In vivo modulation of hypoxia-inducible signaling by topographical helix mimetics

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SI Appendix

Table of contents

Supporting Figures and Tables

Table S1. Computational alanine scanning mutagenesis energies calculated with Rosetta(1) ver. 3.3. Scans were performed on the HIF1α/CBP complex (PDB codes 1L8C and 1L3E).

HELIX B (817-824): ELLRALDQ

Residue	Helix B residue	ΔΔG (kcal/mol)
Leu	818	1.4
Leu	819	0.5
Arg	820	0.1
Ala	821	0.0
Leu	822	1.9
Asp	823	1.4
Gln	824	0.3

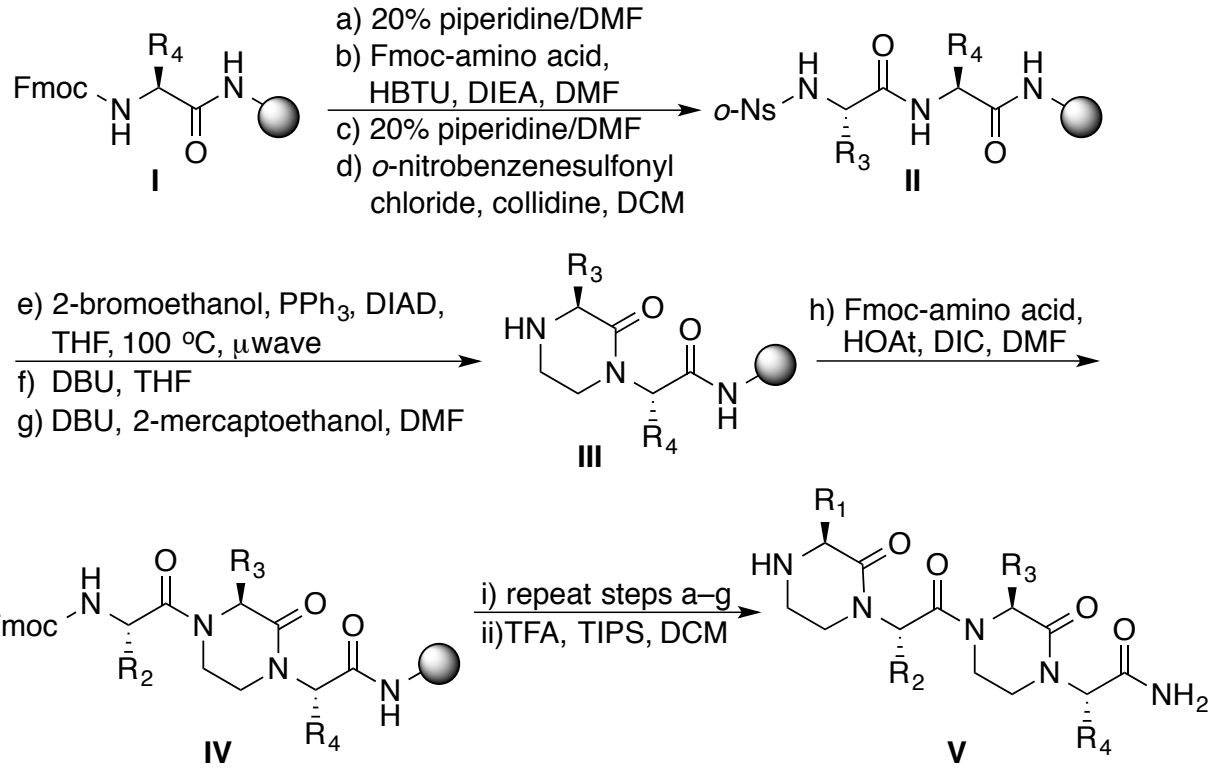
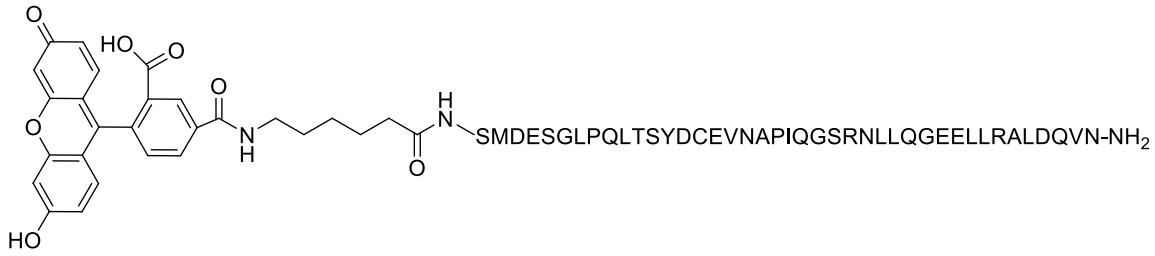


Figure S1. Solid-phase synthesis of oxopiperazine helix mimetics.

(a)



(b)

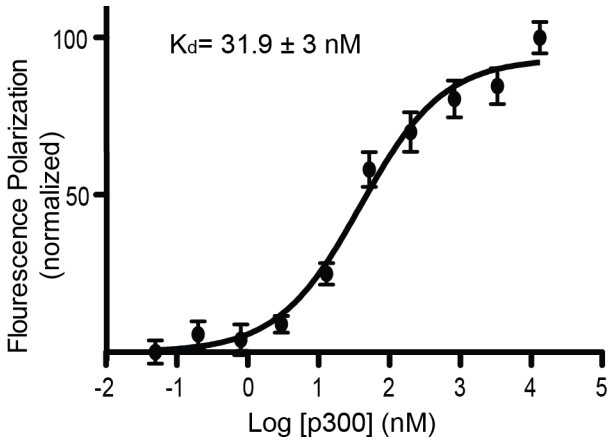
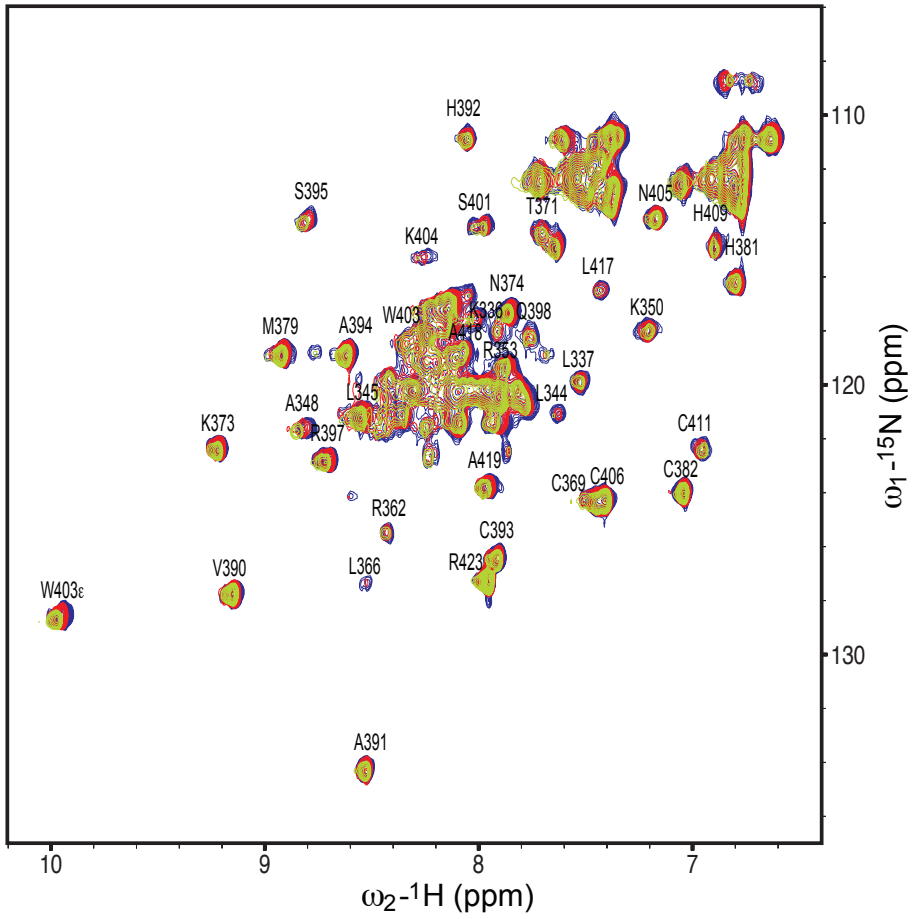


Figure S2. (a) Chemical structure of fluorescein-labeled CTAD (**Flu-HIF-1α CTAD$_{786-826}$**). Mass $[M+H]^+$ calc'd = 4977.1; found = 4976.8. (b) Binding of Flu-HIF CTAD to p300-CH1 as measured by a fluorescence polarization assay.

(a)



(b)

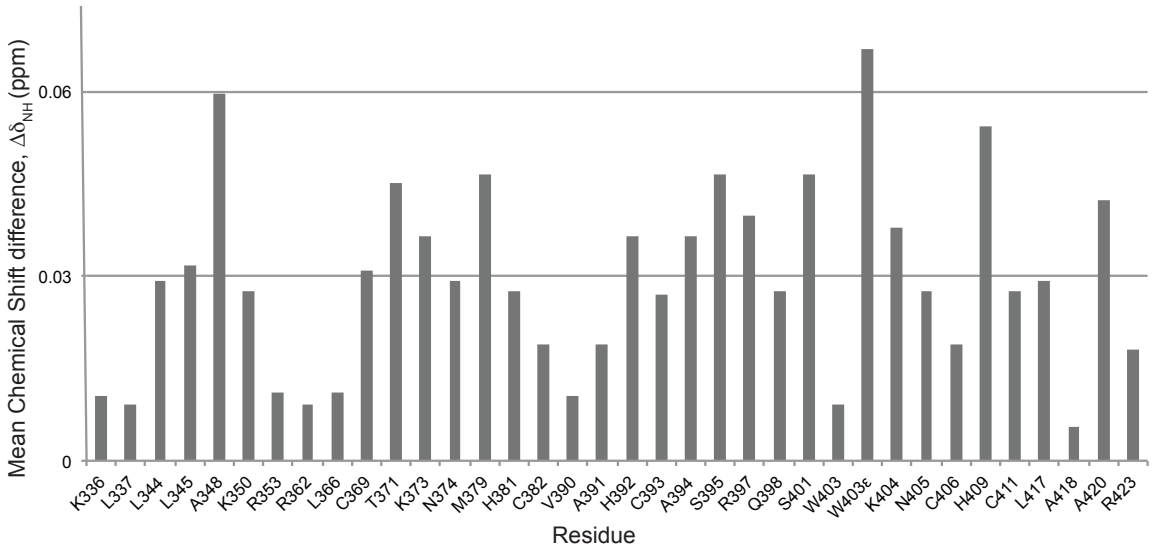


Figure S3. ^{1}H-^{15}N HSQC titration spectra. **(a)** Spectra of p300–CH1 (blue), CH1:OHM **1** (1:1.2, red), and CH1:OHM **1** (1:7, green) are overlaid. **(b)** Mean chemical shift difference (ΔδNH) plot depicting changes in residues chemical shift.

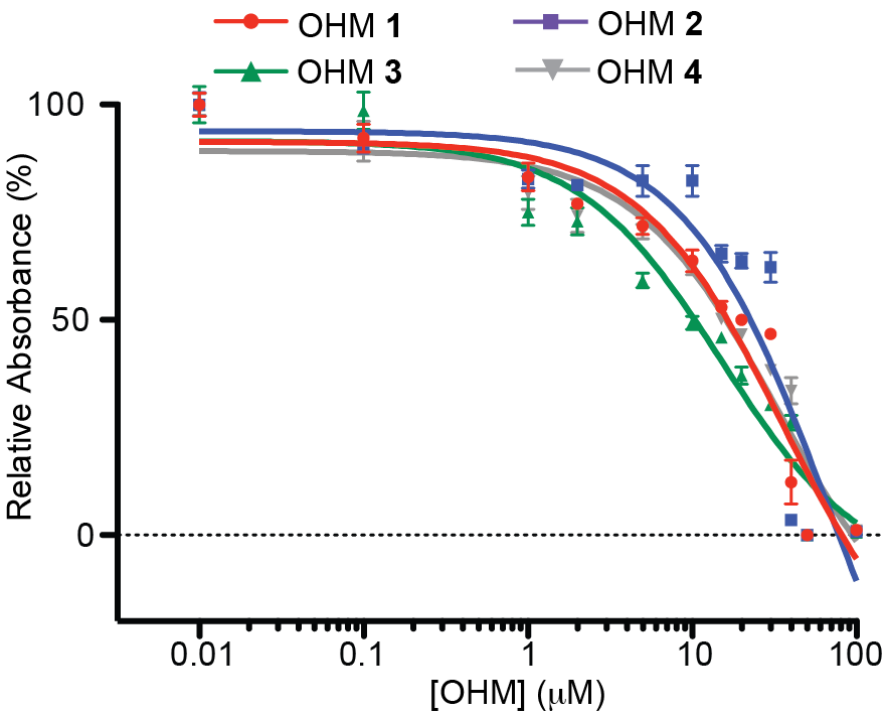


Figure S4. OHMs exhibit low cytotoxicity in A549 cells as evaluated by MTT assay. A549 cells were treated with OHMs **1-4** in the range of concentrations of 1 μM and 100 μM for 48 h.

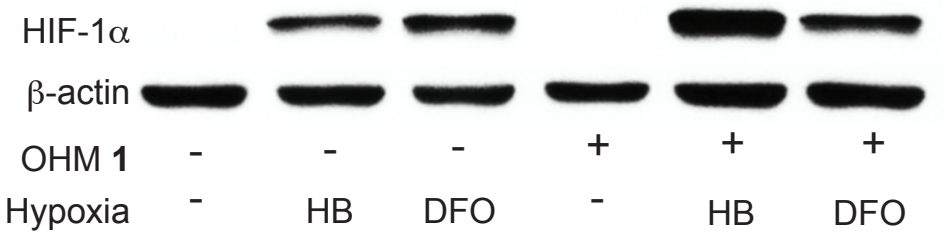


Figure S5. Treatment with OHM 1 does not lead to a decrease in the intracellular levels of HIF1α. Western blot analysis of HIF-1α levels in the whole cell extracts of A549 cells. Cells were incubated for a total of 24 h with OHM **1**: after 6 h, hypoxia was mimicked with DFO (300μM) or GasPak EZ pouch for an additional 18 h.

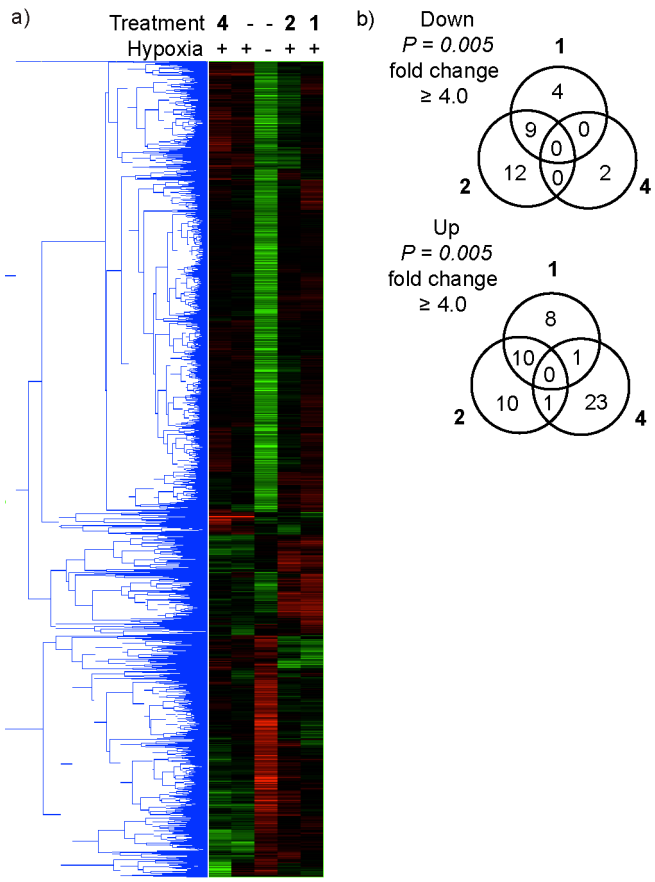


Figure S6. (a) Hierarchical agglomerative clustering of transcripts induced or repressed 4-fold or more (one-way ANOVA, $P \leq 0.005$) by hypoxia alone (GasPak EZ pouch) under the three specified conditions: **-**, no treatment; **1**, OHM **1** (10 μM); **2**, OHM **2** (10 μM); **4**, OHM **4** (10 μM). Clustering was based on a Pearson centered correlation of intensity ratios for each treatment compared to hypoxia-induced cells (controls) using average-linkage as a distance.

Table S2. Number of affected genes in cells treated with the inhibitors of hypoxia-inducible transcription factor complex.

Cell Line	A549		MCF7		U251		
Inhibitor	**OHM 1**	**OHM 2**	**ETP 2**	**ETP 3**	**Echinomycin**	**siRNA**	**Polyamide 1**
Down-regulated 2-fold	449	391	187	113	7426	1811	1575
Up-regulated 2-fold	148	217	142	290	3492	1383	709
Total	597	608	329	403	10918	3194	2284
***P*-value (ANOVA)**	<0.005		<0.01	<0.01		<0.01	
Reference	*Ibid*.		(2)	(3)		(4)	

METHODS

General. Commercial grade solvents and reagents were used without further purification. Fmoc amino acids and peptide synthesis reagents were purchased from Novabiochem. The Hoveyda-Grubbs (second-generation) catalyst, and molecular biology grade salts and buffers were obtained from Sigma.

Synthesis of oxopiperazine dimers. An Fmoc amino acid linked to Knorr Rink Amide resin was extended to a dipeptide using standard Fmoc solid phase peptide synthesis methods in a solid phase reaction vessel.(5) The resultant dipeptide was deprotected with 20% piperidine/dimethylformamide (DMF) and resin was washed sequentially with DMF, dichloromethane (DCM), methanol (MeOH), and diethyl ether and dried under vacuum. *o*-Nitrobenzenesulfonyl chloride (Ns-Cl, 10 eq) and collidine (10 eq) were dissolved in dry DCM and added to the reaction vessel. The mixture was shaken for 2 hours at 23°C to obtain **II**. The resin was washed with sequentially with DMF, DCM, MeOH, and diethyl ether and dried for 12 hours under vacuum. The resin was transferred to a glass microwave tube (CEM). Triphenylphosphine (PPh_3, 10 eq) was added and the tube was flushed with nitrogen gas for 30 minutes. Tetrahydrofuran (THF), diisopropyl azodicarboxylate (DIAD, 10 eq), and 2-bromoethanol (10 eq) were added and the reaction mixture was subjected to microwave irradiation (200 watts, 250 psi) for 10 minutes at 100°C. Resin was washed sequentially with THF, DMF, and DCM. The resin was transferred to a solid phase vessel and treated with 1,8-diazabicyclo[5.4.0]undec-7-ene (DBU) in THF for 2 hours. Resin was washed with THF, DMF, DCM, and diethyl ether, allowed to dry for 30 minutes, and then treated with DBU and 2-mercaptoethanol in DMF for 2 hours. Compound **III** was then washed with DMF, DCM, MeOH and diethyl ether and dried. The desired pre-activated Fmoc-amino acid was added to the resin and the mixture was shaken at 23°C for 12 hours affording **IV**. Nosyl protection and the ring formation steps were repeated to obtain oxopiperazine dimers **V** after cleavage from the resin with 95% trifluoroacetic acid (TFA), 2.5% water, and 2.5% triisopropylsilane (TIPS).

OHM **1** - LLAQ

^{1}H-NMR (600 MHz, d_6-DMSO, 100 °C) δ 6.87 (br, 3H), 6.61 (br, 3H), 5.36 (t, J = 7.47, 1H), 4.90-4.85 (m, 1H), 4.67 (q, J = 6.88, 1H), 3.96 (br, 2H), 3.63-3.24 (m, 8H), 1.95-1.82 (m, 3H),

1.72-1.58 (m, 3H), 1.57-1.49 (m, 1H), 1.38 (s, 3H), 1.06-0.75 (m, 12H). HRMS (ESI) $C_{24}H_{42}N_6O_5$ $[M+H]^+$ calc'd= 494.3217; found= 495.3502

OHM **2** - LLAA

^{1}H-NMR (600 MHz, d_6-DMSO, 100 °C) δ 6.88 (br, 2H), 5.37 (t, *J* = 7.20, 1H), 4.92 (q, *J* = 7.25, 0.8H), 4.87 (q, *J* = 7.25, 0.2H), 4.65 (q, *J* = 6.91, 1H), 4.00 (br, 1H), 3.88 (br, 1H), 3.57-3.34 (m, 7H), 3.30-3.23 (m, 1H), 1.92-1.80 (m, 2H), 1.69-1.57 (m, 3H), 1.56-1.48 (m, 1H), 1.35 (d, *J* = 6.88, 3H), 1.29 (d, *J* = 7.27, 3H), 0.95 (d, *J* = 6.80, 4H), 0.93 (d, *J* = 6.34, 5H), 0.90 (d, 6.34, 3H). HRMS (ESI) $C_{22}H_{39}N_5O_4$ $[M+H]^+$ calc'd= 438.3002; found= 438.3118

OHM **3** - LAAQ

^{1}H-NMR (400 MHz, d_6-DMSO, 100 °C) δ 6.77 (br, 2H), 6.52 (br, 2H), 5.34 (q, *J* = 6.91, 1H), 4.90-4.84 (m, 1H), 4.67 (q, *J* = 6.91, 1H), 4.01-3.91 (m, 1H), 3.76-3.69 (m, 1H), 3.57-3.27 (m, 7H), 3.24-3.14 (m, 1H), 2.19-2.01 (m, 3H), 2.00-1.80 (m, 3H), 1.64-1.55 (m, 1H), 1.38 (d, *J* = 6.92, 3H), 1.27 (d, *J* = 6.92, 3H), 0.94 (t, *J* = 6.02, 6H). HRMS (ESI) $C_{21}H_{36}N_6O_5$ $[M+H]^+$ calc'd= 453.2747; found= 453.2863

OHM **4** - AAAA

^{1}H-NMR (400 MHz, d_6-DMSO, 100 °C) δ 6.71 (br, 2H), 5.34 (q, *J* = 6.87, 1H), 4.91 (q, *J* = 7.13, 1H), 4.65 (q, *J* = 6.94, 13.68, 1H), 4.05-3.91 (m, 1H), 3.82-3.79 (m, 1H), 3.53-3.38 (m, 4H), 3.37-3.33 (m, 2H), 3.31-3.25 (m, 1H), 3.20-3.12 (m, 1H), 1.38 (d, *J* = 6.97, 3H), 1.35 (d, *J* = 6.97, 3H), 1.29 (d, *J* = 7.20, 3H), 1.26 (d, *J* = 6.97, 3H). HRMS (ESI) $C_{16}H_{27}N_5O_4$ $[M+H]^+$ calc'd= 354.2063; found= 354.2139

Plasmids. The DNA sequence of human p300 CH1 domain (amino acid residues 323-423) was subcloned into a pUC57 plasmid by Genscript, Inc. After transformation of JM109 bacteria (Promega) with the plasmid, it was amplified and purified. Then the gene of interest was subcloned between *Bam*HI and *Eco*RI restriction sites of pGEX-4T-2 expression vector (Amersham).

Cloning and Expression of ^{15}N p300–CH1. The pGEX-4T-2-p300 fusion vector was

transformed into BL21(DE3) competent *E.coli* (Novagen) in M9 minimal media with $^{15}NH_4Cl$ as the primary nitrogen source. Protein production was induced with 1 mM IPTG at OD_{600} of 1 for 16 hours at 15 °C. Production of the desired p300-CH1-GST fusion product was verified by SDS-PAGE. Bacteria were harvested and resuspended in the lysis buffer with 20 mM Phosphate buffer (Research Products International, Corp.), 100 μM DTT (Fisher), 100 μM $ZnSO_4$ (Sigma), 0.5% TritonX 100 (Sigma), 1 mg/mL Pepstatin A (Research Products International, Corp.), 10 mg/mL Leupeptin A (Research Products International, Corp.), 500 μM PMSF (Sigma), and 0.5% glycerol at pH 8.0. Pellets were lysed by sonication and centrifuged at 4 ^{o}C and 20,000 rpm for 20 min. Fusion protein was collected from the bacterial supernatant and purified by affinity chromatography using glutathione Sepharose 4B beads (Amersham) prepared according to the manufacturer's directions. GST-tag was cleaved by thrombin and protein was eluted from the resin. Collected fractions were assayed by SDS-PAGE gel; pooled fractions were treated with protease inhibitor cocktail (Sigma) and dialyzed against a buffer containing 10 mM Tris, 50 mM NaCl, 2 mM DTT (Fisher), 3 equivalents $ZnSO_4$ at pH 8.0 to ensure proper folding (*vide supra*).

Tryptophan Fluorescence Binding Assay. Spectra were recorded on a QuantaMaster 40 spectrofluorometer (Photon Technology International) in a 10 mm quartz fluorometer cell at 25 °C with 4 nm excitation and 4 nm emission slit widths from 200 to 400 nm at intervals of 1 nm/s. Samples were excited at 295 nm and fluorescence emission was measured from 200-400 nm and recorded at 335 nm. OHM stock solutions were prepared in DMSO. Aliquots containing 1 μL DMSO stocks were added to 400 μL of 1 μM p300-CH1 in 50 mM Tris and 100 mM NaCl (pH 8.0). After each addition, the sample was allowed to equilibrate for 5 minutes before UV analysis. Background absorbance and sample dilution effects were subtracted by titrating DMSO into p300-CH1 in an analogous manner. Final fluorescence intensity is reported as the absolute value of $[(F_1-F_0)/F_1]*100$, where F_1 is the final fluorescence upon titration, and F_0 is the fluorescence of the blank DMSO titration. EC_{50} values for each compound were determined by fitting the experimental data to a sigmoidal dose-response nonlinear regression model in GraphPad Prism 5.0, and the dissociation constants, K_D, were obtained from equation (**1**).

$$K_D = \frac{(EC_{50}\times(1-F) + P\times F^2)}{F} - P \qquad (\mathbf{1})$$

where,

P = Total concentration of protein

F = Fraction of bound peptide = 0.5

^{1}H-^{15}N HSQC NMR Spectroscopy. Protein samples were prepared as described above. Uniformly ^{15}N-labelled p300-CH1 was concentrated to 69 μM in NMR buffer (10 mM Tris, pH 8, 50 mM NaCl, 2 mM DTT, and 207 μM $ZnSO_4$) using 3 kDa MWCO Amicon Ultra centrifugal filter (Millipore) and supplemented with 5% D_2O. For HSQC titration experiments, data was collected on a 600 MHz Bruker four-channel NMR system at 25 ºC and analyzed with the TopSpin software (Bruker).

For the HSQC titration experiments, 0, 1.2 and 7 molar equivalents of OHM **1** in DMSO were added to ^{15}N-labelled p300-CH1, and the data was collected as described above. Mean chemical shift difference ($\Delta\delta_{NH}$) observed for ^{1}H and ^{15}N nuclei of various resonances were calculated as described,(6) where α is the range of H ppm shifts divided by the range of NH ppm shifts (α = 1/8).

$$d = \sqrt{\frac{1}{2} \cdot [\delta_H^2 + \alpha \cdot \delta_N^2]}$$

Cell lines. Human alveolar basal epithelial adenocarcinoma (A549), human breast adenocarcinoma (MCF7), and human renal cell adenocarcinoma (786-O) cell lines were obtained from ATCC. Human breast epithelial adenocarcinoma cells stably transfected with a hypoxia response element (HRE) luciferase construct (MDA-MB-231-HRE-Luc**)** was a gift of Dr. Robert Gillies.

Cell culture. MCF7 cells were grown in RPMI 1640 (Invitrogen) supplemented with 10% of fetal bovine serum (FBS, Irvine Scientific), 50 units/mL penicillin and 50 μg/mL streptomycin (Pen-Strep, Invitrogen). A549 cells were grown in RPMI 1640 supplemented with 2% or 0.2% of FBS, 50 units/mL penicillin and 50 μg/mL streptomycin. MDA-MB-231-HRE-Luc cells were grown in high glucose Dulbecco's Modified Eagle's Medium (DMEM, Invitrogen) supplemented with 10% FBS and 0.2 g/L geneticin (RPI). All cells were incubated at 37^{o}C in a humidified

atmosphere with 5% CO_2. Hypoxia was induced by GasPak EZ Anaerobe Pouch System (BD Biosciences), unless mentioned otherwise. Cell growth and morphology were monitored by bright field microscopy. Cells were detached using trypsin in PBS (0.05%, Invitrogen).

Preparation of OHM compounds for *in vitro* assays. Stock solutions of OHM compounds (1 mM) were prepared in a buffer, containing 150 mM NaCl, 50 mM Tris, 1 mM DTT, 100 μM $ZnCl_2$, 0.05-0.15% w/v NonidetP40, and 10% v/v glycerol for the preliminary biophysical studies and used without further changes for all *in vitro* assays.

Cell viability assay. MCF7 cells were seeded in a 96-well plate at a density of 1.4×10^4 cells per well in 200 μl of medium per well and allowed to form a monolayer for 48 h. After that, the medium was replaced with 150 μL of fresh medium containing OHM **1** or OHM **2** at a concentration range from 0.5 μM to 60 μM and 0.1 % dimethyl sulfoxide (DMSO). After 48 h of incubation with compounds, a solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) was added to each well (17 μL of 5 mg/mL, in PBS) and incubated at 37°C and 5 % CO_2 for an additional 3 h. After that, the medium was carefully removed and purple formazan crystals were dissolved in DMSO (100 μl per well). The absorbance was measured at 570 nm with a correction at 690 nm in order to quantify the amount of formazan. All experiments were performed in quadruplicate.

A549 cells were seeded in a 96-well plate at a density of 1.5×10^4 cells per well in 200 μl per well of medium with 2% FBS and allowed to form a monolayer for 24 h. After that, the medium was replaced with 150 μL of fresh medium containing 0.2% FBS, OHM **1**, OHM **2**, OHM **3**, and OHM **4** (concentrations ranging from 0.01 μM to 100 μM) and 0.1% DMSO. After 48 h of incubation with compounds, a solution of 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT, Sigma) was added to each well (17 μL of 5 mg/mL, in PBS) and incubated at 37°C and 5 % CO_2 for an additional 3 h. After that, the medium was carefully removed and purple formazan crystals were dissolved in DMSO (100 μL per well). The absorbance was measured at 570 nm with a correction at 690 nm in order to quantify the amount of formazan. All experiments were performed in quadruplicate.

Bicinchoninic acid (BCA) assay. Cell lysate (10 μL) was added to the BCA reagent (200 μL), prepared as per the manufacturer's protocol (Thermo Scientific). BSA standard solutions were prepared at a concentration range of 25 μg/mL to 2000 μg/mL. Absorbance was measured at 562 nm using a Synergy 2 microplate reader (BioTek). Sample concentrations were determined from a calibration curve. All experiments were performed in triplicate.

Analysis of the hypoxia-inducible promoter activity with luciferase assays. MDA-MB-231-HRE-Luc cells were seeded in 24-well plates (BD Falcon) at a density of 6.5×10^4 cells per well in 1 ml of medium. Cells were allowed to adhere and form monolayer for 48 h before the addition of the compounds (~80% confluence). After that, the cells were treated with 1 mL of the fresh medium containing 10% FBS, OHM **1**, OHM **2**, or OHM **4** at a range of concentrations from 0.1 μM to 20 μM, 0.1% DMSO; vehicle samples were treated with cell culture medium containing 0.1% DMSO. Cells were incubated for 6 h at 37°C and 5% CO_2 and then hypoxia was induced for another 18h. The lysates were isolated by using cell culture lysis reagent (Promega), supplemented with Halt protease inhibitor cocktail (Thermo Scientific). Cell lysate from each well was collected into a separate ice-cold polypropylene tube (USA Scientific) and centrifuged at 13,000 rpm for 10 min at 4°C. Supernatant was then transferred into another set of cold polypropylene tubes and the pellet was discarded. Luciferase assay reagent (100 μL, Promega) was added to cell lysate (20 μL) and the luminescence intensity was measured by Turner TD-20e luminometer. The results were normalized to the total protein concentration determined by the BCA assay. All experiments were performed in triplicate.

Isolation of mRNA. A549 cells were seeded in 6-well dishes (Greiner) at a density of 2×10^5 cells per well in 2 mL of medium with 2% FBS and allowed to form a monolayer (~90% confluent) for 72 h. After attachment, cells were treated with 1.5 mL of fresh medium containing 0.2% FBS, OHM **1**, OHM **2**, OHM **3**, and OHM **4** at concentrations of 5 μM and 10 μM, and 0.1% DMSO; vehicle samples were treated with cell culture medium containing 0.1% DMSO. After 6 h of incubation, hypoxia was induced and cells were incubated for another 42 hours. Cells were washed twice with ice-cold PBS and total mRNA was isolated with an RNeasy kit (Qiagen) according to the manufacturer's instructions. The mRNA was further treated with DNase I (Invitrogen, Turbo DNA-*free* kit) to remove any remaining genomic DNA. Then the

mRNA was quantified by UV absorbance at 260 nm. Reverse transcription was performed with Superscript III Reverse Transcriptase (Invitrogen) as recommended by the manufacturer. All experiments were performed in quadruplicate.

Analysis of gene expression. Real-time qRT-PCR was used to determine the effect of OHM **1**, OHM **2**, OHM **3**, and OHM **4** on *VEGF, LOX,* and *SLC2A1 (GLUT1)* genes under normoxia and hypoxia. For *VEGF*, the forward primer 5'-AGG CCA GCA CAT AGG AGA GA-3' and reverse primer 5'-TTT CCC TTT CCT CGA ACT GA-3' were used to amplify a 104-bp fragment. For *GLUT1,* the following primers were used: forward 5'-AGT ATG TGG AGC AAC TGT GTG G-3' and reverse 5'-CGG CCT TTA GTC TCA GGA AC-3' – to yield a product of 106 bp. For *LOX*, we employed the following primer pair: forward 5'-ATG AGT TTA GCC ACT ATG ACC TGC TT-3' and reverse 5'-AAA CTT GCT TTG TGG CCT TCA- 3' – to amplify a product of 73 bp. The mRNA levels were normalized to the expression levels of a housekeeping gene, β-glucuronidase. For β-glucuronidase the following primers was designed and used: forward 5'-CTC ATT TGG AAT TTT GCC GAT T-3' and reverse 5'- CCG AGT GAA GAT CCC CTT TTT A-3'. Reactions were performed with Fast SYBR Green Master Mix (Applied Biosystems). Temperature cycling and detection of the SYBR green emission were performed with an ABI 7900HT Fast Real-Time PCR System. Analysis of the data was performed with Applied Biosystems Sequence Detection System, version 2.3. All experiments were performed in a quadruplicate.

Western blot analysis of HIF1α levels. A549 cells were seeded in a 60 mm dish (VWR) and allowed to reach 80% confluence. Cells were treated with vehicle or OHM **1** at 10 μM concentration in the cell culture medium containing 2% FBS and 0.1% DMSO. Cells were incubated for 6 h and hypoxia was induced with 300 μM of DFO or GasPak EZ Anaerobe Pouch System. After incubation for an additional 18 h, the cells were washed twice with ice-cold PBS and then lysed with the cell culture lysis reagent (Promega). To ensure equal loading, protein concentration was determined by the BCA assay. A 1.5 mm 10% acrylamide denaturing gel was cast and an aliquot of each sample containing 30 μg of protein was loaded into the gel. The SDS-PAGE was carried out and then the gel was electroblotted onto the PVDF membrane (BioRad). After the transfer, the membrane was rinsed with tris-buffered saline with tween-20

(TBST) buffer and incubated with 5 % milk in TBST for 1 h. The membrane was then probed for HIF1α with a monoclonal mouse anti-human HIF-1α antibody (BD Biosciences) or for β-actin (loading control) with a polyclonal rabbit anti-human β-actin antibody (Cell Signaling) overnight at 4°C and gentle rocking. Membrane was further washed three times with TBST for 10 min and incubated with horseradish peroxidase (HRP) conjugated secondary anti-mouse or anti-rabbit antibody (Santa Cruz Biotechnology), respectively. Signals were detected by treating the membrane with Amersham ECL Prime Western Blotting Detection Reagent (GE Healthcare) for 3 min followed up by the exposure of the CL-Xposure film (Thermo Scientific) to the membrane.

Gene expression profiling. Experiments were carried out with A549 cells. The medium, time course, hypoxia induction, small molecule treatment, and mRNA isolation were conducted as described above in the "mRNA isolation" section. Cultured cells contained vehicle, OHM **1**, OHM **2**, or OHM **4** at a concentration of 10 μM. Sample preparation and microarray analysis was performed at the Genome Technology Center, New York University School of Medicine. Labeled mRNA was hybridized to Affymetrix Genechip Human Gene 1.0 ST microarrays. Four data sets were collected: normoxic cells with vehicle, hypoxic cells with vehicle, hypoxic cells with OHM **1** and hypoxic cells with OHM **2**, respectively. Gene expression was analyzed using GeneSpring GX 12.5 software (Agilent). Probe level data have been converted to expression values using a robust multi-array average (RMA) preprocessing procedure on the core probe sets and baseline transformation to median of all samples. A low-level filter removed the lowest 20th percentile of all the intensity values and generated a profile plot of filtered entities. Significance analysis was performed by one-way ANOVA test with Benjamini-Hochberg correction and asymptotic *P*-value computation. Fold change analysis was applied to identify genes with expression ratios above 2-fold between treatments and control set ($P < 0.05$). Hierarchical agglomerative clustering was performed using Pearson's centered correlation coefficient and average-linkage as distance and linkage methods.

Microarray data. Gene expression profiling data reported in this manuscript have been deposited in the Gene Expression Omnibus (GEO) database, www.ncbi.nlm.nih.gov/GEO (accession no. GSE48134).

In vivo toxicity dose-finding study. To determine the maximum tolerated dose of OHM **1**, 4 male BALB/c mice were injected intraperitoneally every other day with the escalating doses of OHM **1** (in mg/kg): 2, 4, 6.6, 10, 14, 24, 32, and 100. The animals were then injected intravenously with 10 mg/kg and then 100 mg/kg of OHM **1**. Toxicity after each injection of OHM **1** was assessed by daily weight measurements and visual inspection of treated mice.

In vivo efficacy testing of OHM 1 in mouse xenograft tumor models. The T-cell deficient mice CrTac:NCr-*Foxn1*^{nu} (Taconic, Inc.) were used. The mice were housed in an A.L.A.C.C. approved barrier facility under the direct supervision of a professional veterinarian. Mice (n=12) were inoculated with MDA-MB-231 cells (5×10^6) into the right flank and allowed to grow into tumors. After the tumors reached 200 mm^3, animals were randomly assigned to the treatment groups. The primary endpoint of efficacy (the tumor volume rate of increase as compared to control) were evaluated when mice (n=4) were treated with OHM **1** at 15 mg/kg dissolved in sterile PBS given parenterally on Days 0, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28 a total of 15 injections. In parallel, a control group (n=4) received injections of PBS (100 µl per animal). Tumor sizes were measured daily using Vernier caliper. To address the question of whether tumor growth is affected by the treatment with OHM **1**, a comparison of the tumor volumes of the control group and group treated with OHM **1** was made.

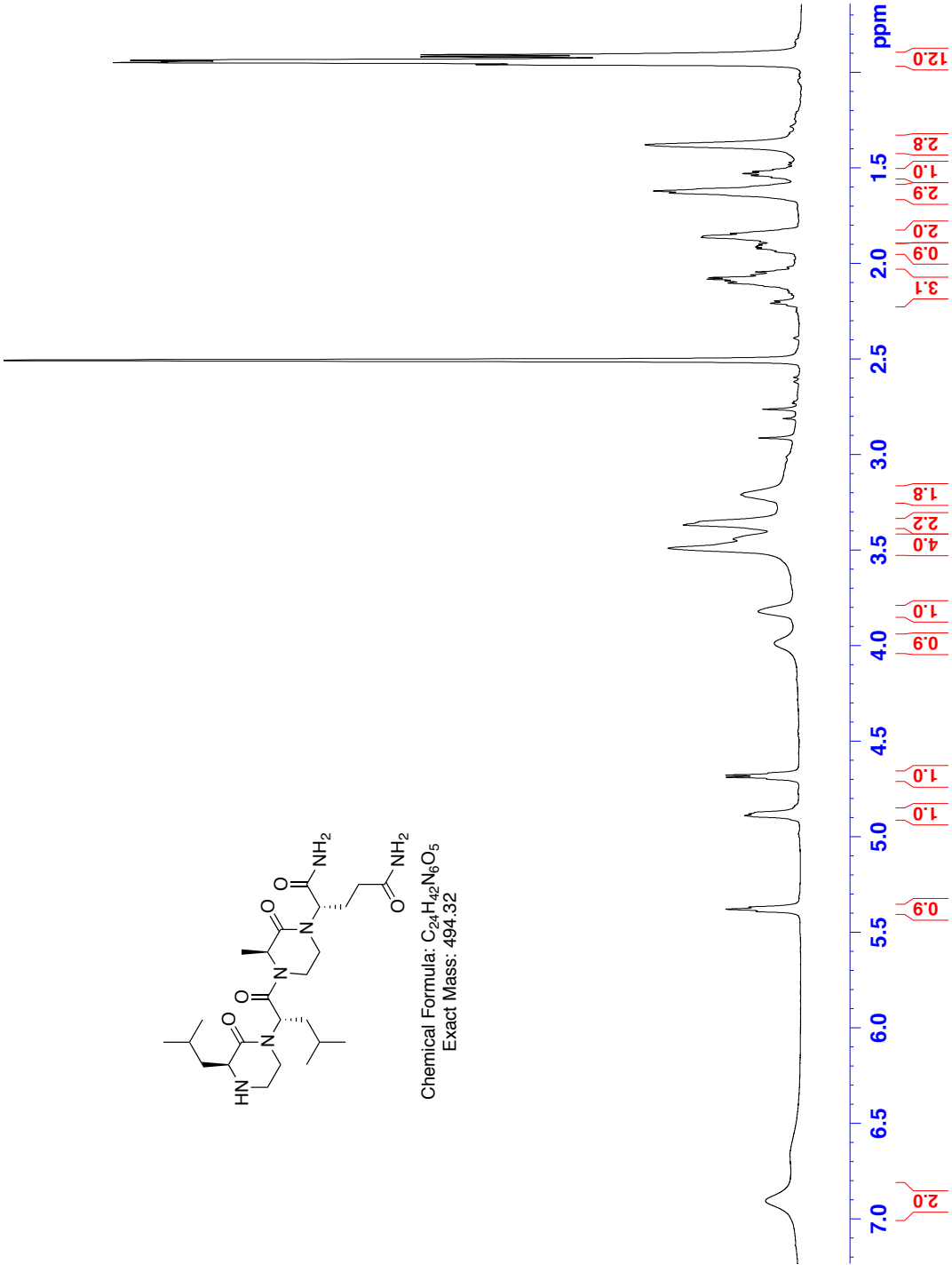
Imaging. At the experimental endpoint of the *in vivo* efficacy study, mice were injected intraperitoneally with the tumor-targeting near-infrared dye IR-783 and imaged using Xenogen IVIS 200 small animal imager. Euthanasia was performed as recommended by the American Veterinary Panel (AVMA 202229-249, 1993). Tumors and organs (liver, kidneys, heart, and lungs) were collected. Tumors were examined in a histopathology study (*vide infra*).

Immunohistochemistry. Tumor tissues were excised and fixed with 10% formalin, embedded in paraffin, and sectioned using a standard histological procedure. For overall morphological observations, the tissue sections were stained with hematoxylin and eosin (H&E). For Ki-67 staining, paraffin sections were deparaffinized in xylene and hydrated in a decreasing concentrations of aqueous ethanol. The slides were immersed in 3% hydrogen peroxide (Sigma) for 20 min to block endogenous peroxidase activity and then washed in PBS. For antigen

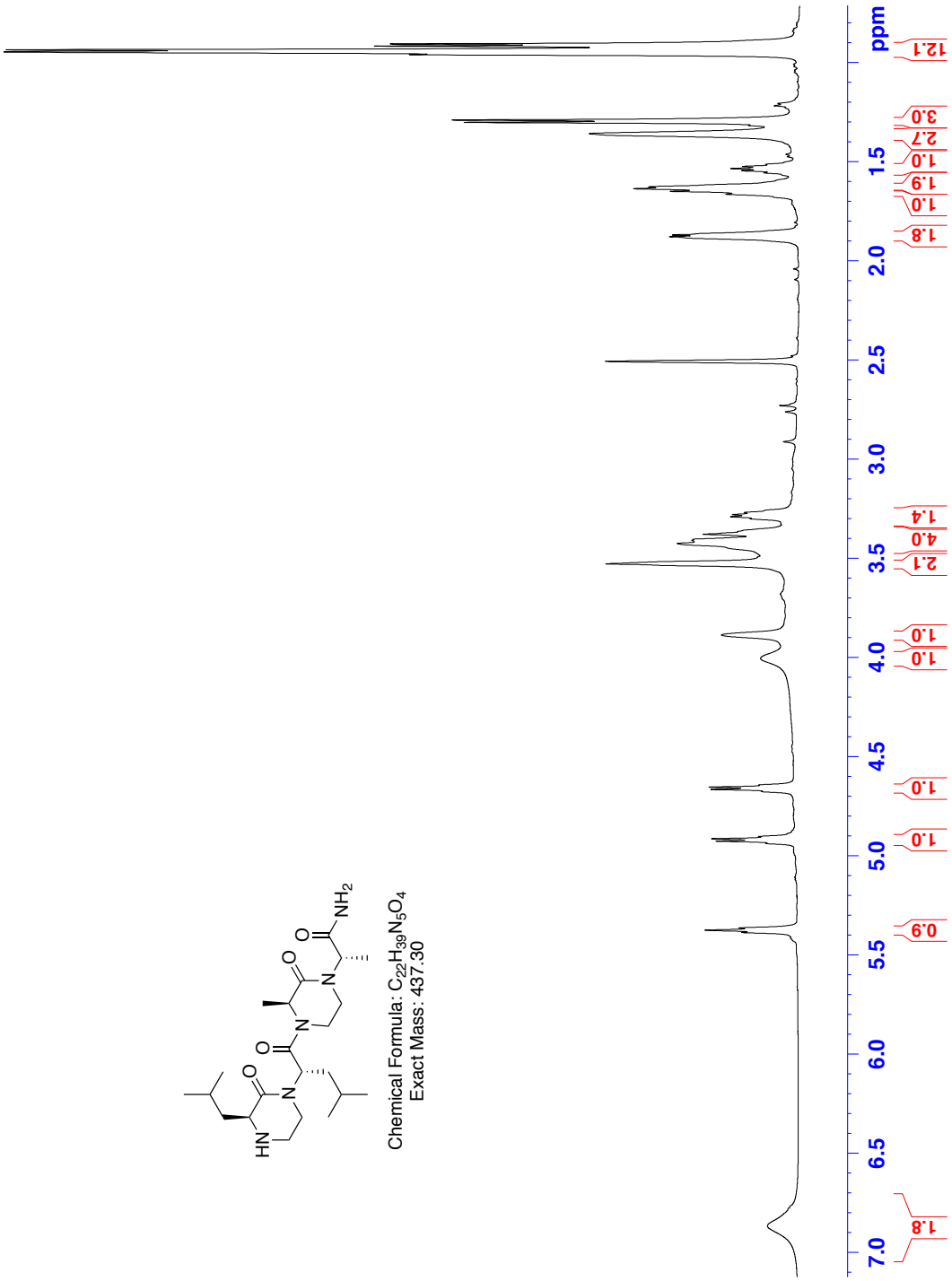
retrieval, the slides were placed in preheated working solution of Retrievagen A (BD Pharmingen, San Jose, CA) and heated in a steamer for 70 min. After cooling for 20 min at room temperature, slides were rinsed with PBS, treated with 10% normal FBS for 30 min, and then incubated with anti-Ki-67 antibodies for 2 h. Slides were then washed with PBS and incubated with the HRP-labeled goat anti-rabbit IgG antibodies for 1 h at room temperature. After washing with PBS, a streptavidin-HRP (BD Pharmingen) was added and incubated for 30 min. Slides were then stained with 3,3'-diaminobenzidine (Vector Laboratories, Burlingame, CA) for 3 min. Counterstaining was performed with hematoxylin (Vector Laboratories). After washing with distilled water, the slides were dehydrated in increasing grades of ethanol, cleared with xylene, and mounted using permanent mounting medium (Vector Laboratories). The proliferation index was determined by measuring the percentage of Ki-67 positive cells. A total of 24 randomly selected fields at 20× objective magnification from the tumors of each treatment group were examined. The pictures were quantified with ImmunoRatio plugin for ImageJ.(7) The data were plotted as a mean ± s.e.m. and analyzed for significance with the unpaired two-tailed t-test.

NMR Spectra.

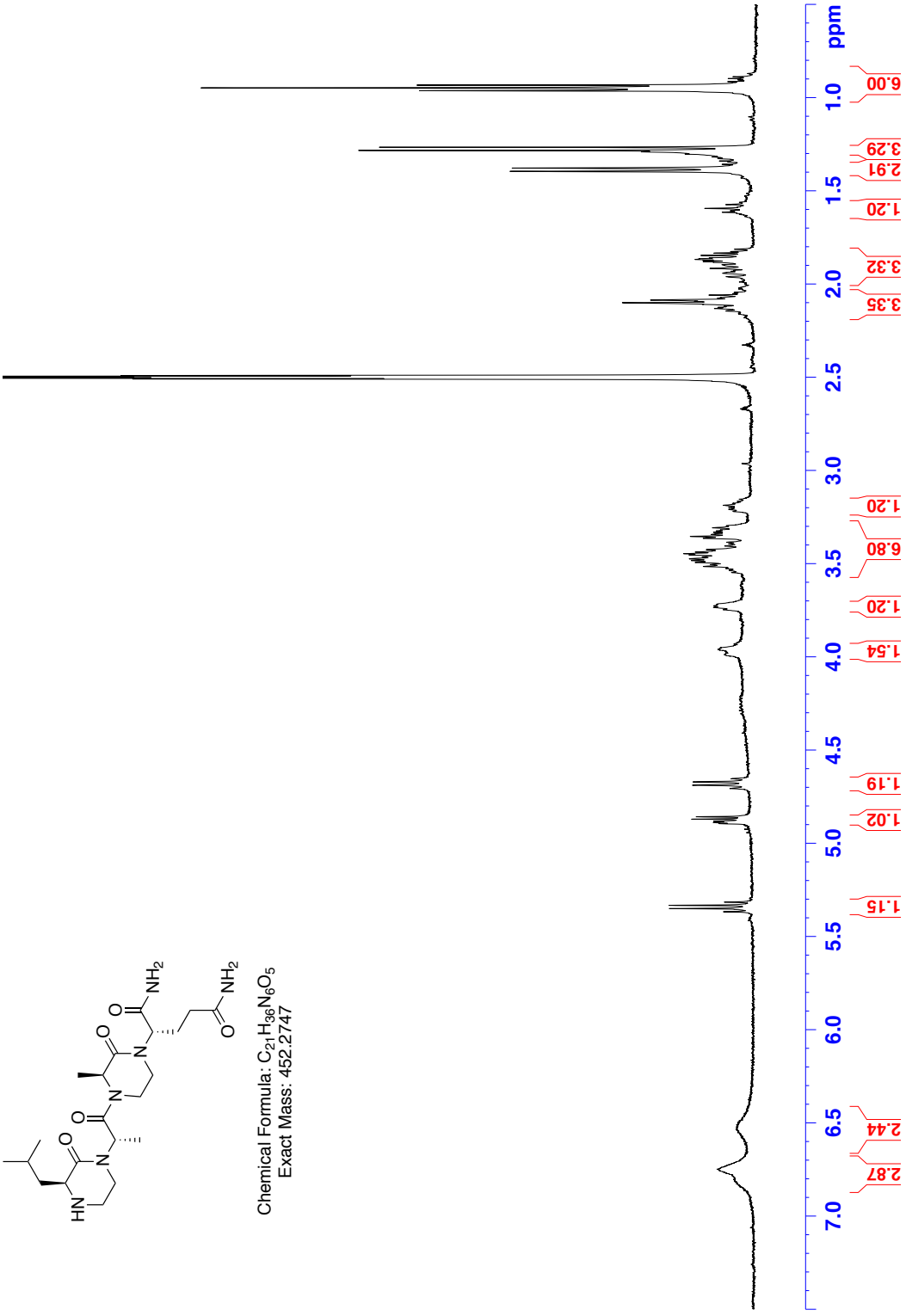
OHM 1 ^{1}H-NMR (600 MHz, d_6-DMSO, 100 °C)



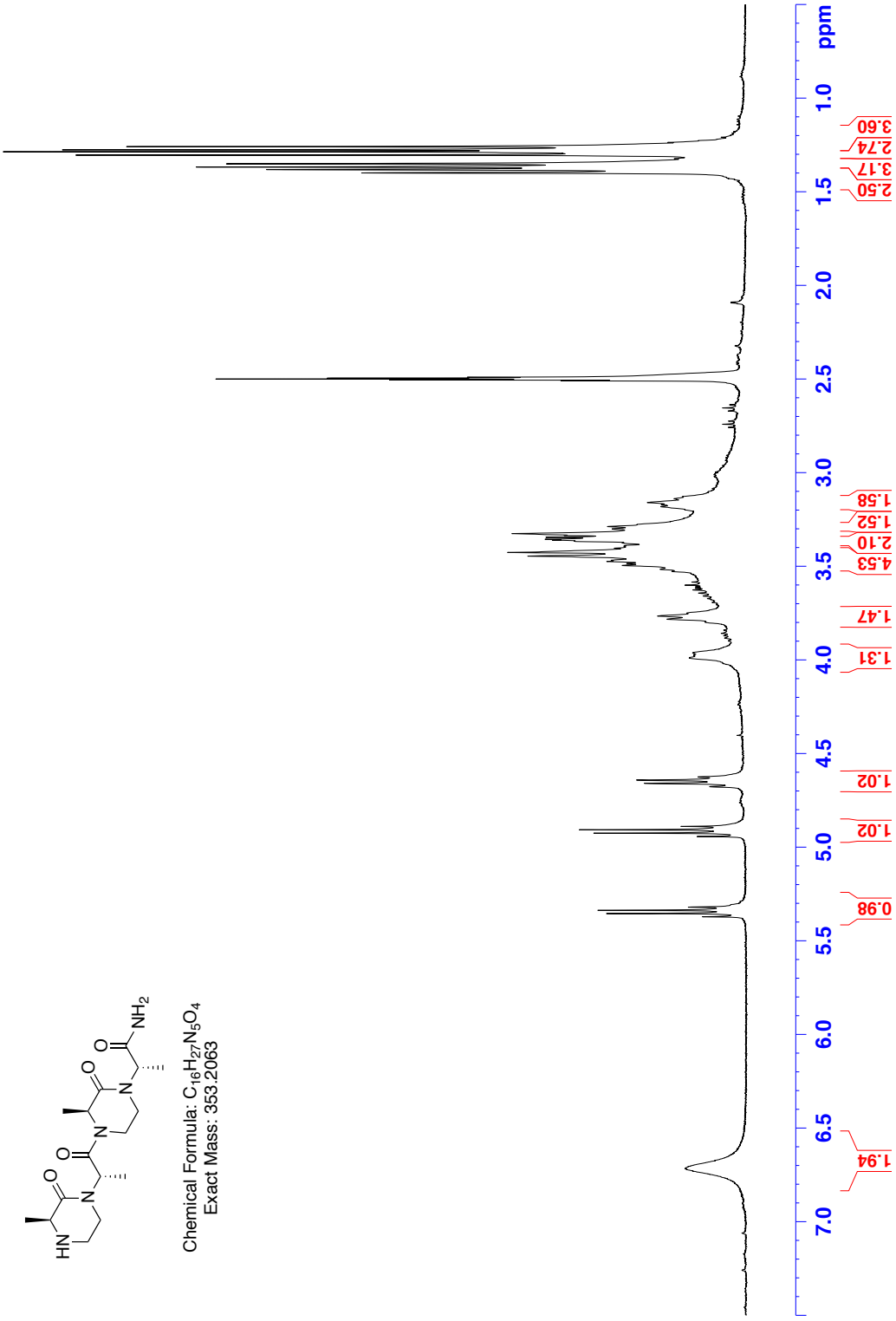
OHM 2 ^{1}H-NMR (600 MHz, d_6-DMSO, 100 °C)



OHM 3 ^{1}H-NMR (400 MHz, d_6-DMSO, 100 °C)



OHM 4 ^{1}H-NMR (400 MHz, d_6-DMSO, 100 °C)



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