in vivo *8:* 231- 236 (1994)

Effects of E-64 (Cysteine - Proteinase Inhibitor) and Pepstatin (Aspartyl-Proteinase Inhibitor) on Metastasis Formation in Mice with Mammary and Ovarian Tumors

G. LETO, G. PIZZOLANTI, F.M. TUMMINELLO and N. GEBBIA

[1]*Institute of Pharmacology, Chair of Chemotherapy, School of Medicine, University of Palermo, Policlinico «P. Giaccone» 90127 Palermo, Italy*

Abstract. *The effects of E-64 (Cathespin B and L inhibitor) and Pepstatin A (Cathepsin D inhibitor) on spontaneous and experimental metastasis formation were investigated in mice with MCa mammary carcinoma, M5076 ovarian sarcoma and L1210 leukemia. Pepstatin induced a marked decrease in the number of* spontaneous *metastasis in MCa or M5076 tumor bearing mice. This phenomenon was also noted with E-64 but only in M5076 tumor bearing mice. On the other hand, both these agents were unable to prevent the formation of* experimental *metastasis in mice injected i.v. with L1210, MCa or M5076 tumor cells or with tumor cells in which Cathepsin B, L and D activities were inhibited by a 24 hour continuous exposure to high non-cytotoxic concentrations of E-64 and / or Pepstatin. These data suggest that Cathepsin B, L and D seem to be involved in the early steps of the metastatic process rather than in the hematogenous spread of tumor cells. However, other pharmacological activities which may account for the discrepant effects of E-64 or Pepsatin on experimental and spontaneous metastasis cannot be ruled out.*

Several experimental and clinical studies suggest that lysosomal proteinases Cathepsin B, L and D may be involved in the malignant progression of some human neoplastic diseases such as breast and ovarian tumors (1-3). Therefore specific inhibitors of these enzymes may provide a tool to assess better their pathophysiological role in tumor progression as well as their value as potential therapeutic agents. In this context we have undertaken a number of *in vivo* and *in vitro* investigations to evaluate, in some experimental murine tumor models, the therapeutic potential of E-64, inhibitor of cystein proteinases Cathepsin B and L (4), and that of Pepstatin, specific inhibitor of the aspartic proteinase Cathepsin D (5). Parallel studies were also carried out with these inhibitors to investigate the role of Cathepsin B, L and D in the hematogenous spread of tumor cells.

Correspondence to: Dr G. Leto.

Key Words: Cathepsins, E-64, metastasis, pepstatin, proteinase inhibitors.

0258-851X/94 $200+.40

Materials and Methods

E-64 (trans - epoxysuccinyl-L-leucylamido (4-guanidino)-butane, Pepsatin A, cell culture reagents and chemicals were purchased from Sigma, St. Louis, MO, USA.

Animals, tumor and tumor cell lines. L1210 leukemia, MCa mammary carcinoma (MCa) and M5076 ovarian reticulum cell carcinoma (M5) were mantained *in vivo* in syngenic DBA, C57BL6 and CBA female mice (Charles River, Calco, Italy) (6,7). L1210 leukemia and M5 tumor cells, obtained from ascitic fluid or by enzymatic disaggregation from primary tumors were also grown *in vitro* as described (6). MCa tumor cells, obtained by mechanical disaggregation from primary tumors, were grown in suspension in DMEM culture medium supplemented with 10% heat inactivated foetal bovin serum, 2 mM glutamine, 1 mM sodium pyruvate and gentamicin (50 mcg/ml). Cells were subcultured 1:1 once a week.

In vitro studies.
Growth inhibitory studies with E-64 and Pepsatin: 1×10^5 L1210, MCa or M5 tumor cells/ml were seeded in 24-well plates (Falcon 3047) on day 0 and further incubated for 24 hours in presence of different concentrations of E-64 or Pepstatin (1×10^{-8} up to 1×10^{-4} M). At the end of the incubation period cells were harvested and counted with a hemocytometer. Cell viability was checked by Trypan blue dye exclusion. To assess whether these agents enter tumor cells and affect the intracellular activity of Cathepsin B,L and D, $2\text{-}5\times10^5$ tumor cells/ml were seeded in 25 cm^2 culture flasks (Falcon 3013) on day 0 and further exposed for 24 hours in presence of non-cytotoxic concentrations of E-64 or Pepstatin (1×10^{-7} up to 1×10^{-5} M). At the end of the incubation period tumor cells were harvested, washed 3 times in cold PBS, and their concentration adjusted to 1×10^6 cells/ml with cold redistilled water containing Triton X-100 (0.2% w/v). Tumor cells were then sonicated at 4° C (Bronson Sonifer 450), at the highest power with two bursts of 30 seconds and an intercooling period of 1 minute, and centrifuged at 3500 rpm for 15 minutes. The supernatant was assayed for Cathepsin B, L and D activities according to Barrett and Kirschke (8) or as described (9), and for protein content according to Bradford (10).

In vivo studies. The dose and schedule of Pepsatin administration were chosen on the basis of previous studies which showed that this agent induces a long-lasting inhibition of Cathepsin D activity in several organ tissues, including target organs for metastatic cells, in both normal and tumor bearing mice (9, 11). Furthermore since *in vivo* studies on the organ toxicity of E-64 are, to our knowledge, currently lacking, this agent was administered in a range of doses usually applied in other studies for experimental or therapeutic purposes (12-14).

Studies with L1210 leukemia. Groups of 10 DBA mice, 6-8 weeks old, were inoculated i.p. or i.v. with 1×10^5 viable L1210 leukemic cells. E-64

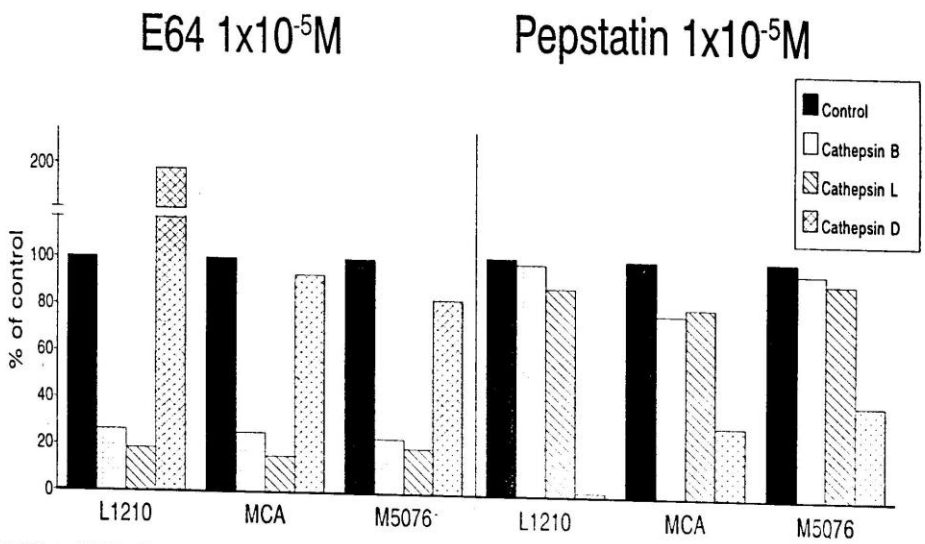

Figure 1. *Effects of E-64 or Pepstatin pretreatment on the intracellular activities of Cathepsin B, L and D in L1210, MCa and M5076 tumor cells. 2-5 × 10^5 tumor cells/ml were exposed for 24 hours to high non-cytotoxic concentrations (1 × 10^{-5} M) of E-64 or Pepstatin. Tumor cells were then harvested, sonicated and centrifuged at 3500 rpm for 15 minutes. Cathepsin B, L and D activities and protein content were determined in the 3500 supernatant as described (8-10). Results are expressed as % of the specific enzyme activities in control samples taken as 100 and are the mean of 3 separate determinations. Standard deviation was less than 15%. Data analysis was computed by Student's «t» test for unpaired samples.*

and/or Pepstatin (50 mg/kg i.p.) were given on days 0,2,4 (L1210 i.p.) or 0,3,5 (L1210 i.v.). The antitumor activity was evaluated according to the National Cancer Institute protocols as described (6,15). In additional experiments, groups of DBA mice were inoculated i.v. with 1×10^5 viable L1210 tumor cells in which Cathepsin activities were markedly inhibited by a 24 hour continous exposure to high non-cytotoxic concentrations of E-64 and Pepstatin. The effects of this pretreatment on tumor cell spreading was evaluated by daily checking of the survival time of mice (15).

Studies with solid tumors. 5×10^5 MCa or M5 viable tumor cells were inoculated i.m. into 6-8 weeks old CBA or C57BL6 female mice. E-64 or Pepstatin (35-50 mg/kg i.p.) were given on days 0,2,4 (E-64) or days 0,3 and 5 (Pepstatin). The effects of these inhibitors on primary tumor growth and spontaneous metastasis formation were evaluated as previously described in detail (6).

Effects of E-64 and Pepstatin on experimental metastasis formation. CBA or C57BL6 mice were injected i.v. with 5×10^4 (MCa) or 2×10^4 (M5) tumor cells. E-64 or Pepstatin (25-35mg/kg i.p.) were given every other day up to day 6 after tumor cell injection. In parallel running experiments, groups of CBA or C57BL6 mice were injected i.v. with 5×10^4 (MCa) or 2×10^4 (M5) viable tumor cells previously exposed for 24 hours to high non cytotoxic concentrations of these agents. Mice were sacrificed on day 19 (MCa) or day 29 (M5) after tumor implant. The number of experimental liver (M5) and lung (MCa) metastasis was counted with a dissection microscope (6).

Statistical analysis. Data analysis, where required, was computed by the Mann-Whitney U test or Student «t» test for unpaired samples assuming the 0.05 level of probability as significant.

Results

In vitro studies

Preliminary *in vitro* studies showed no growth inhibitory effects of E-64 or Pestatin on L1210, MCa and M5 tumor cells in a range of 1×10^{-8} up to 1×10^{-5} M concentrations. Cytotoxic effects (20-40% growth inhibition) were noted with both these agents at concentrations higher than 5×10^{-5} M (Pepstatin), and 10×10^{-4} M (E-64). These studies also showed that, following a 24 hour continous exposure to high non cytotoxic concentrations of E-64 or Pepstatin, these agents enter tumor cells and induce a marked decrease in the intracellular activity of Cathepsin B, D and L (Figure 1). This phenomenon occured to a lesser extent with Pepstatin in M5 tumor cells. Parallel experiments showed that at lower concentrations (10^{-6} - 10^{-7}M) E-64 paradoxically induced an increase of the intracellular activity of Cathepsin B and L as well that of Cathepsin D. Similar effects also occurred with lower concentrations of Pepstatin (1×10^{-8}) but only Cathepsin B and L were affected in this case (data not shown).

In vivo studies

Studies with L1210 leukemia. In L1210 leukemia bearing mice

Table I. *Therapeutic evaluation of E-64 and/or Pepstatin in mice transplanted i.p. with L1210 leukemia*[a].

	Days of treatment	Survival parameters			
		MST[b]	Mean±S.E.	T/C[c]	ABWD5[d]
Control	0,2,4	8.5 (8-9)	8.5±0.5	1.00	+8.8
E64 (50mg/kg i.p.)	0,2,4	9 (7-10)	8.6±0.8	1.06	+10.2
Pepstatin A (50 mg/kg i.p.)	0,2,4	9 (8-9)	8.7±0.5	1.06	+14.3
E64 (50 mg/kg i.p.) + Pepstatin (50 mg/kg i.p.)	0,2,4	9 (8-10)	8.9±0.6	1.06	+1.3

(a) Group of 10 DBA/2 female mice were transplanted i.p. with 1×10^5 viable L1210 leukemic cells on day 0.
(b) MST: Median Survival Time.
(c) T/C: Ratio MST treated mice / MST control mice.
(d) ABWD5: Average body weight variation (%) on day 5 after tumor implant.

Table II. *Therapeutic evaluation of E-64 and/or Pepstatin in mice transplanted i.v. with L1210 leukemia*[a].

	Days of treatment	Survival parameters			
		MST[b]	Mean±S.E.	T/C[c]	ABWD5[d]
Control	0,3,5	7 (7)	7.0±0.0	1.00	+5.4
E64 (50mg/kg i.p.)	0,3,7	7 (7)	6.9±0.3	0.98	-3.5
Pepstatin A (50 mg/kg i.p.)	0,3,5	7.5 (7-8)	7.5±0.5	1.07	+5.1
E64 (50 mg/kg i.p.) + Pepstatin (50 mg/kg i.p.)	0,3,5	7 (7)	7.0±0.0	1.00	-7.6

(a) Group of 10 DBA/2 female mice were transplanted i.p. with 1×10^5 viable L1210 leukemic cells on day 0.
(b) MST: Median Survival Time.
(c) T/C: Ratio MST treated mice / MST control mice.
(d) ABWD5: Average body weight variation (%) on day 5 after tumor implant.

the administration of E-64 or Pepstatin did not result in any antitumor effects, as assessed by the daily checking of the survival time of animals (Table I). Similar results were obtained in mice injected i.v. with L1210 tumor cells or with L1210 tumor cells in which the intracellular activity of Cathepsins were previously inhibited by a 24 hour continuous exposure to high non-cytotoxic concentrations of these agents (Figure 1, Table II and III). No drug-related deaths or evident toxicity, as indicated by body weight variations on day 5 after tumor implant (15), were noted in any group (Tables I and II).

Studies with solid tumors. In mice with MCa mammary tumors the administration of E-64 did not affect either primary tumor growth (T/C 1.1) or spontaneous metastasis formation (-8.7%) (Table IV). Pepstatin was also devoid of

Table III. *Therapeutic evaluation of E-64 and/or Pepstatin in mice transplanted i.v. with 1×10^5 viable L1210 leukemic cell preincubated for 24 h in the presence of these agents.*

	Survival parameters		
	MST[a] (range)	Mean±S.E.	T/C
Control	6 (6-11)	6.8±1.5	1.00
E64 (1×10^{-5}M)	7 (7)	7.0±0.01	1.16
Pepstatin A (1×10^{-5}M)	7 (7-8)	7.5±0.3	1.18
E64 (1×10^{-5}M) + Pepstatin A (1×10^{-5}M)	7 (7-8)	6.9±0.7	1.15

(a) MST: Median Survival Time
(b) T/C: Ratio MST treated mice / MST control mice

Table IV. *Evaluation of antitumor and antimetastatic activity of E-64 and Pepstatin in mice with MCa mammary tumor[a].*

	Days of treatment	Tumor[b] volumes (grams)	T/C[c]	Number of lung metastases[d]		
				Median (range)	% of control	Incidence
Control	0,2,4,6	1.7±0.1	1.00	163.5 (80-205)	100	8/8
Pepstatin (35 mg/kg i.p.)	0,2,4,6	2.0±0.1	1.2	105* (16-181)	64.4	9/9
E64 (35 mg/kg i.p.)	0,2,4,6	1.85±0.1	1.1	149 (15-182)	91.3	9/9

(a) CBA female mice were transplanted i.m. with 5×10^5 viable MCa tumor cells on day 0.
(b) Tumor volume = Π/6×A×B×C, where A = long axis, B = short axis, C = width. Results refer to the measurement made on day 21 (day of sacrifice) and are expressed as mean ±S.E.
(c) T/C = ratio tumor volume treated mice / tumor volume control mice.
(d) Animals were sacrificed on day 21. Metastases were counted in duplicate by a dissection microscope.
* $P<0.05$ versus control (Mann - Whitney U test).

Table V. *Evaluation of antitumor and antimetastatic activity of E-64 and Pepstatin in mice with M5076 ovarian reticulum sarcoma[a].*

	Days of treatment	Tumor[b] volumes (grams)	T/C[c]	Number of hepatic nodules[d]		
				Median (range)	% of control	Incidence
Control	0,7	4.7±0.4	1.00	91 (3-590)	100	14/14
Pepstatin A (50 mg/kg i.p.)	0,7	4.8±0.7	1.02	37* (4-538)	40.6	13/13
E64 (50 mg/kg i.p.)	0,3,5	5.1±0.2	1.08	48 (8-508)	52.7	10/10

(a) C5BL/6J female mice were transplanted i.m. with 5×10^5 tumor cells on day 0.
(b) Tumor volume = Π/6×A×B×C, where A = long axis, B = short axis, C = width. Results refer to the measurement made on day 33 (day of sacrifice) and are expressed as mean±S.E.
(c) T/C = Ratio tumor volume treated mice / tumor volume control mice.
(d) Animals were sacrificed on day 33. Metastases were counted in duplicate by a dissection microscope.
* $P<0.05$ versus control (Mann-Whitney U test).

antitumor activity (T/C=1.2); however this agent induced a significant decrease of spontaneous lung metastasis (-35.6%). Similar results on primary tumor growth were obtained in mice with M5076 ovarian sarcoma (T/C=1.02 and 1.08) (Table V). Although in mice with this tumor both these agents induced a marked decrease in the formation of spontaneous liver metastases (-59.4% with Pepstatin and -48.3% with E64), the inhibiting effects of the E-64 were not statistically singificant. In mice injected i.v. with MCa or M5076 tumor cells or with MCa or M5 tumor cells preincubated with high non-cytotoxic concentrations of E-64 and Pepstatin both of these agents were not able to prevent or decrease the formation of experimental liver (M5) or lung (MCa) metastasis (Tables VI and VII).

Discussion

The present study shows a different pattern of therapeutic response of L1210 leukemia, MCa mammary carcinoma and M5076 ovarian sarcoma to E-64 and/or Pepstatin. L1210 leukemia as well MCa and M5 tumors were unresponsive to these agents either *in vivo* or *in vitro;* however, in MCa and M5 tumor bearing mice a significant decrease in the number of spontaneous lung (MCa) and liver (M5) metastases was induced by Pepstatin. These data further confirm our previous observations which showed similar effects also in mice with Lewis Lung Carcinoma (6). The antimetastatic activity of Pepstatin noted in MCa or M5 tumor bearing mice does not seem to be related to direct cytotoxic effects on tumor cells, as *in vitro* studies showed no growth inhibitory activity of this agent even at concentrations higher than that achieved in the blood of mice (1.1×10^{-5}M) following i.p. administration of 50 mg/kg of Pepstatin (5,6). Thus, conceivably, the inhibition of Cathepsin D activity induced by Pepstatin at tumor cell or at host tissue levels may account for the inhibiting effects on spontaneous metastasis observed in MCa or M5 tumor bearing mice. On the other hand, Pepstatin failed to increase the life span in mice injected i.v. with L1210

Table VI. *Evaluation of the effects of E-64 or Pepstatin on experimental metastases formation in MCa tumor bearing mice*[a].

	Days of treatment	Number of lung metastases[b]			
		Median (range)	% of control	Incidence	P[d]
Control	0,2,4,6	74 (0->200)	100	7/8	
E64 (25 mg/kg i.p.)	0,2,4,6	87.5 (1-120)	118.2	8/8	NS
E64 (2.8×10^{-5} M)[c]		71.5 (26-106)	96.6	8/8	NS
E64 (1×10^{-6} M)[c]		68.5 (0-118)	92.6	7/8	NS
Pepstatin (35 mg/kg i.p.)	0,2,4,6	78 (0->200)	105.4	7/8	NS
Pepstatin (1.1×10^{-5} M)[c]		117 (7-135)	158.1	8/8	NS

(a) CBA female mice were transplanted i.v. with 5×10^4 viable MCa tumor cells on day 0.
(b) Mice were sacrificed on day 19. Lung metastases were counted by a dissection microscope.
(c) Cells were incubated up to 24 hours in the presence of proteinase inhibitors.
(d) NS = not statistically significant (Mann - Whitney U test).

Table VII. *Evaluation of the effects of E-64 or Pepstatin on experimental liver metastasis formation in M5076 tumor bearing mice*[a].

	Days of treatment	Number of liver metastases[b]			
		Median	% of control	Incidence	P[d]
Control	0,2,4,6	81 (52-145)	100	10/10	
E64 (25 mg/kg i.p.)	0,2,4,6	118 (69->200)	145.6	10/10	NS
E64 (2.8×10^{-5} M)[c]		71 (16-106)	87.6	9/10	NS
E64 (1×10^{-6} M)[c]		68.5 (0-118)	84.6	9/10	NS
Pepstatin (35 mg/kg i.p.)	0,3,5	102 (0->200)	125.9	9/10	NS
Pepstatin (1.1×10^{-5} M)[c]		70 (1-142)	86.4	10/10	NS

(a) C57BL6 female mice transplanted i.v. with 2×10^4 viable tumor cells.
(b) Mice were sacrificed on day 29. Liver metastasses were counted by a dissection microscope.
(c) M5076 tumor cells were incubated up to 24 hours in presence of proteinase inhibitors.
(d) NS = not statistically significant (Mann - Whitney U test).

leukemic cells or to prevent or decrease the formation of experimental metastases in mice transplanted i.v. with MCa or M5 tumor cells. Similar results were obtained following i.v. injection of tumor cells in which the intracellular activity of Cathepsin D as well that of Cathepsin B and L were markebly inhibited by a 24 hour continuous exposure to high non-cytotoxic concentrations of Pepstatin and E-64. These data suggest that Cathepsin D does not seem to be involved in the hematogeneous spread of tumor cells. The effects of Pepstatin on spontaneous metastasis, on the other hand, indicate that Cathepsin D may rather be involved in an early step of the metastatic process. This hypothesis is supported by a number of recent studies which showed that Cathepsin D may promote tumor progression by activating latent forms of growth factors or other proteinases involved in the metastatic cascade (16,17). The results obtained following E-64 administration in mice injected i.v. with L1210, MCa or M5 tumor cells or with tumor cells exposed for 24 hours to high non-cytotoxic concentrations of this inhibitor were similar to those obtained with Pepstatin. The lack of inhibiting effects of cysteine proteinase inhibitors on experimental metastasis have already been reported in B16 melanoma bearing mice administered with E-64 analogs or other specific cysteine proteinase inhibitors such as Leupeptin (18,19). Thus these data also seem to rule out a direct involvement of Cathepsin B and L in the hematogeneous spread of tumor cells.

On the other hand, the statistically non-significant effects on spontaneous metastasis noted with E-64 may be related to the dose and schedule of administration used in our studies. It would be likely that, at these dose levels, blood or intracellular concentrations of E-64 which could inhibit tumor cell invasion and metastasis formation or affect tumor cell cycle or parasite growth (13, 19-21) are not achieved. Furthermore, as our *in vitro* studies showed that at 10^{-8}-10^{-7} M concentrations E-64 may paradoxically increase the activity of Cathepsin B and L and, to a greater extent, that of Cathepsin D (data not reported), it would be possible that concentrations of this agent near those inducing this phenomenon might be achieved in tumors cells or organ tissues following the administration of the dose level used. These effects already observed with Leupeptin *in vivo* in organ tissue of mice as well as *in vitro* in B16 melanoma cells (22, 23), might affect the therapeutic potential of this agent. Thus further investigations to better assess the therapeutic potential of E-64 are needed.

These data suggest that proteinase inhibitors may be of therapeutic value in the adjuvant treatment of solid tumors. Nevertheless, because other pharmacological efffects induced by these agents may in addition to the inhibiting proteolytic activity, affect their therapeutic potential as well their selectivity, their use for experimental and therapeutic purposes *in vivo* remains questionable (22-24).

Acknowledgements

Authors wish to thank Prof. G. Sava (Institute of Pharmacology, University of Trieste, Italy) and Dr. R. Giavazzi (Mario Negri Institute, Bergamo, Italy) for kindly providing MCa, M5 and L1210 mouse tumors. This study was supported by an AIRC (Italian Association for Cancer Research) grant and in part by a Ministry of Public Education (MPI 60%) grant.

References

1 Leto G, Gebbia N, Rausa L and Tumminello FM: Cathepsin D in the malignant progression of neoplastic diseases (Review). Anticancer Res *12:* 235-240, 1992.

2 Sloane BF, Moin K, Krepela E and Rozhin J: Cathepsin B and its endogenous inhibitors. The role in tumor malignancy. Cancer Met Rev *9:* 333-352, 1990.

3 Chauhan SS, Goldstein LJ and Gottesman MM: Expression of Cathepsin L in human turmors. Cancer Res *51:* 1478-1481, 1991.

4 Barrett AJ, Kembhavi AA, Brown MA, Kirschke H, Knight GC, Tamai M and Hanada K: L-trans-epoxysuccinil - leucylamido (4-guanidino) – butane (E-64) and its anologues as inhibitors of cysteine proteinases including Cathepsin B, H and L. Biochem J *201:* 189-198, 1982.

5 Umezawa H and Aoyagi T: Pepstatins, pepstanones, hydroxypepstatins - inhibitors of carboxyl proteinases including renin. *In:* (Barrett AJ ed), Proteinases in mammalian cells and tissue. Elsevier, Noth Holland 1979, Chapt 15, pp. 646-651.

6 Leto G, Tumminello FM, Gebbia N, Woynarowska B and Bernacki RJ: Antimetastatic activity of adriamycin in combination with proteinase inhibitors in mice. Anticancer Res *10:* 265-270, 1990.

7 Sava G, Zorzet S, Perissin L, Pacor S, Larsiani L, Nisi C and Varnovas A: Antineoplastic action of p- (3-methyl-1-triazeno) benzoic acid potassium salt, a monomethylderivative of the antimetastatic compound DM-COOK. Cancer Chemother Pharmacol *27:* 424-428, 1991.

8 Barrett AJ and Kirschke H: Cathepsin B, Cathepsin H and Cathepsin L. Methods Enzymol *80:* 535-565, 1982.

9 Leto G, Tumminello FM, Gebbia N and Rausa L: Kinetics of *in vivo* inhibition of tissue Cathepsin D by Pepstatin A. Int J Biochem *20 (9):* 917-920, 1988.

10 Bradford MM: A rapid and sensitive method for the quantitaion of microgram quantities of protein utilizing the principle of protein-dye-binding. Anal Biochem *72:* 248-254, 1976.

11 Greenbaum LM and Sutherland JHR: Host Cathepsin D response to tumor in the normal and Pepstatin-treated mouse. Cancer Res *53:* 2584-2687, 1983.

12 Hanada K, Tamai M, Adachi T, Oguma K, Kashiwagi K, Ohmura S, Kominami E, Towatari T and Katunuma N: Characterization of the three analogs of E-64 and their therapeutic applications. *In:* (Katunuma N *et al* eds), Proteinase inhibitors: Medical and Biological Aspects. Japan Sci Soc Press Tokyo, Springer-Verlag 1983, pp. 26-36.

13 Redwood SM, Liv BC-S, Weiss RE, Hodged and Droller M: Abrogation of the invasion of human bladder tumor cells by using proteinase inhibitors. Cancer *69* (5): 1212-1219, 1992.

14 Tachikura T: The teratogenic effects of E-64 on rat embryogenesis. Acta Pediatr Jpn *32:* 495-501, 1990.

15 Geran RG, Greenberg NH, Mac Donald MM, Schumacher AM and Abbott RJ: Protocols for screening chemical agents and natural products against animal tumors and other biological system. Cancer Chemother Rep *3* (part 3): 1-87, 1972.

16 Mathieu M, Rochefort H, Barenton B, Prebois C and Vignon F: Interaction of Cathepsin-D and Insulin-like growth factor-II (IGF-II) on the IGF/Mannose -6-Phosphate receptor in human breast cancer cells and possible consequence on mitogenic activity of IGFII. Mol Endocrinol *4:* 1327-1335, 1990.

17 Pagano M, Capony F and Rochefort H: *In vitro* activation of pro-Cathepsin B by Cathepsin D both secreted by cancer cells. CR Acad Sci Paris *309* Serie III: 7-12, 1990.

18 Ostrowski LE, Ahsan A, Suthar BP, Pagat P, Bain DL, Wong C, Patel A and Schultz M: Selective inhibition of proteolytic enzymes in an *in vivo* mouse model for experimental metastasis. Cancer Res *46:* 4121-4128, 1986.

19 Kobayashi H, Ohi H, Sigimura M, Shinohara H, Fujii T and Terao T: Inhibition of *in vitro* ovarian cancer cell invasion by modulation of Urokinase-type Plasminogen Activator and Cathepsin B. Cancer Res *52:* 3610-3614, 1992.

20 Shoji-Kasai Y, Senshu M, Ivashita S and Imahori K: Thiol protease-specific inhibitor E-64 arrests human epidermoid carcinoma A431 cells at mitotic methaphase. Proc Natl Acad Sci USA *85:* 146-150, 1988.

21 Bailly E, Jambon R, Savel J and Jaureguiberry G: *Plasmodium falciparum:* Different sensitivity *in vitro* to E-64 (cystein proteinase inhibitor) and Pepstatin (aspartyl-proteinase inhibitor). J Protozool *39:* 593-599, 1992.

22 Sutherland JHR and Greenbaum LH: Paradoxical effect of Leupeptin *in vivo* on Cathepsin B activity. Biochem Biophys Res Comm *110:* 332-338, 1983.

23 Nakao I, Kurita Y, Tsuboi R, Takamori K and Ogawa H: Differences in induction of lysosomal protease activity by protease inhibitors in B16 melanoma cell line. Int J Biochem *21:* 139-142, 1989.

24 Nells LP and Schnebli HP: Are proteinase inhibitors potentially useful in tumor therapy? Invasion Metastasis *2:* 113-124, 1982.

Received October 19, 1993
Accepted December 6, 1993