Metastasis is strongly reduced by the matrix metalloproteinase inhibitor Galardin in the MMTV-PymT transgenic breast cancer model

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Abstract
Matrix metalloproteinases (MMP) have several roles that influence cancer progression and dissemination. However, low molecular weight metalloproteinase inhibitors (MPI) have not yet been tested in transgenic/spontaneous metastasis models. We have tested Galardin/GM6001, a potent MPI that reacts with most MMPs, in the MMTV-PymT transgenic breast cancer model. We followed a cohort of 81 MMTV-PymT transgenic mice that received Galardin, placebo, or no treatment. Galardin treatment was started at age 6 weeks with 100 mg/kg/d, and all mice were killed at age 13.5 weeks. Galardin treatment significantly reduced primary tumor growth. Final tumor burden in Galardin-treated mice was 1.69 cm³ compared with 3.29 cm³ in placebo-treated mice (t test, \( P = 0.0014 \)). We quantified the total lung metastasis volume in the same cohort of mice. The median metastasis volume was 0.003 mm³ in Galardin-treated mice compared with 0.56 mm³ in placebo-treated mice (t test, \( P < 0.0001 \)). Thus, metastasis burden was reduced more than 100-fold, whereas primary tumor size was reduced only 2-fold. We also found that primary tumors from Galardin-treated mice exhibited a lower histopathologic tumor grade, increased collagen deposition, and increased MMP-2 activity. MMPs are known to have tumor-promoting and tumor-inhibitory effects, and several clinical trials of broad-spectrum MPIs have failed to show promising effects. The very potent antimetastatic effect of Galardin in the MMTV-PymT model does, however, show that it may be possible to find broad-spectrum MPIs with favorable inhibition profiles, or perhaps combinations of monospecific MPIs, for future clinical application. [Mol Cancer Ther 2008;7(9):2758–67]

Introduction
Matrix metalloproteinases (MMP) are potentially involved at several stages of cancer progression including tumor initiation, vascularization, invasion, and metastasis (1, 2). The roles of MMPs in cancer reflect their physiologic ability, through limited proteolysis, to restructure the tissue microenvironment and to convey extracellular signaling. In the healthy organism, MMPs are involved in several tissue remodeling processes during ontogenesis and adult life, including embryo implantation (3), bone development (4), mammary gland development (5, 6), and wound healing (7).

The MMPs constitute a large family of 22 extracellular metalloproteinases in mice (24 in humans) and are either secreted (15 MMPs) or membrane-anchored (7 MMPs) (1, 8, 9). The MMPs are synthesized as inactive proforms that require proteolytic activation. Ten of the 22 pro-MMPs, including the membrane-anchored MMPs, have a furin cleavage site and may be activated by furin-like proprotein convertases before they are exported from the cell. The remaining pro-MMPs are activated extracellularly typically by the serine protease plasmin or by other MMPs. The MMP system is counterbalanced by a group of four tissue inhibitors of metalloproteinases (TIMP) that have varying specificities for individual MMPs (10). In addition, the membrane-anchored MMP inhibitor RECK regulates a subgroup of MMPs including MMP-2, -9, and -14 (11). The MMPs are collectively able to degrade any component of the extracellular matrix. Important substrates for the MMPs as a group include the native fibrillar collagens, all major basement membrane components, and several extracellular signaling molecules.

The structural integrity of the stroma is an essential factor in preventing carcinogenesis (12). In the mammary gland, disruption of the extracellular matrix and cellular microenvironment caused by transgenic overexpression of MMP-3 in its active form or of MMP-14 leads to tumor development even in the absence of a transgene-supplied oncogene (13, 14). In the presence of a transgene-supplied oncogene, tumor development, growth, and metastasis are in most cases promoted by increased MMP activity and inhibited by decreased MMP activity (reviewed in ref. 15). A couple of examples, here limited to studies of transgenic breast cancer models, illustrate the general...
finding: (a) tumor onset is promoted by MMP-7 overexpression (16) and delayed by TIMP-2 overexpression (17), (b) tumor growth is reduced by overexpression of either TIMP-1 (18) or TIMP-2 (17), and (c) tumor metastasis is reduced by overexpressing TIMP-1 through a liver-specific transgene (18).

Several small molecular weight metallocproteinase inhibitors (MPI) have been in clinical trials, but none of these compounds have, to our knowledge, been tested in a transgenic metastasis model (15). We have now used the MMTV-PymT transgenic breast cancer model (19) to analyze the effect of Galardin/GM6001/Ilomastat (Fig. 1; ref. 20). Galardin is a potent and broad-spectrum hydroxamate-type MPI that is designed as a molecular mimic of MMP substrates, which allows it to enter the active site of MMPs, where it strongly, but reversibly, binds the critical zinc atom (21). The MMTV-PymT transgene combines the mammary-specific LTR promoter from the MMTV virus with the pluripotent middle T oncogene from the mouse polyomavirus to induce breast tumors with 100% penetrance in virgin females (19). Virtually all MMTV-PymT mice also develop lung metastases (19, 22, 23). The histopathology (19, 24, 25) and expression of breast cancer biomarkers in the MMTV-PymT tumors, including hormone receptors, cytokeratins, ErbB2, and p1-integrin, are consistent with that seen in aggressive (human) breast cancers (24, 25). In contrast to several transplanted tumors, the expression patterns of MMPs (15, 26, 27) in the MMTV-PymT tumors are also similar to ductal mammary adenocarcinomas. In the present study, we show a dramatic reduction in the metastatic burden of MMTV-PymT mice after treatment with Galardin.

Materials and Methods

Galardin

The MMP inhibitor 3-(N-hydroxycarbamoyl)-2(R)-isobutylpropionyl-L-tryptophan methylamide (Galardin/GM6001/Ilomastat; molecular weight: 388.47) was synthesized essentially as described (21). The following IC50 values have been reported for Galardin against human MMPs: MMP-1 (6 nmol/L), MMP-2 (7/17 nmol/L), MMP-3 (28 nmol/L), MMP-7 (41 nmol/L), MMP-8 (1.4 nmol/L), MMP-9 (4.1/15 nmol/L), MMP-12 (23 nmol/L), MMP-13 (3.2 mmol/L), MMP-14 (23/33 nmol/L), MMP-15 (6 nmol/L), MMP-16 (8 nmol/L), and MMP-26 (17 nmol/L; refs. 28, 29). Galardin was administered in one of two ways: by daily injections (i.p.) at 100 mg/kg as a 20 mg/mL slurry in 0.9% saline, 4% clinically formulated carboxymethylcellulose or by surgical insertion of custom-made 60-day slow release tablets containing 150 mg Galardin each (Innovative Research of America). The tablets were inserted s.c. adjacent to the dorsal midline (MMTV-PymT model) or in the dorsal neck region (wound-healing analysis), and the insertion site was closed with wound clips (Roboz Surgical Instrument). The amount of Galardin per tablet corresponds roughly to 100 mg/kg/d for a 25 g mouse [150 mg / (0.025 kg x 60 days)]. Tablets were inserted under general anesthesia induced by s.c. injection of 0.03 mL/10 g of a 1:1 mixture of Dormicum (Midazolam 5 mg/mL) and Hypnorm (Fluanison 5 mg/mL and Fentanyl 0.1 mg/mL).

In vivo Analyses

All animal experiments were conducted according to institutional guidelines and were approved by the Danish Animal Experiments Inspectorate. A concurrent health report compliant with the guidelines of the Federation of European Laboratory Animal Science Associations revealed no infections.

MMTV-PymT Tumor Model. Hemizygous FVB/N-TgN(MMTVpYV/T)634 Mul (hereafter FVB-PymT; ref. 19) female virgins and nontransgenic controls were used throughout the study, all of which were generated from the same cohort of breeding pairs. All animals were weaned and tail-clipped at age 4 weeks, genotyped by PCR using a primer pair specific for the MMTV-PymT transgene (23), and distributed randomly into the treatment groups. Tumor onset and growth were monitored as described previously (22). Slow-release tablets containing Galardin or placebo were inserted at age 43 ± 1 days (range, 41-45 days). The mice receiving tablets were caged individually until sacrifice to allow fast and uniform closure of the tablet insertion site. To terminate the experiment, the mice were anesthetized as above, tumor tissue was removed and immediately placed on dry ice for biochemical analyses, and the mice were perfusion fixed as described (22). The lungs were removed, air-evacuated, fixed, cryoprotected with sucrose, and cryoembedded in OCT compound (Sakura Finetek) for stereologic metastasis quantification (30). Regional lymph nodes and tumor tissue for histopathology were removed and placed in freshly prepared PBS with 4% paraformaldehyde overnight at 4°C before automated paraffin embedding. Tumor tissue was cut into ~ 5-mm-thick slabs for optimal access of fixative.

Wound-Healing Model. Six- to 8-week-old FVB mice were anesthetized as described above, and 20-mm-long full-thickness incisional skin wounds were made mid-dorsally with a scalpel in each of 75 FVB mice randomly distributed with 15 in each of five groups: Galardin or placebo injections, Galardin or placebo tablets, and no treatment. The wounds were neither dressed nor sutured. The mice were caged individually and scored as described previously (7, 31). The administration of Galardin or placebo, as i.p. injections or slow-release tablets, was started 2 days before wounding.

![Galardin](image-url)
Histopathology
Fibers of collagens I, II, and III were stained in 5-μm paraffin sections using PicroSirius Red (32), counterstained with Weigert’s hematoxylin, and visualized using polarizing microscopy. Histopathologic analysis of tumor stage was done by an experienced pathologist (O.D.L.) on coded slides containing two Mayer’s H&E-stained sections made through the center of each (right side) abdominal gland/tumor and using the nomenclature introduced by Lin et al. (25, 33). In short, the development of mammary tumors is divided into five different classes or progression stages: normal gland, hyperplasia, adenoma, early carcinoma, and late carcinoma. The highest grade present in the two sections determined the score.

Stereologic Quantification of Lung Metastasis Volume
For volumetric measurement of total lung metastasis, the cryoembedded lungs were cut transversally to the trachea into uniform 2-mm-thick slabs. The five to eight slabs obtained from each lung were re-embedded in OCT compound into a single block as specified previously (30). From each block, 8-μm cryostat sections were obtained and stained with H&E. Stereologic determination of the metastasis volumes was done by computer-assisted point counting on coded slides (30).

Quantification of Tumor Cell Dissemination to Lymph Nodes
Three sections (3 μm), each separated by 15 μm, of brachial and axillary lymph nodes were stained with an anti-cytokeratin-8 antibody as described previously (23). Coded slides were analyzed under the microscope for the presence of cancer cells. A lymph node specimen was considered positive if one or more cytokeratin-8-positive cells were clearly identified beneath the node capsule in any one of the three sections.

Gelatin Zymography
Frozen tumor tissue from the left side abdominal gland/tumor was crushed using a mortar and pestle, lysed in 5 μL lysis buffer [0.5 mol/L Tris-HCl, 0.2 mol/L NaCl, 10 mmol/L CaCl, 1% Triton-X-100 (pH 7.6) per milligram weight of tissue, and homogenized for 2 × 8 min in an ultrasound bath. The resulting supernatants were collected after centrifugation at 12,000 × g for 30 min at 4°C. Equal amounts of total protein were loaded onto a 10% Bis-Tris Novex Zymogram Gel (Invitrogen) containing 0.1% gelatin as a substrate. The gel was run under nonreducing conditions, renatured, developed, and stained with Coomassie blue according to the manufacturer’s protocol. Mouse pro-MMP-2 and -9 (Calbiochem) were used as markers for gelatinases.

In situ Hybridization
Tissue for in situ hybridization studies was obtained from perfusion-fixed FVB-PymT and nontransgenic FVB females. MMP mRNAs were detected in tissue samples using [35S]UTP-labeled RNA probes at 105 cpm/μL on 3-μm-thick paraffin sections essentially as described (34). To provide positive and negative controls for hybridization specificity, the RNA probes were transcribed from separate plasmids carrying two nonoverlapping cDNA fragments of mouse MMP-2, -3, -7, -9, -10, and -13, and the plasmids were transcribed in both antisense and sense orientation. The six plasmids for MMP-2, -3, and -13 have been described (26). The remaining plasmids were pG7-MMAT-5’ RACE 2-1 (35) containing the 1 to 546 fragment (GenBank L36244) in pGEM7Zf+ and pKSMMATAH (35) containing the 406-ApaLI(1062) fragment in pBluescript II.
KS+ for MMP-7; pmSP6592b (36) containing the BamHI(1999)-2258 fragment (GenBank Z27231) in pSP65, pmSP6492b (36) containing the Avrl(1934)-2258 fragment in pSP64, and pKaA207 containing the 261 to 966 fragment in pBluescript KS+ for MMP-9; and pKaA225 containing the 633-BamHI(1162) fragment (GenBank Y13185) in pBlue- 

kscript II KS- and pKaA222 containing the 1,162 to 1,693 fragment in pCRII for MMP-10. The MMP-3 and -10 plasmids were constructed to avoid the 325-bp-long fragment in pCRII for MMP-10. The MMP-3 and -10 cDNAs.

Statistical Analyses

Hypotheses on differences between treatment groups were tested by two-sided Student’s t tests for tumor volume and with Mann-Whitney U test for metastasis volume due to a nonnormal distribution in the Galardin treatment group. Tumor volumes had a log-normal distribution and were logarithmically transformed before analysis. To allow logarithmic transformation, tumor volumes of zero were assigned a value of 10^{-4} \text{mm}^3. t test on log-transformed data (placebo versus Galardin): P = 0.0014.

Results

Reduced Tumor Growth in Galardin-Treated Mice

To obtain a reproducible administration of the MPI Galardin with minimal trauma to the mice during the 50-day drug exposure, we used slow-release tablets estimated to release 100 mg/kg/d. We have found previously that this dose of Galardin, given as once daily i.p. injections, results in a maximal delay of skin wound healing in mice (7). To validate the in vivo efficacy of the slow-release tablets, we did the same wound-healing assay on 75 mice that were randomly distributed with 15 in each of five groups: Galardin or placebo injections, Galardin or placebo tablets, and an untreated group. We found that the slow-release tablets were at least as effective as the i.p. injection route, causing a somewhat greater delay in wound healing (time to healing delayed from 17.2 ± 4 to 26.5 ± 6 days) compared with the injection route (time to healing delayed from 18.0 ± 2 to 22.9 ± 5 days). Time to healing in untreated mice was 16.4 ± 2 days. The difference between the two Galardin administration routes was not significant (26.5 ± 6 versus 22.9 ± 5 days; t test, P = 0.099).

We then generated a cohort of 81 FVB-PymT mice randomly distributed with 27 in each of three groups receiving Galardin or placebo slow-release tablets or no treatment at all. Treatment was started at age 43 ± 1 days (range, 41-45 days), which was chosen because it is the approximate age of tumor onset in this model and because the mammary epithelium is almost fully developed by age 6 weeks. Importantly, Galardin administration at an earlier age would delay mammary development (6). There were two unscheduled deaths in the cohort leaving 79 FVB- 
PymT mice to be analyzed: one mouse in the Galardin tablet group was found dead and one mouse in the placebo tablet group was killed prematurely due to severe edema surrounding the tablet. Seven nontransgenic control mice were included among the untreated mice.
All the MMTV-PymT transgenic mice developed tumors irrespective of treatment group. Macrosopic tumor onset occurs from age 5 to 8 weeks in FVB-PymT mice, around the same time as treatment was started in this experiment. We are therefore unable to determine whether Galardin has any effect on tumor onset. Galardin reduced the growth rate of the MMTV-PymT tumors, although the tumors still follow an exponential growth pattern in the presence of Galardin (Fig. 2A). All mice were killed at age 13.5 weeks (range, 92-96 days). The geometric mean of the tumor burden measured before sacrifice was 3.37 cm$^3$ in untreated mice, 3.29 cm$^3$ in placebo-treated mice, and 1.69 cm$^3$ in Galardin-treated mice (Table 1). Galardin treatment thus reduced final tumor size significantly by 49% compared with placebo-treated mice ($t$ test, $P = 0.0014$). Tumor size in the placebo-treated group was not significantly different from the untreated group (Fig. 2B; $t$ test, $P = 0.90$).

**Reduced Lung Metastasis in Galardin-Treated Mice**

We quantified the total volume of lung metastases in each mouse using an unbiased stereologic technique (30). The incidence of lung metastases was greatly reduced by Galardin treatment compared with either of the two control groups. The incidence of lung metastases was 100% in untreated mice (27 of 27) and 92% in placebo-treated mice (24 of 26) but only 58% in Galardin-treated mice (15 of 26). Lungs from the nontransgenic control mice ($n = 7$) were included randomly among the analyzed samples; none of these were scored positive for metastases. Galardin treatment thus significantly reduced lung metastasis incidence compared with placebo-treated mice (Fisher’s exact test, $P = 0.009$). The metastatic burden, expressed as total volume of tumor tissue in the lungs, was also significantly reduced by Galardin administration. The median metastasis volume was 1.39 mm$^3$ in untreated mice and 0.56 mm$^3$ in placebo-treated mice but only 0.003 mm$^3$ in Galardin-treated mice (Fig. 2C; Table 1). The effect of Galardin was highly significant compared with the placebo group (Mann-Whitney $U$ test, $P < 0.0001$), whereas the placebo-treated and the untreated mice did not differ significantly (Mann-Whitney $U$ test, $P = 0.27$). We also analyzed the metastasis volume-to-primary tumor volume ratios. This fraction was significantly reduced in Galardin-treated mice. The median of the ratios was $39 \times 10^{-5}$ in untreated mice and $17 \times 10^{-5}$ in placebo-treated mice but only $0.16 \times 10^{-5}$ in Galardin-treated mice (over a 100-fold difference between placebo- and Galardin-treated mice; Mann-Whitney $U$ test, $P < 0.0001$).

**Dissemination to Lymph Nodes in Galardin-Treated Mice**

The MMTV-PymT model exhibits regional lymph node dissemination (23). We recovered the bilateral brachial and axillary lymph nodes from each mouse (total of 4 nodes) in the experimental cohort described above. The small size of the nodes caused a few of them not to be scored for technical reasons. In total, 154 of 158 brachial and 156 of 158 axillary nodes were scored. The incidence of tumor cells was very similar in brachial versus axillary lymph nodes for all treatment groups (Table 1). Treatment with Galardin did not significantly affect the incidence of tumor cells in lymph nodes, although there was a trend toward a lower incidence of positive lymph nodes in the Galardin group (Table 1). Galardin-treated mice thus contained tumor cells in 21% (22 of 103) of all their lymph nodes (brachial + axillary) compared to 29% (30 of 102) of the lymph nodes from placebo-treated mice (Fisher’s exact test, $P = 0.20$). The placebo group was very similar to the group of untreated mice that showed a 30% (31 of 105) incidence of tumor cells in lymph nodes.

**Increased Collagen Deposition in Galardin-Treated Tumors**

Gelatin zymography of tumor extracts from placebo- and Galardin-treated tumors revealed that levels of pro-MMP-2 and active MMP-2 were increased in Galardin-treated tumors. Pro-MMP-9 was seen sporadically in tumors from both groups, whereas active MMP-9 could not be detected (Fig. 3). The increase in MMP-2 enzyme levels is probably a result of substrate accumulation caused by the continuous inhibition of metalloproteinases including the collagenolytic and gelatinolytic MMPs. To directly evaluate the effect of Galardin treatment on collagen turnover, we stained tumors from the no. 4 mammary glands of placebo-treated ($n = 9$) and Galardin-treated ($n = 8$) FVB-PymT mice with PicroSirius Red for the fibrillar collagens type I, II, and III. In normal mammary glands from...
age-matched nontransgenic FVB mice (n = 2), collagen was primarily confined to the wall of milk ducts and to the thin fibrous septae that subdivide the organ (Fig. 4A and B). By comparison, MMTV-PymT tumors contain a very dense but also highly disorganized collagen matrix. In placebo-treated tumors, large cancer cell nodules are surrounded by collagen-rich stroma but are almost devoid of collagen within the nodules (Fig. 4C, C', C''). Similar collagen-free tumor areas are infrequent in tumors of comparable size from Galardin-treated mice (Fig. 4D, D', D''). At high magnification, the collagen fibers also appear thicker in tumors from Galardin-treated mice (data not shown).

Histopathologic Progression in Galardin-Treated MMTV-PymT Primary Tumors

To determine whether Galardin treatment affected the histopathologic progression of the primary tumors, we did a histologic evaluation of the abdominal (no. 4) mammary gland from all mice in the cohort described above. The glands were scored on an ordinal scale as either normal gland (A), hyperplasia (B), adenoma (C), early carcinoma

![Collagen deposition in healthy mammary glands and Galardin-treated mammary tumors.](image)

**Figure 4.** Collagen deposition in healthy mammary glands and Galardin-treated mammary tumors. The no. 4 (right side) mammary glands were obtained from 92- to 96-d-old FVB-PymT mice that had been randomly distributed to receive the MMP inhibitor Galardin or placebo starting at age 41 to 45 d. The no. 4 mammary gland was also obtained from tumor-free nontransgenic mice matched for strain and age. Sections of the tissue samples were stained with PicroSirius Red to label collagen type I (yellow to red) and III (green) under polarizing microscopy. Sections were counterstained with Weigert’s hematoxylin (black nuclei). A and B, no. 4 mammary gland of a tumor-free nontransgenic mouse under bright-field (A) and polarized (B) illumination for comparison. C, C', C'', representative tumors from placebo-treated mice. D, D', D'', representative tumors from Galardin-treated mice. The collagen-rich skin is visible in several panels. Bar, 500 μm.
Growth and metastasis in the MMTV-PymT mouse model completely blocked by Galardin. We now report that tumor development (6), although these processes are not completely inhibited wound healing (7, 39) and mammary gland morphogenesis, and metastasis (2, 9). Collagenase-3 was selected due to its unique expression pattern in human breast cancer (38). Membrane-type MMPs were excluded because they are thought to play key roles in extracellular matrix turnover, mammary gland morphogenesis, and metastasis (2, 9). Collagenase-3 was selected due to its presumed relevance for microinvasion (37). MMP-7 was chosen for its unique expression pattern in human breast cancer (38). Membrane-type MMPs were excluded because their expression patterns have been reported (27). We analyzed primary tumors and lungs with metastases from untreated 13-week-old FVB-PymT mice. In primary tumors, we found that MMP-2, -3, -10, and -13 were expressed in stromal cells. MMP-2 was expressed throughout the tumor stroma, whereas MMP-3 and -13 were restricted to certain subcompartments of the stroma as we have reported previously (26). We found that the expression of MMP-10 (n = 4) was far less abundant than the former three, with prominent expression only in a few narrow streaks of connective tissue in areas of compact tumor tissue (Fig. 6A). Expression of MMP-7 and -9 was even more restricted. We found sporadic expression of MMP-7 (3 of 4 four cases) in a population of cancer cells exclusively located adjacent to necrotic areas (Fig. 6B). We found sporadic expression of MMP-9 (5 of 9 cases) often near or adjacent to necrotic areas in a pattern consistent with mesenchymal or inflammatory cells (Fig. 6C). We also analyzed metastasis-containing lungs (n = 5-7) for expression of these six MMPs. MMP-2 was expressed in the larger bronchii (data not shown) and MMP-9 was expressed throughout the lung tissue in uniformly scattered cells (Fig. 6D). MMP-2 and -9 expression was, however, not particularly associated with lung metastases. We did not detect expression of MMP-3, -7, -10, or -13 in the lungs.

Discussion

We have shown previously that the MPI Galardin efficiently suppresses metalloproteinase activity in vivo, inhibiting wound healing (7, 39) and mammary gland development (6), although these processes are not completely blocked by Galardin. We now report that tumor growth and metastasis in the MMTV-PymT mouse model are also significantly inhibited, but not completely blocked, by continuous systemic administration of Galardin. Importantly, lung metastasis volume was reduced ~100-fold in Galardin-treated MMTV-PymT mice, whereas primary tumor volume was reduced only 2-fold. This suggests that Galardin-sensitive metalloproteinases are directly involved in spontaneous metastasis. Interestingly, there was only a modest reduction in the number of cytokeratin-8-positive tumor cells observed in the regional lymph nodes, suggesting that the effect of Galardin-sensitive metalloproteinases on metastasis is primarily on hematogenous dissemination or perhaps on a later step of the metastatic process after escape of the cancer cells from the primary tumor.

The collagen network of the Galardin-treated MMTV-PymT mammary tumors was much more dense compared with the untreated tumors that contain large areas apparently devoid of collagen matrix. This difference is arguably a direct consequence of reduced collagen degradation in the presence of Galardin, which may limit the expansion of tumor nodules due to physical constraints and lead to preservation of the overall structure of the gland for a longer time. The trend toward lower histopathologic tumor grade in the Galardin-treated mice could thus be an indirect consequence of reduced collagen degradation. We observed a marked increase in pro-MMP-2 and active MMP-2 in the Galardin-treated tumors concomitant with the accumulation of collagen. A corresponding increase in MMP-2 expression was found previously in Galardin-treated skin wounds (7). MMP-2 is one of the major gelatinolytic enzymes. It is effectively inhibited by...
Galardin with an IC₅₀ value in the range of 7 to 17 nmol/L (28, 29) and may thus be up-regulated in the tumors as a consequence of substrate accumulation. The widespread expression of the MMP-2 mRNA in the MMTV-PymT tumor stroma (26) is consistent with an important role of MMP-2 in extracellular matrix turnover. In fact, almost all of the MMPs, which have been analyzed by in situ hybridization, were found to be expressed in the primary MMTV-PymT tumors albeit at greatly varying levels. We have previously submitted data for MMP-2, -3, and -13 (26). In the present report, we add data for MMP-7, -9, and -10, whereas others have submitted data for MMP-14, -15, -16 and -17 (27). From this total of 10 MMPs analyzed by in situ hybridization in the MMTV-PymT tumors, MMP-2, -3, -9, -10, -13, -14, and -16 were found predominantly in the stromal cells and MMP-7 and -15 predominantly in the cancer cells, whereas MMP-17 was not detected. These findings agree well with the mRNA expression data for human breast cancer, because MMP-2 (40), MMP-3 (41), MMP-9 (42), MMP-13 (37), and MMP-14 (43) are all found predominantly in the stroma and MMP-7 predominantly in cancer cells (38), suggesting that the MMTV-PymT model is a highly relevant model (reviewed in ref. 15). Thus far, we have not identified any MMP, whose expression is particularly associated with lung metastases, although MMP-9-expressing cells were widespread in the lung tissue and could be important for tumor cell colonization.

Taken together, our findings indicate that tumor angiogenesis, reorganization of the mammary tissue microenvironment, and even metastasis can occur despite continuous and systemic suppression of the Galardin-sensitive MMPs although with reduced efficiency. All of the protease

![Figure 6](https://example.com/figure6.png)

**Figure 6.** Expression of MMP-7, -9, and -10 mRNA in MMTV-PymT-induced mammary cancers and lung metastases. MMP mRNAs were detected by in situ hybridization in primary tumors (A-C) and lung metastases (D) with antisense RNA probes. Expression is seen as silver grains in the bright-field images (row 1) and white reflections in the dark-field images (row 2). Hybridization with a nonoverlapping antisense RNA probe (row 3) and the corresponding sense RNA probe (row 4) on adjacent sections provided positive and negative controls. A, MMP-10 mRNA was infrequent in the primary breast tumors but was occasionally detected quite prominently in narrow bands of connective tissue in areas of compact tumor tissue (arrows). B, MMP-7 mRNA was practically absent in the primary breast tumors and was restricted to occasional cancer cells (arrows) adjacent to necrotic areas (n). C, MMP-9 mRNA was practically absent in the primary breast tumors but was found in scattered stromal cells (arrows) near or adjacent to necrotic areas. D, MMP-9 mRNA was present in scattered cells throughout the entire lung tissue but was not convincingly associated with lung metastases (compare rows 2 and 3 with row 4). Sections were counterstained with H&E. Bars, 100 μm (A and D), 50 μm (C), and 25 μm (B).
intervention studies done in transgenic breast cancer models report similar partial phenotypes such as delayed carcinogenesis or slower tumor growth, if they report any phenotype at all (15). These partial phenotypes are consistent with individual proteases having mutually overlapping functions. A functional overlap may exist within the MMP family or even between different protease families as illustrated by the synergistic inhibition of wound healing (7) and embryo implantation (3) obtained by superimposing metalloproteinase inhibition (Galardin treatment) and genetic deficiency of the serine protease plasmin.

The MMPs have been linked to invasion and metastasis for some time (reviewed in refs. 1, 44). Especially MMP-2 and -9 have been associated with invasion and metastasis in studies that primarily use colonization assays of injected cells or spontaneous metastasis from transplanted s.c. tumors (44). One such example involves Galardin, which reduces the formation of osteolytic bone colonies from cardially injected breast cancer cells (45). Unfortunately, the direct evidence regarding metastasis in transgenic breast cancer models is rather limited (reviewed in ref. 15). Three examples almost exhaust the available evidence and at the same time illustrate the conflicting results that have been obtained. Metastasis incidence is thus reduced in the MMTV-PymT model by overexpression of TIMP-1 (18). In contrast, MMP-7 overexpression has no effect on lung metastasis in the MMTV-neu model (16), and MMP-11 deficiency unexpectedly results in a higher number of metastases in the MMTV-ras model (46), suggesting a suppressive role of this MMP in cancer metastasis. A similar suppressive role against metastasis has been reported in other models for MMP-3 (47), MMP-8 (48), and MMP-12 (49). Based on the limited evidence available for each MMP, it is probably premature to assign a general metastasis-suppressive role to any of these MMPs. The broader picture is that tumor-suppressive roles of MMPs can be identified at several stages of cancer progression extending from carcinogenesis to metastasis (2), but the bulk of available evidence still identifies MMPs primarily as tumor-promoting enzymes. The dual role of MMPs in tumor progression is probably one of several reasons that several clinical trials of broad-spectrum MPIs have failed in the past (2, 50). The promising effect observed with Galardin in the MMTV-PymT mouse model provides renewed proof of principle and suggests that the potential of broad-spectrum MPIs may not have been fully explored.

The broad inhibition profile of Galardin (see Materials and Methods) does not allow identification of the critical MMP(s), and it will undoubtedly be a challenge to identify a broad-spectrum MPI with a favorable inhibition profile. An alternative approach to selective MMP inhibition would be to use a combination of highly specific MMP inhibitors (e.g., inhibitory monoclonal antibodies). The MMTV-PymT model, or other transgenic models with a well-characterized involvement of MMPs, could be a useful tool for the industry in testing the next generation of MPIs.

MMP Inhibition Reduces Spontaneous Metastasis

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

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