ABSTRACT. Protein tyrosine kinases play a fundamental role in signal transduction pathways. Deregulated tyrosine kinase activity has been observed in many proliferative diseases (e.g., cancer, psoriasis, restenosis, etc.). Tyrosine kinases are, therefore, attractive targets for the design of new therapeutic agents against cancer. We have built up a pharmacophore model of the ATP-binding site of the epidermal growth factor receptor (EGFR) kinase and used it for the rational design of kinase inhibitors. Several examples of the successful use of this model are presented in this review. Amongst these, 4-substituted-pyrrolo[2,3-d]pyrimidines, a new class of highly potent and selective inhibitors of the EGFR kinase, have been identified and further optimized. The most active derivatives inhibited the EGFR tyrosine kinase with IC<sub>50</sub> values between 1 and 5 nM. In EGF-dependent cellular systems, tyrosine phosphorylation, as well as c-fos mRNA expression, was inhibited with similar IC<sub>50</sub> values. Further successful application of this pharmacophore model led to the identification and optimization of phenylamino-pyrazolo[4,3-d]pyrimidines and substituted isoflavones and quinolones, other classes of potent, selective, and ATP competitive EGFR kinase inhibitors with IC<sub>50</sub> values in the low nanomolar range. Structure-activity relationships of both classes are discussed.

KEY WORDS. Epidermal growth factor receptor tyrosine kinase, platelet-derived growth factor receptor tyrosine kinase; pharmacophore model, ATP competitive inhibitors, protein tyrosine kinase inhibitors.

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ABBREVIATIONS. cAMP, cyclic AMP; CDK, cyclin-dependent kinase; EGFR, epidermal growth factor receptor; FGFR, fibroblast growth factor receptor; IGF-IR, insulin-like growth factor-I receptor; IR, insulin receptor; PDGFR, platelet-derived growth factor receptor; PKC, protein kinase C; PTK, protein tyrosine kinase; SAR, structure-activity relationship; VEGFR, vascular endothelial growth factor receptor.

1. INTRODUCTION
Protein kinases play an important role in signal transduction pathways regulating a number of cellular functions, such as cell growth, differentiation, and cell death. A variety of tumor types have dysfunctional growth factor receptor tyrosine kinases, resulting in inappropriate mitogenic signaling. Protein tyrosine kinases (PTKs) are, therefore, attractive targets in the search for therapeutic agents, not only against cancer, but also against many other diseases.

About 10 years ago, when research in the signal transduction field was initiated in many pharmaceutical companies, the EGFR tyrosine kinase was one of the first tyrosine kinases described in the literature, and, therefore, was chosen as a target to start drug discovery projects. Meanwhile, many other receptor and nonreceptor tyrosine kinases have been described and found to be attractive targets for research programs in cancer and noncancer indications. Novel members of the family of the EGFR PTK, such as the p185<sup>erbB2</sup> PTK(c-erbB2 gene product) and the c-erbB3 and c-erbB4 gene products, have been identified and are used as targets for medicinal chemistry programs. In the last few years, a switch from the EGFR target to other receptor and nonreceptor tyrosine kinase targets, such as the platelet-derived growth factor receptor (PDGFR), vascular EGFR (VEGFR), fibroblast growth factor receptor (FGFR), insulin-like growth factor-I receptor (IGF-IR), insulin receptor (IR), Abl, and Src family tyrosine kinases, has been observed. Additional targets under evaluation also include the c-Met, Trk, or the cytoplasmic Jak2 kinases.

In the last decade, hundreds, or even thousands, of tyrosine kinase inhibitors have been described and reviewed in the literature (Bridges, 1995; Burke, 1992; Fry, 1994; Levitzky and Gazit, 1995; Spada and Myers, 1995; Traxler and Lydon, 1995; Traxler, 1997; Traxler, 1998). Most of them only served as tools to set up in vitro assay systems and...
to prove their functionality; only a few inhibitors demonstrated *in vivo* efficacy in relevant tumor models in nude mice. The first compounds, in most cases, inhibitors of the EGFR tyrosine kinases, to enter preclinical development failed to fulfill the pharmacodynamic (efficacy, selectivity), pharmacokinetic (resorption, metabolism), toxicological, and technical (synthesis, formulation) requirements, and never entered clinical trials. Meanwhile, some of these hurdles have been overcome. Currently, there are seven low-molecular weight compounds described as more or less selective tyrosine kinase inhibitors in early phases of clinical trials (Fig. 1). In addition, several candidates are in late-stage preclinical development and are close to entering Phase I trials.

2. THE ATP-BINDING SITE OF TYROSINE KINASES AS A TARGET FOR DRUG DISCOVERY

Within the great number of different structural classes of tyrosine kinase inhibitors, compounds competing with ATP for binding at the catalytic domain of the kinase are considered to be of special interest. Despite the fact that catalytic domains of most protein kinases share significant amino acid sequence homology and conserved core struc-

![Figure 1](image-url)
tures, it is now accepted that the ATP-binding site of protein kinases is an exciting target for rational drug design. In fact, out of the seven tyrosine kinase inhibitors that are currently in clinical trials, five are described to be ATP competitive inhibitors (Fig. 1). Numerous compounds of structurally diverse classes have proven to be highly potent and selective ATP competitive tyrosine kinase inhibitors. Special interest has focused on a special group of compounds containing a phenylamino-pyrimidine moiety in their structure, such as phenylamino-quinazolines (Fry et al., 1994; Rewcastle et al., 1995; Ward et al., 1994), phenylamino-pyrido[1,2-d]pyrimidines (Rewcastle et al., 1996; Thompson et al., 1995), phenylamino-pyrimido[5,4-d]pyrimidines (Rewcastle et al., 1997), phenylamino-pyrrolo[2,3-d]pyrimidines (Traxler et al., 1996), and phenylamino-pyrazolo[3,4-d]pyrimidines (Traxler et al., 1997).

3. PHARMACOPHORE MODEL OF THE ATP-BINDING SITE OF PROTEIN KINASES

Although more than 30 crystal structures of protein kinases complexed with ATP or an ATP competitive inhibitor have been published in the last few years, many attempts to obtain a crystal structure of the EGFR PTK have not been successful. Furet et al. (1995) published the first data of a pharmacophore model for inhibitors competing for the ATP-binding site of the EGFR PTK. In the course of this work, a hypothetical model for the binding mode of the protein kinase inhibitor staurosporine in the ATP-binding site of the cyclic AMP (cAMP)-dependent protein kinase was developed by using the published crystal data of this kinase (Zheng et al., 1993). Recently, a published crystal structure of a complex of staurosporine with cyclin-dependent kinase (CDK)2 fully confirmed the proposed binding mode of staurosporine (Lawie et al., 1997). In addition, our binding hypothesis was fully consistent with a model of the EGFR-PTK constructed by homology to the X-ray crystal structure of the cAMP-dependent protein kinase. This pharmacophore model has then been used successfully for the design and synthesis of 4-phenylamino-pyrrolo[2,3-d]pyrimidines (Traxler et al., 1996) and 4-phenylamino-pyrazolo[3,4-d]pyrimidines (Traxler et al., 1997). As an example, the binding mode of 4-phenylamino-pyrrolo[2,3-d]pyrimidine CGP 59326 (Compound 1) is shown in Fig. 2.

FIGURE 2. CGP 59326 docked at the ATP-binding site of the EGFR kinase. Sulfur-aromatic interaction between the chloro-phenyl ring and Cys773 of the sugar pocket.
Based on our own experience, together with the availability of published crystal data of several protein kinases, the model was refined and is now of general usefulness for the rational design of protein kinase inhibitors (Fig. 3). According to this model, the ATP-binding site in protein kinases can be divided into five regions (P. Furet, manuscript in preparation):

**Adenine region:** This region is of mostly hydrophobic character. In the EGFR kinase, the N1 and N6 nitrogens of the adenine ring of ATP are engaged in a hydrogen bond donor-acceptor system with two amino acid residues, Gln 767 and Met 769, of the hinge region, corresponding to Glu 121 (backbone carbonyl) and Val 123 (backbone NH) in cAMP-dependent protein kinase and anchor the nucleotide. In the pyrrolo-pyrimidine CGP 59326 (Fig. 2), this hydrogen bond donor-acceptor system is formed by the pyrrolo NH (H-donor) and the N1 of the pyrimidine ring (H-acceptor) (Traxler et al., 1996). Many other potent inhibitors use at least one of these two hydrogen bonds (e.g., quinazolines). Although not used by ATP, the backbone carbonyl of the residue corresponding to Val 123 in cAMP-dependent protein kinase can also serve as a hydrogen bond acceptor for inhibitor binding, as shown by X-ray crystallography for the CDK2 inhibitor olomoucine (Schulze-Gahmen et al., 1995).

**Sugar pocket:** This pocket is of hydrophilic character in most protein kinases. It has been exploited in the design of potent and selective EGFR kinase inhibitors of the pyrrolo-pyrimidine or pyrazolo-pyrimidine classes. In CGP 59326 (Fig. 2), a chlorophenyl moiety replaces ribose of ATP. In addition, a sulfur-aromatic interaction between this moiety and residue Cys 773 of the pocket is assumed (Traxler et al., 1996). This Cys 773 is unique to the EGFR family of kinases and provides both potency and selectivity.

**Hydrophobic region I:** This pocket, extending in the direction of the lone pair of the N7 nitrogen of adenine, is present, but not used, by ATP in most protein kinases, thereby offering the medicinal chemist many possibilities for the design of new inhibitors. In the EGFR kinase, its size is controlled by two main amino acid residues, Thr 766 and Thr 860, thus playing an important role in inhibitor selectivity. In other protein kinases, this pocket is relatively small (e.g., in CDKs, a bulky phenylalanine [Phe 80 in CDK2] occupies the position corresponding to Thr 766 in the EGFR kinase). Many potent serine/threonine or tyrosine kinase inhibitors occupy this pocket (e.g., pyrrolo-pyrimidines, pyrazolo-pyrimidines,

---

**FIGURE 3.** Protein kinase pharmacophore.
phenylamino-quinazolines, pyrido-pyrimidines, pyrimido-pyrimidines, oxindoles, etc.). In CGP 59326, the two methyl groups of the pyrrole ring are pointing towards this pocket, thereby providing only a suboptimal occupancy of the available space.

**Hydrophobic region II:** This region corresponds more to a hydrophobic slot opened to solvent, which is not used by ATP and can be exploited to gain binding affinity. In the EGFR kinase, it is formed by residues Leu 694 and Gly 772. The X-ray crystal structures of indolinone inhibitors in complex with the FGFR kinase exploiting this slot have been published recently (Mohammadi et al., 1997). In certain other protein kinases (e.g., mitogen-activated protein and CDK family kinases), the shape of the slot is slightly altered due to the deletion of the amino acid corresponding to Gly 772 in the EGFR kinase.

**Phosphate binding region:** This region has high solvent exposure and is not of primary importance with respect to binding affinity. However, it can be useful to improve the selectivity of inhibitors since it contains nonconserved amino acids.

Independently, Parke-Davis (Ann Arbor, MI, USA) scientists proposed a model for the binding of 4-phenylamino-quinazolines (Palmer et al., 1997) and pyrido[2,3-d]pyrimidines (Trumpp-Kallmeyer et al., 1998) at the ATP-binding site of the EGFR kinase. While there is no other consistent way than their proposed binding mode to accommodate the pyrido[2,3-d]pyrimidine inhibitors in the ATP-binding site of a tyrosine kinase, this is not the case for the phenylamino-quinazolines. According to their model, the phenylamino moiety of these inhibitors occupies hydrophobic region I of the ATP-binding site, while the N1 nitrogen of the pyrimidine ring forms a hydrogen bond with the backbone NH of the residue (Met769) corresponding to Val123 in cAMP-dependent protein kinase. Assuming the same hydrogen bond interaction, many of these inhibitors (with the exception of pyrazolo-quinazoline analogues alkylated at N1) can also be accommodated in a binding mode where the phenylamino moiety occupies the sugar pocket, as we hypothesize for our pyrrolo-pyrimidine inhibitors. The concept that kinase inhibitors belonging to a given chemical class adopt a unique binding mode is not supported by recent X-ray crystallographic structures of purine inhibitors in complex with CDK2 (Schulze-Gahmen et al., 1995). Depending on the types and positions of substituents on a given kinase inhibitor template, changes in the binding mode may occur.

In the following sections, examples of the successful application of our pharmacophore model are described.

### Table 1. SAR of Pyrrolo-Pyrimidine Derivatives

<table>
<thead>
<tr>
<th>Compound</th>
<th>R&lt;sub&gt;1&lt;/sub&gt;</th>
<th>R&lt;sub&gt;2&lt;/sub&gt;</th>
<th>EGFR (IC&lt;sub&gt;50&lt;/sub&gt; μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>CGP 59326</td>
<td>CH₃</td>
<td>CH₃</td>
<td>0.027</td>
</tr>
<tr>
<td>1 (CGP 59326)</td>
<td>C₆H₅</td>
<td>CH₃</td>
<td>0.015</td>
</tr>
<tr>
<td>2</td>
<td>CH₃</td>
<td>C₆H₅</td>
<td>0.230</td>
</tr>
<tr>
<td>3</td>
<td>C₆H₅</td>
<td>C₆H₅</td>
<td>0.096</td>
</tr>
<tr>
<td>4</td>
<td>Biphenyl</td>
<td>CH₃</td>
<td>&gt;100</td>
</tr>
<tr>
<td>5</td>
<td></td>
<td></td>
<td>0.029</td>
</tr>
<tr>
<td>6</td>
<td></td>
<td></td>
<td>0.006</td>
</tr>
<tr>
<td>7</td>
<td></td>
<td></td>
<td>0.006</td>
</tr>
<tr>
<td>PD 153035</td>
<td></td>
<td></td>
<td>0.006</td>
</tr>
</tbody>
</table>

4. **4-SUBSTITUTED-PYRROLO[2,3-d]PYRIMIDINES**

The first successful application of the pharmacophore model for ATP competitive inhibitors interacting with the active site of the EGFR tyrosine kinase led to the identification of 4-phenylamino-7H-pyrrolo[2,3-d]pyrimidines as a novel class of EGFR PTK inhibitors. In an interactive process, this class of compounds was then further optimized (Traxler et al., 1996). In the structure-activity relationship (SAR) study of a first series of compounds (Compound 1, Table 1), the influence of substituents in the anilino moiety was evaluated. The same preference for electron-withdrawing groups (e.g., Cl or Br) at the 3-position was shown, as already described for 4-phenylamino-quinazolines (Rewcastle et al., 1995) or 4-phenylamino-pyrido-pyrimidine series (Rewcastle et al., 1996).

In a further series, the large hydrophobic pocket, as suggested by the pharmacophore model, was explored by replacement of either one or both methyl groups in positions 5 and 6 of the pyrrole ring by bulkier substituents (Compounds 2–7, Table 1). In general, bulky lipophilic groups were tolerated in both positions. Replacement of the 5-methyl group or of both methyl groups by a phenyl moiety (Compounds 3 and 4) only led to a slight decrease of activity, whereas the 6-phenyl analogue 2 was slightly more active compared with Compound 1. However, a limitation was reached, with Compound 5 bearing a biphenyl moiety in position 6 of the pyrrole ring. This compound was inactive. Replacement of both methyl groups by a cyclohexyl ring retained potency (Compound 6). Oxidation of the cyclohexyl ring in 6 to the indolo-pyrimidine derivative 7 further increased the activity against the EGFR kinase. With an IC<sub>50</sub> of 6 nM, this compound was equipotent to the 3-bromophenylamino-quinazoline PD 153035 (Fry et al., 1994; Rewcastle et al., 1995), which, under our assay conditions, had an IC<sub>50</sub> value of 6 nM. Although more potent than Compound 1, Compound 7 was not evaluated further due to its unsatisfactory solubility profile. Instead, 1 (CGP
CGP 59326 inhibited the EGFR tyrosine kinase with an IC$_{50}$ value of 27 nM. Kinetic analysis revealed competitive type kinetics relative to ATP. High selectivity towards a panel of receptor and nonreceptor tyrosine kinases, as well as serine/threonine kinases (protein kinase C [PKC], PKA), was observed. In cells, EGFR-stimulated cellular tyrosine phosphorylation was inhibited by CGP 59326 at an IC$_{50}$ concentration of 0.3 μM, whereas the ligand-induced receptor autophosphorylation of the PDGFR was not affected by concentrations of up to 100 μM. Furthermore, I was able to selectively inhibit c-fos mRNA expression in EGFR-dependent cell lines (IC$_{50}$ ~0.1–1 μM), but not in EGFR-independent cell systems (IC$_{50}$ > 100 μM) (Traxler et al., 1996). When tested on a number of EGF-overexpressing epithelial cell lines (NCI-H596, MDA-MB468, A431, BALB/MK, SK-BR3, BT20, etc.), CGP 59326 inhibited their proliferation with IC$_{50}$ values between 0.5 and 1.9 μM, while having only weak anti-proliferative activity against EGFR negative cell lines (e.g., NCI-H520, NCI-H69, FDC-P1, etc.).

A moderate antitumor effect was described with I in an orthotopic bladder carcinoma (253JB-V) in nude mice at oral doses between 10 and 40 mg/kg. In addition, inhibition of activated EGFR, down-regulation of VEGF in the plasma, and inhibition of metastasis was also seen in these experiments. In pharmacokinetic studies, good bioavailability with high plasma levels was demonstrated after oral administration of the compound to mice, rats, and dogs (P. Traxler et al., unpublished results). CGP 59326 is an interesting candidate for further evaluations.

Further optimization of the pyrrolo-pyrimidine structure by attachment of numerous substituents in the 6-position of the pyrrole ring led to a series of new derivatives with improved biological and physico-chemical properties (Table 2). This includes compounds with acids, ester, or amide groups (e.g., Compound 8); heterocyclic rings (e.g., Compound 9); and especially meta- or para-substituted aromatic rings (Compounds 10–14). Many of these compounds were more potent than CGP 59326 and had IC$_{50}$ values between 1 and 5 nM against the EGFR kinase. Finally, further optimization at the meta- or para-substituent of the aromatic ring and replacement of the m-chloro-anilino moiety at the 4-position of the pyrimidine ring by an (R)-phenethylamino moiety, further improved potency and pharmacokinetic behaviour of the compounds (Compounds 15–22) (Table 3). The most interesting compounds of this series inhibited the EGFR kinase with IC$_{50}$ values between 1 and 5 nM and had a selectivity ratio of >1000 against most tested tyrosine and threonine/serine kinases. Interestingly, the corresponding enantiomeric analogue of 16 with an (S)-phenethylamino moiety was only weakly active (IC$_{50}$ > 0.2 μM). In EGFR-overexpressing A 431 cells, EGF-stimulated phosphorylation was blocked with IC$_{50}$ values between 10 and 50 nM (Compounds 15–18, 20, and 21), whereas PDGF-induced phosphorylation was not inhibited (IC$_{50}$ > 10 μM), thus indicating high selectivity for the inhibition of the ligand-activated EGFR signal transduction pathway. When tested for anti-proliferative activity, these compounds inhibited proliferation of the EGFR-dependent BALB/MK cell line with IC$_{50}$ values between 0.1 and 0.4 μM, but were only weakly active (IC$_{50}$ > 10 μM) against a panel of EGFR-independent cell lines (e.g., FDC-P1 or T24) (Traxler et al., 1998). Extended biological profiling of selected development candidates is currently ongoing at Novartis.

### 5. 4-PHENYLAMINO-PYRAZOLO[3,4-d]PYRIMIDINES

Encouraged by the successful application of our pharmacophore model to the class of the pyrrolo-pyrimidines, we applied it on the pyrazolo-pyrimidines 23 and 24, which

<table>
<thead>
<tr>
<th>Compound</th>
<th>R$_1$</th>
<th>R$_2$</th>
<th>EGFR (IC$_{50}$ μM)</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CH$_3$</td>
<td>CH$_3$</td>
<td>0.027</td>
</tr>
<tr>
<td>8</td>
<td>CONHCH$_3$</td>
<td>H</td>
<td>0.003</td>
</tr>
<tr>
<td>9</td>
<td></td>
<td>H</td>
<td>0.007</td>
</tr>
<tr>
<td>10</td>
<td></td>
<td>H</td>
<td>0.015</td>
</tr>
<tr>
<td>11</td>
<td></td>
<td>H</td>
<td>0.003</td>
</tr>
<tr>
<td>12</td>
<td></td>
<td>H</td>
<td>0.003</td>
</tr>
<tr>
<td>13</td>
<td></td>
<td>H</td>
<td>0.004</td>
</tr>
<tr>
<td>14</td>
<td></td>
<td>H</td>
<td>0.001</td>
</tr>
</tbody>
</table>

---


were identified in a random screening program as potent inhibitors of the EGFR tyrosine kinase, with IC\textsubscript{50} values of 0.22 and 1.2 \textmu M, respectively. In fact, we were able to find an application of our model for both structures (dual fit, Fig. 4). Superimposition of structure 24 with ATP would suggest the bidentate-hydrogen-bond-donor-acceptor system for the 4-amino group (donor) and the N(5) pyrimidine nitrogen (acceptor). In this binding mode, the phenyl ring attached to the N1 of the pyrazole ring would replace ribose of ATP in the “sugar pocket,” whereas the phenyl substituent at the C3 position of the pyrazole ring points towards the large hydrophobic pocket (Fig. 4) (Traxler \textit{et al.}, 1997). Recently, Pfizer (Groton, CT, USA) scientists described a series of 4-amino-pyrazolo[3,4-d]pyrimidines corresponding to this binding mode, with substituents at the pyrazole nitrogen and an aromatic ring at the 3-position of the pyrazole ring. An analogue thereof with a tertiary butyl group at the pyrazole nitrogen was described to be a potent inhibitor of the Src family of tyrosine kinases (Lck and Fyn) (Hanke \textit{et al.}, 1996).

Assuming a similar binding mode of pyrazole 23 (or its equipotent 6-desamino derivative), as has already been demonstrated with 4-phenylamino-pyrrolo-pyrimidines (Traxler \textit{et al.}, 1996), would imply that in this case, (1) the NH1 of the pyrazole ring and N7 of the pyrimidine ring form the bidentate-hydrogen-bond-donor-acceptor system, (2) the 4-amino group points toward the “sugar pocket” not yet filling it, and (3) the anilino substituent at the C3 position of the pyrazole ring again is filling the large hydrophobic pocket. This binding mode suggests the addition of a phenyl or meta-chlorophenyl moiety, respectively, to the 4-amino group of the pyrimidine ring of the lead Compound 23, thus leading to the pyrazolo-pyrimidine analogue 25 as a first target structure (Fig. 4). In fact, Compound 25 had an IC\textsubscript{50} value of 0.033 \textmu M and was almost one order of magnitude more potent than the parent Compound 23 (Traxler \textit{et al.}, 1997). Optimization of this structural class concentrated mainly on derivatives with various substituents at the C3 position of the pyrazole ring, thereby exploiting the cavity of the large hydrophobic pocket of the target enzyme (Comounds 26–39) (Table 4). These SAR studies showed tolerance for rather bulky substituents in this position, as exemplified by the 3-phenylamino derivatives 26–

### TABLE 3. SAR of Pyrrolo-Pyrimidine Derivatives

<table>
<thead>
<tr>
<th>Compound</th>
<th>R</th>
<th>EGFR \text{IC}_{50} \text{ M}</th>
<th>EGF ELISA\textsuperscript{1} \text{IC}_{50} \text{ M}</th>
<th>MK Cell\textsuperscript{2} \text{IC}_{50} \text{ M}</th>
</tr>
</thead>
<tbody>
<tr>
<td>15</td>
<td>p-OCH\textsubscript{3}</td>
<td>0.013</td>
<td>0.002</td>
<td>0.29</td>
</tr>
<tr>
<td>16</td>
<td>p-NH\textsubscript{2}</td>
<td>0.002</td>
<td>0.010</td>
<td>0.26</td>
</tr>
<tr>
<td>17</td>
<td>p-NHCOCH\textsubscript{3}</td>
<td>0.004</td>
<td>0.001</td>
<td>0.34</td>
</tr>
<tr>
<td>18</td>
<td>p-NHSO\textsubscript{2}CH\textsubscript{3}</td>
<td>0.005</td>
<td>0.006</td>
<td>0.19</td>
</tr>
<tr>
<td>19</td>
<td>p-CONH\textsubscript{2}</td>
<td>0.005</td>
<td>0.067</td>
<td>0.18</td>
</tr>
<tr>
<td>20</td>
<td>m-NH\textsubscript{i}</td>
<td>0.001</td>
<td>0.001</td>
<td>0.19</td>
</tr>
<tr>
<td>21</td>
<td>m-NHSO\textsubscript{2}CH\textsubscript{3}</td>
<td>0.004</td>
<td>0.010</td>
<td>0.89</td>
</tr>
<tr>
<td>22</td>
<td>p-CONH\textsubscript{2}</td>
<td>0.005</td>
<td>0.067</td>
<td>0.18</td>
</tr>
</tbody>
</table>

\textsuperscript{1}Inhibition of EGF-stimulated phosphorylation in A431 cells.

\textsuperscript{2}Inhibition of proliferation of EGF-dependent BALB/MK cells.

\textsuperscript{3}Inhibition of phosphorylation of EGFR.

FIGURE 4. Rational design of pyrazolo-pyrimidines.
30, the benzylamino derivatives 31–34, or Compounds 35–39, where a phenyl ring is directly attached to the pyrazole ring. The most potent compounds of this series had IC_{50} values below 10 nM against the EGFR enzyme and a selectivity ratio of 1000 against most tested tyrosine and serine/threonine kinases. In cells, EGF-stimulated cellular tyrosine phosphorylation was inhibited at IC_{50} values below 50 nM, whereas PDGF-induced tyrosine phosphorylation was not affected (IC_{50} = 10 m\muM). In EGF-dependent MK cells, proliferation was blocked with IC_{50} values between 0.4 and 1 m\muM).

Interestingly, the p-NHBoc derivatives 30 (IC_{50} > 10 m\muM) and 37 (IC_{50} > 0.270 m\muM) had markedly decreased activity, indicating that the pocket is limited in size. Docking analyses of Compound 27 in the ATP-binding site of the homology-built model of the EGFR kinase suggests a hydrogen bond interaction between the hydroxy group of the phenol moiety and the backbone amide group of residue Phe832. This amino acid corresponds to Phe185 in the X-ray structure of the cAMP-dependent protein kinase (Zheng et al., 1993), and is known to form a hydrogen bond with a water molecule. It is suggested that expulsion and replacement of this buried water molecule by a hydroxy or amino substituent of the inhibitor could provide additional binding affinity (Traxler et al., 1998).

Further biological profiling of selected 4-phenylamino-pyrazolo[3,4-d]pyrimidines is currently ongoing at Novartis.

### 6. ISOFLAVONES AND 3-PHENYL-4(1H)-QUINOLONES

A quite recent successful application of our pharmacophore model led to a series of isoflavones and 3-phenyl-4(1H)quinolones as potent and selective EGFR tyrosine kinase inhibitors (Traxler et al., 1999). Several flavonoid compounds have been reported to possess protein kinase inhibitory activity. The most prominent of these are the isoflavone genistein, the flavone quercetin, and flavopiridol (Fig. 5). Due to its substantial potency (micromolar) in inhibiting the EGFR PTK, we considered genistein as a possible lead structure and, therefore, were interested in modeling its kinase-binding mode. The three compounds share a 5-hydroxy-chromenone substructure, suggesting similar binding modes. However, when we analyzed the published X-ray data of quercetin in complex with the Hck tyrosine kinase (Sicheri et al., 1997) and of deschloro flavopiridol in complex with CDK2 (Filgueira de Azevedo et al., 1996), it was not possible to dock genistein in the ATP-binding site of the EGFR kinase in analogous ways by giving its 5-hydroxy-chromenone moiety the same orientations as those seen in the above complexes. In both cases, we observed severe steric clashes between the p-hydroxyphenyl moiety in the 3-position and protein residues of the hinge region, suggesting the existence of a different binding mode specific to genistein. A clue to the possible nature of this alternative binding mode came by comparing genistein to the phenylamino-quinazoline inhibitor class reported by Fry et al. (1994) and Rewcastle et al. (1995). In the literature, we found an example of enzyme inhibitors where a salicylic group has been reported to be a successful bioisosteric replacement of a quinazoline moiety (Hodge and Pierce, 1993). Thereby, the pyrimidine ring mimics the pseudo six-membered ring resulting from the hydroxy-keto intramolecular hydrogen bond in salicylic acid. A similar hydroxy-keto function is present in genistein. Assuming the same bioisosteric relationship between the quinazoline-type EGFR kinase inhibitors and genistein led to the hypothesis that they share a common binding mode. In Fig. 6, it can be seen that overlapping the 5-hydroxy-keto system of genistein and the pyrimidine ring of 4-(m-chlorophenyl-
amino)-6,7-dimethoxy-quinazoline gives a very good overall superimposition of the two molecules. In particular, the anilino moiety of the quinazoline superimposes nicely on the \( \text{p-hydroxyphenyl} \) group of genistein. Given the importance of the anilino \( \text{meta-chloro} \) (or \( \text{m-bromo} \)) substituent in conferring high EGFR PTK inhibitory activity to the quinazoline derivatives, as well as to the other classes of phenylamino-pyrimidine inhibitors (e.g., phenylamino-pyrrolo-pyrimidines and phenylamino-pyrazolo-pyrimidines), the overlay immediately suggested that replacing the \( \text{p-hydroxyphenyl} \) moiety of genistein by a \( \text{m-chlorophenyl} \) ring would enhance its potency. This idea motivated the synthesis of Compound 40 (Table 5) as a target structure, which in fact, with an IC\( _{50} \) value of 95 nM, was 10 times more potent than genistein (IC\( _{50} \) 1 \( \mu \)M).

The proposed putative binding mode of 40 in the ATP-binding site of the EGFR kinase model is fully consistent with our pharmacophore model (Fig. 6). The chlorophenyl ring fits in the “sugar pocket,” making a sulfur-aromatic interaction with Cys773. Mimicking ATP, the 5-hydroxy substituent accepts a hydrogen bond from the backbone NH of residue Met769, while the 7-hydroxy substituent donates a hydrogen bond to the backbone carbonyl of Gln767. In accordance with the predicted binding mode, removal of the 5-hydroxy group in Compound 43 or its methylation (together with the 7-hydroxy group) in Compound 42 led to a more than 500-fold loss of enzyme inhibitory activity, thus proving the absolute requirement of this group (Table 5).

It should be noted that 40 can also be docked in the ATP-binding site of the EGFR kinase according to the Parke-Davis model (i.e., with the chlorophenyl ring fitting hydrophobic region I of our general kinase model, the 5-hydroxy group still accepting a hydrogen bond from the backbone NH of Met769). However, in this orientation, one cannot find a hydrogen bond acceptor on the protein to be a partner for the 7-hydroxy group whose hydrogen bond donor functionality clearly contributes to affinity, as testified by the reduced activity of the methoxy analogue 41.

As predicted, replacement of oxygen O1 by a nitrogen, which, according to the model, is facing the part of the cavity binding the triphosphate chain of ATP, further im-
P. Traxler and P. Furet proved potency by a factor of more than 10. With IC_{50} values of 38 and 8 nM, respectively, the quinolones 44 and 45 were the most potent compounds of the series (Table 5).

Again, and in accordance with the proposed binding mode, removal of the 5-hydroxy group (Compound 46) completely abolished enzyme inhibitory activity.

The activity profiles of the isoflavone 40 and especially of the quinolones 44 and 45 show that our pharmacophore model of the ATP-binding site, together with additional rational ideas, can be used successfully for the design of novel classes of highly potent and selective EGFR tyrosine kinase inhibitors.

### 7. PHENYLAMINO-PYRIMIDINES

Although the following example of an ATP competitive inhibitor was not identified with the help of the pharmacophore model, it adds to the fact that the ATP-binding site of protein kinases is an exciting target and that minor modifications of the structure of a molecule can lead to major changes in the selectivity profile. In the course of a random-screening program, Compound 47 was identified as an attractive lead structure, acting as an unselective inhibitor against the serine/threonine kinase PKCα in the submicromolar range. Simply through introduction of a methyl group in the 6-position of the phenyl ring, the inhibition against PKCα was completely lost, whereas the potency for inhibition of PDGFR autophosphorylation was dramatically increased (Fig. 7). The dramatic loss of activity against PKCα was explained by a forced change of the preferred conformation upon the introduction of this “flag-methyl” group, leading to severe clashes with the wall of the ATP-binding pocket within the PKC family of enzymes. The optimization program of the lead structure 47 finally led to Compound 48 (CGP 53716) and its watersoluble analogue 49 (CGP 57148). Both compounds were dual inhibitors of the v-Abl/PDGFR tyrosine kinases. CGP 53716 inhibited PDGFR autophosphorylation and the v-Abl tyrosine kinase with IC_{50}s of 100 and 400 nM, respectively, whereas CGP 57148 was more potent against

![TABLE 5. SAR of Isoflavones and Quinolones](image)

<table>
<thead>
<tr>
<th>Compound</th>
<th>R₁</th>
<th>R₂</th>
<th>EGFR (IC_{50} μM)</th>
<th>Compound</th>
<th>R₁</th>
<th>R₂</th>
<th>EGFR (IC_{50} μM)</th>
</tr>
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<td>40</td>
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<td>OH</td>
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<tr>
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<td>500</td>
<td>46</td>
<td>H</td>
<td>H</td>
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</tr>
<tr>
<td>43</td>
<td>H</td>
<td>OH</td>
<td>58.8</td>
<td></td>
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</tbody>
</table>

![FIGURE 7. Phenylamino-pyrimidines.](image)
the v-Abl kinase (IC$_{50}$ 33 nM) than the PDGFR kinase (IC$_{50}$ 300 nM). Both compounds showed 100- to 1000-fold selectivity against a panel of over 20 serine/threonine and tyrosine kinases (including EGFR, IGF-IR, IR, and c-Src). Based on its more favourable physico-chemical profile, CGP 57148 (49) was selected for further development. In cells, v-Abl and PDGFR autophosphorylation, as well as PDGF-induced c-fos mRNA expression, were blocked with IC$_{50}$ between 100 and 300 nM. Antiproliferative activity of CGP 57148 against v-Abl- or v-Sis-transformed cell lines, but not against EGF-dependent MK or FDC-P1 cells (IL-3-dependent), and a 60–80% decrease in the number of Bcr-Abl colonies in a colony-forming assay of peripheral blood or bone marrow from patients with chronic myelogenous leukemia, was observed. In vivo, CGP 57148 inhibited tumor growth in nude mice inoculated with tumorigenic Bcr-Abl-expressing cells. The compound recently has entered Phase I studies in patients with chronic myelogenous leukemia (Beran et al., 1998; Buchdunger et al., 1996; Carroll et al., 1997; Deininger et al., 1997; Drucker et al., 1996; Gamba-corti-Passerini et al., 1997; Zimmermann et al., 1996, 1997).

8. OUTLOOK

In the last few years, it has been demonstrated with numerous examples that the ATP-binding sites of protein kinases are exciting targets for medicinal chemists. We have demonstrated with a number of examples that a hypothetical pharmacophore model for binding of inhibitors at the ATP-binding site of the EGFR tyrosine kinase can be used successfully for the design of highly potent and selective EGFR tyrosine kinase inhibitors. Independently, other groups have also developed models for the binding of their lead structures in the ATP pocket of tyrosine kinases, such as quinazolines in the EGFR kinase (Palmer et al., 1997), pyrido[2,3-d]pyrimidines in the c-Src tyrosine kinase (Trumpp-Kallmeyer et al., 1998), or oxindoles in FGFR or VEGFR tyrosine kinases (Mohammadi et al., 1997), and used these models for the design of more potent inhibitors. X-ray data of more than 30 published structures of protein kinases complexed with ATP or an ATP competitive inhibitor enabled the refinement of these models and the ability to adapt it to almost any new kinase chosen as a target. Medicinal chemists now have tools in hand that will allow rapid three-dimensional screening of large compound libraries for the identification of hits and new lead structures. Combinatorial chemistry and parallel synthesis probably will accelerate the optimization process of such lead structures. Nevertheless, to overcome the hurdles of in vivo efficacy in animal models, pharmacodynamic parameters (blood and tissues levels or metabolism), or toxicology, it still needs serendipity and creativity of medicinal chemists in the design of single compounds.

**References**


Mohammadi, M., McMahon, G., Sun, L., Tang, C., Hirth, P.,


