Jae Yeol Lee[a)],
Yong Kyu Park[b)],
Seon Hee Seo[a)],
In-Seop So[a)],
Hee-Kyung Chung[a)],
Beom-Seok Yang[a)],
Sook Ja Lee[b)],
Hokoon Park[a)],
Yong Sup Lee[a)]

[a)] Medicinal Chemistry Research Center, Korea Institute of Science & Technology, P.O. Box 131 Cheongryang, Seoul 130-650, Korea

[b)] Department of Chemistry, Hankuk University of Foreign Studies, Yong-in 449-791, Korea

1,4-Dioxane-fused 4-anilinoquinazoline as inhibitors of epidermal growth factor receptor kinase

The 4-anilinoquinazoline PD 153035 (**1**) is a potential antitumor agent which acts by inhibiting tyrosine kinase activity of epidermal growth factor receptor (EFGR) *via* competitive binding at the ATP site of enzyme. A series of cyclic analogues of PD 153035 bearing the 1,4-dioxane ring was prepared by reaction of 6-chloro derivative **5** with several aniline nucleophiles. These were evaluated for their ability to inhibit the EGFR kinase and the growth of primary human tumor cell cultures. All of the new 4-anilinoquinazolines exhibited less potency than PD 153035 against EGFR kinase. However, compounds **2b, 2c, 2e, 2g**, and **2h** showed higher inhibitory activities than PD 153035 against the growth of A431 tumor cell line. The compound **2b** containing 3-chloroaniline ring was as potent as PD 153035 against EGFR kinase and showed about 5.4-fold better potency than PD153035 in the inhibition of growth of A431 cell line with good selectivity.

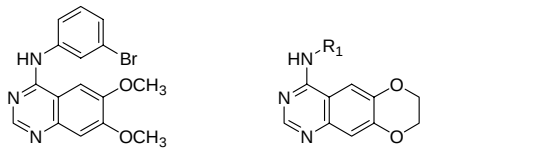
Key Words: EGFR; Tyrosine kinases; 4-Anilinoquinazolines; A431 cell; antitumor agent; 1,4-Dioxane

Received: May 14, 2001 [FP579]

Introduction

Protein tyrosine kinases play a central role in the transduction of proliferative signals in mammalian cells [1]. Tyrosine kinases mainly transduce signals to a target cell by catalyzing the transfer of the terminal phosphate of ATP to tyrosine residues in protein substrates. Abnormal activation of tyrosine kinases has been implicated in many cancers and other proliferative diseases [2]. Therefore, tyrosine kinases are expected to be attractive targets for cancer chemotherapy. Due to the involvement of tyrosine kinases in many signal transduction pathways, the development of selective inhibitors of tyrosine kinases would be important. Recently, several types of synthetic or natural compounds have been reported as potent tyrosine kinase inhibitors [3]. However, most of them have proven to be of limited use due to the low selectivity or weak potency in cellular assays. On the other hand, the 4-anilinoquinazoline class of compounds (e.g. PD 153035, **1**) have proven to be potent and selective inhibitors of tyrosine kinase activity of the epidermal growth factor receptor (EGFR) *via* competitive binding at the ATP site of the enzyme [4]. As a consequence, considerable efforts have been devoted to the synthesis of PD 153035 (**1**) analogues for studying structure-activity relationships to develop tyrosine kinase inhibitory antitumor drugs (Figure 1) [5].

In the present studies, we wish to report the synthesis and *in vitro* evaluation of 1,4-dioxane-fused 4-anilinoquinazolines **2a–2h**, cyclic analogues of PD 153035 (**1**), to probe the effect of the dioxane ring on the inhibition of EGFR kinase and the growth of human tumor cell lines.

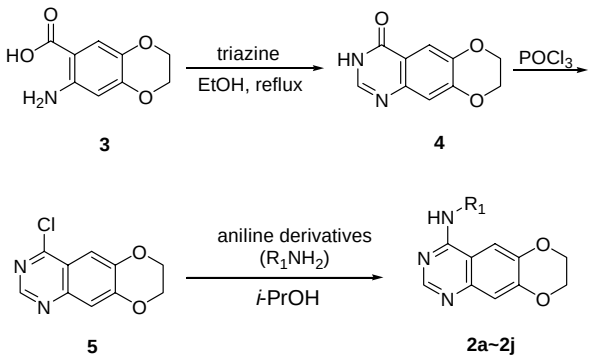


PD 153035 (1) **2a~2h**, R_1 = (un)substituted phenyl

Figure 1. Structures of PD 153035 (**1**) and its cyclic analogue **2**.

Results and discussion

The 1,4-dioxane-fused 4-anilinoquinazolines were prepared by the general method as shown in Scheme 1. 7-Amino-1,4-benzodioxane-6-carboxylic acid (**3**), readily available by the known procedure, was condensed with

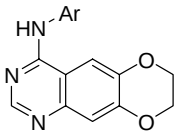


Scheme 1. Synthesis of 1,4-dioxane-fused 4-anilinoquinazolines (**2**).

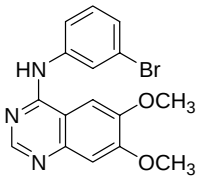
Correspondence: Prof. Yong Sup Lee, Medicinal Chemistry Research Center, Korea Institute of Science & Technology, P.O. Box 131 Cheongryang, Seoul 130-650, Korea.
E-mail: yslee@kist.re.kr
Fax: +82 2 958 5189

 0365-6233/01/1111/0357 $17.50 +.50/0

Table 1. Inhibition of EGFR kinase and inhibition of human tumor cells growth by new quinazoline derivatives (**2a**–**2h**).



(2a~2h)



PD 153035 (1)

Compd[a]	Ar	IC_{50} (nM)[b]	GI_{50} (μM)[c]		
		EGFR	A431	SNU638	A549
2a	phenyl	>100	3.80	54.95	45.71
2b	3-Cl-phenyl	8.3	0.19	4.68	4.27
2c	3-Br-phenyl	17.8	0.20	5.75	4.37
2d	3-I-phenyl	63.0	1.55	6.17	5.62
2e	3-CF_3-phenyl	>100	0.14	2.75	2.24
2f	4-F-phenyl	77.6	1.29	48.98	40.74
2g	3-F-4-Cl-phenyl	>100	0.49	>100	75.86
2h	3,5-di-Me-phenyl	>100	0.12	0.17	0.23
PD 153035 (1)[d]		6.6[e]	1.03	19.50	11.48

[a]These compounds were tested as the free base. [b]Concentration to inhibit by 50% the phosphorylation of the tyrosine kinase substrate 2 by EGFR as determined from the dose-reaponse curve. [c]Dose-response curves were determined at four concentration. The GI_{50} values are the concentrations needed to inhibit cell growth by 50% as determined from these curves. [d]The standard compound was made for comparison for activity. [e]The value was determined by using our assay protocol.

triazine and a catalytic amount of piperidine in refluxing ethanol to afford quinazolinone **4** in 43% yield [6]. The quinazolinone **4** was converted in good yield into 4-chloroquinazoline **5** with $POCl_3$ in the presence of *N,N*-dimethylaniline. The reaction of **5** with appropriate substituted-anilines in refluxing *iso*-propanol gave 1,4-dioxane-fused 4-anilinoquinazolines (**2a–2h**).

The compounds (**2a**–**2h**) shown in Table 1 were evaluated for the ability to inhibit the phosphorylation of EGFR kinase. To compare these data with that of standard, the inhibitory activity of the PD 153035 under our assay conditions was inserted into a data set. The IC_{50} value we obtained with PD 153035 is significantly higher and lower, respectively, than were found by previous workers [7, 8]. It was reported that the difference is at least attributed to the nature of the enzyme and substrate as well as the overall assay conditions [8].

We surveyed a variety of substituents at different positions on the aniline ring of these 1,4-dioxane-fused 4-anilinoquinazolines. In the case of monosubstituted anilide derivatives, all of them (**2c**–**2f**; IC_{50} = 17.8 to >100 nM) were less potent than PD 153035 (IC_{50} = 6.1 nM) except 3-chloroanilinoquinazoline **2b**, which exhibited moderate enzymatic inhibition (IC_{50} = 8.3 nM). The compounds (**2a**, **2g**, and **2h**) containing un- and disubstituted aniline ring showed much lower potency (IC_{50} = >100 nM). This result was consistent with the previous results in that the good enzymatic activity is seen when the aniline ring is monosubstitued with halogen atom at the *meta*-position in the quinazoline-based inhibitors [9].

The compounds were also evaluated for their ability to inhibit the growth of certain cell lines. Three human carcinoma cell lines were used: A431 (uterus cancer), which greatly overexpresses EGFR, and SNU635 (stomach cancer) and A549 (lung cancer) which express low levels of EGFR.

As shown in Table 1, it is immediately obvious that when compared with PD 153035 (GI_{50} = 1.03 μM), most of 1,4-dioxane-fused 4-anilinoquinazolines **2a**–**2h** (GI_{50} = 0.12–3.80 μM) showed similar or better inhibitory activities against A431, irrespective of the potency of the enzymatic activities. This result suggests that the mode of action of these compounds is more complex than originally predicted and it is possible that these compounds inhibit other kinases [10]. Nevertheless, most of compounds except **2h** have the good A431 selectivity over other cell lines which

express low level of EGFR and compound **2g** containing 3-F-4-Cl-aniline ring showed more than 150-fold selectivity for A431 tumor cell line. While all of results are difficult to interpret, there is an approximate correlation between IC_{50} and GI_{50} in the case of compounds (**2b**–**2d**) containing a 3-halogenoaniline ring.

In conclusion, we prepared cyclic analogues **2a**–**2h** of PD 153035 (**1**) to probe the effect of the dioxane ring on the inhibition of EGFR kinase and the growth inhibition of human tumor cell lines. Among them, compound **2b** bearing the 3-chloroaniline ring was as potent as PD153035 against the EGFR but showed about 5.4-fold better potency than PD153035 in the inhibition of growth of A431 cell line with good selectivity. The above results demonstrate that 4-anilinoquinazoline fused with 1,4-dioxane would be effective inhibitor of EGFR when considered on the inhibitory activity against EGFR kinase and the growth of human tumor cell lines. Thus, we will try to prepare the various derivatives of 1,4-dioxane-fused 4-anilinoquinazoline by using this scaffold.

Acknowledgement

This work was supported by Korea Institute of Science and Technology (2E16800).

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. Analytical thin layer chromatography (TLC) was carried out by 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.

*6,7-Ethylenedioxy-4(3H)-quinazolin-4-one (**4**)*

A mixture of 7-amino-1,4-benzodioxane-6-carboxylic acid (**3**, 1.13 g, 5.8 mmol), triazine (0.47 g, 5.8 mmol), and piperidine (0.04 ml, 0.58 mol) in EtOH (120 ml) was heated at reflux for 24 h. The precipitated solid was filtered and washed with EtOH. The filtrate was concentrated and the resulting solid was washed with EtOH. The combined solid was dried *in vacuo* to give **4** (0.51 g, 43%) as a white solid: mp 209 °C (dec.); ^{1}H NMR (DMSO-d_6, 300 MHz) δ 7.95 (1H, s, H-2), 7.45 (1H, s, H-10), 7.06 (1H, s, H-5), 4.36–4.33 (4H, m, 2 × H-7 & 2 × H-8), 1.67 (1H, s, NH); EI-MS (*m/z*): 204 (M^+) (calcd mass for $C_{10}H_8N_2O_3$ 204.18).

*4-Chloro-6,7-ethylenedioxyquinazoline (**5**)*

A solution of **4** (0.56 g, 2.7 mmol) and *N,N*-dimethylaniline (0.64 ml, 5.5 mmol) in $POCl_3$ (25 ml) was heated at reflux for 2 h. $POCl_3$ was removed by distillation and the residue was diluted with CH_2Cl_2 and treated with ice-water. The organic layer was separated and washed with aqueous $NaHCO_3$, brine, dried ($MgSO_4$), and concentrated to give **5** (0.54 g, 89%) as a yellowish solid. The crude **5** was used for the next step without purification: EI-MS (*m/z*): 222 (M^+) (calcd mass for $C_{10}H_7ClN_2O_2$ 222.63).

*General procedure for the preparation of 4-anilino-6,7-ethylenedioxyquinazolines (**2a–2h**)*

A mixture of **5** (16 mg, 0.072 mmol), appropriate (un)substituted aniline (0.144 mmol), and a catalytic amount of conc. HCl in *i*-PrOH (4 ml) was heated at reflux for 24 h. The reaction mixture was cooled to room temperature and the resulting solid was filtered, washed with *i*-PrOH, and dried to give 4-anilino-6,7-ethylenedioxyquinazolines **2** as free base.

*6,7-Ethylenedioxy-(4-phenylamino)quinazoline (**2a**)*

41% Yield; ^{1}H NMR (DMSO-d_6) δ 11.04 (1H, s, NH), 8.76 (1H, s, H-2), 8.68 (1H, s, H-10), 8.25 (1H, s, H-5), 7.70 (2H, d, *J* = 6.6 Hz, H-2′ & H-6′ of aniline), 7.47 (2H, m, H-3′ & H-5′ of aniline), 7.30 (1H, *J* = 7.5 Hz, H-4′ of aniline), 4.51–4.47 (4H, m, 2 × H-7 & 2 × H-8).

*4-[(3-Chlorophenyl)amino]-6,7-ethylenedioxyquinazoline (**2b**)*

60% Yield; ^{1}H NMR (DMSO-d_6) δ 11.78 (1H, s, NH), 9.60 (1H, s, H-2), 9.10 (1H, s, H-10), 8.69 (1H, s, H-2′ of aniline), 8.46 (1H, d, *J* = 6.3 Hz, H-6′ of aniline), 8.28–8.22 (2H, m, H-4′ & H-5′ of aniline), 8.09 (1H, s, H-5), 5.25–5.21 (4H, m, 2 × H-7 & 2 × H-8).

*4-[(3-Bromophenyl)amino]-6,7-ethylenedioxyquinazoline (**2c**)*

73% Yield; mp 230–240 °C; ^{1}H NMR (DMSO-d_6) δ 10.95 (1H, s, NH), 8.80 (1H, s, H-2), 8.27 (1H, s, H-10), 8.07 (1H, s, H-2′ of aniline), 7.78 (1H, d, *J* = 6,6 Hz, H-6′ of aniline), 7.27–7.43 (2H, m, H-4′ & H-5′ of aniline), 7.34 (1H, s, H-5), 4.59–4.41 (4H, m, 2 × H-7 & 2 × H-8); EI-MS (*m/z*): 358 (M^+) (calcd mass for $C_{16}H_{12}BrN_3O_2$ 358.19).

*4-[(3-Iodophenyl)amino]-6,7-ethylenedioxyquinazoline (**2d**)*

95% Yield; mp 242–245 °C; ^{1}H NMR (DMSO-d_6) δ 11.37 (1H, s, NH), 8.89 (1H, s, H-2), 8.48 (1H, s, H-10), 8.15 (1H, s, H-2′ of aniline), 7.66–7.80 (3H, m, H-4′, H-5′ & H-6′ of aniline), 7.46 (1H, s, H-5), 4.69–4.52 (4H, m, 2 × H-7 & 2 × H-8).

*4-[(3-Trifluoromethylphenyl)amino]-6,7-ethylenedioxyquinazoline (**2e**)*

30% Yield; mp 273–275 °C; ^{1}H NMR (DMSO-d_6) δ 11.08 (1H, s, NH), 8.86 (1H, s, H-2), 8.35 (1H, s, H-10), 8.19 (1H, s, H-2′ of aniline), 8.09 (1H, d, *J* = 8.1 Hz, H-6′ of aniline), 7.65–7.74 (2H, m, H-4′ & H-5′ of aniline), 7.34 (1H, s, H-5), 4.50–4.47 (4H, m, 2 × H-7 & 2 × H-8); EI-MS (*m/z*): 347 (M^+) (calcd mass for $C_{17}H_{12}F_3N_3O_2$ 347.29).

*4-[(4-Fluorophenyl)amino]-6,7-ethylenedioxyquinazoline (**2f**)*

47% Yield; mp 282–284 °C; ^{1}H NMR (DMSO-d_6) δ 11.11 (1H, s, NH), 8.80 (1H, s, H-2), 8.31 (1H, s, H-10), 7.72 (2H, d, *J* = 8.0 Hz, H-2′ & H-6′ of aniline), 7.50 (1H, d, *J* = 8.0 Hz, H-3′ & H-5′ of aniline), 7.34 (1H, s, H-5), 4.50–4.46 (4H, m, 2 × H-7 & 2 × H-8); EI-MS (*m/z*): 297 (M^+) (calcd mass for $C_{16}H_{12}FN_3O_2$ 297.28).

*4-[(4-Chloro-3-fluorophenyl)amino]-6,7-ethylenedioxyquinazoline (**2g**)*

27% Yield; ^{1}H NMR (DMSO-d_6) δ 10.33 (1H, s, NH), 8.78 (1H, s, H-2), 8.31 (1H, s, H-10), 8.20 (1H, s, H-2′ of aniline), 8.10 (1H, d, *J* = 7.8 Hz, H-6′ of aniline), 7.56 (1H, d, *J* = 7.8 Hz, H-5′

of aniline), 7.25 (1H, s, H-5), 4.18–4.30 (4H, m, 2 × H-7 & 2 × H-8).

*4-[(3,5-Dimethylphenyl)amino]-6,7-ethylenedioxyquinazoline (**2h**)*

54% Yield; mp >300 °C (dec.); ^{1}H NMR (DMSO-d_6) δ 11.64 (1H, s, NH), 8.90 (1H, s, H-2), 8.30 (1H, s, H-10), 7.57 (2H, s, H-2′ & H-6′ of aniline), 7.30 (1H, s, H-5), 7.10 (1H, s, H-4′ of aniline), 4.52–4.38 (4H, m, 2 × H-7 & 2 × H-8).

Pharmacology

Expression of human recombinant EGFR tyrosine kinase domain protein

The tyrosine kinase domain in the EGFR gene exists in the *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'-CTCGAGATGCATCATCATCATCATCATCGAAGGCGCCACATCGTTCG-3') and 3' primer (GGTACCTCTAGATCATGCTCCAATAAATTCACTGC-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 USA). The overexpression of the recombinant protein was carried in 1L suspension culture of the virus infected SF9 cells. The expressed protein was purified using Ni^{2+} affinity column (from Novagen, USA) 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, USA), 20 µM ATP, 2 µCi p32-gamma-ATP, 5 mM $MgCl_2$, 5 mM DTT, and 1µl 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 0.2N NaCl. The radioactivity from each spot was quantitated using the BAS system (from Kodak).

Cancer cell growth inhibition assay

A431 (uterus cancer), SNU638 (stomach cancer), and A549 (lung 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 0.1M 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] A. J. Bridges, *Chemtracts-Organic Chemistry* **1995**, *8*, 73–107.

[4] 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.

[5] 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.

[6] P. M. Heertjes, B. J. Knape, C. A. van Beek, K. van den Boogaart, *J. Am. Chem. Soc.***1957**, *79*, 3445–3447.

[7] A. J. Bridges, H. Zhou, D. R. Cody, G. W. Rewcastle, A. McMichael, H. D. H. Showalter, D. W. Fry, A. J. Kraker, W. A. Denny, *J. Med. Chem.* **1996**, *39*, 267–276.

[8] 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**, *4*3, 3244–3256.

[9] 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.

[10] A. Gazit, J. Chen, H. App, G. McMahon, P. Hirth, I. Chen, A. Levitzki, *Bioorg. Med. Chem.* **1996**, *8*, 1203–1207.

[11] C. Y. Hsu, M. Mohammadi, M. Nathan, A. Honegger, A. Ullrich, J. Schlessinger, D. R. Hurwitz, *Cell Growth Differ.* **1990**, *1,* 191–200.