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Developing an Irreversible Inhibitor of Human DDAH-1, an Enzyme Upregulated in Melanoma

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Inhibitors of the human enzyme dimethylarginine dimethylaminohydrolase-1 (DDAH-1) can raise endogenous levels of asymmetric dimethylarginine (ADMA) and lead to a subsequent inhibition of nitric oxide synthesis. In this study, N^5-(1-imino-2-chloroethyl)-L-ornithine (Cl-NIO) is shown to be a potent time- and concentration-dependent inhibitor of purified human DDAH-1 ($K_I = 1.3 \pm 0.6$ μM; $k_{inact} = 0.34 \pm 0.07$ min^{-1}), with >500-fold selectivity against two arginine-handling enzymes in the same pathway. An activity probe is used to measure the "in cell" IC_{50} value (6.6 ± 0.2 μM) for Cl-NIO inhibition of DDAH-1 artificially expressed within cultured HEK293T cells. A screen of diverse melanoma cell lines reveals that a striking 50/64 (78%) of melanoma lines tested showed increased levels of DDAH-1 relative to normal melanocyte control lines. Treatment of the melanoma A375 cell line with Cl-NIO shows a subsequent decrease in cellular nitric oxide production. Cl-NIO is a promising tool for the study of methylarginine-mediated nitric oxide control and a potential therapeutic lead compound for other indications with elevated nitric oxide production, such as septic shock and idiopathic pulmonary fibrosis.

Introduction

Biosynthesis of the second messenger nitric oxide is regulated by a number of different processes. One of the more unusual regulatory pathways starts with *S*-adenosyl-L-methionine and the protein arginine methyltransferase-catalyzed posttranslational modification of arginine residues to introduce N^{ω}-methyl and asymmetric N^{ω}-,N^{ω}-dimethylarginine modifications. These modified proteins are eventually degraded by the proteasome and through autophagy pathways[1] to release free methylarginines that accumulate and act as nitric oxide synthase (NOS) inhibitors.[2] The intracellular concentration of accumulated methylarginines is controlled by transporters,[2c,3] through metabolism by alanine:glyoxylate aminotransferase-2,[4] and notably, through metabolism by dimethylarginine dimethylaminohydrolase-1 (DDAH-1),[2a] which catalyzes the hydrolytic conversion into citrulline and (di)methylamine, thus relieving nitric oxide synthesis inhibition.

The efficacy of inhibiting DDAH-1 activity to cause methylarginine accumulation and subsequent downstream inhibition of nitric oxide production in selected cell lines and in animal models has been previously established.[5] Development of DDAH-1 inhibitors is an active area of research, given their potential application as therapeutics for indications including septic shock and idiopathic pulmonary fibrosis.[2a,6] Additionally, DDAH-1 inhibitors are useful biochemical tools for understanding regulatory pathways of nitric oxide production in diverse cell types.

Previously, we developed substrate-mimicking amidines as covalent reversible inhibitors of human DDAH-1 with moderate potency (e.g., IPO: $K_i = 52$ μM; Figure 1).[7] We also found that the fragment-sized compound 2-chloroacetamidine (CAA) is

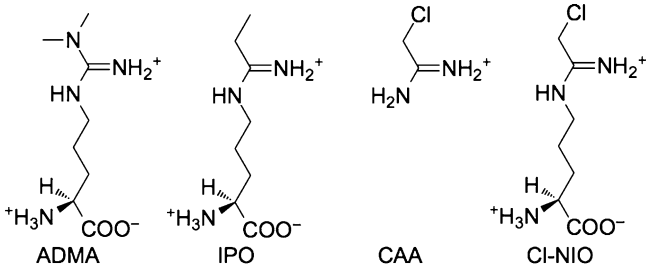

Figure 1. Substrate and inhibitors of DDAH-1: Shown are the substrate N^{ω},N^{ω}-dimethyl-L-arginine (ADMA), the covalent reversible inhibitor N^5-(1-iminopropyl)-L-ornithine (IPO), the covalent irreversible inhibitor chloroacetamidine (CAA), and the covalent irreversible inhibitor N^5-(1-imino-2-chloroethyl)-L-ornithine (Cl-NIO).

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a covalent irreversible inhibitor of *P. aeruginosa* DDAH that selectively modifies the active-site Cys residue, albeit with weak potency ($K_I = 3.1$ mM; $k_{inact} = 1.2$ min^{-1}). Other research groups have demonstrated the utility of 2-haloacetamidines in the design of selective inhibitors for other enzyme targets, and these "warheads" were used in both cell culture and animals, providing precedence for their wider application.[8] Therefore, we combined these two approaches by designing N^5-(1-imino-2-chloroethyl)-L-ornithine (Cl-NIO) as a substrate mimic and as a potential covalent irreversible inhibitor of DDAH-1. Herein, the effect of Cl-NIO is characterized on purified enzymes, in cells artificially expressing DDAH-1, and in melanoma cell lines in which we found DDAH-1 levels to be elevated.

Results and Discussion

Cl-NIO causes time- and concentration-dependent inactivation of purified human DDAH-1 with K_I and k_{inact} values of 1.3 ± 0.6 μM and 0.34 ± 0.07 min^{-1}, respectively (Figure 2). The reversible component of Cl-NIO binding has potency on par with the K_i values of the most potent reversible DDAH-1 inhibitors reported to date (~2 μM),[9] and is enhanced by its subsequent inactivation step. To probe for covalent bond formation, electrospray ionization mass spectrometry (ESIMS) was used to compare the molecular weight of unmodified DDAH-1 ($M_{rcalcd} = 33331$ Da; $M_{robsd} = 33305 \pm 10$ Da) with that of Cl-NIO-treated DDAH-1 before ($M_{robsd} = 33473 \pm 10$ Da; $\Delta M_{robsd} = +168$ Da) and after ($M_{robsd} = 33477 \pm 10$ Da; $\Delta M_{robsd} = +172$ Da) dialysis. These results indicate formation of a covalent adduct with a mass equal to that expected for addition of one equivalent of Cl-NIO minus a Cl^- ion ($\Delta M_{rcalcd} = +173$ Da). These findings are consistent with Cl-NIO as a covalent irreversible inactivator of DDAH-1 that likely works by the same mechanism as CAA.[10]

DDAH-1 inhibitors are sought to raise intracellular concentrations of methylarginines, which subsequently compete with L-arginine for binding to NOS. Therefore, the selectivity of DDAH-1 inhibitors against arginase and NOS is an important consideration that has been noted previously.[7a,11] Cl-NIO does not inhibit endothelial NOS at concentrations up to 500 μM (Figure 3), giving a conservative selectivity ratio ($K_{ieNOS}/K_{iDDAH-1}$) of approximately ≥500:1. NOS isoforms have conserved active sites and do not typically show marked differences in selectivity between arginine-like inhibitors,[12] so only the endothelial isoform was tested here. Cl-NIO does inhibit arginase, but only when used at millimolar concentrations, with a selectivity ratio of ~2400:1 (Figure 3). Because some affinity for arginase was detected, we used HPLC to determine whether elevated concentrations of Cl-NIO can be hydrolyzed by arginase using long reaction times. In an overnight incubation, arginase can catalyze partial conversion of a Cl-NIO stock to L-ornithine, demonstrating that this compound can also act as a slow arginase substrate (Supporting Information Figure S1). These in vitro studies indicate that Cl-NIO is relatively selective for DDAH-1 and selective against these two arginine-handling enzymes that impact the same pathway.

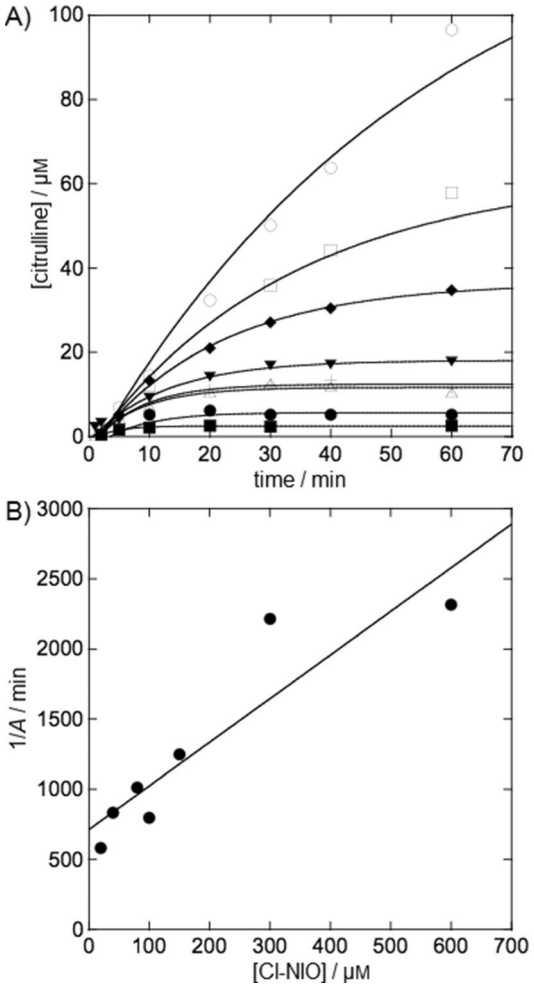

Figure 2. Inactivation of purified DDAH-1 by Cl-NIO: A) Time- and concentration-dependent inactivation of purified DDAH-1 is observed when assayed in the presence of competing substrate and 0 (○), 20 (□), 40 (◆), 80 (▼), 100 (+), 150 (△), 300 (●), and 600 (■) μM Cl-NIO. Solid lines are fits as described in the Experimental Section. B) The inverse of the apparent inactivation rates (*A*) are replotted as described in the Experimental Section to derive K_I (1.3 ± 0.6 μM) and k_{inact} (0.34 ± 0.07 min^{-1}) values for Cl-NIO inactivation of DDAH-1.

At neutral pH, Cl-NIO is a charged amino acid. Compounds of similar structure can gain access to the cytoplasm through transporters.[2c,3,13] To determine cellular availability, we assayed whether Cl-NIO can inhibit DDAH-1 that is artificially expressed within the cytoplasm of cultured human embryonic kidney cells (HEK293T) by using a previously described cell-permeable DDAH-1 activity probe.[14] Increasing concentrations of Cl-NIO effectively block labeling of the active-site DDAH-1 Cys by the competing activity probe within cells (Figure 4), indicating that Cl-NIO can gain access to the cytoplasm, that it can engage its target protein, and that it can compete for active-site binding. After 15 min of exposure, an apparent "in cell" IC_{50} of 6.6 ± 0.2 μM is determined for DDAH-1 inhibition, as measured by competition with the activity probe (Figure 4). This value should not be taken as a true measure of binding potency, because it is impacted by a number of different factors including transport to the site of action, competition with endogenous

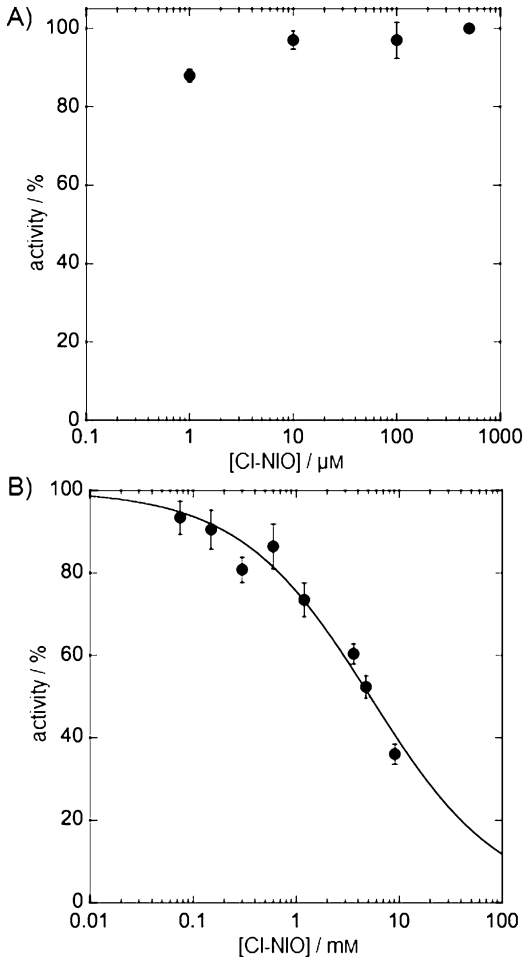

Figure 3. Off-target inhibition tests with purified arginine-processing enzymes: A) The activity of purified human endothelial NOS is not inhibited by Cl-NIO when tested at concentrations $\leq$500 μM. B) The activity of purified Mn^{2+}-containing human arginase is inhibited at millimolar concentrations of Cl-NIO. Concentration-dependent inhibition in the presence of L-Arg (1.5 mM, K_M = 2.3 mM) is fit by $IC_{50} = 5.2 \pm 0.8$ mM and Hill coefficient = 0.7 ± 0.1, which give a calculated K_i value of 3.1 mM, assuming competitive inhibition.

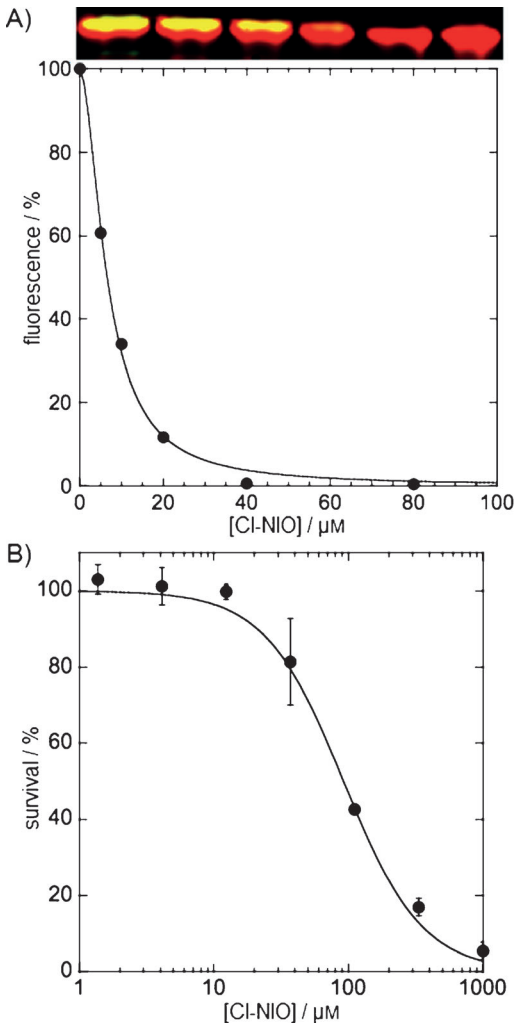

Figure 4. Studies of Cl-NIO-treated HEK293T cells: A) Two-color Western blot (top) shows fluorescence derived from response to a myc-tag genetically encoded into an episomally expressed DDAH-1 (red), and from response to a biotin-tagged activity probe for DDAH-1 (green), when HEK239T cells are treated by increasing concentrations of Cl-NIO (left to right: 0, 5, 10, 20, 40, 80 μM). Normalized fluorescence values for the biotin-derived signal (bottom) are fit to give an apparent "in cell" IC_{50} value for DDAH-1 inhibition of 6.6 ± 0.2 μM and a Hill coefficient of 1.8 ± 0.1. B) Toxicity of Cl-NIO to the HEK293T cell line after 72 h incubation was assessed using an MTS assay (see Experimental Section) to determine an $IC_{50} = 92 \pm 2$ μM for cell survival.

ligands, and time of exposure to the target, among others. Nevertheless, under these conditions, micromolar amounts of Cl-NIO are able to inhibit intracellular DDAH-1. Another consideration for the use of Cl-NIO in cells is the possibility of toxicity. Here, we show that survival of cultured HEK293T cells can be adversely affected by Cl-NIO, but only at much higher concentrations (IC_{50} for cell survival: 92 ± 2 μM) and longer incubation times (72 h) than required for DDAH-1 inhibition. These findings indicate that Cl-NIO can be used as a potent bioavailable DDAH-1 inhibitor, at least in this cultured cell line.

We next sought to identify a cell line in which increased endogenous levels of DDAH-1 may play a functional role, and to use these cells to test downstream effects of Cl-NIO on nitric oxide production. Enhanced production of nitric oxide has previously been shown to play a promoting role in melanomas.[15] Therefore, we chose to screen melanoma cell lines for DDAH-1 levels. Using immunohistochemistry as a semi-quantitative gauge of DDAH-1, DDAH-2, and inducible NOS (iNOS) concentrations, we assayed a panel consisting of two control melanocyte lines and 64 melanoma cell lines that encompass different stages of malignancy, metastases, and mutational status. Strikingly, DDAH-1 was upregulated in 50/64 (78%) of the melanoma lines tested, but was not observed in the control lines (Figure 5, Supporting Information Table S1). In contrast, DDAH-2 was only upregulated in 4/64 (6%) of the lines, and not in the control lines. DDAH-1 levels do not appear to correlate with cell origin, degree of malignancy, metastases, or mutational status of BRaf or NRas. Western blots for DDAH-1 and DDAH-2 in selected cell lines corroborate the immunohistochemical screening results by indicating increased concentrations of DDAH-1, but not DDAH-2, in melanoma lines with respect to a melanocyte control, although there were some dif-

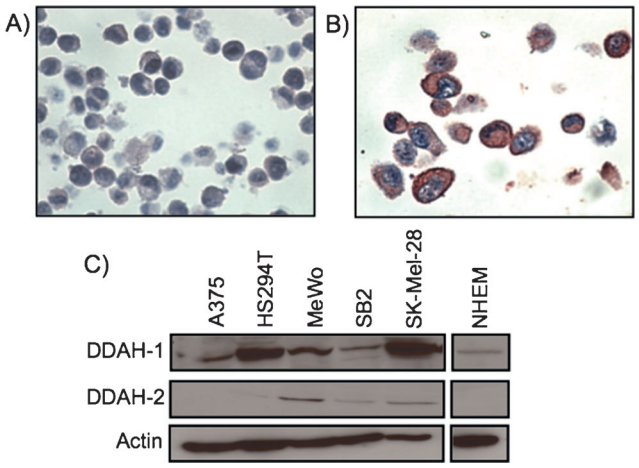

Figure 5. Assays for DDAH expression in melanoma cell lines: Immunohistochemical staining for DDAH-1 is applied in various cell lines to assay for blue color arising from the hematoxylin counter-stain or for a brown color arising from a primary DDAH-1 antibody/peroxidase-linked secondary antibody (see Experimental Section): A) normal human epidermal melanocytes, B) A375; A table summarizing staining for DDAH-1, DDAH-2, and iNOS in >60 cell lines is provided in the Supporting Information (Table S1). C) Western blot for DDAH-1, DDAH-2, and actin (as a control for equal loading) in selected cell lines.

ferences with respect to ranking relative DDAH-1 levels between different melanoma lines (Figure 5). Future studies will be required to better define the role of DDAH-1 in melanoma, but in our opinion, the striking prevalence of upregulated DDAH-1 in these lines and its likely link to enhanced nitric oxide production warrants further investigation.

Using the A375 melanoma cell line, we tested whether Cl-NIO can impact downstream nitric oxide production. Two established proxies[16] were monitored to detect changes in nitric oxide production subsequent to treatment by Cl-NIO: post-translational 3-nitrotyrosine formation in cellular proteins and $NO_2^- + NO_3^-$ levels in culture supernatants (Figure 6). Treatment of A375 cell lines with Cl-NIO clearly inhibited formation of 3-nitrotyrosine. This decrease was not due to decreased levels of DDAH-1 or iNOS, because staining intensities for these enzymes did not change after treatment. Additionally, Cl-NIO treatment resulted in a concentration-dependent decrease in $NO_2^- + NO_3^-$ levels, which reflects decreased nitric oxide production. Both of these results are consistent with a proposed mechanism in which Cl-NIO inhibition of DDAH-1 leads to a downstream inhibition of nitric oxide production in cells.

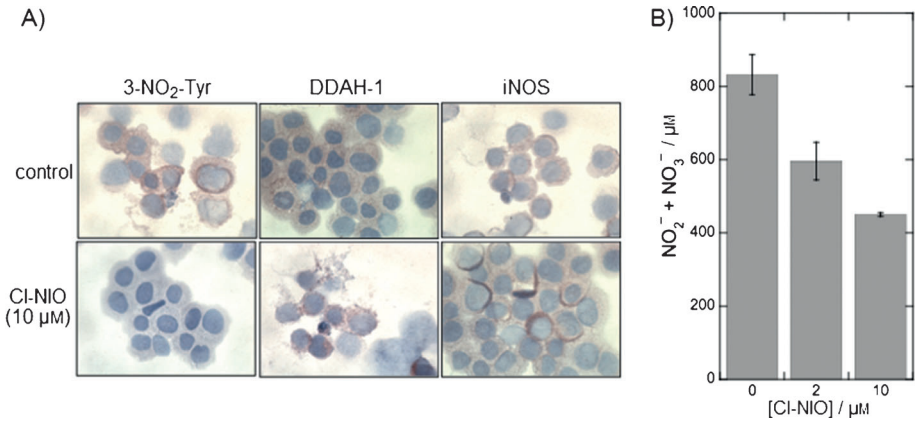

Figure 6. Treatment of A375 cells with Cl-NIO: A) Immunohistochemical staining of A375 cells for 3-nitrotyrosine, DDAH-1, and iNOS, with and without treatment by Cl-NIO. The hematoxylin counterstain (negative result) is blue, and the positive result is brown, due to the activity of a peroxidase-linked secondary antibody bound to separate primary antibodies for each analyte. B) Total nitrate and nitrite were measured in A375 culture supernatants after treatment with various concentrations of Cl-NIO, as described in the Experimental Section.

Conclusions

N^5-(1-Imino-2-chloroethyl)-L-ornithine (Cl-NIO) is found to be a potent time- and concentration-dependent covalent irreversible inactivator of human DDAH-1 with >500-fold selectivity against arginase and endothelial NOS, two arginine-processing enzymes in the same pathway. Cl-NIO inhibits DDAH-1 within cultured human cells and leads to downstream inhibition of nitric oxide biosynthesis. This compound should serve as a suitable tool for studies of methylarginine-mediated regulation of nitric oxide production and as a potential therapeutic lead for a variety of indications with elevated nitric oxide levels. Additionally, DDAH-1 was found to be upregulated in ~80% of melanoma cell lines tested, and represents a putative target for the control of nitric oxide production in these cells.

Experimental Section

Materials: Unless specified otherwise, all chemicals were obtained from Sigma–Aldrich Chemical Co. Wild-type human DDAH-1 bearing an N-terminal His_6 affinity tag was expressed using an expression plasmid with a re-engineered N-terminus (pET28a-hDDAH-1re) to avoid N-terminal non-enzymatic gluconoylation, and purified as described previously.[7a]

Cl-NIO synthesis: N^5-(1-Imino-2-chloroethyl)-L-ornithine (Cl-NIO) was synthesized using procedures similar to those previously described.[7a] Briefly, N^α-BOC-L-ornithine (250 mg, 1.08 mmol) was dissolved in H_2O (4 mL) in an ice bath and treated with NaOH (2.5 M) to adjust the solution to pH 10. 2-Chloro-1-ethoxyethanimine (462 mg, 2.94 mmol) was slowly added in portions while maintaining the mixture at pH 10. The reaction mixture was stirred for 1 h at 0 °C and for 2 h at 25 °C. The resulting solution was adjusted to pH 7.5 by the addition of HCl (1 M) and allowed to react overnight at 25 °C. Volatile solvents were removed under reduced pressure. The protected amino acid was dissolved in H_2O and purified by ion-exchange chromatography, eluted using 10% aqueous pyridine, and further purified by HPLC (10% CH_3CN/H_2O). The product-containing fractions were evaporated to yield a brown foamy solid, which was subsequently treated with HCl (4.6 M in dioxane) at 0 °C. The mixture was slowly warmed to room temperature and stirred for 3 h.

The solvent was evaporated under reduced pressure to yield the desired product (yellow solid, 30 mg, 10% yield for two steps). As an alternative to HPLC purification, a CombiFlash (Rf200, Teledyne/Isco) was used with a reversed-phase C_{18} column (26 g) using the following method: 5% B (B: 0.1%TFA/MeOH, A: 0.1%TFA/H_1O) for 2 min increasing to 50% B over 13 min, hold for 10 min. Fractions containing either the Boc-protected Cl-NIO, or Cl-NIO were pooled and deprotection was completed as described above. ^{1}H NMR (500 MHz, D_2O): $\delta = 4.32$ (s, 2H), 3.82 (t, $J = 6.2$ Hz, 1H), 3.31 (t, $J = 6.9$ Hz, 1H), 1.63–1.92 ppm (m, 4H); ^{13}C NMR (MHz, D_2O): $\delta = 172.9$, 162.9, 53.3, 41.8, 39.1, 27.2, 22.4 ppm; HRMS: $[M+H]^+$ calcd for $C_7H_{15}N_3O_2Cl$: 208.0845; found: 208.0847.

Time-dependent inactivation of DDAH-1 by Cl-NIO: Inactivation parameters were derived from the nonlinear progress curves of incubations with His_6–DDAH-1 and Cl-NIO in competition with the substrate ADMA, which we determined to have a K_M value of 51 μM under these experimental conditions. Briefly, time-dependent inactivation of DDAH-1 by Cl-NIO was measured in a 96-well assay format. Varied concentrations of Cl-NIO (0–600 μM) were mixed with ADMA (10 mM) and DDAH-1 (1.1 μM) in 60 μL assay buffer (100 mM KH_2PO_4, 1 mM EDTA, pH 7.27). Reactions were incubated at 25 °C for 0–60 min. At various time points, the reaction in each well was quenched by the addition of 3 μL trifluoroacetic acid (6 N). Samples were kept at room temperature until the experiment was finished and were analyzed for L-citrulline content by the addition of 200 μL COLDER reagent.[17] The apparent inactivation rates (A) were determined at each concentration of Cl-NIO by using the method of Tsou, and replots of $1/A$ led to determination of the K_I and k_{inact} values, as described.[18]

Mass spectrometry of DDAH-1 after inactivation Cl-NIO: To probe for covalent bond formation, DDAH-1 (40 μM) was incubated with or without Cl-NIO (40 μM) for 30 min at 25 °C in DDAH-1 assay buffer. Samples were buffer exchanged into ammonium acetate (20 mM) at pH 7.4, using an Amicon Microcon YM10 membrane (Millipore) to remove any unreacted inactivator. The treated DDAH-1 protein was collected in a volume of ~100 μL and subjected to ESIMS analysis (Analytical Core Facility, College of Pharmacy, The University of Texas at Austin).

IC_{50} determinations: To determine if manganese-loaded human arginase-I (Mn-hArgI) is inhibited by Cl-NIO, Mn-hArgI (5 μM) was incubated with varying concentrations of Cl-NIO (75 μM–9 mM) in the presence of L-arginine (1.5 mM), $MnSO_4$ (10 μM) in HEPES buffer (100 mM) at pH 7.4 for 15 min at 37 °C. The reaction was quenched with 10 μL trichloroacetic acid (6 M), and the formation of urea was determined as described above for L-citrulline. The IC_{50} value and Hill coefficient were fitted using standard procedures, and the K_i value was calculated by the method of Cheng and Prusoff, assuming competitive inhibition.[19] Purified recombinant endothelial NOS[20] was used in an oxyhemoglobin assay as previously described,[21] using 10 μM of L-arginine along with various concentrations of Cl-NIO, and initiation of the reaction upon addition of NADPH.

Determination of "in cell" IC_{50} values for DDAH-1 inhibition: To determine "in cell" IC_{50} values for inhibition of DDAH-1, a competitive labeling strategy was used, as described elsewhere.[7b,14] Cultured HEK293T cells were seeded in a 12-well polystyrene plate using complete growth medium containing DMEM with 10% FBS (Invitrogen, Carlsbad, CA) and grown to 80% confluency. The pEF6a-hDDAH-1 plasmid[14] was transiently transfected into HEK293T cells using Lipofectamine 2000 (Invitrogen). After 48 h, spent medium was removed, and cells were washed with 1 mL of 0.5 mM PBS at pH 7.2 (Invitrogen). Stock solutions of Cl-NIO (10 mM) were diluted into complete growth medium in the cultures (1000 μL per well) to give final concentrations of 0, 5, 10, 20, 40, and 80 μM. The resulting cultures were subsequently incubated for 15 min at 37 °C in an atmosphere of 5% CO_2 before addition of the activity probe *N*-but-3-ynyl-2-chloroacetamidine (110 μM), followed by an additional 10 min incubation. After treatment, cells were washed with PBS (2×1 mL) and harvested in 500 μL PBS followed by centrifugation at 14000×g for 5 min at 4 °C. Cell pellets were stored at −80 °C. Frozen cell pellets were lysed and labeled with biotin-PEO_3-azide as described earlier.[14] Two-color Western blot detection was used to detect the expression levels of DDAH-1 and the response to the biotin tag, as described previously.[14] Images were scanned using an Odyssey Infrared Imaging System (Li-Cor Biosciences, Lincoln, NE) at the Core DNA Facility (University of Texas, Austin). Fluorescence intensities for both 680 and 800 nm channels were integrated. Fits for the concentration dependence of the normalized I_{800}/I_{680} ratio were determined with the equation: I_{800} (or I_{800}/I_{680}) = 100−(100/1+(IC_{50}/[inhibitor])^h), with h as the Hill coefficient.

Cell toxicity assay: Cultured HEK293T cells were seeded in a 96-well polystyrene plate using complete growth medium containing DMEM with 10% FBS (Invitrogen). Post-seeding (24 h), 1000, 333, 111, 37, 12, 4, 1.3, and 0 μM of Cl-NIO were diluted in growth medium added to the wells in triplicate and incubated for 72 h at 37 °C in an atmosphere of 5% CO_2. MTS reagent (Promega; 20 μL) was added to each well and incubated for an additional 2 h at 37 °C in an atmosphere of 5% CO_2 before absorbance was read at 490 nm. Background absorbance of the media at 490 nm was subtracted from the data before normalization to a positive control (no inhibitor).

Immunohistochemistry: Melanoma cell lines tissue microarrays (MCL-TMAs) were prepared from paraffin-embedded sections of tumor cell lines. This TMA and individual cell line cytospins were examined for iNOS, 3-nitrotyrosine (NT), DDAH-1, and DDAH-2 expression by immunohistochemistry using an anti-iNOS monoclonal antibody (Creative Biolabs, Shirley, NY), an anti-NT polyclonal antibody (EMD Millipore Corp., Billerica MA), anti-DDAH-1 and anti-DDAH-2 antibodies (Abcam Inc., Cambridge, MA). Pre-immune normal mouse IgG (Vector Laboratories, Burlingame, CA) and anti-vimentin antibody (BioGenex Laboratories, San Ramon, CA) were used as negative and positive controls, respectively. Tissue sections were de-paraffinized and rehydrated, then placed in Antigen Unmasking Solution (Vector Laboratories) and microwaved intermittently for a total of 10 min, to maintain boiling temperature. After cooling, the slides were placed in 3% H_2O_2 in cold MeOH for 15 min, and then 0.05% Triton X-100 (Sigma, St. Louis, MO) for 15 min. An avidin–biotin–peroxidase complex (ABC) kit (Vectastain, Vector Laboratories) was then used for antigen detection. After a 30 min incubation with blocking serum, the primary antibody was applied for 2 h at room temperature, followed by 30-min incubations with secondary biotinylated antibody, and the ABC reagent. The immunolabeling was developed with the chromogen 3-amino-9-ethylcarbazole for 10 min. Hematoxylin was applied as a counter-stain. Immunolabeling was scored for the overall intensity of immunoreactivity of the positive cells. Briefly, semi-quantitative scoring for intensity was defined as follows: 0: no staining, 1: light staining, 2: moderate staining, and 3: intense staining.

Western blotting of melanoma lines: Cells were lysed in buffer containing 50 mM Tris (pH 7.9), 150 mM NaCl, 1% NP-40, 1 mM EDTA, 10% glycerol, 1 mM sodium vanadate, and protease inhibitor cocktail (Roche). Proteins were separated by 12% SDS-PAGE, trans-

ferred to a Hybond ECL nitrocellulose membrane (GE Healthcare Biosciences), and blocked in 5% dry milk in PBS. The membrane was then incubated with primary and secondary antibodies, and target proteins were detected with ECL detection reagent (GE Healthcare Biosciences). Antibodies for DDAH-1 and DDAH-2 were purchased from Abcam Inc. (Cambridge, MA) and for β-actin was purchased from Santa Cruz Biotechnology.

Griess assay for nitrate and nitrate: Varying amounts of nitric oxide produced by cultured cells was gauged by monitoring the downstream accumulation of the metabolites nitrate and nitrite found in the supernatant of cultured cells, and was measured using the Griess assay (R&D Systems, Minneapolis, MN) according to the manufacturer's protocol. The assay uses nitrate reductase to catalyze the reduction of nitrate into nitrite. In the final step, the Griess reagents convert the total nitrite into a purple-colored azo compound, which can be quantified by its absorbance at 540 nm. Briefly, cell culture supernatants were assayed by the addition 1:1 (*v*/*v*) of reaction mixture to each well of a 96-well plate (in triplicate). The reaction mixture consisted of 1:1 freshly mixed Griess Reagent A (0.1% *N*-1-naphthyl ethylene diamine dehydrochloride 0.1% in H_2O) and Griess Reagent B (1% sulfanilamide in 5% H_3PO_4). After 5–10 min incubation at room temperature, the resulting increase in absorbance was measured using a microplate reader. The total combined nitrite concentrations (nmol mL^{-1}) were calculated using a standard curve obtained in each assay by using a freshly prepared $NaNO_2$ standard solution.

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Keywords: covalent inhibitors · dimethylaminohydrolase · dimethylarginine · melanoma · nitric oxide

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