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Discovery of indenopyrazoles as EGFR and VEGFR-2 tyrosine kinase inhibitors by in silico high-throughput screening

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Abstract—A series of indenopyrazoles **8** and **9** were designed and synthesized as EGFR tyrosine kinase inhibitors by in silico high-throughput screening. Compounds **8b** and **8d** showed significant inhibition of A431 cell growth (GI_{50} = 0.062 and 0.057 μM, respectively). Compounds **8b** and **9a** showed inhibitory activity toward both EGFR and VEGFR-2 (KDR) tyrosine kinases, whereas **8d** inhibited VEGFR-2 tyrosine kinase, exclusively.

Discovery of a pharmacologically active compound is an essential process for development of new drugs in medicinal chemistry. Despite great advances in high-throughput screening (HTS) technology, much attention has been paid for in silico screening using virtual compound libraries.[1–3] Recently, various X-ray co-crystal structures of a target protein–ligand complex have been reported and computational calculation technology of a docking simulation between proteins and ligands has also been developed based on those co-crystal structures, therefore, it may become possible to find lead compounds toward certain target proteins without using a large number of real chemical libraries.

EGFR (also known as erb-B1 or HER-1) tyrosine kinase is one of the important kinases that play a fundamental role in signal transduction pathways. EGFR and its ligands (EGF and TGF-α) have been implicated in numerous tumors of epithelial origin[4,5] and proliferative disorders of the epidermis such as psoriasis.[6] Therefore, the design of inhibitors toward EGFR-PTK is an attractive approach for the development of new therapeutic agents,[7–10] and gefitinib (ZD-1839, Iressa)[11,12] and erlotinib (OSI-774, Tarceva)[13] have both been approved for the treatment of non-small-cell lung cancer (NSCLC) as EGFR tyrosine kinase inhibitors. Although therapeutic responses to both inhibitors can persist for as long as 2–3 years, the mean duration of response in most cases of NSCLC is only 6–8 months.[14–16] However, the mechanisms underlying acquired drug resistance are not well understood.[17] Therefore, the finding of new lead compounds is still a significant requirement in this area.

In this paper, we carried out the in silico library screening based on X-ray crystal structure of the EGFR tyrosine kinase domain in complex with erlotinib (PDB code 1M17)[18] using Glide, a docking program,[19] against a 400,000 compound library of tyrosine kinase inhibitors from ChemBioBase.[20,21] We focused on an indenopyrazole framework, which was reported as cyclin dependent kinase (CDK) inhibitor[22,23] and found to be one of the common structures among the 100 top scoring compounds. The indenopyrazoles were designed as inhibitors targeting to EGFR tyrosine kinase, based upon the further detailed structure-based drug design (SBDD) using Ligand Fit algorithm, as shown in Figure 1. According to the docking simulation,[24] the hydrogen bonding interaction would be expected between the nitrogen atom (N1) of the indenopyrazoles and the MET769 amide nitrogen, and the hydroxyl group of a benzene ring substituted at C3 position of the indenopyrazoles and the ASP831 carboxylic acid. Therefore, we synthesized a series of indenopyrazoles conjugated with a variety of fragments at C3 and C5 positions.

Keywords: In silico screening; EGFR; VEGFR; Tyrosine kinase inhibitor; Indenopyrazoles.
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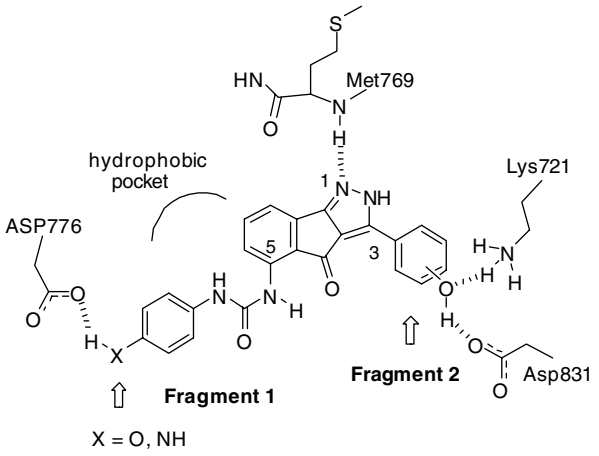
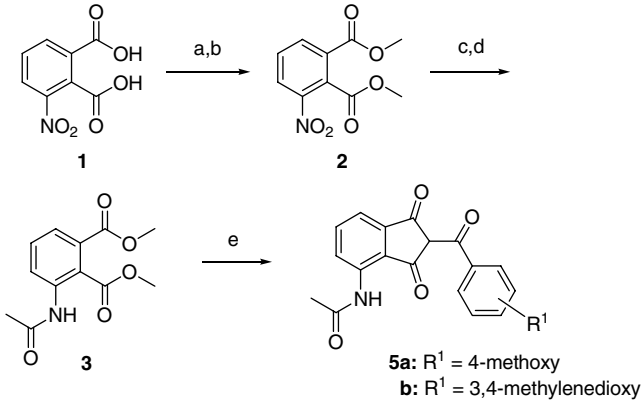


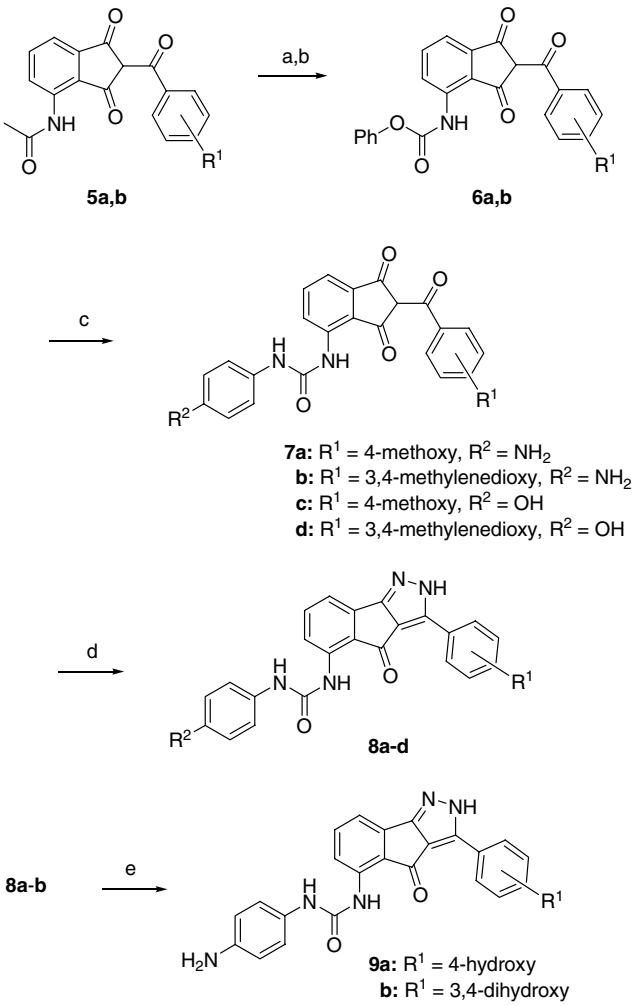
Figure 1. Binding simulation model of the indenopyrazoles into the active site of EGFR tyrosine kinase domain.

The triketone derivatives **5**, as precursors of indenopyrazoles, were synthesized according to the literature procedure with modification as shown in Scheme 1.[23] 3-Nitrophthalic acid **1** was reacted with acetic anhydride, and the 3-nitrophthalic anhydride obtained underwent the esterification in methanol to afford dimethyl 3-nitrophthalate **2**. Direct esterification of **1** gave the monomethyl ester, exclusively. Hydrogenation followed by acetylation gave the corresponding acetamide **3** in high yields. Condensation of **3** with acetophenones, such as 1-(4-methoxyphenyl)ethanone **4a** and 1-(benzo[d][1,3]-dioxol-5-yl)ethanone **4b**, gave the corresponding triketones **5a** and **5b** in 14% and 20% yields, respectively.

Next, phenoxy and anilino groups, as the fragment 1, were introduced into C5 position of indenopyrazoles through a urea bond as shown in Scheme 2. Deacrylation of the amides **5a**, **b** was carried out under acidic methanol in reflux and the resulting anilines were treated with phenyl chloroformate to give the corresponding phenyl carbamates **6a** and **6b** in 64% and 74% yields, respectively. Heating the carbamate **6a** and **6b** in dimethylsulfoxide (DMSO) with the appropriate anilines gave the urea derivatives **7a**–**d**. The pyrazole ring formation was accomplished by treating with hydrazine and a catalytic TsOH in refluxing ethanol to give the indenopyrazoles **8a**–**d** in 35–47% yields. Furthermore, deprotection of methyl and methylenedioxy groups of indenopyrazoles **8a** and **8b** was carried out using $AlCl_3$ and EtSH to give **9a** and **9b** in 22% and 23% yields, respectively.[11]



Scheme 1. Reagents and conditions: (a) Ac_2O, 100 °C; (b) H_2SO_4, MeOH, reflux, 97%; (c) H_2, Pd/C, MeOH, 90%; (d) Ac_2O, Py, 100 °C, 84%; (e) acetophenones (**4a**–**b**), NaH, DMF, 90 °C, 14–20%.



Scheme 2. Reagents and conditions: (a) HCl, MeOH, reflux, 76–82%; (b) PhOC(O)Cl, Na_2CO_3, acetone, reflux, 64–74%; (c) aniline, DMAP, DMSO, 80 °C, 65–91%; (d) Hydradine, *p*-TsOH, EtOH, reflux, 35–47%; (e) $AlCl_3$, EtSH, 0 °C, 22–23%.

We first examined the effects of the compounds on proliferation of A431 human epithelial carcinoma cell line, which overexpresses EGFR on the cell surface. The cells were incubated with compounds at various concentrations for 72 h and cell viability was determined by MTT assay. As shown in Table 1, the indenopyrazoles **8b** and **8d** reduced the proliferation of A431 cells, and their GI_{50} values are 0.062 and 0.056 μM, respectively. These results indicate that indenopyrazoles inhibit cell proliferation at lower concentration of compounds than tarceva (GI_{50} = 0.47 μM). Next, we examined in vitro inhibitory activity of the indenopyrazoles against EGFR, HER-2, Flt-1 (VEGFR-1), and KDR (VEG-

Table 1. Enzyme and cellular inhibitory activity assay results for compounds **8** and **9**

Compound	GI_{50}[a] (μM)	Kinase inhibition[c] (%)			
	A431[b]	EGFR	HER2	Flt-1	KDR[d]
8a	1.59	3	6	16	42
8b	0.062	61	28	60	88 (0.38 μM)
8c	>10	7	8	9	44
8d	0.056	4	2	13	70 (0.70 μM)
9a	>10	65	7	36	73 (0.65 μM)
9b	4.79	50	3	10	58
Tarceva	0.47	81	—	—	38
AAL 993	ND[e]	30	—	34	95 (0.039 μM)

[a] Concentration required to inhibit cell growth by 50%.
[b] EGFR overexpressing human epidermoid carcinoma cell.
[c] Kinase assay was performed at a 1 μM concentration of compounds.
[d] IC_{50} values are indicated in the parenthesis.
[e] Not determined.

FR-2) tyrosine kinases by measuring the levels of phosphorylation of the tyrosine kinase-specific ligand peptides at 1 μM concentration of compounds.[25] Compounds **8b**, **9a**, and **9b** showed moderate inhibition of EGFR tyrosine kinase (~50%), whereas compounds **8a**, **8c**, and **8d** did not show inhibitory activity toward EGFR tyrosine kinase. Interestingly, indenopyrazoles synthesized here possessed relatively selective inhibitory activity toward KDR tyrosine kinase, and compounds **8b** and **8d**, especially, showed high inhibitory activity (88% and 73%, respectively) at 1 μM.

Since a significant growth inhibition of compounds **8b** and **8d** toward A431 cells was observed in Table 1, we next examined CDK inhibitory activity of compounds **8a–d** and **9a–b** by Western blotting analysis of retinoblastoma tumor suppressor gene (Rb), which undergoes phosphorylation by activated CDKs. The results are shown in Figure 2. Compounds **8b** and **8d**, which exhibited a significant growth inhibition toward A431 cells, showed a potent inhibition of the phosphorylation of Rb at 1 μM concentration of compounds in A431 cells. Tarceva did not show a potent inhibition of the phosphorylation of Rb, therefore, the significant growth inhibition of compounds **8b** and **8d** toward A431 cells may be caused by inhibitions of CDKs.

In order to better understand the structure–activity relationship between the VEGFR2 tyrosine kinase inhibition and the possible binding modes of the indenopyrazoles **8b** and **9a**, we performed molecular docking experiments of **8b** and **9a** with the ligand-binding site of VEGFR2 kinase (PDB code 1Y6A).[26] According to our docking simulation as shown in Figure 3,[27,28] hydrogen bond interactions were observed between the N1 nitrogen of **8b** and the backbone-NH of Asn921, and the aniline nitrogen atoms of **8b** and the carboxyl oxygen of Glu883. In the case of the binding mode of **9a**, a hydrogen bond between the phenol group of **9a** and the carboxyl oxygen of Glu848 was observed in addition to two hydrogen bonds observed in the binding mode of **8b**. In both cases, the benzene ring of the indenopyrazole framework was located near Leu1033.

In conclusion, the indenopyrazoles were found as EGFR tyrosine kinase inhibitors by the in silico high-throughput screening using a 400,000 compound library, and a series of indenopyrazoles **8** and **9** were designed and synthesized. Although **8b**, **9a**, and **9b** showed moderate inhibitory activity toward EGFR tyrosine kinase, no inhibitory activity was observed in compounds **8a**, **8c**, and **8d**. Unexpectedly, significant inhibition of VEGFR-2 tyrosine kinase was observed in compounds **8b**, **8d**, and **9a** at 1 μM concentration. The significant growth inhibition toward A431 cells was observed in indenopyrazoles **8b** and **8d**, and their GI_{50} values were much lower than that of tarceva. According to the western blotting analysis, compounds **8b** and **8d** possessed inhibitory activities toward CDKs in A431 cells, therefore the significant cell growth inhibition property

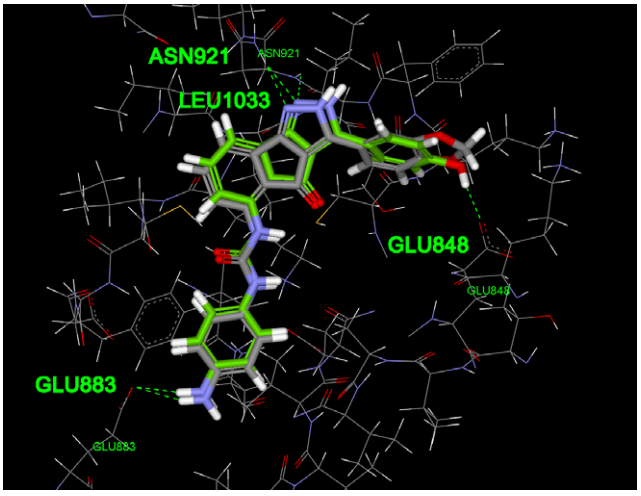


Figure 3. An overlay of indenopyrazoles **8b** (gray) and **9a** (green) docked into the active site of VEGFR2 kinase domain. Docking model was calculated by the DS modeling 1.7 based on the X-ray analysis data of VEGFR2 tyrosine kinase in complex with the ligand[8] (PDB code: 1Y6A).

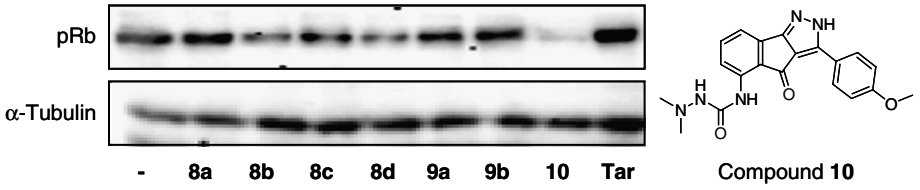


Figure 2. Western blotting analysis of phosphorylation of Rb in A431 cells. A431 cells were incubated for 12 h in the presence of indenopyrazoles or tarceva (Tar) at 1 μM concentration and the levels of each protein were detected with the specific antibody. Compound **10**, which was reported as a CDK inhibitor,[22] was used as a positive control.

of **8b** and **8d** may be mainly caused by regulation of CDK signaling pathway unlike EGFR tyrosine kinase.

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