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Inhibition of epidermal growth factor-induced DNA synthesis by tyrosine kinase inhibitors

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We prepared methyl 2,5-dihydroxycinnamate as a stable analogue of erbstatin, a tyrosine kinase inhibitor. This analogue was about 4 times more stable than erbstatin in calf serum. It inhibited epidermal growth factor receptor-associated tyrosine kinase in vitro with an IC_{50} of 0.15 μg/ml. It also inhibited in situ autophosphorylation of epidermal growth factor receptor in A431 cells. Methyl 2,5-dihydroxycinnamate was shown to delay the S-phase induction by epidermal growth factor in quiescent normal rat kidney cells, without affecting the total amount of DNA synthesis. The effect of erbstatin on S-phase induction was smaller, possibly because of its shorter life time.

Erbstatin; Epidermal growth factor; DNA synthesis; Cell cycle; (Normal rat kidney cell)

1. INTRODUCTION

Erbstatin, isolated as an inhibitor of tyrosine kinase associated with the epidermal growth factor (EGF) receptor [1], inhibits autophosphorylation and internalization of EGF receptors in cultured A431 cells [2], and halts the growth of human breast cancer cell line MCF-7 in nude mice [3]. However, erbstatin is easily inactivated in calf serum [4], and more stable analogues of erbstatin have been looked for. Previously, we synthesized various erbstatin analogues and tested them for their inhibitory activity against tyrosine kinase [5]. Among them only 2,5-dihydroxycinnamic acid has, instead of the *N*-formyl moiety, the carboxylic acid moiety, which would be more stable. This analogue inhibits tyrosine kinase in vitro, but its in situ or in vivo activity is not expected, because, being a polar molecule, it would be difficult to penetrate into the cells. Therefore, we synthesized methyl 2,5-dihydroxycinnamate to reduce the polarity of the molecule and examined its effectiveness as an inhibitor.

As the effect of tyrosine kinase inhibitors on the cell cycle has not been assessed, we also examined this point and found that these tyrosine kinase inhibitors delay EGF-induced DNA synthesis in quiescent NRK cells.

2. MATERIALS AND METHODS

2.1. *Materials*

Erbstatin was isolated from *Streptomyces* MH435-hF3 as described previously [1]. The synthetic peptide RR-SRC and EGF were purchased from the Peptide Institute, Osaka, and Takara Shuzo, Kyoto, respectively. Monoclonal antibody for EGF receptor came from Transformation Research Inc. [γ-^{32}P]ATP (6.64 Ci/mmol), and [^{3}H]thymidine (47 Ci/mmol) were products of Amersham. [^{32}P]Phosphate was purchased from New England Nuclear. The A431 cell line was a gift of Dr S. Kawai, Institute of Medical Science, University of Tokyo. The NRK-49F cells (ATCC CRL-1570) were obtained through Flow Laboratories, Inc.

2.2. *Stability of chemicals in serum*

For the assay of the stability of inhibitors in serum, a given inhibitor (10 μg) was added to 0.1 ml calf serum (Gibco) and the mixture shaken vigorously at 37°C. After the incubation 0.1 ml of ethanol was added to the mixture; which was then left on ice for 30 min and centrifuged at 13000 rpm. The supernatant was applied to an HPLC (nucleosil $_5C_{18}$, 4.6 ∅ × 250 mm) for quantitative analysis.

2.3. *Cell culture*

A431 and NRK cells were grown in a 5% CO_2 atmosphere in Dulbecco's modified Eagle's medium (DMEM) supplemented with 5% and 10% calf serum, respectively.

2.4. *Tyrosine kinase assay*

For the in vitro tyrosine kinase assay [6] the reaction mixture containing synthetic peptide (RR-SRC), [γ-^{32}P]ATP, EGF, and the membrane fraction of A431 cells with or without inhibitor was incubated at 0°C for 30 min in 20 mM Hepes buffer (pH 7.2). The reaction was stopped by the addition of 10% trichloroacetic acid. Then, the mixture was centrifuged (1300 rpm, 5 min), and the supernatant was applied to a piece of P81 phosphocellulose paper. The paper was then washed in 30% acetic acid for 15 min, 3 times in 15% acetic acid, rinsed in acetone, and counted for radioactivity.

For the in situ tyrosine kinase assay, A431 cells (5×10^5) were grown in 35-mm dishes for 18 h before use. The cells were then washed and labeled for 4 h with $^{32}P_i$ in 1 ml of phosphate-free medium. Then, the desired inhibitor and/or EGF were/was added. After 20 min, the cells were extracted with radio-immune precipitation (RIPA) buffer. The cell extract was incubated for 2 h at 4°C with monoclonal anti-EGF receptor antibody, and then a suspension of *Staphylococcus aureus* Cowan I was added. The mixture was allowed to stand at 4°C

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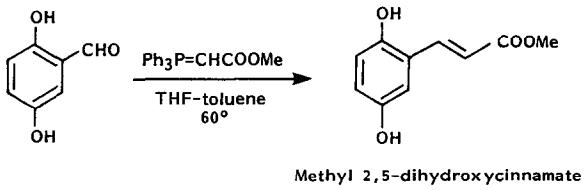

Fig.1. Synthesis of methyl 2,5-dihydroxycinnamate.

overnight. The bacterial cells adsorbing the immune complexes were then washed with RIPA buffer, suspended in electrophoresis loading buffer, boiled for 5 min, and centrifuged at 15000 × *g* for 1 min. The supernatant was electrophoresed on an SDS-polyacrylamide gel.

2.5. *[^{3}H]Thymidine incorporation*

NRK cells (4×10^5) were plated in a 16-mm glassware dish and cultured in 1 ml of DMEM containing 10% calf serum for 60 h. During this period the cells became quiescent by density-dependent inhibition. Then, the cells were washed twice with 0.5 ml of serum-free DMEM and given 1 ml of serum-free DMEM containing inhibitor and/or 100 ng/ml of EGF. After incubation for indicated periods, the cells were washed twice with 0.5 ml of Dulbecco's phosphate-buffered saline, followed by the addition of 1 ml of serum-free DMEM containing 1 μCi/ml of [^{3}H]thymidine. After 1 h the cells were washed twice with 0.5 ml of Dulbecco's phosphate-buffered saline, and 0.5 ml of cold 10% trichloroacetic acid (TCA) was added. After incubation for 10 min at 4°C, the cells were further washed twice with 0.5 ml of 10% TCA. Then, they were solubilized by 0.5 ml of 0.5 N NaOH and an aliquot was counted for radioactivity in a liquid scintillation counter.

3. RESULTS

Synthesis of methyl 2,5-dihydroxycinnamate is shown in fig.1. 2,5-Dihydroxybenzaldehyde (4.3 g) and methyl(triphenylphosphoranilidene) acetate (11.6 g) were mixed in 10 ml of tetrahydrofuran and 120 ml of toluene and stirred for 30 min at 60°C. Then the mixture was evaporated and applied onto a silica gel column (305 g, $CHCl_3$/MeOH 20:1) to give 5.7 g of yellow flakes. The crude product was further purified by LH-20 column chromatography with MeOH to give 4.86 g of methyl 2,5-dihydroxycinnamate as a yellow powder. The structure was confirmed by proton NMR spectroscopy.

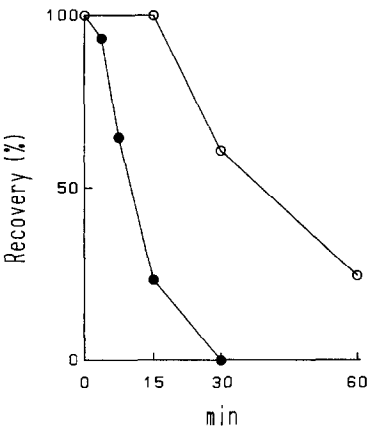

Fig.2. Stability of methyl 2,5-dihydroxycinnamate in calf serum. Methyl 2,5-hydroxycinnamate (○) or erbstatin (●) was incubated of calf serum for the indicated periods. The recovery of each inhibitor was determined by HPLC.

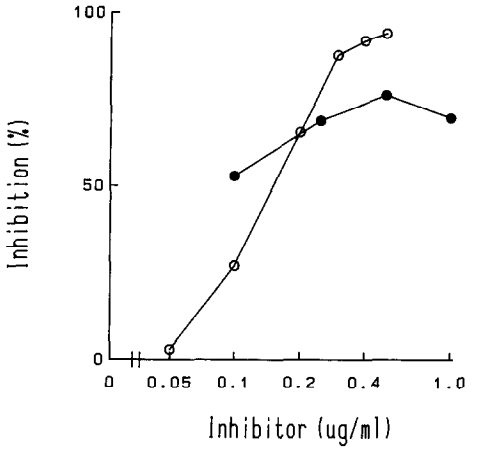

Fig.3. Inhibition of EGF receptor-associated tyrosine kinase by methyl 2,5-dihydroxycinnamate. The reaction mixture was incubated with methyl 2,5-dihydroxycinnamate (○) or erbstatin (●) for 30 min.

As shown in fig.2 methyl 2,5-dihydroxycinnamate was about 4 times more stable than erbstatin in calf serum. Erbstatin disappeared completely by 30 min, while methyl 2,5-dihydroxycinnamate was still recovered in appreciable amount even after a 60-min incubation.

Methyl 2,5-dihydroxycinnamate inhibited EGF receptor tyrosine kinase in vitro with an IC_{50} of 0.15 μg/ml, which is equivalent to the activity of erbstatin, as shown in fig.3. At higher concentrations, it inhibited tyrosine kinase more strongly than did erbstatin. Lineweaver-Burk plotting showed that it inhibits EGF receptor kinase competitively with the peptide and non-

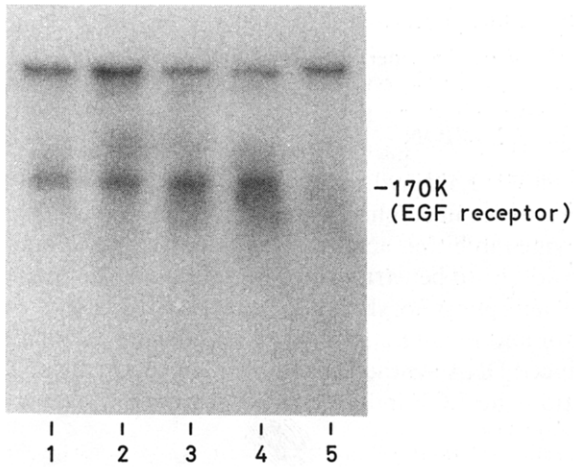

Fig.4. Inhibition by methyl 2,5-hydroxycinnamate of EGF receptor autophosphorylation in cultured A431 cells. The cells were preincubated with 100 μg (lane 1), 50 μg (2), 25 μg (3), or 0 μg (4) of inhibitor for 1 h; and then 0.5 μg/ml of EGF was added and incubation continued for 20 min. For lane 5, no inhibitor or EGF was added to the cells.

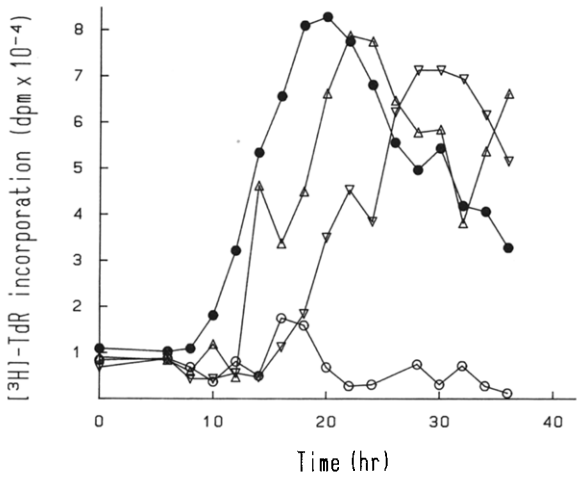

Fig.5. Inhibition of EGF-induced DNA synthesis by tyrosine kinase inhibitors. Quiescent NRK cells were incubated without additives (○) or with EGF (●), EGF and 12.5 μg/ml of erbstatin (△), or EGF and 12.5 μg/ml of methyl 2,5-dihydroxycinnamate (▽). Details are described in section 2.

competitively with ATP (data not shown), as is the case for erbstatin [7]. As shown in fig.4, the methyl ester derivative inhibited EGF receptor autophosphorylation in cultured A431 cells at 25–100 μg/ml. This in situ activity of methyl 2,5-dihydroxycinnamate is comparable to that of erbstatin [2]. The decrease in autophosphorylation is not due to the inhibition of EGF binding, since we confirmed that the inhibitor did not affect EGF-receptor binding in A431 cells at 100 μg/ml.

In quiescent NRK cells, addition of EGF began to induce DNA synthesis after 12 h, and the maximal effect was observed at 16 h, as shown in fig.5. Addition of 12.5 μg/ml of erbstatin delayed the induction of DNA synthesis for about 3 h. Methyl 2,5-dihydroxycinnamate apparently delayed the DNA synthesis induction by EGF for about 10 h. In both cases, the total amount of DNA synthesis did not decrease. Similar results were obtained in 3 repeated experiments.

4. DISCUSSION

Methyl 2,5-dihydroxycinnamate was shown to be more stable in serum than erbstatin, without losing its enzyme inhibition activity. In addition, the analogue is considered to penetrate into the cells, since it inhibits in situ autophosphorylation of the EGF receptor. Erbstatin and methyl 2,5-dihydroxycinnamate delayed the induced DNA synthesis at 12.5 μg/ml at which concentration the EGF-induced DNA synthesis was delayed for about 3 h by erbstatin and for about 10 h by methyl 2,5-dihydroxycinnamate. Neither was effective at 3 μg/ml, and at 50 μg/ml they reduced the total amount of DNA synthesis. For in situ inhibition of tyrosine kinase higher concentration (25–50 μg/ml) of methyl 2,5-dihydroxycinnamate was required. It would be because its penetration into the cell is not sufficient within a short time incubation. Since methyl 2,5-dihydroxycinnamate is about 4 times more stable than erbstatin, the difference noted in the delay until S-phase may be related to the stability of the inhibitor.

Several enzyme inhibitors are known to affect S-phase induction by acting at the G1-phase. W-13, a calmodulin blocker, inhibits S-phase induction acting at G1 and early S-phase [8,9]. Tunicamycin [10], an inhibitor of oligosaccharide synthesis, and bestatin [11], inhibiting aminopeptidase, were shown to inhibit DNA synthesis by acting at the G1-phase. We have demonstrated that tyrosine kinase activity is essential for the S-phase induction by EGF in quiescent NRK cells.

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