

# Antimetastatic Activity of a Novel Mechanism-Based Gelatinase Inhibitor

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## Abstract

**Matrix metalloproteinases (MMPs), and in particular gelatinases (MMP-2 and MMP-9), play a key role in cancer progression. However, clinical trials in which MMP inhibitors were tested in cancer patients have been disappointing. Whereas many reasons have been postulated to explain the failure of the clinical trials, lack of inhibitor selectivity was a major limitation. Thus, despite the consensus opinion that MMP-mediated proteolysis is essential for cancer progression and that certain MMPs represent important targets for intervention, effective and selective inhibition of those MMPs remains a major challenge in drug development. We previously reported the first mechanism-based MMP inhibitor, designated SB-3CT, which is a selective gelatinase inhibitor. Here we report that SB-3CT (5-50 mg/kg/d) is a potent inhibitor of liver metastasis and increases survival in an aggressive mouse model of T-cell lymphoma. This study shows that mechanism-based inhibition of gelatinases represents a novel approach to inhibitor design that promises to be a successful anticancer therapy.** (Cancer Res 2005; 65(9): 3523-6)

## Introduction

Matrix metalloproteinases (MMPs) are zinc-dependent endopeptidases that have been shown to be responsible for many of the proteolytic events leading to cancer progression. The importance of MMPs in cancer, in particular the contribution of gelatinases (MMP-9 and MMP-2) to cancer metastasis and angiogenesis (1), promoted the development of synthetic inhibitors capable of targeting gelatinase activity in tumors (2). Unfortunately, clinical trials in cancer patients with synthetic MMP inhibitors showed lack of therapeutic efficacy and unacceptable side effects (2, 3). Poor inhibitor selectivity has been postulated as one of the major reasons for the disappointing results (3-5). The synthetic MMP inhibitors tested in the clinic thus far were in their majority broad-spectrum zinc-chelator compounds and often incapable of discriminating among related zinc-dependent proteases (5). Other synthetic MMP inhibitors tested were optimized for the purpose of high-affinity binding (2, 6, 7). However, even when high-affinity was attained, the MMP inhibitors lacked selectivity (7, 8). These compounds also failed to show clinical efficacy and they elicited side effects (5, 9). Therefore, mere high-affinity inhibition as an approach for the design of selective MMP (gelatinase) inhibitors has failed to show

promise in treatment of cancer patients. We have described a new approach to MMP inhibition involving covalent mechanism-based inhibition, which has been a point of departure from previous more conventional approaches, and we have focused on inhibition of gelatinases. In this new mechanism of MMP inhibition, we take advantage of a portion of the catalytic mechanism of the targeted gelatinase to generate from the active-site bound inhibitor a reactive chemical entity from an otherwise unreactive functional group, for covalent modification of a residue in the enzyme active site (10). Thereby the principles that impart selectivity in inhibition of gelatinases in this case go beyond mere electrostatic interactions of the classic MMP inhibitors (5, 11, 12). Because the reactive species is formed only within the active site of the targeted enzyme, these inhibitors have great promise for high *in vivo* selectivity, an aspect that is absent in the traditional high-affinity inhibitors for MMPs (7, 9). The prototype compound, designated SB-3CT, was designed based on these principles (10, 13). Whereas SB-3CT is an effective and selective gelatinase inhibitor, it either does not inhibit or inhibits poorly other MMPs (10) and the closely related zinc-dependent metalloprotease ADAM-17 (tumor necrosis factor- $\alpha$  converting enzyme; ref. 14). Here we tested the antitumor activity of SB-3CT in a mouse model of T-cell lymphoma and show that SB-3CT leads to a significant reduction in the growth and number of experimental liver metastases and to an increase in survival.

## Materials and Methods

**Cells, experimental metastasis assays, and SB-3CT treatment.** The generation of the mouse *lacZ*-tagged L-Cl.5s T-cell lymphoma cell line has been described (11, 15, 16). Pathogen-free female DBA/2 mice (8-10 weeks old, purchased from Charles River, Sulzfeld, Germany) were inoculated into the tail vein with  $5 \times 10^3$  *lacZ*-tagged murine L-Cl.5s cells, as previously described (16). Treatment with various doses of SB-3CT (5, 12.5, 25, and 50 mg/kg) diluted in 10% DMSO or vehicle alone started 1 hour before tumor cell inoculation and continued daily. SB-3CT was administered i.p. in a volume of 400  $\mu$ L per dose. Six days after tumor cell inoculation, when macrometastases are already formed (11), the mice were sacrificed and the livers were excised and stained with 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactopyranoside (X-Gal; Roche Diagnostics, Penzberg, Germany), as described (16). Blue metastatic foci on the surface of the livers were counted and confirmed by histology.

**Survival studies.** Tumor-bearing mice treated with 50 mg/kg/d SB-3CT or vehicle alone, as described above, were kept alive until signs of imminent death were evident. Moribund mice were then euthanized. Animal care was done in accordance with the guidelines of the Regierung von Oberbayern (Germany).

**Proliferating cell nuclear antigen immunohistochemistry.** Expression of the proliferating cell nuclear antigen (PCNA) marker in liver tissue sections isolated from three representative treated and untreated

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tumor-bearing mice was carried out by immunohistochemistry using a specific anti-PCNA antibody (Novocastra Laboratories Ltd., Newcastle, United Kingdom) and counterstaining with H&E (11). Metastases were photographed and the ratio of PCNA-positive cells per metastasis was scored.

**Planimetric analysis.** Sections (4  $\mu\text{m}$ ) of paraffin-embedded metastasis bearing livers from SB-3CT (50 mg/kg/d) and vehicle-treated mice were stained with H&E and screened for macrometastases. All metastases found in each of random sections of four animals per group were photographed and the areas of the metastases were measured using MetaMorph 4.6 software (Universal Imaging Cooperation, Downingtown, PA). Mean values and SE of all metastases were plotted.

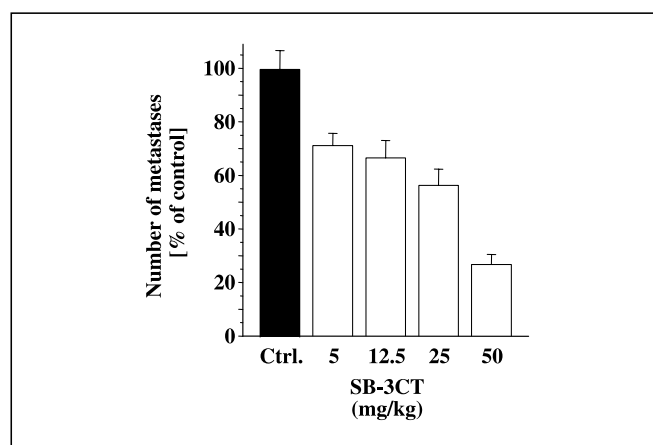
**In situ zymography.** Mice were inoculated with L-Cl.5s cells and treated daily with SB-3CT (50 mg/kg) or vehicle alone as described above. At day 6 after tumor cell inoculation, SB-3CT-treated mice received an additional dose of SB-3CT (50 mg/kg) 1 hour before autopsy and the control mice received vehicle alone (10% DMSO). The livers were then excised, embedded in optimum cutting temperature compound, shock frozen on dry ice, and stored at  $-80^{\circ}\text{C}$  until sectioning for *in situ* gelatin zymography (17). Briefly, liver frozen sections (5  $\mu\text{m}$ ) were quickly dried on microslides and overlaid with a solution containing 1% (w/v) agarose, 0.1 mg/mL fluorescein-conjugated DQ gelatin (Molecular Probes, Leiden, Netherlands), and 2.5  $\mu\text{g}/\text{mL}$  4',6-diamidino-2-phenylindol-dihydrochloride (DAPI; AppliChem, Darmstadt, Germany) in PBS. Parallel sections were overlaid with the same solution with the addition of 10 mmol/L 1,10-phenanthroline. The overlaid sections were incubated (12 hours,  $20^{\circ}\text{C}$ ) in the darkness and photographed using the AxioVert 135 microscope (Zeiss, Jena, Germany) and visualized with the AxioVision LE 4.2 software (Zeiss).

**Statistical analysis.** Data were analyzed using the Mann-Whitney rank sum test. Statistical analysis of survival curves was done with the log-rank test.

## Results and Discussion

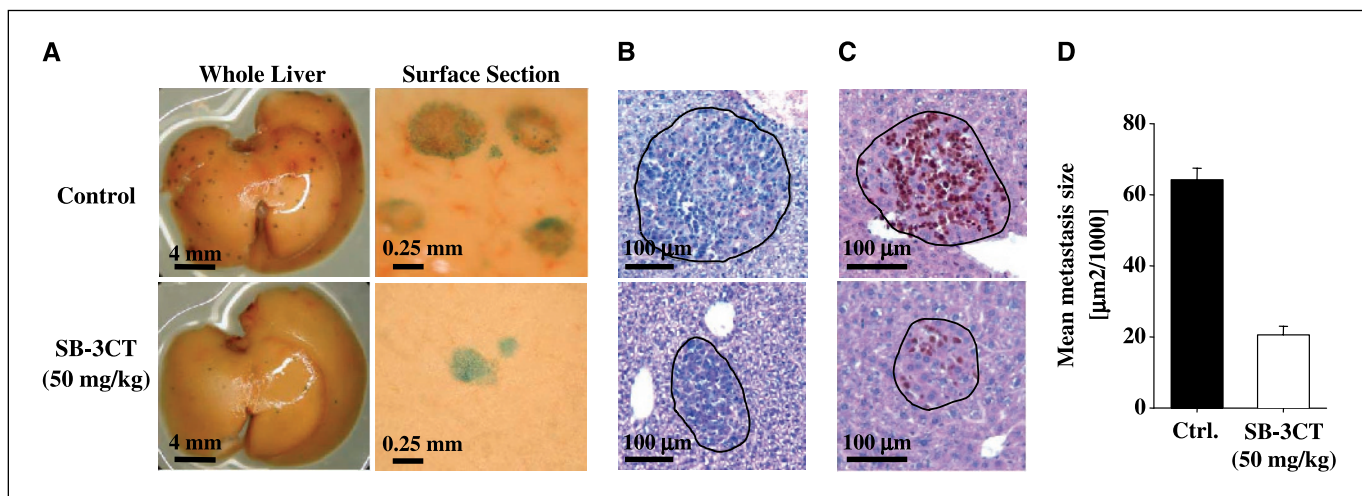
The T-cell lymphoma L-Cl.5s cells form numerous liver colonies when inoculated i.v. into syngeneic DBA/2 mice, a process that has been shown to correlate with enhanced expression of host MMP-9 (11), and thus it mimics certain aspects of human lymphomas (18, 19). This mouse tumor model has been successfully used in the past for rapid screening of MMP inhibitors, providing a measurable and reliable assessment of inhibitor efficacy (11, 12). Therefore, we tested the ability of the gelatinase inhibitor SB-3CT to inhibit experimental metastasis in the L-Cl.5s T-cell lymphoma model. To account for our mouse tumor model, we first carried out inhibition kinetic studies of purified mouse MMP-9 with SB-3CT, as described (10), and found that SB-3CT also behaves as a mechanism-based inhibitor of purified mouse MMP-9 with slow-binding onset for inhibition (data not shown) and an inhibition constant ( $K_i$ ) of  $120 \pm 40$  nmol/L, similar to the human enzyme (10).

Administration of SB-3CT to mice inoculated with L-Cl.5s cells caused a dose-dependent reduction in the number of liver metastases, and at the highest inhibitor dose (50 mg/kg/d) a >70% reduction in the number of tumor colonies was observed (Figs. 1 and 2A). This is in contrast with the effects of Batimastat, a broad-spectrum hydroxamate-based MMP inhibitor (20), which led to the promotion of liver metastasis in this tumor model (12). The reduced number of liver colonies in all treatment groups (5 mg/kg/d:  $71\% \pm 5$ ,  $n = 9$ ; 12.5 mg/kg/d:  $66\% \pm 7$ ,  $n = 7$ ; 25 mg/kg/d:  $56\% \pm 6$ ,  $n = 7$ ; 50 mg/kg/d:  $27\% \pm 4$ ,  $n = 9$ ) compared with the control group was statistically significant (all  $P < 0.014$ ) with 50 mg/kg/d SB-3CT being the most effective dose ( $P = 0.002$ ). In this tumor model, SB-3CT was highly effective in inhibiting liver metastasis when the administration of inhibitor began 1 hour before tumor cell inoculation (Fig. 1). In contrast, the number of liver metastasis was



**Figure 1.** Inhibition of T-cell lymphoma liver metastasis by SB-3CT. X-Gal-stained metastatic foci of L-Cl.5s cells on the surface of the livers were counted and the representative count was confirmed by histology. Relative number of metastases in the different dosage groups as compared with the vehicle-treated control (mean number of macrometastases =  $116 \pm 8$ , equivalent to 100%,  $n = 6$  mice; 5 mg/kg/d:  $71\% \pm 5$ ,  $n = 9$ ,  $P = 0.006$ ; 12.5 mg/kg/d:  $66\% \pm 7$ ,  $n = 7$ ,  $P = 0.014$ ; 25 mg/kg/d:  $56\% \pm 6$ ,  $n = 7$ ,  $P = 0.002$ ; 50 mg/kg/d:  $27\% \pm 4$ ,  $n = 9$ ,  $P = 0.002$ ) on the surface of the livers at day 6 after tumor cell inoculation is shown. Bars, SE. This experiment was repeated thrice with similar results.

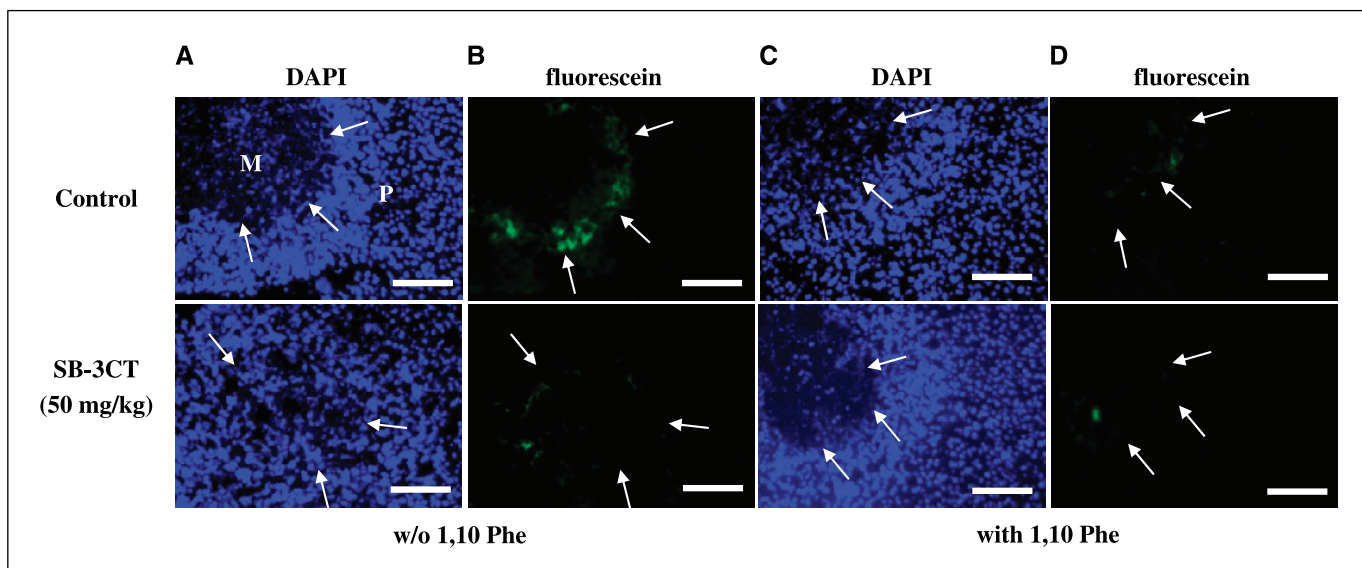
not significantly reduced when the inhibitor was administered 1 day after tumor cell inoculation (data not shown), suggesting that SB-3CT may be more effective at inhibiting tumor cell extravasation. At the highest dose, SB-3CT also caused a significant ( $P < 0.001$ ) reduction in colony size, as determined by gross examination of X-Gal-stained livers (Fig. 2A) and planimetric analysis (Fig. 2B and D) of liver sections. PCNA immunostaining in livers from treated and untreated mice (Fig. 2C) showed a significant decrease in the number of PCNA positive tumor cells per metastases in the livers of SB-3CT-treated animals when compared with control mice (control:  $65.1 \pm 5.8\%$ ,  $n = 7$ ; SB-3CT:  $39.4 \pm 4.4\%$ ,  $n = 7$ ;  $P < 0.05$ ), suggesting that SB-3CT treatment exerts an antiproliferative effect *in vivo*. To determine if SB-3CT directly inhibits L-Cl.5s cell proliferation or cell viability, we examined its effect on *in vitro* cell growth and viability and found no effect with doses up to 12.5  $\mu\text{mol}/\text{L}$  of SB-3CT (data not shown). Because these inhibitor doses are unlikely to be reached in the tissues due to the insoluble nature of SB-3CT and because the liver of treated mice showed lack of evidence of tumor cell necrosis, these data strongly suggest that the effect of SB-3CT on tumor cell proliferation cannot be ascribed to a direct effect on tumor cell proliferation or cytotoxicity and may be mediated by indirect effects on the tumor microenvironment. Indeed, gelatinases, and in particular MMP-9 (21), are known to play a key role in tumor angiogenesis; thus, SB-3CT has the potential to elicit antiangiogenic effects *in vivo*. However, inhibition of angiogenesis by SB-3CT is not likely to be a factor in its antiproliferative effect in this tumor model as we have previously shown that angiogenesis is not involved in the development and growth of L-Cl.5s metastases in highly vascularized liver tissue (11). The *in vivo* antiproliferative effect of SB-3CT is not unique as other synthetic MMP inhibitors were also shown to inhibit tumor cell growth in experimental models of cancer (2, 7). This is consistent with the growing evidence indicating that MMPs, including gelatinases, exert pleiotropic effects on tumor cell behavior, which are partly mediated by their ability to cleave key bioactive proteins in addition to extracellular matrix components (1, 22).



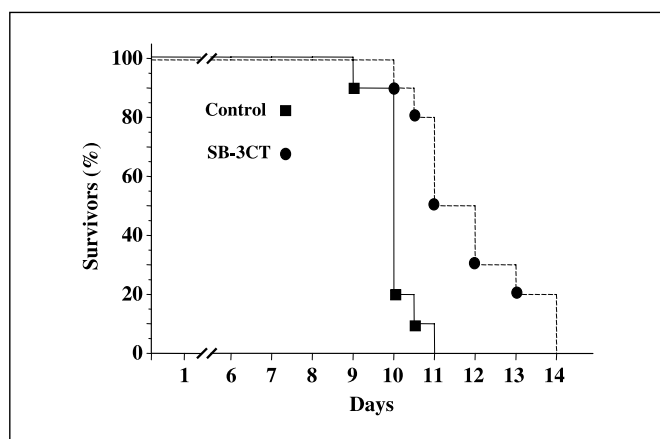
**Figure 2.** Morphologic appearance and size of T-cell lymphoma metastatic foci of livers from SB-3CT-treated and control mice. **A**, photographs of an entire liver (whole liver) and of a close-up (surface section) from representative X-Gal-stained livers from control and SB-3CT (50 mg/kg/d)-treated mice at day 6 after tumor cell inoculation. Note that the diameter of the metastatic foci in the treatment group is smaller than in control livers. **B**, H&E-stained sections of livers from the respective groups. Boundaries of metastatic foci are indicated by a black line. **C**, representative PCNA immunostaining of sections of paraffin-embedded livers. Boundaries of the metastases are indicated by the black lines. Note that the PCNA expression is evident throughout the metastatic foci in the control liver. In contrast, the PCNA signal is weaker and evident only in parts of the metastatic foci in the liver of the SB-3CT-treated mice. **D**, planimetric quantification of metastatic foci of sections of livers from the control ( $64213 \pm 3525 \mu\text{m}^2$ ;  $n = 63$  of evaluated foci) and SB-3CT treated (50 mg/kg/d) mice ( $20575 \pm 2501 \mu\text{m}^2$ ;  $n = 47$  of evaluated foci), as shown in **B**. The difference in metastases size between control and mice receiving 50 mg/kg/d was statistically significant ( $P < 0.001$ ). Differences between lower doses (5 mg/kg/d:  $59026 \pm 4653 \mu\text{m}^2$ ,  $n = 44$ ; 12.5 mg/kg/d:  $66094 \pm 5762 \mu\text{m}^2$ ,  $n = 56$ ; 25 mg/kg/d:  $56939 \pm 4737 \mu\text{m}^2$ ,  $n = 63$ , not shown in the figure) and vehicle-treated mice were not statistically significant (all  $P > 0.1$ ). Bars, SE.

We also did *in situ* zymography, using fluorescein-conjugated DQ gelatin as a substrate, to assess the ability of SB-3CT to inhibit gelatinase activity *in vivo*. As shown in Fig. 3, a representative liver section of vehicle-treated control mice showed punctate green fluorescence at the periphery of a metastatic colony representing gelatinolytic activity (Fig. 3B, top). Indeed, this activity was specifically inhibited by 1,10-phenanthroline consistent with being MMP mediated (Fig. 3D, top). In contrast, liver sections of SB-3CT-treated mice showed little or no MMP-mediated gelati-

olytic activity even when metastatic lesions of similar size were compared or after addition of 1,10-phenanthroline (Fig. 3B and D, bottom). The reduced gelatinolytic activity in livers of SB-3CT-treated mice was not due to differential expression of gelatinases because gelatin zymography of liver tissue extracts showed similar levels of both MMP-9 and MMP-2 expression in SB-3CT- and vehicle-treated mice (data not shown). Thus, these studies indicate that SB-3CT inhibits gelatinolytic activity *in vivo* in metastasis-bearing livers. Because both MMP-2 and MMP-9 are expressed in



**Figure 3.** *In situ* zymography with DQ gelatin. *In situ* zymography was done with frozen sections of L-C1.5s metastasis-bearing liver samples of vehicle-treated controls and SB-3CT-treated (50 mg/kg/d) mice as described in Materials and Methods. Gelatinolytic activity (green fluorescent signal in **B** and **D**) was merged with DAPI staining of the nuclei (blue fluorescent signal in **A** and **C**). As a control, gelatinolytic activity was inhibited by addition of 10 mmol/L 1,10-phenanthroline to the DQ-gelatin overlay. Representative samples are shown. The boundaries of individual metastases are indicated (white arrows), which is equivalent to the tumor-liver parenchyma interface. Note that expansion of metastases resulted in a compression zone of the surrounding liver parenchyma, evident as a higher density of nuclear DAPI staining. P, parenchyma. M, metastasis. Bar, 50  $\mu\text{m}$ .



**Figure 4.** Survival of control and SB-3CT-treated mice. Survival curves of tumor-bearing mice treated IP with 50 mg/kg/d SB-3CT ( $n = 10$ ) or vehicle control ( $n = 10$ ). In the SB-3CT treatment group, the survival time was significantly prolonged compared with the vehicle-treated control group ( $P = 0.005$ ). This experiment was repeated thrice with similar results.

L-Cl.5s liver metastasis (11), the inhibition observed in the *in situ* zymography assay may be a consequence of the ability of SB-3CT to inhibit both enzymes *in vivo*, an activity for which it was designed (10). However, we previously showed that development of liver metastasis by L-Cl.5s lymphoma cells in mice correlates with a specific induction of host MMP-9 but not MMP-2, indicating that MMP-9 is the predominant gelatinase involved in the dissemination of these T-cell lymphoma cells (11) and a likely, but yet unproven, target of SB-3CT in this tumor model.

L-Cl.5s is a highly malignant tumor cell line that causes 100% mortality when inoculated *i.v.* in mice (15). Therefore, survival

studies in this tumor model are a rigorous test of inhibitor efficacy. To this end, mice were inoculated *i.v.* with L-Cl.5s cells as described in Materials and Methods and treated with daily doses of 50 mg/kg/d SB-3CT or with vehicle control until moribund. As shown in Fig. 4, whereas all the mice in the control group died within 11 days after tumor cell inoculation, as expected, 50% of the SB-3CT-treated mice were still alive at that time. Moreover, 20% of the SB-3CT-treated mice survived for 14 days, a statistically significant effect ( $P = 0.005$ ; log-rank test) under these conditions. Neither apparent toxicity nor changes in body weight were observed between the untreated and treated mice. The observed effects on survival are remarkable considering that SB-3CT is a prototype inhibitor with poor solubility and the aggressive nature of this tumor model, which is well documented. In summary, this is the first study to show the antimetastatic activity of the first mechanism-based MMP inhibitor in an aggressive tumor model. The present results suggest that in light of previous disappointments, this approach to inhibitor design holds the promise of a novel potential recourse in anticancer therapy and deserves further exploration.

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