de novo Design and synthesis of *N*-benzylanilines as new candidates for VEGFR tyrosine kinase inhibitors†

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***N*-Benzylanilines were designed and synthesized as vascular endothelial growth factor (VEGF)-2 inhibitors using *de novo* drug design systems based on the X-ray structure of VEGFR-2 kinase domain. Among compounds synthesized, compound 3 showed the most potent inhibitory activity toward VEGFR-2 (KDR) tyrosine kinase and its IC_{50} value was 0.57 μM.**

Vascular endothelial growth factor (VEGF) is a key growth factor in tumor angiogenesis. In general, angiogenesis is tightly regulated and only normally occurs in inflammation, wound healing, and the female reproductive cycle in the adult.[1] Uncontrolled angiogenesis involves pathological states such as atherosclerosis, diabetic retinopathy, rheumatoid arthritis, and solid tumor growth. Vascular endothelial growth factor (VEGF) is a key growth factor in tumor angiogenesis, therefore, its receptor tyrosine kinases known as VEGFR-1 (Flt-1) and VEGFR-2 (KDR/Flk-1) have been considered as attractive targets for the development of anticancer drugs.[2]

The development of receptor-based drug design has profoundly benefited the drug discovery process. Especially, *de novo* design based upon a co-crystal structure of a protein–ligand complex has become a powerful strategy to provide a starting point for design of a lead compound.[3,4] For instance, CDK4 inhibitors,[3a] the HIV-1 integrase inhibitors,[3b] FKBP-12 ligands,[3c] Factor Xa inhibitors,[3d] COX-2 inhibitors,[3e] and DNA gyrase inhibitors[3f] have been developed using *de novo* systems. In this paper, we designed a new type of VEGF tyrosine kinase inhibitor using Ludi, a *de novo* drug design program,[5] based on a crystal structure of tyrosine kinase domain in complex with a ligand.

Fig. 1 shows the *de novo* design of the *N*-benzylbenzene-1,4-diamine derivatives **1–3**. On the basis of an X-ray model of the KDR binding pocket (PDB code: 1Y6A),[6] interaction sites of the ligand were identified. Furthermore, taking into account other co-crystal structures of the KDR kinase domain and ligands, such as AAL993,[2a] AAZ,[6] and 4-amino-furo[2,3-*d*]pyrimidines,[7] we predicted four important interaction sites, Leu840, Arg842, Cys919, and Asn1032, and partitioned off the binding pocket into three partitions, A, B, and C (Fig. 1A). Among the fragment library, phenylurea was chosen as a suitable fragment for partition A with a high Ludi score (= 212) and two hydrogen bonds were predicted between two hydrogens of the urea and the carbonyl group of Arg842. In a similar manner, 3-hydroxybenzylamine (Ludi score = 373) and carboxylic acid (Ludi score = 300) were chosen for partitions B and C, respectively. (Fig. 1B). Finally, linking of these four fragments and modification of the obtained structure due to synthetic compatibility led to candidate molecule **1** (Fig. 1B). According to the docking simulation of **1** toward KDR kinase domain, five hydrogen bonds would be expected between the urea and Arg842, the amine and Asn1032, the hydroxyl group and Leu840, and carboxylic acid and Cys919, and Ligscore2 was calculated as 5.59. Compound **1** showed the highest Ligscore2 value among the compounds conducted with selected fragments (data not shown). Therefore, we designed and synthesized a series of the *N*-benzylbenzene-1,4-diamine derivatives (**1** and **2**), and *N*-(4-aminophenyl)benzamides (**3**) based on these observations (Fig. 2).

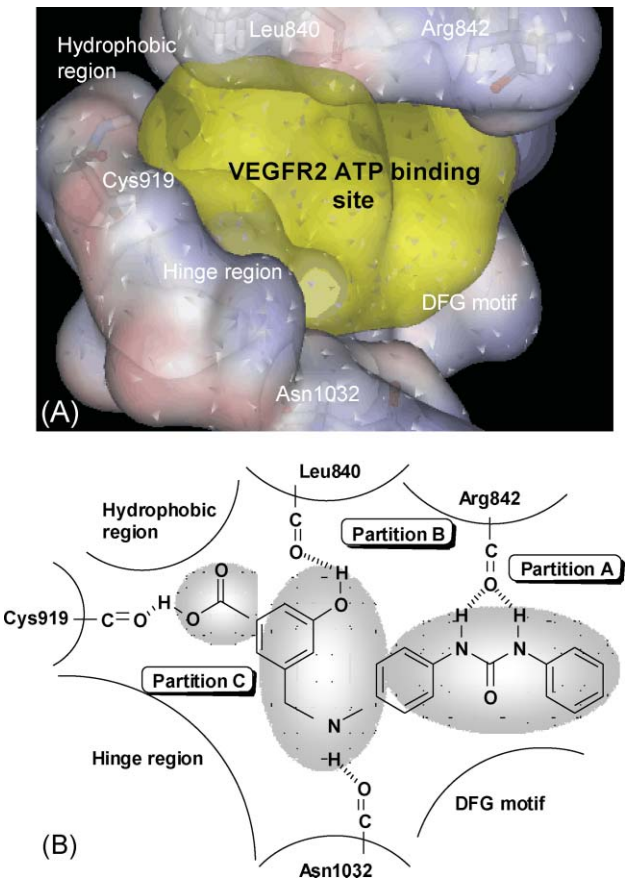

Fig. 1 Design process of VEGFR-2 tyrosine kinase inhibitor using the LUDI *de novo* drug design systems in the DS modeling 1.2 package based on the X-ray analysis data of the kinase domain (PDB: 1Y6A).

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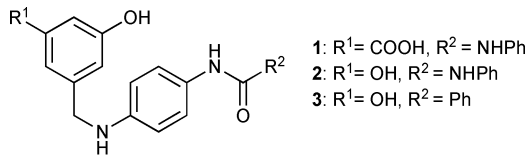
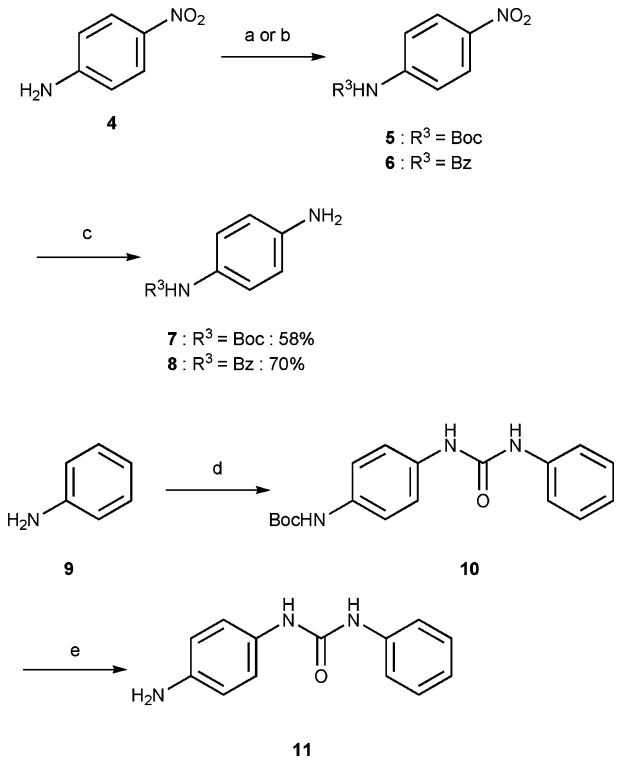


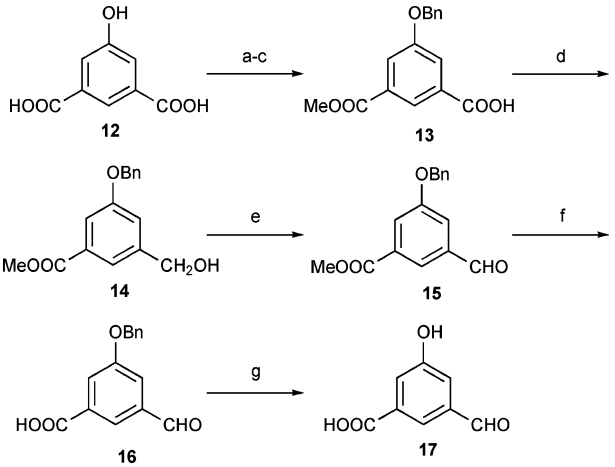
Fig. 2 Design of target compounds.

Synthesis of the aniline fragments that occupied site A is shown in Scheme 1. Amines **7** and **8** were prepared from 4-nitroaniline **4** with Boc_2O or BzCl, and the resulting protected anilines **5** and **6** underwent reduction under Pd/C catalyzed conditions to afford amines **7** and **8** in 58% and 70% yields, respectively. Aniline **9** was treated with phenylisocyanate to give the corresponding urea derivative **10**, and then deprotection of the Boc group with trifluoroacetic acid afforded 1-(4-aminophenyl)-3-phenylurea **11**.



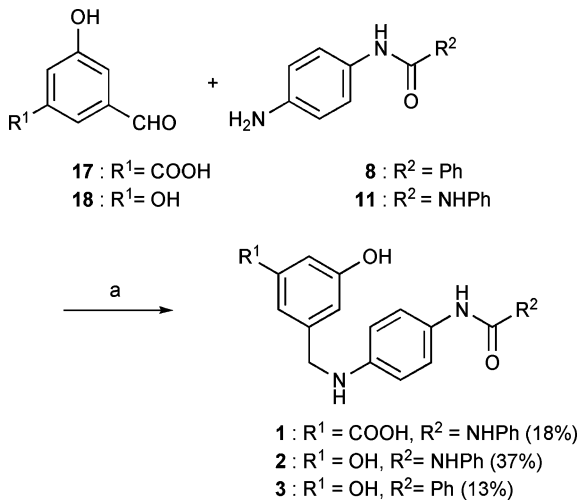
Scheme 1 *Reagents and conditions:* (a) Boc_2O, CH_2Cl_2, reflux, 99%. (b) BzCl, pyridine, cat. DMAP, 60 °C, 99%. (c) H_2, Pd/C, MeOH. (d) $(Cl_3CO)_2CO$, toluene, 80 °C; then **7**, 99%. (e) 4 eq. TFA, CH_2Cl_2, reflux, 91%.

Aldehyde **17** was prepared from 5-hydroxyisophthalic acid **12** according to a previous report.[8] Esterification of 5-hydroxyisophthalic acid with methanol gave the dimethyl ester, which underwent protection of the phenoxy group with benzylbromide, followed by monohydrolysis using 2 equivalents of KOH to afford monomethylester **13**. Selective reduction of the carboxylic acid group on compound **13** was carried out using borane dimethyl sulfide complex to give the benzylalcohol **14**. Oxidation of the hydroxy group on compound **14** with activated MnO_2 in ethyl acetate gave the aldehyde **15**. Hydrolysis of the aldehyde **15** was carried out by treatment with lithium hydroxide in methanol–water to give the benzoic acid **16**, which underwent debenzylation with TFA to give 3-formyl-5-hydroxybenzoic acid **17** (Scheme 2).



Scheme 2 *Reagents and conditions:* (a) MeOH, conc. H_2SO_4, reflux, 90%. (b) BnBr, K_2CO_3, acetone, reflux, 93%. (c) 2 eq. KOH, MeOH, THF, reflux, 60%. (d) $BH_3{\cdot}SMe_2$, THF, 71%. (e) MnO_2, AcOEt, reflux, 87%. (f) LiOH, MeOH–H_2O, 92%. (g) TFA, reflux, 72%.

Finally, reductive amination of formyl-5-hydroxybenzoic acid **17** and 3,5-dihydroxybenzaldehyde **18** with amines **8** and **11** proceeded in the presence of $NaCNBH_3$ to give the *N*-benzylbenzene-1,4-diamine derivatives **1–3** in 13–37% yields (Scheme 3).

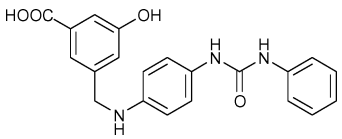
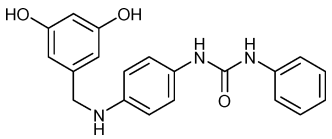
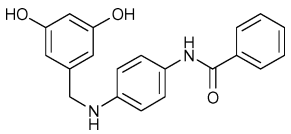


Scheme 3 Synthesis of *N*-benzylbenzene-1,4-diamine derivatives **1–3**: (a) $NaCNBH_3$, MeOH, 60 °C.

Inhibitory activity of the *N*-benzylbenzene-1,4-diamine derivatives **1–3** toward VEGFR2 (KDR) tyrosine kinase was examined by ELISA.[9,10] Among the derivatives synthesized, compound **3** showed the most potent inhibitory activity toward KDR tyrosine kinase and its IC_{50} value was 0.57 μM (Table 1).

We next examined the inhibitory effect of compound **3** on activation of VEGFR-2 kinase in cells. As shown in Fig. 3, immunoblot analysis using HUVEC (human umbilical vein endothelial cells) cells revealed that compound **3** suppressed the VEGF-induced phosphorylation of KDR in a dose-dependent

Table 1 Inhibitory activity of the *N*-benzylbenzene-1,4-diamines **1–3** against KDR

Compound		IC_{50}/μM[a]
1		21
2		15
3		0.57

[a] The inhibitory activity of compounds **1–3** toward KDR tyrosine kinase was determined by ELISA. The drug concentrations required to inhibit the phosphorylation of the poly(Glu:Tyr) substrate by 50% (IC_{50}) were determined from the semilogarithmic dose-response plots.

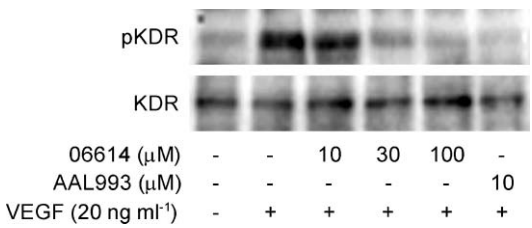


Fig. 3 Inhibition of the VEGF-induced phosphorylation of KDR. HUVEC cells were preincubated with the indicated concentrations of compound **3** or AAL993, and then stimulated with VEGF (20 ng ml^{-1}). The levels of each protein were detected by immunoblot analysis with the KDR-specific antibody.

manner, although AAL993 exhibited more potent inhibition of KDR phosphorylation.

In conclusion, we succeeded in the synthesis of a series of *N*-benzylbenzene-1,4-diamine derivatives as new candidates for KDR inhibitors using *de novo* drug design systems. Among the compounds synthesized, we found that compound **3** possessed significant activity of inhibition of KDR kinase and its IC_{50} value was 0.57 μM. Furthermore, compound **3** suppressed the VEGF-induced phosphorylation of KDR in HUVEC cells. The current findings suggest that compound **3** would be a candidate for a new lead compound of KDR inhibitors.

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10 EIA/RIA stripwell™ plates (Corning) were coated by incubation overnight at 4 °C with 100 μl $well^{-1}$ of 50 μg ml^{-1} poly(Glu:Tyr, 4 : 1) peptide (Sigma) in PBS. The kinase reaction was performed in the plates by addition of 50 μl of kinase buffer (50 mM HEPES, 125 mM NaCl, 10 mM $MgCl_2$, pH 7.4) containing 100 μM of ATP, 10 ng of KDR (Invitrogen, catalytic domain of VEGFR2), and the compound to be tested. After 20 min, the plates were washed three times with wash buffer (0.1% Tween 20 in PBS) and incubated for 20 min with 50 μl $well^{-1}$ of 0.2 μg ml^{-1} HRP conjugated anti-phosphotyrosine antibody (Santa Cruz). After two washes, the plates were developed by addition of 50 μl $well^{-1}$ tetramethylbenzidine (Sigma) and stopped by addition of 50 μl $well^{-1}$ of 2 N H_2SO_4. The absorbance at 450 nm was measured by a 96-well plate reader (Tecan).