Kinetic Analysis of the Inhibition of the Epidermal Growth Factor Receptor Tyrosine Kinase by Lavendustin-A and Its Analogue*

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Lavendustin-A was reported to be a potent tyrosine kinase inhibitor of the epidermal growth factor (EGF) receptor (Onoda, T., Inuma, H., Sasaki, Y., Hamada, M., Isshibi, K., Naganawa, H., Takeuchi, T., Tatsuta, K., and Umezawa, K. (1989) J. Nat. Prod. 52, 1252-1257). Its inhibition kinetics was studied in detail using the baculovirus-expressed recombinant intracellular domain of the EGF receptor (EGFR-IC). Lavendustin-A (RG 14355) is a slow and tight binding inhibitor of the receptor tyrosine kinase. The pre-steady state kinetic analysis demonstrates that the inhibition corresponds to a two-step mechanism in which an initial enzyme-inhibitor complex (EI) is rapidly formed followed by a slow isomerization step to form a tight complex (E1I*). The dissociation constant for the initial rapid forming complex is 370 nM, whereas the overall dissociation constant is estimated to be ≤1 nM. The difference between the two values is due to the tight binding nature of the inhibitor to the enzyme in E1I*. The kinetic analysis using a preincubation protocol to pre-equilibrate the enzyme with the inhibitor in the presence of one substrate showed that Lavendustin-A is a hyperbolic mixed-type inhibitor with respect to both ATP and the peptide substrate, with a major effect on the binding affinities for both substrates.

An analogue of Lavendustin-A (RG 14467) showed similar inhibition kinetics to that of Lavendustin-A. The results of the pre-steady state analysis are also consistent with the proposed two-step mechanism. The dissociation constant for the initial fast forming complex in this case is 3.4 μM, whereas the overall dissociation constant is estimated to be ≤30 nM. It is a partial (hyperbolic) competitive inhibitor with respect to ATP. Its inhibition is reduced to different extents by different peptide substrates, when the peptide is added to the enzyme simultaneously with the inhibitor. When studied with the least protective peptide, K1 (a peptide containing the major autophosphorylation site of the EGF receptor), RG 14467 acts as a hyperbolic noncompetitive inhibitor with respect to the peptide.

Tyrosine protein kinase activity is associated with many cellular and viral oncosomal products (1, 2), including cellular receptors for several growth factors, such as EGF,1 platelet-derived growth factor, and insulin (3, 4). This kinase activity is essential for the growth factor receptor-mediated signal transduction, induction of mitogenesis, and cell transformation (5-8). Specific inhibitors of tyrosine kinases could therefore be potential anti-tumor agents and useful research tools for understanding the mechanisms and physiological functions of the tyrosine protein kinase activity of growth factor receptors.

Lavendustin-A, a potent tyrosine kinase inhibitor, is a natural product isolated from Streptomyces griseolavendus (9). It selectively inhibits the tyrosine protein kinase activity of the EGF receptor with little effect on the activities of cAMP-dependent kinase or protein kinase C. Lavendustin-A was reported to be a competitive inhibitor with respect to ATP and a noncompetitive inhibitor with respect to a peptide substrate (9). We have chemically synthesized this compound (RG 14355) and studied in detail its inhibition mechanism using the purified intracellular domain of the EGF receptor (EGFR-IC) produced by the baculovirus expression system (10) and a synthetic peptide (PLC-1254) representing a major tyrosine phosphorylation site of phospholipase Cγ1, a direct substrate of EGF receptor (11-13), as the substrate in the assay reaction (14). We found that Lavendustin-A inhibited potently only when it was preincubated with the kinase prior to the initiation of the reaction. The pre-steady state analysis showed that Lavendustin-A acts as a slow tight binding inhibitor of the tyrosine kinase domain of the EGF receptor with a two-step mechanism. Moreover, it exhibits a hyperbolic (partial) mixed-type inhibition pattern with respect to both ATP and the peptide substrate. It appears to significantly decrease the binding affinities of the kinase for both substrates. This observation suggests that Lavendustin-A binds at a site in the kinase domain which is distinct from the binding sites for ATP and the peptide, but the interaction of Lavendustin-A with the kinase lowers the binding affinities of the enzyme for its substrates.

An analogue of Lavendustin-A, RG 14467, displayed similar kinetic features to that of Lavendustin-A as a slow binding inhibitor with a two-step mechanism, except that it showed a mode of hyperbolic competitive inhibition with ATP and hyperbolic noncompetitive inhibition with the peptide substrate. The differences between the inhibitory modes of the two compounds are discussed. Although Lavendustin-A and its analogues are not potent inhibitors of cellular mitogenesis, studying their inhibition mechanisms contributes to our understanding of the mechanisms and physiological functions of the tyrosine protein kinase activity of growth factor receptors.

† The abbreviations used are: EGF, epidermal growth factor; EGFR-IC, intracellular domain of the epidermal growth factor receptor; PLC, phospholipase Cγ1; Hepes, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; SDS, sodium dodecyl sulfate.

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understanding of the interactions of the receptor tyrosine kinase with its substrates and inhibitors. Derivatives of these inhibitors may prove to be useful reagents to study in detail the nature of the catalytic center of the enzyme.

EXPERIMENTAL PROCEDURES

Materials—Lavendustin-A (RG 14355) was synthesized according to the published procedure (9). RG 14467 was synthesized as outlined in Scheme I. The synthesis and sequences of the peptides: K1, PLC-1254, PLC-783, and PLC-771 were described previously (14). The number of each PLC peptide indicates the position of the tyrosine residue in the phospholipase Cγ1 (from bovine brain) that is phosphorylated by EGFR. [γ-32P]ATP (6000 Ci/mmol) was purchased from Du Pont-New England Nuclear.

Tyrosine Kinase Assay of EGFR-IC—Each reaction was performed with 40 ng of EGFR-IC (21 nM) (except where indicated otherwise), 10 mM MnCl₂ in 30 μl of HNTG (50 mM Hepes, pH 7.5, 150 mM NaCl, 0.1% Triton X-100, and 10% glycerol). The concentrations of the phosphate substrate, [γ-32P]ATP (2–10 × 10⁶ cpm/pmol) and the inhibitor, as well as the preincubation time and the order of the additions of the components are as indicated in each respective figure legend. The kinetic experiments with ATP as the substrate with varying concentrations (Figs. 6 and 10) were performed with the prephosphorylated EGFR-IC (P-EGFR-IC), which is the more active form of the enzyme (14), prepared by preincubating the enzyme, MnCl₂, and ATP (7.0–10.0 × 10⁴ cpm/pmol) at the appropriate concentration (1.0, 2.0, 5.0, 10.0, or 20.0 μM) on ice for 30–40 min. This step is necessary since without prephosphorylation, different ATP concentrations in the reaction mixtures may lead to different levels of autophosphorylation, and subsequently different kinase activities of the enzyme. The inhibitor, at the indicated concentration, was then added to the mixture, and the incubation was allowed to continue for 10 min more on ice. The reaction was then initiated by addition of PLC-1254 at a final concentration of 2 nM. The experiments with the peptide as the substrate with varying concentrations (Figs. 7 and 11) were performed by preincubating mixtures containing the enzyme, MnCl₂, the peptide at varying concentrations, and the inhibitor at the indicated concentration on ice for 10 min, followed by addition of 10 μM [γ-32P]ATP (2.5–3.6 × 10⁶ cpm/pmol). Each reaction was allowed to proceed for 2 or 3 min on ice and then stopped by addition of a half-volume of 3 X 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.

RESULTS

Inhibitory Potency of Lavendustin-A Is Maximal When Preincubated with EGFR-IC—The first observation that suggested that Lavendustin-A (RG 14355) may exert its tyrosine kinase inhibition by a complex mechanism was the demonstration that the extent of inhibition varied considerably, depending on the order of addition of the inhibitor with respect to ATP and the peptide substrate. Fig. 1, left, shows that when Lavendustin-A (1–4 μM) was added to the prephosphorylated EGFR-IC 5 min prior to the addition of the substrate PLC-1254, more than 90% of the peptide phosphorylation activity was inhibited. In contrast, when the inhibitor was added simultaneously with the peptide (the rightmost lane), the extent of inhibition was greatly reduced. To test whether the peptide protects the enzyme from being inhibited by Lavendustin-A, we added the inhibitor (2 μM) to EGFR-IC either 5 min prior to, or together with the peptide, followed by ATP addition. In both cases, we observed a strong inhibition of both EGFR-IC autophosphorylation and peptide phosphorylation (Fig. 1, right). Thus, simultaneous additions of the peptide and the inhibitor to the enzyme does not prevent the inhibition of the kinase activity by Lavendustin-A. The results shown in Fig. 1, left and right, and similar experiments with varying orders of additions of the inhibitor and substrates indicate that Lavendustin-A significantly inhibits the EGFR-IC kinase activity only when it is added to the enzyme in the absence of at least one of the substrates. We also observed that Lavendustin-A inhibited the prephosphorylated and the unphosphorylated EGFR-IC equally well.

Another experiment to test the inhibitory potency of Lavendustin-A was performed as shown in Fig. 2. Lavendustin-A was added to the kinase together with the two substrates, ATP and PLC-1254, at varying concentrations. The Kᵢ values of EGFR-IC for ATP and PLC-1254 are 2 (10) and 350 μM (14), respectively. As expected, when the concentrations of both substrates are high (10 μM for ATP and 1 mM for PLC-1254), Lavendustin-A (4 μM) exhibited only minimal inhibition on the kinase activity. When the ATP concentration was dropped to 1 μM, which is below its Kᵢ, value, the inhibition was significantly enhanced. When the concentration of PLC-1254 was dropped to 50 μM, which is 7-fold lower than its Kᵢ, value, the inhibition was slightly enhanced. When both the concentrations of ATP and PLC-1254 were reduced, the inhibition was stronger than in the previous two situations.

The results shown in Figs. 1 and 2 suggest that the formation of the enzyme-inhibitor complex is slow. A time course study for the inhibition by Lavendustin-A was therefore performed as shown by Fig. 3. When the kinase was preincubated with 100 nM Lavendustin-A in the presence of PLC-1254, the extent of inhibition increased with the preincubation time, indicating that Lavendustin-A is a slow binding inhibitor of EGFR-IC.

Reversibility of the Enzyme-Inhibitor Complex—To test whether the formation of the enzyme-inhibitor complex is
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with Lavendustin-A at a 1:2 stoichiometry in a concentrated solution containing 5 PM \(\gamma^3P\)ATP and 1 mM PLC-1254, so that the final inhibitor concentration as 7.5 nM. Samples of 40 \(\mu\)l at the indicated time points were taken and added to 20 \(\mu\)l of 3 \(\times\) SDS sample buffer. Another mixture of control (\([I] = 0\)) was treated the same way. The results were analyzed by electrophoresis and autoradiography as described under “Experimental Procedures.” The peptide bands were cut and counted by Cerenkov counting.

For Lavendustin-A, \(k_b\) or \(k_o\) is negligible (if not really zero). Mechanism A describes a slow binding rate of inhibitor to the enzyme (a low \(k_b\) value). Mechanism B describes a rapid formation of EI complex followed by a slow isomerization step to form a stable EI* complex (a low \(k_b\) value). The pseudo-first order rate constant of inhibition (\(k_{obs}\)) can be obtained by the equation:

\[
v = v_u + (v_0 - v_u)\exp(-k_{obs}t)
\]

where \(v, v_u,\) and \(v_0\) represent velocities at time \(t,\) steady state, and time 0, respectively. When \([S] = 0,\) namely when enzyme is incubated with inhibitor but in the absence of the substrate, \(k_{obs} = k_b[I]\) for mechanism A, and \(k_{obs} = k_b[I]/(K_i + [I])\) for mechanism B. \(K_i\) in mechanism B is the true dissociation constant and equals \(k_b/k_o\) (16, 19–21). Therefore, mechanisms A and B can be distinguished by plotting \(1/(k_{obs})\) versus \([I]\).

For both mechanisms, the plot will be linear, but for mechanism A, the line will pass through the origin, while for mechanism B, the line will intercept at \(1/(k_{obs}) = 1/k_b\) and \(1/[I] = -1/K_i.\) Fig. 5A shows the plot of \(\ln(v - v_u)/(v_0 - v_u)\) versus time from a time course experiment to determine \(k_{obs}\) at increasing concentrations of inhibitor. Fig. 5B shows that the plot of \(1/(k_{obs})\) versus \([I]\) does not pass through the origin and therefore is consistent with the two-step mechanism (mechanism B). The \(K_i\) and \(k_b\) values are hence determined.
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Fig. 5. Pre-steady state analysis of the inhibition by RG 14355. EGFR-IC (20 ng which equals a final concentration of 11 nM) was preincubated with Lavendustin-A at the indicated concentration on ice for various times. The reaction was initiated by addition of a mixture containing PLC-1254 and ATP at a final concentration of 1 nM and 6.7 µM, respectively. Each reaction was allowed to proceed on ice for 2 min. The results were analyzed by electrophoresis and autoradiography as described under "Experimental Procedures." A, plots of $\ln([v - v_i]/(v_0 - v_i))$ versus time to determine $k_{obs}$ at each [I] from the slope of each line. B, plot of $1/(k_{obs})$ versus $1/[I]$ to determine $k_a$ and $K_I$.

**Table I**

Kinetic parameters for RG 14355 and RG 14467

<table>
<thead>
<tr>
<th>Inhibitor</th>
<th>$K_i$</th>
<th>$k_{obs}$</th>
<th>$t_{50}$, Inactivation</th>
<th>$K_i$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Lavendustin-A</td>
<td>0.37</td>
<td>2.01</td>
<td>0.34</td>
<td>$\leq1^*$</td>
</tr>
<tr>
<td>RG 14467</td>
<td>3.40</td>
<td>0.75</td>
<td>0.92</td>
<td>$\leq30^*$</td>
</tr>
</tbody>
</table>

*Estimated by assuming $t_{50}$ of reactivation is $\geq100$ min, therefore $k_a \leq0.007$ and $K_i = K_{obs}(k_0 + k_a)$.

by the intercepts and shown in Table I. It is noteworthy that $v_0$ at each concentration of the inhibitor is smaller than $v_i$ (velocity of the control sample, data not shown) which is consistent with an initial step of rapid formation of EI. The maximal $k_{obs}$ value (when $[I] \rightarrow \infty$) equals $k_o$ and the $t_{50}$ of inactivation at $[I] \rightarrow \infty$ can therefore be calculated by $v_0 = 0.693/k_o$ (Table I). (Similar experiments were repeated and the results were reproducible.)

The overall dissociation constant, $K_i^*$, is generally obtained from equation: $K_i^* = K_{obs}(k_o + k_h)$ (16, 19–21) or a Henderson plot of $[I]_o/(1 - v/v_i)$ versus $v/v_i$ for the tightly bound inhibitors (22). However, in the case of Lavendustin-A, it is difficult to determine $k_o$ since no reactivation was detected, as described in the previous section, during 80 min after the total inhibitor concentration was diluted to 5–7 nM (repeated experiments). Moreover, a Henderson plot analysis cannot be directly applied either since the EI* complex is not a "dead end" complex, shown by the kinetic analysis for the mode of inhibition described in the following section. An upper limit for the value of $K_i^*$ was estimated to be in the nanomolar range (see Table I) using an estimated upper limit of the rate of reactivation based on the observation of the result shown in Fig. 4.

The Mode of Inhibition of Lavendustin-A with Respect to the Binding of Each Substrate—Conventional steady state kinetic analysis of Lavendustin-A inhibition in the presence of both ATP and the peptide substrate seems inapplicable, since the formation of the E-ATP-peptide complex is much faster than the formation of the stable EI* complex as described above. We therefore examined the effect of Lavendustin-A on the binding of ATP or PLC-1254 after pre-equilibration of EGFR-IC with the inhibitor and the alternative substrate, respectively. The strategy was to preincubate the enzyme with the inhibitor at fixed concentrations and one substrate at varying concentrations for 10 min on ice. Then an excess amount of the second substrate was added to initiate the reaction. The reaction was then allowed to proceed on ice for 3 min. Since EI* is a stable complex and the substrate concentrations are in excess in comparison with the enzyme concentration, the reaction kinetics after preincubation should be close to a steady state kinetics. Double-reciprocal plots were therefore used to reveal the effect of the inhibitor on the apparent $K_m$ and $V_m$ values under such conditions. Fig. 6 shows that Lavendustin-A inhibits the kinase activity by a marked decrease of the binding affinity for ATP (4-fold at 300 nM) and only a slight decrease (20%) of the maximal reaction velocity. The slope versus $[I]$ replot is hyperbolic as shown in Fig. 6, inset, suggesting a hyperbolic (partial) mixed-type inhibition with respect to ATP. The result in Fig. 7 shows that Lavendustin-A is also a hyperbolic mixed-type inhibitor with respect to PLC-1254 with a 3-fold decrease of the binding affinity and a 40% decrease of the maximal reaction velocity at 300 nM. These observations suggest that Lavendustin-A is a nonexclusive inhibitor with respect to ATP and peptide as defined by Segel (23). It binds at a site distinct from either one of the binding sites for ATP and the peptide, and this binding significantly lowers the binding affinities of the enzyme for both substrates. The effect on the $V_m$ values may simply be due to the changed affinity for the second substrate or/and that Lavendustin-A may also affect the overall rate of product formation as expressed by the model of hyperbolic (partial) mixed-type inhibition (24). An analysis of the effect of substrate concentration on $k_{obs}$ also suggested that the initial binding of the inhibitor is not affected by the substrate (data not shown). This observation is also inconsistent with a mode of pure competitive inhibition and is consistent with the model of a partial inhibitory mechanism.

Inhibition of the Tyrosine Kinase Activity of EGFR-IC by RG 14467—RG 14467 bears a structural similarity to a portion of Lavendustin-A (Scheme I). A time course study, similar to the experiment shown in Fig. 3, showed that RG 14467 is also a slow binding inhibitor. The pre-steady state time interval analysis shown in Fig. 8A determined that the $k_{obs}$ is a function of $[I]$. Fig. 8B shows that the result of the plot of $1/(k_{obs})$ versus $1/[I]$ also corresponds to the two-step mechanism, as described for Lavendustin-A. The determined values of the corresponding kinetic parameters are summarized in Table I. Reactivation studies were also performed as described for Lavendustin-A and showed that RG 14467 also acts like an irreversibly bound inhibitor (data not shown). The overall dissociation constant $K_i^*$ was estimated to be at the 10th micromolar range (Table I).

One interesting feature of RG 14467, that was not observed with Lavendustin-A, is that the inhibition was reduced to different extents when the inhibitor was added to the kinase in the presence of different peptides. As shown by Fig. 9, the
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Fig. 6. The kinetic analysis for RG 14355 versus ATP. The experiment was performed with prephosphorylated EGFR-IC by preincubating the enzyme, MnCl₂, and [γ-32P]ATP (at varying concentrations) for 30 min on ice as described under “Experimental Procedures.” The inhibitor at the indicated concentration was then added to the mixture and allowed to incubate with prephosphorylated EGFR-IC for 10 min on ice before the addition of 2 mM PLC-1254. The reaction was then allowed to proceed on ice for 3 min. The results were analyzed as described above. Shown are the double-reciprocal plots of the data and the determined apparent $V_\text{m}$ and $K_\text{m}$ values at each inhibitor concentration. The inset shows the slope versus [I] replot.

Fig. 7. The kinetic analysis for Lavendustin-A versus PLC-1254. The experiments were performed as described under “Experimental Procedures.” The inhibitor at the indicated concentration was incubated with the enzyme, MnCl₂, PLC-1254 at varying concentrations (100, 200, 500, 1000, or 1500 μM) on ice for 10 min. [γ-32P]ATP (10 μM) was then added to initiate the reaction. Reaction time was 3 min. The results were analyzed as described above. The double-reciprocal plots and the resultant apparent $V_\text{m}$ and $K_\text{m}$ values are shown. The slope versus [I] replot is shown in the inset.

addition of any peptide, PLC-1254, PLC-783, PLC-771, or K1 (14), to the kinase before the addition of RG 14467 (1 μM) (ATP was added last to initiate the reaction) fully protects the enzyme from being inhibited. However, if the peptide was added together with the inhibitor during the preincubation time, the inhibition with PLC-1254 and PLC-783 (20–30%) was still less than the results of the nonprotection experiments (50–60% inhibition), in which the inhibitor was added prior to the peptide (data not shown), but with PLC-771 and K1, the inhibition (~60%) is comparable with that of the nonprotection experiments. Possible explanations for these different protective effects induced by different peptides are discussed under “Discussion.”

The kinetic analyses of the reactions with addition of one substrate during the preincubation of enzyme and inhibitor were performed as described above for Lavendustin-A. Fig. 10 shows that the double-reciprocal plot and the slope replot indicate that RG 14467 is a hyperbolic (partial) competitive inhibitor with respect to ATP. The apparent $K_\text{m}$ value for ATP was increased 4-fold by 3 μM RG 14467. When the analysis was performed with respect to the binding of the peptide substrate, the double-reciprocal plot for PLC-1254 in the presence of the inhibitor was parabolic-like (data not shown), which was consistent with the observed protective effect of PLC-1254 against the inhibition by RG 14467. The nonprotective peptide K1 was therefore used in the kinetic
petitive inhibitor with respect to the peptide K1. The apparent
than other reported tyrosine kinase inhibitors,
atin-A is competitive with ATP and noncompetitive with the
were analyzed as described for Fig. 5. Each reaction was
allowed to proceed for 2 min on ice. The results
indicated concentration on ice for various times. The reaction was then
initiated by addition of a mixture containing PLC-1254 and
[γ-32P]ATP at a final concentration of 0.5 mM and 5 μM, respectively.
There was an allowed to proceed for 2 min on ice. The results
were analyzed as described for Fig. 5. A, plots of ln[(v − v)/v] versus t for the determination of kobs. B, plot of 1/(kobs) versus 1/[I] for the determination of k0 and K4 (Table I).

studies. Fig. 11 shows that RG 14467 is a hyperbolic noncompetitive inhibitor with respect to the peptide K1. The apparent Vm value was 75% reduced by 1 μM RG 14467.

DISCUSSION

Lavendustin-A is a potent and selective tyrosine kinase inhibitor. Its potency in a cell-free reaction is much higher than other reported tyrosine kinase inhibitors, i.e. erbstatin (25) and its derivative (26), genistein (27), and tyrphostin molecules (28–30). However, its effects on growth factor-stimulated cell proliferation and tyrosine phosphorylation in intact cells are much weaker. It was reported that Lavendus-
A is competitive with ATP and noncompetitive with the peptide substrate (9). We have studied in detail its kinetic characteristics of inhibition using the baculovirus-expressed tyrosine kinase-active intracellular domain of the EGF recep-
tor (10). It was found that Lavendustin-A is a slow and tight binding inhibitor to the receptor tyrosine kinase domain and its mode of binding can be fitted by a two-step mechanism, in which an EI complex is first rapidly formed followed by a slow isomerization to form a tight and stable EI* complex. Kinetic analysis using a preincubation protocol to pre-equilib-
rate the enzyme with the inhibitor and one substrate suggests that Lavendustin-A is a hyperbolic (partial) mixed-type inhibitor with a major effect on the binding affinities of EGFR-IC for its substrates (Figs. 6 and 7). The discrepancy between the conclusions of this study and the previous report (9) is possibly due to: (i) the difference of the experimental systems with respect to the preparation of the receptor kinase and the nature of the substrate, and (ii) the recognition of the slow binding feature of Lavendustin-A and consequently the use of the appropriate experimental approach to analyze the

kinetic data. The mode of a partial mixed-type inhibition suggests that Lavendustin-A binds to the kinase domain at a site distinct from either the ATP site or the peptide site. It is possible that the kinase active site is extended since it usually recognizes a protein substrate rather than a small peptide, and therefore Lavendustin-A binds to the kinase domain simultaneously with ATP and the small peptide substrate. Alternatively, it may bind to an allosteric site and induce a conformational change which reduces the binding affinities for the substrates. The conformational change may well be the result of the slow isomerization process from EI to EI*

It is worth mentioning that the behavior of Lavendustin-A being able to affect the binding of either one of the substrates during the preincubation before the addition of the second substrate supports a recent observation of a sequential random Bi – Bi mechanism for the EGF receptor tyrosine kinase2 and is inconsistent with the previous report of a sequential ordered Bi – Bi mechanism (31).

RG 14467 is an analogue of Lavendustin-A that lacks a phenyl ring and has a different substituent group. It also exhibits the characteristics of a slow binding inhibitor following the two-step mechanism. However, several major differences were observed between the two compounds. (i) RG 14467 is a less potent inhibitor to the tyrosine kinase than Lavendustin-A shown by the determined K5 and k5 values that correlate with the initial step of the rapid formation of EI and the second step of isomerization. (ii) The inhibition by RG 14467 was completely blocked by the preincubation of the kinase with any of the peptide substrates prior to the addition of the inhibitor. However, protection was only observed with PLC-1254 and PLC-783, but not with PLC-771 and K1, when the peptide was added together with the inhibitor to the

2 I. Posner, personal communication.
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Fig. 10. The kinetic analysis for RG 14467 versus ATP. The experiments were performed similarly to that described in the legend to Fig. 6. The double-reciprocal plots and the determined apparent \( V_\text{a} \) and \( K_\text{a} \) values are shown. The inset shows that the slope versus [I] replot is hyperbolic.

Fig. 11. The kinetic analysis for RG 14467 versus K1. The experiments were performed similarly to that described for Fig. 7, except the peptide (K1) concentrations applied were 40, 80, 150, 300, and 500 \( \mu \text{M} \). The double-reciprocal plots and the effects of the inhibitor at each concentration on the apparent \( V_\text{a} \) and \( K_\text{a} \) values are shown. The inset shows the slope versus [I] replot.

greatly the binding affinity of the EGFR-IC kinase for PLC-771, but has essentially no effect on the binding affinities for PLC-1254 or PLC-783 (14). (iii) The mode of inhibition of RG 14467 is shown to be partial competitive with ATP and partial noncompetitive with the peptide K1. Therefore, RG 14467 interacts with the kinase domain in a way that results in reduced binding affinity for ATP, but no effect on the binding of K1. The large effect on the apparent \( V_\text{a} \) value in the study versus K1 (75\% reduced by only 1 \( \mu \text{M} \) RG 14467 at [ATP] = 10 \( \mu \text{M} \)) indicates that the overall rate of product formation following the formation of the \( E^* \cdot \text{RG 14467-K1} \) complex is significantly reduced in comparison with the control rate of the \( E \cdot \text{K1} \) complex.

Taken together, the described differences between Lavendustin-A and RG 14467, it is possible that these two compounds actually bind at different sites in the kinase domain. The kinase may have multiple sites accessible for the binding of molecules with different functional groups. The existence of different side chains of the two compounds may contribute to the selective orientations of the inhibitors in the kinase domain. This information is worth bearing in mind for future development and study of related compounds as tyrosine kinase inhibitors. The complexity of the interactions of the enzyme with its inhibitors is also a manifestation of the dynamic nature of the catalytic center of the kinase domain of the EGF receptor. Derivatives of the slow tight binding inhibitors may be useful modification reagents to identify the functional groups in the catalytic center of the enzyme.

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REFERENCES
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