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Selective Inhibition of Proteolytic Enzymes in an *in Vivo* Mouse Model for Experimental Metastasis[1]

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ABSTRACT

Peptide aldehyde transition state analogue inhibitors of serine and cysteine proteases have been used to selectively inhibit proteases for which prior evidence supports a role in tumor cell metastasis. These enzymes include cathepsin B, urokinase plasminogen activator (PA), and thrombin. The inhibition constants of the peptidyl aldehyde inhibitors show that they are highly selective for a particular targeted serine or cysteine protease. The inhibitors are introduced by i.p. injection or by miniosmotic pumps into syngeneic C57BL/6 mice also given injections of B16-F10 melanoma cells, and the number of metastatic foci in the lung was determined. While the injection protocol gave an initially high but changing *in vivo* concentration of inhibitor over time, the minipump implant gave a constant steady state concentration of inhibitor over 5–7 days. Minipump infusion of leupeptin (acetylleucylleucylargininal), a strong inhibitor of cathepsin B at a steady state plasma concentration 1000-fold greater than its $K_{i\,(\text{cathepsin B})}$, gave no significant decrease in lung colonization by the B16 tumor cells. Ep475, a stoichiometric irreversible peptide inhibitor of cathepsin B-like proteases, also did not significantly inhibit metastatic foci formation. Introduction of selective inhibitors of urokinase PA, *tert*-butyloxycarbonylglutamylglycylargininal and H-glutamylglycylargininal at concentrations near its K_i, produced no significant decrease in mouse lung colonization. The selective thrombin inhibitor D-phenylalanylprolylargininal infused to a steady state concentration 100-fold greater than its K_i dramatically increased B16 melanoma colonization of mouse lung. The results indicate that neither secreted cathepsin B-like nor urokinase PA have roles in B16 colonization of mouse lung, while thrombin may have a role in preventing metastasis. These experiments do not eliminate roles for a cathepsin B-like enzyme or urokinase PA in the initial steps of the metastatic process.

INTRODUCTION

A variety of serine and cysteine type proteases have been implicated in the mechanism by which cancer cells metastasize. In the initial stages of metastasis roles have been postulated for proteolytic enzymes in the detachment and invasion of the metastasizing cell through the extracellular protein matrices and the basement membrane surrounding the capillaries of the primary tumor. In addition proteolytic enzymes may be essential for the penetration of the tumor cell through the fibrin clot often found around a primary neoplasm. In later stages of the metastatic process, proteolytic enzymes are believed to be essential to the formation of microthrombi incorporating tumor cells and platelets in the capillaries of the target organ, which can form a stationary phase giving the tumor cells time to attach to the capillary wall and invade. Then again, proteolytic enzymes may participate in the extravasation of the metastasizing tumor cell from the vascular system through the basement membrane of the capillaries and into the target organ (for reviews see Refs. 1 and 2). Particular proteolytic enzymes of significant interest in metastasis include PA[4] (3, 4), cathepsin B (5, 6), collagenase (7), and fibrinogenic enzymes (8).

In this work we have developed specific proteolytic enzyme inhibitors that have a high selectivity in their inhibition constants among the serine and cysteine type proteases implicated in the metastatic process. In most previous studies of metastasis with proteolytic inhibitors, the inhibitors utilized were often general protease inhibitors which acted as strong inhibitors to a wide variety of proteases and proteolytic pathways, or the specificity of their inhibition of particular proteases was not known (1, 2, 8). Thus the role of specific proteases could not be easily assessed. In order to study the role of a protease *in vivo* with protease inhibitors, one must have a knowledge of the *in vivo* inhibitor concentration with respect to the known K_i of the inhibitor to the protease. The enzyme will be significantly inhibited at inhibitor concentrations equal to or greater than the equilibrium K_i. In addition the inhibitor should be highly selective to one particular protease so its inhibition can be clearly differentiated from other possible inhibitory effects. Accordingly, the inhibitor concentration *in vivo*, while at a greater concentration than the K_i of the targeted protease, must at the same time be at a lower concentration than the K_i of other nontargeted proteases in order to have a selectivity of inhibition.

The transition state analogue inhibitors used in this study are peptides in which the normally COOH-terminal carboxyl group is transformed to an aldehyde group. The peptide aldehydes reversibly associate as hemiacetals with the serine nucleophile of serine proteases or as thiohemiacetals with the cysteine nucleophile in cysteine proteases (9, 10). In these complexes of inhibitor and enzyme, the carbonyl carbon of the inhibitor takes on a tetrahedral configuration like that of the peptide carbonyl carbon in the transition state for peptide hydrolysis (9). Thus these inhibitors act as transition state analogues [for mechanistic details, refer to Kennedy and Schultz (9) and Frankfater and Kupy (10)]. Because of their transition state-like mode of association, these inhibitors show a strong selectivity in their inhibition even among proteases of identical primary site specificity in substrates (11). We have utilized these highly selective protease inhibitors, as well as the peptide epoxide inhibitor of cysteine protease Ep453 (12), to selectively inhibit *in vivo* targeted proteases that are believed to have a role in the metastatic process.

MATERIALS AND METHODS

Leupeptin (acetyl-Leu–Leu–argininal) was a gift of Dr. Walter Troll (New York University Medical Center and the Japanese-American Friendship Committee). Ep453 and Ep475 were gifts of Dr. Wataru Tanaka (Institute of Microbial Chemistry, Tokyo, Japan).

Boc'-L-glutamylglycyl-L-argininal was synthesized by procedures to be published in detail in a later publication. Briefly, Boc'-(O^γ-Bzl)-

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[2] Research comprises a part of a Dissertation to be submitted in partial fulfillment of the requirements for the Ph.D. degree at Department of Biochemistry and Biophysics, Loyola University of Chicago.

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[4] The abbreviations used are: PA, plasminogen activator; Boc', *tert*-butyloxycarbonyl; Bzl, benzyl; PBS, phosphate buffered saline, pH 7.4.

glutamyl-*N*-hydroxysuccinimide ester was joined to glycine by standard procedures to form the dipeptide Boc^t-(O^γ-Bzl)-Glu–Gly. This dipeptide was then joined to (N^G-NO_2)-argininal semicarbazone, prepared in a way similar to procedures described previously (11), to form Boc^t-(O^γ-Bzl)-Glu–Gly–(N^G-NO_2)–argininal semicarbazone. The semicarbazone group was removed and then the N^G-NO_2 and Bzl protecting group were removed by catalytic hydrogenation over palladium black, similar to procedures described previously (11), yielding Boc^t-Glu–Gly–argininal.

^{1}H nuclear magnetic resonance and IR spectra are in agreement with the proposed structure, confirming the final products and all intermediate products of the synthesis.

$C_{18}H_{32}N_6O_7 \cdot 0.25H_2O$ (449.0)

Calculated: C 48.15, H 7.19, N 18.72
Found: C 47.72, H 7.38, N 19.17

Analysis was performed by Spang Microanalytical, Eagle Harbor, MI.

The ditrifluoroacetate salt of H-glutamylglycylargininal was prepared from the above compound by treatment with trifluoroacetic acid to remove the Boc^t protective group. Spectral and elemental analyses are in agreement with the proposed product.

R-D-Phe–Pro–argininal (R = H, Boc^t) inhibitors were synthesized according to procedures of Bajusz *et al.* (13). H-D-Phe–Pro–argininal at 3.3×10^{-7} M increased the clotting time for bovine fibrinogen 5-fold over a control in the absence of inhibitor. This is a result similar to that observed by Bajusz *et al.*

Equilibrium Inhibition Constants (K_i values). The K_i values for Boc^t-Glu–Gly–argininal and leupeptin inhibitors were determined by Dixon plots of $1/v$ *versus* $[I]$ at various substrate concentrations in the region of substrate K_m. The K_i values for R-D-Phe–Pro–argininal inhibitors (R = Boc^t, H) were determined in most cases in the same manner, except to thrombin to which the binding was very tight. In the later cases the K_i values were obtained under first order conditions $[S] \ll K_m$ and the K_i was calculated by the equation

$$K_i = \frac{[I]}{\frac{v_u}{v_i} - 1}$$

where $[I]$ is the inhibitor concentration, v_i is the first order rate constant in the presence of inhibitor, and v_u is the rate constant in the absence of inhibitor. The K_i reported represents the average and standard error of the K_i for multiple runs and different inhibitor concentrations. In these cases the K_i was also calculated by the plot of Kézdy and Perlstein for tight binding inhibitors (10), and the K_i values calculated by this procedure were in agreement with the K_i values calculated by the equation above. The K_i values for Factor Xa were also calculated by the first order procedure and the equation above, because the K_m for its substrate was too high for the Dixon procedure.

Enzymes and substrates were as follows: bovine thrombin (Miles Laboratories) assayed against H-D-phenylalanylpipecolylarginine-*p*-nitroanilide (KABI S-2238), human urokinsae (Abbott Laboratories; Abbokinase) assayed against pyroglutamylglycylarginine-*p*-nitroanilide (KABI S-2444), B16a mouse melanoma cathepsin B (gift of A. Bajkowski, B. Sloane, and K. Honn, Wayne State University School of Medicine) assayed against carbobenzoxy-arginylarginine-4-methoxy-β-naphthylamide (Sigma Chemical Co.). Factor Xa (Sigma; activated by Russel's venom) assayed against Bz-isoleucylglutamylglycylarginine-*p*-nitroanilide (KABI S-2222), and bovine plasmin (Sigma) assayed against H-D-valylleucyllysine-*p*-nitroanilide (KABI S-2251). Urokinase stock solutions contained 0.9% NaCl and 5% albumin. Factor Xa stock solutions contained 0.04 M sodium citrate, pH 5.8. Plasmin stock solutions were in 50% glycerol and contained 5% Carbowax 1000. Cathepsin B stock solutions were activated by diluting 1:1 with a solution of 30 mM dithiothreitol and 15 mM EDTA at pH 5.2. All *p*-nitroanilide substrate reactions were carried out at pH 7.8 in 0.2 M sodium phosphate buffer at 37°C, by following the appearance of *p*-nitroaniline at 410 nm in a Perkin-Elmer 320 spectrophotometer. Cathepsin B was assayed in 0.3 M sodium citrate-sodium phosphate buffer at pH 6.2 and 37°C, in a Perkin-Elmer MPF-44B spectrofluorimeter with excitation at 292 nm and the emission of the 4-methoxy-β-naphthylamine product followed at 410 nm.

Assay of Protease Inhibitor Concentrations *in Vivo*. Mice were given i.p. injections of inhibitor solution and then sacrificed at selected times by CO_2 suffocation. Blood samples were immediately collected by cardiac puncture using a 22-gauge needle. The blood collected was centrifuged in an Eppendorf microfuge for 15 min in the cold. The supernatant plasma was collected, diluted (usually 1:2) with the buffer used in the enzyme assay, and filtered through an Amicon YM-5 membrane (M_r 5000 cutoff) in an Amicon Micropartition system with centrifugation for 30 min at 4°C. This filtration removed natural protein proteinase inhibitors of the plasma, but the peptide argininal inhibitor of low molecular weight easily passed through the filter. A 200-μl sample of the filtrate was assayed for the presence of inhibitor by incubating for 3 min with the appropriate assay enzyme in the assay buffer, adding the *p*-nitroanilide substrate specific for the assay, and following the rate of substrate hydrolysis at 410 nm in a spectrophotometer. The percentage of inhibition observed was normalized by controls using plasma from mice obtained in a similar manner in which the mouse was given an injection of the carrier vehicle only. Control plasma filtrates gave either no observable inhibition or very minor inhibition in this assay. Finally, the observed inhibition from filtered plasma was compared to a standard curve in which known concentrations of peptide inhibitor were utilized. Inhibitions were adjusted by dilution to obtain inhibitions in the range of 50 to 80%. If inhibition was >80%, dilutions were made and the concentration of inhibitor was back calculated to obtain the concentration of inhibitor in the original solution.

Leupeptin was measured against bovine trypsin with the substrate *N*-benzoyl-DL-arginine-*p*-nitroanilide in 0.2 M phosphate buffer, pH 7.8.

Ep475 was assayed against papain (Sigma). In this assay 100 μl of 2.8×10^{-6} M papain were activated by a 1:1 dilution with 100 μl of 30 mM dithiothreitol and 15 mM EDTA, pH 5.2, and incubation for 30 min at 37°C. A plasma filtrate aliquot and 100 μl of the activated papain were added to 1.0 ml of 0.3 M sodium citrate and 0.3 M sodium phosphate buffer, pH 6.2, in a cuvet to which were added 30 μl of substrate *N*-benzoyl-DL-arginine-*p*-nitroanilide.

Boc^t-Glu–Gly–argininal hydrochloride and Glu–Gly–argininal ditrifluoroacetate were assayed with human urokinase (Abbokinase) against the substrate KABI S-2444 (pyroglutamylglycylargininyl-*p*-nitroanilide) in 0.1 M Tris 0.1 M NaCl, pH 7.8, in a buffer containing 0.1% Triton X-100.

Boc-D-Phe–Pro–argininal hemisulfate and D-Phe–Pro–argininal hemisulfate were assayed with 20 milliunits bovine thrombin (Pentax) against KABI S-2238 (D-Phe–Pro–Arg-*p*-nitroanilide) in 0.2 M sodium phosphate buffer, pH 8.0.

***In Vivo* Experimental Metastasis Assay: Procedure for Introduction of Inhibitors in Mouse Assay Experiments.** The effect of the protease inhibitors on metastasis was assessed with the B16 melanoma model described in detail by Fidler (14). B16 F10 melanoma cells (obtained from the Division of Cancer Treatment, Tumor Repository, NCI-Frederick Cancer Research Facility, Frederick, MD) (2×10^5 to 3×10^5) were seeded in 10 ml of complete media (Dulbecco's modifed Eagle's medium containing 10% fetal calf serum and 100 units penicillin and 100 μg streptomycin per ml). All media solutions were purchased from GIBCO. After 3 days semiconfluent cells were collected by a mild trypsinization (0.25% trypsin in PBS containing 1 mM EDTA for less than 1 min), washed in complete media, resuspended in PBS, counted, and diluted to 500,000 cells/ml. Cells treated in this way were always greater than 85% viable by the trypan blue exclusion test. After all the mice had been given injections, cells were often used to initiate new cultures and showed no apparent decrease in viability.

Female 6–8-week C57BL/6 mice were obtained from Harlan, Cumberland View Farms, or Charles River Laboratories. The drug treatment was always started prior to the injection of tumor cells, so that an optimum amount was present initially. The injection protocol was similar to that described by Fidler (14). Alternate injections of tumor cells were made in control animals and then in inhibitor treated animals

so that one group did not exclusively receive "early" harvested cells. The cell suspension was repeatedly inverted during the course of the injections, and microscopic examination after the conclusion of the injections revealed no cell clumping. We noticed differences in the number of metastatic foci formed from the controls of one experiment to another. The differences seemed to be due to a number of factors including animal supplier, number of passages in culture, and the exact time of harvesting the cells.

Animals were usually sacrificed after 15 days, the lungs were removed and rinsed in PBS, the lungs were dissected into lobes, and the black metastatic foci were counted under a ×30 binocular dissecting scope. Only surface colonies were counted on both sides of the lung lobes. When amelanotic colonies were present, lungs were dissected into lobes as above, stained with Bouin's solution, and then counted. Animals were routinely examined for other sites of metastasis and no significant colonization of other sites than the lung were observed. Complete autopsies were occasionally performed on randomly selected animals from the different experiments, and a few (approximately 1 to 5) individual metastatic foci were observed in approximately 25% of the animals at locations other than the lungs. This always constituted a significantly much lower invasion number than at the lung (see Tables 2 and 3).

Implantation of Osmotic Pumps for Drug Infusion. The miniosmotic pumps (Alzet, Model 2001; rate of flow, 1 μl/h for 7 days) were implanted in the mice similar to procedures described by manufacturer (Alza Corp., Palo Alto, CA). Mice were anesthetized with an i.p. injection of 0.2 ml of pentobarbital solution (6.5 mg/ml in PBS) for insertion of the pumps. Occasionally, when the skin was pulled tight around the pump, the wound would reopen to the sides of the wound clips. In these cases, the animal was anesthetized again and the wound was repaired. Pumps were removed after 1 week. The pumps were handled with sterile gloves, and none of the mice showed any sign of infection or discomfort.

The solutions used within the minipumps were: (*a*) 0.1 M leupeptin in PBS with controls containing PBS alone; (*b*) 0.08 M Ep453 in propylene glycol with controls containing propylene glycol; (*c*) 0.25 M H-D-Phe-Pro-argininal in 40% dimethyl sulfoxide-PBS with controls containing 40% dimethyl sulfoxide-PBS. These concentrations in the pumps gave steady state concentration in plasma of 3×10^{-6} M for leupeptin, 3×10^{-7} M for Ep475, and 4×10^{-7} for M H-D-Phe-Pro-argininal. Inhibitor concentrations *in vivo* were determined by procedures described above.

RESULTS

Selective Inhibition of Cysteine and Serine Proteases Implicated in the Metastatic Process by Peptidyl Aldehyde Inhibitors. The equilibrium dissociation constants, K_i, were determined for peptidyl aldehyde inhibitors against particular proteolytic enzymes of interest and are given in Table 1. The K_i values were determined by Dixon plots against the appropriate specific peptidyl chromogenic or fluorophoric substrates (15). If the binding was extremely tight, plots from the first order rate constants determined under conditions of $[S] \ll K_m$ were made, according to Perlstein and Kézdy (10), in order to obtain an accurate K_i under the necessary conditions of inhibitor concentration in only slight excess of enzyme.

A comparison of K_i values shows in each case more than 1 order of magnitude better binding to one of the proteases of Table 1. Thus leupeptin is a particularly strong inhibitor of the cysteine protease cathepsin B ($K_i = 4 \times 10^{-9}$ M), which is 3 orders of magnitude greater than any of the other targeted proteases of Table 1. The peptide aldehyde H-D-Phe-Pro-argininal has a 2.5 to 3 orders of magnitude stronger affinity to thrombin ($K_i = 1 \times 10^{-8}$ M) than other targeted proteases possibly having a role in the metastatic process. While the affinity of H-Glu-Gly-argininal towards urokinase is not as strong as H-D-Phe-Pro-argininal to thrombin or leupeptin to cathepsin B, it is highly selective towards urokinase (Table 1). It is concluded that the peptidyl aldehyde inhibitors are highly selective for particular proteases and have a potential for use as selective inhibitors of targeted proteases *in vivo*.

Effect of the Inhibition of Cathepsin B-like Activity on B16 Metastasis. The procedure in the B16 melanoma cell metastatic lung colonization assay is similar to that described previously by Fidler (14). In the assay, 6–8-week-old female C57BL/6 mice were given injections of variable amounts of dispersed B16-F10 melanoma cells in the tail vein. The mice were concurrently treated with protease inhibitors, while control animals received the carrier vehicle alone. After 15 days the animals were sacrificed, the lungs were removed and sectioned into lobes, and the melanoma foci were counted under a dissecting microscope.

Leupeptin is a strong inhibitor of cathepsin B ($K_i = 4 \times 10^{-9}$ M; Table 1). As discussed above, this inhibitor shows at least a 3 orders of magnitude better K_i towards cathepsin B than other potential metastatic promoting proteases of Table 1. A protocol was initially used in which i.p. injections of leupeptin were made every 12 h from 1 to 2 h just prior to the introduction of the B16 tumor cells to 5.5 days after the injection of B16 tumor cells. The dose per injection varied with the experiment from 5 to 50 mg/kg (Table 2). This protocol is similar to that by Saito *et al.* (16) for leupeptin injections in rats also given Yoshida rat ascites hepatoma cells in the rat tail vein, in which a 50% decrease of lung colonization was observed. Table 2 shows that in all six of our experiments with leupeptin, the average number of metastatic lung colonies formed in the leupeptin treated animals was below that of its matched controls. The decrease from matched controls averaged 64%. However, in only one of the experiments was the value significantly lower at a probability of 95% by Student's *t* test ($P < 0.05$) (Table 2, Experiment 1). The other five experiments showed an overlap between leupeptin treated mice and control, resulting in no differences even at $P = 0.1$ although in all cases the leupeptin treated mice gave a lower average.

In order to determine whether leupeptin is present *in vivo* in sufficient amounts to inhibit cathepsin B-like enzyme activity,

Table 1 *Inhibition constants of peptidyl aldehydes against selected proteases*

K_i values were determined *in vitro* against specific chromogenic or fluorophoric substrates (see "Materials and Methods"). Standard error averaged ±30% of reported K_i values.

	K_i (M)				
Enzyme	Ac-Leu-Leu-argininal[a]	D-Phe-Pro-argininal	Boc-D-Phe-Pro-argininal	Glu-Gly-argininal	Boc-Glu-Gly-argininal
Thrombin	4.7×10^{-6}	1.1×10^{-8}	1.5×10^{-8}	$>2 \times 10^{-4}$	$>2 \times 10^{-4}$
Factor Xa	5.7×10^{-6}	5.2×10^{-5}	3.9×10^{-6}	$>2 \times 10^{-4}$	3.1×10^{-4}
Urokinase	7.7×10^{-5}	2.0×10^{-4}	2.2×10^{-6}	2.1×10^{-5}	5.4×10^{-5}
Plasmin	1.8×10^{-6}	2.6×10^{-5}	2.6×10^{-6}	$>2 \times 10^{-4}$	1.6×10^{-4}
Cathepsin B	3.7×10^{-9}	$>2 \times 10^{-5}$	$>2 \times 10^{-5}$	$>2 \times 10^{-5}$	

[a] Ac, acetyl.

Table 2 *Effect of i.p. injected proteinase inhibitors on experimental metastasis*

Experimental treatment	No. of lung metastases/animal	Av. ± SE
1. Control	13, 17, 21, 21, 37, 47, 51	30 ± 6
Leupeptin (50)[a]	0, 7, 10, 15, 17, 18, 22, 31	15 ± 3[b]
Boc-D-Phe-Pro-arginal (1 × 50, then 12.5)	9, 29, 47	28
2. Control	66, 68, 69, 88, 108, 180	97 ± 18
Leupeptin (5)	32, 38, 66, 72, 83, 125, 146	80 ± 16
Leupeptin (50)	26, 32, 36, 46, 69, 111, 168	70 ± 20
3. Control	3, 8, 18, 22, 25, 41, 54, 60, 70, 73, 84, 92, 120, 133, 299	74 ± 19
Leupeptin (5)	4, 12, 14, 18, 28, 33, 47, 66, 70, 76, 77, 79, 81, 86, 101, 106, 120	60 ± 9
4. Control	1, 23, 33, 41, 54, 59, 121	47 ± 14
Leupeptin (50)	5, 6, 23, 44, 103	36 ± 18
Boc-D-Phe-Pro-arginal (37.5)	12, 25, 26, 31, 36, 48, 62, 79, 118, 180	62 ± 16
5. Control	15, 31, 44, 46, 48, 54, 97, 113, 131, 139, 142	78 ± 14
Ep453 (50)	8, 36, 37, 42, 42, 51, 60, 60, 80, 127, 129, 153, 180	77 ± 15
6. Control	122, 165, 177, 180, 220, 230, 260, >300, >300, >300	226 ± 20
Boc-Glu-Gly-argininal (30 mg i.p./animal 8 times over 36 h)	62, 68, 154, 160, 178, >300, >300	174 ± 37
7. Control	87, 89, 122, 122, 128, 149, 189	127 ± 13
H-Glu-Gly-argininal (30 mg i.p./ animal 6 times over 25 h)	214, 279, >300, >300, >300	279 ± 17[c]
8. (20,000 cells)		
Control	0, 0, 0, 1, 1, 1, 9, 10, 10	3.6 ± 1.5
Leupeptin (25)	0, 0, 0, 1, 1, 1, 1, 3	0.9 ± 0.4
Boc-D-Phe-Pro-arginal (25)	0, 1, 1, 1, 1, 2, 2, 2, 2, 3, 6, 14, 17, 36	6.3 ± 2.7

[a] Numbers in parentheses, mg/kg injected i.p. every 12 h for 5.5 days. Control animals received injections of the drug vehicle, usually PBS.
[b] Value is significantly different from control by Student's t test at $P < 0.05$.
[c] Significantly different from control at $P < 0.001$.

we measured the concentration of leupeptin in mouse plasma at various time points after a 50-mg/kg i.p. injection. It was found that 30 min after an injection a very high 5×10^{-5} M concentration in plasma was present which decreased to 1.5×10^{-5} M in plasma at 2 h and to 2×10^{-7} M in 4 h. The concentration of leupeptin was below our limits of detection (1×10^{-7} M) in 6 h. Our data are in general agreement with the leupeptin turnover data of Tanaka (17). Thus the initial concentrations of leupeptin after each injection were higher than the K_i values of all the targeted proteases of Table 1 but by 4 h they were below all the targeted proteases except for cathepsin B. Because cathepsin B will be inhibited at very low concentrations (10^{-9} M, which was below our limit of detection), it may be that this enzyme was inhibited throughout the protocol. In support of a continued inhibition of cathepsin B over the time course of the protocol is the observation that a 15-mg/kg dose leupeptin injected i.p. 3 days/week prevents the destructive actions of proteolytic enzymes in a mouse genetic model for muscular dystrophy (18). In addition, Saito *et al.* (16) had reported significant decreases in hepatoma cell colonization of rat lung by this same injection protocol (50 mg/kg every 12 h) (16).

However, in order to address the criticism of too low a concentration of inhibitor over the time course for melanoma cell invasion, we carried out experiments with surgically inserted miniosmotic pumps that added leupeptin at a constant flow rate over 5 to 7 days. By this method a known constant steady state *in vivo* inhibitor concentration was achieved. Infusions were initiated 12 h prior to the tail vein injection of the B16 cells and maintained for 5–7 days after the B16 tumor cell injection. Assay of the leupeptin concentrations in mouse plasma over the 5–7-day period showed a constant steady state concentration of 3×10^{-6} M in mouse plasma. The results of the minidiffusion pump protocol (Table 3) clearly show that leupeptin does not inhibit B16 F10 lung colonization even at 3 orders of magnitude higher concentration than its K_i towards B16 melanoma cathepsin B.

The concentration of the inhibitor was taken from the plasma from which the tumor cells must extravasate and not from the interstitial space into which the tumor cells must propagate and invade after extravasation. However, leupeptin, Ep475, and presumably other small peptide inhibitors can easily exit the plasma into interstitial space. Measurements by Hanada *et al.* (12), Tanaka (17), and Aoyagi *et al.* (19) show a wide extravascular tissue distribution after introduction into animals of small peptide protease inhibitors by a variety of injection modalities.

Single injections of leupeptin have reportedly caused later increases of cathepsin B in treated animals over normal values, as a recompensatory overshoot apparently occurring after the inhibition by leupeptin ceases (20). However, the continuous infusion at a steady state level of 3×10^{-6} M in plasma as in this protocol should be sufficient to inhibit over 99.9% of the extracellular cathepsin B activity and prevent any extracellular activity while present. While the *in vivo* leupeptin steady state concentration achieved is highly effective against cathepsin B, it was near or below the K_i values of the other targeted proteases of Table 1 and therefore selective for cathepsin B-like activity among the targeted proteases. Other major proteases implicated in the metastatic process such as PA or the fibrinogenic enzymes Factor Xa and thrombin (Table 1) will not be significantly inhibited at 3×10^{-6} M leupeptin.

In order to confirm the conclusion that secreted cathepsin B-like proteases are not essential in B16 melanoma cell lung colonization, a second potent cathepsin B inhibitor was studied in the metastatic model. Hanada *et al.* (12) have developed a series of epoxide peptide derivatives that are potent stoichiometric inhibitors of cysteine proteases such as cathepsins B and L. The peptide epoxide Ep453 of Hanada was injected i.p. When it reaches plasma it is rapidly converted to the more soluble Ep475 inhibitor by esterases present *in vivo* (12). In our laboratory we found the conversion to Ep475 to occur within 30 min after the addition of Ep453 to mouse plasma, which directly inhibited a stoichiometric concentration of the cysteine protease papain. The injection of Ep453 (50 mg/kg every 12 h), as in the protocol used with leupeptin, had no significant effect on the number of metastatic foci formed (Table 2). More clear-cut is the experiment in Table 3 showing that the infusion of Ep453 by the surgically implanted miniosmotic pumps, leading to a constant steady state concentration of Ep475 in mouse plasma of 3×10^{-7} M, also showed the lack of a significant effect on lung colonization. Thus two cathepsin B inhibitors at high *in vivo* concentrations relative to their K_i values showed no significant effect on the B16 melanoma lung colonization process.

Effect of a Selective Thrombin Inhibitor on B16 Metastasis. The peptide aldehyde H-D-Phe-Pro-argininal is a strong inhibitor of thrombin and the thrombosis pathway (13). The K_i observed against bovine thrombin (1×10^{-8} M) is 2 orders of magnitude lower than for other proteases implicated in the metastatic process such as B16 melanoma cell cathepsin B ($K_i = >2 \times 10^{-5}$ M), plasmin ($K_i = 2.6 \times 10^{-5}$ M), and urokinase PA ($K_i = 2.0 \times 10^{-4}$ M) (Table 1). Thus as leupeptin is a highly

Table 3 *Effect of continuously infused protease inhibitors on experimental metastasis*

Solution infused	*In vivo* concentration[a] (M)	No. of lung metastases/animal	Av. ± SE
9. Control (PBS)[b]		34, 46, 64, 71, 80, 96, 111, 132, 133, 153, 157, 236	109 ± 16
Leupeptin (0.1 M in PBS)	3×10^{-6}	74, 91, 102, 109, 109, 121, 127, 137, 155, 250	128 ± 15
10. Control (PBS)		1, 2, 2, 3, 4, 4, 5, 6, 7, 7, 7, 7, 8, 8, 10	5.4 ± 0.7
Leupeptin (0.1 M in PBS)	3×10^{-6}	0, 0, 1, 2, 2, 3, 4, 6, 20	4.2 ± 2.1
11. Control (40% DMSO[c])		4, 4, 4, 6, 8, 9, 12, 13	7.5 ± 1.3
D-Phe-Pro-argininal (0.25 M in 40% DMSO-PBS)	4×10^{-7}	229, >300, >300, >300, >300, >300, >300, >300, >300, >300	>300[d]
12. Control (40% DMSO)		7, 8, 11, 12, 13, 18	12 ± 1.4
D-Phe-Pro-argininal (0.25 M in 40% DMSO-PBS)	4×10^{-7}	104, >300, >300, >300, >300, >300	>300[d]
13. Control (propylene glycol)		13, 22, 27, 33, 44, 49, >300, >300	98 ± 44
Ep453 (0.08 M in propylene glycol)	3×10^{-7}	17, 25, 25, 43, 66, 79, 90, 105	56 ± 12

[a] Steady state concentration determined in mouse plasma during infusion of inhibitor.
[b] Solution in parentheses infused into animal from Alzet miniosmotic pumps surgically implanted into mice. Rates of infusion were 1 μl/h over 5–7 days.
[c] DMSO, dimethyl sulfoxide.
[d] Value is significantly different from control at $P < 0.001$.

selective inhibitor of cathepsin B among the targeted proteases, H-D-Phe-Pro-argininal is a selective inhibitor of thrombin. We found that Boc-D-Phe-Pro-argininal is also a potent inhibitor of thrombin ($K_i = 1.5 \times 10^{-8}$ M) although its selectivity for thrombin over other implicated proteases is less dramatic (Table 1).

The injection of 37.5 mg/kg i.p. every 12 h from just prior to 5.5 days after melanoma cell introduction consistently showed the lack of any inhibition of B16 lung colonization. However, the turnover *in vivo* of the R-D-Phe-Pro-argininal inhibitors appeared faster than the turnover observed for leupeptin. We found after an i.p. injection of Boc'-D-Phe-Pro-argininal (37.5 mg/kg) that the plasma concentration was approximately 1×10^{-6} M at 0.5 h and 4×10^{-7} M at 1 h, after which it decreased below our limits of detection. Thus any conclusions from the 12-h injection protocol would be speculative.

With surgically inserted miniosmotic pumps a constant steady-state level in plasma of 4×10^{-7} M H-D-Phe-Pro-argininal was achieved from 12 h before to 5–7 days after B16 introduction, which is 36-fold greater than the K_i for thrombin yet below the other major serine and cysteine proteases implicated in the metastatic process. Under these conditions of inhibitor, a dramatic increase in metastasis was repeatedly observed (Table 3). In Experiment 11, 9 of the mice treated with H-D-Phe-Pro-argininal contained greater than 300 metastatic foci, whereas all control mice showed 13 or less metastatic foci per lung. In Experiment 12 one mouse contained 104 foci and 5 greater than 300 foci in Phe-Pro-argininal infused mice, whereas the controls averaged 12 foci (9 mice). This statistically significant promotion of metastasis by the selective thrombin inhibitor may indicate that thrombin has a role in retarding metastasis.

Effect of a Selective PA Inhibitor on B16 Metastasis. Boc-Glu-Gly-argininal and the trifluoroacetate salt of H-Glu-Gly-argininal were synthesized as plasminogen activator inhibitors based on an analogy to the R-Glu-Gly-arginine chloromethyl ketone inhibitors (R = H, dansyl) of both tissue PA and urokinase PA synthesized by Coleman *et al.* (21). Synthesis of the argininal inhibitors are required as the chloromethyl ketone compounds are highly toxic *in vivo*. The K_i values obtained for the argininal derivatives towards urokinase by *in vitro* assays were not as strong as for leupeptin with cathepsin B or Phe-Pro-argininal with thrombin (Table 1). However, the K_i values obtained with R-Glu-Gly-argininals (R = H, Boc') were highly selective for urokinase over the other implicated proteases (Table 1).

Because of the relatively high K_i towards PA, use of the miniosmotic pumps was not possible as the concentrations required within the pump to achieve a steady state concentration higher than the K_i of the Glu-Gly-argininals for urokinase is greater than the inhibitors solubility. Turnover studies showed that after a 30-mg/kg i.p. injection the Boc'-Glu-Gly-argininal was present at a concentration after 1 h of 5×10^{-5} M in plasma, which decreased below our limit of detection (>1×10^{-5} M) at 2 h. Accordingly, we chose to give maximum doses i.p. of 30 mg/mouse (approximately 2000 mg/kg) in 0.2-ml aliquots every 2 to 4 h from just before to 1.5 days after B16 cell injection, rather than once every 12 h for 5.5 days, in order to saturate the animal with the highest possible concentrations of inhibitor during the early period after i.v. tumor cell injection. If extracellular urokinase PA was essential to the colonization process of B16 melanoma cells, an inhibition of metastasis would be observed. However, Experiment 6 (Table 2) with Boc'-Glu-Gly-argininal shows that no statistically significant effect is observed. Experiment 7 with H-Glu-Gly-argininal shows a significant promotion of metastasis. However, because there were observations that the high doses of this derivative were beginning to make the mice ill, we believe that interpretations as to reasons for a promotion of metastasis in this experiment must be considered speculative.

DISCUSSION

The metastasis of tumor cells from a primary neoplasm to distant sites in target organs may be divided into several stages: (*a*) the invasion of the primary tumor into the surrounding tissue, with penetration through the basement membrane into the capillaries; (*b*) the penetration of single or multiple tumor cells through the fibrin "cage" usually surrounding a tumor growth in the blood vessels and into the circulation; (*c*) the attachment of the circulatory tumor cell(s) to the capillary wall of the target organ; (*d*) the infiltration of the arrested tumor cell through the capillary and basement membrane surrounding the capillary and into the target organ; and (*e*) the multiplication of the tumor cells and growth of the tumor into its new host tissue. Our experimental model tests only the later stages of the metastatic process (Steps *c* through *e*) because the tumor cells are injected directly into the circulation.

Two selective inhibitors of cathepsin B, which act by different

inhibitory mechanisms (leupeptin and Ep453), gave no significant change of experimental metastasis in the B16 melanoma cell lung colonization model. A decrease is expected if an extracellular cathepsin B-like protease has an essential role in lung colonization by B16 melanoma cells. The inhibitors were present in plasma, and presumably in interstitial space (12, 17–19), at orders of magnitude higher concentrations than their K_i to cathepsin B, under which conditions the inhibitors were highly selective for inhibition of cathepsin B-like proteases over other serine proteases implicated in the metastatic process. Thus the lack of an inhibition of experimental metastasis indicates that extracellular cathepsin B does not have an essential role in B16 melanoma extravasation and lung colonization, which contradicts a large body of indirect evidence indicating a role for extracellular cathepsin B in metastatic processes. Evidence in support of a role of cathepsin B-like protease in metastasis includes observations of increased amounts of a cathepsin B-like protease found in the more metastatic B16 melanoma cell lines (5) and the observation that cathepsin B can activate latent collagenase and degrade the proteoglycans of the basement membrane (5). However, it may be that other factors are rate determining in the extravasation and lung colonization process rather than the activity of a cathepsin B-like protease.

Our result showing a lack of inhibition of mouse lung colonization by leupeptin is in disagreement with the earlier results of Saito *et al.* (16) which found leupeptin significantly inhibited by 50% rat lung colonization by Yoshida ascites hepatoma cells. In the work of Saito *et al.*, 50-mg/kg i.p. injections of leupeptin were made every 12 h, in a protocol identical to that used in Table 2 of this work. As discussed previously, we appeared to also observe an average decrease in lung colonization by the injection protocol because in all experiments the leupeptin treated animals averaged fewer metastases, but in only Experiment 1 (Table 2) did the result approach statistical significance. The determination of the *in vivo* decrease of leupeptin after a 50-mg/kg i.p. injection showed that the concentration of leupeptin was varying by at least 2 orders of magnitude during the first 4 h (from 5×10^{-5} to 2×10^{-7} M in plasma), to amounts below our limits of detection after 6 h. It may be that the extremely high concentration of leupeptin initially present inhibited additional enzymes other than cathepsin B, which caused the inhibition of experimental metastasis observed by Saito *et al.* Accordingly variations in the plasma leupeptin concentrations during the injection protocol, the differences between the animal species utilized, and/or differences in the mechanism of metastasis between the two different tumor cell lines utilized could explain the statistical differences observed between our work and that of Saito *et al.* However, our result is in agreement with the findings of Giraldi *et al.* (22) that leupeptin had no effect on the spontaneous metastasis of Lewis lung carcinoma. In this work the minipump diffusion protocol achieves a constant steady state concentration of leupeptin orders of magnitude higher than its K_i towards cathepsin B yet lower than the K_i of other targeted proteases and is thus a more direct experiment. Our experiments thus specifically appear to show the nonessentiality of cathepsin B-like proteases to the B16 colonization process. The experiments with Ep453 confirm this conclusion for B16 colonization.

The results with R-Glu–Gly–argininal inhibitors are less clear-cut, because *in vivo* inhibitor concentrations significantly greater than the equilibrium binding constant (K_i) to urokinase were not achievable by the osmotic pump method. However, the plasminogen activator urokinase should be significantly inhibited by the large amounts of inhibitor introduced by the injection protocols, and the lack of a decrease in experimental metastasis indicates a nonessential role in lung colonization for urokinase PA. These results may be in agreement with the hypothesis that PA is essential in the early stages of a spontaneous metastasis, especially Stage *b* in which the tumor cells must penetrate through a fibrin matrix surrounding the primary neoplasm, but not in the later stages (Steps *c* and *d*) (3, 8, 23). In the later stages, a current hypothesis argues that coagulation and the formation of microthrombi acts to promote metastasis (3, 8, 23). Thus the result showing a possible promotion of experimental metastasis with H-Glu–Gly–argininal is in agreement with the hypothesis that argues that a decrease in PA activity will increase the concentration of microthrombi (3, 8, 23). Ossowski and Reich (24) have shown that an antibody to human urokinase decreases metastasis of human HEp3 epidermal carcinoma cells placed on a chick embryo chorioallantoic membrane to the lung of the chick embryo. These authors argue that the effect of the anti-urokinase antibody on the inhibition of metastasis is due to an intervention of the inhibitor at an early stage of the metastatic process (Steps *a* or *b*).

Thrombin activation will also increase microthrombus formations and thus by the current hypothesis (discussed above) should promote metastasis in the later stages (Steps *c* and *d*). Evidence in support of a role of thrombus formation in the promotion of metastasis comes from pictures showing the formation of thrombi with tumor cells in the capillaries of target organs of metastasis (8, 16), the observation that many tumor cells contain thrombolytic factors (8), and the sometimes observed inhibition of metastasis in animals receiving heparin or warfarin (8). However, there are contrary reports on the effect of heparin and warfarin (25–27). In addition both heparin and warfarin have a large number of *in vivo* effects other than the selective inhibition of thrombin (8), making interpretations from their inhibition data mechanistically not as clear-cut as for a reagent acting uniquely on a particular enzyme. For example, in certain combinations, heparin can either promote or facilitate the inhibition of angiogenesis (28). Warfarin in turn inhibits the synthesis of a large number of both thrombolytic and fibrinolytic proteases and by decreasing γ-carboxyglutamate formation also decreases calcium binding to a wide variety of γ-carboxyglutamate calcium binding sites (29).

Our result is that a direct thrombin inhibitor, H-D-Phe–Pro–argininal, dramatically promoted B16 melanoma cell metastasis. It may be that fibrin formation actually inhibits metastasis and is a part of the vascular system's defense for preventing cells from migrating from the vascular system into specific organs. Encircling the tumor cells within a thrombus will physically act to prevent migration of cells from the vascular system, giving scavanger systems an opportunity to respond. By this rationale, the reason for the observation of large amounts of thrombi with tumor cell metastasis is that under the experimental conditions a large number of cells are attempting extravasation and a correspondingly high activity of a defensive mechanism acting against the extravasation process is observed. The clot probably must be degraded before the tumor cell can leave the vascular system, and this is a delaying effect giving other systems time to act against the tumor cells.

Supporting a hypothesis that thrombin inhibits metastasis is the recent report that thrombin directly stimulates the synthesis and secretion of collagenase inhibitor from platelets (30). Collagenase has been clearly shown to be essential to the invasion of cells through the basement membrane (7) and thus should be essential to the extravasation process (Step *d*). The inhibition

of collagenase inhibitor secretion by the inhibition of thrombin could then directly lead to a promotion of a high collagenase activity and correspondingly high rate of lung invasion by the tumor cells, as observed.

In addition, recent evidence has shown that purified thrombin initiates cell proliferation by itself or in combination with epidermal growth factor or platelet derived growth factor, through its stimulation of phosphoinositide turnover (31). Furthermore, thrombin can activate fibrinolysis when associated with thrombomodulin receptors on the endothelial cells of the microcapillaries (32, 33). Thus the effect of our thrombin inhibitor may be to inhibit fibrinolysis in the microcapillaries, in agreement with the current hypothesis that microthrombus formation promotes extravasation (8). These actions of thrombin point to the complex roles of thrombin in hemostasis, any one of which may be crucial to the process of extravasation and invasion.

In other work Persky *et al.* (34) have shown that peptidyl aldehyde serine and cysteine protease inhibitors do not decrease the invasion of the B16 cells through a human amniotic membrane. The amniotic membrane serves as a model for the study of basement membrane invasion, and a collagenase inhibitor does significantly inhibit the penetration of tumor cells across the membrane (7). The amnion data support the hypothesis that serine and cysteine proteases may not be essential in extravasation (Step *d*).

The extracellular protease activities observed to be secreted in high concentrations from tumor cells by prior workers (1–6), which are inhibited in our model, may simply represent an excess of enzyme shed from the plasma membrane of the tumor cells (4, 5). The site of proteolytic actions in invasion processes may be not in extracellular space with secreted proteases but on the plasma membrane of the invading cell. In such a mechanism, the "effective concentration" of substrates in a membrane associated protease active site will be extremely high relative to that of inhibitor free in solution. The situation is analogous to the increase in effective concentration of reactants observed when a bimolecular reaction is converted to a unimolecular reaction (35). For example, experiments show that tying together a catalytic site and substrate prior to the rate determining catalytic step will increase the effective concentrations of substrate within the catalytic site by factors as high as 10^8 M (35). If this process of substrate binding prior to catalysis is occurring during tumor cell migration, the inhibitors (which act competitively), even at 3 orders of magnitude higher solution concentration than their equilibrium K_i values determined against the non-membrane bound forms of the enzymes, may not be able to compete against the effective active site concentration of the substrate. In support of the possible importance of membrane associated proteases over secreted protease activities, a PA enzymatic activity, a cathepsin B-like activity, and general trypsin-like protease activities have been reported in high relative concentrations in the plasma membranes of transformed cells (36–40). Campbell *et al.* (41) have shown that elastase is not inhibitable by elastase inhibitors after the elastase has bound to a protein substrate. Nicolson *et al.* (42) have shown that basement membrane is not degraded by the cell culture media of B16 melanoma cells but is degraded only when direct cell contact is made with the basement membrane. It has recently been shown that the appearance of a tumor plasma membrane protease activity is a direct expression of the $pp60^{src}$ oncogene product (43). Accordingly, our proteolytic enzyme inhibitors may not show an inhibition of metastasis due to an inability by the free inhibitors to inhibit proteolytic activities on the cell surface which also binds substrate tightly.

In conclusion, we have shown that selective inhibitors of cathepsin B-like proteases at steady state *in vivo* concentrations significantly higher than the enzyme inhibitor K_i values failed to inhibit B16 melanoma cell colonization of mouse lung. A selective urokinase inhibitor also had no significant effect on lung colonization. A selective thrombin inhibitor present at steady state concentrations specific for thrombin inhibition had a dramatic effect in increasing the number of B16 metastatic foci in the lung. The results point to the need to further investigate the role of thrombin and thrombus formation in the later stages of metastasis, the possible relationship between thrombin activity in other processes than coagulation, as well as the possible importance of tumor cell membrane associated protease activities in tumor cell metastasis. The evidence strongly indicates that secreted forms of cathepsin B-like proteases do not have a role in B16 melanoma cell lung colonization. The role of proteases in the initial steps of metastasis (Steps *a* and *b*) were not tested in this model for lung colonization and also should be further explored.

REFERENCES

1. Liotta, L. A., and Hart, I. R. (eds.). Tumor Invasion and Metastasis. The Hague: Martinus Nijhoff, 1982.
2. Strauli, P., Barrett, A. J., and Baici, A. (eds.). Proteinases and Tumor Invasion. New York: Raven Press, 1980.
3. Dano, K., Andreasen, P. A., Grondahl-Hansen, J., Kristensen, P., Nielsen, L. S., and Skriver, L. Plasminogen activators, tissue degradation, and cancer. Adv. Cancer Res., *44:* 139–266, 1985.
4. Quigley, J. P. Proteolytic enzymes of normal and malignant cells. *In:* R. O. Hynes (ed.), Surfaces of Normal and Malignant Cells, pp. 247–285. New York: John Wiley & Sons, Inc., 1979.
5. Sloane, B. F., and Honn, K. V. Cysteine proteinases and metastasis. Cancer Metastasis Rev., *3:* 249–263, 1984.
6. Recklies, A. D., Tiltman, K. J., Stoker, T. A. M., and Poole, A. R. Secretion of proteinases from malignant and nonmalignant human breast tissue. Cancer Res., *40:* 550–556, 1980.
7. Thorgeirsson, U. P., Liotta, L. A., Kalebic, T., Margulies, I. M., Thomas, K., Rios-Candelore, M., and Russo, R. G. Effect of natural protease inhibitors and a chemoattractant on tumor cell invasion *in vitro*. J. Natl. Cancer Inst., *69:* 1049–1054, 1982.
8. Markus, G. The role of hemostasis and fibrinolysis in the metastatic spread of cancer. Semin. Thromb. Hemostasis, *10:* 61–70, 1984.
9. Kennedy, W. P., and Schultz, R. M. Mechanism of association of a specific aldehyde transition-state analogue to the active site of α-chymotrypsin. Biochemistry, *18:* 349–356, 1979.
10. Frankfater, A., and Kuppy, T. Mechanism of association of *N*-acetyl-L-phenylalanyl-glycinal to papain. Biochemistry, *20:* 5517–5524, 1981.
11. Patel, A. H., Ahsan, A., Suthar, B. P., and Schultz, R. M. Transition-state affinity chromatography of trypsin-like proteinases with dipeptidyl argininal ligands. Biochim. Biophys. Acta, *748:* 321–330, 1983.
12. Hanada, K., Tamai, M., Adachi, T., Oguma, K., Kashiwagi, K., Ohmura, S., Kominami, E., Towateri, T., and Katunuma, N. Characterization of the three new analogs of E-64 and their therapeutic application. *In:* N. Katunuma, H. Umezawa, and H. Holzer (eds.), Proteinase Inhibitors, Medical and Biological Aspects, pp. 25–36. Tokyo: Japan Scientific Societies Press, 1983.
13. Bajusz, S., Barabas, E., Tolnay, P., Szell, E., and Bagdy, D. Inhibition of thrombin and trypsin by tripeptide aldehydes. Int. J. Peptide Protein Res., *12:* 217–221, 1978.
14. Fidler, I. J. General considerations for studies of experimental cancer metastasis. Methods Cancer Res., *15:* 399–439, 1978.
15. Svendsen, L. G., Fareed, J., Walenga, J. M., and Hoppensteadt, N. Newer synthetic peptide substrates in coagulation testing: some practical considerations for automated methods. Semin. Thromb. Hemostasis, *9:* 250–262, 1983.
16. Saito, D., Sawamura, M., Umezawa, K., Kanai, Y., Furihata, C., Matsushima, T., and Sugimura, T. Inhibition of experimental blood-borne lung metastasis by protease inhibitors. Cancer Res., *40:* 2539–2542, 1980.
17. Tanaka, W. Absorption, distribution, metabolism, and excretion of leupeptin. *In:* N. Katunuma, H. Umezawa, and H. Holzer (eds.), Proteinase Inhibitors, Medical and Biological Aspects, pp. 17–24. Tokyo: Japan Scientific Societies Press, 1983.
18. Sher, J. H., Stracher, A., Shafiq, S. A., and Hardy-Stashin, J. Successful treatment of murine muscular dystrophy with the proteinase inhibitor leupeptin. Proc. Natl. Acad. Sci. USA, *78:* 7742–7744, 1981.
19. Aoyagi, T., Wada, T., Umezawa, K., Kojima, F., Nagai, M., and Umezawa, H. Relation between *in vivo* effects and *in vitro* effects of serine and thiol proteinase inhibitors. J. Pharmacobio-dyn., *6:* 643–653, 1983.

20. Sutherland, J. H. R., and Greenbaum, L. M. Paradoxical effect of leupeptin *in vivo* on cathepsin B activity. Biochem. Biophys. Res. Commun., *110:* 332–338, 1983.
21. Coleman, P., Kettner, C., and Shaw, E. Inactivation of the plasminogen activator from HeLa cells by peptides of arginine chloromethyl ketone. Biochim. Biophys. Acta, *569:* 41–51, 1979.
22. Giraldi, T., Nisi, C., and Sava, G. Lysosomal enzyme inhibitors and antimetastatic activity. Eur. J. Cancer, *13:* 1321–1323, 1977.
23. Columbi, M., Barlati, S., Magdelenat, H., and Fiszer-Szafarz, B. Relationship between multiple forms of plasminogen activator in human breast tumors and plasma and the presence of metastases in lymph nodes. Cancer Res., *44:* 2971–2975, 1984.
24. Ossowski, L., and Reich, E. Antibodies to plasminogen activator inhibit human tumor metastasis. Cell, *35:* 611–619, 1983.
25. Gasic, G. J. Role of plasma, platelets, and endothelial cells in tumor metastasis. Cancer Metastasis Rev., *3:* 99–116, 1984.
26. Annegers, J. F., and Zacharski, L. R. Cancer morbidity and mortality in previously anticoagulated patients. Thromb. Res., *18:* 399–403, 1980.
27. Lorenzet, R., Bottazzi, B., Locati, D., Colucci, M., Mantovani, A., Semeraro, N., and Donati, M. B. Failure of warfarin to affect the tissue factor activity and the metastatic potential of murine fibrosarcoma cells. Eur. J. Cancer Clin. Oncol., *21:* 263–265, 1985.
28. Folkman, J. Tumor angiogenesis. Adv. Cancer Res., *43:* 175–203, 1985.
29. Hilgard, P. Cancer and vitamin K. Lancet, *2:* 403, 1977.
30. Cooper, T. W., Eisen, A. Z., Stricklin, G. P., and Welgus, H. G. Platelet-derived collagenase inhibitor: characterization and subcellular localization. Proc. Natl. Acad. Sci. USA, *82:* 2779–2783, 1985.
31. Carney, D. H., Scott, D. L., Gordon, E. A., and LaBelle, E. F. Phosphoinositides in mitogenesis: neomycin inhibits thrombin-stimulated phosphoinositide turnover and initiation of cell proliferation. *Cell, 42:* 479–488, 1985.
32. Esmon, N. L., Owen, W. G., and Esmon, C. T. Isolation of a membrane-bound cofactor for thrombin-catalyzed activation of protein C. J. Biol. Chem., *257:* 859–864, 1982.
33. Sakata, Y., Curriden, S., Lawrence, D., Griffin, J. H., and Loskutoff, D. J. Activated protein C stimulates the fibrinoytic activity of cultured endothelial cells and decreases antiactivator activity. Proc. Natl. Acad. Sci. USA, *82:* 1121–1125, 1985.
34. Persky, B., Ostrowski, L. E., Pagast, P., Ahsan, A., and Schultz, R. M. Inhibition of proteolytic enzymes in the *in vitro* amnion model for basement membrane invasion. Cancer Res., *46:* 4129–4134, 1986.
35. Jencks, W. P. Binding energies, specificity and enzymatic catalysis: the Circe effect. Adv. Enzymol., *43:* 219–410, 1975.
36. DiStefano, J. F., Beck, G., Lane, B., and Zucker, S. Role of tumor cell membrane-bound serine proteases in tumor-induced cytolysis. Cancer Res., *42:* 207–218, 1982.
37. Fraser, J. D., and Scott, G. K. Membrane proteinases from normal and neoplastic tissues in man and rat. Comp. Biochem. Physiol., *79B:* 105–111, 1984.
38. Pietras, R. J., and Roberts, J. A. Cathepsin B-like enzymes, subcellular distribution and properties in neoplastic and control cells from human ectocervix. J. Biol. Chem., *256:* 8536–8544, 1981.
39. Sloane, B. F., Rozhin, J., Johnson, K., Taylor, H., Crissman, J. D., and Honn, K. V. Cathepsin B: association with plasma membrane in metastatic tumors. Proc. Natl. Acad. Sci. USA, *83:* 2483–2487, 1986.
40. Fairbairn, S., Gilbert, R., Ojakian, G., Schwimmer, R., and Quigley, J. P. The extracellular matrix of normal chick embryo fibroblasts: its effects on transformed chick fibroblasts and its proteolytic degradation by the transformants. J. Cell Biol., *101:* 1790–1798, 1985.
41. Campbell, E. J., Senior, R. M., McDonald, J. A., and Cox, D. L. Proteolysis by neutrophils. Relative importance of cell-substrate contacts and oxidative inactivation of proteinase inhibitors *in vitro*. J. Clin. Invest., *70:* 845–852, 1982.
42. Kramer, R. H., Vogel, K. G., and Nicolson, G. L. Solubilization and degradation of subendothelial matrix glycoproteins and proteoglycans by metastatic tumor cells. J. Biol. Chem., *257:* 2678–2686, 1982.
43. Chen, W. T., Chen, J. M., Parsons, S. J., and Parsons, J. T. Local degradation of fibronectin at sites of expression of the transforming gene product pp60^{src}. Nature (Lond.), 316: 156–158, 1985.