

## INHIBITION OF GELATINASE A (MMP-2) BY BATIMASTAT AND CAPTOPRIL REDUCES TUMOR GROWTH AND LUNG METASTASES IN MICE BEARING LEWIS LUNG CARCINOMA

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We have examined the effects of the synthetic matrix metalloproteinase inhibitor, batimastat (BB-94) and the angiotensin-converting enzyme inhibitor, captopril, on metalloproteinase activity of murine Lewis-lung-carcinoma cells (3LL) *in vitro*, and on local growth and lung metastasis of the same tumor implanted intramuscularly in syngeneic C57BL/6 mice. The effect of BB-94 and captopril on the survival of the 3LL-tumor-bearing mice was also examined. Here we report that captopril treatment resulted in decreased transcription and protein levels of gelatinase A by 3LL cells. Both BB-94 and captopril also prevented substrate degradation by gelatinase A and B released in conditioned medium by cultured cells. Treatment of tumor-bearing animals with BB-94 (i.p.) or captopril (in drinking water) resulted in significant inhibition of the mean tumor volume (25 and 33% respectively) and of the mean lung metastasis number (26 and 29% respectively). When both agents were given, they acted in synergy, resulting in 51 and 80% inhibition of tumor growth and metastasis. The survival time of the mice treated with both BB-94 and captopril was also significantly longer compared with the groups treated with each agent alone or with the vehicle. Our data support the hypothesis of an essential role of metalloproteinase(s) in the metastatic process. Moreover, blockade of invasion, angiogenesis and other processes mediated by metalloproteinases may underlie the anti-tumor and anti-metastatic effect of BB-94 and captopril and their combination. It is conceivable that this combination could be tested in selected clinical conditions as an adjuvant modality to cytotoxic therapy. *Int. J. Cancer* 81:761–766, 1999.

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Matrix metalloproteinases (MMPs) are a multigene family of enzymes involved in extracellular-matrix (ECM) degradation and secreted by various cell lines in normal and pathological conditions. The expression of MMPs and of their specific tissue inhibitors (TIMPs) is controlled by growth factors and cytokines, which either induce or repress transcription of MMP genes (Birkedal-Hansen, 1995; Prontera *et al.*, 1996). Several classes of enzymes, such as MMPs, that have been studied have been implicated in tumor invasion. Among the MMPs, attention has been focused on gelatinase A and gelatinase B, (also known as MMP-2/MMP-9 and 72-kDa/92-kDa gelatinases) which specifically degrade the main structural component of the basement membranes, type-IV collagen, considered to play a crucial role in metastasis formation (Duffy, 1992). MMPs are involved in cell invasion, migration and angiogenesis, and may be produced by tumor cells or by stromal cells that degrade the extracellular matrix around tumor cells (Liotta *et al.*, 1991; Brown and Giavazzi, 1995). MMPs are secreted in a soluble pro-enzyme form. The overall activity of MMPs is regulated as a sequence of events which includes pro-enzyme activation and interaction with the specific inhibitors, TIMPs. The inhibition of invasion and metastasis in cells transfected with the tissue inhibitors, TIMP-1 and TIMP-2, have indirectly shown the role of MMPs in those processes (DeClerck *et al.*, 1992). Thus, MMP-inhibiting drugs may be able to suppress invasive and metastatic behavior of tumor cells.

Batimastat, known as BB-94, is a synthetic inhibitor of MMPs, and was designed as an anti-invasive and anti-metastatic drug (Brown and Giavazzi, 1995). It has been shown to inhibit lung metastasis of B16 melanoma in mice (Chirivi *et al.*, 1994) and local and metastatic spread of human colon and breast cancers in nude mice (Watson *et al.*, 1995; Sledge *et al.*, 1995). Moreover, BB-94

has been reported to reduce the angiogenic potential of murine hemangioma (Taraboletti *et al.*, 1995).

Captopril, an inhibitor of the angiotensin-converting enzyme, is widely used in the treatment of several cardiovascular diseases, due to its ability to improve left ventricular function and reduce blood pressure (ISIS-4, 1995). In addition, captopril has been reported to affect atherogenesis, diabetic retinopathy and various tumor processes by inhibiting new blood vessel growth (Jackson *et al.*, 1992). Indeed, captopril inhibits endothelial-cell migration by blocking the activity of Zn<sup>2+</sup>-dependent metalloproteinases, such as 72-kDa and 92-kDa gelatinases, required by endothelial cells to respond to angiogenic stimuli (Volpert *et al.*, 1996). Agents that inhibit angiogenesis and extracellular-matrix degradation may complement other anti-tumor therapies, such as chemotherapy, to further inhibit tumor growth and metastatic spread (Teicher *et al.*, 1994).

Here, we have studied the *in vitro* and *in vivo* effects of BB-94 and captopril on MMPs secreted by 3LL cells. Our data show that captopril decreased gelatinase-A protein level and gene expression. Furthermore, captopril also prevented gelatin-substrate degradation by gelatinase A and B released in conditioned medium of cells such as BB-94. The *in vivo* effects of combined therapy with BB-94 and captopril on 3LL tumor were also studied to establish whether the combination of 2 different MMP inhibitors could be relevant in reducing tumor invasion. Indeed, treatment with both BB-94 and captopril significantly reduced 3LL-tumor growth and lung-metastasis number and significantly prolonged survival in syngeneic C57BL/6 mice implanted intramuscularly (i.m.) with 3LL cells.

### MATERIAL AND METHODS

#### Tumor cells

Lewis-lung-carcinoma cells (3LL) were propagated by i.m. injection in female C57BL/6 mice. The 3LL cells were isolated from primary tumor by digestion with collagenase (1 mg/ml) and DNase (0.1 mg/ml) and were maintained in Dulbecco's modified Eagle's medium (DMEM) plus 10% FCS, penicillin (100 units/ml), streptomycin (100 units/ml) and L-glutamine (2 mM), all obtained from GIBCO (Grand Island, NY). 3LL cells at the third to fourth *in vitro* passage were seeded in 25-cm<sup>2</sup> flasks (Costar, Cambridge, MA) at a density of  $2 \times 10^5$  and grown to confluence. The cells were incubated for 24 hr in serum-free-DMEM with or without captopril dissolved in sterile water, at the doses of 0.1 to 5 mM or BB-94, dissolved in absolute ethanol at the concentration of 3 mM (stock solution) and then diluted at the doses of 5 to 500 nM with DMEM.

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### *Lewis-lung-carcinoma in vivo model*

3LL cells ( $5 \times 10^4$  cells/mouse) were injected i.m. in 5-week-old female C57BL/6 mice, obtained from Charles River, Milan, Italy). Animals were matched for age and weight in each experiment.

### *Drug preparation and schedule of treatment*

BB-94 was generously provided by British Biotech (Oxford, UK). It was suspended by sonication with PBS and 0.01% Tween-80 (pH 7.2) at 3.0 mg/ml and given at a volume of 0.2 ml/mouse, corresponding to 30 mg/kg, and injected i.p. daily. This dose gives therapeutic plasma concentration of about 20 ng/ml after 24 hr (Brown and Giavazzi, 1995). Control mice received the same volume of vehicle. Captopril was a generous gift from Dr. G.B. Leproux (Bristol Meyers/Squibb, Rome, Italy). It was given at the concentration of 0.4 mg/ml in drinking water, resulting in a dose of 50 mg/kg per day. This dose has been reported to inhibit angiogenesis and the growth of experimental tumors in rats (Volpert *et al.*, 1996).

Groups of 5 to 10 mice implanted i.m. with 3LL cells ( $5 \times 10^4$  cells/mouse) were treated with BB-94 (30 mg/kg), captopril (50 mg/kg) or with both agents at the same doses, from day 0 to day 21. Control mice were treated with the vehicle alone. On days 15 and 21, 5 mice per group were killed by ether inhalation. Primary tumor and lungs were then surgically resected, and tissue specimens were snap-frozen in liquid nitrogen, then analyzed by gelatin zymography and Western blot to detect 72-kDa-gelatinase activity and protein expression. Lungs were fixed in Bouin's solution and the metastases were macroscopically counted. In parallel experiments, diameter of the primary tumor was measured in groups of 10 mice, twice a week with a caliper. At the end of treatments, the same mice were left for evaluation of survival time.

### *Gelatin zymography*

3LL cells ( $2 \times 10^5$ /ml) were grown to confluence and then incubated 24 hr in serum-free DMEM with appropriate treatments. Conditioned medium was concentrated by centrprep MW 10,000 Da (Amicon, Beverly, MA). Specimens of tumor, muscle and lungs of tumor-bearing and control mice were homogenized in non-reducing sample buffer and centrifuged; the supernatant was collected. They were also incubated for 3 hr in culture with DMEM containing 10% of serum, then treated with captopril and BB-94 for 24 hr in serum-free DMEM. Protein concentrations in supernatants and in conditioned medium were quantified using the Pierce method. Equal amounts of protein were analyzed by zymography on 10% polyacrylamide gels containing 1 mg/ml gelatin (Merck, Darmstadt, Germany) as substrate. After electrophoresis, the gels were rinsed twice in 2.5% Triton X-100 and incubated overnight in substrate buffer (50 mM Tris-HCl, pH 8, containing 5 mM  $\text{CaCl}_2$  and 0.02%  $\text{NaN}_3$ ). Gels were stained with Coomassie brilliant blue R-250 and de-stained with 20% methanol and 10% acetic acid in distilled water.

*In vitro* inhibition of gelatinases A and B was obtained by adding BB-94 and captopril to the buffer during the overnight incubation, to prevent gelatinolytic activity. Gelatinases A and B were identified by molecular weight and inhibition of gelatinolytic activity with the MMP inhibitor 1, 10-phenanthroline (Fluka, Buchs, Switzerland) dissolved in the incubation buffer at a concentration of 10 mM.

### *Western-blot analysis*

Equal amounts of proteins from the cell or tumor-tissue supernatants were run on sodium-dodecyl-sulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and electroblotted onto nitrocellulose membranes. The membranes were pre-incubated with TBS buffer (Tris 20 mM, NaCl 500 mM, pH 7.5) containing 5% skim-milk powder, for 2 hr at room temperature, and then incubated overnight at 4°C with a polyclonal antibody (2 µg/ml) against human 72-kDa gelatinase, diluted in TBS containing 5% skim-milk powder, kindly provided by Dr. W.G. Stetler-Stevenson (NCI, NIH, Bethesda, MD). After extensive washing with TBS containing 0.1% skim-

milk powder and 0.1% Tween-20, membranes were incubated with anti-rabbit-immunoglobulin peroxidase-conjugated antibodies (Calbiochem, La Jolla, CA) diluted 1:1000 for 1 hr at room temperature. Immunoblots were visualized by the enhanced chemoluminescence system (Amersham, Aylesbury, UK).

### *Northern-blot analysis*

Total cellular RNA was isolated using the guanidinium-isothiocyanate method. For Northern-blot analysis, 10 µg of total RNA was separated by size on formaldehyde-agarose gels and transferred overnight onto nitrocellulose filters. The filters were pre-hybridized at 42°C for 3 hr in 50% formamide,  $5 \times \text{SSC}$ , 0.1 M sodium phosphate,  $1 \times \text{Denhardt's}$  solution (0.02% polyvinylpyrrolidone, 0.02% Ficoll and 0.02% BSA), and 250 µg/ml denatured salmon sperm. Hybridization was performed at the same temperature, in buffer containing human  $^{32}\text{P}$ -labeled cDNA probes, overnight. Probes were radiolabeled with ( $\alpha$ - $^{32}\text{P}$ )dCTP using a random primer labeling kit (Amersham), specific for 72-kDa gelatinase, TIMP-2 and 28 S. MMP-2-cDNA (1,582 bp) cloned into the KpnI site of pGEM4Z vector (Promega, Madison, WI) was kindly provided by British Biotech. TIMP-2 (675-bp), cloned into pGEM vector, kindly provided by Dr. W.G. Stetler-Stevenson, was digested with HindIII-EcoRI enzymes. 28S RNA was used as a control for the amount of RNA transferred onto the nitrocellulose filters. Membranes were washed in up to  $0.1 \times \text{SSC}$  1% SDS at 55°C and blots were exposed to Kodak XAR film at -80°C with intensifying screens. The relative mRNA levels on the nitrocellulose filters were quantitated by Instant Imager (Canberra-Packard, Zurich, Switzerland).

### *Statistical analysis*

Data were examined using analysis of variance with Dunnett's *t*-test. Survival-time analysis was performed using the Kaplan-Meier test. Hazard ratios, along with their 95% confidence intervals (CI), were performed by the Cox proportional-hazards analysis. The SAS (Cary, NC) statistical package was used.

## RESULTS

### *Effect of captopril and BB-94 on gelatin-substrate degradation by gelatinases A and B released in conditioned medium of 3LL cells*

Samples from conditioned serum-free medium of 3LL cells were run on a gelatin-zymography gel. Gelatinolytic activity was analyzed *in vitro* by overnight gel incubation in a buffer containing increasing concentrations of BB-94 (5 to 500 nM) and captopril (5 to 20 mM). Gelatinase-A and -B activity, released in 3LL conditioned medium, was reduced by BB-94 at the concentrations of 50 and 500 nM, and by captopril at 20 mM (Fig. 1). Moreover, the 64- and 62-kDa bands, representing the active forms of gelatinase A, were also reduced in comparison with the control (Fig. 1).

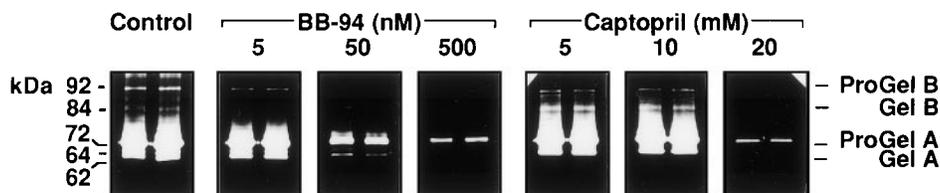
### *Gelatinase-A expression*

Cells treated with captopril at the dose of 5 mM showed a decrease of the gelatinolytic activity of the gelatinase-A proenzyme and of the 64- and 62-kDa active enzymes as compared with untreated control samples (Fig. 2a, lanes 4, 1). Western-blot analysis, using a specific polyclonal antibody, showed inhibition of pro-gelatinase-A and gelatinase-A protein levels induced by captopril at the same dose (Fig. 2b, lanes 4, 1). Complete inhibition of gelatinase-A activity and of protein levels was achieved by incubation of 3LL cells with 10 to 20 mM of captopril (data not shown).

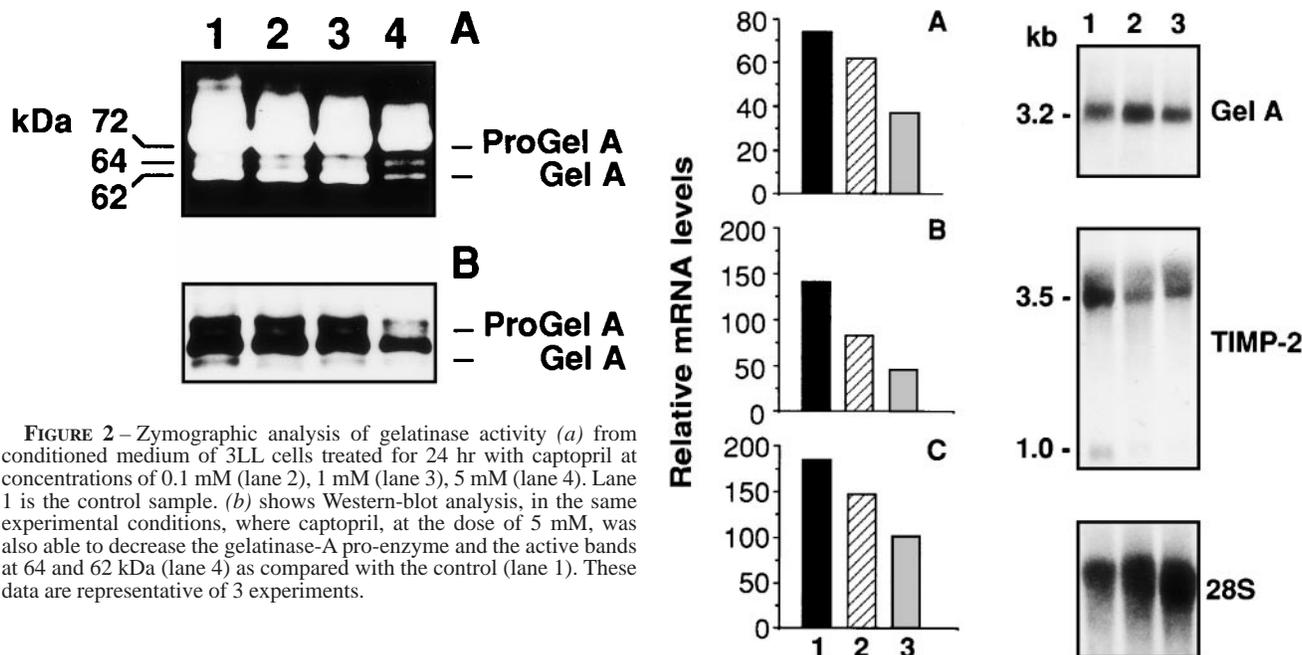
Northern-blot analysis, in the same experimental conditions, showed inhibition of gelatinase-A expression with 5 and 10 mM of captopril (Fig. 3a, lanes 2, 3) compared with the control (lane 1). TIMP-2 mRNA of 3.5 kb and 1.0 kb was also decreased (Fig. 3b, lanes 2, 3).

### *Tissue gelatinase activity and protein levels*

We compared gelatinolytic activity and protein levels of gelatinase A (72-, 64- and 62-kDa bands) in the supernatants of primary



**FIGURE 1** – Gelatinolytic activity from 3LL-cell-conditioned medium was inhibited by BB-94 at concentrations between 5 and 500 nM, and by captopril at concentrations between 5 and 20 mM. During the gelatinolytic incubation period, the gels were incubated with buffer containing BB-94 or captopril, at the above-indicated doses. These data are representative of 3 experiments.



**FIGURE 2** – Zymographic analysis of gelatinase activity (*a*) from conditioned medium of 3LL cells treated for 24 hr with captopril at concentrations of 0.1 mM (lane 2), 1 mM (lane 3), 5 mM (lane 4). Lane 1 is the control sample. (*b*) shows Western-blot analysis, in the same experimental conditions, where captopril, at the dose of 5 mM, was also able to decrease the gelatinase-A pro-enzyme and the active bands at 64 and 62 kDa (lane 4) as compared with the control (lane 1). These data are representative of 3 experiments.

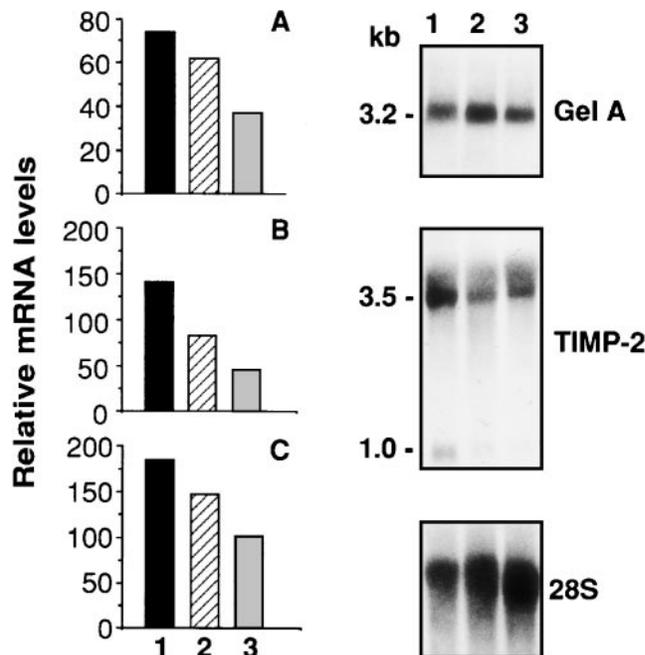
tumors and normal muscle specimens (Fig. 4, lanes 1, 2), lungs with metastatic tumor deposits and normal lung specimens (lanes 3, 4) of tumor-bearing or normal mice respectively, at day 15 after 3LL-cell implantation and treatments.

Enzymatic activity of gelatinases A and B, as well as the amount of activated enzyme, was higher in the untreated tumor than in adjacent normal muscle. Lung specimens, containing metastatic tumor deposits, had increased gelatinase B compared with normal lungs, as assessed by zymography (Fig. 4*a*).

Western-blot analysis showed higher gelatinase A protein levels in tumors compared to muscles (Fig. 4*b*). Gelatinase A activity and protein levels in the lungs were unchanged (Fig. 4*a,b*). Incubation with the specific gelatinase inhibitor 1,10 phenanthroline (10 mM) inhibited the activity of all the samples (Fig. 4*c*). To determine whether both agents were effective in the Lewis-lung-carcinoma model, we treated tumor and lung specimens containing metastatic tumor deposits for 24 hr with BB-94 (50 to 250 nM) and captopril (10 to 20 mM). BB-94 did not modify 3LL tumor and lung-associated gelatinolytic activity, confirming its direct and reversible action on the zinc-related active site of MMPs. In contrast, captopril at 20 mM decreased gelatinolytic activity of both gelatinase A (40% by 64 kDa and 80% by 62 kDa) and pro-gelatinase B (30%) compared with the control tumor specimens (Fig. 5*a*, lanes 3, 1), *in vitro*. At the same concentration, captopril decreased gelatinolytic activity of lung specimens containing metastatic deposits (Fig. 5*b*, lanes 3, 1).

#### Tumor growth

Female C57BL/6 mice were injected i.m. with 3LL cells ( $5 \times 10^4$  cells/mouse). Mice were randomized to 4 experimental groups of 10 mice, and treated as follows: vehicle (controls),

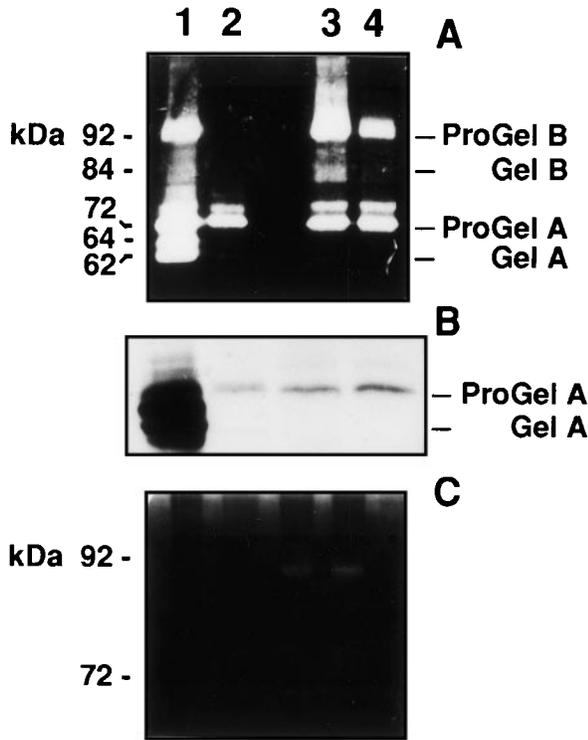


**FIGURE 3** – mRNA expression of gelatinase A and TIMP-2 by 3LL cells treated with captopril up to 24 hr. Treated and untreated cells were harvested after 24 hr and RNA was analyzed by Northern blot to detect gelatinase-A and TIMP-2 expression. Equal amounts of total RNA (10  $\mu$ g) were loaded on 15% agarose gel, blotted to membrane and probed with gelatinase A, TIMP-2 and 28S. Lane 1, control; lane 2, captopril 5 mM; lane 3, captopril 10 mM. The relative mRNA levels were quantitated by Instat Imager (Canberra-Packard), and values are shown after correction for variations in rRNA (28 S). (*a*) Gelatinase A; (*b*) TIMP-2/3.5 kb; (*c*) TIMP-2/1.0 kb. The data shown are representative of 3 experiments.

batimastat (30 mg/kg i.p.), captopril (50 mg/kg in the drinking water) and association of BB-94 and captopril, at the same doses, from day 0 to day 21 after 3LL-tumor implantation, as shown in Figure 6. At the end of treatments, we observed a mean tumor volume reduction of 25% by BB-94 ( $2.9 \text{ cm}^3 \pm 0.22$ ,  $p < 0.001$ ) and 33% by captopril ( $2.61 \text{ cm}^3 \pm 0.11$ ,  $p < 0.001$ ), as compared with the vehicle-treated group (control,  $3.9 \text{ cm}^3 \pm 0.26$ ). The combined therapy with both agents was significantly more effective (51%) than with either agent alone (BB-94/captopril,  $1.89 \text{ cm}^3 \pm 0.11$ ,  $p < 0.001$  compared with the vehicle-treated mice). Moreover, the combined therapy was statistically significant compared with single agents. Mean tumor-volume reduction with both agents was 35% as compared with BB-94 and 28% as compared with captopril ( $p < 0.05$ ). These results are representative of 2 different experiments.

#### Spontaneous lung metastases

The murine Lewis lung carcinoma is known to produce spontaneous lung metastases. To determine whether treatment with



**FIGURE 4**—Zymographic analysis of surnatant from homogenized specimens of tumor, muscle and lungs of tumor-bearing and control mice (a). We compared gelatinase-A and -B activity of tumor specimens (lane 1) with that of normal muscle (lane 2) and of normal lung specimens (lane 4) and lungs with metastatic deposits (lane 3). In the same experimental condition (b), we analyzed, by Western blot, protein levels of gelatinase A in primary tumor (lane 1) and in muscle (lane 2) also in normal lungs (lane 4) and in lungs containing metastatic deposits (lane 3). (c) shows the gel incubated in the presence of the broad-spectrum MMP inhibitor, 1, 10 phenanthroline (10 mM). These data are representative of 3 experiments.

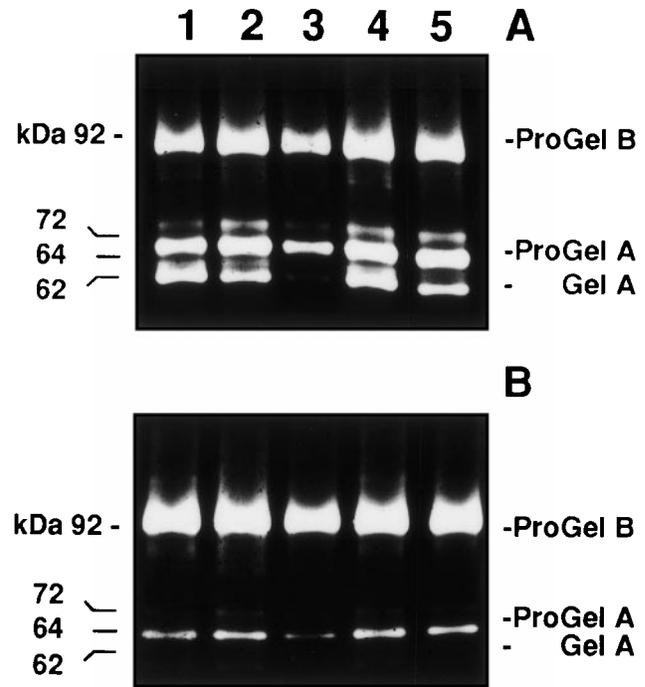
batimastat and captopril also reduced the number and/or growth of lung metastases, groups of 5-mice were implanted with 3LL cells and killed on day 21 to count lung-metastasis number by the macroscopic counting method. Figure 7 shows that captopril reduced the mean lung-metastasis number by 26% ( $60 \pm 19.4$  S.E.) and BB-94 reduced it by 29% ( $58 \pm 13.5$  S.E.), as compared with vehicle-treated mice ( $81.4 \pm 10.3$  S.E.). Combined therapy with batimastat and captopril reduced the mean number of lung metastases by 80% ( $16.4 \pm 3.2$  S.E.,  $p < 0.05$ ) as compared with the control group.

**Survival time**

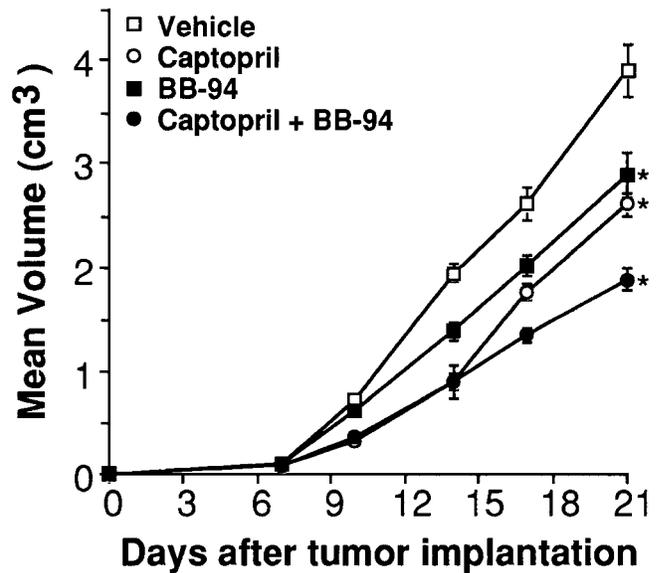
At the end of treatments, the groups of 10 mice implanted with 3LL cells, after measurements of tumor volume, were left for evaluation of survival time. As shown in Figure 8, treatment with captopril or BB-94 alone resulted in a slight prolongation of survival time, whereas, when given in combination, they significantly increased survival time (Kaplan-Meier approach). Hazard ratios of the group of mice treated with both agents were 0.27 (95% CI, 0.14 to 0.54;  $p < 0.001$ ). The 50% mortality rate was at day 28 for the vehicle-treated group and the BB-94-treated group, at day 30 for the captopril-treated group and at day 35 for the group that received combined treatment.

**DISCUSSION**

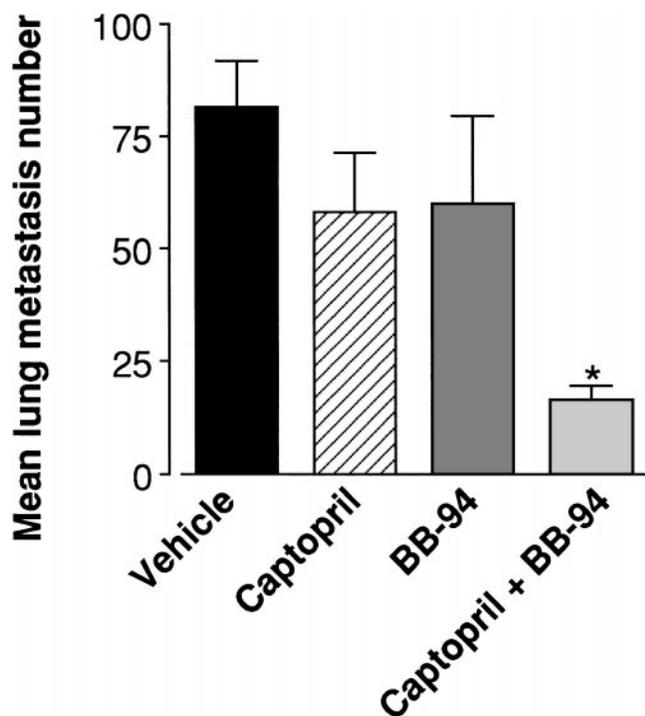
Matrix metalloproteinases are considered to play a crucial role in tumor invasion and metastasis as well as in new blood-vessel



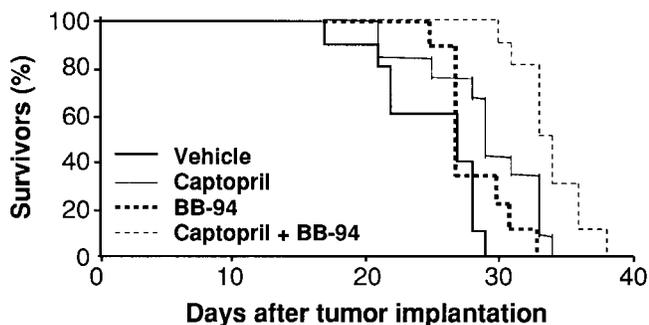
**FIGURE 5**—Zymographic analysis of gelatinases A and B from conditioned medium of 3LL tumor specimens (a) and lung specimens containing metastatic tumor deposits (b), treated for 24 hr with captopril at concentrations of 10 mM (lane 2), 20 mM (lane 3) and with BB-94 at concentrations of 50 nM (lane 4) and 250 nM (lane 5). Lane 1 is the control sample. Results are representative of 2 different experiments.



**FIGURE 6**—Inhibition of i.m. injected 3LL cells ( $5 \times 10^4$  cells/mouse). Tumor-bearing mice (10 mice per group) were treated with vehicle ( $\square$ ), BB-94 (30 mg/Kg i.p.  $\blacksquare$ ), captopril (50 mg/kg in drinking water  $\circ$ ), and with BB-94 and captopril combined ( $\bullet$ ) at the same doses from day 0 to day 21 after tumor implantation. Results are the mean volume of tumor + S.D. Both single and combined therapy, at day 21, resulted in significant reduction in the mean tumor volume (\*Dunnett's  $t$ -test:  $p < 0.001$ ). Results are representative of 2 different experiments.



**FIGURE 7** – Inhibition of lung metastasis number induced by murine Lewis lung carcinoma in mice. Tumor-bearing-mice were treated with BB-94 (30 mg/kg i. p.) and captopril (50 mg/kg in drinking water) and with both agents at the same doses, their combined effect being compared with control mice. Results are the mean of lung metastasis number  $\pm$  ES. of 5 lungs per group removed at day 21 of therapy (\*Dunnett's *t*-test:  $p < 0.01$ ). These data are representative of 2 different experiments.



**FIGURE 8** – Survival curve for mice bearing murine Lewis lung carcinoma. Mice were treated with vehicle, with BB-94 (30 mg/kg i.p.), with captopril (50 mg/kg in drinking water) or with both BB-94 and captopril daily from day 0 to day 21 after tumor implantation (as described in “Material and Methods”); subsequently the animals were observed to evaluate survival. Results are the mean of 10 animals per group and are representative of 2 experiments. Hazard ratios of the group treated with both BB-94 and captopril were 0.27 (95% C.I 0.14 to 0.54;  $p < 0.001$ ).

formation (Birkedal-Hansen, 1995). Here we have studied the effect of BB-94, a MMP inhibitor, and of captopril, an angiotensin-converting enzyme inhibitor, on *in vitro* and *in vivo* MMP expression and tumor growth of Lewis lung carcinoma. The well-characterized Lewis-lung-carcinoma model was chosen for these studies because the tumor is relatively resistant to many cancer therapies, and spontaneously metastasizes to the lungs.

BB-94 is a broad-spectrum reversible inhibitor, active at nanomolar concentrations against the Zn-related site of MMPs, and shows little activity against the angiotensin-converting enzyme (Brown and Giavazzi, 1995). Moreover, BB-94 was able to reduce the angiogenic potential of a murine hemangioma (Tarabozetti *et al.*, 1995). The ability of captopril, mainly known as an inhibitor of the angiotensin-converting enzyme, to inhibit cell-associated gelatinase activity and new blood-vessel formation, has been demonstrated in capillary endothelial cells (Volpert *et al.*, 1996).

In agreement with these authors, we found that the gelatinolytic activity of 3LL cells was inhibited by both BB-94 and captopril *in vitro*, showing that they were able to block the zinc-related active site of MMPs.

It is important to note that captopril reduced expression of gelatinase A mainly released by 3LL cells at transcriptional and post-transcriptional level, as shown by Western-blot and Northern-blot analyses *in vitro*. Therefore, in contrast with BB-94, captopril appears to be an inhibitor of gelatinase-A synthesis. The catalytic activity of gelatinase A is tightly regulated. Like other MMPs, it is secreted as an inactive pro-enzyme, and tumor spread is correlated with increased levels of the activated enzyme. Gelatinase A is produced at low levels by normal cells, but is dramatically over-expressed in many invasive, metastatic human cancers and murine tumors, such as the Lewis lung carcinoma (Anderson *et al.*, 1996).

We show that the 3LL primary tumor increased gelatinases A and B and their active forms, as compared with normal muscle. Lung specimens containing metastatic tumor deposits had higher levels of gelatinase B than normal lungs, whereas gelatinase A was unchanged. Only traces of gelatinase B were released in conditioned medium of 3LL cells. On the other hand, high levels of gelatinase B were found in tumor tissue and metastatic lung. This observation could be explained by the necessity of 3LL cells to be in contact with other stromal cells in the tumor mass, in order to express gelatinase B. Once the gelatinase B is activated by gelatinase A and set free from its inhibitor TIMP, it may operate within the connective-tissue compartment as well as in the basement membrane of the extracellular matrix (Bernhard *et al.*, 1994; Fridman *et al.*, 1995). Indeed, gelatinase A has been closely linked to the invasive phenotype of cancer cells (Ray and Stetler-Stevenson, 1995).

Noel *et al.* (1998) have demonstrated a crucial role of stromal MMPs in tumor progression. This hypothesis was supported by observations that several MMPs, including interstitial collagenase, stromelysin-3 and gelatinases A and B, involved in tumor progression, are produced not only by cancer cells, but also by stromal cells (Heppner *et al.*, 1996). Moreover, the interaction of cells with the extracellular matrix, and the localization of gelatinase A to the surface of invasive cells by interaction with integrin  $\alpha\beta 3$ , play an important role in the regulation of cell behavior (Brooks *et al.*, 1996).

On the basis of our *in vitro* data, we investigated the effects on murine Lewis lung carcinoma of single and combined therapy with BB-94 and captopril *in vivo*. Combined therapy with BB-94 and captopril was able to affect 3LL primary tumor volume, metastasis number and survival time. The strong anti-tumor effect of the combination of BB-94 and captopril in the Lewis-lung-carcinoma model may be due to the inhibition of tumor-cell-associated gelatinases A and B, but also to the inhibition of neo-angiogenesis. In fact, we observed a reduction of new vessel formation around the tumor in the groups of mice treated with both agents, as compared with control mice. Although it has been demonstrated that metalloproteinase activity is essential for new vessel formation (Tarabozetti *et al.*, 1995; Volpert *et al.*, 1996), it is difficult to evaluate the relative contribution of metalloproteinase inhibition in the ability of captopril to inhibit angiogenesis. The mechanism(s) underlying the important effects of combined therapy with captopril and BB-94 has/have not yet been defined. In this context, it is worth

mentioning that, in a retrospective cohort study, the risk of cancer was found to be reduced in patients receiving acetyl-converting enzyme inhibitors (Lever *et al.*, 1998).

The most straightforward explanation for our findings is that both agents act by inhibiting the breakdown of the extracellular matrix by tumor-induced MMPs. Agents that either decrease angiogenesis and/or inhibit MMPs can offer an alternative approach to arresting neoplastic progression at the stage of hyper-proliferative non-invasive cancer. Cytotoxic therapy is often followed by relapse; on the contrary, cytostatic therapy can potentially retard recurrence and prolong survival (Kohn and Liotta, 1995).

In conclusion, we have observed that captopril inhibits the expression of gelatinase A released by 3LL cells. Moreover, like BB-94, it blocks the zinc-related active site of gelatinases A and B. We have shown, in contrast, that the combination of BB-94 and

captopril *in vivo* induced strong inhibition of 3LL primary tumor, reduced lung metastasis number and prolonged survival time. It is therefore suggested that the effects detected *in vitro* on gelatinase production by tumor cells may represent one of the possible mechanisms (inhibition through metalloproteases of angiogenesis) of the anti-tumor effect observed *in vivo*.

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