Disruption of matrix metalloproteinase 2 binding to integrin $\alpha_v\beta_3$ by an organic molecule inhibits angiogenesis and tumor growth *in vivo*

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Matrix metalloproteinase 2 (MMP2) can associate with integrin $\alpha_v\beta_3$ on the surface of endothelial cells, thereby promoting vascular invasion. Here, we describe an organic molecule (TSRI265) selected for its ability to bind to integrin $\alpha_v\beta_3$ and block $\alpha_v\beta_3$ interaction with MMP2. Although disrupting $\alpha_v\beta_3$/MMP2 complex formation, TSRI265 has no effect on $\alpha_v\beta_3$ binding to its extracellular matrix ligand vitronectin and does not influence MMP2 activation or catalytic activity directly. However, TSRI265 acts as a potent antiangiogenic agent and thereby blocks tumor growth *in vivo*. These findings suggest that activated MMP2 does not facilitate vascular invasion during angiogenesis unless it forms a complex with $\alpha_v\beta_3$ on the endothelial cell surface. By disrupting endothelial cell invasion without broadly suppressing cell adhesion or MMP function, the use of compounds such as TSRI265 may provide a novel therapeutic approach for diseases associated with uncontrolled angiogenesis.

Invasion of vascular cells into tissues requires the coordinated interplay of numerous factors including proteases, which remodel the extracellular matrix architecture, as well as cell adhesion receptors that recognize this provisional matrix. Recent reports have implicated that the 72-kDa matrix metalloproteinase (MMP2) is a key player in vascular development and angiogenesis. For example, MMP2 and its activator membrane type 1 (MT1)–MMP are coordinately expressed by mesenchymal cells almost exclusively during embryonic development, indicating specific matrix remodeling constraints in these tissues (1). In addition, angiogenesis and corresponding tumor growth are reduced in MMP2 knockout mice (2). Interestingly, ligation of the integrin $\alpha_v\beta_3$, itself a known mediator of angiogenesis, induces MMP2 production (3, 4), suggesting a coordinated interplay of these two molecules during the vascular remodeling associated with blood vessel formation. In fact, direct interaction between MMP2 and integrin $\alpha_v\beta_3$ has been demonstrated (5), and the negative regulation of MMP2 during vascular invasion and maturation has been shown to depend on expression of $\alpha_v\beta_3$ (6).

Although the inhibition of angiogenesis and concomitant suppression of tumor growth by natural as well as synthetic inhibitors of MMPs (including MMP2) have been thoroughly documented, the translation of such strategies into clinical modalities has met with limited success, primarily because of the deleterious side effects of such broad-spectrum inhibitors. Because MMP function in general may be required for many processes in the adult organism, active-site inhibition of enzymatic function is likely to have far-reaching effects on various biological processes involving tissue remodeling, such as wound healing. In fact, it has been documented in clinical studies that therapy of various cancer types with broad-spectrum MMP inhibitors causes severe side effects, including inflammatory tendonitis, polyarthritis, and musculoskeletal pain syndromes, which are dose limiting and often persist after discontinuation of therapy (7, 8). However, given the limited distribution of integrin $\alpha_v\beta_3$ in the adult, one would predict that targeting the interaction between MMP2 and $\alpha_v\beta_3$ to the overlapping area of expression exclusively (i.e., areas of neovascularization or cellular invasion) should correspondingly limit the effects of such treatment-related toxicities. Indeed, application of the recombinant noncatalytic carboxyl-terminal hemopexin domain of MMP2 (PEX), which mediates MMP2 binding to integrin $\alpha_v\beta_3$, has shown antiangiogenic and antitumor activity *in vivo* (6), demonstrating the potential utility of such a targeted strategy. However, the limitations of attempting a treatment protocol with such a large protein fragment (e.g., large-scale production problems, Food and Drug Administration quality and safety control issues, and antigenicity) suggested the need for a more practical solution to this problem.

In an effort to develop an organic inhibitor selective in this manner, we screened a chemical library of potential antagonist compounds for inhibition of MMP2 binding to $\alpha_v\beta_3$ in a solid-phase binding assay. The compounds examined were derived by a combinatorial chemistry approach involving a library of 600 different compounds, which was designed to mimic potential protein–protein interactive moieties and will be described in a future publication. After extensive analysis, a candidate compound with favorable solubility and stability characteristics was synthesized (TSRI265; Fig. 1). This molecule is homobifunctional, comprising a pair of identical subunits separated by a benzene linker moiety. Each side chain subunit is a substituted lysine diamide, thus yielding a bivalent derivatized dilysine tetraamide final structure. As an inactive control, a compound was produced that contained minor modifications to the ends of the structure and a subtle alteration in the subunit linker (TSRI359; Fig. 1). Examination of compound TSRI265 demonstrated that the suppression of MMP2/integrin $\alpha_v\beta_3$ binding observed *in vitro* translated into antiangiogenic and antitumor effects, which were independent of direct effects on MMP2 activity *in vivo*. Thus, TSRI265 or derivative compounds may prove useful in the newly emerging field of selective inhibitors of protein–protein interaction, molecules whose mechanism of action may provide a therapeutic alternative that presents less incidence of deleterious side effects.

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Abbreviations: CAM, chorioallantoic membrane; MMP, matrix metalloproteinase; VN, vitronectin; bVN, biotinylated VN; HRP, horseradish peroxidase; vWF, von Willebrand factor.

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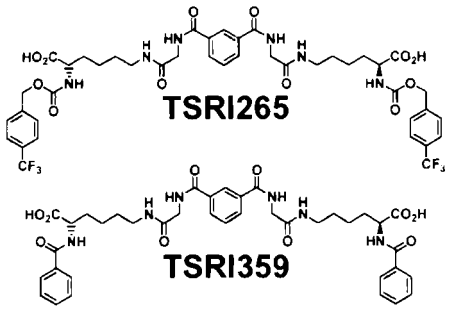


Fig. 1. Chemical structure of organic compounds TSRI265 and TSRI359. The differences between the active compound TSRI265 and the control compound TSRI359 are limited to an additional oxygen in the benzyl spacer arm of TSRI265, yielding a benzoyl carbamate instead of the benzoyl amide linkage observed in TSRI359, and the presence of a trifluoromethyl substitution in the para position of the terminal benzyl ring on TSRI265 in place of an unsubstituted benzyl moiety in TSRI359.

Materials and Methods

Antibodies, Cells, and Reagents. CS-1 hamster melanoma cells and CS-1 cells transfected with the human β_3-integrin subunit (β_3CS-1 cells) have been described previously (5, 6). The horseradish peroxidase (HRP)-conjugated antibiotin mAb BN-34 and the anti-actin mAb AC-40 are from Sigma. The anti-von Willebrand factor (vWF) polyclonal antibodies are from Dako. The cyclic peptides 66203 (cRGDfV) and 69601 (cRADfV) and integrin $\alpha_v\beta_3$ were generously provided by Merck. Purified proMMP2 and integrin $\alpha_5\beta_1$ were generously provided by Chemicon. Purified active MMP2 is from Calbiochem. Basic fibroblast growth factor was kindly provided by Scios (Mountain View, CA).

Synthesis of Unlabeled and ^{14}C-Labeled Compounds. Agents were synthesized in a four-step sequence starting with N^{ε}-Boc-lysine methyl ester (Calbiochem–Novabiochem). The α-amino group was first reacted with 4-(trifluoromethyl)benzyl alcohol and *N*,*N*′-disuccinimidyl carbonate, installing the carbamate functionality (TSRI265) or benzoyl chloride providing the benzoyl amide substitution (TSRI359). Each lysine derivative was then subjected to Boc-deprotection (HCl) and coupled to *N*-Boc-glycine, followed by a second deprotection (HCl) and coupled to isophthaloyl dichloride, providing the penultimate dimeric intermediates that were purified by silica gel chromatography. The synthesis was completed by saponification (LiOH) of the two methyl ester groups, providing TSRI265 in 60% overall yield or TSRI359 in 32% overall yield as white powders that were found to be >97% pure by ^{1}H NMR spectroscopy and HPLC analysis. Solubility was determined to be >100 μM in 0.1% DMSO/PBS. [^{14}C]TSRI265 (specific activity 110 mCi/mmol) was prepared following the same procedure outlined above by using *N*-Boc-[1-^{14}C]Gly (American Radiolabeled Chemicals, St. Louis) in 25% overall yield. A more detailed description of the synthesis of these agents, as well as characterization data for all products and intermediates, will be described in a future publication.

Solid Phase Integrin-Binding Assays. Purified integrins were adsorbed overnight onto microtiter wells (1–5 μg/ml, 50 μl/well) before blocking with Caseinblocker (Pierce). Purified biotinylated MMP2 (bMMP2, 3–5 nM) in binding buffer (50 mM Tris, pH 8/150 mM NaCl/1 mM $MgCl_2$/1 mM $CaCl_2$/0.5 mM $MnCl_2$) was added to the wells in the presence or absence of TSRI265, TSRI359, cyclic RGD or RAD peptides, or buffer vehicle alone. Control wells received no integrin, and biotinylated vitronectin (bVN, 1 μg/ml) was used as a reference. Bound protein was detected with an HRP–antibiotin mAb and quantitated at 450 nm with TMB (Bio-Rad).

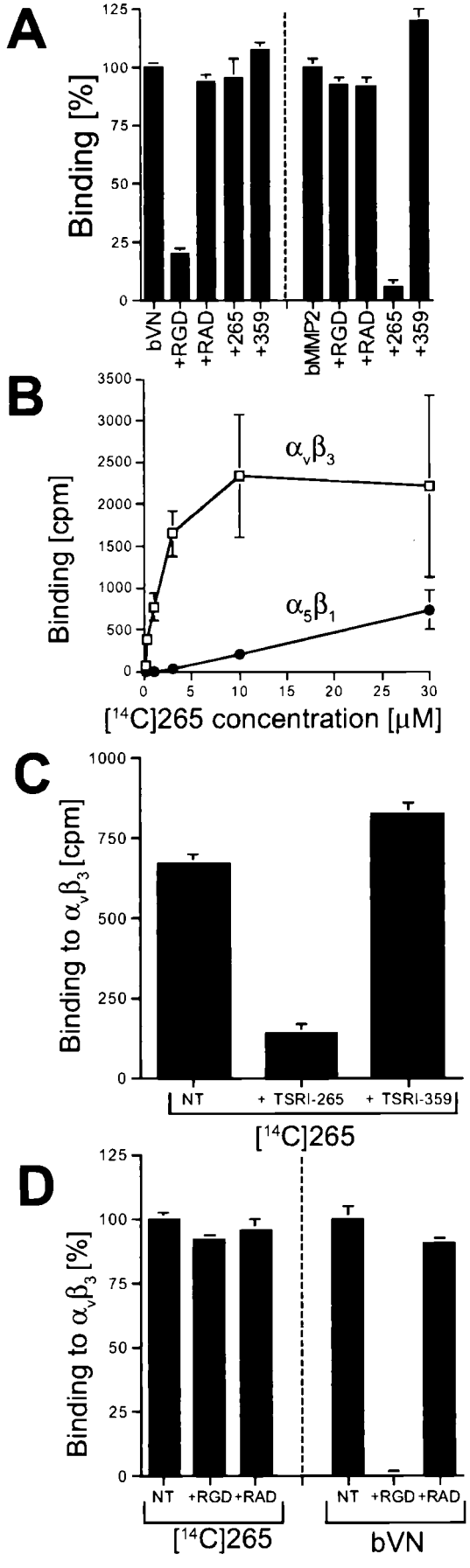
For the assessment of direct integrin binding by TSRI265, $\alpha_v\beta_3$ and $\alpha_5\beta_1$ (10 μg/ml, 50 μl/well) were coated onto Immulon 4-microtiter wells (Dynatech). As a control for integrin coating, fibronectin binding as well as antiintegrin antibody binding showed equivalent levels for both integrins (data not shown). Blocked wells were incubated with a titration of [^{14}C]TSRI265 before the addition of 150 μl of binding buffer containing 0.1% Tween-20 and aspiration of all liquid. Dried wells were separated and immersed in BetaMax liquid scintillation mixture (ICN) for quantification. From this binding curve, a subsaturating concentration (3 μM) of [^{14}C]TSRI265 was examined in the presence or absence of a 25-fold molar excess (75 μM) of unlabeled TSRI265 or TSRI359, or 100 μM cyclic RGD or RAD peptide. bVN was used as a control and detected as described above.

MMP2 Cell Binding and [^{3}H]Collagen IV Degradation Assays. CS-1 cells or β_3CS-1 cells were incubated in adhesion buffer (Fibroblast Basal Medium supplemented with 0.5% BSA/0.4 mM $MnCl_2$/10 μg/ml aprotinin) containing either 4 nM purified active MMP2 alone or in combination with 10 μM TSRI265 or TSRI359 for 45 min at 37°C before washing and addition to the [^{3}H]collagen IV-coated wells. Wells had been coated overnight with 50 μl of [^{3}H]collagen IV (specific activity 0.19 mCi/mmol; ICN) and washed extensively until the radioactivity in the recovered wash solution reached background. Alternatively, cells were treated as above in the absence of MMP2, or the MMP2 solutions were added directly to the wells without cells as controls. Collagen IV degradation was quantitated by measuring the radioactivity released into 50 μl of culture medium as determined in a liquid scintillation counter. For the assessment of biotinylated MMP2 binding to CS-1 cells, cells were suspended in adhesion buffer and incubated with 12 nM bMMP2 for 45 min at 37°C in the presence or absence of 10 μM TSRI265 or TSRI359. Cells were subsequently washed before lysis and processing for SDS/PAGE and immunoblotting with an antibiotin mAb.

Chick Chorioallantoic Membrane (CAM) Angiogenesis Assay. Angiogenesis was assessed essentially as described previously (5, 6). After stimulation with 3 μg/ml basic fibroblast growth factor, 10-day chicken embryo CAMs were treated with 20 μl of 3 μM TSRI265 or TSRI359. Three days after induction, the CAMs were quantitated in a blind fashion. CAMs from each group were pooled, minced, and extracted with 50 mM Tris/150 mM NaCl/0.1% Triton X-100 containing Complete protease inhibitor mixture without EDTA (Boehringer Mannheim) before analysis by zymography.

SDS/PAGE, Immunoblotting, and Zymography. *Immunoblotting.* Equal quantities of protein were separated by SDS/PAGE under reducing conditions and electroblotted to an Immobilon-P membrane (Millipore). The membrane was blocked, and immobilized proteins were detected by incubation with an antigen-specific primary antibody, followed by an HRP-conjugated secondary antibody as required. Bands were visualized with the chemiluminescent substrate PS-3 (Lumigen).

Zymography. Chick CAM lysates were prepared as described above, and equal quantities of protein were separated in the absence of reducing agents or boiling at 30 mA on polyacrylamide gels embedded with 0.2% gelatin. Gels were washed with 2% Triton X-100, followed by extensive washing with water before overnight incubation at 37°C in collagenase buffer (50 mM Tris 7.4/200 mM NaCl/10 mM $CaCl_2$). Gelatinolytic activity was visualized by staining the gels with 0.5% Coomassie blue.



Tumor Growth Assay. Primary tumors were grown on the CAMs of 9-day embryos by implantation of 5×10^6 CS-1 cells and incubation for 7 days. At this point, 50-mg sections of these tumors were subcultured onto fresh 9-day CAMs and allowed to implant for 24 h before a single i.v. injection with 100 μl of 100 μM compound in Hanks' balanced salt solution. Buffer alone was used as a control. Tumors were incubated for a total of 10 days, harvested, and trimmed free of excess stromal tissue before determination of wet weight and processing for histology.

Immunofluorescence. Snap-frozen CS-1 tumor sections were fixed with 4% paraformaldehyde and permeabilized with 0.1% Triton X-100. Sections were blocked with 5% BSA in PBS before staining with an anti-vWF polyclonal antibody and visualization with an Alexa 568-conjugated anti-rabbit secondary antibody. Samples were analyzed on an MRC1024 confocal microscope (Bio-Rad). Blood vessel density was quantitated with a 20× objective on 4 fields per section and 4 tumors per condition. Data shown are the mean ± SEM.

Results

TSRI265 Disrupts the RGD-Independent Interaction Between Integrin $\alpha_v\beta_3$ and MMP2. The recent observation that the carboxyl-terminal hemopexin-like domain of MMP2 can interfere with MMP2 binding to integrin $\alpha_v\beta_3$ and thus block angiogenesis prompted us to search for organic inhibitors of this interaction, which might be more amenable to therapeutic administration. To identify a specific inhibitor of the binding interaction between MMP2 and integrin $\alpha_v\beta_3$, solid phase receptor-binding assays were performed with immobilized integrins and biotinylated MMP2. The binding of purified MMP2 was found to be entirely RGD independent in this system, as evidenced by the lack of effect of cRGDfV on MMP2 binding to integrin $\alpha_v\beta_3$, even though this peptide inhibited the interaction of $\alpha_v\beta_3$ with its extracellular matrix ligand, vitronectin (VN; Fig. 2*A*). Importantly, the binding of MMP2, but not that of VN, was completely abrogated by TSRI265, demonstrating the specificity of TSRI265 for the interaction between MMP2 and $\alpha_v\beta_3$. Furthermore, the binding between MMP2 and TIMP2 was not inhibited by TSRI265 (data not shown), supporting the contention that the effect of this compound is restricted to the binding interaction between MMP2 and integrin $\alpha_v\beta_3$, and demonstrating a distinction between the binding sites for the MMP2 PEX domain on TIMP2 and integrin $\alpha_v\beta_3$. It is important to note that neither the control compound TSRI359 nor the control peptide cRADfV interfered with MMP2 binding to integrin $\alpha_v\beta_3$ (Fig. 2*A*).

TSRI265 Binds Directly to Integrin $\alpha_v\beta_3$ and Not to MMP2. To further address the mechanism of action of TSRI265, additional solid phase receptor-binding assays were performed with immobilized $\alpha_v\beta_3$ and ^{14}C-labeled TSRI265, or biotinylated VN as a

Fig. 2. TSRI265 binds directly to integrin $\alpha_v\beta_3$, suppressing the interaction between MMP2 and integrin $\alpha_v\beta_3$. (*A*) TSRI265 specifically blocks integrin $\alpha_v\beta_3$ binding to MMP2 without affecting interaction of $\alpha_v\beta_3$ with its classical ligand, vitronectin. Solid phase receptor binding of biotinylated MMP2 (bMMP2) or bVN to integrin $\alpha_v\beta_3$ was performed in the presence or absence of 3 μM TSRI265/TSRI359 or 100 μM cyclic RGD or RAD peptide. Binding was determined colorimetrically with an HRP-conjugated antibiotin mAb as described in the Materials and Methods. (*B–D*) TSRI265 binds specifically and saturably to integrin $\alpha_v\beta_3$ in an RGD-independent manner. Purified integrins $\alpha_v\beta_3$ and $\alpha_5\beta_1$ were coated onto microtiter wells, which were subsequently blocked and incubated with ^{14}C-labeled TSRI265 alone (*B*) or in the presence or absence of a 25-fold molar excess of unlabeled TSRI265 or TSRI359 (*C*) or 100 μM cyclic RGD or RAD peptide (*D*). bVN was used as a control and detected colorimetrically with an HRP-conjugated antibiotin mAb as described in *Materials and Methods*.

control. As can be seen in Fig. 2*B*, TSRI265 bound directly to integrin $\alpha_v\beta_3$ in a solid phase receptor-binding assay. This interaction was dose dependent, saturable, and specific, demonstrating minimal interaction of TSRI265 with the unrelated control integrin $\alpha_5\beta_1$ (Fig. 2*B*). Indeed, negligible binding to integrin $\alpha_5\beta_1$ was observed at higher concentrations of compound (data not shown). In addition, no binding of TSRI265 was observed when MMP2 was coated on the microtiter well (data not shown), suggesting that the effects observed in the MMP2/integrin $\alpha_v\beta_3$ binding assay are due to TSRI265 binding to integrin $\alpha_v\beta_3$. Estimates of stoichiometry on the basis of data presented in Fig. 2*B* suggest that less than one TSRI265 binds per molecule. However, these estimates are influenced by several factors that indicate such calculations are likely to be an underestimate. Specifically, TSRI265 exhibits a high intrinsic off-rate of binding, and it is likely that much of the adsorbed $\alpha_v\beta_3$ is conformationally inaccessible to TSRI265. Therefore, it is likely that no more than one TSRI265-binding site is present per molecule. It is significant that this interaction was inhibited by the presence of a 25-fold molar excess of unlabeled TSRI265 but not the related control compound TSRI359 (Fig. 2*C*). Furthermore, TSRI265 bound to integrin $\alpha_v\beta_3$ in an RGD-independent manner, as demonstrated by the inability of the cRGDfV peptide to inhibit the interaction of radiolabeled TSRI265 with integrin $\alpha_v\beta_3$, even as cRGDfV completely abolished the binding of biotinylated VN to the immobilized integrin in the same system (Fig. 2*D*). The control peptide cRADfV is shown as a control for the specificity of the binding inhibition. Thus, TSRI265 binding to $\alpha_v\beta_3$ exhibits comparable specificity, selectivity, and lack of susceptibility to RGD inhibition to that of MMP2.

Cell-Mediated Collagen IV Degradation via MMP2 Is Blocked by TSRI265. Prevention of MMP2 binding to integrin $\alpha_v\beta_3$ on melanoma cells was shown previously to inhibit cell-mediated collagen IV degradation in an $\alpha_v\beta_3$-dependent manner (6). Therefore, we assessed whether melanoma cells expressing or lacking $\alpha_v\beta_3$ could use activated MMP2 to degrade immobilized [^{3}H]collagen IV. Importantly, neither cell produces detectable quantities of MMP2 endogenously. Whereas both cell types were capable of some level of basal collagen degradation, only the β_3-transfected CS-1 cells were able to use the exogenous MMP2, demonstrating significantly more release of substratum radioactivity into the culture medium after preincubation with purified MMP2 (Fig. 3*A*). This enhanced substrate degradation in response to treatment with MMP2 was specifically abolished by inclusion of TSRI265, whereas TSRI359 had a negligible effect (Fig. 3*A*). Significantly, the effect of TSRI265 on cell-mediated collagen degradation was not because of a direct inhibition of MMP2 activity, as purified active MMP2 in the absence of cells was still able to degrade the immobilized [^{3}H]collagen IV irrespective of the presence or absence of either compound. To demonstrate that the reduced cell-mediated collagen degradation observed in Fig. 3*A* was the result of inhibition of MMP2 interaction with integrin $\alpha_v\beta_3$ by TSRI265 on the cell surface, CS-1 cells and their $\alpha_v\beta_3$-bearing counterpart were examined in a biotinylated MMP2-binding assay. As expected, the β_3-negative CS-1 cells were capable of binding some level of MMP2; however, their capacity to do so was not diminished by the presence of either compound (Fig. 3*B*). In contrast, β_3CS-1 cells bound significantly greater quantities of MMP2, and this enhanced MMP2 binding was specifically suppressed by TSRI265. In fact, when corrected for the loading of the lanes as demonstrated by staining with an antiactin mAb, TSRI265 effectively reduced the binding of MMP2 by the β_3CS-1 cells to the level observed in the absence of $\alpha_v\beta_3$ (i.e., parental CS-1 cells; Fig. 3*B*, lane 2).

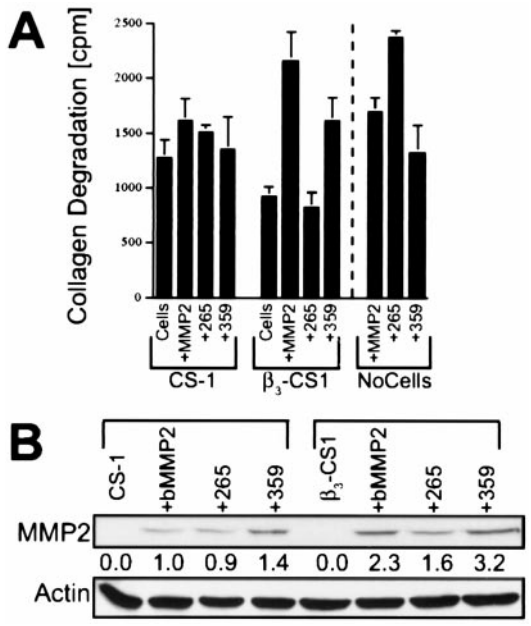


Fig. 3. TSRI265 suppresses cell-mediated collagenolytic activity by interfering with MMP2 binding to integrin $\alpha_v\beta_3$. (*A*) TSRI265 blocks cell-mediated β_3-dependent utilization of active MMP2 to degrade collagen IV. CS-1 melanoma cells or CS-1 cells transfected with integrin β_3 (β_3CS-1) were incubated with active MMP2 in the presence or absence of either 10 μM TSRI265 or TSRI359, washed, and plated onto wells coated with [^{3}H]collagen IV. As a control, active MMP2 was examined in the absence of cells (denoted by dotted line). After 36 h, a sample of media was removed and quantitated in a liquid scintillation counter. (*B*) TSRI265 blocks MMP2 binding to integrin $\alpha_v\beta_3$ on the cell surface. CS-1 or β_3CS-1 cells were incubated with biotinylated MMP2 in the presence or absence of either 10 μM TSRI265 or TSRI359, washed twice, and lysed for analysis by SDS/PAGE and immunoblotting with an HRP-conjugated antibiotin mAb. Cells that had not been treated with bMMP2 are shown for comparison. Biotin-reactive bands corresponding to MMP2 were analyzed by scanning densitometry, and the relative intensities are shown under each lane. The blot was reprobed with an antiactin mAb to ensure equal loading of lysates.

TSRI265 Disrupts Angiogenesis *in Vivo* Without Suppressing MMP2 Activation. Suppression of $\alpha_v\beta_3$–MMP2 interaction by exogenously applied recombinant MMP2 PEX domain was shown previously to impair angiogenesis in animal models (6). Therefore, we examined the effects of TSRI265 on growth factor-induced angiogenesis on the 10-day-old chick CAM. Application of TSRI265 to CAMs that had been stimulated with basic fibroblast growth factor almost completely abolished the development of new blood vessels in response to this stimuli (Fig. 4 *A* and *B*), whereas the control compound TSRI359 was ineffective in this regard. Importantly, the abrogation of angiogenic infiltration in response to TSRI265 was not associated with suppression of MMP2 activation because equivalent levels of active MMP2 (62 kDa) were detected in CAM tissues from treated and untreated embryos (Fig. 4*C*). This is in stark contrast to the effect of exogenous MMP2 PEX domain, which suppressed MMP2 activation in this system (6). These data are consistent with the notion that TSRI265 specifically interferes with the binding of MMP2 to integrin $\alpha_v\beta_3$, without impacting the activation of MMP2 directly. Indeed, the overall levels of MMP2 observed in the CAM lysates were unaffected by TSRI265 treatment as well, ruling out a potential effect on the expression level of MMP2 in the angiogenic tissues (Fig.4*C*). These results suggest that the antiangiogenic effects of TSRI265 likely result from the suppression of MMP2 binding to integrin $\alpha_v\beta_3$ on the cell surface as demonstrated in Fig. 3*B*. These data

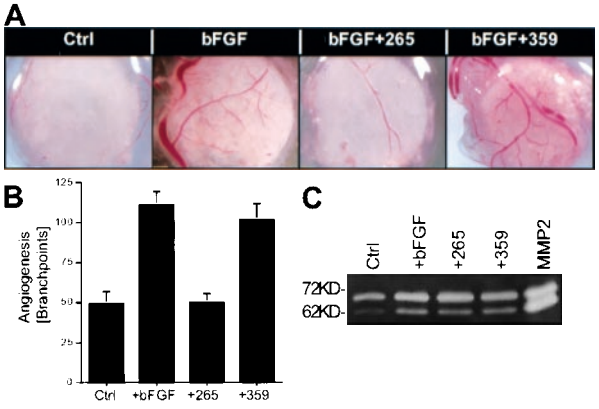


Fig. 4. TSRI265 blocks angiogenesis on the chick CAM without inhibiting the activation of MMP2. (*A*) Angiogenesis was induced with basic fibroblast growth factor (bFGF) on the CAMs of 10-day chick embryos, and CAMs were treated with either 3 μM TSRI265 or TSRI359. After 72 h, CAMs were harvested and photographed before quantification of the number of branch points within the treatment field (*B*) and processing of pooled CAMs for SDS/PAGE and zymography (*C*). The relative migration of the proenzyme and activated MMP2 species is indicated (*Left*) in kilodaltons. Purified enzyme is shown (*Right*) as a control (MMP2).

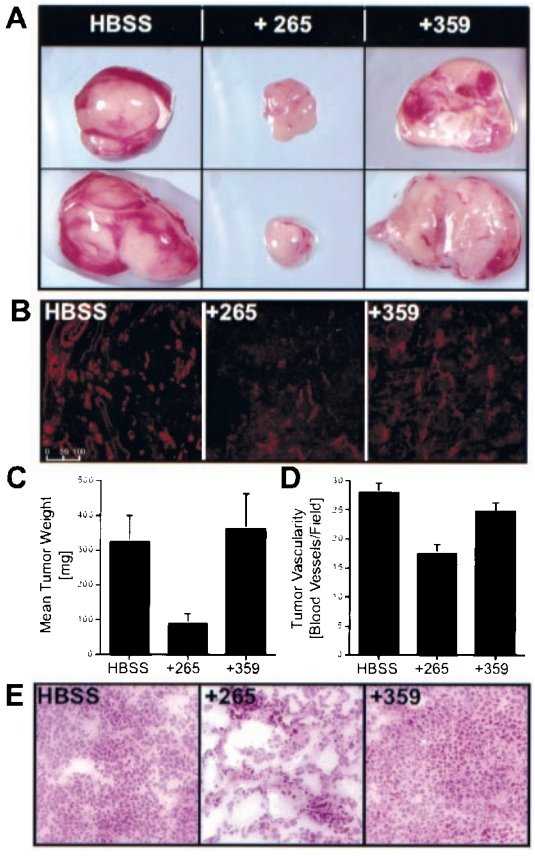


Fig. 5. TSRI265 suppresses tumor growth on the chick CAM via impairment of angiogenesis. (*A*) Primary tumors were grown on the CAMs of 9-day embryos by implantation of 5×10^6 CS-1 cells and incubation for 7 days. At this point, 50-mg sections of these tumors were subcultured onto fresh 9-day CAMs. After 24 h, embryos were injected i.v. with 100 μl of 100 μM ($\approx$10 μg) TSRI265 or TSRI359, or Hanks' balanced salt solution vehicle alone. Tumors were harvested 10 days later, trimmed of adjacent stromal tissue, and weighed wet (*C*). (*B* and *D*) Quantification of blood vessel density in treated and control tumors. (*B*) Tumors harvested as above were snap frozen, sectioned, and stained with anti-vWF polyclonal antibodies and an Alexa 568-labeled secondary antibody. Representative anti-vWF staining of treated and control tumors. (Bar = 100 μm.) (*D*) Quantification of the number of blood vessels per field as defined by anti-vWF reactivity. Data shown are the mean $\pm$ SE. (*E*) Serial sections of tumors were stained with hematoxylin and eosin for standard histological analysis. Representative photomicrographs are shown.

also indicate that MMP2 that has been fully activated is not used for angiogenesis in this system unless coupled to integrin $\alpha_v\beta_3$ on the cell surface.

TSRI265 Abrogates Tumor Growth *in Vivo*. Disruption of angiogenesis has been shown to inhibit tumor growth in numerous systems (6, 9, 10). As a result, blocking the invasive properties of endothelial cells by inhibiting MMPs suppresses angiogenesis and tumor growth in animal models as well (11). In fact, a number of MMP inhibitors have shown promise as antiangiogenic agents in humans (7, 8). Therefore, we assessed whether the inhibition of angiogenesis associated with blockade of MMP2–$\alpha_v\beta_3$ interactions observed in this study might be sufficient to suppress the growth of an $\alpha_v\beta_3$-negative tumor. The use of the $\alpha_v\beta_3$-negative tumor allowed the assessment of the effect of TSRI265 on vascular $\alpha_v\beta_3$ selectively. As shown in Fig. 5, growth of transplanted $\alpha_v\beta_3$-negative CS-1 melanoma tumors on the chick CAM was significantly retarded by a single i.v. injection of TSRI265. It is likely that this effect did not result from a direct impact of TSRI265 on the tumor, as the melanoma cells used in this assay lack integrin $\alpha_v\beta_3$. In fact, their growth *in vitro* is not affected by coculture with the compound (data not shown). A gross reduction in the surface vasculature (Fig. 5*A*) as well as the overall blood vessel density (Fig. 5 *B* and *D*) was evident in the tumors that had been treated with TSRI265. Importantly, this reduction in tumor vasculature was associated with significant cell death within the tumor mass, even as the control tumors showed a 6-fold increase in mass during the 10-day time frame of the assay (Fig. 5*E*).

Discussion

MMPs have been implicated in processes involving angiogenesis as well as the cellular invasion associated with tumor growth and metastasis (11). MMP2, in particular, has been identified as a central player important for these processes. For example, angiogenesis and corresponding tumor growth are reduced in MMP2 knockout mice (2). In addition, the potential coordinate regulation of MMP2 with the angiogenic mediator $\alpha_v\beta_3$ was suggested by the retarded vascular invasion and concomitant tumor growth observed in Id transcription regulator-deficient animals (12). These animals demonstrated a lack of $\alpha_v\beta_3$ and MMP2 expression specific to the tumor-invading endothelial cells. Indeed, MMP2 has been shown to localize in a proteolytically active form on the surface of invasive cells via interaction of its PEX domain with integrin $\alpha_v\beta_3$ (5, 11). Accordingly, exogenous application of recombinant MMP2 PEX domain, which is required for activation of MMP2 at the cell surface (11), was shown previously to inhibit angiogenesis and concomitant tumor growth by suppressing the activation of MMP2 (6). Although the PEX domain is involved in the localization of MMP2 to $\alpha_v\beta_3$, it is also critically involved in binding to other MMP2 partners, including most notably TIMP2, which localizes MMP2 to MT1-MMP for initiation of the MMP2 activation cascade (11, 13). Thus, the inhibition of angiogenesis by PEX may result from a combined effect involving both blocking the initial MMP2 interaction with the TIMP2/MT1-MMP complex at the cell surface (11) and the abrogation of active MMP2 binding to integrin $\alpha_v\beta_3$ at points of cellular invasion (14). Indeed, recent data suggest that the expression of $\alpha_v\beta_3$ facilitates MMP2 activation by recruitment of active MT1-MMP on the surface of melanoma cells both *in vitro* and *in vivo* (15). As such, it would be expected

that specifically inhibiting only the interaction between MMP2 and integrin $\alpha_v\beta_3$ might impact the invasive events associated with angiogenesis. In this report, evidence is provided that specific disruption of the binding interaction between MMP2 and integrin $\alpha_v\beta_3$, without effect on the interactions of either MMP2 or $\alpha_v\beta_3$ with their classical ligands, blocks cell-associated MMP2-mediated collagenolytic activity, thereby suppressing angiogenesis and concomitant tumor growth.

That TIMP2/MMP2 complex formation is not inhibited by TSRI265 suggests a disparity in the mechanisms used by TIMP2 and $\alpha_v\beta_3$ for binding to MMP2. Indeed, although the MMP2 docking site on TIMP2 does not appear to be affected by TSRI265, whereas that on integrin $\alpha_v\beta_3$ is affected, this does not necessarily indicate that the region(s) of PEX involved in these binding events need be distinct. Also, that blocking the interaction of MMP2 with integrin $\alpha_v\beta_3$ does not suppress MMP2 activation on the cell surface suggests the primary binding interaction between the two molecules may take place only once MMP2 is fully activated. In the least, these results indicate that integrin $\alpha_v\beta_3$ may not be actively involved in MMP2 processing in this system. Although this is contrary to what had been postulated previously (11, 16, 17), those reports used significantly different cell systems that could account for the disparity in findings.

Although it is tempting to speculate that all of the antiangiogenic and antitumor effects of TSRI265 stem directly from the inability of invading endothelial cells to use MMP2 to remove constraining barriers, the TSRI265 effect may result from the contribution of an alternate mechanism as well. Classical integrin ligation is known to initiate intracellular signaling pathways (18), and as such, signaling events may be triggered by MMP2 occupancy of integrin $\alpha_v\beta_3$. Indeed, it has been suggested that the specificity of $\alpha_v\beta_3$ for either its natural ligands (e.g., vitronectin) or those that arise during invasion (e.g., cryptic sites within proteolyzed collagen; refs. 19 and 20) may stem from whether a secondary site on the integrin is occupied by MMP2 (11). As such, blockade of the secondary binding site by TSRI265 may mimic MMP2 binding, perhaps suppressing the ability of $\alpha_v\beta_3$ to recognize provisional matrices that it would otherwise find capable of supporting cell survival. Thus, the mechanism of action of TSRI265 may consist of both direct and indirect components.

The list of novel compounds approved by the Food and Drug Administration for antitumor use in the last 20 years is a relatively short one largely composed of compounds identified by their toxicity to dividing cells (21). With the current explosion of molecular biological advances in the processes of cancer, it is now within the grasp of researchers to begin tailoring their approach to antitumor agents with ever narrowing selectivity. Herein, we describe an organic compound that should display a vastly reduced incidence of extraneous side effects, on the basis of the selectivity with which it was derived as an antitumor agent. Application of similar goal-oriented screening recently yielded one of the first examples of the emerging group of inhibitors whose efficacy is based on disrupting protein–protein interactions without effect on the individual function of the constituent molecules. Using an affinity-driven selection process, Aramburu *et al.* (22) demonstrated enhanced immunosuppressive selectivity with lessened toxicity by prohibiting the interaction of calcineurin with the NFAT transcription factors, leaving intact all other processes requiring calcineurin within the cell. This demonstrates the potential advantage of the targeted inhibition of protein–protein interactions within the intracellular space over conventional strategies that block protein function directly.

In this report, we demonstrate that this approach, disruption of protein–protein binding, is also amenable to interactions in the extracellular space. Although the use of active site inhibitors including peptides or mimetics derived from the binding sequences of ligand/receptor complexes has been in practice for many years, this approach abrogates any and all functions relating to the respective partners in any and all cells encountered. By targeting a protein–protein interaction that is limited temporally and spatially, the incidence of undesired effects should be minimized. The results presented in this study demonstrate that prevention of a single binding event between two significant players in the process of angiogenesis can interfere with the complex processes of tumor growth and angiogenic recruitment *in vivo*. Thus, the development of additional compounds that selectively disrupt binding interactions restricted to pathological conditions should present a new strategy for therapeutic intervention in the future.

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