: A web resource to identify exonic splicing enhancers.Nucleic Acids Res, 2003, vol. 31, 133568-71 [0147]
- **BRAASCH DACOREY DR.**Locked nucleic acid (LNA): fine-tuning the recognition of DNA and RNAChem Biol, 2001, vol. 8, 11-7 [0147]
- **BRAASCH DACOREY DR.**Novel antisense and peptide nucleic acid strategies for controlling gene expression.Biochemistry, 2002, vol. 41, 144503-10 [0147]
- **ELAYADI AN COREY DR.**Application of PNA and LNA oligomers to chemotherapyCurr Opin Investig Drugs, 2001, vol. 2,

4558-61 [0147]

- **LARSEN HJBENTIN TNIELSEN PE.**Antisense properties of peptide nucleic acid.Biochim Biophys Acta, 1999, vol. 1489, 1159-66 [0147]
- **SUMMERTON J.**Morpholino antisense oligomers: the case for an RNase H-independent structural type.Biochim Biophys Acta, 1999, vol. 1489, 1141-58 [0147]
- **SUMMERTON JWELLER D.**Morpholino antisense oligomers: design, preparation, and properties.Antisense Nucleic Acid Drug Dev, 1997, vol. 7, 3187-95 [0147]
- **WAHLESTEDT CSALMI PGOOD L et al.**Potent and nontoxic antisense oligonucleotides containing locked nucleic acids.Proc Natl Acad Sci U S A, 2000, vol. 97, 105633-8 [0147]
- **DE ANGELIS FGSTHANDIER 0BERARDUCCI B et al.**Chimeric snRNA molecules carrying antisense sequences against the splice junctions of exon 51 of the dystrophin pre-mRNA induce exon skipping and restoration of a dystrophin synthesis in Delta 48-50 DMD cellsProc Natl Acad Sci U S A, 2002, vol. 99, 149456-61 [0147]
- **DENTI MAROSA AD'ANTONA G et al.**Chimeric adeno-associated virus/antisense U1 small nuclear RNA effectively rescues dystrophin synthesis and muscle function by local treatment of mdx mice.Hum Gene Ther, 2006, vol. 17, 5565-74 [0147]
- **GORMAN LSUTER DEMERICK VSCHUMPERLI DKOLE R.**Stable alteration of pre-mRNA splicing patterns by modified U7 small nuclear RNAsProc Natl Acad Sci U S A, 1998, vol. 95, 94929-34 [0147]
- **SUTER DTOMASINI RREBER UGORMAN LKOLE RSCHUMPERLI D.**Double-target antisense U7 snRNAs promote efficient skipping of an aberrant exon in three human beta-thalassemic mutations.Hum Mol Genet, 1999, vol. 8, 132415-23 [0147]
- **WAGNER KRHAMED SHADLEY DW et al.**Gentamicin treatment of Duchenne and Becker muscular dystrophy due to nonsense mutationsAnn Neurol, 2001, vol. 49, 6706-11 [0147]
- **AARTSMA-RUS A et al.** Entries in the leiden Duchenne Muscular Dystrophy mutation database: an overview of mutation types and paradoxical cases that confirm the reading-frame ruleMuscle Nerve, 2006, vol. 34, 135-144 [0147]
- **HODGETTS S. et al.**Neuromuscular Disorders, 2006, vol. 16, 591-602 [0147]
- Glucocorticoid corticosteroids for Duchenne muscular dystrophy (review)**MANZUR AY et al.** The Cochrane collaborationWiley publishers20080000 [0147]
- **VAN DEUTEKOM JC et al.**Antisense Oligonucleotide PRO051 Restores Local Dystrophin in DMD PatientsN Engl J Med., 2007, vol. 357, 262677-86 [0147]
- **YUAN HTAKEUCHI ETAYLOR GAMCLAUGHLIN MBROWN DSALANT DJ.**Nephrin dissociates from actin, and its expression is reduced in early experimental membranous nephropathyJ Am Soc Nephrol, 2002, vol. 13, 946-56 [0147]
- **KOOP KBAKKER RCEIKMANS M et al.**Differentiation between chronic rejection and chronic cyclosporine toxity by analysis of renal cortical mRNAKindney Int, 2004, vol. 66, 2038-46 [0147]
- **MERCURI EBUSHBY KRICCI E. et al.**Muscle MRI findings in patients with limb girdle muscular dystrophy with calpain 3 deficiency (LGMD2A) and early contractures.Neuromuscul Disord, 2005, vol. 15, 164-71 [0147]
- **DORPH CNENNESMO ILUNDBERG IE.**Percutaneous conchotome muscle biopsy: a useful diagnostic and assessment tool.J Rheumatol, 2001, vol. 28, 1591-9 [0147]
- **HAVENGA MJ LEMCKERT AAOPHORST OJ et al.**Exploiting the natural diversity in adenovirus tropism for therapy and prevention of diseaseJ Virol, 2002, vol. 76, 4612-20 [0147]
- **ROEST PAVAN DER TUIJN AC GINJAAR HB et al.**Application of in vitro Myo-differentation of non-muscle cells to enhance gene expression and facilitate analysis of muscle proteinsNeuromuscul Disord, 1996, vol. 6, 195-202 [0147]
- **JOHN J.**Grading of muscular power: comparison of MRC and analogue scales by physiotherapistsInt J Rehabil Res, 1984, vol. 7, 173-81 [0147]
- **ROEST PAROBERTS RGVAN DER TUIJN AC HEIKOOP JCVAN OMMEN GJDEN DUNNEN JT.** Protein truncation test (PTT) to rapidly screen the DMD gene for translation terminating mutationsNeuromuscul Disord, 1993, vol. 3, 391-4 [0147]
- **CULLEN MJWALSH JROBERDS SLCAMPBELL KP.**Ultrastructural localization of adhalin, alpha-dystroglycan and merosin in normal and dystrophic muscleNeuropathol Appl Neurobiol, 1996, vol. 22, 30-7 [0147]

Patentkrav

1. Kombination af:
- et antisenseoligonukleotid, omfattende en sekvens, der er komplementær til en del af human dystrofin-præ-mRNA exon 51, og hvilket oligonukleotid er gengivet ved SEQ ID NO:204
og
- en tilsætningsforbindelse til reduktion af inflammation, hvor denne forbindelse omfatter en steroid, hvilken kombination er til anvendelse som et medikament til lindring af et eller flere symptom(er) på Duchennes muskeldystrofi hos individet.

2. Kombination til anvendelse ifølge krav 1, hvor fraværet af exonet fra mRNA fremstillet ud fra dystrofin-præ-mRNA'et genererer en kodende region til et funktionelt dystrofinprotein.

3. Kombination til anvendelse ifølge krav 1 eller 2, hvor oligonukleotidet omfatter RNA.

4. Kombination til anvendelse ifølge krav 3, hvor RNA'et indeholder en 2'-*O*-methyl-modificeret ribose (RNA).

5. Kombination til anvendelse ifølge krav 3 eller 4, hvor oligonukleotidet er et 2'-*O*-methylphosphorothioat-oligoribonukleotid.

6. Kombination til anvendelse ifølge et hvilket som helst af kravene 1 til 5, hvor oligonukleotidet er en peptidnukleinsyre, låst nukleinsyre, morpholinophosphorodiamidat eller en hvilken som helst kombination deraf.

7. Farmaceutisk præparat omfattende
- en kombination som defineret i et hvilket som helst af kravene 1 til 6
og
- en farmaceutisk acceptabel bærer, adjuvans, fortyndingsmiddel og/eller excipiens.

DRAWINGS

Fig 1a

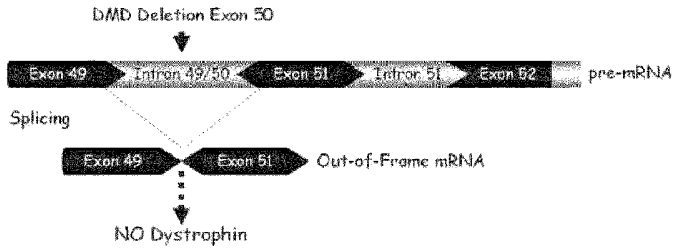


Fig 1b

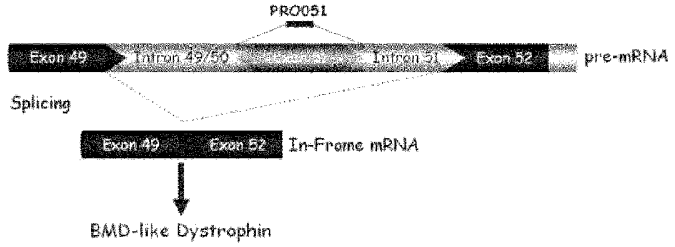


Fig 2

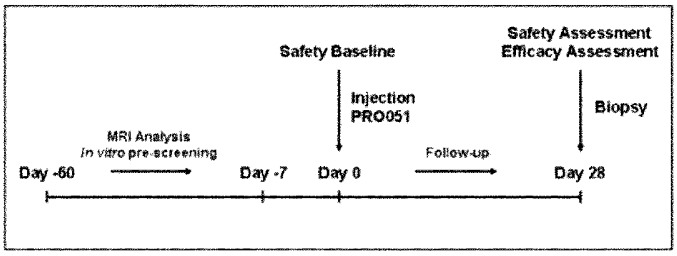


Fig 3a

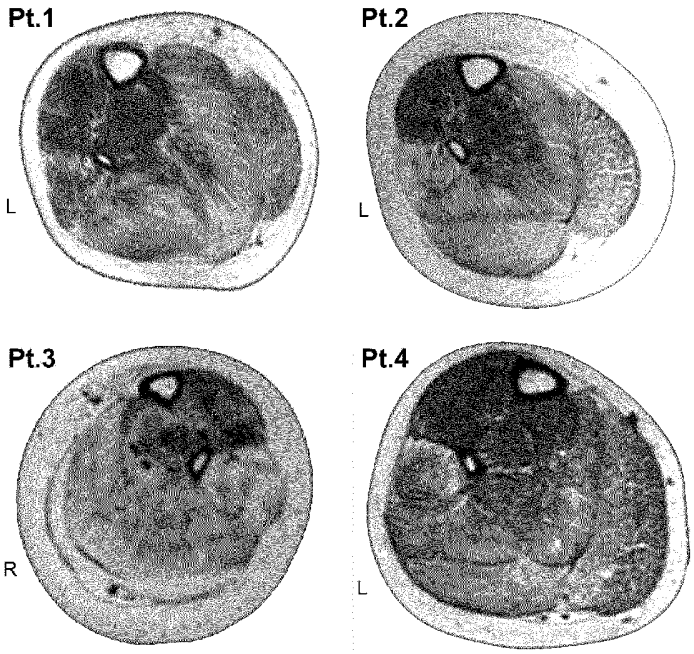


Fig 3b

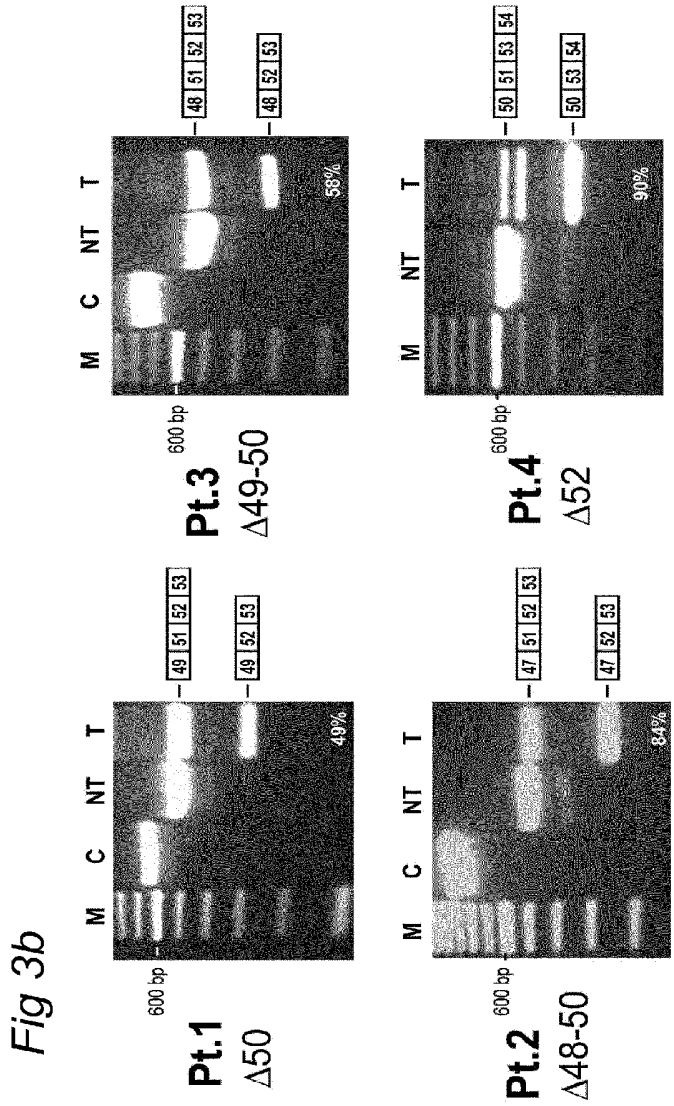

M
C
NT
T
600 bp
49%
49 51 52 53
49 52 53
Pt.1
Δ50
56%
48 51 52 53
48 52 53
Pt.3
Δ49-50
64%
47 51 52 53
47 52 53
Pt.2
Δ48-50
90%
50 51 53 54
50 53 54
Pt.4
Δ52

Fig 3c

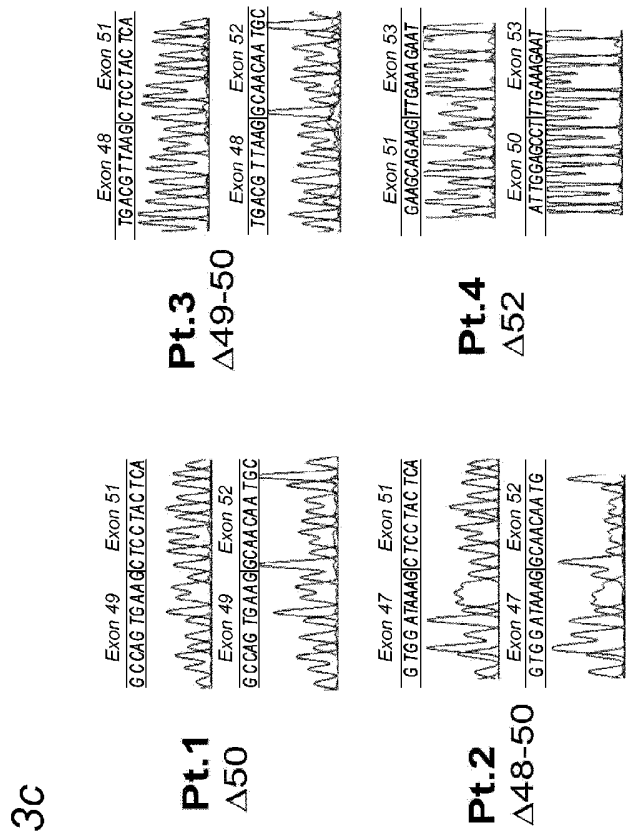
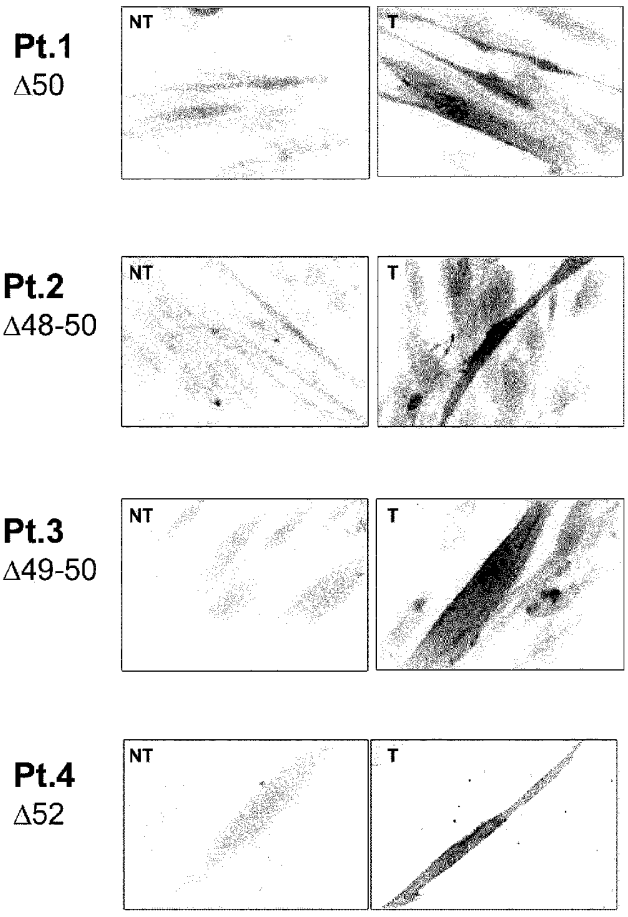

Exon 49 Exon 51
GCCAG TGAAGC TCC TAC TCA
Pt.1
Δ50
Exon 49 Exon 52
GCCAG TGAAGGCAACAA TGC
Exon 47 Exon 51
G TG G ATAAAGC TCC TAC TCA
Pt.2
Δ48-50
Exon 47 Exon 52
GTG G ATAAAGGCAACAA TG
Exon 48 Exon 51
TGACG TTAAGC TCC TAC TCA
Pt.3
Δ49-50
Exon 48 Exon 52
TGACG TTAAGGCAACAA TGC
Exon 51 Exon 53
GAAGCAGAAGTTCAAA GAAT
Pt.4
Δ52
Exon 50 Exon 53
ATTGGAGCCTTTGAAAGAAT

Fig 3d



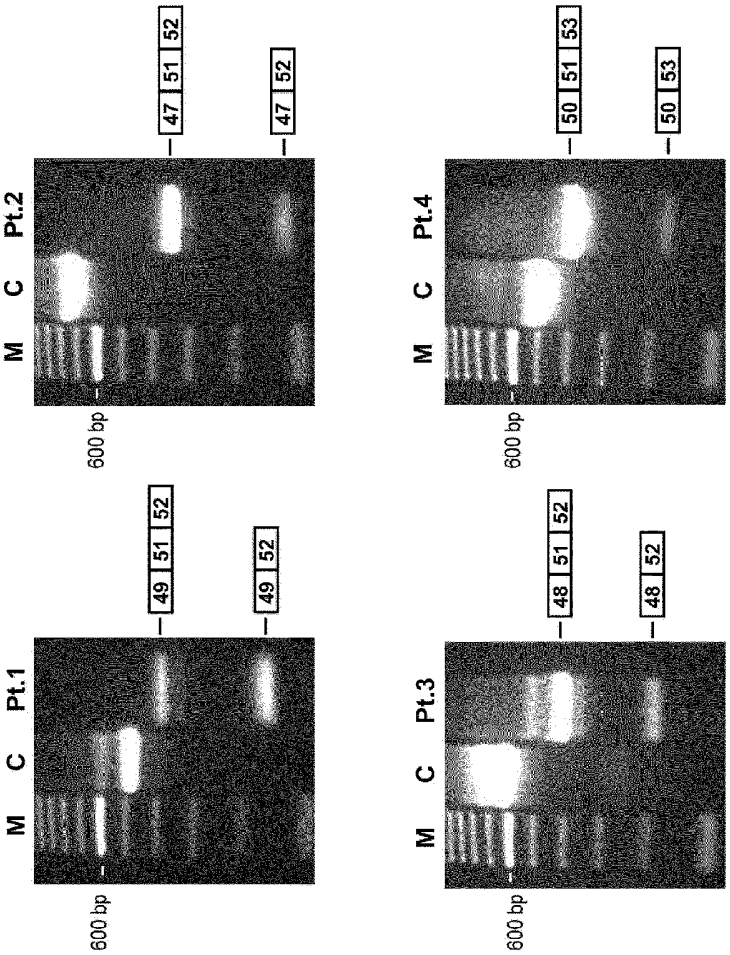

M
C
Pt.1
600 bp
49
51
52
49
52
M
C
Pt.2
600 bp
47
51
52
47
52
M
C
Pt.3
600 bp
48
51
52
48
52
M
C
Pt.4
600 bp
50
51
53
50
53

Fig 4a

Fig 4b

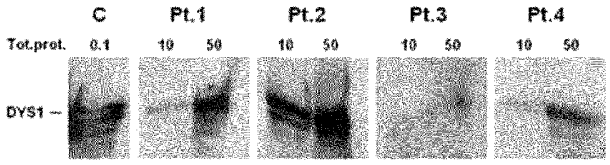

C
Pt.1
Pt.2
Pt.3
Pt.4
Tot.prot.
0.1
10
50
10
50
10
50
10
50
DYS1

Fig 4c

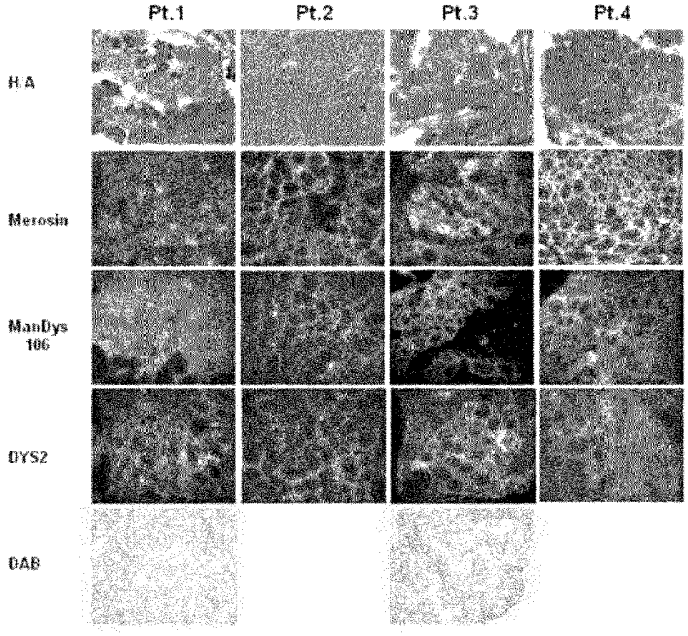

Pt.1
Pt.2
Pt.3
Pt.4
H.A
Merosin
ManDys 106
DYS2
DAB

Fig 5

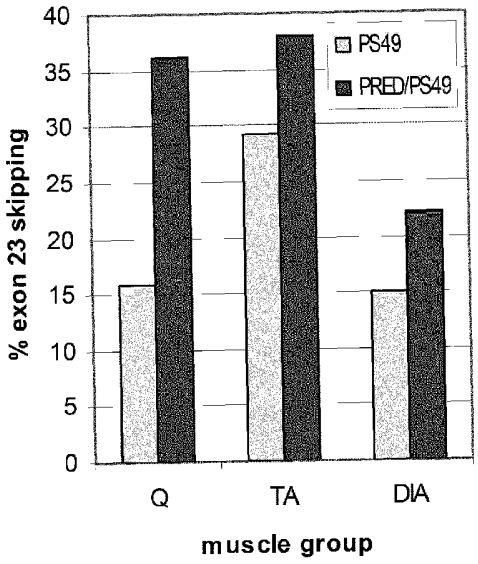

40
35
30
25
20
15
10
5
0
% exon 23 skipping
PS49
PRED/PS49
Q
TA
DIA
muscle group

Fig 6a

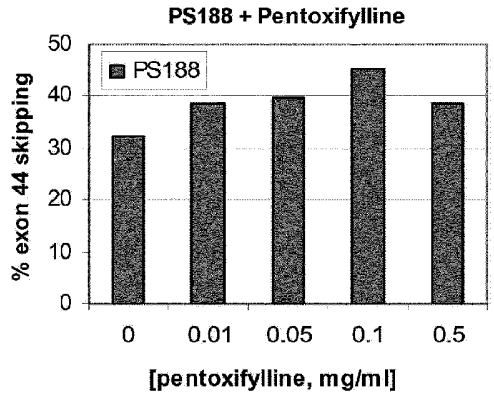

PS188 + Pentoxifylline
PS188
% exon 44 skipping
50
40
30
20
10
0
0
0.01
0.05
0.1
0.5
[pentoxifylline, mg/ml]

Fig 6b

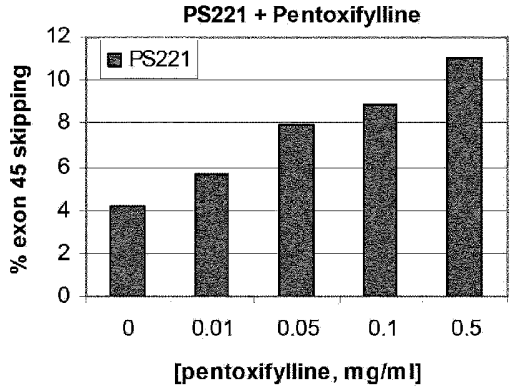

PS221 + Pentoxifylline
PS221
% exon 45 skipping
12
10
8
6
4
2
0
0
0.01
0.05
0.1
0.5
[pentoxifylline, mg/ml]

Fig 6c

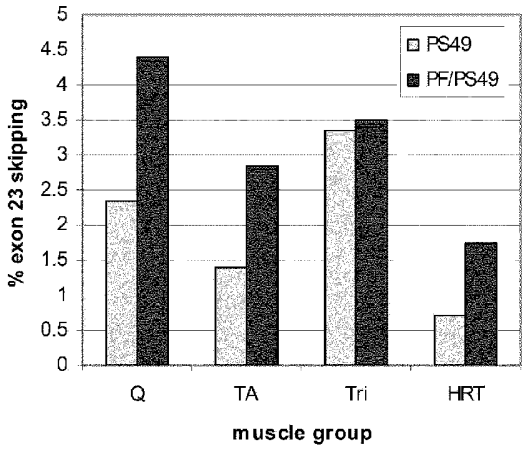

5
4.5
4
3.5
3
2.5
2
1.5
1
0.5
0
% exon 23 skipping
PS49
PF/PS49
Q
TA
Tri
HRT
muscle group

Fig 7

SEQ ID NO 1:

MLWWEEVEDCYEREDVQKKTFTKWVNAQFSKFGKQHIENLFSDLQDGRRLLDLLEGLTG
QKLPKEKGSTRVIIALNNVNKALRVLQNNNVDLVNIGSTDIVDGNIIKLTLGLIWNILIIWQ
VKNVMKNIMAGLQQTNSEKILLSWVRQSTRNYPQVNVINFTTSWSDGLALNALIHSHRPDL
FDWNSVVCQQSATQRLEHAFNIARYQLGIEKLLDPEDVDTTYPDKKSILMYITSLFQVLPQQ
VSIEAIQEVEMLPRPPKVTKEEHFQLHHQMHYSQQITVSLAQGYERTSSPKPRFKSYAYTQ
AAYVTTSDPTRSPFPSQHLEAPEDKSFGSSLMESEVNLDRYQTALEEVLSWLLSAEDTLQA
QGEISNDVEVVKDQFITHEGYMMDLTAHQGRVGNILQLGSKLIGTGKLSEDEETEVQEQM
NLLNSRWECLRVASMEKQSNLHRVLMDLQNQKLKELNDWLTKTEERTRKMEEEPLGPDL
EDLKRQVQQHKVLQEDLEQEQVRVNSLTHMVVVVDESSGDHATAALEEQLKVLGDRWAN
ICRWTEDRWVLLQDILLKWQRLTEEQCLFSAWLSEKEDAVNKIHTTGFKDQNEMLSSLQK
LAVLKADLEKKKQSMGKLYSLKQDLLSTLKNKSVTQKTEAWLDNFARCWDNLVQKLEKS
TAQISQAVTTTQPSLTQTTVMETVTTVTTREQILVKHAQEELPPPPPQKKRQITVDSEIRKRL
DVDITELHSWITRSEAVLQSPEPAIERKEGNFSDLKEKVNAIEREKAEKFRKLQDASRSAQA
LVEQMVNEGVNADSIKQASEQLNSRWIEFCQLLSERLNWLEYQNNIIAFYNQLQQLEQMT
TTAENWLKIQPTTPSEPTAIKSQLKICKDEVNRLSCLQPQIERLKIQSIALKEKGQGPMFLDA
DFVAFTNHFKQVFSDVQAREKELQTIFDTLPPMRYQETMSAIRTWVQQSETKLSIPQLSVT
DYEIMEQRLGELQALQSSLQEQQSGLYYLSTTVKEMSKKAPSEISRKYQSEFEEIEGRWKK
LSSQLVEHCQKLEEQMNKLRKIQNHIQTLKKWMAEVDVFLKEEWPALGDSEILKKQLKQC
RLLVSDIQTIQPSLNSVNEGGQKIKNEAEPEFASRLETELKELNTQWDHMCQQVYARKEAL
KGGLEKTVSLQKDLSEMHEWMTQAEEEYLERDFEYKTPDELQKAVEEMKRAKEEAQQKE
AKVKLLTESVNSVIAQAPPVAQEALKKELETLTTNYQWLCTRLNGKCKTLEEVWACWHEL
LSYLEKANKWLNEVEFKLKTTENIPGGAEEISEVLDSLENLMRHSEDNPNQIRILAQTLTD
GGVMDELINEELETFNSRWRELHEEAVRRQKLLEQSIQSAQETEKSLHLIQESLTFIDKQLA
AYIADKVDAAQMPQEAQKIQSDLTSHEISLEEMKKHNQGKEAAQRVLSQIDVAQKKLQDV
SMKFRLFQKPANFEQRLQESKMILDEVKMHLPALETKSVEQEVVQSQLNHCVNLYKSLSE
VKSEVEMVIKTGRQIVQKKQTENPKELDERVTALKLHYNELGAKVTERKQQLEKCLKLSR
KMRKEMNVLTEWLAATDMELTKRSAVEGMPSNLDSEVAWGKATQKEIEKQKVHLKSITE
VGEALKTVLGKKETLVEDKLSLLNSNWIAVTSRAEEWLNLLLEYQKHMETFDQNVDHITK
WIIQADTLLDESEKKKPQQKEDVLKRLKAELNDIRPKVDSTRDQAANLMANRGDHCRKLV
EPQISELNHRFAAISHRIKTGKASIPLKELEQFNSDIQKLLEPLEAEIQQGVNLKEEDFNKD
MNEDNEGTVKELLQRGDNLQQRITDERKREEIKIKQQLLQTKHNALKDLRSQRRKKALEIS
IIQWYQYKRQADDLLKCLDDIEKKLASLPEPRDERKIKEIDRELQKKKEELNAVRRQAEGL
SEDGAAMAVEPTQIQLSKRWREIESKFAQFRRLNFAQIHTVREETMMVMTEDMPLEISYVP
STYLTEITHVSQALLEVEQLLNAPDLCAKDFEDLFKQEESLKNIKDSLQQSSGRIDIIHSKKT
AALQSATPVERVKLQEALSQLDFQWEKVNKMYKDRQGRFDRSVEKWRRFHYDIKIFNQW
LTEAEQFLRKTQIPENWEHAKYKWYLKELQDGIGQRQTVVRTLNATGEEIIQQSSKTDASIL
QEKLGSLNLRWQEVCKQLSDRKKRLEEQKNILSEFQRDLNEFVLWLEEADNIASIPLEPGK
EQQLKEKLEQVKLLVEELPLRQGILKQLNETGGPVLVSAPISPEEQDKLENKLKQTNLQWI
KVSRALPEKQGEIEAQIKDLGQLEKKLEDLEEQLNHLLLWLSPIRNQLEIYNQPNQEGPFD
VQETEIAVQAKQPDVEEILSKGQHLYKEKPATQPVKRKLEDLSSEWKAVNRLLQELRAKQP
DLAPGLTTIGASPTQTVTLVTQPVVTKETAISKLEMPSSLMLEVPALADFNRAWTELTDWLS
LLDQVIKSQRVMVGDLEDINEMIIKQKATMQDLEQRRPQLEELITAAQNLKNKTSNQEART
IITDRIERIQNQWDEVQEHLQNRRQQLNEMLKDSTQWLEAKEEAEQVLGQARAKLESWKE
GPYTVDAIQKKITETKQLAKDLRQWQTNVDVANDLALKLLRDYSADDTRKVIIMITENINAS
WRSIHKRVSEREAALEETHRLLQQFPLDLEKFLAWLTEAETTANVLQDATRKERLLEDSKG
VKELMKQWQDLQGEIEAHTDVYHNLDENSQKILRSLEGSDDAVLLQRRLDNMNFKWSEL
RKKSLNIRSHLEASSDQWKRLHLSLQELLVWLQLKDDELSRQAPIGGDFPAVQKQNDVHR
AFKRELKTKEPVIMSTLETVRIFLTEQPLEGLEKLYQEPRELPPEERAQNVTRLLRKQAEEV
NTEWEKLNLIISADWQRKIDETLERLQELQEATDELDLKLRQAEVIKGSWQPVGDLLIDSL

QDHLEKVKALRGEIAPLKENVSHVNDLARQLTTLGIQLSPYNLSTLEDLNTRWKLLQVAVE
DRVRQLHEAHRDFGPASQHFLSTSVQGPWERAISPNKVPYYINHETQTTCWDHPKMTELY
QSLADLNNVRFSAYRTAMKLRRLQKALCLDLLSLSAACDALDQHNLKQNDQPMDILQIINC
LTTIYDRLEQEHNNLVNVPLCVDMCLNWLLNVYDTGRTGRIRVLSFKTGIISLCKAHLEDK
YRYLFKQVASSTGFCDQRRLGLLLHDSIQIPRQLGEVASFGGSNIEPSVRSCFQFANNKPEIE
AALFLDWMRLEPQSMVWLPVLHRVAAAETAKHQAKCNICKECPIIGFRYRSLKHFNYDICQ
SCFFSGRVAKGHKMHYPMVEYCTPTTSGEDVRDFAKVLKNKFRTKRYFAKHPRMGYLPV
QTVLEGDNMETPVTLINFWPVDSAPASSPQLSHDDTHSRIEHYASRLAEMENSNGSYLNDS
ISPNESIDDEHLLIQHYCQSLNQDSPLSQPRSPAQILISLESEERGELERILADLEEENRNLQ
AEYDRLKQQHEHKGLSPLPSPPEMMPTSPQSPRDAELIAEAKLLRQHKGRLEARMQILED
HNKQLESQLHRLRQLLEQPQAEAKVNGTTVSSPSTSLQRSDSSQPMLLRVVGSQTSDSMGE
EDLLSPPQDTSTGLEEVMEQLNNSFPSSRGRNTPGKPMREDTM

Fig 8

Human IGF-1 Isoform 4 amino acid sequence

SEQ ID NO 2:
MGKISSLPTQLFKCCFCDFLKVKMHTMSSSHLFYLALCLLTFTSSATAGPETLCGAELVDAL
QFVCGDRGFYFNKPTGYGSSSRRAPQTGIVDECCFRSCDLRRLEMYCAPLKPAKSARSVRA
QRHTDMPKTQKEVHLKNASRGSAGNKNYRM

Fig 9

DMD Gene Exon 43

SEQ ID NO 3	CGACC UGAGC UUUGU UGUAG
SEQ ID NO 4	CGACC UGAGC UUUGU UGUAG ACUAU
SEQ ID NO 5	CCUGA GCUUU GUUGU AGACU AUC
SEQ ID NO 6	CGUUG CACUU UGCAA UGCUG CUG
SEQ ID NO 7	CUGUA GCUUC ACCCU UUCC
SEQ ID NO 8	GAGAG AGCUU CCUGU AGCUU CACC
SEQ ID NO 9	GUCCU UGUAC AUUUU GUUAA CUUUU UC
SEQ ID NO 263	GGA GAG AGC UUC CUG UAG CU
SEQ ID NO 264	UCA CCC UUU CCA CAG GCC UUG CA
SEQ ID NO 265	UGCACUUUGCAAUGCUGCUGUCUUCUUG CUAU

DMD Gene Exon 44

SEQ ID NO 10	UCAGCUUCUGUUAGCCACUG	SEQ ID NO 35	AGCUUCUGUUAGCCACUGAUUAAA
SEQ ID NO 11	UUCAGCUUCUGUUAGCCACU	SEQ ID NO 36	CAGCUUCUGUUAGCCACUGAUUAAA
SEQ ID NO 12	UUCAGCUUCUGUUAGCCACUG	SEQ ID NO 37	AGCUUCUGUUAGCCACUGAUUAAA
SEQ ID NO 13	UCAGCUUCUGUUAGCCACUGA	SEQ ID NO 38	AGCUUCUGUUAGCCACUGAU
SEQ ID NO 14	UUCAGCUUCUGUUAGCCACUGA	SEQ ID NO 39	GCUUCUGUUAGCCACUGAUU
SEQ ID NO 15	UCAGCUUCUGUUAGCCACUGA	SEQ ID NO 40	AGCUUCUGUUAGCCACUGAUU
SEQ ID NO 16	UUCAGCUUCUGUUAGCCACUGA	SEQ ID NO 41	GCUUCUGUUAGCCACUGAUUA
SEQ ID NO 17	UCAGCUUCUGUUAGCCACUGAU	SEQ ID NO 42	AGCUUCUGUUAGCCACUGAUUA
SEQ ID NO 18	UUCAGCUUCUGUUAGCCACUGAU	SEQ ID NO 43	GCUUCUGUUAGCCACUGAUUAA
SEQ ID NO 19	UCAGCUUCUGUUAGCCACUGAUU	SEQ ID NO 44	AGCUUCUGUUAGCCACUGAUUAA
SEQ ID NO 20	UUCAGCUUCUGUUAGCCACUGAUU	SEQ ID NO 45	GCUUCUGUUAGCCACUGAUUAAA
SEQ ID NO 21	UCAGCUUCUGUUAGCCACUGAUUA	SEQ ID NO 46	AGCUUCUGUUAGCCACUGAUUAAA
SEQ ID NO 22	UUCAGCUUCUGUUAGCCACUGAUA	SEQ ID NO 47	GCUUCUGUUAGCCACUGAUUAAA
SEQ ID NO 23	UCAGCUUCUGUUAGCCACUGAUUAA	SEQ ID NO 48	CCAUUUGUAUUUAGCAUGUUCCC
SEQ ID NO 24	UUCAGCUUCUGUUAGCCACUGAUUAA	SEQ ID NO 49	AGAUACCAUUUGUAUUUAGC
SEQ ID NO 25	UCAGCUUCUGUUAGCCACUGAUUAAA	SEQ ID NO 50	GCCAUUUGUCAACAGAUCU
SEQ ID NO 26	UUCAGCUUCUGUUAGCCACUGAUUAAA	SEQ ID NO 51	GCCAUUUCUCAACAGAUCUGUCA

SEQ ID NO 27	CAGCUUCUGUUAGCCACUG	SEQ ID NO 52	AUUCUCAGGAAUUUGUGUCUUUC
SEQ ID NO 28	CAGCUUCUGUUAGCCACUGAU	SEQ ID NO 53	UCUCAGGAAUUUGUGUCUUUC
SEQ ID NO 29	AGCUUCUGUUAGCCACUGAUU	SEQ ID NO 54	GUUCAGCUUCUGUUAGCC
SEQ ID NO 30	CAGCUUCUGUUAGCCACUGAUU	SEQ ID NO 55	CUGAUUAAAUAUCUUUAUAU C
SEQ ID NO 31	AGCUUCUGUUAGCCACUGAUUA	SEQ ID NO 56	GCCGCCAUUUCUCAACAG
SEQ ID NO 32	CAGCUUCUGUUAGCCACUGAUUA	SEQ ID NO 57	GUAUUUAGCAUGUUCCCA
SEQ ID NO 33	AGCUUCUGUUAGCCACUGAUUAA	SEQ ID NO 58	CAGGAAUUUGUGUCUUUC
SEQ ID NO 34	CAGCUUCUGUUAGCCACUGAUUAA	SEQ ID NO 267	UUU GUG UCU UUC UGA GAA AC
SEQ ID NO 266	UCAUAAUGAAAACGCCGCCAUUUCUCAACAG AUCU	SEQ ID NO 268	UUUAGCAUGUUCCCAAUUCUCAGGA AUUUG

DMD Gene Exon 45

SEQ ID NO 59	UUUGCCGCUGCCCAAUGCCAUCCUG	SEQ ID NO 89	GUUGCAUUCAAUGUUCUGACAACAG
SEQ ID NO 60	AUUCAAUGUUCUGACAACAGUUUGC	SEQ ID NO 90	UUGCAUUCAAUGUUCUGACAACAGU
SEQ ID NO 61	CCAGUUGCAUUCAAUGUUCUGACAA	SEQ ID NO 91	UGCAUUCAAUGUUCUGACAACAGUU
SEQ ID NO 62	CAGUUGCAUUCAAUCUUCUCAC	SEQ ID NO 92	CCAUUCAAUCUUCUCACAACAGUUU
SEQ ID NO 63	AGUUGCAUUCAAUGUUCUGA	SEQ ID NO 93	CAUUCAAUGUUCUGACAACAGUUUG
SEQ ID NO 64	GAUUGCUGAAUUAUUUCUUCC	SEQ ID NO 94	AUUCAAUGUUCUGACAACAGUUUGC
SEQ ID NO 65	GAUUGCUGAAUUAUUUCUUCCCCAG	SEQ ID NO 95	UCAAUGUUCUGACAACAGUUUGCCG
SEQ ID NO 66	AUUCCUCAAUUAUUUCUUCCCCAGU	SEQ ID NO 96	CAAUCUUCUCACAACAGUUUGCCCC
SEQ ID NO 67	UUGCUGAAUUAUUUCUUCCCCAGUU	SEQ ID NO 97	AAUGUUCUGACAACAGUUUGCCGCU
SEQ ID NO 68	UGCUGAAUUAUUUCUUCCCCAGUUG	SEQ ID NO 98	AUGUUCUGACAACAGUUUGCCGCUG
SEQ ID NO 69	GCUGAAUUAUUUCUUCCCCAGUUGC	SEQ ID NO 99	UGUUCUGACAACAGUUUGCCGCUGC
SEQ ID NO 70	CUGAAUUAUUUCUUCCCCAGUUGCA	SEQ ID NO 100	GUUCUGACAACAGUUUGCCGCUGCC
SEQ ID NO 71	UGAAUUAUUUCUUCCCCAGUUGCAU	SEQ ID NO 101	UUCUGACAACAGUUUGCCGCUGCCC
SEQ ID NO 72	GAAUUAUUUCUUCCCCAGUUGCAUU	SEQ ID NO 102	UCUGACAACAGUUUGCCGCUGCCCA
SEQ ID NO 73	AAUUAUUUCUUCCCCAGUUGCAUUC	SEQ ID NO 103	CUGACAACAGUUUGCCGCUGCCCAA
SEQ ID NO 74	AUUAUUUCUUCCCCAGUUGCAUUCA	SEQ ID NO 104	UGACAACAGUUUGCCGCUGCCCAAU
SEQ ID NO 75	UUAUUUCUUCCCCAGUUGCAUUCAA	SEQ ID NO 105	GACAACAGUUUGCCGCUGCCCAAUG
SEQ ID NO 76	UAUUUCUUCCCCAGUUGCAUUCAAU	SEQ ID NO 106	ACAACAGUUUGCCGCUGCCCAAUGC
SEQ ID NO 77	AUUUCUUCCCCAGUUGCAUUCAAUG	SEQ ID NO 107	CAACAGUUUGCCGCUGCCCAAUGCC

SEQ ID NO 78	UUUCUUCCCCAGUUGCAUUCAAUGU	SEQ ID NO 108	AACAGUUUGCCGCUGCCCAAUGCCA
SEQ ID NO 79	UUCUUCCCCAGUUGCAUUCAAUGUU	SEQ ID NO 109	ACAGUUUGCCGCUGCCCAAUGCCAU
SEQ ID NO 80	UCUUCCCCAGUUGCAUUCAAUGUUC	SEQ ID NO 110	CAGUUUGCCGCUGCCCAAUGCCAUC
SEQ ID NO 81	CUUCCCCAGUUGCAUUCAAUGUUCU	SEQ ID NO 111	AGUUUGCCGCUGCCCAAUGCCAUCC
SEQ ID NO 82	UUCCCCAGUUGCAUUCAAUGUUCUG	SEQ ID NO 112	GUUUGCCGCUGCCCAAUGCCAUCCU
SEQ ID NO 83	UCCCCAGUUGCAUUCAAUGUUCUGA	SEQ ID NO 113	UUUGCCGCUGCCCAAUGCCAUCCUG
SEQ ID NO84	CCCCAGUUGCAUUCAAUGUUCUGAC	SEQ ID NO 114	UUGCCGCUGCCCAAUGCCAUCCUGG
SEQ ID NO 85	CCCAGUUGCAUUCAAUGUUCUGACA	SEQ ID NO 115	UGCCGCUGCCCAAUGCCAUCCUGGA
SEQ ID NO 86	CCAGUUGCAUUCAAUGUUCUGACAA	SEQ ID NO 116	GCCGCUGCCCAAUGCCAUCCUGGAG
SEQ ID NO 87	CAGUUGCAUUCAAUGUUCUGACAAC	SEQ ID NO 117	CCGCUGCCCAAUGCCAUCCUGGAGU
SEQ ID NO 88	AGUUGCAUUCAAUGUUCUGACAACA	SEQ ID NO 118	CGCUGCCCAAUGCCAUCCUGGAGUU
SEQ ID NO 269	UCC UGU AGA AUA CUG GCA UC	SEQ ID NO 272	UGU UUU UGA GGA UUG CUG AA
SEQ ID NO 270	UGC AGA CCU CCU GCC ACC GCA GAU UCA	SEQ ID NO 273	UGUUCUGACAACAGUUUGCCGCUGCCC AAUGCCAUCCUGG
SEQ ID NO 271	UUGCAGACCUCCUGCCACCGCAGAUUCAGGCU UC		

DMD Gene Exon 46

SEQ ID NO 119	GCUUUUCUUUUUAGUUGCUGCUCUUU	SEQ ID NO 147	AGGUUCAAGUGGGAUACUAGCAAUG
SEQ ID NO 120	CUUUUCUUUUAGUUGCUGCUCUUUU	SEQ ID NO 148	GGUUCAAGUGGGAUACUAGCAAUGU
SEQ ID NO 121	UUUUCUUUUAGUUGCUGCUCUUUUC	SEQ ID NO 149	GUUCAAGUGGGAUACUAGCAAUGUU
SEQ ID NO 122	UUUCUUUUAGUUGCUGCUCUUUCC	SEQ ID NO 150	UUCAAGUGGGAUACUAGCAAUGUUA
SEQ ID NO 123	UUCUUUUAGUUGCUGCUCUUUUCCA	SEQ ID NO 151	UCAAGUGGGAUACUAGCAAUGUUAU
SEQ ID NO 124	UCUUUUAGUUGCUGCUCUUUUCCAG	SEQ ID NO 152	CAAGUGGGAUACUAGCAAUGUUAUC
SEQ ID NO 125	CUUUUAGUUGCUGCUCUUUUCCAGG	SEQ ID NO 153	AAGUGGGAUACUAGCAAUGUUAUCU
SEQ ID NO 126	UUUUAGUUGCUGCUCUUUUCCAGGU	SEQ ID NO 154	AGUGGGAUACUAGCAAUGUUAUCUG
SEQ ID NO 127	UUUAGUUGCUGCUCUUUUCCAGGUU	SEQ ID NO 155	GUGGGAUACUAGCAAUGUUAUCUGC
SEQ ID NO 128	UUAGUUGCUGCUCUUUUCCAGGUUC	SEQ ID NO 156	UGGGAUACUAGCAAUGUUAUCUGCU
SEQ ID NO 129	UAGUUGCUGCUCUUUUCCAGGUUCA	SEQ ID NO 157	GGGAUACUAGCAAUGUUAUCUGCUU
SEQ ID NO 130	AGUUGCUGCUCUUUUCCAGGUUCAA	SEQ ID NO 158	GGAUACUAGCAAUGUUAUCUGCUUC
SEQ ID NO 131	GUUGCUGCUCUUUUCCAGGUUCAAG	SEQ ID NO 159	GAUACUAGCAAUGUUAUCUGCUUCC
SEQ ID NO 132	UUGCUGCUCUUUUCCAGGUUCAAGU	SEQ ID NO 160	AUACUAGCAAUGUUAUCUGCUUCCU
SEQ ID NO 133	UGCUGCUCUUUUCCAGGUUCAAGUG	SEQ ID NO 161	UACUAGCAAUGUUAUCUGCUUCCUC
SEQ ID NO 134	GCUGCUCUUUUCCAGGUUCAAGUGG	SEQ ID NO 162	ACUAGCAAUGUUAUCUGCUUCCUCC
SEQ ID NO 135	CUGCUCUUUUCCAGGUUCAAGUGGG	SEQ ID NO 163	CUAGCAAUGUUAUCUGCUUCCUCCA
SEQ ID NO 136	UGCUCUUUUCCAGGUUCAAGUGGGA	SEQ ID NO 164	UAGCAAUGUUAUCUGCUUCCUCCAA

SEQ ID NO 137	GCUCUUUUCCAGGUUCAAGUGGCAC	SEQ ID NO 165	AGCAAUGUUAUCUGCUUCCUCCAAC
SEQ ID NO 138	CUCUUUUCCAGGUUCAAGUGGGAUA	SEQ ID NO 166	GCAAUGUUAUCUGCUUCCUCCAACC
SEQ ID NO 139	UCUUUUCCAGGUUCAAGUGGGAUAC	SEQ ID NO 167	CAAUGUUAUCUGCUUCCUCCAACCA
SEQ ID NO 140	CUUUUCCAGGUUCAAGUGGGAUACU	SEQ ID NO 168	AAUGUUAUCUGCUUCCUCCAACCAU
SEQ ID NO 141	UUUUCCAGGUUCAAGUGGGAUACUA	SEQ ID NO 169	AUGUUAUCUGCUUCCUCCAACCAUA
SEQ ID NO 142	UUUCCAGGUUCAAGUGGGAUACUAG	SEQ ID NO 170	UGUUAUCUGCUUCCUCCAACCAUAA
SEQ ID NO 143	UUCCAGGUUCAAGUGGGAUACUAGC	SEQ ID NO 171	GUUAUCUGCUUCCUCCAACCAUAAA
SEQ ID NO 144	UCCAGGUUCAAGUGGGAUACUAGCA	SEQ ID NO 172	GCUGCUCUUUUCCAGGUUC
SEQ ID NO 145	CCAGGUUCAAGUGGGAUACUAGCAA	SEQ ID NO 173	UCUUUUCCAGGUUCAAGUGG
SEQ ID NO 146	CAGGUUCAAGUGGGAUACUAGCAAU	SEQ ID NO 174	AGGUUCAAGUGGGAUACUA
SEQ ID NO 274	CUCUUUUCCAGGUUCAAGUGGGAUACUAGC	SEQ ID NO 276	UAUUCUUUUGUUCUUCUAGCCUGGAGAAAG
SEQ ID NO 275	CAAGCUUUUCUUUUAGUUGCUGCUCUUUUCC	SEQ ID NO 277	CUGCUUCCUCCAACCAUAAAACAAAUUC

DMD Gene Exon 50

SEQ ID NO 175	CUCAGCUCUUGAAGUAAACG
SEQ ID NO 176	CCUCAGCUCUUGAAGUAAAC
SEQ ID NO 177	CCUCAGCUCUUGAAGUAAACG
SEQ ID NO 178	AUAGUGGUCAGUCCAGGAGCU
SEQ ID NO 179	CAGUC CAGGA GCUAG GUCAGG
SEQ ID NO 180	UAGUGGUCAGUCCAGGAGCUAGGUC
SEQ ID NO 278	CCACUCAGAGCUCAGAUCUUCUAACUUCC
SEQ ID NO 279	CUUCCACUCAGAGCUCAGAUCUUCUAA
SEQ ID NO 280	CAGUCCAGGAGCUAGGUCAGGCUGCUUUGC
SEQ ID NO 281	UCUUGAAGUAAACGGUUUACCGCCUUCCACU CAGAGC

DMD Gene Exon 51

SEQ ID NO 181	AGAGCAGGUACCUCCAACAUCAAGG	SEQ ID NO 203	UCAAGGAAGAUGGCAUUUCUAGUUU
SEQ ID NO 182	GAGCAGGUACCUCCAACAUCAAGGA	SEQ ID NO 204	UCAAGGAAGAUGGCAUUUCU
SEQ ID NO 183	AGCAGGUACCUCCAACAUCAAGGAA	SEQ ID NO 205	CAAGGAAGAUGGCAUUUCUAGUUUG
SEQ ID NO 184	GCAGGUACCUCCAACAUCAAGGAAG	SEQ ID NO 206	AAGGAAGAUGGCAUUUCUAGUUUGG
SEQ ID NO 185	CAGGUACCUCCAACAUCAAGGAAGA	SEQ ID NO 207	AGGAAGAUGGCAUUUCUAGUUUGGA
SEQ ID NO 186	AGGUACCUCCAACAUCAAGGAAGAU	SEQ ID NO 208	GGAAGAUGGCAUUUCUAGUUUGGAG
SEQ ID NO 187	GGUACCUCCAACAUCAAGGAAGAUG	SEQ ID NO 209	GAAGAUGGCAUUUCUAGUUUGGAGA
SEQ ID NO 188	GUACCUCCAACAUCAAGGAAGAUGG	SEQ ID NO 210	AAGAUGGCAUUUCUAGUUUGGAGAU
SEQ ID NO 189	UACCUCCAACAUCAAGGAAGAUGGC	SEQ ID NO 211	AGAUGGCAUUUCUAGUUUGGAGAUG
SEQ ID NO 190	ACCUCCAACAUCAAGGAAGAUGGCA	SEQ ID NO 212	GAUGGCAUUUCUAGUUUGGAGAUGG
SEQ ID NO 191	CCUCCAACAUCAAGGAAGAUGGCAU	SEQ ID NO 213	AUGGCAUUUCUAGUUUGGAGAUGGC
SEQ ID NO 192	CUCCAACAUCAAGGAAGAUGGCAUU	SEQ ID NO 214	UGGCAUUUCUAGUUUGGAGAUGGCA
SEQ ID NO 193	CUCCAACAUCAAGGAAGAUGGCAUU UCUAG	SEQ ID NO 215	GGCAUUUCUAGUUUGGAGAUGGCAG
SEQ ID NO 194	UCCAACAUCAAGGAAGAUGGCAUUC	SEQ ID NO 216	GCAUUUCUAGUUUGGAGAUGGCAGU
SEQ ID NO 195	CCAACAUCAAGGAAGAUGGCAUUUC	SEQ ID NO 217	CAUUUCUAGUUUGGAGAUGGCAGUU
SEQ ID NO 196	CAACAUCAAGGAAGAUGGCAUUUCU	SEQ ID NO 218	AUUUCUAGUUUGGAGAUGGCAGUUU
SEQ ID NO 197	AACAUCAAGGAAGAUGGCAUUUCUA	SEQ ID NO 219	UUUCUAGUUUGGAGAUGGCAGUUUC
SEQ ID NO 198	ACAUCAAGGAAGAUGGCAUUUCUAG	SEQ ID NO 220	UUCUAGUUUGGAGAUGGCAGUUUCC

SEQ ID NO 199	ACAUCAAGGAAGAUGGCAUUUCUAG UUUGG		
SEQ ID NO 200	ACAUCAAGGAAGAUGGCAUUUCUAG		
SEQ ID NO 201	CAUCAAGGAAGAUGGCAUUUCUAGU		
SEQ ID NO 202	AUCAAGGAAGAUGGCAUUUCUAGUU		

DMD Gene Exon 52

SEQ ID NO 221	CCUCUUGAUUGCUGGUCUUGUUUUU	SEQ ID NO 285	UUUUGGGCAGCGGUAAUGAGUUCUU
SEQ ID NO 222	CUCUUGAUUGCUGGUCUUGUUUUUC	SEQ ID NO 286	UUUGGGCAGCGGUAAUGAGUUCUUC
SEQ ID NO 223	UCUUCAUUGCUGGUCUUGUUUUUCA	SEQ ID NO 287	UUGCGCAGCGCUAAUGAGUUCUUCC
SEQ ID NO 224	CUUGAUUGCUGGUCUUGUUUUUCAA	SEQ ID NO 288	UGGGCAGCGGUAAUGAGUUCUUCCA
SEQ ID NO 225	UUGAUUGCUGGUCUUGUUUUUCAAA	SEQ ID NO 289	GGGCAGCGGUAAUGAGUUCUUCCAA
SEQ ID NO 226	UGAUUGCUGGUCUUGUUUUUCAAAU	SEQ ID NO 290	GGCAGCGGUAAUGAGUUCUUCCAAC
SEQ ID NO 227	GAUUGCUGGUCUUGUUUUUCAAAUU	SEQ ID NO 291	GCAGCGGUAAUGAGUUCUUCCAACU
SEQ ID NO 228	ACUGCUGGUCUUGUUUUUCAAAUUU	SEQ ID NO 292	CAGCGGUAAUGAGUUCUUCCAACUG
SEQ ID NO 229	UUGCUGGUCUUGUUUUUCAAAUUUU	SEQ ID NO 293	AGCGGUAAUGAGUUCUUCCAACUGG
SEQ ID NO 230	UGCUGGUCUUGUUUUUCAAAUUUUG	SEQ ID NO 294	GCGGUAAUGAGUUCUUCCAACUGGG
SEQ ID NO 231	GCUGGUCUUGUUUUUCAAAUUUUGG	SEQ ID NO 295	CGGUAAUGAGUUCUUCCAACUGGGG
SEQ ID NO 232	CUGGUCUUGUUUUUCAAAUUUUGGG	SEQ ID NO 296	GGUAAUGAGUUCUUCCAACUGGGGA
SEQ ID NO 233	UGGUCUUGUUUUUCAAAUUUUGGGC	SEQ ID NO 297	GUAAUGAGUUCUUCCAACUGGGGAC
SEQ ID NO 234	GGUCUUGUUUUUCAAAUUUUGGGCA	SEQ ID NO 298	UAAUGAGUUCUUCCAACUGGGGACG
SEQ ID NO 235	GUCUUGUUUUUCAAAUUUUGGGCAG	SEQ ID NO 299	AAUGAGUUCUUCCAACUGGGGACGC
SEQ ID NO 236	UCUUGUUUUUCAAAUUUUGGGCAGC	SEQ ID NO 300	AUGAGUUCUUCCAACUGGGGACGCC
SEQ ID NO 237	CUUGUUUUUCAAAUUUUGGGCAGCG	SEQ ID NO 301	UGAGUUCUUCCAACUGGGGACGCCU
SEQ ID NO 238	UUGUUUUUCAAAUUUUGGGCAGCGG	SEQ ID NO 302	GAGUUCUUCCAACUGGGGACGCCUC

SEQ ID NO 239	UGUUUUUCAAAUUUUGGGCAGCGGU	SEQ ID NO 303	AGUUCUUCCAACUGGGGACGCCUCU
SEQ ID NO 240	GUUUUUCAAAUUUUGGGCAGCGGUA	SEQ ID NO 304	GUUCUUCCAACUGGGGACGCCUCUG
SEQ ID NO 241	UUUUUCAAAUUUUGGGCAGCGGUAA	SEQ ID NO 305	UUCUUCCAACUGGGGACGCCUCUGU
SEQ ID NO 242	UUUUCAAAUUUUGGGCAGCGGUAAU	SEQ ID NO 306	UCUUCCAACUGGGGACGCCUCUGUU
SEQ ID NO 243	UUUCAAAUUUUGGGCAGCGGUAAUG	SEQ ID NO 307	CUUCCAACUGGGGACGCCUCUGUUC
SEQ ID NO 244	UUCAAAUUUUGGGCAGCGGUAAUGA	SEQ ID NO 308	UUCCAACUGGGGACGCCUCUGUUCC
SEQ ID NO 245	UCAAAUUUUGGGCAGCGGUAAUGAG	SEQ ID NO 309	GAUUG CUGGU CUUGU UUUC
SEQ ID NO 246	CAAAUUUUGGGCAGCGGUAAUGAGU	SEQ ID NO 310	CCUCU UGAUU GCUGG UCUUG
SEQ ID NO 247	AAAUUUUGGGCAGCGGUAAUGAGUU	SEQ ID NO 311	GGUAA UGAGU UCUUC CAACU GG
SEQ ID NO 248	AAUUUUGCGCAGCGCUAAUCACUUC	SEQ ID NO 312	ACUCG GCACC CCUCU GUUCC
SEQ ID NO 249	AUUUUGGGCAGCGGUAAUGAGUUCU	SEQ ID NO 283	ACUGGGGACGCCUCUGUUCCA
SEQ ID NO 282	UCCAACUGGGGACGCCUCUGUUCC AAAUCC	SEQ ID NO 284	CCGUAAUCAUUGUUCUAGCC

DMD Gene Exon 53

SEQ ID NO 250	CCAUUGUGUUGAAUCCUUUAACAUU
SEQ ID NO 251	CCAUUGUGUUGAAUCCUUUAAC
SEQ ID NO 252	AUUGUGUUGAAUCCUUUAAC
SEQ ID NO 253	CCUGUCCUAAGACCUGCUCA
SEQ ID NO 254	CUUUUGGAUUGCAUCUACUGUAUAG
SEQ ID NO 255	CAUUCAACUCUUGCCUCCGGUUCUG
SEQ ID NO 256	CUGUUGCCUCCGGUUCUGAAGGUG
SEQ ID NO 257	CAUUCAACUGUUGCCUCCGGUUCUGAAGGUG
SEQ ID NO 258	CUGAAGGUGUUCUUGUACUUCAUCC
SEQ ID NO 259	UGUAUAGGGACCCUCCUUCCAUGACUC
SEQ ID NO 260	AUCCCACUGAUUCUGAAUUC
SEQ ID NO 261	UUGGCUCUGGCCUGUCCUAAGA
SEQ ID NO 262	AAGACCUGCUCAGCUUCUUCCUUAGCUUCCAG CCA