

Inhibition of Membrane-Type 1 Matrix Metalloproteinase Tyrosine Phosphorylation Blocks Tumor Progression in Mice

CARINE NYALENDO¹, HERVÉ SARTELET², DENIS GINGRAS¹ and RICHARD BÉLIVEAU¹

¹Laboratoire de Médecine Moléculaire, Université du Québec à Montréal,
C.P. 8888, Succ. Centre-ville, Montréal, Québec H3C 3P8, Canada;

²Département de Pathologie, CHU Sainte-Justine,
3175, Chemin Côte-Sainte-Catherine, Montréal, Québec H3T 1C5, Canada

Abstract. We recently reported that membrane-type 1 matrix metalloproteinase (MT1-MMP) is phosphorylated on its unique cytoplasmic tyrosine residue but the contribution of this event to tumor progression remains unclear. In this work, we show that the non phosphorylatable cell-permeable peptide antennapedia-coupled cytoplasmic MMP-14 (ACM-14), consisting of the mutated (Y573F) cytoplasmic domain of MT1-MMP coupled to antennapedia, inhibits tyrosine phosphorylation of the enzyme and markedly reduces tumor cell proliferation within 3D type I collagen matrices. Interestingly, administration of ACM-14 to mice markedly delays tumor progression and increases survival, these antitumor actions being associated with the induction of extensive tumor necrosis. Overall, these findings suggest that inhibition of MT1-MMP tyrosine phosphorylation may represent an attractive strategy for the development of novel anticancer drugs.

Membrane-type 1 matrix metalloproteinase (MT1-MMP; MMP-14) plays essential roles in tumor cell migration and invasion by acting as a potent matrix-degrading protease that proteolyzes a broad spectrum of cell surface-associated and extracellular matrix (ECM) proteins (reviewed in (1)). MT1-MMP is overexpressed in many types of tumor (2, 3) and pericellular proteolysis of the dense cross-linked meshwork of type I collagen fibrils mediated by the enzyme confers tissue-invasive activity on neoplastic cells (4) and sustains tumor cell growth in otherwise growth-restrictive three-dimensional (3-D) matrices (5).

Correspondence to: Richard Béliveau, Laboratoire de Médecine Moléculaire, Université du Québec à Montréal, C.P. 8888, Succ. Centre-ville, Montréal, Québec H3C 3P8, Canada. Tel: +1 5149873000 ext.8551, Fax: +1 5149870246, e-mail: oncomol@nobel.si.uqam.ca

Key Words: MT1-MMP, MMP-14, tyrosine phosphorylation, antennapedia, tumor progression, 3D matrix.

In addition to its important matrix-degrading activity, MT1-MMP contains a short cytoplasmic sequence whose function seems important for tumor cell invasion. Cells expressing cytoplasmic domain-deleted MT1-MMP mutants retain the ability to proteolyse the ECM but have a markedly reduced migratory potential (6-10), suggesting a role of this domain in linking extracellular proteolysis to intracellular signaling events involved in cell locomotion. Accordingly, the cytoplasmic domain of MT1-MMP actively participates in the internalization of the enzyme (7), the activation of the ERK signaling pathway (9, 11), as well as in its interaction with tyrosine-phosphorylated caveolin-1 (10), and these events may all contribute to efficient cell locomotion triggered by the enzyme. We also showed that MT1-MMP is phosphorylated on its unique cytoplasmic tyrosine residue and that this event may also participate in tumor and endothelial cell migration (12, 13). More recently, we reported that overexpression of a dominant-negative, non phosphorylatable mutant of MT1-MMP (Y573F) in the highly invasive HT-1080 fibrosarcoma cell line markedly reduces the proliferation of these cells within 3D collagen matrices and completely inhibits their growth in mice, suggesting that the inhibition of tyrosine phosphorylation of MT1-MMP may represent an unexpected strategy to block tumor progression (14).

To investigate this possibility, we designed a cell-permeable peptide, termed antennapedia-coupled cytoplasmic MMP-14 (ACM-14), consisting of a mutated non phosphorylatable version of the cytoplasmic domain of MT1-MMP coupled to the cell-penetrating third helix of the homeodomain of the *Drosophila* transcription factor antennapedia.

Materials and Methods

Peptides and cell lines. Biotinylated peptides consisting of the homeodomain of antennapedia transcription factor (RQIKIWFQN RRMKWKK) coupled to sequences corresponding to the non-phosphorylatable cytoplasmic domain of MT1-MMP (RRHGTPRRL LFCQRSLLDKV) (ACM-14 peptide) or its scrambled version (TLRQRRCLPHFDSGLRKRKVR) (scACM-14) were synthesized by 21st Century Biochemicals (Marlboro, MA, USA).

All cell lines (HT-1080 fibrosarcoma, SK-Mel-28 melanoma, DAOY medullablastoma, U-87 MG glioblastoma, PC-3 prostate adenocarcinoma, A-549 lung carcinoma, MDA-MB-231 breast adenocarcinoma and MG-63 osteosarcoma cells) were obtained from the American Type Culture Collection (ATCC, Manassas, VA, USA). Cells were cultured at 37°C in a humidified atmosphere composed of 95% air and 5% CO₂ and grown as recommended by the ATCC.

Uptake of peptides. ACM-14 and scACM-14 uptake by tumor cells was determined by fluorescence or Western blot. For fluorescence, HT-1080 fibrosarcoma cells were seeded on type I collagen-coated cover-slides and incubated with peptides. Intracellular peptides were detected using FITC-conjugated streptavidin (Invitrogen, Burlington, ON, Canada). For Western blotting, cells were seeded atop of a type I collagen film (2D) or embedded within type I collagen gels (3D) and incubated with peptides, followed by protein extraction as described previously (10). Briefly, equal amounts of proteins were solubilized in Laemmli sample buffer, boiled for 5 min, separated by SDS-PAGE containing 15% acrylamide and blotted with HRP-conjugated streptavidin (Chemicon–Millipore, Billerica, MA, USA).

In vitro phosphorylation of MT1-MMP. A glutathione-S-transferase (GST) fusion protein containing the cytoplasmic domain of MT1-MMP was incubated for 2 h at 30°C with recombinant His6-Src (Upstate-Millipore, Billerica, MA, USA) in 100 µl of phosphorylation buffer (20 mM HEPES pH 7.5, 1 mM dithiothreitol (DTT), 10 mM MgCl₂, 0.1 mM ATP) containing equivalent molar concentrations (0.35 nmole) of either the ACM-14 or scACM-14 peptides. The reaction was stopped by boiling for 5 min in Laemmli buffer and samples (20 µl) were subjected to immunoblotting using the monoclonal pY99 antibody (Santa Cruz Biotechnology, Santa Cruz, CA, USA) or polyclonal antibodies specific to the tyrosine phosphorylated form of MT1-MMP (12).

2D/3D Cell culture. Type I collagen was extracted from rat tail and resuspended at 2.7 mg/ml in acetic acid. For 2D culture, cells were seeded on plates coated with 100 µg/ml type I collagen. For 3D culture, cells were mixed with 10× Eagle's minimal essential medium (EMEM), 0.17 N NaOH and 2.7 mg/ml type I collagen. The mixture was allowed to gel for 45 min at 37°C and culture medium (EMEM) containing 10% fetal bovine serum (FBS) was added atop. For growth inhibition, 1 to 10 µM of ACM-14 or scACM-14 were added to culture medium (for 2D growth), or incorporated into the collagen gels (for 3D growth). Cell growth was followed for 5 days. Collagen gels were dissolved using 2 mg/ml bacterial collagenase (Sigma-Aldrich, Oakville, ON, Canada) and viable cells were counted by trypan blue exclusion using a hemacytometer. For FACS analysis, cells extracted from collagen gels were processed as described (14).

Flow cytometric analysis of MT1-MMP. HT-1080 fibrosarcoma cells embedded within type I collagen gels (3D) for 5 days were extracted from collagen gels using bacterial collagenase and washed twice with binding buffer (BB) containing 0.1% bovine serum albumin (BSA) in phosphate-buffered saline (PBS). Cells (10⁶) were incubated for 1 h at 4°C with 1 ml of 1 µg/ml MT1-MMP (MAB3328) antibody or a control mouse IgG (Sigma) in BB, washed twice with BB and incubated with anti-mouse FITC-

conjugated antibody for 30 min at 4°C in the dark. After two washes, cells were resuspended in 1 ml BB and analyzed using FACS Calibur (BD Biosciences, Mississauga, ON, Canada).

Gelatin zymography. Conditioned media (10 µl) from cells grown within collagen gels were resuspended in non-reducing sample buffer and subjected to SDS-PAGE, using polyacrylamide gels containing 1 mg/ml gelatin. Gels were then washed twice for 30 min in 2.5% Triton X-100 and rinsed with nanopure water. Gels were incubated at 37°C in zymography buffer (50 mM Tris pH 7.6, 200 mM NaCl, 5 mM CaCl₂, 0.02% Brij 35) for 18 h and digested areas were visualized by coloration of the gels with Coomassie blue.

Fluorescence and confocal microscopy. Following incubation with the peptides (for 5 minutes to 1 hour) fluorescence was assessed as described previously (14), using antibodies against phosphoMT1-MMP (12) and MT1-MMP (MAB3328; Chemicon – Millipore, Billerica, MA, USA). Biotinylated peptides were stained with fluorescein isothiocyanate (FITC)-conjugated streptavidin. Fluorescence was visualized and photographed using a Zeiss LSM 510 Meta confocal microscope.

Immunoprecipitation and Western blotting. The procedures have been described previously (10). Briefly, equal amounts of proteins were incubated overnight at 4°C in the presence of 1 µg/ml of MT1-MMP MAB3328 monoclonal antibody and the immune complexes were collected with Protein G-coupled Sepharose beads (Amersham Biosciences – GE Healthcare, Baie d'Urfe, QC, Canada). Bound material was solubilized in Laemmli sample buffer, boiled for 5 min, separated by SDS-PAGE and blotted with horse radish peroxidase (HRP)-conjugated streptavidin (for detection of ACM-14 and scACM-14) or with antibodies against tyrosine phosphorylated MT1-MMP (12).

Detection of apoptosis. Cells, treated or not with the pan caspase inhibitor z-Val-Ala-Asp(Ome)-fluoromethylketone (z-VAD-fmk, 50 µM) (ICN Biomedical, Irvine, CA, USA) were harvested with 0.5 mM ethylenediaminetetraacetic acid/PBS, washed twice with PBS, double stained with FITC-annexin V and propidium iodide (PI) using the ApoAlert® Annexin V Apoptosis Kit (BD Biosciences–Clontech, Oakville, ON, Canada) according to the manufacturer's instructions and analyzed using the FACS Calibur (BD Biosciences). Double staining of cells with FITC-annexin V and PI permits the discrimination of living cells (FITC⁻PI⁻), early apoptotic (FITC⁺PI⁻), late apoptotic (FITC⁺PI⁺) or necrotic cells (FITC⁻PI⁺). Molecular markers of apoptosis (cleaved caspase-3 and PARP) as well as others proteins were evaluated by Western Blot using antibodies against cleaved caspase-3 and PARP (Cell signaling Technology, Beverly, MA, USA); ERK, pERK and caveolin-1 antibodies (Cell signaling Technology); catenin, vinculin and GAPDH antibodies (Santa Cruz Biotechnology)

In vivo tumor growth. Six-week-old female athymic nude mice (CrI: CD-1® -Foxn^{nu}) were purchased from Charles River Laboratories (Wilmington, MA, USA). Experiments were performed according to the guidelines of the Canadian Council of Animal Care. Mice were anesthetized with O₂/isoflurane inhalation and 5×10⁶ HT-1080 fibrosarcoma viable cells (in 100 µl of 1% methylcellulose in

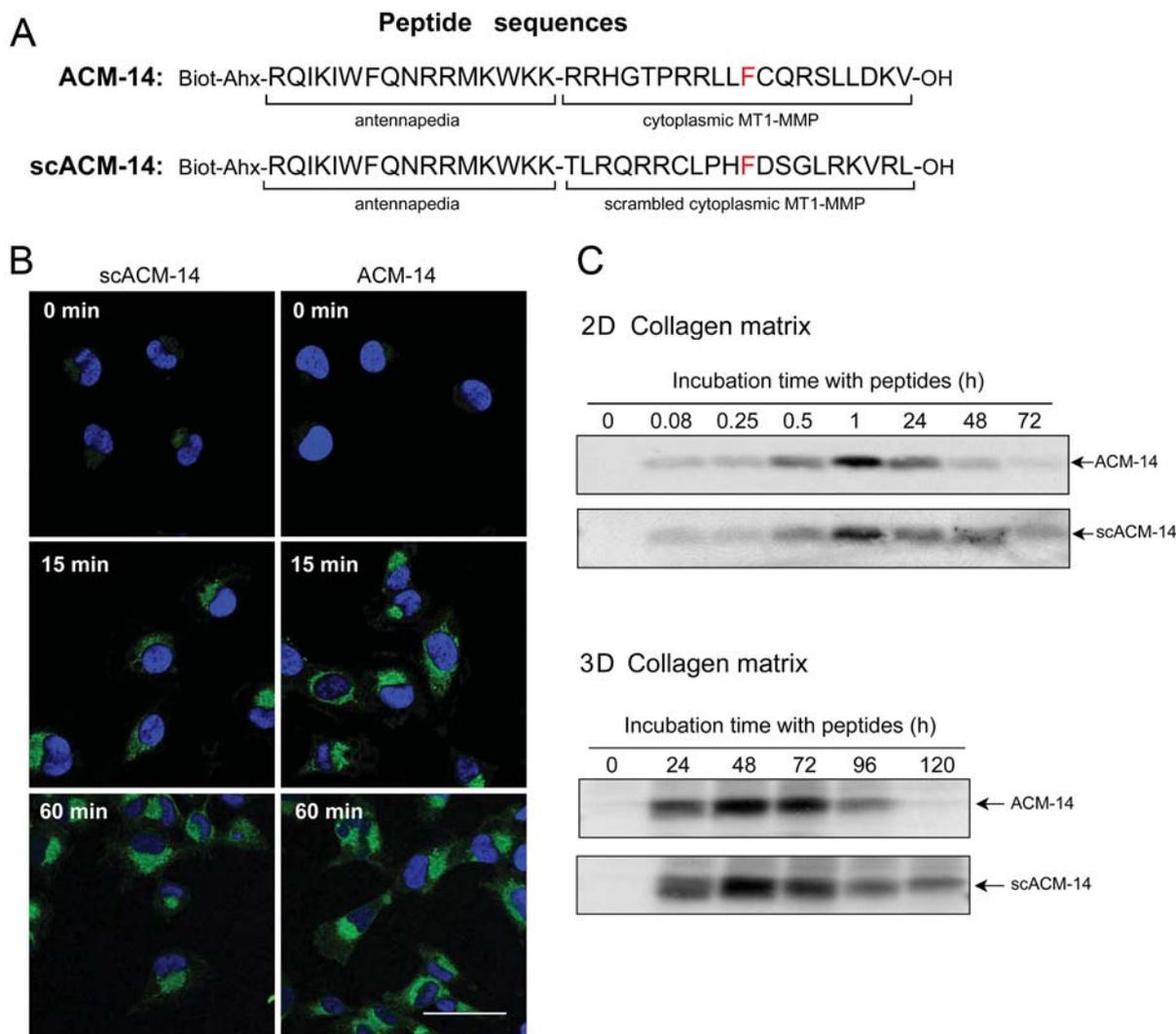


Figure 1. Cellular uptake of ACM-14 and scACM-14 peptides. A: Peptides used in this study. ACM-14: Antennapedia-coupled cytoplasmic MMP-14; scACM-14: scrambled ACM-14; Biot: Biotin; Ahx: amino hexanoic acid. B: HT-1080 cells were seeded on type I collagen-coated slides, and incubated with ACM-14 and scACM-14 peptides (1 μ M) for 15 or 60 minutes. Confocal images of fluorescence were obtained following intracellular staining of peptides using FITC-conjugated streptavidin. The white scale bar represents 50 μ m. C: HT-1080 cells were incubated with ACM-14 and scACM-14 (1 μ M) for the indicated periods of time. For 2D growth, cells were seeded atop of a type I collagen film, and for 3D growth, cells were embedded within type I collagen gels. Cellular content of peptides was assessed by Western blotting, using HRP-conjugated streptavidin.

serum-free EMEM) were implanted subcutaneously into the flank of mice. Once tumors reached 100 mm³, animals were randomly separated into three groups for treatment with vehicle (0.85% NaCl; n=5), scACM-14 (n=5) or ACM-14 (n=6); and were given daily subcutaneous administrations of the peptides ACM-14 or scACM-14 (10 mg/kg), or vehicle, distant of tumor site (*i.e.* in the area of the head, between the ears). Tumor size was measured every 2 days using a digital caliper and calculated using the formula $\pi/6 \times \text{length} \times \text{width}^2$. For kinetic studies, mice were sacrificed after 26 days. For survival studies, animals were sacrificed once tumors reached either 700 mm³ or 50 days post-implantation. Kaplan-Meier survival curves were derived using Graphpad Prism (GraphPad Software, La Jolla, CA, USA). Histological analysis was performed as previously described (14).

Statistical analysis. All statistical analyses were performed by one- or two-way analysis of variance (ANOVA) followed by Bonferroni post tests. Analyses were performed using GraphPad Prism. $P < 0.05$ was considered statistically significant.

Results

ACM-14 peptide inhibits tyrosine phosphorylation of MT1-MMP. The ACM-14 peptide consists of the mutated (Y573F) cytoplasmic domain of MT1-MMP fused to antennapedia (Figure 1A). This peptide, as well as its scrambled version (scACM-14), rapidly entered in tumor cells, being detectable as early as 15 minutes after their addition to the cell media

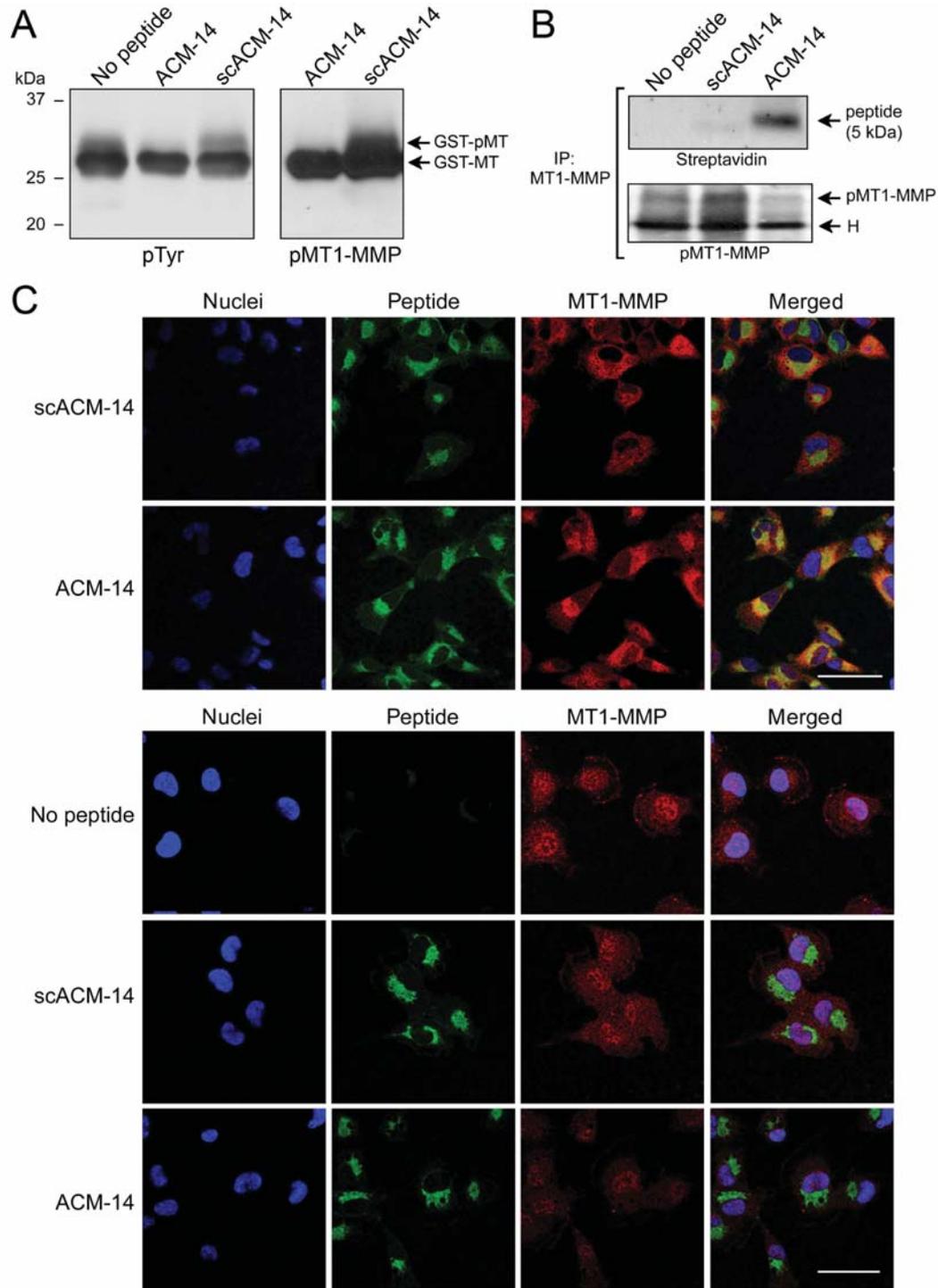


Figure 2. Inhibition of MT1-MMP tyrosine phosphorylation by ACM-14. **A:** In vitro phosphorylation of MT1-MMP cytoplasmic domain by recombinant Src. The cytoplasmic domain of MT1-MMP fused to glutathione-S-transferase (GST-MT) was incubated with Src in the presence of either ACM-14 or scACM-14. Monoclonal antibody pY99 was used to identify phosphotyrosine (pTyr), while pMT1-MMP polyclonal antibodies recognized phosphorylated MT1-MMP. **B:** HT-1080 cells were grown within 3D collagen matrices containing 1 μ M of either ACM-14 or scACM-14 during 5 days. MT1-MMP was immunoprecipitated from cell lysates and MT1-MMP-associated peptides were detected using HRP-conjugated streptavidin, while MT1-MMP (pMT1-MMP) was immunodetected with polyclonal antibodies against phosphorylated MT1-MMP. **C:** HT-1080 cells were seeded on type I collagen-coated slips and incubated with ACM-14 or scACM-14 peptides (1 μ M) for 1 h. Confocal images were obtained by staining the peptides with streptavidin-FITC followed by staining of MT1-MMP and phosphorylated MT1-MMP using primary antibodies specific to these proteins followed by rhodamine-conjugated secondary antibodies. The scale bar represents 50 μ m.

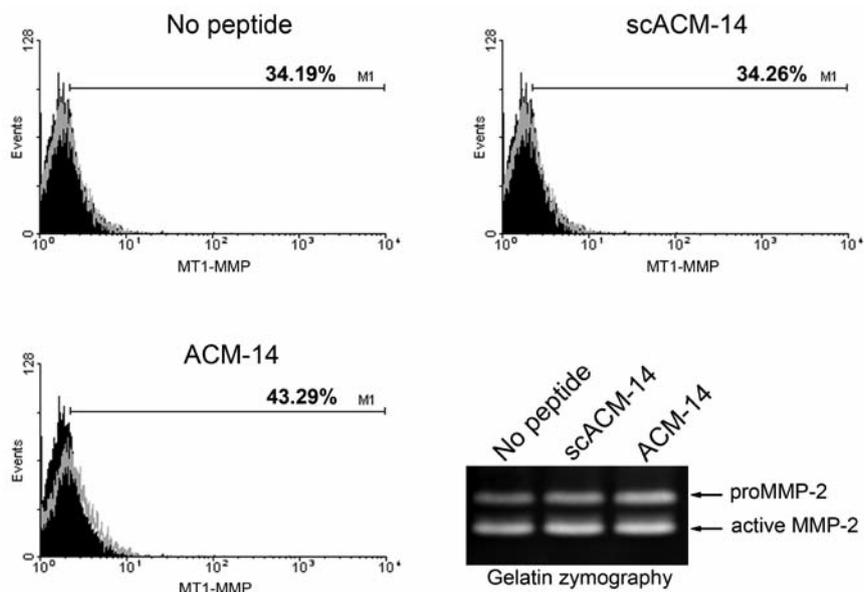


Figure 3. *HT-1080* cells were incubated with *ACM-14* and *scACM-14* ($1 \mu\text{M}$) and were allowed to grow within a 3D collagen matrix for 24 hours. *MT1-MMP* cell surface expression was monitored by flow cytometry. Black areas represent the control isotype (mouse IgG), while grey lines represent *MT1-MMP*. Conditioned media were analyzed for *MT1-MMP*-dependent activation of *MMP-2* by gelatin zymography.

(Figure 1B-C). *In vitro* phosphorylation assays using a GST fusion protein containing the *MT1-MMP* cytoplasmic sequence (GST-MT) showed that *ACM-14* completely inhibited the Src-mediated tyrosine phosphorylation of this domain, whereas the scrambled control peptide had no effect (Figure 2A). Interestingly, we observed that in tumor cells, *ACM-14*, but not its scrambled counterpart, co-immunoprecipitated and co-localized with *MT1-MMP*, this association occurring predominantly in the perinuclear compartment (Figure 1B and C, upper panels). The interaction between *MT1-MMP* and *ACM-14* was correlated with a marked decrease in the levels of tyrosine phosphorylated *MT1-MMP* (Figure 1B and C, lower panels). The peptides had, however, no inhibitory effect on either the cell surface expression of *MT1-MMP* or on catalytic activity of the enzyme towards pro*MMP-2* (Figure 3), suggesting that the interaction of the peptide with the enzyme does not alter its subcellular localization.

ACM-14 reduces tumor cell proliferation within 3D matrices through a caspase-dependent mechanism. In addition to the crucial role of *MT1-MMP*-dependent proteolysis for tumor cell proliferation in 3D environments (5), our recent results suggest that tyrosine phosphorylation within the intracellular domain of the enzyme may also participate in this process (14). As shown in Figure 4A, while *ACM-14* did not affect tumor cell proliferation under 2D conditions, it significantly reduced the proliferation of several tumor cell lines within 3D collagen gels. Indeed, *ACM-14* reduced the proliferation

of glioblastoma (40%), medulloblastoma (35%), prostate and breast adenocarcinoma (83% and 29% respectively), osteosarcoma (68%) and fibrosarcoma (50%) cells, while the scrambled version of the peptide had no significant inhibitory effect. *ACM-14* showed however no inhibitory effect on the proliferation of lung carcinoma or melanoma cells (Figure 4A). However, low levels of *MT1-MMP* expression were observed in these two cell lines (Figure 4B) and their proliferation was largely independent of the enzyme's activity, as reflected by their insensitivity to tissue inhibitor of *MMP-2* (*TIMP-2*) and to the *MMP* inhibitor *GM6001* (results not shown).

Interestingly, the inhibitory effect of *ACM-14* on tumor cell proliferation was correlated with an increase of apoptosis. Approximately 40% of untreated *HT-1080* fibrosarcoma cells grown within the collagen matrix underwent apoptosis, possibly reflecting cell death induced by the growth-restrictive 3D environment (Figure 5A). *ACM-14* (but not its control peptide *scACM-14*) induced a small but significant increase (15%) in the number of apoptotic cells which was correlated with the cleavage of caspase-3 and *PARP* (Figure 5B), two classical markers of apoptosis (15). We also observed that *ACM-14* inhibited *ERK1/2* phosphorylation (Figure 5B), an effect that may also contribute to the reduction of cell survival induced by the peptide. Interestingly, the pan caspase inhibitor *z-VAD-fmk* completely reversed *ACM-14*-induced apoptosis of tumor cells (Figure 5C) as well as the effect of the peptide on tumor cell proliferation (Figure 5D), further implying

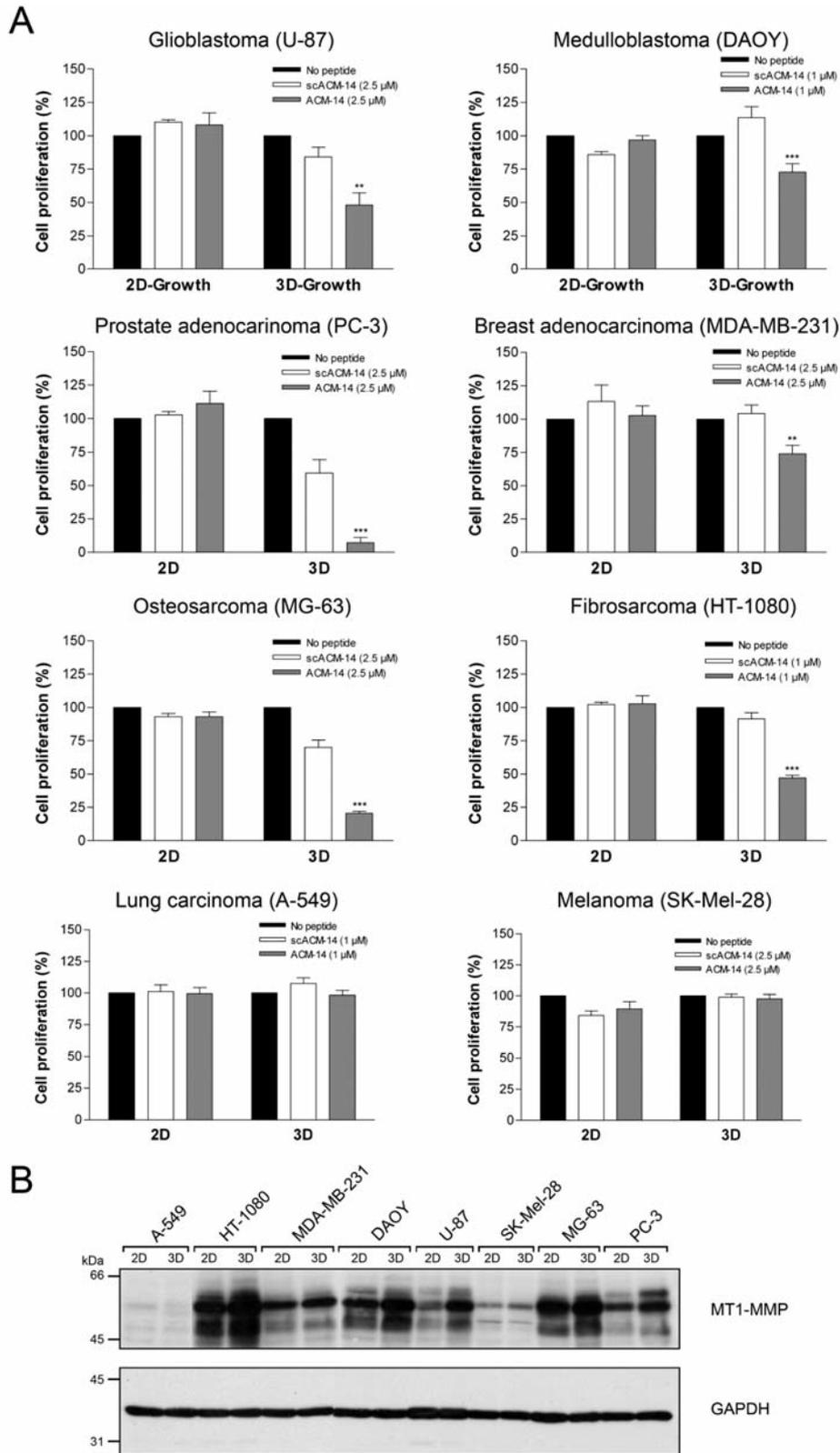


Figure 4. Reduction of cell proliferation within a 3D collagen matrix by ACM-14. A: Cells were allowed to grow atop of collagen film (2D) or within collagen gel (3D) during 5 days in the presence of scACM-14 or ACM-14 peptides, as indicated. B: Total cellular content of MT1-MMP was assessed by Western blot. ** $p < 0.01$, *** $p < 0.001$ versus the control (no peptide).

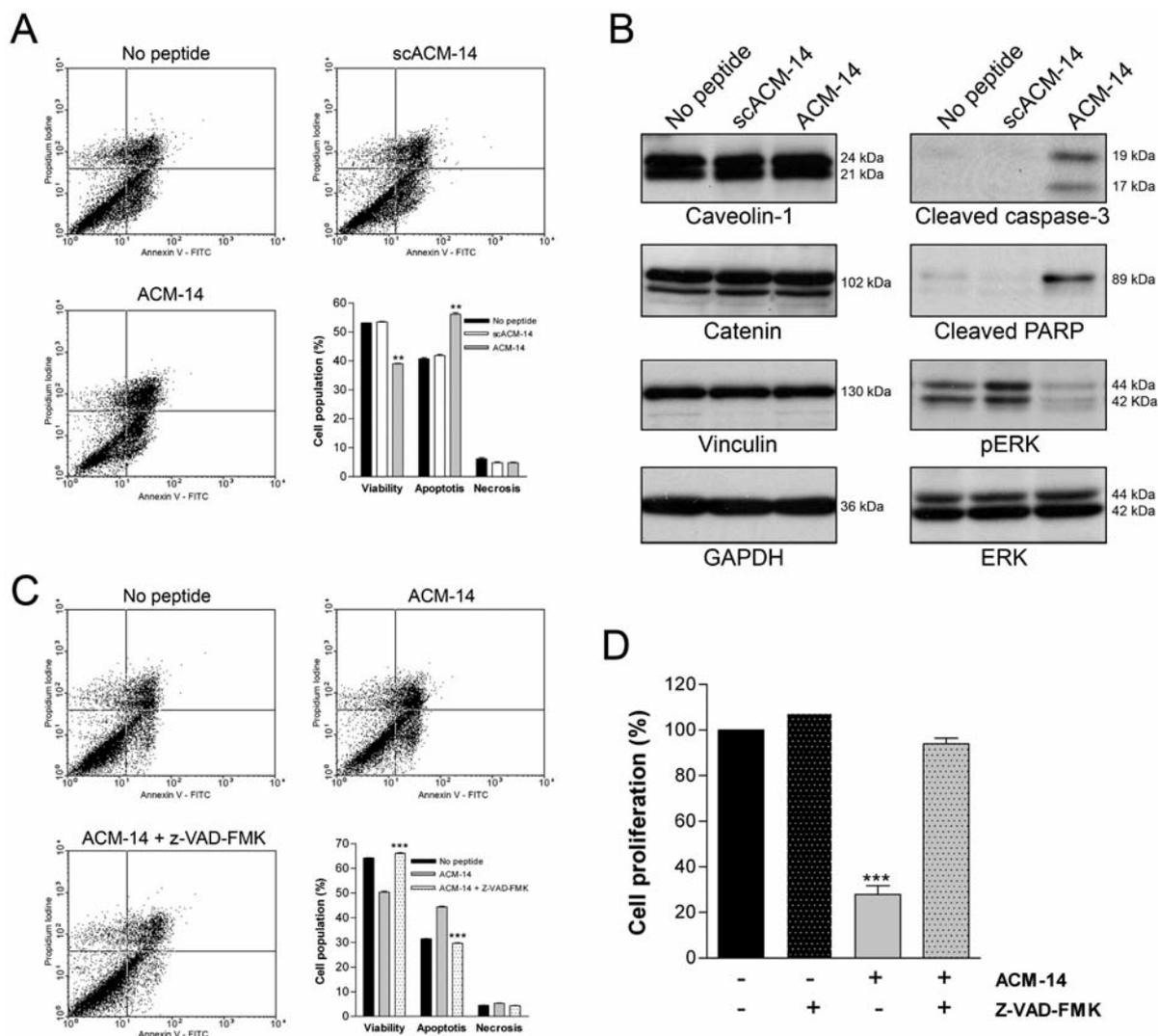


Figure 5. ACM-14 induces apoptosis of tumor cells. HT-1080 fibrosarcoma cells were grown for 5 days within 3D collagen matrices with or without 1 μ M of the peptides (ACM-14 or scACM-14) (A), or with ACM-14 in the absence or in the presence of pan-caspase inhibitor z-VAD-fmk (C). Cells were extracted from collagen gels and double stained with annexin V and propidium iodine for apoptosis analysis by flow cytometry. B: Total cellular content of some apoptosis markers was assessed by Western blot. (D) Cells were allowed to grow within 3D collagen matrices and proliferation was determined by counting.

caspase activation as an important mechanism involved in the inhibitory action of the peptide.

ACM-14 increases the survival of mice bearing fibrosarcoma xenografts. In order to examine the impact of ACM-14 on tumor growth, HT-1080 fibrosarcoma cells were implanted subcutaneously in athymic mice. After 12 days, animals were given daily subcutaneous injections of the peptides (ACM-14 or scACM-14, 10 mg/kg each) or vehicle and tumor size was monitored every two days. As shown in Figure 6A, administration of ACM-14 strongly reduced (70%) tumor growth 26 days post-implantation. The median survival of mice that received ACM-14 was increased by 10 days, with

one tumor that completely regressed following treatment with the peptide (Figure 6B). The antitumor effect of the peptide was well illustrated by low magnification histological examination of paraffin sections which showed that ACM-14 induced extensive necrosis of the tumor tissue, in agreement with its cytotoxic effects observed *in vitro* (Figure 6C).

Discussion

Overall, the results presented in this study indicate that ACM-14 is a cell-permeable peptide that inhibits MT1-MMP tyrosine phosphorylation under 3D growth conditions, possibly by physically interacting with the enzyme.

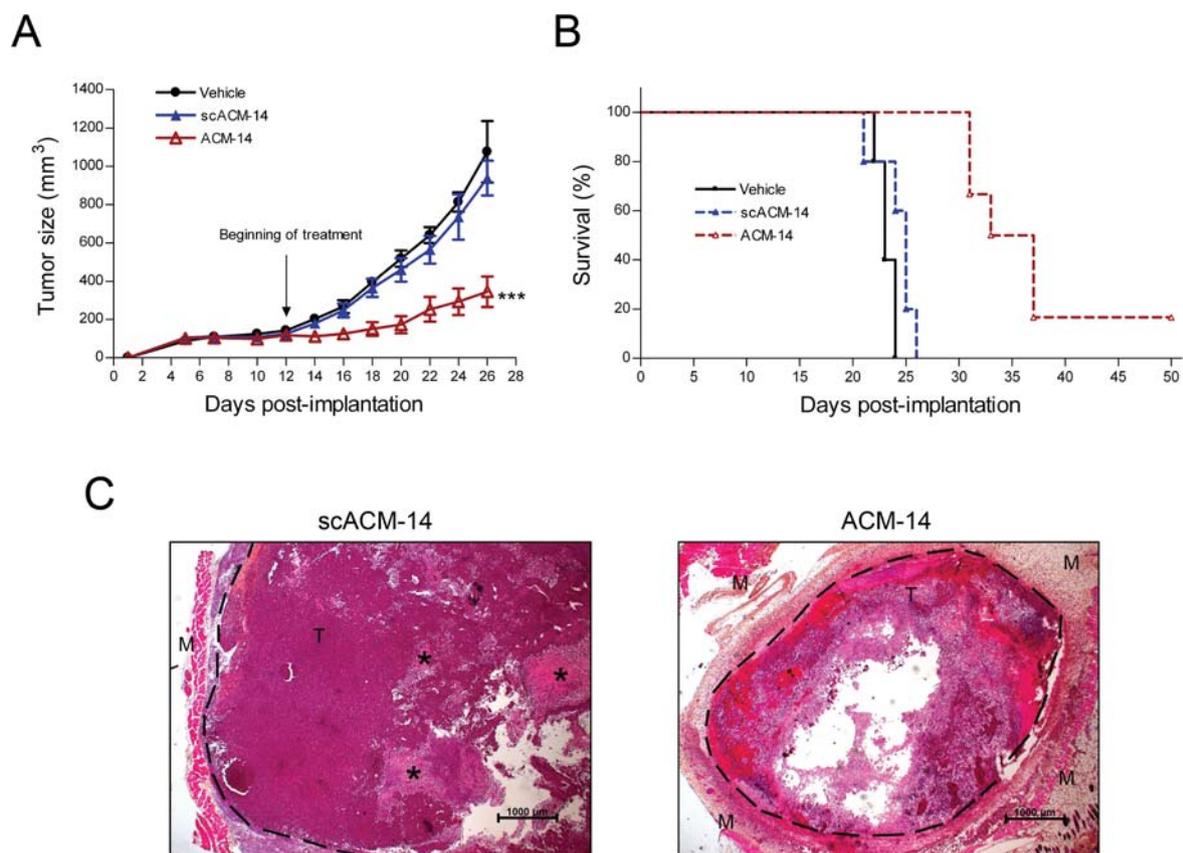


Figure 6. Inhibition of tumor growth by the ACM-14. A total of 5×10^6 HT-1080 fibrosarcoma cells were implanted subcutaneously in athymic nude mice and after 12 days, peptide (ACM-14 or its control scACM-14; 10 mg/kg) or vehicle control was subcutaneously administered daily. A: Kinetics of tumor growth. $n=5$ for vehicle, $n=5$ for scACM-14, $n=6$ for ACM-14. $***P < 0.001$. B: Kaplan-Meier survival curves. C: Histological analysis of paraffin-embedded xenografts. Dotted lines show the tumor zones. Necrotic tumor cells show a pink staining, while viable tumor cells show a purple staining. M, Mouse tissue; T, tumor; *small necrotic zones. Black scale bars represent 1000 μm .

Although the mechanisms involved in this interaction remain to be established, it is noteworthy that the PRXXLYC⁵⁷⁴XRS XXXXV motif located in the cytoplasmic tail of MT1-MMP has been suggested to be involved in the dimerization of the enzyme and this sequence may thus interact with the peptide (16). Such a dimerization of ACM-14 with the cytoplasmic tail of MT1-MMP may result in the inaccessibility of the cytoplasmic tyrosine 573 for phosphorylation reactions. Interestingly, since this dimerization motif is also present in other transmembrane MMPs involved in tumor cell invasion within 3D collagen matrix, such as MT2- and MT3-MMP (17) and that these enzymes also possess a conserved tyrosine residue adjacent to the cysteine residue (18), these MT-MMPs may also undergo tyrosine phosphorylation in their cytoplasmic tail and may thus be sensitive to ACM-14 inhibition. Further studies are currently underway in our laboratory in order to investigate this possibility.

Owing to its ECM-degrading activity and important role in tumor progression, MT1-MMP represents an attractive target for anticancer therapies. However, broad-spectrum inhibitors of MMP activity described to date have been largely unsuccessful in clinical trials and, in some patients, also induced musculoskeletal side-effects (19, 20). Although the exact mechanisms underlying these effects remain incompletely understood, they may involve an inhibition of MT1-MMP catalytic activity since mice lacking this enzyme present severe skeletal effects (19, 20). In this context, the identification of molecules that impair tumor cell proliferation *in vitro* and significantly delay tumor progression in mice by interfering with the tyrosine phosphorylation of MT1-MMP, such as the ACM-14 peptide described in this study, may represent a novel and promising approach to inhibiting this important enzyme. Further studies aimed at the molecular characterization of the pathways involved in the tyrosine phosphorylation of MT1-MMP as

well as their participation in tumor progression could thus provide interesting new targets for the development of anticancer therapeutics.

Acknowledgements

We would like to thank Dr. Yannève Rolland for helpful advices and technical assistance during animal manipulations. This study was supported by a grant (MOP62918) from the Canadian Institutes for Health Research to R.B. and D.G.

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Received November 24, 2009

Revised April 27, 2010

Accepted April 30, 2010