Interaction of a Novel Biotin-tagged Photoaffinity Probe with Its Target Protein, VEGFR-2

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Chemical proteomics is a branch of proteomics in order to decipher protein function and mechanism at the level of directly observed protein-ligand (or small molecule) interactions by using chemical probes. Recently, chemical probes have been shown to have great potential to facilitate the processes of identifying target proteins or lead drug.[1]

To identify target protein(s) that can directly interact with biologically active small molecules, several approaches such as the use of nanoparticle, photoaffinity probe or chemical affinity matrix have been developed.[2-4] One of powerful tools is to use a tag-conjugated photoaffinity probe, in which a photoactive moiety is for UV-crosslinking bioactive partners and a specific tag for fishing out the labeled complex.[5,6] Photoactive moieties such as an azide or a diazirine group have been used as precursors for the highly reactive intermediates, which can be generated upon photolysis. Perfluorophenyl azides are the most widely applied photolabeling reagents and belong to a new class of photolabeling reagents with improved C-H insertion efficiency compared with their nonfluorinated analogues.[7,8] Biotin has been employed as a useful tag for highly sensitive and radioisotope-free detection of photolabeled complex.

The vascular endothelial growth factor receptors (VEGFRs) have been implicated in a variety of human diseases including tumor angiogenesis, tumor-dependent ascites formation, metastasis, inflammatory diseases such as rheumatoid arthritis and psoriasis, hyperthyroidism and atherosclerosis.[9-11] VEGFR-2 is a potent regulator of vascular endothelial cells and has been directly linked to tumor angiogenesis and blood vessel-dependent metastasis. There are several approaches to block or inhibit the function of VEGFRs by using neutralizing antibodies to the ligands as well as to the receptors, small molecular tyrosine kinase inhibitors and inhibitors to suppress the downstream signal pathway. However, the development of improved VEGFR inhibitors has been still required. Therefore, the precise understanding for the interaction between VEGFRs and their ligands, and the cellular function of receptors by using chemical probes could be a promising way to speed up and further industrialize target-based drug discovery.

In previous study, a novel biotin-tagged photoaffinity probe **1** (Fig. 1) was synthesized based on the structure of 4-[(4'-chloro-2'-fluoro)phenylamino]-6,7-dimethoxyquinazoline (CB676475),[12] which is a potent inhibitor of VEGFR tyrosine kinase.[13] The probe **1** was shown to inhibit the VEGF-stimulated proliferation of human umbilical vein endothelial cells (HUVECs) and to be photodecomposited by UV irradiation at 254 nm.[12] These results made us evaluate the possibility of direct interaction of the probe **1** with VEGFR-2 by using Western blot analysis.[14] Using VEGFR-2 specific antibody, VEGFR-2 (*ca.* 220-kDa) proteins were detected in all lines – 1) proteins only, 2) UV-irradiated proteins, 3) proteins mixed with the probe **1**, and 4) after mixed with the probe **1**, proteins UV-irradiated at 254 nm twice for 2 min (as shown at the middle image in Fig. 2). There were two bands detected by VEGFR-2 antibody; the higher might be the post-translationally modified (or phosphorylated) form of VEGFR-2.[15] When used either HRP-conjugated streptavidin or biotin antibody, the immunoreactivities were detected only in the fourth line at similar molecular weight position with that of VEGFR-2 as shown at the left or right images in Figure 2. There were several bands with weak immunoreactivities; it could result from the nonspecific binding of the probe **1** with proteins. However, the major immunoreactivity was shown in ~220-kDa; this could be identical with the higher in the molecular weight detected by VEGFR-2 antibody. It suggested that the probe **1** might

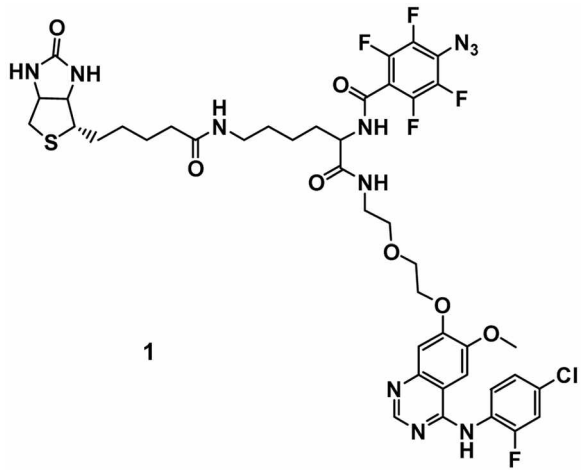

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Figure 1. Structure of a novel biotin-tagged photoaffinity probe for VEGFR-2.

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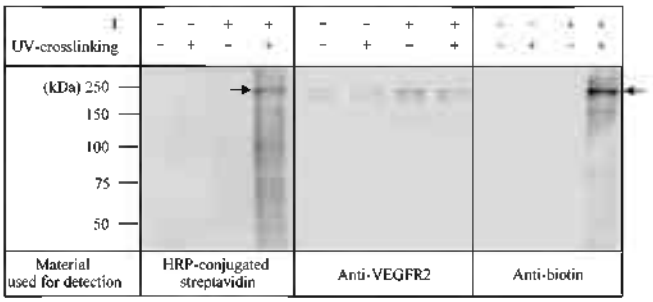

Figure 2. Western blot analysis showing the interaction of the probe **1** with VEGFR-2. Proteins in the absence or presence of the probe **1** were irradiated at 254 nm twice for 2 min. After the SDS-PAGE gel was blotted onto a nitrocellulose membrane, the membrane was immersed in blocking buffer containing HRP-conjugated streptavidin (left image), VEGFR-2 antibody (middle image), or biotin antibody (right image) for 2 hr, and the immunoreactivity was detected using LAS-3000 luminescent image analyzer.

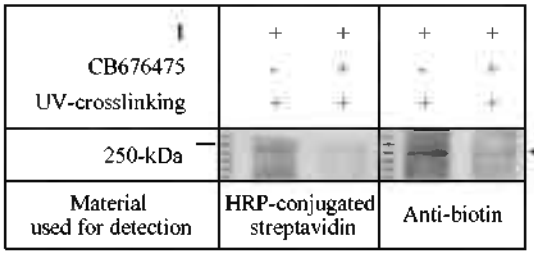

Figure 3. Competitive cross-linking with proteins between the probe **1** and CB676475. For the competition experiment, protein samples mixed with the probe 1 were incubated with or without 200 μM of CB676475 before UV-cross linking. Samples were irradiated, blotted onto a nitrocellulose membrane, and the immunoreactivity was detected with HRP-conjugated streptavidin (left image) or biotin antibody (right image).

mostly interact with the post-translationally modified (or phosphorylated) form of VEGFR-2. The specific binding of the probe **1** with VEGFR-2 was confirmed by the competitive binding assay under the condition in the presence of 2-fold molar excess of unlabeled mother compound, CB676475 (Fig. 3).

In conclusion, a novel biotin-tagged photoaffinity probe **1** can be used in photoaffinity crosslinking to label VEGFR-2 in cell-based studies on the receptor expression/localization/internalization and VEGF/VEGFR-2 signaling pathway; for example, since VEGFR-2 internalization might desensitize endothelial cells to VEGF activation and thus regulate the rate and degree of neovascularization, the probe **1** could be used in order to trace the real-time cellular localization of VEGFR-2. Further deciphering the interaction between target protein and its direct binding inhibitor could provide invaluable information for the exploration of new therapeutic strategies in the treatment of patients. The biochemical application of this probe is currently under investigation.

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14. Photoaffinity labeling experiment – HUVECs were homogenized in buffer consisting of 10 mM Tris–HCl (pH 7.5), 150 mM NaCl, 0.05% (v/v) Tween 20, 1 mM PMSF, and one protease inhibitor cocktail tablet (Roche, Germany) at 4 °C and centrifuged at 10,000 × *g* for 15 min. The BCA protein assay Kit (Pierce, IL) was used to determine the concentration of protein in the supernatant. Proteins (100 μg in 50 μL) were incubated with the probe **1** (100 μM) at 4 °C for 2 hr, and placed on an ice tray under a UV light source (BIO-LINK with 5 × 8 W tubes, 254 nm, Vilber Lourmat, France) twice for 2 min. The probe **1** was first dissolved in dimethyl sulfoxide (DMSO; Sigma, MO), and then diluted with homogenate buffer, so that the final DMSO concentration was 1% in the samples. For the competition experiment, 200 μM of CB676475 was incubated with samples before UV-cross linking. Irradiated protein samples (20 μg) were mixed with sample buffer (100 mM Tris-HCl, 2% sodium dodecyl sulfate, 1% 2-mercapto-ethanol, 2% glycerol, 0.01% bromophenol blue, pH 7.6), incubated at 50 °C for 10 min, and loaded onto 8% polyacrylamide gels.[16] Electrophoresis was performed using the Mini Protean 3 Cell (Bio-Rad). Proteins separated on the gels were transferred onto nitrocellulose membrane (Scheicher & Schnell BioScience, Germany), and the membrane was incubated in blocking buffer (10 mM Tris-HCl, pH 7.5, 150 mM NaCl, 0.1% Tween 20, 3% nonfat dry milk). The membrane was incubated for 2 hr at room temperature with 1:1000 diluted primary antibodies. Horse radish peroxidase(HRP)-conjugated anti-VEGFR-2 was purchased from Santa Cruz Biotechnology Inc. (CA). HRP-conjugated streptavidin was purchased from Merck and the goat anti-biotin purchased from Pierce. After washing three times for 15 min with blocking buffer, and developed with SuperSignal West Femto Maximum Sensitivity Substrate (Pierce). When used the goat anti-biotin, membrane was incubated for 1 hr at room temperature with 1:2000 diluted HRP labeled anti-goat IgG (Pierce) before the development. The immunoreactivity was detected using LAS-3000 luminescent image analyzer (Fuji Photo Film Co., Ltd., Japan).
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