Regulation of a Notch3-Hes1 Pathway and Protective Effect by a Tocopherol-Omega Alkanol Chain Derivative in Muscle Atrophy

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ABSTRACT

Muscle atrophy, a physiopathic process associated with severe human diseases such as amyotrophic lateral sclerosis (ALS) or cancer, has been linked to reactive oxygen species (ROS) production. The Notch pathway plays a role in muscle development and in muscle regeneration upon physical injury. In this study, we explored the possibility that the Notch pathway participates in the ROS-related muscular atrophy occurring in cancer-associated cachexia and ALS. We also tested whether hybrid compounds of tocopherol, harboring antioxidant activity, and the omega-alkanol chain, presenting cytoprotective activity, might reduce muscle atrophy and impact the Notch pathway. We identified one tocopherol-omega alkanol chain derivative, AGT251, protecting myoblastic cells against known cytotoxic agents. We showed that this compound presenting antioxidant activity counteracts the induction of the Notch pathway by cytotoxic stress, leading to a decrease of Notch1 and Notch3 expression. At the functional level, these regulations correlated with a repression of the Notch target gene Hes1 and the atrophy/remodeling gene MuRF1. Importantly, we also observed an induction of Notch3 and Hes1 expression in two murine models of muscle atrophy: a doxorubicin-induced cachexia model and an ALS murine model expressing mutated superoxide dismutase 1. In both models, the induction of Notch3 and Hes1 were partially opposed by AGT251, which correlated with ameliorations in body and muscle weight, reduction of muscular atrophy markers, and improved survival. Altogether, we identified a compound of the tocopherol family that protects against muscle atrophy in various models, possibly through the regulation of the Notch pathway.

Introduction

Muscle atrophy is associated with severe human pathologies, such as degenerative diseases and cancer (von Haehling et al., 2010). For example, amyotrophic lateral sclerosis (ALS) is characterized by the death of motor neurons and the atrophy of skeletal muscles, which leads to paralysis and death due to a lack of an efficient cure (Ludolph et al., 2012). During cancer, muscle atrophy may develop as a side effect of the chemotherapy (cisplatin, doxorubicin) (Gilliam et al., 2013). In particular, elevated ROS production has been linked to the presence of mutations in the ROS detoxification enzyme superoxide dismutase 1 (SOD1) associated with hereditary ALS (Beckman et al., 2001). Similarly, cisplatin and doxorubicin have been shown to induce the production of ROS (Chirino and Pedraza-Chaverri, 2009; Gilliam and St Clair, 2011; Hydock et al., 2011; Garcia et al., 2013) or at the late stage of the pathology (Fearon et al., 2012). This muscle-wasting syndrome (or cachexia) drastically reduces the patient’s quality of life and may force the clinician to stop the treatment. The lack of an efficient cure against muscle atrophy correlates with a poor knowledge of the mechanisms involved.

Although the origin of muscle atrophy differs between pathologies, common features can be identified that play an important role in fiber and myoblast alterations, such as an abnormal production of reactive oxygen species (ROS) (Barbieri and Sestili, 2012; Gilliam and St Clair, 2011; Ray et al., 2012). In particular, elevated ROS production has been linked to the presence of mutations in the ROS detoxification enzyme superoxide dismutase 1 (SOD1) associated with hereditary ALS (Beckman et al., 2001). Similarly, cisplatin and doxorubicin have been shown to induce the production of ROS (Chirino and Pedraza-Chaverri, 2009; Gilliam and St Clair, 2011; Hydock et al., 2011; Garcia et al., 2013; Gilliam et al., 2013). ROS production has also been linked to the muscular atrophy induced by the presence of a tumor, likely mediated by cytokines, such as tumor necrosis factor-α (Adams et al., 2008). At the cellular level, production of ROS induces lipid peroxidation, protein oxidation, DNA damage, and mitochondrial dysfunction. These cellular alterations activate specific signaling pathways, such as the unfolded protein response, and/or transcription factors, such as p53/p73, which in turn lead to cell death and tissue degeneration (Gonzalez de Aguilar et al., 2000; Benosman et al., 2007, 2011; Barbieri and Sestili, 2012).

To counteract the atrophy process, small molecules have been tested for their cytoprotective properties. Two examples...
of these drugs are tocopherol and flavonoid derivatives. Flavonoid derivatives can reduce ROS effects and present neuroprotective or cardioprotective activity (Hauss et al., 2007; Thuaud et al., 2011; Ribeiro et al., 2012). Several molecules that act on regeneration and neuroprotection are derived from tocopherol, a member of the vitamin E family, which is widely used in clinical practice because of its antioxidant and anti-inflammatory properties (Singh and Jialal, 2004). Moreover, α-tocopherol derivatives are considered disease modulators in multiple sclerosis and show beneficial outcomes after white matter damage in experimental models (Coowar et al., 2004; Blanchard et al., 2013).

The notch pathway is an intercellular developmental signaling cascade with fundamental roles in muscles (Buas and Kadesch, 2010; Bjornson et al., 2012; Guruharsha et al., 2012). This pathway comprises four receptors (Notch 1–4) and several ligands (δ-like, Jagged) that mediate their biologic function through induction of target genes, like Hes1 and HeyL (Buas and Kadesch, 2010; Bjornson et al., 2012). Several studies have shown that Notch signaling favors myoblast proliferation, and when sufficient myoblasts are produced, Notch signaling needs to be switched off again to allow myoblast differentiation (Buas and Kadesch, 2010). This sequential regulation of the Notch pathway plays a central role in muscle development and muscle regeneration. For example, Notch3 silencing leads to muscle hypertrophy due to excess regenerative processes (Kitamoto and Hanaoka, 2010). Inversely, the expression of a general dominant negative Notch peptide leads to severe muscle atrophy. We chose gastrocnemius muscles were sampled, immediately frozen in liquid nitrogen, and stored at −80°C for later use. We used 1 ml of TRIzol (Invitrogen/Life Technologies) per 150 mg of muscle to extract RNA according to the manufacturer’s instructions. RNA samples were ethanol-precipitated twice, and 1 μg was used for reverse transcription (High-Capacity cDNA Reverse Transcription Kit; Applied Biosystems, Foster City, CA). We performed quantitative polymerase chain reaction (qPCR) using 2 ng/μl cDNA (RNA equivalent) according to the manufacturer’s instructions (SYBR Green PCR Master Mix; Applied Biosystems) and with 400 μM of each primer (Supplemental Table 2). Expression levels were normalized using an average of 18S.

SODI+ Mice. SODI+ mice are FBV transgenic mice expressing the missense mutation G85R (human G85R equivalent) in the SOD1 gene (Ripps et al., 1995). Transgene expression was monitored by PCR on SOD1 (forward GACATCATTGTTCATCC; reverse ATTGATGGAAATGCCTCTCCTGAA), Fsp1 digestion, and agarose electrophoresis (Ripps et al., 1995). We distinguished several experimental groups based on age and the presence of symptoms: asymptomatic 60-day-old mice, asymptomatic 75-day-old mice, early symptomatic mice (90 days; presenting signs of denervation as indicated by upregulation of the mRNA for acetylcholine receptor α-subunit), and symptomatic mice (105–110 days) when full paralysis is in.

Mice Treatment. SODI+ mice were injected intraperitoneally 3 times a week beginning at an age of 65 days (Rene et al., 1996). AGT251 (20 mg/kg) was administered in 0.9% NaCl with 6% propylene glycol, 4% dimethylsulfoxide, and 2% Cremophor EL. Vehicle-treated littersmates received the same formulation without AGT251. For doxorubicin treatment, 8-week-old mice were injected once intraperitoneally with doxorubicin (BioAustralis, Smithfield, NSW, Australia) diluted at 18 mg/kg in 0.9% NaCl. AGT251 treatment was performed as described earlier. In vivo experiments were repeated at least twice with a number of animals (between 5 and 12) recommended to optimize statistical analyses according to the regional and national animal ethics committee. All animal manipulations were performed under appropriate supervision and observing protocols validated by the regional and national animal ethics committee.

Statistical Analyses. Statistical analyses were performed using a one-way analysis of variance test followed by a Tukey post-test to allow a comparison between all the conditions. In the graphs, an asterisk indicates a statistically significant difference. For in vivo survival analysis of SOD mice, two statistical protocols were used: Mantel-Cox test and Gehan-Breslow-Wilcoxon. Both tests confirmed a statistically significant difference between control and AGT251-treated mice. Statistical analyses were performed using Prism (GraphPad Software, San Diego, CA).

Transfection and Luciferase Assays. Cells were transfected by a polyethylenimine-based or JetPrim (Polyplus, Strasbourg, France) as previously described elsewhere (Gaiddon et al., 1998). For luciferase assays, cells were seeded in 24-well plates and transfected with the indicated expression vectors (200 ng) and reporter constructs (250 ng). Luciferase activity was measured in each well 24 hours later, and the results were normalized with a cytomeglovirus-driven reporter gene (Bensman et al., 2011). The MuRF1-luc constructs were previously described by Waddell et al. (2008). The Hes1 reporter gene contains the 3-kb promoter of Hes1. Hes1 reporter gene and NICD and CBF expression vectors were a generous gift of Dr. Kadesch (Ross and Kadesch, 2001).
Results

Tocopherol and Flavonoid Derivatives Impact on Myoblastic Cell Survival. The effect on cell survival of several flavonoid or tocopherol derivatives (AGT) was assayed on C2C12 cells, a commonly used in vitro model for myoblast cells. Dose-response survival relationships were established by means of an MTT assay and using a large window of concentrations (1 nM–125 μM) (Fig. 1A). Most compounds were reducing survival, with an IC_{50} between 50 and 125 μM, although three compounds were already active at lower concentrations (Fig. 1A; Table 1). The toxic effect of these compounds at high concentrations correlated with the upregulation of p21 and noxa expression (Fig. 1B). We chose p21 and noxa as they represent markers for cell growth arrest and apoptosis, respectively (Benosman et al., 2011). Interestingly, two compounds significantly increased cell survival by 22% (AGT048, 25 μM) or 41% (AGT251, 15 μM) at concentrations preceding toxicity. AGT031 did not show any prosurvival effect and was more toxic than most compounds. Thus, it was used as a control in subsequent experiments.

AGT048 and AGT251 Protect against Oxidative and DNA Damage-Induced Stresses. As AGT048 and AGT251 compounds were increasing cell survival, we assessed whether these compounds could also improve viability upon stresses associated with degenerative syndromes. Therefore, C2C12 were treated with 1) menadione that induces a strong production of anion superoxide (Rosen and Freeman, 1984) and 2) cisplatin that induces DNA damage and mild ROS production (Supplemental Fig. 1A) (Vidimar et al., 2012). We observed that both AGT048 and even more AGT251 were able to increase cell survival in the presence of cisplatin and to a lesser extent of menadione (Fig. 2, A and B). Under the same conditions, AGT251 reduced the ROS production induced by cisplatin and menadione (Supplemental Fig. 1B). As expected, AGT031 did not increase cell survival.

To further assess the prosurvival activity of AGT251 at the molecular level, we analyzed the expression of p21 and noxa. We observed that in C2C12, cisplatin induced p21 and noxa expression (Fig. 2C). At the dose of AGT251 that favored survival (10 μM), the cisplatin-induced expression of p21 was...
unchanged, whereas the expression of the proapoptotic gene noxa was decreased. Taken together, these experiments demonstrated that AGT251 protects myoblast cells against DNA-damaging stress in vitro.

**AGT251 Modulates the Notch Pathway.** To further understand the biologic properties of the AGT251 tocopherol derivative, we then focused on the Notch pathway for two reasons. First, previous work has shown that compounds sharing as imilars structu tears to our compound swere able to repress Notch4 RNA expression in neurospheres correlating with an increased neuronal survival (Coowar et al., 2004; Blanchard et al., 2013). Second, it has been shown that the notch pathway plays a key role in muscle regeneration and myoblast survival (Buas and Kadesch, 2010; Bjornson et al., 2012) and is regulated by the physiologic status of the mitochondria (Arthur and Cooley, 2012; Kasahara et al., 2013).

Before evaluating the effect of AGT251 on the Notch pathway in stressed myoblastic cells, we first characterized the impact of cisplatin on the expression of Notch receptors (Notch 1–4) using reverse-transcription real-time quantitative polymerase chain reaction (Fig. 3A). A cytotoxic dose of cisplatin (7.5 μM) induced upregulation of Notch1, Notch3, and Notch4 mRNA levels. It is of note that the expression level of Notch4 in C2C12 cells was low (threshold cycle ≥ 30). To assess the functionality of these regulations, we also evaluated the expression of Notch target genes and observed an increase of Hes1 and HeyL mRNA upon cisplatin treatment.

We then tested how AGT251 might affect the cisplatin-induced activation of the Notch pathway. We found that prosurvival doses of AGT251 (Figs. 1A and 3B) caused a decrease of cisplatin-induced expression of Notch1 and Notch3 expression while Notch4 was further induced (Fig. 3C). Importantly, the repression of Notch1 and Notch3 correlated with a diminished expression of Notch target genes, such as Hes1.

Taken together, these results indicated that the Notch pathway was induced by a cytotoxic dose of cisplatin, and that this induction was reduced by AGT251. In particular, we observed a correlation between the expression of Notch1, Notch3, and Hes1 upon treatment with AGT251.

**AGT251 Protects Muscles from Doxorubicin-Induced Cachexia.** To assess whether our observations with C2C12 cells in vitro were physiologically relevant, we decided to use a murine model for cachexia. Cachexia is a severe syndrome including muscle atrophy observed in cancer patients with certain cancer localizations (such as pancreatic cancer) and in patients treated with doxorubicin (Fearon et al., 2012). In patients, doxorubicin treatment is associated with muscle weakness and fatigue and with muscle atrophy (fiber types 1 and 2) (Bonifati et al., 2000; Tozer et al., 2008). The exact causes of muscle weakness and atrophy induced by doxorubicin remain poorly understood, and doxorubicin-treated rodents have been used to investigate these phenomena. In these models, muscle weakness and fiber alterations (proteolysis, autophagy, apoptosis) have been described, with variations depending on the mode of administration (intraperitoneal versus intravenous) and the type of muscle (soleus, extensor digitorum longus, heart, diaphragm) (Gilliam et al., 2009, 2011, 2012, 2013; De Angelis et al., 2010; Smuder et al., 2011a,b; Dirks-Naylor et al., 2013; Kavazis et al., 2014; Yu et al., 2014). These studies show evidence that muscle fibers as well as the
A week with AGT251 (20 mg/kg). The AGT251 dose was established as the maximal dose tolerated by the mice without noticeable adverse effects based on the body weight and behavior. After 15 days of treatment, the total mouse weight and skeletal muscle weight were measured, and RNA was extracted from muscles to quantify the expression of muscle atrophy markers (MuRF1 and Atrogin-1). We observed a loss of total animal and muscle weight with doxorubicin treatment (Fig. 4A and B), confirming its atrophic effect on the muscle. This effect correlated with an upregulation of the two effectors and markers for muscle atrophy, MuRF1 and Atrogin-1 (Fig. 4C). It was very interesting that we observed that cotreatment with AGT251 partially reversed the muscle atrophy, as assessed by muscle weight (Fig. 4A). This effect was further confirmed by the repression of MuRF1 and Atrogin-1 expression after AGT251 treatment (Fig. 4C). Based on our results, AGT251 appeared to protect muscles against atrophy under conditions inducing cachexia.

To investigate the impact of muscle atrophy and AGT251 on the Notch signaling pathway in vivo, we measured the expression of Notch receptors and Notch target genes. Doxorubicin treatment induced expression of Notch1 and Notch3 (Fig. 4D). Expression of the Notch target gene Hes1 was also increased by doxorubicin, in contrast with other Notch target genes, such as HeyL, which remained unaffected (Fig. 4E). Importantly, administration of AGT251 led to a reduction of Notch1, Notch3, and Hes1 expression in doxorubicin-treated mice (Fig. 4, D and E). To further investigate the potential functional relationship between the Notch pathway and muscular atrophy, we used a reporter gene containing the MuRF1 promoter and a reporter gene containing the promoter of the Notch target gene Hes1. As expected, overexpression of the intracellular domain of Notch3 (NICD), alone or with its coactivator CBF, led to an increase in the Hes1 promoter activity (Fig. 4F). Interestingly, NICD also stimulated the activity of the MuRF1 promoter.

Taken together, these results confirmed that the activity of the Notch pathway is induced upon muscle injury, such as occurs with doxorubicin treatment, and that AGT251 is able to partly counteract this effect.

AGT251 Increases Survival in a Murine Model of ALS. Given its protective effect on myoblasts in vitro and the protection against doxorubicin-induced muscular atrophy, we wondered whether treatment with AGT251 could be beneficial in mice showing progressive muscle degeneration leading to death. In addition, by using another model we wanted to assess whether AGT251 might have a broad protective effect independent of the etiology of the disease. Thus, we next studied mice with a substitution mutation in the Cu/Zn superoxide dismutase 1 gene (SOD1*), which is used as a model of the human pathology ALS (Ripps et al., 1995; Gonzalez de Aguilar et al., 2000). Although the G86R mutation in the SOD1 (SOD1*) does not affect SOD1 activity, the mice carrying the SOD1* transgene develop oxidative stress (Ripps et al., 1995; Gonzalez de Aguilar et al., 2000). On average, SOD1* mice die at around 105 days following muscular atrophy and motoneuronal death. SOD1* mice of 60 or 75 days of age are in the asymptomatic stage; their physical symptoms start at around 90 days, with some mice already showing a marked muscular denervation (group 90S) while others do not (group 90NS) (Ripps et al., 1995; Gonzalez de Aguilar et al., 2000). The denervation of the animal can be followed by the expression of the acetylcholine receptor gene in the muscles (Fig. 5A). In addition, the level of muscular...
atrophy can be assessed using markers such as the MuRF1 and Atrogin-1 genes (Foletta et al., 2011) (Fig. 5B).

We first assessed whether in this model the expression of components of the Notch pathway were altered in the muscles during the ALS pathology (Fig. 5, C and D). We observed a marked increase in Notch3 expression at the early stage of the pathology (60 and 70 days), followed by a decrease upon disease progression (90S, animals with denervation at 90 days; 105 days) (Fig. 5, A and C). Interestingly, expression of Hes1 also increased at 90 days in all animals, regardless of their denervation status, while other Notch target genes, such as HeyL, remained unchanged (Fig. 4D). These observations established that in ALS atrophic muscle a Notch-dependent response takes place that might participate in the development of the pathology or in an attempt of a regenerative process.

We used then this mouse model to assess whether AGT251 could prolong the life span. We treated SOD1* mice 3 times a week with AGT251 (20 mg/kg) starting at an asymptomatic age of 65 days. The treatments were performed until death. The efficacy of the AGT251 treatment was indicated by a decreased expression of the muscle atrophy marker MuRF1 and Notch3-Hes1 components (Supplemental Fig. 3). But, more importantly, the results showed a mild but statistically significant increase in survival of AGT251-treated SOD1* mice compared with the vehicle-treated mice (Fig. 4E). Indeed, the vehicle-injected animals had a median survival of 105 days, but this number rose to 112 days (+8%) for AGT251-injected animals.

Altogether, this result indicated that AGT251 exerts a protective effect on diseases with muscle atrophy and that it also inhibits Notch3-Hes1 during this process.

**Discussion**

Muscular atrophy occurs in several lethal human diseases, including neuromuscular syndromes such as ALS or cancer. In cancer, it has been estimated that about 30% of the mortality is due to the cachexia that includes muscle atrophy. In the case of ALS, respiratory muscle atrophy is also the cause of death. This dramatic clinical status is a direct reflection of the absence of a curative treatment for muscle atrophy. In this study, we investigated the beneficial effect of flavonoid and tocopherol derivatives on skeletal muscles based on their antioxidant and anti-inflammatory properties, which have previously been shown to have protective activity in the nervous system and the heart. In addition, we investigated some of the molecular mechanisms potentially involved in muscle protection by focusing on the Notch pathway.

**Cellular Effects of Novel Flavonoid and Tocopherol Derivatives.** The synthesized flavonoid and tocopherol derivatives and hybrids with an omega-alkanol chain showed in vitro biologic activity on the C2C12 myoblast cell line. Above a specific concentration, each compound reduced cell survival as observed by MTT tests and by the induction of cell growth arrest and the proapoptotic genes p21 and noxa (Fig. 1; Table 1). However, a moderate concentration of two tocopherol derivatives, AGT048 and AGT251, sharing a similar pharmacophore, stimulated survival. Importantly, at these subtoxic concentrations AGT048 and AGT251 partly counteracted the toxic effect of cisplatin or menadione on C2C12 cells (Fig. 2), which correlated with a reduced level of the proapoptotic gene noxa (Fig. 2C). In addition, AGT251 restored the expression level of two myoblastic cell markers, Pax7 and MyoD, which were both downregulated by cisplatin (Supplemental Fig. 4).

All the tested flavonoid and tocopherol derivatives share the same omega-alkanol chain. However, only two tocopherol derivatives present the protective effect on C2C12 cells, which
suggests that this effect is not mediated by the omega-alkanol chain but more likely by an intact tocopherol core. This is further supported by the fact that the protective effect of AGT251 correlates with the reduction of ROS production in C2C12 cells (Supplemental Fig. 1B).

Tocopherol Derivatives Diminish the Stress-Induced Expression Levels of Notch1, Notch3, and Hes1 in Muscles. A recent study showed that the Notch pathway is involved in muscle repair during aging or physical damage (Arthur and Cooley, 2012). In addition, it was previously...
shown that tocopherol derivatives repressed mRNA level for Notch4 in neuronal cells (Coowar et al., 2004). Our study brings novel information on these two aspects.

Concerning the first aspect, we uncovered the first evidence of regulation of the Notch pathway during ALS or cachexia. Indeed, we observed both in vitro (C2C12 myoblastic cells) and in vivo (murine models of ALS and doxorubicin-induced cachexia) the induction of the Notch pathway after various stressors. In particular, we showed a correlation between the upregulation of Notch1 and Notch3 expression and their target gene Hes1. Based on the complex nature of the muscle—which includes fibers, muscle progenitors, vessels, and neuromuscular junctions, for example—it is difficult to definitively conclude that the regulation of Notch observed in vivo is solely due to muscle cells. We can only hypothesize that part of this regulation does indeed involve muscle cells, based on our results with C2C12 and on the literature that has indicated that the Notch pathway is regulated in myoblastic cells and

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**Fig. 5.** AGT251 favors mice survival in a model of ALS. The mRNA levels of markers of ALS disease progression—acetyl choline receptor α, AcR-α (A) MuRF1, and atrogin-1 (B)—and components of the Notch signaling pathway—Notch1, -3, and -4 (C), Hes1, HeyL (D)—were assayed in muscles of SOD1* mice using reverse-transcription real-time quantitative polymerase chain reaction. mRNA were extracted at different ages. Curves are the mean of fold induction versus the condition of wild-type littermate 60-day-old mice and of three experiments containing animals of matching age (60, 75, 90NS, 90S, 105-day-old, n = 5). 90NS and 90S populations are differentiated based on the level of expression of the AcR-α gene. *P < 0.05, **P < 0.01 compared with control, as calculated by a one-way analysis of variance test followed by a Tukey post test. (E) SOD1* 650-day-old mice were injected 3 times a week with AGT251 (20 mg/kg) until death. Survival curves are relative to the day of death of the untreated animals (n = 12). *Statistically significant differences established by log-rank (Mantel-Cox) test (P = 0.0053) and Gehan-Breslow-Wilcoxon test (P = 0.0185).
participates in the control of muscle development and regeneration.

The consequences of this regulation in the muscles, and more specifically in the progenitors and the fibers, also remain to be established. As indicated by the literature, the Notch pathway seems to play a role in maintaining muscle stem cell quiescence (Bjornson et al., 2012; Mourikis et al., 2012). Therefore, the increase of Hes1 expression during the pathologies, or after in vitro treatment with anticancer drugs, might contribute to muscle atrophy by maintaining at least a subpopulation in a quiescent state. The exact and respective contribution of Notch1 and Notch3 in this process needs to be more clearly established.

In addition, our observation that NICD regulates the promoter of the atrophic/remodeling factor MuRF-1 might suggest that the Notch pathway could play a role in fibers. We also observed some differences in response between the models. For example, we found in vitro an induction of HeyL expression that we did not see in vivo. This is surprising because two recent publications indicated that HeyL might play a more important role than Hes1 in the maintenance of satellite cell number in the muscles (Buas and Kadesch, 2010; Fukada et al., 2011). In contrast, our results might indicate that in injury/repair processes, such as those taking place in response to doxorubicin and in ALS-related syndromes, the opposite is the case, with Hes1 being more important than HeyL. However, the exact role of Notch1, Notch3, and Hes1 in the atrophic process and whether they are related to the regulation of the differentiation and proliferation equilibrium of satellite cells or fiber atrophy remains to be established. Interestingly, our study also suggested that Notch might be involved in muscle atrophy via induction of the atrophic/remodeling effector MuRF1, which provides a novel possible avenue in the overall function of the Notch pathway in muscle development and repair.

Concerning the second aspect, our data confirmed that tocopherol derivatives such as AGT251 affect the activity of the Notch pathway. Indeed, AGT251 treatment partly counteracted both in vitro and in vivo stress-induced Notch pathway activation. However, in C2C12 myoblastic cells and in muscles of ALS or doxorubicin-treated animals, the repression was mostly observed on Notch1 and Notch3 but not on Notch4 as previously seen in neurons (Coowar et al., 2004). In addition, here we also provided the first evidence that regulation of the Notch receptor by tocopherol derivatives impacts the Notch target genes.

It is not yet precisely understood how AGT251 might affect the Notch pathway. As indicated earlier, one possibility is that due to its antioxidant properties, AGT251 protects the cells against the oxidative stress induced by cisplatin, doxorubicin, or mutant SOD1 (Supplemental Fig. 1B), hence reducing the induction of Notch. This suggests that AGT251 might help to restore the properties of the myoblastic cells. This is partly supported by the fact that AGT251 reduces the inhibition of Pax7 and MyoD expression caused by cisplatin in C2C12 cells (Supplemental Fig. 4). Alternatively, the antioxidant properties of AGT251 might also protect the fibers from atrophy. In addition, one recent interesting observation has been that the Notch pathway is a relay toward myocyte differentiation induced by variations in mitochondria physiologic activity (Kasahara et al., 2013). Therefore, based on the antioxidant activity of the tocopherol derivative (Supplemental Fig. 1B), one hypothesis might be that by controlling the mitochondrial redox status, AGT251 might regulate the Notch pathway. In addition, a more recent study indicates that the Notch pathway is partly controlled partly by Nrf2, which is a Redox sensitive factor (Paul et al., 2014). Therefore, one hypothesis might be that regulation of the Notch pathway by the antioxidant AGT251 or during ROS-related muscle atrophy could involve Nrf2.

A Tocopherol Derivative Ameliorates Mouse Survival in ALS Model and Reduces Muscle Cachexia. The in vitro trophic effect of tocopherol derivatives on myoblast cell line survival observed in vitro was validated using in vivo murine models. Indeed, sustained injection of AGT251 in a model of cachexia (doxorubicin-treated mice) ameliorated mice health, both on the overall weight of the mice and more specifically on the muscular weight, going along with the downregulation of effectors and markers of muscular atrophy (MuRF1 and Atrogin-1) and an upregulation of muscle fiber diameter (Supplemental Figs. 3 and 5). The protective effect of the tocopherol derivative AGT251 on muscles was further confirmed as AGT251 prolonged slightly but significantly the survival of mice developing ALS with severe muscle atrophy.

Of note, the observed beneficial effect is close to what was previously observed for Riluzol in mice, the only treatment for ALS approved by the US Food and Drug Administration (Gurney et al., 1996). This effect might be linked to an improved resistance of muscle fibers and/or motoneurons, which are both primary tissues affected by the pathology. More generally, the proatrophic effect of doxorubicin and mutated SOD1 as well as the protective effect of AGT251 might involve several components of the muscle that ultimately lead to an improvement or alteration of muscle and fiber size, respectively. For example, it is likely that the changes in the levels of ROS caused by doxorubicin, mutant SOD1, and AGT251 in the fibers and the progenitors might impact muscle atrophy.

Altogether, our in vitro and in vivo data indicate that specific tocopherol derivatives can improve syndromes that involve skeletal muscle atrophy, including in ALS or cancer-related cachexia. It also points out that the Notch pathway is regulated during muscle atrophy, with specificities depending on the pathology. Our data suggest that Notch1, Notch3, and Hes1 seem to be potential players in these processes. However, the exact contribution of Notch1, Notch3, and Hes1 in the atrophic and regenerative processes remains to be more precisely established. Consequently, designing molecules that target the Notch pathways might be an interesting avenue in the development of drugs aimed at the protection of muscle against various stresses.

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Authorship Contributions

Participated in research design: von Grabowiecki, Coowar, Mellitzer, Guiddon.

Conducted experiments: von Grabowiecki, Licena, Palamiuc, Abreu, Vidimar.

Performed data analysis: von Grabowiecki, Licena, Palamiuc, Abreu, Vidimar, Coowar, Mellitzer, Guiddon.

Wrote or contributed to the writing of the manuscript: von Grabowiecki, Mellitzer, Guiddon.

References

Regulation of a Notch3-Hes1 pathway and protective effect by a tocopherol-omega alkanol chain derivative in muscle atrophy

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Supplementary data #1

A. ROS production

B. ROS production

A, B. Production of reactive oxygen species. Cells were treated with cisplatin (Cispl 10 µM) or Menadione (1µM) for the indicated time and labeled with carboxy-H2DCFDA for 1 h. Then fluorescence was quantified.
### Supplemental Data, Table 2

#### qPCR primers

All 5' to 3'

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Sequences of primers use for RT-qPCR experiments
Expression of MuRF1, Notch3 and Hes1 in SOD1* mice treated or not with AGT251. Mice treatment started with 60 days old mice and mice were scarified at 95 days to extract RNA and performed RT-qPCR analyses. *: p< 0.01 compared to control, as calculated by a one-way ANOVA test followed by a Tukey post-test (n=6).

HES1 reporter gene was co-transfected with control, NICD or CBF expression vector. Cells were then treated for 8 hours (cisplatin 7.5 μM, Cis; AGT251 15 μM, AGT) and luciferase was quantified. Fluorescence from a CMV-GFP-expressing vector was used as internal control in all condition. Graphs are means of fold induction versus Ct in absence of cisplatin with SD (n=3). *: p< 0.01 as calculated by a one-way ANOVA test followed by a Tukey post-test.
Expression of Pax7 and MyoD in C2C12 cells treated or not with Cisplatin or/and AGT251. Cells were treated for 24h before RNAs were extracted and RT-qPCR performed. *: p<0.01 compared to control, as calculated by a one-way ANOVA test followed by a Tukey post-test (n=6).
Histochemistry analysis on gastrocnemius muscle section of mice (A, control; B, doxorubicin 18μg/Kg 5 days; C, doxorubicin 18μg/Kg and AGT251 20μg/Kg). Section show also nuclei labeled with DAPI. C. Quantification of number of fibre per surface unit indicated as % of the number observed in control mice.*: p< 0.01 compared to control, as calculated by a one-way ANOVA test followed by a Tukey post-test (n=4).