KINETIC STUDIES OF TYROSINE KINASE INHIBITION BY ERBSTATIN

Sir:

Erbstatin was isolated from the culture filtrate of *Streptomyces* sp. MH435-hF3 as a potent inhibitor of epidermal growth factor (EGF) receptor associated tyrosine protein kinase[1]. It inhibits EGF receptor-autophosphorylation with IC_{50} of 0.55 μg/ml *in vitro*, but does not inhibit either cyclic AMP dependent protein kinase[1] or protein kinase C (Dr. H. HIDAKA, personal communication). It was also shown to inhibit EGF-stimulated receptor-autophosphorylation in cultured A431 cells and the autophosphorylation of $p60^{src}$ in Rous sarcoma virus-infected rat kidney cells[2]. Besides autophosphorylation of the above proteins, the synthetic peptide (Arg-Arg-Leu-Ile-Glu-Asp-Ala-Glu-Tyr-Ala-Ala-Arg-Gly (RR-SRC)) was found to be a good substrate for EGF receptor associated tyrosine kinase[3], therefore, using this peptide as a substrate we have studied the mechanism of enzyme inhibition by erbstatin.

EGF receptor associated tyrosine kinase was partially purified from human epidermoid carcinoma A431 cells[4]. A431 cells were grown in DULBECCO's modified EAGLE's medium supplemented with 5% calf serum, washed with cold DULBECCO's phosphate buffered saline, scraped off with rubber policeman, and centrifuged. Then, the cells were homogenized in 20 mM *N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, purchased from Sigma (HEPES) buffer (pH 7.4) containing 1 mM $MgCl_2$ and 5 mM KCl. The homogenate was centrifuged at $15,000 \times g$ for 5 minutes, and the supernatant was recentrifuged at $100,000 \times g$ for 30 minutes. The pellet obtained as a membrane fraction was solubilized for 60 minutes at 4°C in 25 mM HEPES buffer (pH 7.4) containing Triton X-100 1%, glycerol 10%, leupeptin 0.15 mg/ml, aprotinin 0.15 mg/ml and phenylmethylsulfonylfluoride (PMSF) 0.2 mM. Insoluble material was removed by centrifugation at $100,000 \times g$ for 60 minutes. The supernatant was then diluted so that the Triton X-100 concentration was 0.05% and passed four times through a column containing 2 ml of wheat germ agglutinin-agarose (Seikagaku Kogyo Co., Ltd.) at 2°C. The column was extensively washed with the Triton X-100 solution containing Triton X-100 0.05%, glycerol 10%, PMSF 0.2 mM, NaCl 0.5 M and HEPES buffer 40 mM (pH 7.4). The EGF receptor was eluted with the buffer above added with 0.3 M *N*-acetylglucosamine, and dialyzed against the Triton X-100 solution. Tyrosine kinase reactions were carried out in a final volume of 60 μl containing HEPES-NaOH 20 mM, pH 7.4, $MnCl_2$ 1 mM, EGF 100 ng, the purified EGF receptor (12 μg protein) and indicated concentrations of inhibitors, RR-SRC (Peninsula Laboratory) and [γ-^{32}P]ATP. The kinase activities were measured by a slight modification of the method described by GLASS *et al.*[5]. The EGF receptor was first incubated with EGF at 23°C for 30 minutes before assay of kinase activity. The kinase reactions were initiated by the addition of RR-SRC and [γ-^{32}P]ATP, and the reaction mixture was incubated for 10 minutes at 23°C. The reactions were terminated by addition of 25 μl of 10% TCA and 6 μl of bovine serum albumin (10 mg/ml). Precipitated proteins were removed by centrifugation and 40 μl aliquot of the supernatant was spotted on a Whatman P81 paper (2×2 cm) which were immediately immersed in 30% AcOH at 25°C. The papers were washed four times for 15 minutes in 15% AcOH, for 5 minutes in Me_2CO, and dried. The radioactivity was counted with a liquid scintillation counter.

As shown in Fig. 1(A), the inhibitory pattern in the Lineweaver-Burk plot of erbstatin vs. peptide was a typical competitive inhibition, in which each line met together on the *y*-axis. The inhibitory pattern of erbstatin vs. ATP was noncompetitive as shown in Fig. 1(B), in which each line met on the *x*-axis. From the Dixon plot analysis the *Ki* value was found to be about 5.58 μM. (*E*)-2-(2,3-dihydroxyphenyl)vinylformamide, a structural analogue of erbstatin, gave the same pattern of kinetics as erbstatin in tyrosine protein kinase inhibition with *Ki* value of 2.23 μM (data not shown). Orobol was first isolated from the *Streptomyces* culture broth as an inhibitor of dopa decarboxylase[6] and then isolated again in the course of screening for tyrosine protein kinase inhibitor[1]. It has an isoflavonoid structure and inhibits EGF receptor tyrosine protein kinase with IC_{50} of 3.0 μg/ml *in vitro*. As shown in Fig. 2(A) inhibition of tyrosine protein kinase by orobol was competitive to ATP. The Lineweaver-Burk plot of orobol vs. the peptide showed neither noncom-

Fig. 1.

(A) A Lineweaver-Burk plot of enzyme kinetics showing competitive inhibition by erbstatin against peptide substrate. Inhibitor concentrations are 0 (○), 11.16 (●), 22.3 (△) and 33.5 (□) μM, respectively.

(B) A Lineweaver-Burk plot of enzyme kinetics showing noncompetitive inhibition by erbstatin against ATP. Inhibitor concentrations are same as (A).

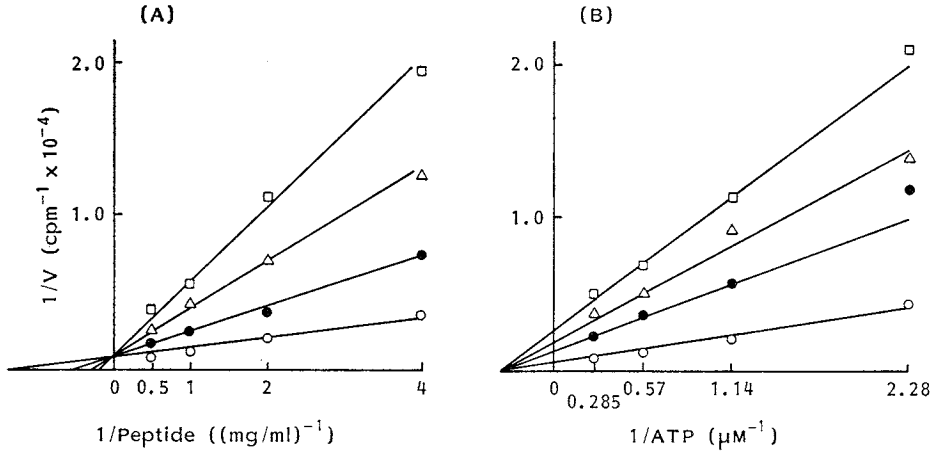
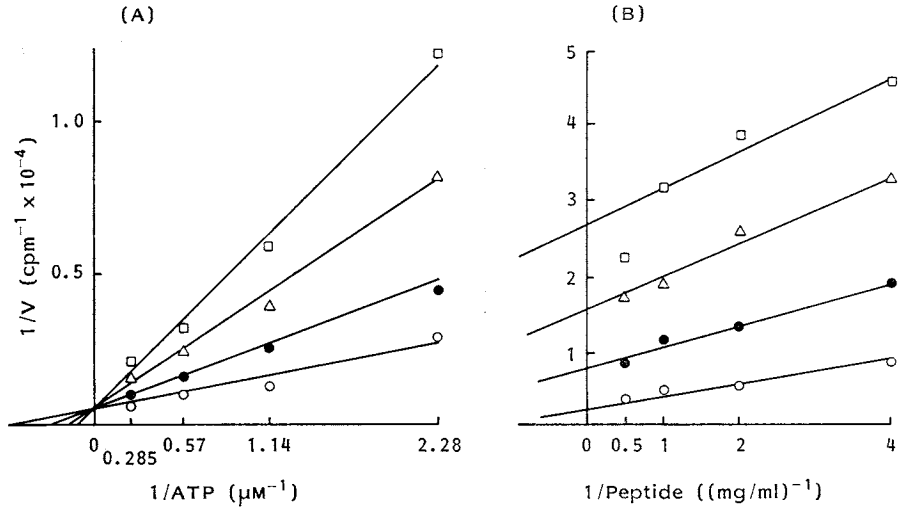


Fig. 2.

(A) A Lineweaver-Burk plot showing competitive inhibition by orobol against ATP. Inhibitor concentrations are 0 (○), 1.75 (●), 3.49 (△) and 5.24 (□) μM, respectively.

(B) A Lineweaver-Burk plot showing not competitive inhibition by orobol against peptide substrate. Inhibitor concentrations are same as (A).



petitive nor uncompetitive as shown in Fig. 2(B).

Thus, erbstatin competes with the peptide substrate, and the mechanism of inhibition is clearly different from that of orobol which competes with ATP. The EGF receptor associated tyrosine kinase was reported to act in the Ordered Bi Bi mechanism, in which the peptide came first and ATP second to the enzyme active site, since the dipeptide Tyr-Arg, a weak tyrosine protein kinase inhibitor (*Ki* 600 μM) competed with the synthetic peptide and noncompeted against ATP, and since ADP, a product of the kinase reaction, was a linear noncompetitive inhibitor with respect to ATP and a linear competitive inhibitor with respect to peptide[7]. Our result confirmed this mechanism using the more potent inhibitor

erbstatin, and also erbstatin was confirmed to attack the enzyme directly.

The structure of erbstatin apparently resembles tyrosine, and it is possible that erbstatin resembles to a greater extent the specific conformation of tyrosine within the peptide. Genistein[8,9] and amiloride[10] which inhibit tyrosine kinase and H-8[11] and K-252 compounds[12] which inhibit protein kinase C are all competitive to ATP. Erbstatin is a unique tyrosine protein kinase inhibitor competing with the peptide substrate.

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