21. Supported by NIH grants HD-20197 and R37-CA21923. P.L.S. was supported by USPHS grant T32-ES07066. We thank the National Institute of Diabetes and Digestive and Kidney Diseases and the National Hormone and Pituitary Program, University of Maryland School of Medicine, for providing hCGα. We also thank S. Kornfeld, R. Kornfeld, C. Frieden, and members of the Baenziger lab for critical review and suggestions.

20 June 1988; accepted 2 September 1988

Blocking of EGF-Dependent Cell Proliferation by EGF Receptor Kinase Inhibitors

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A systematic series of low molecular weight protein tyrosine kinase inhibitors were synthesized; they had progressively increasing affinity over a 2500-fold range toward the substrate site of epidermal growth factor (EGF) receptor kinase domain. These compounds inhibited EGF receptor kinase activity up to three orders of magnitude more than they inhibited insulin receptor kinase, and they also effectively inhibited the EGF-dependent autophosphorylation of the receptor. The most potent compounds effectively inhibited the EGF-dependent proliferation of A431/clone 15 cells with little or no effect on the EGF-independent proliferation of these cells. The potential use of tyrosine protein kinase inhibitors as antiproliferative agents is demonstrated.

MANY ONCOGENE PRODUCTS EXhibit protein tyrosine kinase (PTK) activity that is essential for their biological function (*1*). Similarly, early events in mitogenesis induction by growth factors are the ligand-induced autophosphorylation of the receptor and the phosphorylation of a host of intracellular substrates (*1, 2*). Insulin-induced autophosphorylation of its receptor, as well as hormone-induced tyrosine phosphorylation of specific intracellular target proteins, are the earliest identifiable biochemical events triggered by the hormone (*3*). The nullification of the PTK activity of insulin receptor (*3*) and of EGF receptor (*4*) by site-directed mutagenesis resulted in the elimination of their biological activity. Furthermore, low molecular weight PTK inhibitors that blocked insulin-dependent PTK activity of insulin receptor also inhibited insulin-induced lipogenesis and its antilipolytic effect in rat adipocytes (*5*). The involvement of PTK activity of many oncogene products in the expression of the transformed phenotype identifies these proteins as potential targets for selective chemotherapy and thus, for rational drug design. A series of low molecular weight compounds that inhibited EGF receptor PTK activity at concentrations that were a factor of 100 to 700 less than the concentrations needed to inhibit the PTK activity of insulin receptor were synthesized. The most potent EGF receptor kinase (EGFRK) blockers inhibited the EGF-dependent proliferation of A431/clone 15 cells.

Umezawa and colleagues (*6*) identified in the medium of an actinomycete a compound they termed erbstatin (Fig. 1), which inhibits the autophosphorylation of the EGF receptor in membranes of A431 epidermoid carcinoma cells. Low concentrations of erbstatin were required to inhibit the EGF receptor autophosphorylation (inhibition concentration $IC_{50} < 2.5$ μg/ml; 14 μ*M*), whereas its potency in inhibiting cyclic adenosine 3′,5′-monophosphate (cAMP)–dependent protein kinase was rather weak ($IC_{50} = 100$ μg/ml; 558 μ*M*).

We designed PTK inhibitors whose structures were initially patterned after erbstatin. Three criteria that guided us were as follows. (i) The compounds should be competitive with the substrate of EGFRK and not with adenosine triphosphate (ATP). The PTK inhibitors quercetin (*7*) and genistein (*8*), which compete with ATP, inhibit other protein kinases and are highly cytotoxic. (ii) As a test of selectivity we chose compounds that inhibited EGFRK much better than the insulin receptor kinase (IRK), which is structurally and functionally highly homologous to EGFRK in its kinase domain (*9*). (iii) The compounds should be soluble both in water and in mildly hydrophobic solvents such as alcohols and dimethyl sulfoxide. These solubility properties enhance the probability that such compounds will be

Table 1. Potency of EGFRK inhibitors. The inhibition constants quoted in the table were determined from Dixon plots with three substrate concentrations for each inhibitor, as described in legend to Fig. 2. Together with Fig. 2 the benzylydene compounds represent a series in which their affinity toward EGFRK is increased 2940-fold (2500/0.85) through specific substitutions.

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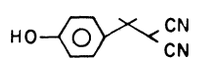
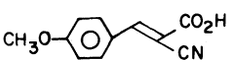
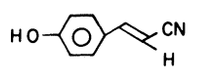
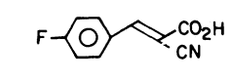
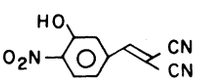
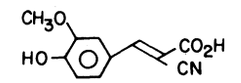
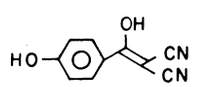
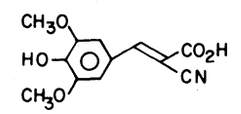
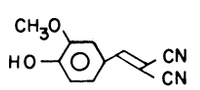
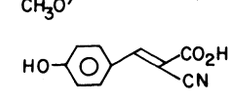
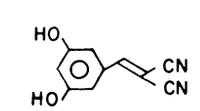
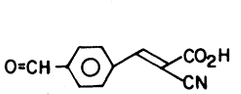
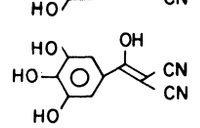
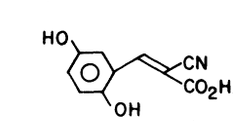
13		2500	20		833
14		1333	21		833
15		150	22		267
16		77	23		233
17		67	24		166
18		10	25		47
19		3.3	26		24

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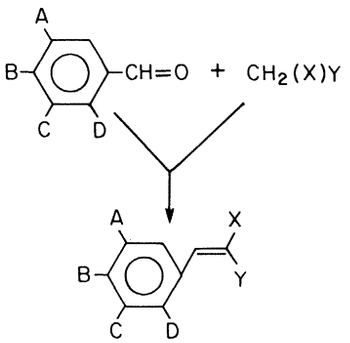


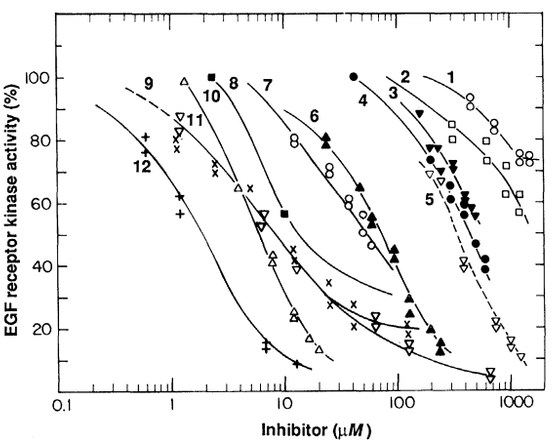
Fig. 1. Design and synthesis of EGFRK inhibitors. For erbstatin, A and D are OH; B, C, and Y are H; and X is NHCHO. The detailed protocols for the synthesis of all of the compounds described in this study are available on request.

able to traverse the cell membrane. We prepared compounds derived from the benzylidene malononitrile (BMN) nucleus (Fig. 1). These compounds resembled both the phenolic moiety of tyrosine as well as erbstatin. The direction of the amide bond in erbstatin is inverted to give the cyano group in BMN. The second cyano group in BMN increases biological activity and simplifies the synthetic protocol. The BMN analogs were prepared by a straightforward synthesis (Fig. 1).

The substrate poly(Glu_6-Ala_3-Tyr) was used as a substrate for EGFRK and poly(Glu_4-Tyr) for IRK (*10*). The benzylidene compounds depicted display a series in which the affinity is increased 2500-fold through specific substitutions on the aromatic ring and modifications of the side chain of the compounds (Fig. 2 and Table 1). In contrast, the effect of these compounds on IRK activity is in the millimolar range with no particular trend (Fig. 2). For both receptors the compounds were found to be competitive with the substrate, as is revealed by the Dixon plots; a representative example is shown in Fig. 3. Some of the compounds were tested as inhibitors of protein kinase C and cAMP-dependent protein kinase and were found to inhibit these enzymes only partially at the millimolar range. Similar results were reported for erbstatin (*6*).

The substructure responsible for the potency of the BMN analogs as PTK inhibitors appears to be hydroxy *cis*-cinnamonitrile. Saturation of the double bond or replacement of the *cis*-cyano group by the *trans* configuration lowered the activity. Introduction of electron-withdrawing or electron-donating substituents on the aromatic ring had little effect on the potency. Addition of hydroxyl substituent on the phenolic ring increased the potency 10- to 15-fold for each hydroxyl group added. Transsubstituents on the double bond showed an increase of potency from COOH to CN to $CONH_2$ to $CSNH_2$.

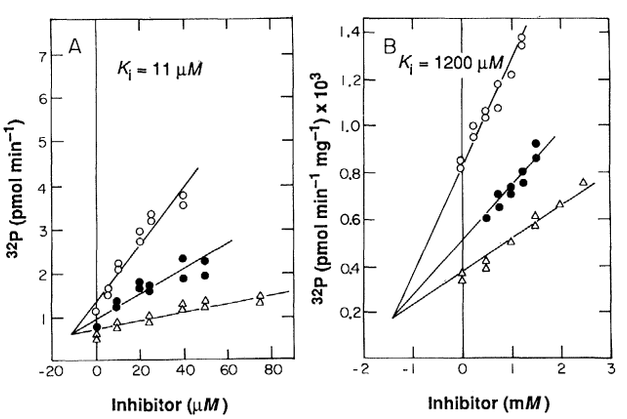
The autophosphorylation of EGF receptor as well as of other growth factor receptors is believed to be the first step in signal transduction and a prerequisite for the phosphorylation of exogenous substrates by the receptor (*2, 11–13*). The EGF receptor autophosphorylation was inhibited effectively by the competitive inhibitors that inhibited the EGF receptor–catalyzed phosphorylation of the exogenous substrate (Fig. 4). The inhibitors were seemingly less effective (approximately tenfold) as blockers of EGF-receptor autophosphorylation than as blockers of exogenous-substrate phosphorylation. This difference may result because autophosphorylation probably represents intermolecular transphosphorylation within the EGFRK dimer formed on binding of EGF to its receptor (*14*). Therefore, the inhibitor must compete against a high local substrate concentration. Because of this "proximity effect," the substrate concentration can be estimated to be in the high molar range (*15*).



Compound	Substituents						K_i (μM)		K_i^{EGFRK}/K_i^{IRK}
	A	B	C	D	X	Y	EGFRK	IRK	
1	H	OH	H	H	CO_2H	H	1000	4100	0.24
2	H	OH	H	H	CO_2H	CO_2H	500		
3	H	OH	H	H	CN	CN	166	1500	0.11
4	OH	OH	H	H	CO_2H	H	150	2800	0.053
5	H	H	OH	H	CN	CN	123		
6	OH	H	H	OH	CN	CO_2H	24		
7	H	OH	OH	H	CO_2H	CN	18		
8	H	OH	OH	H	CN	CN	11	1200	0.0091
9	OCH_3	OH	OH	H	CN	CN	2		
10	OH	OH	OH	H	CN	CN	1		
11	H	OH	OH	H	$CONH_2$	CN	2.3	410	0.0056
12	H	OH	OH	H	$CSNH_2$	CN	0.85	640	0.0013

Fig. 2. Inhibition of EGFRK by BMN (arylidene) derivatives. The receptor was purified from A431 epidermoid carcinoma cells grown in culture, as described (*11*). Purified EGF receptor (0.25 μg per assay) was activated with EGF (0.85 μ*M*) in 50 m*M* tris–2-(*N*-morpholino)ethanesulfonic acid (MES) buffer, *p*H 7.6, for 20 min at 4°C. The assay was initiated by addition of a mixture containing magnesium acetate (60 m*M*), [γ-^{32}P]ATP (125 μ*M*), poly(Glu_6-Ala_3-Tyr) (0.0625 to 0.25 mg/ml), EGF (300 n*M*), and inhibitors at the indicated concentrations. The temperature of the assay was 22°C, and production of phosphorylated polymer was linear for 10 to 20 min according to the substrate concentration. Other details are in (*10*). Detailed kinetic analysis according to Dixon (*17*) was performed for all the compounds depicted. The substrate concentrations chosen were 0.0625, 0.125, and 0.25 mg/ml. A total of 40 to 50 experimental points were used to obtain each inhibition constant (K_i) value. The Michaelis constant (K_m) for the polymer was 0.069 mg/ml, and the K_m for ATP in this assay was 7.2 μ*M*. The assay of IRK and its inhibition was conducted as described elsewhere with poly(Glu_4Tyr) as substrate (*10*). The concentrations of substrate used to construct the Dixon plots were 0.125, 0.25, and 0.5 mg/ml. A total of 40 to 50 experimental points were used to construct the Dixon plots. The standard errors in the K_i values depicted are in the range of ≤11% of the value for both EGFRK and IRK.

Fig. 3. The competitive action of EGFRK blockers. Dixon plots for (**A**) EGFRK and (**B**) IRK inhibition for a representative example are shown. Values for ^{32}P are quantities incorporated per assay; in (B), mg refers to milligrams of protein. Details as in Fig. 2. The data shown are for compound 8. The symbols △, ●, and ○ in (A) correspond to substrate concentrations of 0.0625, 0.125, and 0.25 mg/ml, respectively; in (B), to 0.125, 0.25, and 0.5 mg/ml, respectively.



Accurate inhibition constants, however, could not be calculated since the true concentration of the autophosphorylated sites cannot be precisely determined. These considerations revealed that the PTK inhibitors block autophosphorylation at least as effectively as they inhibited the EGF receptor–catalyzed phosphorylation of exogenous substrate.

Proliferation of A431/clone 15 cells induced by EGF is mediated by the EGF receptor (*16*). This clone was used to evaluate whether the potent EGFRK inhibitors also acted as inhibitors of EGF-dependent proliferation. Potent inhibitors of the EGFRK also inhibited the EGF-dependent proliferation of these cells (Fig. 5). These compounds exhibited a much smaller inhibitory effect or no effect at all on the EGF-independent growth, thus revealing their specificity and the absence of adverse toxic effects. The inhibitory effect of the EGFRK blockers is fully reversible, since upon removal of the inhibitors, cell growth resumes its normal rate. These findings correlate with the high specificity of these compounds as revealed by their ability to inhibit EGFRK at concentrations that were a factor of 750 less than those required to inhibit IRK (Table 1 and Fig. 2).

Our findings corroborate other observations that demonstrate that the tyrosine kinase activity of the EGF receptor is essential for the EGF-mediated proliferation (*2, 4*). Furthermore, the relation between the potency of the PTK inhibitors in blocking EGFRK activity and their ability in blocking EGF-dependent proliferation suggests a general biochemical screening method for selective antiproliferative drugs aimed at oncogene proteins with PTK activity. These findings also strengthen the hypothesis that selective blockers for each of the protein tyrosine kinases can be developed by rational design. Such compounds have the potential to become antiproliferative drugs aimed at specific pathological conditions that involve cell proliferation and that can be linked to the expression of specific protein tyrosine kinases. We propose the general name "tyrphostins" for such compounds.

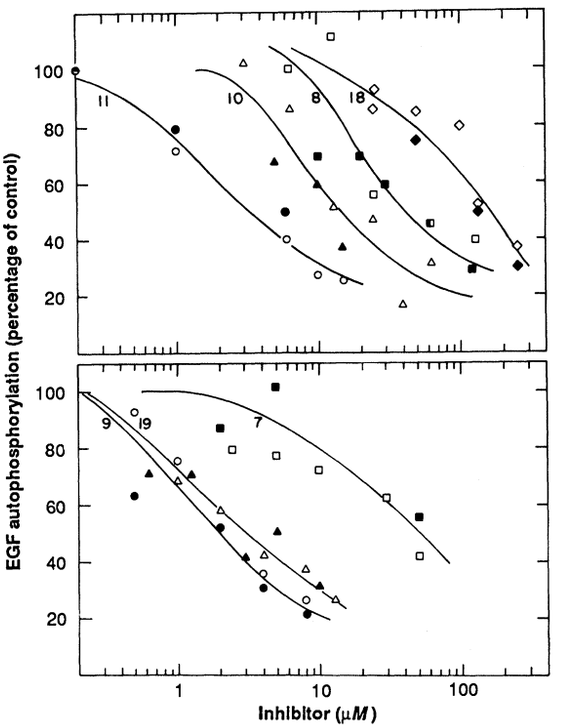


Fig. 4. Inhibition of EGF receptor autophosphorylation. Purified EGF receptor from A431 cells (0.5 μg per assay) was activated with EGF (0.85 μ*M*) for 20 min at 4°C. The assay was performed at 15°C and initiated by addition of magnesium acetate (60 m*M*), tris-MES buffer, *p*H 7.6 (50 m*M*), [γ-^{32}P]ATP (10 μ*M*; 5 μCi per assay), and various inhibitors at increasing concentrations. The assay was terminated after 10 s by the addition of SDS–polyacrylamide gel electrophoresis (PAGE) sample buffer. The samples were run on a 6% SDS-polyacrylamide gel and the gel was dried. Autoradiography was performed with an Agfa Curix RP2 x-ray film. The relevant radioactive bands were cut and counted in Cerenkov mode. The fast phase of autophosphorylation continued for another 10 min. The extent of phosphorylation completed in the first 10 s comprises one-third of the total autophosphorylation signal and probably reflects the phosphorylation of the first site on the receptor. Filled symbols and open ones with identical shape represent two independent experiments. Numbers refer to compounds listed in Table 1 and Fig. 2.

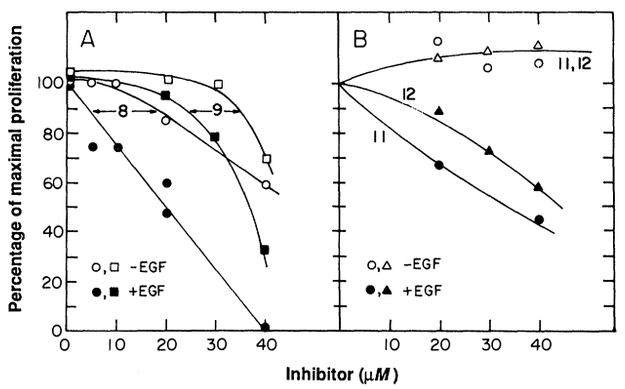


Fig. 5. Inhibition of EGF-dependent proliferation of A431/clone 15 cells. (**A**) Compounds that inhibit preferentially EGF-dependent growth. (**B**) Compounds that inhibit exclusively EGF-dependent growth. Cells were plated at high dilution (1000 cells per 90-mm dish) and supplied with Dulbecco's minimum essential medium (DMEM) containing 10% fetal bovine serum (FBS) and EGF (100 ng/ml) for reclonning. Two of the surviving clones were propagated in 10% FBS with EGF (100 ng/ml), and clone 15/2 was chosen for further work. To determine EGF-dependent growth of A431/clone 15/2, 25,000 cells per well were plated in 24-well plates (Costar) and supplied with DMEM containing 10% FBS and increasing concentrations of EGF. The medium was replaced with fresh medium every other day. The cells were counted every other day for 6 days. To assess the inhibition of EGF-dependent cell growth, 25,000 cells per well were plated in 24-well plates (Costar) containing 10% FBS, with EGF (10 ng/ml) (filled symbols) or with no added EGF (open symbols). EGFRK inhibitors at various concentrations were added to the cells 2 hours after plating. The medium containing the inhibitors was replaced with fresh inhibitor-containing medium every other day. On the fifth day, the number of cells in the presence of EGF and in the absence of EGF was determined; 100% refers to the number of cells in the absence of inhibitor for each mode of cell growth (without EGF: 100,000 ± 10,000 cells; with EGF: 260,000 ± 30,000 cells for seven experiments). Filled symbols refer to inhibition of EGF-stimulated growth, whereas open symbols depict inhibition of EGF-independent growth. Each experimental point represents the average of triplicate determination where the variance was less than 5%. Numbers refer to compounds in Fig. 2.

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18. We thank R. Bravo for donating the A431/clone 15 cells and we acknowledge the encouragement given by Rorer Biotechnology, King of Prussia, PA.

31 May 1988; accepted 18 August 1988