Pergamon

SCIENCE DIRECT

Bioorganic & Medicinal Chemistry Letters 13 (2003) 3587–3592

BIOORGANIC & MEDICINAL CHEMISTRY LETTERS

Adenosine-Anchored Triphosphate Subsite Probing: Distinguishing between HER-2 and HER-4 Tyrosine Protein Kinases

Fei Liu,[a] Eric F. Johnson,[b] David J. Austin[a,*] and Karen S. Anderson[b,*]

[a]*Department of Chemistry, 225 Prospect Street, Yale University, New Haven, CT 06520, USA*
[b]*Department of Pharmacology, 333 Cedar Street, Yale University School of Medicine, New Haven, CT 06520, USA*

Received 30 January 2003; accepted 9 June 2003

Abstract—A strategy of full-site occupancy and stereospecific recognition in the triphosphate subsite was used to specifically inhibit two protein kinases HER-2 and HER-4 from the EGFR family. The SAR profiles of a panel of adenosine-anchored bicyclic heterocycles against HER-2 and HER-4 indicated that specificity can be derived for highly homologous protein kinases from stereospecific recognition in the triphosphate-subsite.

The regulation of protein kinases (PKs) is vital to many cellular events such as gene expression, immune response, signal transduction and cell division.[1] The aberrant activities of these enzymes have been linked to diseases such as cancer and diabetes.[2] The two protein kinases of interest in this report are HER-2 and HER-4, two highly similar human tyrosine protein kinases. These two are members of the epidermal growth factor receptor (EGFR) tyrosine PK (TPK) family, whose signaling network is most frequently implicated in human cancers of the brain, lung, breast, ovary, pancreas and prostate, making this family one of the most intensely studied classes of protein kinases.[3] The importance of HER-2 as a valid molecular target in breast cancer has been established by the success of Herceptin®.[4] It is the only member of the EGFR family that still does not yet have its own extracellular growth factor identified.[5] It has been proposed that HER2 heterodimerizes with the other members of the EGFR family for altering their existing signaling characteristics.[6] Along with HER-2, HER-4 is also implicated in some human cancers but not others.[7] The exact role of HER4 in the EGFR signaling pathway remains unclear due to the complexity of this network which utilizes combinatorial dimerization or oligomerization to achieve the signaling specificity and potency.[8]

The human HER-2 TPK is a 185-kDa protein of 1255 amino acid residues, and HER-4 is a 180-kDa protein of 1308 amino acid residues.[9] The tyrosine kinase catalytic domains of HER-2 and HER-4 are 77% identical.[9] There is no structural data currently available for the kinase domain of either HER2 or HER4.[10]

Various approaches exist for studying specific recognition of individual PKs in order to understand their roles in cancer biology and develop subsequently therapeutic strategies.[11] Inhibition of PKs at the ATP-binding site is an approach particularly suitable for small-molecule based therapeutics,[12] but the specificity of such recognition remains a challenge, because PKs exhibit a high degree of sequence homology at this site.[13] This challenge has been met with substantial success as evidenced by the development of highly potent and family-specific small-molecule inhibitors of a few protein kinases.[14] However, achieving specificity within a given family, such as the case of HER2 and HER4, continues to be a difficult task. As illustrated in Figure 1, the sequence homology for the catalytic domains of PKs within a family is substantially higher than that between different PK families.[15] Between Protein Kinase A (PKA), a prototypical serine/threonine protein kinase, and Src, a prototypical tyrosine protein kinase, the sequence homology level is less than 50%. Between two tyrosine protein kinases, such as Src and HER-2, the sequence homology level rises to be greater than 60%. The sequence homology level is highest between HER-2 and HER-4, with their sequence similarity being greater

*Corresponding author. Tel.: +1-203-785-4526; fax: +1-203-785-7670; e-mail: karen.anderson@yale.edu; (K. S. Anderson); tel.: +1-203-432-3962; e-mail: david.austin@yale.edu (D. J. Austin).

0960-894X/$ - see front matter © 2003 Published by Elsevier Ltd.
doi:10.1016/S0960-894X(03)00761-3

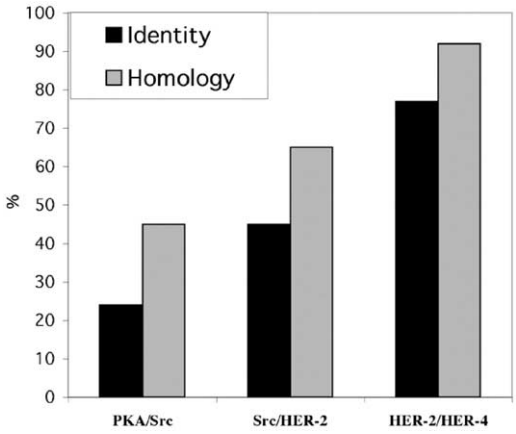


Figure 1. Homology levels of the catalytic domains of protein kinases.

than 90%. The ability to differentially inhibit these two highly similar protein kinases will provide new chemical tools for studying the cancer biology of these two signaling proteins.

For enhancing specific recognition of the ATP-site of PKs, a full-site-occupancy strategy has been proposed that not only targets the hydrophobic inner subsites of the ATP-binding pocket but also explores the conformationally flexible loops of the triphosphate subsite.[16] The importance of the triphosphate subsite in terms of specificity is exemplified by balanol, a potent natural product inhibitor of PKA,[17] and STI-571, the first FDA-approved antitumor drug targeting the Abl kinase.[18] In both cases as ascertained by X-ray structural analysis, these compounds achieve an exquisite level of specificity through characteristic loop interactions in the triphosphate subsite. Figure 2a illustrates the triphosphate subsite interactions in a STI-571 and c-Abl kinase binary complex.[18b] In this report, we describe our approach of probing the triphosphate subsites of HER2 and HER4, using an adenosine-anchored series of small molecules to ask the question of whether this region of the kinase can be utilized to enhance specificity between highly homologous kinases. An ancillary issue that has not previously been addressed is whether there is a chiral preference at this subsite. Neither Balanol nor STI-571 confers the triphosphate subsite recognition through stereospecific interactions. Structural probes that are capable of stereospecific interactions in the triphosphate subsite can be used to examine if further specificity can be derived from the potential chiral preference of a given triphosphate subsite (Fig. 2b). This is especially relevant to protein kinases such as HER2- and HER-4 that are highly similar. The Gly-X-Gly-X-X-Gly motif that has been recognized as a consensus sequence for orienting the phosphates of ATP[9c] is the same in both proteins (GSGAFG). Furthermore, the region that contains the catalytic Asp residue, the activation loop and the catalytic loop, which are all involved in the transfer of the γ-phosphoryl group,[10] is 90% identical in these two kinase domains.[15]

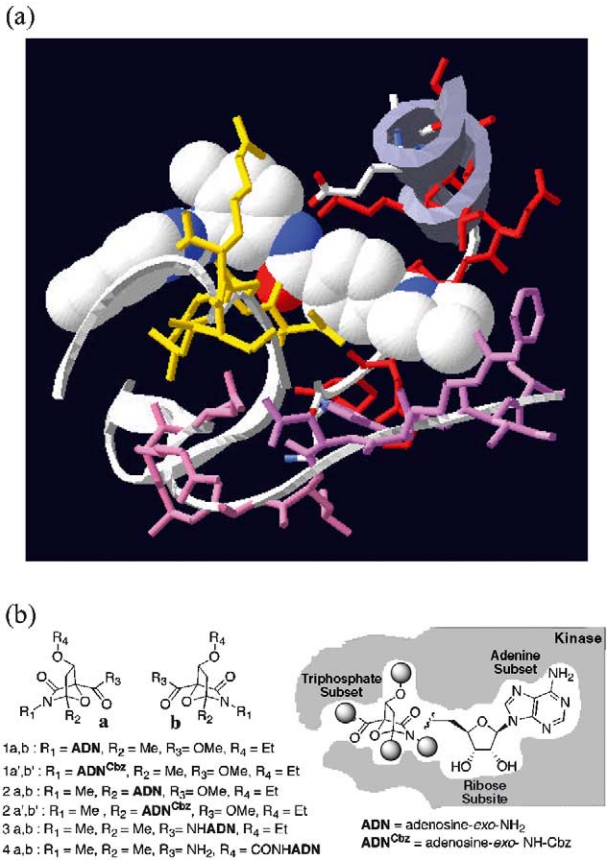
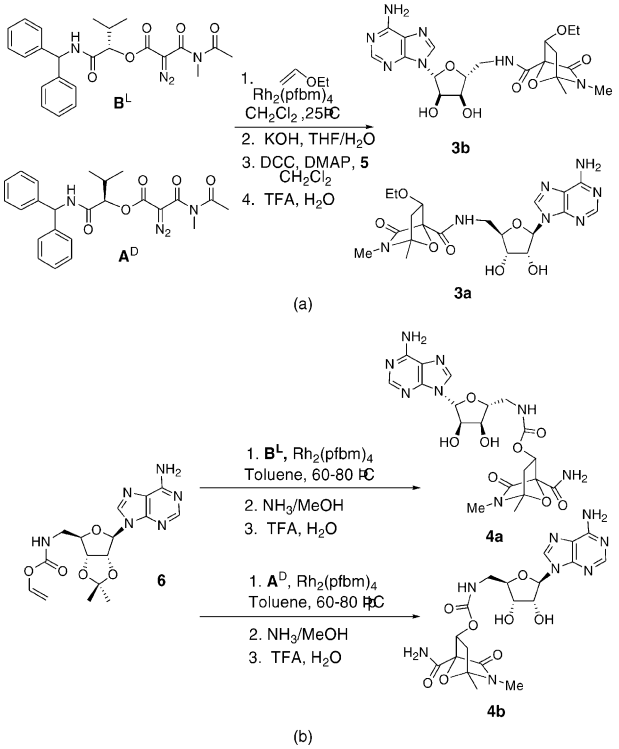


Figure 2. (a) STI-571 bound to C-Abl kinase domain, re-created using pdb file 1IEP in Swisspdbviewer. The triphosphate subsite is shown in color. STI-571 is shown with space-filling. The activation loop (FIHRLAARN) and catalytic loop (DFGLRLMT) are colored in yellow and purple respectively. Shown in red are side-chain residues (AVMKEIKHP) of the helix-turn from the small-lobe domain. (b) The anchored library consists of six pairs of four positional subsets. Positions 1 and 2 were anchored by either just adenosine or *exo*-Cbz adenosine to examine the effect of anchor alteration. The anchor occupies the adenine and sugar subsites, leaving the attached diversity platform in the conformationally flexible triphosphate subsite. In each pair, the anchor is constant while the two bicyclic cores are related as mirror images.

We have previously reported the development of a [2.2.1] bicyclic scaffold for the synthesis of chemically diversity using a rhodium(II) catalyzed 1,3-dipolar-cycloaddition reaction.[19] Using this methodology nucleoside-derivatized molecules[20] that combine both natural and diversity characteristics can be constructed.[21] The [2.2.1] bicyclic diversity platform was chosen for its small size and ability to project functionalities spherically, allowing stereospecific space sampling in a three-dimensional manner. Rather than use hydrophobic interactions to magnify nonspecific affinity, the goal of this first-generation panel is to address whether or not the presence of the bicyclic core extended from a non-specific adenosine anchor would satisfy the following criteria: (1) maintain or improve the affinity with respect to that of adenosine alone and (2) introduce additional specificity to adenosine that by itself displays undiscriminating inhibitory activity for many kinases.

A panel of 12 adenosine derivatives, containing a [2.2.1] bicyclic platform for probing the triphosphate subsite, were synthesized. The triphosphate subsite probing bicyclic platform that extends from the adenosine anchor of these molecules was each synthesized stereospecifically. Adenosine was chosen as the anchor since it is universally recognized by all protein kinases in a conserved way, although its absolute affinity for protein kinases is in the micromolar range. However, this anchor ensures the proper spatial locale of the triphosphate subsite probe, even for HER2 and HER4 whose structures are not currently available.

The synthetic sequence for stereospecifically constructing these adenosine-attached bicyclic molecules features a Rh(II) mediated 1,3-dipolar cycloaddition reaction.[19] The synthesis of members **3a**,**b** and **4a**,**b** are shown in Scheme 1 while the others of the library have been previously reported.[21] Both enantiomers of the [2.2.1] bicyclic core were synthesized using the valine-based chiral auxiliary strategy.[20] The two auxiliary-biased diazo precursors $\mathbf{A^D}$ and $\mathbf{B^L}$ were synthesized individually following previous procedures[20] starting from commercially available L- and D-valine. $\mathbf{A^D}$ and $\mathbf{B^L}$ underwent diastereofacial selective cycloaddition with vinyl ether, and the resulting auxiliary-attached cycloadducts were then saponified with NaOH to give the bicyclic acids that were immediately coupled with 5′-amino-5′-deoxy-2′3′-acetonideadenosine **5**[21] and deprotected in wet TFA to give **3a** and **3b**.[22] The synthesis of **4a** and **4b** required the use of an adenosine-derived dipolarophile **6**[21] in the auxiliary-based cyclization-cycloaddition strategy. Dipolarophile **6** was reacted with each of the two diazo ketones $\mathbf{A^L}$ and $\mathbf{B^D}$ in the presence of $Rh_2(pfbm)_4$ in toluene at 80 °C. In each case, only one cycloadduct was isolated from the diastereofacial and *endo* selective cycloaddition, providing the fully protected adenosine-linked cycloadducts that were advanced to **4a** and **4b** after the removal of the chiral auxiliaryby by aminolysis and TFA deprotection of the acetonide group.[22] The weak nucleophilicity of the free *exo*-amino group on the adenine ring does not interfere with the Rh(II) catalyst. In addition, the chiral auxiliary led to high facial selectivity, which was not compromised at the relatively high temperature required for the reaction.



Scheme 1. (a) Preparation of **3a**,**b**; (b) preparation of **4a**,**b**.

The inhibitory activities of each molecule against HER-2 and HER-4 are measured as apparent K_i's.[23] Adenosine, a nonspecific inhibitor without the ability to interact with the triphosphate subsite, serves as the baseline for the affinity and specificity improvement comparison. As expected, the K_i of adenosine was found to be identical for HER-2 and HER-4 in the high μM range. In the case of HER-2, all molecules of the panel exhibited inhibitory activities with a wide range of apparent K_i's, and two members showed improvement of affinity relative to that of adenosine. The panel was in general less active for HER4 with a smaller range of apparent K_i's. None of the molecules for HER-4 showed improvement of affinity, and three members exhibited no inhibitory activity. As illustrated in Figure 3, the inhibition profiles of HER2 and HER4 by this panel of adenosine derivatives are different, even though these two protein kinases share greater than 90% sequence homology.

In the case of HER-2, linking adenosine from position 2 or 4 improves the original adenosine anchor affinity while linking through position 1 or 3 has an adverse effect relative to adenosine alone. In the case of **2b**, where adenosine is linked at the bridged carbon, the small molecule shows a 3-fold enhancement in affinity ($K_i = 88\ \mu M$) with respect to that of adenosine). In terms of chiral preference within the diastereomeric pair, a more dramatic selectivity is observed in that **2a**, which

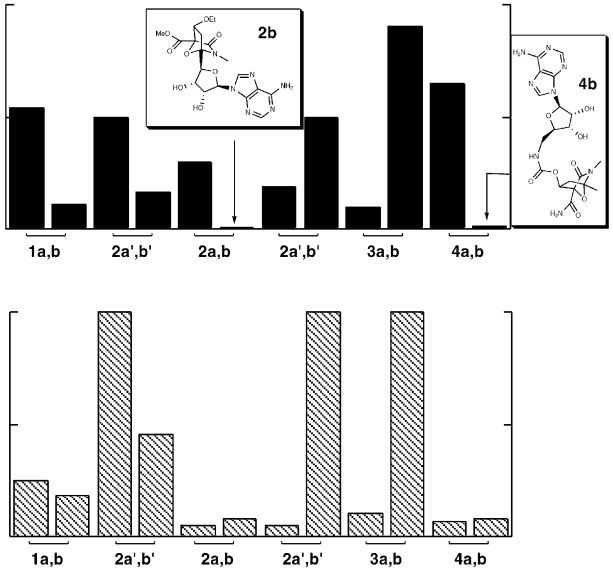


Figure 3. Profiling HER-2 and HER-4 by apparent K_i's of the six pairs.

only differs from **2b** as the mirror image at the bicyclic moiety, is almost 40-fold less active. The selectivity is even more pronounced for **4a** and **4b**, as the difference is about 50-fold, although the absolute K_i value (190 μM) of the active isomer **4b** is only slightly lower to that of the adenosine-anchor alone and twice that of **2b**.

The improvement in affinity by **2a**, though small in its absolute value, is significant for the triphosphate subsite that is solvent-exposed and hydrophilic in nature. The level of affinity enhancement provided by the bicyclic heterocyle in this subsite is unlikely to rival that seen in the hydrophobic adenine subsite where additional hydrophobic interactions, if not sterically hindering and often buried, can readily provide affinity enhancement of hundreds of fold. The adenine subsite, though a well-known area for enhancing affinity, is likely to be limited in providing specificity for 'sibling' kinases from the same family that are highly homologous. The value of the triphosphate subsite is not primarily for conferring high affinity improvement but rather specificity that arises from conformational induction, as seen in the case of balanol and STI-571, or the diastereomeric preference of this subsite, as suggested by the differential K_i's exhibited by **2a** and **2b**.

The effect of altering the adenosine anchor by the addition of a Cbz group is demonstrated by comparing results for **2b** and **2b′**. The two molecules differ only in the *exo*-amino group of adenosine, which in **2b′** is modified with a Cbz group. **2b′** exhibits a K_i that is higher by two orders of magnitude. This suggests that the *exo*-Cbz group has altered the anchoring specifications of just adenosine by itself and hence resulted in an incompatibility with the diversity platform.

The two profiles, though different, shared two features: positional preference for anchor attachment and stereospecific recognition between diastereomeric pairs that distinguish these two highly homologous kinases. Molecules **2b** and **4b**, the most active members of the panel against HER-2, indicate positions 2 and 4 on the bicyclic platform are favored for the anchor attachment. The same two positions are also relatively favored by HER-4. While none of the adenosine-anchored probes improves upon the affinity of adenosine itself against HER-4, the profile of inhibition and diastereomeric preference observed for this kinase suggest that the triphosphate subsite, similar to HER-2, is also a viable location for further development of selective inhibitors.

Table 1. Selectivity ratios[a] of the apparent activities of each diastereomeric pair against HER-2 and HER-4

Pairs	HER-2	HER-4
1a,b	4.8	1.4
1a′,b′	1.7	—[b]
2a,b	**34.4**	**1.6**
2a′,b′	3.1	—[b]
3a,b	9.1	—[b]
4a,b	**54.7**	**1.2**

[a]The ratio of a given diastereomeric pair is taken by dividing the K_i of the more active diastereomer by that of the less active one.
[b]The ratio is designated with—because one member of the pair is not inhibitory.

By comparing the activity ratios within each of the six diastereomeric pairs, a correlation between affinity and chiral preference is observed. As illustrated in Table 1, in the case of HER-2, **2b** and **4b** exhibit the highest activity, and their diastereomeric pairs also display the highest degree of diastereomeric preference as indicated by the inhibitory diastereomeric selectivity ratios of 34 and 55, respectively. The same two diastereomeric pairs containing **2b** and **4b** showed virtually no selectivity in the case of HER-4. Similar to the case of HER-2, the most active diastereomer against HER-4, **2a′**, also comes from the diastereomeric pair with the highest selectivity ratio, in which case the other member of the pair exhibited no inhibitory activity. This suggests that stereospecific interactions in the triphosphate subsite of the ATP binding pocket is a viable strategy for distinguishing highly homologous protein kinases.

To further characterize the inhibition mode of this class of compounds, **4b**, as one of the more synthetically available members of the panel, was subjected to a Lineweaver–Burk reciprocal analysis using pre-phosphorylated HER2 (Fig. 4).[24] This analysis indicates a largely competitive mode of inhibition with some indication of complex inhibition characteristic.[25]

In summary, we have shown that stereospecific probing of the triphosphate subsite of the ATP-binding pocket can be used to enhance specificity, particularly in the case of highly homologous protein kinases HER-2 and HER-4. The activity exhibited by a panel of structurally related molecules, designed to asymmetrically probe the triphoshphate subsite, demonstrates the first known example where HER-2 and HER-4 are inhibited with selective differentiation. Given the selectivity exhibited by the first-generation series, further generations

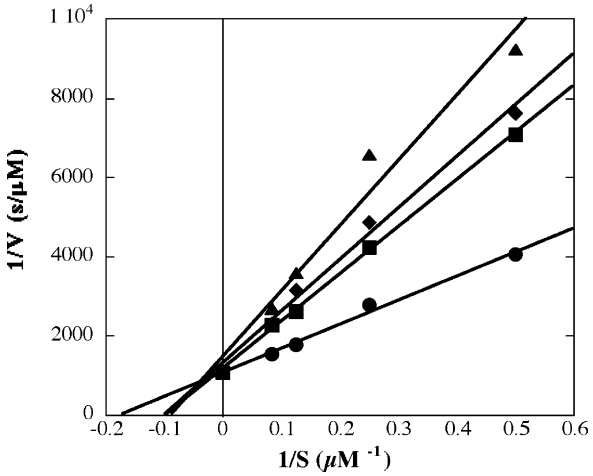


Figure 4. Lineweaver–Burk analysis of the inhibition of pHER-2 by **4b** with respect to ATP. The control experiment was done in 4% DMSO (●). Three concentrations of **4b** (500 μM (■), 800 μM (◆) and 1200 μM (▲) were used in the presence of various concentrations of ATP (2, 4, 8, and 12 μM), 1 mM substrate peptide, and 100 nM HER-2.

of compounds can be designed by using high affinity adenine-ribose mimetics to replace adenosine as better affinity anchors from which additional diversity elements can be extended to probe the triphosphate subsite with stereospecificity. This modular approach is complementary to previous strategies. Ongoing work includes screening against homologous members of other PK families and the synthesis of further generations of asymmetric triphosphate-subsite probes for better selective affinity.

Acknowledgements

The work is supported by ACS RPG-98-027-03 CDD and NIH R01GM61413 to K.S.A. and NIH R01GM059673 to D.J.A. We thank Kermit L. Carraway III for a generous gift of HER-4.

References and Notes

1. (a) Sallese, M.; Iacovelli, L.; De Blasi, A. *Curr. Top. Pharmacol.* **1997**, *3*, 193. (b) Siraganian, R. P.; Zhang, J.; Kimura, T. *Signal Transduct. Mast Cells Basophils* **1999**, 115.
2. (a) King, G. L. *Endocr. Updates* **1998**, *1*, 181. (b) Shankland, S. J.; Wolf, G. *Am. J. Physiol.* **2000**, *278*, F515.
3. (a) van der Geer, P.; Hunter, T.; Lindberg, R. A. *Annu. Rev. Cell Biol.* **1994**, *10*, 251. (b) Carraway, K. L., III; Cantley, L. C. *Cell (Cambridge, Mass.)* **1994**, *78*, 5. (c) Aaronson, S. A. *Science* **1991**, *254*, 1146.
4. (a) Perez, E. A. *Cancer Invest.* **2001**, *19*, 155. (b) Gibbs, J. B. *Science (Washington, D. C.)* **2000**, *287*, 1969.
5. Karunagaran, D.; Tzahar, E.; Beerli, R. R.; Chen, X.; Graus-Porta, D.; Ratzkin, B. J.; Seger, R.; Hynes, N. E.; Yarden, Y. *EMBO J.* **1996**, *15*, 254.
6. (a) Graus-Porta, D.; Beerli, R. R.; Daly, J. M.; Hynes, N. E. *EMBO J.* **1997**, *16*, 1647. (b) Nagy, P.; Jenei, A.; Damjanovich, S.; Jovin, T. M.; Szollosi, J. *Pathol. Oncol. Res.* **1999**, *5*, 255.
7. (a) Junttila, T. T.; Sundvall, M.; Maatta, J. A.; Elenius, K. *Trends Cardiovasc. Med.* **2001**, *10*, 304. (b) Robinson, D.; He, F.; Pretlow, T.; Kung, H.-J. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 5958.
8. (a) Gassmann, M.; Casagranda, F.; Orioli, D.; Simon, H.; Lai, C.; Klein, R.; Lemke, G. *Nature (London)* **1995**, *378*, 390. (b) Alroy, I.; Yarden, Y. *FEBS Lett.* **1997**, *410*, 83. (c) Riese, D. J., 2nd; Stern, D. F. *Bioessays* **1998**, *20*, 41. (d) Woodburn, J. R. *Pharmacol. Ther.* **1999**, *82*, 241.
9. (a) Shih, C.; Padhy, L. C.; Murray, M.; Weinberg, R. A. *Nature (London)* **1981**, *290*, 261. (b) Plowman, G. D.; Culouscou, J. M.; Whitney, G. S.; Green, J. M.; Carlton, G. W.; Foy, L.; Neubauer, M. G.; Shoyab, M. *Proc. Natl. Acad. Sci. U.S.A.* **1993**, *90*, 1746. (c) Cox, S.; Radzio-Andzelm, E.; Taylor, S. S. *Curr. Opin. Struct. Biol.* **1994**, *4*, 893.
10. The X-ray structure of EGFR kinase domain has recently become available. Stamos, J.; Silwkowski, M. X.; Eigenbrot, C. *J. Biol. Chem.* **2002**, *48*, 46265.
11. (a) Hamby, J. M.; Showalter, H. D. H. *Pharmacol. Ther.* **1999**, *82*, 169. (b) Bishop, A. C.; Kung, C.-y.; Shah, K.; Witucki, L.; Shokat, K. M.; Liu, Y. *J. Am. Chem. Soc.* **1999**, *121*, 627. (c) Mendel, D. B.; Laird, A. D.; Smolich, B. D.; Blake, R. A.; Liang, C.; Hannah, A. L.; Shaheen, R. M.; Ellis, L. M.; Weitman, S.; Shawver, L. K.; Cherrington, J. M. *Anti-Cancer Drug Des.* **2000**, *15*, 29. (d) Lawrence, D. S.; Jinkui, N. *Pharmacol. Ther.* **1998**, *77*, 81.
12. (a) Fry, D. W.; Kraker, A. J.; McMichael, A.; Ambroso, L. A.; Nelson, J. M.; Leopold, W. R.; Connors, R. W.; Bridges, A. J. *Science (Washington, D. C.)* **1994**, *265*, 1093. (b) Boschelli, D. H. *Drugs Future* **1999**, *24*, 515. (c) Gray, N. S.; Wodicka, L.; Thunnissen, A. W. H.; Norman, T. C.; Kwon, S.; Espinoza, F. H.; Morgan, D. O.; Barnes, G.; Leclerc, S.; Meijer, L.; Kim, S. H.; Lockhart, D. J.; Schultz, P. G. *Science* **1998**, *281*, 533.
13. Johnson, L. N.; Lowe, E. D.; Noble, M. E. M.; Owen, D. J. *FEBS Lett.* **1998**, *430*, 1.
14. (a) Wedge, S. R.; Ogilvie, D. J.; Dukes, M.; Kendrew, J.; Curwen, J. O.; Hennequin, L. F.; Thomas, A. P.; Stokes, E. S. E.; Curry, B.; Richmond, G. H. P.; Wadsworth, P. F. *Cancer Res.* **2000**, *60*, 970. (b) Sedlacek, H. H. *Drugs* **2000**, *59*, 435.
15. Sequence analyses were performed using the Blast program (http://www.ncbi.nlm.nih.gov). The accession codes for proteins analysed are: P04626(HER-2); Q15303(HER-4); 1ATP_E(PKA); and 1BYG_A (Src).
16. (a) Taylor, S. S.; Radzio-Andzelm, E. *Curr. Opin. Chem. Biol.* **1997**, *1*, 219. (b) Traxler, P.; Furet, P. *Pharmacol. Ther.* **1999**, *82*, 195.
17. Narayana, N.; Diller, T. C.; Koide, K.; Bunnage, M. E.; Nicolaou, K. C.; Brunton, L. L.; Xuong, N.-H.; Eyck, L. F. T.; Taylor, S. S. *Biochemistry* **1999**, *38*, 2367.
18. (a) Schindler, T.; Bornmann, W.; Pellicena, P.; Miller, W. T.; Clarkson, B.; Kuriyan, J. *Science* **2000**, *289*, 1938. (b) Nagar, B.; Bornmann, W. G.; Pellicena, P.; Schindler, T.; Veach, D. R.; Miller, W. T.; Clarkson, B.; Kuriyan, J. *Cancer Res.* **2002**, *62*, 4236.
19. (a) Savinov, S. N.; Austin, D. J. *Chem. Commun.* **1999**, *18*, 1813. (b) Savinov, S. N.; Austin, D. J. *Org. Lett.* **2002**, *4*, 1415. (c) Savinov, S. N.; Austin, D. J. *Org. Lett.* **2002**, *4*, 1419.
20. (a) Liu, F.; Austin, D. J. *Tet. Lett.* **2001**, *42*, 3153. (b) Liu, F.; Austin, D. J. *J. Org. Chem.* **2001**, *66*, 8643.
21. Liu, F.; Austin, D. J. *Org. Lett.* **2001**, *3*, 2273.
22. **3a**: ^{1}H NMR (500 MHz, MeOH-d_4) δ 0.91 (t, J = 7.2 Hz, 3H), 1.56 (s, 3H), 1.65 (dd, J = 2.6 Hz, 12.5 Hz, 1H), 2.15 (dd, J = 8.1 Hz, 12.5 Hz, 1H), 2.71 (s, 3H), 3.30–3.49 (m, 3H), 3.85 (dd, J = 5.3 Hz, 14.1 Hz, 1H), 4.12–4.17 (m, 2H), 4.26 (dd, J = 2.6 Hz, 8.2 Hz, 2H), 4.66 (t, J = 5.8 Hz, 1H), 5.86 (d, J = 6.0 Hz, 1H), 8.12 (s, 1H), 8.21 (s, 1H); ^{13}C NMR (125 MHz, MeOH-d_4) δ 15.7, 18.1, 25.8, 42.2, 68.0, 73.0, 75.4, 82.0, 85.4, 90.6, 97.4, 142.3, 154.4, 167.8; HRMSFAB [M + H$^+$]: 478.2051, calcd for $C_{20}H_{28}N_7O_7$: 478.1972. **4a**: ^{1}H NMR (500 MHz, MeOH-d_4) δ 0.88 (t, J = 7.0 Hz, 3H), 1,54 (s, 3H), 1.61 (q, J = 2.5 Hz, 12.5 Hz, 1H), 2.71 (q, J = 8.1 Hz, 12.5 Hz, 1H), 2.72 (s, 3H), 3.31–3.45 (m, 2H), 3.49 (dd, J = 4.1 Hz, 14.1 Hz, 1H), 3.83 (dd, J = 4.1 Hz, 14.1 Hz, 1H), 4.10 (dd, J = 4.2 Hz, 9.1 Hz, 1H), 4.23–4.27 (m, 2H), 4.65 (t, J = 5.3 Hz, 1H), 5.87 (d, J = 5.3 Hz, 1H), 8.09 (s, 1H), 8.23 (s, 1H); ^{13}C NMR (125 MHz, MeOH-d_4) δ 15.7, 18.1, 25.8, 41.7, 42.1, 68.1, 72.7, 75.4, 82.3, 84.8, 90.7, 97.2, 142.3, 154.4, 167.9; HRMSFAB [M + H$^+$]: 478.2051, calcd for $C_{20}H_{28}N_7O_7$: 478.1972. **4a**: ^{1}H NMR (500 MHz, MeOH-d_4) δ 1.60 (s, 3H), 1.81 (dd, J = 1.2 Hz, 13.0 Hz, 1H), 2.36 (dd, J = 8.6 Hz, 13.0 Hz, 1H), 2.76 (s, 3H), 3.21 (s, 3H), 3.59 (dd, J = 14.5 Hz, 14.1 Hz, 1H), 4.10–4.12 (m, 2H), 4.72 (t, J = 6.0 Hz, 1H), 5.41 (dd, J = 1.2 Hz, 8.6 Hz, 1H), 5.78 (d, J = 6.4 Hz, 1H), 8.13 (s, 1H), 8.14 (s, 1H); ^{13}C NMR (125 MHz, $CDCl_3$) δ 17.8, 25.9, 42.2, 44.3, 73.1, 74.8, 75.5, 86.0, 86.8, 91.4, 97.1, 121.5, 142.7, 150.5, 154.3, 157.9, 158.0, 169.0, 171.2; HRMSFAB [M + H$^+$]: 493.1796, calcd for $C_{20}H_{28}N_7O_7$: 492.1717; $[\alpha]_D^{20}$ −143.1° (*c* 0.23, MeOH). **4b**: ^{1}H NMR 500 MHz, MeOH-d_4) δ 1.72 (s, 3H), 1.84 (dd, J = 2.6 Hz, 13.3 Hz, 1H), 2.49 (dd, J = 8.2 Hz, 13.1 Hz, 1H), 2.86 (s, 3H), 3.42 (dd, J = 2.4, 14.5 Hz, 1H), 3.59 (dd, J = 3.9, 14.2 Hz, 1H), 4.20 (m, 2H), 4.66 (dd, J = 5.1, 6.4 Hz, 1H), 5.35 (dd, J = 2.8 Hz, 8.6 Hz, 1H), 5.98 (d, J = 6.3 Hz, 1H), 8.46 (s, 1H), 8.72 (s, 1H); ^{13}C NMR ($CDCl_3$,

125 MHz) δ 17.9, 25.9, 42.4, 44.8, 73.2, 74.9, 75.5, 86.0, 86.4, 91.4, 97.1, 142.7, 154.6, 157.9, 158.0; HRMSFAB $[M+H^+]$: 493.1796, calcd for $C_{20}H_{28}N_7O_7$: 492.1717; $[\alpha]_D^{20}$ −42.9° (*c* 0.20, MeOH).

23. The kinase domains of the two proteins were used: HER-2 (G704-V1025) and HER-4 (R676-V1308). Screening was performed at 1–2 mM concentration of the small molecules. The kinase activity assay was performed as follows: the small molecule and enzyme were allowed to incubate on ice for 5 min prior to initiating the reaction by the addition of a substrate mixture. The resulting 4-μL reaction contained 50 mM Tris, pH 7.6, 20 mM $MnCl_2$, 4 μM ATP, 0.1 μCi/μL γ^{32}P-ATP, and 2 mM peptide substrate GGMEDIY-FEFNGGKKK, 0.1 μM enzyme, and 1–2 mM inhibitor. Following 10–15 min of reaction time corresponding to 5–15% ATP turnover, the mixture was quenched by the addition of 2.67 μL EDTA (0.5 M). All reactions were performed in triplicates and analyzed on 15% SDS-polyacrylamide gels. The apparent K_i of each small molecule was calculated using the following equation: $K_i = [I]^* K_m/((F-1)(K_m + [S]))$. [I] is the inhibitor concentration, K_m is 5 μM, F is the ratio between the initial rates of phosphorylated peptide formation without or with the presence of the inhibitor, and [S] is the ATP concentration. See: Jan, A. Y.; Johnson, E. F.; Diamonti, J. A.; Carraway, K. L., III; Anderson, K. S. *Biochemistry* **2000**, *39*, 9786.

24. HER-2 and phosphorylated HER-2 have different kinase activities. Autophosphorylation of HER-2 results in heterogeneity in enzyme population during the course of assay. The prephosphorylation of HER-2 was performed in excess of ATP for 30 min on ice and quenched with 0.5 M EDTA. The phosphorylated HER-2 was purified by using Bio-Spin™ columns.

25. The complex kinetic characteristics may arise from the small population of HER-2 still remaining from the prephosphorylation reaction of HER-2. See ref 18a.