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Orally active anti-proliferation agents: novel diphenylamine derivatives as FGF-R2 autophosphorylation inhibitors

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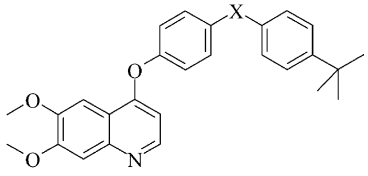
Abstract—(6,7-Disubstituted-quinolin-4-yloxy-phenyl)(4-substituted-phenyl)amine derivatives were synthesized and evaluated by a cellular autophosphorylation assay for FGF-R2 in the human scirrhous gastric carcinoma cell line, OCUM-2MD3. We also performed metabolic stability studies showing that substitutions at the 7-position of quinoline affect its biological stability. In this study, we achieved a remarkable improvement in the solubility and metabolic stability of the diphenylamine derivative. The most promising compound **15e** showed a significant decrease in tumor volume when orally administered.

Fibroblast growth factors (FGFs) are thought to play important roles in angiogenesis, wound healing as well as some malignancies.[1] It has been reported that an inappropriate expression of FGFs or their receptors (FGFRs) could contribute to a variety of pathologies, such as tumor neovascularization, diabetic retinopathy, atherosclerosis and rheumatoid arthritis.[2] FGFRs consist of four related receptor tyrosine kinases, FGF-R1 (Flg), -R2 (Bek), -R3 and -R4. Among them, the overexpression of FGF-R2 has been implicated with the poor prognosis in some types of cancers, including scirrhous gastric carcinoma.[3] Some groups have reported small molecule compounds that inhibit FGF-R1,[4] but no small molecule inhibitor of FGF-R2 has been reported.

We previously reported[5] the synthesis and biological evaluation of 6,7-disubstituted-4-phenoxyquinolines and 6,7-disubstituted-4-phenoxyquinazolines. In these studies, we found novel selective inhibitors of PDGFR kinase and showed their potency as novel therapeutic agents. During the course of the investigation on PDGFR tyrosine kinase inhibitors, we found that **1** inhibited the autophosphorylation of FGF-R2 and PDGFR with about the same IC_{50} value.[5c] After synthesizing and evaluating some derivatives of **1**, we found that replacement of the benzophenone moiety of **1** into the diphenylamine moiety **2** increased kinase inhibitory activity (Table 1). Furthermore, **2** did not inhibit the EGFR kinase activity, which indicated **2** is not a non-specific kinase inhibitor. The ex vivo inhibitory effects on the autophosphorylation of FGF-R2 were tested using blood serum collected from mice 5 h after the oral administration of these compounds. As shown in Table 1, only the diphenylamine derivative **2** showed potent inhibitory activity. Encouraged by these results, we investigated the potency of the diphenylamine

Table 1. Inhibition of FGF-R2 autophosphorylation



Compd	X	In vitro (IC_{50}, nM)	Ex vivo (% inhibition at 100 mg/kg)
1	CO	190	3
2	NH	40	70
3	O	440	NT
4	CH_2	>1000	5

NT, not tested.

Keywords: Kinase inhibitor; Quinoline; Anti-proliferation; FGF-R2; Bek.
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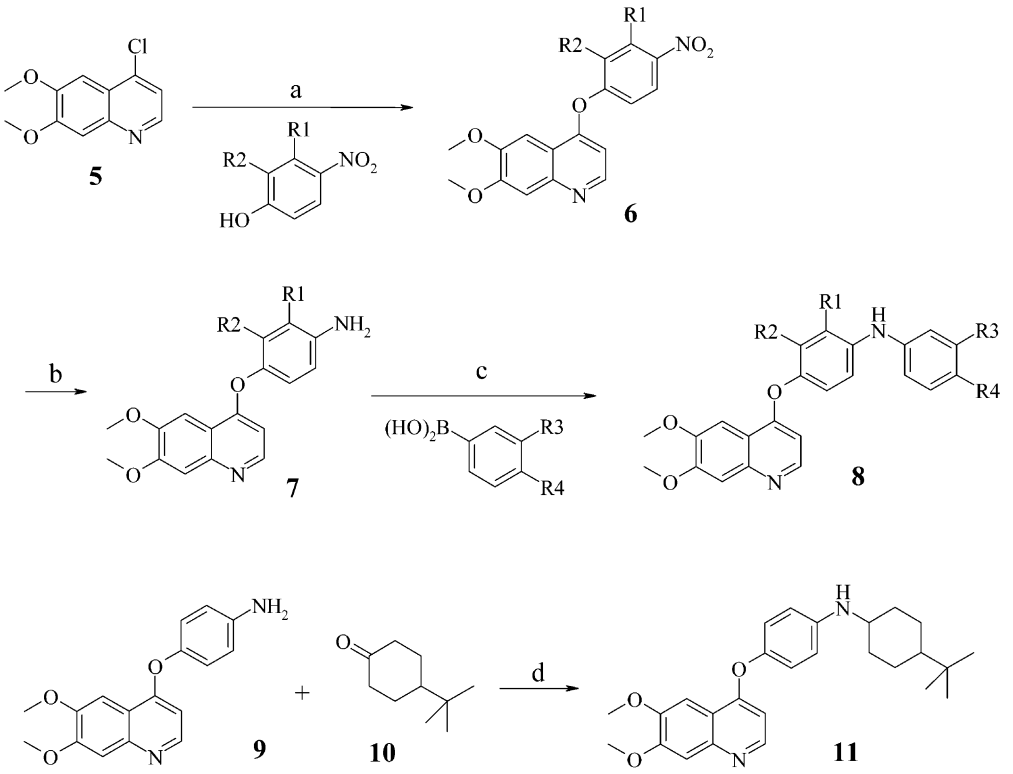


Scheme 1. (a) Chlorobenzene, 120 °C, 32–100%; (b) $Pd(OH)_2$, H_2 gas, Et_3N, DMF or Zn, NH_4Cl, $EtOH/H_2O$, 55-79%; (c) $Cu(OAc)_2$, Et_3N, $CHCl_3$, 4–79%; (d) $NaBH(OAc)_3$, DMF, 11%.

derivatives as novel anti-tumor agents. In this report, we describe the synthesis and biological evaluation of the diphenylamine derivatives as FGF-R2 inhibitors. We also present the in vivo data of the optimized compound and its potency as an anti-proliferation agent.

We previously established an excellent synthetic route of the 4-chloroquinoline derivatives.[6] We adopted this route to prepare the 6,7-disubstituted chloroquinolines **5**. A typical synthetic Scheme for the diphenylamine derivatives is shown in Scheme 1. Heating **5** with nitrophenols in chlorobenzene afforded the 4-nitrophenoxy derivatives **6**. The nitro groups of **6** were reduced in DMF using palladium or zinc to give the anilines **7**, which were reacted with boronic acids in the presence of copper diacetate and triethylamine to afford the diphenylamines **8**. **11** was synthesized by the reductive alkylation of the aniline **9**.

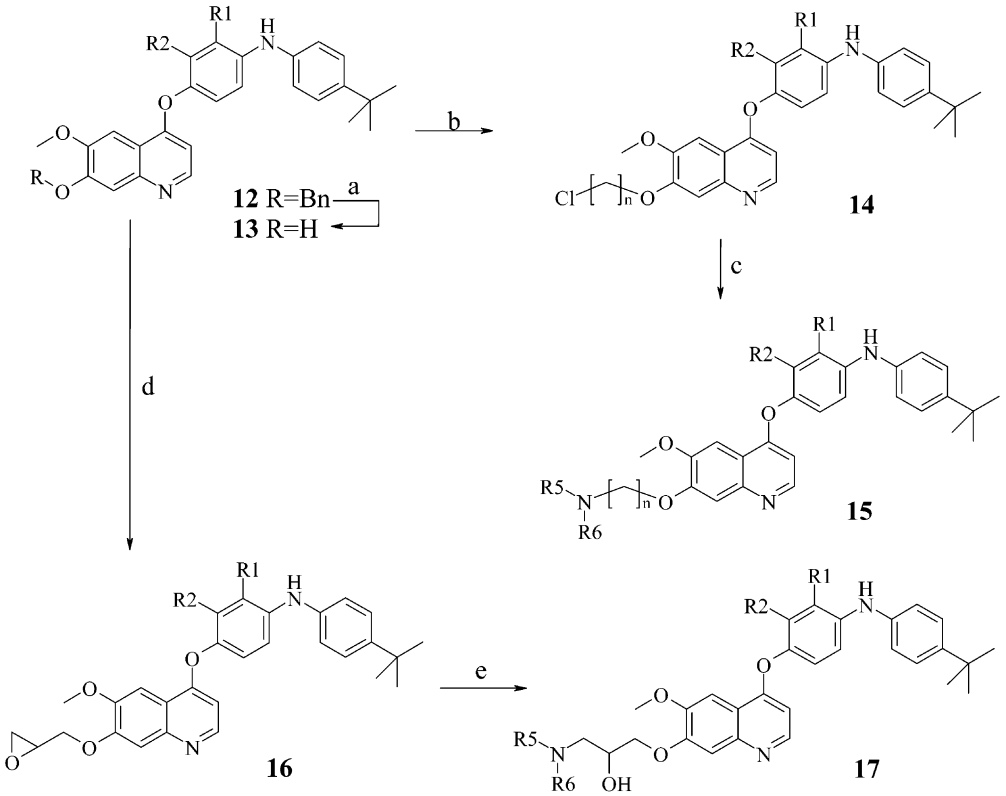
Scheme 2 shows the synthetic route of the compounds containing a substituted alkoxy group at the 7-position. After the preparation of **12**, the benzyl group was removed using methanesulfonic acid in trifluoroacetic acid. The treatment of **13** with bromochloroalkanes or epibromohydrin, followed by nucleophilic substitution with amines gave the amino-substituted alkoxy compounds **15** and **17**, respectively.

Biological evaluations of the diphenylamine derivatives were carried out using OCUM-2MD3, a human scirrhous gastric carcinoma cell line, established by Yashiro et al.[7] This cell line is reported to constitutively express activated FGF-R2, and to be tumorigenic in athymic animals.

Table 2 shows the inhibitory effects of these compounds on the autophosphorylation of FGF-R2, assayed with the cellular ELISA system. First, the substituent effects on both phenyl rings of diphenylamine were investigated. These results are shown in Table 2. The results of **2** and **8a–e** strongly supported the necessity of a hydrophobic bulky group in R4 to retain the inhibitory activity. This part of the molecules could be considered to be recognized by FGF-R2 so strictly that a minor change would not be allowed to provide any inhibitory activity. Similar results were obtained regarding the R1 and R2 substituents. Although retention in the IC_{50} value was observed with the fluoro-substituted compounds **8i** and **8j**, none of the compounds showed a significant increase in inhibitory activity.

After we found that substitutions on these phenyl rings were severely restricted, our focus was shifted to modification of the methoxy group at the 7-position of quinoline.

It has been demonstrated that the metabolic stability is drastically changed depending on substituents at the 7-position on quinoline or quinazoline.[8] For this reason, we evaluated the stability in the presence of microsomes at the same time. In addition to this issue, we needed to increase the solubility of the compounds because **2** was only slightly soluble in water. Therefore, we selected secondary and tertiary amines to introduce at the 7-position in order to obtain compounds with both good metabolic stability and good solubility. These results are summarized in Table 3. During the investigation, we observed a tendency that compounds with a



Scheme 2. (a) MsOH, TFA, DMF, 80–92%; (b) $Br(CH_2)_nCl$, K_2CO_3, DMF, 90–100%; (c) R_5R_6NH, K_2CO_3, DMF, 80 °C, 47–94%; (d) epibromohydrin, K_2CO_3, DMF; (e) R_5R_6NH, K_2CO_3, DMF, 80 °C, 81–89% (2 steps).

Table 2. Inhibitory activity on autophosphorylation of FGF-R2

Compd	R_1	R_2	R_3	R_4	IC_{50} (nM)
2	H	H	H	*t*Bu	40
8a	H	H	H	H	8900
8b	H	H	H	Ph	4600
8c	H	H	Ph	H	>10,000
8d	H	H	H	OMe	780
8e	H	H	H	*i*Pr	200
8f	Me	H	H	*t*Bu	100
8g	H	Me	H	*t*Bu	>10,000
8h	Me	Me	H	*t*Bu	130
8i	F	H	H	*t*Bu	63
8j	H	F	H	*t*Bu	54
8k	CF_3	H	H	*t*Bu	>10,000
11	—	—	—	—	3400

terminal hydroxyl group (**15c–g**) were metabolically stable. Compound **15e** had the best stability as well as good inhibitory activity. Furthermore, **15e** was stable under incubation with human liver microsomes for 10 min (Table 4). It was noteworthy that 78 and 89% of **15e** existed after a 40-min incubation with rat and human liver microsomes, respectively. Therefore, **15e** was selected to investigate its potency as an anti-tumor agent.

Table 5 shows the solubility of **15e**. A drastic improvement in the solubility from the initial compound **2** was achieved with **15e**, which was soluble even into water for injection (WFI; JP grade) at 108μg/mL.

The kinase specificity profile of **15e** was also demonstrated, and these results are shown in Table 6. Autophosphorylation of each receptor tyrosine kinase was

Table 3. Inhibition of candidates to FGF-R2 autophosphorylation assay and their metabolic stabilities

Compd	R_1	R_2	*n*	R_5	R_6	IC_{50} (nM)	Metabolization[a] (%)
2	H	H	—	—	—	40	90
15a	H	H	3	$-(CH_2)_5-$		350	28
15b	H	H	2	$-(CH_2)_2O(CH_2)_2-$		200	9
15c	H	H	2	$-(CH_2)_2OH$	$-(CH_2)_2OH$	71	46
15d	H	H	2	$-(CH_2)_2CH(CH_2OH)(CH_2)_2-$		23	51
15e[b]	H	H	2	$-(CH_2)_2OH$	H	88	97
15f	F	H	2	$-(CH_2)_2OH$	H	260	92
15g	H	F	2	$-(CH_2)_2OH$	H	70	60
17a	H	H	—	$-(CH_2)_5-$		200	78
17b[b]	H	H	—	$-(CH_2)_2O(CH_2)_2-$		71	72

[a] Percentage of parent compounds remaining after incubation with rat liver microsomes for 10 min at 37 °C.
[b] Hydrochloride salt was used.

measured in intact cells.[9] **15e** showed a decent inhibitory activity for the autophosphorylation of KDR, PDGF-Rβ, c-Kit as well as FGF-R2. On the contrary, no activity was observed against the EGFR, IGF-1R and c-Met tyrosine kinases at 10 μM. These results indicate that **15e** possesses a favorable specificity for the PDGF-R family.

The effects of **15e** on the proliferation of cultured cells were evaluated using the WST-1 colorimetric assay system[10] after a three-day incubation of cells with 10% FBS. As shown in Figure 1, **15e** potently inhibited the proliferation of the human scirrhous gastric carcinoma cell line OCUM-2MD3 in a concentration-dependent manner. It is noteworthy that **15e** did not inhibit the proliferation of IEC-6, the rat ileac epithelial cell line, which indicates that the inhibitory effect of **15e** is selective among cell lines.

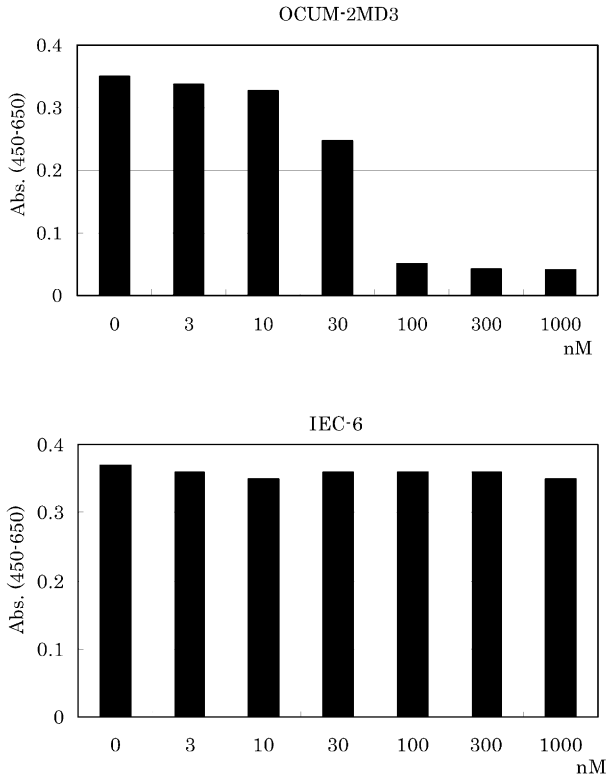


Figure 1. Inhibitory effects of **15e** on the proliferation of cultured cells: (1) Abs. (450-650) values were in proportion to the number of cells. (2) Hydrochloride salt of **15e** was used.

With these encouraging data in hand, the anti-tumor effects of compound **15e** were evaluated in a nude mice xenograft model bearing OCUM-2MD3.[11] **2** did not show any anti-tumor activity at 30 mg/kg, presumably due to its poor solubility. On the contrary, as shown in Figure 2, the oral administration of **15e**, once daily for five consecutive days, potently inhibited the growth of established tumors in a dose-dependent manner. In the

Table 4. Metabolic stability profiles of **15e** to human or SD rat microsomes

Species	Time (min)	Metabolization[a] (%)
Rat	10	97
	40	78
Human	10	99
	40	89

[a] Percentage of parent compounds remaining after incubation with rat or human liver microsomes at 37 °C.

Table 5. Comparison of solubilities

Compd	WFI (μg/mL)	pH1.2[a] (μg/mL)
2	0	3
15e	108	802

[a] An aqueous solution of NaCl (0.2%, w/v) and HCl (0.07N) was used.

Table 6. Kinase specificity of **15e**

RTK	IC_{50} (nM)
FGF-R2	88
KDR	83
PDGFR	100
c-Kit	480
EGFR	> 10,000
IGF-1R	> 10,000
c-Met	> 10,000

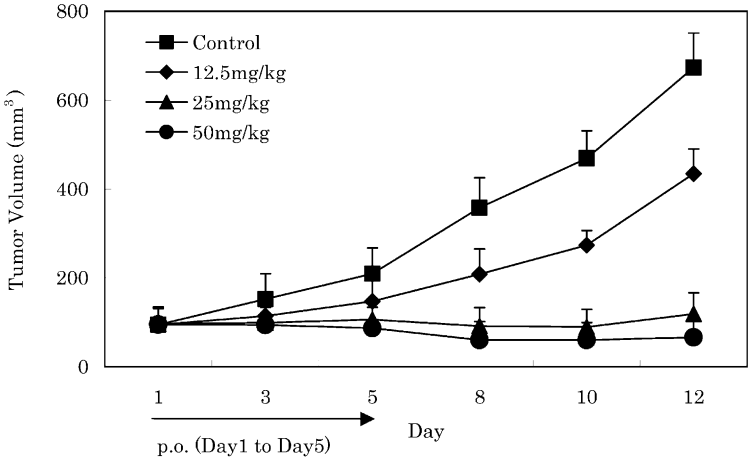


Figure 2. Anti-tumor effects of **15e**: (1) hydrochloride salt of **15e** was used.

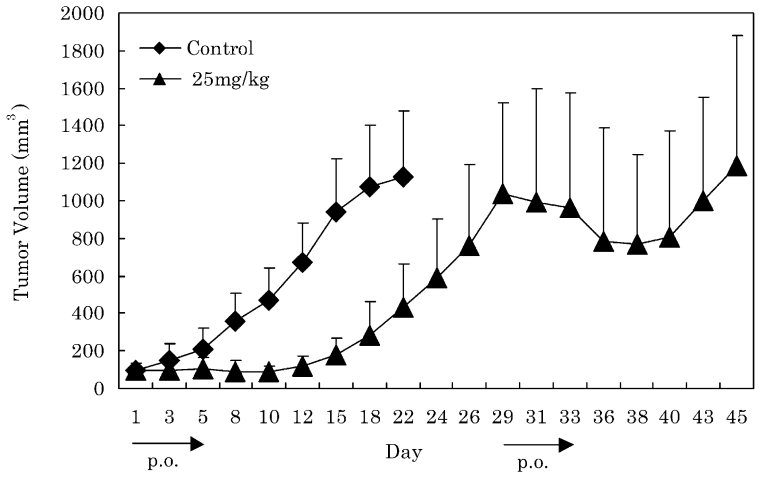


Figure 3. Anti-tumor effects of **15e**: (1) hydrochloride salt of **15e** was used.

25 mg/kg and 50 mg/kg treatment groups, the anti-tumor effects of **15e** were sustained with a tendency of tumor regression for nearly one week after discontinuation of the administration. In the 25 mg/kg treatment group, the administration of **15e** was restarted 24 days after the discontinuation of the initial administration (Fig. 3). Re-administration of **15e**, 25 mg/kg once daily for five consecutive days, induced tumor regression. This effect was sustained for nearly one week after the discontinuation of the re-administration. In all treatment groups, no abnormal body weights were detected. These data suggest that the oral administration of **15e** may be able to decrease the volume of tumors in cancer patients.

In summary, we described the synthesis of diphenylamine derivatives and their inhibitory activity on the autophosphorylation of FGF-R2. We also showed that **15e** possessed anti-tumor activity in vivo. As shown in Table 5, **15e** possesses kinase inhibitory activity against other receptor tyrosine kinases, which have been reported to correlate with cell proliferation.[12] With its encouraging anti-tumor effects data in a nude mice xenograft model bearing OCUM-2MD3, **15e** can be expected to be an effective therapeutic agent against a variety of tumors. Investigations on its potency will be separately reported.

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