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Identification of HSP60 as a Primary Target of *o*-Carboranylphenoxyacetanilide, an HIF-1α Inhibitor

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Abstract: We succeeded in the design and synthesis of multifunctional chemical probes of the HIF-1α inhibitor carboranylphenoxyacetanilide (**1**) that combine photoaffinity labeling and click reaction to identify the target protein. HSP60 was identified as a primary target protein of **1** using the chemical probes **2** and **3**. Furthermore, HSP60 inhibitor **4** suppressed hypoxia-induced HIF activation, indicating that HSP60 affects HIF-1α accumulation directly or indirectly.

The hypoxia-inducible factor (HIF) is a basic helix−loop−helix heterodimeric transcription factor composed of one of three subunits (HIF-1α, -2α, or -3α) and a HIF-1β subunit.[1−3] Under aerobic conditions, HIF-1α is rapidly degraded via a 26S proteasome-dependent pathway, whereas under hypoxic conditions, HIF-1α is stabilized and translocated into the nucleus, where it dimerizes with the constitutively expressed HIF-1β.[4−7] The HIF-1α/β dimer binds to specific nucleotide sequences [hypoxia-responsive elements (HREs)] in the promoter of hypoxia-responsive genes such as vascular endothelial growth factor (VEGF), insulin-like growth factor, heme oxygenase-1, and inducible nitric oxide synthase.[8,9] Among the HIF-regulated genes, VEGF plays a pivotal role in pathological angiogenesis and tumor growth;[10,11] therefore, the inhibition of the VEGF inducer HIF is recognized as an attractive strategy for cancer therapy.[8,12] Our efforts have been focused on the development of HIF inhibitors[13−17] as antiangiogenesis agents. Recently, we reported that *o*-carboranylphenoxyacetanilide (**1**) induced a potent inhibitory effect on HIF-1α activation under hypoxic conditions.[18] In the present study, we clarified the action mechanism of **1** against HIF inhibition.

Standard chemical biology techniques, including photoaffinity labeling, click conjugation, and biotinylation, are very useful tools for detecting target proteins of biologically active molecules having undefined action mechanisms.[19−21] On the basis of this approach, we designed and synthesized multifunctional chemical probes of **1** substituted with benzophenone to covalently bond with a target protein by UV (photoaffinity labeling) and an acetylene moiety to conjugate with azide-linked fluorescence by click reaction (Figure 1A). The chemical probes of **1** (**2** and **3**) were synthesized from 4-ethynylphenoxyacetic acid (**5**) and aminobenzophenones **6a**−**c** (Scheme S1 in the Supporting Information). To confirm whether the synthesized probes exhibit HIF inhibitory activity, we examined the effects of the probes on the hypoxia-induced activation of HIF in HeLa human cervical cancer cells by HRE reporter gene assay and immunoblot analysis. As shown in Figure 1B,C, although the inhibitory effects of probes **2** and **3** against HIF transcriptional activity were decreased almost 10-fold relative to **1** (Figure 1B), the reducing property of the probes on the HIF-1α protein level were parallel to that of **1** (Figure 1C). These results indicate that

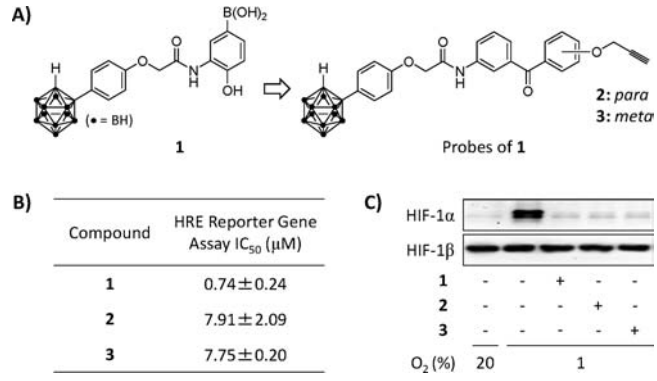

Figure 1. Chemical probes of *o*-carboranylphenoxyacetanilide **1** and their HIF inhibitory activities under hypoxic conditions. (A) Chemical structures of **1** and the chemical probes **2** and **3**. (B) Inhibitory effects of **1** and the probes **2** and **3** on transcriptional activity of HIF were determined by HeLa cell-based HRE reporter gene assay. (C) Effects of **1** (10 μM) and probes **2** and **3** (30 μM) on the hypoxia-induced increase in HIF-1α protein were determined by immunoblot analysis in HeLa cells.

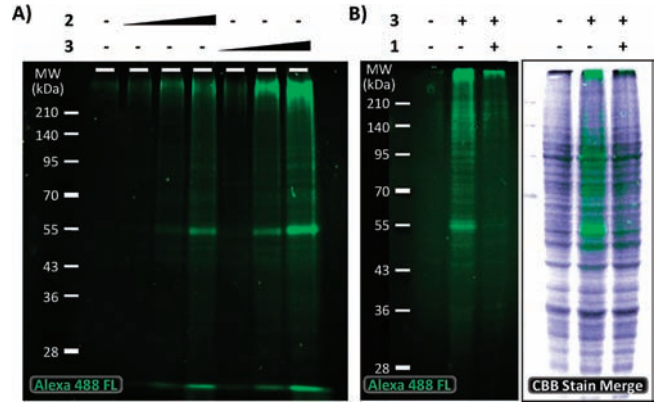

Figure 2. Fluorescence imaging of target protein bound to the probe. (A) HeLa cell lysate was irradiated for 30 min at 360 nm with various concentrations (30, 100, and 300 μM) of each probe. The conjugation of probe and Alexa Fluor 488 azide was performed by click reaction. (B) Total cell lysates from HeLa cells were photoaffinity-labeled with **3** (100 μM) in the presence or absence of **1** (500 μM).

the boronic acid in **1** is an efficient functional group for HIF inhibition, a finding similar to the results of our previous study.[18]

Using these chemical probes, we performed photoaffinity labeling in HeLa cell lysate and click conjugation with Alexa Fluor 488 azide to visualize the target protein (see the Supporting Information). After sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE), proteins bound to the probe Alexa Fluor 488 were visualized by direct in-gel fluorescence detection. As shown in Figure 2A, a major fluorescent band above the 55 kDa molecular weight marker was detected by both probes in a concentration-dependent manner, and binding of **3** with the protein was slightly

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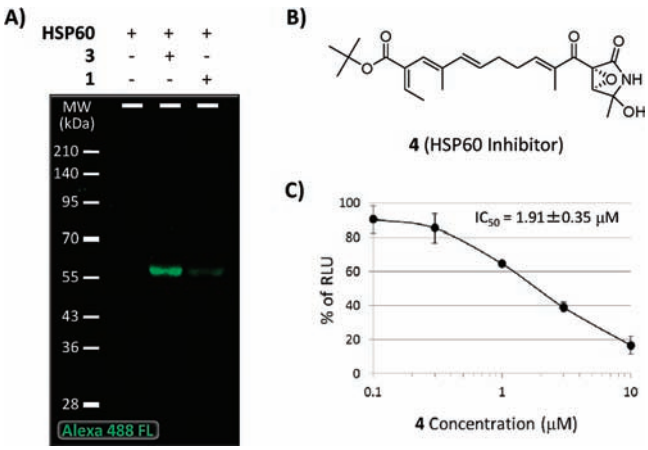

Figure 3. Fluorescence imaging of probe-labeled recombinant HSP60 and HIF inhibition by **4**. (A) Recombinant human HSP60 (2 μg) was irradiated with **3** (10 μM) in the presence or absence of **1** (100 μM) and click-conjugated with Alexa Fluor 488 azide. (B) Structure of HSP60 inhibitor **4**. (C) Inhibition of HIF transcriptional activity by **4** in HeLa cells.

more sensitive than that of **2**. To confirm the specificity of probe binding, we performed competition assays with **1**. As shown in Figure 2B, competition with **1** resulted in almost complete abrogation of the level of the fluorescent band at ~55 kDa. Similar results were also observed in a competition assay with **8c**, an acetylene-free analogue of the chemical probes **2** and **3** (Figure S1 in the Supporting Information). These results raise the possibility that the band at ~55 kDa may be the main target protein of **1** and its probes.

To identify the protein manifested at above 55 kDa, we next performed two-dimensional electrophoresis (Figure S2) followed by in-gel digestion and peptide mass fingerprinting analysis (PMF) by liquid chromatography/electrospray ionization time-of-flight mass spectrometry (LC/ESI-TOF MS). As shown in Figure S3, PMF analysis searched in Mascot revealed that the protein bound to the probe was heat shock protein 60 (HSP60) with a significant score value of 245, sequence coverage of 73%, and 28 matching peptides (Table S1). To confirm the direct binding of chemical probe **3** and HSP60, in-gel fluorescence imaging was performed with recombinant human HSP60 protein, and a fluorescent band was observed in the presence of **3** (Figure 3A, lane 2). Under these conditions, an excess of **1** suppressed the binding of HSP60 and the chemical probe **3** (Figure 3A, lane 3). In addition, **3** selectively bound to HSP60 in a mixture of recombinant HSP60, -70, and -90 (Figure S5). Moreover, an immunoprecipitation assay demonstrated that HSP60 interacts with HIF-1α in HeLa cells (Figure S6A). These results suggest that the binding of **1** to HSP60 appears to be implicated in the inhibition of HIF-1α. To further clarify the relationship between HSP60 and HIF-1α, the effects of the known HSP60 inhibitor epolactaene *tert*-butyl ester (**4**)[22] (Figure 3B) on the hypoxia-induced activation of HIF were examined. As shown in Figure 3C, **4** inhibited the hypoxia-induced transcriptional activation of HIF with an IC_{50} value of 1.91 μM (Figure 3C) and accumulation of HIF-1α similar to **1** (Figure S6C). Furthermore, **4** showed the competitive property against binding of **3** to recombinant HSP60 (Figure S6E, lane 3). These results show that HSP60 inhibitor **4** inhibits activation of HIF as well as **1**, indicating that HSP60 is involved in the activation of HIF-1α. Moreover, **1** inhibited HSP60 chaperone activity similar to **4** (Figure S7A). Interestingly, **1** suppressed HSP60 ATPase activity (Figure S7B), but **4** did not affect the activity.

It has been reported that HIF-1α is one of the client proteins of molecular chaperon HSP90 and geldanamycin, an HSP90 inhibitor that stimulates HIF-1α degradation by affecting folding and maturation.[23−25] Unlike HSP90, the interaction of HSP60 and HIF-1α has not been established. To our knowledge, this is the first report demonstrating the implication of HSP60 in HIF activation. However, details of the functional association between HSP60 and HIF-1α remain to be elucidated.

In conclusion, we have succeeded in the design and synthesis of multifunctional molecular probes of the HIF-1 inhibitor *o*-carboranylphenoxyacetanilide **1** that combine photoaffinity labeling and click reaction to identify the target protein. Using the chemical probes **2** and **3**, we identified that HSP60 is the target protein of **1**. Furthermore, HSP60 inhibitor **4** suppressed hypoxia-induced HIF activation, indicating that HSP60 affects HIF-1α accumulation directly or indirectly.

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Supporting Information Available: Detailed experimental procedures for syntheses and biological assays, analytical data for compounds, and results of biological experiments. This material is available free of charge via the Internet at http://pubs.acs.org.

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