Biochemical Pharmacology, Vol. 32, No. 6, pp. 1051–1057, 1983.
Printed in Great Britain.

0006-2952/83/061051-07 $03.00/0

IDENTIFICATION AND PROPERTIES OF THE CELL MEMBRANE BOUND LEUCINE AMINOPEPTIDASE INTERACTING WITH THE POTENTIAL IMMUNOSTIMULANT AND CHEMOTHERAPEUTIC AGENT BESTATIN

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(*Received* 14 *July* 1982; *accepted* 25 *October* 1982)

Abstract—Bestatin was found to be a competitive inhibitor (with respect to the Leu-NA substrate) not only of the isolated microsomal and cytosolic leucine aminopeptidases (Leu-APm and Leu-APc) but also of the aminopeptidases (APs) present in membrane preparations (from mouse liver) and on the cell surface of L5178Y cells. Kinetic parameters indicate that cellular AP is identical to Leu-APm. To rule out the possibility that AP-B is involved in the inhibition reactions, comparable studies with amastatin were performed. Electrophoretical studies revealed the solubilized cell membrane bound AP to co-migrate with Leu-APm in polyacrylamide gels. The activity of the separated membrane AP was inhibited by bestatin *in situ*. The cell membrane bound AP activity was found to be lowest in lymphocytes, higher in tumor cells and highest in bone marrow cells and macrophages. Using synchronized L5178Y cells, the AP activity changes during the cell division cycle; the lowest activity was determined during the G_1-phase and 35% higher values were measured during the S/G_2-phase. The fluctuation of the cell surface associated AP activity parallels with changes in the number of binding sites for bestatin.

The importance of cell surface bound enzymes in the modulation of genetic activity of mammalian cells is widely recognized; e.g. glycosyltransferases and glycosidases during cell–cell interaction [1, 2] and exopeptidases in the immune response [3]. The latter aspect was intensively studied by the group of Umezawa [3, 4]. Aoyagi *et al.* [4] found unexpectedly high exopeptidase activity on the surface of mammalian cells. Two of these exopeptidases, leucine aminopeptidase (Leu-AP) and aminopeptidase B (AP-B),** were found to be strongly inhibited by bestatin, a dipeptide [(2*S*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl-L-leucine], which had been isolated from culture filtrates of *Streptomyces olivoreticuli* [5].

Bestatin possesses the potentially chemotherapeutically useful property of acting *in vivo* both as an antitumor agent [6, 7] and as an immunomodifier [6, 8]. Bestatin alone, as well as in combination with, for example, bleomycin or adriamycin, shows a synergistic effect in the treatment of animal [6, 8] and human tumors [9]. Furthermore, bestatin restores the reduced delayed-type hypersensitivity caused by intravenous injection of cyclophosphamide or by intraperitoneal inoculation of Ehrlich carcinoma cells into mice [8]. This cell-mediated immune response is due to an activation of T-cells and pre T-cells [10]. Recent results indicate that bestatin increases the activity of human natural killer cells both *in vitro* and *in vivo* [11]. Based on cell culture studies [6, 12], it has been suggested that bestatin causes the proliferation of T-cells through the activation of macrophages.

In spite of the knowledge that bestatin binds to the cell surfaces of various mammalian cells and is not sequestered with time inside the cell [12], no direct data about the inhibition of the cell surface bound Leu-APc and AP-B by the drug are available. In this study experimental evidence is given which indicates that bestatin interacts with cell membrane bound Leu-APm.

MATERIALS AND METHODS

Compounds

The following materials were obtained: ammediol from Serva (Heidelberg, F.R.G.); Leu-APc (from swine kidney) (100 units/mg) and α_2-macroglobulin

† This paper is part of a dissertation by this author in partial fulfilment of the requirements for the degree of Doctor of Natural Sciences at the Johannes Gutenberg-Universität.

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** Abbreviations: Leu-APc, cytosolic leucine aminopeptidase (EC 3.4.11.1); Leu-APm, microsomal leucine aminopeptidase (EC 3.4.11.2); AP-B, aminopeptidase B (EC 3.4.11.6); Leu-NA, L-leucine-β-naphthylamide; Arg-NA, L-arginine-β-naphthylamide.

(from bovine serum) from Boehringer (Mannheim, F.R.G.); Leu-APm (from swine kidney) (25 units/mg), Leu-NA·HCl, Arg-NA·HCl and *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid (HEPES) from Sigma (St. Louis, MO); sterile nylon wool in LP-1 Leuko-Pak Leucocyte Filters from Fenwal Lab (Morton Grove, FL).

The commercial enzymes were determined to be pure with respect to other aminopeptidases (APs) when checked *in situ* after gel electrophoresis.

Epibestatin [(2*R*,3*R*)-3-amino-2-hydroxy-4-phenylbutanoyl-L-leucine], amastatin [(2*S*,3*R*)-3-amino-2-hydroxy-5-methylhexanoyl-L-valyl-L aspartic acid] and bleomycin (55–70% A_2, 25–32% B_2, <7% A_2', and <1% B_4) were prepared by Nippon Kayaku Co. Ltd. (Tokyo, Japan).

Bestatin. Unlabeled bestatin was prepared by Nippon Kayaku Co. Ltd. following the instruction of one of us [13]. Radioactively labeled bestatin was prepared as described previously (12); the sp. act. of [^{3}H]bestatin was 55 mCi/nmole.

Cells

L5178Y mouse lymphoma cells, a Thy-1,1-bearing, Fc receptor positive T-lymphoma [14, 15], P815 mast cells (mouse leukemic cells) [16] and X_2-(P815-X_2) cells [17] were obtained through the courtesy of Dr G. A. Fischer, Yale University (New Haven, CT). The cells were grown in Spinner medium for leukemic cells, supplemented with 10% horse serum [18]. The generation time of logarithmically growing L5178Y cells was 8.5 hr and that of P815 cells 12.1 hr. Cell vol. and concn were determined electronically [18]. L5178Y cells were synchronized by the double thymidine block method as described previously [19]. The durations of the cell periods were determined as reported previously [12, 19].

L5178Y cells were also maintained in NMRI mice. 2×10^5 cells were inoculated intraperitoneally to form ascites tumor. On day 5 after inoculation, the cells were obtained by aspiration and assayed for AP activity.

Macrophages were harvested from 10–15-week-old male inbred CBA/J mice (Ivanovas, Kisslegg, F.R.G.) by peritoneal washing 3 days after intraperitoneal injection with 2 ml thioglycolate medium (E. Merck, Darmstadt, F.R.G.). They were cultivated in Hanks medium containing 5% calf serum [20]. T- and B-cell-enriched preparations were obtained from a splenic cell suspension (CBA/J mice) by filtration through a nylon wool column [21]. Bone marrow cells were obtained from femurs as described previously [22].

Cell membranes

The cell membranes were isolated from mouse liver according to the "Tris method" described by Warren *et al.* [23]. The preparation had the typical chemical and enzymatic composition described previously [1]. The resulting membrane fraction was dialyzed (24 hr, 2°) against 50 mM Tris–HCl (pH 7.2). The membranes were dissolved in Tris–saline at a concn of 0.5 mg protein/ml and assayed for enzyme activity. For detection and identification of cell membrane bound AP by gel electrophoresis, 1 ml of the membrane preparation (0.5 mg protein/ml) was solubilized (0°, 15 min) in 1% (w/v) Triton X-100 (in 100 mM Tris–HCl, pH 8.5, supplemented with 10% sucrose). After centrifugation (5 min, 20,000 *g*), the supernatant was analyzed by gel electrophoresis.

Enzyme assays

The activities of the soluble Leu-APc [24] and of the particle-bound Leu-APm [25] were determined using 0.5 mM Leu-NA or 0.5 mM Arg-NA as substrate, dissolved in 20 mM HEPES, supplemented with 100 mM NaCl as described earlier [26]. These substrates were used in order to compare the presented data with previous ones [26]. The pH value of the reaction mixture for the isolated Leu-APm was adjusted to 7.2 and the one in the Leu-APc assay to 8.2. In a typical experiment 0.18 μg Leu-APm or 2.5 μg Leu-APc were added to a 1-ml assay. Incubation was performed at 37° for 30 min; during this period the increase in the reaction velocity is linear. The experiments revealed that Leu-APc accepts the Leu-NA substrate only.

AP activities on the surface of intact cells and in preparations from cell membranes were determined by the same method as used for the isolated Leu-APs, using routinely 3×10^5–6×10^5 cells or 14–80 μg of membrane protein per 1-ml assay. The pH value in the assays was adjusted to 7.2. During the period of incubation (37°, 30 min) the cells remained vital, as determined by the dye-exclusion test with trypan blue [2].

The sp. acts are expressed as pmoles of substrate hydrolyzed per mg of protein × min or per 10^5 cells × min.

In situ *assay for AP after gel electrophoresis*

A solubilized mouse liver membrane fraction (100 μl) was subjected to a separation by electrophoresis on 7% acrylamide gel using the Tris–HCl (pH 8.9) system described by Dewald *et al.* [27]; Tris–glycine (pH 8.2) was used as the running buffer. Electrophoresis was carried out at 22° with 2.5 mA per tube (8 cm). After removal from the tube, the enzyme activity in the gel was detected after incubation (5–30 min, 22°) in 10 ml of the reaction mixture, containing as substrate either 0.3 mM Leu-NA or 0.3 mM Arg-NA, dissolved in Tris–maleate (pH 7.2) and 0.1% in 100 mM Fast Garnet GBC [27]. The reaction was terminated by transferring the gels into 7% acetic acid. The gels showing the scarlet-dye band [28] were stored in distilled water. The R_f values of the purified enzymes were as follows: Leu-APc: 0.35; and Leu-APm: 0.25.

Binding assay

The determination of the number of binding sites for bestatin was performed as described previously [12], using 5×10^6 L5178Y cells/ml and 17 μM [^{3}H]bestatin (0.96 μCi).

Protein was measured according to the method described by Lowry *et al.* [29].

RESULTS

It is the aim of this contribution to identify that

Table 1. Influence of different compounds on the activities of isolated leucine aminopeptidases and cellular aminopeptidases

Substance	Leucine aminopeptidases			Cellular aminopeptidases			
	Cytosolic (substrate Leu-NA)	Microsomal substrate		Cell surface bound substrate		Cell membrane bound substrate	
		Leu-NA	Arg-Na	Leu-NA	Arg-NA	Leu-NA	Arg-NA
Control	100	100	100	100	100	100	100
$ZnCl_2$ (5 mM)	17	9	20	48	27	21	12
EDTA (15 mM)	69	90	88	89	62	83	68
Leucine (5 mM)	92	50	26	96	86	60	52
α_2-Macroglobulin (0.1 mg/ml)	96	98	97	99	100	105	105
Bleomycin (50 μm)	59	86	92	105	98	98	92
Bestatin (32 μM)	4	48	31	65	59	53	44
Epibestatin (32 μM)	98	98	96	92	92	99	99
Amastatin (22 μM)	19	1	3	79	55	1	9

The determinations were performed in the standard assay with Leu-APc, Leu-APm, L5178Y cells and mouse liver membranes, using the substrates Leu-NA or Arg-NA. The activity is given as per cent of the control. The values represent the means of five samples each. S.D. does not exceed 5%.

enzyme which interacts with bestatin on the cell membrane. This goal was achieved by comparable enzymatic studies with purified enzymes (Leu-APc and Leu-APm) and by comparative inhibition studies with bestatin (which inhibits Leu-AP and AP-B) and amastatin (which inhibits Leu-AP but not AP-B) [3].

Inhibition of Leu-APc and Leu-APm by bestatin

Purified Leu-APc and Leu-APm were strongly inhibited by 5 mM $ZnCl_2$, while other divalent cations tested ($CaCl_2$, $MgCl_2$, $MnCl_2$) caused no or only a slight effect on their activities (Table 1). Leucine, which is formed during the enzymic reactions, preferentially inhibits Leu-APm at a concn of 5 mM. α_2-Macroglobulin was found not to interact with the two enzymes. However, 50 μM bleomycin markedly inhibits Leu-APc and only slightly Leu-APm. Bestatin (32 μM) and amastatin (22 μM) are strong inhibitors of the two Leu-AP species, while the bestatin epimer (2*R*,3*R* steric configuration) epibestatin is ineffective (Table 1). Detailed kinetic analyses revealed both enzymes to be competitively inhibited by bestatin (Table 2). As a measure for the relative affinities of the enzymes to inhibitor and substrate (Leu-NA) in competitive inhibition, the $K_i : K_m$ ratio can be adopted [30]; the lower this ratio the higher is the potency of the inhibitor to reduce the enzyme activity. By far the highest affinity of bestatin was observed with Leu-APc; the $K_i : K_m$ ratio was determined to be 6.3×10^{-6}. The affinity of the inhibitor is lower for Leu-APm; $K_i : K_m$ ratio = 4.2×10^{-2}.

Inhibition of cellular APs

In comparative studies, the parameters of the cellular APs have been elucidated. The activities were determined with the two substrates Leu-NA and Arg-NA using either intact cells (L5178Y cells, cell surface bound activity) or membranes (mouse liver, cell membrane bound activity) as the enzyme source (Tables 1 and 2). The experiments show that the cellular APs are affected by the four microbial products to a comparable extent to Leu-APm: no inhibition by bleomycin and epibestatin, 50% inhibition by bestatin and 20–40% (cell surface bound activity) or over 90% inhibition (cell membrane bound activity) by amastatin (Table 1). The suggestion that cell surface bound and cell membrane bound APs are closer related to Leu-APm than to Leu-APc is supported by the fact that the two cellular enzymes respond to these drugs with the same activity change, irrespective of the nature of the substrate used. In this context it must be stressed that Leu-APc accepts only Leu-NA as substrate [31]. The observed inhibition of cellular APs by amastatin already excludes the existence of AP-B in our preparations.

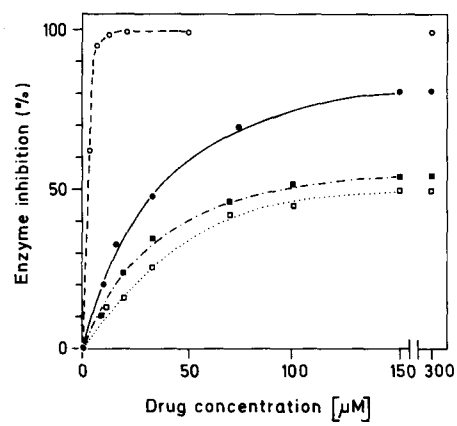

Fig. 1. Inhibition of cellular aminopeptidases (APs) by bestatin and amastatin. Cell surface bound AP activity (5×10^5 L5178Y cells/assay) and cell membrane bound AP activity (20 μg of liver membranes/ml) were determined in the standard assay containing 0.5 mM Leu-NA in the presence of increasing concns of bestatin or amastatin. The AP activity in the absence of inhibitor was 115pmoles/10^5 cells × min or 21 nmoles/mg membrane protein × min. Curves for the inhibition of the cell surface bound activity by bestatin (■ – · – ■) or amastatin (□ · · · · · □) and of the cell membrane bound activity by bestatin (● —— ●) or amastatin (○ – – ○).

Table 2. Kinetic properties of isolated leucine aminopeptidases and cellular aminopeptidases

Enzyme preparation	Substrate	pH optimum	K_m (μM)	V_{max} (μmoles/mg × min or pmoles/10^5 cells × min)	K_i bestatin (μM)	K_i:K_m ratio
Leucine aminopeptidase (cytosolic)	Leu-NA	8.5–9.1	1420	4.26	0.009	6.3×10^{-6}
Leucine aminopeptidase (microsomal)	Leu-NA	6.3–7.4	73	5.84	3.03	4.2×10^{-2}
Aminopeptidase (cell surface bound)	Leu-NA	7.2	63	330.2	13.6	2.2×10^{-1}
	Arg-NA	7.2	92	112.7	11.9	—
Aminopeptidase (cell membrane bound)	Leu-NA	6.3–7.3	58	32.4×10^{-3}	4.1	7.1×10^{-2}
	Arg-NA	6.5–7.4	196	7.3×10^{-3}	9.2	—

For the determination of the pH optimum two buffer systems were used: 100 mM Tris–maleate (pH range 5–8.5) and 100 mM ammediol (8.5–10). The K_m values were determined in assays containing 0.02–1 mM substrate [38], the K_i values in assays containing 0.004–32 μM bestatin [38]. The reaction velocities of the isolated and cell membrane bound activities are given in μmoles/mg protein × min and of the cell surface bound activities in pmoles/10^5 cells × min. The S.D.s of the kinetic parameters are less than 7%. Further details for the activity determinations of Leu-APc, Leu-APm, L5178Y cells (cell surface bound activity) and mouse liver membranes are given in Materials and Methods. Using Leu-NA as substrate, the bestatin inhibition is of the competitive type; with Arg-NA as substrate, a non-competitive inhibition was determined.

Dose–response experiments revealed that bestatin does not totally inhibit AP activity associated with the cell membrane (Fig. 1). In assays with intact cells the drug reduced the AP activity by 52% (maximum), and in experiments with isolated membranes by 81%. The inhibitory effect caused by amastatin was strong in these two systems; maximal inhibitions of 49% (cell surface bound activity) and nearly 100% (cell membrane bound activity) respectively were reached. Similarly the isolated Leu-APs, the cell surface bound and the cell membrane bound APs are competitively inhibited by bestatin using the substrate Leu-NA (Table 2). As examples, the inhibition kinetics for the cell membrane bound AP are shown in Fig. 2. Comparing the K_i:K_m ratios (Table 2) it is obvious that the values for the cellular APs (2.2×10^{-1} for the cell surface bound enzyme and 7.1×10^{-2} for the cell membrane bound enzyme) are approximately of the same order of magnitude as the ratio determined for Leu-APm (4.2×10^{-2}) and they differ greatly from the K_i:K_m for Leu-APc (6.3×10^{-6}). As already noted (Fig. 1), amastatin is a stronger inhibitor of cellular Leu-APs than bestatin; the kinetic analysis revealed for the membrane-bound enzyme a K_i:K_m ratio of 5.9×10^{-4} (Fig. 2). The replacement of the Leu-NA substrate by Arg-NA in the reaction mixture resulted in a change in the kinetic parameters. Using cell membranes as the enzyme source, the K_m value increases from 58.3 ± 4.2 μM (Leu-NA substrate) to 196.4 ± 14 μM (Arg-NA substrate). Furthermore, the inhibition of the AP by bestatin changes from competitive (Leu-NA substrate) to non-competitive (Arg-NA substrate) (Table 2). The inhibitor constant of bestatin in the Arg-NA assays was determined to be 9.2 μM. This means that 9.2 μM bestatin causes

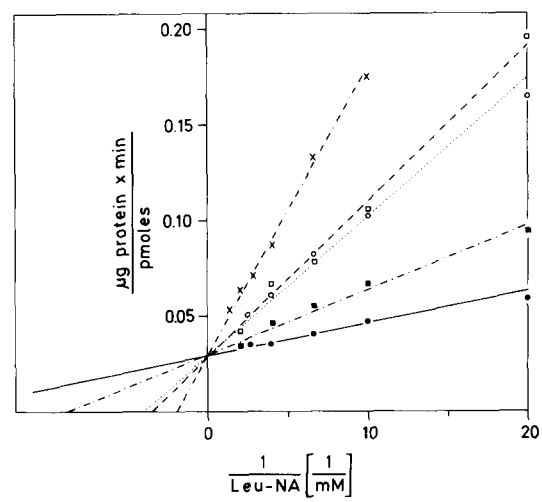

Fig. 2. Competitive inhibition of cell membrane bound aminopeptidase by bestatin and amastatin: Lineweaver–Burk [38] plot. The inhibitor constants (K_i) were determined with 3.4 (■), 16 (□) and 32 μM (×) bestatin as well as with 0.11 μM (○) amastatin; controls in the absence of inhibitor (●): $K_{m(\text{Leu-NA})} = 58 \pm 4$ μM; $K_{i(\text{bestatin})} = 4.1 \pm 0.4$ μM and $K_{i(\text{amastatin})} = 0.034 \pm 0.002$ μM. The enzyme assay containing 50 μg protein of liver membranes was composed as described in Materials and Methods.

Table 3. Activity of cell surface bound aminopeptidase of different cell types

Cells	Aminopeptidase (pmoles/10^5 cells × min) (substrate Leu-NA)	
	Minus bestatin	32 μM bestatin
L5178Y (cell culture)	115.1 ± 9	93.8 ± 5
L5178Y (mouse ascites)	183.8 ± 5	143.9 ± 7
X_2 (cell culture)	118.3 ± 5	90.7 ± 4
P815	113.2 ± 8	88.2 ± 4
T-Lymphocytes (spleen)	33.8 ± 2	20.8 ± 2
B-Lymphocytes (spleen)	49.9 ± 2	30.9 ± 2
Macrophages Day 0	791.3 ± 23	205.7 ± 8
Day 1	1140.7 ± 30	402.6 ± 13
Bone marrow	330.4 ± 13	240.9 ± 9

The cells were incubated in the standard assay, using Leu-NA as substrate. In one series, the reaction mixture was supplemented with 32 μM bestatin.

a 50% reduction of the maximum reaction velocity. The same changes in the inhibition characteristics are observed with the cell surface bound AP. Using the substrate Leu-NA, bestatin inhibited the enzyme in a competitive way with a K_i:K_m ratio of 2.2×10^{-1}, while in the experiments with Arg-NA a non-competitive inhibition type (K_i: 11.9 μM) was observed (Table 2).

Identification of membrane-bound leucine AP

We succeeded in identifying the membrane-bound AP by applying the method of gel electrophoresis and a subsequent *in situ* localization. The liver membrane fraction was solubilized with Triton X-100 (see Materials and Methods) and analyzed. Comparing the R_f value of the visualized band ($R_f = 0.25$), *in situ* stained for Leu-AP activity [Fig. 3A(5)], with the values for the marker enzymes Leu-APc {R_f = 0.35 [Fig. 3A(4)]} and Leu-APm {$R_f = 0.25$ [Fig. 3A(1 and 2)]} it becomes obvious that the membrane-bound AP co-migrates with Leu-APm. On top of the gel ($R_f = 0.08$–0.18), loaded with the liver membrane extract, a Leu-AP-positive zone was traced. At present, we assume that this slow migrating activity is due to partially solubilized Leu-APm. The *in situ* activity pattern of solubilized membrane-bound AP is detectable both with Leu-NA. [Fig. 3A(5)] and with Arg-NA substrate (not shown). This property is shared with Leu-APm but not with Leu-APc; as documented in Fig. 3A(3), Leu-APc activity is not demonstrable if Arg-NA is used as substrate.

In a further experiment it was established that the Leu-AP solubilized from the membrane fraction is almost totally inhibited *in situ* by bestatin (Fig. 3B).

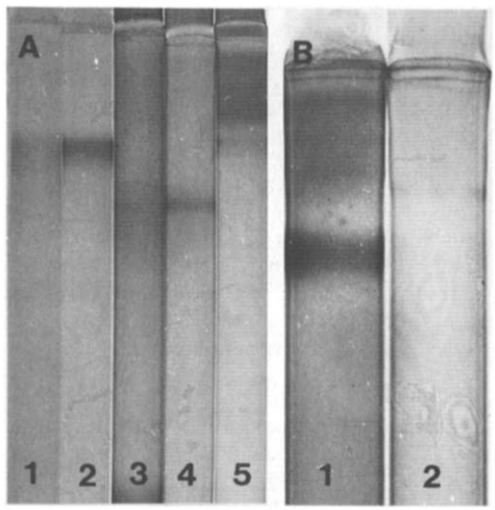

Fig. 3. Identification of cell membrane bound aminopeptidase by gel electrophoresis. (A) Separation of purified and cell membrane bound aminopeptidase: 1 and 2, purified Leu-APm (10 μg/gel) incubated with Arg-NA (1) or Leu-NA (2); 3 and 4, purified Leu-APc (40 μg/gel) incubated in the presence of Arg-NA (3) or Leu-NA (4); 5, solubilized membrane fraction (supernatant obtained after Triton extraction of 500 μg isolated membranes) incubated with Leu-NA. (B) Inhibition of membrane-bound aminopeptidase by bestatin; incubation in the absence (1) or in the presence of 320 μM bestatin (2). Disc electrophoresis and staining for *in situ* detection of enzyme activity were performed as described in Materials and Methods.

Cell type dependent activity of AP

The cell surface associated AP activity varies depending on the cell type (Table 3). The lowest activities were determined for T- and B-lymphocytes which had been isolated from spleen (40 pmoles/10^5 cells × min). Higher are the activities of the tumor cell lines leukemia L5178Y (derived from reticuloendothelial tissue) and mastocytoma P815 and X_2-P815 (derived from connective tissue); their sp. acts are around 115 pmoles/10^5 cells × min. Bone marrow cells and macrophages show high levels of AP activity (up to 1140 pmoles/10^5 cells × min). The cell surface associated activities were found to be correlated with the physiological state of the cells. As two examples we have chosen L5178Y cells and macrophages (Table 3). L5178Y cells growing *in vivo* show a 60% higher activity compared to the activity of the same cell line cultivated *in vitro*. Noteworthy is the fact that the activity of the cell surface associated AP of macrophages increases by 45% during an *in vitro* cultivation period of 1 day.

Table 4. Cell surface associated aminopeptidase activity and bestatin binding during the cell cycle of synchronized L5178Y cells

Cell cycle phase	Time after release from Thd block (hr)	Cell surface associated aminopeptidase activity (pmoles/min)				Bestatin binding (molecules)	
		Substrate Leu-NA		Substrate Arg-NA			
		Per 10^5 cells	Per surface area ($10^{-8} \times \mu m^2$)	Per 10^5 cells	Per surface area ($10^{-8} \times \mu m^2$)	Per 10^5 cells ($\times 10^{10}$)	Per surface area ($10^2 \times \mu m^2$)
S-Phase Beginning	0	115 ± 6	164 ± 9	62 ± 2	89 ± 3	17 ± 2	24 ± 3
Mid	2	153 ± 8	265 ± 14	93 ± 5	161 ± 9	32 ± 2	55 ± 3
S/G_2-phase	4	151 ± 7	228 ± 11	85 ± 4	128 ± 6	48 ± 2	73 ± 4
M/G_1-phase	7	111 ± 5	181 ± 8	n.d.	n.d.	29 ± 2	47 ± 3
S-Phase Beginning	8.5	129 ± 7	214 ± 12	60 ± 2	100 ± 3	18 ± 1	30 ± 2
Mid	10	150 ± 8	267 ± 14	71 ± 3	127 ± 5	31 ± 2	50 ± 4

The methods used are described in the text. The average (± S.D.) of five separate experiments is given. n.d. = not determined.

Using a bestatin concn of 32 μM, the lowest inhibition of the AP was determined for bone marrow cells (25%), tumor cells (20–25%) and lymphocytes (37%), while almost 70% of the activity associated with macrophages was inhibited by the drug.

Alterations of cell surface bound AP activity during the cell cycle

During the cell cycle of L5178Y cells, the cell surface associated AP activity (determined with Leu-NA) increases from 111–115 pmoles/10^5 cells × min (G_1- and onset of S-phase) to 150 pmoles/10^5 cells × min (during S- and G_2-phases) (Table 4). This change is not only due to an increase in the cell surface area which occurs during the S- and G_2-phases, but must be attributed to a higher enzyme density on the surfaces of S- and G_2-cells as well (Table 4). The cell cycle dependent alteration of the AP activity is also observed in assays containing Arg-NA as substrate (Table 4); on average the activity is 45% lower compared to the one determined in the presence of Leu-NA.

The alteration of the cell surface bound AP activity during the cell cycle correlates well with the phase-specific changes of bestatin binding sites present on the cell surface (Table 4). The highest receptor density was determined during the S/G_2-phase ($7300/\mu m^2$) and the lowest density at the onset of the S-phase ($2400/\mu m^2$).

DISCUSSION

It is well proven that bestatin is an inhibitor of both AP-B and Leu-AP (of cytoplasmic and microsomal origin) *in vitro* [32] and that it interacts with cell surface components [12]. The results in the present contribution show that the potential immunostimulant and chemotherapeutic agent bestatin interacts with the cell membrane bound Leu-APm.

In the central part of the present contribution, the identification of the target enzyme for bestatin on the cell surface was approached by comparison with the purified enzymes Leu-APc and Leu-APm. The two Leu-APs were found to be competitively inhibited by bestatin (substrate Leu-NA); the stronger effect has been determined for Leu-APc ($K_i:K_m = 6.3 \times 10^{-6}$) compared to Leu-APm ($K_i:K_m = 4.2 \times 10^{-2}$). The cell surface bound and cell membrane bound AP activity is inhibited by bestatin as well. Using Leu-NA as substrate, the inhibition is of the competitive type with a potency comparable to that determined for isolated Leu-APm ($K_i:K_m = 7.1 \times 10^{-2}$ and 2.2×10^{-1}). Using the Arg-NA substrate, which is hydrolyzed by Leu-APm as well [31], the cellular AP is inhibited non-competitively by bestatin. Considering the fact that the affinity of this substrate to the AP is much lower ($K_m = 196\ \mu M$) compared with the affinity of the Leu-NA substrate ($K_m = 58\ \mu M$), we assume that the non-competitive inhibition is due to an interaction of bestatin with the enzyme–substrate complex. The point here is that bestatin inhibits a leucine-splitting cellular activity as well as an arginine-hydrolyzing activity. Therefore, it had to be clarified whether AP-B, which hydrolyzes the basic amino acids arginine and lysine [33], is involved in the bestatin-caused inhibition of the cell surface associated AP. To prove this possibility, we have applied the microbial product amastatin as a tool; this compound has previously been shown to inhibit Leu-AP but not AP-B [3]. Our experimental data show that the cell membrane bound AP is strongly inhibited by this compound ($K_i:K_m = 5.8 \times 10^{-4}$). This fact excludes the possibility that AP-B participates in the inhibition reactions studied in the present contribution. We have also ruled out the possibility that the novel bleomycin-inactivating AP-B [34, 35] is present on the cell membrane preparations used; both the cell surface bound and the cell membrane bound AP activity are not influenced by bleomycin. The observed inefficacy of α_2-macroglobulin, a potent inhibitor of a wide variety of endopeptidases [36], on leucine- and arginine-hydrolyzing cellular AP activities excludes the possibility that endopeptidases interfere with the observed bestatin effect on cell surface associated APs.

A direct identification of the cell surface associated AP as Leu-APm was achieved by *in situ* detection of the solubilized enzyme in polyacrylamide gels. The activity released from cell membrane preparations co-migrated with the isolated Leu-APm, while its mobility in the gel differed from that characteristic for Leu-APc. Furthermore, this activity was proven to be inhibited by bestatin.

The determinations of the AP-activity detectable on the surface of a series of mammalian cells revealed the lowest activity on normal lymphocytes obtained from mouse spleen. Higher activities have been

measured on the surface of three tumor cell lines (L5178Y, X_2 and P815). Impressively high are the activities on bone marrow cells and macrophages. The finding that the level of cell surface associated Leu-APm activity is dependent on the cell type confirms earlier data [37]. We now additionally present the fact that the enzyme level is also dependent on the physiological state of the cells. *In vitro* cultivation of activated macrophages resulted in a 45% increase in measurable Leu-APm activity. Furthermore, the activity of the cell surface associated AP alters during the cell cycle. Using synchronized L5178Y cells, the lowest activity was determined during the G_1-phase. The activity increases to 140% during the S/G_2-phase. The cell cycle dependent alteration of membrane-associated Leu-APm activity correlates well with the fluctuation of the number of bestatin receptors during the cell division cycle supporting again the already substantiated assumption that Leu-APm is the target enzyme for bestatin.

In conclusion, inhibitors of cell surface associated enzymes (like bestatin) are among the most useful and promising new drugs developed to improve cancer chemotherapy at the level of enhancement of immune responses. Consequently, the knowledge of the target enzyme (e.g. Leu-APm in the present study) for a particular inhibitor will serve as an objective guideline in drug selection for the treatment of a specific human tumor.

Acknowledgements—We thank Dr F. M. Schabel (Southern Research Institute, Birmingham, AL) for helpful discussions. This work was supported by a grant from the Deutsche Krebshilfe e.V. The expert technical assistance by Ms. R. Steffen is gratefully acknowledged.

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