VOL. 52, NO. 6, JUNE 1999 THE JOURNAL OF ANTIBIOTICS pp. 542 — 547

WF14861, a New Cathepsins B and L Inhibitor Produced by *Colletotrichum* sp.

II. Biological Properties

TAKANAO OTSUKA, YUKO MURAMATSU, KAZUAKI NIIKURA, MASANORI OKAMOTO, MOTOHIRO HINO and SEIJI HASHIMOTO

Exploratory Research Laboratories, Fujisawa Pharmaceutical Co., Ltd., 5-2-3 Tokodai, Tsukuba, Ibaraki 300-2698, Japan

(Received for publication March 16, 1999)

WF14861, 3-(*N*-(1-(*N*-(4-aminobutyl)-*N*-(3-aminopropyl)carbamoyl)-2-(4-hydroxyphenyl)ethyl)carbamoyl)oxirane-2-carboxylic acid, was obtained from the culture mycelium of *Colletotrichum* sp. as a novel cathepsins B and L inhibitor. WF14861 also showed inhibitory activities against bone derived crude protease and other cysteine proteases *in vitro*. The compound ameliorated the tissue damage and the bone destruction models of low-calcium-diet-fed mouse and adjuvant arthritis rat model.

It has been widely known that lysosomal cysteine proteases such as cathepsins B, L and K contribute to matrix turnovers in mammals. Abnormal expression and action of these enzymes lead to tissue-destructing diseases including osteoporosis, osteoarthritis, chronic inflammatory arthritis and so on[1,2]. Proper control of these proteolytic enzyme activities by a cysteine protease inhibitor could perhaps benefit the patients suffering from such diseases.

As described in a previous paper, we found that the culture of *Colletotrichum* sp. produced a novel *trans*-epoxysuccinyl type of cathepsins B and L inhibitor[3]. This paper describes the biological properties of WF14861 both *in vitro* and *in vivo*.

Materials and Methods

Materials

Human liver cathepsin B (Athens Research & Technology), bovine liver cathepsin B (Sigma) and human liver cathepsin L (Calbiochem) were purchased. Enzyme inhibitory activities were determined by measuring the release of 7-amino-4-methylcoumarin (AMC) from the fluorogenic substrate *N*-carbobenzoxy-L-Arg-L-Arg-AMC (*Z*-Arg-Arg-AMC, Peptide Institute) for cathepsin B and *N*-carbobenzoxy-L-Phe-L-Arg-AMC (*Z*-Phe-Arg-AMC, Peptide Institute) for cathepsin L. Porcine μ calpain (Nacalai tesque), Papain (Sigma), protein assay reagent (Bio-Rad) and BCA protein assay kit (PIERCE) were purchased. A low calcium diet for mice was obtained from CLEA Japan. Calcium concentration in the plasma was measured by the MXB method (Calcium E-test wako, WAKO). Fluorescence was determined using a Titertek Fluoroscan II spectrophotometer (Flow Laboratories). Absorbance was measured using a Titertek Multiscan plate reader. All other chemicals were of analytical grade.

Enzyme Assay

The methods for measuring the inhibition of cathepsins B and L activity were described in a previous paper[3]. Briefly, human liver cathepsin B inhibition was determined using 10 μM *Z*-Arg-Arg-AMC in 100 mM sodium phosphate buffer (pH 5.5) containing 5 mM dithiothreitol and 4 mM EDTA for 60 minutes at 37°C. Human liver cathepsin L inhibition was determined using 10 μM *Z*-Phe-Arg-AMC in a similar manner to that of cathepsin B inhibition assay.

Crude mouse cathepsin activity inhibition was determined by femur bone suspension of 6 week old female ICR mouse. After the removal of soft tissues, each

femur was minced and ground in a mortar with one milliliter of Hanks' balanced salt solution with calcium and magnesium. These procedures were carried out on ice. The liberation of AMC from the fluorogenic substrate Z-Phe-Arg-AMC by the mouse bone powder suspension (diluted with assay buffer to 1/12 w/v to a final, protein concentration was 32.6 μg/ml) was estimated as bone cathepsin activity. Reaction conditions were the same as the assay methods of cathepsins B and L.

The activity of μ calpain was determined by the casein degradation. The reaction was started by the addition of porcine μ calpain (0.25 μg/50 μl of reaction mixture without casein) to 50 μl of reaction mixture in 10 mM Tris-HCl buffer (pH 7.4) containing serially diluted inhibitors, 0.8 mg/ml of casein sodium salt, 10 mM of dithiothreitol and 4 mM $CaCl_2$. After 15 minutes of reaction at ambient temperature, 20 μl of reaction mixture was transferred to a new plate containing 40 μl of protein assay reagent (Bio-Rad) and 140 μl of distilled water. The reaction was monitored by measuring the absorbance at 600 nm which derived from remnant casein.

Papain inhibition was also determined by the degradation of casein. All constituents for the reaction were diluted or dissolved in 50 mM Borate-KCl buffer (pH 7.4). Final concentrations of constituents needed for the assay were as follows: papain (10 μg/ml), casein sodium salt (10 mg/ml, adjusted to pH 7.4 with 1 N NaOH), cysteine (0.2 mg/ml). Reaction mixtures in microtest tubes were incubated for 30 minutes at 37°C. The reaction was stopped by the addition of 0.44 M trichloroacetic acid (TCA, 500 μl). After the TCA precipitation of residual casein for 1 hour at room temperature, test tubes were centrifuged at 12,000 rpm for 5 minutes. To monitor the degradation of casein, 20 μl of supernatant was withdrawed and diluted five times with water and measured by the BCA protein assay kit.

All reactions using synthetic substrates *in vitro* were performed in microtiter plates. The fluorescence or the absorbance of the reagent blanks (without enzyme) was subtracted from test readings. Calibration was performed with different concentration of free AMC. All data are the average of at least duplicate determinations.

Inhibition against degradation of natural substrate using rat cartilage by bovine cathepsin B was also evaluated. Femoral heads were removed from 9~10 weeks old female Lewis rats and steamed for 60 minutes to inactivate the endogeneous proteases. After washing twice with PBS (−) and removing the soft tissues, femoral heads were used as substrate. Each femoral head was incubated with inhibitor and bovine cathepsin B in a 24 well plate for 17 hours at 37°C ($n=3$). Total volume was 500 μl and the final concentrations of the constituents in sodium-acetate buffer (100 mM, pH 5.5) were as follows: bovine cathepsin B (50 μg/ml), dithiothreitol (5 mM) and EDTA (1 mM). The release of glycosaminoglycan (GAG) into the buffer by bovine cathepsin B from femoral head was measured by the method reported by YAMAMOTO *et al.*[4]. Results were expressed as means ± SE.

Low-calcium-diet-fed Mouse Model

Inhibition of bone resorption in low-calcium-diet-fed mice were evaluated by the modified method of J.-M. DELAISSÉ *et al.*[5]. Three week old female ICR mice, fed for 6 days with a 0.003% calcium diet, were injected subcutaneously with WF14861 or E-64 ($n=5$ for each dose and $n=6$ for the control). After the injection of sample, peripheral blood was withdrawn from eye socket by heparinized capillary tube under halothane anesthesia. Calcium concentration in the plasma was monitored at 0, 1, 2, 4, 6 hours by calcium assay kit. Results were expressed as means ± SE.

Rat Adjuvant Arthritis Model

Induction and evaluation of adjuvant arthritis were performed following the method by YAMAMOTO *et al.*[4]. Briefly, arthritis was induced on day 0 by the injection of 0.05 ml of adjuvant (suspension of heat killed *Mycobacterium tuberculosis* H37RA in heavy mineral oil) into the right hind foot pad of female Lewis rats (150~200 g, $n=10$). WF 14861 was subcutaneously administered every day throughout the experiment. On day 17, body weights and the volume of foot pads were measured. In addition, serological parameters, bone mineral densities, glycosaminoglycans of articular cartilages and bone destruction scores were also measured. Results were expressed as means ± SE.

Student's t test ($^{*}p<0.05$, $^{**}p<0.01$, $^{***}p<0.001$) and paired Student's t test ($^{¶}p<0.05$, $^{¶¶}p<0.01$) were used for statistical analysis of the results. Dunnett Multiple Comparison test ($^{\#}p<0.05$, $^{\#\#}p<0.01$) and Wilcoxon test ($^{\dagger\dagger\dagger}p<0.01$) were also used for statistical analysis of the results.

Results

Enzyme Inhibition *in vitro*

As shown in Fig. 1, WF14861 inhibited human

cathepsins B and L dose-dependently with IC_{50} values of 1.6×10^{-8} M and 1.1×10^{-9} M respectively. IC_{50} values of WF14861 on cysteine proteases were listed in Table 1. E-64, Leupeptin and Chymostatin were used as reference compounds. WF14861 strongly inhibited the action of cathepsins B and L. In addition, WF14861 inhibited mouse crude bone cathepsin with IC_{50} value of 4.0×10^{-8} M when *Z*-Phe-Arg-AMC was used as substrate. This assay system may reflect the actual *in vivo* bone resorption inhibitory activity evoked by proteases such as cathepsins B, L and K. On the other hand, it weakly inhibited μ calpain and papain comparing with cathepsins tested. Furthermore, WF14861 and E-64 didn't inhibit serine proteases including bovine chymotrypsin, bovine trypsin, human cathepsin G and human elastase even at 2.4×10^{-4} M (data not shown). These results suggest that WF14861 is likely to inhibit cysteine proteases specifically.

Bovine cathepsin B released 287.3 ± 31.0 μg/ml of GAG from each rat femoral head. Spontaneous release of GAG without cathepsin B in our assay condition was 34.0 ± 2.00 μg/ml. Fig. 2 shows that both WF14861 and E-64 inhibited the GAG release dose-dependently with IC_{50} values of 0.277 μg/ml (6.56×10^{-7} M) and 0.274 μg/ml (7.68×10^{-7} M) respectively. These results suggest that cathepsin B can attack natural cartilage and WF14861 and E-64 can inhibit its action. In place of femoral head, fluorescein isothiocyanate (FITC)-labeled type I collagen was used as natural substrate. Degradation of collagen was monitored by the release of FITC into reaction buffer. Bovine cathepsin B

Fig. 1. Inhibiton of human cathepsins B and L by WF14861.

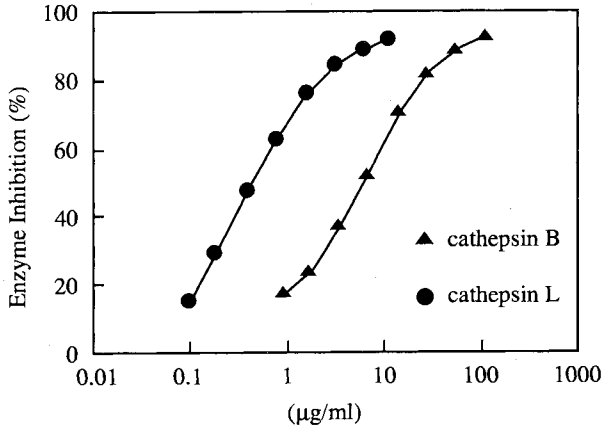
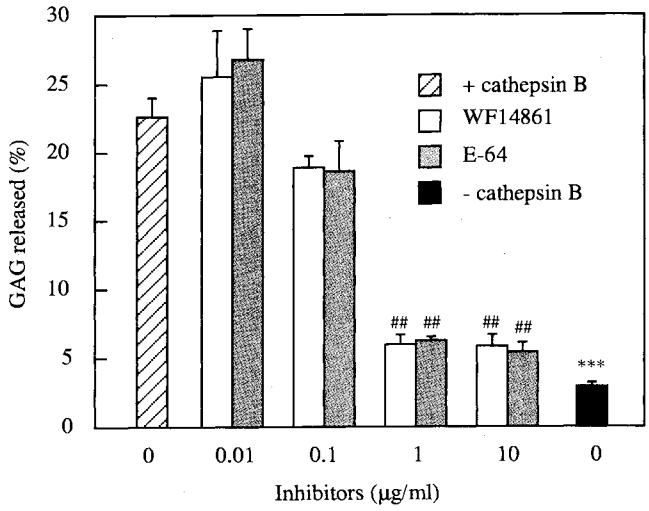


Fig. 2. Inhibition of cathepsin B-induced GAG release from rat femoral head by WF14861 and E-64.

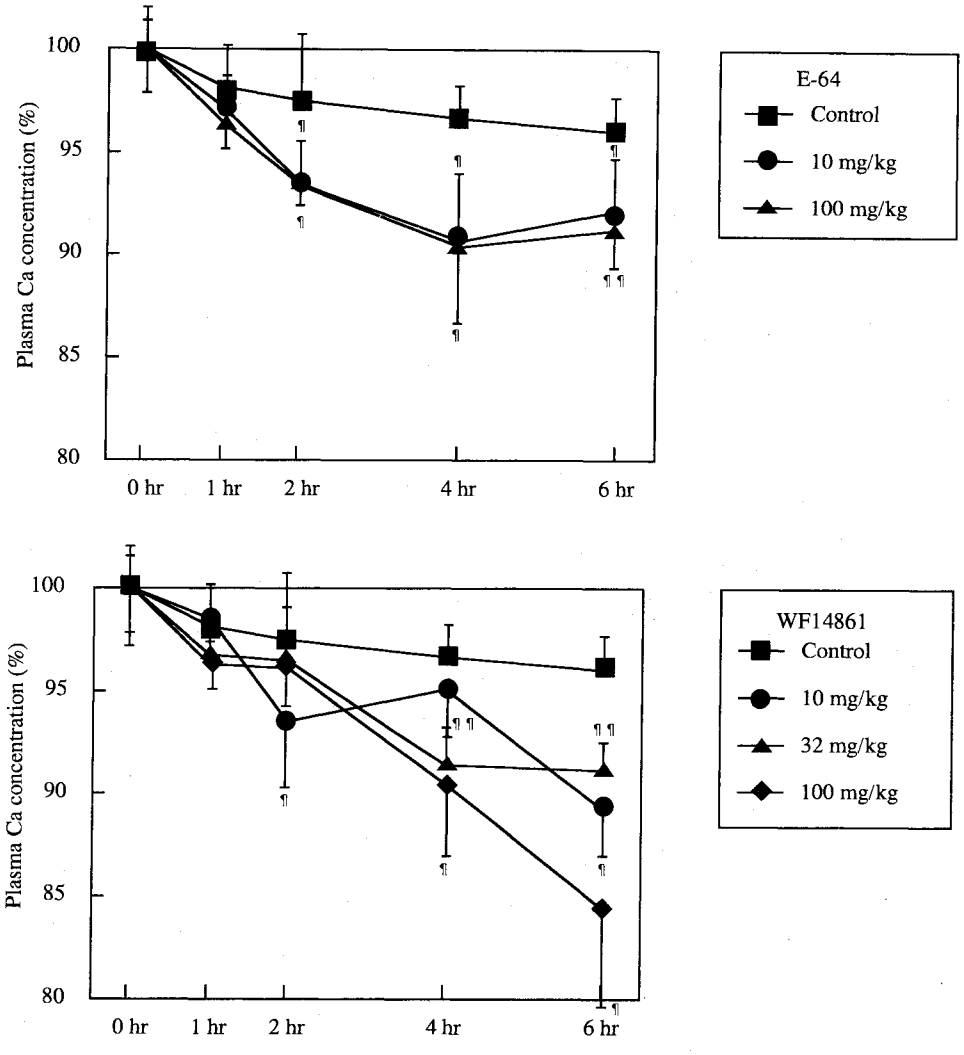


Statistically different from the + cathepsin B group by Student's t test (*** $p<0.001$). The significance of the difference between WF14861 or E-64 *versus* + cathepsin B by Dunnett Multiple Comparison test (## $p<0.01$).

Table 1. IC_{50} values of WF14861 and reference compounds against cysteine proteases ($\times 10^{-9}$ M).

	Human Cathepsin B	Human Cathepsin L	Mouse Cathepsins	Porcine μ Calpain	Papain
WF14861	16	1.1	40	95000	850
E-64	55	68	6.1	4100	580
Chymostatin	8300	12	200	28000	86000
Leupeptin	19	1.5	8.0	1700	2200

Fig. 3. Effects of WF14861 and E-64 on low-calcium-diet-fed mouse model.



Statistically different from the initial plasma calcium concentration by paired Student t test ($¶p<0.05$, $¶¶p<0.01$).

degradaded collagen and both WF14861 and E-64 inhibited the release of FITC at similar extent as GAG assay (data not shown).

Effect of WF14861 on *In vivo* models

The homeostasis of plasma calcium concentrations of mice fed with low-calcium-diet depend on their bone calcium turnovers. Normal diet contains 1.25% calcium, whereas a 0.003% calcium diet was used in this experiment. Inhibition of bone resorption will certainly decrease the plasma calcium level. The results of injection of WF14861 and E-64 to low-calcium-diet fed mice are shown in Fig.3. The control group of animals did not show good statistical validity throughout this experiment. Both compound showed a time-dependent decrease of plasma calcium concentration. Compared with an initial plasma calcium level (11.98 ± 2.17 mg/dl), the group injected with 100 mg/kg of E-64 lowered its level to 10.92 ± 0.22 mg/dl (91.15 ± 1.84%) in 6 hours. Similarly, the group injected with 100 mg/kg of WF14861 lowered the plasma calcium concentration from 11.70 ± 0.33 mg/dl to 9.88 ± 0.57 mg/dl (84.0 ± 4.87%) in 6 hours. The effect of WF14861 was stronger than that of E-64 in this model.

Since WF14861 exerted its effect in an *in vivo* model,

Table 2. Effect of WF14861 on rat adjuvant arthritis model.

	Body weight [a, b] (g)	Bone mineral density [a, b] (mg/cm^3)	Increased paw volume [a,b] (ml) injected	uninjected	Bone destruction [a, c] (score)
Normal	195 ± 2 ***	562.6 ± 3.6 ***	0.00 ± 0.04 ***	0.00 ± 0.04 ***	0 ± 0***
Control	162 ± 2	314.8 ± 15.5	3.12 ± 0.14	2.30 ± 0.13	7 ± 1
10 mg/kg	161 ± 2	336.1 ± 14.8	3.33 ± 0.16	2.03 ± 0.19	7 ± 1
32 mg/kg	165 ± 1	349.4 ± 10.2	2.54 ± 0.17 #	1.80 ± 0.08 #	5 ± 1
100 mg/kg	174 ± 3 ##	406.5 ± 13.1 ##	0.92 ± 0.11 ##	0.66 ± 0.07 ##	0 ± 0†††

	ESR [a, b] (mm/hr)	Fibrinogen [a, b] (mg/dl)	Sialic acid [a, b] (mg/dl)	GAG [a, b] (mg/femoral condyle)
Normal	0.3 ± 0.0 ***	111.5 ± 2.3 ***	56.4 ± 0.6 ***	448.8 ± 8.9 ***
Control	11.7 ± 1.0	494.1 ± 3.5	129.4 ± 4.0	347.8 ± 8.2
10 mg/kg	9.5 ± 0.9	478.4 ± 6.0	124.8 ± 2.5	381.0 ± 9.3
32 mg/kg	9.2 ± 1.1	468.4 ± 7.0	123.6 ± 4.5	357.8 ± 13.0
100 mg/kg	1.5 ± 0.4 ##	274.8 ± 21.4 ##	74.5 ± 4.1 ##	399.5 ± 8.2 ##

[a, b]; Statistically different from the arthritis control group by Student's t test (*** $p < 0.001$). The significance of the difference between WF14861-treated rats and arthritis control group by Dunnett Multiple Comparison test (# $p < 0.05$, ## $p < 0.01$).

[a, c]; Statistically different from the arthritis control group by Student's t test (*** $p < 0.001$). The significance of the difference between WF14861-treated rats and arthritis control group by Wilcoxon test (††† $p < 0.01$).

it was then evaluated in a chronic inflammation model. The effect of WF14861 on several parameters in the rat adjuvant arthritis model was shown in Table. 2. The group injected with 100 mg/kg of WF14861 responded positively in all of the parameters we tested. It reversed the decrease of body weight and bone mineral density and suppressed edema formation. Bone destruction was scored by the X-ray photograph of each heel. The group injected with 100 mg/kg of WF14861 showed complete inhibition when the bone destruction score was used. WF14861 improved the general indicators of inflammation such as erythrocyte sedimentation rate (ESR), plasma fibrinogen and sialic acid. The decrease of GAG content in articular cartilage was also improved in the 100 mg/kg injected group. These results suggest the usefulness of WF14861 against chronic inflammation.

Acute toxicity of WF14861 was also determined. WF14861 was intraperitoneally given (300 mg/kg in saline) to five ICR strain mice (female, 5 weeks old). After one-week of observation, no abnormal symptoms were recognized.

Discussion

A variety of *trans*-epoxysuccinyl peptides and their derivatives have been reported as cysteine protease inhibitors from microbial origins. However, their *in vivo* biological properties, especially their effects on pathological models, have not been extensively reported[5~7].

At first, we confirmed the *in vitro* properties of WF14861 against several cysteine proteases including cathepsins B, L, bone-derived cathepsin like protease, μ calpain and papain using synthetic or natural substrates. WF14861 specifically inhibited cathepsins which had been reported as key enzymes for bone resorption. Since these results showed the possibility that WF14861 may be active *in vivo*, we then evaluated its effects in two bone destructing animal models.

Subcutaneous injection of E-64 and WF14861 lowered plasma calcium levels in low-calcium-diet fed mouse model. This result suggests that these compounds are bioavailable and that the inhibition of cysteine protease activities prevent bone resorption.

As a result of the evaluation of WF14861 in the rat adjuvant arthritis model, we showed that it reduced the cartilage and bone destruction as expected by its inhibition of cathepsins B and L. In addition to these essential effects, WF14861 inhibited even acute and chronic inflammation. Certainly, it has been reported that synthetic peptidyl fluoromethyl ketones also inhibited cathepsin B *in vitro* and lowered the severity of inflammation as well as the disorder of cartilage and bone in adjuvant-induced arthritis[8,9]. Inhibition of WF14861 against pure cathepsin K has not yet been determined. Since E-64 inhibits cathepsin K, WF14861 may also show similar effect[10].

These results suggest that cathepsins B and L or other enzymes, in other words, *trans*-epoxysuccinyl peptides sensitive enzymes, are likely to participate in bone resorption *in vivo*.

References

1) MACIEWICZ, R. A. & S. F. WOTTON: Degradation of cartilage matrix components by the cysteine proteinases, cathepsins B and L. Biomed. Biochim. Acta 50: 561～564, 1991
2) FLIPO, R.-M.; G. HUET, F. MAURY, F. ZERIMECH, C. FONTAINE, B. DUQUESNOY, P. DEGAND & B. DELCAMBRE: Cysteine-proteolytic activities (cathepsins B, L and calpains) in rheumatoid synovium. Arthritis & Rheum. 36: S252, 1993
3) OTSUKA, T.; Y. MURAMATSU, T. HIGAKI, T. NAKANISHI, S. TAKASE, H. HATANAKA, M. OKAMOTO, M. HINO & S. HASHIMOTO: WF14861, a new cathepsins B and L inhibitor produced by *Colletotrichum* sp. I. Taxonomy, production, purification and structure elucidation. J. Antibiotics 52: 536～541, 1999
4) YAMAMOTO, N.; F. SAKAI, H. YAMAZAKI, Y. KAWAI, K. NAKAHARA & M. OKUHARA: Effect of FR133605, a novel cytokine suppressive agent, on bone and cartilage destruction in adjuvant arthritic rats. J. Rheumatol. 23: 1778～1783, 1996
5) DELAISSÁ, J.-M.; Y. EECKHOUT & G. VAES: *In vivo* and *in vitro* evidence for the involvement of cysteine proteinases in bone resorption. Biochem. Biophys. Res. Commun. 125: 441～447, 1984
6) GOUR-SALIN, B.; P. LACHANCE, C. PLOUFFE, A. C. STORER & R. MÉNARD: Epoxysuccinyl dipeptides as selective inhibitors of cathepsin B. J. Med. Chem. 36: 720～725, 1993
7) TOWATARI, T.; T. NIKAWA, M. MURATA, C. YOKOO, M. TAMAI, K. HANADA & N. KATUNUMA: Novel epoxysuccinyl peptides. A selective inhibitor of cathepsin B, *in vivo*. FEBS 280: 311～315, 1991
8) AHMED, N. K.; L. A. MARTIN, L. M. WATTS, J. PALMER, L. THORNBURG, J. PRIOR & R. E. ESSER: Peptidyl fluoromethyl ketones as inhibitors of cathepsin B. Biochem. Pharmacol. 44: 1201～1207, 1992
9) ESSER, R. E.; L. M. WATTS, R. A. ANGELO, L. P. THORNBURG, J. J. PRIOR & J. T. PALMER: The effects of fluoromethyl ketone inhibitors of cathepsin B on adjuvant arthritis. J. Rheumatol. 20: 1176～1183, 1993
10) AIBE, K.; H. YAZAWA, K. ABE, K. TERAMURA, M. KUMEGAWA, H. KAWASHIMA & K. HONDA: Substrate specificity of recombinant osteoclast-specific cathepsin K from rabbits. Biol. Pharm. Bull. 19: 1026～1031, 1996