Omigapil ameliorates the pathology of muscle dystrophy caused by laminin-α2 deficiency

Michael Erb*, Sarina Meinen*, Patrizia Barzaghi, Lazar T. Sumanovski, Isabelle Courdier-Früh, Markus A. Rüegg and Thomas Meier

Santhera Pharmaceuticals, Hammerstrasse 49, 4410 Liestal, Switzerland (M.E., P.B., L.T.S., I.C.F., T.M.); Biozentrum, University of Basel, Klingelbergstrasse 70, 4056 Basel, Switzerland (S.M., M.A.R)
a) Running Title:
Omigapil ameliorates laminin-α2 deficient muscular dystrophy

b) Corresponding Author:
Markus A. Rüegg, Ph.D.
Biozentrum, University of Basel
Klingelbergstrasse 70
CH-4056 Basel, Switzerland
Phone: +41 61 267 22 23
Fax: +41 61 267 22 08
E-Mail: markus-a.ruegg@unibas.ch

c) Manuscript Information:
Number of text pages: 26
Number of tables: 0
Number of figures: 5
Number of references: 37
Words in Abstract: 159
Words in Introduction: 750
Words in Discussion: 1157

d) List of non-standard abbreviations:
CK, creatine kinase; DAPI, 4',6-diamidino-2-phenylindole; dyW-omigapil, omigapil treated dyW/dyW mice; dyW-vehicle, vehicle-treated dyW/dyW mice; GAPDH, glyceraldehyde 3-phosphate dehydrogenase; GOSPEL, GAPDH's competitor of Siah1 Protein Enhances Life; H&E, hematoxylin & eosin; LM, laminin; Omigapil (also referred to as TCH346 or CGP3466), N-(dibenz(b,f)oxepin-10-ylmethyl)-N-methyl-N-prop-2-ynylamine maleate; PTPC, permeability transition pore complex; PUMA, p53–upregulated modifier of apoptosis; TUNEL, TdT-mediated dUTP-biotin nick end labelling; WGA, wheat-germ agglutinin; WT, wild-type
e) Section Assignment:

Cellular and Molecular
Abstract

Laminin alpha2-deficient Congenital Muscular Dystrophy, called MDC1A, is a rare, devastating genetic disease characterized by severe neonatal hypotonia (“floppy infant syndrome”), peripheral neuropathy, inability to stand or walk, respiratory distress and premature death in early life. Transgenic overexpression of the apoptosis inhibitor protein BCL-2, or deletion of the pro-apoptotic Bax gene in a mouse model for MDC1A prolong survival and mitigate pathology, indicating that apoptotic events are involved in the pathology. Here we demonstrate that the pro-apoptotic glyceraldehyde 3-phosphate dehydrogenase (GAPDH)-Siah1-CBP/p300-p53 pathway is activated in a mouse model for MDC1A. Moreover, we show that omigapil, which inhibits GAPDH-Siah1 mediated apoptosis, ameliorates several pathological hallmarks in the MDC1A mouse model. Specifically, we demonstrate that treatment with omigapil inhibits apoptosis in muscle, reduces body weight loss and skeletal deformation, increases locomotive activity and protects from early mortality. These data qualify omigapil, which is in late phase of clinical development for human use, as a drug candidate for the treatment of MDC1A.
Introduction

Laminin-α2 is an extracellular matrix protein expressed in skeletal muscle and peripheral nerve that associates with the β1 and γ1 subunit to form the heterotrimeric laminin-211 (LM-211). LM-211 is tightly attached to the basement membrane, interacts with α-dystroglycan and α7β1 integrin on the muscle fiber surface (Colognato and Yurchenco, 2000), and thus confers mechanical stability to the contracting muscle. Thus, mutations in the LAMA2 gene coding for laminin-α2 disrupt the linkage of the basement membrane to the plasma membrane. In humans, this results in a severe form of Congenital Muscular Dystrophy, called MDC1A, which is characterized by neonatal hypotonia (“floppy infant syndrome”), peripheral neuropathy, inability to stand or walk, respiratory distress and premature death (Muntoni and Voit, 2004; Schessl et al., 2006). The dy/W/dyW mouse model (Kuang et al., 1998) of MDC1A largely recapitulates the human disease. It is characterized by a severe degeneration and incomplete regeneration of damaged muscle fibers (Miyagoe et al., 1997; Kuang et al., 1999; Bentzinger et al., 2005; Meinen et al., 2007). Histology of affected muscles typically shows variation in muscle fiber size, extensive fibrosis, infiltration of adipose tissue, and high levels of creatine kinase (CK) in the blood. In addition, the hindlegs of laminin-α2-deficient mice are paralyzed within a few weeks from birth due to progressing demyelination of the peripheral nervous system (Kuang et al., 1998). Finally, like human patients, these mice die prematurely. Apoptosis has been reported as a pathological hallmark at the cellular level of affected muscle tissue both in dy/W/dyW mice (Kuang et al., 1999; Girgenrath et al., 2004; Bentzinger et al., 2005; Dominov et al., 2005) and MDC1A patients (Hayashi et al., 2001).

Several transgenic approaches in dy/W/dyW mice have shown that proteins sharing biochemical binding properties with laminin-α2 can ameliorate the pathology. These approaches include transgenic expression of laminin-α1 (Gawlik et al., 2004), of a miniaturized form of the extracellular matrix molecule agrin (Moll et al., 2001; Bentzinger et al., 2005; Qiao et al., 2005; Meinen et al., 2007) or of perlecan (Meinen et al., 2007). All these attempts ameliorated the pathology of dy/W/dyW mice in several aspects. However, because of largely technical obstacles, protein replacement strategies are currently difficult to be translated into a treatment of human patients.

An alternative is offered by pharmacological intervention into the apoptotic pathways underlying the disease. This approach aims at preventing cell death of muscle fibers, which in the laminin-α2 deficient muscle is a consequence of muscle fiber detachment from the disrupted basement membrane (Miyagoe et al., 1997), possibly due to loss of intracellular signaling mediated by α7 integrin and/or dystroglycan.
(Langenbach and Rando, 2002; Laprise et al., 2003). Indeed, a first proof-of-concept for this treatment approach was accomplished by transgenic overexpression of the anti-apoptotic protein BCL-2, or deletion of the pro-apoptotic Bax gene (Girgenrath et al., 2004; Dominov et al., 2005). Both approaches prolong survival and mitigate disease-specific parameters in dyW/dyW mice, suggesting that pharmacological inhibition of apoptosis could be a valid strategy to overcome the pathological manifestations of laminin-α2 deficiency. Indeed, minocycline and doxycycline have recently been shown to alleviate some of the disease parameters in dyW/dyW mice (Girgenrath et al., 2009).

However, the underlying signaling pathways leading to apoptosis in laminin-α2 deficient muscle are still poorly understood. One of the newly described apoptotic pathways involves the glycolytic housekeeping enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) and its S-nitrosylation via inducible or neuronal nitric oxide synthase (Hara et al., 2005). S-nitrosylation abolishes the catalytic activity of GAPDH and, upon binding of the E3 ubiquitin ligase Siah1, GAPDH is translocated into the nucleus. Nuclear GAPDH binds to the acetyltransferase CBP/p300 and enhances acetylation and transcription of various targets including pro-apoptotic genes, such as p53, p53–upregulated modifier of apoptosis (PUMA) and p21 (Sen et al., 2008). This pro-apoptotic signaling cascade can be interrupted by omigapil, an orally available chemical derivative of (-) deprenyl, which exhibits virtually no monoamine oxidase type B or type A-inhibiting properties and is not metabolized to amphetamines (Tatton et al., 2003; Hara et al., 2006; Waldmeier et al., 2006).

We provide evidence that the GAPDH-Siah1-CBP/p300 signaling pathway (Chuang et al., 2005) is dysregulated in muscle tissue of dyW/dyW mice and that application of omigapil once per day results in reduced apoptosis in muscle. Omigapil also reduces body weight loss and skeletal deformation, increases locomotive activity and protects from early mortality in dyW/dyW mice. Based on these encouraging results, combined with the advanced non-clinical and clinical development status, omigapil holds the potential to offer a valid treatment option for MDC1A.
Methods

Gene expression analysis. Muscle biopsies were snap frozen in liquid nitrogen and stored at -80°C. Total RNA was extracted using the RNeasy Fibrous Tissue Mini Kit (Qiagen, Hombrechtikon, Switzerland). The initial homogenization step was carried out using the gentleMACS™ M Tubes (Miltenyi Biotec, Bergisch Gladbach, Germany). One µg of total RNA was reverse transcribed into cDNA using the QuantiTect Rev. Transcription Kit (Qiagen, Hombrechtikon, Switzerland). QPCR was carried out on the LightCycler® 480 System (Roche, Rotkreuz, Switzerland) using the LightCycler® 480 SYBR Green I Master (Roche, Rotkreuz, Switzerland). The housekeeping genes were amplified using the primer pairs: β-actin fwd TGTTACCACTGGGAGCAGCA and rev GGGGTGTTGAAGGTCTCAAA; β-2 microglobulin fwd ATGGGAAGCGCAATCCTGT and rev GGGGGTGAGATGCTGAG; GAPDH fwd ATTTCGCAATGCTCCTGT and rev ATGGACTGTGGTCATGAG; the genes of interest were amplified using the primer pairs: GOSPEL fwd AGAGGGTCAGACCCAC and rev TCCAGCTCTTTTATGCTT; p53 fwd AGGGCTCACTCCAGCTACC and rev CCCACCTTTTTGACCATTG; PUMA fwd CCAATGCAGAGAGTGAG; p21 fwd TGAATGGAGACAGAGCC and rev CGAAGTCAAAGTTCCACCGT. The primers were designed using the NCBI/Primer-BLAST software.

Relative levels of mRNA are expressed after normalization to all three housekeeping genes.

Preparation and analysis of nuclear extracts. The cytosolic and nuclear fractions were prepared from freshly isolated diaphragma muscles from 4 week-old dy/w/dy/w mice using the NE-PER Nuclear and Cytoplasmic Extraction Kit (Pierce, Lausanne, Switzerland) according to the manufacturer's protocol. The initial homogenization of the fresh muscles was carried out at 4°C using the gentleMACS™ M Tubes (Miltenyi Biotec, Bergisch Gladbach, Germany). The protein concentrations of the cytosolic and nuclear fractions were determined by the BCA Protein Assay Kit (Pierce, Lausanne, Switzerland). The GAPDH levels were analyzed by Western blot using a rabbit mAb (#2118 from CST, Danvers, MA, USA). The blots were read and quantified using the Odyssey Infrared Imaging System (LI-COR Biosciences, Bad Homburg, Germany). The relative amount of nuclear GAPDH protein was calculated as ratio of the densitometric values from the nuclear and the cytosolic fractions.
Treatment of dyW/dyW mice with omigapil. Homozygous dyW/dyW mice (Kuang et al., 1998) were treated with 0.1 or 1 mg/kg omigapil dissolved in 0.5% ethanol as vehicle. For all experiments, treatment started at the age of 15 days, except for the one week treatment shown in Figure 2b. For the first week of drug treatment, omigapil was administered once daily by intraperitoneal injection. Thereafter omigapil was given orally by gavage once daily. Age-matched animals treated with vehicle only were used as controls. To ensure optimal access to water and food, cages were supplied with long-necked water bottles and wet food was placed inside the cage, which resulted in substantially prolonged survival of dyW/dyW mice in our colony compared to survival data presented by others (e.g. Kuang et al., 1998; Girgenrath et al., 2004; Dominov et al., 2005; Girgenrath et al., 2009). At the end of the treatment period, mice were sacrificed by CO2 asphyxiation. Genotyping of dyW/dyW mice was performed as previously described (Kuang et al., 1998). All procedures were performed in accordance with the Swiss regulations for animal experimentation and under the required licenses.

Body weight and survival. Body weight and death events were recorded daily. Kaplan-Meier survival curves were generated and compared using the Peto-Peto Wilcoxon test. The body weight was recorded for each animal from day 15 (onset of the experiment) onwards. For each animal, the average weight gain per week was calculated. Two sets of body weight curves were recorded: a curve only for animals that survived the entire observation period of 18 weeks and a separate curve including all animals irrespective of when they died during the observation period. Both sets of data produced similar results.

Histological analysis and CK assay. Muscle cross-sections were prepared and analyzed for fiber size distribution as described previously (Briguet et al., 2004). The triceps brachii muscle was chosen for histological analysis to exclude muscles that are affected by the secondary atrophic effect due to hindlimb paralysis. Briefly, frozen muscle cross-sections were stained using Alexa-488-conjugated wheat-germ agglutinin (WGA-Alexa, Molecular Probes, Eugene OR, USA) to stain membrane-bound and extracellular epitopes and 1 μg/ml 4',6-diamidino-2-phenylindole (DAPI; Molecular Probes, Eugene OR, USA) to stain nuclei. Apoptotic myonuclei were detected by "TdT-mediated dUTP-biotin nick end labelling" (TUNEL) using the 'In Situ Cell Death Detection Kit, Fluorescein' according to the manufacture's protocol (Roche Diagnostics Ltd., Rotkreuz, Switzerland). Pictures of TUNEL/WGA/DAPI-stained cross-sections were collected using a fluorescence microscope (DM5000B; Leica; Heerbrugg, Switzerland), a digital camera (F-
View; Soft Imaging System/Olympus; Hamburg, Germany), and analySIS software (Soft Imaging System). Only TUNEL- and DAPI-positive nuclei that were located within muscle fibers were counted as apoptotic myonuclei. Values are expressed relative to the total number of myonuclei. General histology was performed using Hematoxylin & Eosin (H&E; Merck, Rayway, NJ, USA) according to standard histological techniques. Blood levels of creatine kinase (EC 2.7.3.2) were determined with 2 µl of serum using the CK-NAC Liqui-UV kit (Rolf Greiner Biochemica, Flacht, Germany).

**Locomotion and examination of spine deformation.** Locomotive behavior was determined as previously described (Moll et al., 2001). In brief, mice were placed into a new cage and motor activity (walking, digging, and standing upright) was measured during 10 minutes. To determine spine deformation, X-ray pictures were taken from anesthetized mice. The extent of kyphosis was quantified for mice at the age of 11 weeks by visual inspection at the same day by an investigator blinded to the treatment status of the animals. The degree of kyphosis was scored and allocated to one of the following categories: 1, barely detectable; 2, mild; 3, moderate; 4, severe.

**Statistical analysis.** Quantitative data are expressed as mean ± SEM. Significance between two groups were determined using the unpaired Student’s t-test. Significance for differences in the fiber size distribution was determined using cumulative distribution curves on which the Kolmogorov–Smirnov goodness-of-fit test (Chakravarti et al., 1967) was applied. Comparison of survival curves between treatment groups and vehicle controls was performed by the Peto-Peto Wilcoxon test. The Wilcoxon Rank Sum test was used to compare the kyphosis score of omigapil and vehicle-treated animals.
Results

The GAPDH-Siah1-CBP/p300 pro-apoptotic signaling pathway is activated in dyW/dyW mice. As a consequence of the laminin-α2 deficiency muscle fibers in dyW/dyW mice are prone to undergo apoptosis (Miyagoe et al., 1997; Girgenrath et al., 2004). As the underlying signaling cascade(s) causing muscle cell apoptosis are still poorly understood we investigated whether the GAPDH-Siah1-CBP/p300 pro-apoptotic pathway could be involved in this marked loss of muscle mass. We first tested whether the expression of the downstream pro-apoptotic genes of this pathway was increased, which was to be expected if the GAPDH-Siah1-CBP/p300 pathway was activated in dyW/dyW mice. Indeed, expression of p53 and its downstream genes PUMA and p21 was markedly elevated in triceps brachii and diaphragm muscle of the dyW/dyW mice compared to wild-type mice (Fig. 1a-c). The increased expression of these pro-apoptotic genes was already seen at young age, indicating that the activation of the GAPDH-Siah1-CBP/p300 pathway precedes tissue apoptosis. Specifically, already in 4 week-old dyW/dyW mice the increase in all three genes was evident in diaphragm and triceps brachii muscle (Fig. 1a, b). At this young age, the expression of p53, PUMA and p21 was generally higher in the diaphragm compared to the triceps brachii. Expression of p53, PUMA and p21 continued to increase over time as seen in their expression levels in triceps brachii at 7 weeks of age (Fig. 1c). As the pro-apoptotic GAPDH-Siah1-CBP/p300 pathway has recently been shown to be antagonized by GAPDH’s competitor GOSPEL (Sen et al., 2009), we also examined the expression level of this neuroprotective protein, which is predominantly expressed in brain and muscle. In contrast to the upregulation of pro-apoptotic genes, GOSPEL was strongly reduced in muscle of 4 week-old dyW/dyW mice (Fig. 1d), indicating early depletion of this anti-apoptotic component. GOSPEL inhibits the GAPDH-Siah1-CBP/p300 pathway by preventing the nuclear accumulation of GAPDH (Sen et al., 2009). Consistent with this notion and the observed reduction in GOSPEL, the amount of GAPDH in the nuclear fraction of muscle tissue of dyW/dyW mice was increased by ~40% relative to the cytosolic fraction (Fig. 1e). These results therefore indicate that the pro-apoptotic GAPDH-Siah1-CBP/p300 pathway is activated in muscle of laminin-α2 deficient dyW/dyW mice and that the sustained activation parallels disease progression.

Omigapil reduces apoptosis in the dyW/dyW mice. Omigapil (Fig. 2a) has been reported to inhibit the pro-apoptotic GAPDH-Siah1-CBP/p300 pathway by its binding to GAPDH and inhibiting the interaction of GAPDH and Siah1 (Hara et al., 2006). Consistent with this activity, omigapil when given to dyW/dyW mice (designated here dyW-omigapil) for 1 week at a dose of 1 mg/kg starting at 21 days of age consistently
reduced the levels of p53, PUMA and p21 compared to vehicle-treated $dy^W/dy^W$ littermates ($dy^W$-vehicle) (Fig. 2b). In addition, treatment of $dy^W/dy^W$ mice with omigapil for 4 weeks starting at 15 days of age significantly reduced the number of apoptotic myonuclei (Fig. 2c, d). Thus, omigapil by its well described binding to GAPDH inhibits nuclear accumulation of GAPDH, thereby reducing the expression of pro-apoptotic genes p53, PUMA, and p21 and decreasing the number of apoptotic myonuclei in $dy^W/dy^W$ mice.

Omigapil ameliorates muscle histology, improves locomotion and overall health in $dy^W/dy^W$ mice. A hallmark of laminin-α2 deficiency is a shift of the myofiber size distribution to small-caliber fibers. Treatment of $dy^W/dy^W$ mice with omigapil for 4 weeks at a dose of 0.1 mg/kg starting at 15 days of age normalized the size distribution in triceps brachii muscle by reducing the proportion of small-caliber and increasing that of large-caliber fibers (Fig. 3a, b). Furthermore, omigapil treatment reduced the relative amount of fibrotic tissue (Fig. 3a). Accordingly, treatment with omigapil also normalized the elevated density of muscle fibers in cross-sections from triceps brachii (Fig. 3c). Finally, omigapil lowered CK levels in the blood compared to age-matched vehicle-treated $dy^W/dy^W$ animals (Fig. 3d), indicative of reduced muscle cell damage. In summary, these data demonstrate that omigapil markedly reduces apoptotic events and protects muscle fibers from degeneration caused by laminin-α2 deficiency.

The effect of omigapil on the overall health and behavior of $dy^W/dy^W$ mice were studied next. In these experiments, mice were treated with two different doses of omigapil (0.1 and 1 mg/kg/day) starting at 15 days of age. Body weight was measured daily up to an age of 18 weeks. In an analysis that included only animals that did not die before the age of 18 weeks, vehicle-treated $dy^W/dy^W$ mice gained weight until ~7 weeks of age after which no further increase was measured (Fig. 4a). In contrast, animals treated with either dose of omigapil continued to gain weight up to ~9 weeks of age and thus reached a higher weight gain than vehicle-treated animals. After 12 weeks of age, animals treated with omigapil at 0.1 mg/kg/day slowly lost weight and eventually reached body weights equivalent to vehicle-treated $dy^W/dy^W$ mice. In contrast, $dy^W/dy^W$ mice treated with 1 mg/kg omigapil ended the study period with a higher body weight gain than vehicle-treated animals. A comparable pattern of weight gain was seen when all animals were included in the analysis, irrespective of when they died (data not shown). In this latter group the peak body weight reached by $dy^W/dy^W$ mice allocated to either one of the omigapil treatment groups differed significantly from the peak body weight reached by animals in the vehicle group ( $dy^W$-vehicle: 8.00 ± 0.50 g, mean ± SEM, N=33; $dy^W$-omigapil 0.1 mg/kg: 9.91 ± 0.48 g, N=33, p=0.008 vs vehicle; $dy^W$-omigapil 1.0 mg/kg: 9.75 ± 0.56 g, N=28,
p=0.020 vs vehicle). As body weight is known to be a confounding factor for survival, we also determined the percentage of animals in each treatment group reaching a threshold of 12 grams over time (Fig. 4b). In all age groups, a higher percentage of omigapil-treated animals were above this threshold body weight compared to vehicle-treated animals. In contrast to omigapil-treated animals, none of the vehicle-treated animals maintained this threshold body weight from 15 weeks of age onwards. Again, the effect of omigapil was dose-dependent favoring the higher dose, which becomes particularly evident at 12 weeks of age and above.

To assess whether omigapil treatment not only preserved muscle histology and body weight but also affected locomotive behavior, an open field test was performed with 5-6 week-old and 10 week-old mice. Omigapil treatment indeed improved locomotion in the younger mice (Fig. 4c, left panel) with a similar efficacy for the two doses of omigapil (see also Supplemental Data; Movie). Although there was still a slight improvement in locomotion in omigapil-treated mice at 10 weeks of age, the effect did not reach statistical significance (Fig. 4c, right panel).

**Effect of omigapil on skeletal deformation and overall survival.** Muscle weakness and degeneration results in the deformation of the spine, which is a major complication in MDC1A patients as it causes respiratory distress. Spine deformation, identified as hunchback, is also a very obvious phenotype in laminin-α2 deficient dyW/dyW mice and became apparent in our mouse colony at around 11 weeks of age. The degree of scoliosis and kyphosis was clearly visible by X-ray imaging in a lateral view (Fig. 5a, left panel). Significant loss of lean tissue in dyW/dyW animals, which causes the rib cage to collapse, was seen in dorso-ventral X-ray images (Fig. 5a, right panel, white arrows). Treatment with 1 mg/kg/day omigapil starting at 15 days of age conspicuously reduced tissue loss, scoliosis, kyphosis, and the collapse of the rib cage. A visual assessment of the severity of spine deformation using a kyphosis score confirmed that omigapil-treated dyW/dyW mice were less affected than vehicle-treated animals. A total of 77% of vehicle-treated dyW/dyW mice had moderate to severe kyphosis, while a comparable percentage of omigapil-treated animals had barely detectable or mild kyphosis (Fig. 5b; p<0.05, Wilcoxon Rank Sum test).

Loss of muscle tissue, locomotive immobility, spinal deformation and rib cage collapse as a consequence of laminin-α2 deficiency causes early death of dyW/dyW mice. The administration of omigapil starting at 15 days of age exhibited a dose-dependent and significant effect on the survival of dyW/dyW mice (Fig. 5c). Overall survival of vehicle-treated dyW/dyW mice showed a bi-phasic pattern with approximately half
of the population dying by ~35 days. Both doses of omigapil largely prevented the death of dy/dyw mice in this early phase and resulted in a 50% survival time of ~85 days (0.1 mg/kg omigapil) and ~105 days (1 mg/kg omigapil), respectively. While the 0.1 mg/kg dose of omigapil had only a small effect on maximum survival time, the 1 mg/kg dose of omigapil prolonged survival for a higher proportion of animals, with more than 25% of animals surviving beyond the age of 28 weeks.
Discussion

Neuromuscular diseases are rare genetic disorders that are characterized by the loss of muscle control and subsequent loss of muscle mass. While the primary cause for this group of diseases can originate in different cell types (e.g. motor neurons or skeletal muscle) and can be triggered by mutations in many different genes, there are certain commonalities to the diseases that may open new avenues for treatment. For example, apoptosis and necrosis of muscle fibers or the invasion of fibrotic cells are hallmarks of several muscular dystrophies. Thus, drugs that affect such secondary consequences of a disease have promise for the treatment of several neuromuscular disorders irrespective of their primary cause.

Here we show that the apoptosis inhibitor omigapil ameliorates many of the pathological symptoms in the dyw/dyw mouse, a well accepted animal model for MDC1A. Specifically, we show that omigapil inhibits apoptosis and thus protects muscle tissue, reduces body weight loss and skeletal deformation and increases locomotive activity. Importantly, loss of muscle mass, impaired mobility and skeletal deformation are also disease hallmarks seen in MDC1A patients and any improvement in such parameters is of therapeutic relevance. We also find that omigapil protects dyw/dyw mice from early mortality and prolongs overall survival although the latter effect was less strong. However, comparison of our data with published survival curves of dyw/dyw mice indicates that our vehicle treated dyw/dyw mice appear to generally survive much longer, especially after an early phase of mortality. For example, in a recent study reporting on the use of doxycycline for the treatment of dyw/dyw mice (Girgenrath et al., 2009), all the vehicle treated dyw/dyw mice had died after 56 days and those treated with doxycycline after 140 days. In our experiments, approximately 47% of vehicle-treated mice were still alive after 56 days and approximately 18% of the mice were still alive after 140 days. Interestingly, our current study and the study by Girgenrath et al. (2009) show an almost identical early phase of mortality that lasts until around day 40. While almost all mice died in the Grigenrath study around this age, many mice in our colony survived this crisis period. One of the reasons for the prolonged survival in vehicle-treated animals in our cohort might be the use of wet food and long-necked drinking bottles to ascertain adequate food and water intake in this fragile mouse strain. In summary, the markedly different survival times in untreated or vehicle-treated dyw/dyw mice reported in studies from different laboratories will have to be taken into consideration when interpreting treatment effects of experimental drugs for this indication. Nevertheless, the 50% survival time in animals treated with omigapil at a dose of 1 mg/kg/d was ~3 times longer than for vehicle treated animals and more than 20% of the omigapil-treated animals survived past 28 weeks.
Omigapil has originally been developed for the treatment of neurodegenerative diseases (Waldmeier et al., 2000; Waldmeier et al., 2006) and the therapeutic efficacy of omigapil has been tested in models of several neurological diseases both in vitro and in vivo. For example, omigapil has neuroprotective effects in cellular and animal models of Parkinson’s disease (Andringa et al., 2000) and enhances survival in an animal model of motoneuron disease (Sagot et al., 2000). Based on these positive pre-clinical results, omigapil was tested in clinical trials for Parkinson's disease and amyotrophic lateral sclerosis. While omigapil was shown to be safe, no significant difference in key outcome measures could be demonstrated between placebo and omigapil treated patients (Olanow et al., 2006; Miller et al., 2007) and the possible reasons for this failure were explored and discussed (Waldmeier et al., 2006).

The anti-apoptotic effect of omigapil has been proposed to be due to its binding to GAPDH (Kragten et al., 1998). Independent of its enzymatic function in glycolysis, several lines of evidence strongly indicate that GAPDH plays an important role in apoptosis and that this activity requires its translocation into the nucleus (Dastoor and Dreyer, 2001; reviewed in Chuang et al., 2005). The nuclear translocation of GAPDH is mediated by Siah1, an E3 ubiquitin ligase with a nuclear localization signal (Hara et al., 2005). Specifically, it has been demonstrated that the GAPDH-Siah1 complex forms upon S-nitrosylation of GAPDH and then the complex translocates into the nucleus (Hara et al., 2005; Hara et al., 2006). Once in the nucleus, GAPDH becomes acetylated through the acetyltransferase CBP/p300 which in turn further stimulates CBP/p300 activity. Consequently, downstream targets of CBP/p300 such as p53 are induced, which triggers apoptosis through the activation of the pro-apoptotic genes PUMA and p21 (Sen et al., 2008). The protein GOSPEL can act as a modifier of this pro-apoptotic pathway by preventing the initial formation of the GAPDH-Siah1 complex (Sen et al., 2009). Omigapil has been shown to directly bind to GAPDH (Carlile et al., 2000). This omigapil-GAPDH interaction prevents the formation of the GAPDH-Siah1 complex in a similar manner as GOSPEL (Hara et al., 2006; Sen et al., 2009). In muscle from dy/W/dy/W mice we found that the anti-apoptotic protein GOSPEL is significantly reduced, while we find GAPDH to be accumulated in the nuclear fraction. Furthermore, the pro-apoptotic genes that are activated downstream in the GAPDH-Siah1-CBP/p300 pathway (p53, PUMA, p21) are upregulated. This provides evidence that apoptosis in laminin-α2 deficient muscle may be caused by activation of GAPDH-Siah1-CBP/p300 signaling pathway. Omigapil may thus compensate for the loss of GOSPEL and block the activation of this pro-apoptotic GAPDH-Siah1-CBP/p300 pathway thereby preventing apoptosis.
Alternatively, omigapil could also interfere with an alternative pathway that causes apoptosis. This pathway is based on the "permeability transition" of mitochondria as a response to cellular insults that cause a strong increase in intracellular calcium. This process results in the regulated formation of a large pore complex that spans the outer and inner mitochondrial membranes, leading to the swelling and long-lasting opening of this permeability transition pore complex (PTPC) and causing necrotic and/or apoptotic cell death. GAPDH has recently been shown to accumulate in mitochondria during apoptosis and to induce PTPC-dependent pro-apoptotic mitochondrial membrane permeabilization via its association with VDAC, one of the key components of the PTPC (Tarze et al., 2007). A role of the PTPC in muscle dystrophies has recently been suggested by the finding that deletion of the gene encoding cyclophilin D, another key component of the PTPC, reduces muscle fiber necrosis in mouse models for several muscle dystrophies including MDC1A (Millay et al., 2008; Palma et al., 2009).

Taken together, our study demonstrates for the first time the activation of the GAPDH-Siah1-CBP/p300 pro-apoptotic signaling pathway in muscle of dy/dy mice. Further, we show that omigapil inhibits apoptosis in muscle, protects from body weight loss, reduces skeletal deformation, increases locomotive activity and protects from early mortality. As omigapil is well advanced in the non-clinical and clinical development and was proven to be safe in large clinical trials with Parkinson’s disease and ALS patients, this orally bioavailable drug candidate is well suited to be tested as a potential therapy for MDC1A.
Acknowledgments

We thank Drs. J. Dubach and S. Possekel for their comments on an earlier version of the manuscript.
References


Footnotes

M.E. and S.M. contributed equally to this study

Financial support:

This work was supported by grants to M.A.R. by the Muscular Dystrophy Association (USA) [Grant MDA4168] and the Swiss Foundation for Research on Muscle Diseases.

Contact for reprint requests:

Markus A. Ruegg, Ph.D.
Biozentrum, University of Basel
Klingelbergstrasse 70
CH-4056 Basel, Switzerland
Phone: +41 61 267 22 23
Fax: +41 61 267 22 08
E-Mail: markus-a.ruegg@unibas.ch
Legends for Figures

Fig. 1. The GAPDH-Siah1-CBP/p300 pathway is activated in dyW/dyW mice. a, b, quantitative PCR (qPCR) analysis of pro-apoptotic genes in triceps brachii (a) and diaphragm (b) muscle in 4 week-old wild-type (WT) and dyW/dyW mice. The transcriptional levels of p53, PUMA and p21 are increased in both muscles with a higher relative increase in the diaphragm compared to the triceps muscle (WT: N=5; dyW/dyW: N=6). c, qPCR analysis of pro-apoptotic genes in triceps brachii muscle of 7 week-old mice demonstrating sustained activation of pro-apoptotic genes (WT: N=5; dyW/dyW: N=6). d, qPCR analysis of GOSPEL in the diaphragm muscle of 4 week-old mice. GOSPEL levels are significantly reduced in dyW/dyW mice to less than 50% of the WT levels (WT: N=5; dyW/dyW: N=6). e, Accumulation of GAPDH in nuclear preparations. Quantitative assessment of GAPDH protein by Western blot in nuclear preparations isolated from 4 week-old WT and dyW/dyW diaphragm. In muscle from dyW/dyW mice a ~40% increase in GAPDH protein in nuclear preparations is detected (WT: N=3; dyW/dyW: N=3). a-e, Data is mean ± SEM; **: p<0.01, Student’s t-test.

Fig. 2. Omigapil reduces apoptosis in dyW/dyW mice. a, Chemical structure of omigapil, N-(dibenz(b,f)oxepin-10-ylmethyl)-N-methyl-N-prop-2-ynylamine maleate (also referred to as TCH346 or CGP3466). b, qPCR analysis of pro-apoptotic genes in diaphragm of 4 week-old dyW/dyW mice. The transcriptional levels of p53, PUMA and p21 are decreased in dyW/dyW mice treated for 1 week with 1 mg/kg omigapil compared to vehicle treated animals (dyW-vehicle: N=12; dyW-omigapil: N=13). c, Quantitative assessment of apoptotic myonuclei in triceps brachii muscle. The percentage of apoptotic myonuclei is significantly lower in dyW/dyW mice treated for 4 weeks with 0.1 mg/kg omigapil compared to animals treated with vehicle (WT: N=6; dyW-vehicle: N=12; dyW-omigapil: N=13). d, TUNEL staining of triceps brachii muscle of 6 week-old mice. Arrows: TUNEL-positive myonuclei. b-c: data is mean ± SEM; **: p<0.01, Student’s t-test. d: Scale bar: 50 μm.

Fig. 3. Omigapil ameliorates muscle histology and reduces blood CK levels in dyW/dyW mice. a, as detected by H&E staining, treatment of mice with 0.1 mg/kg/day omigapil reduces fibrosis in triceps brachii muscle of 6 week-old dyW/dyW mice compared to muscle from vehicle-treated mice. b, muscle fiber size distribution in triceps brachii muscle of dyW/dyW and wild-type (WT) animals. Values represent relative numbers of fibers in a given diameter class determined as minimum Feret’s diameter (Briguet et al., 2004). In vehicle-treated dyW/dyW mice a higher proportion of muscle fibers have small diameters. Treatment with 0.1 mg/kg omigapil
shifted the fiber size distribution towards a higher percentage of fibers with larger diameters (WT: N=6; dyW-vehicle: N=12; dyW-omigapil: N=13). There is a significant difference between dyW-vehicle and dyW-omigapil mice by comparison of the cumulative distribution curves of muscle fiber diameters (p < 0.001; Kolmogorov-Smirnov analysis). c. Number of muscle fibers in triceps brachii muscle per cross-sectional area. The increased number of muscle fibers per mm² in vehicle-treated dyW/dyW mice is normalized to wild-type levels by treatment with 0.1 mg/kg omigapil (WT: N=6; dyW-vehicle: N=12; dyW-omigapil: N=13). d. Relative creatine kinase (CK) levels in 6 week-old mice. Blood CK levels are significantly lower in omigapil-treated dyW/dyW mice compared to vehicle-treated mice (WT: N=9, dyW-vehicle: N=5, dyW-omigapil: N=9). a: scale bar: 50 μm; b-d: data is mean ± SEM; c, d: **: p<0.01, Student's t-test.

Fig. 4. Omigapil treatment significantly improves weight gain and locomotive behavior. a, absolute weight gain relative to weight at the age of 15 days (onset of treatment). For each week, body weight data were averaged from daily measurements. Body weights at 15 days of age were 6.28 ± 0.40 g (dyW-vehicle; N=9), 6.66 ± 0.15 g (dyW-omigapil 0.1 mg/kg; N=7) and 5.99 ± 0.22 g (dyW-omigapil 1 mg/kg; N=11). Only mice that survived for the entire time period were included. b, Relative number of dyW/dyW mice with a mean body weight of >12 grams, using data from all animals irrespective of the time of their death. A maximum of 14% of vehicle-treated dyW/dyW mice reached this cut-off body weight. The number was considerably higher in omigapil-treated (dyW-omigapil) mice: 26% (in the 0.1 mg/kg group) and 27% (in the 1 mg/kg group). Number of animals treated were dyW-vehicle: N=33, dyW-omigapil 0.1 mg/kg: N=33; dyW-omigapil 1.0 mg/kg: N=28 at start of the experiment. c, Locomotion time measured in dyW/dyW mice within a 10-minute period after placing animals into a new cage (Moll et al., 2001). Number of animals analyzed: 5-6 week-old mice (left panel): dyW-vehicle: N=13; dyW-omigapil 0.1 mg/kg: N=15; dyW-omigapil 1.0 mg/kg: N=10. Ten week-old mice (right panel): dyW-vehicle: N=11, dyW-omigapil 0.1 mg/kg: N=17; dyW-omigapil 1.0 mg/kg: N=7. Omigapil at both doses increases locomotion time in 5-6 week-old dyW/dyW mice compared to vehicle-treated animals. a, c: data is mean ± SEM; *: p < 0.05; **: p < 0.01, Student’s t-test.

Fig. 5. Effect of omigapil on skeletal deformation and survival. a, X-ray examination of 11 week-old wild-type (WT) and dyW/dyW mice in lateral (left panel) and dorso-ventral (right panel) view. The angle of spine curvature (kyphosis) is indicated in the lateral view (dotted line). While the curvature of the spine in a wild-type mouse was >120° between the costal and abdominal region, vehicle-treated dyW/dyW mice often show...
very pronounced dorso-ventral curvature (hunchback) with angles between the costal and abdominal spinal region being as small as 66°. Kyphosis and scoliosis is ameliorated upon treatment with omigapil, resulting in reduced dorso-ventral spine curvature reaching ~ 90°. The body dimension is indicated by the vertical arrows depicting the loss of lean and fat mass in vehicle-treated animals and the preservation thereof in omigapil-treated dyw/dyw mice. Horizontal arrows indicate the antero-posterior dimension of the rib cage, which is enlarged after omigapil treatment compared to vehicle-treated dyw/dyw mice. Note: paralysis of hindlimbs is seen in both the vehicle-treated and omigapil-treated animals. b, Quantification of kyphosis by visual inspection performed at the same day using a kyphosis score (1: barely detectable; 2: mild; 3: moderate; 4: severe). Dots: scoring of individual 11 week-old dyw/dyw mice. c, Kaplan-Meier cumulative survival plot shows that omigapil-treated dyw/dyw mice survive longer than vehicle-treated animals (0.1 mg/kg omigapil vs vehicle: p<0.05; 1 mg/kg omigapil vs vehicle: p<0.01; Peto-Peto Wilcoxon test). Omigapil treatment particularly protects from early death (dyw-vehicle: N=36, dyw-omigapil 0.1 mg/kg: N=33; dyw-omigapil 1.0 mg/kg: N=28).
Figure 1
Figure 2

(a) Structure of the compound.

(b) mRNA levels of p53, PUMA, and p21.

(c) Apoptotic myonuclei (%).

(d) Images of WT, dy^-vehicle, and dy^-omigapil (0.1 mg/kg).
Figure 3
Figure 4
Figure 5

(a) Lateral and dorso-ventral views of WT, dy^w-vehicle, and dy^w-omigapil (1 mg/kg) mice. Kyphosis score:
1: barely detectable
2: mild
3: moderate
4: severe

(b) Graph showing survival probability over age (weeks) for dy^w-vehicle, dy^w-omigapil (1 mg/kg), and dy^w-omigapil (0.1 mg/kg) mice.