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Chemoimmunotherapy with cyclophosphamide and bestatin in experimental metastasis in mice*

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Summary. Bestatin has significant therapeutic activity (even following oral administration) for the treatment of metastatic disease, an activity which is limited by tumor burden. Therefore, the therapeutic potential of bestatin was examined in combination with chemotherapy to determine if there is additive activity for heavy tumor burdens. Bestatin significantly increased therapeutic activity and decreased the myelotoxicity of cyclophosphamide following a single injection of cyclophosphamide or split daily doses. In immune function studies, in tumor-bearing animals, bestatin increased the number of colony-forming units (granulocyte-macrophage) (CFU) and alveolar macrophage tumoricidal activity. However, when bestatin was combined with cyclophosphamide, which depressed bone marrow and macrophage activity, it did not show apparent augmentation of macrophage and NK cell activity, but did significantly increase bone marrow CFU activity. Thus, in combined chemoimmunotherapy, bestatin appears to enhance therapeutic activity by accelerating the recovery of hematopoiesis. We suggest, therefore, that a combination chemotherapy protocol, with oral bestatin, may facilitate myelorestoration following aggressive chemotherapy. The majority of biological response modifiers require parental administration; thus, the identification of an orally active, synthetic immunoaugmenting agent with a defined receptor is of particular interest.

Introduction

Bestatin is a low-molecular-mass dipeptide obtained from *Streptomyces olivoreticuli* [30, 35]. This metabolite is a potent inhibitor of leucine aminopeptidase and aminopeptidase B of mammalian cells [18, 29, 30] and immune cells both in vitro [17, 19, 22, 36] and in vivo [15]. Aminopeptidase activity has been associated with macrophage activation and differentiation [21, 37], the inflammatory process [20, 23, 28] and was shown in a cell-free system to correlate with enhancing effects in delayed-type hypersensitivity in vivo [36]. We, as well as others, have undertaken a variety of immunomodulatory and immunotherapeutic studies with bestatin including therapeutic studies for metastases, solid tumors and autochthonous tumors [1, 3–5, 10, 13, 27, 31, 32, 33, 34]. In addition bestatin has shown therapeutic activity for acute nonlymphocytic leukemia as determined by a significant prolongation of survival [16, 25] and for transitional cell carcinoma of the bladder on the basis of disease-free survival but not prolongation of survival [7]. However, additional studies on the optimal dose are needed for clarification of the therapeutic profile of bestatin in the clinic. As reported previously, bestatin is a macrophage-augmenting agent; it has adjuvant activity for suboptimal tumor vaccines and no apparent activity for natural killer (NK) cell augmentation or interferon induction, but does appear to increase interleukin-1 and -2 (IL-1 and IL-2) activity [2]. However, the therapeutic activity of bestatin is limited by its pharmacokinetics and the tumor burden. Therefore, we have examined the therapeutic activity of bestatin in combination with chemotherapy and investigated the immune function of the treated mice.

Materials and methods

Animals. Specific pathogen-free female C57BL/6 mice (H-2^b), 8 weeks of age, were obtained from the Animal Production Area of the National Cancer Institute-Frederick Cancer Research Facility.

Tumors. These studies used the Moloney-virus-induced lymphoma YAC-1 of A/SN (H-2^a) origin and mastocytoma P815 of DBA/2 (H-2^d) origin. Adherent cell lines included the metastatic variant B16-BL6 [11] from the B16 melanoma, which arose spontaneously in a C57BL/6N (H-2^b) mouse, and the spontaneous lung carcinoma 3LL, which is syngeneic to the C57BL/6 mice. All adherent cell lines were maintained as monolayers in Eagle's minimum essential medium (MEM) supplemented with 5% fetal bovine serum (Gibco, Grand Island, NY); twofold-concentrated vitamin solution, glutamine, sodium pyruvate, and nonessential amino acids. The YAC-1 and P815 tumor cell lines were grown in Roswell Park Memorial Institute (RPMI) 1640 medium supplemented with 10% fetal bovine serum and the same medium supplements used with MEM. All cell lines were free of mycoplasma and pathogenic murine viruses.

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Agents. Bestatin [24], *N*-[(2*S*, 3*R*)-3-amino-2-hydroxy-4-phenylbutyrl]-L-leucine was obtained from Nippon Kayaku Co. Ltd, Chiyoda-ku, Tokyo, Japan. Cyclophosphamide (cytoxan) was obtained from Dr. Ven Narayanan, NCI, Bethesda, Md. Bestatin and cyclophosphamide were dissolved in Hanks' balanced salt solution (HBSS) without magnesium and calcium, and sterilized by passing the solutions through a Millipore filter (0.22 µm pore size). All media, salt solutions, and agents were endotoxin-negative, as determined by the *Limulus* lysate assay ($\leqslant 0.125$ EU/ml).

Assay of macrophage-mediated cytotoxicity. We performed the macrophage cytotoxicity assay as previously described [32]. Briefly, 100000 macrophages were plated into each well of a 96-well microtiter plate, nonadherent cells were removed by washing 2 h after incubation. [^{125}I]Iododeoxyuridine-radiolabeled B16–B20 target cells (10000) were then added to each well. In some wells lipopolysaccharide, 5 ng/ml, a dose which had no effect on tumoricidal activity alone, was added with the tumor cells as a second signal. The cytotoxicity assays were terminated 72 h after the addition of target cells by washing the monolayers to remove the radiolabel released from dead cells, lysing the remaining viable adherent cells, and monitoring radioactivity with a gamma counter. Under these conditions of assay, untreated macrophages are not cytotoxic to neoplastic cells. The cytotoxic activity of the macrophages was calculated according to the formula: cytotoxicity (%) = $100 \times (A-B)/A$, where A equals ^{125}I (cpm) in target cells cultured with normal macrophages and B equals ^{125}I (cpm) in target cells cultured with test macrophages.

Cell suspension. Mice were sacrificed at different times in the course of therapy, as indicated; and femurs, spleens, and lungs were removed. Alveolar macrophages were collected by broncho-alveolar lavage before removal of the lungs. Single-cell suspensions from femoral bone marrow and spleen were prepared as described previously [9] in RPMI 1640 supplemented with 0.5% fetal bovine serum and gentamicin, and incubated for 2 h at 37° C to remove adherent cells. After the incubation, the number of viable, nucleated cells per femur was counted in white blood cell counting solution using a hemocytometer. The alveolar macrophages were purified by adherence as previously described [32].

Augmentation of NK cell activity. NK cell activity was assessed in a 4-h ^{51}Cr-release assay using YAC target cells, as previously described [32]. In this assay, spleen cells are admixed in U-bottom 96-well plates with YAC tumor cells prelabeled with ^{51}Cr. The tumor cell cytotoxicity was determined from cultures with an effector-to-target cell ratio of 50:1, 25:1, 12:1, and 6:1. Spontaneous release of radiolabel did not exceed 8% of the total radioactivity. Specific release (percentage cytotoxicity) was calculated as $100 \times$ (^{51}Cr released from the target cells admixed with effector cells $-$ ^{51}Cr released spontaneously/total ^{51}Cr released by Triton X), where ^{51}Cr release was measured as cpm.

Assay of granulocyte-macrophage colony-forming unit frequency. The number of granulocyte-macrophage colony-forming units (CFU) was determined as described previously [9]. Briefly, fractionated bone marrow cells (10000, 5000, 1000, and 100/well) were suspended with CMRL-1066 medium supplemented with sodium pyruvate, equine serum (5%), fetal bovine serum (10%), and tryptic soy broth (0.3%). For colony growth, medium was also supplemented with 0.33% bacto agar. Each dilution of the cell suspensions was plated in 24 wells of 96-well microtest plates. The cultures were incubated at 37° C in a fully humidified atmosphere at 5% CO_2 in air. The wells with colonies (more than 40 cells each) were scored 10 days after the incubation by using a dissecting microscope at $100\times$/magnification. Morphological examination of colony-forming cells was done on Wright-Giemsa-stained preparations.

Calculation of frequency of granulocyte-macrophage colony-forming units. The frequency of CFU was calculated from the limiting dilution data using a probability distribution, which was estimated with a multiple linear regression equation, the calculation being performed with the BMDP-1R program on an IBM-AT computer.

Therapy of established metastases. Experimental lung metastases were established in 8-week-old C57BL/6 mice by i.v. injection of 40000 in-vitro-propagated B16-BL6 melanoma cells in 0.2 ml HBSS lacking Ca^{2+}, Mg^{2+}. The schedule for therapy of metastases by biological response modifiers varied in each experiment and is described within the text. Determinations of therapeutic efficacy were based on the prolongation of survival.

Statistical analyses. The NK and macrophage assays used three samples per group. The paired Student's *t*-test was used to determine the significance of any differences from the appropriate control groups. The difference between the extent of metastasis of the control (saline-treated animals) and experimental groups was determined using the non-parametric Mann-Whitney *U*-test, and ten animals were included in each group. Correlations of therapeutic activity and effector cell function or hematological parameters were determined using the Pearsons correlation coefficient.

Results

Chemoimmunotherapy in the early stage of experimental metastasis

We examined the therapeutic effect on experimental metastases of bestatin in combination with cyclophosphamide when cyclophosphamide was administered 4 days after i.v. tumor cell inoculation. Bestatin was administered by i.v. daily injection for 4 weeks starting 24 h after cyclophosphamide injection. As shown in Table 1, cyclophosphamide exhibited therapeutic activity in a dose-dependent manner against pulmonary metastases when used for the treatment of comparatively minor tumor burden, and the dose of 200 mg/kg cured a large number of the animals. Note, however, as is the case with most biological response modifiers, that bestatin had only minor therapeutic activity for other than the most minimal metastatic disease. The combination of cyclophosphamide at 50 mg/kg and bestatin showed significant therapeutic activity compared to cyclophosphamide alone. However, the combination of cyclophosphamide at 200 mg/kg and either dose of bestatin cured all of the pulmonary metastases in all mice. While curing all the animals of their neoplastic disease is impressive, one must consider that this was a relatively

Table 1. Combination therapy of experimental metastases[a]

CTX[b] (mg/kg)	Bestatin[b] (mg/kg)	Metastasis		P versus[c]	
		Median	Range	HBSS	CTX
–	–	>300	(81–300)	–	
50	–	67	(19–217)	0.04	
200	–	0	(0–2)	0.00	
–	15	261	(3–300)	0.755	
–	50	289	(0–300)	0.725	
50	15	3	(0–300)	0.0008	0.08
50	50	5	(0–172)	0.001	0.01
200	15	0	(0–0)	0.0000	0.06
200	50	0	(0–0)	0.0000	0.06

[a] B16-BL6 melanoma cells (40 000) were injected i.v. into 8-week-old C57BL/6 female mice on day 0

[b] Cyclophosphamide (CTX) was injected on day 4, i.p., and bestatin was administered daily by i.v. injection beginning on day 5, for 4 weeks

[c] Probability of no difference between groups, as determined by Mann-Whitney *U*-test. HBSS, Hanks' balanced salts solution

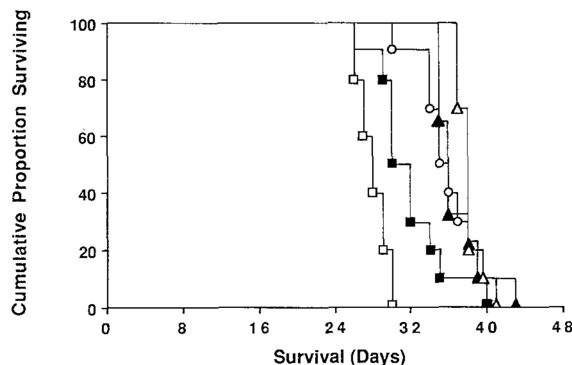

Fig. 1. Mice (ten/group) received i.v. injections of 40000 B16-BL6 tumor cells. Fifteen days later, cyclophosphamide (cytoxan) was injected once i.p. at 300 mg/kg. Bestatin treatment was initiated 48 h following cyclophosphamide injection and was given i.v. each day at 50 mg/kg. □, Hanks' balanced salts solution (HBSS); ○, cyclophosphamide 300 mg/kg on day 15; ■, bestatin 50 mg/kg, i.v. each day starting on day 17; △, cyclophosphamide and bestatin for 4 wks; ▲, bestatin for 2 wks starting on day 17

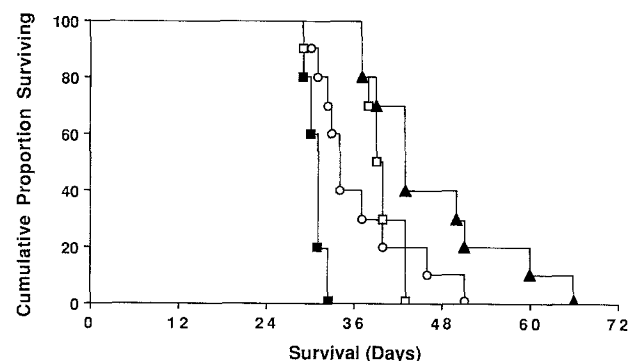

Fig. 2. Mice (ten/group) received i.v. injections of 40000 B16-BL6 tumor cells. After 15 days, cyclophosphamide was injected once i.p. at 300 mg/kg. Bestatin treatment was initiated 3 days following cyclophosphamide injection and was given orally twice daily at a dose of 50 mg/kg until death. ■, HBSS; □, cyclophosphamide at 300 mg/kg on day 15; ○, bestatin at 50 mg/kg per os, twice daily beginning on day 18; ▲, cyclophosphamide and bestatin combined protocols

minor tumor burden and certainly does not accurately represent the current clinical challenge of patients with more advanced disease.

Chemoimmunotherapy in the advanced stage of experimental metastasis

The therapeutic effect of bestatin in combination with cyclophosphamide at 300 mg/kg, injected 15 days after the i.v. tumor cell inoculation, was examined since it represented a greater tumor burden. Metastatic colonies in lung are grossly visible 15 days after i.v. tumor injection, and thus represent a heavy tumor burden. Bestatin at a dose of 50 mg/kg i.v., when administered daily for 4 weeks, significantly prolonged survival compared to HBSS ($P = 0.007$). Cyclophosphamide also significantly prolonged survival compared to HBSS ($P \leq 0.001$) and bestatin alone ($P \leq 0.001$). However, multiple injections of bestatin administered for 4 weeks, beginning 48 h following cyclophosphamide injection, significantly prolonged the host survival compared with cyclophosphamide ($P = 0.027$) or bestatin alone ($P = 0.041$). In contrast, the combination of cyclophosphamide and bestatin did not significantly prolong host survival ($P = 0.689$) if bestatin was administered for only 2 weeks (Fig. 1). As shown in Fig. 2, a similar therapeutic effect was observed following oral administration of bestatin twice a day. In this study, both bestatin (administered per os twice a day) and cyclophosphamide at 300 mg/kg significantly prolonged survival compared to the HBSS control ($P = 0.0025$ and 0.0009, respectively). However, the combination of bestatin and cyclophosphamide together significantly prolonged survival compared to either one alone ($P = 0.0081$ and 0.0105, respectively). We also assessed the immune function of the treated mice 5 days after cyclophosphamide injection (day 20). The numbers of CFU in the femur and alveolar macrophage tumoricidal activity were significantly increased in the group treated with bestatin alone (Table 2). Note that bestatin augments macrophage tumoricidal activity [32] and CFU (results not shown) in normal mice. The CFU number in the femur in the group treated with cyclophosphamide and bestatin combined were also increased compared to the group treated with cyclophosphamide alone. However, alveolar macrophage activity, which was augmented in tumor-bearing animals treated with bestatin alone, was depressed to background levels 5 days following cyclophosphamide administration. When the effector cell function and myelopoietic analyses (Table 2) were correlated with median survival time (Fig. 2), there was a significant correlation between therapeutic efficacy, as measured by increased median survival, and total/number of CFU ($P = 0.016$) and their frequency ($P = 0.0442$). In agreement with the suggestion that the myelodepressive/cellular toxicity activity of cyclophosphamide was responsible for the loss of macrophage function, there was also a significant correlation between bone marrow cellularity and macrophage activity in the absence of lipopolysaccharide ($P = 0.0002$) and macrophage activity in its presence ($P = 0.0504$). As one would expect, there was also a correlation between the total number and frequency of CFU ($P = 0.0074$) and macrophage activity in the presence or absence of lipopolysaccharide ($P = 0.0451$). Note that there was no correlation between NK cell activity and survival, this may have been associated with the sensitivity of the assay since very low levels of NK cell activity were

Table 2. Immune function of tumor-bearing mice following bestatin and cyclophosphamide therapy

Treatment[a]			Bone marrow			Alveolar macrophage tumoricidal activity (%)	
Tumor	CTX	Bestatin	Cells × 10^{-6}	Freq. × 10^{-6}	CFU[b] femur	− LPS	+ LPS
–	–	–	7.4	157	1162	0	0
+	–	–	6.9	312	2153	18.1	8.1
+	–	+	9.9	352	3485	31.2	27.9
+	+	–	2.5	3520	8800	0	0
+	+	+	2.4	4660	11184	0	0

[a] Cyclophosphamide (CTX; 300 mg/kg) was injected (i.p.) 15 days following i.v. inoculation of 40000 B16-BL6 cells. Bestatin was initiated (p.o., twice daily at 50 mg/kg, 3 days after CTX injection. Immune function was assessed 20 days after B16-BL6 inoculation
[b] CFU, colony-forming unit (culture)

observed. Bestatin has not been shown to augment NK activity in normal mice [31, 32] and we suggest that bestatin, via its leukorestorative properties, may accelerate the lymphocytic reconstitution including NK cells resulting in increased numbers of NK cells as opposed to NK cell augmentation. The statistical analyses strongly support the suggestion that, at 5 days following cyclophosphamide administration, the therapeutic activity of bestatin was due to its ability to increase the frequency of bone marrow stem cells and thereby to increase both the frequency and the total number of CFU.

Bestatin therapy following short-term concentrated chemotherapy. Cyclophosphamide, at a total dose of 450 mg/kg, was injected in divided doses on 1 (day 15), 2 (days 15 and 16), or 3 consecutive days (days 15, 16, and 17) after tumor injection. Bestatin at a dose of 50 mg/kg was administered orally twice daily, beginning on day 18, until death. A single injection of cyclophosphamide at 450 mg/kg was toxic, and this dose seemed to be greater than the maximum tolerated dose (Table 3) for animals bearing bulky tumor burdens. Four out of ten mice that were injected with a single dose of cyclophosphamide at 450 mg/kg died as a result of pneumonia secondary to myelotoxicity. However, the mice receiving a single dose of cyclophosphamide (450 mg/kg) in combination with bestatin had a significantly prolonged survival in the absence of treatment-related mortality. A similar increased therapeutic effect was observed with two divided cyclophosphamide doses combined with bestatin. Three divided cyclophosphamide treatments significantly prolonged survival compared to HBSS alone ($P \leqslant 0.001$) but the addition of bestatin to the treatment protocol did not significantly prolong survival compared to three split doses of cyclophosphamide.

Discussion

In the present report, we examined the combination-therapeutic activity of cyclophosphamide and bestatin. As reported here, bestatin has additive therapeutic properties when used in combination with chemotherapy. This combination was most effective with moderate tumor burden, in which case aggressive chemotherapy combined with immunotherapy totally eliminated pulmonary metastases and cured all animals. Perhaps more significant for clinical utilization was the increased therapeutic activity for large tumor burdens when bestatin was administered after a single injection or divided consecutive injections of cyclophosphamide.

Bestatin was found to restore the lowered immune responses of tumor-bearing mice [6, 8, 12, 26, 38] undergoing chemotherapy. Bestatin also increased CFU activity in bone marrow stem cells in tumor-bearing and cyclophosphamide treated mice and augmented the tumoricidal activity of alveolar macrophages in tumor-bearing animals. Thus, the combination-therapeutic effect is associated with the reduction in tumor burden and correlates with the ability of bestatin to increase stem cell activity and to augment macrophage activity following cyclophosphamide injec-

Table 3. Chemoimmunotherapy of experimental metastasis with injection of cyclophosphamide (CTX) and bestatin[a]

CTX		Bestatin[a]	Toxicity	Survival time (days)	
Dose (mg/kg)	Schedule			Range	Median
–	–	–	0/10	23–32	26
450	day 15	–	4/10	16–41	35
225	day 15, 16	–	0/10	33–44	36
150	day 15, 16, 17	–	0/10	35–50	38
–	–	+	0/10	25–31	26
450	day 15	+	0/10	35–40	37[b]
225	day 15, 16	+	0/10	38–42	39[b]
150	day 15, 16, 17	+	0/10	35–42	39

[a] Bestatin, 50 mg/kg, was administered per os twice daily beginning on day 18. CTX was injected in split doses for a total of 450 mg/kg on days 15, 15 and 16, or 15, 16 and 17 following the i.v. injection of 40000 B16-BL6 tumor cells
[b] $P = 0.05$ as compared to the appropriate CTX-injected animals using the Mann-Whitney U-test

Table 4. Immune function of tumor-bearing mice following bestatin and cyclophosphamide (CTX) therapy

Treatment[a]			Bone marrow		Alveolar macrophage activity (%)	NK[b] activity (LU)	MST[c] (days)
Tumor	CTX	Bestatin	Cells/femur ($\times 10^{-6}$)	CFU/femur			
–	–	–	22.1	1808	0	0.2	
+	–	–	22.3	3875	0	0.1	26
+	–	+	21.0	6846	2.4	0.2	26
+	450	–	16.3	145	16.6	0.2	35
+	225 × 2	–	14.7	131	10.2	0.4	36
+	150 × 3	–	10.0	279	7.2	0.3	38
+	450	+	14.8	413	4.4	0.3	38
+	225 × 2	+	12.8	357	10.5	0.4	39
+	150 × 3	+	13.8	123	16.2	0.3	39

[a] CTX was injected as split doses (i.p.) 15, 16, or 17 days after tumor inoculation. Bestatin was administered p.o., twice daily beginning on day 16. Immune functions were measured 27 days after tumor inoculation
[b] One lytic unit (LU) is defined as the 30% point for 10^6 effector cells. NK, natural killer
[c] MST, mean survival time

tion. We suggest that the optimal clinical utilization of bestatin will depend on a logical protocol, addressing concerns of schedule of administration, scheduling of cycles of chemo-immunotherapy and duration of administration. The greatest therapeutic benefit of bestatin will be derived from combination protocols with aggressive chemotherapy. One would expect that chemotherapy could potentially reduce the tumor burden to a level where the immunotherapeutic properties of bestatin might be expected to be active. In addition, bestatin may reduce the life-threatening neutropenia associated with aggressive chemotherapy by limiting secondary infections and the associative iatrogenic mortality. On the basis of these and other studies from our laboratories we believe that moderate to high doses of bestatin, administered orally following a short course of aggressive chemotherapy, might be expected to provide clinical benefits.

References

1. Abe F, Shibuya K, Uchida M, Takahashi K, Horinishi H, Matsuda A, Ishizuka M, Takeuchi T, Umezawa H (1984) Effect of bestatin on syngeneic tumors in mice. Gann 75: 89
2. Abe F, Shibuya K, Ashizawa J, Takahashi K, Horinishi H, Matsuda A, Ishizuka M, Takeuchi T, Umezawa H (1985) Enhancement of antitumor effect of cytotoxic agents by bestatin. J Antibiot (Tokyo) 38: 411
3. Abe F, Shibuya K, Ashizawa J, Takahashi K, Horinishi H, Matsuda A, Ishizuka M, Takeuchi T, Umezawa H (1985) Enhancement of antitumor effect of cytotoxic agents by bestatin. J Antibiot (Tokyo) 38: 411
4. Abe F, Hayashi M, Horinishi H, Matsuda A, Ishizuka M, Umezawa H (1986) Enhancement of graft-versus-host reaction and delayed cutaneous hypersensitivity in mice by ubenimex. J Antibiot (Tokyo) 39: 1172
5. Abe F, Alvord G, Koyama M, Matsuda A, Talmadge JE (1989) Optimal therapeutic protocol and pharmacokinetics of bestatin treatment against experimental metastases. Cancer Immunol Immunother 28: 29–33
6. Blomgren H, Edsmyr F, Esposti P-L, Näslund I (1984) Immunological and hematological monitoring in bladder cancer patients receiving adjuvant bestatin treatment following radiation therapy. A prospective randomized trial. Biomed Pharmacother 38: 143
7. Blomgren H, Näslund I, Esposti P-L, Johansen L, Aaskoven O (1987) Adjuvant bestatin immunotherapy in patients with transitional cell carcinoma of the bladder. Cancer Immunol Immunother 25: 40
8. Bryley-Rosset M, Florentin L, Kiger N, Schulz J, Mathe G (1984) Restoration of impaired immune functions of aged animals by chronic bestatin treatment. Immunology 38: 75
9. Castelli MP, Black PL, Schneider M, Pennington R, Abe F, Talmadge JE (1988) Protective, restorative and therapeutic properties of recombinant human Il-1 in rodent models. J Immunol 140: 3830
10. Ebihara K, Abe F, Yamashita T, Shibuya K, Hayashi E, Takahashi K, Horinishi H, Enomoto M, Ishizuka M, Umezawa H (1986) The effect of ubenimex on *N*-methyl-N′-nitro-*N*′-nitrosoguanidine-induced stomach tumor in rats. J Antibiot (Tokyo) 39: 955
11. Hart IR (1979) The selection and characterization of an/invasive variant of the B16 melanoma. Am J Pathol 97: 587
12. Ishizuka M, Masuda T, Kanbayashi N, Fukazawa S, Takeuchi T, Ayoyagi T, Umezawa H (1980) Effect of bestatin on mouse immune system and experimental murine tumors. J Antibiot (Tokyo) 33: 642
13. Ishizuka M, Masuda T, Mizutani S, Takeuchi T, Umezawa H (1987) Antitumor cells found in tumor-bearing mice given ubenimex. J Antibiot (Tokyo) 40: 697
14. Kaya G, Akin C, Altug T, Devrim S (1988) The antitumor effect of bleomycin combined with bestatin against Ehrlich ascites carcinoma in mice (42667). Proc Soc Exp Biol Med 187: 292
15. Kuramochi H, Motegi A, Iwabuchi M, Takahashi K, Horinishi H, Umezawa H (1987) Action of ubenimex on aminopeptidase activities in spleen cells and peritoneal macrophages from mice. J Antibiot (Tokyo) 40: 1605
16. Kurita S, Ota K, Yamada K, Masaoka T, Uzuka Y, Ogawa N (1984) Immunotherapy with bestatin for acute non-lymphocytic leukemia (ANLL) in adults. Jpn J Cancer Chemother 11(12): part II, 2442
17. Leyhausen G, Gramzow M, Zahn RK, Steffen R, Umezawa H, Müller WEG (1983) Immunochemical identification of the cell surface bound leucine aminopeptidase, the target enzyme for the immunostimulant bestatin. J Antibiot (Tokyo) 36: 728
18. Leyhausen G, Schuster DK, Vaith P, Zahn RK, Umezawa H, Falke D, Müller WEG (1983) Identification and properties of the cell membrane bound leucine aminopeptidase interacting with the potential immunostimulant and chemotherapeutic agent bestatin. Biochem Pharmacol 32: 1051
19. Reference deleted
20. Makinen KK, Oksala E (1973) Evidence on the involvement in inflammation of an enzyme resembling aminopeptidase B. Clin Chim Acta 49: 301
21. Morahan PS, Edelson PJ, Gass K (1980) Changes in macrophages ectoenzymes associated with antitumor activity. J Immunol 125: 1312

22. Müller WEG, Schuster DK, Zahn RK, Maidhof A, Leyhausen G, Falke D, Koren R, Umezawa H (1982) Properties and specificity of binding sites for the immunodulater bestatin on the surface of mammalian cells. Int J Immunopharmacol 4: 393
23. Nagaoka I, Yamashita T (1981) Inactivation of phagocytosis-stimulating activity of tuftsin by polymorphonuclear neutrophils. A possible role of leucine aminopeptidase as an ectoenzyme. Biochim Biophys Acta 675: 85
24. Nishizawa R, Saino T, Suzuki M, Fujii T, Shirai T, Aoyagi T, Umezawa H (1983) A facile synthesis of bestatin. J Antibiot (Tokyo) 36: 695
25. Ota K, Kurita S, Yamada K, Masaoka T, Uzuka Y, Ogawa N (1986) Results of investigation into prognosis after immunotherapy with bestatin for acute nonlymphocytic leukemia in adults. Jpn J Cancer Chemother 13: 1017
26. Schorlemmer HU, Bosslet K, Dickneite G, Luben G, Sedlacek HH (1984) Studies on the mechanism of action of the immunomodulator bestatin in various screening test systems. Behring Inst Mitt 74: 157
27. Shibuya K, Hayashi E, Abe F, Takahashi K, Horinishi H, Ishizuka M, Takeuchi T, Umezawa H (1987) Enhancement of interleukin 1 and interleukin 2 release by ubenimex. J Antibiot (Tokyo) 40: 363
28. Soderling E, Knuuttila M (1980) Release of the chloride-dependent arginine aminopeptidase from PMN leukocytes and macrophages during phagocytosis. Life Sci 26: 303
29. Suda H, Aoyagi T, Takeuchi T, Umezawa H (1976) Inhibition of aminopeptidase B and leucine aminopeptidase by bestatin and its stereoisomer. Arch Biochem Biophys 177: 196
30. Suda H, Takita T, Aoyagi T, Umezawa H (1976) The structure of bestatin. J Antibiot (Tokyo) 29: 100
31. Talmadge JE (1985) Immunomodulatory and therapeutic characteristics of bestatin: In: Recent results of bestatin, 1985. Jpn Antibiot Res Assoc, Tokyo, p 55
32. Talmadge JE, Lenz BF, Pennington R, Long C, Phillips H, Schneider M, Tribble H (1986) Immunomodulatory and therapeutic properties of bestatin in mice. Cancer Res 46: 4505
33. Talmadge JE, Koyama M, Matsuda A, Long C, Abe F (1987) Immunotherapeutic properties of bestatin: mechanism of activity: In: recent results of bestatin, 1986. Jpn Antibiot Res Assoc, Tokyo, p 8
34. Tsuruo T, Naganuma K, Iida H, Yamori T, Tsukagoshi S, Sakurai Y (1981) Inhibition of lymph node metastasis of P388 leukemia by bestatin in mice. J Antibiot (Tokyo) 34: 1206
35. Umezawa H, Aoyagi T, Suda H, Hammda M, Takeuchi T (1976) Bestatin, an inhibitor of aminopeptidase B, produced by actinomycete. J Antibiot (Tokyo) 29: 97
36. Umezawa H, Ishizuka M, Aoyagi T, Takeuchi T (1976) Enhancement of delayed-type hypersensitivity by bestatin, an inhibitor of aminopeptidase B and leucine aminopeptidase. J Antibiot (Tokyo) 29: 857
37. Wachsmuth ED (1975) Aminopeptidase as a marker for macrophage differentiation. Exp Cell Res 96: 409
38. Yamakura Y, Shimbo T, Yata J (1981) Effect of an aminopeptidase inhibitor (bestatin) on human lymphocytes. I. Effect on pokeweed mitogen-induced in vitro immunoglobulin production: In: Small molecular immunomodifiers of microbial origin. Pergamon Press, New York, p 109

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