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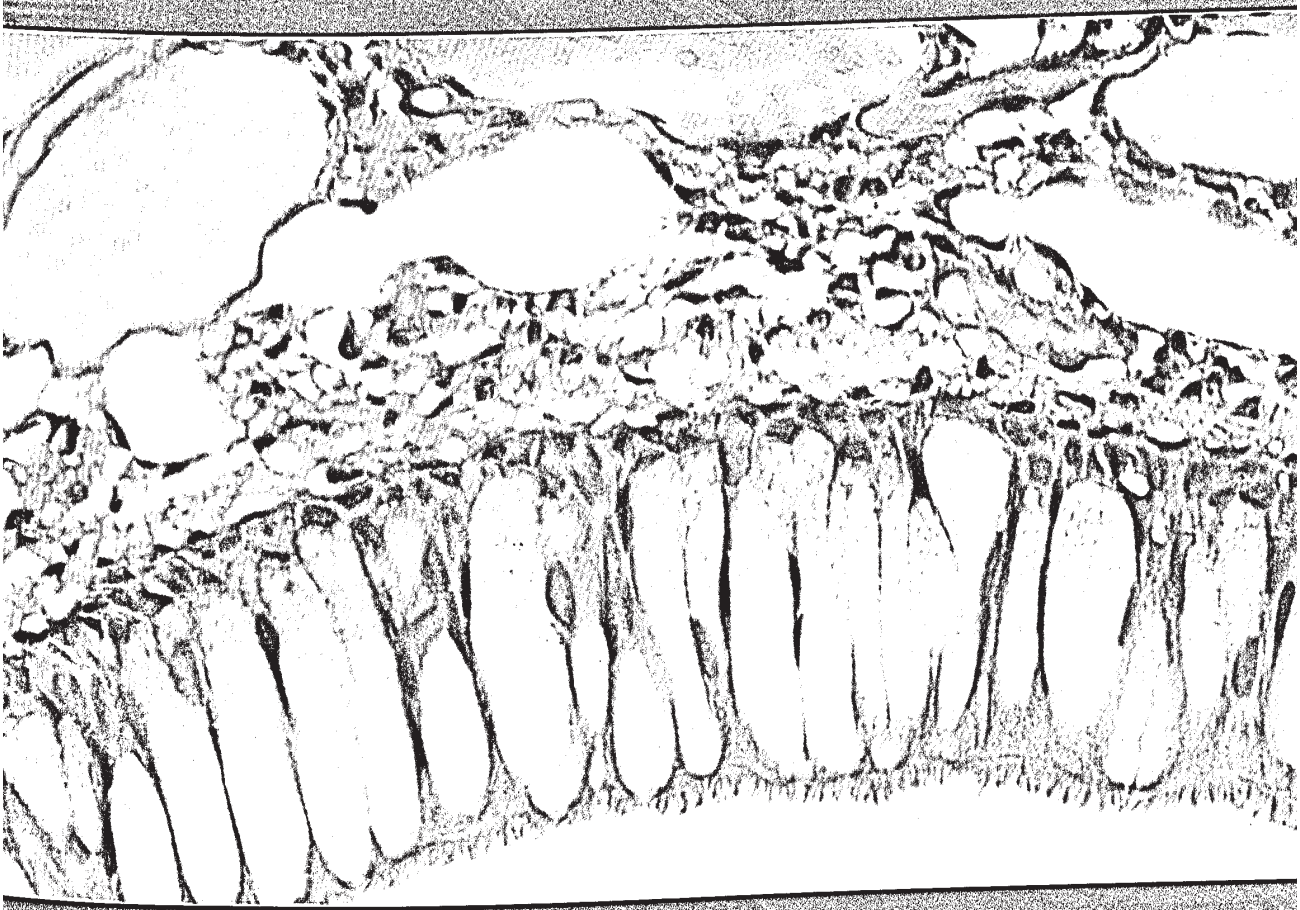

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Safety Pharmacology and Genotoxicity Evaluation of AVI-4658

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Abstract

Duchenne muscular dystrophy (DMD) is caused by dystrophin gene mutations. Restoration of dystrophin by exon skipping was demonstrated with the phosphorodiamidate morpholino oligomers (PMO) class of splice-switching oligomers, in both mouse and dog disease models. The authors report the results of Good Laboratory Practice–compliant safety pharmacology and genotoxicity evaluations of AVI-4658, a PMO under clinical evaluation for DMD. In cynomolgus monkeys, no test article–related effects were seen on cardiovascular, respiratory, global neurological, renal, or liver parameters at the maximum feasible dose (320 mg/kg). Genotoxicity battery showed that AVI-4658 has no genotoxic potential at up to 5000 μg/mL in an in vitro mammalian chromosome aberration test and a bacterial reverse mutation assay. In the mouse bone marrow erythrocyte micronucleus test, a single intravenous injection up to 2000 mg/kg was generally well tolerated and resulted in no mutagenic potential. These results allowed initiation of systemic clinical trials in DMD patients in the United Kingdom.

Keywords

Exon skipping, phosphorodiamidate morpholino oligomer, genotoxicity, antisense oligonucleotide, safety pharmacology

Duchenne muscular dystrophy (DMD) affects 1 in every 3500 male and, in rare cases, female newborns worldwide[1] and results from mutations of the dystrophin gene. Lack of dystrophin leads to reduced sarcolemmal stability with actin filament contraction and increased intracellular calcium influx followed by muscle fiber degeneration. The clinical effect of a disrupted reading frame in the dystrophin gene is dramatic and lethal.[1,2] In DMD patients, the first symptoms involve the lower limbs and appear between the third and fifth year. These boys develop hypertrophic calves, show difficulty in running and climbing stairs, run on their tiptoes, and frequently fall. Muscle weakness progresses to the shoulder girdle upper arm and trunk muscles and loss of ambulation before the age of 12 years. Histological changes are readily apparent with light microscopy analysis of cross sections from patient muscle biopsies. They involve variation in fiber size with atrophic and hypertrophic fibers, degeneration and regeneration of the muscle fibers, infiltration of inflammatory cells and fibrosis, and characteristic central location of the nuclei within muscle cells. The fiber membrane destabilization results in leakage of the enzyme creatine kinase (CK), resulting in very high serum CK levels (20 000 to 50 000 U/L compared with 80 to 250 U/L in unaffected individuals). These levels decline as the patients get older, and the overall muscle mass decreases progressively. One-third of all affected boys are mentally impaired, and learning difficulties are common. Due primarily to the loss of muscle strength and integrity, DMD patients usually die in their 20s from cardiorespiratory failure.[1,2]

Despite extensive effort, no effective disease-modifying therapy for DMD is yet available. However, a delay of the onset of disease manifestations and an improvement in quality of life can be achieved by drugs that decelerate progression of the DMD pathology. These include glucocorticoids,[3] most commonly prednisolone and deflazocort, which can improve muscle strength and delay loss of ambulation by up to 2 to 3 years.[1] The mechanisms of their beneficial effect are not well understood but likely include anti-inflammatory activity, which may prevent the additional damage caused by the infiltration of mononuclear cells into the muscle upon necrosis.[4] However, the benefits of glucocorticoid therapy come at a price of frequent side effects, which can include obesity, spine deformities, bone loss, and growth retardation.[5,6] To date, the most effective treatment for prolonging the life of DMD boys has been assisted ventilation with portable ventilators. Ventilation has been shown to increase the average life expectancy of DMD boys from 19 to 24 years. All of the above treatments are palliative and do not address the underlying cause of the disease: loss of dystrophin expression. No current treatment reverses or arrests the progression of DMD.

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In addition to DMD, a milder, allelic form of muscular dystrophy called Becker muscular dystrophy (BMD) exists.[1,2] In BMD, unlike DMD, the reading frame is not disrupted, and an internally truncated yet functional dystrophin protein is produced. Most BMD patients remain ambulant for life and have a near-normal life expectancy. Work by Kole and others proposed the use of antisense oligonucleotides to modulate dystrophin mRNA splicing and convert out-of-frame DMD mutations into the nearest in-frame BMD-like mutation, to produce an internally deleted Becker-like functional dystrophin protein.[7-10] The mechanism of splice-switching oligomer (SSO) modulation of dystrophin pre-mRNA splicing involves hybridization to specific motifs involved in splicing and exon recognition in the pre-mRNA. This prevents normal spliceosome assembly and results in skipping of the target exon in the mature RNA transcript.[11,12] In the case of out-of-frame dystrophin gene deletions, selective removal of specific flanking exons should result in in-frame mRNA transcripts that may be translated into an internally deleted, BMD-like, and functionally active dystrophin protein.[9,10]

Unmodified DNA and RNA oligonucleotides have poor in vivo stability and therefore are ineffective as drugs. The most common chemical modification used in early-generation oligonucleotides to improve the stability and pharmacokinetics was the introduction of the phosphorothioate linkage in place of the natural phosphodiester linkage. These phosphorothioate compounds have been used extensively in both preclinical and clinical evaluations, and the dose-limiting toxicities and adverse effects are well established.[13] Initial evaluations in primates led to mortality following intravenous bolus injections of phosphorothioate oligonucleotides at doses as low as 10 mg/kg,[14] while other effects included lethargy, central hypotension, and reduced cardiac output. Other notable toxic effects associated with phosphorothioate oligonucleotides include complement activation and prolonged coagulation times.[15] The latter, specifically linked to the phosphorothioate component of the oligonucleotides, is therefore considered a class effect,[16] as were observed hepatotoxic effects.[17] These dose-limiting toxicities are most likely due to high C_{max} achieved with bolus injections. Studies subsequent to those in which mortality was observed have typically been limited to lower doses and increased infusion times to reduce the toxic effects.[13]

To be effective in modulating splicing, SSOs must not activate RNA cleavage by RNase H, which would destroy the pre-mRNA before splicing can occur.[18] Phosphorodiamidate morpholino oligomers (PMOs) productively compete with the splicing factors for target sequences in pre-mRNA during splicing, and in addition, their stability and in vivo uptake and bioavailability are improved compared with natural oligonucleotides. PMOs have standard nucleic acid bases attached to the morpholino-phosphoroamidate backbone (Figure 1), which, unlike other sugar-phosphate backbone oligonucleotides, is uncharged.[19] PMOs are very resistant to enzymatic degradation in vivo, providing unparalleled stability and somewhat different biodistribution than other oligonucleotides. Other modifications that can be used as SSOs include previous-generation chemistries such as the 2′-O–substituted 2′-O-methyl phosphorothioate (2′OMe), and locked nucleic acids/phosphorothioate.[20]

AVI-4658 is a PMO drug with the general structure described in Figure 1, with the base sequence CTC CAA CAT CAA GGA AGA TGG CAT TTC TAG. It is designed to skip exon 51 of human dystrophin and thus restore dystrophin expression in DMD patients having certain mutations.[21] AVI-4658 targets the pre-mRNA transcripts of the dystrophin gene, causing exon 51 to be skipped from the mature, spliced mRNA. In cells from DMD patients with deletions in exons 50, 52, 52-63, 45-50, 48-50, or 49-50, exon skipping restored or is expected to restore the reading frame and produce an internally truncated, BMD-like form of dystrophin. Here, we report the results of a safety pharmacology evaluation of the PMO AVI-4658 in cynomolgus monkeys following intravenous and subcutaneous administration at doses up to the maximum feasible dose of 320 mg/kg. We also report the results of a standard genotoxicity battery evaluation using AVI-4658 at concentrations up to 5000 µg/mL in an in vitro mammalian cell chromosome aberrations test, up to 5000 µg/plate in a bacterial reverse mutation assay, and up to 2000 mk/kg as a single intravenous administration in a mouse micronucleus assay.

Materials and Methods

The safety pharmacology evaluation was performed by MDS Pharma Services (Lyon, France). The testing facility is Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC) accredited, and the study plan was reviewed by the ethical committee, according to the following animal health and welfare guidelines: guide for the care and use of laboratory animals, NRC, 1996, Decree no. 2001-464 regarding the experiments with laboratory animals described in the *Journal Officiel de la République Française* on May 29, 2001, Decree no. 2001-486 relating to the protection of animals used in scientific experiments described in the *Journal Officiel de la République Française* on June 6, 2001. The study was conducted according to the following: guideline on safety pharmacology studies for human pharmaceuticals (November 8, 2000, issued as CPMP/ICH/539/00–ICH S7A, published in the *Federal Register*, vol 66, no. 135, July 13, 2001, pp 36791-36792) and guideline on nonclinical evaluation of the potential for delayed ventricular repolarization (QT interval prolongation) by human pharmaceuticals (May 12, 2005, issued as CPMP/ICH/423/02–ICH S7B, published in the *Federal Register*, vol 70, no. 202, October 20, 2005, pp 61133-61134). All phases of this study performed at the testing facility were conducted in compliance with the following Good Laboratory Practice (GLP) regulations: OECD *Principles of Good Laboratory Practice* concerning mutual acceptance of data in the assessment of chemicals, dated November 26, 1997, (C[97] 186 Final), "Principles of Good Laboratory Practice" described in the *French Official Journal* on March 23, 2000, Organization for Economic Co-operation and Development (OECD) GLP consensus document (the application of the

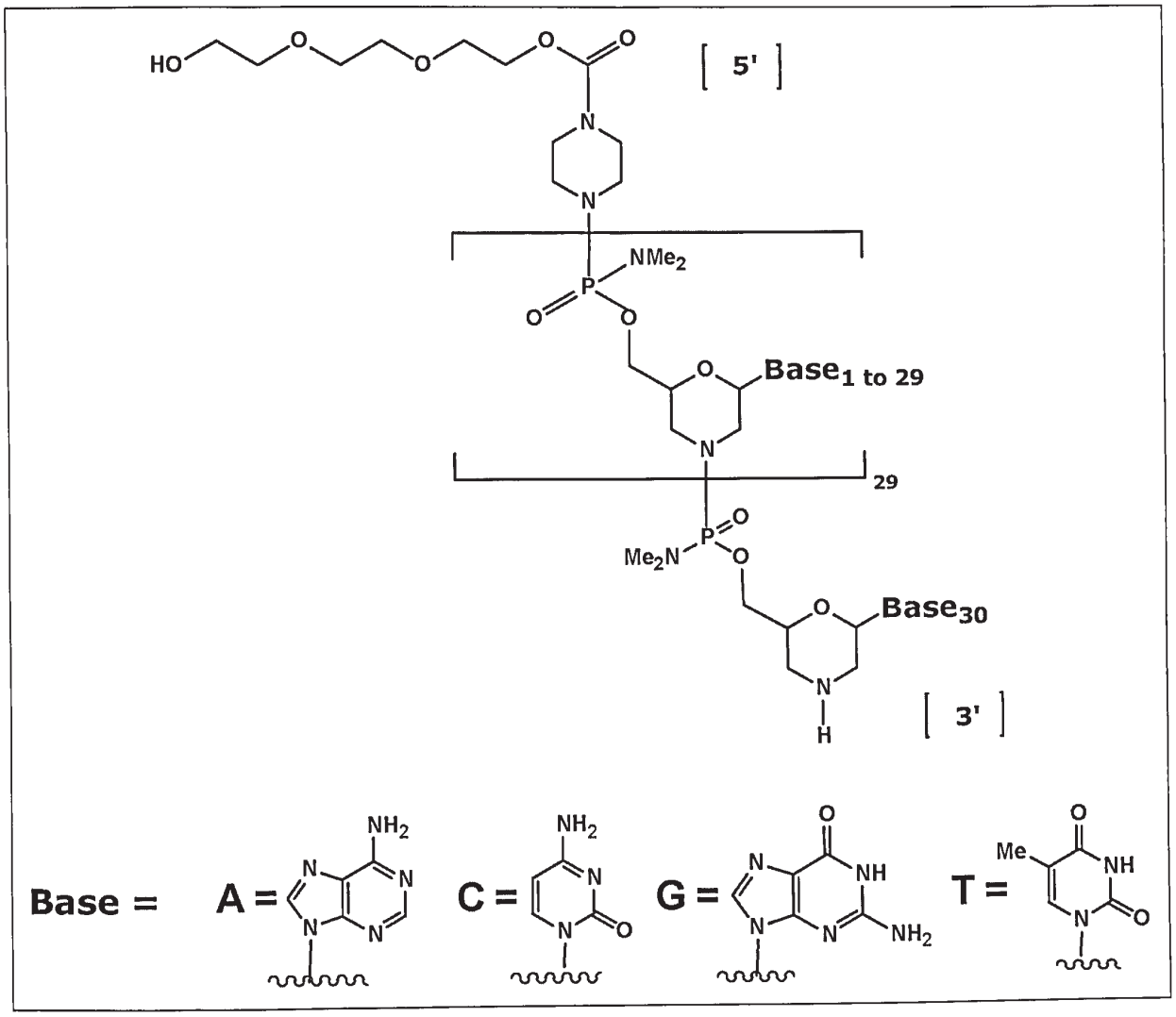

Figure 1. Structure of phosphorodiamidate morpholino oligomers.

OECD principles of GLP to the organization and management of multisites studies, ENV/JM/MONO [2002]9, June 25, 2002).

The genotoxicity battery was performed by BioReliance (Rockville, MD). This study was conducted in compliance with the most recent version of the US Food and Drug Administration GLP regulations, 21 CFR part 58, and the OECD *Principles of Good Laboratory Practice*, C(97)186/Final, and in compliance with the testing guidelines ICH S2A (1996), ICH S2B (1997), and OECD 474 (1998). The number of mice and the procedures and experimental design used for this study have been reviewed and were approved by the BioReliance Institutional Animal Care and Use Committee 8 and 10. All procedures involving mice performed at BioReliance follow the specifications recommended in *The Guide for the Care and Use of Laboratory Animals* (National Academy Press, Washington, DC, 1996). The mice were housed in an AAALAC-accredited facility.

Safety Pharmacology Evaluation of AVI-4658

Animals and animal husbandry. Six male cynomolgus monkeys (*Macaca fascicularis*) were used in this study, with a weight range of 2.7 to 2.9 kg and an age range of 2 to 3 years. Animals were housed in 1 room for the study in an air-conditioned building with a target temperature of 22°C ± 2°C, relative humidity >40%, a minimum 10 air changes per hour, and 12 hours light (artificial)/12 hours dark. Animals were housed singly in stainless steel mesh cages. Animals were fed expanded complete commercial primate diet at approximately 100 g diet/animal per day. In addition, animals received fruit or vegetable daily (apple, banana, or carrot). Certificates of analysis for the diet and drinking water are maintained in the archives of the testing facility, which conducted the tests according to current animal welfare guidelines. The normal dark cycle was interrupted on occasions (for up to 45 minutes)

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