Comparison of the Biochemical and Kinetic Properties of the Type 1 Receptor Tyrosine Kinase Intracellular Kinase Domains

DEMONSTRATION OF DIFFERENTIAL SENSITIVITY TO KINASE INHIBITORS*

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Epidermal growth factor receptor (EGFR), ErbB-2, and ErbB-4 are members of the type 1 receptor tyrosine kinase family. Overexpression of these receptors, especially ErbB-2 and EGFR, has been implicated in multiple forms of cancer. Inhibitors of EGFR tyrosine kinase activity are being evaluated clinically for cancer therapy. The potency and selectivity of these inhibitors may affect the efficacy and toxicity of therapy. Here we describe the expression, purification, and biochemical comparison of EGFR, ErbB-2, and ErbB-4 intracellular domains. Despite their high degree of sequence homology, the three enzymes have significantly different catalytic properties and substrate kinetics. For example, the catalytic activity of ErbB-2 is less stable than that of EGFR. ErbB-2 uses ATP-Mg as a substrate inefficiently compared with EGFR and ErbB-4. The three enzymes have very similar substrate preferences for three optimized peptide substrates, but differences in substrate synergies were observed. We have used the biochemical and kinetic parameters determined from these studies to develop an assay system that accurately measures inhibitor potency and selectivity between the type 1 receptor family. We report that the selectivity profile of molecules in the 4-anilinoquinazoline series can be modified through specific aniline substitutions. Moreover, these compounds have activity in whole cells that reflect the potency and selectivity of target inhibition determined with this assay system.

The type 1 receptor tyrosine kinase family contains four members, epidermal growth factor receptor (EGFR1 or ErbB-1), ErbB-2, ErbB-3, and ErbB-4 (reviewed in Ref. 1). These receptors consist of an extracellular ligand binding domain, a single transmembrane domain, an intracellular tyrosine kinase catalytic domain, and a tyrosine-rich cytoplasmic tail. The biological activity of these receptors is mediated through the following signal transduction mechanism. A complex pattern of ligand/receptor interactions results in the formation of homo- and heterodimers among type 1 receptor family members. Dimerization activates the catalytic domains and allows one receptor monomer to phosphorylate tyrosine residues on the cytoplasmic tail of the other monomer in the pair. Phosphorylation of tyrosine residues on the cytoplasmic tail creates specific binding sites for Src homology 2 or phosphotyrosine binding domain containing proteins. The recruitment of these proteins to the receptor activates signal transduction pathways that produce the response of the cell to the ligand. EGFR, ErbB-2, and ErbB-4 all possess active tyrosine kinase catalytic domains. ErbB-3 is catalytically impaired (2), but it is biologically active because it can form heterodimers with the other family members.

Members of the type 1 family of receptors have been implicated in cancer. Specifically, overexpression of ErbB-2 and EGFR is correlated with poor prognosis and reduced overall survival (3). The role of ErbB-3 and ErbB-4 in cancer is not as well described. In pre-clinical models of tumor formation, however, co-expression of these receptors with EGFR or ErbB-2 results in a more aggressive transformed phenotype (4, 5). The therapeutic potential of the type 1 receptors as targets has been clinically proven for ErbB-2 through the use of HerceptinTM, an anti-ErbB-2 antibody (6). Inhibitors of the tyrosine kinase activity of EGFR have demonstrated pre-clinical activity in animal tumor models and are being evaluated clinically (7).

The design and discovery of future generations of kinase inhibitors directed at the type 1 receptor family are progressing at a rapid rate (8). The catalytic domains of these receptors have very similar amino acid sequences, and it is likely that some inhibitors will affect more than one member of the family. A clear understanding of how these molecules affect the different type 1 receptors is necessary to interpret biological effects and develop the structure-activity relationships of inhibitor enzyme selectivity. Biochemical characteristics of the enzyme assay system such as substrate kinetics and concentration, peptide substrate sequence, and enzyme activation state can significantly influence the apparent affinity of a kinase inhibitor. For this reason it is important to have well characterized enzyme and assay systems for inhibitor analysis.

We describe the expression and purification of the intracellular domains of EGFR, ErbB-2, and ErbB-4 using a baculovirus system. We have directly compared several biochemical properties of the enzymes including steady-state substrate kinetics, metal preferences, and peptide substrate selectivity. We also report that the three enzymes are significantly different in...
their sensitivity to kinase inhibitors in the 4-anilinoquinoline class. The structure-activity relationship of a subset of these compounds with respect to type 1 receptor potency and selectivity is presented.

**EXPERIMENTAL PROCEDURES**

Cloning—Recombinant baculoviruses expressing the intracellular domains for each family member were made from full-length human clones by PCR. During PCR, oligonucleotides were used that introduced restriction sites for subcloning as follows: for EGFR (GenBank^*TM* accession number X06858) amino acids 671–1210 with Stu I, Xho I ends; for ErbB-2 (GenBank^*TM* accession number X16743) amino acids 691–1255 with Xba I, HindIII ends; and for ErbB-4 (GenBank^*TM* accession number L07686) amino acids 690–1309 with Not I, Xho I ends. All constructs were fused to a N-terminal His tag (MKKGGHHHHHHH) and subcloned into pFastBac (Invitrogen). Each construct was verified by DNA sequence analysis. The recombinant baculoviruses were made following Bac-To-Bac^®^*TM* baculovirus expression system manual (Invitrogen), which utilizes the method developed at Monsanto/Searle (9).

Fermentation—Large scale (2-liter) virus preparations for fermentation were made by infecting Spodoptera frugiperda (SF-9) cells in 6-liter shake flasks at 27.5 °C and 120 rpm at a multiplicity of infection = 0.1. The virus-containing culture supernatants were harvested at 72 h post-infection, centrifuged at 2000 rpm for 20 min, and stored at 4 °C. Cells were harvested 48 h post-infection with a cell count, and viability. Infection was allowed to proceed at 30 °C with 5% CO2. After 48 h, the culture was dosed with compounds diluted in the appropriate growth medium. After incubating with compounds for 6 h, cells were rinsed with ice-cold PBS and quick frozen in dry ice/ethanol after discarding the supernatant. The virus-containing culture supernatants were harvested at 72 h post-infection, centrifuged at 2000 rpm for 20 min, and stored at −80 °C.

**Type 1 Receptor Tyrosine Kinase Assays—**Reactions were performed in 96-well polystyrene round-bottom plates in a final volume of 45 μl. Reaction mixtures contained 50 mM Mops (pH 7.5), 2 mM MnCl2, 10 μM ATP, 10 μCi of [γ-32P]ATP per reaction, 50 μM peptide substrate, and 1 mM dithiothreitol. We have used three peptide substrates. Peptide A (b.rotin-aminoacidic-hexaplex)-EEEYFEVAKKCONH2 was optimized for EGFR phosphorylation using a synthetic library approach. Peptide B (b.rotin-aminoacidic-hexaplex)-SGMEDIYFEFKKKGKC was optimized for ErbB-2 phosphorylation in a similar fashion (11). Peptide C (b.rotin-aminoacidic-hexaplex)-RAHEEYYHHFFKCONH2 was optimized for phosphorylation by ErbB-2 (12). All peptides were synthesized by Quality Controlled Biochemicals Inc., Hopkinton, MA. The concentrations of dissolved peptide stocks were determined by absorbance at 280 nm. Peptide reactions were initiated by adding 10 nM peptide to a reaction mixture containing 50 μM ATP, and 20 μM peptide A. The reaction was allowed to proceed for 30 min, terminated, and quantified as described above. VEGFR2 assays contained 10 nM enzyme, 50 μM MOPS, pH 7.5, 10 μM MgCl2, 20 μM ATP, and 200 μM peptide A. The reaction was allowed to proceed for 40 min, terminated, and quantified as described above. VEGFR2 assays contained 10 nM enzyme, 100 μM HEPES, pH 7.5, 0.1 mg/ml bovine serum albumin, 0.1 mM dithiothreitol, 360 mM peptide A, 75 μM ATP, and 5 μM MgCl2. The reaction was allowed to proceed for 40 min. Product was detected using a homogeneous time-resolved fluorescence procedure (13). Briefly, the reactions were quenched by adding 10 μM of 100 μM HEPES, pH 7.5, 100 μM EDTA, 45 mM streptavadin-linked allopoycyonin (Molecular Probes, Eugene, OR), and 3 mM europium-conjugated anti-phosphotyrosine antibody (Wallac, Turku, Finland). The product was detected using a Victor plate reader (Wallace, Turku, Finland) with a time delay at 665 nm. VEGFR2 was optimized for phosphorylation by ErbB-2 (12). After incubating with compounds for 6 h, cells were rinsed with ice-cold phosphate-buffered saline and were lysed in RIPA (150 mM NaCl, 50 mM Tris-HCl, pH 7.5, 0.25% deoxychylate, 0.1% Nonidet P-40) containing 10 μg/ml leupeptin (Roche Molecular Biochemicals). An equal volume of 2 XSDPAGE loading buffer was added, the mixture was boiled for 5 min, centrifuged at 30,000 g for 1 h, and the supernatant was used for gel electrophoresis. Whole cell extracts were separated on a 10–16% gradient gel and bands were quantified by densitometric analysis. The data analysis was performed using the program Quantity One (BioRad) and was expressed as percentages of control. Reaction products were detected by autoradiography, and quantified by densitometric analysis.
amount of phosphorylated peptide formed in 10 min, since this time period produced adequate amounts of product for all three enzymes and was within the linear portion of the reaction progress curve. Three experiments were performed for all kinetic studies, and the average data were globally fit to the equations described under results. Nonlinear regressions were conducted using the program Sigma Plot (Jandel Scientific, San Rafael, CA).

RESULTS

Enzyme Expression and Purification—The intracellular domains of EGFR, ErbB-2, and ErbB-4 were cloned into a baculovirus expression system (see “Experimental Procedures”). The first purification step for each enzyme was nickel chelate affinity chromatography. Further purification was performed using ceramic hydroxyapatite chromatography (HA) followed by HIC (see under “Experimental Procedures”). The protein eluting from HIC was analyzed for purity by SDS-PAGE (Fig. 1). Each protein migrated as predicted from its molecular weight. We estimate the purity of these samples to be (>95%). The identity of each enzyme was confirmed by N-terminal sequencing (data not shown).

For each enzyme, analysis of the activity following chelating Sepharose revealed it was devoid of any contaminating kinase activities and had kinetic characteristics identical to the pure enzyme (data not shown). Because additional purification steps resulted in a significant loss in yield, we used enzyme purified through the chelating Sepharose stage for the remainder of our studies. Typical protein yields from fermentation after chelating Sepharose were 4 mg/liter for ErbB-2, 6 mg/liter for ErbB-4, and 10 mg/liter for EGFR.

Time Course of Substrate Phosphorylation—The kinase activity of the purified type 1 receptor intracellular domains was evaluated by measuring the phosphorylation of an exogenous peptide substrate (see “Experimental Procedures”). The production of product as a function of time is shown in Fig. 2. In this experiment the amount of product formed is less than 10% of the total substrate available for all three enzymes. Therefore, the observed decrease in the rate of the reaction as a function of time is not due to substrate consumption. The experiment was analyzed by fitting the data to a model of single exponential enzyme decay (Equation 1).

\[ P = v(e^{-kt}) \cdot t \] (Eq. 1)

In Equation 1, \( v \) represents the initial rate of product (\( P \)) formation that decays at a rate (\( k \)) over a period of time (\( t \)).

EGFR was linear over the course of the experiment (\( v = 0.5 \) pmol/min, \( k = 0 \)). ErbB-2 and ErbB-4 had higher initial rates but demonstrated significant decay as follows: ErbB-2, \( v = 0.85 \) pmol/min, \( k = 0.014 \) min; ErbB-4, \( v = 1.25 \) pmol/min, \( k = 0.007 \) min. Approximately 10% of the total enzyme has lost activity in 10 min at the calculated rates of ErbB-2 and ErbB-4 decay. All subsequent experiments, therefore, were terminated at 10 min to obtain a good estimate of the initial rate.

We have found that purified ErbB-2 is highly stable when maintained in a concentrated solution, even at room temperature. However, the enzyme rapidly loses activity upon dilution. These observations suggest that enzyme inactivation after dilution results from alterations in the tertiary structure of the enzyme or alterations in its aggregation state. Our observations are consistent with previous results demonstrating that aggregation of the EGFR catalytic domain significantly affects the rate of catalysis (15, 16).

Divalent Cation Preferences—The interaction between protein kinases and divalent cations is complex. All phosphotransferases require a divalent cation that coordinates the phos-
phate groups of the nucleotide triphosphate substrate (17). Kinases have a similar requirement, but they also have an additional site of interaction that can be distinguished from the primary site by affinity. Kinases can be activated or inactivated by binding of a cation to this second site depending upon the specific kinase and identity of the cation (18, 19). The effect of divalent cation concentration on the rate of peptide substrate phosphorylation catalyzed by the type 1 receptor kinases is shown in Fig. 3. The type 1 receptor kinases are different with respect to their ability to utilize Mg$^{2+}$. ErbB-2 is almost inactive in the presence of Mg$^{2+}$, whereas EGFR and ErbB-4 can use this cation efficiently (Fig. 3A). Another key difference is the optimum concentration of Mn$^{2+}$ used by each enzyme (Fig. 3B). In this experiment ATP was held constant at 10 μM. Under these conditions any concentration of cation above 10 μM is essentially free from bound nucleotide. Peak activity for ErbB-2 was observed at 2 mM Mn$^{2+}$. ErbB-4, on the other hand, exhibits optimal activity at 0.2 mM Mn$^{2+}$, and EGFR exhibits optimal activity at 0.6 mM Mn$^{2+}$. Presumably these differences reflect different affinities of Mn$^{2+}$ for binding to the second cation site.

To evaluate further the interaction between the type 1 receptor kinases, ATP, and divalent cations, we determined the apparent $K_m$ and $V_{max}$ values for ATP-Mg and ATP-Mn at a fixed concentration of peptide C (Fig. 4). These experiments were performed at a concentration of free Mg$^{2+}$ or Mn$^{2+}$ equivalent to the optimum concentration determined in the experiment shown in Fig. 3. ATP-Mg and ATP-Mn titrations were performed in parallel, and the reactions were initiated with the same diluted enzyme mixture to ensure that the results are directly comparable. The substrate kinetic parameters were determined by nonlinear least squares analysis of the averaged
ATP-metal described for Fig. 3. The steady-state kinetic parameters were determined by fitting the averaged initial velocity data to Equation 2 (see "Results"). The standard errors of the parameter estimates were all less than 10% of the value shown.

<table>
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<th>Enzyme</th>
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<th>ATP-Mn</th>
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<tr>
<td></td>
<td>KmCl2</td>
<td>kcat</td>
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<tr>
<td>ErbB-2</td>
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</tr>
<tr>
<td>EGFR</td>
<td>10 m</td>
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</tr>
<tr>
<td>ErbB-4</td>
<td>10 m</td>
<td>0.045</td>
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</table>

Comparison of Type 1 Receptor Tyrosine Kinases

Peptide substrate phosphorylation reactions were performed using the indicated enzyme and peptide C (50 µM). MgCl₂ and MnCl₂ were added at the optimum concentration as indicated. Three experiments were performed for each enzyme substrate combination using the concentration of ATP-metal described for Fig. 3. The steady-state kinetic parameters were determined by fitting the averaged initial velocity data to Equation 2 (see "Results"). The standard errors of the parameter estimates were all less than 10% of the value shown.

Steady-state Substrate Kinetics—We evaluated the steady-state kinetic parameters of three different peptide substrates for each of the type 1 receptor kinases. Peptide phosphorylation was measured at 8 fixed concentrations of peptide and 12 varied concentrations of ATP-Mn. Autophosphorylation contributes to the overall velocity to a varying extent depending upon the enzyme-peptide substrate pair under evaluation. The contribution of autophosphorylation was eliminated from our analysis by subtracting the amount of product formed in the absence of peptide substrate from the amount formed in the presence of peptide substrate at each concentration of ATP. The results of ErbB-2 phosphorylation of peptide A are shown in Fig. 5A. Reciprocal velocity as a function of reciprocal ATP-Mn concentration was plotted for each fixed peptide concentration. The data were globally fit to the equation for a sequential two-substrate reaction (Equation 3) (20).

\[ v = \frac{V_{AB}}{k_{cat}K_{mcat} + K_{cat}A + K_{mB}B + AB} \]  

In this equation, \( v \) is the measured initial velocity; \( V \) is the maximum velocity; \( A \) is the concentration of ATP-metal; and \( K_{m(app)} \) is the apparent Michaelis constant for ATP-metal. Similar experiments were performed for EGFR and ErbB-4, and the results are summarized in Table I. The \( k_{cat} \) values were calculated by dividing \( V_{max} \) by the total enzyme concentration. It is likely that this procedure significantly underestimates \( k_{cat} \) since the fraction of active enzyme is unknown (11). For all three enzymes, \( K_{m(app)} \) was slightly lower for ATP-Mn. For EGFR the \( k_{cat} \) value was similar for either form of the ATP substrate. However, for ErbB-2 the \( k_{cat} \) value was 7.5-fold higher for ATP-Mn. The relative efficiency of various substrates can be determined by comparing the ratio of the turnover number (\( k_{cat} \)) and \( K_{m(app)} \). For ErbB-2 (the \( k_{cat}/K_{m(app)} \) value for ATP-Mn (4615 M⁻¹ s⁻¹) is 16 times greater than that for ATP-Mg (296 M⁻¹ s⁻¹). EGFR and ErbB-4 have a modest preference for ATP-Mn.

![Fig. 5. Two substrate steady-state kinetics.](image)

A similar pattern was observed when the reciprocal velocity was plotted as a function of reciprocal peptide concentration (data not shown).

The negative substrate synergy discerned from the Line-weaver-Burke analysis using peptide A is readily apparent from the calculated kinetic parameters (Table I). The ratio of \( K_{m(app)}/K_{m(cat)} \) is an estimate of the degree of binding synergy between substrates in a two-substrate kinase reaction (22). If binding of one substrate has no effect on the \( K_{m} \) of the other substrate,
Comparison of Type 1 Receptor Tyrosine Kinases

TABLE II

ATP and peptide substrate steady-state kinetic parameters

Peptide substrate phosphorylation reactions were performed using the indicated enzyme and peptide substrate. Substrate concentrations were varied as described for Fig. 4. Three experiments were performed for each enzyme substrate combination. The steady-state kinetic parameters were determined by fitting the averaged initial velocity data to Equation 3 (see “Results”). The standard errors of the parameter estimates are shown.

<p>|</p>
<table>
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<th>k&lt;sub&gt;cat&lt;/sub&gt;</th>
<th>K&lt;sub&gt;ma&lt;/sub&gt;</th>
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<th>K&lt;sub&gt;mb&lt;/sub&gt;</th>
<th>K&lt;sub&gt;cb&lt;/sub&gt;</th>
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<td>μM</td>
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<tr>
<td>ErbB-2</td>
<td>Peptide A</td>
<td>0.065</td>
<td>19 ± 2</td>
<td>53 ± 8</td>
<td>25 ± 8</td>
<td>100</td>
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<td>Peptide B</td>
<td>0.037</td>
<td>84 ± 17</td>
<td>157 ± 40</td>
<td>31 ± 17</td>
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<td>Peptide C</td>
<td>0.024</td>
<td>50 ± 8</td>
<td>65 ± 11</td>
<td>65 ± 17</td>
<td>414</td>
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<tr>
<td>EGFR</td>
<td>Peptide A</td>
<td>0.230</td>
<td>34 ± 4</td>
<td>316 ± 38</td>
<td>1.9 ± 1.4</td>
<td>73</td>
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<td>Peptide B</td>
<td>0.118</td>
<td>76 ± 19</td>
<td>899 ± 200</td>
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<td>Peptide C</td>
<td>0.057</td>
<td>21 ± 3</td>
<td>128 ± 16</td>
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<td>ErbB-4</td>
<td>Peptide A</td>
<td>0.031</td>
<td>44 ± 7</td>
<td>270 ± 38</td>
<td>13 ± 4</td>
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<td>Peptide B</td>
<td>0.076</td>
<td>29 ± 4</td>
<td>144 ± 22</td>
<td>9 ± 5</td>
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<td>Peptide C</td>
<td>0.105</td>
<td>42 ± 3</td>
<td>42 ± 3</td>
<td>1.1 ± 2.1</td>
<td>2500</td>
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then K<sub>cb</sub>/K<sub>ma</sub> is equal to 1. The EGFR K<sub>cb</sub>/K<sub>ma</sub> values range from 0.020 (peptide B) to 0.10 (peptide C). By comparison, the ErbB-2 K<sub>cb</sub>/K<sub>ma</sub> values range from 0.6 (peptide B) to 1.3 (peptides A and C). This lack of negative substrate synergy for ErbB-2 is graphically demonstrated by lines that intersect to the left of the vertical axis and near the horizontal axis (Fig. 5A).

Posner et al. (23) observed similar negative substrate synergy for EGFR, and Jan et al. (11) observed no substrate synergy for ErbB-2. These two studies are not directly comparable with each other, however, because dissimilar enzyme constructs and different peptide substrates were used. In our work, we have used comparable intracellular domain constructs and identical peptide substrates for EGFR and ErbB-2. Our results confirm the substrate synergy observations made by Posner et al. (23) and Jan et al. (11) and suggest that the presence or absence of negative substrate synergy is an inherent property of the particular type 1 receptor intracellular domain.

Peptide Substrate Selectivity—The catalytic efficiency of an enzyme substrate combination is described by the ratio k<sub>cat</sub>/K<sub>ma</sub>. This ratio is the best measure for comparison of the efficiency of product formation catalyzed by related enzymes. It also captures the effects of substrate molecular structure on either kinetic constant. Thus, the ratio is a good measure of different substrate selectivity for the same enzyme (24). The kinetic parameters for all three enzymes and all three peptides were determined, and the k<sub>cat</sub>/K<sub>ma</sub> ratios were calculated (Table II). Peptide A was optimized for phosphorylation by EGFR (10). Peptide B was optimized for phosphorylation by ErbB-2 (11). Peptide C was also optimized for phosphorylation by ErbB-2 (12).

All three enzymes phosphorylated peptide A with similar efficiency (k<sub>cat</sub>/K<sub>ma</sub> = 100 μM<sup>-1</sup> s<sup>-1</sup>). Peptide B was a better substrate for EGFR, ErbB-2, and ErbB-4 even though it was optimized for phosphorylation by ErbB-2. Jan et al. (11) analyzed the kinetics of ErbB-2 phosphorylation of peptide B at a single fixed concentration of ATP (50 μM) and found a k<sub>cat</sub>/K<sub>ma(app)</sub> of 309 μM<sup>-1</sup> s<sup>-1</sup>. We varied each substrate at several fixed concentrations of second substrate and obtained kinetic parameters by globally fitting the entire data set. We determined k<sub>cat</sub>/K<sub>ma</sub> to be 235 μM<sup>-1</sup> s<sup>-1</sup> for the ErbB-2 peptide B combination. Our result is similar to that reported by Jan et al. (11) because ErbB-2 exhibits very little substrate binding synergy. The k<sub>cat</sub>/K<sub>ma</sub> for peptide C was better than peptides A or B for all three enzymes. This effect was primarily K<sub>ma</sub>-mediated.

The three peptide substrates are used with vastly different efficiencies ranging from the EGFR peptide A combination (k<sub>cat</sub>/K<sub>ma</sub> = 73) to the ErbB-4 peptide C combination (k<sub>cat</sub>/K<sub>ma</sub> = 2500). However, all three kinases select the three peptide substrates with the same rank order of k<sub>cat</sub>/K<sub>ma</sub>; peptide A < peptide B < peptide C. By this criterion, the different members of the type 1 receptor family have very similar downstream substrate preferences.

Inhibitor Potency and Selectivity—Members of the 4-anilinoquinazolone class of compounds have been shown to be potent type II kinase inhibitors (25). Many molecules in this class have been shown to inhibit EGFR (26). These molecules bind in the ATP-binding pocket and compete with this substrate. EGFR, ErbB-2, and ErbB-4 have very similar catalytic domains, so members of this class of kinase inhibitors may affect more than one member of the type 1 receptor family.

A representative set of compounds was selected to demonstrate key structure-activity relationships in the optimized assay system for the type 1 receptor kinases (Table III). The design and synthesis of these compounds has been fully described elsewhere (27). The aniline modifications to a core 6,7-dimethoxyquinazolone were previously shown to affect dramatically the tyrosine kinase inhibition profile (26). Of the type 1 receptor family members, the inhibition of EGFR appeared to be the least affected by the aniline changes. The range of EGFR IC<sub>50</sub> values was less than 5-fold, despite the broad size of the aniline substitutions. The 4-(3-hydroxyanilino)-substituted quinazolines (compounds 1 and 2) showed general tyrosine kinase inhibition. When the hydroxyl of compound 1 was replaced with a 3-methoxy group (compound 3), a significant loss in inhibition of VEGFR2 was observed, but the compound retained a similar type 1 inhibitor inhibition profile. AG1478 (compound 4) demonstrated the reported profile with a marked potency against EGFR (28). Bulky aniline substituents, represented by compounds 5–8, confer improved potency against ErbB-2 while retaining EGFR inhibition. The benzoxoxyaniline (compound 5) and halogenated benzoxoxyaniline (compound 6) substituents appeared to confer significant selectivity for dual inhibition of EGFR and ErbB-2 compared with the other kinase enzymes tested.

Intracellular Inhibition of EGFR and ErbB-2—The ability of potent dual ErbB-2/EGFR inhibitors to block receptor auto-phosphorylation in whole cells was analyzed in two cell lines, HN5 and BT474 (Fig. 6). HN5 cells overexpress EGFR (29), and BT474 cells overexpress ErbB-2 (30). Logarithmically growing cells were incubated with various concentrations of the indicated compound for 6 h. The cells were lysed and the indicated receptor was captured by immunoprecipitation. The relative receptor content was determined by immunoblot with a separate anti-receptor antibody, and the state of phosphorylation...
was analyzed by immunoblot with an anti-phosphotyrosine antibody. Treatment of the HN5 cell line with all three compounds did not affect the total EGFR content, but there was a dose-dependent decrease in tyrosine-phosphorylated EGFR (Fig. 6A). The approximate IC_{50} value for inhibition of EGFR for these compounds ranges from 0.5 to 5 \mu M. Similar results were obtained for inhibition of ErbB-2 autophosphorylation in the BT474 cell line (Fig. 6B).

DISCUSSION

The discovery and clinical development of inhibitors of EGFR catalytic activity is progressing at a rapid rate (7). These molecules are promising for the treatment of tumors that overexpress this receptor. The discovery of dual inhibitors of ErbB-2 and EGFR may be the next step in the progression of this therapeutic approach (31, 32). The catalytic domains of the active members of the type 1 receptor family are very similar.

### TABLE III

Structure-activity relationship of quinazoline aniline substitutions

Peptide substrate phosphorylation reactions were performed using peptide A and the indicated enzyme. The indicated compound was serially diluted 1 to 3 and added to the reactions to produce an 11-point dose-response curve ranging from 0.0001 to 10 \mu M. The IC_{50} values were estimated from data fit to the equation $y = V_{\text{max}} \cdot \frac{1}{1 + \frac{x}{K}}$. cFms, VEGFR2, cSrc, and PKCγ assays were performed as described under "Experimental Procedures." n.d., not determined.

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<thead>
<tr>
<th>Compound</th>
<th>ErbB-2</th>
<th>EGFR</th>
<th>ErbB-4</th>
<th>VEGFR2</th>
<th>cFms</th>
<th>cSrc</th>
<th>PKCγ</th>
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<tr>
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The amino acid sequences of the catalytic domains of EGFR, ErbB-2, and ErbB-4 are 84–86% identical to one another depending upon the specific comparison. Because of this high degree of similarity, it is possible that compounds designed to inhibit one family member will have some effect on the other receptors. The interpretation of the biological effects of type 1 receptor kinase inhibitors will be enhanced by a detailed understanding of the potency profile of the inhibitor versus all three catalytically active enzymes. The potency of a kinase inhibitor in a biological system depends upon several biochemical properties. These properties include inhibitor $K_i$, substrate identity, enzyme substrate kinetic parameters, enzyme activation state, enzyme reaction mechanism, and inhibitor mode of action.

The evaluation of the biochemical and kinetic properties of members of the type 1 receptor family has been greatly facilitated by the expression and purification of defined domains using baculovirus expression systems. EGFR has been most extensively studied (15, 33–36). The catalytic domain is subject to a regulatory mechanism that is driven by the formation of higher order complexes (15, 36). The divalent metal ion requirement seems to reflect aspects of EGFR catalytic activity associated with the activation state (37). Autophosphorylation of EGFR does not seem to affect catalytic activity (36). Biochemical and kinetic properties of human ErbB-2 and the rat homologue, c-Neu, have been characterized using purified baculovirus-expressed proteins (11, 38–41). ErbB-2 seems to be similar to EGFR in some respects. A higher order structure such as dimerization was correlated with increased enzyme activity (40, 41), and divalent metal requirement seems to reflect the activation state (38). ErbB-3 has been expressed and purified from baculovirus and shown to have impaired tyrosine kinase activity (2). Purified ErbB-4 has not been characterized in this manner, and little is known about the biochemical and kinetic properties of this protein. Differences in the biochemical properties of the type 1 receptor kinases that influence inhibitor potency and selectivity are hard to determine from the studies described above due to differences in protein construct and experimental design. We have used comparable constructs and identical experimental design to accurately assess similarities and differences between these enzymes.

**ATP-Metal Substrate Preferences**—Protein kinases have diverse responses to divalent metal concentration. cAMP-dependent protein kinase binds ATP in association with one or two Mg$^{2+}$ ions. The first ion binds with high affinity and coordinates the $\beta$- and $\gamma$-phosphate of ATP. The second Mg$^{2+}$ ion binds with lower affinity and coordinates $\alpha$- and $\gamma$-phosphates of ATP (42). All protein kinases require binding by the first metal ion for activity. The consequences of second metal ion binding vary. The $k_{cat}$ of cAMP-dependent protein kinase is reduced by second ion binding (43), but the $K_m$ value for ATP is decreased. c-Src and C-terminal Src kinase have increased $k_{cat}$ values in response to second site binding, but the $K_m$ value for ATP is unchanged (19). ErbB-2, EGFR, and ErbB-4 all have optimal Mg$^{2+}$ concentrations far above the concentration bound to ATP (Fig. 3A). This suggests that Mg$^{2+}$ occupancy of a second site is required for optimal activity. We cannot determine from these studies, however, if the effect is $K_m$ or $k_{cat}$-mediated.

Several reports (11, 33, 34, 38) indicate that EGFR and ErbB-2 intracellular domains exhibit a preference for ATP-Mg relative to ATP-Mn. We observe a similar ATP-Mn preference, but the optimal free concentration of Mn$^{2+}$ is different for each enzyme (Fig. 3B). For example, ErbB-4 appears to have a 10-fold higher affinity for Mn$^{2+}$ compared with ErbB-2. This experiment and other published cation preference experiments were conducted at a fixed concentration of ATP, so the kinetic nature of the preference cannot be established. We conducted ATP-metal substrate kinetic studies in the presence of a fixed concentration of free divalent cation equal to the optimal concentration (Fig. 4). Presumably, the optimal concentration represents equal binding site occupancy for each metal-enzyme pair. This way the true effect of metal identity can be determined rather than a concentration-dependent phenomenon associated with differential metal site occupancy. The $k_{cat}/K_m(app)$ is a good overall measure of substrate preference. ErbB-4 and EGFR have very similar $k_{cat}/K_m(app)$ values for ATP-Mg and 4-fold higher $k_{cat}/K_m(app)$ values for ATP-Mn. The kinetic nature of the preference for ATP-Mn for the enzymes is different, however. EGFR has a similar $k_{cat}$ value for either cation, but ATP-Mn has a significantly lower $K_m(app)$ value. The increased ATP-Mn $k_{cat}/K_m(app)$ value for ErbB-4 results from an effect on both kinetic parameters. ErbB-2 demonstrates the greatest preference for ATP-Mn. The $k_{cat}/K_m(app)$ value is 16-fold higher than for ATP-Mg. This effect is almost entirely $k_{cat}$-mediated.

It is remarkable that two enzymes as closely related as...
EGFR and ErbB-2 would exhibit such dramatic differences in ATP-metal substrate kinetic parameters. The biggest kinetic contributor to this difference is the very low $k_{cat}$ value for ErbB-2 and ATP-Mg. ATP-Mg is believed to be the natural biological substrate for protein kinases. The inability of ErbB-2 to utilize this substrate suggests that ATP-Mn may mimic a physiologically relevant activation mechanism. Higher order enzyme structure may be one such mechanism that is affected by ATP-Mn (15).

Steady-state Kinetics—We determined the steady-state kinetic parameters of the type 1 receptor kinases by measuring initial velocities at varied concentrations of both substrates. The plot of reciprocal velocity versus reciprocal ATP-Mn concentration for ErbB-2 at different fixed concentrations of peptide (Fig. 4A) reveals lines that intersect to the left of the vertical axis near the horizontal axis. This relationship is consistent with a sequential Bi-Bi steady state ordered or a sequential Bi-Bi rapid equilibrium random mechanism. Our results do not distinguish between these two possibilities, but a sequential Bi-Bi rapid equilibrium random mechanism was demonstrated previously for EGFR (23). An analysis of burst-phase kinetics for ErbB-2 demonstrated that chemical catalysis occurs 3-fold faster than the rate-limiting step of the reaction. The rate-limiting step appeared to be ADP release (11).

A striking feature of our analysis of EGFR is the presence of a strong negative effect of one substrate binding on the affinity of the other. A similar effect was observed in an analysis of EGFR holoenzyme (23). EGF binding to the holoenzyme significantly reduced the negative substrate binding synergy. Posner et al. (23) hypothesized that ligand binding causes a conformational change that increases the stability of the tertiary enzyme-ATP-peptide complex. Our work was conducted using just the intracellular domain of EGFR. The fact that we observed such significant negative substrate binding synergy suggests that the intracellular domain adopts a confirmation similar to the nonactivated holoenzyme.

Peptide Selectivity—Receptor protein tyrosine kinases have unique peptide substrate specificity, and the specificity of the kinase has been proposed to influence downstream signal transduction events (10). Substrate specificity is determined by the amino acid sequence within 5 residues on either side of the phosphorylated tyrosine and corresponding side chains in the catalytic domain of the kinase. Single amino acid substitutions within the peptide-binding region of the catalytic domain have been found to affect significantly the peptide substrate amino acid sequence preferences (44, 45). The type 1 receptor kinases have a number of amino acid sequence differences in the C-terminal lobe that could affect peptide substrate selection.2

We evaluated the peptide substrate steady-state kinetic parameters for each of the type 1 receptor kinases using three different peptide substrates. One peptide was optimized for phosphorylation by EGFR (10), and two peptides were optimized for phosphorylation by ErbB-2 (11, 12). It is difficult to compare peptide substrate selectivity for two different enzymes by comparing the $K_m$ or $k_{cat}$ values for a single substrate because the intrinsic catalytic capability of the enzymes may differ. Comparison of the $k_{cat}/K_{m}$ for the three peptides reveals that each enzyme selects the three different peptides with the same rank order: peptide A < peptide B < peptide C. The three different type 1 receptor tyrosine kinases, therefore, have very similar peptide-substrate preferences for these three optimized substrates. Other amino acid sequence substitutions around the phosphorylation site or additional protein-protein interactions may still allow differential downstream signaling by these enzymes.

Sensitivity to Kinase Inhibitors and Biological Activity—We have used the purified type 1 receptor intracellular domains, substrates, and kinetic parameters determined above to design an assay system that will allow us to assess accurately the structure-activity relationship of inhibitor potency and selectivity. A variety of inhibition profiles can be obtained within the 4-anilinoquinazoline template. Potent relatively nonselective inhibitors, selective EGFR inhibitors, and selective dual ErbB-2/EGFR inhibitors were characterized. EGFR seems to be the most promiscuous member of the family because relatively small or bulky aniline substitutions retain potent activity. Dual ErbB-2/EGFR inhibition is conferred by bulky aniline substitutions, but these molecules are not as potent versus ErbB-4.

We have used purified intracellular domains to create our type 1 receptor enzyme assay system for practical reasons such as expression level and ease of purification. The biologically relevant forms of the receptors also contain the transmembrane and extracellular domains, and the conformation of these forms might be influenced by ligand binding. We evaluated the effect of kinase inhibitors that we characterized using our assay system in whole cells to determine whether the potency and selectivity that we obtain in the enzyme studies translates to a true biological effect. Inhibitors in this class are competitive with ATP, and our enzyme studies are conducted using relatively low ATP concentrations (10 μM) to approximate the compound $K_i$. Therefore, the $IC_{50}$ of the inhibitor in whole cells will differ from the enzyme $IC_{50}$ due to the higher intracellular concentration of ATP and other unknown factors such as membrane permeability. Three potent dual ErbB-2/EGFR inhibitors were characterized. All three were very effective at specifically reducing both ErbB-2 and EGFR autophosphorylation without affecting receptor levels (Fig. 6). The enzyme assay system therefore faithfully reflects dual activity in a biological system for ErbB-2 and EGFR inhibitors.

We have characterized the biological activity of a subset of these molecules more completely (31, 32). The inhibition of receptor autophosphorylation that we observe in tissue culture translates into inhibition of tumor cell proliferation and growth in mouse models. Future compound design and inhibitor analysis may lead to the discovery of different compounds with a variety of type 1 receptor inhibition profiles. The biological evaluation of such molecules should lead to a better understanding of the role of these enzymes in normal and tumor cell growth, and ultimately to improved therapies for patients with cancers that are linked to the type 1 receptor family.

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2. D. Vanderwall, personal communication.