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Syntheses and EGFR kinase inhibitory activity of 6-substituted-4-anilino [1,7] and [1,8] naphthyridine-3-carbonitriles

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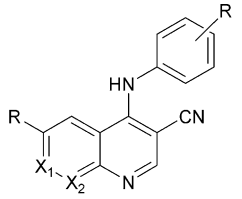
Abstract—The syntheses and EGFR kinase inhibitory activity of a series of 6-substituted-4-anilino [1,7] and [1,8] naphthyridine-3-carbonitriles are described. Both reversible and irreversible binding inhibitors were prepared. These series were compared with each other and with the corresponding 4-anilinoquinoline-3-carbonitriles. Compounds having a 1,7-naphthyridine core structure can retain high potency while those with a 1,8-naphthyridine core are significantly less active. These results are consistent with molecular modeling observations.

Receptor protein tyrosine kinases play an important role in signal transduction pathways that regulate cell division and differentiation. Overexpression of certain growth factor receptors such as Epidermal Growth Factor Receptor (EGFR) is known to predict poor prognosis in many human cancers.[1–5] Compounds that inhibit the kinase activity of EGFR after binding of the growth factor are of potential interest as new therapeutic antitumor agents.[6]

Previously, we have described our research on a series of kinase inhibitors based on compounds with a 4-anilinoquinoline-3-carbonitrile core structure.[7] We reported that these compounds are effective inhibitors of EGFR and Her2 kinases. We described compounds that function in this way as conventional reversible ATP-competitive inhibitors as well as those that function as irreversible binding inhibitors.[8,9] One example of the latter type, EKB-569,[10] is currently in phase II clinical trial for the treatment of EGFR dependent cancers. In additional reports, members of our laboratory have described the activity of a number of 4-anilinoquinoline-3-carbonitriles that inhibit kinases other than EGFR which are involved along the signal transduction pathway.[11]

In this present report, we would like to describe our efforts to extend this work. In particular, we will comment on the synthesis and EGFR kinase activity of some 6-substituted 1,7 and 1,8-naphthyridine-3-carbonitriles represented by the formula shown below.



1,7-naphthyridines: X_1 = N, X_2 =CH
1,8-naphthyridines: X_2 = N, X_1 =CH

The goal of this study was to determine the effect on the EGFR kinase activity resulting from incorporation of an additional nitrogen atom at various positions on the left-hand ring of the cyanoquinoline core structure. The different naphthyridine derivatives will be compared with each other and with the corresponding quinoline-3-carbonitriles.

Compounds in the 1,8-naphthyridine series were prepared as shown in Scheme 1. 2-Chloro-5-nitronicotinic acid, **1**,[12] was converted to its acid chloride in an excess of refluxing thionyl chloride. After removal of the excess reagent and without additional purification, **2** was condensed with the cyanoenamine **3**[13] using Hunig's base in refluxing CH_2Cl_2 to furnish the intermediate **4** in

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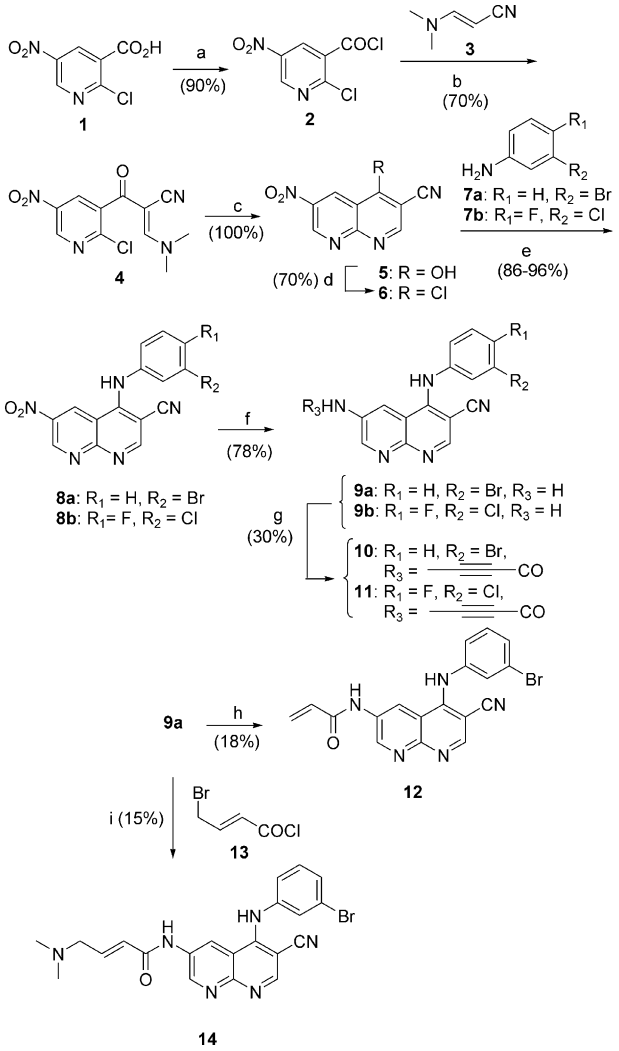
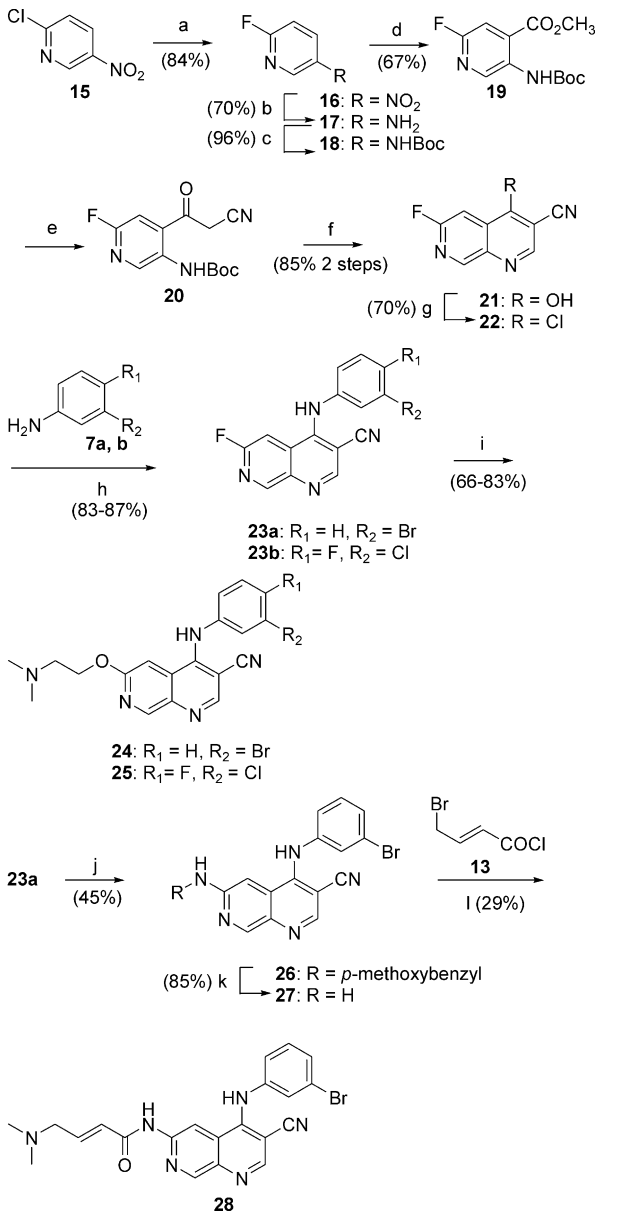
good yield. Cyclization of **4** to the intermediate **5** having the 1,8-naphthyridine core was accomplished quantitatively by refluxing in a mixture of EtOH and NH_4OH. This compound was chlorinated by refluxing in an excess of $POCl_3$, and after removal of the excess reagent and aqueous KOH extractive work up at 0 °C, **6** was obtained as a yellow solid. Condensation of **6** with either aniline **7a** or **7b** gave the desired 4-anilino-1,8-naphthyridine-3-carbonitriles **8a** and **8b**, respectively, as the hydrochloride salts. The nitro groups of these intermediates were reduced by using iron powder in a refluxing mixture of aqueous NH_4Cl and MeOH. Inhibitors **10** and **11** bearing the butynamide Michael acceptor side chain were prepared by the reaction of the mixed anhydride of butynoic acid, prepared from the acid using isobutyl chloroformate and *N*-methylmorpholine in THF at 0 °C, and adding this solution to a mixture of **9a** or **9b** in pyridine, containing a catalytic amount of DMAP. The inhibitor **12** with the acrylamide side chain was prepared in poor yield by acylation with acryloyl chloride in a THF–DMF mixture. The inhibitor with the 4-dimethylaminocrotonamide side chain, **14**, was prepared in low yield by acylation with acid chloride **13**[14] at 0 °C using Hunig's base in THF, and adding enough dry *N*-methyl pyrolidone to dissolve the solids. This was followed by the addition of 15 equiv of 2M dimethylamine in THF to displace the bromide and incorporate the dimethylamino group. The product was purified by chromatography.



Scheme 1. (a) $SOCl_2$, reflux, 5 h; (b) *i*-Pr_2NEt, CH_2Cl_2, reflux, 16 h; (c) NH_4OH, EtOH, reflux, 4 h; (d) $POCl_3$, reflux, 24 h; (e) *i*-PrOH, reflux, 3 h; (f) NH_4Cl, Fe, H_2O, MeOH, reflux, 1 h; (g) *1*. butynoic acid, *i*-ButOCOCl, *N*-methylmorpholine, THF, 0 °C and *2*. **9a** or **9b**, DMAP (cat), pyridine, rt, 1 h.; (h) acryloyl chloride, *N*-methylmorpholine, THF, DMF, 3 h; (i) *i*-Pr_2NEt, THF, *N*-methyl pyrolidone, 0 °C, 1.5 h and then 2M Me_2NH in THF, 30 min.

Compounds in the 1,7-naphthyridine series were prepared as shown in Scheme 2. 2-Chloro-5-nitropyridine



Scheme 2. (a) KF, DMSO, 70 °C, 18 h; (b) Raney Ni, H_2, EtOAc; (c) $(Boc)_2O$, *t*-BuOH, 40 °C, 4 h; (d) *1*. *n*-BuLi, TMEDA, ether, −78 °C, *2*. Dry CO_2, *3*. $TMSCHN_2$ in hexanes, MeOH–$CHCl_3$ (1:3), 0 °C then rt 2 h; (e) *1*. *n*-BuLi, CH_3CN, THF, −78 °C, 1 h, *2*. HOAc, warm to rt; (f) DMF–DMA, DMF (1:1), 5 h; (g) CO_2Cl_2, DMF (cat), CH_2Cl_2, reflux 2 h; (h) EtOH reflux 8 h; (i) $NaOCH_2CH_2N(CH_3)_2$, THF, reflux 2 h; (j) *p*-MeO-$PhCH_2NH_2$, EtOH, reflux 8 days; (k) TFA–CH_2Cl_2 (1:1), rt 20 h; (l) *1*. *i*-Pr_2NEt, THF, 0 °C, *2*. NaBr, THF–DMF (10:1), 3 days and then 2M Me_2NH in THF.

was converted to the fluoro derivative **16** by displacement, using KF in hot DMSO. The nitro group was then reduced with Raney nickel to give **17**. The amino group was then protected as the Boc derivative. Lithiation of **18** in ether at −78 °C was regioselective and was accomplished with *n*-BuLi and tetramethyethylenediamine (TMED). The lithiated species was then quenched with dry CO_2 and the resulting acid was methylated using (trimethylsilyl)diazomethane to give **19**. Cyclization of **19** to intermediate **21** having a 1,7-naphthyridine core structure was accomplished in good yield using a multi-step process beginning with the formation of the lithium anion of acetonitrile in THF at −78 °C using *n*-BuLi. A solution of **19** was added to an excess (3 equiv) of the anion solution and after 1 h, the mixture was quenched with an excess of acetic acid and then warmed to room temperature giving **20**. The reaction of **20** with dimethylformamide dimethylacetal (DMA–DMF) at room temperature for 5 h gave the 1,7-naphthyridine derivative **21**. This compound was chlorinated using oxalyl chloride in refluxing CH_2Cl_2 in the presence of a catalytic amount of DMF. Condensation of **22** with either aniline **7a** or **7b** gave the desired 4-anilino-3-cyano 1,7-naphthyridines **23a** and **23b**, respectively. Inhibitors **24** and **25** with the 6-(2-dimethylaminoethoxy) side chain were prepared by refluxing a THF solution of **23a** or **23b** with sodium 2-dimethylamino-ethoxide. The inhibitor **28** in the 1,7-naphthyridine series, having the 4-dimethylamino crotonamide Michael acceptor side chain, was prepared by the reaction of **23a** with 4-methoxy-benzylamine in refluxing EtOH for 8 days to give **26**. The deprotection of the amino group was accomplished in good yield using TFA in CH_2Cl_2.

The side chain was introduced by acylation of **27** with acid chloride **13** as described above. A mixture of the 4-bromo and 4-chloro butenamides was obtained. This was treated with NaBr in THF–DMF for 3 days to convert the chloro derivative to the bromide. The mixture was quenched with a large excess of $(CH_3)_2NH$ to give **28** in modest yield after chromatographic purification.

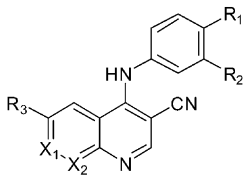
While the compounds such as **24** and **25** with non-reactive side chains at the 6-position were designed to function as conventional reversible binding ATP-competitive inhibitors, those compounds with Michael acceptor functionality at the 6-position such as **10**, **11**, **12**, **14**, and **28** were designed to function as irreversible binding inhibitors. It is assumed that these latter compounds will function in this manner by virtue of the fact that they can form a covalent bond to a Cys residue (Cys 773) located in the ATP binding pocket of EGFR. Other groups,[15] as well as ourselves,[8,14,16] have previously shown that 4-anilinoquinazolines and 4-anilino-quinoline-3-carbonitriles with appropriate Michael acceptor functionality at the 6-position do bind covalently to the target protein. In this earlier work, we found that compounds bearing 2-butynamide, acrylamide, or 4-dimethylamino crotonamide groups at the 6-position were particularly effective in this regard; it is for this reason that these functionalities were chosen for the present study. In addition, we also know from our earlier studies that the 3-bromoanilino and the 3-chloro-4-fluoroanilino substituents at the 4-position of the quinazoline and quinoline-3-carbonitrile cores are among the optimal choices when targeting EGFR and, consequently, only these substituents have been used in the present work.

These naphthyridine derivatives have been evaluated for their ability to inhibit EGFR kinase. These data are shown in Table 1. For comparison, we have also included similar data for several quinoline-3-carbonitriles (**29**–**32**)[8] that correspond in structure to some of the naphthyridines. In addition, data is provided for our clinical lead EKB-569.[8] We measured the ability of these compounds to inhibit the phosphorylation of a peptide substrate by EGFR kinase, as well as the ability to inhibit EGFR autophosphorylation, using a protocol and enzyme source that is different than that described in our earlier studies.[17] We find that the IC_{50} for inhibiting autophosphorylation is always significantly greater than the IC_{50} for inhibiting phosphorylation of a peptide substrate. However, the relative activities of the inhibitors are highly correlated between these different measurements. In earlier reports,[8,14] we already commented on some of the pitfalls involved in the interpretation of IC_{50} measurements when it comes to irreversible binding inhibitors. Such measurements reflect a combination of the ability of an inhibitor to fit at the active site as well as its reactivity towards the target Cys residue.

It is evident that in the 1,7-naphthyridine series, the 6-(2-dimethylaminoethoxy) side-chain is not conducive for good enzyme inhibitory activity as shown by the results obtained for **24** and **25**. In contrast, the compound in this series, **28**, which was designed to function as an irreversible binder, is an extremely potent inhibitor of the enzyme with an IC_{50} of 1.9 nM for inhibiting phosphorylation of the substrate. This compound appears to be more potent than the corresponding inhibitor, **32**, in the quinoline-3-carbonitrile series. It is also evident that **28** is much more potent than the corresponding analogue **14** in the 1,8-naphthyridine series, where the IC_{50} values differ by 1000-fold. In comparing **14** and **32**, it is clear that placing a nitrogen atom at the 8-position is detrimental to activity. This is also evident when compounds with the other types of Michael acceptors are compared. In each case, the 1,8-naphthyridine **10**, **11**, or **12** are significantly (11- to 37-fold) less potent than the respective quinoline-3-carbonitrile **29**, **30**, or **31**.

It was also of interest to compare the cellular activities of some of these inhibitors, in particular, those with the 4-dimethylaminocrotonamide side-chains. These data are shown in Table 1. Three human carcinoma cell lines were used: A431 (epidermoid) which over-expresses EGFR, SKBR3 (breast) which over-expresses HER-2 and to a lesser extent EGFR, and SW620 (colon) which is believed not to express either EGFR or HER-2 to a significant extent. Full experimental details for these assays have already been reported.[14] EGFR and HER-2 show a very high sequence homology in the region of

Table 1. Inhibition of EGFR kinase[17] and inhibition of cell growth



Compd[a]	R_1	R_2	X_1	X_2	R_3	Substrate[b] IC_{50} (±SD) nM	Autophos[c] IC_{50} (±SD) nM	A431[d] IC_{50} (μg/mL)	SKBR3[d] IC_{50} (μg/mL)	SW620[d] IC_{50} (μg/mL)
10	H	Br	CH	N	—≡—CONH	6266.2	> 10,000			
11	F	Cl	CH	N	—≡—CONH	3762.4	6794.8			
12	H	Br	CH	N	CONH	374.8±4.8	975.7			
14	H	Br	CH	N	$(H_3C)_2N$ CONH	2043.6	4128.6	2.76	4.83	> 5
24	H	Br	N	CH	$(H_3C)_2NCH_2CH_2O$	> 10,000	> 10,000			
25	F	Cl	N	CH	$(H_3C)_2NCH_2CH_2O$	> 10,000	> 10,000			
28	H	Br	N	CH	$(H_3C)_2N$ CONH	1.9±0.6	8.6±4.1	0.088	0.036	0.356
29	H	Br	CH	CH	—≡—CONH	258.9±212.9	> 1000			
30	F	Cl	CH	CH	—≡—CONH	330.2±289	1027.8±195			
31	H	Br	CH	CH	CONH	9.7±2.9	86.9±50.6			
32	H	Br	CH	CH	$(H_3C)_2N$ CONH	37.2±26.5	122.9±98.1	0.230	0.089	2.80
EKB-569	F	Cl	C-OEt	CH	$(H_3C)_2N$ CONH	14.5±15.7	97.6±84.1			

[a] All new compounds were fully characterized by NMR and MS. Purity was assessed by elemental analysis.
[b] Concentration in nM needed to inhibit the phosphorylation of a peptide substrate by 50% as determined from the dose–response curve. Where more than one test was run, IC_{50}'s are averages of multiple determinations and the standard deviation (SD) is reported.
[c] Concentration in nM needed to inhibit the autophosphorylation of EGFR by 50% as determined from the dose–response curve. Where more than one test was run, IC_{50}'s are averages of multiple determinations and the standard deviation (SD) is reported.
[d] Dose–response curves were determined at 7 concentrations. The IC_{50} values are the concentrations in μg/mL needed to inhibit cell growth by 50% as determined from these curves.

the active site. The Cys residue targeted in EGFR kinase is conserved in HER-2 kinase and, therefore, we expect (and earlier work has confirmed) that our inhibitors will be effective in inhibiting the growth of cell lines that express either of these growth factor receptors. With respect to the cell assays, it is evident that each compound is a much better inhibitor of the A431 and SKBR3 cell lines than the SW620 line. This is consistent with the mechanism of cell growth inhibition being reliant, to a degree, on the target kinases. The fact that some of these compounds do inhibit the growth of the SW620 line at higher concentrations could suggest that this line has some dependence on EGFR or HER-2, even though these receptors are not expressed to a significant degree, or that these compounds inhibit cell growth by an undefined mechanism at these higher concentrations. We find that the order of activity in inhibiting cell growth is **28** > **32** > > **14**. It can be seen that this order parallels the potency we see in the enzyme assay.

The above observations can be nicely rationalized from the results of molecular modeling experiments. In our previous EGFR kinase modeling efforts,[8,9,14] we made use of a homology model of the catalytic domain of the enzyme that was constructed using a combination of two different crystal structures as templates, FGF Receptor-1 for the N-terminal lobe, and Hematopoietic Cell Kinase (Hck) for the C-terminal lobe. Recently the X-ray crystal structure of EGFR kinase having a bound quinazoline-based ligand was reported.[18] For the present study, we decided to use the published coordinates[19] from this X-ray structure as the starting point for the modeling studies. The publication of this structure also allows us to retrospectively evaluate our earlier homology model. Overall, our model and the reported structure agree with respect to most of the important features. We find a RMS difference of 4.95 Å when comparing C^{α} atoms of the full catalytic domains. In the region surrounding the ATP-binding site, the RMS difference is 1.68 Å.

The ligand was removed from the published X-ray structure and replaced with inhibitor **28**. The inhibitor was oriented in a manner similar to the original ligand, and similarly to the orientation that we used in some of

our earlier modeling studies of the quinazolines and quinoline-3-carbonitriles. The complex was energy minimized using the CharmM forcefield as implemented in Quanta™ software.[20] As in our previous work, we allowed the entire system of protein, ligand, and water molecules to move during minimization. In the final model (Fig. 1) the N1 atom of **28** is hydrogen-bonded to the backbone NH of Met 769. The 3-cyano group of **28** has displaced the water molecule that bridged the N3 atom of the original quinazoline ligand to the hydroxyl group of Thr 766. The nitrogen atom of the 3-cyano group now interacts with that same residue. The 4-(3-bromoaniline) moiety lies in a hydrophobic pocket containing Leu 820, Thr 830, Asp 831, Val 702, Ala 719, Ile 720, Lys 721, Thr 766, and Leu 764. Most significantly, the β-carbon atom of the Michael acceptor side chain of **28** is located 4.13Å from the sulfhydryl group of Cys 773, and should be easily accessible for covalent interaction. Additionally, the N-atom of the dimethylamino group on **28** is located 3.56 Å away from the sulfhydryl hydrogen of Cys 773. In our earlier work on inhibitors in the quinazoline and quinoline-3-carbonitrile series, we proposed (and presented experimental evidence in support of the fact) that this dimethylamino group can serve as an intramolecular base catalyst for Michael additions to these types of inhibitors. We are now proposing that the dimethylamino group of **28** is playing a similar role. Also, given the arrangement of functionalities predicted by this binding model, we suggest that this intramolecular catalysis operates after **28** binds at the active site of EGFR and that the Michael addition reaction of the sulfhydryl group of Cys 773 to bound **28** is accelerated (relative to reactions of other nucleophiles that may be in the cytosol) due to the close proximity of the reactive center, nucleophile, and base catalyst.

Of particular significance to the case at hand, the C8 atom of **28** is located 3.18 Å from the backbone carbonyl oxygen of Met 769. We believe that this might account for the large difference in activity we see between the 1,7- and 1,8-naphthyridines. For **28**, the interaction of the hydrogen atom attached to C8 and the carbonyl oxygen of Met 769 is expected to be stabilizing[21–23] via a weak CH–O hydrogen bond. If the 1,8-naphthyridine analogue **14** adopted a similar binding orientation compared to **28**, not only would the favorable aromatic hydrogen–carbonyl interaction be lost, but it would be replaced by an unfavorable interaction resulting from the repulsion of the lone pairs on N8 of the inhibitor and the carbonyl oxygen of Met 769. In reality, it is likely that **14** would bind in a manner that would mitigate this unfavorable interaction, but this might orient the molecule in a less favorable position for the covalent interaction to occur at an efficient rate. Additionally, one would expect that this aromatic hydrogen–carbonyl interaction would be stronger the more polarized the C–H bond. The C–H bond at the 8-position is expected to be more polarized in **28** compared to **32** and this might account for the slightly better potency of **28** compared **32**.

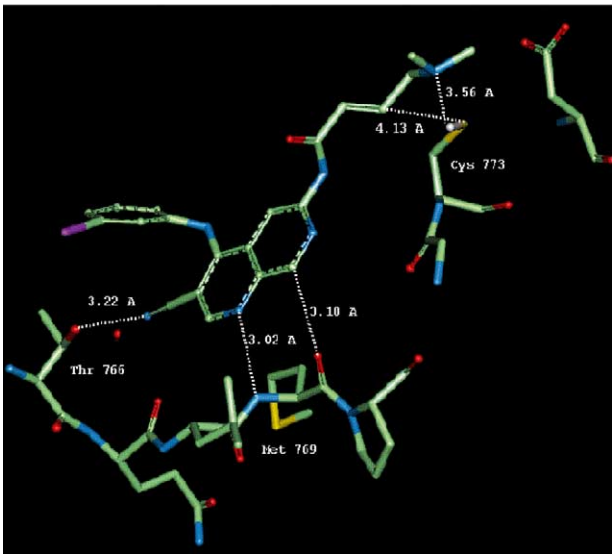


Figure 1. Binding model of **28** at the active site of EGFR kinase.

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