Communication to the Editor

BIOSYNTHETIC PREPARATION OF RADIOACTIVELY LABELED ERBSTATIN

Sir:

Erbstatin (Fig. 1) was isolated from the culture filtrate of *Streptomyces* sp. MH435-hF3 as a specific inhibitor of tyrosine protein kinase[1]. It inhibits autophosphorylation of EGF receptors in cultured human epidermoid carcinoma A431 cells[2] and delays S phase entry induced by EGF in quiescent NRK cells for 3 hours[3]. The inhibitor also induces the normal phenotype in RSV^{ts}-NRK cells, but its effect lasts only for about 15 hours[4]. These temporary effects of erbstatin in cell culture are most probably due to a short half-life of the drug in the medium. To confirm this possibility, we biosynthetically prepared radioisotope labeled erbstatin using the erbstatin-producing strain and studied the stability of erbstatin in culture medium using this preparation.

We looked for effectively incorporated precursors of erbstatin. First, we looked for the minimum concentration of fish meal in the medium necessary for the effective conversion of precursor to erbstatin. The MH435-hF3 strain was cultured in a 500-ml Erlenmeyer flask containing 100 ml of medium consisting of 3% glycerol, 2% fish meal, and 0.2% $CaCO_3$ on a rotary shaker at 27°C for 48 hours. Then, 3.0 ml of cultured broth thus prepared was inoculated into a 500-ml flask containing 100 ml of medium comprising 3% glycerol, 0.2% $CaCO_3$, and various amounts (2.0, 1.0, 0.50, 0.25, or 0.05%) of fish meal. An aliquot of the cultured broth was sampled every day, and the amount of erbstatin produced in the broth sample was measured (Fig. 2). In the medium containing 2.0% fish meal, the amount of erbstatin increased gradually and reached a plateau after 4 days and decreased sharply after 5 days. Reduction of the fish meal content lowered production of erbstatin. In the medium containing 0.05% fish meal, erbstatin was almost undetectable. Although the amount of production of erbstatin in 0.5% fish meal medium was half of that in 2.0% fish meal medium, it was sufficient to study the precursors of erbstatin. Therefore, we decided to use the medium containing 0.5% fish meal for the experiment described below.

The structure of erbstatin is similar to that of tyrosine. Therefore, we used L-[*U*-^{14}C]tyrosine (ICN Radiochemicals; 13.87 GBq/mmol) and its biosynthetic intermediates, L-[*U*-^{14}C]phenylalanine (Amersham; 18.98 GBq/mmol) and [*G*-^{14}C]-shikimic acid (NEN; 0.81 GBq/mmol) to prepare radioactive erbstatin. The seeding broth prepared as described above was inoculated into a 500-ml flask containing 100 ml of medium consisting of 0.5% fish meal, 3% glycerol, and 0.2% $CaCO_3$. After fermentation for 48 hours, each labeled chemical was added to the medium. Further fermentation was carried out for 24 hours, and then the culture broth was extracted with an equal volume of ethyl acetate. For isolation of erbstatin the extract was con-

Fig. 1. Structure of erbstatin.

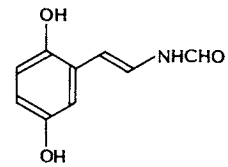
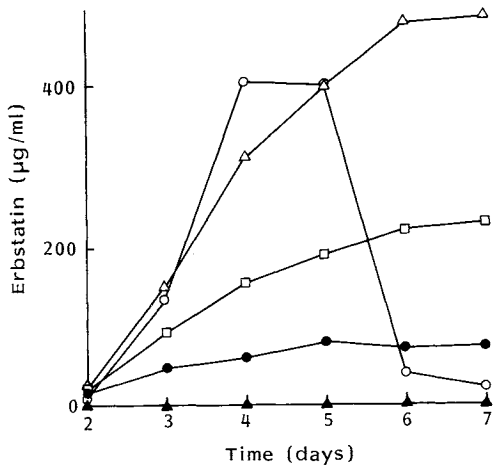


Fig. 2. Effect of fish meal on the production of erbstatin.

The seeding broth was inoculated into a 500-ml flask containing 100 ml of the medium. Fish meal concentrations in the medium were 2.0% (○), 1.0% (△), 0.50% (□), 0.25% (●), and 0.05% (▲).



An aliquot of the culture broth was sampled every day. The sample (1 ml) was extracted with an equal volume of EtOAc, and the amount of erbstatin was estimated spectrophotometrically.

Table 1. Radioactivity of erbstatin biosynthesized from various precursors.

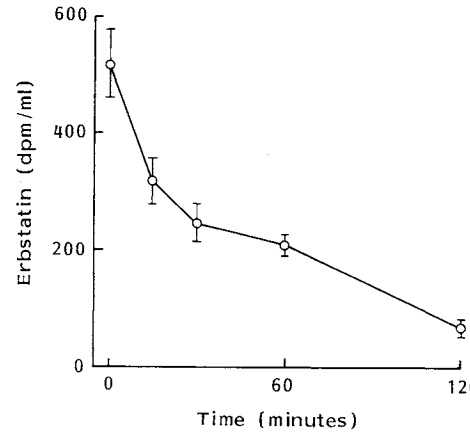
Precursor	Labeled erbstatin (dpm/25 ml broth)[a]
Shikimic acid	626
Phenylalanine	1,444
Tyrosine	16,435

[a] After fermentation for 48 hours, labeled shikimic acid, phenylalanine, or tyrosine was added, and labeled erbstatin was extracted 24 hours later.

centrated and spotted onto a silica gel thin-layer plate which was developed with chloroform-methanol (10:2). The erbstatin fraction was scraped off, and the radioactivity of this fraction was counted in a liquid scintillation counter. Shikimic acid, L-phenylalanine, and L-tyrosine were all converted to erbstain; and as shown in Table 1, the strongest radioactive spot was observed when labeled tyrosine was used as the precursor. The specific activity of the erbstatin thus obtained was 7.1×10^3 dpm/mg. Similar result was obtained when erbstatin was separated by silica gel thin-layer chromatography using toluene-acetone (1:1) as a developing solvent. When L-[2,3,5,6-^{3}H]tyrosine (Amersham; 2.59 TBq/mmol) was used instead of L-[*U*-^{14}C]-tyrosine, the specific activity of erbstatin was 1.3×10^4 dpm/ mg.

To prepare more highly radioactive erbstatin, we tried to further decrease the amount of possible cold precursors contained in the medium. Thus, we prepared a completely synthetic medium consisting of inorganic salts, amino acids, vitamins, and other components, based on DULBECCO's modified EAGLE's medium (DMEM). L-Phenylalanine, L-tyrosine, glucose, succinic acid, sodium succinate, sodium hydrogen carbonate, and phenol red were all eliminated from the components of DMEM, and glycerol (30 g/liter) and HEPES (4.76 g/liter) were added as new components. The seeding broth prepared as described above was inoculated into a 500-ml flask containing 100 ml of the synthetic medium, and the fermentation was carried out at 27°C for 48 hours. After that [^{3}H]labeled L-tyrosine (3 μCi) was added. After 48 hours, the culture broth filtrate was extracted with an equal volume of ethyl acetate. The extract was concentrated and applied to a silica gel column. The fraction containing erbstatin was eluted with chloroform-methanol (100:5) and spotted onto a preparative silica gel thin-layer plate, which was then developed with chloroform-methanol (10:2). Erbstatin was scrapped off from the plate and eluted from the silica gel with chloroform-methanol (2:1). The specific activity of the [^{3}H]erbstatin was 1.4×10^6 dpm/mg or 110 times greater than that of the one prepared from the medium containing fish meal.

Fig. 3. Stability of erbstatin in mammalian cell culture medium.

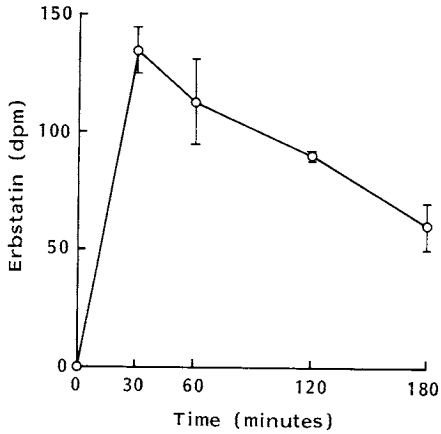


Labeled erbstatin (5 μg/ml, 908 dpm/μg) was added to serum-free DMEM. After incubation at 37°C for the indicated periods, the labeled erbstatin recovered from the medium was counted in a liquid scintillation counter. The results are mean $\pm$SD of triplicate determinations.

The stability of erbstatin in culture medium was analyzed as follows: [^{3}H]Erbstatin (908 dpm/μg) was added to 2 ml of serum-free DMEM at 5 μg/ml. After incubation at 37°C for the indicated periods, the medium was extracted with 2 ml of butanol and the extract was concentrated under reduced pressure. The dried material was dissolved in 20 μl of methanol, and 10 μl of the solution was then spotted onto a silica gel thin-layer plate and the plate developed with chloroform-methanol (10:2). The erbstatin fraction was scraped off, and [^{3}H]erbstatin was counted in a liquid scintillation counter. As shown in Fig. 3, the amount of erbstain in the medium quickly decreased, and after 2 hours only 13% of the initial amount remained. The half-life of erbstatin in this medium was about 20 minutes. This decrease of erbstatin should be due to decomposition in the medium.

The accumulation of erbstatin in A431 cells was examined as follows: Almost confluent A431 cell cultures plated in 145 mm^2 dishes were incubated at 37°C with 250 μg [^{3}H]erbstatin (25 μg/ml) in 10 ml of serum-free DMEM for the indicated periods. The medium was removed on ice and the plates were

Fig. 4. Accumulation of erbstatin in A431 cells.



Labeled erbstatin (25 μg/ml, 908 dpm/μg) was added to each of several cultures of A431 cells in 10 ml of serum-free DMEM. After incubation at 37°C for the indicated periods, cell-associated erbstatin was recovered and counted in a liquid scintillation counter. The results are mean ± SD of triplicate determinations.

washed with cold DULBECCO's phosphate-buffered saline (PBS) 3 times. Then the cells were scraped off, homogenized in PBS, and extracted with an equal volume of butanol, and erbstatin was recovered as described above. The radioactivity of erbstatin in A431 cells was measured in a liquid scintillation counter. As shown in Fig. 4, the intracellular amount of erbstatin in A431 cells was maximum before or around 30 minutes; and after that, the amount of erbstatin decreased gradually, becoming about 45% of the peak amount after 3 hours of incubation.

Erbstatin was shown to be unstable in calf serum[5]. Using radioactively labeled erbstatin, the rate of degradation in medium or in cells was determined. The labeled compound should be useful in further studies of the subcellular distribution of erbstatin.

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