Jae Yeol Lee,
Yong Kyu Park,
Seon Hee Seo,
Beom-Seok Yang,
Hokoon Park,
Yong Sup Lee

Medicinal Chemistry Research Center, Korea Institute of Science & Technology, P.O. Box 131, Cheongryang, Seoul 130–650, Korea

7-Substituted-[1,4]dioxano[2,3-*g*]quinazolines as Inhibitors of Epidermal Growth Factor Receptor Kinase

With the aim of developing inhibitors of EGFR tyrosine kinase, the 7-methoxymethyl-[1,4]dioxano[2,3-*g*]quinazolines (**3a**–**b**) and 7-mono- or di-alkylaminomethyl-[1,4]dioxano[2,3-*g*]quinazolines (**4a**–**i**) were prepared and evaluated for the inhibition of EGFR tyrosine kinase and the growth inhibition of human tumor cell lines. Among them, compounds **4d** and **4h** showed potencies against both EGFR tyrosine and the A431 cell line similar to that of PD153035 with greater aqueous solubilities of their HCl salts.

Keywords: EGFR; Tyrosine kinase; Inhibitor; Water solubility; Dioxane; Quinazoline; A431; Antitumor

Received: March 12, 2002 [FP683]

Introduction

The epidermal growth factor receptor (EGFR) plays a central role in growth signaling and regulation of mammalian cells [1]. Like other protein kinases, it mainly transduces signals to a target cell by catalyzing the transfer of the terminal phosphate of ATP to tyrosine residues in protein substrates. Overexpression of EGFR has been implicated in a significant proportion of human tumors and other proliferative diseases [2]. EGFR is therefore expected to be an attractive target for cancer chemotherapy. Due to the relative conservation of the ATP binding site of protein kinases, the development of selective inhibitors of protein kinases of the EGFR has been an important concern. As a result of extensive research, the 4-anilinoquinazoline class of compounds (e.g. PD153035, **I**) have recently proven to be potent and selective inhibitors of tyrosine kinase activity of EGFR *via* competitive binding at the ATP site of the enzyme [3]. Based on these results, considerable efforts have been devoted to the synthesis of PD153035 analogues for studying structure-activity relationships to develop tyrosine kinase inhibitory antitumor drugs [4–7].

While PD153035 (**1**) has an EGFR tyrosine kinase inhibitory activity with IC_{50} = 25 pM (K_i 6 pM) and shows little effect on the activity of other kinase [3], its poor water solubility complicated its *in vivo* evaluation in tumor models [8]. With a view to overcoming the poor aqueous solubility of the parent compound, pyrido[*d*]pyrimidines and fused tricyclic quinazolines have therefore been prepared where various solubilizing groups were appended to amine substituents [9–11]. Recently, we have also reported that 4-anilino-[1,4]dioxano[2,3-*g*]quinazolines (**2**) are effective inhibitors of EGFR as judged by their inhibitory activity against EGFR kinase and the growth of human tumor cell lines [12].

With a view to the further investigation of structure-activity relationships (SAR) and improving water solubility in this series of [1,4]dioxano[2,3-*g*]quinazoline inhibitors, we report here the synthesis and biological activity of a series of [1,4]dioxano[2,3-*g*]quinazolines substituted with methoxymethyl (**3a**–**b**) or mono- or dialkylaminomethyl group (**4a**–**i**) at 7-position, based on the our recent report that a [1,4]dioxano[2,3-*g*]quinazoline ring would be a new scaffold for EFGR kinase inhibitor [12].

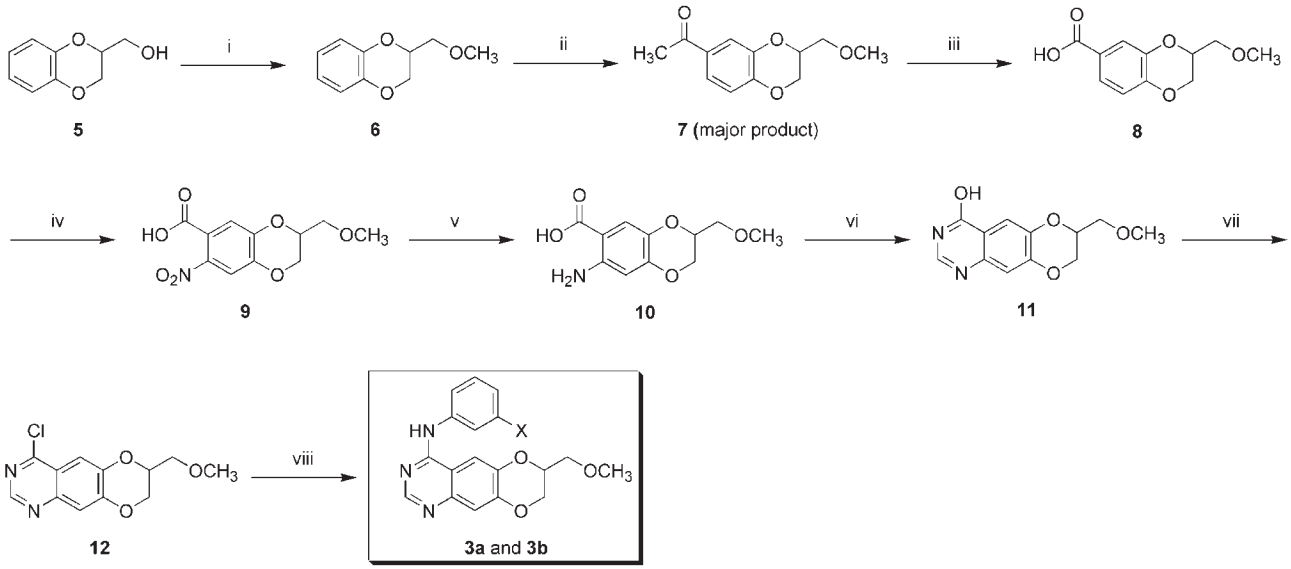
Synthesis

First, compounds of the 7-methoxymethyl-[1,4]dioxano[2,3-*g*]quinazoline series (**3a**–**b**) were prepared by the known procedures as shown in Scheme 1 [8, 12–14]. Commercially available 2-hydroxymethylbenzo[1,4]-dioxane (**5**) was protected with NaH and CH_3I to afford 2-methoxymethylbenzo[1,4]dioxane (**6**) in 78 % yield. Friedel-Crafts acylation of compound **6** using acetyl chloride gave 7-acylbenzodioxane **7** (70 %) along with 6-acyl isomer (30 %) in 84 % yield. Because 6-acyl isomer could not be separated by column chromatography, the isomeric mixture was used for the next reactions [13]. Oxidation of the isomeric mixture with NaOCl (48 %), nitration (54 %), and reduction (65 %) provided a [1,4]dioxane-fused anthranilic acid (**10**) as a precursor

Correspondence: Yong Sup Lee, Medicinal Chemistry Research Center, Korea Institute of Science & Technology, P.O. Box 131 Cheongryang, Seoul 130–650, Korea. Phone: +8229585167, Fax: +8229585189, e-mail: yslee@kist.re.kr.

Full Paper

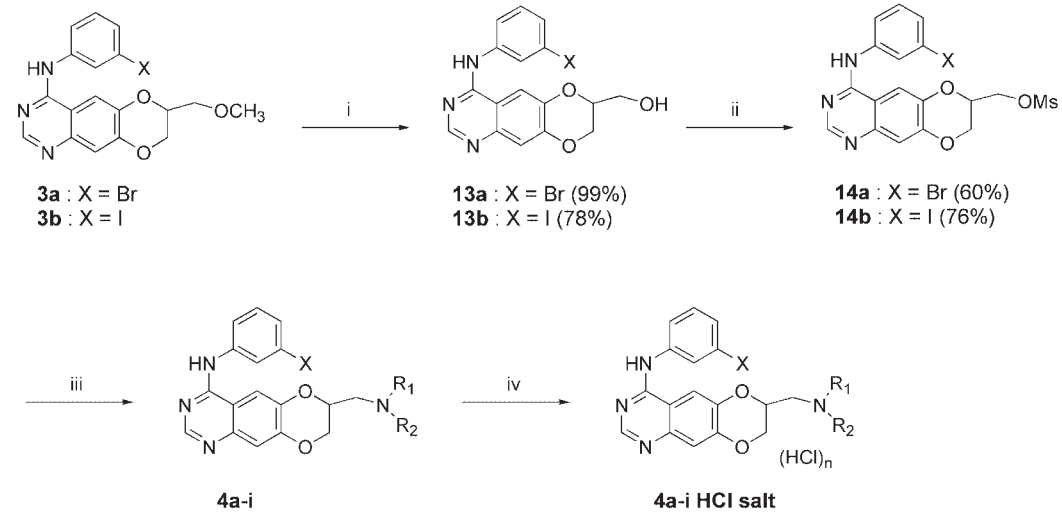
 0365-6233/010/0487 $ 17.50+.50/0



[a] (i) NaH/CH_3I/THF/rt/48 h, 78%; (ii) $AlCl_3$/AcCl/DMA/reflux/5 h, 84%; (iii) NaOCl/65 °C, 48%; (iv) HNO_3/H_2SO_4/AcOH/rt/1 h, 54%; (v) H_2/Pd-C/MeOH/rt/24 h, 65%; (vi) triazine/piperazine/MeOH/reflux/4 h, 63% ; (vii) $POCl_3$/reflux/2 h, 90%; (viii) aniline/*iso*-PrOH/reflux.

Scheme 1[a]

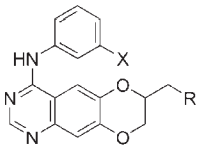
for cyclization. At this stage, the impurity from 6-acyl isomer could be removed by recrystallization with EtOAc. The cyclization of pure compound **10** with triazine was conducted in the presence of piperazine to afford 4-hydroxyquinzaoline (**11**) in 63 % yield, which was easily converted into 4-chloroquinazoline (**12**) with $POCl_3$ in 90 % yield. The coupling reaction between 4-chloroquinazoline (**12**) and appropriate anilines was finally conducted to provide 4-anilino-7-methoxymethyl-[1,4]dioxano[2,3-*g*]quinazolines (**3a**–**b**) in *iso*-propanol under reflux in 63 and 94 % yields. The structures and yields of these compounds are summarized in Table 1.The 7-mono or di-alkylaminomethylquinazoline



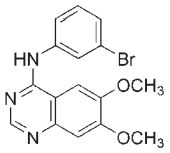
[a] (i) BBr_3/CH_2Cl_2/-78 °C-rt/24 h; (ii) CH_3SO_2Cl/TEA/CH_2Cl_2/rt/4 h; (iii) amines/THF/reflux/3 h; (iv) EtOH/AcCl/rt/24 h

Scheme 2[a]

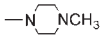
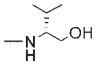
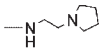
Table 1. Structures, yields, physical and biological properties of 7-substituted-[1,4]-dioxano[2,3-*g*]quinazolines.



(3a-3b, 4a~i)[a]



PD153035 (1)

No.	R	X	M. W.	Yield[b] (%)	mp	IC_{50} (nM)[c]	GI_{50} (μM)[d] A 431	Solubility (mM)[e]
3a	OCH_3	Br	402.24	63[f]	268-230	35.5	0.59	0.60[g]
3b	OCH_3	I	449.24	94[f]	175-177	28.2	0.85	0.30[g]
4a		Br	457.32	68	229-230	63.1	1.01	>200
4b		Br	475.34	40	188-191	>100	2.57	>200
4c		Br	431.36	42	168-172	28.2	1.50	>200
4d		Br	470.36	80	182-188	8.9	0.98	>200
4e		Br	479.36	84	170-175	>100	1.21	>200
4f		I	531.39	20	195-205	100	0.71	>200
4g		I	504.32	44	238-240	93.3	1.12	>200
4h		I	522.32	66	174-175	9.5	2.21	>200
4i		I	478.28	77	200-201	>100	1.01	>200
PD153035[h]			360.21			6.6[i]	1.03[i]	0.06[g]

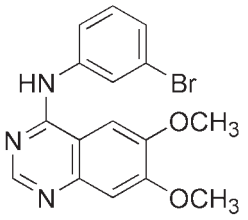
[a] All compounds are free bases; [b] Yields from the reaction of compound **14a** or **14b** and amines; [c] IC_{50}, concentration needed to inhibit the phosphorylation of the tyrosine kinase substrate 2 by EGFR by 50% as determined from the dose-response curve at four concentrations. Determination was done in three separate experiments and each was performed in triplicate; [d] GI_{50}, concentration needed to inhibit cell growth by 50% as determined from the dose-response curve. Determination was done in three separate experiments and each was performed in triplicate; [e] Value of HCl salt; [f] Yields from from the raction of compounds **12** and anilines; [g] free base compounds [h] The standard compound was made for comparison for activity; [i] The value was determined by using our assay protocol.

derivatives (**4a**–**i**) were prepared from the selected 7-methoxymethyl-[1,4]-dioxano[2,3-*g*]quinazolines (**3a**–**b**) by demethylation of 7-methoxymethyl group of compound (**3a**–**b**) with BBr_3, mesylation of the resultant free hydroxy group with MsCl/TEA, and subsequent displacement of mesylate (**14a**–**b**) with various amines as shown in Scheme 2. In order to improve the aqueous solubility of parent compounds, all compounds **4a**–**i** were also prepared as hydrochloride salts (>99 %) by treating the parent compounds with EtOH/AcCl. The structures and yields of these compounds **4a**–**i** are also summarized in Table 1.

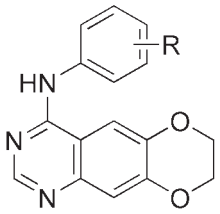
Results and discussion

The compounds (**3a**–**b**) and (**4a**–**i**) shown in Table 1 were evaluated for their ability to inhibit the phosphorylation of tyrosine kinase substrate 2 by EGFR and the growth of A431 cell line (uterus cancer), which highly overexpresses EGFR. To compare these data with that of standard, the inhibitory activity of the PD153035 under our assay conditions was inserted into the data set. The IC_{50} value of PD153035 we obtained is significantly higher and lower, respectively, than reported by previous workers [3, 15]. It was reported that the difference is at least partly attributable to the nature of the enzyme and substrate as well as the overall assay conditions [16].

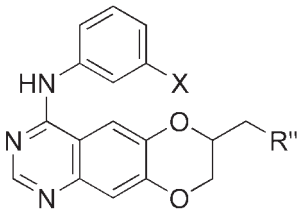
In the case of 7-methoxymethylquinazoline compounds (**3a** and **3b**), both compounds showed better potencies (GI_{50} = 0.59 and 0.85 nM, respectively) than the standard compound, PD153035 (GI_{50} = 1.03 nM) against the growth of the A431 cell line, but less inhibitory activities



PD 153035 (1)



[1,4]dioxano[2,3-*g*]quinazoline (2)



(3) R'' = OCH_3; (4) R'' = NR_1R_2

Figure 1. PD153035 (**1**), its cyclic analogue (**2**), and 7-substituted-[1,4]dioxano[2,3-*g*]quinazolines (**3** and **4**).

(**3a** and **3b**; IC_{50} = 35.5 and 28.2 nM, respectively) than PD153035 (IC_{50} = 6.6 nM) against the isolated enzyme. Among the synthesized compounds, compound **3a**, containing a 3-bromoaniline ring at 4-position, was the most potent compound against the growth of A 431 cells.

In the case of 7-mono- or di-alkylaminomethylquinazoline derivatives (**4a**–**i**), several points are apparent from the data shown in Table 1: First, all compounds generally showed similar activities to PD153035 against the growth of the A 431 cell line, while the range of enzyme inhibitory activities (IC_{50} = 8.9 to >100 nM) against the isolated enzyme are relatively broad. As expected, secondly, HCl salts of all compounds showed much greater aqueous solubilities (>200 mM) than PD153035 (0.056 mM) under those experiments. With regard to isolated enzyme, compound **4d**, containing (4-methylpiperazino)methyl group at 7-position, was the most potent compound (IC_{50} = 8.9 nM) and compound **4h**, bearing bis(ethanol)aminomethyl group, showed an activity as effective (IC_{50} = 9.5 nM) as compound **4d**. In the case of other compounds, the substitution of 7-mono- or -dialkylamino side chain generally resulted in decreasing inhibitory potency (IC_{50} = 28.2 to >100 nM) against the isolated enzyme when compared with their parent compounds (**3a** and **3b**; IC_{50} = 35.5 and 28.2 nM, respectively).

With regard to cellular assay, most compounds showed similar potencies (GI_{50} = 0.71 to 1.21 μM) to PD153035 (GI_{50} = 1.03 μM) against the A431 cell line with the exception of compounds **4b** and **4h** (GI_{50} = 2.57 and 2.21 μM, respectively), irrespective of enzyme inhibitory activity. Therefore, it is difficult to interpret the structure-activity relationship of compounds in Table1 between enzyme inhibitory activity and cell growth inhibitory activity. Among this series, compound **4f**, having a 2-(pyrrolidino)ethylamino group at 7-position, was the most potent compound (GI_{50} = 0.71 μM).

Some compounds (**4a**–**d**) were further evaluated against human cancer cell lines for their selectivity:

Table 2. Inhibition of the growth of selected cell lines.

No.	GI50 (μM)[a]		
	A 431[b]	HCT 116[c]	SNU 638[d]
4a	1.01	28.25	>100
4b	2.57	20.72	18.00
4c	1.50	14.81	13.22
4d	0.98	14.10	12.00
PD153035	1.03	19.50	11.48

[a] GI_{50}, concentrations needed to inhibit cell growth by 50% as determined from the dose-response curve. Determination was done in three separate experiments and each was performed in triplicate; [b] Uterus cancer; [c] Colon cancer; [d] Stomach cancer

HCT116 (colon cancer) and SNU638 (stomach cancer), which express low levels of EGFR. As a result, all compounds showed more than 10-fold selectivities for the inhibition of growth of A431 over HCT116 and SNU638, as shown in Table 2. This suggests that the mechanism of growth inhibition is largely due to the targeting of EGFR.

With regard to water solubility, the parent compound **3a** was 10-fold more soluble (0.6 mM) than PD153035 (0.06 mM) and compounds **4a**–**i** as HCl salts showed much greater aqueous solubilities (>200 mM) in those experiments. Meanwhile, the formation of **4c** as HCl salt resulted in slight loss of activity against the isolated enzyme and A431 cells, when compared with **4c** as a free base (IC_{50}'s of 42.7 and 28.2 nM; GI_{50}'s 2.48 and 1.50 μM, respectively – the data for HCl salt compounds are not shown in Table 1). In spite of poor enzyme inhibitory activities of some compounds, therefore, their high potency against the growth of cell lines would be explained by good cellular uptake of compounds.

In conclusion, we prepared new 7-methoxymethyl-[1,4]dioxano[2,3-*g*]quinazolines (**3a**–**b**) and 7-mono- or di-alkylaminomethyl-[1,4]dioxano[2,3-*g*]quinazolines (**4a**–**i**) derivatives to probe the effect of 7-substituents on the inhibition of EGFR tyrosine kinase and the growth inhibition of human tumor cell lines. Most compounds were as potent as PD153035 against the A431 cell line and some compounds (**4a**–**d**) showed good selectivities for A431 over other tumor cell lines such as HCT116 and SNU638. HCl salts of most compounds showed much greater aqueous solubilities (>200 mM) than PD153035. In particular, compounds **4d** and **4h** showed similar potencies to PD153035 against both EGFR and the A431 cell line. The above results demonstrate that 7-mono- or dialkylaminomethylquinazoline derivative would be an effective inhibitor of EGFR when considered in the light of the inhibitory activity against EGFR kinase, the growth of human tumor cell lines, and the aqueous solubility of its HCl salt.

Acknowledgement

This work was supported by Korea Institute of Science and Technology (2E17073).

Experimental part

Chemistry

^{1}H NMR spectra were recorded on a Gemini Varian-300 (300 MHz). Mass spectra (EI) were determined on HP GC 5972 and HP MS 5988A system at 70 eV. HPLC was performed on a Waters pump model 501, with a UV detector (λ = 254 nm) model 486 system using a LiChrosorb RP-18 (10 mm i.d. × 25 cm, Merck). Analytical thin layer chromatography (TLC) was carried out on precoated silica gel (E. Merck Kiesegel $60F_{254}$ layer thickness 0.25 mm). Flash column chromatography was performed with Merck Kiesegel 60 Art 9385 (230–400 mesh). All solvents used were purified according to standard procedures.

2-Methoxymethyl-benzo[1,4]dioxane (**6**)

To a susupension of NaH (5.0 g, 0.20 mol) in 60 mL of anhydrous THF was added 2-hydroxymethylbenzo[1,4]dioxane (28.9 g, 0.17 mol) at 0 °C, and then the mixture was stirred at room temperature for 30 min followed by addition of methyl iodide (16.3 mL, 0.26 mol). The reaction mixture was stirred at room temperature for 48 h, quenched with 10 mL of water, and extracted with ethyl acetate. The combined organic layer was dried over $MgSO_4$ and concentrated *in vacuo* to give **6** (24.0 g, 78 %) as an oil: ^{1}H NMR ($CDCl_3$) δ 6.96–6.85 (m, 4H), 4.32–4.26 (m, 2H), 4.05 (m, 1H), 3.67–3.54 (m, 2H), 3.42 (s, 3H).

7-Acetyl-2-methoxymethyl-benzo[1,4]dioxane (**7**)

A mixture of anhydrous $AlCl_3$ (4.4 g, 33.3 mmol) and *N,N*-dimethylacetamide (1.3 mL, 16.7 mmol) was stirred at room temperature for 30 min. To the mixture was added the above compound **6** (0.5 g, 3.8 mmol), and the mixture was stirred at the same temperature for 15 min, followed by addition of acetyl chloride (0.3 mL, 3.3 mmol). After stirring at room temperature for 5 h, the reaction mixture was cooled to 0 °C, treated with ice-water, neutralized with saturated $NaHCO_3$ solution, and extracted with ethyl acetate. The combined organic layer was dried over $MgSO_4$ and concentrated *in vacuo* to give **7** (520 mg, 84 %) as an isomeric mixture, which was used for the next reaction without further purification: ^{1}H NMR ($CDCl_3$) δ 7.53 (s, 1H), 7.50 (d, *J* = 8.4 Hz, 1H), 6.90 (d, *J* = 8.4 Hz, 1H), 4.37–4.23 (m, 2H), 4.11 (m, 1H), 3.67–3.57 (m, 2H), 3.42 (s, 3H), 2.51 (s, 3H).

3-Methoxymethyl-benzo[1,4]dioxane-6-carboxylic acid (**8**)

A solution of 13 % NaOCl (140 mL; Fluka®) was added to **7** (12.9 g, 57.8 mmol). The reaction mixture was heated at 65 °C for 30 min, cooled to room temperature, and further stirred at the same temperature for 30 min. The insoluble precipitate was filtered off and the filtrate was acidified to pH 3–4 with *c*-HCl to give a solid. The filtered solid was washed with water and recrystallized with water/ethanol (*v/v* = 2/3) to afford **8** (6.3 g, 48 %) as a white solid: ^{1}H NMR (CD_3OD_3) δ 7.48 (d, *J* = 8.5 Hz, 1H), 7.45 (s, 1H), 6.85 (d, *J* = 8.5 Hz, 1H), 4.35–5.25 (m, 2H), 4.05 (m, 1H), 3.62–3.58 (m, 2H), 3.25 (s, 3H).

3-Methoxymethyl-7-nitro-benzo[1,4]dioxane-6-carboxylic acid (**9**)

To a solution of **3** (0.8 g, 3.5 mmol) in 2 mL of glacial acetic acid was added a mixture of H_2SO_4 (3 mL) and HNO_3 (3 mL) at room temperature during a period of 30 min. After further stirring for 30 min, the reaction mixture was treated with 10 mL of water to produce a solid. The solid was filtered, washed with water and ether, and dried *in vacuo* to afford **9** (0.5 g, 54 %) as a white solid: ^{1}H NMR ($CDCl_3$) δ 7.41 (s, 1H), 7.34 (m, 1H), 4.52–4.37 (m, 2H), 4.16 (m, 1H), 3.70–3.61 (m, 2H), 3.44 (s, 3H).

7-Amino-3-methoxymethyl-benzo[1,4]dioxane-6-carboxylic acid (**10**)

To a solution of **9** (1.2 g, 4.53 mmol) in 10 mL of methanol was added 10 % Pd/C (50 mg), and the reaction mixture was stirred at room temperature for 24 h. After filtering through Celite 545, the filtrate was concentrated *in vacuo* and the residue was recrystallized with EtOAC to afford pure **10** (0.7 g, 65 %) as a pale yellow solid: mp 161–167 °C; ^{1}H NMR ($CDCl_3$) δ 7.50 (s, 1H), 7.28 (s, 2H), 6.17 (s, 1H), 4.34–4.24 (m, 2H), 4.12 (m, 1H), 3.67–3.59 (m, 2H), 3.44 (s, 3H).

7-Methoxymethyl-[1,4]dioxano[2,3-g]quinazolin-4-ol (**11**)

A solution of **10** (0.4 g, 1.53 mmol), triazine (0.2 g, 2.30 mmol), and piperidine (0.015 mL, 0.15 mmol) in 10 mL of methanol was heated at reflux for 4 h. After cooling to room temperature, the resultant solid was filtered, washed with methanol, and dried *in vacuo* to afford **11** (0.24 g, 63 %) as a white solid: mp 246–251 °C; ^{1}H NMR (DMSO-d_6) δ 7.92 (s, 1H), 7.46 (s, 1H), 7.09 (s, 1H), 4.46–4.19 (m, 2H), 4.13 (m, 1H), 3.62–3.60 (m, 2H), 3.32 (s, 3H).

4-Chloro-7-methoxymethyl-[1,4]dioxano[2,3-g]quinazoline (**12**)

To a solution of **11** (0.5 g, 2.0 mmol) in 25 mL of $POCl_3$ was added *N,N*-dimethylaniline (0.64 mL, 5.49 mmol), and the reaction mixture was heated at reflux for 2 h. After excess of $POCl_3$ was distilled off under reduced pressure, the residue was dissolved in 30 mL of CH_2Cl_2 and treated with 50 mL of ice-water. The separated organic layer was washed with saturated $NaHCO_3$, dried over $MgSO_4$, and concentrated *in vacuo* to provide **12** (0.5 g, 90 %), which was used for the next reaction without further purification: ^{1}H NMR (DMSO-d_6) δ 8.88 (s, 1H), 7.46 (s, 1H), 7.10 (s, 1H), 4.59–4.55 (m, 2H), 4.26 (m, 1H), 3.67–3.66 (m, 2H), 3.25 (s, 3H).

4-[(3′-Bromophenyl)amino]-7-methoxymethyl-[1,4]dioxano[2,3-g]quinazoline (**3a**): *General procedure of coupling reaction in Scheme 1*

A solution of **12** (40 mg, 0.15 mmol) and 3-bromoaniline (33 mg, 0.3 mmol) in 2.5 mL of 2-propanol was treated with a catalytic amount of *c*-HCl, and the reaction mixture was heated at reflux for 24 h. After cooling to room temperature. the resultant solid was filtered, washed with a small volume of *iso*-propanol, and dried *in vacuo* to afford **3a** (24 mg, 47 %) as a white solid: mp 268–274 °C; ^{1}H NMR (DMSO-d_6) δ 11.23 (s, 1H), 8.89 (s, 1H), 8.49 (s, 1H), 8.07 (m, 1H), 7.78 (m, 1H), 7.50–7.40 (m, 2H), 7.46 (s, 1H), 4.62–4.58 (m, 2H), 4.30 (m, 1H), 3.68 (d, *J* = 4.5 Hz, 2H), 3.34 (s, 3H).

4-[(3′-Iodophenyl)amino]-7-methoxymethyl-[1,4]dioxano-[2,3-g]quinazoline (**3b**)

By reaction of **12** with 3-iodoaniline (2 equiv): 94 % (yellow solid); mp 175–177 °C; ^{1}H NMR (DMSO-d_6) δ 10.70 (s, 1H), 9.19 (s, 1H), 8.31 (s, 1H), 8.21 (s, 1H), 7.81 (d, *J* = 8.2 Hz, 1H), 7.64 (d, *J* = 8.4 Hz, 1H), 7.30 (s, 1H), 7.27 (dd, *J* = 8.2, 8.4 Hz, 1H), 4.61–4.58 (m, 2H), 4.28 (m, 1H), 3.68 (d, *J* = 4.7 Hz, 2H), 3.34 (s, 3H).

4-[(3′-Bromophenyl)amino]-7-hydroxymethyl-[1,4]dioxano[2,3-g]quinazoline (**13a**): *General procedure of demethylation of* **3a** *and* **3b** *in Scheme 2*

To a solution of 4-[(3′-iodophenyl)amino]-7-methoxymethyl-[1,4]dioxano[2,3-*g*]quinazoline (**3a**; 1 g, 2.5 mmol) in 50 mL of CH_2Cl_2 was added 1 M solution of BBr_3 in hexane (4.0 mL, 4.0 mmol) under N_2 atmosphere at –78 °C. The reaction mixture was stirred at the same temperature for 1 h and allowed to warm to room temperature for 24 h. After treatment with saturated $NaHCO_3$ solution, the reaction mixture was extracted with ethyl acetate. The combined organic layer was dried over $MgSO_4$ and concentrated *in vacuo* to afford **13a** (960 mg, 99 %) as red solid: ^{1}H NMR (CD_3OD) δ 8.42 (s, 1H), 8.10 (s, 1H), 7.87 (s, 1H), 7.70 (m, 1H), 7.29–7.21 (m, 3H), 4.49 (m, 1H), 4.35 (m, 1H), 4.25 (m, 1H), 3.86 (d, *J* = 4.8 Hz, 2H), 3.42 (s, 3H).

{4-[(3′-Bromophenyl)amino]-[1,4]dioxano[2,3-g]quinazolinyl}methyl methanesulfonate (**14a**): *General procedure for mesylation of* **13a** *and* **13b** *in Scheme 2*

To a solution of **13a** (200 mg, 0.52 mmol) in 10 mL of CH_2Cl_2 was added triethylamine (0.72 mL, 5.15 mmol) followed by addition of methanesulfonyl chloride (0.40 mL, 5.15 mmol) at 0 °C. The reaction mixture was stirred at the same temperature for 1 h and further stirred at room temperature for 4 h. After treating with saturated with $NaHCO_3$ solution, the reaction mixture was extracted with CH_2Cl_2. The combined organic layer was dried over $MgSO_4$, concentrated, and purified with column chromatography (EtOAc/hexane = 2/1) to provide **14a** (144 mg, 60 %) as a pale yellow solid: ^{1}H NMR ($CDCl_3$) δ 8.42 (s, 1H), 8.10 (s, 1H), 7.91 (s, 1H), 7.70 (m, 1H), 7.28–7.27 (m, 2H), 7.21 (s, 1H), 4.65 (m, 1H), 4.60–4.50 (m, 3H), 4.26 (m, 1H), 3.10 (s, 3H).

{4-[(3′-Iodophenyl)amino]-[1,4]dioxano[2,3-g]quinazolin-7-yl}methyl methanesulfonate (**14b**): Demethylation and successive mesylation of 4-[(3′-iodophenyl)amino]-7-methoxymethyl-[1,4]dioxano[2,3-*g*]quinazoline **3b** (960 mg, 2.47 mmol) afforded **14b** (756 mg, 59 %) as a pale yellow solid: ^{1}H NMR ($CDCl_3$) δ 8.69 (s, 1H), 8.19 (s, 1H), 8.02 (s, 1H), 7.75 (d, *J* = 8.3 Hz, 1H), 7.49 (d, *J* = 8.7 Hz, 1H), 7.43 (s, 1H), 7.38 (s, 1H), 7.12 (dd, *J* = 8.3, 8.7 Hz, 1H), 4.60 (m, 1H), 4.55-4.44 (m, 3H), 4.25 (m, 1H), 3.13 (s, 3H).

7-(4-Morpholino)methyl-4-[(3′-bromophenyl)amino]-[1,4]-dioxano[2,3-g]quinazoline (**4a**): *General procedure of substitution in Scheme 2*

To a stirred solution of **14a** (64 mg, 0.13 mmol) in 10 mL of THF was added morpholine (113 mL, 1.3 mmol) and triethylamine (0.36 mL, 1.3 mmol) at 0 °C, and the reaction mixture was heated at reflux for 3 h. After concentration, the residue was washed successively with water and ethyl ether and purified by column chromatography (EtOAc/hexane = 2/1) to afford **4a** (42 mg, 68 %) as a white solid: mp 229–230 °C; IR (KBr) 3358, 2826, 2362, 1506, 1426, 1270, 1228 cm^{-1}; ^{1}H NMR (CD_3OD) δ 8.41 (s, 1H), 8.11 (s, 1H), 7.83 (s, 1H), 7.70 (m, 1H), 7.28–7.27 (m, 2H), 7.17 (s, 1H), 4.52–4.45 (m, 2H), 4.16 (m, 1H), 3.72–3.66 (m, 4H), 2.72–2.55 (m, 6H).

7-(Diethanolamino)methyl-4-[(3′-bromophenyl)amino]-[1,4]dioxano[2,3-g]quinazoline (**4b**)By reaction of **14a** with diethanolamine (10 equiv): 40 % (a pale yellow slid); mp 188–191 °C; IR (KBr) 2978, 1506, 1428, 1274, 1234 cm^{-1}; ^{1}H NMR (CD_3OD) δ 8.41 (s, 1H), 8.10 (s, 1H), 7.85 (s, 1H), 7.72 (d, *J* = 8.2 Hz, 1H), 7.28–7.26 (m, 2H), 7.18 (s, 1H), 4.54–4.43 (m, 2H), 4.20 (m, 1H), 3.67–3.59 (m, 4H), 3.30 (m, 1H), 2.92 (m, 1H), 2.89–2.73 (m, 4H).

7-(Ethanolamino)methyl-4-[(3′-bromophenyl)amino]-[1,4]-dioxano[2,3-g]quinazoline (**4c**)

By reaction of **14a** with 2-ethanolamine (10 equiv): 42 % (a yellow solid); mp 168–172 °C; IR (KBr) 2910, 2834, 1602, 1570 cm^{-1}; ^{1}H NMR (CD_3OD) δ 8.42 (s, 1H), 8.10 (s, 1H), 7.88 (s, 1H), 7.71 (m, 1H), 7.28–7.27 (m, 2H), 7.20 (s, 1H), 4.52–4.44 (m, 2H), 4.17 (m, 1H), 3.69 (t, *J* = 5.2 Hz, 2H), 2.97–2.84 (m, 2H), 2.82 (t, *J* = 5.2 Hz, 2H).

7-(4-Methyl-1-piperazino)methyl-4-[(3′-bromophenyl)amino]-[1,4]dioxano[2,3-g]quinazoline (**4d**)

By reaction of **14a** with 1-methylpiperazine (10 equiv): 80 % (a pale yellow solid); mp 182–189 °C; IR (KBr) 3270, 2940, 2796, 1510, 1424, 1278 cm^{-1}; ^{1}H NMR (CD_3OD) δ 8.42 (s, 1H), 8.11 (s, 1H), 7.87 (s, 1H), 7.71 (m, 1H), 7.28–7.27 (m, 2H), 7.20 (s, 1H), 4.51–4.45 (m, 2H), 4.18 (m, 1H), 2.78–2.69 (m, 10H), 2.50 (s, 3H).

7-[(R)-1-Isopropyl-2-ethanolamino]methyl-4-[(3′-bromophenyl)amino]-[1,4]dioxano[2,3-g]quinazoline (**4e**)

By reaction of **14a** with (*R*)-valinol (10 equiv): 84 % (a yellow solid); mp 170–175 °C; ^{1}H NMR (CD_3OD) δ 8.41 (s, 1H), 8.10 (s, 1H), 7.86 (s, 1H), 7.71 (m, 1H), 7.28–7.26 (m, 2H), 7.18 (s, 1H), 4.51–4.38 (m, 2H), 4.19 (m, 1H), 3.70–3.62 (m, 2H), 3.48 (m, 1H), 3.01–2.96 (m, 2H), 1.95 (m, 1H), 0.96–0.89 (m, 6H).

7–[(1-pyrrodino)ethylamino]methyl-4-[(3′-iodophenyl)amino]-[1,4]dioxano[2,3-g]quinazoline (**4f**)

By reaction of **14b** with 1-(2-aminoethyl)pyrrolidine (10 equiv): 20 % (a yellow solid); mp 195–205 °C; ^{1}H NMR (CD_3OD) δ 8.42 (s, 1H), 8.22 (s, 1H), 7.87 (s, 1H), 7.75 (d, *J* = 8.2 Hz, 1H), 7.50 (d, *J* = 8.0 Hz, 1H), 7.21 (s, 1H), 7.14 (dd, *J* = 8.0, 8.2 Hz, 1H), 4.53–4.44 (m, 2H), 4.23 (m, 1H), 3.20–2.90 (m, 10H), 2.00–1.97 (m, 4H).

7-(4-Morpholino)methyl-4-[(3′-iodophenyl)amino]-[1,4]-dioxano[2,3-g]quinazoline (**4g**)

By reaction of **14b** with morpholine (10 equiv): 44 % (a white solid); mp 238–240 °C; IR (KBr) 2966, 2806, 1512, 1424, 1274, 1228 cm^{-1}; ^{1}H NMR (CD_3OD) δ 8.41 (s, 1H), 8.25 (s, 1H), 7.86 (s, 1H), 7.76 (d, *J* = 8.1 Hz, 1H), 7.48 (d, *J* = 7.9 Hz, 1H), 7.19 (s, 1H), 7.13 (dd, *J* = 7.9, 8.0 Hz, 1H), 4.53–4.47 (m, 2H), 4.24 (m, 1H), 3.73–3.69 (m, 4H) 2.73–2.56 (m, 6H).

7-(Diethanolamino)methyl-4-[(3′-iodophenyl)amino]-[1,4]-dioxano[2,3-g]quinazoline (**4h**)

By reaction of **14b** with diethanolamine (10 equiv): 66 % (a white solid); mp 174–175 °C; IR (KBr) 2822, 2234, 1568, 1508, 1426, 1274, 1232 cm^{-1}; ^{1}H NMR (CD_3OD) δ 8.40 (s, 1H), 8.24 (s, 1H), 7.85 (s, 1H), 7.76 (d, *J* = 8.2 Hz, 1H), 7.48 (d, *J* = 8.5 Hz, 1H), 7.19 (s, 1H), 7.13 (dd, *J* = 8.2, 8.5 Hz, 1H), 4.87–4.45 (m, 2H), 4.22 (m, 1H), 3.71–3.49 (m, 4H), 2.93–2.90 (m, 2H), 2.80–2.77 (m, 4H).

7-(2-Ethanolamino)methyl-4-[(3′-iodophenyl)amino]-[1,4]-dioxano[2,3-g]quinazoline (**4i**)

By reaction of **14b** with 2-ethanolamine (10 equiv): 77 % (a white solid); mp 200–201 °C; IR (KBr) 3094, 2910, 2838, 1568, 1508, 1424, 1268, 1240 cm^{-1}; ^{1}H NMR (DMSO-d_6) δ 8.48 (s, 1H), 8.37 (s, 1H), 8.12 (s, 1H), 7.98 (d, *J* = 8.2 Hz, 1H), 7.44 (d, *J* = 8.1 Hz, 1H), 7.21 (s, 1H), 7.17 (dd, *J* = 8.1, 8.2 Hz, 1H), 4.54–4.39 (m, 2H), 4.22–4.18 (m, 1H), 3.69 (t, *J* = 5.3 Hz, 2H), 2.89–2.84 (m, 2H), 2.82 (t, *J* = 5.2 Hz, 2H).

7-(4-Morpholino)methyl-4-[(3′-bromophenyl)amino]-[1,4]-dioxano[2,3-g]quinazoline hydrochloride (**4a HCl**): *General procedure of preparation of hydrochloride*

*To a solution of 7-(4-morpholino)methyl-4-[(3′-bromophenyl)amino]-[1,4]dioxano[2,3-g]*quinazoline **4a** (14 mg, 0.03 mmol) in 3 mL of ethanol was added 1 mL of acetyl chloride at 0 °C, and the reaction mixture was stirred at room temperature for 24 h. The reaction mixture was poured into 15 mL of diethyl ether to give a precipitate. The solid was filtered, washed with ether and dried *in vacuo* to provide **4a** (15 mg, 99 %) as HCl salt (a white solid): mp 230–255 °C (decomp); IR (KBr) 2974, 2596, 1616, 1530, 1446, 1294 cm^{-1}; ^{1}H NMR (D_2O) δ 8.52 (s, 1H), 7.98 (s, 1H), 7.77 (s, 1H), 7.50 (d, *J* = 8.2 Hz, 1H), 7.45 (d, *J* = 8.0 Hz, 1H), 7.39 (dd, *J* = 8.0, 8.2 Hz, 1H), 7.36 (s, 1H), 5.07 (m, 1H), 4.64 (m, 1H), 4.34 (m, 1H), 3.96–3.90 (m, 4H), 3.80–3.70 (m, 2H), 3.60–3.50 (m, 4H).

Determination of water solubility

The water solubility of the compounds was determined as follows: Stock solutions of compound were made up in DMSO and used to calibrate the HPLC (peak area in mmol, assuming a linear response). Conditions of HPLC were as follows: eluant = CH_3CN/H_2O (4/1) and flow rate = 1 mL/min. An accurately weighed amount of compound (5 mg) was supersaturated in 1 mL of distilled water at room temperature and the resultant supersaturated solution was sonicated for 30 min at the same temperature. After standing for an additional 30 min at room temperature, the samples were centrifuged at 14,000 rpm for 5 min. The concentration of compound in the supernatant was determined by HPLC using the calibration curve determined [11]. The data of solubility were recorded in Table 1.

Pharmacology

Expression of human recombinant EGFR tyrosine kinase domain protein

Tyrosine kinase domain in EGFR gene exists in *C*-terminal cytoplasmic domain and the recombinant *C*-terminal protein was verified to possess the tyrosine kinase activity which is identical to the purified natural EGFR protein [11]. To express EGFR tyrosine kinase domain protein in SF9 insect cells, the expression vector using pBacPAK8 vector (from Clontech, U.S.A.) was constructed. The EGFR tyrosine kinase domain (amino acids 668–1210) was PCR amplified using 5′ primer (5′-CTCGAGATGCATCATCATCATCATCGAA-GGCGCCA-CATCGTTCG-3′) and 3′ primer (GGTACCTCTAGATCAT-GCTCCAATAAATTCACTGC-3′) and subcloned into XhoI and KpnI restriction enzyme cloning sites in pBacPAK8 vector. The amplified gene was sequenced to confirm the absence of mutation. The expressed protein was engineered to have hexahistidine tag in its N-terminal. The baculoviral stock from the vector was generated using the virus generation kit (from Clontech U.S.A.). The overexpression of the recombinant protein was carried in 1 L of suspension culture of the virus infected SF9 cells. The expressed protein was purified using Ni^{2+} affinity column (from Novagen, U.S.A.) to over 95 % purity following the protocol provided by the manufacturer. The purified recombinant EGFR tyrosine kinase domain protein has nearly identical K_m value for ATP and V_{max} compared to those of purified natural EGFR protein under our assay condition (unpublished observation).

Tyrosine kinase assays

Total enzymatic inhibition assay was done in total 20 µL reaction mixture containing 10 ng of purified human recombinant EGFR tyrosine kinase domain protein, 250 µM biotinylated tyrosine kinase substrate 2 (purchased from Promega, U.S.A.), 20 µM ATP, 2 µCi p32-gamma-ATP, 5 mM $MgCl_2$, 5 mM DTT, and 1 µL of appropriate dilution of inhibitor. The reaction was carried out for 1 h at 30 °C. The reaction was terminated by adding 10 µL of 30 % phosphoric acid and spotted in avidin coated PVDF membrane (purchased from Promega). The membrane was washed five times with 20 mM Tris-HCl (pH 8.0) containing 0.2 N NaCl. The radioactivity from each spot was quantitated using the BAS system (from Kodak).

Cancer cell growth inhibition assay

A431 (uterus cancer), HCT116 (colon cancer), and SNU638 (stomach cancer) human cancer cells were maintained using RPMI 1640 medium containing 10 % fetal calf serum in 37 °C and 5 % CO_2 incubator. A thousand cells were plated in 96 well plate and incubated overnight. Cells were treated with inhibitor and left for two days. Cells were fixed with formalin solution (SIGMA) and washed with tap water. The cells were dried and stained with 0.1 % sulforhodamine B (SIGMA) for 30 min. The cells were washed with 1 % acetic acid and the dye was eluted from cells by adding 0.1 M Tris-HCl (pH 8.0). The absorbance was measured at 520 nm wavelength using microplate reader (Molecular Dynamics). The inhibitor concentration, which inhibits cell growth by 50 %, was assigned as GI_{50} value.

References

[1] M. Yamada, T. Ikeuchi, H. Hatanaka, *Prog. Neurobiol.* **1997**, *51*, 19–37.

[2] K. Hickey, D. Grehan, I. M. Reid, S. O'Briain, T. N. Walsh, T. P. J. Hennessy, *Cancer* **1994**, *74*, 1693–1698.

[3] D. W. Fry, A. J. Kraker, A. McMichael, L. A. Ambroso, J. M. Nelson, W. R. Leopold, R. W. Connors, A. J. Bridges, *Science* **1994**, *265*, 1093–1095.

[4] G. W. Rewcastle, W. A. Denny, A. J. Bridges, H. Zhou, D. R. Cody, A. McMichael, D. W. Fry, *J. Med. Chem.* **1995**, *38*, 3482–3487.

[5] W. H. J. Ward, P. N. Cook, A. M. Slater, D. H. Davies, G. A. Holdgate, L. R. Green, *Biochem. Pharmacol.* **1994**, *48*, 659–666.

[6] A. Wissner, B. D. Johnson, M. B. Floyd, D. B Kitchen, U.S. Patent 5,760,041, **1998**; CAN 127:190750.

[7] C. M. Discafani, M. L. Carroll, M. B. Floyd Jr., I. J. Hollander, Z. Husain, B. D. Johnson, D. Kitchen, M. K. May, M. S. Malo, A. A. Minnick Jr., R. Nilakantan, R. Shen, Y.-F. Wang, A. Wissner, L. M. Greenberger, *Biochem. Pharmacol.* **1999**, *57*, 917–925.

[8] M. W. Kunkel, K. E. Hook, C. T. Howard, S. Przybranowski, B. J. Roberts, W. L. Elliott, W. R. Leopold, *Invest. New Drugs* **1996**, *13*, 295–302.

[9] A. M. Thompson, A. J. Bridges, D. W. Fry, A. J. Kraker, W. A. Denny, *J. Med. Chem.* **1995**, *38*, 3780–3788.

[10] G. W. Rewcastle, B. D. Palmer, A. M. Thompson, A. J. Bridges, D. J. Fry, A. J. Kraker, W. A. Denny, *J. Med. Chem.* **1996**, *39*, 1823–1835.

[11] A. M. Thompson, D. K. Murray, W. L. Elliott, D. W. Fry, J. Nelson, H. D. H. Showalter, B. J. Roberts, P. W. Vincent, W. A. Denny, *J. Med. Chem.* **1997**, *40*, 3915–3925.

[12] J. Y. Lee, Y. K. Park, S. H. Seo, I.-S. So, H.-K. Chung, B.-S. Yang, S. J. Lee, H. Park, Y. S. Lee, *Arch. Pharm. Pharm. Med. Chem.* **2001**, *334*, 357–360.

[13] A. G. Suárez, *Tetrahedron Lett.* **1999**, *40*, 3523–3526.

[14] P. M. Heertjes, B. J. Knape, H. C. A. van Beek, K. van den Boogaart, *J. Chem. Soc.* **1957**, 3445–3447.

[15] A. Wissner, D. M. Berger, D. H. Boschelli, M. B. Floyd, Jr. L. M. Greenberger, B. C. Gruber, B. D. Johnson, N. Mamuya, R. Nilakantan, M. F. Reich, R. Shen, H-R. Tsou, E. Upeslacis, Y. F. Wang, B. Wu, F. Ye, N. Zhang, *J. Med. Chem.* **2000**, *43*, 3244–3256.

[16] B. D. Smaill, B. D. Palmer, G. W. Rewcastle, W. A. Denny, D. J. McNamara, E. M. Dobrusin, A. J. Bridges, H. Zhou, H. D. H.Showalter, R. T. Winters, W. R. Leopold, D. W. Fry, J. M. Nelson, V. Slintak, W. L. Elliot, B. J. Roberts, P. W. Vincent, S. J. Patmore, *J. Med. Chem.* **1999**, *42*, 1803–1815.