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# Contortrostatin, a Snake Venom Disintegrin, Induces αvβ3-mediated Tyrosine Phosphorylation of CAS and FAK in Tumor Cells

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**Abstract** Contortrostatin is a homodimeric disintegrin that inhibits platelet aggregation and cell adhesion to extracellular matrix proteins by blocking integrins. The effect of contortrostatin on integrin-mediated signaling in tumor cells was investigated by studying tyrosine phosphorylation events and activation of specific signaling molecules. We found that at concentrations as low as 1 nM, soluble contortrostatin activates integrin signals leading to increased tyrosine phosphorylation of FAK and CAS, and that these signals are abolished by inhibiting Src family kinases. Using transfected 293 cells expressing specific integrins, it was determined that contortrostatin-generated signals are mediated exclusively by the αvβ3 integrin. This observation was extended by showing that cells lacking αvβ3, but expressing αvβ5 and α5β1, do not respond in this way to contortrostatin treatment. In cells expressing αvβ3, blocking contortrostatin binding with antibodies against αvβ3 completely abrogates contortrostatin signals. Monovalent disintegrins echistatin and flavoridin were incapable of affecting tyrosine phosphorylation alone, but when added simultaneously with contortrostatin, completely inhibited contortrostatin-initiated signals. We propose that the homodimeric nature of contortrostatin imparts the ability to crosslink αvβ3 integrins, causing Src activation and hyperphosphorylation of FAK and CAS. This activity may represent a novel mechanism by which tumor cell motility can be inhibited. J. Cell. Biochem. 79:28–37, 2000. 

**Key words:** integrin; integrin signaling; Src; αvβ3; vitronectin receptor; motility

Much of what is known about integrin signaling is the product of studies carried out in platelets [Huang et al., 1993], fibroblasts, and epithelial cell lines [Giancotti and Ruoslahti, 1999; Schlaepfer and Hunter, 1998]. Significant progress has been made in the area of integrin-mediated signaling and the role of integrins in cell motility, but this area remains understudied in tumor cells. Focal adhesion kinase (FAK) is a non-receptor tyrosine kinase that localizes with integrins and is phosphorylated on tyrosine residues upon cellular adhesion to the extracellular matrix (ECM) [Burridge et al., 1992]. FAK plays an important role in allowing integrins, which lack catalytic activity, to convert extracellular stimuli into intracellular signals. Cell binding to the ECM causes integrin clustering and association of a number of cytoskeletally associated proteins into complexes known as focal adhesions [Burridge et al., 1988]. FAK associates with the cytoplasmic domains of the β integrin subunits and can undergo *trans*-autophosphorylation at specific tyrosine residues, creating a binding site for the Src family of protein tyrosine kinases [Schaller et al., 1994]. Src then can phosphorylate tyrosines in the FAK activation loop, resulting in full catalytic activity. The function of FAK is complex and is not yet fully appreciated, however, a role of FAK in cell migration has recently been established [Gilmore and Romer, 1996; Sieg et al., 1999]. In one study, overexpression of FAK in CHO cells lead to enhanced migration [Cary et al., 1996], and association of the adapter protein CAS via its SH3 domain with the proline-rich region of FAK mediated this enhancement [Cary et al.,

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1998]. In addition to its SH3 domain, CAS contains multiple tyrosine residues that undergo phosphorylation in response to cell adhesion to the ECM, and this phosphorylation is dependent on FAK and Src [Vuori et al., 1996]. CAS is bound by SH2-domain containing proteins Crk and Nck. The CAS/Crk complex has been shown to serve as a "molecular switch" in the regulation of motility in carcinoma cells [Klemke et al., 1998]. Thus, FAK and CAS are important participants in integrin signaling and in the regulation of motility.

Disintegrins are a family of snake venom proteins that were first identified as inhibitors of platelet aggregation by virtue of their ability to block fibrinogen binding to the αIIbβ3 integrin [Huang et al., 1987]. This interaction was shown to be dependent on the RGD (Arg-Gly-Asp) sequence present in the C-terminal half of the disintegrins. This sequence is also found in fibrinogen and extracellular matrix proteins. The disintegrins are also characterized by the presence of highly conserved cysteine residues that are critical to maintaining the structure and activity of these molecules through disulfide bonds. Disintegrins have subsequently been shown to block cell adhesion to various ECM components by binding to different β1 and β3 integrins on various cell types [Niewiarowski et al., 1994]. A subfamily is emerging which includes several dimeric disintegrins with varying integrin binding specificities. All previously described disintegrins contain the tripeptide sequence RGD or KGD, but the recently described heterodimeric disintegrin EC3, has VGD in the EC3A subunit and MLD in the EC3B subunit substituted into this position [Marcinkiewicz et al., 1999].

We previously described the isolation of a distinct member of the growing family of dimeric disintegrins [Trikha et al., 1994b]. Contortrostatin, purified from the venom of *Agkistrodon contortrix contortrix,* has a molecular weight of 13.5 kDa and is unique since it contains two identical 6.75 kDa subunits [Trikha et al., 1994a]. Each subunit contains an RGD motif and 10 cysteines at conserved positions. However, contortrostatin appears to have a truncation at the amino terminus and lacks two cysteines present in other monomeric disintegrins. EC3 similarly lacks these two amino-terminal cysteines [Marcinkiewicz et al., 1999], suggesting that the dimeric structure is a result of the absence of these residues which leaves cysteines elsewhere in the protein available for interchain disulfide bond formation. Contortrostatin was shown to inhibit adhesion of melanoma cells to type I collagen, vitronectin, and fibronectin [Trikha et al., 1994a], and we are currently studying the antitumor and antiangiogenic properties of this disintegrin. Contortrostatin was found to inhibit human platelet aggregation and to block binding of an anti-αIIbβ3 antibody to platelets. Importantly, in addition to its ability to block aggregation, contortrostatin caused an increase in tyrosine phosphorylation of a subset of platelet proteins [Clark et al., 1994]. This activity distinguished contortrostatin from a monomeric disintegrin which blocked platelet aggregation but was unable to induce protein tyrosine phosphorylation. These findings led to further investigations into the functional consequences of contortrostatin structure and prompted a study of the role of contortrostatin in regulating integrin-mediated signaling in tumor cells. Here we report that contortrostatin has the unique ability to act as an integrin agonist by stimulating αvβ3-mediated tyrosine phosphorylation of important signaling molecules in tumor cells, an activity not found in monomeric disintegrins. We provide evidence that this activity may represent a novel mechanism through which tumor cell motility can be inhibited.

## MATERIALS AND METHODS

### Materials

MDA-MB-435 human mammary carcinoma cells were obtained from Janet Price (M.D. Anderson Cancer Center, Houston, TX) [Price et al., 1990]. T24 human bladder carcinoma cells were purchased from ATCC (Manassas, VA). Human embryonic kidney cells (line 293) transfected with cDNA for β3 and β5 integrin subunits and parental 293 cells were provided by Dr. Jeffrey Smith (The Burnham Institute, La Jolla, CA) [Lin et al., 1997]. OVCAR-5 human ovarian carcinoma cells were from Dr. Thomas Hamilton (Fox Chase Cancer Center, Philadelphia, PA) [Schilder et al., 1990]. Contortrostatin was purified from venom of the Southern Copperhead Snake (*Agkistrodon contortrix contortrix*) as described previously [Trikha et al., 1994a,b]. The monomeric disintegrins echistatin and flavoridin, and the general protease inhibitor cocktail

used in lysis buffers were obtained from Sigma (St. Louis, MO). Vitronectin and Matrigel were purchased from Becton Dickinson (Bedford, MA). PP1, a Src family inhibitor, was from Calbiochem (La Jolla, CA). Anti-phosphotyrosine monoclonal antibody (mAb) PY99 was obtained from Santa Cruz Biotechnology (Santa Cruz, CA). Anti-FAK and anti-CAS mAbs were purchased from Transduction Laboratories (Lexington, KY) and 7E3 mAb was provided by Dr. Marian Nakada from Centocor (Malvern, PA).

### Cell Culture, Preparation, and Stimulation

T24 and OVCAR-5 cells were maintained in RPMI 1640 medium containing 5% fetal bovine serum, and MDA-MB-435 and 293 cells were maintained in Dulbecco's modified Eagle's medium with 10% serum at 37°C in 5% $CO_2$. Prior to immunoblot analysis, cells were washed with phosphate-buffered saline (PBS) and starved in the appropriate serum-free medium for 6 h at 37°C. Cells were detached by brief treatment with 0.05% trypsin/0.02% EDTA in PBS and collected by centrifugation, resuspended in soybean trypsin inhibitor (1 mg/ml in serum-free medium), and washed in 2% bovine serum albumin/serum-free medium. Cells were maintained in suspension for 1 h in 2% bovine serum albumin/serum-free medium at 37°C with end-over-end agitation. Quiescent cells ($3 \times 10^6$/ml) were treated with disintegrins or other reagents while in suspension, or were allowed to adhere to Matrigel diluted 1:100 with serum-free medium during treatment.

### Lysate Preparation and Immunoprecipitation

Suspended and adherent cells were washed twice with cold PBS and lysed in cold lysis buffer (50 mM Tris, pH 8.0, 150 mM NaCl, 1% Nonidet P-40, 0.5% deoxycholate, 0.1% SDS, protease inhibitor cocktail, 1 mM sodium pyrophosphate, 1 mM sodium orthovanadate, 50 mM sodium fluoride). After 10–15 min incubation on ice, insoluble material was removed by centrifugation at 14,000 RPM in a microcentrifuge for 15 min. Supernatants were collected and total protein concentrations standardized by the BCA protein assay (Pierce, Rockford, IL). Immunoprecipitation was carried out by incubating whole cell lysates (200 μg total protein) with 1.25 μg anti-FAK or anti-CAS mAb 4–6 h at 4°C followed by 20 μl protein G-agarose overnight at 4°C. Immunoprecipitates were washed four times in lysis buffer without inhibitors and dissociated by adding SDS-PAGE sample buffer and boiling 5 min. Whole cell lysates (30 μg total protein) or immunoprecipitates were resolved by 7.5% SDS-polyacrylamide gel electrophoresis and transferred to nitrocellulose membranes.

### Immunoblotting

Membranes were blocked with 5% nonfat milk/Tris-buffered saline/0.1% Tween 20 (blocking buffer) 1 h at room temperature or overnight at 4°C. Primary antibody incubations were performed in blocking buffer for 1 h at room temperature. After washing in Tris-buffered saline/0.1% Tween 20, membranes were incubated with horseradish peroxidase-conjugated secondary antibody in blocking buffer 1 h at room temperature. Membranes were washed extensively. Immunoblots were developed using Super Signal® West Pico Chemiluminescent Substrate from Pierce. Densitometry was performed using UN-SCAN-IT$_{TM}$ software (Silk Scientific, Orem, UT).

### Motility Assays

Tumor cell motility was quantitated using a modified Boyden chamber [Repesh, 1989]. Transwell chambers with 12 μm pore size (Corning Costar, Cambridge, MA) were coated with Matrigel diluted 1:100 with serum-free medium. Treated or untreated cells were added to the upper chamber, and the lower chamber was filled with HT1080 conditioned medium. Cells were incubated at 37°C for 10 h and after removal of non-migrating cells, the number of cells migrating to the bottom side of the coated membrane were fixed, stained, and quantitated using digital image analysis (NIH Image).

## RESULTS

### Contortrostatin Treatment Induces Protein Tyrosine Phosphorylation in Tumor Cells

To investigate the role of contortrostatin in regulating overall tyrosine phosphorylation in tumor cells, MDA-MB-435 human breast carcinoma cells were treated for 10 min with various concentrations of soluble contortrostatin while in suspension. The cells showed a dramatic increase in tyrosine phosphorylation of proteins with molecular weights from 120–140

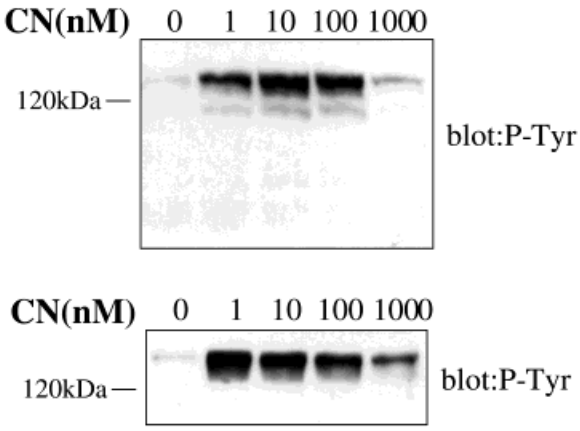


**Fig. 1.** Phosphotyrosine levels in tumor cells after contortrostatin treatment. MDA-MB-435 cells (**upper**) or T24 cells (**lower**) were treated in suspension with the indicated concentrations of soluble contortrostatin (CN) for 10 min. Whole cell lysates (30 μg total protein) were subjected to SDS-PAGE and immunoblotting with antibody against phosphotyrosine (PY99) as described in Materials and Methods.

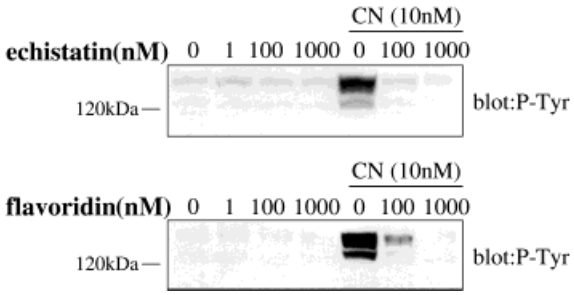


**Fig. 2.** Effects of monomeric disintegrins on tyrosine phosphorylation in tumor cells. Suspended MDA-MB-435 cells were treated with the indicated concentrations of echistatin (**upper**) or flavoridin (**lower**) or were treated simultaneously with monomeric disintegrins and 10 nM contortrostatin (CN) as indicated for 10 min. Lysates were analyzed for phosphotyrosine content by immunoblot as described in Materials and Methods.

kDa following contortrostatin treatment (Fig. 1). Maximal levels of tyrosine phosphorylation were observed at 10 nM contortrostatin, with reduced levels observed at higher concentration (1,000 nM). Tyrosine phosphorylation of proteins in this size range was also observed in T24 human bladder carcinoma cells (Fig. 1) and KSY-1 Kaposi's sarcoma cells (data not shown) following contortrostatin treatment using the same methods, indicating that this phenomenon is not cell-type specific.

### Monomeric Disintegrins, Echistatin, and Flavoridin, do not Induce Tyrosine Phosphorylation in Tumor Cells

In an effort to determine whether the ability to stimulate tyrosine phosphorylation in tumor cells was related to the homodimeric structure of contortrostatin, we tested two well-characterized monomeric disintegrins, echistatin [Gan et al., 1988] and flavoridin [Niewiarowski et al., 1994], for their ability to affect integrin signaling in suspended MDA-MB-435 cells. In sharp contrast to the observed effects of contortrostatin, the monomeric disintegrins alone had no effect on tyrosine phosphorylation at concentrations up to 1 μM. However, when echistatin or flavoridin (1 μM) were added simultaneously with contortrostatin (10 nM), contortrostatin-induced tyrosine phosphorylation was completely abrogated (Fig. 2). These findings indicate that the monomeric disintegrins competitively inhibit contortrostatin binding to specific signal-generating integrins.

### Contortrostatin-Induced Signaling Events are Mediated by the αvβ3 Integrin

The integrin-binding specificities of echistatin and flavoridin have been determined previously [Niewiarowski et al., 1994; Pfaff et al., 1994]. Both monomeric disintegrins interact with αIIbβ3, αvβ3, and α5β1. We have shown that contortrostatin binds these same integrins [Markland and Zhou, 1999; Trikha et al., 1994a,b], as well as αvβ5 [Zhou et al., 2000]. With the knowledge that αIIbβ3 is not expressed on MDA-MB-435 cells, as shown by a lack of staining with 10E5, a specific anti-αIIbβ3 mAb [Markland and Zhou, 1999], this implicated αvβ3, α5β1, or both, in contortrostatin-induced signaling events, based on the monomeric disintegrin vs. contortrostatin observations presented above. In order to determine which receptor(s) was involved, we employed transfected 293 cell lines with specific integrin profiles [Lin et al., 1997]. The parental 293 cells express the αv subunit but have no detectable αvβ3 and only trace levels of αvβ5 expression. Cells transfected with cDNAs encoding the β3 or β5 integrin subunits show significant levels of the αvβ3 or αvβ5 heterodimers, respectively [Lin et al., 1997]. Integrin expression was confirmed in our laboratory by flow cytometry. When these cell lines are treated with contortrostatin using established methods, only the αvβ3-expressing cells show the robust induction of tyrosine phos-

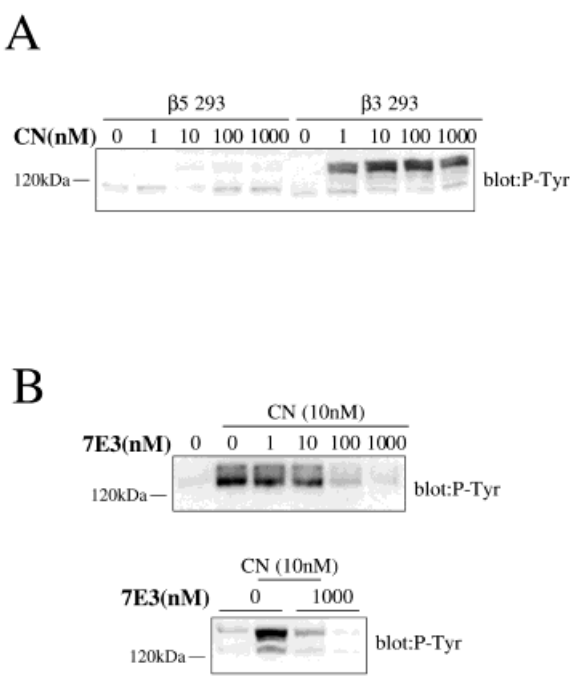


**Fig. 3.** Contortrostatin-induced tyrosine phosphorylation is mediated by the αvβ3 integrin. **A:** Suspended 293 cells expressing either αvβ5 (β5 293) or αvβ3 (β3 293) were treated for 10 min with the indicated concentrations of contortrostatin (CN). β3 293 cells responded to contortrostatin treatment with increased tyrosine phosphorylation while β5 293 cells showed no response. Lysates were analyzed for phosphotyrosine content by immunoblot as described in Materials and Methods. **B:** T24 cells (**upper**) or MDA-MB-435 cells (**lower**) were treated simultaneously for 10 min with contortrostatin (CN) and the indicated concentrations of the anti-αvβ3 mAb 7E3. Whole cell lysates were analyzed by phosphotyrosine immunoblot.

phorylation observed in other cell types (Fig. 3A). Importantly, the proteins undergoing tyrosine phosphorylation in the β3 transfected cells are in the 120–140 kDa range, the same molecular weights as those observed in other cell lines tested. These findings demonstrate the involvement of αvβ3 in contortrostatin signaling but do not directly address the potential contribution of α5β1, since echistatin and flavoridin are known to bind α5β1. This possibility was ruled out by studies using 7E3, a mAb generated against αIIbβ3 that has equal affinity for αvβ3 [Tam et al., 1998]. Contortrostatin-induced tyrosine phosphorylation was completely blocked when MDA-MB-435 cells, which express αvβ3, αvβ5, and α5β1 [Chandrasekaran et al., 1999; Wong et al., 1998], are treated simultaneously with 1 μM 7E3 and 10 nM contortrostatin for 10 min (Fig. 3B, lower panel). This result was duplicated in T24 cells (Fig. 3B, upper panel), providing convincing evidence that αvβ3 is solely responsible for mediating contortrostatin-induced tyrosine phosphorylation. Further proof of this finding is found in OVCAR-5, a human ovarian carcinoma cell line that is negative for αvβ3 expression but expresses αvβ5 and α5β1. These cells do not show significant enhancement of tyrosine phosphorylation when treated with contortrostatin (data not shown). Thus, despite the ability to interact with other integrins, contortrostatin induces protein tyrosine phosphorylation in tumor cells exclusively via the αvβ3 integrin.

### Contortrostatin Binding to αvβ3 Results in Tyrosine Phosphorylation of CAS and FAK

In order to identify the specific proteins that are tyrosine phosphorylated in response to contortrostatin treatment, lysates prepared from contortrostatin-treated cells were subjected to immunoprecipitation with CAS or FAK monoclonal antibodies followed by anti-phosphotyrosine immunoblotting. CAS and FAK were selected as likely candidates based on the similarity of their molecular weights (130 and 125 kDa, respectively) with those observed on the anti-phosphotyrosine immunoblots with whole cell lysates. We found that CAS and FAK are both tyrosine phosphorylated in response to contortrostatin treatment (Fig. 4), and immunoprecipitated CAS and FAK co-migrate with the major bands observed after anti-phosphotyrosine immunoblot with whole cell lysates.

### Activity of Src Family Kinases is Necessary for Contortrostatin-Induced Tyrosine Phosphorylation Events

The Src family of tyrosine kinases is known to play a central role in integrin signaling. In order to determine if Src family kinases participate in transmitting contortrostatin-induced signals from αvβ3, T24 cells were pretreated in suspension for 30 min with the Src family inhibitor PP1 [Hanke et al., 1996; Schlaepfer et al., 1998], prior to stimulation with 10 nM contortrostatin. As shown in Figure 5 (upper panel), PP1 demonstrates a dose-dependent inhibition of tyrosine phosphorylation with complete elimination of contortrostatin-induced

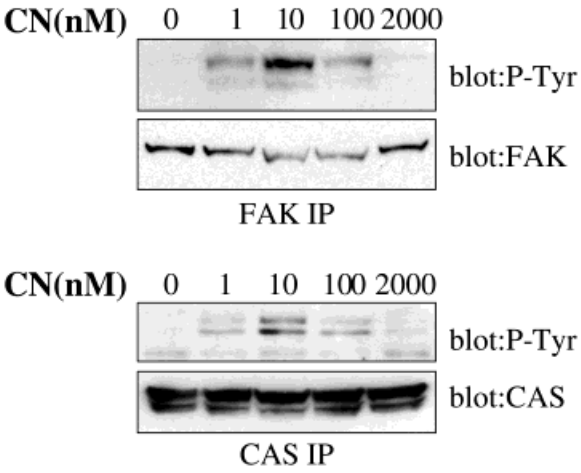


**Fig. 4.** Contortrostatin treatment causes tyrosine phosphorylation of CAS and FAK. Lysates from MDA-MB-435 cells were immunoprecipitated with antibodies specific for CAS or FAK followed by anti-phosphotyrosine immunoblotting, or were immunoblotted with the same antibody used for immunoprecipitation to demonstrate equal loading. FAK (**upper**) and CAS (**lower**) are maximally tyrosine phosphorylated at 10 nM contortrostatin (CN), which corresponds to the peak in tyrosine phosphorylation with varying contortrostatin concentrations (Fig. 1).

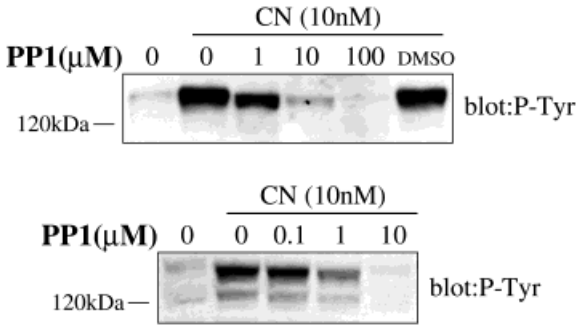


**Fig. 5.** Involvement of Src family kinases in contortrostatin-induced tyrosine phosphorylation. T24 cells (**upper**) or MDA-MB-435 cells (**lower**) were pretreated with the indicated concentrations of the Src family inhibitor PP1 for 30 min prior to stimulation with 10 nM contortrostatin (CN). Lysates were prepared and analyzed by anti-phosphotyrosine immunoblot. Stock solutions of PP1 are prepared in DMSO, but DMSO alone had no effect on contortrostatin-induced tyrosine phosphorylation (**upper**).

signals at a concentration of 100 μM. Similar results were obtained following PP1 treatment of MDA-MB-435 cells, although complete inhibition was achieved at 10 μM PP1 (Fig. 5, lower panel). These findings implicate the Src family kinases as being involved in integrin signaling stimulated by contortrostatin.

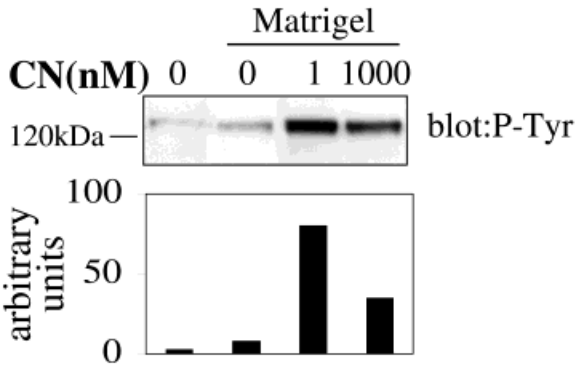


**Fig. 6.** Contortrostatin-induced tyrosine phosphorylation is independent of cellular adhesion. T24 cells were pretreated with the indicated concentrations of contortrostatin (CN) for 5 min prior to addition to Matrigel-coated plates. Control cells were maintained in suspension. Lysates were prepared after incubating cells on Matrigel for 20 min and analyzed by anti-phosphotyrosine immunoblot (**upper**). The **lower** panel shows the relative intensity of the corresponding bands as determined by densitometry.

### Contortrostatin-Induced Tyrosine Phosphorylation Is Independent of Cellular Adhesion

To determine if contortrostatin is able to affect tyrosine phosphorylation in adherent cells where integrin ligation and cytoskeletal structure exist, T24 cells were pretreated with contortrostatin before allowing them to adhere to Matrigel-coated plates. It should be noted that contortrostatin does not significantly inhibit cellular binding to laminin. Therefore, contortrostatin does not inhibit cellular binding to Matrigel, which is rich in laminin. Phosphotyrosine immunoblotting revealed that T24 cells showed modest increases in tyrosine phosphorylation after adhesion to Matrigel, but contortrostatin treatment of adherent cells causes a significant additional increase in these signals, including the 120–140 kDa bands shown to contain CAS and FAK (Fig. 6). It was again observed that high concentrations of contortrostatin (1,000 nM) generated reduced levels of tyrosine phosphorylation. In similar experiments, T24 cells were allowed to adhere to Matrigel for 30 min prior to treatment with contortrostatin. Following an additional 30 min incubation on Matrigel in the presence of contortrostatin, cells showed similar increased tyrosine phosphorylation of the 120–140 kDa bands. Thus, contortrostatin induction of tyrosine phosphorylation can occur in

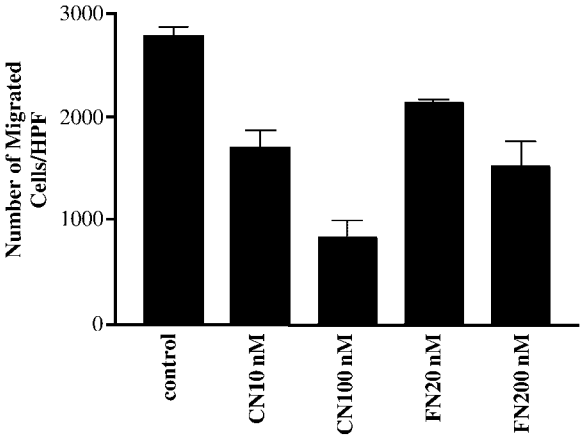


**Fig. 7.** Contortrostatin inhibits tumor cell motility more potently than the monomeric disintegrin flavoridin. Flavoridin (FN) was used at twice the concentration of contortrostatin (CN) to equalize the number of integrin-binding RGD sites present. Contortrostatin significantly inhibits motility when compared to control cells, and at 100 nM shows nearly double the inhibitory activity of flavoridin at 200 nM. Data shown represents the average number of cells per randomly selected high power field (HPF). Error bars indicate SEM. Experiments were independently performed four times to confirm results.

adherent cells, in the presence of stimuli from ECM proteins, as well as in non-adherent cells.

### Contortrostatin Inhibits MDA-MB-435 Cell Migration on Matrigel

The functional effect of contortrostatin treatment on tumor cells was investigated by studying the ability of cells to migrate on Matrigel in the presence of the disintegrin. Contortrostatin had a significant negative effect on migration and, importantly, showed comparatively higher inhibitory activity relative to the monomeric disintegrin flavoridin at twice the concentration (Fig. 7). By removing the possibility that contortrostatin achieves this increased inhibition simply through more integrin antagonism due to the presence of two RGD sites vs. one in flavoridin, this data suggests that contortrostatin possesses additional activity not present in monovalent disintegrins.

## DISCUSSION

Since their discovery, disintegrins have been studied almost exclusively for their ability to block the function of various integrins. Disintegrins have been used extensively to investigate the function of αIIbβ3 on platelets, and more recent work has been conducted with endothelial cells in the study of the role of αvβ3 in angiogenesis [Kang et al., 1999; Sheu et al., 1997; Yeh et al., 1998; Zhou et al., 2000a]. In these reports, the antiangiogenic effects of the disintegrins are described as a function of their ability to block αvβ3, an integrin which has been shown to be involved in induction of endothelial cell apoptosis [Brooks et al., 1994b]. However, the direct effects of disintegrins on integrin-mediated signaling remains largely unstudied. In one report, echistatin was shown to cause a decrease in FAK phosphorylation and disassembly of focal adhesions prior to melanoma cell detachment from fibronectin [Staiano et al., 1997]. In contrast to these descriptions of disintegrins as passive integrin-blocking agents, the present work shows that the disintegrin, contortrostatin, has a structure that enables it to function as an integrin agonist, initiating signals that are usually observed only after cellular binding to natural ECM ligands or artificial crosslinking with anti-integrin antibodies. Our studies suggests that contortrostatin actively regulates the function of αvβ3 in tumor cells. The effects of contortrostatin on integrin signaling have been studied previously in platelets where it was found that the contortrostatin dimer and the monomeric disintegrin, multisquamatin, both inhibited αIIbβ3-mediated platelet aggregation and aggregation-dependent tyrosine phosphorylation of numerous proteins including FAK [Clark et al., 1994]. A distinct set of platelet proteins have been shown to become tyrosine phosphorylated upon αIIbβ3 crosslinking with fibrinogen or αIIbβ3 antibodies [Huang et al., 1993]. Contortrostatin was shown to activate tyrosine phosphorylation of these same proteins, presumably by virtue of its dimeric structure, since the monomeric disintegrin did not activate tyrosine phosphorylation. However, there are notable differences between the present study and the work performed in platelets, particularly the tyrosine phosphorylation status of FAK following contortrostatin treatment. This discrepancy is likely the result of differences in the regulatory mechanisms of FAK tyrosine phosphorylation in platelets and tumor cells. In platelets, FAK phosphorylation is dependent on platelet aggregation, and does not occur after fibrinogen binding to αIIbβ3 alone, indicating that events occurring during platelet-platelet interaction, and not integrin crosslinking, are critical in

regulating FAK phosphorylation [Lipfert et al., 1992]. In contrast, studies in fibroblasts show that FAK undergoes tyrosine phosphorylation after integrin clustering with non-inhibitory mAbs in the absence of integrin ligation [Miyamota et al., 1995]. Thus, simple dimerization of integrins is sufficient to cause FAK phosphorylation in fibroblasts, and this mechanism is expected to function through αvβ3 crosslinking during contortrostatin-induced signaling in tumor cells.

From the observations presented in this report, we propose that each subunit of contortrostatin binds to a separate αvβ3 integrin, bringing the integrins into close proximity allowing *trans*-autophosphorylation of integrin-associated FAK, creating a binding site for Src [Schaller et al., 1994]. Binding of Src leads to further tyrosine phosphorylation of FAK [Schlaepfer and Hunter, 1996] and to Src-mediated phosphorylation of CAS [Vuori et al., 1996]. We observed in this study that at high concentrations of contortrostatin (1 μM), the levels of tyrosine phosphorylation decreased. This apparent paradox might be explained when taking into account two possible binding orientations of the contortrostatin dimer. At low concentrations, each of the two chains of the dimer binds to a different αvβ3 molecule on a single cell, bringing them into close proximity and allowing for the initiation of signaling cascades. At high concentrations, only one of the two chains of the dimer binds to αvβ3 due to competitive binding between dimers, and integrin clustering will not occur. Under these conditions, contortrostatin acts as a monovalent antagonist, and will not initiate tyrosine phosphorylation, nor will it display the additional functional inhibitory activity. This interpretation is consistent with our biochemical observations (Figs. 1 and 4) and with the observations made in the tumor cell motility assays where at high concentrations ($> 1$ μM) contortrostatin and the monomer flavoridin behave similarly, both inhibiting motility by ~90% (data not shown). The overall effect of contortrostatin on motility thus appears to be a summation of passive integrin antagonism and active disruption of integrin-mediated signaling. In another study, soluble monomeric and multimeric vitronectin were studied for their ability to differentially regulate tyrosine phosphorylation in bovine pulmonary artery endothelial cells [Bhattacharya et al., 1995]. Multimeric vitronectin was shown to mediate enhanced tyrosine phosphorylation of several proteins, including FAK, yet monomeric vitronectin did not produce this effect. This finding was confirmed in our studies with MDA-MB-435 cells in which monomeric vitronectin failed to stimulate tyrosine phosphorylation at 10 μg/ml (data not shown). These studies support our findings that contortrostatin possesses the ability to initiate αvβ3-mediated signaling by crosslinking integrins at the surface of tumor cells, resulting in dramatic stimulation of tyrosine phosphorylation of the important signaling molecules FAK and CAS. Attention has been directed to these two molecules recently with respect to their roles in cell motility, and their physiological importance has been highlighted in a report investigating the role of a protein tyrosine phosphatase, PTP-PEST [Angers-Loustau et al., 1999]. Fibroblasts lacking expression of PTP-PEST show severe defects in motility. Biochemical and immunocytochemical analysis revealed that this defect was due in part to a constitutive increase in tyrosine phosphorylation of CAS, FAK, and paxillin and to an increase in the number of focal adhesions present. These similarities suggest a functional consequence of tumor cell treatment with contortrostatin where a disruption, through αvβ3-mediated hyperphosphorylation of CAS and FAK, occurs in the delicate and highly regulated machinery that drives cell motility. The involvement of αvβ3 and FAK in tumor cell motility is emphasized in a recent report showing that the presence of αvβ3 on human prostatic carcinoma cells generated a migratory phenotype that is modulated by pathways involving FAK [Zheng et al., 1999]. In addition to its involvement in motility, αvβ3 has been shown to be critical to other events in tumor progression, including localization of MMP-2 and degradation of the surrounding matrix [Brooks et al., 1996], and in tumor-induced angiogenesis [Brooks et al., 1994a,b]. Conclusive proof that the homodimeric structure of contortrostatin is what imparts this activity, and not some other unidentified properties, can be provided through use of a monomeric form of contortrostatin. At present, this form of contortrostatin is not available, however, efforts are underway to generate monomeric contortrostatin.

In conclusion, this work identifies activity novel to the disintegrin family through which

integrin signaling can be modulated in tumor cells. This activity appears to be unique to contortrostatin, as three other disintegrins have been found to lack the ability to stimulate tyrosine phosphorylation [Clark et al., 1994]. Recently, a number of new dimeric disintegrins have been purified from various snake venom [Marcinkiewicz et al., 1999]. It is unlikely that these molecules would demonstrate activity similar to contortrostatin because they are heterodimers lacking the RGD sequence, and they do not appear to interact with αvβ3. Our report identifies contortrostatin as a useful reagent for the further study of αvβ3 function, and identifies a novel integrin-mediated mechanism that may negatively effect tumor cell motility. We propose that the combined effects of blocking the binding of αvβ5, α5β1, and αvβ3 to the ECM, and the initiation of inappropriate signals leading to hyperphosphorylation of critical signaling molecules, leads to immobilization of otherwise motile and invasive tumor cells.

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