[CANCER RESEARCH 61, 1272–1275, February 15, 2001]

Advances in Brief

Hydroxamate-Type Matrix Metalloproteinase Inhibitor Batimastat Promotes Liver Metastasis[1]

Achim Krüger,[2] Rita Soeltl, Igor Sopov, Charlotte Kopitz, Matthias Arlt, Viktor Magdolen, Nadia Harbeck, Bernd Gänsbacher, and Manfred Schmitt

Klinische Forschergruppe der Frauenklinik [A. K., R. S., I. S., V. M., N. H., M. S.], and Institut für Experimentelle Onkologie und Therapieforschung [A. K., C. K., M. A., B. G.], Technische Universität München, D-81675 Munich, Germany

Abstract

Overexpression of matrix metalloproteinases (MMPs) facilitates tumor cell invasion. Synthetic MMP inhibitors such as batimastat have been designed to treat cancer. We report that because of batimastat treatment, human breast carcinoma cells metastasized to the liver in nude mice and that an increase of liver metastases of murine T-cell lymphoma cells was observed in syngeneic mice. Batimastat treatment also caused liver-specific overexpression of MMPs-2, -9, and mRNA up-regulation of angiogenesis factors and caspase-1, even in tumor-free animals. Induction of organ-specific side effects need to be taken into account regarding further development and clinical use of synthetic MMP inhibitors.

Introduction

Tumor invasion and metastasis requires proteolytic degradation of components of the extracellular matrix such as collagens, proteoglycans, laminin, elastin, and fibronectin. MMPs[3] are the only enzymes known to degrade fibrillar collagens and are able to degrade essentially all of the extracellular matrix components. MMPs also substantially contribute to other steps in the metastatic cascade, such as angiogenesis, differentiation, proliferation, and apoptosis. Therefore, MMPs are considered to be important regulators of tumor growth, both at the primary site and in distant metastases (1). In the clinical setting, increase of some MMPs is associated with poor prognosis of cancer patients (1). Consequently, synthetic MMP inhibitors have been developed and administered to patients to reduce cancer spread (2).

The synthetic hydroxamate-type inhibitor batimastat inhibits tumor growth and lung metastasis in different mouse tumor models (2) and has been tested in clinical therapy trials (3, 4). Still, it remains to be shown that hydroxamate-type of MMP inhibitors have statistically significant advantages over placebo regarding either primary or secondary end points of the respective therapy trials in human cancer.

In the present study, we investigated the effect of batimastat treatment on distant metastasis in two different mouse tumor models. Applying cDNA-array technology, we identified batimastat-induced changes of expression of several tumor-promoting genes.

Materials and Methods

Experimental Metastasis Assay. Pathogen-free, female athymic (*nu/nu*, CD1) mice and female DBA/2 mice (both 4–6 weeks of age; Charles River, Sulzfeld, Germany) were inoculated with either 10^6 *lacZ*-tagged human MDA-MB-231 BAG cells (5) or 10^4 DBA/2-syngeneic *lacZ*-tagged murine L-CI.5 cells (6) into the tail vein of each mouse, respectively. Fifty-two days after inoculation of MDA-MB-231 BAG cells or 6 days after inoculation of L-CI.5s cells when macrometastases (>0.2 mm) were already formed in this model (7), mice were sacrificed, lungs and liver were removed, and these organs were stained with X-Gal (Ref. 6; Roche Diagnostics, Penzberg, Germany). After the respective time points, blue macrometastatic foci on the surface of the organs were counted. This method allows assessment of the metastatic pattern even of lymphoma cells (7). [4-(*N*-Hydroxyamino)-2R-isobutyl-3S-(thiopen-2-ylthiomethyl)-succinyl]-L-phenylalanine-*N*-methylamide, which is identical to batimastat and will be referred to as batimastat in this study (kindly provided by Dr. H-W. Krell, Roche Diagnostics; synthesized according to the PCT Patent Application WO 90/05719), was suspended in sterile PBS/0.01% Tween 80 (Sigma, Munich, Germany) and administered i.p. at a daily dose of 30 mg/kg (previously shown to be tolerable and effective; Ref. 8), from the day after tumor cell inoculation until the day before organ removal.

Zymography. Frozen tissue (0.5 cm^3 of a liver lobe or half of a lung) was homogenized for 10 s in a Mini-beadbeater in the presence of zirconium beads (1.0-mm diameter; Biospec Products, Inc., Bartlesville, Oklahoma) and 300 μl of extraction buffer [50 mM Tris/HCl, 5 mM $CaCl_2$, 200 mM NaCl, and 1% Triton X-100 (pH 7.5)]. After centrifugation of the tissue homogenate (4°C; 12,000 × *g*; 10 min), the supernatant was collected, snap-frozen in liquid nitrogen, and stored in aliquots at −80°C. Zymographic detection of gelatinolytic activity in 15% SDS-polyacrylamide gels was performed according to Edwards *et al.* (9). Recombinant MMP-2 and MMP-9 (Roche Diagnostics) served as standards and were used after a 2-h activation with amino-phenyl mercury acetate (Sigma).

RNA Isolation, Atlas-Array, and Northern Blot Analysis. Mice were treated with batimastat (30 mg/kg/day in PBS/0.01% Tween 80) for 12 consecutive days; the control mice received PBS/Tween only. On day 13, mice were sacrificed, and livers and lungs were collected, snap frozen in liquid nitrogen, and stored at −80°C. Isolation of total tissue RNA and subsequent Northern blot analysis were performed as described (10). Differentially expressed mRNA in organs of batimastat-treated mice *versus* control mice were identified by Atlas Mouse cDNA Expression Arrays (Clontech, Heidelberg, Germany). For this purpose, we isolated mRNA from the lung (as an internal control) and liver tissue of tumor-free mice, which had received daily i.p. injections of batimastat or, as a control, vehicle only. cDNA was synthesized and hybridized to the arrays according to the manufacturer's protocol. For the generation of specific cDNA probes (HGF, bFGF, angiogenin, and caspase-1), primers designed by Clontech and synthesized by MWG-Biotech AG (Ebersbach, Germany) were used for PCR amplification, and the products were used in the subsequent Northern blot analysis.

Statistical Analysis. Kruskal-Wallis One Way ANOVA on Ranks was performed to assess differences between groups. In case of a statistically significant difference ($P < 0.05$), pairwise comparisons of groups were done by Dunn's method.

Results and Discussion

The resulting metastatic pattern in lungs and liver of mice as an effect of treatment with the synthetic hydroxamate-type MMP inhibitor batimastat is presented, using *lacZ*-tagged tumor cells (7). Experimental metastasis was generated by injection of *lacZ*-tagged MDA-

Received 8/8/00; accepted 12/21/00.

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[1] Supported by the Deutsche Forschungsgemeinschaft, Sonderforschungsbereich 469, project B13.

[2] To whom requests for reprints should be addressed, at Institut für Experimentelle Onkologie und Therapieforschung der Technischen Universität München, Ismaninger Strasse 22, D-81675 Munich, Germany. Phone: 49-89-4140-4463. Fax: 49-89-4140-6182; E-mail: achim.krueger@lrz.tu-muenchen.de.

[3] The abbreviations used are: MMP, matrix metalloproteinase; X-Gal, 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; HGF, hepatocyte growth factor; SF, scatter factor; bFGF, basic fibroblast growth factor.

MB-231 BAG breast cancer cells into the tail veins of nude mice, and, as a result, metastases were formed in the lung but not in the liver. After tumor cell administration, one group of mice ($n = 6$) was subjected to daily i.p. injections with batimastat, whereas the group of control mice ($n = 18$) was treated with the vehicle only. Mice were sacrificed, lungs and livers collected, X-Gal stained, and macrometastases quantified. In the batimastat-treated group, a slight trend in reduction of lung metastases was observed as compared with the control group (Fig. 1*A*). A different metastatic pattern of the breast cancer cells was observed in the batimastat-treated animals; in addition to the lung metastases, all but one animal of the batimastat-treated group developed liver metastases, whereas no liver metastases were seen in the control group (Fig. 1*A*).

L-CI.5s murine T-cell lymphoma cells form liver metastases and, therefore, allow examination of the effect of batimastat on preexisting liver metastases. These cells directly colonize the liver upon i.v. injection without forming lung metastases. The day after tumor cell injection, one group of mice ($n = 6$) was treated with daily i.p. injections of batimastat, whereas the control group ($n = 9$) received the vehicle only. Six days after tumor cell inoculation, the mice were sacrificed, and the livers were removed and examined for number of metastases. Compared with the control group, a significant increase of liver metastases in the batimastat-treated group of mice was observed (Fig. 1*B*). Even a 3-day batimastat treatment before tumor cell inoculation did not prevent the significant increase of liver metastases (data not shown).

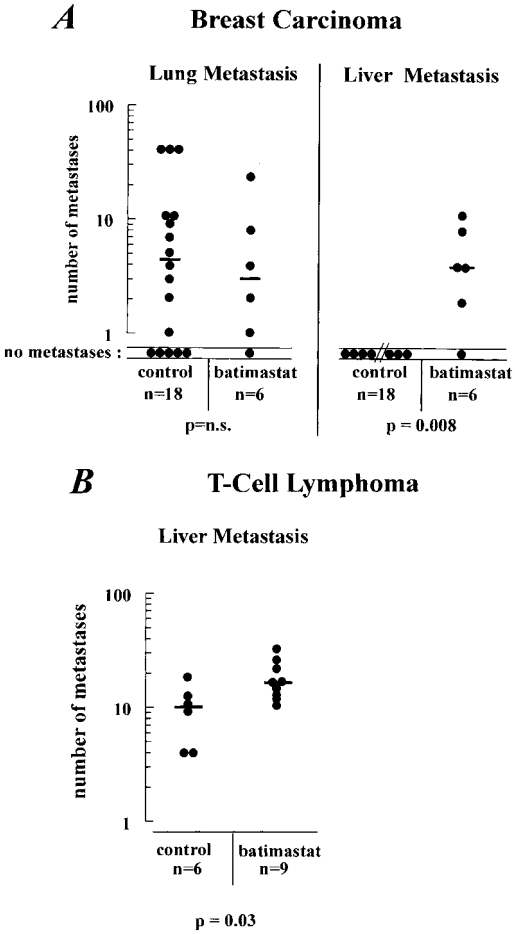

Fig. 1. Effects of batimastat treatment on experimental metastasis in mice. *A*, number of X-Gal-stained macrometastases on the surface of lungs or liver of nude mice present 52 days after inoculation of 10^6 MDA-MB-231 BAG breast carcinoma cells. *Left panel*, number of lung metastases in the batimastat-treated group ($n = 6$; median = 4.5) and in the control group ($n = 18$; median = 3.0). *Right panel*, number of liver metastases in the batimastat-treated group ($n = 6$; median = 4.0) and in the control group ($n = 10$; median = 0). *B*, number of macrometastases on the surface of the livers of syngeneic DBA/2 mice 6 days after inoculation of 10^4 L-CI.5s T-cell lymphoma cells in the batimastat-treated group ($n = 9$; median = 17) *versus* the control group ($n = 6$; median = 10).

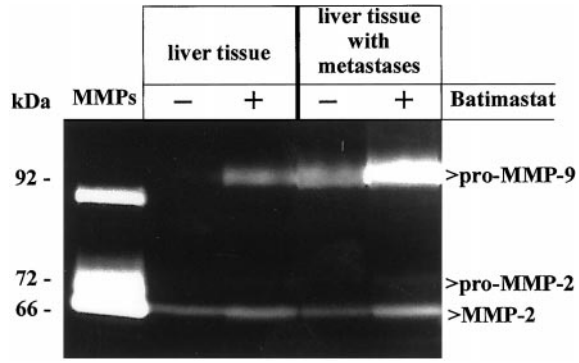

Fig. 2. Zymographic detection of MMP-2 and -9 protein expression in livers of batimastat-treated DBA/2 mice. Liver tissue was isolated from batimastat-treated (+) or vehicle-treated (−) tumor-free (*Lanes 2* and *3*) or lymphoma metastases-bearing (*Lanes 4* and *5*) mice, respectively. Lanes were equally loaded with 36 μg of protein each. The experiment shown is representative of three independent experiments. Recombinant MMPs were used as standards.

In DBA/2 mice that did not receive tumor cells, liver tissue revealed a baseline expression of active MMP-2 and no MMP-9 (Fig. 2, *Lane 2*). Interestingly, in mice that were never inoculated with tumor cells, batimastat treatment led to expression of pro-MMP-9 and increased the level of MMP-2 (Fig. 2, *Lane 3*). In mice inoculated with LCI.5s T-cell lymphoma cells, expression of pro-MMP-9 but no elevation of the baseline level of MMP-2 was detected in liver tissue afflicted with metastases (Fig. 2, *Lane 4*). Both liver cells and tumor cells could be responsible for this increase in pro-MMP-9. Batimastat treatment for 6 days after lymphoma cell inoculation led to further elevation of pro-MMP-9 and also of MMP-2 expression in the liver (Fig. 2, *Lane 5*). Higher numbers of metastases were found in these livers. However, in lungs, batimastat treatment did not lead to elevation of MMP-2 or MMP-9 levels (data not shown). In a recent *in vitro* study, Maquoi *et al.* (11) also reported on the stimulation of pro-MMP-9 expression in a fibrosarcoma cell line upon treatment with another broad-spectrum hydroxamate-type MMP inhibitor. In our *in vivo* study, the MMP inhibitor-induced MMP expression is host organ specific as induction of MMP-2 and MMP-9 was observed even in the absence of tumor cells (Fig. 2). This up-regulation of MMPs in the batimastat-treated mice is quite contradictory to the rationale of cancer therapy based on MMP inhibitors, because overexpression of MMPs in tumor tissues has even been linked to poor patient prognosis in cancer (1).

To reveal what further changes in organ-specific gene expression are induced by batimastat, cDNAs of livers and lungs from control nude mice or mice that were treated with batimastat for 12 consecutive days were subjected to microarrays. To exclude interference with the gene expression associated with the presence of tumor cells, the mice were not inoculated with tumor cells. Assessment of differentially expressed genes in the liver, compared with the lungs, allowed detection of those genes that revealed liver-specific alteration of transcription (at least a 2-fold difference in expression). In addition to the increased MMP expression because of batimastat treatment, we observed that liver-specific mRNA expression of HGF/SF, bFGF, angiogenin, and caspase-1 was significantly elevated, as revealed by Mouse-Atlas™ Arrays and confirmed by Northern blot analysis (Fig. 3). HGF/SF, an

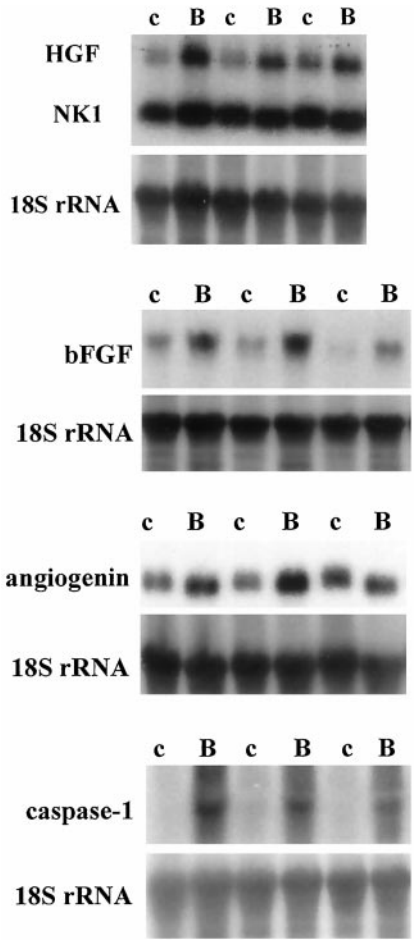

Fig. 3. Increases in mRNA expression of angiogenesis factors and caspase-1 in livers of batimastat-treated nude mice. Total RNA was isolated from the livers of three control mice (*c*) and three batimastat-treated mice (*B*), and 15 μg of RNA were analyzed on a Northern blot. Increased expression of HGF/SF, bFGF, and caspase-1 was observed in all of the three batimastat-treated mice, whereas angiogenin was increased in two of three mice. In the *upper panel*, the upper band is the complete HGF-transcript, whereas the lower band, with unaltered expression pattern, is the splice-variant NK1, a HGF antagonist. 18S rRNA served as a loading and transfer control.

intrinsic factor produced in liver tissue, is a metastasis-promoting molecule that enhances the motility of carcinoma cells (12). mRNA expression of the HGF-antagonist NK1, a splice-variant of HGF/SF (Ref. 13; Fig. 3), as well as of the HGF-receptor (*c-met*), was not altered. HGF, bFGF, and angiogenin are factors involved in angiogenesis, which is a crucial step in tumor progression (14, 15). Caspase-1 (interleukin-converting enzyme) is an immunomodulatory molecule that regulates a cascade of cytokines in tissue (16), including soluble interleukin-1, which can be associated with elevation of MMP levels (17). The observed up-regulation of angiogenesis-promoting factors, in our model produced by normal liver cells upon treatment with batimastat, is in accordance with the results of Patterson and Sang (18), who pointed out that MMP inhibitors prevent MMP-induced generation of the angiogenesis inhibitor angiostatin. Thus, MMP inhibitors may even facilitate tumor progression by promotion of angiogenesis (19) which could favor the successful establishment and growth of metastases.

In the literature, conflicting data are presented. Several authors evaluating liver metastasis after batimastat treatment (Refs. 20, 21 and previous work summarized in Ref. 2) found reduction of tumor burden (pancreatic carcinoma, melanoma, hepatic carcinoma, and colon carcinoma) in the liver. In contrast, other authors using lymphoma cells in severe combined immunodeficient mice found no reduction of metastasis in the liver (22). We found previously that batimastat treatment inhibits primary tumor growth of i.p. inoculated human esophagus and ovarian carcinoma cells in nude mice but at the same time leads to formation of secondary tumors in the liver (23). These findings provide further evidence that batimastat is able to induce liver-specific changes, which could lead to promotion of tumor progression.

In cancer patients, marimastat, an p.o. administrated derivative of batimastat, has been tested in Phase II and Phase III clinical studies. Three of these studies have recently been closed without reaching their primary end point, *i.e.*, a reduction in mortality, attributable to marimastat treatment (24). Our data on the liver-specific batimastat-induced metastasis, together with the evidence for induction of gelatinases and angiogenesis-promoting genes during treatment with batimastat in tumor-free mice, indicate the need for further research before synthetic MMP inhibitors are used for treatment of cancer patients.

Acknowledgments

We thank Dr. Reingard Senekowitsch-Schmidtke (Institute for Nuclear Medicine, TU, Munich, Germany) for allowing us to perform the animal studies in her facility, Dr. Ulrike Kliebsch (Munich, Germany) for expert help in the statistical analysis, Dr. N. Brünner (Copenhagen, Denmark) for the generous gift of the MDA-MB-231 BAG cell line, and Katja Honert (Institut für Onkologie und Therapieforschung, TU, München) for her expert technical assistance.

References

1. Yu, A. E., Hewitt, R. E., Connor, E. W., and Stetler-Stevenson, W. G. Matrix metalloproteinases. Novel targets for directed cancer therapy. Drugs Aging, *11:* 229–244, 1997.
2. Rasmussen, H. S., and McCann, P. P. Matrix metalloproteinase inhibition as a novel anticancer strategy: a review with special focus on batimastat and marimastat. Pharmacol. Ther., *75:* 69–75, 1997.
3. Wojtowicz-Praga, S. M., Dickson, R. B., and Hawkins, M. J. Matrix metalloproteinase inhibitors. Investig. New Drugs, *15:* 61–75, 1997.
4. Macaulay, V. M., O'Byrne, K. J., Saunders, M. P., Braybrooke, J. P., Long, L., Gleeson, F., Mason, C. S., Harris, A. L., Brown, P., and Talbot, D. C. Phase I study of intrapleural batimastat (BB-94), a matrix metalloproteinase inhibitor, in the treatment of malignant pleural effusions. Clin. Cancer Res., *5:* 513–520, 1999.
5. Brünner, N., Thompson, E. W., Spang-Thomsen, M., Rygaard, J., Dano, K., and Zwiebel, J. A. lacZ transduced human breast cancer xenografts as an *in vivo* model for the study of invasion and metastasis. Eur. J. Cancer, *12:* 1989–1995, 1992.
6. Krüger, A., Umansky, V., Rocha, M., Hacker, H. J., Schirrmacher, V., and von Hoegen, P. Pattern and load of spontaneous liver metastasis dependent on host immune status studied with a lacZ transduced lymphoma. Blood, *84:* 3166–3174, 1994.
7. Krüger, A., Schirrmacher, V., and Khokha, R. The bacterial lacZ gene: an important tool for metastasis research and evaluation of new cancer therapies. Cancer Metastasis Rev., *17:* 285–294, 1998.
8. Eccles, S. A., Box, G. M., Court, W. J., Bone, E. A., Thomas, W., and Brown, P. D. Control of lymphatic and hematogenous metastasis of a rat mammary carcinoma by the matrix metalloproteinase inhibitor batimastat (BB-94). Cancer Res., *56:* 2815–2822, 1996.
9. Edwards, D. R., Leco, K. J., Beaudry, P. P., Atadja, P. W., Veillette, C., and Riabowol, K. T. Differential effects of transforming growth factor-β1 on the expression of matrix metalloproteinases and tissue inhibitors of metalloproteinases in young and old human fibroblasts. Exp. Gerontol., *31:* 207–223, 1996.
10. Krüger, A., Fata, J. E., and Khokha, R. Altered tumor growth and metastasis of a T-cell lymphoma in Timp-1 transgenic mice. Blood, *90:* 1993–2000, 1997.
11. Maquoi, E., Munaut, C., Colige, A., Lambert, C., Frankenne, F., Noel, A., Grams, F., Krell, H. W., and Foidart, J. M. Paradoxical stimulation of matrix metalloproteinase-9 expression in HT1080 cells by a broad-spectrum hydroxamate-based matrix metalloproteinase inhibitor. Ann. NY Acad. Sci., *878:* 744–746, 1999.
12. To, C. T., and Tsao, M. S. The roles of hepatocyte growth factor/scatter factor and met receptor in human cancers (Review). Oncol. Rep., *5:* 1013–1024, 1998.
13. Jakubczak, J. L., LaRochelle, W. J., and Merlino, G. NK1, a natural splice variant of hepatocyte growth factor/scatter factor, is a partial agonist *in vivo*. Mol. Cell. Biol., *18:* 1275–1283, 1998.
14. Schmidt, N. O., Westphal, M., Hagel, C., Ergun, S., Stavrou, D., Rosen, E. M., and Lamszus, K. Levels of vascular endothelial growth factor, hepatocyte growth factor/scatter factor and basic fibroblast growth factor in human gliomas and their relation to angiogenesis. Int. J. Cancer, *84:* 10–18, 1999.

15. Hartmann, A., Kunz, M., Kostlin, S., Gillitzer, R., Toksoy, A., Brocker, E. B., and Klein, C. E. Hypoxia-induced up-regulation of angiogenin in human malignant melanoma. Cancer Res., *59:* 1578–1583, 1999.
16. Khatib, A. M., Kontogiannea, M., Fallavollita, L., Jamison, B., Meterissian, S., and Brodt, P. Rapid induction of cytokine and E-selectin expression in the liver in response to metastatic tumor cells. Cancer Res., *59:* 1356–1361, 1999.
17. Vecil, G. G., Larsen, P. H., Corley, S. M., Herx, L. M., Besson, A., Goodyer, C. G., and Yong, V. W. Interleukin-1 is a key regulator of matrix metalloproteinase-9 expression in human neurons in culture and following mouse brain trauma *in vivo*. J. Neurosci. Res., *61:* 212–224, 2000.
18. Patterson, B. C., and Sang, Q. A. Angiostatin-converting enzyme activities of human matrilysin (MMP-7) and gelatinase B/type IV collagenase (MMP-9). J. Biol. Chem., *272:* 28823–28825, 1997.
19. Sang, Q. X. Complex role of matrix metalloproteinases in angiogenesis. Cell Res., *8:* 171–177, 1998.
20. Jimenez, R. E., Hartwig, W., Antoniu, B. A., Compton, C. C., Warshaw, A. L., and Fernandez-Del Castillo, C. Effect of matrix metalloproteinase inhibition on pancreatic cancer invasion and metastasis: an additive strategy for cancer control. Ann. Surg., *231:* 644–654, 2000.
21. Wylie, S., MacDonald, I. C., Varghese, H. J., Schmidt, E. E., Morris, V. L., Groom, A. C., and Chambers, A. F. The matrix metalloproteinase inhibitor batimastat inhibits angiogenesis in liver metastases of B16F1 melanoma cells. Clin. Exp. Metastasis, *17:* 111–117, 1999.
22. Zubair, A. C., Ali, S. A., Rees, R. C., Goepel, J. R., and Goyns, M. H. Investigation of the effect of BB-94 (batimastat) on the colonization potential of human lymphoma cells in SCID mice. Cancer Lett., *107:* 91–95, 1996.
23. Della Porta, P., Soeltl, R., Krell, H. W., Collins, K., O'Donoghue, M., Schmitt, M., and Krüger, A. Combined treatment with serine protease inhibitor aprotinin and matrix metalloproteinase inhibitor Batimastat (BB-94) does not prevent invasion of human esophageal and ovarian carcinoma cells *in vivo*. Anticancer Res., *19:* 3809–3816, 1999.
24. Fox, S. European roundup: no anticancer benefit in trials of marimastat. Genet. Eng. News, *20:* 30, 2000.