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INHIBITION KINETICS AND SELECTIVITY OF THE TYROSINE KINASE INHIBITOR ERBSTATIN AND A PYRIDONE-BASED ANALOGUE

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Abstract—The inhibition mechanisms of the epidermal growth factor (EGF) receptor tyrosine kinase and the cAMP-dependent kinase activities by erbstatin and its analogue, RG 14921, were studied by kinetic analysis. Both compounds were slow-binding inhibitors of the EGF receptor kinase. Erbstatin inhibited the EGF receptor kinase as a partial competitive inhibitor with respect to both ATP and the peptide substrate, suggesting that it binds at a site distinct from the ATP and peptide binding sites of the enzyme, and thus lowers the binding affinities of the enzyme for both substrates. In contrast, the analogue RG 14921 inhibited EGF receptor kinase activity as a non-competitive inhibitor with respect to both ATP and the peptide substrate. The distinct modes of inhibition by structurally related compounds suggest a dynamic and possibly extended structure of the catalytic center of the kinase domain of the receptor. Erbstatin and RG 14921 exerted similar effects on cAMP-dependent protein kinase activity. In this system, both compounds displayed potent inhibition and acted by a mode of competitive inhibition with respect to ATP and non-competitive with the peptide substrate.

Growth factor receptor associated tyrosine protein kinase (TPK†) activity has been shown to be essential for the growth factor-mediated signal transduction, cell proliferation and cell transformation [1–4]. Several classes of compounds have been isolated and shown to be tyrosine kinase inhibitors. However, the inhibition mechanisms are different among these inhibitors. Genistein, quercetin and the related flavonoids are competitive inhibitors with ATP binding [5–7]. Lavendustin-A was first reported as an ATP competitor [8]; however, we have studied its inhibition kinetics in detail and reported recently that it is actually a hyperbolic mixed-type inhibitor with respect to both ATP and the peptide substrate [9]. The tyrphostin class of molecules showed various types of inhibition, and most of them are mixed-type inhibitors of the receptor tyrosine kinase [10, 11]. Thiazolidine-diones also exhibit mixed-type inhibition with respect to ATP [12]. Erbstatin (RG 15016), a natural product isolated from *Streptomyces* sp., and its synthetic analogue, methyl 2,5-dihydroxycinnamate, are potent inhibitors of tyrosine protein kinases [13–15]. It was reported that both of them inhibit the epidermal growth factor (EGF) receptor tyrosine kinase competitively with the peptide substrate and non-competitively with ATP [15, 16]. We have studied in detail the inhibition kinetics of erbstatin using a recombinant baculovirus-expressed intracellular domain of the EGF receptor (EGFR-IC) [17]. We found that erbstatin was actually a slow-binding inhibitor of the EGF receptor kinase. It exhibited a mode of partial competitive inhibition with respect to both ATP and the peptide substrate. The results of selectivity studies of inhibition by erbstatin against different kinases in different reports are also inconsistent. Some reported that erbstatin selectively inhibits the EGF receptor kinase [12, 13], while others reported that it inhibits the EGF receptor kinase, protein kinase C (PKC) and cAMP-dependent protein kinase (PKA) with similar potencies [18]. We found that erbstatin was also a slow-binding inhibitor of PKA. The results of the kinetic analysis, using an appropriate preincubation protocol, indicate that erbstatin inhibits PKA very effectively and is competitive with ATP and non-competitive with the substrate kemptide. This result is similar to the observations by Bishop *et al.* [18]. A pyridone-based analogue of erbstatin, RG 14921, also inhibited the EGFR-IC kinase, but the mode of inhibition was very different from that of erbstatin. Diverse modes of inhibition of EGFR-IC kinase activity by analogous compounds were previously observed and reported for Lavendustin-A and its analogue RG 14467 [9], and suggest a dynamic and possibly extended catalytic center of the receptor kinase domain.

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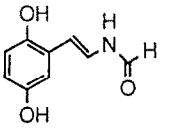
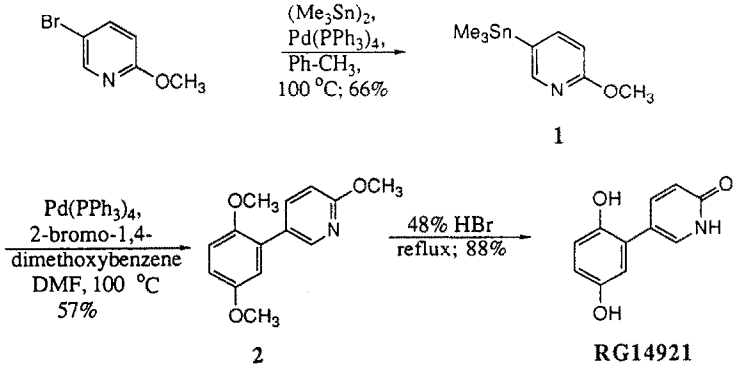
† Abbreviations: TPK, tyrosine protein kinase; EGFR-IC, intracellular domain of the epidermal growth factor receptor; PKA, cAMP-dependent protein kinase; PKC, protein kinase C; PLC, phospholipase C; HEPES, 4-(2-hydroxyethyl)-1-piperazine-ethanesulfonic acid; and SDS, sodium dodecyl sulfate.

EXPERIMENTAL PROCEDURES

Materials and enzymes. The tyrosine kinase-active EGFR-IC was produced and purified from a baculovirus expression system [17]. The catalytic



erbstatin (RG 15016)



Scheme 1.

subunit (from bovine heart) and the holoenzyme (from rabbit muscle) of PKA were from Sigma.

Erbstatin (RG 15016) was synthesized according to a published procedure [14]. RG 14921 was synthesized as outlined in Scheme 1. Briefly, palladium-catalyzed reaction of 5-bromo-2-methoxypyridine [19] with hexamethylditin gave the stannylpyridine derivative **1** in 66% yield. Coupling of **1** with 2-bromo-1,4-dimethoxybenzene again via palladium catalysis produced the trimethoxy compound **2** in 57% yield. Removal of the methoxyl groups was accomplished by refluxing **2** in 48% hydrobromic acid to give RG 14921 in 88% yield.*

The synthesis and sequence of the peptide phospholipase C-783 (PLC-783) and PLC-1254 derived from the phospholipase C-γ1 sequence were described previously [20]. Kemptide (Leu-Arg-Arg-Ala-*Ser*-Leu-Gly), a synthetic peptide containing the sequence of the phosphorylation site of pig liver pyruvate kinase, was purchased from Penninsula Laboratories. [γ-^{32}P]ATP (6000 Ci/mmol) was purchased from DuPont-New England Nuclear.

Tyrosine kinase assay of EGFR-IC. Each reaction was performed with 40 ng EGFR-IC (21 nM), 10 mM $MnCl_2$ in 30 μl HNTG (50 mM HEPES, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, and 10% glycerol). The concentrations of the peptide substrate, [γ-^{32}P]ATP (2–5 × 10^4 cpm/pmol) and the inhibitor, as well as the preincubation time, are as indicated in each respective figure legend. The kinetic experiments with ATP as the substrate at varying concentrations (Figs. 3 and 5) were performed with the pre-phosphorylated EGFR-IC, which is the more active form of the enzyme [20], prepared by preincubating the enzyme, $MnCl_2$ and ATP (3–4 × 10^4 cpm/pmol) at the appropriate concentration (in the range of 1–20 μM as the final concentrations) on ice for 30–40 min. The inhibitor, at the indicated concentration, was then added to the mixture, and the incubation was allowed to continue for 10 more min on ice. The reaction was then initiated by the addition of 1 mM peptide PLC-1254 (Fig. 3) or PLC-783 (Fig. 5). The experiments with the peptide as the substrate at varying concentrations (Figs. 4 and 6) were performed by preincubating the pre-phosphorylated enzyme (using unlabeled ATP) and the inhibitor at the indicated final concentration for 10 min on ice. This mixture was then added to a solution containing the peptide at varying concentrations and [γ-^{32}P]ATP at a final concentration of 20 μM (~2 × 10^4 cpm/pmol) to initiate the reaction. Each reaction was allowed to proceed for 3 min on ice (product formation is linear up to 10 min) and then stopped by the addition of a half volume of 3× sodium dodecyl sulfate (SDS)-sample buffer. Peptide phosphorylation was analyzed by electrophoresis on a 5–15% linear gradient (total of 16 mL) over a 20% (12 mL) polyacrylamide gel followed by autoradiography. The peptide bands were excised and quantitated by Cerenkov counting.

PKA assay. Rabbit muscle holoenzyme was used in all the kinetic experiments because it was found to be more stable than the bovine heart catalytic subunit of PKA. The assay conditions were similar

* Data for RG 14921: recrystallized from methanol, m.p. 303–306°; IR (KBr) 1647, 1616, 1330, 830 cm^{-1}; ^{1}H-NMR (300 MHz, DMSO-d_6) δ 6.36 (1 H, d, J = 9.5 Hz), 6.53 (1 H, dd, J = 2.9, 8.6 Hz), 6.62 (1 H, d, J = 2.9 Hz), 6.71 (1 H, d, J = 8.6 Hz), 7.55 (1 H, d, J = 2.4 Hz), 7.65 (1 H, dd, J = 2.4, 9.5 Hz), 8.80 (1 H, br s), 8.96 (1 H, br s), 11.62 (1 H, br s); exact mass calc. for $C_{11}H_9NO_3$ (M^+) 203.0582, found 203.0585. Anal. calc. for $C_{11}H_9NO_3$: C, 65.02; H, 4.46; N, 6.89. Found: C, 64.58; H, 4.56; N, 6.69.

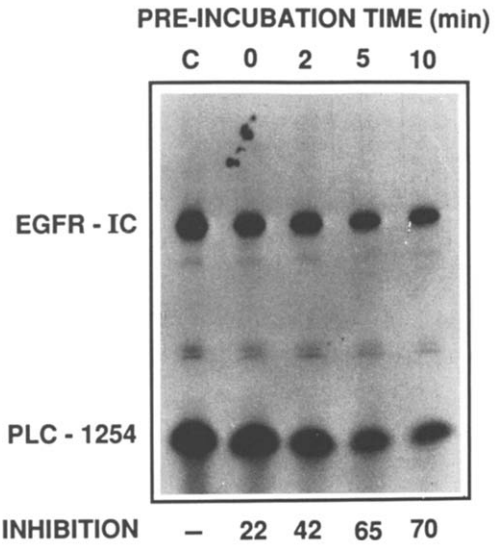


Fig. 1. Effect of preincubation on the inhibition of EGFR-IC kinase activity by RG 15016. EGFR-IC was preincubated with 20 μM RG 15016 (final concentration) on ice for various times as indicated before the initiation of the reaction by the addition of a mixture containing PLC-1254 and [γ-^{32}P]ATP at 500 μM and 3 μM as final concentrations, respectively. The reaction proceeded on ice for 3 min. The results of the autoradiogram after electrophoresis, performed as described in the Experimental Procedures, are shown.

to those employed in the reported procedure [18]. The reaction mixture contained 50 mM Tris (pH 7), 10 mM magnesium-acetate, 50 μg bovine serum albumin, 1 μM cAMP and 1.25 μg rabbit muscle holoenzyme in a final volume of 200 μL. When ATP was the substrate with varying concentrations in the kinetic experiments, a mixture containing the enzyme and ATP at varying concentrations (~0.4×10^4 cpm/pmol) was preincubated with the inhibitor at the indicated concentration for 15 min at room temperature. The reaction was then initiated by the addition of 16 μM kemptide. When kemptide was used as the substrate with varying concentrations, the mixture containing the enzyme and kemptide at various concentrations was incubated with the inhibitor at the indicated concentration for 15 min at room temperature, followed by the addition of 16 μM ATP (~0.4×10^4 cpm/pmol) to initiate the reaction. All reactions were allowed to proceed for 3 min at 30° and terminated by the addition of 100 μL of ice-cold 375 mM H_3PO_4. A 50-μL portion of each sample was spotted onto phosphocellulose P81 paper, washed three times (20 min each) with 75 mM H_3PO_4, air dried, and Cerenkov counted.

RESULTS

Slow-binding inhibitor character of erbstatin and its analogue RG 14921. It was first observed that erbstatin and RG 14921 did not inhibit EGFR-IC kinase activity significantly without preincubation with the enzyme. Therefore, time course experiments were performed, as shown by Fig. 1, for erbstatin (RG 15016). Results show that the extent of inhibition increased with the preincubation time and, therefore, indicate that both erbstatin and RG 14921 are slow-binding inhibitors. This preincubation effect was observed for both the pre-phosphorylated and non-phosphorylated EGFR-IC (data not shown). It is worth mentioning that the addition of substrate did not reverse the inhibition in the 3-min reaction time, indicating that the compounds may also be called tight-binding inhibitors according to the definition by Cha *et al.* [21–23]. We also observed that the compounds exhibited marked inhibition only when the enzyme was conjugated with Mn^{2+} during the preincubation time as shown in Fig. 2 for RG 14921. When Mg^{2+} was used, no significant inhibition was observed (data not shown).

Mode of inhibition of EGFR-IC kinase by erbstatin and RG 14921. Preincubation and the duration play crucial roles in determining the modes of inhibition by kinetic analysis for slow-binding and slow, tight-binding inhibitors [22–25]. We have previously described a preincubation protocol to study the steady-state inhibition kinetics for the slow-binding inhibitors Lavendustin-A and its analogue RG 14467 [9]. The strategy is to pre-equilibrate the enzyme with the inhibitor at fixed concentrations in the presence of one substrate at varying concentrations by a 10-min incubation on ice. An excess amount of the second substrate is then added to initiate the

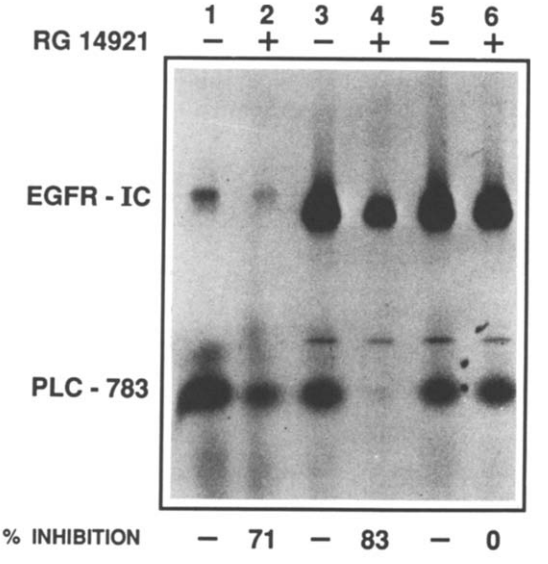


Fig. 2. Effect of Mn^{2+} in the preincubation mixture on the inhibition of EGFR-IC kinase activity by RG 14921. EGFR-IC pre-phosphorylated in a mixture containing Mn^{2+} and unlabeled ATP (lanes 1 and 2), or conjugated with Mn^{2+} (lanes 3 and 4), or alone (lanes 5 and 6) was preincubated in the presence (+) or absence (–) of 20 μM RG 14921 on ice for 10 min, followed by the addition of [γ-^{32}P]ATP (to render the final specific activity the same as that of the other samples) and 0.5 mM PLC-783 (final concentration) (lanes 1 and 2), or 10 μM [γ-^{32}P]ATP and 0.5 mM PLC-783 (lanes 3 and 4), or 10 μM [γ-^{32}P]ATP, 10 mM $MnCl_2$ and 0.5 mM PLC-783 (lanes 5 and 6) to initiate the reaction. The reaction was allowed to proceed on ice for 3 min and was terminated by the addition of a half volume of 3× SDS-sample buffer. The results were analyzed by electrophoresis and shown by the autoradiogram.

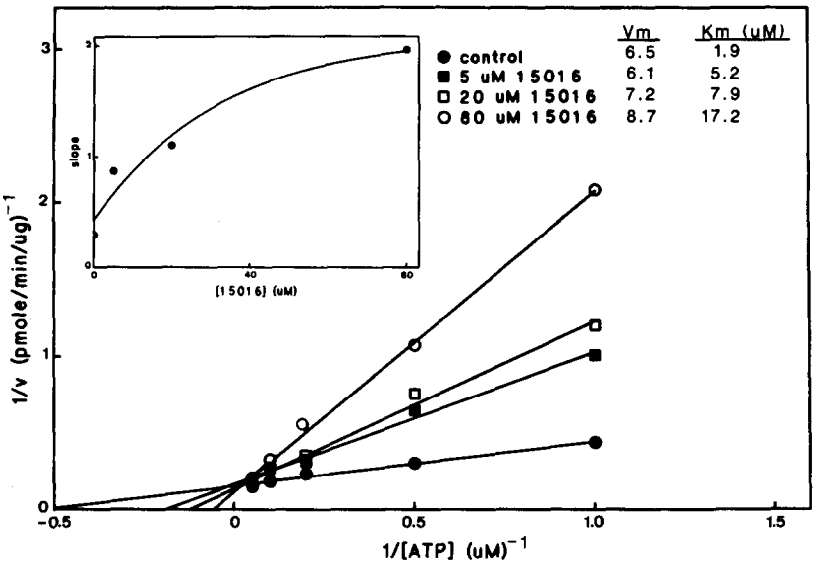


Fig. 3. Kinetic analysis for the inhibition of EGFR-IC by erbstatin versus ATP. Erbstatin (RG 15016) at the indicated concentration was preincubated with pre-phosphorylated EGFR-IC in the presence of $MnCl_2$ and [γ-^{32}P]ATP at various concentrations (1–20 μM) for 10 min on ice. The reaction was initiated by the addition of 1 mM PLC-1254 and allowed to proceed for 3 min on ice. The results were analyzed by electrophoresis and autoradiography as described in Experimental Procedures. The double-reciprocal plots and the determined apparent V_m and K_m values at each inhibitor concentration are shown. The V_m values are expressed in pmol phosphate incorporated/min/μg. The inset shows the slope versus [I] replot.

reaction. A similar protocol was used in the present study, as described in Experimental Procedures. We have noticed that preincubating the enzyme with the inhibitor alone, or in the presence of the peptide before the initiation of the reaction (for Figs. 4 and 6) did not make any difference for the extent and the mode of inhibition. Figure 3 shows the double-reciprocal plot of the kinetic results of RG 15016 versus ATP. The binding affinity for ATP was reduced dramatically, whereas the reaction velocity

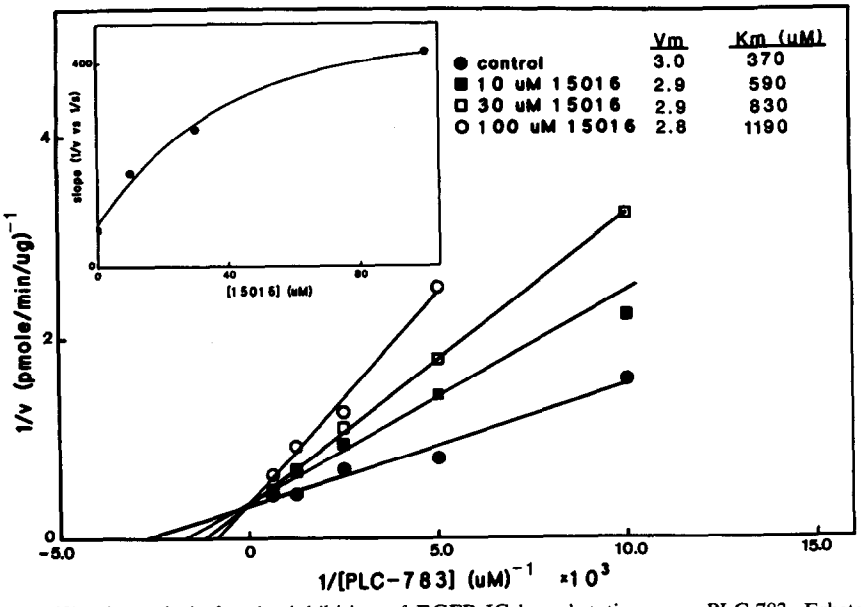


Fig. 4. Kinetic analysis for the inhibition of EGFR-IC by erbstatin versus PLC-783. Erbstatin was preincubated on ice for 10 min with pre-phosphorylated EGFR-IC, in the presence of 10 mM $MnCl_2$ and 20 μM unlabeled ATP (as final concentrations). This mixture was then added to a solution containing [γ-^{32}P]ATP and PLC-783 at various concentrations (100, 200, 400, 800 and 1600 μM). The reaction condition and method of data analysis are as described in Experimental Procedures. The double-reciprocal plots and the slope versus [I] replot are shown. The V_m values are expressed in pmol phosphate incorporated/min/μg.

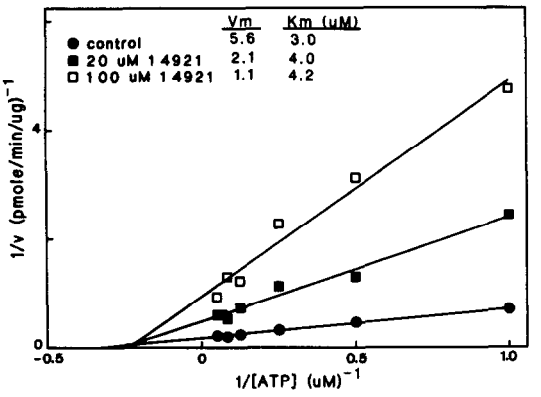


Fig. 5. Kinetic analysis for the inhibition of EGFR-IC by RG 14921 versus ATP. The experiment was performed and analyzed similarly as described for Fig. 3 except that the peptide substrate used was PLC-783. The double-reciprocal plots of the data are shown.

was not affected much by RG 15016. The slope versus [I] replot was hyperbolic, suggesting a hyperbolic (partial) competitive inhibition with respect to ATP by RG 15016. Figure 4 shows the result with PLC-783 as the substrate at varying concentrations. The binding affinity for PLC-783 was decreased 3-fold at 100 μM RG 15016, whereas the reaction velocity was not changed significantly. The slope versus [I] replot was also hyperbolic, therefore suggesting a partial competitive inhibition with respect to the peptide substrate, too. Results with another PLC-γ1-derived peptide, PLC-1254, were similar (data not shown). These results suggest that erbstatin binds to the receptor kinase at a site distinct from either one of the binding sites for ATP and the peptide substrate and its binding markedly lowers the binding affinities of the enzyme for both substrates.

A similar kinetic analysis was performed for RG 14921. Interestingly, the results were clearly different from those observed with erbstatin. Figure 5 shows that 100 μM RG 14921 reduced the reaction velocity 5-fold, whereas the binding affinity for ATP was not affected significantly. Figure 6 shows the result versus peptides. When either PLC-783 or PLC-1254 was used as the substrate at varying concentrations, RG 14921 reduced the reaction velocity markedly without much effect on the binding affinities for the peptides. Therefore, RG 14921 is a non-competitive inhibitor with respect to both ATP and the peptide substrate.

Inhibitory effect of erbstatin and RG 14921 *on PKA*. We have used the rabbit muscle PKA holoenzyme (more stable than the bovine heart catalytic subunit) and kemptide (sequence derived from pig liver pyruvate kinase) as the peptide substrate to study the effect of erbstatin and RG 14921 on PKA activity. Time course studies, similar to the study shown in Fig. 1, showed that both compounds were slow-binding inhibitors to PKA (data not shown). The inhibitory potencies of RG 15016 and RG 14921 with PKA were about one order of magnitude higher than that with EGFR-IC (see Figs. 3–6 in comparison with Figs. 7 and 8) under our experimental conditions. Kinetic experiments using a preincubation protocol, as described in Experimental Procedures, showed that RG 15016 and RG 14921 were competitive inhibitors with ATP and non-competitive inhibitors with kemptide against PKA, as shown in Figs. 7 and 8 for RG 15016.

DISCUSSION

Erbstatin was described originally as a potent inhibitor of EGF receptor tyrosine kinase activity and reported to be a competitive inhibitor with respect to the peptide substrate and a non-competitor with ATP. It was also reported that erbstatin inhibits tyrosine kinases selectively, because its inhibitory effect on PKA is very weak [12, 13]. We have studied in detail the inhibition kinetics of erbstatin using the tyrosine kinase-active intracellular domain of the EGF receptor produced from a recombinant baculovirus [17]. Our results show that erbstatin was a slow-binding inhibitor to the receptor kinase, and it inhibited the EGF receptor kinase by acting as a partial competitor with respect to both ATP and the peptide. Furthermore, our studies with PKA using kemptide as a substrate show that erbstatin exhibited relatively potent inhibition against PKA. It acted as an ATP competitor and a non-competitor with kemptide in the PKA reactions. These results are in

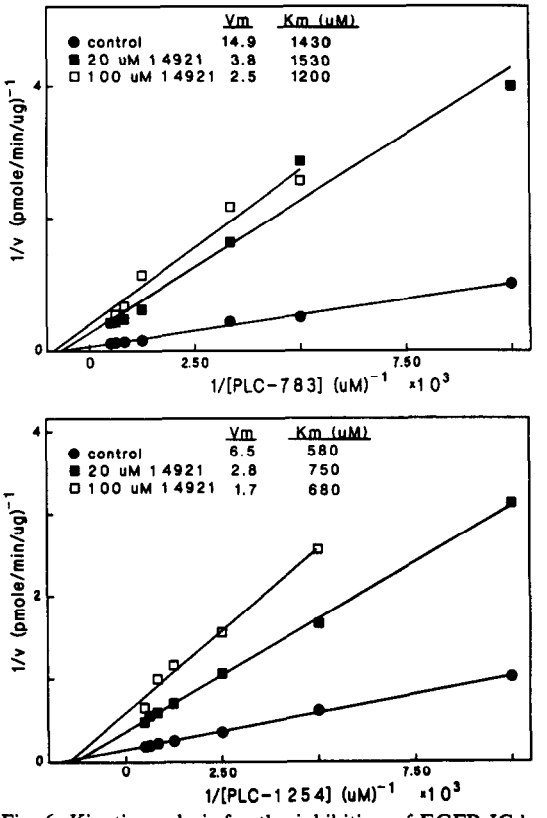


Fig. 6. Kinetic analysis for the inhibition of EGFR-IC by RG 14921 versus peptide substrate. The experiment was performed and analyzed similarly as described for Fig. 4. The peptide substrate used was PLC-783 (upper) or PLC-1254 (lower) at various concentrations (100–2000 μM). The resultant double-reciprocal plots are shown.

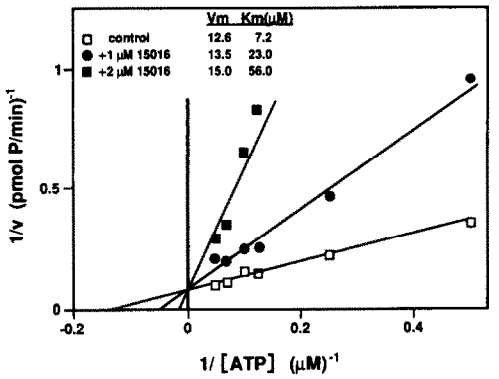


Fig. 7. Kinetic analysis for the inhibition of PKA by RG 15016 versus ATP. A mixture containing PKA, [γ-^{32}P]ATP at various concentrations (2–20 μM) and RG 15016 at the indicated concentration was preincubated for 15 min at room temperature, followed by addition of kemptide (16 μM) to initiate the reaction. The reaction was performed and analyzed as described in Experimental Procedures. The resultant double-reciprocal plots are shown. The V_m values are expressed in pmol P/min.

agreement with the observations of Bishop *et al.* [18] with PKC and PKA.

The slow-binding nature of inhibitors to the EGF receptor kinase was presented and discussed previously for Lavendustin-A and its analogue RG 14467 [9]. It seems likely that the receptor kinase has a dynamic catalytic center, which is subject to various subtle conformational changes upon the initial interaction with each individual effector, and subsequently forms a stable enzyme–inhibitor complex. The finding that erbstatin and RG 14921 inhibited the receptor kinase by distinct mechanisms suggests that these two compounds may bind at different sites in the kinase domain and form different enzyme–inhibitor complexes. Different inhibition mechanisms of the EGF receptor kinase exhibited by compounds of analogous structures were also observed in studies with Lavendustin-A and its analogue RG 14467 [9]. This phenomenon further manifests the dynamic and possibly extended catalytic center of the receptor kinase domain.

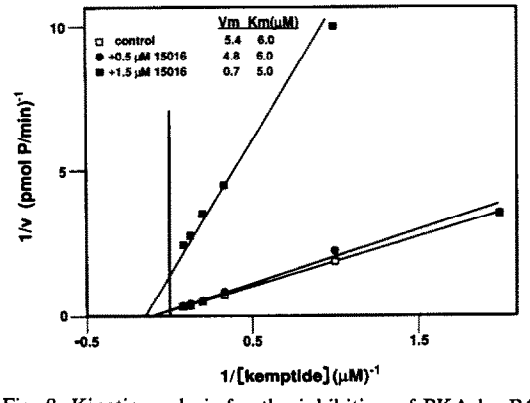


Fig. 8. Kinetic analysis for the inhibition of PKA by RG 15016 versus kemptide. PKA was preincubated with kemptide at various concentrations (0.5 to 12 μM) and RG 15016 at the indicated concentration for 15 min at room temperature, followed by the addition of 16 μM [γ-^{32}P]-ATP to initiate the reaction. The reaction conditions and method of analysis are described in Experimental Procedures. The resultant double-reciprocal plots are shown. The V_m values are expressed in pmol P/min.

The finding that erbstatin and RG 14921 only inhibited the Mn^{2+}-conjugated form is interesting. Mn^{2+} is an activator of the tyrosine kinase of the EGF receptor and the insulin receptor [26–28]. It was shown that Mn^{2+} decreases the apparent K_m for ATP of the insulin receptor kinase [29] by inducing an activating conformational change [30]. We did not observe any inhibition when incubating erbstatin with EGFR-IC in the presence of Mg^{2+} (data not shown). The inhibition observed with the Mn^{2+}-conjugated EGFR-IC, therefore, suggests a unique conformation of the kinase in the presence of Mn^{2+}, that is accessible to the binding of erbstatin and its analogue, in contrast to the Mg^{2+}-induced conformation.

The observation that structurally related analogs inhibited the EGF receptor kinase by different inhibition mechanisms adds a challenge for the development of selective synthetic TPK inhibitors.

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