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SUPPRESSION OF SPLENIC ENZYME ACTIVITIES BY ADMINISTRATION OF AMINOPEPTIDASE N (CD13) INHIBITORS: RELATIONSHIP BETWEEN ACTIONS *IN VIVO* AND *IN VITRO*

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The enzymatic changes in murine spleen caused by the administration for 20 successive days of various inhibitors of aminopeptidase N (leucocyte antigen CD13) have been compared. When compared with the control (saline), most of the inhibitors significantly suppressed splenic enzyme activities including those of ectoenzymes. A multivariate study indicated that the *in vivo* effects of the inhibitors were closely related to their inhibitory actions *in vitro*.

KEY WORDS: Probestin, actinonin, leuhistin, bestatin, amastatin, arphamenine A, proteases, glycosidases, ectoenzymes

INTRODUCTION

Our previous reports suggested that ectoenzymes play important roles in maintaining the homeostasis in the body and that the networks around the ectoenzymes are significantly altered in various pathologic states including malignancies, infections, and immunological diseases, as well as in aging processes.[1]

Among various ectoenzymes, special interest has recently been directed at aminopeptidase N (AP-N),[2–5] (found to be coincident with CD13) which plays an important role in melanoma invasion into basement membranes,[6] and whose activity has been correlated to the degree of malignancy of fresh myeloid leukemia,[7] and is known to act as a cell receptor for viral infections.[8]

Based on the hypothesis that the development of inhibitors of ectoenzymes could lead to the discovery of therapeutic agents for pathologic states, we have been searching for various ectoenzyme inhibitors. Some of the inhibitors we discovered

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were actually found to have immunomodifying actions.[1,9–14] Hence, the present study was undertaken to relate the *in vitro* actions of AP-N inhibitors to their actions *in vivo*.

MATERIALS AND METHODS

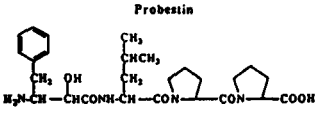
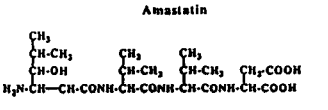
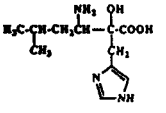
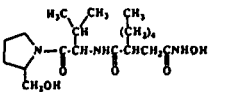
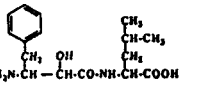
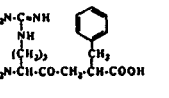
Experimental animals

Nine-week-old male mice (SPF-Balb/c Crslc, body weight 24–28 g) were obtained from Sankyo Labo Service Corporation, Tokyo, Japan. Twenty-four hours after the final injection the mice were anesthetized with ethyl ether. After the blood was drawn by cardicenthesis, various organs including the spleen were removed. Spleen homogenates were prepared in phosphate-buffered saline (PBS, pH 7.2) by use of a tissue homogenizer (Ultraturrax) at maximum speed for 1 min. The homogenate was centrifuged (3,000 × *g* for 20 min) and the supernatant fluid withdrawn for measurement of enzyme activity.

Inhibitors

The inhibitors used in this study were probestin,[9] actinonin,[10] leuhistin,[12] bestatin,[12] amastatin,[13] and arphamenine A,[14] whose structure and activity are shown in Table 1. Probestin (0.4, 2.0, 10.0 mg/kg), actinonin (0.4 mg/kg), leuhistin (0.4 mg/kg), bestatin (0.4 mg/kg), amastain (0.4 mg/kg), and arphamenine A (0.4 mg/kg) dissolved in 0.2 ml of PBS were given daily for 20 days by intraperitoneal injection. After a preliminary study using three doses of probestin, the adequate dose for administration was determined as 0.4 mg/kg for all of inhibitors. Control animals were given 0.2 ml of PBS for 20 days. Six mice were used for each group and killed on the day after the last injection.

TABLE 1
Inhibitory activities of aminopeptidase inhibitors

	Probestin	Amastatin	Leuhistin	Actinonin	Bestatin	Arphamenine A
						

	IC_{50} (μg/ml)					
	Probestin	Leuhistin	Actinonin	Amastatin	Bestatin	Arphamenine A
AP-A	>100	10.3	>100	0.54	>100	>100
AP-B	37	12.5	>100	>100	0.05	0.006
AP-N	0.06	0.12	0.24	0.58	6.2	>100

TABLE 2
The proteases studied and their substrates

Enzyme	Abbreviation	Substrate	Reference for assay method
Aspartate aminopeptidase (EC 3.4.11.7)	AP-A	Glu·NA	15
Arginine aminopeptidase (EC 3.4.11.6)	AP-B	Arg·NA	15
Leucine aminopeptidase (EC 3.4.11.1)	Leu-AP	Leu·NA	15
Aminopeptidase N (EC 3.4.11.2)	AP-N	Leu·NA	15
Proline iminopeptidase (EC 3.4.11.5)	Pro-IP	Pro·NA	16
Dipeptidyl peptidase I (EC 3.4.14.1)	DAP-I	Gly-Arg·NA	17
Dipeptidyl peptidase II (EC 3.4.14.2)	DPP-II	Lys-Ala·NA	18
Dipeptidy peptidase III (EC 3.4.14.4)	DPP-III	Arg-Arg·NA	19
Dipeptidyl peptidase IV (EC 3.4.14.5)	DAP-IV	Gly-Pro·NA	20
Prolyl endopeptidase (EC 3.4.21.26)	PEP	Z-Gly-Pro·NA	16
Trypsin (EC 3.4.22.4)	Trypsin	Boc-Gln-Ala-Arg·MCA	21
Tissue kallikrein (EC 3.4.21.35)	Kallikrein	Pro-Phe-Arg·MCA	22
Cathepsin B (EC 3.4.22.1)	Cathepsin B	Z-Arg-Arg·NA	23
α-D-Glucosidase (EC 3.2.1.20)	Glc-ase	NP-Glc	15
β-D-Galactosidase (EC 3.2.1.23)	Gal-ase	NP-Gal	15
α-D-Mannosidase (EC 3.2.1.24)	Man-ase	NP-Man	15
α-L-Fucosidase (EC 3.2.1.51)	Fuc-ase	NP-Fuc	24
N-Acetyl-β-D-glucosaminidase (EC 3.2.1.30)	GlcNAc-ase	NP-GlcNAc	15

Abbreviations used: Glu·NA, L-glutamic acid β-naphthylamide hydrochloride; Arg·NA, L-arginine β-naphthylamide hydrochloride; Leu·NA, L-leucine β-naphthylamide hydrochloride; Pro·NA, L-proline β-naphthylamide hydrochloride; Gly-Arg·NA, glycyl-L-arginine β-naphthylamide; Lys-Ala·NA, L-lysyl-L-alanine β-naphthylamide; Arg-Arg·NA, L-arginyl-L-arginine β-naphthylamide; Gly-Pro·NA, glycyl-L-proline β-naphthylamide; Z-Gly-Pro·NA, benzyloxycarbonyl-glycyl-L-proline β-naphthylamide; Boc-Gln-Ala-Arg·MCA, *t*-butyloxycarbonyl-L-glulaminyl-L-alanyl-L-arginine 4-methylcoumaryl-7-amide; Pro-Phe-Arg·MCA, L-prolyl-L-phenylalanyl-L-arginine 4-methylcoumaryl-7-amide; Z-Arg-Arg·NA, benzyloxycarbonyl-L-arginyl-L-arginine β-naphthylamide; NP-Glc, *p*-nitrophenyl-α-D-glucopyranoside; NP-Gal, *p*-nitrophenyl-β-D-galactopyranoside; NP-Man, *p*-nitrophenyl-α-D-mannopyranoside; NP-Fuc, *p*-nitrophenyl-α-L-fucopyranoside; NP-GlcNAc, *p*-nitrophenyl-N-acetyl-β-D-glucosaminide.

Substrates for enzyme assay

The sources of substrates were as follows (see Table 2)[15-24]: Glu·NA, Arg·NA, Leu·NA, Pro·NA, Gly-Arg·NA, Lys-Ala·NA, Arg-Arg·NA, and Gly-Pro·NA were from Backem Feichemi-kalien AG, Budendorf, Switzerland; Boc-Gln-Ala-Arg·MCA and Pro-Phe-Arg·MCA were from Peptide Institute Inc., Osaka, Japan; NP-Glc, NP-Gal, NP-Man,

NP-Fuc, and NP-GlcNAc were from Sigma Chemical Company, St. Louis, MO. Z-Gly-Pro·NA and Z-Arg-Arg·NA were synthesized in our laboratory, according to the method of L. Zervas *et al.*[25]

Determination of enzyme activities

The supernatant fluids of homogenates were dispensed into microwell plates (nunclone, F96, Copenhagen, Denmark) for aminopeptidases (AP) and for glycosidases and into test tubes (1.5 × 10 cm) for endopeptidases, to which the respective substrates were added, followed by incubation for 1 h at 37°C. For the AP assay, 50 μl of 2.5 mM β-naphthylamide derivative was used as the substrate, and the absorbance at 525 nm was determined using a BIO-RAD microplate reader model 3550 (Seiko-Epson Co., Ltd., Suwa, Japan). The same substrates were used for the assays of Leu-AP and AP-N, and then the activities were described in the tables as those of AP-N. For the endopeptidase assay, 20 μl of 2.5 mM 4-methylcoumaryl-7-amide derivative was used, and the fluorescence (Ex 380 nm, Em 460 nm) was determined by a HITACHI MDF-4 fluorimeter (Hitachi Co., Ltd., Hitachi, Japan). For the glycosidase assay, 50 μl of 10 mM *p*-nitrophenyl derivative was used, and the absorbance at 405 nm was determined using the microplate reader. The references for the assay methods and substrates used are listed in Table 2.[15–24]

The time course of the enzyme reaction in the spleen homogenate increased linearly for at least 60 min and the enzymatic activities in the homogenate were linear with respect to the protein concentration in the assay medium.[15] As the synthetic substrates used for the assay of endopeptidase activities can be cleaved by miscellaneous enzymes different from the target enzymes, in order to exclude such nonspecific effects bestatin was utilized to suppress extra enzyme reactions.[26] This manoeuver enabled to linearity in the endopeptidase assays.

All the enzyme assays were done in triplicate, and their standard deviation was within 10% of the average values.[12,23] For the assays, the units of enzyme activities were expressed as nmols of reaction products generated during 1 min of incubation per mg protein (nmol/min/mg protein). Protein was determined by the method of Lowry *et al.*[27]

Statistical analysis

For statistical examination of the enzymatic changes, analysis of variance (ANOVA) was used initially. Only with regard to those which were judged as significant, Bonferroni's method[28] was further applied to test the difference between the control group and each experimental group. This statistical process is known to be much more rigid when compared to the student's *t*-test, which is commonly used. The computer programs for these procedures were according to Tanaka and Tarumi.[28] Factor analysis was chosen as the method of multivariate analysis.[29] The principle of factor analysis is to assume that the observational values of each variable are composed of two parts: one specific to each variable and one related to several independent "factors," i.e., F1, F2, F3, etc. Usually we assume that the number of main factors is less than four because the assumption of too many factors, including minor ones, complicates the

analysis. For example, one may assume that the observational values of AP-A, which are standardized to make their mean zero and their variance 1.0, are regarded as the sum of the components specific to AP-A and the scores for the three main factors, a1 × F1, a2 × F2, a3 × F3, where a1, a2, and a3 are the weighing coefficients named "factor loadings." The factor loadings can be estimated from the matrix of correlation coefficients between each variable. The matrix of the scores corresponding to each sample and to each factor can be computed by multiplication of the factor loading matrix, the inverse matrix of correlation coefficients and the matrix of standardized observational values.

RESULTS

Tables 3 and 4 show the changes in various enzymatic activities in spleen after the treatment with the AP-N inhibitors. Experiments were divided into two parts because of the difficulty of performing a simultaneous study on 6 agents. Hence the results are

TABLE 3
Enzymatic changes relative to the controls in spleen induced by probestin, actinonin and leuhistin

	Specific activity±SE (nmol/min/mg protein) n=6			
Enzyme	Control	Probestin	Actinonin	Leuhistin
AP-A	0.76±0.03	0.68±0.08	0.65±0.05	0.67±0.05
AP-B	19.14±0.50	16.85±0.79*	15.10±0.23**	15.33±0.39**
AP-N	9.20±0.43	8.27±0.46	7.79±0.22	6.21±0.25**
Pro-IP	1.77±0.05	1.46±0.07**	1.50±0.04*	1.62±0.05
DPP-I	6.85±0.75	6.87±0.99	9.64±0.75	8.84±0.78
DPP-II	7.67±0.41	6.70±0.21	6.98±0.38	7.02±0.15
DPP-III	5.16±0.27	4.61±0.08	4.63±0.16	4.84±0.15
DPP-IV	2.63±0.08	2.32±0.12	2.27±0.05	2.12±0.13
PEP	3.67±0.08	3.26±0.06**	3.17±0.05**	3.25±0.09**
Trypsin	0.97±0.12	0.98±0.13	0.71±0.05	0.74±0.08
Kallikrein	0.16±0.01	0.15±0.01	0.13±0.004	0.13±0.01
Cathepsin B	34.45±1.63	36.77±2.07	33.03±0.99	33.20±1.69
Glc-ase	1.65±0.07	1.34±0.09*	1.15±0.02**	1.22±0.07**
Gal-ase	9.10±0.49	7.56±0.31	7.92±0.22	7.38±0.19*
Man-ase	1.74±0.21	1.42±0.09	1.28±0.07**	1.21±0.08**
Fuc-ase	0.54±0.07	0.42±0.07	0.51±0.08	0.46±0.04
GlcNAc-ase	83.99±4.61	68.78±1.33**	68.68±2.21**	70.50±1.07*

*$p<0.05$; **$p<0.01$; according to Bonferroni's test; SE: Standard error of mean

TABLE 4
Enzymatic changes relative to the controls in spleen induced by amastatin, bestatin and arphamenine A

Enzyme	Specific activity±SE (nmol/min/mg protein) n=5			
	Control	Amastatin	Bestatin	Arphamenine A
AP-A	1.47±0.12	1.30±0.04**	1.41±0.07	1.38±0.09
AP-B	20.76±1.45	20.35±0.82	20.93±0.68	28.12±1.49**
AP-N	11.03±0.26	9.66±0.14*	10.99±0.29	10.03±0.37
Pro-IP	2.16±0.04	2.00±0.04	2.06±0.05	1.53±0.09**
DPP-I	12.83±0.98	10.86±0.73	11.41±0.35	9.64±1.22
DPP-II	15.19±0.75	12.85±0.45	13.64±0.46	13.55±0.66
DPP-III	7.05±0.29	6.50±0.37	7.00±0.16	7.14±0.62
DPP-IV	5.42±0.18	4.62±0.10**	4.78±0.14*	4.10±0.12**
PEP	1.83±0.04	1.61±0.04**	1.58±0.02**	1.63±0.06*
Trypsin	2.71±0.06	2.11±0.14*	2.61±0.13	2.07±0.15*
Kallikrein	0.48±0.01	0.32±0.01**	0.33±0.01**	0.34±0.03**
Cathepsin B	45.94±1.61	47.62±0.90	47.98±1.42	50.16±3.33*
Glc-ase	2.14±0.07	1.87±0.04*	1.80±0.05**	1.78±0.08**
Gal-ase	14.09±0.69	12.25±0.49	11.96±0.46	10.57±0.35**
Man-ase	2.03±0.07	1.74±0.02**	1.84±0.03*	1.74±0.04**
Fuc-ase	0.73±0.03	0.73±0.04	0.64±0.02	0.63±0.05
GlcNAc-ase	98.57±1.84	86.72±4.33	90.25±1.48	87.03±4.64

*$p<0.05$; **$p<0.01$; ***$p<0.001$, according to Bonferroni's test; SE: Standard error of mean

separately shown in Tables 3 and 4. In these two tables it should be noted that the statistical significance shown (marked by asterisks) is relative to the control groups.

Table 3 shows the results of the study using probestin, actinonin, and leuhistin. The administration of probestin significantly suppressed the activities of AP-B, Pro-IP, PEP, Glc-ase, and GlcNAc-ase. The administration of actinonin suppressed the activities of AP-B, Pro-IP, PEP, Glc-ase, Man-ase, and GlcNAc-ase, while leuhistin suppressed the activities of AP-B, AP-N, PEP, Glc-ase, Gal-ase, Man-ase, and GlcNAc-ase. No significant increases in the enzymatic activities were caused by the administration of any of the above inhibitors.

Table 4 shows the results of the studies on amastatin, bestatin, and arphamenine A. The administration of amastatin significantly suppressed the activities of AP-A, AP-N, DPP-IV, PEP, trypsin, kallikrein, Glc-ase and Man-ase. Bestatin suppressed DPP-IV, PEP, kallikrein, Glc-ase, and Man-ase. Arphamenine A suppressed Pro-IP, DPP-IV, PEP, trypsin, kallikrein, cathepsin B, Glc-ase, Gal-ase, and Man-ase, but that of AP-B was significantly increased.

TABLE 5
Factor loadings showing relations to each enzyme in spleen

Enzyme	Factor 1	Enzyme	Factor 2	Enzyme	Factor 3
Kallikrein	0.86	Cathepsin B	0.77	DPP-IV	0.81
Trypsin	0.75	PEP	0.68	Gal-ase	0.76
Man-ase	0.75	DPP-I	0.67	Pro-IP	0.65
Glc-ase	0.70	DPP-III	0.64		
GlcNAc-ase	0.67				

In order to compare the inhibitory actions of the six inhibitors altogether, the differences in the enzymatic levels between the experimental animals and control animals were calculated and were analyzed by factor analysis, a method of multivariate analysis.[28,29] Namely, before the analysis the mean value of each enzymatic activity was calculated for the control groups in the two studies, and then this value was substracted from each enzymatic value in each animal. In the multivariate analysis, the data of the two studies were put together.

Table 5 shows the factor loadings, which correspond to the correlation coefficients of each factor to each enzymatic activity. It can be seen that the first factor (F1) is closely correlated to kallikrein, trypsin, Man-ase, Glc-ase and GlcNAc-ase, whereas the second (F2) is correlated to cathepsin B, PEP, DPP-I, and DPP-III. The third factor (F3) was closely correlated to DPP-IV, Gal-ase, and Pro-IP.

Of these three main factors extracted, F1 and F3 were found to be meaningful from the biological point of view as demonstrated in Figure 1 where the animal groups

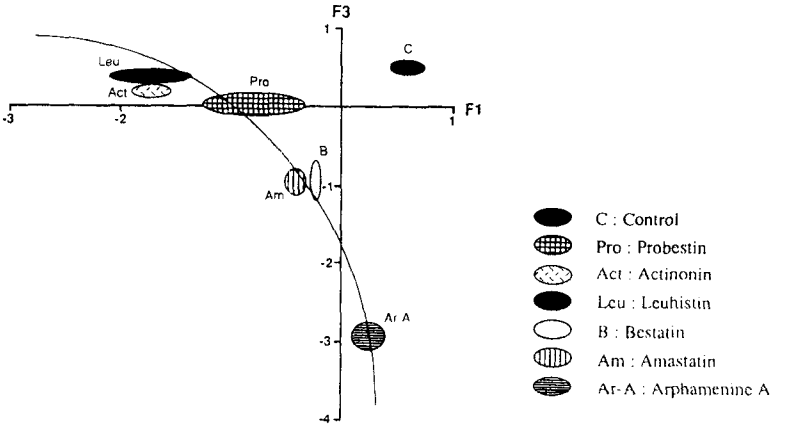


FIGURE 1 Changes in enzyme networks in spleen caused by administration of aminopeptidase inhibitors.

corresponding to the 6 inhibitors are neatly arrayed along a hyperbolic curve, whereas the control group (in which two control groups are put together) is located apart. Note that the animal groups are arrayed in the sequence arphamenine A, amastatin, bestatin, probestin, leuhistin, and actinonin. This sequence is roughly compatible with the order of their inhibitory actions *in vitro* as shown in Table 1.

DISCUSSION

In contrast to the inhibitors' actions *in vitro*, it is difficult to elucidate their actions *in vivo* because of the complexity of the environment wherein these actions occur.

Of the actions of various inhibitors of aminopeptidases *in vivo*, probably the best known are those of bestatin. It has been shown to possess immunomodulatory effects, presumably through the inhibition of multiple aminopeptidases, including the augmentation of humoral and cell-mediated immune responses and the activation of macrophages and natural killer cells to become cytotoxic against tumor cells.[30,31] Several investigators have revealed that bestatin inhibited tumor-cell invasion and the degradation of type-IV collagen by tumor cells, possibly through a mechanism involving its inhibitory action on aminopeptidases in tumor cells.[32]

In the present study, all of the six types of inhibitors of AP-N tended to suppress the hydrolytic enzyme activities in spleen. Judging from the spectrum of enzyme suppression, it is not likely that these effects were due to the direct inhibitory actions of the inhibitors. Rather, it seems reasonable to assume that the effects came from some functional changes of splenic cells induced by the immunomodulatory actions of the inhibitors. Such a notion may be endorsed by the results of the multivariate analysis.

Although the exact meaning of F1, F2, and F3 were not obtainable in the present study, the approximate arrangement of the experimental groups along a hyperbolic curve (see Figure 1) strongly suggests the actual existence of such bio-physiological factors. Moreover, the serial order of the experimental groups were roughly in agreement with the order of the inhibitory actions *in vitro*. So, the overall results were taken to indicate that the splenic enzymatic changes induced by the administration of AP-N inhibitors were closely related to their inhibitory actions seen *in vitro*.

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