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# NEW SYNTHETIC INHIBITORS OF C1$\bar{r}$, C1 ESTERASE, THROMBIN, PLASMIN, KALLIKREIN AND TRYPSIN

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**p-Guanidinobenzoate derivatives were prepared and their inhibitory effects on trypsin, plasmin, pancreatic kallikrein, plasma kallikrein, thrombin, C1$\bar{r}$ and C1 esterase were examined. Among the various inhibitors tested, 6'-amidino-2-naphthyl-4-guanidinobenzoate dihydrochloride, 4-(β-amidinoethenyl)phenyl-4-guanidinobenzoate dimethanesulfonate and 4-amidino-2-benzoylphenyl-4-guanidinobenzoate dimethanesulfonate were the most effective inhibitors of trypsin, plasmin, pancreatic kallikrein, plasma kallikrein and thrombin and they strongly inhibited the esterolytic activities of C1$\bar{r}$ and C1 esterase, and then strongly inhibited complement-mediated hemolysis.**

## Introduction

Previously [1–4], we reported the strong reversible inhibitory effects of various ω-guanidinino acid esters, ω-guanidinocaproate and *p*-guanidinobenzoate derivatives on trypsin, plasmin, plasma kallikrein, thrombin, C1$\bar{r}$ and C1 esterase. Among the various inhibitors examined, *p*-guanidinobenzoate derivatives were more inhibitory than ω-guanidinino acid esters and ω-guanidinocaproate derivatives. Baker and coworkers [5,6] reported the irreversible inhibitory effects of *meta*-substituted benzamidines and pyridines substituted with benzoyl halides on complement-mediated hemolysis, and Markwardt [7] reported those of benzamidine derivatives. Geratz [8] reported the inhibitory effects of aromatic amidino compounds on thrombin, and Okamoto [9] reported those of $N^{\alpha}$-naphthalenesulfonyl-L-arginine derivatives.

This paper describes the strong inhibitory effects of several *p*-guanidinobenzoate derivatives on trypsin, plasmin, plasma kallikrein, pancreatic kallikrein, thrombin, C1$\bar{r}$ and C1 esterase. Studies on the inhibitory effects of these compounds on complement-mediated hemolysis are also described.

## Materials and Methods

### *Enzymes and reagents*

Human C1$\bar{r}$ [10] and C1 esterase [11] were prepared as described previously. Bovine trypsin (type I) and bovine α-chymotrypsin were purchased from Sigma Chemical Co., and dissolved in 50 mM sodium phosphate buffer, pH 7.4. Human plasmin and thrombin were purchased from Green Cross Co., Osaka, Japan and dissolved in 50 mM sodium phosphate buffer, pH 7.4. Porcine plasma and pancreatic kallikrein in 50 mM sodium phosphate buffer, pH 7.4, were obtained from the Research Laboratory of Ono Pharmaceutical Co., Osaka, Japan. Sheep and rabbit

Abbreviations: TAME, $N^{\alpha}$-tosyl-L-arginine methyl ester; ATEE, $N^{\alpha}$-acetyl-L-tyrosine ethyl ester; AAME, $N^{\alpha}$-acetyl-L-arginine methyl ester; FUT-175, 6-amidino-2-naphthyl-4-guanidinobenzoate dihydrochloride; FUT-5895, 4-(β-amidino ethenyl)phenyl-4-guanidinobenzoate dimethanesulfonate; FUT-5897, 4-amidino-2-benzoylphenyl-4-guanidinobenzoate dimethanesulfonate; *p'*-GB-DBiG, *N,N*-dimethylamino-4-(4'-guanidinobenzoyloxy)benzylcarbonyloxy glycolate.

erythrocytes were purchased from the Research Foundation for Microbial Diseases of Osaka University, Osaka, Japan. Guinea pig serum and rabbit antiserum against boiled sheep erythrocyte stroma were purchased from Nippon Biotest Laboratory, Tokyo, Japan.

*Substrates and inhibitors*

TAME, ATEE and AAME were purchased from the Foundation for Protein Reserach Institute of Osaka University, Osaka, Japan. Casein was purchased from E. Merck, Japan Ltd. FUT-175, FUT-5895 and FUT-5897 were prepared in the research Laboratories of Torii and Co., Ltd., Tokyo, Japan. *p'*-GB-DBiG was obtained from the Research Laboratory of Ono Pharmaceutical Co., Osaka, Japan. The structural formulae of these compounds are shown in Fig. 1.

*Experiments on inhibition of various enzymes*

The rates of hydrolysis of TAME by trypsin, plasmin, plasma kallikrein, pancreatic kallikrein and thrombin, and of ATEE by chymotrypsin, were determined as described previously [12], at a substrate concentration of 10 mM. The rates of hydrolysis of AAME by $C1\bar{r}$ and of ATEE by C1 esterase were determined as described previously [10], at a substrate concentration of 10 mM. The caseinolysis of trypsin was determined as described previously [13]. The final concentration of casein was 2%. For measurement of inhibitory effects, mixtures of enzyme solution and inhibitor were preincubated at 37°C for 5 min and then residual enzyme activity was determined, as described above. $K_m$ values were determined from Lineweaver-Burk [14] plots of the results. $K_i$ values were determined as described by Dixon [15].

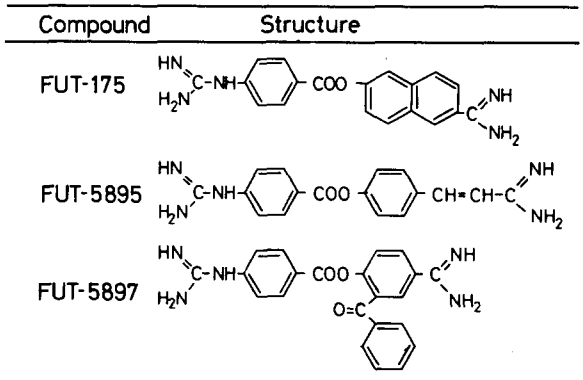


Fig. 1. Structural formulae of FUT-175, 5895 and 5897.

*Experiments on inhibition of complement-mediated hemolysis*

Complement-mediated hemolytic activities were determined as described by Baker and Erickson [16] for the classical hemolytic reaction system, and as by Platts-Mills and Ishizaka [17] for the alternative hemolytic reaction system. In the hemolytic reaction system for the classical pathway, sensitized sheep erythrocytes at a concentration of $2.5 \cdot 10^8$ cells/ml were lyzed with 1/128 diluted guinea-pig serum, and by the alternative pathway, unsensitized rabbit erythrocytes at a concentration of $1 \cdot 10^7$ cells/ml were lyzed with 1/10 diluted normal human serum. For measurement of inhibitory effects, mixtures of the hemolytic reaction system and inhibitor were preincubated at 37°C for 5 min and then hemolytic activity was determined, as described above.

## Results and Discussion

The effects of *p*-guanidinobenzoate derivates on the esterolysis of various enzymes were examined. The concentrations for 50% inhibition are shown in Table I. The concentration of FUT-175 required for 50% inhibition of the caseinolysis of trypsin was $2.9 \cdot 10^{-8}$ M. This indicates that the inhibitory activities on esterolysis and proteolysis were almost the same.

Previously, we reported [4] that *p'*-GB-DBiG was a strong inhibitor and that the concentrations causing 50% inhibition of AAME hydrolysis of $C1\bar{r}$ and ATEE hydrolysis of C1 esterase were $4.4 \cdot 10^{-6}$ and $3.4 \cdot 10^{-5}$ M, respectively. We also showed that it was weakly inhibitory to chymotrypsin. As shown in Table I, FUT-175, 5895 and 5897 inhibited $C1\bar{r}$, C1 esterase and chymotrypsin more than did *p'*-GB-DBiG.

FUT-175 inhibited C1 esterase more than $C1\bar{r}$; the concentrations of this compound causing 50% inhibitions of ATEE hydrolysis by C1 esterase and AAME hydrolysis by $C1\bar{r}$ were $5.1 \cdot 10^{-8}$ and $2.1 \cdot 10^{-7}$ M, respectively, as shown in Table I.

The $K_i$ values of these inhibitors on esterolysis with various substrates were next determined. Table

TABLE I

CONCENTRATIONS IN INHIBITORS FOR 50% INHIBITION OF ESTEROLYSIS

Incubations were carried out in 50 mM sodium phosphate buffer, pH 7.5, at 37°C. TAME (10 mM) was used as substrate for trypsin, plasmin, kallikrein, and thrombin, AAME (10 mM) for C1$\bar{r}$, and ATEE (10 mM) for C1 esterase and chymotrypsin.

| Inhibitor | Inhibitor concentration for 50% inhibition (M) | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | Trypsin | Chymotrypsin | Thrombin | Plasmin | Plasma kallikrein | Pancreatic kallikrein | C1$\bar{r}$ | C1 esterase |
| FUT-175 | $1.9 \cdot 10^{-8}$ | $5.4 \cdot 10^{-5}$ | $3.3 \cdot 10^{-7}$ | $4.1 \cdot 10^{-7}$ | $3.1 \cdot 10^{-7}$ | $8.4 \cdot 10^{-6}$ | $2.1 \cdot 10^{-7}$ | $5.1 \cdot 10^{-8}$ |
| FUT-5895 | $1.9 \cdot 10^{-7}$ | $8.4 \cdot 10^{-5}$ | $1.4 \cdot 10^{-6}$ | $9.0 \cdot 10^{-7}$ | $5.6 \cdot 10^{-7}$ | $5.1 \cdot 10^{-5}$ | $3.9 \cdot 10^{-7}$ | $1.0 \cdot 10^{-7}$ |
| FUT-5897 | $8.4 \cdot 10^{-8}$ | $7.3 \cdot 10^{-6}$ | $1.8 \cdot 10^{-7}$ | $9.0 \cdot 10^{-7}$ | $1.3 \cdot 10^{-7}$ | $2.4 \cdot 10^{-6}$ | $3.1 \cdot 10^{-7}$ | $3.8 \cdot 10^{-7}$ |
| *p'*-GB-DBiG | $3.4 \cdot 10^{-8}$ | $1.0 \cdot 10^{-3}$ | $5.0 \cdot 10^{-5}$ | $1.9 \cdot 10^{-7}$ | $3.2 \cdot 10^{-7}$ | $4.1 \cdot 10^{-6}$ | $4.4 \cdot 10^{-6}$ | $3.4 \cdot 10^{-5}$ |

II shows that the $K_i$ value of FUT-175 for trypsin hydrolytic activity was $1.5 \cdot 10^{-8}$ M, the $K_i$ value of FUT-5895 was $8.5 \cdot 10^{-8}$ M and the $K_i$ value of FUT-5897 was $2.9 \cdot 10^{-8}$ M. The $K_i$ values of FUT-175 for thrombin, C1$\bar{r}$ and C1 esterase were $8.4 \cdot 10^{-7}$, $1.4 \cdot 10^{-7}$ and $3.8 \cdot 10^{-8}$ M, respectively. These compounds were all competitive inhibitors. The $K_m$ values for trypsin, thrombin, C1$\bar{r}$ and C1 esterase were estimated. As shown in Table III, the $K_m$ value was $3.1 \cdot 10^{-3}$ M for trypsin with TAME as substrate, $8.7 \cdot 10^{-3}$ M for thrombin with TAME, $1.2 \cdot 10^{-2}$ M for C1$\bar{r}$ with AAME, and $4.2 \cdot 10^{-2}$ M for C1 esterase with ATEE. The various compounds described above were reversible inhibitors of trypsin, plasmin, plasma kallikrein, pancreatic kallikrein, thrombin, C1$\bar{r}$ and C1 esterase. For example, as shown in Fig. 2, FUT-175 was not removed from the inhibited enzyme by dialysis, but after dialysis it was completely removed by incubating the inhibited enzyme at 37°C for 6 h. The effects of FUT-175, 5895, 5897 and *p'*-BG-DBiG on complement-mediated hemolysis were examined.

TABLE II

$K_i$ VALUES FOR TRYPSIN, THROMBIN, C1$\bar{r}$ AND C1 ESTERASE

$K_i$ values were estimated in 50 mM sodium phosphate buffer, pH 7.4, at 37°C. TAME was used as substrate for trypsin and thrombin, AAME for C1$\bar{r}$, and ATEE for C1 esterase.

| Inhibitor | $K_i$ (M) | | | |
|---|---|---|---|---|
| | Trypsin | Thrombin | C1$\bar{r}$ | C1 esterase |
| FUT-175 | $1.5 \cdot 10^{-8}$ | $8.4 \cdot 10^{-7}$ | $1.4 \cdot 10^{-8}$ | $3.8 \cdot 10^{-8}$ |
| FUT-5895 | $8.5 \cdot 10^{-8}$ | $2.1 \cdot 10^{-6}$ | $2.9 \cdot 10^{-7}$ | $1.9 \cdot 10^{-7}$ |
| FUT-5897 | $2.9 \cdot 10^{-8}$ | $6.1 \cdot 10^{-7}$ | $3.4 \cdot 10^{-7}$ | $2.1 \cdot 10^{-7}$ |

TABLE III

$K_m$ VALUES FOR TRYPSIN, THROMBIN, C1$\bar{r}$ AND C1 ESTERASE

$K_m$ values were estimated in 50 mM sodium phosphate buffer, pH 7.4, at 37°C.

| Enzyme | Substrate | $K_m$ (M) |
|---|---|---|
| Trypsin | TAME | $3.1 \cdot 10^{-3}$ |
| Thrombin | TAME | $8.7 \cdot 10^{-3}$ |
| C1$\bar{r}$ | AAME | $1.2 \cdot 10^{-2}$ |
| C1 esterase | ATEE | $4.2 \cdot 10^{-2}$ |

TABLE IV

CONCENTRATIONS OF INHIBITORS FOR 50% INHIBITION OF COMPLEMENT-MEDIATED HEMOLYSIS

Inhibitory effects of inhibitors on complement-mediated hemolysis were measured as described in Materials and Methods.

| Inhibitor | Inhibitor concentration for 50% inhibition (M) | |
|---|---|---|
| | Classical pathway | Alternative pathway |
| FUT-175 | $6.9 \cdot 10^{-8}$ | $5.1 \cdot 10^{-7}$ |
| FUT-5895 | $1.0 \cdot 10^{-7}$ | $7.1 \cdot 10^{-7}$ |
| FUT-5897 | $2.4 \cdot 10^{-7}$ | $3.1 \cdot 10^{-7}$ |
| *p'*-GB-DBiG | $2.1 \cdot 10^{-4}$ | $4.0 \cdot 10^{-4}$ |

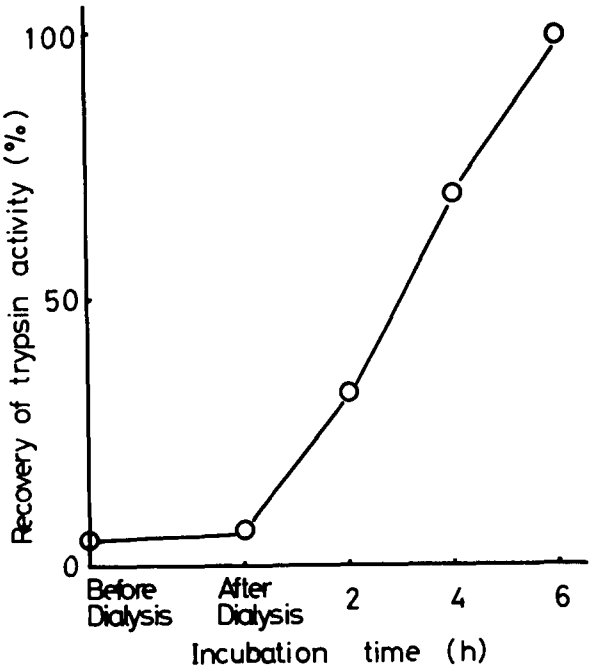


Fig. 2. Effects of dialysis and incubations on esterolysis of FUT-175-treated trypsin. A mixture of trypsin (20 μg/ml) and FUT-175 ($10^{-3}$ M) and 0.1 M Tris-HCl buffer containing 1 mM $CaCl_2$, pH 8.0, was incubated at 37°C for 5 min and then dialyzed against the same buffer overnight at 4°C. Samples of 0.1 ml of the dialyzate were incubated at 37°C for the indicated times and then esterase activities of trypsin were determined by incubation of the mixtures at 37°C for 30 min after addition of 10 mM TAME.

The concentrations required for 50% inhibition are shown in Table IV. The concentration of FUT-175 required for 50% inhibition of the complement-mediated hemolysis in a classical reaction system was $6.9 \cdot 10^{-8}$ M. This indicates that the inhibitor activities on $C1\bar{r}$ and C1 esterase with esterolysis and complement-mediated hemolysis were almost the same. The stronger inhibitory effects of these new derivatives than of *p'*-GB-DBiG might be due to their more potent effects on $C1\bar{r}$ and C1 esterase.

FUT-175, 5895 and 5897 also inhibited complement-mediated hemolysis in the alternative reaction system. This suggests that these compounds inhibit some enzymes, such as Factor B or Factor D, in the alternative pathway of complement activation. In vivo experiments are now in progress on this problem.

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