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INHIBITION OF PROTEIN KINASE C BY THE TYROSINE KINASE INHIBITOR ERBSTATIN

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Abstract—We examined the tyrosine kinase inhibitor erbstatin and several derivatives for their ability to inhibit serine/threonine protein kinases *in vitro*. Erbstatin was found to inhibit protein kinase C (PKC) with an IC_{50} of 19.8 ± 3.2 μM. A trihydroxy derivative of erbstatin inhibited PKC with similar potency, whereas the corresponding methoxy derivatives were inactive. Inhibition by erbstatin was competitive with ATP (K_i = 11.0 ± 2.3 μM) and non-competitive with the phosphate acceptor, either histone or the synthetic peptide kemptide. Action of erbstatin at the catalytic stie of PKC was further indicated by the findings that it inhibited the catalytic fragment of PKC but did not inhibit the interaction of phorbol ester with the intact enzyme. Erbstatin had a similar potency against three PKC isozymes (α, β, and γ) examined. In addition, erbstatin was found to inhibit other serine/threonine kinases (assayed at their K_m for ATP). The greatest potency was observed versus the cyclic nucleotide-dependent kinases, while lower potency was seen versus myosin light chain kinase. These observations are discussed in terms of the structure and kinetic properties of PKC and the epidermal growth factor receptor tyrosine kinase.

Protein kinases play essential roles in intracellular signal transduction. Cellular protein kinases can be categorized based on their amino specificity as tyrosine kinases or serine/threonine kinases. Many growth factor receptors, including the receptors for insulin, epidermal growth factor (EGF§) and platelet-derived growth factor, possess intrinsic tyrosine kinase activity. The tyrosine kinase activity of these receptors is required to elicit the biological response to growth factor (reviewed in Ref. 1). The products of a number of transforming oncogenes (e.g. *src* and *fes*) are non-receptor protein tyrosine kinases [1]. Currently, considerable research is directed towards defining the link between tyrosine phosphorylation and cellular proliferation and transformation.

Serine/threonine kinases are a diverse group of enzymes, many of which are regulated by intracellular second messengers. These include cAMP-dependent protein kinase, cGMP-dependent protein kinase, a family of Ca^{2+} calmodulin-dependent protein kinases and the Ca^{2+}/phospholipid-dependent protein kinase, protein kinase C. Multiple isozymes of each of these serine/threonine kinases exist; in the case of protein kinase C (PKC), seven related isozymes have been discovered [2]. PKC is activated during cell stimulation by receptor-coupled increases in two second messengers derived from phospholipid hydrolysis: inositol-1,4,5-trisphosphate (leading to mobilization of intracellular Ca^{2+}) and *sn*-1,2-diacylglycerol. PKC is also activated by the tumor-promoting phorbol esters which interact with high affinity at the diacylglycerol binding site on the enzyme [3, 4]. PKC has been implicated in a wide array of cellular responses, including a variety of secretion reactions and cell proliferation and growth control (reviewed in Ref. 5).

A great deal of effort has been made to identify inhibitors of specific protein kinases. These would be useful in defining physiological function and may be of therapeutic value. Many protein kinase inhibitors have been described which act by competing with ATP for binding to the enzymes. Some selectivity for various kinases has been observed with compounds of this type; however, they typically inhibit a number of serine/threonine and tyrosine protein kinases. This can be attributed to the highly conserved amino acid sequence of the ATP binding sites of various protein kinases.

Erbstatin, a novel compound recently isolated from the culture filtrate of a *Streptomyces* sp., was reported to inhibit the EGF-receptor tyrosine kinase (K_i = 3.4 μM) competitive with peptide (or protein) substrate and noncompetitive with ATP [6–8]. Antiproliferative properties of erbstatin-related compounds have been attributed to tyrosine kinase inhibition (e.g. Ref. 9).

In contrast to earlier reports [6, 7], we have found that erbstatin and a trihydroxy derivative of erbstatin inhibit serine/threonine kinases over the same concentration range required for inhibition of the EGF-receptor kinase. Erbstatin inhibited both intact PKC and its catalytic fragment with equal potency and did not interfere with [^{3}H]phorbol dibutyrate binding to PKC, indicating that it acts on the catalytic (rather than the regulatory) domain of this enzyme. Kinetic

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§ Abbreviations: EGF, epidermal growth factor; PKC, protein kinase C; MLCK, myosin light chain kinase; PDBu, phorbol-12,13-dibutyrate; OAG, 1-oleoyl-2-acetylglycerol; and PS, phosphatidylserine.

analysis showed that erbstatin inhibition of PKC is competitive with ATP rather than with the peptide substrate. These results indicate that caution must be used in interpreting data obtained using erbstatin in intact cells since it does not display absolute specificity for tyrosine kinases.

EXPERIMENTAL PROCEDURES

Materials and enzymes. cAMP-dependent protein kinase (catalytic subunit purified from bovine heart) and cGMP-dependent protein kinase (heart) were obtained from Dr. J. Corbin (Vanderbilt University). Myosin light chain kinase (native, calmodulin-dependent enzyme from chicken gizzard) was prepared by Dr. M.Cable (Schering-Plough Research).

The synthetic peptide substrates were obtained from Peninsula Laboratories (Belmont, CA). Phosphatidylserine and diacylglycerol cofactors for PKC were from Avanti Polar Lipids (Birmingham, AL). [^{3}H]Phorbol-12,13-dibutyrate (19.1 Ci/mmol) and [^{32}P]ATP (3000 Ci/mmol) were from New England Nuclear (Boston, MA).

Erbstatin and derivatives. Erbstatin and its derivatives were synthesized using previously described methods [10, 11]. The identity and purity of the compounds were determined by spectroscopic and chromatographic methods as well as elemental analysis. Detailed protocols for the preparation of the compounds described in this study are available upon request.

Preparation of protein kinase C and its catalytic fragment. Protein kinase C was partially purified from rat brain by chromatography on DEAE-Sephacel according to Shearman *et al.* [12]. The specific activity of DEAE-purified PKC ranged from 0.5 to 2.2 nmol/min/mg under standard assay conditions (see below). Purification to homogeneity was achieved by chromatography of the DEAE purified material on threonine-Sepharose and phenyl-Sepharose columns as described [13]. Homogeneous rat brain PKC was also purchased from Sphinx Biotechnologies, Inc. (Durham, NC). Purified enzyme had a specific activity between 2 and 2.5 μmol/min/mg.

The lipid-independent proteolytic fragment of PKC was formed by limited trypsinization essentially as described by Lee and Bell [14]. Reactions were carried out in 20 mM Tris–HCl (pH 7.5)/1 mM dithiothreitol and contained 12 μg PKC/mL. Trypsin (40 μg) was added, and reactions were stopped after 1 min by addition of 200 μg of soybean trypsin inhibitor. The catalytic fragment was purified by chromatography on a Pharmacia FPLC system equipped with a Mono Q column as described [15].

PKC Types I, II and III were resolved from DEAE-purified enzyme (50 mg protein) by chromatography on a 2.5 × 12.5 cm column of Bio-Rad Biogel HTP hydroxyapatite using the method described by Jaken and Kiley [16]. Isozyme separation was also achieved using an HPLC hydroxyapatite column (0.78 × 10 cm; Bio-Rad) as described [12]. Fractions of PKC activity eluted from the column were identified as PKC Type I, Type II or Type III by their elution position (0.07–0.08, 0.09–0.11 and 0.14–0.16 M potassium phosphate respectively) and by Western analysis using polyclonal antipeptide antisera specific for each isozyme. These antisera were a gift of Drs. Mary Makowske and Ora Rosen, Memorial Sloan-Kettering Cancer Research Center [17].

Protein kinase C assays. Protein kinase C was assayed in a reaction mixture (0.25 mL total volume) containing: 20 mM Tris–HCl (pH 7.5); 200 μg/mL histone III-S; 10 mM $MgCl_2$; 5 μM [γ-^{32}P]ATP (4×10^6 cpm/nmol); 200 μM $CaCl_2$; 32 μg/mL phosphatidylserine; 1.6 μg/mL 1-oleoyl-2-acetylglycerol and the indicated concentration of erbstatin [added from dimethyl sulfoxide (DMSO) stock solution]. The final concentration of DMSO in the reaction was 2%, and activity was compared to DMSO controls in each experiment. Reactions were started by addition of approximately 7 μg of DEAE-purified enzyme or 7 ng of homogeneous enzyme and allowed to proceed at 23° for 15 min. Reactions were terminated by collection of trichloroacetic acid (TCA)-precipitated phosphoproteins on GF/C filters [12]. In some experiments, the concentration of ATP or histone was varied as indicated. Where kemptide was employed as phosphate acceptor, it was used at a final concentration of 0.125 to 1 mM, and reactions were terminated by addition of 50 μL of 3.75 M H_3PO_4. Incorporation of [^{32}P] into kemptide was quantitated by spotting the reaction mixture on Whatman P81 phosphocellulose paper according to standard procedures (e.g. Ref. 18).

In all experiments, controls were run in which the lipid cofactors (phosphatidylserine and oleoylacetylglycerol) were omitted from the reaction mixture to assess lipid-independent kinase activity. This value (generally 10% or less of the total activity) was subtracted from the total to determine lipid-stimulated PKC activity. Lipid cofactors were omitted for assay of the catalytic fragment.

Mixed micellar PKC assays were performed as described by Hannun *et al.* [19] using 12 mol% PS and 2 mol% *sn*-1,2-dioleoylglycerol.

Other kinase assays. cAMP-dependent protein kinase (catalytic subunit) was assayed using a reaction mixture (0.25 mL final volume) containing: 20 mM Tris–HCl (pH 7.4), 2 mM Mg^{2+} acetate, 60 μg/mL bovine serum albumin, 16 μM ATP (1.7×10^6 cpm/nmol), and 16 μM kemptide (Leu-Arg-Arg-Ala-Ser-Leu-Gly). Erbstatin was added to the indicated concentrations from DMSO stocks (final concentration of DMSO: 2%). Assays were initiated by adding enzyme and allowed to proceed for 10 min at 30°. Assays were terminated by the addition of 100 μL of 375 mM H_3PO_4 and spotting on phosphocellulose P81 paper.

cGMP-dependent protein kinase was assayed by a similar method, except that the reactions included cyclic GMP (2 μM). ATP (25 μM) and 29 μM phosphate acceptor peptide (Arg-Lys-Arg-Ser-Arg-Lys-Glu) were used. Reaction time was 30 min.

Myosin light chain kinase (MLCK) was assayed in a reaction mixture containing: 30 μM myosin light chain kinase substrate (Lys-Lys-Arg-Pro-Gln-Arg-Ala-Thr-Ser-Asn-Val-Phe-Ser-NH_2), 10 nM calmodulin, 25 μM [^{32}P]ATP, 0.1 mM Ca^{2+} acetate, and 10 mM Mg^{2+} acetate. Reactions were stopped

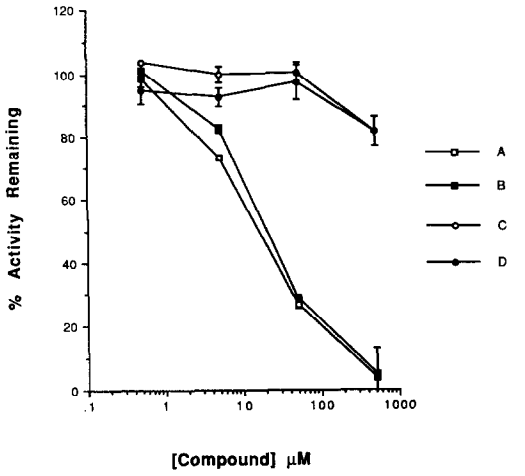


Fig. 1. Inhibition of PKC by erbstatin and derivatives. Compounds were added to the reaction mixture prior to addition of enzyme. DEAE-purified PKC was assayed under standard conditions as described in Experimental Procedures (5 μM ATP; 200 μg/mL histone III-S; 32 μg/mL phosphatidylserine; 1.6 μg/mL 1-oleoyl-2-acetylglycerol). Structures of compounds A–D are as indicated in Table 1. Data are expressed relative to a DMSO control (63 pmol phosphate incorporated). Data for erbstatin are representative of ten independent experiments, while compounds B, C and D were run twice with similar results. Values are means ± SD.

by acidification and spotting on phosphocellulose as described above.

PDBu binding. Binding of [^{3}H]phorbol dibutyrate (PDBu) to PKC was measured using the procedure described by Sando and Young [20].

RESULTS

Protein kinase C inhibition by erbstatin and its analogs. Protein kinase C was assayed using cosonicated phosphatidylserine and diacylglycerol as lipid cofactors, ATP at its K_m (5 μM), and histone III-S as phosphate acceptor. Under these assay conditions erbstatin was found to inhibit PKC with an IC_{50} of 19.8 ± 3.2 μM (mean ± SE, N = 10) (Fig. 1). Similar results were obtained using partially (DEAE)-purified rat brain PKC or rat brain PKC purified to homogeneity.

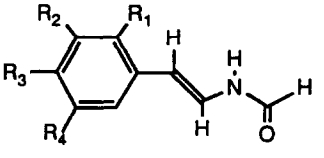
Several erbstatin derivatives were synthesized and assayed for PKC inhibition. These data and published data for inhibition of the EGF receptor tyrosine kinase are shown in Fig. 1 and Table 1. Erbstatin (A) and a trihydroxy derivative (B) inhibited both PKC and the EGF receptor kinase. For each compound the inhibitory potency was slightly greater against the EGF receptor kinase. Corresponding methoxy derivatives of each compound (C and D) did not inhibit PKC at 50 μM, and were only slightly (20%) inhibitory at 500 μM, indicating that the presence of free hydroxyl groups is a critical feature.

Inhibition of PKC by erbstatin was modulated considerably by the assay conditions employed. When Triton X-100–phosphatidylserine–diacylglycerol mixed micelles were used to support PKC activity the potency of erbstatin was reduced about 10-fold (data not shown). This suggests that erbstatin is subject to surface dilution in the detergent/phospholipid mixed micelles, decreasing its effective concentration in the assay [21].

Mechanism of protein kinase C inhibition by erbstatin. Using the standard vesicular assay, we examined the kinetics of erbstatin inhibition of PKC. Erbstatin inhibited PKC competitively with respect to ATP (Fig. 2). Secondary plots of the double-reciprocal data indicate a K_i of 11.0 ± 2.3 μM (N = 2).

A similar kinetic analysis with histone as the varied substrate indicated that erbstatin was not competitive with the phosphate acceptor for binding to PKC (data not shown). Kinetics of histone phosphorylation are complicated by the presence of multiple phosphorylation sites; therefore, kinetic analysis was

Table 1. Inhibition of protein kinase C and the EGF receptor tyrosine kinase by erbstatin derivatives



Compound	R_1	R_2	R_3	R_4	IC_{50} (μM) PKC	IC_{50} (μM) EGF-R*
A (erbstatin)	OH	H	H	OH	19.8 ± 3.2	3.35
B	H	OH	OH	OH	20.0 ± 0.9	7.20
C	OCH_3	H	H	OCH_3	>500	ND
D	H	OCH_3	OCH_3	OCH_3	>500	ND

Erbstatin (A), a trihydroxy derivative (B), and their corresponding methoxy derivatives were tested for inhibition of PKC.

* Data for inhibition of the EGF receptor kinase (EGF-R) are from Isshiki *et al.* [8]. ND = not determined.

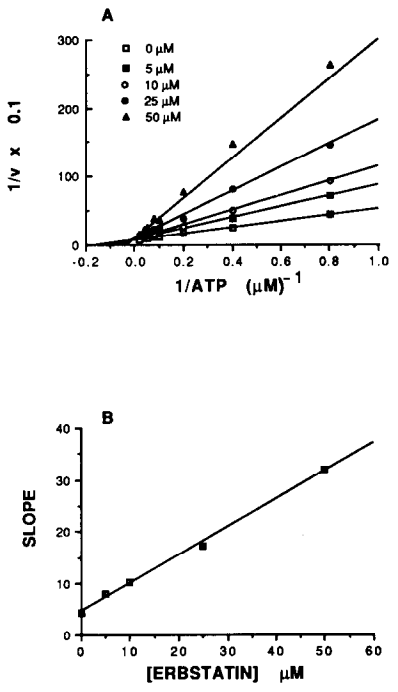


Fig. 2. Kinetics of PKC inhibition by erbstatin with respect to ATP. PKC (purified to homogeneity) was assayed under standard conditions in the presence of the indicated concentration of erbstatin. The ATP concentration was varied from 1.25 to 50 μM. (A) Data are presented as double-reciprocal plots of 1/reaction velocity × 0.1 (expressed in nmol/min) versus 1/[ATP]. (B) The slopes of the double-reciprocal lines are plotted versus the erbstatin concentration. K_i was calculated from this plot according to Dixon and Webb [22]. Lines were fit by least-squares and yielded linear correlation coefficients of 0.999, 0.999, 0.997, 0.998 and 0.997 for 0, 5, 10, 25 and 50 μM erbstatin respectively. The line in panel B has a linear correlation coefficient of 0.997. Similar results were obtained in two independent experiments.

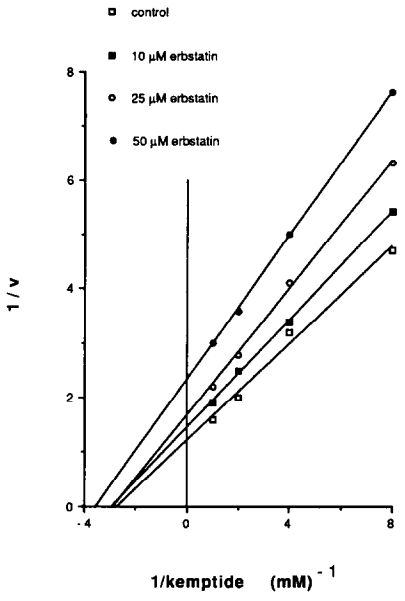


Fig. 3. Kinetics of PKC inhibition by erbstatin with respect to phosphoacceptor. DEAE-purified PKC was assayed under standard conditions in the presence of various concentrations of erbstatin. The kemptide concentration was varied from 0.125 to 1 mM. Data are presented as double-reciprocal plots of 1/reaction velocity (expressed in nmol/15 min) versus 1/[kemptide]. Lines were fit by least-squares and yielded linear correlation coefficients of 0.989, 0.999, 0.999 and 0.999 for 0, 10, 25 and 50 μM erbstatin respectively.

also performed using the synthetic peptide kemptide as phosphate acceptor. As reported by O'Brian *et al.* [23], we found that rat brain PKC will utilize kemptide as a phosphate acceptor (with a K_m of approximately 375 μM).* Phosphorylation of kemptide was fully dependent on the presence of lipid cofactors (data not shown). Erbstatin inhibited kemptide phosphorylation. A similar IC_{50} (55–60 μM) was observed over the range of 0.125–1 mM kemptide. This IC_{50} was higher than that observed using histone, indicating that the potency of erbstatin varies slightly with different phosphoacceptors. Inhibition of PKC by erbstatin appeared non-competitive with respect to kemptide up to 25 μM, although at higher concentrations some effect on the K_m for kemptide was also apparent (Fig. 3).

These data indicate that erbstatin inhibits PKC by interacting at a site on the catalytic domain of the enzyme where it prevents ATP binding. To confirm that erbstatin interacts with the catalytic (and not the regulatory domain) of PKC, the catalytic fragment was prepared by limited trypsinization. Erbstatin inhibited both intact PKC and its lipid-independent catalytic fragment with equal potency (Fig. 4). In addition, erbstatin at concentrations up to 1 mM did not inhibit the binding of [^{3}H]phorbol-12,13-dibutyrate to PKC, whereas sphingosine, a compound which inhibits PKC through an interaction with the regulatory domain did [21] (Fig. 5).

Specificity of erbstatin for serine/threonine protein kinases. We further explored the specificity of erbstatin inhibition. Protein kinase C Types I, II and III (corresponding to the products of the PKC γ, β, and α genes) were resolved from partially-purified rat brain PKC by hydroxyapatite chromatography. Erbstatin inhibited all three PKC isozymes with similar potency (Fig. 6). The observed IC_{50} values were: 17, 22 and 28 μM for Types I, II, and III respectively.

Inhibition of several other serine/threonine kinases by erbstatin was also examined (Fig. 7). All kinases were assayed with ATP present at its apparent K_m (16 μM for cAMP-dependent protein

* In experiments where phosphorylation of kemptide and histone III-S were directly compared, kemptide (1 mM) supported approximately 3-fold greater PKC activity than histone (200 μg/mL). In Fig. 3 (performed with DEAE-purified PKC) the PKC activity was 42 pmol/min at 10 μM ATP, 1 mM kemptide, and no erbstatin. In Fig. 2 (performed with homogeneous PKC), the PKC activity was 8 pmol/min at 10 μM ATP, 200 μg/mL histone, and no erbstatin. This difference can be attributed to the difference in phosphoacceptor used and the difference in enzyme preparation.

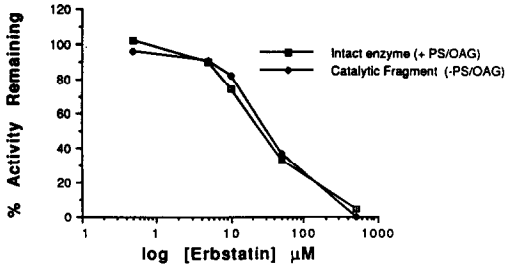


Fig. 4. Inhibition of the catalytic fragment of PKC by erbstatin. The catalytic fragment of PKC was prepared by limited proteolysis as described in Experimental Procedures. Inhibition of intact (homogeneous) enzyme and catalytic fragment was assessed using standard assay conditions, except that the lipid cofactors, phosphatidylserine (PS) and oleoylacetylglycerol (OAG), were omitted from assays of the catalytic fragment. The total activity in the absence of inhibitor was 100 pmol/15 min for intact enzyme and 20 pmol/15 min for the fragment. Similar results were obtained in two independent experiments.

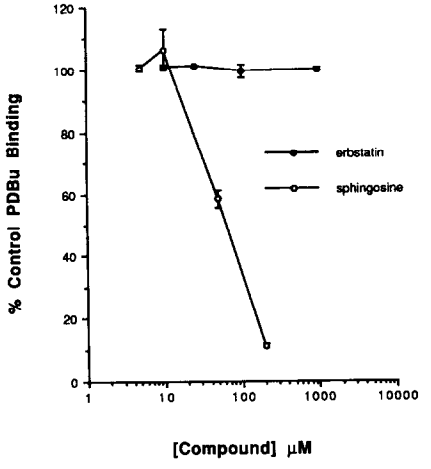


Fig. 5. Effect of erbstatin on [^{3}H]phorbol dibutyrate binding to PKC. Phorbol dibutyrate (PDBu) binding to DEAE-purified PKC was measured by the method of Sando and Young [20]. [^{3}H]PDBu (25 nM) and the indicated concentration of erbstatin or sphingosine were added to the reaction mixture. Reactions were initiated by addition of enzyme, and binding was allowed to proceed to equilibrium (2 hr at 4°). Total binding in the absence of inhibitor was 34,485 cpm in this experiment. Non-specific binding accounted for less than 2% of the total as assessed by inclusion of 30 μM unlabeled PDBu in the reaction. Values are means ± range of two experiments.

kinase and 25 μM for cGMP-dependent protein kinase and myosin light chain kinase). Erbstatin was found to be active against all kinases examined. The most potent inhibition was observed against the catalytic subunit of the cAMP-dependent protein kinase (IC_{50} = 0.78 ± 0.11 μM). Erbstatin inhibited cGMP-dependent protein kinase with an IC_{50} of 4.5 μM. Myosin light chain kinase was the least sensitive among the kinases examined (IC_{50} = 210 μM).

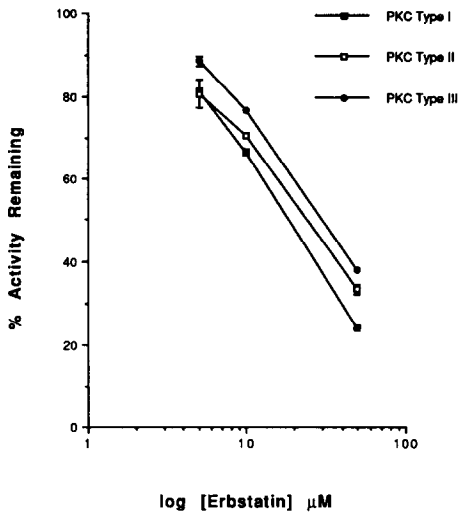


Fig. 6. Inhibition of PKC isozymes by erbstatin. PKC Types I, II and III were resolved by hydroxyapatite chromatography as described in Experimental Procedures. Type III enzyme was free of contamination by the other isoforms, while poorer resolution of Types I and II resulted in some cross contamination. Inhibition of these fractions by erbstatin was determined using standard assay conditions.

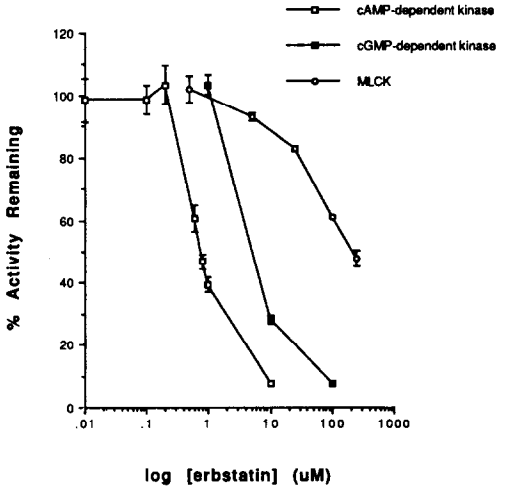


Fig. 7. Effect of erbstatin on other serine/threonine kinases. Inhibition of cAMP- and cGMP-dependent protein kinases and myosin light chain kinase by erbstatin was determined as described in Experimental Procedures. Data points represent the means ± range of duplicate determinations. Similar results were obtained in three separate experiments for cAMP-dependent protein kinase and two experiments for myosin light chain kinase (MLCK) and cGMP-dependent protein kinase.

DISCUSSION

The data reported here indicate that the tyrosine kinase inhibitor erbstatin also inhibits protein kinase C. The mechanism of inhibition of protein kinase C was competitive with ATP and non-competitive with the peptide (protein) substrate. Since most protein

kinases have ATP binding sites with high sequence homology, it is not surprising that erbstatin was also found to inhibit cyclic nucleotide-dependent protein kinases and myosin light chain kinase. A similar lack of specificity has been encountered with other known kinase inhibitors. The similar potency of erbstatin for PKC and the cyclic nucleotide-dependent kinases and the lower potency versus myosin light chain kinase is consistent with the closer evolutionary relationship between PKC and cyclic nucleotide-dependent kinases than between PKC and the Ca^{2+}/calmodulin-dependent kinases (e.g. Ref. 24). These results suggest that caution must be exercised in interpreting results obtained with erbstatin in cell-based model systems.

Erbstatin was originally isolated as an inhibitor of the EGF receptor autophosphorylation reaction with an IC_{50} of 0.55 μg/mL (3 μM) [6]. It was reported to lack inhibitory activity against cAMP-dependent protein kinase [6] and PKC [7] and to inhibit tyrosine kinases by competing with peptide substrate (K_i = 5.6 μM). Our data clearly indicate that erbstatin inhibition of PKC is competitive with ATP. PKC and the EGF receptor display sequence homology not only at the consensus ATP binding site (Gly-X-Gly-X-X-Gly-X_{15}-Lys) but over a longer stretch of amino acids on the carboxy terminal side of this site [24, 25]. While the precise binding sites for protein (peptide) substrates have not been identified, important determinants for protein binding to PKC lie in this region (e.g. Ref. 26). It is possible that erbstatin interacts with a site on various protein kinases where it can compete for binding with either the nucleotide or peptide substrate. The differences in kinetic profiles of erbstatin inhibition observed with PKC and the EGF receptor may then be due to differences in the order of substrate binding. Substrate binding to the EGF receptor occurs in an ordered fashion with peptide binding first [27], while ATP is the first substrate to bind to cAMP-dependent protein kinase [28]. The kinetic profiles obtained with PKC using ATP competitive compounds are consistent with an ordered mechanism with ATP being the first substrate to bind (e.g. Ref. 29).

The reason for the discrepancy between our work and previous studies suggesting that erbstatin lacks inhibitory activity versus serine/threonine kinases is not clear. Differences in PKC assay protocol, however, can have dramatic effects on erbstatin potency. First, as mentioned above, use of detergent-phospholipid mixed micelles to deliver the PKC lipid cofactors decreased the potency of erbstatin about 10-fold, suggesting that this compound is subject to surface dilution. Therefore, the concentration of lipid cofactors and their delivery method are important variables. Second, the potency of erbstatin will be modulated by tho concentration of ATP employed in the assay. We used ATP at its K_m to assay all of the serine/threonine kinases; therefore, the assays were sensitive to ATP competitive compounds.

In several preliminary experiments we have been unable to detect inhibition of phosphorylation of the 40 kD PKC substrate in thrombin-stimulated human platelets following short (10 min) preincubations with erbstatin (5–100 μM). Inhibition of EGF-receptor autophosphorylation in NIH 3T3 fibroblasts by two erbstatin-related compounds (RG50810 and RG50864) is optimal following a 16-hr incubation with these agents [30]. This result was attributed to a slow rate of entry of these compounds into cells [30]. Lack of inhibition of PKC-mediated phosphorylation in platelets is likely due to this limited entry into cells. However, since intracellular ATP concentrations are considerably higher than the K_m for ATP of the kinases tested here, inhibition of serine/threonine kinases by erbstatin *in vivo* may be attenuated.

Since the initial reports on erbstatin, a number of similar compounds have been synthesized and examined for tyrosine kinase inhibition. These include ST 638 which is reported to inhibit the EGF receptor autokinase reaction with an IC_{50} below 1 μM and to be inactive against PKC and cAMP-dependent kinase up to 100 μM [31, 32]. A series of such compounds were reported by Yaish *et al.* [9] to have up to 700-fold selectivity for the EGF receptor kinase versus the insulin receptor kinase and to inhibit EGF-dependent proliferation of human epidermal carcinoma A431 cells. These compounds were also reported to lack serine/threonine kinase inhibitory activity.

Other classes of non-ATP competitive tyrosine kinase inhibitors have been discovered. Shechter *et al.* [33] reported a series of dicarboxylic acid hydroxyphenyl derivatives (e.g. succinyl tyrosine) which have weak inhibitory activity against the insulin receptor kinase. A more potent inhibitor of the insulin receptor kinase is (hydroxy-2-naphthalenylmethyl) phosphonic acid (IC_{50} = 200 μM) which was demonstrated to have no effect on PKC activity at concentrations up to 420 μM [34].

Classes of compounds which inhibit a variety of serine/threonine protein kinases primarily through an ATP competitive mechanism include the flavones (e.g. quercetin) [35], isoquinolinesulfonamides (e.g. H7) [36] and indolocarbazoles (e.g. K252a) [37]. It has proven to be difficult to build selectively for certain protein kinases into compounds acting by this mechanism. However, some success has been reported recently [38]. The indolocarbazole CGP 41 251 was reported to inhibit PKC with an IC_{50} of 50 nM, but to inhibit the EGF receptor tyrosine kinase and a number of other serine/threonine kinases with micromolar potency [38].

Finding highly specific protein kinase inhibitors remains a challenging task. The finding that compounds which inhibit tyrosine kinases presumably due to their structural similarity to tyrosine also inhibit serine/threonine kinases through a distinct kinetic mechanism further complicates the search for specificity.

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