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Hydroxy group requirement for halofuginone-dependent inhibition of muscle fibrosis and improvement of histopathology in the mdx mouse model for Duchenne muscular dystrophy

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* Equal contribution

Running title: Deoxyhalofuginone and mdx histopathology

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Summary

In Duchenne muscular dystrophy (DMD), the progressive loss of muscle and its ability to function is associated with significant fibrosis, representing the major disease complication in patients. Halofuginone, a halogenated analog of the naturally occurring febrifugine, has been shown to prevent fibrosis in various animal models, including those of muscular dystrophies. Here, two optically active enantiomers of deoxyhalofuginone—a halofuginone analogue in which the hydroxy group in position 3 was removed from the piperidinyl entity—were evaluated with respect to their effect on muscle histopathology in mdx mice. Male mdx mice were treated with either deoxyhalofuginone (as single enantiomers or in racemic form), or halofuginone, for 10 weeks, starting at the age of 4 weeks. Halofuginone caused a significant reduction in total collagen content, degenerative areas, as well as in utrophin and phosphorylated-Smad3 levels in the mdx diaphragms. However, neither the deoxyhalofuginone enantiomers, nor its racemic form had any effect on these parameters. A positive effect of the deoxyhalofuginone (+)-enantiomer was observed on myofiber diameters; however, it was lesser than that of halofuginone. It is concluded that the hydroxy group plays a key role in halofuginone’s effects related to fibrosis in DMD, and points towards the transforming growth factor β/Smad3 signaling pathway being involved in this inhibition. Elucidation of the structure–function relationship of halofuginone, in relation to inhibiting fibrosis in muscular dystrophies, is of the utmost importance for creating the next generation of anti-fibrotic therapies that will be more efficacious and less toxic, hence improving life quality of patients.

Key words: Skeletal muscle; Duchenne muscular dystrophy; Fibrosis; Utrophin; Halofuginone
Introduction

Muscular dystrophies (MD) are genetic disorders characterized by the progressive loss of muscle strength and integrity. Dystrophic muscles show variation in myofiber size, infiltration of connective and fatty tissue, and centrally located nuclei. The most common form of MD is Duchenne MD (DMD) in which there is a near absence of dystrophin protein in skeletal muscles. This causes repeated cycles of myofiber degeneration-regeneration and the progressive replacement of the muscle tissue with fibrotic tissue consisting of mainly collagen type I and III (Dunace et al., 1980; Spector et al., 2013; Gaiad et al., 2014), ultimately leading to loss of ambulation and death from respiratory or cardiac failure (reviewed in Lessa et al., 2016). Utrophin, a 395-kDa protein with a high degree of homology with dystrophin is elevated in early stages of the disease (Pearce et al., 1993; Grady et al., 1997, Moorwood and Khurana, 2013). Utrophin was found to be expressed in a reverse correlation with fibrosis levels in muscles of DMD patients and mdx mice – the most common model for DMD (Levy et al., 2015).

The tumor growth factor-β (TGF-β) is a central mediator of fibrogenesis; it is upregulated in fibrotic diseases and is a main inducer of the myofibroblast phenotype (Prud'homme, 2007). The autocrine release of TGF-β from necrotic myofibers contributes to the increasing loss of muscle regeneration and the increase in fibrosis (Melone et al., 1999). Conversely, inhibition of TGF-β signaling restores muscle strength (Heydemann et al., 2009; Acuña et al., 2014).

Halofuginone serves as a novel anti-fibrotic therapy (Pines, 2008, 2014; Luo et al., 2017). Its inhibitory effect on fibrosis has been reported in numerous animal models for diseases with excess fibrosis (Nagler et al., 1996; Bruck et al., 2001; Zion et al., 2009), as well as in clinical trials, such as for Kaposi sarcoma (de Jonge et al., 2006) and scleroderma (Pines et al., 2003). Several mechanisms for halofuginone's effects against fibrosis have
been suggested, one of which is inhibiting Smad3 phosphorylation downstream of TGF-β signaling, thereby inhibiting the activation of fibroblasts and their ability to synthesize extracellular matrix - regardless of their origin or location (Pines, 2008; Pines and Spector, 2015; Luo et al., 2017). In addition, halofuginone was reported to inhibit Th17 cell differentiation via association with and reduction of prolyl-tRNA synthetase (ProRS) activity leading to activation of the amino acid starvation response (Sundrud et al., 2009; Keller et al., 2012).

In murine models of MDs, halofuginone decreased collagen levels with improvement of cardiac function and enhanced motor coordination and balance (Turgeman et al., 2008; Huebner et al., 2008; Nevo et al., 2010; Halevy et al., 2013). The inhibitory effect of halofuginone on fibrosis was accompanied with elevation of utrophin levels in mdx mice (Levy et al., 2015). In addition, halofuginone has been shown to exert direct effects on muscle cells. Halofuginone enhanced cultured fusion of myotubes derived from various MD mouse models (Nevo et al., 2010; Roffe et al., 2010; Halevy et al., 2013). Recently, we have reported the promotive effect of halofuginone on the cell-cycle activity of myofiber-attached satellite cells (Barzilai-Tutsch et al., 2016).

Halofuginone \{7-bromo-6-chloro-3-[3-(3-hydroxy-2-piperidinyl)-2-oxopropyl]-4(3H)-quinazolinone\} is a halogenated analog of the naturally occurring quinazoline-type alkaloid (+)-febrifugine (Fig. 1A)—the latter was originally isolated from Dichroa febrifuga (Koepfli et al., 1947; McLaughlin et al., 2014). Because the initial activities of halofuginone were reported on the racemic material, most subsequent biological work appears to have been performed on this mixture of optical isomers. Moreover, halofuginone did not arise from a specific fibrosis discovery program (Linder et al., 2007), or any other program aiming at targeting specifically MDs; it possesses multifaceted actions (Pines and Spector, 2015). Notably, at high levels, halofuginone proved to have some toxicity and/or adverse
effects (Van de Casteele et al., 2004). Linder et al., (2007) resolved a racemic mixture of halofuginone into its enantiomeric forms and reported a different activity of each against Cryptosporidium parvum. Therefore, our hypothesis was that halofuginone’s overall structure may not be optimal for inhibiting fibrosis in general and in MDs in particular—other structural analogues may well possess improved activities. In addition, it could well be that the single enantiomers may possess different, or even opposite effects on muscle fibrosis and overall histopathology. Here, the efficacy of two optically active enantiomers of deoxyhalofuginone, an analogue of halofuginone in which the hydroxy group has been removed from the piperidinyl ring (Fig. 1B; Zaidan et al., 2015), were evaluated on muscle histopathology in mdx mice.

**Materials and Methods**

*Materials*

Halofuginone hydrobromide was obtained from Akashi Therapeutics, (Newton, MA, USA). Hematoxylin and eosin were purchased from Surgipath Medical Industries (Richmond, Canada). Sirius red F3B was obtained from BDH Laboratory Supplies (Poole, UK).

*Animals and experimental design*

Male mdx mice [C57BL/10ScSn-Dmdmdx/J (Stock 001801), dystrophin-deficient)] (Jackson Laboratories, Bar Harbor, ME, USA) were housed in cages under constant photoperiod (12 L:12 D) with free access to food and water. The experimental design was based on previous studies on mdx mice in which halofuginone effects were tested in a time-course manner at different doses (Turgeman et al., 2008). Mice were divided into 5 groups (n = 4-5) and were injected intraperitoneally (ip) with either saline, or halofuginone as a positive control, or with the deoxyhalofuginone enantiomers - either alone or as a
combination (racemate) - 10 µg per mouse, three times per week for 10 weeks starting at 4 weeks of age (Turgeman et al., 2008). As previously described (Zaidan et al., 2015), the optically active deoxyhalofuginone enantiomers [termed, (+)-Deoxyhalo and (−)-Deoxyhalo], consisted of halofuginone in which the hydroxy (OH) group in the 3-position of the piperidine ring was deleted (see Fig. 1B). Biopsies from diaphragm muscles were collected for further analyses. All animal experiments were carried out according to the guidelines of the Volcani Center Institutional Committee for Care and Use of Laboratory Animals (IL-558/14).

**Histology and fibrosis evaluation**

Diaphragm samples were fixed with 4% paraformaldehyde in PBS at 4°C overnight, dehydrated and embedded in paraffin as previously described (Turgeman et al., 2008), and serial sections (5 µm) were prepared. For fibrosis evaluation, the sections (five sections per mouse, n = 4-5) were stained with Sirius red as previously described (Turgeman et al., 2008) and analyzed with ImagePro software (Media Cybernetics, Inc., Silver Spring, MD, USA). Photographs (at least twenty evenly distributed photographs from each group) were taken for analysis at x 20 magnification under a light microscope (Olympus, Hamburg, Germany) with a DP-11 digital camera (Olympus). The results were calculated as the red area divided by the total area (red + green) and presented as the proportion of fibrotic muscle area (mean ± SE). Special care was taken to exclude blank areas, which were assumed artefactual.

**Immunohistochemistry**

Paraffin-embedded muscle sections were deparaffinized and rehydrated with ethanol and citrate buffer. The sections were then incubated with 3% normal donkey serum,
10% bovine serum albumin in 0.05% Tween 20-PBS (v/v) for 1 h at room temperature followed by overnight incubation at 4°C with either polyclonal rabbit anti-phosphorylated-Smad3 (pSmad3) antibody (1:50, Santa Cruz Biotechnology, Dallas, TX, USA), or polyclonal rabbit anti-utrophin (1:50, Santa Cruz Biotechnology). The secondary antibody was Cy3 goat anti-rabbit IgG (1:200, Jackson Laboratories, Bar Harbor, ME, USA). Nuclei were then stained with 4’,6-diamidino-2-phenylindole (DAPI; Sigma Aldrich, St. Louis, MO, USA) in PBS (1:1000). For Smad3 evaluation, the sections were visualized under a light microscope (Olympus) with a DP-11 digital camera (Olympus). The number of Smad3-positive nuclei was calculated per total nuclei and at least 4000 nuclei were counted in 18 pictures taken from at least four independent mice per group. For utrophin detection, microscope observations and image acquisition were performed with the Leica sp8 inverted laser-scanning confocal microscope, equipped with a 405-nm diode laser, 488-nm OPLS, 552-nm OPLS and the HC PL APO CS2 63X/1.40 oil objective. “RED” was excited at 552 nm. U tropin quantification analysis was performed as described (Levi et al., 2015); 10 sections along the diaphragm from three to four independent mice were acquired and utrophin expression levels were calculated using ImageJ software and presented as percent of red pixel area out of total image area (pixel/area).

Myofiber diameter analysis

Muscle sections were stained with hematoxylin and eosin and the myofiber diameter was determined by analyzing the lesser diameters of myofibers as described (Dubowitz, 1985; Halevy et al., 2004). At least 10 arbitrary fields in two to three serial sections of each muscle sample from each mouse were photographed under a light microscope (Olympus) with a DP-11 digital camera (Olympus). Myofiber diameter was then determined with Adobe Photoshop software. In each muscle sample, the lesser myofiber
diameter was measured for individual myofibers, analyzing between 4,300 and 4,500 myofibers from four to five mice per treatment. Since there were no statistically significant differences among mice of the same treatment group, all data for the same treatment were pooled.

Statistical analysis

The data were subjected to one-way analysis of variance (ANOVA) and to all-pairs Tukey-Kramer HSD test. All statistical analyses were conducted using JMP® software (SAS Institute, 2009). Results were considered statistically significant at $p \leq 0.05$.

Results

Effect of deoxyhalofuginone on fibrosis

Halofuginone (serving as a positive control) treatment of male $mdx$ mice from the age of 4 weeks revealed lower collagen staining in their diaphragms at 14 week-age compared with those in the non-treated mice (termed, Control). The latter exhibited wide bundles of collagen surrounding the myofibers as opposed to much thinner collagen bundles in the halofuginone-treated mice (Fig. 2A, red stain). In contrast, a picture similar to the Control was observed in the diaphragms of the mice treated with deoxyhalofuginone, either as single enantiomers, (+)-Deoxyhalo and (−)-Deoxyhalo, or as the racemic form (±)-Deoxyhalo (2A). A quantitative analysis revealed a significant reduction in collagen content in the halofuginone-treated group, whilst no reductive effect by the deoxyhalofuginone enantiomers and the racemic compound was observed relative to the Control (Fig. 2B).
Effect of deoxyhalofuginone on myofiber diameter and myotube fusion

Many areas of degeneration as well as infiltration of inflammatory cells were observed in the diaphragms of the non-treated mdx mice at 14 weeks of age (Fig. 3A, white arrows). This pathology was improved by the halofuginone treatment, with the appearance of more uniform myofibers and less degenerative areas. The histopathology of the diaphragms of the deoxyhalofuginone-treated mice remained quite similar to those of the control non-treated mice (Fig. 3A). Analysis of diameter distribution of the myofibers in the experimental mdx mice revealed a higher total range of myofiber-diameter values in the halofuginone-treated group than in the non-treated one (between 0 and 60 µm vs. 0 and 45 µm, respectively). The total range of myofiber-diameter values of the enantiomers was similar to that of the Control mice, as was the value obtained for the mice treated with racemic material (between 0 and 45 µm). Compared to the Control, only the halofuginone treated group had a curve shift to the right, i.e. towards the higher diameter bins (Fig. 3B). Mean myofiber diameter was significantly higher in the halofuginone-treated mdx mice vs. the non-treated mice (Fig. 3C). While the mean myofiber diameter was increased in the group that was treated with the (+)-enantiomer, it was profoundly lower than that of the halofuginone group. In the groups that received the (−)-enantiomer and the racemic compound, the mean myofiber diameter remained similar to the control, non-treated group.

Utrophin expression

Utrophin levels, known to be increased in mdx mice compared to wild type mice, were recently reported to be further elevated by halofuginone (Levi et al., 2015). Here, an immunostaining for utrophin in the diaphragms of the experimental mice at 14 weeks of age, revealed a few myofibers with positive staining for utrophin in the control mdx diaphragm (Fig. 4, white arrows). As expected, more utrophin-positive myofibers were observed in the
diaphragm of the halofuginone-treated mice. However, hardly any myofibers with utrophin staining were observed in the diaphragms of mice that were treated with deoxyhalofuginone, either as single enantiomers, or as the racemic compound (Fig. 4).

*The phosphorylated form of Smad3 expression is not affected by deoxyhalofuginone*

The lack of deoxyhalofuginone effects on collagen content and histopathology of *mdx* diaphragms could be explained, at least in part, by abolishing the halofuginone-dependent inhibition of Smad3 phosphorylation. To verify this assumption, diaphragm sections of 14-week-old mice were immunostained for the phosphorylated form of Smad3 with a specific antibody. Whereas numerous nuclei were found with positive staining for phospho-Smad3 in the control group (Fig. 5, white arrows), only a few positively stained nuclei were found in the halofuginone-treated experiment. In contrast, hardly any effect on the levels of Smad3 phosphorylation was observed in the deoxyhalofuginone, single enantiomer, or racemic-treated, groups; all of which presented profound numbers of phospho-Smad3-positive nuclei (Fig. 5). Quantitative analysis of the percent of phospho-Smad3-expressing nuclei out of total nuclei showed a minor and non-significant reduction in these values in the deoxyhalofuginone treated mice, relative to control mice, whereas almost fourfold reduction was noted in response to halofuginone treatment (Fig. 6).

**Discussion**

The anti-fibrotic activity of halofuginone and its ameliorating effects on tissue pathology have been shown in numerous types of diseases including MDs, in which excess collagen is the hallmark of the disease. Yet, these promotive effects of halofuginone—a halogenated analog of febrifugine, were demonstrated using racemic halofuginone. Moreover, other analogues of halofuginone have not been analyzed, at least in the context of
MDs. Here, for the first time, a halofuginone analogue in which the hydroxy group has been removed from the piperidinyl ring has been tested for its efficacy in the \textit{mdx} mouse model for DMD. The results of this study emphasize the importance of the hydroxy site on the piperidinyl ring in exerting halofuginone's effects on fibrosis in the \textit{mdx} mice, regardless of whether the analogue is given as a racemic mixture, or as single optical forms (enantiomers).

In \textit{mdx} mice, fibrosis is already apparent at 4 weeks of age and continues to develop. At 14 weeks of age it is recognized by large fibrotic bundles surrounding the myofibers (Fig. 2; Turgeman et al., 2008). While, in agreement with previous studies, the racemic form of halofuginone significantly inhibited the collagen content in the diaphragms (Turgeman et al., 2008; Bodanovsky et al., 2014), the removal of hydroxy group from the piperidinyl ring profoundly reduced halofuginone's promotive effects, regardless as to whether deoxyhalofuginone was used in a racemic form, or as single enantiomers. Similarly, in these mice significant areas of inflammatory cells and myofibers with smaller diameters in the diaphragms remained as in the non-treated, controls (Fig. 3). Moreover, hardly any effect of deoxyhalofuginone was observed on utrophin levels in the diaphragms (Fig. 4). Utrophin levels are elevated in the early stages of DMD and are believed to partially compensate for dystrophin absence in DMD patients and \textit{mdx} mice (Davies and Nowak, 2006; Moorwood and Khurana, 2013). Recently, a reverse correlation between fibrosis and utrophin levels has been established in both DMD patients, or in \textit{mdx} mice that were treated with halofuginone (Levi et al., 2015). Here, in agreement with the previous study, this reverse correlation was noticed in all treatments: utrophin levels in the diaphragms proved to be directly opposite to collagen levels and were negligible in the control and deoxyhalofuginone-treated mice, whilst in the halofuginone-treated animals these levels were significantly higher (Fig. 4). Together with the previous report, the data support our notion that common links between the fibrotic and utrophin-synthesis pathways exist, and
illustrate the requirement for the piperidinyl ring's hydroxy group for halofuginone's action in elevating utrophin levels and reducing fibrosis in DMD.

The inhibitory effect of halofuginone on Smad3 phosphorylation downstream to TGF-β is believed to be a canonical pathway in which halofuginone exerts its functions on fibrosis (McGaha et al., 2002; Pines and Halevy, 2011; Pines and Spector, 2015; Luo et al., 2017), and also directly on muscle cell apoptosis (Bodanovsky et al., 2014; Barzilai-Tutsch et al., 2016). In contrast to halofuginone, deoxyhalofuginone that was injected either as single enantiomers, or in racemic form, did not have any significant effect on Smad3 phosphorylation (Fig. 5), directly supporting a loss-of-function mechanism for halofuginone. The profoundly reduced effect on Smad3 phosphorylation by the removal of a single hydroxy group from the piperidinyl ring clearly demonstrates that this group is crucial for halofuginone's action on Smad3 phosphorylation downstream to TGF-β, and thereby its effect on fibrosis.

The negative effect of deoxyhalofuginone vs. halofuginone was less dramatic with respect to myofiber hypertrophy; in fact, all forms had some positive effect on myofiber diameter relative to control with the (+)-Deoxyhalo enantiomer demonstrating a more pronounced effect. This indicates that the hydroxy group on the piperidine ring is less essential for halofuginone's effect on myotube fusion. These results raise the possibility that the mechanism by which deoxyhalofuginone affects myofiber fusion and fibrosis are distinct, and that additional to the Smad3 signaling pathway other pathways may be involved in mediating the deoxyhalofuginone and halofuginone pathways. For example, our previous studies have demonstrated the direct effects of halofuginone on muscle cell cycle events, survival and myotube fusion via the MAPK/ERK and PI3K/Akt signaling pathways (Roffe et al., 2010; Bodanovsky et al., 2014; Barzilai-Tutsch et al., 2016). The Smad3 signaling pathway was reported to be inhibited, at least in part, via direct association with
phosphorylated Akt and MAPK/ERK (Roffe et al., 2010). Nevertheless, it may well be that MAPK/ERK and Akt signaling, in addition to their effect on Smad3 phosphorylation, directly mediate halofuginone's effects on muscle cells. Together, it is conceivable that additional sites on the halofuginone molecule are crucial for mediating its effects via different signaling pathways. Deciphering the various effects of these sites will be beneficial in understanding the structure-function relationship of the halofuginone molecule and will assist the design of second-generation molecules for therapeutics. Notably, to date, the standard care therapy for DMD patients is steroid treatment, and hence is included in all clinical trials in these patients. Thus, the combination of steroids with successful second-generation of halofuginone analogues warrants future studies.

Based on crystallographic analysis, a recent study has suggested that halofuginone (or febrifugine) inhibits ProRS by forming a ternary complex with the enzyme and ATP; the α-phosphate group of ATP forms two hydrogen bonds with the piperidinyl ring hydroxy group, hence mimicking the activated form of proline in its typical mechanism of action (Zhou et al., 2013). Halofuginone was reported to inhibit T17 cell differentiation by activating the amino acid response pathway (Sundrud et al., 2009) through inhibiting human ProRS (Keller et al., 2012). More recently, it was reported that halofuginone, as well as other ATP-competitive inhibitors, demonstrated anti-fibrotic activity that was associated with their ProRS inhibitory activity (Shibata et al., 2017). Additionally, a study on cardiac stress showed that the antifibrotic effects of halofuginone are mediated by activation of the amino acid response pathway (Qin et al., 2017). In agreement with these studies, the evidence that deoxyhalofuginone nearly eliminated halofuginone's inhibitory effect on fibrosis and its promotive effects on utrophin levels and histopathology in mdx mice, suggest and support the importance of ProRS inhibition in post-transcriptional events.
related to fibrosis in DMD, and points towards the TGF-β/Smad3 signaling pathway being involved in this inhibition.

In summary, the efficacy of an analogue for halofuginone has been tested for the first time in the context of MDs. The data demonstrate the requirement of the hydroxy group in the halofuginone molecule for halofuginone's promotive effects on fibrosis in mdx mice, emphasizing the need for additional studies in the search for second-generation halofuginone analogues with better efficacy and less adverse effects on MD patients.
Acknowledgments

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References


Legends:

**Figure 1:** Chemical structure of racemic halofuginone (A), and the (+)-Deoxyhalo and (–)-Deoxyhalo enantiomers of deoxyhalofuginone (B).

**Figure 2:** Collagen deposition in *mdx* mice 14 weeks of age. (A) Sirius red staining of diaphragms taken from untreated *mdx* mice (Control), *mdx* mice treated with racemic halofuginone and *mdx* mice treated with deoxyhalofuginone, either with enantiomer (+)-Deoxyhalo and (–)-Deoxyhalo, or their equal combination (Racemic). Mice were injected (ip) with halofuginone, or each of the enantiomers (10 µg/mouse), or a combination of the two (5 µg each), 3 times a week for 10 weeks starting from the age of 4 weeks. The untreated control mice were injected with saline. Fibrilar collagens (types I and III) are stained red. Bar, 50 µm. Inserts depict the diaphragms at low magnification. Bar, 500 µm. (B) Quantitative analysis of collagen content in diaphragms. Results are presented as mean ± SE of twenty pictures from each of 3 different mice (n = 60). * Significantly different when compared with Control (P < 0.05).

**Figure 3:** Histopathology of diaphragms of untreated *mdx* mice (Control), or *mdx* mice treated with racemic halofuginone and *mdx* mice treated with deoxyhalofuginone, either with enantiomer (+)-Deoxyhalo and (–)-Deoxyhalo, or their racemic combination (Racemic). The experiment course was as described in Figure 2. (A) Hematoxylin and eosin staining of diaphragms derived from experimental mice. White arrows depict degenerated areas. Bar, 50 µm. (B) Myofiber diameter distribution in diaphragms. Myofibers are clustered in bin intervals of 0.5 µm, and results are presented as percentage of total myofibers. Between 4,300 to 4,500 myofibers were counted for each treatment group. (C) Average of myofiber diameter of the experimental groups. Results are presented as mean ± SE (n = 4,300-4,500). Different letters indicate significant difference between treatments (P < 0.05).
**Figure 4:** Utrophin expression in the diaphragms sections from *mdx* mice, nontreated or treated with halofuginone and deoxyhalofuginone as described in Fig. 2. (A) The muscle sections were immunostained with an antibody specific to utrophin (red, white arrows). Nuclei were stained with DAPI (blue) and all were visualized by confocal microscopy. Bar, 50 µm. (B) Image analysis quantification of utrophin levels presented as mean pixels/unit area ± SE of 3 mice with 10 sections/diaphragm (n = 30). * Significantly different when compared with Control ($P < 0.05$).

**Figure 5:** Smad3 phosphorylation is not inhibited by deoxyhalofuginone. Diaphragm sections of the experimental *mdx* mice were immunostained with anti-pSmad3-antibody. Nuclei were stained with DAPI (blue) and all were visualized by confocal microscopy. White arrows depict positive-pSmad3 cells. Bar, 50 µm.

**Figure 6:** Quantitative analysis of the number of pSmad3-positive cells out of total nuclei in the diaphragm sections. Results are presented as mean ± SE (n = 4000). * Significantly different when compared with Control ($P < 0.05$).
A

Control  Halofuginone

(+)-Deoxyhalo  (-)-Deoxyhalo  Racemic

B

Utrophin expression levels (Pixel/area)

Control  Halo  (+)  (-)  Racemic