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A Click Chemistry Mediated *in Vivo* Activity Probe for Dimethylarginine Dimethylaminohydrolase

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Asymmetric N^{ω},N^{ω}-dimethyl-L-arginine (ADMA) is an endogenously produced inhibitor of human nitric oxide synthase and an emerging biomarker for endothelial dysfunction in cardiovascular disease.[1] Concentrations of ADMA are tightly controlled by two isoforms of its catabolic enzyme dimethylarginine dimethylaminohydrolase (DDAH-1 and DDAH-2) that have different tissue distributions.[2] Homozygous disruption of the *Ddah1* gene in mice results in embryonic lethality; heterozygous mice have elevated concentrations of plasma ADMA and display endothelial dysfunction.[3] Accordingly, dysregulation of DDAH activity has been studied as a mediating factor for endothelial dysfunction in a number of disease states including hyperhomocysteinemia,[4] renal failure,[5] and diabetes.[6] However, the existing methodology has shortcomings. Measurements of transcript mRNAs and immunoblots by DDAH-selective antibodies have been used to monitor changes in DDAH expression, but these values do not always correlate well with the activity of DDAH because the enzyme can be inhibited by physiologically relevant modulators such as homocysteine,[7] *S*-nitroso-L-homocysteine,[8−10] zinc(II),[11] 4-hydroxy-2-nonenal,[12] nitric oxide,[13,14] and other reactive nitrogen and oxygen species. Likewise, enzyme activity assays are also problematic because they are performed after cell lysis and homogenation, which removes DDAH from its cellular environment and alters the concentrations of modulators. A cell permeable probe to detect enzyme activity would likely prove more relevant for the study of biological function. Therefore, to better understand the regulation of DDAH in normal and pathophysiology, we developed a two-part click-chemistry mediated activity-based probe that labels active DDAH-1 in intact mammalian cells but does not label an inactive mutant or inhibited enzyme.

In prior work, we discovered that 2-chloroacetamidine (CAA) selectively modifies the active site Cys of DDAH.[15] Subsequently, a similar 2-haloamidine activity probe specific for a related enzyme, peptidylarginine deiminase-4, was demonstrated in *Escherichia coli* cell lysates.[16] As a candidate DDAH probe for *in vivo* use with mammalian cell cultures, we synthesized *N*-but-3-ynyl-2-chloroacetamidine (**1**, Supporting Information). The simple butynyl chain was appended to CAA to minimize its impact on selectivity and bioavailability yet still provide a handle for variable functionalization using a click reaction after cell lysis, here a Cu (I) catalyzed 1,3-dipolar cycloaddition[17] with an azide labeled-biotin (Figure 1), a strategy proven successful with other enzyme superfamilies.[18]

By transient transfection, human DDAH-1 bearing an *N*-terminal Myc-tag was expressed in HEK 293T cells. Addition of **1** (150 μM) to the growth medium was followed by a short incubation, cell washing, and harvesting. Cells were subsequently lysed by multiple freeze/thaw cycles, and the resulting lysate was incubated

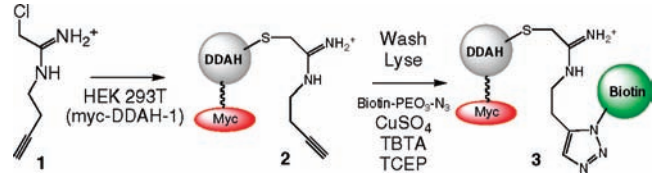

Figure 1. *In vivo* activity probe for DDAH-1. HEK 293T cells expressing myc-tagged DDAH-1 are treated with **1**, washed, lysed, and reacted with biotin-PEO_3-azide and catalysts to biotinylate the active fraction of DDAH-1. The *syn*/*anti* ratio of **3** has not been determined.

with biotin-PEO_3-azide (Supporting Information) and catalysts for the cycloaddition reaction followed by SDS-PAGE to resolve the crude protein mixture. The presence of myc and biotin was demonstrated by Western blot using rabbit antimyc and mouse antibiotin primary antibodies and two near-infrared dye labeled secondary antibodies with emissions at 680 nm (antirabbit, red) and 800 nm (antimouse, green) (Figure 2, Supporting Information) that were chosen for their linear concentration-dependent response.[19] Response to the myc tag (red) indicated consistent levels of

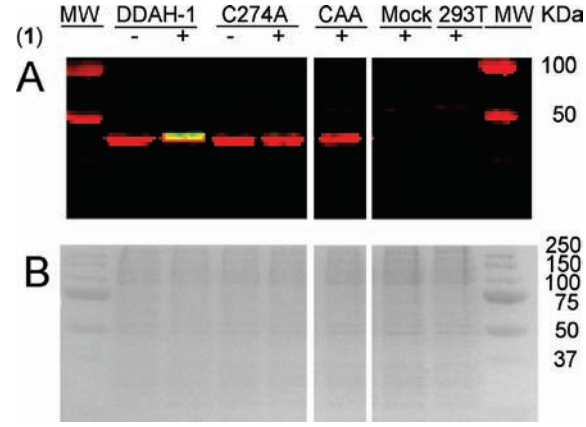

Figure 2. Two-color imaging for DDAH-1 activity. (A) Red and green coloring correspond to antimyc and antibiotin Western blot signals, respectively. (B) Coomassie staining of the same samples used for (A). The MW_{calcd} of myc-DDAH-1 is 35 894 Da.

DDAH-1 expression and loading. Response to the biotin tag (green) was observed when cells were treated by **1**, but not when **1** was omitted. An inactive DDAH-1 in which the active-site Cys_{274} is mutated to Ala is also not labeled by **1**, despite the presence of five other Cys residues in its primary sequence. Addition of CAA (25 μM) prior to addition of **1** blocks biotinylation, indicating that CAA has sufficient bioavailability to react with DDAH-1 in 293T cells and that CAA and **1** compete for the same site. Mock transfection with an empty expression vector or 293T cells alone did not result in a labeled band at the expected position for DDAH-

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1. These experiments clearly demonstrate that **1** can label active DDAH-1 in cultured mammalian cells.

To determine whether **1** is capable of detecting reversible inhibition of DDAH-1 in cells, a challenge experiment[20] was performed using a competitive reversible inhibitor of DDAH-1, N^5-(1-iminopropyl)-L-ornithine (**4**) ($K_i = 50$ μM), that we previously developed as a nonhydrolyzable analogue of the substrate N^{ω}-methyl-L-arginine.[21] Cells were preincubated with varying concentrations of **4**, followed by addition of **1**, washing, harvesting, and lysis. For dose dependent studies, response to the biotin tag was normalized for expression levels of myc-DDAH-1. In the absence of inhibitor, DDAH-1 was effectively biotinylated, indicating that the active site Cys_{274} was available to react with **1** (Figure 3).

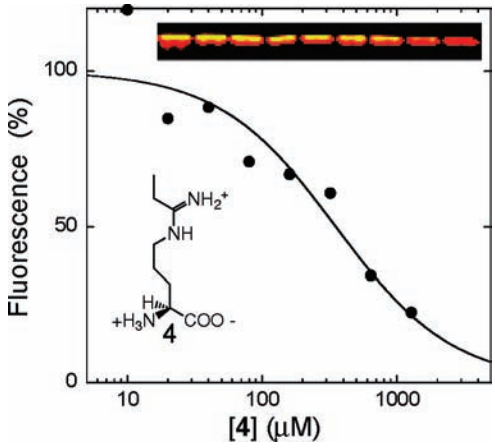

Figure 3. *In vivo* inhibition assay. Normalized fluorescence intensities for the biotin-derived signal are fit with an *in vivo* IC_{50} of 350 ± 90 μM. (Inset) Two-color Western blots reflect presence of myc (red) and biotin (green) tags after labeling of intact HEK 293T cells in the presence of 0, 10, 20, 40, 80, 160, 320, 640, and 1280 μM **4** (left to right).

Biotinylation of DDAH-1 diminished with increasing concentrations of **4**, indicating both the bioavailability of **4** and its competition with **1** for binding to the active site of DDAH-1, resulting in a level of inhibition that defines an *in vivo* IC_{50} value of 350 ± 90 μM. The *in vivo* IC_{50} is approximately 7-fold higher than the *in vitro* K_i value, with the difference in potency representing a combination of factors including bioavailability, protein binding, competition with endogenous DDAH-1 modulators, and other variables not easily mimicked during *in vitro* tests.

In conclusion, the data presented herein demonstrate that **1** serves as a novel click chemistry mediated *in vivo* activity probe that labels the active fraction of DDAH-1 in intact mammalian cells and that can be blocked by the presence of competitive reversible and irreversible inhibitors. Incorporation of the alkyne tag allows the flexibility to derivatize with a variety of reagents after *in vivo* tagging.[22] The two-color imaging system enables normalization to account for variable protein expression when determining *in vivo* IC_{50} values of inhibitors. Additionally, the small size and simplicity of **1** suggest its use as a broad-specificity probe for labeling endogenous DDAH isoforms and enzymes with similar pharmacophores, the subject of ongoing studies. This probe provides a novel tool for the analysis of DDAH-1 activity in normal and pathophysiological states relevant to cardiovascular disorders and should allow more meaningful studies of the etiology of endothelial dysfunction.

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Supporting Information Available: Synthetic procedure for (**1**) and biotin-PEO_3-azide, cloning procedure for myc-tagged human DDAH-1, and conditions for biotinylation and Western blot analysis. This material is available free of charge via the Internet at http://pubs.acs.org.

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