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Inhibition and Reversal of Myogenic Differentiation by Purine-Based Microtubule Assembly Inhibitors

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Summary

Using a muscle cell differentiation screen, we have identified myoseverin from a 2,6,9-trisubsituted purine library as a purine-based microtubule binding molecule [1]. Structure-activity relation studies of myoseverin identify positions N2 and N6 to be critical for inhibiting muscle differentiation. Inhibition of microtubule polymerization induced the reversion of terminally differentiated myotubes to mononucleated cells that were responsive to both growth and differentiation conditions, without any observable cytotoxicity. Comparison of myoseverin derivatives to taxol, vinblastine, nocodazole, and colchicine identify myoseverin's effect as being selectively reversible in addition to lacking the cytotoxic effects of these non-purine-based microtubule-disrupting molecules. Myoseverin, as a purine-based microtubule inhibitor, reverted terminal muscle-differentiated cells to a state that was responsive to environmental cues. These results suggest that myoseverin may have applications in muscle regeneration and stem cell differentiation.

Introduction

Chemical genetic approaches have gained precedence for both drug discovery and as tools for probing biological systems [2]. The feasibility of finding bioactive molecules for a desired phenotype has been greatly enhanced by the combinatorial chemistry techniques that allow the parallel synthesis of molecules on the order of a hundred thousand or more - of which only a select few compounds will display interesting properties in relation to their biological target [3–5]. Our laboratory has synthesized a targeted chemical library of purine-based kinase inhibitors (2,6,9-trisubstituted purines) around the molecular scaffold of olomoucine, a known CDK2 inhibitor (Figure 1A) [6–8].

The activity of the compounds was assayed in C2C12 muscle cells—a mouse cell line that retains the ability to differentiate in culture. Myogenic differentiation requires a cell cycle arrest to induce expression of the proper phenotype. Inhibition of cell cycle progression induces myogenic differentiation [9, 10]. The screening of the purine-based library sought compounds that promoted or inhibited the terminal differentiation of C2C12 myogenic precursors. Using such a screen, we previously identified myoseverin as a novel purine tubulin binding molecule that augmented the terminal differentiation of muscle cells and induced expression of immunomodulatory and stress-responsive genes [1].

We report here the reversal and inhibition of terminal myogenic differentiation by exogenous application of myoseverin at low micromolar concentrations. Treatment of myoseverin reverted multinucleated myotubes to a mononuclear state that was responsive to growth factors and proliferation. Mononuclear cells derived from terminally differentiated muscle cells (referred to as myotubes) by treatment with myoseverin were also responsive to differentiation conditions and retained the capability to form multinucleated tubes after treatment. Functional characterization of the cellular effects identifies the downregulation of differentiation markers Myf5, MyoD, and myosin heavy chain, with a concomitant upregulation of cell cycle proteins cyclin A and CDK2 after myoseverin treatment and addition of growth media. These cells in this state can also be induced to differentiate for a second time if they are placed under differentiation conditions rather than growth conditions. Structure-activity relation (SAR) studies of synthesized myoseverin derivatives identify positions N2 and N6 as critical for the bioactivity of myotube disassembly. Analysis of derivatizations at position N9 identifies a structural requirement for a branched/cyclic moiety for the reversibility of myotube formation and correlated with the inhibition of microtubule polymerization in vitro. Comparison with other microtubule interfering agents, taxol, vinblastine, nocodazole and colchicine, mediated a similar myotube disassembly; however, only myoseverin was nontoxic and reversible. Thus, it resulted in responsive cells that can be induced to either proliferate or differentiate. These results further characterize myoseverin's effect on terminal-muscle cell differentiation and demonstrate the induction of a reversible state of terminally differentiated muscle cells. Furthermore, the noncytotoxic effects displayed by myoseverin and some of its derivatives might have useful applications for both muscle cell biology and muscle regeneration.

Results and Discussion

Morphological and Phenotypic Characterization of Myoseverin's Effect on Muscle Cells

Myoseverin had been identified from a morphological screen with the 2,6,9-trisubstituted purine library [1]. A

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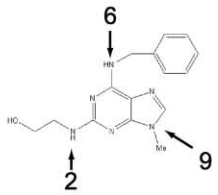
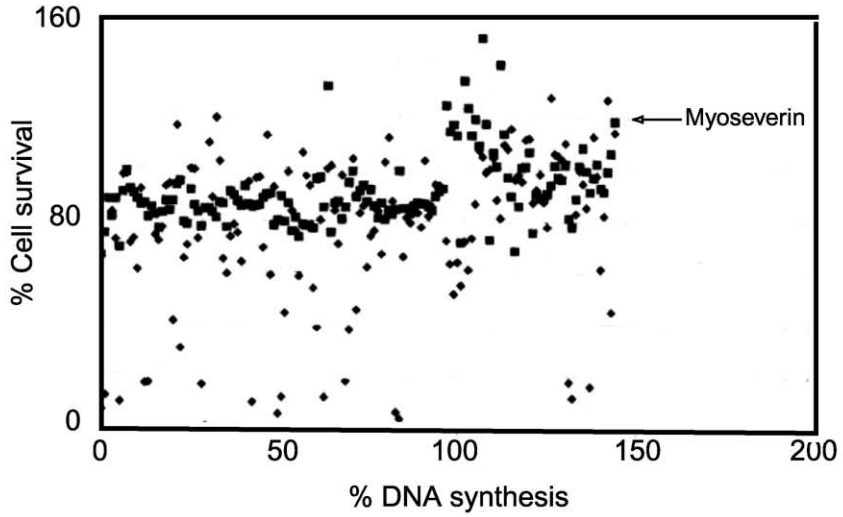
A



Olomoucine

B



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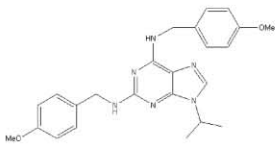
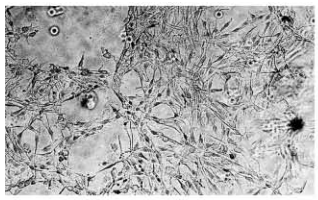
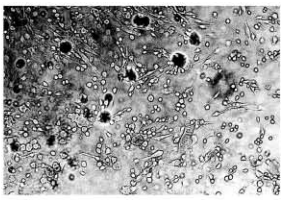


Myoseverin



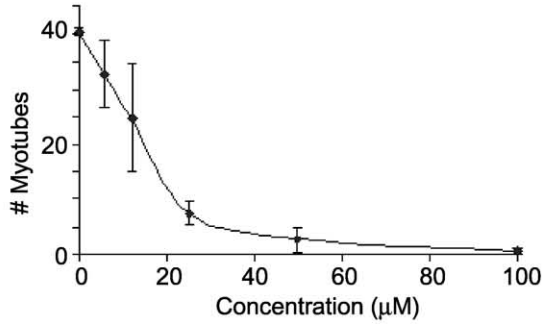
Myocytes

Myotubes



Myoseverin treated Myotubes (24 hrs)

D



series of cell-based assays were used to categorize the effects the purine library members would have on C2C12 cells. Although the original screen was designed to identify molecules that induced cell cycle arrest and therefore myogenic differentiation in the C2C12 system, the unexpected morphological effects and lack of any observable cytotoxicity of myoseverin (Figure 1B) prompted further investigation. The screens took advantage of the facts that muscle differentiation can be assayed morphologically (Figure 1C) and that several inhibitors of the yeast Cdk homolog were identified in the library of purines that was screened [6, 11]. Muscle cells grown in the presence of growth factors will remain in a proliferative single-cell state [12]. Switching the cells to a deprived media of growth factors induces myogenic differentiation [13]. Myogenic differentiation is characterized by the formation of multi-nucleated myotubes that result from cell fusion and elongation. This morphological difference is readily observed with phase contrast and immunofluorescence microscopy and is also monitored by the expression of specific differentiated-muscle cell markers such as myosin heavy chain, MyoD, Myf5, and surface marker acetycholine receptor (AchR) [14–16].

Myotube Fragmentation Generates Mononuclear Cells Derived from Multinucleated Myotubes

A dose response curve of myoseverin's effect reported a bioactivity for myotube disassembly for a 24 hr treatment to be 11 $\pm$ 4 μM, referred to as the intracellular concentration at which 50% of the myotubes had disassembled (IC_{50}). Immunofluorescence microscopy was performed in order to assess how the morphological changes observed were related to changes in cytoskeletal organization. Staining for tubulin revealed a uniform filamentous network of microtubule structure in the multinucleated myotube (Figure 2A). Punctate AchR staining identified a terminally differentiated myotube (Figure 2A). Treatment of terminally differentiated myotubes with myoseverin resulted in a fragmentation of the myotube structure [1], and occasionally the expulsion of a single nucleus was observed with the complete disruption of the clustered AchR (Figure 2A). Extraction of monomeric tubulin from intact cells revealed a disorganized microtubule networks, suggesting a possible mechanism for myoseverin-induced myotube disassembly (Figure 2B).

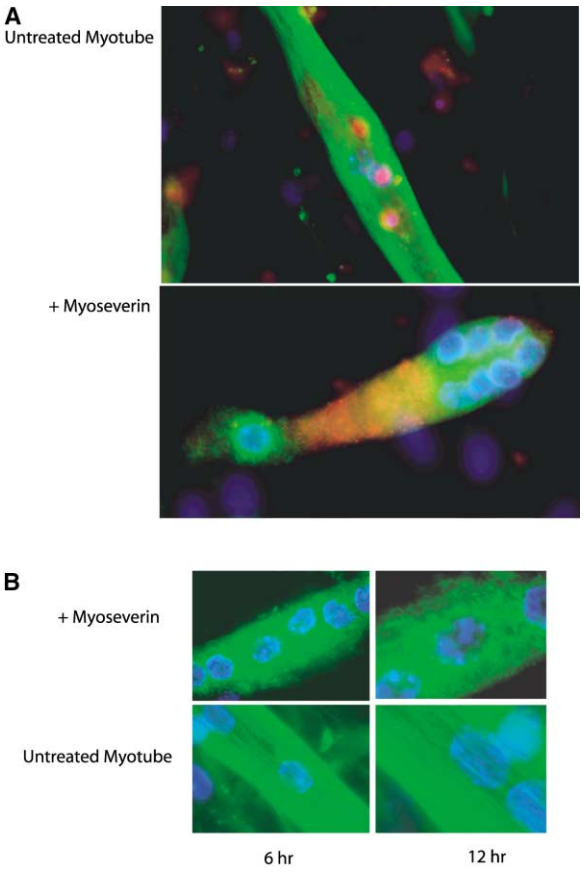


Figure 2. Immunofluorescence Microscopy of Myotube Structure and Consequential Effects of Myoseverin Treatment

(A) Terminally differentiated myotubes were stained for tubulin (green), nuclei (blue), and acetylcholine receptor (red) by the use of anti-tubulin antibodies, Hoescht, or rhodamine-labeled α-bungarotoxin, respectively. Myoseverin-treated (10 μM, 24 hr) (bottom panel) and untreated (top panel) myotubes are shown.

(B) Mild permeabilization of myotubes reveals microtubule disorganization after the treatment of myoseverin. Myotubes were stained as described above in the presence or absence of myoseverin (10 μM) at the indicated times preceeding a mild permeabilization step.

Induction of Monouclear Cells Derived from Disassembled Myotubes to Proliferate and Differentiate

The fragmentation of myotubes was quantitated by counting the number of nuclei in myotubes, as stained for myosin heavy chain and nuclear stain hoescht dye, as a function of myoseverin treatment (10 μM) over time. The decrease in nuclei count per myotube over time is consistent with the fragmentation pattern observed (Figure 3A). Interestingly, removal of myoseverin (by thorough washing) and replenishment with differentiation media induces the mononuclear cells derived from disassembled myotubes to differentiate over the typical 3–4 days required for normal monocyte differentiation

Figure 1. Scaffold of 2,6,9-Trisubistitued Purine Library and Cellular Effects of Myoseverin, a Library Member

(A) Structure of olomoucine, a CDK2 inhibitor.

(B) Compound library cellular effects reported as percent survival and percent DNA synthesis effect on proliferating C2C12 muscle cells (relative to DMSO control). Cell-based assays that were used to index compound effects are described in the Experimental Procedures.

(C) Structure of myoseverin and morphological effect on terminally differentiated muscle cells as visualized by phase microscopy (10 μM, 24 hr treatment, n $\geq$ 100).

(D) Dose response curve of myotube fragmentation to varying concentrations of myoseverin for a 24 hr period. Myotube count was defined as 1/4 the field of view with a 40$\times$ objective lens.

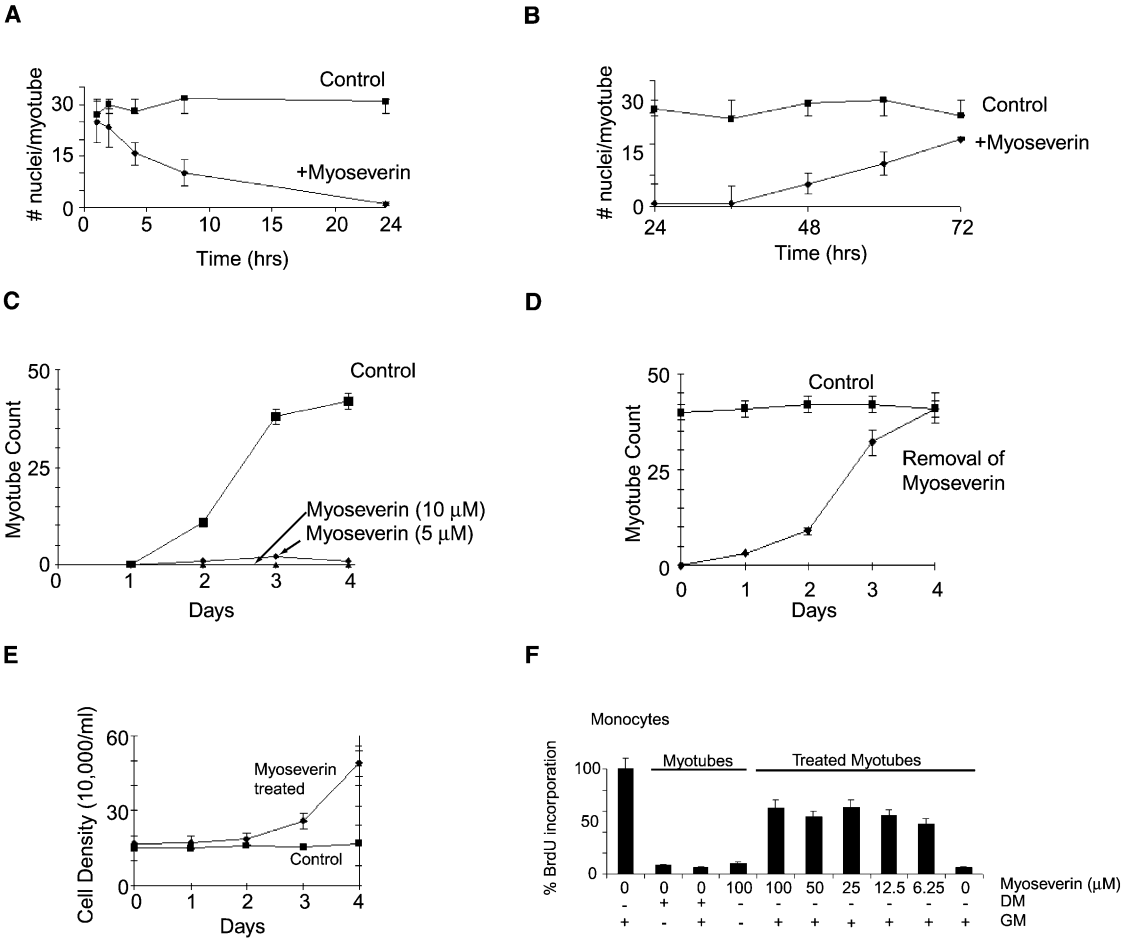


Figure 3. Morphological Assessment of Myotube Disassembly and Reformation after Myoseverin Treatment

(A) Terminally differentiated muscle cells were treated with myoseverin (10 μM), and the number of nuclei per myotube was counted after immunofluorescence staining for myosin heavy chain and nuclei. The number of nuclei/myotube is plotted as a function of time for at least five different fields of view (n = 8). Control cells were terminally differentiated muscle cells that underwent DMSO vehicle treatment.

(B) The removal of myoseverin from treated myotubes (fragmented mononuclear cells) and the addition of differentiation media induces myotube reformation. Myoseverin-treated myotubes (10 μM, 24 hr) were washed thoroughly in PBS and replenished with differentiation media. The number of nuclei/myotube was counted as described above and plotted as a function of time. Control cells were terminally differentiated muscle cells that underwent DMSO vehicle treatment.

(C) Myoseverin inhibits muscle cell differentiation. Proliferating muscle cells treated with myoseverin (10 μM and 5 μM, 24 hr) were induced to differentiate in the presence or absence of myoseverin, and the number of myotubes was counted per day. Control cells reached full myotube formation at day 4, in contrast with the failure of myotube formation in myoseverin-treated monocytes (n = 10). Myoseverin was replenished daily to prevent rapid turnover (our unpublished data).

(D) Removing myoseverin from monocytes that failed to differentiate under differentiation conditions allowed the cells to regain the capability to form myotubes, as monitored by myotube count as a function of time. Myoseverin was removed from 4 day-treated monocytes and replenished with fresh differentiation media. Control cells were terminally differentiated muscle cells (n = 40).

(E) Clonal expansion of mononuclear cells derived from myoseverin-treated myotubes. Mononuclear cells derived from myoseverin-treated myotubes (10 μM, 24 hr) were washed and replenished with growth media. Cell density was monitored over time and compared to growth media-treated myotubes.

(F) BrdU incorporation of myoseverin-treated myotubes after replenishment with growth media. DNA synthesis was monitored for myoseverin-treated (concentrations as indicated for 24 hr period) myotubes, and nontreated cells were switched to growth media for 24 hr. Values were made relative to cycling monocytes.

in culture (Figure 3B). This reversible effect was continuously repeated (up to ten times) with no observable cellular damage to the muscle cells (our unpublished data). In the continuous presence of myoseverin, the monocytes fail to differentiate into myotubes (Figure 3C), in contrast with the results when the compound is removed for cultures treated in parallel (Figure 3D). Myoseverin-treated, myotube-derived monocytes were also susceptible to growth factors and induced proliferation as detected by clonal expansion of the cellular population (Figure 3E) and induction of DNA synthesis (Figure 3F). These observations indicate the significant role the microtubule architecture has in myogenic differentiation and suggest that the interference of microtubule

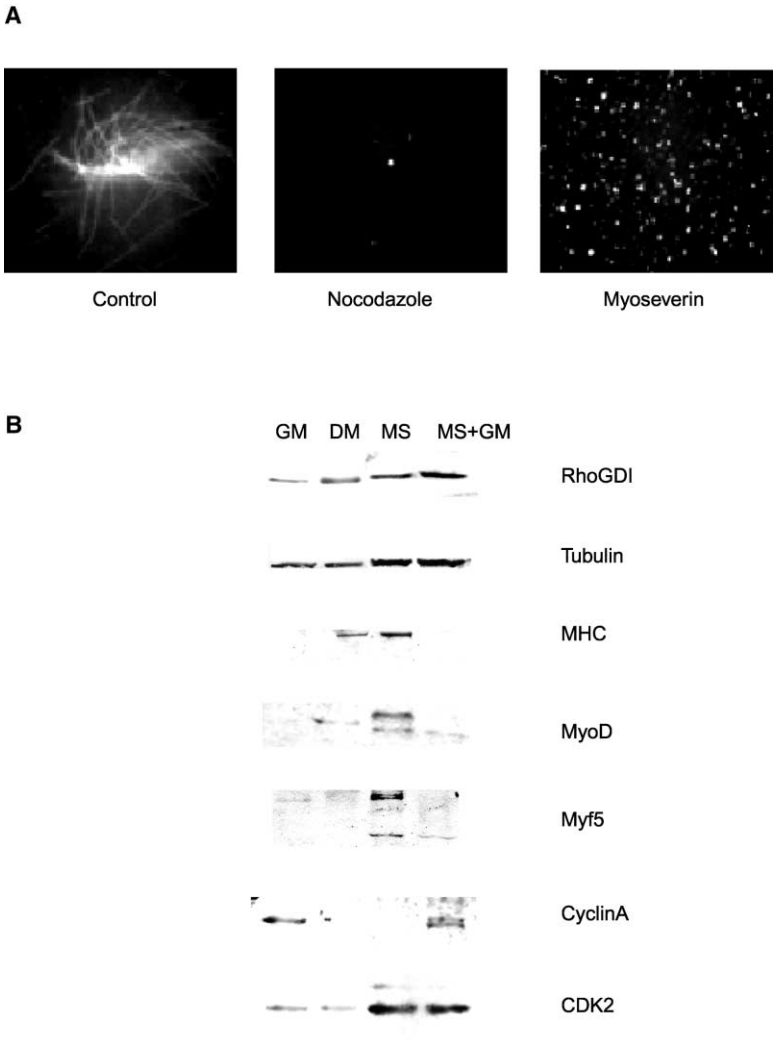


Figure 4. Myoseverin Effect on Microtubule Polymerization and Expression of Differentiation and Proliferation Markers

(A) In vitro microtubule polymerization assay in the absence (control) and presence of 50 μM myoseverin. Nocodazole (50 μM) served as a negative control.

(B) Immunoblot analysis of differentiation markers (MyoD, Myf5, MHC) and proliferation markers (cyclin A, CDK2, RhoGDi) in monocytes (GM), myotubes (DM), myoseverin-treated myotubes (10 μM, 24 hr; MS), and myoseverin-treated myotubes replenished with growth media for 24 hr (MS+GM). Equal quantities of protein were loaded and immunoblotted with antibodies as indicated. Tubulin served as a loading control, although myoseverin-treated cells had slightly higher amounts for an equivalent protein content.

assembly may be a mechanism for exogenously controlling muscle cell differentiation.

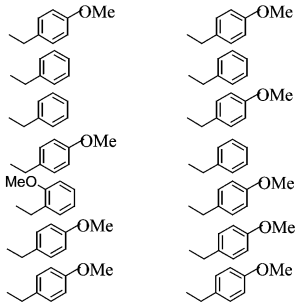
In Vitro Assessment of Myoseverin's Effect on Microtubule Polymerization

For the verification of myoseverin's effect on microtubule polymerization, an in vitro microtubule polymerization assay was performed. Purified rhodamine-labeled tubulin and 500 μM GTP were introduced to 50 μM myoseverin and to 50 μM nocodazole, a known microtubule depolymerizer [17]. Figure 4A shows polymerized microtubules (control). Nocodazole was used as a negative control to inhibit microtubule polymerization and is similar to myoseverin's effect (Figure 4A).

Functional Characterization of Muscle-Specific Differentiation and Proliferation Markers

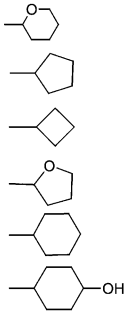
For the assessment of the cell state of myoseverin-treated cells, immunoblot analyses were performed to monitor the expression of myosin heavy chain, tubulin, CDK2, cyclin A, RhoGDI, MyoD, and Myf5 (Figure 4B). Terminal differentiation of skeletal myoblasts requires cell cycle withdrawal and the initiation of myogenesis. The myogenic basic loop helix (bhlh) factors—MyoD, Myf5, myogenin, and MRF4—control terminal differentiation by both temporal and spatial regulation [15, 18, 19]. Myoblast differentiation requires irreversible cell cycle withdrawal and is mediated partly by MyoD expression [20]. Thus, MHC, MyoD, and Myf5 markers serve as differentiation markers [18]. In the presence of myoseverin, MHC is present, with a subsequent increase in the expression of both MyoD and Myf5 (Figure 4B). In the terminally differentiated myotubes, the apparent decrease in MyoD mobility and the accompanying loss of Myf5 detection (Figure 4B) could be a result of the temporal regulation of these muscle-specific transcription factors. However, CDK2 expression is also upregulated (Figure 4B). In the presence of growth factors after myoseverin treatment, the expression of differentiation markers MHC, Myf5, and MyoD is down-regulated in conjunction with an upregulation of cyclin A and CDK2 (Figure 4B). These results suggest that the myogenic program is overridden in the presence of growth factors after myoseverin treatment, as suggested by the upreg-

2, 6 position derivatives



N2	N6	N9	N6'	IC_{50} Myotube Dissasembly	IC_{50} Tubulin Polymerization
OMe	OMe	$CH(CH3)_2$	H	11	8
		$CH(CH3)_2$	H	-	-
	OMe	$CH(CH3)_2$	H	-	-
OMe		$CH(CH3)_2$	H	75	-
MeO	OMe	$CH(CH3)_2$	H	-	-
OMe	OMe	$CH(CH3)_2$		-	-
OMe	OMe	$CH(CH3)_2$	CH_3	70	N/A

9 position deritives



	IC_{50} Myotube dissasembly	IC_{50} Tubulin Polymerization
	10	N/A
	15	3
	20	4
	25	5
	48	2
	30	3
$OCH_2CH_2Si(CH3)_3$	-	-
$(CH_2)_5NH_2$	-	-
$(CH_2)_4NH_2$	-	-
$(CH_2)_3NH_2$	-	-
$(CH_2)_2NH_2$	-	-
CH_2CH_3	90	25
CH_3	-	25
H	-	25

Figure 5. Structure-Activity Relation Studies of Myoseverin Derivatives at the N2, N6, an N9 Positions and Correlation with Muscle Cell Bioactivity and In Vitro Inhibition of Microtubule Assembly

Chemical modifications were made as described in the Experimental Procedures. Functional groups are displayed for the N2, N6, and N9 positions. The N6′ position is the incorporation of a second functional group at the N6 position. IC_{50} values for myotube disassembly were the intracellular concentration for which 50% of the myotubes had fragmented, relative to DMSO control-treated myotubes and defined as stated. IC_{50} values for tubulin polymerization were defined from the in vitro microtubule polymerization assay. Values correspond to micromolar concentrations and were verified in at least three independent experiments. N/A means "not available," and "-" means that no value was discovered to have an effect. Compounds were tested up to 100 μM.

ulation of CDK2 and cyclin A. Differentiated cells placed in proliferating media do not display a similar effect (our unpublished data). RhoGDI, which has recently been shown to inhibit myogenic differentiation, displays increased levels after growth factor replenishment post-myoseverin treatment (Figure 4B; [21]). The underlying mechanism by which the cells are shunted from the myogenic program to reenter into the cell cycle is not clearly understood. Recently, it has been shown that MyoD can be positively regulated by serum response factor and contains several CDK phosphorylation sites that commit it to proteolysis upon phosphorylation [21, 22]. This suggests that the cell cycle machinery can influence muscle-specific proteins needed for differentiation.

Structure-Activity Relationships of Chemically Synthesized Myoseverin Derivatives

Myoseverin is unique in its effects on myotubes, and studies of analogs might shed information on the speci-

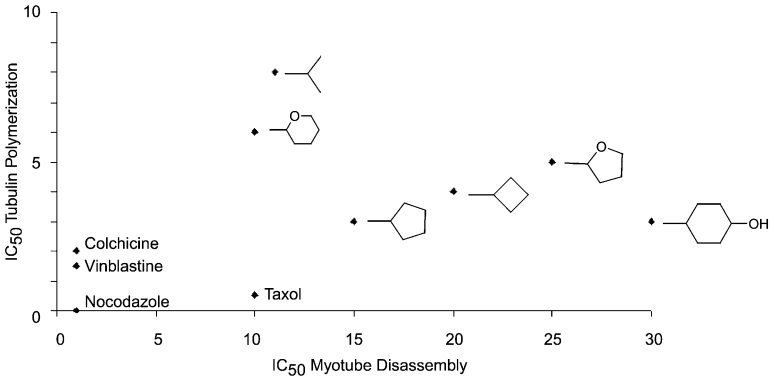


Figure 6. Analysis of the N9 Functional Group on Cellular Effects in Myotube Fragmentation and Inhibition of Tubulin Polymerization as Compared to Microtubule-Interfering Agents

The IC_{50} for myotube values for myotube disassembly and the IC_{50} values for in vitro inhibition of tubulin polymerization were plotted and compared to taxol, nocodazole, vinblastine, and colchicine (1 μM, myoseverin and derivative compounds at 10 μM). Structures of N9 position derivatives are overlayed on data points (isopentyl group corresponds to myoseverin). Taxol, vinblastine, nocodazole, and colchicine had an LD_{50} of 1–5 μM (lethal dose at which 50% of the cells were dead) on muscle cells, in contrast with no observable cytotoxicity of myoseverin or its derivatives as tested up to 100 μM.

ficity/activity of the compound. To see if it was possible to correlate a structure with the molecule's effect, several myoseverin derivatives were synthesized by solution phase chemistry by a Mitsunobo reaction at N9-position and subsequent amination with p-methoxybenzylamine at the N2 and N6 positions (Figure 5; [6, 7]). This myoseverin-analog library was rescreened in differentiated muscle cells, IC_{50} values were derived for myotube disassembly, and the compounds were tested for inhibition of in vitro microtubule polymerization (Figure 5). Elimination of the paramethoxy functional moieties on the benzyl groups or substitution at the ortho and meta positions by methoxy groups completely abolished the bioactivity of the parent molecule in both cellular and in vitro assays (Figure 5). Derivatization of the para, meta, or ortho position on the benzyl ring with amine derivatives, halides, bulky substituents, and alkyl chains also abolished all bioactivity (our unpublished data). Additional substituents synthesized at the N6 or N2 positions by the addition of functional groups similarly affected the cellular activity of the compound. Thus, positions N2 and N6 of myoseverin were absolutely required for cellular activity, and any functional group tested augmented all myotube bioactivity (for more than 200 derivatives synthesized).

Derivitization at position N9 yielded some promising results. Hydrophobic substituents at the N9 position had potent inhibition of microtubule assembly (Y.-T.C.'s unpublished data); smaller alkyl substituents diminished activity when compared to the parent myoseverin molecule (isopropyl [myoseverin] > ethyl > methyl > H; [Figure 5]). Larger substituents enhanced the microtubule effect in vitro but were less active in the muscle cells (myoseverin < cyclopentyl < cyclobutyl < cyclohexl; [Figure 5]). The insertion of heteroatoms into the cyclohexyl ring had similar effects to myoseverin in the muscle cell-based assays (Figure 5).

Comparison of Purine-Based Microtubule Polymerization Inhibitors to Taxol, Nocodazole, Colchicine, and Vinblastine

Comparisons of myoseverin N9 derivatives to known microtubule interfering agents such as taxol, vinblastine, nocodazole, and colchicines was performed by plotting IC_{50} values for myotube disassembly against IC_{50} values for inhibition of microtubule assembly. There is an apparent correlation with microtubule interference and myotube disassembly, supporting the notion that microtubules are implicated in maintenance of the terminally differentiated state of the muscle cell in vivo (Figure 6). However, the cytotoxicity of these classic microtubule-interfering compounds (LD_{50}, 1–10 μM) as well as their nonreversible effects largely contrasts the nontoxicity and reversibility of myoseverin and its derivatives (90% survival tested up to 100 μM). This suggests that myoseverin (and its analogs) are strong candidates for potential therapeutic drugs for the treatment of degenerated muscle tissue and can also be used as biological tools to study myogenic differentiation.

Fluorophore-conjugated myoseverin compounds were made to study intercellular localization of myoseverin. Fluorescein-conjugated and rhodamine-conjugated myoseverin analogs at N9 position were cell permeable only to proliferating cells but failed to produce the desired effect when the cells were induced to differentiate, again illustrating the importance of the N9 position. A biotin linker has been conjugated to the N9 position and has been used to purify tubulin as a myoseverin binding protein [1]; however, the chemical synthesis at this position indicates the inaccessibility to other potentially interacting protein molecules by this approach. Therefore, although tubulin may contribute to the observed effect, the identity of other cellular constituents with which myoseverin interacts may illuminate the connection between the myogenic differentiation program and the microtubule network.

Significance

The major findings of the present study on C2C12 myogenic cells have extended the functional characterization of myoseverin and illustrate that (1) mononuclear cells derived from myoseverin-disassembled myotubes are susceptible to both growth and differentiation conditions, (2) myoseverin treatment can inhibit myogenic differentiation without promoting cellular toxicity, and (3) myoseverin depolymerizes microtubules, and this effect correlates with the observed morphological phenotype. The results so far do not exclude myoseverin from interacting with a target that

may be responsible either for regulating the gene expression during muscle cell differentiation or for coordinating the organization of the cytoskeletal structure that is needed for myotube formation. The results do indicate that myoseverin as a novel microtubule depolymerization agent can be used to induce terminally differentiated C2C12 muscle cells to proliferate and may possess pharmacological activity in the treatment of skeletal muscle-associated disorders or when the regeneration of muscle cell interfaces is needed. Additionally, the nontoxicity of these purine-based microtubule-interfering agents may give them potential applications as anti-cancer agents because typical microtubule-disrupting agents such as taxol and vinblastine have had clinical efficacy with acceptable toxicities.

Experimental Procedures

Cell Culture

C2C12 myoblast cells (CRL 1772 stock, American Type Culture Collection, Rockville, MD) were kept in a proliferating state by maintaining them in Dulbecco's modified Eagle's media (DMEM, GIBCO, Grand Island, NY) and 20% fetal bovine serum (Hyclone, Logan, UT). Proliferating myoblasts were induced to differentiate into myotubes by replacement of the above serum-enriched media with serum-deficient media, DMEM, and 2% horse serum (GIBCO) for 4 days. Differentiated and proliferating C2C12 control cells were treated with 1% DMSO, which was used to solubilize the compounds. All assays were relative to this control.

Cell-Based Assays

Cytotoxicity Assays

Compound toxicity was determined against both proliferating and differentiated C2C12 cells via the microtiter MTT assay (Boehringer Mannheim, IN). Cytotoxicity was also assayed morphologically by the staining of cells with Trypan Blue (Sigma; MO), a dye that stains necrotic cells (as a positive control, cells were permeabilized with 0.1% Triton X-100 in PBS). The percent cell survival index is the MTT value of compound-treated cells relative to the MTT value of control cells. Triplicate measurements were performed per experiment.

Differentiation Assay

Cells were assessed morphologically by direct observation under the microscope, and the number of myotubes was counted in the presence and absence of the compounds. A myotube is defined as a multinucleated structure of at least 1/4 the field of view in a 40× objective lens. The percent differentiation index is the number of myotubes after compound treatment relative to the number of myotubes of control cells.

DNA Synthesis Assay

Bromodeoxyuridine (BrdU) incorporation was used to measure the rate of DNA synthesis relative to that in control cells. Anti-BrdU-alkaline phosphatase and ABS substrate (Boehringer Mannheim, IN) were used for detection of BrdU after a 24 hr incorporation. Absorbance was measured with a Spectramax plate reader at 570 nm.

Apoptosis Assay

Apoptosis was monitored by the TUNEL assay (Boehringer Mannheim) according to the manufacturer's instructions. Another compound from the library served as a positive control that displayed a characteristic apoptosis DNA ladder pattern on an agarose gel (our unpublished data).

Immunofluorescence Microscopy

Proliferating and differentiated C2C12 cells were grown on sterile cover slips (autoclaved 30 min, coated with 20% serum for 1 hr) in 24 wells in a 5% CO_2/95% air, humidified environment. Cells were washed twice in ice-cold phosphate-buffered saline (PBS) and then fixed in ice-cold 2.7% paraformaldehyde for 10 min. Cells were then permeabilized with 0.1% Triton X-100 for 5 min. Nuclei were stained with Hoechst 33258 (Sigma). Myosin heavy chain (MHC) was stained with a mouse MF20 anti-MHC antibody, and tubulin was stained with a mouse anti-tubulin antibody (see below); a fluorescein-conjugated horse anti-mouse IgG (Vector Laboratories) was used as a secondary antibody. The number of nuclei (stained with Hoechst) in the presence and absence of compound was determined from at least five microscope fields. Acetylcholine receptor was stained with α-bungarotoxin Texas red conjugate (Molecular Probes, OR; 1:100 in 1× PBS) for 15 min. Mild permeabilization to myotubes prior to fixation was performed as described [1]. Phase contrast pictures were visualized with a Carl Zeiss Axioskop microscope and photographed onto film. Images were visualized with a Nikon epifluorescence microscope and collected with a Sony digital CCD camera. The anti-tubulin antibody developed by Michael Klymkowsky was obtained from the Developmental Studies Hybridoma Bank developed under the auspices of the NICHD and maintained by the University of Iowa, Department of Biological Sciences, Iowa City, IA 52242.

Immunoblotting

Whole-cell extracts were prepared by lysing the cells in buffer (50 mM Tris [pH 8.0], 150 mM NaCl, 5 mM EDTA, and protease inhibitor cocktail tablet [Boehringer Mannheim]). Lysates were cleared of insoluble material by centrifugation at 4°C and 10,000 × g for 10 min. Protein concentrations of lysates were determined by the Bradford assay (Biorad, Hercules, CA). Proteins (20 μg) were separated by electrophoresis through a 12% SDS-PAGE gel and transferred to nitrocellulose membranes (0.2 μm; Biorad). Membranes were blocked in buffer (1× PBS, 0.1% Tween-20, 5% nonfat dry milk) overnight at 4°C and then incubated with anti-CDK2 (Santa Cruz Biotechnology), anti-tubulin, anti-RhoGDI, anti-MyoD, anti-Myf5, anti-cyclin A, or anti-myosin heavy chain (1:200 dilution in 1× PBS, 0.1% Tween-20, and 2% nonfat milk) for 6 hr at 4°C. After incubations, membranes were washed five times with 1× PBS for 20 min. Membranes were then incubated with secondary antibody (alkaline-phosphatase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG at 1:500 dilution; or horse peroxidase-conjugated goat anti-mouse IgG or goat anti-rabbit IgG in 1× PBS, 0.1% Tween-20, and 2% nonfat milk; Sigma Chemicals) for 1 hr at 4°C. Membranes were then washed five times (with 1× PBS and 0.1% Tween-20) and developed by BCIP/NBT colorimetric detection (Sigma Chemicals).

Microtubule Polymerization Assay

The microtubule polymerization assay was developed by the use of rhodamine-labeled tubulin (Cytoskeleton), 500 μM GTP, and 5 μM Mg^{2+} and incubated at 37°C for 30 min. Rhodamine-tubulin was incubated in the presence of the compound (50 μM) or its absence (DMSO control 1%). Visualization was done with a Carl Zeiss Axioskop microscope and photographed onto film.

Structure-Activity Studies

For the correlation of the selected compound's structure with its observed effect, specific analogs were synthesized. Extrapolation of a general structure moiety directed synthesis to a subset classification of the 2,6,9-substituted purines. Solid phase and glycinamide-based synthetic techniques were used to synthesize specific 2,6,9-substituted purines as described [6, 7]. The position of the group necessary for bioactivity of lead compounds was determined by selective removal of the groups at the different positions and a repeat of the cell-based bioassays. All other reagents were obtained from Sigma/Aldrich (St. Louis, MO) unless otherwise indicated. The synthesized compounds were purified by the use of preparative TLC and characterized by reverse-phase HPLC, ^{1}H NMR, and high-resolution mass spectrometry. The final purity of the compounds was >98%.

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