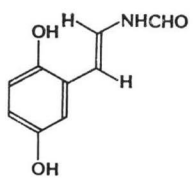
STUDIES ON A NEW EPIDERMAL GROWTH FACTOR-RECEPTOR KINASE INHIBITOR, ERBSTATIN, PRODUCED BY MH435-hF3

Sir:

The binding of epidermal growth factor (EGF) to its receptor, which has an associated tyrosine protein kinase (TPK) activity, stimulates the phosphorylation of tyrosine residue in EGF receptor, and the phosphorylated receptor transfers the terminal phosphate of ATP to the tyrosine residue of other proteins[1~3]. Recently, receptors for platelet-derived growth factor (PDGF)[4,5], insulin[6,7], and insulin-like growth factor (IGF-I)[8] were found to have tyrosin-specific protein kinase activity, suggesting the involvement of tyrosine phosphorylation in triggering cell proliferation. Moreover, it has been observed that the protein products of malignant genes of most retroviruses have tyrosine protein kinase activity[9]. Furthermore, the amino acid sequence of v-erb-B protein of avian erythroblastosis virus (AEV) has been confirmed to be closely related to that of EGF receptor[10]. We have undertaken the screening of culture filtrates of actinomycetes for inhibitors of tyrosin protein kinase, using the membrane fraction of human epidermoid carcinoma cell line A-431 as the source of this enzyme, and we found two strains capable of producing inhibitors. One of these strains (MD88-A6), as previously reported[11], has been classified as *Streptomyces neyagawaensis* var. *orobolere*, produced an isoflavon compound which was identified to be oroborl. The concentration of orobol exhibiting 50% inhibition of tyrosine protein kinase was 3 μg/ml. It inhibited the growth of rat kidney cells transformed by a temperature sensitive mutant (src^{ts}-NRK) of Rous sarcoma virus: 50% inhibition concentrations were 4 μg/ml at 33°C and 16 μg/ml at 39°C. The inhibitor produced by the other strain (No. MH435-hF3) is a novel compound, and we named it erbstatin.

Fig. 1. Structure of erbstatin.



Taxonomic studies indicated that the strain producing erbstatin was closely related to *S. viridosporus*. In this paper, we report on production, isolation and properties of erbstatin. The study of the structure (Fig. 1) determination will be reported in another paper[12].

The strain MH435-hF3 was cultured in a 500-ml Erlenmeyer flask containing 110 ml of the medium consisted of glycerol 3%, fish meal 2% and $CaCO_3$ 0.2%, pH 7.4 (before sterilization), on a rotary shaker at 27°C for 48 hours, and 3.0 ml of the cultured broth thus prepared was inoculated in to a 500-ml flask containing 110 ml of the medium described above. The fermentation was carried out at 27°C for 4 days. The production of erbstatin was followed by testing for inhibition of tyrosine protein kinase activity using the method of CARPENTER *et al.*[13]. Erbstatin in culture filtrates (5 liters) was extracted with 5 liters of butyl acetate, and the extract was concentrated under reduced pressure to give a yellowish powder (370 mg). The dried material was dissolved in $CHCl_3$, and subjected to a silicic acid column chromatography. After washing with $CHCl_3$ - MeOH (100: 2), the active fraction was eluted with $CHCl_3$ - MeOH (100: 5) and concentrated *in vacuo* to give a yellow powder (110 mg). It was dissolved in MeOH - $CHCl_3$

Fig. 2. UV spectra of erbstatin.

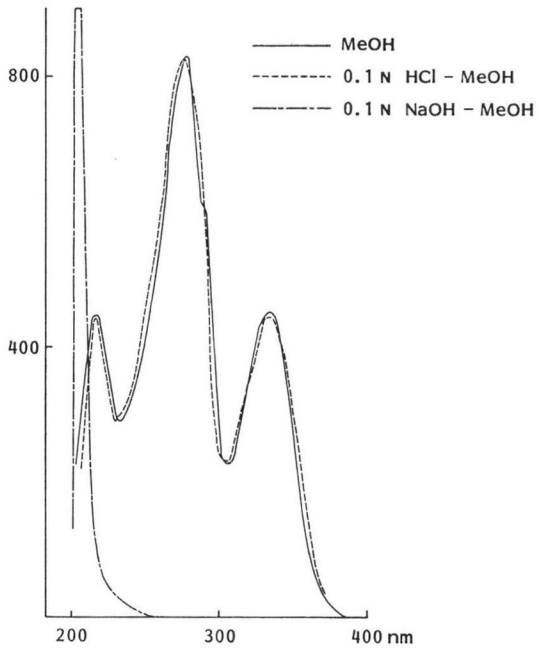
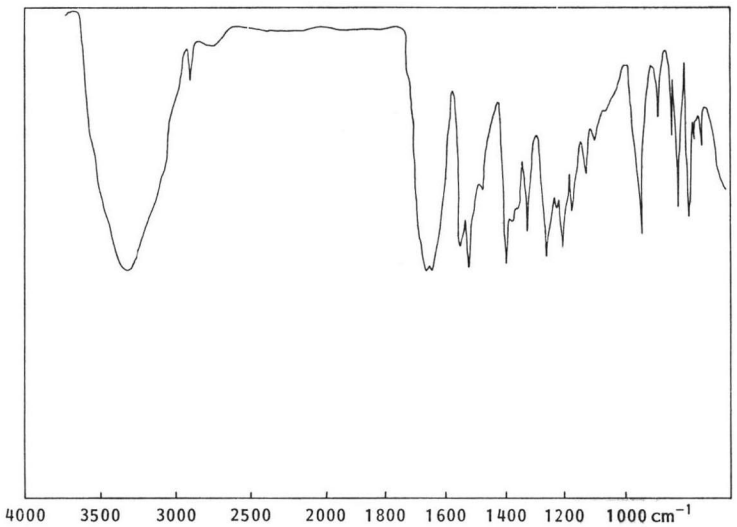


Fig. 3. IR spectrum of erbstatin (KBr).



(1:1) and kept at 4°C to yield 60 mg of crude crystals of erbstatin. It was recrystallized from MeOH - $CHCl_3$, mp 78~82°C. It is soluble in MeOH, ethanol and acetone, slightly soluble in $CHCl_3$, and EtOAc, substantially insoluble in H_2O and *n*-hexane. The molecular formula, $C_9H_9NO_3$ was determined by high resolution mass spectrometry. $[\alpha]_D^{25}$ is 0° (*c* 1.0, MeOH). The UV spectra are shown in Fig. 2; λ_{max}^{MeOH} nm ($E_{1cm}^{1\%}$) 215 (460), 278 (830), 330 (450); $\lambda_{max}^{0.1N\ HCl}$ nm ($E_{1cm}^{1\%}$) 215 (460), 278 (830), 330 (450); $\lambda_{max}^{0.1N\ NaOH}$ nm ($E_{1cm}^{1\%}$) 208 (6,000). The IR spectrum is shown in Fig. 3, suggesting the presence of a double bond (1655 cm^{-1}) and amide bond (1670 cm^{-1}). As will be reported by NAKAMURA *et al.*[12], the structure [(*E*)-2-(2-formamidovinyl)-1,4-hydroquinone] shown in Fig. 1 was determined by X-ray crystallography.

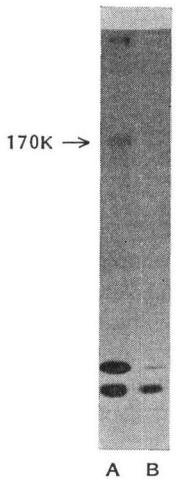
The 50% inhibition concentration of erbstatin against tyrosine protein kinase was 0.55 μg/ml, when it was examined as follows: The reaction mixture contained 1 mM $MnCl_2$, 100 ng EGF, 40 μg protein of A-431 membrane fraction (prepared by method of THOM[14]), 75 μg of albumin, 3 μg of histone and HEPES (*N*-2-hydroxyethylpiperazine-*N'*-2-ethanesulfonic acid, purchased from Sigma) buffer (20 mM, pH 7.4) in a final volume of 50 μl. The reaction tubes were placed on ice and incubated for 10 minutes in the presence or absence of erbstatin. The reaction was initiated by the addition of labeled ATP (10 μl) and the incubation was continued for 30 minutes at 0°C. Then aliquots of 50 μl were pipetted onto Whatman No. 3MM filter paper and dropped immediately into a beaker of cold 10% TCA containing 0.01 M sodium pyrophosphate. The filter papers were washed extensively with TCA solution containing 0.01 M sodium pyrophosphate at room temperature, extracted with alcohol, ether and dried. Radioactivity was measured by a scintillation counter.

The inhibition of tyrosine kinase by erbstatin was further confirmed by SDS-gel electrophoretic (7% acrylamide, unless otherwise noted) analysis of the A-431 membrane components by the method of LAEMMLI[15]. Membranes of A-431 cells were phosphorylated in the absence and presence of erbstatin (2.5 μg/ml). The gels were fixed and stained with Coomassie blue. The gels were dried under vacuum and subjected to autoradiography. As shown in Fig. 4, 170K EGF receptor phosphorylation was not seen in the presence of erbstatin. The action of erbstatin against tyrosine kinase seems to be specific, because its effect against cAMP-dependent protein kinase was much weaker; IC_{50} was around 100 μg/ml.

Erbstatin also has weak antibacterial activity: MIC on nutrient agar was as follows; *Staphylococcus aureus* Smith (MIC 25 μg/ml), *Pseudomonas aeruginosa* A3 (25), *Micrococcus lysodeikticus* IFO 3333 (50), *S. aureus* 209P (50), *Proteus mirabilis* IFM OM-9 (50). Erbstatin inhibited the growth of human epidermoid

Fig. 4. Electrophoresis and autoradiography of A-431 membrane components incubated with [γ-^{32}P]ATP in the presence and absence of erbstatin.

A-431 cell membrane fraction was prepared and subjected to phosphorylation by the procedures of CARPENTER *et al.*[13]. The phosphorylation was carried out in the absence or presence of erbstatin for 30 minutes at 0°C. Thereafter LAEMMLI's SDS sample buffer[15] was added, and the reaction mixture was boiled for 10 minutes and subjected to SDS-gel electrophoresis and autoradiography. A indicates the result of autoradiography in the absence of erbstatin, and B in the presence of erbstatin.



carcinoma (A-431 cells) (IC_{50} 3.6 μg/ml) and IMC-carcinoma cells (IC_{50} 3.01 μg/ml). It is not certain whether the cytotoxic action is related to inhibition of tyrosine kinase. Antibacterial action should not be due to the inhibition of tyrosine kinase. The acute toxicity LD_{50} in *dd*Y mice was greater than 200 mg/kg by intraperitoneal injection.

As shown by the structure, erbstatin is a new compound and we reported here its discovery, isolation and its actions.

Acknowledgments

We are grateful to Dr. SADAAKI KAWAI, Institute of Medical Science, University of Tokyo, for his generous gift of the A-431 cell line. This research was partially supported by a Contract No. NO1-CM-47593 with the Division of Cancer Treatment, National Cancer Institute, U.S.A. and by a Grant-in-Aid for Cancer Research from the Ministry of Education, Science and Culture, Japan and from the Ministry of Health and Welfare, Japan.

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(Received September 9, 1985)

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