INHIBITION OF TYROSINE KINASE AND *src* ONCOGENE FUNCTIONS BY STABLE ERBSTATIN ANALOGUES

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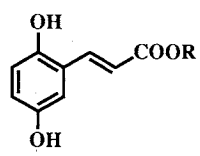
Erbstatin was isolated as an inhibitor of tyrosine kinase from *Streptomyces*[1]. It inhibits epidermal growth factor (EGF) receptor and v-*src*-associated tyrosine kinase[1,2] and EGF-induced DNA synthesis in quiescent NRK cells[3], and induces normal phenotypes in Rous sarcoma virus-transformed NRK (RSV-NRK) cells[4]. Erbstatin also inhibits growth of human breast carcinoma MCF-7 transplanted in nude mice[5]. However, it is unstable and easily degraded in serum[6] and in cell culture medium (K. UMEZAWA; unpublished results). Therefore, we synthesized methyl 2,5-dihydroxycinnamate (2,5-MeC) as a stable analogue of erbstatin[3]. It inhibited EGF-induced S-phase entry[3], cellular *src* functions[4], and *in vivo* growth of MCF-7 tumors (Dr. M. TOI, Hiroshima University; personal communication) more prominently than erbstatin.

In this presently reported study we synthesized several new alkyl dihydroxycinnamates and an aldehyde derivative as more stable analogues of erbstatin and examined their inhibitory activity on tyrosine kinase and cellular *src* oncogene functions. We synthesized alkyl 2,5-dihydroxycinnamates[7] and 2,3-dihydroxycinnamates[8]. The structures of these new erbstatin derivatives are shown in Fig. 1. The inhibition of EGF receptor-associated tyrosine kinase by them and their cytotoxicity toward A431 cells are summarized in Table 1. The tyrosine kinase activity was assayed as described before[3] with the peptide RR-SRC as substrate and the A431 cell membrane as an enzyme source. Cell growth was assayed by counting the cell number after 3 days of incubation of the cells in DULBECCO's modified EAGLE's medium supplemented with 5% calf serum. The ethyl and propyl 2,5-esters showed similar enzyme inhibitory activity to those of erbstatin and 2,5-MeC. Further elongation of the aliphatic structure slightly decreased the inhibitory activity. The 2,3-dihydroxy derivative of erbstatin was shown to be more potent than erbstatin in inhibition of

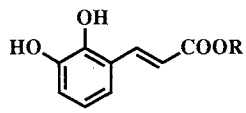
Table 1. Inhibition of tyrosine kinase and of A431 cell growth by erbstatin analogues.

Chemical	IC_{50} μg/ml (μM)	
	Tyrosine kinase	Cell growth
Erbstatin	0.09 (0.60)	3.7 (24.80)
2,5-MeC	0.15 (0.77)	0.34 (1.75)
2,5-EtC	0.17 (0.81)	0.26 (1.25)
2,5-PrC	0.22 (0.99)	2.0 (8.98)
2,5-BuC	0.34 (1.44)	3.8 (16.04)
2,5-HeC	0.72 (2.72)	8.0 (30.20)
2,5-BzC	0.31 (1.14)	6.0 (22.15)
2,3-BuC	0.30 (1.27)	1.5 (6.33)
2,3-HeC	1.5 (5.66)	0.30 (1.13)
2,5-Cal	0.50 (3.73)	1.0 (7.45)

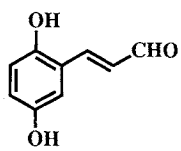
Fig. 1. Structure of erbstatin analogues.



Methyl 2,5-dihydroxycinnamate (2,5-MeC) R = Me
Ethyl 2,5-dihydroxycinnamate (2,5-EtC) R = Et
Propyl 2,5-dihydroxycinnamate (2,5-PrC) R = Pr
Butyl 2,5-dihydroxycinnamate (2,5-BuC) R = Bu
Hexyl 2,5-dihydroxycinnamate (2,5-HeC) R = He
Benzyl 2,5-dihydroxycinnamate (2,5-BzC) R = Bz



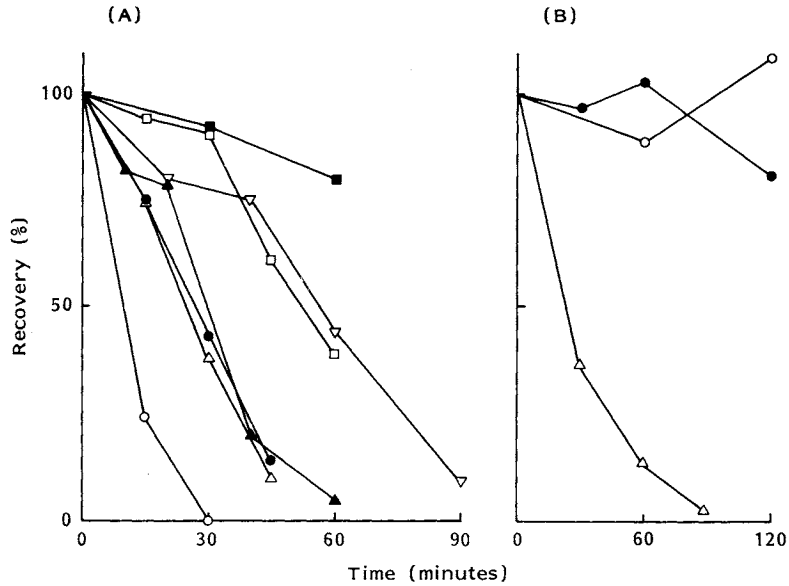
Butyl 2,3-dihydroxycinnamate (2,3-BuC) R = Bu
Hexyl 2,3-dihydroxycinnamate (2,3-HeC) R = He



2,5-Dihydroxycinnamic aldehyde (2,5-Cal)

Fig. 2. Stability of erbstatin analogues in calf serum.

A: Erbstatin (○), 2,5-MeC (●), 2,5-EtC (△), 2,5-PrC (▲), 2,5-BuC (□), 2,5-HeC (■) or 2,5-BzC (▽) was incubated for the indicated periods in calf serum and then recovered. B: 2,3-BuC (○), 2,3-HeC (●), or 2,5-Cal (△) was incubated as in A. The percent recovery in B was assayed by thin-layer chromatography.



tyrosine kinase[9]; but it showed no biological effect in cell culture, possibly because of poor permeability through the plasma membrane. So we prepared butyl and hexyl esters of 2,3-dihydroxycinnamic acid. The hexyl derivative showed weaker inhibitory potency than 2,3-BuC. As shown in Table 1, large aliphatic or aromatic ester showed weaker cytotoxicity toward A431 cells.

The stability of 2,5- and 2,3-dihydroxycinnamates and 2,5-Cal in calf serum is shown in Fig. 2. The stability was assayed as described before[3]. The butyl, hexyl, or benzyl esters were more stable than the esters with a shorter aliphatic chain. Also, the 2,3-derivatives were more stable than the 2,5-derivatives.

Induction of normal morphology in RSV^{ts}-NRK cells was determined by phase-contrast microscopy as described earlier[4]. Normal morphology was most effectively induced by 2,5-MeC and 2,5-EtC among the 2,5-dihydroxycinnamates, as shown in Table 2. Elongation of the aliphatic chain reduced the activity. For the 2,3-derivatives, 2,3-HeC was more effective than 2,3-BuC in induction of normal phenotypes.

Although erbstatin and 2,5-MeC show antitumour activity against transplanted human tumours, their effective dosage (1~4 mg/mouse) is so high that further development will be difficult. It is highly desirable to find more effective and stable tyrosine kinase inhibitors. The new erbstatin analogues described here were not more effective in inhibition of the enzyme, but their stability in serum was greatly enhanced.

Table 2. Induction of normal morphology in RSV^{ts}-NRK cells by erbstatin analogues.

Chemical	Concentration μg/ml				
	0.1	0.3	1	3	10
Erbstatin		−[b]	+[a]	+	T[d]
2,5-MeC	±[c]	+	+	+	T
2,5-EtC	±	+	+	+	T
2,5-PrC		−	+	+	T
2,5-BuC		−	±	+	+
2,5-HeC			−	T	T
2,5-BzC			±	+	T
2,3-BuC			−	−	±
2,3-HeC			+	+	T
2,5-Cal		±	+	+	T

[a] Normal morphology induced after 8 hours; [b] normal morphology not induced; [c] normal morphology partly induced; [d] toxic.

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