

Discovery of EGFR Selective 4,6-Disubstituted Pyrimidines from a Combinatorial Kinase-Directed Heterocycle Library

Qiong Zhang,[†] Yi Liu,[†] Feng Gao,[†] Qiang Ding,[†] Charles Cho,[‡] Wooyoung Hur,[‡] Yunho Jin,[†] Tetsuo Uno,[†] Claudio A. P. Joazeiro,[†] and Nathanael Gray^{*,†}

Departments of Chemistry and Cell Biology, Genomics Institute of the Novartis Research Foundation, 10675 John Jay Hopkins Drive, San Diego, California 92121, and Department of Chemistry, The Scripps Research Institute, 10550 North Torrey Pines Road, La Jolla, California 92037

Received October 2, 2005; E-mail: gray@gnf.org

High-throughput screening of small molecule gene-family targeted libraries has been the most efficient method to discover new lead compounds from combinatorial libraries.¹ We recently described an efficient method to prepare libraries of kinase-directed heterocycles.² Our approach consists of the synthesis of libraries where the heterocyclic core is considered as a combinatorial input, thereby allowing the parallel synthesis of multiple kinase-privileged scaffolds (2,4- and 4,6-pyrimidines, triazines, purines, quinazolines, pyrazines, etc.) in a single combinatorial synthesis. These libraries were plated in a 1536-well format and screened in a variety of cellular and biochemical assays using a robotic HTS platform. Here we wish to describe the identification of a class of 4,6-disubstituted pyrimidines (represented by compound **1**) that are highly selective inhibitors of the epidermal growth factor receptor (Her-1, also erbB-1, EGFR). Inhibitor **1** possesses an enzymatic IC₅₀ = 21 nM against EGFR kinase in vitro and blocks receptor autophosphorylation in cells. Inhibitor **1** was serendipitously discovered using a cell-based reporter gene assay (RGA) for modulators of protein stability (to be described elsewhere). Structure–activity relationship (SAR) with respect to inhibition of EGFR kinase activity allowed the essential pharmacophore to be identified, a binding mode to EGFR proposed, and the high degree of kinase selectivity rationalized.

EGFR was one of the first receptor tyrosine kinases to be targeted for inhibitor development by the pharmaceutical industry due to its overexpression in a variety of tumors.³ This research has culminated in the recent approval of two highly related anilinoquinazolines, gefitinib (Iressa) and erlotinib (Tarceva), which target the intracellular tyrosine kinase domain, as well a chimeric monoclonal antibody, erbitux, which targets the extracellular portion of the receptor. In addition to the anilinoquinazolines, a variety of EGFR small molecule inhibitors from other scaffold classes are undergoing clinical evaluation.⁴ Curiously, despite years of intensive research on EGFR inhibitors, there is a surprising dearth of chemically distinct small molecule inhibitors with a high degree of selectivity. There is also a need for new scaffolds due to the recent finding of EGFR mutations which render the kinase resistant to gefitinib and erlotinib.⁵

Pyrimidines substituted at the 2 and 4 positions are an extremely well studied class of inhibitors for diverse kinases, including Cdks, p38, Aurora, KDR, and Gsk3.⁶ Crystallography has revealed that 2,4-dianilino-pyrimidines typically form a bidentate hydrogen bonding interaction with the hinge amino acid using the pyrimidine N1 and the aniline NH at the C2 position.⁷ Despite their fairly generic mode of recognizing the kinase active site, pyrimidines are capable of displaying a wide range of kinase selectivity profiles depending

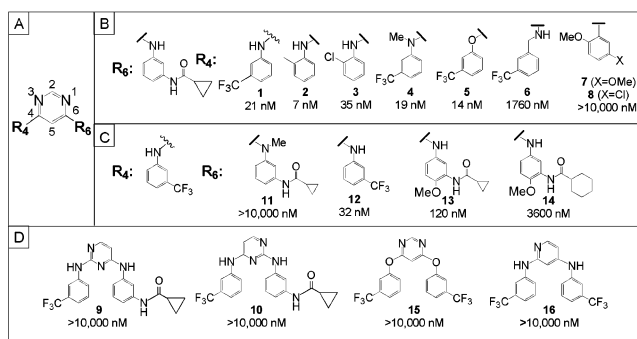


Figure 1. EGFR enzyme IC₅₀ values for lead compound **1** and its analogues: (A) 4,6-pyrimidine scaffold, (B) R₆ fixed, (C) R₄ fixed, (D) additional inhibitors.

on the substitution pattern on the two pendant aniline rings. In contrast, the 4,6-disubstituted pyrimidines represent a significantly less explored class of kinase inhibitors because they are generally not as potent as their corresponding 2,4-regioisomers.⁸ This may be due to a greater energetic penalty required to attain the *s*-cis conformation required for the hinge region interaction.

Despite the low molecular weight of inhibitor **1**, it exhibited exclusive selectivity against EGFR when tested against a panel of 55 recombinant kinases at a concentration of 10 μM (data in Supporting Information). Inhibitor **1** also potently inhibits two EGFR mutants associated with clinical response to gefitinib: L858R (IC₅₀ = 63 nM) and L861Q (IC₅₀ = 4 nM), but displays much weaker activity against Her 4 (IC₅₀ = 7640 nM). To understand the SAR with respect to EGFR kinase inhibition, several derivatives based on the structure of compound **1** were made (Figure 1). Removal of the trifluoromethyl from the 4-aniline and introduction of an ortho methyl (**2**) or chloro (**3**) substituent resulted in approximately equipotent compounds. The NH at the 4-position was not essential and could be replaced with an *N*-methyl (**4**) or oxygen (**5**), suggesting that this group is not involved in an H-bonding interaction with the enzyme. Addition of a methylene group to form the benzylamino compound **6** resulted in an 88-fold loss of activity. Removal of the aniline to form carbon-linked bicyclic compounds **7** and **8** resulted in a complete loss of activity. Replacement of the 4,6-pyrimidine core with the two possible regioisomeric 2,4-pyrimidines (**9**, **10**) or with a pyridine (**16**) also resulted in loss of EGFR inhibitory activity, suggesting that the correct positioning of both pyrimidine nitrogens is essential (Figure 1). A hydrogen bond donor was essential at the 6-position as the *N*-methylated compound was completely inactive (**11**). Introduction of a 3-trifluoromethylaniline (**12**) resulted in a compound equipotent to **1**, while introduction of a methoxy (**13**) or a larger cyclohexylamide (**14**) resulted in a significant loss of activity.

[†] Genomics Institute of the Novartis Research Foundation.

[‡] The Scripps Research Institute.

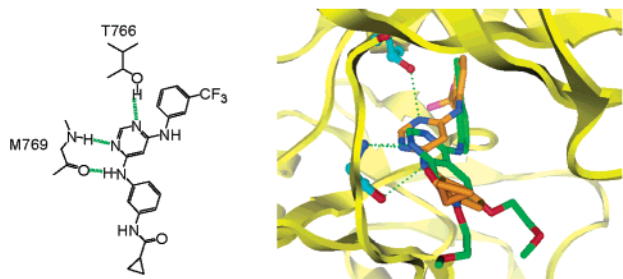


Figure 2. Chemical structure version of docking model of **1** bound to the ATP site of the EGFR kinase domain (left). Superimposition of model of **1** (gold) with erlotinib (green) in the ATP site of EGFR kinase domain (right).

In an effort to rationalize the high degree of kinase selectivity exhibited by the 4,6-pyrimidines against the EGFR kinase family, we employed the SAR data in conjunction with comparisons to known EGFR co-crystal structures to propose a binding mode (Figure 2). We propose that the pyrimidine N1 and 6-NH form a pair of hydrogen bonds with the “hinge” amino acid (Met 769). The corresponding hydrogen bonding interaction is formed by the quinazoline N1 of erlotinib.⁹ The trifluoromethyl phenyl is proposed to be situated in a hydrophobic binding pocket which is also occupied by the ethynyl-substituted aniline group of erlotinib. The pyrimidine N3 appears to mimic the quinazoline N3 by forming a hydrogen bonding interaction to the side chain hydroxyl of the “gatekeeper” threonine 766. The pyrimidine 6-anilino substitution partially overlaps the site occupied by the quinazoline phenyl, and the amide substitution is directed toward solvent. The proposed binding mode is fully consistent with the observed SAR: the requirement for correct positioning of the pyrimidine N1, N3, and 6-NH, the tolerance for substitution of the 6-NH, and the ability to tolerate a variety of the substitutions to the 4-aniline.

We hypothesized that the selectivity of compound **1** is derived from the ability to form three hydrogen bonding interactions while occupying a hydrophobic cavity made accessible due to the small gatekeeper threonine 766. To test this idea and the proposed binding mode, we replaced the gatekeeper threonine with a methionine since primary sequence alignment revealed that Met is normally present in several kinases in the equivalent position of EGFR T766, such as in Jak, Syk, Fak, and Csk. Moreover, Csk and Syk are resistant to inhibition by compound **1**. Thus, the T766M substitution was anticipated to cause resistance to both compound **1** and gefitinib due to steric blockage of the hydrophobic binding pocket and removal of a key hydrogen bond interaction without affecting kinase activity. Indeed, a recent report shows that T766M mutation to EGFR induces resistance to gefitinib.¹⁰ Treatment of U-2OS cells transfected with either WT or T766M EGFR with epidermal growth factor (EGF) resulted in receptor autophosphorylation (Figure 3, lanes 2 and 5). Pretreatment of cells with either compound **1** (10 μ M) or gefitinib (1 μ M) resulted in complete inhibition of WT receptor autophosphorylation (lanes 3 and 4). The ability of both the compounds to inactivate WT EGFR is also evident from the observation that greater levels of receptor were immunoprecipitated from compound-treated compared to untreated cells, consistent with reports that EGFR activation leads to its rapid degradation.¹¹ As expected, the T766M mutant receptor was completely resistant to inhibition by either compound (lanes 6 and 7). Thus, we were able to successfully design an inhibitor-resistant allele of EGFR by

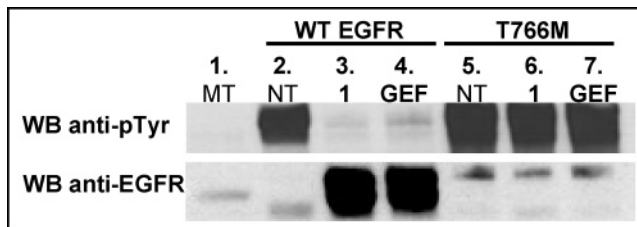


Figure 3. EGF-induced autophosphorylation of wild-type (WT) EGFR, but not the T766M mutant, is sensitive to inhibition by compound **1** (10 μ M) and gefitinib (1 μ M, GEF). MT, mock transfected; NT, not treated with inhibitor.

mutation of its gatekeeper Thr residue to Met. To investigate whether the 4,6-substitution pattern was a key component of kinase selectivity, both 2,4-pyrimidine regioisomers (**9**, **10**) were tested against a panel of 55 kinases at a concentration of 10 μ M. As expected, both 2,4-pyrimidine inhibitors were considerably less selective with compound **9** inhibiting Aurora A (IC_{50} = 931 nM) and compound **10** inhibiting Aurora A (IC_{50} = 42 nM), Bmx (IC_{50} = 386 nM), Btk (IC_{50} = 3550 nM), Lck (IC_{50} = 131 nM), IGF-1R (IC_{50} = 591 nM), cSrc (IC_{50} = 1980 nM), TrkB (IC_{50} = 2510 nM), and Syk (IC_{50} = 887 nM). Neither inhibitor **9** nor **10** inhibited EGFR significantly at a concentration of 10 μ M.

In conclusion, we have demonstrated that screening a combinatorial library based on a privileged class of 4,6-dianilino-pyrimidines allowed the efficient identification of potent and highly selective inhibitors of both enzymatic and cellular EGFR kinase activity. Docking, SAR, and mutagenesis studies suggest that a key H-bonding interaction is required to the gatekeeper residue T766.

Supporting Information Available: Experimental details and characterization data of all the reported compounds. This material is available free of charge via the Internet at <http://pubs.acs.org>.

References

- Bredel, M.; Jacoby, E. *Nat. Rev. Genet.* **2004**, *5*, 262–275.
- Ding, S.; Gray, N. S.; Wu, X.; Ding, Q.; Schultz, P. J. *Am. Chem. Soc.* **2002**, *124*, 1594–1596.
- Hynes, N.; Lane, H. *Nat. Rev. Can.* **2005**, *5*, 341–354.
- El-Rayes, B. F.; LoRusso, P. M. *Brit. J. Can.* **2004**, *91*, 418–424.
- Kobayashi, S.; Boggon, T. J.; Dayaram, T.; Jänne, P. A.; Kocher, O.; Meyerson, M.; Johnson, B. E.; Eck, M. J.; Tenen, D. G.; Halmos, B. N. *Engl. J. Med.* **2005**, *352*, 786–792.
- (a) Anderson, M.; Beattie, J. F.; Breault, G. A.; Breed, J.; Byth, K. F.; Culshaw, J. D.; Ellston, R. P.; Green, S.; Minshull, C. A.; Norman, R. A.; Pauptit, R. A.; Stanway, J.; Thomas, A. P.; Jewsbury, P. J. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 3021–3026. (b) Cumming, J. G.; McKenzie, C. L.; Bowden, S. G.; Campbell, D.; Masters, D. J.; Breed, J.; Jewsbury, P. J. *Bioorg. Med. Chem. Lett.* **2004**, *14*, 5389–5394. (c) Tavares, F. X.; Boucheron, J. A.; Dickerson, S. H.; Griffin, R. J.; Preugschat, F.; Thomson, S. A.; Wang, T. Y.; Zhou, H.-Q. *J. Med. Chem.* **2004**, *47*, 4716–4730.
- Wang, S.; Meades, C.; Wood, G.; Osnowski, A.; Anderson, A.; Yuill, R.; Thomas, M.; Mezna, M.; Jackson, W.; Midgley, C.; Griffiths, G.; Fleming, I.; Green, S.; McNaie, I.; Wu, S.-Y.; McInnes, C.; Zheleva, D.; Walkinshaw, M. D.; Fischer, P. M. *J. Med. Chem.* **2004**, *47*, 1662–1675.
- (a) Beattie, J.; Breault, G.; Ellston, R.; Green, S.; Jewsbury, P.; Midgley, C.; Naven, R.; Minshull, C.; Pauptit, R.; Tucker, J.; Pease, J. *Bioorg. Med. Chem. Lett.* **2003**, *13*, 2955–2960. (b) Thomas, A. **1995**, WO 95/15952.
- Stamos, J.; Sliwkowski, M. X.; Eigenbrot, C. *J. Biol. Chem.* **2002**, *277*, 46265–46272.
- Carter, T. A.; Wodicka, L. M.; Shah, N. P.; Velasco, A. M.; Fabian, M. A.; Treiber, D. K.; Milanov, Z. V.; Atteridge, C. E.; Biggs, W. H., III; Edeen, P. T.; Floyd, M.; Ford, J. M.; Grotzfeld, R. M.; Hergard, S.; Insko, D. E.; Mehta, S. A.; Patel, H. K.; Pao, W.; Sawyers, C. L.; Varmus, H.; Zarrinkar, P. P.; Lockhart, D. J. *Proc. Natl. Acad. Sci. U.S.A.* **2005**, *102*, 11011–11016.
- Levkowitz, G.; Waterman, H.; Zamir, E.; Kam, Z.; Oved, S.; Langdon, Y.; Beguinot, L.; Geiger, B.; Yarden, Y. *Genes Dev.* **1998**, *12*, 3663–3674.

JA0567485