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Derivatives of Iressa, a Specific Epidermal Growth Factor Receptor Inhibitor, are Powerful Apoptosis Inducers in PC3 Prostatic Cancer Cells

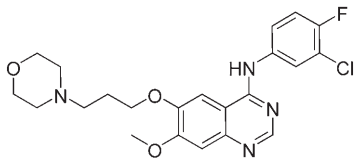
Aurélie Telliez,[a] Matthieu Desroses,[a] Nicole Pommery,[a] Olivier Briand,[b] Amaury Farce,[c] Guillaume Laconde,[a] Amélie Lemoine,[a] Patrick Depreux,[a] and Jean-Pierre Hénichart*[a]

The tyrosine kinase activity of the epidermal growth factor receptor (EGFR) is widely involved in signaling pathways and often deregulated in cancer. Its role in the development of prostate cancer is well established, and therapeutic strategies such as blockade of the intracellular tyrosine kinase domain with small-molecule tyrosine kinase inhibitors have been proposed. Herein we describe the synthesis and in vitro pharmacological properties of C6- and C7-substituted 4-anilinoquinazolines, analogues of Iressa and powerful proapoptotic inducers in hormone-independent prostate cancer PC3 cell lines.

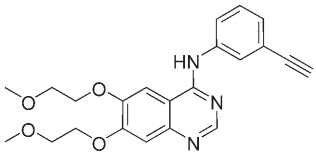
Introduction

Prostate cancer is the most invasive and frequently diagnosed malignancy and is the second leading cause of death by cancer in men. For advanced and aggressive prostate cancer, endocrine therapy induces dramatic growth arrest and cell death in sensitive prostate cells, but mutations of the androgen receptor (AR) and alterations in the relative expression of AR co-regulators contribute to the continued progression of the cancer.[1] Moreover, certain cellular changes, including increased growth factor production and overexpression of oncogenic protein tyrosine kinases with altered downstream signaling, contribute to the spread of cancer.[2–5] Aberrant kinase signaling leads to increased proliferation and invasion and a decrease in cancer cell apoptosis. Furthermore, it has also been implicated in the process of angiogenesis in cancerous tissues. Therefore, the development of therapeutic agents that inhibit the abnormal activation of protein tyrosine kinases is now a major field of research in both academic institutions and pharmaceutical companies.[6,7] Evidence that these enzymes are deregulated in prostate cancer along with their possible role in the development of hormone-refractory prostate cancer[8,9] has led to the consideration of tyrosine kinases, particularly those of the human epidermal growth factor receptor (HER) family, as prime targets for anticancer drug therapy.

Blockade of the intracellular domain of the epidermal growth factor receptor (EGFR) is one strategy that has been adopted to limit aberrant signaling, and this was recently carried out successfully with low-molecular-weight tyrosine kinase inhibitors. Among such inhibitors is the selective EGFR inhibitor Iressa (gefitinib, ZD 1839), which induces cell-cycle arrest along with inhibition of cell proliferation and invasion in both androgen-sensitive and insensitive human prostatic cancer cells.[10–12] These effects are mainly mediated by suppression of EGF-stimulated activation of the PI3K/Akt pathway,[13] which is



ZD 1839, gefitinib (Iressa)



OSI-774, erlotinib (Tarceva)

[a] *Dr. A. Telliez, Dr. M. Desroses, Dr. N. Pommery, Dr. G. Laconde, A. Lemoine, Prof. P. Depreux, Prof. J.-P. Hénichart*
Institut de Chimie Pharmaceutique Albert Lespagnol
EA 2692, Université de Lille 2, 59006 Lille (France)
Fax: (+33) 3-2096-4906
E-mail: jean-pierre.henichart@univ-lille2.fr

[b] *Dr. O. Briand*
Institut Pasteur de Lille, Département d'Athérosclérose
Inserm U545, 59019, Lille (France)
and
Faculté des Sciences Pharmaceutiques et Biologiques
Université de Lille 2, 59006, Lille (France)

[c] *Dr. A. Farce*
Faculté des Sciences Pharmaceutiques et Biologiques
Laboratoire de Chimie Thérapeutique, EA 1043, 59006 Lille (France)

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perturbed in cancers associated with somatic deletions or mutations of the PTEN gene.[14,15] Iressa is an orally active anilinoquinazoline derivative currently under evaluation in patients with various tumor types. However, a randomized phase II study showed only minimal efficacy with the drug in hormone-refractory prostate cancer subjects.[16] In addition, cases of interstitial lung disease have been reported in Japan in patients with non-small-cell lung cancer under treatment with Iressa.[17,18]

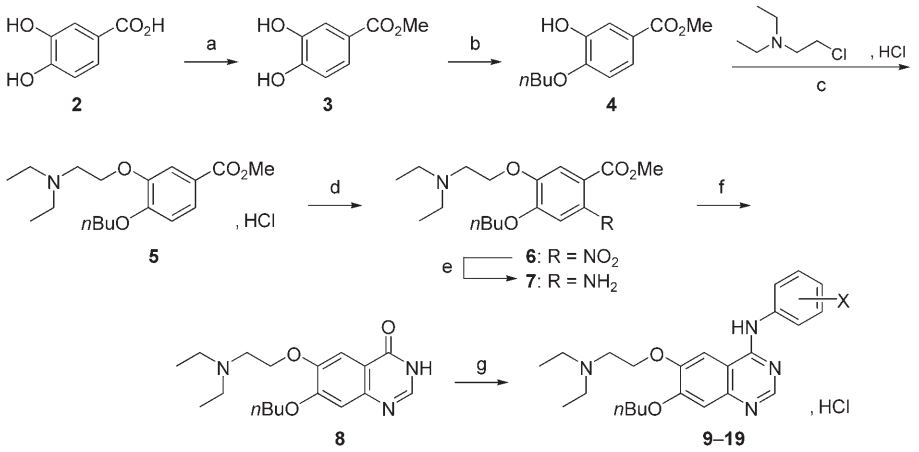
Based on our previous findings,[19] we describe herein the synthesis and in vitro properties of C6- and C7-substituted 4-anilinoquinazolines. As with Iressa and Tarceva (erlotinib), another EGFR inhibitor, the quinazoline ring is believed to mimic ATP and interact with its binding pocket in the kinase. The positioning of the aniline into the selectivity pocket, which is rather small and lipophilic in the case of EGFR, is well described;[20] in this study various substituents have been introduced into the anilino ring in an attempt to increase the binding affinity. In addition, two linear side chains (either butoxy or diethylaminoethoxy) at positions 6 and 7 of the quinazoline ring were introduced. The butoxy group could interact with a hydrophobic portion of the ATP site, and the basic atom incorporated into one of the two linear side chains could improve the physicochemical properties (solubility and bioavailability) of these structures to render them more druglike. It is known that quinazolines, especially 4-anilinoquinazolines with basic side chains at C7, are potent inhibitors of VEGFR-1 (Flt-1) and VEGFR-2 (KDR) tyrosine kinases in both in vitro and in vivo assays and may be useful in limiting angiogenesis.[21–24] We therefore preferentially studied these regioisomers of Iressa and other C7-substituted anilinoquinazolines, because we expected that this might favor greater inhibition of cell growth and invasion.

The 4-anilinoquinazolines described herein were first evaluated for their ability to inhibit EGFR with respect to other tyrosine kinases and, as binding to the ATP pocket of EGFR was observed with some of the compounds, this was compared with the binding of the reference compounds Iressa and Tarceva. We also report the effects of these new tyrosine kinase inhibitors on cell proliferation, survival, and invasion in hormone-independent PC3 prostate cancer cell lines. As EGFR is involved in the activation and expression of Akt and cyclooxygenase-2 (COX-2), and these proteins also play a role in prostate cancer development[25] through antiapoptotic, proliferative, and invasion pathways, the effect of the new derivatives on these enzymes was also evaluated in an attempt to study their overall mechanism of action.

Results and Discussion

Chemistry

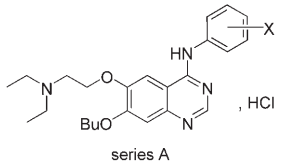
The synthesis of 4-anilinoquinazoline analogues **9**–**19** is described in Scheme 1. 3,4-Dihydroxybenzoic acid was converted into its methyl ester. Selective introduction of the *n*-butyl substituent was performed using one equivalent of 1-iodobutane and potassium carbonate in acetone at room temperature over 18 h. Alkylation of the free hydroxy group of compound **4** with 2-diethylaminoethylchloride hydrochloride and potassium carbonate in acetone at reflux gave **5** in quantitative yield. Selective nitration at C6 was carried out by treating **5** with fuming nitric acid and tin(IV) chloride in dichloromethane at −25 °C to give **6** in 65% yield. Reduction of the nitro group with tin(II) chloride in concentrated hydrochloric acid at 100 °C, followed by quinazolinone formation using ammonium formate in formamide at 140 °C furnished **8** in 66% yield. The 4-chloro substituent was introduced by treating **8** with phosphorus pentachloride in an excess of phosphorus oxychloride at reflux. Nucleophilic displacement of the chlorine atom with anilines then yielded the corresponding 4-anilino-7-*n*-butoxy-6-(2-diethylaminoethoxy)quinazolines **9**–**19** (Table 1).



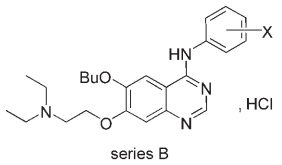
Scheme 1. Reagents and conditions for the synthesis of 4-anilinoquinazolines **9**–**19**: a) $SOCl_2$, MeOH, reflux, >99%; b) *n*-C_4H_9I, K_2CO_3, acetone, 64%; c) K_2CO_3, acetone, reflux, >99%; d) HNO_3 (100%), $SnCl_4$, CH_2Cl_2, −25 °C, 65%; e) $SnCl_2$, HCl (12 N), 100 °C, 91%; f) $HCOONH_4$, $HCONH_2$, 140 °C, 66%; g) 1: PCl_5, $POCl_3$, reflux; 2: R-NH_2, *i*PrOH, reflux, 25–99%.

We decided to use the same synthetic route to prepare isomers **27**–**37** (Scheme 2). This synthesis was accomplished by benzylation of **3** in acetone at room temperature using one equivalent of potassium carbonate and benzyl bromide. The position of the benzyl group was confirmed by COSY and ROESY experiments. In the next step, the free phenol group of the intermediate **20** was alkylated with 1-iodobutane by using potassium carbonate in acetone at reflux to give **21** in 96% yield. After hydrogenolysis of the benzyl protecting group (94% yield), the free hydroxy group of compound **22** was alkylated with 2-diethylaminoethylchloride hydrochloride using potassium carbonate in acetone at reflux. Intermediate **23** was

Table 1. Structures and yields of compounds **9**–**19** and **27**–**37**.

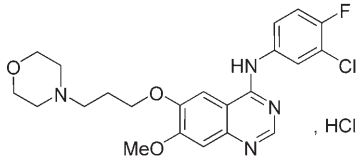


series A



series B

Compd	X	Yield [%]	Compd	X	Yield [%]
9	H	30	**27**	H	52
10	3-Cl, 4-F	50	**28**	3-Cl, 4-F	60
11	3-Cl	100	**29**	3-Cl	83
12	4-Cl	60	**30**	4-Cl	49
13	4-Br	32	**31**	4-Br	89
14	4-F	95	**32**	4-F	14
15	3-Cl, 4-COOH	25	**33**	3-Cl, 4-COOH	51
16	4-COOH	61	**34**	4-COOH	82
17	3-Br	51	**35**	3-Br	38
18	3-Br, 4-CH_3	43	**36**	3-Br, 4-CH_3	12
19	3-Cl, 4-Br	83	**37**	3-Cl, 4-Br	65



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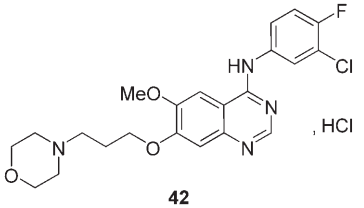


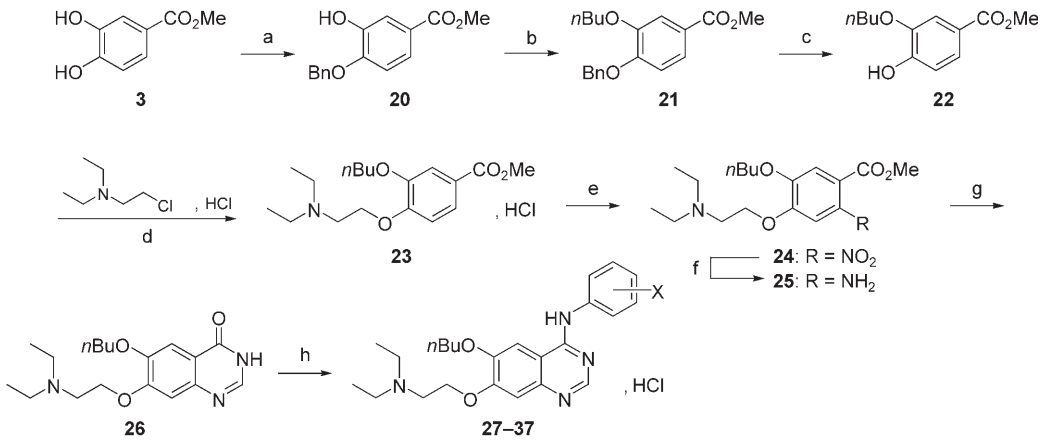
42

obtained in 91% yield. The quinazolinone **26** was then prepared with a similar strategy to that used to afford **8**. Nitration of compound **23** with fuming nitric acid and tin(IV) chloride in dichloromethane at −25 °C followed by reduction of the nitro group with tin(II) chloride in concentrated hydrochloric acid at 100 °C furnished **25** in 79% yield. This compound was cyclized to the quinazolinone **26** using ammonium formate in formamide at 140 °C in 60% yield. The 4-chloro substituent was then introduced by treating **26** with phosphorus pentachloride in an excess of phosphorus oxychloride at reflux. Nucleophilic displacement of the chlorine atom with various anilines then yielded the corresponding 4-anilino-6-*n*-butoxy-7-(2-diethylaminoethoxy)quinazolines **27**–**37** (Table 1).

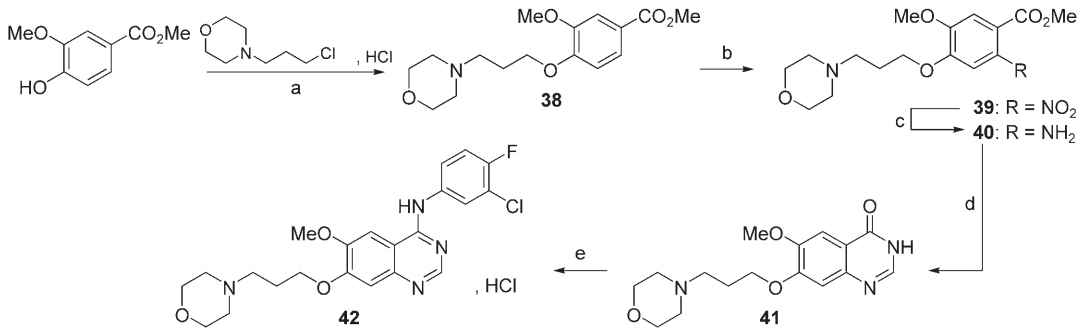
The synthesis of compound **42** is described in Scheme 3. The reaction of methyl vanillate with 4-chloropropylmorpholine hydrochloride under basic conditions in acetone at reflux provided methyl-3-methoxy-4-(4-morpholinopropyl)benzoate **38** in quantitative yield. Reaction of **38** with fuming nitric acid and tin(IV) chloride in dichloromethane at −78 °C led to compound **39** (91% yield). On heating with tin(II) chloride and hydrochloric acid (12 N) at 100 °C, **39** was converted into the methyl-6-amino-3-methoxy-4-(4-morpholinopropyl)benzoate **40**. The treatment of **40** with ammonium formate and formamide at 140 °C gave quinazolinone **41** in 90% yield. Chlorination of **41** was carried out with phosphorus pentachloride and phosphorus oxychloride at 105 °C to yield the 4-chloroquinazoline intermediate, which was immediately reacted with 3-chloro-4-fluoroaniline in isopropanol at reflux. Compound **42** was obtained in 19% yield (Table 1).

Biological data: inhibition of EGFR tyrosine kinase activity and PC3 membrane-associated tyrosine kinase activity

The reference compound Iressa (compound **1**, a selective EGFR inhibitor) and compounds **9**–**19** (series A), **27**–**37** (series B), and **42** were tested for their ability to inhibit tyrosine kinase activity of the EGFR (purified from A431 cells) and other tyrosine kinases present in PC3 membranes (Tables 2 and 3). Compound **1** is a highly potent EGFR inhibitor (IC_{50} = 72 nM). This activity is lost if the morpholino side chain is moved to the 7-position (compound **42**, IC_{50} > 1 μM). The compounds of series A (**9**–**19**) appear to be more effective than compounds belonging to series B (**27**–**37**). Compounds substituted by a fluorine alone or a carboxylic group are unfavorable for EGFR inhibition (compounds **14**–**16** and **32**–**34**). Bromo and chloro



Scheme 2. Reagents and conditions for the synthesis of 4-anilinoquinazoline analogues **27**–**37**: a) K_2CO_3, BnBr, acetone, >67%; b) *n*-C_4H_9I, K_2CO_3, acetone, reflux, 96%; c) H_2, Pd/C, MeOH, 94%; d) K_2CO_3, acetone, reflux, 91%; e) HNO_3 (100%), $SnCl_4$, CH_2Cl_2, −25 °C, 67%; f) $SnCl_2$, HCl (12 N), 100 °C, 79%; g) $HCOONH_4$, $HCONH_2$, 140 °C, 60%; h) 1: PCl_5, $POCl_3$, reflux; 2: R-NH_2, *i*PrOH, reflux, 12–89%. Bn = benzyl.



Scheme 3. Reagents and conditions for the synthesis of compound **42**: a) K_2CO_3, acetone, reflux, >99%; b) HNO_3 (100%), SnCl4, CH_2Cl_2, −78 °C, 91%; c) $SnCl_2$, HCl (12 N), 100 °C, 40%; d) $HCOONH_4$, $HCONH_2$, 140 °C, 90%; e) 1: PCl_5, $POCl_3$, reflux; 2: 3-chloro-4-fluoroaniline, *i*PrOH, reflux, 19%.

Table 2. Enzymatic activities for compounds **1** and **9**–**19** (series A).

	EGFR[a]		PC3[b]	
Compd	Inhibition [%][c]	IC_{50} [μM][d]	Inhibition [%][e]	IC_{50} [μM][d]
1	nd[f]	0.072	68.0	7.3
9	11.5	>1	23.0	>10
10	69.6	0.660	48.0	>10
11	67.5	0.669	45.0	>10
12	51.3	0.937	86.2	7.3
13	54.1	0.875	57.0	8.1
14	11.5	>1	2.9	>10
15	20.0	>1	4.9	>10
16	118	>1	2.0	>10
17	65.2	0.735	37.0	>10
18	76.2	0.587	42.5	>10
19	59.1	0.887	64.1	7.5

[a] Inhibition of EGFR (purified from human carcinoma A431 cells) tyrosine kinase activity. [b] Inhibition of whole PC3 cell membrane-associated tyrosine kinase activity. [c] Compounds tested at a concentration of 1 μM. [d] Values correspond to $n=3$ (SD<10%). [e] Compounds tested at a concentration of 10 μM. [f] nd = not determined.

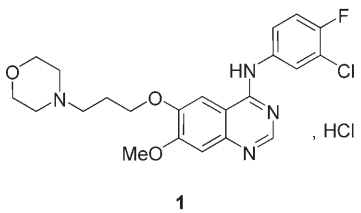
Table 3. Enzymatic activities for compounds **42** and **27**–**37** (series B).

	EGFR[a]		PC3[b]	
Compd	Inhibition [%][c]	IC_{50} [μM][d]	Inhibition [%][e]	IC_{50} [μM][d]
42	33.0	>1	100	1.8
27	20.7	>1	69.2	6.9
28	55.8	0.895	90.0	4.3
29	43.4	>1	74.5	6.5
30	46.6	>1	95.0	4.2
31	6.2	>1	59.1	7.9
32	13.7	>1	29.0	>10
33	11.3	>1	0	>10
34	24.9	>1	15.0	>10
35	33.7	>1	64.6	6.0
36	21.4	>1	73.6	4.5
37	71.9	0.634	62.1	6.6

[a] Inhibition of EGFR (purified from human carcinoma A431 cells) tyrosine kinase activity. [b] Inhibition of whole PC3 cell membrane-associated tyrosine kinase activity. [c] Compounds tested at a concentration of 1 μM. [d] Values correspond to $n=3$ (SD<10%). [e] Compounds tested at a concentration of 10 μM.

derivatives are the most active compounds, but remain less active than reference compound **1**.

To study the potential of these different compounds to inhibit other tyrosine kinases, they were incubated with PC3 membranes. The reference compound **1**, which is known to be a powerful EGFR inhibitor, manifests tyrosine kinase inhibition on PC3 membranes ($IC_{50}=7.3$ μM). Compound **42**, the isomeric analogue of compound **1**, was slightly more active ($IC_{50}=1.8$ μM) than compound **1**. As compound **42** is not a good EGFR inhibitor, it is plausible that it is active toward alternate kinase targets. Compounds that belong to series B are more potent tyrosine kinase inhibitors than compounds of series A.



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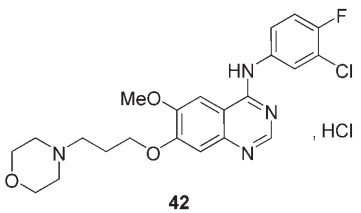

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Chloro or bromo substitutions produced relatively good tyrosine kinase inhibition, but activity is lost with fluoro-substituted derivatives (compounds **14** and **32**) and carboxylic acids (compounds **15**, **16** and **33**, **34**). As noted for **42**, it is clear that compounds that are not EGFR inhibitors but which maintain tyrosine kinase inhibitory activity may act upon other intracellular targets. As a basic side chain at the 7-position of the quinazoline ring has previously been described to produce potent VEGFR tyrosine kinase inhibitors,[21–24] we evaluated the activity of compounds **1**, **42**, **9**–**19** (series A), and **27**–**37** (series B) against VEGFR-2, a tyrosine kinase widely involved in angiogenesis. SU5416[26] ($IC_{50}=1.2$ μM) is the reference compound for the test. Compound **1** and derivatives belonging to series A manifest a weak inhibition of VEGFR-2 tyrosine kinase at 5 μM (Table 4), if any, whereas the isomeric analogue is a better VEGFR-2 inhibitor ($IC_{50}=5.0$ μM). Moreover, most of compounds belonging to series B manifest VEGFR-2 tyrosine kinase inhibition at 5 μM, and the most active compound is substituted at the 4′ position of the aniline by a bromine atom (compound **31**, $IC_{50}=3.4$ μM).

Table 4. Enzymatic activities against VEGFR-2[a] for compounds **1**, **42**, and derivatives belonging to series A and B.

Series A			Series B		
Compd	Inhibition [%][b]	IC_{50} [μM][c]	Compd	Inhibition [%][b]	IC_{50} [μM][c]
1	10.8	>5	**42**	50.0	5.0
9	0	>5	**27**	59.7	4.2
10	0	>5	**28**	57.0	3.7
11	0	>5	**29**	40.6	>5
12	55.3	4.7	**30**	33.4	>5
13	0	>5	**31**	63.2	3.4
14	0	>5	**32**	28.5	>5
15	0	>5	**33**	30.7	>5
16	0	>5	**34**	0	>5
17	0	>5	**35**	50.2	4.7
18	13.1	>5	**36**	31.0	>5
19	15.5	>5	**37**	44.2	>5

[a] Inhibition of VEGFR-2 (recombinant human protein) tyrosine kinase activity. [b] Compounds tested at a concentration of 5 μM. [c] Values correspond to $n=3$ (SD < 10%).

Molecular modeling

To better understand the binding modes of these inhibitors, a model of compounds belonging to series A (compounds **10**, **16**, and **19**) interacting with the ATP site of EGFR was conceived.[27] The model used the reported crystal structure of the EGFR domain in complex with Tarceva[20,28] and previously described procedures[29–31] in which the binding site was defined by a sphere of 10 Å radius around the center of the co-crystallized EGFR–Tarceva structure. The most active (compounds **10** and **19**) and least active (compound **16**) molecules were docked into this active site and compared with the binding mode already published for Tarceva and Iressa. Tarceva and Iressa lie with the N1-/C8-containing edge of the quinazoline ring directed toward the peptide segment with the ether linkages projecting into solvent, and the anilino substituent, at the opposite end of the molecule, sequestered into a hydrophobic pocket in the enzyme (Figure 1 a). The N1 nitrogen atom of the quinazoline ring accepts an H bond from the Met 769 amide nitrogen. The other quinazoline nitrogen atom (N3) is not within H-bonding distance of the Thr 766 side chain; a water molecule is thought to bridge this gap.

Compounds **10** and **19** appear to bind to the active site in a similar way (Figure 1 b), with their aniline rings protruding into the hydrophobic pocket, the N1 atom of the quinazoline rings being hydrogen bonded to the backbone nitrogen atom of Met 769, whereas the N3 atom is hydrogen bonded to the side chain hydroxy group of Thr 766 through a water molecule. However, compound **16** appears to bind to the active site in another manner (Figure 1 c). In compound **16**, the N1 nitrogen atom of the quinazoline ring forms a hydrogen bond with Thr 766 (through a water molecule), and the N3 atom accepts an H bond from Met 769. Furthermore, the hydrophilic 4-COOH anilino moiety cannot interact with the hydrophobic region of the receptor. Consequently, the ether side chains occupy this active site, and the anilino moiety faces the solvent-exposed region. These opposing directions of the anilino group may explain the important loss of activity observed with compound **16**, and a similar hypothesis can also be made when trying to understand the poor activity of compound **15**.

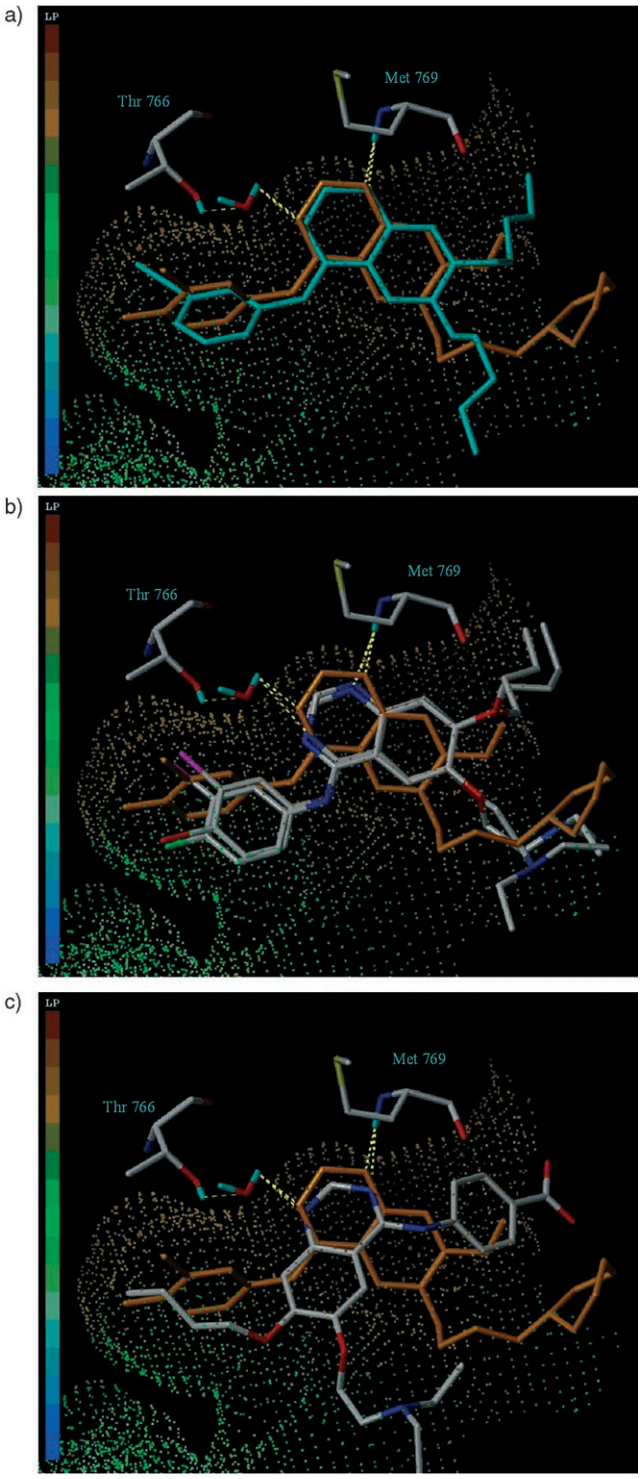

Figure 1. Binding mode of compound **1** (Iressa), Tarceva, and compounds **10**, **16**, and **19** to the ATP site of EGFR. In each case, hydrophobic regions are shown in brown. a) Active site of EGFR with Iressa (orange) and Tarceva (cyan) docked. b) Active site of EGFR with Iressa (orange) and compounds **10** and **19** docked. c) Active site of EGFR with Iressa (orange) and compound **16** docked.

Cellular activity

The antiproliferative activity of compounds **1**, **42**, **9**–**19** (series A), and **27**–**37** (series B) was evaluated against human androgen-independent prostate carcinoma PC3 cell line (Figure 2). Compound **1** has an inhibitory effect on the growth of PC3 cells (52% proliferation at 10 μM) and this activity is augmented when the quinazoline is substituted at the 7-position by a morpholino side chain (compound **42**) (37.8% proliferation at 10 μM). Compounds **10** and **28**, which only differ from compound **1** by the butoxy and diethylaminoethoxy side chains, are more effective than the morpholino compounds (0% proliferation at 10 μM). An aniline ring lacking substituents maintains antiproliferative activity, as demonstrated by compounds **9** and **27**, which abolished cell proliferation at 10 μM. However, chloro- or bromo-anilinoquinazolines provide the most effective antiproliferative agents because most of them decrease PC3 proliferation at 1 μM, whereas carboxylic acid derivatives are less active (compounds **15**, **33** and **34**). These results show that most of these analogues are effective antiproliferative compounds.

Cell-cycle analysis and detection of apoptosis

To examine whether the growth inhibition induced by the different compounds is associated with cell-cycle regulation, the cell-cycle distribution of PC3 cells was analyzed by using flow cytometry (Tables 5 and 6). Compounds that increased the sub-G1 fraction (indicative of cell death) by at least 10% were analyzed with the TUNEL assay, which detects the apoptotic cell population, but not necrotic cells (Figure 3).

The reference compound (compound **1**) caused an accumulation of cells in the G1 phase (57.8%), whereas the isomeric compound **42** produced a sub-G1 fraction at an equivalent concentration (10 μM). The sub-G1 peak is in agreement with the apoptosis index (62%, Figure 3) induced by compound **42** at 10 μM. A higher concentration (50 μM) of compound **1** is re-

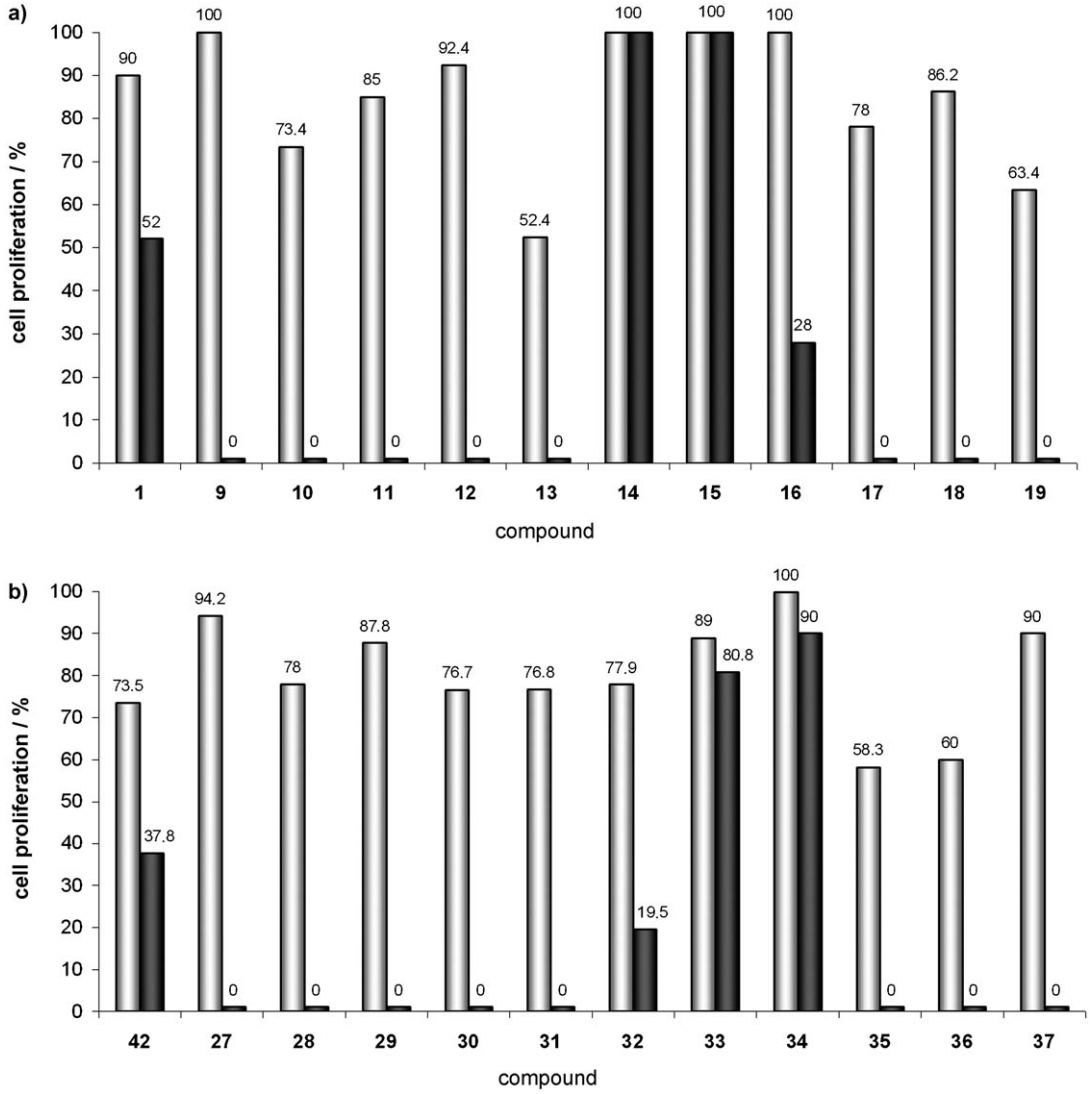

Figure 2. Activity of a) compounds **1** and **9**–**19** (series A), and b) compounds **27**–**37** (series B) and **42** on the proliferation of the PC3 cancer cell line. Light bars: 1 μM; dark bars: 10 μM. Cell proliferation was measured by MTT assay from at least three independent determinations (SD < 10%).

Table 5. Inhibitory effect of compounds **1** and **9**–**19** on the cell-cycle progression of PC3 cells.[a]

Compd	Population in cell-cycle phase [%] Sub-G1	G1	S	G2–M
untreated control	1.5	53.4	15.3	30.4
1	1.0	57.8	14.1	27.5
9	26.6	29.6	11.4	33.5
10	98.1	0.9	0.4	0.6
11	94.7	4.2	0.9	0.2
12	96.6	2.0	1.2	0.1
13	67.2	13.4	10.2	9.0
14	nd[b]	nd	nd	nd
15	nd	nd	nd	nd
16	49.3	27.3	8.7	10.1
17	95.8	3.0	0.9	0.4
18	99.1	0.2	0.5	0.1
19	98.3	0.9	0.5	0.3

[a] Flow cytometric cell-cycle analysis; compound concentration: 10 μM. [b] nd = not determined.

Table 6. Inhibitory effect of compounds **42** and **27**–**37** on the cell-cycle progression of PC3 cells.[a]

Compd	Population in cell-cycle phase [%] Sub-G1	G1	S	G2–M
untreated control	1.5	53.4	15.3	30.4
42	22.2	34.9	12.4	32.3
27	4.8	52.8	13.2	29.5
28	19.5	49.9	8.2	22.8
29	41.1	29.0	7.8	21.0
30	14.4	41.1	10.5	34.3
31	30.6	29.7	13.9	26.6
32	15.2	51.3	9.7	23.8
33	2.0	54.0	14.7	29.4
34	0.9	52.3	13.6	27.7
35	49.3	24.8	7.3	19.2
36	27.2	38.1	7.9	27.3
37	58.4	21.0	4.2	16.7

[a] Flow cytometric cell cycle analysis; compound concentration: 10 μM.

quired to induce a smaller effect (apoptosis index: 20.5 %, data not shown).

The effect of compounds at 5 μM (data not shown) belonging to series A and B was determined by flow cytometry and show that only derivatives of series A increased the sub-G1 fraction. 6-Diethylaminoethoxy-7-butoxy compounds (series A) are active at lower concentrations than their isomeric counterparts (6-butoxy-7-diethylaminoethoxy) (series B). For the TUNEL assay, compounds belonging to series A were evaluated at 5 μM, whereas those of series B were tested at 10 μM.

Results of PC3 cell-cycle distribution after treatment with compounds at 10 μM are compared in Tables 5 and 6. Because PC3 proliferation was not affected by compounds **14** and **15**, their activity was not determined. Most of the compounds produced an increase of hypodiploid cells (sub-G1 phase). Compounds **10** and **28**, which only differ from the morpholino compounds by the butoxy and diethylaminoethoxy side chains, possess a high apoptosis index. The positions of the side chains have, however, no impact on the increase of the sub-G1 phase. As unsubstituted compounds (compounds **9** and **27**) are inactive, we can conclude that substitution of the aniline ring contributes to proapoptotic activity. Amongst the substituted compounds, 3-bromo derivatives are very effective (com-

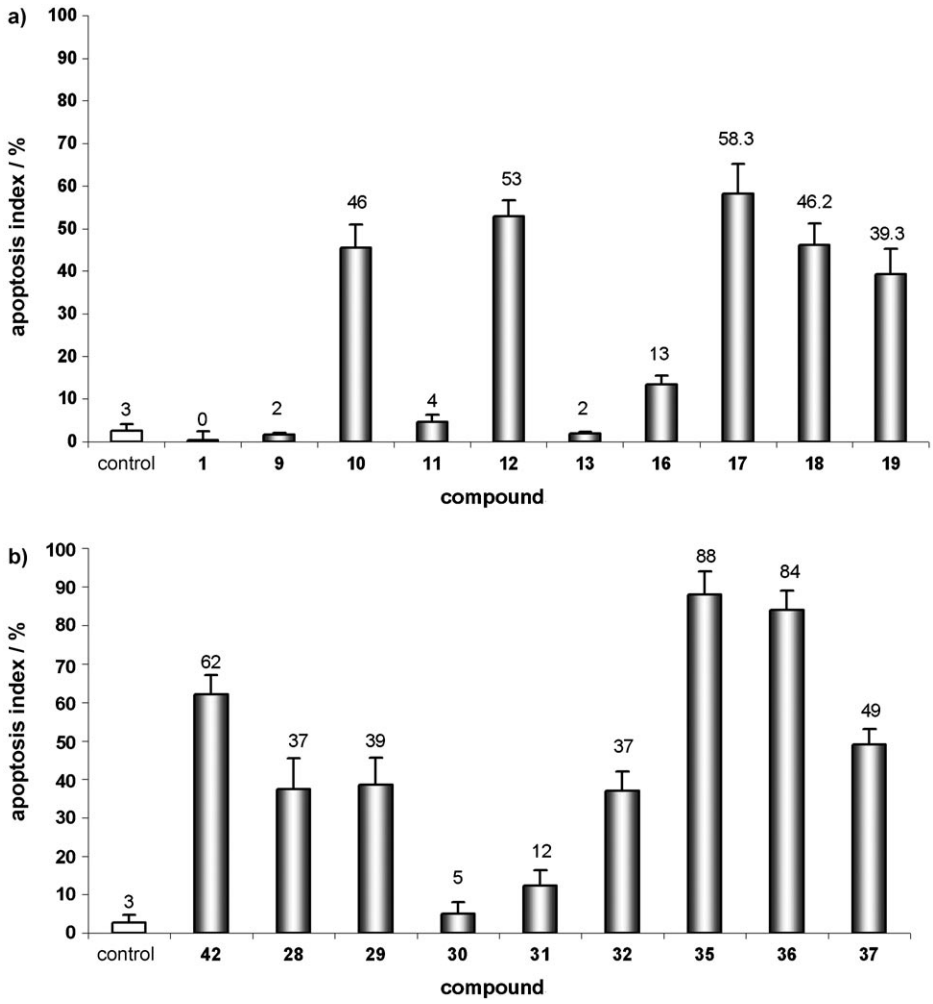

Figure 3. Apoptosis index induced a) by 5 μM of compound **1** and compounds belonging to series A, and b) by 10 μM of compound **42** and compounds belonging to series B on PC3 cell line.

pounds **17**, **18**, **35**, and **36**), 4-bromo compounds do not induce apoptosis (compounds **13** and **31**), and activity is restored in chloro-bromo-substituted compounds (compounds **19** and **37**). Carboxylic acid substitution, however, provides compounds that have no effect on either cell-cycle progression or apoptosis.

The G1 block induced by compound **1** has been well documented for different solid tumors, including head and neck squamous carcinoma cells[32] and prostate cancer cells.[11] Compound **1** decreases $p27^{Kip1}$ degradation by the ubiquitin proteasome pathway, leading to accumulation of cyclin-dependent kinase inhibitor (CDKI), inactivation of CDK2, and G1-phase cell-cycle arrest. This mechanism was elucidated using DU-145 androgen-independent cells, but has not been described previously for PC3 cells. Moreover, the proapoptotic role of compound **1** can be explained by different mechanisms. For example, it inhibits the expression of the antiapoptotic protein Bcl-2 in prostate cancer cells[33] and, by EGFR blockade and consequently MAPK pathway inactivation, restores the proapoptotic function of Bad in mammary cancer cells.[34] Other studies have shown that compound **1** increases the expression of Bax without affecting its mRNA levels.[35] In addition, compound **1** promotes Bax cleavage, thus producing the active form of Bax.

In summary, the novel and active compounds described herein can be considered as apoptosis inducers. Therefore, great interest will be placed on the future study of their effects on the expression of pro- and anti-apoptotic cell proteins.

Activation of Akt

As most of these new derivatives induce cell death, the active (phosphorylated) form of Akt, a central protein of the cell-survival pathway,[36] was also studied. Influence of the position and nature of the side chains (morpholino or butoxy and diethylaminoethoxy) can be carried out by comparing the effects of compounds **1**, **10**, **28**, and **42**. The chloro- and bromo-substituted compounds (compounds **12**, **19**, **30**, and **37**) that showed the greatest activity in apoptosis and in the tyrosine kinase assays were also examined. All compounds were evaluated at concentrations of 1 μM and 10 μM.

Akt is phosphorylated in PC3 cells after 10 min incubation with EGF (20 ng mL^{-1}, Figure 4). Compound **1** at 1 μM attenuates Akt phosphorylation, whereas the isomeric compound **42** must be present at 10 μM to observe such an effect. A concentration of 10 μM is also required for compounds **10**, **19**, **28**, and **37** to decrease Akt phosphorylation. Compounds **12** and **30** decrease the phosphorylated (active) form of Akt at concentrations of 1 μM and higher. These results agree with published data. The phosphorylation of Akt is inhibited by compound **1** in human esophageal squamous cell carcinoma,[37] in nasopharyngeal cancer cells,[38] and in human neuroblastomas.[39] Our results are important because PC3 cells are PTEN-deficient and consequently show constitutively activated Akt.[40] PTEN is a phosphatase that antagonizes PI3K activity and regulates Akt activation. Thus the compounds described herein are able to decrease Akt phosphorylation and have an impact on the cell-survival pathway.

COX-2 expression

COX-2 is known to play a crucial role in carcinogenesis, and is overexpressed in many malignant and pre-malignant organs such as the prostate.[41] As its expression is partially regulated through the EGFR pathway,[42] western-blot analysis was performed after treatment of PC3 cells with compounds **1**, **10**, **12**, **19**, **28**, **30**, **37**, and **42** (at 1 and 10 μM, Figure 5). Published data[43,44] report a decrease of COX-2 expression after treatment with compound **1**, and our results confirm this observation. The isomeric compound **42** is less active than the reference compound **1**. However, it is important to note that compound **10** is active from 1 μM, whereas a higher concentration (10 μM) of compound **1** is required for activity. In summary, a basic side chain is preferred at the 6-position to influence COX-2 expression, and fluoro–chloro compounds manifest better activity than the chloro-substituted and chloro-bromo-substituted derivatives.

Invasion assay

EGFR is involved in the invasion process through different mechanisms, including COX-2 expression and activation of the PI3K/Akt pathway. The capacity of compounds **1**, **10**, **12**, **19**, **28**, **30**, **37**, and **42** (1 and 10 μM) to decrease invasion of PC3 cells was determined (Figure 6). Compound **1** is only slightly active at 1 μM in this assay, but decreases the invasion of PC3 cells by approximately 50% at 10 μM. These results agree with published reports in which compound **1** was found to inhibit

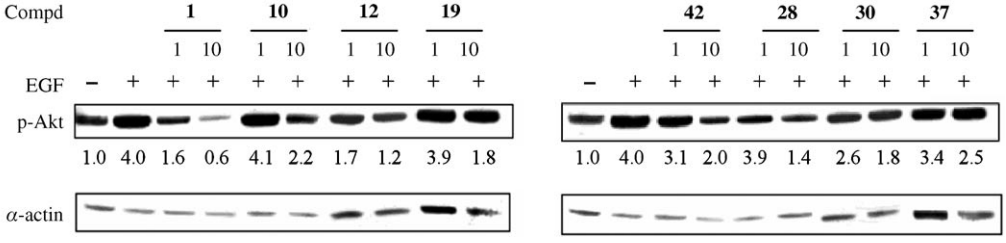

Figure 4. Akt activation in PC3 cells after treatment with compounds **1**, **42**, compounds belonging to series A (**10**, **12**, and **19**), and compounds belonging to series B (**28**, **30**, and **37**) at 1 and 10 μM (as indicated). Quantitation of bands was done by densitometric analysis, and is shown as the fold change normalized to α-actin.

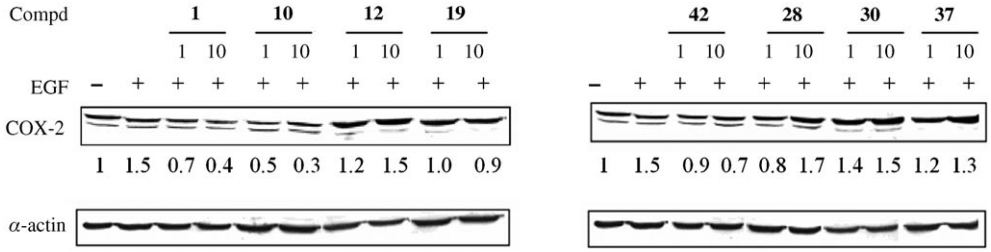

Figure 5. Expression of COX-2 in PC3 cells after treatment with compounds **1**, **42**, compounds belonging to series A (**10**, **12**, and **19**), and compounds belonging to series B (**28**, **30**, and **37**) at 1 and 10 μM (as indicated). Quantitation of bands was done by densitometric analysis, and is shown as the fold change normalized to α-actin.

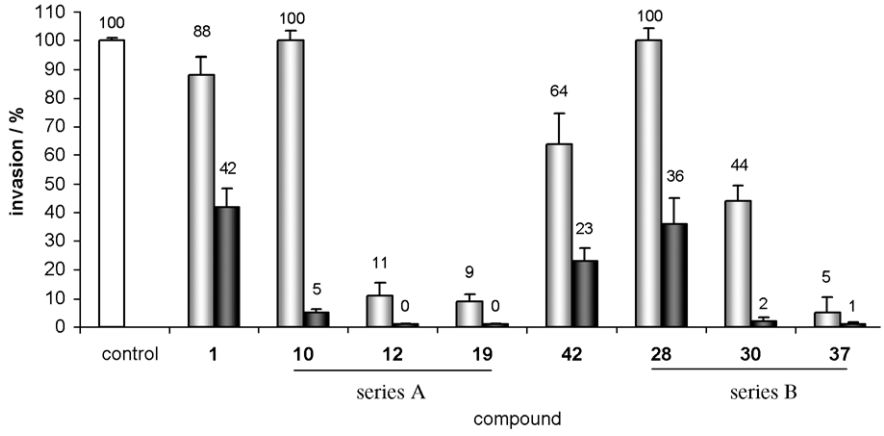

Figure 6. Inhibition of PC3 cell invasion by compounds **1**, **42**, compounds belonging to series A (**10**, **12**, and **19**), and compounds belonging to series B (**28**, **30**, and **37**) at 1 μM (light bars) and 10 μM (dark bars).

invasion of pancreatic cells,[45] ovarian clear-cell adenocarcinoma,[46] and prostate cancer cells.[12,47] The isomeric compound **42** shows better activity than the reference compound and decreases invasion of PC3 cells by 77% at a concentration of 10 μM. Compounds **10** and **28** are as effective as the morpholino compounds. However, it should be noted that the chloro (compounds **12** and **30**) and bromo–chloro derivatives (compounds **19** and **37**) are more active than the chloro–fluoro compounds (compounds **1**, **10**, **28**, and **42**). Activity, therefore, may depend on the aniline substitutions, but less on the nature of the side chain.

Conclusions

Twenty-three anilinoquinazolines, analogues of Iressa, were synthesized, and certain structure–activity relationships have been proposed. Suppression of the side chains on the quinazoline ring leads to a loss of activity, but substitution by a diethylamino side chain confers EGFR inhibition. A basic side chain is preferred at the 6-position (series A) for EGFR activity, although the isomers of series B gave better tyrosine kinase inhibitors than EGFR inhibitors. As these compounds are able to block tyrosine kinases present in PC3 membranes and because a basic side chain at the 7-position has previously been described to produce potent VEGFR tyrosine kinase inhibitors,[21–24] VEGFR-2 was considered as a target. Our results show that some derivatives of series B are VEGFR-2 tyrosine kinase inhibitors. Although the different compounds are less potent EGFR inhibitors than Iressa, most of the derivatives described herein (except for compounds bearing a carboxylic group) decrease PC3 proliferation and invasion. Moreover, they are interesting powerful inducers of apoptosis, and this is of particular interest because PC3 cells possess a constitutively active antiapoptotic Akt. Finally, certain compounds, particularly compound **10**, have been found to downregulate Akt activation and COX-2 expression, and this could be of therapeutic significance as these two proteins play major roles in cancer cell development and the acquisition of hormone independence.[48] Additional studies (DNA microarray and quantitative PCR) are expected to help elucidate the mechanism of action of these proapoptotic derivatives.

Experimental Section

General methods: Melting points were determined in open capillary tubes on a Büchi B-530 melting point apparatus and are uncorrected. Analytical thin-layer chromatography (TLC) was performed on precoated Kieselgel 60 F_{254} plates (Merck); compounds were visualized by UV light and/or with iodine. Column chromatography was performed with silica gel Kieselgel Si 60, 0.063–0.200 mm (Merck). All reagents and solvents obtained from commercial suppliers were used without further purification. The structures of all compounds were supported by IR (using a Bruker VECTOR 22 instrument) and ^{1}H NMR spectra were recorded using a Bruker AC 300P spectrometer in [D_6]DMSO or in $CDCl_3$ at ambient temperature. Chemical shifts (δ) are reported in ppm referenced to tetramethylsilane, *J* values are in Hertz, and the splitting patterns are designed as follows: s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet. APCI+ (atmospheric pressure chemical ionization) mass spectra were obtained on an LC–MS system, Thermo Electron Surveyor MSQ. Compounds **1** (Iressa) and **2** were synthesized according to described procedures.[49,50]

Methyl-4-butoxy-3-hydroxybenzoate (4): Potassium carbonate (8.30 g, 0.06 mol) was added to a solution of **2** (10 g, 0.06 mol) in acetone (400 mL). The mixture was stirred 15 min, and then a solution of 1-iodobutane (7 mL, 0.06 mol) in acetone (100 mL) was added slowly dropwise. The mixture was stirred 2 days at room temperature and filtered. The filtrate was concentrated in vacuo, and the oily residue was washed with water. The resulting precipitate was collected, washed successively with water and petroleum ether, and dried in vacuo. Recrystallization from cyclohexane gave **4** (8.62 g, 64%) as white crystals, mp: 113–115 °C. IR: $\tilde{\nu}$ = 3700–3000 (OH), 1699 cm^{-1} (CO); ^1H NMR ($CDCl_3$): δ = 0.99 (t, 3H, J = 7.40 Hz), 1.52 (m, 2H), 1.84 (m, 2H), 3.88 (s, 3H), 4.11 (t, 2H, J = 6.45 Hz), 5.70 (s, 1H), 6.88 (d, 1H, J = 9.10 Hz), 7.58–7.64 ppm (m, 2H); anal. ($C_{12}H_{16}O_4$) C, H.

Methyl-4-butoxy-3-(2-diethylaminoethoxy)benzoate hydrochloride (5): A mixture of **4** (5.47 g, 0.024 mol), potassium carbonate (16.6 g, 0.122 mol), and 2-diethylaminoethyl chloride hydrochloride (8.6 g, 0.048 mol) in acetone (80 mL) was held at reflux for 16 h. The inorganic solid was filtered off, and the filtrate was concentrated in vacuo. The oily residue was dissolved in diethyl ether (50 mL) and a solution of diethyl ether saturated with gaseous HCl was added (30 mL). The resulting precipitate was collected, washed with diethyl ether, and dried in vacuo to afford **5** (8.78 g, 100%) as an HCl salt (a white solid), mp: 148.5–150 °C. IR: $\tilde{\nu}$ = 2588 and 2488 (NH), 1716 cm^{-1} (CO); ^1H NMR ($CDCl_3$): δ = 0.99 (t, 3H, J = 7.20 Hz), 1.42–1.54 (m, 8H), 1.83 (m, 2H), 3.33 (m, 4H), 3.53 (t, 2H, J = 4.60 Hz), 3.89 (s, 3H), 4.05 (t, 2H, J = 6.70 Hz), 4.53 (t, 2H, J = 4.60 Hz), 6.89 (d, 1H, J = 8.70 Hz), 7.55 (d, 1H, J = 2.0 Hz), 7.64 (dd, 1H, J = 8.70 and 2.0 Hz), 12.46 ppm (s, 1H); anal. ($C_{18}H_{30}ClNO_4$) C, H, N.

Methyl-4-butoxy-5-(2-diethylaminoethoxy)-2-nitrobenzoate hydrochloride (6): A solution of tin(IV) chloride (2.7 mL, 0.025 mol) and fuming nitric acid (1 mL, 0.025 mol) in CH_2Cl_2 (30 mL) was added dropwise to a solution of **5** (3 g, 0.0083 mol) in CH_2Cl_2 (111 mL) cooled at −25 °C. After stirring at −25 °C for 5 h, water (75 mL) was added. The layers were separated by decantation. The aqueous layer was extracted with CH_2Cl_2. The combined organic layers were washed with saturated sodium bicarbonate solution, dried over magnesium sulfate, and concentrated in vacuo. The oily residue was dissolved in diethyl ether and a solution of diethyl ether saturated with gaseous HCl (30 mL) was added. The resulting precipitate was collected, washed with diethyl ether, and dried in vacuo to provide compound **6** (2.20 g, 65%) as an HCl salt (a white solid), mp: 124.2–124.8 °C. IR: $\tilde{\nu}$ = 1728 (CO), 1520 cm^{-1} (NO_2); ^1H NMR ($CDCl_3$): δ = 0.99 (t, 3H, J = 7.57 Hz), 1.45 (m, 8H), 1.80 (m, 2H), 3.25 (m, 4H), 3.55 (m, 2H), 3.85 (s, 3H), 4.05 (t, 3H, J = 6.62 Hz), 4.62 (m, 2H), 7.10 (s, 1H), 7.38 ppm (s, 1H); anal. ($C_{18}H_{29}ClN_2O_6$) C, H, N.

Methyl-2-amino-4-butoxy-5-(2-diethylaminoethoxy)benzoate (7): A suspension of **6** (1 g, 0.0025 mol) in HCl (concd, 25 mL) was heated at 50–60 °C for 5 min. A solution of tin(II) chloride (2.33 g, 0.015 mol) in HCl (concd, 20 mL) was added dropwise. The reaction mixture was heated at 100 °C for 45 min. The solid formed was collected and dissolved in water (300 mL). A solution of sodium hydroxide (2 N) was added to obtain pH 8–9. The aqueous solution was then extracted with ethyl acetate (3×100 mL). The combined organic layers were dried over magnesium sulfate and concentrated in vacuo to give **7** (0.78 g, 91%) as a brown oil. IR: $\tilde{\nu}$ = 3571 (NH_2), 1684 cm^{-1} (CO); ^1H NMR ($CDCl_3$): δ = 0.95 (t, 3H, J = 7.22 Hz), 1.12 (t, 6H, J = 7.22 Hz), 1.48 (m, 2H), 1.80 (m, 2H), 2.52 (q, 4H, J = 7.22 Hz), 2.85 (t, 2H, J = 6.24 Hz), 3.80 (s, 3H), 3.95 (m, 4H), 5.55 (s, 2H), 6.10 (s, 1H), 7.32 ppm (s, 1H); anal. ($C_{18}H_{30}N_2O_4$) C, H, N.

7-Butoxy-6-(2-diethylaminoethoxy)-3*H*-quinazolin-4-one (8): A mixture of **7** (1 g, 0.003 mol), ammonium formate (0.9 g, 0.009 mol), and formamide (1 mL, 0.015 mol) was heated at 140 °C for 16 h. The mixture reaction was hydrolyzed with water (50 mL) and extracted with CH_2Cl_2. The separated aqueous layer was neutralized with a saturated solution of potassium carbonate. The resulting precipitate was collected to afford **8** (0.66 g, 66%) as a white solid, mp: 201.8–202.2 °C. IR: $\tilde{\nu}$ = 1659 (CO), 1611 cm^{-1} (NH); ^1H NMR ($CDCl_3$): δ = 0.99 (t, 3H, J = 7.22 Hz), 1.10 (t, 6H, J = 7.22 Hz), 1.52 (m, 2H), 1.92 (m, 2H), 2.68 (q, 4H, J = 7.22 Hz), 2.98 (t, 2H, J = 5.58 Hz), 4.10 (m, 2H), 4.20 (m, 2H), 7.10 (s, 1H), 7.55 (s, 1H), 7.98 ppm (s, 1H); anal. ($C_{18}H_{27}N_3O_3$) C, H, N.

General procedure for the preparation of anilinoquinazolines 9–19: A mixture of **8** (0.10 g, 0.30 mmol), phosphorus pentachloride (0.75 g, 0.36 mmol), and phosphorus oxychloride (5 mL, 9 mmol) was held at reflux for 16 h. The mixture was cooled to room temperature, and the excess of phosphorus oxychloride was evaporated under reduced pressure. The residue was dissolved in *i*PrOH (3 mL) and the corresponding aniline was added. The reaction mixture was held at reflux for 2 h.

4-Anilino-7-butoxy-6-(2-diethylaminoethoxy)quinazoline hydrochloride (9): The reaction mixture was concentrated in vacuo, and the residue was purified by column chromatography on silica gel, eluting with CH_2Cl_2/MeOH (9:1) saturated with NH_3 to yield **9** (135 mg, 30%) as an HCl salt (a yellow solid), mp: 189.5–192 °C. IR: $\tilde{\nu}$ = 3380 cm^{-1} (NH); ^1H NMR ($CDCl_3$): δ = 0.98 (t, 3H, J = 7.18 Hz), 1.05 (t, 6H, J = 7.18 Hz), 1.48 (q, 2H, J = 7.69 Hz, J = 7.18 Hz), 1.78 (m, 2H), 2.60 (q, 4H, J = 7.18 Hz), 2.85 (t, 2H, J = 6.16 Hz), 3.98 (t, 2H, J = 6.66 Hz), 4.12 (m, 2H), 4.6 (m, 1H), 7.08 (m, 2H), 7.25 (m, 2H), 7.48 (s, 1H), 7.70 (d, 2H, J = 7.7 Hz), 8.42 (s, 1H), 8.60 ppm (s, 1H); LC–MS (APCI$^+$) *m/z* 409.2–411.2 [MH^+]; anal. ($C_{24}H_{33}ClN_4O_2$) C, H, N.

4-(3-Chloro-4-fluoroanilino)-7-butoxy-6-(2-diethylaminoethoxy)-quinazoline hydrochloride (10): The reaction mixture was concentrated under reduced pressure, and the residue was treated with petroleum ether. The oily residue was dissolved in diethyl ether, and a solution of diethyl ether saturated with gaseous HCl was added to afford **10** (150 mg, 50%) as an HCl salt (a brown solid), mp: 172.7–175 °C. IR: $\tilde{\nu}$ = 3600–3200 (NH), 1224 (C–F), 1064 cm^{-1} (C–Cl); ^1H NMR (DMSO): δ = 0.98 (t, 3H, J = 7.48 Hz), 1.30 (t, 6H, J = 7.11 Hz), 1.50 (m, 2H), 1.80 (m, 2H), 3.30 (m, 4H), 3.65 (m, 4H), 4.18 (t, 2H, J = 5.99 Hz), 4.70 (m, 2H), 7.38 (s, 1H), 7.55 (m, 1H), 7.82 (m, 1H), 8.10 (m, 1H), 8.62 (s, 1H), 8.85 ppm (s, 1H); LC–MS (APCI$^+$) *m/z* 461.6–463.6 [MH^+]; anal. ($C_{24}H_{31}Cl_2FN_4O_2$) C, H, N.

4-(3-Chloroanilino)-7-butoxy-6-(2-diethylaminoethoxy)quinazoline hydrochloride (11): The reaction mixture was concentrated in vacuo. The residue was dissolved in ethanol, and petroleum ether was added slowly dropwise. The resulting precipitate was collected to yield **11** (160 mg, 100%) as an HCl salt (a brown solid), mp: 203.5–205.5 °C. IR 3432–3200 and 1633 (NH), 1068 cm^{-1} (C–Cl); ^1H NMR (DMSO): δ = 0.98 (t, 3H, J = 7.24 Hz), 1.30 (t, 6H, J = 7.24 Hz), 1.50 (m, 2H), 1.80 (m, 2H), 3,30 (m, 4H), 3.65 (m, 2H), 4.20 (m, 2H), 4.70 (m, 2H), 7.38 (d, 1H, J = 7,24 Hz), 7.45 (s, 1H), 7.52 (t, 1H, J = 7.76 Hz), 7.70 (d, 1H, J = 7.76 Hz), 7.90 (s, 1H), 8.70 (s, 1H), 8.90 (s, 1H), 10.80 (m, 1H), 11.90 ppm (s, 1H); LC–MS (APCI$^+$) *m/z* 443.5 [MH^+]; anal. ($C_{24}H_{32}Cl_2N_4O_2$) C, H, N.

4-(4-Chloroanilino)-7-butoxy-6-(2-diethylaminoethoxy)quinazoline hydrochloride (12): The reaction mixture was concentrated in vacuo, and the residue was purified by column chromatography on silica gel, eluting with CH_2Cl_2/MeOH (8:2) to yield **12** (120 mg, 60%) as an HCl salt (a yellow solid), mp: 168.5–170.3 °C. IR: $\tilde{\nu}$ =

3600–3300 and 1633 (NH), 1067 cm^{-1} (C–Cl); ^1H NMR (DMSO): $\delta =$ 0.98 (t, 3H, $J = 7.30$ Hz), 1.30 (t, 6H, $J = 7.30$ Hz), 1.50 (m, 2H), 1.80 (m, 2H), 3.30 (m, 6H), 4.18 (t, 2H, $J = 6.26$ Hz), 4.70 (m, 2H), 7.42 (s, 1H), 7.55 (d, 2H, $J = 8.87$ Hz), 7.82 (d, 2H, $J = 8{,}87$ Hz), 8.62 (s, 1H), 8.85 (s, 1H), 10.65 (m, 1H), 11.75 ppm (s, 1H); LC–MS ($APCI^+$) *m/z* 443.4 [MH^+]. Anal ($C_{24}H_{32}Cl_2N_4O_2$) C, H, N.

4-(4-Bromoanilino)-7-butoxy-6-(2-diethylaminoethoxy)quinazoline hydrochloride (13): The reaction mixture was concentrated under reduced pressure. The residue was purified by column chromatography on silica gel, eluting with CH_2Cl_2/MeOH (9:1) to yield **13** (50 mg, 32%) as an HCl salt (a yellow solid), mp: 135–137 °C. IR: $\tilde{\nu} = 1638$ (NH), 1072–1046 cm^{-1} (C–Br); ^1H NMR (DMSO): $\delta = 0.98$ (t, 3H, $J = 7.30$ Hz), 1.28 (t, 6H, $J = 7.30$ Hz), 1.45 (m, 2H), 1.80 (m, 2H), 3.28 (m, 4H), 3.85 (m, 2H), 4.18 (m, 2H), 4.68 (m, 2H), 7.40 (s, 1H), 7.65 (d, 2H, $J = 8.80$ Hz), 7.75 (d, 2H, $J = 8.80$ Hz), 8.62 (s, 1H), 8.85 (s, 1H), 10.65 ppm (m, 1H); LC–MS ($APCI^+$) *m/z* 487.2–489.2 [MH^+]; anal. ($C_{24}H_{32}BrClN_4O_2$) C, H, N.

4-(4-Fluoroanilino)-7-butoxy-6-(2-diethylaminoethoxy)quinazoline hydrochloride (14): The reaction mixture was concentrated in vacuo. The residue was purified by column chromatography on silica gel, eluting with CH_2Cl_2/MeOH (9:1) to yield **14** (132 mg, 95%) as an HCl salt (a brown oil). IR: $\tilde{\nu} = 1600$ (NH), 1224 cm^{-1} (C–F); ^1H NMR (DMSO): $\delta = 0.98$ (m, 3H), 1.15 (m, 6H), 1.45 (m, 2H), 1.78 (m, 2H), 3.12 (m, 4H), 3.75 (m, 2H), 4.15 (m, 2H), 4.58 (m, 2H), 6.55 (m, 2H), 6.80 (m, 2H), 7.30 (s, 1H), 7.38 (s, 1H), 8.62 ppm (s, 1H); LC–MS ($APCI^+$) *m/z* 427.4 [MH^+]. Anal ($C_{24}H_{32}ClFN_4O_2$) C, H, N.

4-(4-Carboxy-3-chloroanilino)-7-butoxy-6-(2-diethylaminoethoxy)quinazoline hydrochloride 15): The reaction mixture was concentrated in vacuo. The residue was purified by column chromatography, eluting CH_2Cl_2/MeOH (7:3) to yield **15** (40 mg, 25%) as an HCl salt (a yellow solid), mp: 191–193 °C. IR: $\tilde{\nu} =$ 3600–3200 and 3100–2400 (OH), 1721 (C=O), 1633 cm^{-1} (NH); ^1H NMR (DMSO): $\delta =$ 0.98 (t, 3H, $J = 7{,}37$ Hz), 1.28 (t, 6H, $J = 7{,}37$ Hz), 1.50 (m, 2H), 1.80 (m, 2H), 3.30 (m, 2H), 3.65 (m, 4H), 4.18 (m, 2H), 4.65 (m, 2H), 7.35 (s, 1H), 7.95 (d, 2H, $J = 7{,}89$ Hz), 8.18 (s, 1H), 8.55 (s, 1H), 8.90 (s, 1H), 10.40 ppm (m, 1H); LC–MS ($APCI^+$) *m/z* 487.5–489.5 [MH^+]; anal. ($C_{25}H_{32}Cl_2N_4O_4$) C, H, N.

4-(4-Carboxyanilino)-7-butoxy-6-(2-diethylaminoethoxy)quinazoline hydrochloride (16): The reaction mixture was concentrated in vacuo. The residue was dissolved in CH_2Cl_2, and the resulting precipitate was collected to yield **16** (180 mg, 61%) as an HCl salt (a yellow solid), mp: 220–222 °C. IR: $\tilde{\nu} =$ 3400 and 3100–2400 (OH), 1709 (C=O), 1635 cm^{-1} (NH); ^1H NMR (DMSO): $\delta = 0.98$ (t, 3H, $J =$ 7,57 Hz), 1.30 (t, 6H, $J = 7{,}10$ Hz), 1.50 (m, 2H), 1.80 (m, 2H), 3.30 (m, 4H), 3.65 (m, 2H), 4.15 (m, 2H), 4.72 (m, 2H), 7.42 (s, 1H), 8,00 (m, 4H), 8.68 (s, 1H), 8.91 (s, 1H), 10.80 ppm (m, 1H); LC–MS ($APCI^+$) *m/z* 453.2 [MH^+]; anal. ($C_{25}H_{33}ClN_4O_4$) C, H, N.

4-(3-Bromoanilino)-7-butoxy-6-(2-diethylaminoethoxy)quinazoline hydrochloride (17): The reaction mixture was concentrated in vacuo. The residue was dissolved in CH_2Cl_2, and the resulting precipitate was collected to yield **17** (80 mg, 51%) as an HCl salt (a yellow solid), mp: 99–102 °C. IR: $\tilde{\nu} =$ 3100–2500 and 1632 (NH), 1072 cm^{-1} (C–Br); ^1H NMR (DMSO): $\delta = 0.94$ (t, 3H, $J = 7{,}50$ Hz), 1.27 (t, 6H, $J = 7{,}49$ Hz), 1.42 (m, 2H), 1.75 (m, 2H), 3.25–3.35 (m, 6H), 4.16 (m, 2H), 4.65–4.75 (m, 2H), 6.91 (m, 2H), 7.42 (s, 1H), 7.44 (m, 1H), 8.09 (s, 1H), 8.92 (s, 1H), 10.70–10.80 (m, 1H), 11.88 ppm (s, 1H); LC–MS ($APCI^+$) *m/z* 487.1–489.1 [MH^+]; anal. ($C_{24}H_{32}BrClN_4O_2$) C, H, N.

4-(3-Bromo-4-methylanilino)-7-butoxy-6-(2-diethylaminoethoxy)quinazoline hydrochloride (18): The solid was collected and afford **18** (70 mg, 43%) as an HCl salt (a brown solid), mp: 144.5–147.5 °C. IR: $\tilde{\nu} =$ 3000–2800 and 1633 cm^{-1} (NH); ^1H NMR (DMSO): $\delta = 0.94$ (t, 3H, $J = 7.21$ Hz), 1.27 (m, 9H), 1.44 (m, 2H), 1.77 (m, 2H), 3.60–3.70 (m, 6H), 4.16 (t, 2H, $J = 6{,}10$ Hz), 4.65–4.75 (m, 2H), 7.37 (s, 1H), 7.44 (d, 1H, $J = 8.32$ Hz), 7.71 (m, 1H), 8.07 (s, 1H), 8.55 (s, 1H), 8.85 (s, 1H), 10.55–10.65 (m, 1H), 11.45–11.55 ppm (m, 1H); LC–MS ($APCI^+$) *m/z* 503.1–504.1 [MH^+]; anal. ($C_{25}H_{34}BrClN_4O_2$) C, H, N.

4-(4-Bromo-3-chloroanilino)-7-butoxy-6-(2-diethylaminoethoxy)quinazoline hydrochloride (19): The solid was collected and afford **19** (140 mg, 83%) as an HCl salt (a yellow solid), mp: 163.5–166.5 °C. IR: $\tilde{\nu} =$ 1633 (NH), 1071 (C–Br), 1020 cm^{-1} (C–Cl); ^1H NMR (DMSO): $\delta =$ 0.90–1.00 (m, 3H), 1.25–1.35 (m, 6H), 1.43–1.53 (m, 2H), 1.71–1.85 (m, 2H), 3.25–3.40 (m, 6H), 4.15–4.25 (m, 2H), 4.70–4.80 (m, 2H), 7.42 (s, 1H), 7.80–7.95 (m, 2H), 8.25 (s, 1H), 8.70 (s, 1H), 8.82 (s, 1H), 10.55–10.70 (m, 1H), 11.65–11.80 ppm (m, 1H); LC–MS ($APCI^+$) *m/z* 521.0–523.1 [MH^+]; anal. ($C_{24}H_{31}BrCl_2N_4O_2$) C, H, N.

Methyl-4-benzyloxy-3-hydroxybenzoate (20): As described for **4**, recrystallization from diisopropyl ether gave **20** (10.42 g, 67%) as a light-yellow solid from compound **2** (10 g, 0.06 mol) in acetone (400 mL), potassium carbonate (8.30 g, 0.06 mol), and benzyl bromide (7 mL, 0.06 mol) in acetone (100 mL), mp: 127.6–129 °C. IR: $\tilde{\nu} =$ 3392 (OH), 1693 cm^{-1} (CO); ^1H NMR ($CDCl_3$): $\delta =$ 3.89 (s, 3H), 5.18 (s, 2H), 5.70 (s, 1H), 6.96 (d, 1H, $J = 8.20$ Hz), 7.38–7.47 (m, 5H), 7.58–7.64 ppm (m, 2H); anal. ($C_{15}H_{14}O_4$) C, H.

Methyl-4-benzyloxy-3-butoxybenzoate (21): Potassium carbonate (1.10 g, 0.0078 mol) was added to a solution of **20** (1 g, 0.0039 mol) in acetone (50 mL). The reaction mixture was stirred for 10 min, and 1-iodobutane (0.91 mL, 0.0078 mol) was added. The reaction mixture was held at reflux for 5 h. The inorganic solid was filtered off, and the filtrate was concentrated in vacuo. The resulting solid residue was washed successively with water and petroleum ether. Recrystallization from ethanol/water (95:5) gave **21** (1.18 g, 96%) as white crystals, mp: 54.7–55.8 °C. IR: $\tilde{\nu} = 1716$ cm^{-1} (CO); ^1H NMR ($CDCl_3$): $\delta = 1.01$ (t, 3H, $J = 7.75$ Hz), 1.55 (m, 2H), 1.86 (m, 2H), 3.89 (s, 3H), 4.09 (t, 2H, $J = 6.70$ Hz), 5.20 (s, 2H), 6.92 (d, 1H, $J = 8.30$ Hz), 7.27–7.48 (m, 5H), 7.56–7.65 ppm (m, 2H); anal. ($C_{19}H_{22}O$) C, H.

Methyl-3-butoxy-4-hydroxybenzoate (22): Pd/C (0.2 g) was added to a solution of **21** (1 g, 0.0032 mol) in methanol (50 mL). The reaction mixture was stirred under hydrogen atmosphere at room temperature for 2 days, filtered, and evaporated in vacuo. The oily residue was treated with petroleum ether, and the resulting precipitate was collected. Recrystallization from petroleum ether gave **22** (0.67 g, 94%) as white crystals, mp: 61–62.2 °C. IR: $\tilde{\nu} =$ 3405 (OH), 1701 cm^{-1} (CO); ^1H NMR ($CDCl_3$): $\delta = 0.99$ (t, 3H, $J = 7.30$ Hz), 1.50 (m, 2H), 1.82 (m, 2H), 3.88 (s, 3H), 4.10 (t, 2H, $J = 6.85$ Hz), 5.70 (s, 1H), 6.93 (d, 1H, $J = 8.30$ Hz), 7.54 (s, 1H), 7.62 ppm (d, 1H, $J =$ 8.30 Hz); anal. ($C_{12}H_{16}O_4$) C, H.

Methyl-3-butoxy-4-(2-diethylaminoethoxy)benzoate hydrochloride (23): Compound **23** was synthesized by using the same procedure as that for **5**, starting from compound **21** (2 g, 0.009 mol). Recrystallization from toluene gave white crystals (2.55 g, 91%), mp: 116–118 °C. IR: $\tilde{\nu} =$ 2608 and 1483 (NH^+), 1717 cm^{-1} (CO); ^1H NMR ($CDCl_3$): $\delta = 0.98$ (t, 3H, $J = 7.35$ Hz), 1.38–1.60 (m, 8H), 1.77 (m, 2H), 3.30 (m, 4H), 3.53 (m, 2H), 3.89 (s, 3H), 4.02 (m, 2H), 4.57 (t, 2H, $J = 4.20$ Hz), 6.89 (d, 1H, $J = 8.35$ Hz), 7.53 (d, 1H, $J = 2.00$ Hz), 7.63 (dd, 1H, $J = 8.35$ and 2.00 Hz), 12.44 ppm (s, 1H); anal. ($C_{18}H_{30}ClNO_4$) C, H, N.

Methyl-5-butoxy-4-(2-diethylaminoethoxy)-2-nitrobenzoate hydrochloride (24): Compound **24** was obtained by using the same procedure as that for **6**. Starting from **23** (5 g, 0.014 mol), a white solid was prepared (3.8 g, 67%), mp: 119–120 °C. IR: $\tilde{\nu}$ = 1734 (CO), 1530 cm^{-1} (NO_2); ^1H NMR ($CDCl_3$): δ = 1.00 (t, 3H, J = 7.30 Hz), 1.50 (m, 8H), 1.80 (m, 2H), 3.30 (q, 4H, J = 7.23 Hz), 3.60 (t, 2H, J = 6.24 Hz), 3.80 (s, 3H), 4.10 (t, 2H, J = 6.30 Hz), 4.60 (t, 2H, J = 6.24 Hz), 6.80 (s, 1H), 7.40 (s, 1H), 12.50 ppm (m, 1H); anal. ($C_{18}H_{29}ClN_2O_6$) C, H, N.

Methyl-2-amino-5-butoxy-4-(2-diethylaminoethoxy)benzoate (25): As described for **7**, **25** (0.33 g, 79%) was obtained as an oil from **24** (0.50 g, 0.0012 mol) and tin(II) chloride (0.93 g, 0.006 mol) in HCl (concd, 23 mL). IR: $\tilde{\nu}$ = 3480 and 3385 (NH_2), 1685 (CO), 1624 cm^{-1} (NH_2); ^1H NMR ($CDCl_3$): δ = 0.91 (t, 3H, J = 7.20 Hz), 1.01 (t, 6H, J = 7.20 Hz), 1.40 (m, 2H), 1.68 (m, 2H), 2.58 (m, 4H), 2.88 (t, 2H, J = 6.26 Hz), 3.85 (s, 3H), 3.88 (t, 2H, J = 6.57 Hz), 4.00 (t, 2H, J = 6.26 Hz), 5.50 (s, 2H), 6.10 (s, 1H), 7.30 ppm (s, 1H); anal. ($C_{18}H_{30}N_2O_4$) C, H, N.

6-Butoxy-7-(2-diethylaminoethoxy)-3*H*-quinazolin-4-one (26): Similar to the procedure described for **8**, the title compound (0.53 g, 60%) was prepared starting from **25** (0.89 g, 0.0026 mol) as a white solid, mp: 154–157 °C. IR: $\tilde{\nu}$ = 1689 (CO), 1609 cm^{-1} (NH); ^1H NMR ($CDCl_3$): δ = 0.95 (t, 3H, J = 7.20 Hz), 1.06 (t, 6H, J = 7.00 Hz), 1.45 (m, 2H), 1.80 (m, 2H), 2.64 (q, 4H, J = 7.18 Hz), 2.96 (t, 2H, J = 6.11 Hz), 4.08 (t, 2H, J = 6.46 Hz), 4.17 (t, 2H, J = 6.11 Hz), 7.11 (s, 1H), 7.54 (s, 1H), 8.01 ppm (s, 1H); anal. ($C_{18}H_{27}N_3O_3$) C, H, N.

General procedure for the preparation of anilinoquinazolines 27–37: A mixture **26** (0.10 g, 0.30 mmol), phosphorus pentachloride (0.75 g, 0.36 mmol) and phosphorus oxychloride (1.5 mL, 9 mmol) was held at reflux for 16 h. The mixture was cooled to room temperature, and the excess phosphorus oxychloride was evaporated under reduced pressure. The residue was dissolved in *i*PrOH (3 mL), and the corresponding aniline was added. The reaction mixture was held at reflux for 2 h.

4-Anilino-6-butoxy-7-(2-diethylaminoethoxy)quinazoline hydrochloride (27): The reaction mixture was concentrated in vacuo, and the residue was purified by column chromatography on silica gel, eluting with CH_2Cl_2 saturated with NH_3 to yield **27** (70 mg, 52%) as an HCl salt (a white solid), mp: 129–132 °C. IR 1618 cm^{-1} (NH); ^1H NMR (DMSO): δ = 0.98 (m, 9H), 1.46 (m, 2H), 1.75 (m, 2H), 2.56 (q, 4H, J = 7.03 Hz), 2.84 (m, 2H), 4.12 (m, 4H), 7.09 (m, 1H), 7.17 (s, 1H), 7.38 (m, 2H), 7.77 (m, 2H), 8.43 (s, 1H), 9.44 ppm (s, 1H); LC–MS ($APCI^+$) m/z 409–411 [MH^+]; anal. ($C_{24}H_{33}ClN_4O_2$) C, H, N.

4-(3-Chloro-4-fluoroanilino)-6-butoxy-7-(2-diethylaminoethoxy)-quinazoline hydrochloride (28): The reaction mixture was treated acetone, and the resulting precipitate was collected to afford **28** (90 mg, 60%) as an HCl salt (a white solid), mp: 203–205 °C. IR: $\tilde{\nu}$ = 1630 (NH), 1222 (C–F), 1064 cm^{-1} (C–Cl); ^1H NMR (DMSO): δ = 0.95 (t, 3H, J = 7.35 Hz), 1.25 (t, 6H, J = 7.32 Hz), 1.45 (m, 2H), 1.75 (m, 2H), 3.20 (m, 4H), 3.55–3.65 (m, 2H), 4.15 (t, 2H, J = 6.07 Hz), 4.50–4.60 (m, 2H), 7.25 (s, 1H), 7.40 (m, 1H), 7.75–7.95 (m, 1H), 8.10 (s, 1H), 8.17–8.25 (m, 1H), 8.55 (s, 1H), 10.18–10.28 (m, 1H), 10.60–10.70 ppm (m, 1H); LC–MS ($APCI^+$) m/z 461.4–463.74 [MH^+]; anal. ($C_{24}H_{31}Cl_2FN_4O_2$) C, H, N.

4-(3-Chloroanilino)-6-butoxy-7-(2-diethylaminoethoxy)quinazoline hydrochloride (29): The reaction mixture was concentrated in vacuo. The residue was purified by column chromatography on silica gel, eluting with CH_2Cl_2/MeOH (7:3) to yield **29** (120 mg, 83%) as an HCl salt (a brown solid), mp: 193–196 °C. IR: $\tilde{\nu}$ = 3600–3200 and 1633 (NH), 1070 cm^{-1} (C–Cl); ^1H NMR (DMSO): δ = 0.94 (t, 3H, J = 7.24 Hz), 1.29 (t, 6H, J = 7.24 Hz), 1.43 (m, 2H), 1.53 (m, 2H), 3.20–3.35 (m, 4H), 3.55–3.70 (m, 2H), 4.26 (t, 2H, J = 6,72 Hz), 4.55–4.70 (m, 2H), 7.32 (d, 1H, J = 8.27 Hz), 7.46–7.52 (m, 2H), 7.82 (d, 1H, J = 7.76 Hz), 8.0 (s, 1H), 8.65 (s, 1H), 8.85 (s, 1H), 11.20 (s, 1H), 11.80 ppm (s, 1H); LC–MS ($APCI^+$) m/z 443.4 [MH^+]; anal. ($C_{24}H_{32}Cl_2N_4O_2$) C, H, N.

4-(4-Chloroanilino)-6-butoxy-7-(2-diethylaminoethoxy)quinazoline hydrochloride (30): The reaction mixture was concentrated in vacuo, and the residue was treated with acetone. The resulting precipitate was collected to yield **30** (70 mg, 49%) as an HCl salt (a brown solid), mp: 186–189 °C. IR: $\tilde{\nu}$ = 1067 cm^{-1} (C–Cl); ^1H NMR (DMSO): δ = 0.95 (t, 3H, J = 7.28 Hz), 1.29 (t, 6H, J = 7.15 Hz), 1.48 (m, 2H), 1.77 (m, 2H), 3.28 (m, 4H), 3.55–3.70 (m, 2H), 4.25 (m, 2H), 4.55–4.70 (m, 2H), 7.53 (s, 1H), 7.56 (d, 2H, J = 8.79 Hz), 7.80 (d, 2H, J = 8.79 Hz), 8.60 (s, 1H), 8.85 (s, 1H), 10.95–11.05 ppm (m, 1H); LC–MS ($APCI^+$) m/z 443.4 [MH^+]; anal. ($C_{24}H_{32}Cl_2N_4O_2$) C, H, N.

4-(4-Bromoanilino)-6-butoxy-7-(2-diethylaminoethoxy)quinazoline hydrochloride (31): The reaction mixture was concentrated under reduced pressure. The residue was purified by column chromatography on silica gel, eluting with CH_2Cl_2/MeOH (8:2) to yield **31** (140 mg, 89%) as an HCl salt (a yellow solid), mp: 193–195 °C. IR: $\tilde{\nu}$ = 1632 (NH), 1077 cm^{-1} (C–Br); ^1H NMR (DMSO): δ = 0.98 (t, 3H, J = 7.30 Hz), 1.18 (t, 3H, J = 7.30 Hz), 1.45 (m, 2H), 1.75 (m, 2H), 3.55–3.70 (m, 4H), 3.80 (m, 2H), 4.15 (m, 2H), 4.52 (m, 2H), 7.25 (s, 1H), 7.52 (d, 2H, J = 8.80 Hz), 7.80 (d, 2H, J = 8.80 Hz), 8.08 (s, 1H), 8.52 (s, 1H), 10.05 (m, 1H), 10.75 ppm (s, 1H); LC–MS ($APCI^+$) m/z 487.0–489.0 [MH^+]; anal. ($C_{24}H_{32}BrClN_4O_2$) C, H, N.

4-(4-Fluoroanilino)-6-butoxy-7-(2-diethylaminoethoxy)quinazoline hydrochloride (32): The reaction mixture was concentrated in vacuo. The residue was purified by column chromatography on silica gel, eluting with CH_2Cl_2/MeOH (9:1) saturated with NH_3 to yield **32** (20 mg, 14%) as an HCl salt (a white solid). IR: $\tilde{\nu}$ = 1618 (NH), 1215 cm^{-1} (C–F); ^1H NMR ($CDCl_3$): δ = 0.95 (t, 3H, J = 7.24 Hz), 1.09 (t, 6H, J = 7.24 Hz), 1.46 (m, 2H), 1.79 (m, 2H), 2.69 (q, 4H), 3.00 (m, 2H), 4.00 (t, 2H, J = 6.21 Hz), 4.15 (m, 2H), 7.06 (m, 2H), 7.20 (s, 1H), 7.49 (s, 1H), 7.60 (m, 2H), 8.62 ppm (s, 1H); LC–MS ($APCI^+$) m/z 427.3 [MH^+]. Anal ($C_{24}H_{32}ClFN_4O_2$) C, H, N.

4-(4-Carboxy-3-chloroanilino)-6-butoxy-7-(2-diethylaminoethoxy)quinazoline hydrochloride (33): The precipitate was collected to afford **33** (80 mg, 51%) as an HCl salt (a brown solid), mp: 240–245 °C. IR: $\tilde{\nu}$ = 3600–3200 and 3000–2800 (OH), 1700 (C=O), 1635 cm^{-1} (NH); ^1H NMR (DMSO): δ = 0.95 (t, 3H, J = 7.63 Hz), 1.29 (t, 6H, J = 7.12 Hz), 1.48 (m, 2H), 1.75 (m, 2H), 3.20–3.40 (m, 4H), 3.55–3.70 (m, 2H), 4.25 (t, 2H, J = 6.10 Hz), 4.60–4.70 (m, 2H), 7.46 (s, 1H), 7.90–8.00 (m, 2H), 8.16 (s, 1H), 8.57 (s, 1H), 8.96 (s, 1H), 10.90–11.00 (m, 1H), 11.79 ppm (s, 1H); LC–MS ($APCI^+$) m/z 487.4–489.4 [MH^+]; anal. ($C_{25}H_{32}Cl_2N_4O_4$) C, H, N.

4-(4-Carboxyanilino)-6-butoxy-7-(2-diethylaminoethoxy)quinazoline hydrochloride (34): The precipitate was collected to afford **34** (120 mg, 82%) as an HCl salt (a white solid), mp: 235–237 °C. IR: $\tilde{\nu}$ = 3100–2400 (OH), 1698 (C=O), 1631 cm^{-1} (NH); ^1H NMR (DMSO): δ = 0.95 (t, 3H, J = 7.31 Hz), 1.29 (t, 6H, J = 7.30 Hz), 1.47 (m, 2H), 1.77 (m, 2H), 3.28 (m, 4H), 3.60–3.70 (m, 2H), 4.24 (t, 2H, J = 6.26 Hz), 4.60–4.70 (m, 2H), 7.43 (s, 1H), 7.93 (d, 2H, J = 8.87 Hz), 8.02 (d, 2H), 8.42 (s, 1H), 8.88 (s, 1H), 10.65–10.75 (m, 1H), 11.40–11.50 ppm (m, 1H); LC–MS ($APCI^+$) m/z 453.2 [MH^+]; anal. ($C_{25}H_{33}ClN_4O_4$) C, H, N.

4-(3-Bromoanilino)-6-butoxy-7-(2-diethylaminoethoxy)quinazoline hydrochloride (35): The resulting precipitate was collected to afford **35** (60 mg, 38%) as an HCl salt (a white solid), mp: 125–128 °C. IR: $\tilde{\nu}$ = 1634 (NH), 1070–1025 cm^{-1} (C–Br); ^1H NMR (DMSO): δ = 0.95 (t, 3H, J = 7.49 Hz), 1.28 (t, 6H, J = 7.49 Hz), 1.46 (m, 2H), 1.76 (m, 2H), 3.28 (m, 4H), 3.60–3.70 (m, 2H), 4.23 (t, 2H, J = 6.15 Hz), 4.55–4.65 (m, 2H), 7.41 (s, 1H), 7.45 (m, 2H), 7.79 (m, 1H), 8.05 (s, 1H), 8.42 (s, 1H), 8.88 (s, 1H), 10.65–10.75 (m, 1H), 11.40–11.50 ppm (m, 1H); LC–MS (APCI$^+$) m/z 487.0–489.0 [MH^+]; anal. ($C_{24}H_{32}BrClN_4O_2$) C, H, N.

4-(3-Bromo-4-methylanilino)-6-butoxy-7-(2-diethylaminoethoxy)-quinazoline hydrochloride (36): The solid was collected and afford **36** (20 mg, 12%) as an HCl salt (a brown solid), mp: 150–153 °C. IR: $\tilde{\nu}$ = 1632 (NH), 1072–1038 cm^{-1} (C–Br); ^1H NMR (DMSO): δ = 0.95 (t, 3H, J = 7.45 Hz), 1.28 (t, 6H, J = 6.99 Hz), 1.46 (m, 2H), 1.79 (m, 2H), 3.29 (m, 4H), 3.60–3.70 (m, 2H), 4.21 (t, 2H, J = 6.05 Hz), 4.55–4.65 (m, 2H), 7.38 (s, 1H), 7.45 (d, 1H, J = 8.38 Hz), 7.68 (d, 1H, J = 8.38 Hz), 8.34 (s, 1H), 8.85 (s, 1H), 10.55–10.65 (m, 1H), 11.20–11.30 ppm (m, 1H); LC–MS (APCI$^+$) m/z 501.1–503.1 [MH^+]; anal. ($C_{25}H_{34}BrClN_4O_2$) C, H, N.

4-(4-Bromo-3-chloroanilino)-6-butoxy-7-(2-diethylaminoethoxy)-quinazoline hydrochloride (37): The solid was collected and afford **37** (110 mg, 65%) as an HCl salt (a white solid), mp: 72–75 °C. IR: $\tilde{\nu}$ = 3394 and 1635 (NH), 1071 (C–Br), 1021 cm^{-1} (C–Cl); ^1H NMR (DMSO): δ = 0.95 (t, 3H, J = 7.21 Hz), 1.28 (t, 6H, J = 7.22 Hz), 1.46 (m, 2H), 1.78 (m, 2H), 3.28 (m, 4H), 3.60–3.70 (m, 2H), 4.24 (t, 2H, J = 6.66 Hz), 4.55–4.65 (m, 2H), 7.42 (s, 1H), 7.76 (d, 2H, J = 8,57 Hz), 8.18 (s, 1H), 8.47 (s, 1H), 8.91 (s, 1H), 10.60–10.70 (m, 1H), 11.55–11.65 ppm (m, 1H); LC–MS (APCI$^+$) m/z 521.0–522.9 [MH^+]; anal. ($C_{24}H_{31}BrCl_2N_4O_2$) C, H, N.

Methyl-3-methoxy-4-(4-morpholinopropoxy)benzoate (38): Potassium carbonate (5.69 g, 0.0417 mol) and 4-chloropropylmorpholine hydrochloride (3.30 g, 0.0165 mol) were added to a solution of methyl vanillate (1.5 g, 0.0082 mol) in acetone (30 mL). The reaction mixture was held at reflux for 2 days. The inorganic solid was filtered off, and the filtrate was concentrated in vacuo. The oily residue was dissolved in diethyl ether, and a solution of diethyl ether saturated with gaseous HCl was added. The precipitate was collected, washed with diethyl ether, and dried in vacuo to afford **38** (2.8 g, 98%) as an HCl salt (a white solid), mp: 79–82 °C. IR: $\tilde{\nu}$ = 1698 cm^{-1} (C=O); ^1H NMR ($CDCl_3$): δ = 2.06 (m, 2H), 2.50–2.60 (m, 6H), 3.70–3.80 (m, 4H), 3.89 (s, 3H), 3.91 (s, 3H), 4.14 (t, 2H, J = 6.64 Hz), 6.90 (d, 1H, J = 8.62 Hz), 7.54 (s, 1H), 7.64 ppm (dd, 1H, J = 1.99 Hz); anal. ($C_{16}H_{24}ClNO_5$) C, H, N.

Methyl-3-methoxy-4-(4-morpholinopropoxy)-6-nitrobenzoate (39): Starting from **38** (1 g, 3.23 mmol), the title compound (1.04 g, 91%, a yellow solid) was obtained using the same procedure as that for **6** at −78 °C, mp: 139–142 °C. IR: $\tilde{\nu}$ = 1733 cm^{-1} (C=O); ^1H NMR ($CDCl_3$): δ = 2.12 (m, 2H), 2.64 (m, 6H), 3.78 (m, 4H), 3.81 (s, 3H), 3.96 (s, 3H), 4.16 (t, 2H, J = 6.63 Hz), 7.07 (s, 1H), 7.48 ppm (s, 1H); anal. ($C_{16}H_{22}N_2O_7$) C, H, N.

6-Amino-3-methoxy-4-(4-morpholinopropoxy)benzoate (40): As described for **7**, **40** (0.37 g, 40%) was obtained as a yellow solid from compound **39** (1 g, 2.82 mmol); mp: 147–151 °C. IR: $\tilde{\nu}$ = 2925 (NH), 1618 cm^{-1} (C=O); ^1H NMR ($CDCl_3$): δ = 2.10 (m, 2H), 2.55–2.65 (m, 6H), 3.80 (s, 3H), 3.85 (s, 3H), 4.06 (t, 2H, J = 6.44 Hz), 5.60–5.70 (m, 2H), 6.18 (s, 1H), 7.30 ppm (s, 1H); anal. ($C_{16}H_{24}N_2O_5$) C, H, N.

6-Methoxy-7-(4-morpholinopropoxy)-3*H*-quinazolin-4-one (41): Similar to the procedure described for **8**, the title compound (0.66 g, 90%) was prepared starting from **40** (0.69 g, 2.13 mmol) as a brown oil. IR: $\tilde{\nu}$ = 3324 (NH), 1679 cm^{-1} (C=O); ^1H NMR (DMSO): δ = 2.15 (m, 2H), 2.71 (m, 6H), 3.60–3.70 (m, 4H), 3.83 (s, 3H), 4.05 (m, 2H), 7.35 (s, 1H), 7.73 (s, 1H), 8.39 ppm (s, 1H); LC–MS (APCI$^+$) m/z 320.0 [MH^+]; anal. ($C_{16}H_{21}N_3O_4$) C, H, N.

4-(3-Chloro-4-fluoroanilino)-6-methoxy-7-(4-morpholinopropoxy)quinazoline hydrochloride (42): A mixture of **41** (650 mg, 2.035 mmol), phosphorus pentachloride (510 mg, 2.44 mmol), and phosphorus oxychloride (0.51 mL, 5.47 mmol) was held at reflux for 16 h. The mixture was cooled to room temperature, and the excess of phosphorus oxychloride was evaporated under reduced pressure. The residue was dissolved in *i*PrOH (5 mL), and 3-chloro-4-fluoroaniline (325.18 mg, 2.24 mmol) was added. The reaction mixture was held at reflux for 2.5 h and concentrated under reduced pressure. The residue was purified by column chromatography on silica gel, eluting with CH_2Cl_2/MeOH (9:1) saturated with NH_3 to yield **42** (190 mg, 19%) as an HCl salt (a white solid), mp: 199–202 °C. IR: $\tilde{\nu}$ = 1628 (NH), 1221 (C–F), 1055 cm^{-1} (C–Cl); ^1H NMR (DMSO): δ = 1,93 (m, 2H), 2.38 (m, 6H), 3.58–3.65 (m, 4H), 3.95 (s, 3H), 4.15 (m, 2H), 7.19 (s, 1H), 7.41 (t, 1H, J = 9.06 Hz), 7.75–7.85 (m, 2H), 8.11 (m, 1H), 8.49 (s, 1H), 9.50–9.60 ppm (m, 1H); anal. ($C_{22}H_{25}Cl_2FN_4O_3$) C, H, N.

In vitro kinase assays: Kinase assays were performed in 96-well plates (Multiscreen Durapore, Millipore) using [γ-^{32}P]ATP (Amersham Biosciences) and the synthetic polymer poly(Glu_4/Tyr) (Sigma) as a phosphate-acceptor substrate. Tested compounds were dissolved in DMSO (the final concentration of DMSO in assay solutions was 0.1% (total kinase activity) or 0.01% (EGFR kinase activity), which was shown to have no effect on kinase activity).

EGFR tyrosine kinase activity: EGFR purified from human carcinoma A431 cells (20 ng, Sigma) was incubated for 1 h at 28 °C using various concentrations of tested compounds in kinase buffer (HEPES 50 mM pH 7.5, BSA 0.1 mg mL^{-1}, $MnCl_2$ 10 mM, $MgCl_2$ 5 mM, Na_3VO_4 100 μM, DTT 0.5 mM, poly(Glu_4/Tyr) 250 μg mL^{-1}, ATP 5 μM, and [γ-^{32}P]ATP 0.5 μCi).

Whole-cell membrane-associated tyrosine kinase activity: Cytoplasmic membranes (20 μg) from PC3 cells were preincubated for 5 min at 37 °C using various concentrations of tested compounds. Membranes were then incubated for 2 h at 37 °C in kinase buffer (Tris-HCl 50 mM pH 7.5, $MgCl_2$ 20 mM, Na_3VO_4 100 μM, poly(Glu_4/Tyr) 1 mg mL^{-1}, ATP 200 μM, and [γ-^{32}P]ATP 1 μCi).

VEGFR-2 tyrosine kinase activity: VEGFR-2 recombinant human protein (10 ng, Invitrogen) was incubated for 1 h at 27 °C using various concentrations of tested compounds in kinase buffer (Tris 50 mM pH 7.5, BSA 25 μg mL^{-1}, $MnCl_2$ 1.5 mM, $MgCl_2$ 10 mM, Na_3VO_4 100 μM, DTT 2.5 mM, β-glycerophosphate 5 mM, poly(Glu_4/Tyr) 250 μg mL^{-1}, ATP 5 μM, and [γ-^{32}P]ATP 0.5 μCi). The reaction was stopped by adding trichloroacetic acid (100%, 20 μL). Wells were screened out and washed 10 times with trichloroacetic acid (10%). Plates were counted in a Top Count (Packard/PerkinElmer). SU5416 was used as reference compound.

Cell culture and cell proliferation assay: Human prostate cancer cells PC3 were grown at 37 °C in a humidified atmosphere containing 5% CO_2 in RPMI-1640 medium supplemented with 10% fetal bovine serum, glutamine (2 mM), penicillin (100 IU mL^{-1}), and streptomycin (100 μg mL^{-1}). In the cell proliferation assay, cells were plated in triplicate on 96-well plates (3×10^3 cells per well) and incubated for 72 h. The cell medium was changed to serum-free medium, and the cells were starved for 24 h for culture synchronization. Cells were then incubated in culture medium that contained various concentrations of tested compounds, each dis-

solved in $<0.1\%$ DMSO. After 72 h, cell growth was estimated by the colorimetric MTT test.

Cell-cycle analysis: For flow cytometry analysis of DNA content, PC3 cells were grown in 25 cm^2 flasks for 72 h, synchronized for 24 h in serum-free medium, and incubated for 72 h with various concentrations of tested compounds. After incubation, the medium was aspirated off and pooled with the adherent cell layer, which was trypsinized. Cell pellets were washed in PBS, fixed in 70% ethanol, and stored at 4 °C for at least one night. For the analysis, cells were collected by centrifugation, washed in PBS, and the pellets were resuspended in PBS containing RNase (100 $\mu g\,mL^{-1}$) and propidium iodide (50 $\mu g\,mL^{-1}$) and incubated in the dark at room temperature for 30 min. Cytometric analysis was then performed on a FACScan flow cytometer (BD Biosciences) using the Cellquest software. Cell-cycle phase distribution was determined using WinMDI software to analyze DNA content histograms.

Terminal deoxynucleotidyl transferase-mediated dUTP nick-end labeling (TUNEL) assay: DNA fragmentation was assessed by TUNEL assay. The apoptosis detection system fluorescein (Promega) was used according to the supplier's recommended protocol. PC3 cells (10^6) were treated with various concentrations of tested compounds for 72 h. After incubation, the medium was aspirated off and pooled with the adherent layer, which was trypsinized. Cells were collected and centrifuged at 200 *g* for 5 min at 4 °C. Cell pellets were washed twice in PBS and centrifuged at 300 *g* for 10 min at 4 °C. Pellets were resuspended in PBS (500 μL) and fixed in ice-cold paraformaldehyde (1%) for 20 min at 4 °C. Fixed cells were centrifuged, and pellets were washed twice in PBS and made permeable in 70% ethanol at −20 °C for at least one night. Cells were centrifuged at 300 *g* for 10 min at 4 °C, washed in PBS, and centrifuged. Pellets were resuspended in PBS and incubated in equilibration buffer (80 μL) for 5 min at room temperature. DNA strand breaks (3′-OH ends of fragmented DNA) were labeled with fluorescein–dUTP by adding incubation buffer containing equilibration buffer (45 μL), nucleotide mix (5 μL), and terminal deoxynucleotidyl transferase (1 μL) (volumes for one slide). For each experimental set, a negative control was prepared by omitting the TdT from the reaction mixture. After incubation in the dark at 37 °C for 1 h, the reaction was stopped with EDTA (20 mM). After two successive washes, propidium iodide (5 $\mu g\,mL^{-1}$) was added to stain all cells. Apoptotic cells were detected at 515–555 nm using an FL-1 detector of a FACScan flow cytometer (BD Biosciences) and the Cellquest software.

Invasion assay: The invasive ability of PC3 was determined using BD BioCoat Matrigel invasion chamber (8 μm porosity polyethylene terephthalate track-etched membranes, Becton Dickinson). Inserts were rehydrated with serum-free medium for 2 h and transferred to 24-well plates containing medium supplemented with 10% serum. Cells (0.5×10^5) were added to each insert in the presence of compounds and were incubated for 24 h at 37 °C. Noninvading cells were then removed by scraping the upper surface of the insert. The cells that migrated to the lower surface of the insert were detected using the kit Hemacolor. Inserts were dried, and cells were counted under a microscope. The invasion fraction was determined by dividing the number of cells that invaded the Matrigel by the number of cells counted in the control inserts.

Western blotting: After being serum-starved for 24 h, cells were incubated with tested compounds diluted in medium supplemented with EGF for 10 min (Akt phosphorylation) or 24 h (COX-2 expression). Cells were scraped in lysis buffer (PBS containing Triton X100 1%, deoxycholate 0.5%, sodium pyruvate phosphate 10 mM, sodium vanadate 2 mM, NaF 100 mM, PMSF 0.5 mM, protease inhibitor cocktail). Western blot analysis was performed after SDS-PAGE of cell lysates (20 μg protein) in the Mini-protean 3 gel box (BioRad). Proteins were transferred to Hybond ECL nitrocellulose membranes (Amersham Biosciences), which were blocked for at least 1 h with a buffer containing 10 mM Tris/150 mM NaCl (pH 7.4), 0.05% Tween 20, and 5% (*w/v*) nonfat dried milk. The blot was then incubated for 1 h at room temperature with primary antibodies specific for the following proteins: phosphorylated Akt (Santa Cruz Biotechnology; dilution 1:1000), COX-2 (Cayman; dilution 1:1000), and α-actin (Santa Cruz Biotechnology; dilution 1:500), washed three times with 10 mM Tris/150 mM NaCl (pH 7.4), 0.05% Tween 20, and incubated with a secondary antibody conjugated to horseradish peroxidase (dilution 1:10000) for 1 h at room temperature. After being washed three times for 15 min, the blot was visualized with a chemiluminescence kit ECL (Amersham Biosciences). Bands were normalized to α-actin.

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