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# Short Communication

# Antiretroviral Protease Inhibitors Prevent L6 Muscle Cell Fusion by Reducing Calpain Activity

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## ABSTRACT

**The antiretroviral protease inhibitors indinavir (IDV) and ritonavir (RTV) are used in highly active antiretroviral therapies (HAART). Side effects from long-term HAART therapy include loss of muscle mass. Myoblasts when cultured in media low in growth factors withdraw from the cell cycle, express muscle-specific differentiation inducers and proteins, and fuse to form myotubes. The neutral protease, calpain, is required for myotube formation and RTV decreased calpain activity *in vitro*. We found lower calpain activity, but not protein, in homogenates of RTV-treated L6 cells than in control cultures. Importantly, L6 and C2C12 myoblasts did not form myotubes when cultured with 10 or 20 $\mu$M IDV or RTV. Control and drug-related L6 myoblasts showed identical decreases in proliferating cell nuclear antigens expression indicating proliferation arrest. Similarly, muscle differentiation inducers MyoD and myogenin and their downstream target, myosin heavy chain, were expressed at similar levels in control and drug-treated cells. Thus, whereas muscle differentiation was unaffected by protease inhibitors, calpain activity was reduced and myotube formation prevented. We conclude that RTV and IDV reduced myotube formation by reducing calpain activity. Our data suggest that protease inhibitors included in HAART might be directly involved in muscle wasting by reducing muscle remodeling.**

## INTRODUCTION

The use of antiretroviral protease inhibitors (PI) with either nucleoside reverse transcriptase inhibitors or nonnucleoside reverse transcriptase inhibitors has proven most effective in treating human immunodeficiency virus (HIV)-infected patients and maintaining low viral loads.[1,2] Highly active antiretroviral therapy (HAART) combines antireverse transcriptase with protease inhibitors and is a significant advance in the treatment of HIV infections.[3,4] However, side effects arise from long-term HAART therapy. These include muscle wasting, fat redistribution, insulin resistance, and significant modulation of blood lipids.[5–11] The mechanism, direct or indirect, for the effects of PIs on muscle wasting is unknown.

Skeletal muscles are damaged and repaired repeatedly throughout life.[12–14] Satellite cells, between the basal lamina and the sarcolemma of mature myofibers, are responsible for muscle repair.[15,16] Upon injury, these normally quiescent cells proliferate and then differentiate to form new muscle fibers.[14–17] *In vitro* muscle cell lines, such as the rat L6 or mouse C2C12 myoblast cells lines, have been used extensively to model these events. When cultured in low growth factor-con-

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taining media these myoblast cell lines permanently withdraw from the cell cycle and express an ordered series of muscle-specific differentiation inducers, such as MyoD and myogenin. These inducers direct expression of skeletal muscle-specific contractile proteins and eventually the myoblasts fuse to form multinucleated myotubes.[17–21]

Protease activity is necessary for myotube formation. The activities of cathepsins B, L, and H as well as $\mu$-calpain and m-calpain increase during L6 myoblast differentiation.[22] Inhibition of calpain, but not cathepsin, activity reduced myotube formation[15,22] suggesting that calpain activity is critical for myotube formation. Calpains are calcium-dependent cysteine proteases present in all mammalian cells.[23] In the rat muscle cell line L8, calpain protein levels do not vary with differentiation *in vitro*, but calpain activity is increased transiently during myoblast fusion.[16] Ritonavir (RTV), an HIV protease inhibitor and a component of HAART therapies, competitively inhibited calpain activity in PC12 cells in *in vitro* and *in situ* assays.[24] Conversely, calpain inhibitors reduced HIV protease activity.[25] Since muscle wastage is a common feature in therapy that includes PIs, and RTV was shown to reduce calpain activity *in vitro*, we questioned whether PI could reduce calpain activity, and perhaps other features of muscle cell myoblast to myotube differentiation, in *in vitro* muscle culture models.

In this study, we investigated the effects of two commonly used protease inhibitors, RTV and indinavir (IDV), on the *in vitro* differentiation of L6 and C2C12 muscle cells. We found both protease inhibitors, at physiologically relevant concentrations, markedly reduced calpain activity and myotube formation. The decrease in myotube formation was not a consequence of PI-mediated upstream changes in differentiation or muscle-specific protein expression.

## MATERIALS AND METHODS

### *Materials*

IDV was a gift from Merck and RTV was a gift from Abbott Pharmaceuticals. Mouse monoclonal antimyosin MF20 was a gift from Dr. Michael Rudnicki, McMaster University (Hamilton, Canada). Antisera to MyoD, myogenin, and proliferating cell nuclear antigen (PCNA) were purchased from Santa Cruz Biotechnology, Inc. (Santa Cruz, CA). Anti-calpain antibodies were purchased from Calbiochem (San Diego, CA). Secondary antibodies complexed to horseradish peroxidase and SuperSignal Enhanced Chemiluminescence detection kits were purchased from Pierce Biotechnology Inc. (Rockford, IL). Immobilon P membrane was purchased from Millpore Corp. (Bedford, MA). All other chemicals were purchased from Sigma Chemical Co., Inc. (St. Louis, MO).

### *Cells*

L6 myoblasts were grown in growth media, Dulbecco's modified Eagle's medium (DMEM) containing 10% (v/v) fetal calf serum (FCS).[26,27] To induce myoblast differentiation, growth medium was removed and replaced with an equal volume of fusion medium [$\alpha$-MEM containing 2% (v/v) FCS]. All experiments were done between 2 and 5 days later when myotube formation became apparent (>85% myotubes, >5 nuclei per myotube) at 5 days in control cultures.

### *Calpain activity*

Cells were treated as above and homogenized in 50 mM Tris, pH 7.4, 10 mM ethylenediaminetetraacetic acid (EDTA), and 10 mM dithiothreitol (DTT). The homogenate was clarified by centrifugation at top speed in an Eppendorf centrifuge for 15 min in the cold. Calpain activity was measured using the method of Buroker-Kilgore and Wang[28] and casein as the protein substrate. Clarified rat heart homogenate served as a positive con-

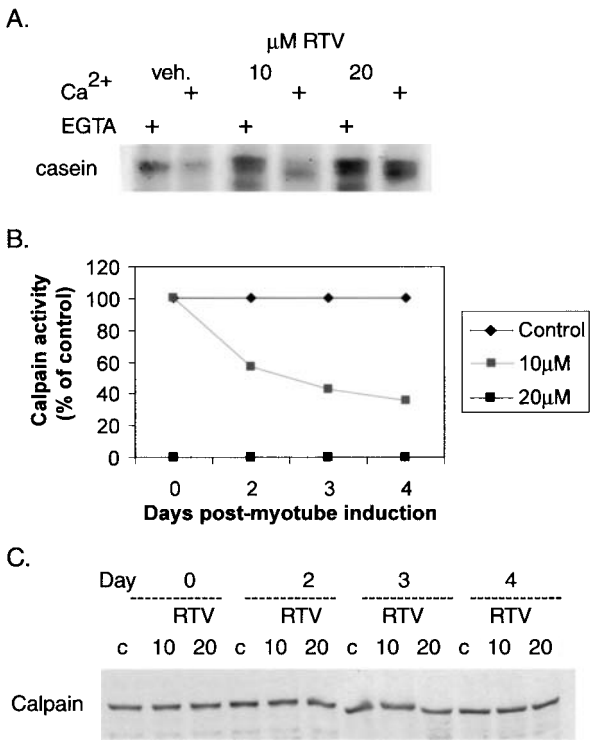


**FIG. 1.** Effect of RTV on $Ca^{2+}$-mediated calpain activity. Calpain activity was measured in homogenates of control or L6 cells treated with either 10 or 20 $\mu$M RTV. Cells were homogenized at the time of induction and 2, 3, and 4 days post-myotube induction as indicated. (**A**) Equal aliquots of the casein incubation reaction that included homogenates from cells 4 days after myotube induction in the presence of EGT or calcium were separated by SDS–PAGE and stained with Coomassie brilliant blue are shown. (**B**) The amount of casein in the stained gels was scanned and hydrolysis calculated as the amount of casein left in incubations containing 5 mM calcium divided by the amount of casein in companion incubations containing 5 mM EGTA for each sample. Hydrolysis in the control samples was artificially designated as 100% and the amount of casein hydrolyzed by the drug-treated samples was compared to this level of hydrolysis. (**C**) Equal amounts of protein from the above homogenates were separated on SDS–PAGE; the proteins were transferred to membranes and immunoblotted using anti-m-calpain.

trol in calpain activity assays. Activity was measured in the absence of added calcium (background) and in the presence of 5 mM calcium (calpain activity).

### *Immunoblots*

Cells were homogenized in RIPA buffer (50 mM Tris, pH 7.8, 150 mM NaCl, 1% NP40, 0.1% sodium dodecyl sulfate (SDS), 0.25% deoxycholate, 10 $\mu$g/ml aprotinin, and 1 mM AEBSF and clarified by centrifugation in an Eppendorf microfuge for 15 min in the cold. Proteins were measured using the Bradford assay against a standard curve of bovine serum albumin. Protein, 40 $\mu$g per lane, was separated using SDS–PAGE and transferred to an Immobilon P membrane using standard techniques. Membranes were stained with Ponceau S to confirm transfer and equal loading, and destained. Membranes were blocked in TBST + 5% milk (50 mM Tris, pH 7.4, 200 mM NaCl, 0.05% Tween 20, 5% fat-free milk) for 1 hr at room temperature (RT). Primary antibody was diluted 1:200 in the same buffer and incubated overnight at 40°C. Membranes were washed, incubated with a 1:20,000 dilution of the appropriate horseradish peroxidase complexed secondary antibody for 1 hr, washed, and incubated with chemiluminescence substrates for 5 min. Membranes were exposed to film for between 1 and 10 min. Films were scanned and analyzed using U SCAN IT gel software (Silk Science).

### *Histological staining*

At the times indicated, cell monolayers were fixed with formalin (15 min) followed by staining with hematoxylin and eosin (H&E).

### *Statistical analysis*

The level of significance chosen for any analysis employed was 5%. The tests employed were one-way ANOVA or two-tailed *t* test. All data are ± SEM.

## RESULTS AND DISCUSSION

L6 myoblasts were plated and 24 hr later the medium was replaced with growth medium containing vehicle, 10 $\mu$M IDV, or 10 $\mu$M RTV. Three days after the addition of drug, the medium was changed to fusion medium, also containing vehicle, 10 $\mu$M IDV, or 10 $\mu$M RTV. To determine if a reduction in calpain activity was present, calpain activity was measured in cell homogenates of PI-treated versus untreated L6 myotubes (Fig. 1A and B). We found L6 cells treated with 10 $\mu$M RTV had reduced calpain activity at all time points and at higher concentrations, 20 $\mu$M RTV, no calpain activity was detectable. To determine if the decrease in calpain activity could be ascribed to a decrease in calpain protein we measured the level of calpain in immunoblots. We found no change in calpain protein levels when vehicle versus protease inhibitor-treated cell homogenates was compared (Fig. 1C). This suggests that RTV inhibits calpain activity in muscle cells.

To determine if the reduced calpain activity also reduced myoblast fusion we cultured L6 and C2C12 cells with vehicle, RTV, or IDV and examined the cells with time for the formation of myotubes (Fig. 2). As expected, L6 myoblasts cultured in growth medium did not develop multinucleated myotubes (Fig. 2A), whereas L6 myoblasts cultured in fusion medium had multinucleated myotubes first evident on Day 2 (data not

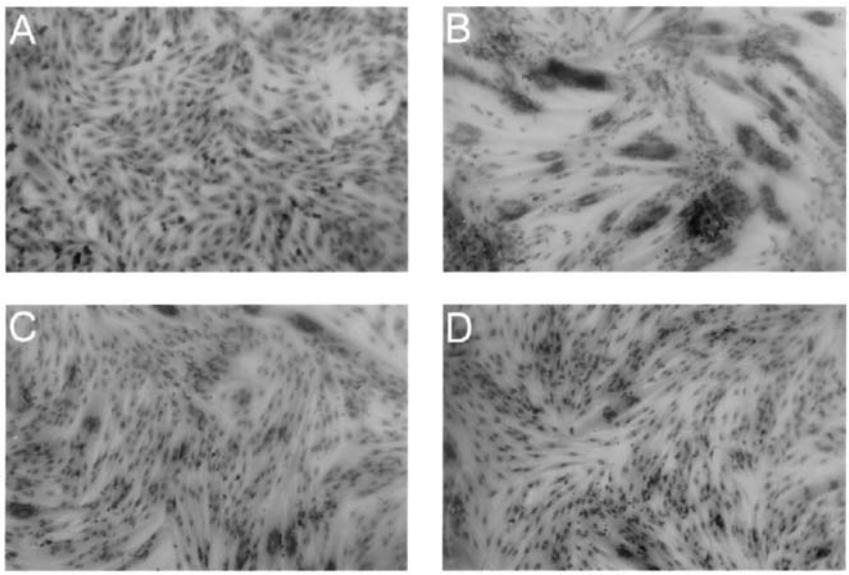


**FIG. 2.** Histological staining of L6 cells during induction. L6 myoblasts were grown to confluence in 10% DMEM in the presence or absence of protease inhibitors. (**A**) Control—Day 0 of induction; myogenesis was induced with 2% $\alpha$-MEM for 4 days. (**B**) Control. (**C**) IDV 10 $\mu$M. (**D**) RTV 10 $\mu$M. Cells were then fixed and stained with hematoxylin and eosin. Syncytia with greater than five nuclei represent myotubes (**B**).

shown) with the number and the extent of multinucleation (≥5 nuclei per syncytium) further increased on Day 4 (Fig. 2B). Thus, as previously reported, culture in reduced serum medium induced muscle differentiation *in vitro* in L6 myoblasts. In contrast, L6 myoblasts incubated with either IDV (Fig. 2C) or RTV (Fig. 2D) did not develop multinucleated myotubes by Day 4 of fusion medium incubation. Similar results were obtained when C2C12 cells were cultured with IDV or RTV (data not shown). To determine if myotube formation was delayed we continued culture in fusion media to Day 6, however, longer times did not result in evident myotube formation in the drug-treated muscle cells. These data suggest that the addition of these HIV protease inhibitors prevented morphologically evident muscle-specific differentiation. Both drugs successfully prevented myotube formation in both mouse (C2C12) and rat (L6) myoblast cell lines suggesting that this inhibition is not cell line specific and might be a class effect.

*In vitro* differentiation of L6 myoblasts to myotubes requires cessation from proliferation, expression of muscle differentiation inducers, and the accumulation of muscle-specific proteins.[18] To determine if the protease inhibitors affected upstream pathways leading to differentiation, we isolated whole cell protein homogenates from L6 myoblasts cultured in the presence or absence of RTV or IDV for 1, 2, and 4 days and measured the expression of cell cycle, muscle differentiation inducers, and muscle-specific proteins.

To begin differentiation myoblasts must permanently withdraw from the cell cycle. PCNA protein is elevated in cycling cells and reduced in quiescent cells. All samples had similar levels of PCNA protein initially that decreased with increasing time in fusion media (Fig. 3A). This indicates that the reduction in myotube formation was not due to continued cell proliferation in IDV- or RTV-treated cells. The concentrations of PI employed did not affect cell viability and apoptosis was not detected. We conclude that PI treatment permitted cell cycle arrest.

Muscle differentiation inducer expression is critical for myotube formation.[14] MyoD expression is typically expressed before myogenin expression and this sequential expression identifies early (MyoD) and late (myogenin) stages in muscle development. Expression of MyoD (Fig. 3B) and myogenin (Fig. 3C) was similar in control, IDV-, and RTV-treated cells. This suggests that activation of the sequential stages of the differentiation program was essentially identical in control and drug-treated cells. Our data indicate that the inhibition of myotube formation in the drug-treated cells was not due to the absence of these muscle-specific activating proteins.

The differentiation-inducing proteins might be present, but inactive. To determine if the differentiation inducers were active we measured expression of the major muscle protein, myosin heavy chain (MHC)[14] in vehicle, and drug-treated cell homogenates (Fig. 3D). MHC expression was undetectable at the time of change to fusion media on Day 1, however, MHC increased in amount with time in fusion medium in control and PI-treated cells. This suggests that the accumulation of muscle-specific contractile proteins is present in all samples. The data also suggest that the differentiation inducers, MyoD or myogenin, are active. We conclude that PIs do not reduce muscle-specific differentiation.

Our data indicate that drug-treated L6 myoblasts are able to exit the cell cycle, express muscle-differentiation inducers, and accrue differentiation-specific muscle proteins. The accumulated data indicate that the HIV protease inhibitors did not prevent the *in vitro* muscle-differentiation program, but inhibited myotube formation downstream of these major differentiation events, likely by reducing calpain activity. RTV was shown to competitively inhibit calpain activity in PC12 cells *in vitro* and *in situ* assays.[24] We show in the present studies that calpain activity is reduced in RTV-treated muscle cells in the absence of any decrease in calpain protein. Others have recently shown that IDV and RTV directly inhibit m-calpain.[29] That both inhibitors show the same pattern suggests that their method of inhibition is likely to be similar and that is a class effect. The HIV protease is an aspartyl protease and RTV and IDV are competitive inhibitors of the HIV protease.[1,2] We suggest that both RTV and IDV inhibit calpain activity and that calpain activity inhibition is responsible for the lack of myotube formation in these drug-treated cells. In support of this, calpain has been shown to be instrumental in brush border microvillous assembly during differentiation.[30] RTV was shown to inhibit calpain at clinically relevant concentrations.[30] It was concluded

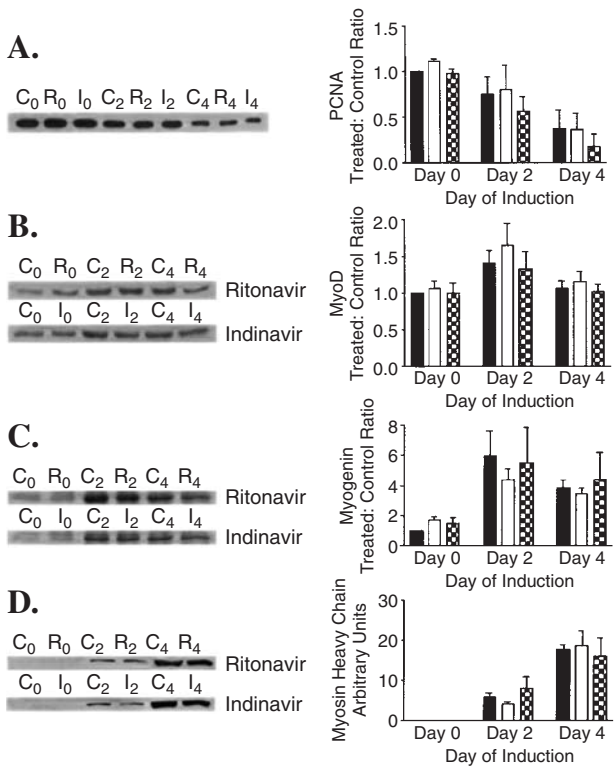


**FIG. 3.** Expression of muscle-specific proteins in L6 cells during myogenesis. L6 myoblasts grown in the absence or in the presence of RTV or IDV were harvested on Days 0, 2, and 4 following initiation of differentiation. Whole cell lysates were analyzed for (**A**) PCNA, (**B**) MyoD, (**C**) myogenin, and (**D**) myosin heavy chain by SDS–PAGE and immunoblotting. Results bands were quantified by scanning and densitometry. Filled bars represent control cells, open bars represent RTV 10 $\mu$M, and hatched bars represent IDV 10 $\mu$M.

that an RTV-mediated decrease in assembly might be involved in the diarrhea associated with high-dose RTV.[30]

Despite the use of HAART, wasting, which includes depletion of fat free mas, body cell mass, and fat mass, is still a problem for people with HIV-1 infection and wasting remains an independent prognosticator of mortality. Patients on HAART have reduced "lean body mass" or muscle.[4,5] When muscle versus adipose tissue-free body mass was assessed by multiscan magnetic resonance imaging, HAART-treated female and male AIDS patients had significantly decreased skeletal muscle mass suggestive of selective muscle atrophy.[31] Moreover, weight loss has either not been ameliorated by HAART, or if weight gain was reported did not include fat free mass.[32–34] This suggests that the reduction in viral load and/or increased ability to combat secondary infections expected to occur as a result of HAART did not result in a decrease in wasting. The finding of new cases of wasting only after HAART therapy initiation suggests that HAART therapy components contribute to wasting. Our *in vitro* data suggest that the PIs present in HAART might contribute to the muscle wasting by reducing muscle repair and remodeling. Thus, whereas HAART reduces viral load and promotes well-being we suggest that the PI components of HAART are part of the mechanism initiating or maintaining wasting. We suggest that the wasting of HV-positive patients before drug therapy is initiated due to the actions of the virus on the patient and the accumulated toll of secondary infections and malnutrition. We suggest that the wasting after HAART therapy is initiated, when the viral load and secondary infections are reduced, is due to the combined effects of the remaining viral load compounded by the effects of the PI drugs reducing muscle repair.

As a cautionary comment, these results are preliminary and demonstrably linked to therapy-related calpain inhibition *in vitro* only. Therefore, considering any changes in currently accepted antiretroviral therapeutic regimens based on this report would be highly premature and inappropriate in the absence of additional evidence.

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