Functional resolution of fibrosis in mdx mouse dystrophic heart and skeletal muscle by halofuginone

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Huebner KD, Jassal DS, Halevy O, Pines M, Anderson JE. Functional resolution of fibrosis in mdx mouse dystrophic heart and skeletal muscle by halofuginone. Am J Physiol Heart Circ Physiol 294: H1550–H1561, 2008. First published February 8, 2008; doi:10.1152/ajpheart.01253.2007.—The effect of halofuginone (Halo) on established fibrosis in older mdx dystrophic muscle was investigated. Mice (8 to 9 mo) treated with Halo (or saline in controls) for 5, 10, or 12 wk were assessed weekly for grip strength and voluntary running. Echocardiography was performed at 0, 5, and 10 wk. Respiratory function and exercise-induced muscle damage were tested. Heart, quadriceps, diaphragm, and tibialis anterior muscles were collected to study fibrosis, collagen I and III expression, collagen content using a novel collagenase-digestion method, and cell proliferation. Hepatocyte growth factor and α-smooth muscle actin were measured by Western blot and immunofluorescence. Halo decreased fibrosis (diaphragm and quadriceps), collagen I and III expression, collagen protein, and smooth muscle actin content after 10 wk treatment. Muscle-cell proliferation increased at 5 wk, and hepatocyte growth factor increased by 10 wk treatment. Halo markedly improved both cardiac and respiratory function and reduced damage and improved recovery from exercise. Overall, Halo markedly improved both cardiac and skeletal muscle mass by 10.2 ± 0.3 mm, and hepatocyte growth factor increased by 10 wk treatment. Halo markedly improved both cardiac and skeletal muscle mass by 10.2 ± 0.3 mm. Marked improvements in vital-organ functions implicated Halo as a strong candidate drug to reduce morbidity and mortality in Duchenne muscular dystrophy.

Fibrosis in skeletal and cardiac muscles leads to significant functional deterioration and reduced quality of life in Duchenne muscular dystrophy (DMD). DMD is a lethal disorder that results in exercise-induced muscle damage, inflammation, fibrosis, and progressive dysfunction and weakness (7). Initially, damage is followed by regeneration, but eventually muscle is replaced by collagen and adipose tissue in the extracellular matrix (ECM). As the disease progresses, wheelchair and ventilatory assistance are required, and patients often succumb to cardiac dysfunction and respiratory failure (26).

DMD is caused by a mutation of the dystrophin gene (29); the mdx-mouse model of DMD also has X-linked myopathy due to a dystrophin mutation (30). As mdx mice age, the muscles become increasingly similar to those in DMD. Large increases in the amount of fibrosis replace damaged fibers in dystrophic muscles in an age-related fashion in skeletal muscle (49, 50) and in cardiomyopathy (3, 59). Ultimately, dystrophy has a significant negative impact on functional capacity in mdx mice.

Fibrosis is demonstrated by large increases in collagens types I and III in muscle ECM (18). Fibrosis is regulated by signaling through transforming growth factor-β (TGF-β) and offset by upstream hepatocyte growth factor (HGF) signaling, which acts as a TGF-β antagonist. HGF reduces fibrosis by decreasing the activation of fibroblasts into the myofibroblasts that synthesize α-smooth muscle actin (SMA) at the site of injury (14, 57). HGF also plays a role in the development of renal and pulmonary fibrosis (14, 28). Interestingly, HGF has a major role in satellite cell activation and proliferation mediated via c-met receptor (5, 19, 34, 66). The distinct roles of HGF in myogenesis and fibrosis have not been explored in relation to muscle fibrosis in muscular dystrophy.

Halofuginone hydrobromide (Halo) has potent antifibrotic activity due to inhibition of TGF-β-mediated collagen synthesis (43, 45, 51, 52) and phosphorylation and activation of TGF-β-dependent Smad3 (39) that inhibited collagen I expression without changing collagen content. It prevented collagen synthesis and fibrosis in mice with tight skin, chronic graft versus host disease, and scleroderma (35, 45, 52) and decreased fibrosis in rats with pulmonary fibrosis (43), hepatic fibrosis (8, 22), urethral stricture (45), and peritoneal adhesions (44, 46). Therefore, Halo has antifibrotic effects in acute and chronic nonmuscle conditions.

These reports led to our experiments on young mdx mice treated with Halo from the onset of dystrophy for 8 wk (Turgeman et al., unpublished data). Halo induced a dose-dependent and reversible reduction in diaphragm collagen content and fibrosis. Halo also prevented cyclosporine-induced fibrosis in the mdx mouse diaphragm, abrogated Smad3 phosphorylation, and partially prevented the cardiac hypertrophy that is typical of mdx mouse cardiomyopathy (2, 59). Most importantly, Halo treatment alleviated the progression of dystrophy in diaphragm muscle (indicated by central nucleation) and improved function in both limb muscle (endurance, coordination and balance) and the heart [preventing ventricular wall motion abnormalities (WMAs)]. These animal studies therefore suggest Halo as a putative antifibrotic agent for treating dystrophic muscles in an age-related fashion in skeletal muscle (49, 50) and in cardiomyopathy (3, 59). Ultimately, dystrophy has a significant negative impact on functional capacity in mdx mice.

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early DMD. However, the outcome of Halo treatment on established dystrophy, where function has already deteriorated, remains unexplored.

Based on the severe functional impact of chronic fibrosis on muscle and cardiac function in DMD, experiments were designed to test the hypothesis that Halo would be efficacious in resolving fibrosis in older mdx mice. Results demonstrated that decreased SMA content and collagen gene expression, increased HGF content, increased muscle cell proliferation, and changes in fibrosis were associated with improved functional outcome in limb, respiratory, and cardiac muscles in mdx dystrophic mice.

MATERIALS AND METHODS

Materials. Halo was obtained from Collarg Biopharmaceuticals (Tel Aviv, Israel). Sirius red F3B was obtained from ESBE Laboratory supplies (Toronto, ON, Canada). Bouin’s fixative and Weigert’s iron hematoxylin were obtained from Fisher (Hampton, NH). Biebrick scarlet acid fuschin and light green SF yellowish were obtained from Matheson Coleman and Bell (Norwood, OH). The polyclonal anti-collagen α1(I)-antibody and HGF antibody were from Sigma (St. Louis, MO), anti-GAPDH and anti-Ki67 antibodies (ab15580, which reacts against mouse Ki67) were from Abcam (Cambridge, UK), and the α-SMA antibody was from Santa Cruz (Santa Cruz, CA). Secondary antibodies were from Santa Cruz and Abcam. Collagen I and III sequences in plasmids were obtained from ATCC (Manassas, VA).

Animals and experimental design. Mdx mice were intraperitoneally injected with 3x107 Halo cells in 10 μl PBS (10 μg Halo/animal) on 3 consecutive days beginning at 1 wk of age. Control mice were injected with 10 μl PBS and were age-matched to the Halo-treated animals. Mice were monitored for growth and mortality. Animals were euthanized after 4 to 5 weeks of treatment. A central nucleation index (CNI) was also calculated for the TA and EDL muscles by observing three fields at 100 magnification/animal was analyzed) using a 10x10 ocular grid. Squares filled by blue-green stain over 25% or more were considered positive, and fibrosis was represented by the number of positive squares as a proportion of 500 in each section.

Assays for collagen, HGF, and SMA content. Collagen content was assayed by immunoblotting and radiolabeling extracts. Aliquots of equal protein concentration (100 μg) were digested (20 min at 37°C) in 0.2% collagenase, and the reaction was stopped with sample buffer and boiling (Huebner and Anderson, unpublished). Samples were loaded on 8% reducing gels. One lane was loaded with Vitrogen (a commercial solution of 98% collagen I and 2% collagen III) as a positive control. Gels were blotted and the collagen band (190 kDa) was detected using an antibody against collagen α1(I) (1:4,000), appropriate secondary antibody, and the chemiluminescence substrate phenylphosphate disodium (CSPD, Roche Diagnostics, Indianapolis, IN). HGF and SMA contents were determined from protein samples of quadriceps muscle using standard Western blot analysis protocols using immunodetection with anti-HGF (1:500) or anti-SMA (1:2,500) antibodies. Blots for collagen α1(I) were stripped and reprobed for GAPDH. For all blots, the optical density of each band was normalized to the optical density of GAPDH (collagen) or per milligram muscle (HGF and SMA) represented by the same band.

Proliferation assay. Cell proliferation was determined using immunostaining for Ki67 (1:4,000), which identifies a protein in nuclei from G1 through M phase. Ki67+ cells were counted separately at 10×400 for regions of muscle. Cells were counted (per field) as myogenic cells in the satellite-cell position (immediately adjacent to fibers) and in the ECM (cells in the interstitium between fibers and in larger areas of fibrosis). Counts were made in five fields along the long axis of each section (one from every animal) and were represented as the distribution of proliferating muscle and ECM cells per five fields.

Functional measures of treatment outcome. Forelimb-grip strength was measured weekly with a calibrated strain gauge. Muscle performance was measured as the mean peak pull from five trials, as previously reported (4).

Overall endurance was assessed weekly with a running wheel device, as previously reported (6). Data were normalized to body weight and compared over time between groups. The final running trial was 4 days after the previous trial to test the functional impact of repeated bouts of exercise. The extent of exercise-induced muscle damage was determined in the final 24-h period of voluntary exercise using Evans blue dye (EBD), as described (6). EBD+ fibers were counted and expressed as a proportion of the total fibers for each section.

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Overall respiratory function was measured in vivo 1 to 2 days before euthanasia using barometric plethysmography (Buxco, Troy, NY). Responsiveness to a methacholine challenge was calculated as $P_{	ext{e-hal}}$, a value derived by computer from the changes in chamber pressure caused by respiratory effort to overcome the bronchoconstriction effect of methacholine (27, 54). Aerosol methacholine was administered in increasing stepped doses (3.1, 6.25, 12.5, 25, and 50 mg/ml) beginning with saline as a baseline. Between doses of methacholine, tidal volume was allowed to return to baseline or a stable minimum value. Peak $P_{	ext{e-hal}}$ at each dose of methacholine and the dose at which $P_{	ext{e-hal}}$ did not return to baseline were determined.

Echocardiographic studies were conducted to assess in vivo cardiac function in control and Halo-treated mice, as previously reported (17, 47). With the use of a noninvasive 13-MHz probe (Vivid 7, General Electric Medical Systems, Milwaukee, WI), mice were imaged awake to maintain heart rate of 550–700 beats/min (48, 56) at baseline and 5 and 10 wk after treatment. Hearts were imaged in the two-dimensional parasternal short-axis view, and three different M-mode echocardiograms were recorded. Left ventricular (LV) end-diastolic diameter, end-systolic diameter, posterior wall thickness, and fractional shortening were measured. End-systolic and end-diastolic volumes were measured from a parasternal long-axis view using the prolate-ellipsoid geometric model, and the LV ejection fraction was calculated. Cardiac output was calculated as the product of stroke volume and simultaneous heart rate; heart rate was >550 beats/min for all tracings. Tissue-Doppler imaging (TDI) was acquired on a parasternal short-axis view at the level of the papillary muscles at a rate of 483 frames per second. For peak-systolic endocardial velocity, a region of interest (0.2 mm × 0.2 mm) in the posterior wall was analyzed. Radial strain rate was measured over a distance of 0.6 mm (Echopac PC, General Electric Medical Systems). Temporal smoothing filters were turned off for all measurements. Values obtained in five consecutive cardiac cycles were averaged. LV wall-motion abnormalities (WMAs) were graded from 0 to 4 by an expert observer (D. S. Jassal) without knowledge of the treatment group, to indicate normal wall motion (0), mild hypokinesia (1), hypokinesia (2), akinesia (3), and dyskinesia (4). The distribution of WMAs was compared between groups. Statistical analysis. Data were analyzed using two-way analysis of variance (ANOVA) or repeated-measures ANOVA to determine the knowledge of the treatment group, to indicate normal wall motion (0), minimum value. Peak Penh at each dose of methacholine and the dose at which $P_{	ext{e-hal}}$ did not return to baseline were determined.

RESULTS

Fibrosis and collagen gene expression. Halo treatment did not result in any toxicity or change in body weight. Collagen I and III gene transcripts were detected in mdx dystrophic mouse muscle by ISH (Fig. 1A). The majority of collagen-expressing nuclei were located in the interstitial compartment between muscle fibers. There was differential collagen expression among muscles for both collagens: TA had the most ISH+ cells, followed by the diaphragm, quadriceps, and heart. This pattern was the same in each treatment group. The distribution of mononuclear cells expressing collagen types I and III in all four tissues was reduced by Halo at 5 and 10 wk ($P < 0.001$ and $P < 0.02$, respectively; Fig. 1, B and C). Between 5 and 10 wk treatment, there was an increase in collagen expression in control (untreated mdx mouse) heart; Halo treatment reduced collagen expression. Central nuclei in regenerating fibers were also noted to express collagen I (Fig. 1A, bottom) and III (data not shown) in quadriceps and TA.

The number of collagen-expressing central nuclei decreased after Halo treatment for 5 and 10 wk compared with control-untreated mdx mice ($P \leq 0.02$ for both muscles) (Table 1). Halo did not change fiber CNI in TA or quadriceps muscles.

Histological observations showed variable fibrosis within each muscle section, among muscles in each animal, and among animals in each group. Staining displayed consistently more intense Sirius red staining, indicative of fibrillar collagen, in the diaphragm than in quadriceps (Fig. 2A). The calculated total average intensity of Sirius red staining at the four sampling angles revealed an increase in diaphragm fibrosis in both treated and untreated mdx mice between the 5- and 10-wk time points. There were also similar levels of birefringent collagen (average intensity per square micrometer) in the two muscles at the 10-wk time point. Some of the central myonuclei in regenerated fibers were stained pink to red with Sirius red when viewed under bright-field optics; however, none of these nuclei demonstrated birefringence when viewed under polarized light at any angle (Fig. 2B).

Halo treatment for 10 wk induced a modest but significant reduction in Sirius red staining in both diaphragm and quadriceps muscles compared with mdx-control muscles ($P < 0.001$; Fig. 2C). An assessment of fibrosis with Masson’s trichrome staining did not show a change with treatment (data not shown).

Collagen, HGF, and SMA content. Collagen type I content was analyzed in partially digested protein extracts of muscle (Fig. 3). Collagen content varied significantly by muscle in all groups for untreated controls ($P < 0.001$ at both 5 and 10 wk) and Halo-treated mice ($P < 0.05$ at both 5 and 10 wk). By this collagenase-digestion method, collagen protein content was higher in TA and heart than in diaphragm and quadriceps for both treated and untreated mdx mice after 5 ($P < 0.001$) and 10 wk ($P < 0.01$). Halo treatment for 5 wk reduced collagen content in the quadriceps, TA, and heart ($P < 0.01$ for each) compared with untreated mdx controls, but there was no significant change in collagen content in the diaphragm with treatment. There was no significant effect of treatment on collagen content after 10 wk of Halo, likely due to large within-group variability at that age.

HGF was increased in quadriceps muscle after 10 wk of Halo ($P = 0.03$; Table 1). SMA content in quadriceps was decreased after 10 wk of Halo ($P = 0.01$).

Cell proliferation. Halo reduced the number of Ki67+-proliferating cells in ECM regions of diaphragm, quadriceps, heart, and TA compared with untreated mdx mice ($P < 0.05$, $P < 0.01$, $P < 0.05$, and $P < 0.05$, respectively) after 5 wk (Fig. 4). There was a concurrent increase in muscle cell proliferation in the quadriceps and TA ($P < 0.01$ and $P < 0.05$, respectively). After 10 wk of Halo, ECM cell proliferation in quadriceps was increased ($P < 0.05$) and muscle-cell proliferation in TA was decreased ($P < 0.05$) compared with respective age-matched mdx control muscles.

Functional outcomes of treatment. Performance in grip strength and running tests was less variable among mdx mice during and after Halo treatment than in the untreated mdx control mice. However, neither strength nor endurance was changed by Halo.

Voluntary running was used to induce damage as a separate indicator of treatment outcome on dystrophic muscle that is
characteristically susceptible to injury by exercise. In the final running trial, mice were exercised only 4 days after the previous run. In this test, Halo-treated mice ran more than four times farther than \( mdx \) control mice (\( P < 0.01 \)) (Fig. 5A). The drop in distance run between the penultimate and final run was significantly lower in the Halo-treated group than for controls (\( P < 0.01 \)).

The proportion of EBD\(^+\) fibers (dye permeable as a result of exercise-induced damage) served as a measure of treatment effects on membrane stability. After 12 wk of Halo treatment,
to methacholine, since Penh rose less with increases in metha-
dose of methacholine increased. Halo attenuated the response
increases in distance run by Halo-treated mice (Fig. 5
fiber damage after running did not change (0.02– 0.03) with
to 0.10) increased as a function of distance run. By contrast, the
mice showed that the proportion of damaged fibers (from 0.05
choconstrictor. Control mice showed increasing Penh as the
tory response (inspiratory and expiratory excursion) to a bron-
control quadriceps (Quads, %)

HGF

SMA

CNI

Central nuclei expressing collagen I mRNA

Central nuclei expressing collagen III mRNA

Values are means ± SE; n, number of mice. *Data are tabulated as
percentage; statistics were run using data as proportion. Control, control-
untreated mdx; HGF, hepatocyte growth factor; SMA, smooth muscle actin;
CNF, centrally nucleated fiber; CNI, central nucleation index; OD, optical
density; TA, tibialis anterior. †P < 0.05; ‡P < 0.01.

The quadriceps muscle exhibited fewer EBD+ fibers than in mdx
control quadriceps (P < 0.02) (Fig. 5B). Muscle from control
mice showed that the proportion of damaged fibers (from 0.05
to 0.10) increased as a function of distance run. By contrast, the
fiber damage after running did not change (0.02–0.03) with
increases in distance run by Halo-treated mice (Fig. 5C).

Barometric plethysmography quantified the overall respira-
tory response (inspiratory and expiratory excursion) to a bron-
choconstrictor. Control mice showed increasing Pems as the
dose of methacholine increased. Halo attenuated the response
to methacholine, since Pems rose less with increases in metha-
choline compared with controls (P < 0.01) (Fig. 6). Halo-
treated mice also maintained a greater baseline tidal volume at
higher doses of methacholine (25–50 mg/ml) than mdx controls
(6.25 mg/ml) (P < 0.01). Findings were identical for mice after
10 and 12 wk of treatment.

Cardiovascular function was measured in Vivo in Halo-
treated and control mdx mice (n = 8 to 9/group) before
treatment and after 5 and 10 wk treatment. Heart rate remained
unchanged over the treatment period (baseline: control, 649 ±
10, and Halo, 671 ± 9; 5 wk: control, 680 ± 14, and Halo,
678 ± 8; and 10 wk: control, 684 ± 10, and Halo, 678 ± 9
beats/min). There were no significant changes in LV end-
diastolic diameter, LV end-systolic diameter, interventricular
septum, posterior wall thickness, fractional shortening, or eje-
tion fraction between mdx control and Halo groups at all three
time points (Table 2; 5-wk data not shown). All mice demon-
strated significant LV hypertrophy that did not change with
Halo treatment. However, treatment significantly improved the
peak endocardial systolic velocity (P < 0.01) and strain rate

Fig. 2. Sirius red staining for fibrosis. A: micrographs of diaphragm from
mdx-control (left) and Halo-treated (right) mice after 5 wk (top) or 10 wk
(bottom), showing collagen birefringence by Sirius red staining under polar
illumination. Original magnification is ×130; bar = 80 μm. B: micrographs
from the same field of regenerated fibers in diaphragm muscle from a
representative mdx mouse treated with Halo for 5 wk. Under the bright-field
view (BF), muscle shows red-stained connective tissue between fibers (yellow-
tinted from picric acid) and a red, centrally located nucleus (black arrow) in a
small fiber. Under polarized light (Pol), connective tissue collagen is birefrin-
gent, whereas the central nucleus is not (white arrow). In situ staining of the
same field shows the centrally nucleated fiber is expressing transcripts for
collagen I (Coll I, white arrow) but not collagen III (Coll III). Original
magnification ×130; bar = 80 μm. C: graph of tissue fibrosis in sections,
measured as overall intensity of Sirius red birefringence, in mdx-control and
Halo-treated mdx mouse diaphragm (5 and 10 wk) and quadriceps (10 wk
only). Fibrosis was reduced in diaphragm and quadriceps (Quads) after 10 wk
of Halo treatment (*P < 0.001).
Halo treatment for 5 wk reduced (P < 0.01) collagen content in quadriceps, TA, and heart but not diaphragm. *P < 0.01, from 5-wk control of same muscle.

(P < 0.01). In addition, Halo treatment induced a significant functional improvement in the qualitative measure of ventricular wall motion (Fig. 7, supplemental videos S1 and S2; note: all supplemental material may be found posted with the online version of this article). Echocardiography demonstrated LV WMAs in all mdx mice at baseline, from hypokinetic to dyskinetic walls. In contrast, Halo reduced the number and severity of WMAs after 5 wk of treatment, when the LV demonstrated either normal wall motion or only mild hypokinesis, compared with untreated mdx-control mice where akinesis and dyskinesis persisted (P < 0.01; Fig. 7E). The effect of Halo on cardiac function was sustained up to 10 wk, and only hypokinesis was observed compared with a wide range of WMAs in untreated controls.

**DISCUSSION**

Halo treatment for 10–12 wk improved the structural character and functional performance of striated muscles in old dystrophic mice with well-established disease and dysfunction (followed up to the age of 10 to 11 mo). After 5 wk, Halo had decreased expression of collagen types I and III in four muscles (TA, diaphragm, quadriceps, and heart) and decreased collagen protein content in three muscles (TA, heart, and quadriceps). Halo also decreased ECM cell proliferation and increased skeletal muscle cell proliferation after 5 wk treatment. After 10 wk of Halo treatment, in addition to further decreases in collagen I and III expression in all muscles, fibrosis was reduced in the diaphragm and quadriceps muscles, and the level of HGF was increased and SMA was decreased in quadriceps muscle. These changes to muscles of older mdx mice did not affect CNI, an index of the overall progression of dystrophy. Most importantly, the changes in limb, respiratory, and cardiac muscle were sufficient for significant improvements in performance. The functional gains in limb muscle included reduced susceptibility to exercise-induced injury and faster restoration of function following an exercise challenge. Respiratory muscle showed better compensation for a methacholine challenge, and cardiac muscle demonstrated a marked improvement in ventricular WMAs. A new approach to measuring collagen content using collagenase predigestion of protein samples, novel observations of collagen expression by central nuclei in regenerated muscle fibers, and new information on differential treatment effects between muscles extend our understanding of basic processes in mouse muscular dystrophy. Overall, Halo is a potent antifibrotic agent that induces a functionally significant attenuation of preexisting fibrosis in skeletal and cardiac muscle while reducing new collagen synthesis. Our findings have significant implications for translation of basic research findings on an animal model to clinical application in the treatment of DMD and possibly other conditions where muscle fibrosis restricts function.

Halo treatment reduced the expression of gene transcripts for collagen types I and III decreased in all muscles. This is consistent with previous reports of Halo effects in new and established fibrosis in nonmuscle tissues and in dystrophic muscles of younger mdx mice. Since there is a reciprocal relationship between the differentiation of mononuclear myogenic cells and the expression and deposition of collagen in the interstitium by cells that diverge from the myogenic lineage (1), the distribution of ISH* cells was used as an indicator of collagen synthesis (and subsequent secretion) in the different muscles. Similarly, the expression of collagen transcripts is also reported as a screen for the level of fibrosis developing in an mdx mouse muscle (23, 42). Therefore, although the mechanisms of Halo effects are under investigation, the current findings of decreased collagen I and III expression in older mdx mice treated with Halo and the findings in younger Halo-treated mdx mice are consistent with the notion that Halo is a potent antifibrotic agent. In younger mdx mice, reduced collagen expression was accompanied by reduced Smad3 phosphorylation and reduced fibrosis, suggesting that similar pathways may be targeted by Halo in reducing collagen expression and content in older mdx mice.

*Mdx muscle is reported to show signs of fibrosis very early [starting at 3 wk in the diaphragm and becoming conspicuous after 5 wk (49, 50)]. Fibrosis increases with increasing age (23, 25) and as a result of greater muscle activity, causing fiber injury and inflammation (42). Collagen in mdx diaphragm increases progressively throughout the life span (24), as demonstrated in diaphragm muscle in this study. Studies indicating that mdx muscle lacks or has minimal fibro-fatty change in connective tissue are typically interpreted compared with the severe changes observed in DMD muscle (13, 15). However, most studies, including a Halo study on younger mdx mice (Turgeman et al., unpublished), show that fibrosis and collagen expression (collagens I and III) and deposition rise with age in many mdx mouse muscles, even between 4 and 12 wk of age.
The variability between and within groups of muscles may have been lower if collagen were assayed by studying the content of hydroxyproline (which is also correlated to collagen transcript expression), although collagen contains only 13.5% hydroxyproline (10, 11), which may not be representative of total collagen content. Alternatively, variations in daily animal activity (resulting in exercise-induced fiber damage and subsequent inflammation) or the variable time course by which fibrosis develops and matures in particular muscles would also contribute variability in the data. Halo induced a decrease in fibrosis in both diaphragm and quadriceps muscles after 10 wk treatment, according to studies of the overall average intensity of Sirius red staining sampled under polar illumination at four different angles. These changes were consistent with studies in younger mdx mice, despite the different doses of Halo (5 μg/g in younger mice vs. 3 μg/g in older mice).

A new protocol for Western blot analysis to detect collagen protein was developed for further investigation of Halo treatment effects on muscle in older mdx mice given the known antifibrotic effects on other tissues and the findings that Halo reduced fibrosis in muscles of young mdx mice. The modified procedure included brief collagenase digestion of glycine bonds in the triple-helical region of native collagen. After synthesis, collagen is deposited in the ECM where it matures through a process of cross-linking that increasingly stabilizes the assembly of collagen fibrils, especially in aging and fibrosis (64, 65). Since collagen gene expression and the content of 190-kDa collagen protein both decreased after 5 wk treatment, before the reduced fibrosis observed at 10 wk treatment, Halo may affect the molecular, fibrillar conformation of collagen or prevent cross-linking at lysine residues (32) before changing the stable structure of collagen that is detected with birefringence. In a microarray study of liver fibrosis, Halo inhibited the expression of lysyl oxidase, an enzyme regulated by TGF-β that is responsible for collagen lysyl cross-linking (21). Halo may also remove cross-links by activating metalloproteinase activity: a preliminary study of matrix metalloproteinase (MMP)9 activity (assayed by zymography) showed increases over control levels in diaphragm muscle from 7-mo-old mdx mice after 4 wk treatment with Halo (Pines et al.; unpublished data), whereas MMP9 activity was undetectable in young Halo-treated mdx mice (Turgeman et al., unpublished data).

The decrease in collagen protein in older mdx mice would be consistent with a similar effect to increase MMP9 activity in 7-mo-old mice. Here, the intermuscle variation may represent differences in maturation, stability, or cross-linkage of collagen, rather than total ECM collagen content. This would account for finding the highest level of collagen in the TA using the digestion method: fibrous connective tissue in TA of older mice may contain the least mature forms of collagen.
compared with other muscles such as diaphragm where fibrosis is extensive (61). Extensive fibrosis in the diaphragm is more resistant to collagenase digestion in the preparation of single fiber cultures (Anderson, unpublished observations). Additionally, immature collagen may be more susceptible to the action of MMPs than highly cross-linked collagen in long-standing fibrosis. Since the level of fibrosis has a complex relationship to collagen expression, synthesis, secretion, and degradation, the activity of MMPs (or tissue inhibitors of MMPs) and TGF-β signaling may be involved in the mechanism by which Halo reduces collagen expression and content in dystrophic muscle. The novel approach to assaying collagen protein was developed for this investigation to account for changes in collagen gene expression that only modestly reduced fibrosis according to histochemical staining. The method has the potential to reveal previously inaccessible details of the time course of collagen deposition and maturation and the changes due to disease or treatment in tissues with primary and secondary fibrosis.

Table 2. Echocardiographic parameters at baseline and after 10 wk treatment for control and halofuginone-treated groups

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Normal Value</th>
<th>Baseline</th>
<th>Week 10</th>
<th>P Value</th>
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</thead>
<tbody>
<tr>
<td>Heart rate, beats/min</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control mdx</td>
<td>649±10</td>
<td>684±10</td>
<td>NS</td>
<td></td>
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<tr>
<td>Halofuginone</td>
<td>671±9</td>
<td>678±9</td>
<td>NS</td>
<td></td>
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<td>PWT, mm</td>
<td>&lt;0.7</td>
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<tr>
<td>Control mdx</td>
<td>1.0±0.1</td>
<td>1.0±0.1</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Halofuginone</td>
<td>1.0±0.1</td>
<td>1.0±0.1</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>LVEDD, mm</td>
<td>3.0–3.2</td>
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<td></td>
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<tr>
<td>Control mdx</td>
<td>3.0±0.4</td>
<td>3.1±0.6</td>
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<td>Halofuginone</td>
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<td>3.1±0.8</td>
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<td>Fractional shortening, %</td>
<td>55–60</td>
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<td>Control mdx</td>
<td>54±0.4</td>
<td>52±1.0</td>
<td>NS</td>
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<td>Halofuginone</td>
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<td>52±0.7</td>
<td>NS</td>
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<td>Ejection fraction, %</td>
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<td>Control mdx</td>
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<td>NS</td>
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<td>Halofuginone</td>
<td>82±1</td>
<td>82±1</td>
<td>NS</td>
<td></td>
</tr>
<tr>
<td>Vendo, cm/s</td>
<td>3.0–3.5</td>
<td></td>
<td></td>
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<tr>
<td>Control mdx</td>
<td>1.3±0.3</td>
<td>1.2±0.2</td>
<td>NS</td>
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<tr>
<td>Halofuginone</td>
<td>1.4±0.2</td>
<td>2.4±0.1</td>
<td>&lt;0.01</td>
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<tr>
<td>Strain rate, s⁻¹</td>
<td>20–25</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>Control mdx</td>
<td>12±2</td>
<td>11±2</td>
<td>NS</td>
<td></td>
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<tr>
<td>Halofuginone</td>
<td>11±3</td>
<td>16±2</td>
<td>&lt;0.01</td>
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</table>

Values are means ± SE; n = 8–10 mice/group. Baseline, before the start of treatment; PWT, posterior wall thickness; LVEDD, left ventricular end-diastolic diameter; Vendo, peak endocardial systolic velocity; NS, not significant.
Declining muscle function as DMD progresses is closely associated with significant fibrosis, marked by abnormally high levels of collagens I and III (18), and contractures chronically limit range of motion, postural stability, and respiratory and cardiac function (12, 53). Fibrotic tissue accumulates as muscle tissue and architecture are damaged through inflammatory and dystrophic processes. Indeed, normal muscle is also affected by fibrosis that develops during and after injury and repair events, and fibrosis is known to delay the return of function (9). Based on the effect of Halo treatment to reduce collagen content and collagen I and III gene expression in dystrophic mouse muscle, tissue contractures in DMD and other conditions may also respond to Halo by changes that would improve muscle range of motion and function.

The reduction in SMA is further evidence of the antifibrotic effect of Halo. As the cells synthesize SMA upon activation, myofibroblasts participate in mechanical or biophysical remodeling of muscle, and SMA⁺ myofibroblasts disappear when there is no longer any active contraction of ECM collagen (16, 63). These changes are consistent with the report that Halo inhibits the activation of fibroblasts into myofibroblasts (58). After 10 wk Halo treatment, SMA was reduced to the level in normal, wild-type mouse quadriceps. Interestingly, HGF is itself an antifibrotic protein that antagonizes TGF-β (36, 37) and inhibits SMA expression (67). HGF treatment successfully reduced fibrosis in skin, lung, and renal tissue via TGF-β inhibition (20, 28, 31). Although previous work showed that Halo acts downstream to TGF-β (38), the increase in quadriceps HGF after 10 wk of Halo suggests that Halo may also act upstream to TGF-β to induce HGF synthesis by cross-talk with other signaling pathways. Further study of HGF and cell proliferation in the diaphragm and other muscles is needed to confirm the notion that the upregulation of HGF and proliferation of cells in the ECM and muscle are correlated. Increased numbers of Ki67⁺ cells in muscle after 5 wk of Halo and regeneration from exercise-related damage are consistent with the high levels of HGF that would promote myogenic cell proliferation and migration. Furthermore, since damage from exercise was reduced (Ebd data) without changing CNI, Halo may have promoted more effective repair via HGF: reduced collagen expression and accu-
mulation, and fewer myofibroblasts to contract in the ECM would alleviate the mechanical strain on fiber membranes during exercise, notwithstanding the progression of dystrophy.

Surprisingly, central nuclei in fibers in two muscles regenerating from dystrophic injury were noted to express collagen I and III. Notably, Halo decreased the expression of collagen genes by central myonuclei. To our knowledge there are no reports to indicate that central nuclei inside regenereated muscle fibers of the TA or quadriceps express collagen. A recent report found that proliferating primary myoblasts and C2C12 cells can express collagen I until they differentiate and that cells surrounding cultured myofibers from old dystrophic mice express collagen (1). Since there was no difference in central nucleation itself, the studies of collagen expression suggest Halo has direct effects on muscle fibers as well as fibroblasts. The interesting appearance of red, nonbirefringent central nuclei in regenerating fibers and the reduced level of collagen expression by central nuclei in mononucleus cells after Halo treatment suggest that there may be a relationship between the staining (acidic residues in euchromatin that bind stain and/or due to intranuclear, nonfibrillar collagen) and the actively expressed genes in fibers regenerating during improvements in structure and function in the dystrophic muscles.

Muscle function studies showed Halo-reduced exercise-induced damage. Since the final two running tests were separated by only 4 days, a dramatic drop in distance run was expected in control mice since that interval time is too short for complete repair (40). Similar improvements in muscle repair were reported for the antifibrotic effects of the TGF-β inhibitor, suramin, in mice with a muscle laceration injury (9) and after combined steroid and l-arginine treatment in mdx mice (6). The results of the final running test, therefore, implicate Halo in improving muscle repair, possibly through reduced collagen deposition and cross-linking that would increase ECM flexibility and greater activation and migration of the myoblasts proliferating during treatment. Halo treatment also reduced the variation in grip strength and voluntary running, although there was no increase in strength or distance run. Similar stabilization was reported after long-term steroid treatment (33).

In DMD, dystrophy leads to severe deficits in respiratory function. The current study used plethysmography to study respiratory function in mdx mice. Halo-treated mice had a smaller response to a methacholine challenge than controls, suggesting that the decreased collagen I and III expression in the diaphragm during treatment was effective in improving respiratory function, since that muscle has such an important role in respiration and is most severely affected by dystrophy in mdx mice. Functional improvement of Halo-treated mice, demonstrated using plethysmography, was consistent with the reduced level of fibrosis in the diaphragm of the same animals.

Cardiac function was also significantly improved by Halo treatment. Murine echocardiography effectively demonstrated improvements in ventricular wall motion, which evolved from severe dyskinesis toward more synchronous, concentric contractions of the ventricle. Echocardiography is a highly sensitive tool for assessment of structural and physiological indexes that can reveal disease onset and progression (53a, 60). The progression of cardiomyopathy was also arrested, since wall motion did not worsen from 5–10 wk treatment (according to the observations of LV wall motion), as did control mice. Although there were no quantitative changes in typical measures of LV systolic function with treatment, TDI indexes including endocardial velocity and strain rate were examined to detect subtle regional WMAs that can appear before a drop in ejection fraction (47). TDI parameters were abnormal at baseline in both groups. In mdx control mice, TDI stayed abnormal, whereas in Halo-treated mice, TDI improved, attesting to the treatment-induced gains in LV systolic function using these novel measures. Although there was no gross evidence of a dilated cardiomyopathy, regional wall motion improved with Halo treatment. Notably, in young mdx mice, Halo partly prevented the LV hypertrophy (Turgeman et al., unpublished) that is typical of mdx mouse cardiomyopathy, along with fibrosis and ventricular dysfunction (2, 53, 59). Although mdx mouse cardiac pathology may not be regulated by myostatin, a member of the TGF-β superfamily (12), cardiac changes induced by Halo (improved function and reduced collagen content and gene expression) are consistent with Halo preventing TGF-β-dependent gene activation and inflammation (21), thus preventing further progression of cardiomyopathy. With the high frequency of LV dysfunction in DMD (20% at 10 yr of age) and the goals for early prevention and effective resolution of fibrosis in overt clinical cardiomyopathy (41), Halo is highly attractive for clinical trials in DMD. This might be particularly valuable to investigate relative to cardiomyopathy that can progress rapidly and appear even in carriers of DMD. The serious, ultimately lethal impact of DMD is due to weakness in cardiac, respiratory and limb muscles, progressive fibrosis, and sequelae thereof. Present findings suggest that Halo has a significant potential to be a successful pharmacological approach to the cardiac and respiratory dysfunction observed in DMD. The results encourage a rapid testing of Halo as a potential therapeutic agent for established muscle fibrosis in human disease. A positive outcome of clinical trials on Halo as an antifibrotic agent in DMD would, at the very least, expand the window and extent of opportunity for effective therapies using anti-inflammatory and regenerative drugs, precursor and stem cells, and/or genetic constructs.

In conclusion, Halo treatment of older mdx dystrophic mice contributed to significant structural remodeling to reduce fibrosis, which in turn supported important functional gains in muscles in the limbs, diaphragm, and the heart. Functional deterioration was attenuated or reversed, and collagen expression and synthesis were reduced. This increased the resistance to exercise-induced muscle damage and improved muscle repair, respiratory functional capacity, and cardiac function. These findings are especially important because the drug acted concurrently to resolve preexisting limitations on function related to fibrosis and to reduce new collagen synthesis. Together with our findings in young mdx mice, the present results in older dystrophic animals with established fibrosis and advanced disease demonstrate the strong potential for Halo as an antifibrotic treatment for DMD. It will be critical to understand the pathways by which Halo acts on mature fibrotic connective tissue in the different muscles, while rapidly translating these findings toward clinical trials.

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REFERENCES


