Cell permeable NM23 blocks the maintenance and progression of established pulmonary metastasis

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Commercialization right on the intellectual property (CELL PERMEABLE NM23 RECOMBINANT PROTEINS, POLYNUCLEOTIDES ENCODING THE SAME, AND ANTI-METASTATIC COMPOSITION COMPRISING THE SAME, PCT Application PCT/KR2008/005221 (Patent Pending)) presented in this article has been acquired by ProCell Therapeutics, Inc. from Chonnam National University in Gwangju, Korea. Daewoong Jo was the founding scientist of ProCell Therapeutics, Inc., and is affiliated to Vanderbilt University at present. Junghee Lim, Giyong Jang, Seeun Kang and Kyuwha Lee are employees of ProCell Therapeutics, Inc. Hereby; these authors disclose a financial interest in the company. The other authors disclosed no potential conflicts of interest.

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Abstract

Occult metastases are a major cause of cancer mortality, even among patients undergoing curative resection. Therefore, practical strategies to target the growth and persistence of already-established metastases would provide an important advance in cancer treatment. Here, we assessed the potential of protein therapy using a cell permeable NM23-H1 metastasis suppressor protein. Hydrophobic transduction domains developed from a screen of 1,500 signaling peptide sequences enhanced the uptake of the NM23 protein by cultured cells and systemic delivery to animal tissues. The cell permeable (CP)-NM23 inhibited metastasis associated phenotypes in tumor cell lines and blocked both the establishment of lung metastases and cleared already established pulmonary metastases, significantly prolonging the survival of tumor-bearing animals. Therefore, these results establish the potential use of cell-permeable metastasis suppressors as adjuvant therapy against disseminated cancers.

PRÉCIS: This study offers a preclinical proof of concept that protein-based therapies can be used to target malignancies that have spread into the lung, a common site of metastasis in human cancers.
Introduction

Metastasis is an acquired and separately evolving phenotype that enables cancer cells to disseminate and grow at locations distant from the primary tumor site. For many tumors, the molecular changes responsible for initiating metastatic spread have already occurred by the time of initial diagnosis, and are ultimately responsible for most cancer deaths (1, 2). Effective strategies to target disseminated tumors are therefore expected to have tremendous therapeutic benefit.

In principle, anti-metastasis therapies could either block activities required for the growth or survival of disseminated cancer cells, or restore the expression and/or activity of proteins that function to suppress metastasis. The latter includes over 20 metastasis suppressors; proteins that selectively inhibit the seeding, growth or persistence of metastatic foci while having only limited effects on primary tumors (3). NME1, the first reported metastasis suppressor gene, was initially characterized as nucleoside diphosphate kinase (NDK), an enzyme required to maintain cellular pools of nucleoside triphosphates. Interest in NDK as a metastasis suppressor (alternatively named NM23-H1 or NM23) was prompted by studies describing inverse correlations between NM23 expression and metastatic potential, first in melanoma cells (4) and later in other types of tumors (5). Subsequent gene transfer experiments documented the ability of NM23 to suppress metastasis-associated phenotypes both in cultured cells and in animal metastasis models (6-10). The precise mechanism by which NM23 influences metastasis is not understood, in part because the protein possesses multiple enzymatic activities that directly or indirectly suppress mitogen activated protein kinase (MAPK) signaling (11, 12); regulate small G-protein functions important in cell motility, cytoskeletal reorganization and cell adhesion(13-15); and influence genome maintenance (16, 17). Nevertheless, clinical trials based on hormonal activation of endogenous NM23 expression are currently in progress (4).
In the present study, we describe an anti-metastasis therapy based on the systemic delivery of a cell penetrating NM23-H1 protein. For this, we developed novel macromolecule transduction domains (MTD) modeled after hydrophobic signal peptides previously shown to promote protein uptake by cultured cells and animal tissues (18). The MTD-NM23 inhibited metastasis-associated phenotypes in tumor cell lines and not only suppressed the establishment of lung metastases but also cleared previously established metastases, significantly prolonging the survival of animals harboring disseminated tumor cells.
Materials and Methods

Preparation of recombinant MTD-fused proteins. MTD-76 and MTD-77 were identified from a screen of 1,500 signaling peptides for sequences with protein transduction activity. Sequences spanning amino acids 8-30 of NP631283 (FLIAGVIVALLA VFTVVRA VRIV) and 1-23 of NP003231 (MWPLWCWLEVLPLAGPGAALT) were subsequently modified (Jo et al., manuscript in preparation), generating ALVLPLAP and AVALLILAV, respectively. Coding sequences for NM23 and EGFP fusion proteins were cloned into pET-28a(+) (Novagen) from PCR-amplified DNA segments (Supplementary Table S1). The recombinant proteins were purified from *E. coli* BL21-CodonPlus (DE3) cells grown at an A600 of 0.6 and induced for 2 hrs with 0.7 mM IPTG. Denatured 6xHistidine-tagged recombinant proteins were purified by Ni$^{2+}$ affinity chromatography as directed by the supplier (Qiagen). After purification, they were then dialyzed against a refolding buffer (0.55 M guanidine HCl, 0.44 M L-arginine, 50 mM Tris-HCl, 150 mM NaCl, 1 mM EDTA, 100 mM NDSB, 2 mM reduced glutathione, and 0.2 mM oxidized glutathione) and then against a physiological buffer such as RPMI 1640 medium. H, N and M stand for the His tag, NM23 and MTD, respectively. Histidine-tagged recombinant NM23 proteins were HN (His-NM23), HM$_{76}$N (His-MTD76-NM23), HNM$_{76}$ (His-MTD76-NM23), HM$_{77}$N (His-MTD77-NM23) and HNM$_{77}$ (His-NM23-MTD77).

Analysis of protein uptake in cultured cells. Recombinant proteins were conjugated to 5/6-FITC, according to the manufacturer’s instructions (Pierce Chemical). RAW 264.7 cells were treated with 10 μM FITC-labeled proteins for 1 hr at 37°C, washed three times with cold PBS, treated with proteinase K (10 μg/ml) for 20 min at 37°C to remove cell-surface bound proteins and subjected to FACS analysis (FACSCalibur; Becton, Dickinson). Each experiment was conducted at least three times.
Experiments to visualize protein uptake were conducted in much the same manner except NIH3T3 cells were exposed to 10 µM FITC-proteins for 30 min and then nuclei or plasma membranes were counter stained with 1 µg/ml propidium iodide (Sigma-Aldrich) or 5 µg/ml FM4-64 (Molecular Probes), respectively. The cells were washed three times with cold PBS and examined by confocal laser scanning microscopy.

**Systemic delivery of MTD fusion proteins.** 6-week-old Balb/c female mice were injected intraperitoneally (300 µg/head) with FITC only, FITC-conjugated EGFP or NM23 recombinant proteins. After 2 hrs, the liver, kidney, spleen, lung, heart, and brain were isolated, infused with O.C.T. compound (Sakura) and frozen on dry ice. Cryosections (20 µM thickness) were analyzed by fluorescence microscopy. Alternatively, the uptake of FITC-NM23 proteins was assessed after 1 hr and 2 hrs by FACS analysis of unfixed blood leukocytes/lymphocytes or total splenocytes.

To determine the tissue distribution of CP-NM23 proteins, mice were injected I.V. with diluent, HN or MTD-NM23 (600 µg/mouse). The spleen and liver were removed after 3 hrs, embedded in paraffin, sectioned at 5 µM and immunostained with NM23 (Santa Cruz Biotechnology) or 6xHis tag (Abcam) monoclonal antibodies, and followed by HRP-conjugated goat anti-mouse secondary antibody (Santa Cruz).

**Immunoblot analysis.** MDA-MB-435, MDA-MB-231 and A549, HCT116 cell lines were purchased from the Korean Cell Line Bank (Seoul, Korea) and ATCC (Manassas, USA), respectively and maintained as recommended by the suppliers. The biological activities of the cell lines (MDA-MB-435, MDA-MB-231 and HCT116) were authenticated by *in vivo* metastasis and/or tumor growth in Balb/c nu/nu mice. All cell lines were negative for mycoplasma as assessed by using MycoALERT (2009; Lonza). Cells were treated at 37°C
with 10 μM recombinant proteins (HN, HM\textsubscript{76}N, HNM\textsubscript{76}, HM\textsubscript{77}N, or HNM\textsubscript{77}) or with buffer only. After 1 hr, cells were processed for immunoblot analysis to detect the phosphorylation of MEK, ERK and p53 or incubated for an additional 8 hrs to monitor changes in EDG2 and VEGF expression. Western blots were probed with antibodies against EDG2, VEGF and β-actin (Santa Cruz) and phospho-ERK, phospho-MEK, and phospho-p53 (Cell Signaling). The secondary antibody was goat anti-mouse IgG-HRP (Santa Cruz).

**Invasion assay.** MDA-MB-435 human breast-cancer cells were treated with HN, HM\textsubscript{76}N, HNM\textsubscript{76}, HM\textsubscript{77}N, or HNM\textsubscript{77} proteins (10 μM) or with buffer alone for 1 hr at 37°C. 1×10\textsuperscript{5} cells were added to the top of a trans-well chamber partitioned with a Matrigel-coated (40 µg per well; BD Biosciences) polycarbonate membrane filter (BD Falcon) with a pore size of 3 μM. After 24 hrs at 37°C, the filters were washed with PBS and the non-invasive cells remaining on the surface of the upper part were removed with a cotton swab. The invasive cells that passed through the Matrigel and migrated to the lower part of the filter were fixed with 4% paraformaldehyde for 10 min, stained with 0.5% (w/v) hemacolor for 20 min and counted with an optical microscope. Statistical significance was determined by a Student’s \( t \)-test.

**Wound-healing assay.** Confluent monolayers of MDA-MB-435 cells were treated with HN, HM\textsubscript{76}N and HNM\textsubscript{76}, HM\textsubscript{77}N or HNM\textsubscript{77} proteins (10 μM) or with buffer alone for 1 hr at 37°C. After the cells were washed with PBS, they were wounded with a sterile yellow micropipette tip and cultured for 24 hrs at 37°C. The cells were fixed in methanol for 1 min, stained with Giemsa (Chameleon Chemical) for 5 min and were washed with distilled water. The migration was quantified by counting the number of cells that migrated from the wound edge into the clear area. Representative data from three experiments are presented.
**Adhesion assay.** 96-well plates were coated with either 10 μg/mL fibronectin (BD Biosciences), 10 μg/mL mouse collagen IV (BD Biosciences) or 1 mg/mL BSA for 16 hrs at 4°C. Wells were then washed with 1 mg/mL BSA RPMI and blocked for 1 hr with 5 mg/mL BSA RPMI at 37°C. MDA-MB-435 or MDA-MB-231 cells (2×10^4 well) were allowed to adhere for 30 min following treatment with 10 μM of recombinant proteins (HN or HM77N) for 24 hrs. The wells were washed carefully, and adherent cells were fixed, stained with crystal violet and dried overnight. Cells were lysed in 2% SDS solution. Cell number was estimated with a SpectraMax microplate reader (Molecular Devices) at 550 nm. Background absorbance from a BSA-coated well was subtracted. Statistical significance was determined by a Student’s t-test.

**Anchorage-independent growth assay.** A bottom agar (Difco) of 0.5% in DMEM + 10% FBS was allowed to harden in all wells of a 24-well plate. Cells (MDA-MB-435) that were treated for 24 hrs with 10 μM of recombinant proteins (HN or HM77N) were suspended in a 0.3% agar/RPMI + 10% FBS medium at a concentration of 1×10^4/mL. 0.5 mL of each cell suspension was plated on top of the bottom agar in triplicate wells. Colony number was assessed after 14 days of growth at 37°C. Colonies larger than 3 mm in diameter were counted. This assay was performed in triplicate. Statistical significance was determined by a Student’s t-test.

**Tube-formation assay.** Endothelial cell tube-forming assays were performed in 24-well Matrigel (BD Bioscience) coated plates. 5×10^4 human umbilical-vein endothelial cells (HUVECs) were treated with 5 μM of recombinant protein (HN or HM77N) for 1 hr or 12 hrs and tube-formation was monitored after 24 hrs.
**Xenograft metastasis models.** Female Balb/c nu/nu mice were intravenously inoculated (5×10⁶) with human breast-cancer cells (MDA-MB-435) through the lateral tail vein. After 5 weeks, tumor metastasis-induced mice were intravenously administered with protein (300 μg/mouse) for a period of 3 weeks. There were an additional 2 weeks of observation by our group (ProCell R&D Institute, Korea) or a professional CRO (Biotoxtech, Korea), and maintenance for 24 or 40 weeks for the determination of the survival rate of the mice following the termination of treatment. After protein treatment, half of the mice were killed, and six organs (brain, heart, lung, liver, kidney, and spleen) from each were collected and kept in a suitable fixation solution until the next step. Lung tissues were kept in Bouin’s fixative solution overnight to detect metastasis. 5 μm formalin-fixed, paraffin-embedded lung sections were immunostained with mouse anti-vimentin antibody (Abcam) followed by goat anti-mouse IgG-HRP (Santa Cruz Biotechnology).

**Statistical analysis.** All experimental data obtained from cultured cells are expressed as the means ± S.D. from at least three independent experiments. For the invasion assays, annexin V assays, caspase-3 assays, anchorage-independent growth assays and adhesion assays, statistical significance was evaluated using a one-tailed Student’s *t*-test. For animal tests, paired *t*-tests were used for comparisons between and within groups to determine the significance of the differences in survival *in vivo*. Statistical significance was established as *p* < 0.05.
Results

Protein sequences with enhanced MTD activity. Previous studies using hydrophobic MTD to deliver protein cargoes into cultured cells and animals almost exclusively used an MTD derived from the signal peptide of fibroblast growth factor 4 (FGF-4) (19-21). To identify sequences with enhanced MTD activity, we screened a library of predicted hydrophobic signal peptides for their ability to promote the uptake of fluorescein isothiocyanate (FITC)-labeled EGFP fusion proteins into cultured cells (Jo et al. manuscript in preparation). High scoring peptides were further modified and included two [MTD-76 (ALVLPLAP: M76) and MTD-77 (AVALLILAV: M77)] with greater (~2.2- and 4.0-fold respectively; Supplementary Fig. S1A and B) protein transduction activity than FGF-4 MTD (AAVLLPVLLAAP: Mm). Conversely, a random hydrophobic peptide (SANVEPLERL: S) did not increase EGFP uptake. Moreover, MTD-76 and MTD-77 enhanced the systemic delivery of EGFP fusion proteins to multiple tissues following intra-peritoneal injection (Supplementary Fig. S1C), suggesting that both MTDs were effective in vitro and in vivo.

CP-NM23 Proteins. NM23 proteins containing MTD-76 or MTD-77 fused to either the N- or C-terminus along with an amino-terminal 6xHis tag were expressed in E. coli and purified by Ni\textsuperscript{2+} affinity chromatography (Supplementary Fig. S2A), with yields of soluble protein ranging from 4 to 15 mg/L. Higher levels of all MTD-NM23 fusion proteins (HM\textsubscript{76}N, HNM\textsubscript{76}, HM\textsubscript{77}N, and HNM\textsubscript{77}) accumulated in RAW cells, as compared to a control protein (His tagged NM23, HN) without an MTD sequence (Supplementary Fig. S2B); however, MTD-77 had greater transduction activity, regardless of whether the MTD was positioned on the amino or carboxyl terminal end. Given this difference, subsequent experiments focused primarily on MTD-77, which also enhanced the uptake of NM23 by RAW cells (Supplementary Fig. S2C) and systemic delivery of NM23 to various organs in mice.
CP-NM23 suppresses MAPK signaling and cell migration. The metastasis suppressor NM23 has pleiotropic effects on MAPK signaling, EDG2 (lysophosphatidic acid receptor) expression and cell migration. We, therefore, examined the effects of CP-NM23 on steady-state MEK and ERK phosphorylation and EDG2 expression in several human tumor cell lines. Both MTD-76- and MTD-77-NM23 proteins suppressed MEK and ERK phosphorylation in MDA-MB-435 breast cancer cells (Fig. 1A), although MTD-77-NM23 proteins (HM77N and HNM77) were more potent suppressors, consistent with higher levels of protein uptake (Supplementary Fig. S2B). The effects of HM77N were even more striking; with near complete suppression of MEK and ERK phosphorylation and EDG2, expression in MDA-MB-231 and A549 cells (Fig. 1B and C). These results suggest that MTD-76 and MTD-77 can deliver biologically active NM23 protein into human tumor cells.

We next used a matrigel invasion assay to assess the effects of CP-NM23 proteins on cell migration. Tumor cells with high mobility [MDA-MB-435 (Fig. 2A, left panel) and MDA-MB-231 (Fig. 2A, right panel)] were treated with recombinant proteins for 1 hr, and their migration was compared to controls treated with media alone or with NM23 lacking an MTD. HM76N, HM77N and HNM77 significantly inhibited migration in both cell lines (by 68 and 56%; 79 and 58%; and 74 and 58%, respectively). Similar results were obtained in a wound-healing assay using the same cell lines (Fig. 2B). HM77N, which displayed the greatest uptake and activity suppressing MAPK signaling and cell migration, was selected as the best candidate to test for anti-metastasis activity.

CP-NM23 suppresses metastasis-associated phenotypes. The metastatic phenotype relies on the ability of tumor cells to adhere to distal sites, survive, and promote angiogenesis. We
therefore evaluated the impact of HM77N on these aspects of the metastatic phenotype. First, we examined the adhesion of cancer cell lines to fibronectin and type 4 collagen, two major components of the extracellular matrix. HM77N significantly inhibited the attachment of MDA-MB-435 (Fig. 3A) and MDA-MB-231 (Fig. 3B) cells to fibronectin, and suppressed the attachment of MDA-MB-435 cells to type IV collagen. MDA-MB-231 cells adhered poorly to type 4 collagen, and attachment was not reduced further by HM77N. HM77N also inhibited anchorage independent growth of MDA-MB-435 cells (Fig. 3C) and induced significant apoptosis as assessed by caspase-3 activity and annexin V staining (Fig. 3D and E). However, HM77N was not overtly toxic to untransformed (NIH3T3) cells (Supplementary Fig. S4). To determine the effects of CP-NM23 on p53-dependent apoptosis and angiogenesis, three different human cancer cell lines originating from breast (MDA-MB-435), colon (HCT116) and lung (A549) cells were treated with CP-NM23. HM77N positively regulated the phosphorylation of p53 and negatively controlled the expression of metastasis-specific markers, EDG2 and VEGF (Supplementary Fig. S5). Thus, CP-NM23 suppressed multiple aspects of the metastatic phenotype.

Since metastasis also involves processes extrinsic to tumor cells, including tissue remodeling and angiogenesis, HM77N was also tested for anti-angiogenic activity (Supplementary Fig. S6). Primary human vascular endothelial cells (HUVACs) treated with buffer alone or control NM23 protein (HN) spontaneously organized into tubes when cultured on a matrigel substrate whereas, HM77N effectively blocked tube formation.

Systemic delivery of CP-NM23. Systemic delivery of CP-NM23 was examined in mice following intravenous (IV) injection of fluorescein isothiocyanate (FITC)-labeled HM77N. High levels of FITC-HM77N were observed in peripheral blood leukocytes/lymphocytes, total splenocytes and in liver and spleen cryosections within 1 hr post-injection. The protein
persisted for several hours as assessed by flow cytometry (Fig. 4A), fluorescence microscopy (Fig. 4B) and immunostaining with antibodies specific for either NM23 (Fig. 4C, left panel) or the His-peptide tag (Fig. 4C, right panel). In contrast, the same cells and tissues failed to accumulate FITC alone or FITC-labeled NM23 protein lacking the MTD-77 sequence (FITC-HN). The half-lives of internalized MTD-fused recombinant NM23 proteins in PBMC and spleen cells were 4.5 and 2.8 hrs, respectively (Jo et al., manuscript in preparation). These results demonstrate the ability of MTD-77 to deliver a biologically active NM23 fusion protein to multiple cells and tissues.

**CP-NM23 enhances survival of mice with disseminated metastases.** We next tested CP-NM23 for anti-tumor activity in xenograft metastasis models. MDA-MB-435 cells, a metastatic human breast-cancer cell line, were injected (5x10^6) into the lateral tail veins of 5-week-old, MHC-deficient Balb/c nu/nu mice, and after 5 weeks, 10 mice each were treated daily with diluent alone or with 300 μg HN, HM77N, or HNM77 proteins for three weeks (week 5 ~ 8). Animal survival was monitored for 24 weeks (Fig. 5A). HM77N and HNM77 proteins both greatly enhanced survival as compared to controls (p<0.05). Consistent with differences in survival, mice treated with CP-NM23 proteins contained far fewer lung metastases at the end of the treatment period at week 8 (Fig. 5B, top panel) and two weeks later (Fig. 5B, bottom panel) than lungs from mice treated with diluent alone or with NM23 protein without a MTD sequence. These macroscopic differences were confirmed by a histologic examination of lung sections immunostained for vimentin, a marker of tumor invasiveness (Fig. 5C). Similar or greater survival differences between HM77N treatment and controls were obtained when the experiment was extended for 40 weeks (Fig. 6A; p<0.001) and when mice were treated twice weekly instead of daily (Fig. 6B; p<0.001). As before, lungs from control mice contained greater numbers of visible vimentin-expressing...
metastases at week 8 as compared with HM77N treated mice (Fig. 6C and D). Similar results were obtained following tail vein injection of $5 \times 10^6$ MDA-MB-231 cells in fewer animals (5 per treatment group, data not shown).

These results demonstrate that protein therapy with CP-NM23 significantly inhibits the growth and persistence of well-established breast cancer metastases in vivo, resulting in increased survival. To test whether CP-NM23 can inhibit the seeding of tumor metastases, protein therapy was initiated at the same time mice were injected with MDA-MB-435 tumor cells. Mice injected daily with HM77N exhibited enhanced survival (90%) after 40 weeks as compared with control mice (20%; Fig. 7A), and remained virtually free of metastases at weeks 3, 8, 13, and 20 (Fig. 7B and C). These results suggest that CP-NM23 inhibits the initial seeding of metastatic cancer cells as well as the growth and persistence of established tumors.
Discussion

Metastasis is an important clinical problem responsible for the vast majority of all cancer deaths, including a high percentage of therapeutic failure following curative resection. For example, the 5-year recurrence rate for lymph node negative colon, lung and breast/prostate cancers is 30%, 40%, and 10-20%, respectively (2). Adjuvant therapies capable of arresting or reversing the metastatic process are therefore expected to have a significant impact on cancer mortality. In the present study, we demonstrate the effectiveness of protein-based therapy to deliver cell permeable (CP) -NM23 protein as an anti-metastatic agent. The CP-NM23 inhibited metastasis associated phenotypes in tumor cell lines and not only suppressed the establishment of lung metastases but also cleared already established metastases, greatly prolonging the survival of animals harboring disseminated tumor cells. Our approach made use of new macromolecule transduction domains (MTDs) designated MTD-76 and MTD-77, which were selected from a screen of 1,500 hydrophobic signaling peptides and subsequently modified to eliminate charged and polar amino acids, increase predicted $\alpha$-helical content and limit the number of consecutive hydrophobic residues (Jo, et al., manuscript in preparation). In the present study, MTD-76 and MTD-77 significantly improved (up to 4-fold) the delivery of recombinant proteins into cultured cells and tissues as compared to the hydrophobic transduction domain based on the FGF-4 signal peptide. These data establish MTD-76 and MTD-77 as potential vectors for protein-based therapeutics. Our decision to investigate hydrophobic MTDs rather than use basic protein transduction domains (e.g. HIV tat) was based on several considerations. First, previous studies had made only a limited effort to identify hydrophobic sequences with enhanced protein transduction activity (18). Second, despite this limitation, the FGF-4 MTD has been successfully used to deliver biologically active peptides and proteins systemically in animals including dramatic protection to lethal pro-inflammatory conditions (20, 22-24). Finally, the
hydrophobic MTDs are thought to enter cells directly by penetrating the plasma membrane; (18) by contrast, the basic protein transduction domains bind to the cell surface and bulk entry occurs by endocytosis (25). While these distinctions are not absolute and uptake mechanisms may vary depending on the cargo, we were concerned that endocytosed proteins that fail to reach the cytosol (26) could limit the bioavailability of proteins with basic transduction domains (27).

CP-NM23 protein suppressed multiple metastasis-associated phenotypes in cultured tumor cells including cell migration, adhesion and matrigel invasion, and blocked angiogenic tube formation by vascular endothelial cells. These effects were accompanied by reductions in MAPK signaling (notably MEK and ERK phosphorylation), EDG2 expression and enhanced apoptosis, consistent with the effects of augmented NM23 gene expression in cultured cells (11, 13-15, 28). In principle, such activities are expected to suppress multiple early events in the metastatic process such as invasion, attachment, colonization and neovascularization. Indeed, CP-NM23 blocked the seeding of pulmonary metastases when administered at the time tumor cells were introduced into the blood stream. Moreover, CP-NM23 also targeted already established metastases, in some cases clearing the lungs of tumors and greatly increasing survival.

The levels of metastasis suppression achieved by CP-NM23 were comparable to if not greater than those reported after enforced expression of the NM23 gene in tumor cell lines (6-10). This suggests that the activity of systemically delivered MTD-77-NM23 approaches theoretical limits determined by the biology of the NM23 function in tumor cells. MTD-77-NM23 also outperformed gene therapy (29, 30) and hormonal activation of the endogenous NM23 gene (12). The latter study, which provided the basis for human trials of medroxyprogesterone acetate, reported 55% fewer lung metastasis in treated mice after 14 weeks; whereas, most mice treated with cell permeable NM23 remained free of lung
metastases even after 20 weeks. Moreover, while medroxyprogesterone-treated mice maintained weight better (by 18% after 14 weeks), we observed far more dramatic survival differences after 40 weeks (80-100% treated animals survive vs. 0-25% of mice in the control groups). These results underscore the ability of MTD-77 to systemically deliver biologically active proteins into blood-borne tumor cells and metastases. Moreover, in addition to targeting tumor cells, the efficacy of CP-NM23 as a metastasis suppressor may benefit from targeting other cells and processes, required to establish and maintain metastases in ectopic tissue niches.

Although NM23-H1 was initially characterized as a metastasis suppressor, the protein functions in normal hematopoiesis (31, 32) and plays complex roles in the development of different malignancies (33). Moreover, NM23-H1 functions are not always intracellular, judging from activities mediated by extracellular NM23 (34, 35). In particular, the protein is overexpressed in some tumors, including hematologic malignancies, and is present at elevated levels in patient sera where the protein appears to promote tumor cell growth and survival by autocrine and/or paracrine mechanisms (36-39). In the present study, we show that NM23-H1 lacking an MTD sequence does not efficiently enter cells. This underscores the idea that the biological effects of externally applied NM23 protein originate from outside the cell and not from internalized protein. Even so, considering the widespread ability of proteins to enter cells (40), studies investigating extracellular HM23 should examine this issue more carefully. Conversely, the anti-metastatic function of HM23, which strictly required an MTD sequence, appears to be mediated by intracellular protein. However, while the MTD sequence and protein internalization appear necessary, they may not be sufficient for the full anti-metastatic response. Additional experiments will be required to determine if extracellular NM23, for example acting on myeloid cells, contributes to the anti-metastatic response.
In summary, despite widespread interest in metastasis as a therapeutic target, most anti-metastatic drugs currently in development focus on tumor cell migration and invasion with uncertain utility against disseminated disease (41). Our results describe a potential therapeutic strategy to target occult metastases that are resistant to conventional chemotherapy.
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FIGURE LEGENDS

Figure 1. Inhibition of MAPK signaling and EDG2 expression by CP-NM23. A to C, MDA-MB-435 (A), MDA-MB-231 (B) and A549 cells (C) were treated for 1 hr with 10 μM of the indicated recombinant NM23 proteins. Cell lysates, prepared immediately (P-MEK and P-ERK) or after 8 hrs (EDG2) were immunoblotted with antibodies against the indicated proteins.

Figure 2. CP-NM23 suppresses cell invasion and migration. A, Matrigel invasion assay. Tumor cells were treated for 1 hr with the indicated proteins (10 μM); and after 24 hrs, cells penetrating and migrating to the basal side of matrigel-coated membranes were visualized (upper panels) and counted (lower panels). The data are presented as means ± s.d. (n = 3), * p < 0.01, ** p < 0.05, as determined by a Student’s unpaired t-test. B, Wound-healing assay. Cell monolayers, treated with NM23 proteins as in (A), were scraped to create a “wound” devoid of cells (dashed lines) and photographed after 24 hrs. Data shown here are representative of five independent assays.

Figure 3. CP-NM23 inhibits cell adhesion and anchorage-independent growth, and induces apoptosis in cancer cells. A and B, Adhesion assay. MDA-MB-435 (A) and MDA-MB-231 (B) cells were treated with 10 μM of recombinant NM23 proteins (HN: gray bar or HM77N: black bar) or with a vehicle (cells only: white bar). Cells adhering to fibronectin or type IV collagen were assessed by absorbance at 560 nm. The data are presented as means ± s.d. (n = 3), * p < 0.05, ** p < 0.005, Student’s unpaired t-test. C, Anchorage-independent cell-growth. MDA-MB-435 cells were treated with 10 μM of recombinant NM23 proteins (HN: gray bar or HM77N: black bar) or vehicle (cell only: white bar). Colonies larger than 3 mm in diameter were counted and presented as means ± s.d. (n
= 3), *p < 0.005, as determined by a Student’s unpaired t-test. D and E, Apoptosis induction by CP-NM23. CP-NM23-mediated activation of caspase-3 in cancer cells (MDA-MB-435) treated with 10 μM control protein (HN, gray), CP-NM23 (HM77N, black) or vehicle (cell only: white) for 4 or 8 hrs (D). Apoptosis as assessed by annexin V staining was determined in the cells treated with HM77N for 2 or 4 hrs (E). The data are represented as a mean ± s.d. of three independent experiments. *p < 0.05, as determined by a Student’s unpaired t-test.

**Figure 4.** Systemic delivery of CP-NM23. A, Mice were injected I.V. with FITC-conjugated HM77N, HN or with diluent alone (solid grey) and protein uptake by peripheral blood leukocytes/lymphocytes (left panel) or splenocytes (right panel) was assessed by flow cytometry after 1 (blue line) or 2 (red line) hrs. B and C, Tissue distribution of HM77N at the indicated times after injection was assessed by fluorescence microscopy (B) and after 3 hrs by immunostaining (C).

**Figure 5.** CP-NM23 suppresses established metastases. A, Kaplan-Meier plots comparing MDA-MB-435-injected mice treated with diluent (black square) or 300 μg/mouse/day of recombinant NM23 proteins (HN: blue triangle, HM77N: red circle or HNM77: magenta square). n = 10, *p < 0.05, as determined by a Student’s t-test. B, Lungs from mice were fixed in Bouin’s solution. C, Immunohistology of vimentin expression.

**Figure 6.** CP-NM23 promotes long-term survival in mice with established pulmonary metastases. A and B, Kaplan-Meier plots comparing MDA-MB-435-injected mice treated daily (A) or semi-weekly (B). n = 10, *p < 0.001, as determined by a Student’s t-test. Start
and Stop arrows indicate the start and stop of protein treatment (from week 5 to 8 after IV injection of cancer cells). C, Lungs isolated at weeks 0, 5 and 8 were photographed showing metastases (indicated by an arrow). D, immunohistology of vimentin expression.

**Figure 7. CP-NM23 blocked the establishment of metastases.** A, Kaplan-Meier plots of mice treated in a prophylactic protocol. Mice were injected with MDA-MB-435 tumor cells and were immediately treated with recombinant NM23 proteins daily for 3 weeks as in (A). n = 10, *p < 0.05, as determined by a Student’s *t*-test. B, Lungs fixed in Bouin’s solution. C, visible tumor colonies (> 1 mm length) were counted at week 3 and 13. n = 5, *p < 0.01, as determined by a Student’s *t*-test.
Figure 1

A

<table>
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<tr>
<th>Treatment (10μM)</th>
<th>MDA-MB-435</th>
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<tr>
<td>Proteins</td>
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<tr>
<td>P-MEK Ratio</td>
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<tr>
<td>P-ERK Ratio</td>
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<tr>
<td>β-actin</td>
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B

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<td>β-actin</td>
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C

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<td>Proteins</td>
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<tr>
<td>β-actin</td>
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</table>
Figure 2

A

Cell only

HM76N  HNM76  HM77N  HNM77

HN

Cell only

HM76N  HNM76  HM77N  HNM77

HN

B

Invaded Cell Number

0  100  200  300  400  500  600

Cell only  HN  HM76N  HNM76  HM77N  HNM77

MDA-MB-435

0h  24h

Invaded Cell Number

0  100  200  300  400  500  600

Cell only  HN  HM76N  HNM76  HM77N  HNM77

MDA-MB-231

0h  24h

**  *  *  *  *  *  *

0h  24h
Figure 4

A

blood leukocytes/lymphocytes

spenocytes

Counts

FITC-HM$_{77}$N

FITC-HM$_{77}$N

FITC-HN

FITC-HN

Diluent

1h

2h

FITC

B

1h

2h

FITC only

FITC-HN

FITC-HM$_{77}$N

FITC only

FITC-HN

FITC-HM$_{77}$N

Liver

Spleen

C

anti-Nm23 antibody

anti–His tag antibody

Diluent

HN

HM$_{77}$N

Diluent

HN

HM$_{77}$N

Liver

Spleen

(x400)
Figure 6

A

Diluent
HN
HM 77N

Survival (%)

weeks after injection of tumor cells

0 4 8 12 16 20 24 28 32 36 40 44

Start Stop

B

Diluent
HN
HM 77N

Survival (%)

weeks after injection of tumor cells

0 4 8 12 16 20 24 28 32 36 40 44

Start Stop

C

No Treatment

HN

HM 77N

5 weeks after xenografting

At week 8

D

No Treatment

HN

HM 77N

5 weeks after xenografting

At week 8
Figure 7

A

Survival (%)

Weeks after injection of tumor cells

B

C

Diluent

HN

HM77N

Weeks

0

3 8 13 20

Tumor colonies in lung

Weeks

3 13
Cell permeable NM23 blocks the maintenance and progression of established pulmonary metastasis

Junghee Lim, Giyong Jang, Seeun Kang, et al.

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