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PEPTIDYL FLUOROMETHYL KETONES AS INHIBITORS OF CATHEPSIN B

IMPLICATION FOR TREATMENT OF RHEUMATOID ARTHRITIS

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Abstract—Peptidyl fluoromethyl ketones (FMKs), with the amino acid sequence Phe-Ala held constant but with variable N-terminal groups, were synthesized and tested for inhibition of the cysteine proteinase cathepsin B. The FMKs were effective in inhibiting cathepsin B activity *in vitro*. The inhibition was time dependent and was not reversed by dialysis, suggesting covalent modification of the enzyme. Cathepsin B activity present in livers and kidneys of rats treated with FMKs was reduced by 22–91% 4 hr after a single oral dose of 25 mg/kg. The FMKs inhibited the severity of inflammation and the extent of cartilage and bone damage in adjuvant-induced arthritis. These effects were seen during the late-stage of the disease with no effect on onset or incidence of disease. This is consistent with inhibition of protease-mediated damage. These FMKs or derivatives may be of clinical value in the treatment of arthritis.

In the extracellular matrix of cartilage, assemblies of proteoglycan and collagen molecules interact to produce a rigid gel on which normal joint function depends. Destruction of the extracellular matrices of articular cartilage and bone may lead to loss of joint function. In arthritis cathepsins and other lysosomal proteases are released from polymorphonuclear leukocytes, synoviocytes and chondrocytes into the synovial fluid and adjacent inflamed tissues. These proteinases are considered important mediators in the degradation of collagen and proteoglycan [1–5]. Immunocytochemical studies revealed the presence of cathepsins B and D in rheumatoid articular cartilage [6]. Additionally, the synovial lining tissue and synovial fluid of rheumatoid arthritis patients have been found to contain high concentrations of cathepsin B [7, 8]. Other studies have demonstrated increased cathepsin B activity in synoviocytes, fibroblasts, and chondrocytes after experimentally induced arthritis [9–11]. It may therefore be of therapeutic value to develop selective cathepsin B inhibitors.

Microbial compounds such as antipain and leupeptin are effective but less specific acting inhibitors of both serine and thiol proteases. Peptidyl diazomethyl ketones are potent, irreversible inhibitors of cysteine proteinases, but are known to have toxic effects on protein synthesis and are mutagenic.

Peptidyl chloromethyl ketones inhibit cathepsin B, but have shown a lack of promise in the treatment of disease. They are strongly electrophilic and indiscriminately alkylate non-target molecules present under *in vivo* conditions [12, 13]. It would be desirable to provide inhibitors which do not produce this indiscriminate alkylation. It has been hypothesized that peptidyl fluoromethyl ketones (FMKs§) would possess the desired reduced alkylating potential [14].

We have examined a series of six peptidyl FMKs with a fixed amino acid sequence (Phe-Ala) and varying N-terminal blocking groups for inhibition of cathepsin B activity. A variety of blocking groups were tested since they can greatly affect the overall hydrophobocity of the compounds as well as *in vivo* stability, adsorption, metabolism, and distribution. The FMKs were shown to be potent irreversible inhibitors of cathepsin B activity *in vitro*, and were active in an *ex vivo* model of cathepsin B activity. In addition, two of the compounds were shown to decrease the severity of adjuvant-induced arthritis in rats.

MATERIALS AND METHODS

Two cathepsin B sources were used for kinetic analysis and determinations of k_{app}/I. Purified human liver cathepsin B was obtained from Enzyme Systems Products (Dublin, CA), specific activity 8330 mU/mg protein. For other *in vitro* studies, bovine spleen cathepsin B (Sigma Chemical Co., St. Louis, MO) was used. The substrate Z-Arg-Arg-7-amino-4-methylcoumarin (Z-Arg-Arg-AMC) was from Enzyme Systems Products. Dithiothreitol (DTT), purchased from the Sigma Chemical Co., and all other chemicals were of analytical reagent grade.

Animals used in this study were female Sprague–Dawley rats obtained from Sasco (Omaha,

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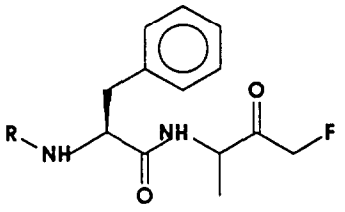
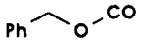
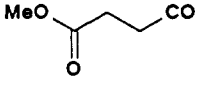
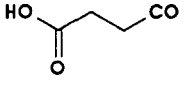
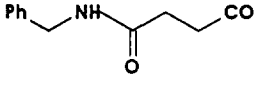
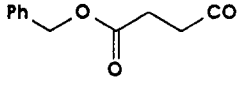
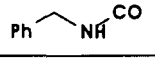
§ Abbreviations: FMKs, peptidyl fluoromethyl ketones; Z-Arg-Arg-AMC, Z-Arg–Arg-7-amino-4-methylcoumarin; DTT, dithiothreitol; and PBS, phosphate-buffered saline.

Table 1. Summary of cathepsin B/fluoromethyl ketone inhibition kinetics



MDL	R	[I] (μM)	k_{app}/I^* (I/mol/sec)
201,000		0.3	12,300
201,003		0.2	3000
201,004		0.3	900
201,005		0.3	3900
201,006		0.2	21,000
201,022		0.5	2800

* The percent standard deviation when multiple determinations were made was 36% (N = 6). (The rate of hydrolysis of substrate in the absence of inhibitor was 25 pmol/min).

NE) or from Charles River Breeding Laboratories (Portage, MI).

Ex vivo *inhibition of tissue cathepsin B activity.* FMKs were suspended in phosphate/buffered saline (PBS), sonicated briefly to achieve an even suspension, and then administered by gavage using an 18-gauge stainless steel feeding needle. The FMKs were administered at a dose of 25 mg/kg body weight in a total volume of 1 mL. Control rats were given an equal volume of PBS alone. Four hours after FMK administration, the rats were killed by CO_2 asphyxiation, and the liver and kidneys were harvested. The tissues were stored at −70° until processed. Liver and kidney tissues at a concentration of 2% (w/v) were homogenized in 100 mM phosphate buffer (pH 6.0) containing 2 mM EDTA and 0.2% (v/v) Triton X-100 at 4°. The homogenates were centrifuged for 20 min at 4° at 13,000 *g* and supernatants were stored in aliquots at −20° for determination of cathepsin B activity.

Determination of cathepsin B activity. Cathepsin B activity was determined by following the release of 7-amino-4-methylcoumarin (AMC) from the fluorogenic peptide Z-Arg-Arg-AMC at an excitation of 360 nm and an emission at 460 nm [12]. The reaction mixture, in a final volume of 1 mL, contained 5 mM DTT, 2.5 mM EDTA, sodium phosphate buffer (pH 6), and 10 ng/mL bovine spleen cathepsin B or 2–10 μL of tissue homogenate. FMKs were present as indicated. The reaction was initiated by the addition of Z-Arg-Arg-AMC. The mixture was incubated at 37° for 30 min and the reaction was stopped by the addition of 1 mL of 100 mM sodium monochloroacetate in a buffer containing 30 mM sodium acetate and 70 mM acetic acid (pH 4.3). The fluorescence was determined using a Perkin–Elmer LS-3 fluorescence spectrophotometer. The fluorescence of the reagent blanks (without enzyme) was subtracted from test readings. Calibration was performed with different concentrations of free

AMC (0.05 to 5.0 μM). A milliunit of enzyme activity is defined as the amount of enzyme which converts 1 nanomole of substrate per minute under the standard conditions of assay. Specific activity was expressed as milliUnits per milligram of protein.

Determination of pseudo-first order rate of inactivation for a typical experiment. One hundred microliters (50 mU/mL) of human cathepsin B were added to 1.87 mL of buffer A (buffer A: 50 mM sodium phosphate buffer, pH 6.2, containing 2 mM EDTA and 5 mM DTT), at 22°. The reaction was initiated by the addition of 30 μL of a 20 μM inhibitor stock in buffer A at time 0 (final inhibitor concentration = 0.3 μM). Immediately after initiating the reaction, and then at approximately 2-min intervals, the remaining cathepsin B activity was measured by adding 10 μL of the enzyme inhibitor mixture to 440 μL of 0.5 mM Z-Arg-Arg-AMC in buffer A in a 0.5-mL cuvette at 22°. The activity was followed using a spectrofluorometer as described above. The observed pseudo-first order rate constant was determined from the slope of the line of a plot of ln (initial velocity) versus time. Dividing by inhibitor concentration gives k_{app}/I [15].

Dialysis study. Two enzyme preparations containing 12.5 ng/mL cathepsin B in phosphate buffer (pH 6) with DTT and EDTA were incubated for 30 min at 37°. No FMK was present in the control solution, while the test solution contained 0.0625 μM MDL 201,000. The solutions were then dialyzed for 19 hr in buffer with DTT and EDTA at 4°. Dialysis buffer was replaced every 6 hr. When dialysis was discontinued, the enzyme solutions were assayed for cathepsin B activity.

Induction and evaluation of arthritis. Rats (120 g) were injected intradermally at the base of the tail with 0.25 mg of heat-killed *Mycobacterium tuberculosis* H37Ra (Difco Laboratories, Detroit, MI) suspended in light paraffin oil. At periodic intervals following adjuvant injection, the severity of inflammation in each hind paw was scored clinically on a scale of 9 (normal) to 4 (severe inflammation) based on the number of joints involved, the severity and extent of periarticular erythema and edema, and the enlargement and distortion of the joints. Clinical scores were expressed as the means of the right and left hind paws [16]. Hind paw volumes were measured using a Ugo Basile plethysmometer (Stoelting Co., Wood Dale, IL). At the termination of each experiment, the hind paws were removed and placed in 10% buffered formalin. The tissues were decalcified, embedded in paraffin, cut in 5 μm sections, and stained with hematoxylin and eosin. Each tissue section was evaluated for evidence of destruction of articular cartilage and bone by three different criteria: (1) the percent of articular surfaces with focal ulcers which resulted in exposure of the underlying subchondral bone; (2) the percent of articular cartilage lost from the joint formed by the articulation of the tibia, tibial tarsal, and fibular tarsal bone; (3) the percent of articular surfaces with destruction in the subchondral bone. FMKs were administered in certified ground rodent diet (Purina, Richmond, IN). FMK treatment was initiated the day of adjuvant injection and continued until the termination of the experiment. Food consumption was measured weekly, and the average daily dose of MDL 201,000 and MDL 201,003 was calculated from the quantity of food consumed.

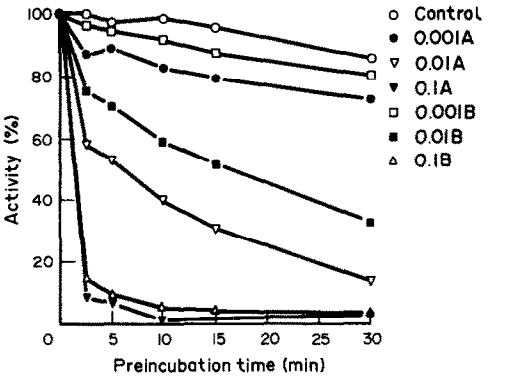


Fig. 1. *In vitro* change in cathepsin B activity by MDL 201,000 (A) and MDL 201,003 (B) as a function of incubation time of enzyme with inhibitor. Activity was measured in the presence of 0.001, 0.01, and 0.1 μM concentrations of (A) and (B). The assay was conducted as described in Materials and Methods using 200 μM Z-Arg-Arg-AMC. Control activity = 0.055 mU.

Fluoromethyl ketone synthesis. The carbobenzoxy (Z) protecting group on MDL 201,000 was removed and new acyl groups were introduced at the phenylalanine nitrogen by standard methods. All compounds were characterized on the basis of ^{1}H-NMR, ^{19}F-NMR, and IR. All compounds exist as a mixture of diastereomers at the alanine residue. The chemical and diastereometic purity of all compounds synthesized was ≥98.5% as determined by high-performance liquid chromatography.

Protein determination. Protein concentration was measured by the Pierce (BCA) standard essay using bovine serum albumin as a standard [17].

RESULTS

Effect of FMKs on cathepsin B activity in vitro. A series of FMKs were synthesized in which the Phe-Ala sequence was held constant, but the structure of the N-terminal blocking group was varied. The structures of the N-terminal groups of each member of the series and the effects of the FMKs on purified cathepsin B *in vitro* are shown in Table 1. The values for k_{app}/I varied over an approximately 20-fold range, suggesting that the N-terminal blocking group contributes to the interaction of the FMKs with cathepsin B. The low apparent rates are probably not due to insolubility of the inhibitor. The solubility of the slower reacting MDL: 201,003 was found to be 60 times that of MDL 201,000. Figure 1 illustrates the increase in inhibition of enzyme activity over time with several concentrations of MDL 201,000 and MDL 201,003. After 15 min of preincubation of cathepsin B with 0.01 μM MDL 201,000 and MDL 201,003, the inhibition was 68 and 43%, respectively. After 30 min of preincubation of cathepsin B with 0.01 μM MDL 201,000 and MDL 201,003, inhibition

Table 2. Inhibition of cathepsin B activity by MDL 201,000 after dialysis

	Activity (mU)*	% Activity	% Inhibition
Control	0.044	100	0
MDL 201,000 (0.0625 μM)	0.0036	8	92

Reaction mixtures contained 12.5 ng/mL cathepsin B (bovine spleen from Sigma), 5 mM DTT, 2.5 mM EDTA, and 250 mM sodium phosphate buffer, pH 6. The control solution contained no MDL 201,000; the experimental solution contained 0.0625 μM MDL 201,000. After 19 hr of dialysis, solutions were assayed for cathepsin B activity (in triplicates) as described under Materials and Methods.

* A milliUnit (mU) is defined as the amount of cathepsin B required to hydrolyze 1 nmol of Z-Arg-Arg-AMC/min.

was 89 and 63%, respectively. This pattern of time-dependent inhibition is suggestive of irreversible inhibition. The irreversibility of cathepsin B by FMKs was confirmed by dialysis of the enzyme–inhibitor complex. After 19 hr of dialysis, the enzyme incubated with MDL 201,000 had 8% of the activity of that of control enzyme dialyzed for the same amount of time (Table 2).

Effect of FMKs on cathepsin B activity ex vivo. All six of the FMKs were evaluated for their effectiveness as inhibitors of cathepsin B *ex vivo*. It is known that the highest level of cathepsin B activity in rats is seen in the kidney, liver, and spleen while low activity has been observed in other organs [10]. Accordingly, rat liver and kidney tissue homogenates were measured for cathepsin B activity 4 hr after a single 25 mg/kg oral dose of FMK. The effectiveness of the FMKs as cathepsin B inhibitors was determined by comparing cathepsin B activity in the tissues of FMK-treated animals to cathepsin B activity of control animals treated with vehicle (PBS) alone. As shown in Table 3, orally administered FMKs inhibited cathepsin B activity in liver and kidney homogenate from 22 to 91%. There was no obvious direct correlation between inhibition of purified cathepsin B *in vitro* and *ex vivo* activity of the FMKs. This may be due to differences in absorption, stability *in vivo*, metabolism or other characteristics of FMKs *in vivo*.

Effect of FMK treatment on the severity of adjuvant-induced arthritis. The effects of MDL 201,000 and MDL 201,003 on the course and severity of adjuvant-induced arthritis are shown in Figs. 2 and 3. Clinical scores of FMK-treated rats were comparable to those of control rats during the early phase of disease, but were lower later in the course of inflammation. Statistically significant decreases in the paw volumes of FMK-treated rats were consistently observed during the late-stage of the disease. The effects of FMK treatment on histological measures of cartilage and bone destruction are shown in Table 4. Both MDL 201,000 and MDL 201,003 inhibited tissue destruction, which decreases in histological measures of articular cartilage and bone loss ranging from 51 to 77% in FMK-treated rats. However, due to the variability in the severity of histological lesions, statistically significant differences between FMK-treated and control groups were observed in only four of the six histological comparisons. FMK administration did not affect average daily food consumption or body weight over the 32-day treatment period (Table 5), suggesting that the effects of treatment on the severity of arthritis were not due to overt FMK toxicity.

Table 3. Cathepsin B activity in rat liver and kidney tissue homogenates after treatment with 25 mg/kg FMKs

		Liver homogenates			Kidney homogenates		
		Specific activity (mU/mg protein)*	% Inhibition	CV†	Specific activity (mU/mg protein)*	% Inhibition	CV†
Control	N = 30‡ x = 134§	3.29		16.0	21.15		16.7
MDL 201,000	N = 16 x = 62	0.97	70.5	89.3	12.89	39.0	21.9
MDL 201,003	N = 19 x = 74	0.43	86.9	32.9	9.02	57.3	26.8
MDL 201,004	N = 14 x = 54	2.36	28.3	21.3	16.03	24.2	26.3
MDL 201,005	N = 13 x = 52	0.60	81.8	52.2	5.21	75.4	40.4
MDL 201,006	N = 19 x = 76	2.56	22.2	22.9	14.85	29.8	23.5
MDL 201,022	N = 13 x = 52	0.29	91.2	49.5	4.48	78.8	51.0

* A milliUnit (mU) is defined as the amount of cathepsin B required to hydrolyze 1 nmol of Z-Arg-Arg-AMC/min.
† CV: coefficient of variance as determined by SAS.
‡ N = number of animals.
§ x = number of observations.

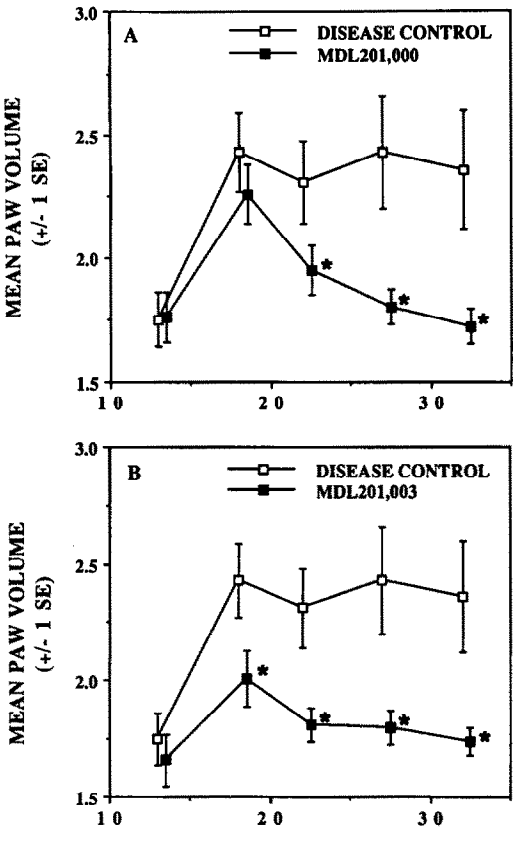


Fig. 2. Effects of MDL 201,000 (A) and MDL 201,003 (B) on paw volume increases in adjuvant-induced arthritis. Rats were injected with adjuvant on day 0 and treated with either MDL 201,000 (N = 16) or MDL 201,003 (N = 15) mixed with ground diet from the time of adjuvant injection being killed on day 32. Control rats (N = 16) were fed ground diet without added FMKs. The average daily dose of the FMKs was 20 mg/kg for MDL 201,000 and 21 mg/kg for MDL 201,003. Mean paw volumes ±1 SE are shown. Mean values were compared by the *t*-test for independent means, and statistically significant differences ($P < 0.05$) are indicated by an asterisk.

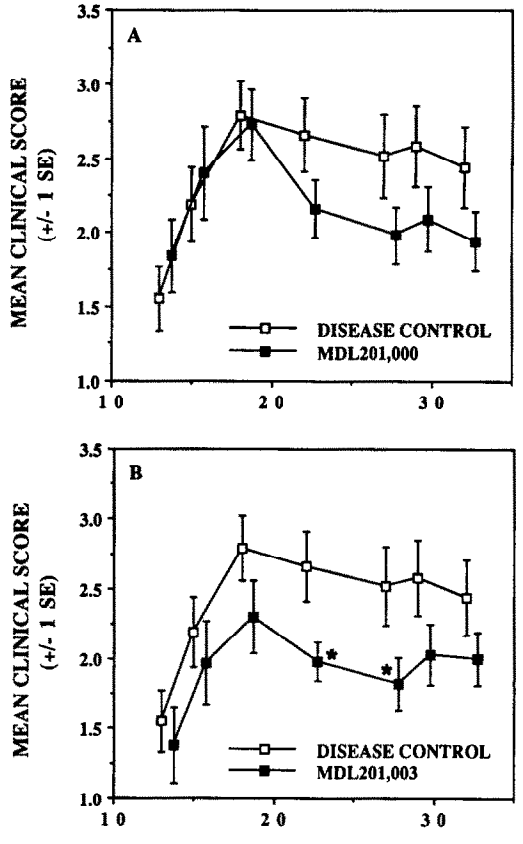


Fig. 3. Effects of MDL 201,000 (A) and MDL 201,003 (B) on gross clinical scores of rats with adjuvant-induced arthritis. Rats were injected with adjuvant and treated with FMKs as described in the legend of Fig. 2. Mean clinical scores ±1 SE are shown. Statistically significant differences ($P < 0.05$) are indicated by an asterisk.

DISCUSSION

A possible mechanism for cartilage and bone damage in arthritis may be due to an imbalance between degradative enzymes and their inhibitors. Both are secreted from the cells of the inflamed synovium [18]. One of the attractive hypotheses proposed to explain matrix destruction implicates a role for lysosomal cysteine proteases including cathepsin B which can degrade extracellular matrix and a variety of proteins and as such has a role in disease states such as arthritis.

Our data show that the FMKs used in this study were effective irreversible inhibitors of cathepsin B activity. Our values for k_{app}/I of 12,300 I/mol/sec for MDL 201,000 is in reasonable agreement with the value of 13,700 reported previously [14]. MDL 201,000 and MDL 201,003 both were shown to inhibit cathepsin B activity through time-dependent covalent modification of the active site cysteine residue of the enzyme. The inactivation of cathepsin B via the active site is suggested by the *in vitro* kinetics with other compounds that we have studied (unpublished observations). The inactivation was stereospecific and one would not expect nonspecific alkylation to be stereospecific. The six FMKs studied were all effective in inhibiting tissue cathepsin B activity *in vivo* after a single oral dose of 25 mg/kg.

MDL 201,000 and MDL 201,003 were also evaluated in adjuvant-induced arthritis and shown to significantly decrease the severity of grossly apparent inflammation and the destruction of cartilage and bone. The effects of FMK treatment appeared to be most prominent during the late-stage of the disease, and no effect on the incidence or the time of onset of joint inflammation was noted in FMK-treated rats. This pattern of activity is consistent with direct inhibition of proteinase-mediated degradation of articular cartilage and bone which is most prominent during late-stage adjuvant-

Table 4. Effects of MDL 201,000 and MDL 201,003 on histological measures of bone and cartilage destruction in adjuvant-induced arthritis*

Treatment group	N	Cartilage destruction		Bone destruction§
		Focal ulcers†	Tibiotarsal joint‡	
Control	16	13.5 ± 5.9	9.9 ± 4.3	26.9 ± 8.9
MDL 201,000		3.5 ± 3.1‖	4.9 ± 3.4	7.6 ± 4.4‖
% Decrease	16	74	51	72
MDL 201,003		3.1 ± 1.8‖	3.8 ± 2.1	6.6 ± 2.6‖
% Decrease	15	77	62	76

* Rats were injected with adjuvant and treated with FMKs as described in the legend of Fig. 2. Stained tissue sections from both right and left hind paws of each rat were examined for histological lesions. Values shown are means ± SEM.

† Percent of articular cartilage surfaces with focal destruction of articular cartilage which exposed underlying subchondral bone.

‡ Percent of articular cartilage lost from tibiotarsal joint.

§ Percent of articulating surfaces with cavities in the subchondrial bone.

‖ Statistically different ($P < 0.05$) from control value by the *t*-test for independent means, or by nonparametric analysis of variance.

Table 5. Food consumption and body weights in FMK-treated and untreated arthritis control rats*

Treatment group	Food consumption† (g/day)	FMK dose‡ (mg/kg/day)	Body weight§ (g)	N
Normal control	ND		200 ± 8	4
Arthritis control	12.9		178 ± 4	16
MDL 201,000	12.7	20	176 ± 4	16
MDL 201,003	13.0	21	181 ± 5	15

* Rats were injected with adjuvant and treated with FMKs as described in the legend of Fig. 2.

† Average daily food consumption over the 32-day treatment period. ND = not determined.

‡ Average daily FMK dose calculated from food consumption and average body weights.

§ Mean body weight + 1 SEM at the termination of the experiment 32 days after adjuvant injection.

induced inflammation. Furthermore, the inhibition of cartilage and bone destruction could decrease the release of phlogistic degradation products which may act to promote the inflammatory process. Other types of anti-rheumatic drugs have distinctly different patterns of activity [19, 20]. Both anti-inflammatory steroids and nonsteroidal anti-inflammatory agents inhibit all phases of adjuvant-induced arthritis and immunosuppressive/cytotoxic agents delay the time of onset and decrease the incidence of disease.

Other investigators have also examined the effects of cysteine proteinase inhibitors on the severity of arthritis in animal models. It has been reported that FMK treatment inhibits the acute inflammation that occurred within 7 days of antigen challenge, and the extent of cartilage damage in acute antigen-induced arthritis [10, 11]. These investigators also reported that the level of cathepsin B activity present in inflamed tissue is reduced in FMK-treated rats. Our data confirm these observations in a model of chronic joint inflammation.

In conclusion, the combination of reduced electrophilicity, as compared to chloromethyl ketones, and the high inhibitory capacity of the FMKs towards cathepsin B suggests that they may be of clinical value in the treatment of protease-associated diseases including arthritis. Currently, we are pursuing other modifications of the dipeptidyl FMKs to evaluate their efficacy *in vitro* and *in vivo*.

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