1862 J. Med. Chem. 2010, 53, 1862–1866
DOI: 10.1021/jm901338g

Journal of Medicinal Chemistry
Brief Article

Exploring Epidermal Growth Factor Receptor (EGFR) Inhibitor Features: The Role of Fused Dioxygenated Rings on the Quinazoline Scaffold

Adriana Chilin,*,† Maria Teresa Conconi,† Giovanni Marzaro, Adriano Guiotto, Luca Urbani, Francesca Tonus, and Pierpaolo Parnigotto

Dipartimento di Scienze Farmaceutiche, Università di Padova, via Marzolo 5, 35131 Padova, Italy.
†First two authors contributed equally to this work and should be considered co-first authors.

Received September 9, 2009

A number of dioxolane, dioxane, and dioxepine quinazoline derivatives have been synthetized and evaluated as EGFR inhibitors. Their cytotoxic activity has been tested against two cell lines overexpressing and not expressing EGFR. Most derivatives were able to counteract EGF-induced EGFR phosphorylation, and their potency was comparable to the reference compound PD153035. The size of the fused dioxygenated ring was crucial for the biological activity, the dioxane derivatives being the most promising class of this series.

Introduction

The epidermal growth factor receptor (EGFR[a]) is a transmembrane tyrosine kinase receptor that belongs to the ErbB family. In the absence of a ligand EGFR exists in an inactive monomeric form, whereas after interaction with its growth factor ligands, the formation of homo- or heterodimers occurs,[1] leading to signal transduction pathways that regulate cell differentiation, growth, migration, and apoptosis.[2] EGFR is often mutated and/or overexpressed in many tumors, representing therefore a valuable target for the design of anticancer agents. A number of ATP-mimetic tyrosine kinase inhibitors (TKIs) have so far received approval by FDA for cancer therapy[3] as Erlotinib,[4] Gefitinib,[5] and Lapatinib.[6]

Among several TKIs, the 4-anilinoquinazoline class of compounds plays a key role and has been extensively studied in the past few years. These TKIs generally show high potency in vitro, inhibiting EGFR at nanomolar concentration.[7] However, a major problem of these compounds is their poor in vivo activity, and many analogues of PD153035,[7] the most potent inhibitor on isolated EGFR (IC_{50} = 25 pM), have been developed by inserting solubilizing groups to address this issue.[8] Moreover, it has been established that TKIs can bind their target both in the active and in the inactive forms, leading to different activity profiles.[9] For example, Lapatinib, which binds only the inactive EGFR form, is a slow off-rate inhibitor, leading to a longer inhibition effect with respect to Erlotinib or Gefitinib, which bind only the active EGFR form.[6] This is due to Lapatinib ability to occupy a deep pocket only present in the inactive form of the receptor. To investigate the structural determinants that could positively modulate the interaction with EGFR and consequently the biological activity, we have modified the molecular structure of PD153035 in two specific sites: the moiety involved in the interaction with the ATP-binding site of EGFR, incorporating both the 6- and 7-methoxy groups into rings of various size (dioxolane, dioxane, and dioxepine rings), and the 4-aniline moiety responsible for the interaction with an hydrophobic pocket of EGFR, substituting the bromine atom with other functional groups of different lipophilicity and/or steric hindrance (Table 1).

The antiproliferative activity of the synthesized compounds has been evaluated against A431 and NIH3T3 cells overexpressing and not expressing EGFR, respectively. Moreover, the inhibition of ligand-induced receptor phosphorylation and the abrogation of EGF-mediated growth effect and reversibility were all assessed on A431 cells.

Chemistry

All the designed compounds were synthesized according to a novel synthetic strategy, with a quinazoline oxidation step leading to the key quinazolinone intermediates[10] (Scheme 1).

The starting quinazolines **3a**−**c** have been synthesized from the appropriate aniline derivative **1a**−**c** using the HMTA/TFA/$K_3Fe(CN)_6$ method.[11] Compounds **3a**−**c** were oxidized to quinazolin-4(3*H*)-ones with CAN in aq AcOH and then submitted to chlorination with $POCl_3$ in presence of TEA and finally condensed with the suitable substituted aniline to give the final products **6a**−**c**, **7a**−**c**, **8a**−**c**, **9a**−**c**, and **10a**−**c**, directly as hydrochlorides. The final compounds are summarized in Table 1.

Biology

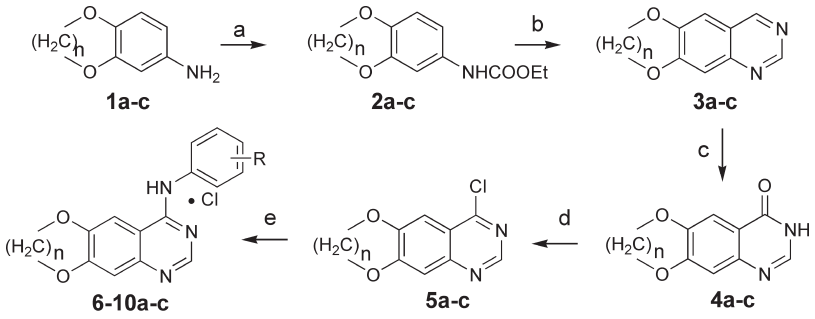
The inhibitory effects on cell proliferation were determined by MTT assay (Table 2 and Figure S1 in the Supporting Information). The results obtained on A431 cells (overexpressing EGFR) showed that the dioxane derivatives were more cytotoxic than the corresponding dioxolane and dioxepine derivatives. IC_{50} values of compounds **6b**, **7b**, and **8b** were about 6 times lower than that determined for PD153035.

*To whom correspondence should be addressed. Phone: +39 0498275349. Fax: +39 0498275366. E-mail: adriana.chilin@unipd.it.

[a] Abbreviations: AP, alkaline phosphatase; CAN, cerium ammonium nitrate; EGF, epidermal growth factor; EGFR, epidermal growth factor receptor; ELISA, enzyme-linked immunoassorbent assay; FDA, Food and Drug Administration; HMTA, hexamethylenetetramine; HRMS, high resolution mass spectrometry; HRP, horseradish peroxidase; MAbs: monoclonal antibodies; MOE, Molecular Operating Environment; PDB, Protein Data Bank; SD, standard deviation; TEA, triethylamine; TFA, trifluoroacetic acid; THF, tetrahydrofuran; TKI, tyrosin kinase inhibitors; VEGFR, vascular endothelial growth factor receptor.

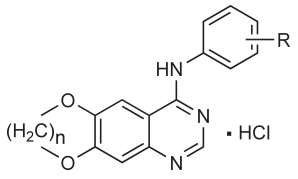
 Published on Web 01/22/2010

Scheme 1[a]



[a] Reagents and conditions: (a) ClCOOEt, TEA, THF, rt, 30 min; (b) (i) HMTA, TFA, MW, 110 °C, 10 min, (ii) $K_3Fe(CN)_6$, KOH 10% EtOH/H_2O 1/1, reflux, 4 h; (c) CAN, AcOH, H_2O, rt, 5 min; (d) $POCl_3$, TEA, reflux, 3 h; (e) $ArNH_2$, ^{i}PrOH, MW, 80 °C, 15−30 min. See Table 1 for R specification.

Table 1. Structures of Synthesized Compounds



n = 1	*n* = 2	*n* = 3	R
6a[a]	**6b**[a]	**6c**[a]	3′-Br
7a[a]	**7b**	**7c**	3′-Me
8a	**8b**[a]	**8c**	3′-CF_3
9a	**9b**[a]	**9c**	3′-Cl, 4′-F
10a	**10b**	**10c**	2′-F, 4′-Br

[a] These compounds have already been synthesized (see references in the Supporting Information) but using a different strategy, and only data about cytotoxicity and inhibition of isolated EGFR have been published.

Table 2. IC_{50} Values for 72 h Exposure of A431 and NIH3T3 Cells to the Compounds

	IC_{50} (μM)[a]	
compd	A431	NIH3T3
PD153035	4.40 ± 1.3	>10[b]
6a	7.16 ± 1.11	>10[b]
7a	6.69 ± 0.92	>10[b]
8a	8.46 ± 0.87	>10[b]
9a	>10[b]	nd[c]
10a	>10[b]	nd[c]
6b	0.75 ± 0.09	>10[b]
7b	0.67 ± 0.11	5.80 ± 0.58
8b	0.77 ± 0.09	7.10 ± 0.60
9b	>10[b]	nd[c]
10b	6.28 ± 0.46	5.40 ± 0.20
6c	8.17 ± 0.76	>10[b]
7c	6.29 ± 0.62	>10[b]
8c	4.73 ± 0.56	>10[b]
9c	7.13 ± 0.63	>10[b]
10c	>10[b]	nd[c]

[a] The values are the mean ± SD of at least three independent experiments. [b] IC_{50} not determined because less than 50% inhibition was observed at the highest tested concentration (10 μM). Higher concentrations were not used to avoid precipitation of the compounds in the culture medium. [c] nd: not determined. Cell proliferation assay was not carried out on NIH3T3 cells because the compound was quite ineffective on A431 cells.

Because the 4-aniline moiety interacts with a hydrophobic pocket of EGFR,[12] the presence of lipophilic groups in specific positions improves the interaction with this site, leading to an increase in the affinity toward EGFR. Thus, the compounds bearing a bromine atom or methyl (compounds **6a**−**c** and **7a**−**c**) or trifluoromethyl (compounds **8a**−**c**) groups at the 3′ position are more active than those substituted with two halogen atoms.

To assess whether the antiproliferative activity of the compounds could be only due to inhibition of EGFR, the same test was repeated with NIH3T3 cells, not expressing EGFR (Table 2). In general, almost all the compounds, including PD153035, showed a lower but not negligible cytotoxicity in NIH3T3 cells, demostrating that other cell targets, probably other kinases, may be affected by these compounds (particularly in the case of dioxane derivatives). The interaction with other tyrosine kinase receptors, such as the vascular endothelial growth factor receptor (VEGFR), could improve the antitumor activity via an antiangiogenetic mechanism.[13] It is known that the pharmacological inhibition of EGFR can decrease VEGF expression and consequently angiogenesis in many tumor types.[14] Nevertheless, VEGF production is controlled by many other factors and the prolonged administration of EGFR inhibitors can lead to resistance. Thus, several inhibitors of both EGFR and VEGFR have been designed and studied.[15] It is noteworthy that compounds **10a**−**c** bear two halogen atoms (F and Br) in the 4-aniline moiety like Vandetanib, a dual inhibitor able to slow down the growth of tumors nonresponsive to EGFR inhibitors.[16] Among these three compounds, compound **10b** was the most effective, inducing similar antiproliferative effects in both cell lines. We are planning in vitro studies on endothelial cells to assess if this compound could interact also with VEGFR and exert antiangiogenic effects.

On the basis of the inhibitory effect on A431 cell proliferation, the more active compounds were selected for further studies. The antiproliferative effects of 4-anilinoquinazoline derivatives seems to be related to a pro-apoptotic activity. All the compounds, as well as PD153035, induced strong increases in the apoptotic rate when compared to nontreated cultures (Figure 1).

The reversibility of the antiproliferative effects of the compounds was also studied. Almost all the compounds seems to be cytostatic, like PD153035,[17] showing on A431 cells a reversible growth inhibitory activity (see Figure S2 in the Supporting Information). Only compound **6a** partially

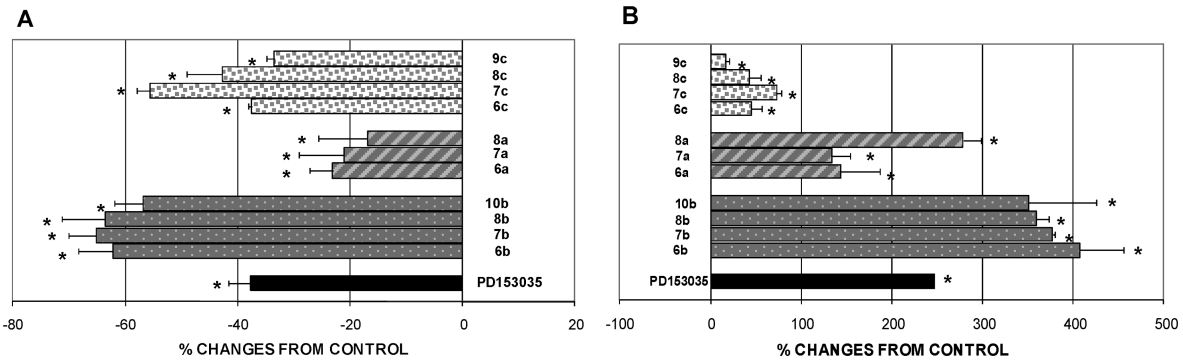


Figure 1. Pro-apoptotic effects of compounds on A431 cells. A431 cells were treated for 24 h with compounds (10 μM). At the end of the incubation period, both cell viability (A) and apoptotic rate (B) were determined by MTT assay and assessment of mono- and oligonucleosomes formation, respectively. Results, expressed as percent change from control nontreated cultures (taken as 0), are the mean ± SD of at least three independent experiments. $p < 0.05$ vs control cultures, Student t test.

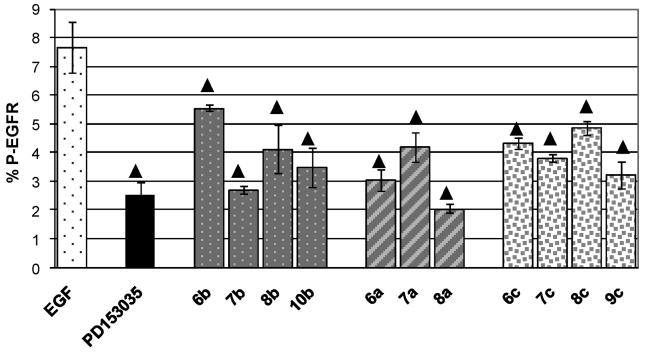


Figure 2. Effects on EGFR phosphorylation. A431 cells were pretreated for 30 min with 1 μM of compounds and then incubated with 50 ng/mL EGF for 5 min. Phosphorylated EGFR was determined by a sandwich ELISA using a phospho-specific anti-EGFR antibody and a normalization antibody that recognizes the pan-protein regardless of phosphorylation status. The results were expressed as ratio between phosphorylated EGFR and total EGFR. $\Delta = p < 0.05$ vs cultures treated with EGF.

maintained its antiproliferative activity after the recovery period.

The ability of the compounds to inhibit EGFR phosphorylation was assessed using a cell-based ELISA experiment able to discriminate EGFR from other tyrosine kinases. As shown in Figure 2, all the compounds significantly decreased EGF-induced EGFR phosphorylation.

To evaluate whether the synthesized compounds could be able to counteract the increase in cell growth induced by exogenous EGF, A431 cells were pretreated for 24 h with 4-anilinoquinazoline derivatives and then, after removal of media, incubated for 72 h with either EGF alone (Figure 3A) or both compounds and EGF added to culture media (Figure 3B). On the basis of MTT data, the experiments were carried out by treating the cultures with the highest noncytotoxic concentrations of the tested compounds, thus avoiding direct cytotoxic effects. Although all compounds inhibited EGFR phosphorylation induced by exogenous EGF, only PD153035, **8a**, **8b**, and **10b** were able to counteract the proliferogenic effects of the cytokine. In both experimental conditions, PD153035 and compound **8a** reversed the EGF-induced cell growth. Compound **10b** was able to counteract the EGF stimulatory effects when it was simultaneously administered with the growth factor, suggesting a preferential interaction of **10b** with the active form of EGFR. On the contrary, compound **8b** seems to be effective on the inactive form because only preincubation reversed the EGF-induced growth effects.

A docking study was performed on the two structurally related compounds **8a** and **8b** to explain the reason of such a different behavior for so similar derivatives. The structures of **8a** and **8b** were docked in the crystal structure of inactive EGFR form (PDB ID: 2GS7): in this structure, a magnesium ion, a well-known cofactor for the ATPase activity,[18] was present near the interaction pocket. Only compound **8b** was able to interact with the ion through the oxygen at the 6 position (Figure 4 and Figure S3 in the Supporting Information).

Conclusion

The syntheses and biological evaluation of three classes of fused tricyclic quinazolines as EGFR inhibitors have been described. The cytotoxic activity of all the compounds was assessed against two cell lines overexpressing and not expressing EGFR, respectively. Most derivatives were able to counteract EGF-induced EGFR phosphorylation and showed better or at least comparable potency with respect to PD153035, used as reference compound. The size of the fused dioxygenated ring was crucial for the cytotoxic activity and for the biological profile. In particular, the dioxane derivatives showed an interesting profile: among these, the most promising derivative is the one bearing the 3′-trifluoromethylaniline substituent (**8b**) due to its preferential binding to the inactive form of EGFR. Further studies on the VEGFR inhibition ability of this compound are in progress to investigate its potential dual behavior.

Experimental Section

Chemistry. See Supporting Information for general synthetic methods, for synthesis of **1c**, and for the analytical details (mp, NMR, HRMS, elemental analyses) of all compounds.

General Procedure for Carbamates 2a−c. A mixture of **1** (10.0 mmol), ethyl chloroformate (20.0 mmol), and TEA (20.0 mmol) in anhydrous THF (200 mL) was stirred at room temperature for 30 min. The solid was filtered off, and the solvent was evaporated under reduced pressure. The residue was dissolved in $CHCl_3$ (200 mL), and the organic layer was washed with water (2 × 100 mL). The organic phase was evaporated under reduced pressure to give **2** (yields 97−98%).

General Procedure for Quinazolines 3a−c. A mixture of ethyl carbamate **2** (10.0 mmol) and HMTA (10.0 mmol) in TFA (30 mL) was microwave irradiated at 110 °C (power set point 80 W; ramp time 1 min; hold time 10 min). After cooling, the mixture was diluted with aq ethanolic (water/EtOH: 1/1) 10% KOH

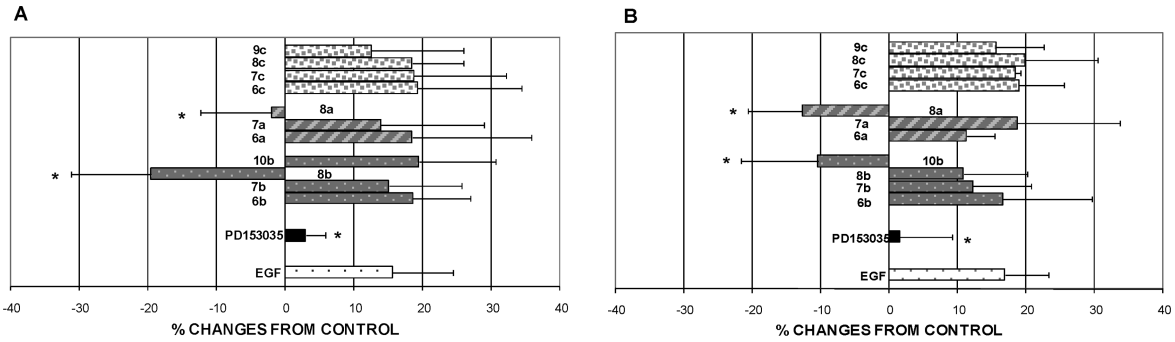


Figure 3. Effects on EGF-mediated growth stimulation. (A) After 24 h of treatment with the compounds at the highest concentration not inducing decrease in cell viability (0.01 μM PD153035, **6b**, **7b**, **10b**, **6c**, and **8c**; 0.1 μM **6a**, **7a**, **8b**, **7c**, and **9c**; 1 μM **8a**), medium from A431 cells was removed and replaced with fresh one containing 5 ng/mL EGF. Then, cultures were incubated for 72 h. (B) A431 cells were treated for 72 h with 5 ng/mL EGF and noncytotoxic concentrations of compounds. At the end of the incubation period, cell proliferation was determined by MTT assay. Results, expressed as percent change from control not treated cultures (taken as 0), are the mean ± SD of at least three independent experiments. * = $p < 0.05$ vs EGF-treated cultures, Student t test.

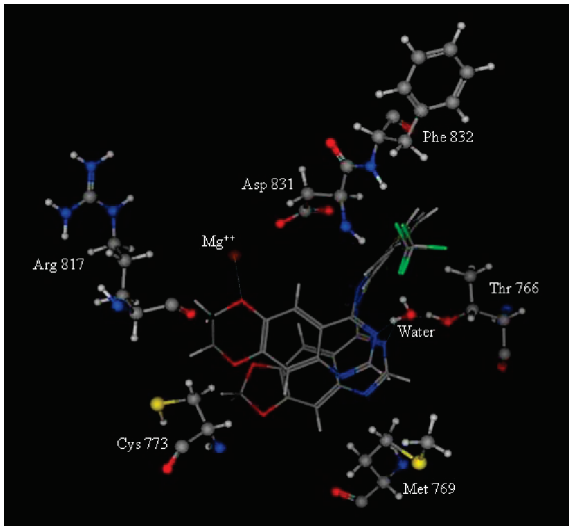


Figure 4. Superposition of docked structure of compounds **8a** and **8b** in the crystal structure of inactive EGFR (PDB ID: 2GS7).

(300 mL), and $K_3Fe(CN)_6$ (80.0 mmol) was added to the solution, which was refluxed under stirring for 4 h. After cooling, the mixture was diluted with water (300 mL) and extracted with $CHCl_3$ (3 × 200 mL). The organic phase was evaporated under reduced pressure to give **3** (yields 83−89%).

General Procedure for Quinazolinones 4a−c. To a solution of **3** (5.0 mmol) in AcOH (2.0 mL), a solution of CAN (20.0 mmol) in water (24.0 mL) was added dropwise. The white precipitate formed was collected to give **4** (yields 65−73%).

General Procedure for Chloroquinazolines 5a−c. A suspension of **4** (4.0 mmol) in $POCl_3$ (8.0 mL) and TEA (2.0 mL) was refluxed for 3 h. After cooling, the mixture was concentrated under reduced pressure and the solid residue was dissolved in EtOAc (50 mL) and washed with a satd aq $NaHCO_3$ solution (2 × 20 mL). The organic phase was evaporated under reduced pressure to give **5** (yields 70−93%).

General Procedure for Anilinoquinazolines 6−10a−c. A mixture of **5** (1.0 mmol) and substituted aniline (1.0 mmol) in ^{i}PrOH (2 mL) was microwave irradiated at 80 °C (power set point 60 W; ramp time 1 min; hold time 15 min). After cooling, further aniline (1.0 mmol) was added and the mixture was microwave irradiated at 80 °C for another cycle. After cooling, the obtained precipitate was collected by filtration to give **6−10a−c** as hydrochlorides (yields 72−86%).

Biology. See Supporting Information for evaluation of antiproliferative and pro-apoptotic activity, reversibility of the antiproliferative activity, EGFR-phosphorylation, and effects on EGF-induced cell growth.

Acknowledgment. The present work has been carried out with the financial support of the University of Padova "Progetto di Ricerca di Ateneo 2008".

Supporting Information Available: Detailed characterization data for all the compounds, experimental procedures for biochemical assays, and computational methodologies. This material is available free of charge via the Internet at http://pubs.acs.org.

References

(1) Bazley, L. A.; Gullick, W. J. The epidermal growth factor receptor family. *Endocr. Relat. Cancer* **2005**, *12* (Suppl 1), S17–S27.
(2) Citri, A.; Yarden, Y. EGF-ERBB signalling: towards the systems level. *Nat. Rev. Mol. Cell Biol.* **2006**, *7*, 505–516.
(3) Ellis, P. M.; Morzycki, W.; Melosky, B.; Butts, C.; Hirsh, V.; Krasnoshtein, F.; Murray, N.; Shepherd, F. A.; Soulieres, D.; Tsao, M. S.; Goss, G. The role of the epidermal growth factor receptor tyrosine kinase inhibitors as therapy for advanced, metastatic, and recurrent non-small-cell lung cancer: a Canadian national consensus statement. *Curr. Oncol.* **2009**, *16*, 27–48.
(4) Dowell, J.; Minna, J. D.; Kirkpatrick, P. Erlotinib hydrochloride. *Nat. Rev. Drug Discovery* **2005**, *4*, 13–14.
(5) Cohen, M. H.; Williams, G. A.; Sridhara, R.; Chen, G.; Pazdur, R. FDA drug approval summary: gefitinib (ZD1839) (Iressa) tablets. *Oncologist* **2003**, *8*, 303–306.
(6) Wood, E. R.; Truesdale, A. T.; McDonald, O. B.; Yuan, D.; Hassell, A.; Dickerson, S. H.; Ellis, B.; Pennisi, C.; Horne, E.; Lackey, K.; Alligood, K. J.; Rusnak, D. W.; Gilmer, T. M.; Shewchuk, L. A unique structure for epidermal growth factor receptor bound to GW572016 (Lapatinib): relationships among protein conformation, inhibitor off-rate, and receptor activity in tumor cells. *Cancer Res.* **2004**, *64*, 6652–6659.
(7) Bridges, A. J.; Zhou, H.; Cody, D. R.; Rewcastle, G. W.; McMichael, A.; Showalter, H. D.; Fry, D. W.; Kraker, A. J.; Denny, W. A. Tyrosine kinase inhibitors. 8. An unusually steep structure−activity relationship for analogues of 4-(3-bromoanilino)-6,7-dimethoxyquinazoline (PD 153035), a potent inhibitor of the epidermal growth factor receptor. *J. Med. Chem.* **1996**, *39*, 267–276.
(8) Liu, G.; Hu, D. Y.; Jin, L. H.; Song, B. A.; Yang, S.; Liu, P. S.; Bhadury, P. S.; Ma, Y.; Luo, H.; Zhou, X. Synthesis and bioactivities of 6,7,8-trimethoxy-*N*-aryl-4-aminoquinazoline derivatives. *Bioorg. Med. Chem.* **2007**, *15*, 6608–6617.
(9) Liao, J. J. Molecular recognition of protein kinase binding pockets for design of potent and selective kinase inhibitors. *J. Med. Chem.* **2007**, *50*, 409–424.
(10) Marzaro, G.; Guiotto, A.; Pastorini, G.; Chilin, A. A novel approach to quinazolin-4(3H)-one via quinazoline oxidation: an improved synthesis of 4-anilinoquinazolines. *Tetrahedron* **2010**, *66*, 962–968.

(11) Chilin, A.; Marzaro, G.; Zanatta, S.; Guiotto, A. A microwave improvement in the synthesis of the quinazoline scaffold. *Tetrahedron Lett*. **2007**, *48*, 3229–3231.
(12) Roskoski, R. The ErbB/HER receptor protein-tyrosine kinases and cancer. *Biochem. Biophys. Res. Commun*. **2004**, *319*, 1–11.
(13) Cao, Y. H. Antiangiogenic cancer therapy. *Semin. Cancer Biol*. **2004**, *14*, 139–145.
(14) Dutta, P. R.; Malty, A. Cellular responses to EGFR inhibitors and their relevance to cancer therapy. *Cancer Lett*. **2007**, *254*, 165–177.
(15) Tabernero, J. The role of VEGF and EGFR inhibition: Implications for combining anti-VEGF and anti-EGFR agents. *Mol. Cancer Res*. **2007**, *5*, 203–220.
(16) Naumov, G. N.; Nilsson, M. B.; Cascone, T.; Briggs, A.; Straume, O.; Akslen, L. A.; Lifshits, E.; Byers, L. A.; Xu, L.; Wu, H. K.; Janne, P.; Kobayashi, S.; Halmos, B.; Tenen, D.; Tang, X. M.; Engelman, J.; Yeap, B.; Folkman, J.; Johnson, B. E.; Heymach, J. V. Combined vascular endothelial growth factor receptor and epidermal growth factor receptor (EGFR) blockade inhibits tumor growth in xenograft models of EGFR inhibitor resistance. *Clin. Cancer Res*. **2009**, *15*, 3484–3494.
(17) Bos, M.; Mendelsohn, J.; Kim, Y. M.; Albanell, J.; Fry, D. W.; Baselga, J. PD153035, a tyrosine kinase inhibitor, prevents epidermal growth factor receptor activation and inhibits growth of cancer cells in a receptor number-dependent manner. *Clin. Cancer Res*. **1997**, *3*, 2099–2106.
(18) Saylor, P.; Wang, C.; Hirai, T. J.; Adams, J. A. A second magnesium ion is critical for ATP binding in the kinase domain of the oncoprotein v-Fps. *Biochemistry* **1998**, *37*, 12624–12630.