SUPPLEMENTAL INFORMATION

Selective *N*-hydroxyhydantoin carbamate inhibitors of mammalian serine hydrolases

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Supplemental Figures and Tables

Table S1. (See accompanying Excel file). Representative proteomic data (Related to **Figures 3** and **4**). Data represent median SILAC ratios for all quantified tryptic peptides per protein. Extracted SH activities and complete proteomic data are shown in separate tabs and provided information includes peptide sequences and masses, charge states, and corresponding SILAC ratios. Additional datasets are available upon request.

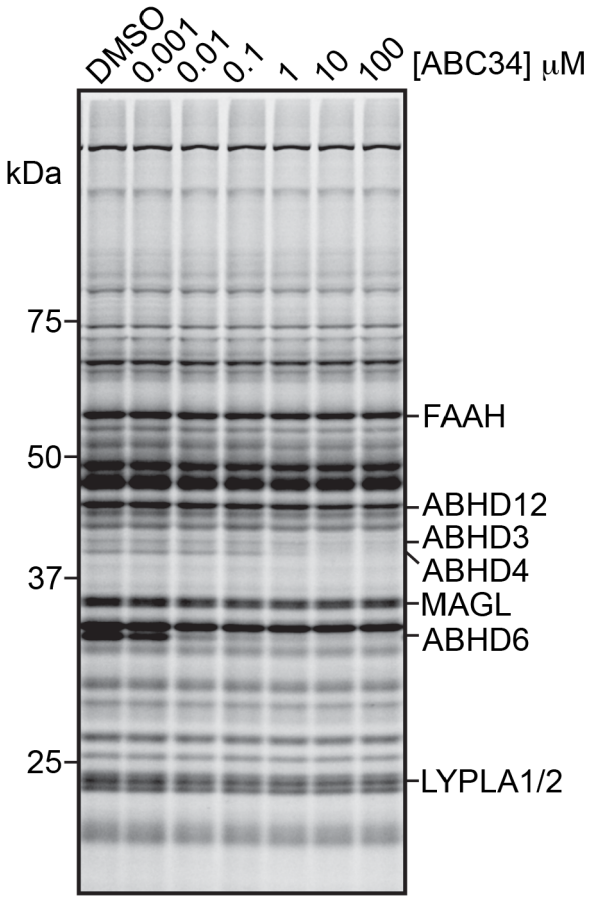


Figure S1. Concentration-dependent inhibition of mouse brain membrane SHs by NHH carbamate ABC34 (**13**) as measured by gel-based competitive ABPP (related to **Figure 1**). ABC34 (**13**) inhibited FP-Rh labeling of ABHD4 and ABHD6, but not other detected mouse brain serine hydrolases.

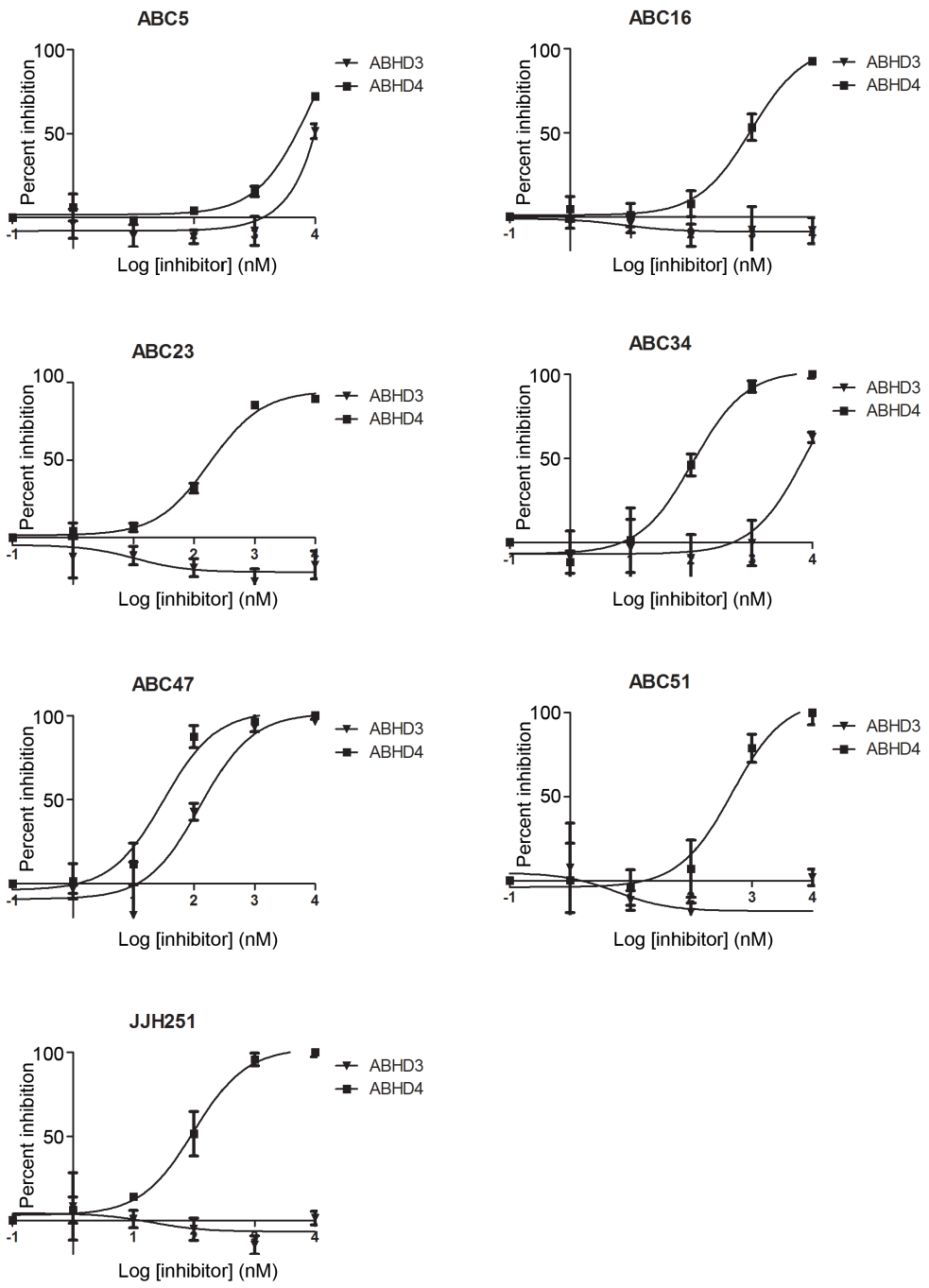
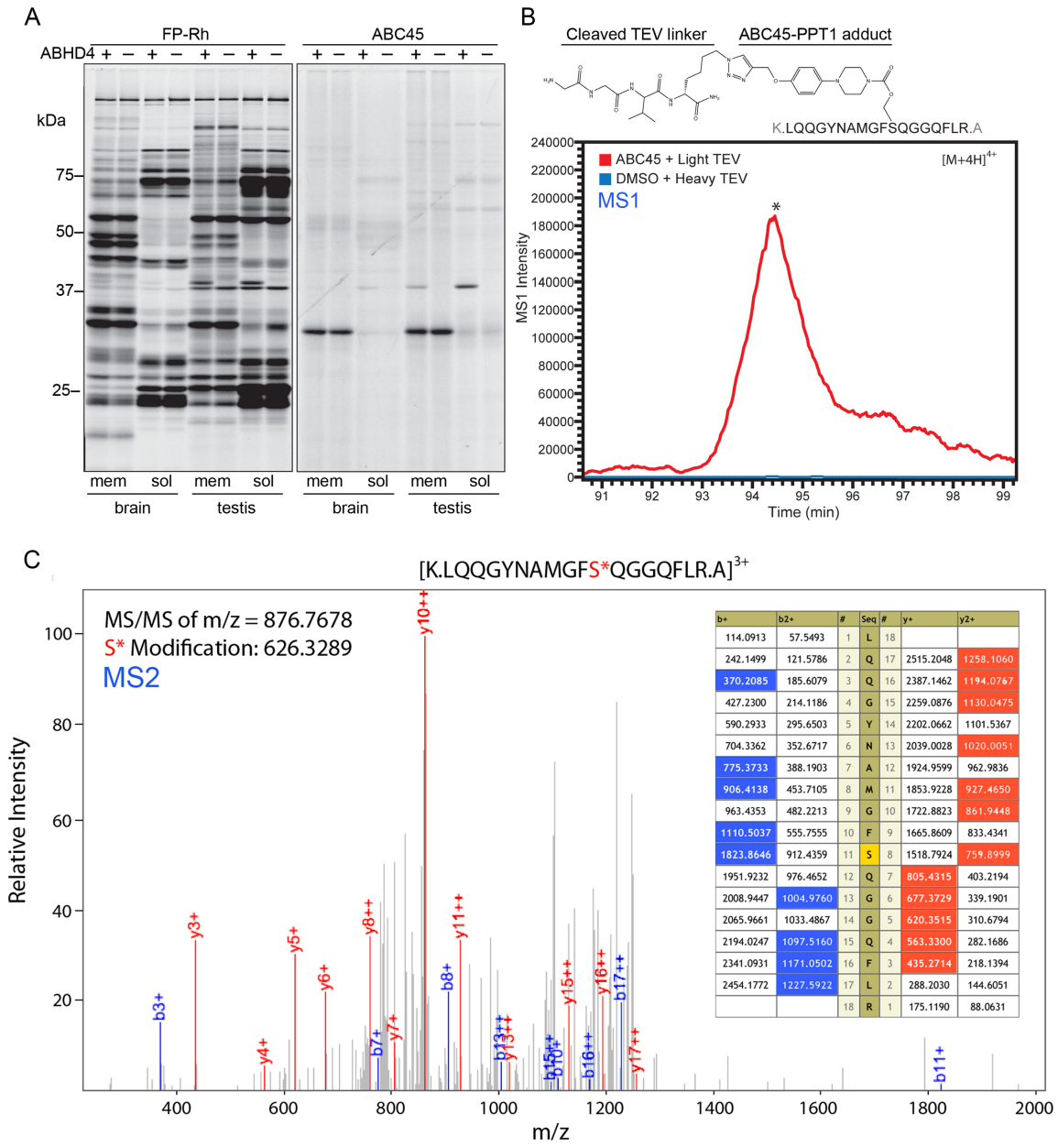


Figure S2. Concentration-dependent inhibition of ABHD3 and ABHD4 by NHH carbamates (related to **Figure 2**). Data represent competitive gel-based ABPP signals for recombinant ABHD3 and ABHD4 expressed by transient transfection in HEK293T cells. See **Figure 2C** for representative competitive gel-based ABPP data and calculated IC_{50} values. Data represent average values ± S.E. for three independent experiments.



b+	b2+	#	Seq	#	y+	y2+
114.0913	57.5493	1	L	18		
242.1499	121.5786	2	Q	17	2515.2048	1258.1060
370.2085	185.6079	3	Q	16	2387.1462	1194.0767
427.2300	214.1186	4	G	15	2259.0876	1130.0475
590.2933	295.6503	5	Y	14	2202.0662	1101.5367
704.3362	352.6717	6	N	13	2039.0028	1020.0051
775.3733	388.1903	7	A	12	1924.9599	962.9836
906.4138	453.7105	8	M	11	1853.9228	927.4650
963.4353	482.2213	9	G	10	1722.8823	861.9448
1110.5037	555.7555	10	F	9	1665.8609	833.4341
1823.8646	912.4359	11	S	8	1518.7924	759.8999
1951.9232	976.4652	12	Q	7	805.4315	403.2194
2008.9447	1004.9760	13	G	6	677.3729	339.1901
2065.9661	1033.4867	14	G	5	620.3515	310.6794
2194.0247	1097.5160	15	Q	4	563.3300	282.1686
2341.0931	1171.0502	16	F	3	435.2714	218.1394
2454.1772	1227.5922	17	L	2	288.2030	144.6051
		18	R	1	175.1190	88.0631

Figure S3. Characterization of ABHD4 and PPT1 as targets of NHH carbamates (related to **Figure 3**). (A) The clickable NHH carbamate ABC45 probe detects ABHD4 in mouse brain and testis tissues. ABHD4$^{+/+}$ and $^{-/-}$ tissues were treated with FP-Rh (1 μM, 30 min) or ABC45 (1 μM, 60 min) and then analyzed by gel-based ABPP. A 37 kDa band matching the predicted molecular mass of ABHD4 was detected by both probes in ABHD4$^{+/+}$, but not ABHD4$^{-/-}$ tissues. (B) isoTOP-ABPP experiment identifies the catalytic serine S115 as the site of ABC45 labeling in PPT1. Shown is the structure of the

ABC45-PPT1 adduct (connected by CuAAC to the azide-tag used to enrich and release ABC45-reactive peptides in isoTOP-ABPP experiments). Note that an MS1 peak corresponding to an adduct between ABC45 and the tryptic peptide containing S115 was detected in ABC45-, but not DMSO-treated proteomes from PPT1-transfected HEK293T cells. (C) Representative MS2 spectrum for the MS1 peak shown in (B) corresponding to the adduct between the tryptic PPT1 peptide containing S115 and the probe ABC45. The MS/MS fragmentation spectrum indicates that the tryptic peptide is modified by ABC45 on S115 (adduct shown in part B). These MS2 data were derived by IP2 (http://www.proteomicswiki.com/wiki/index.php/IP2).

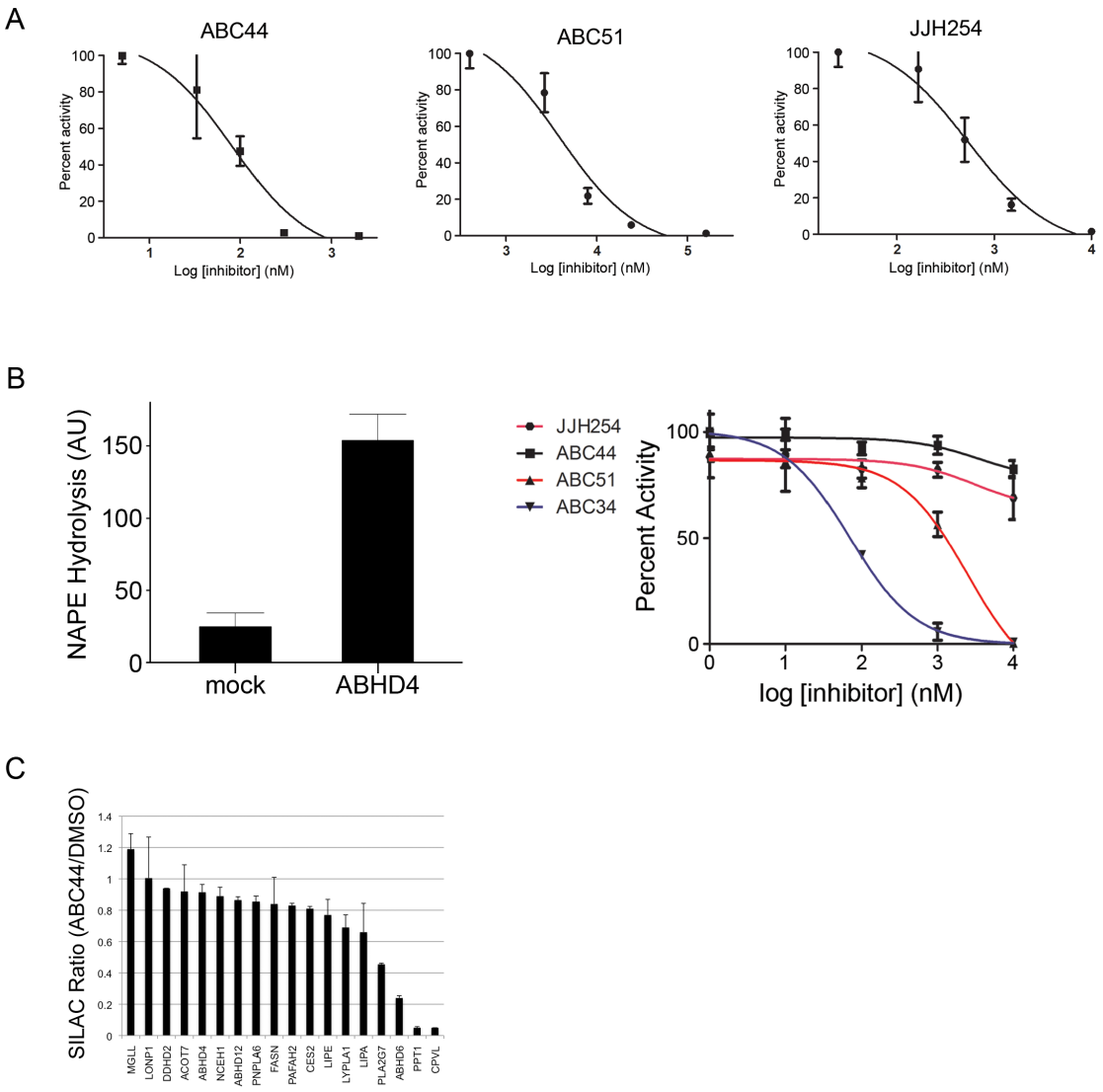
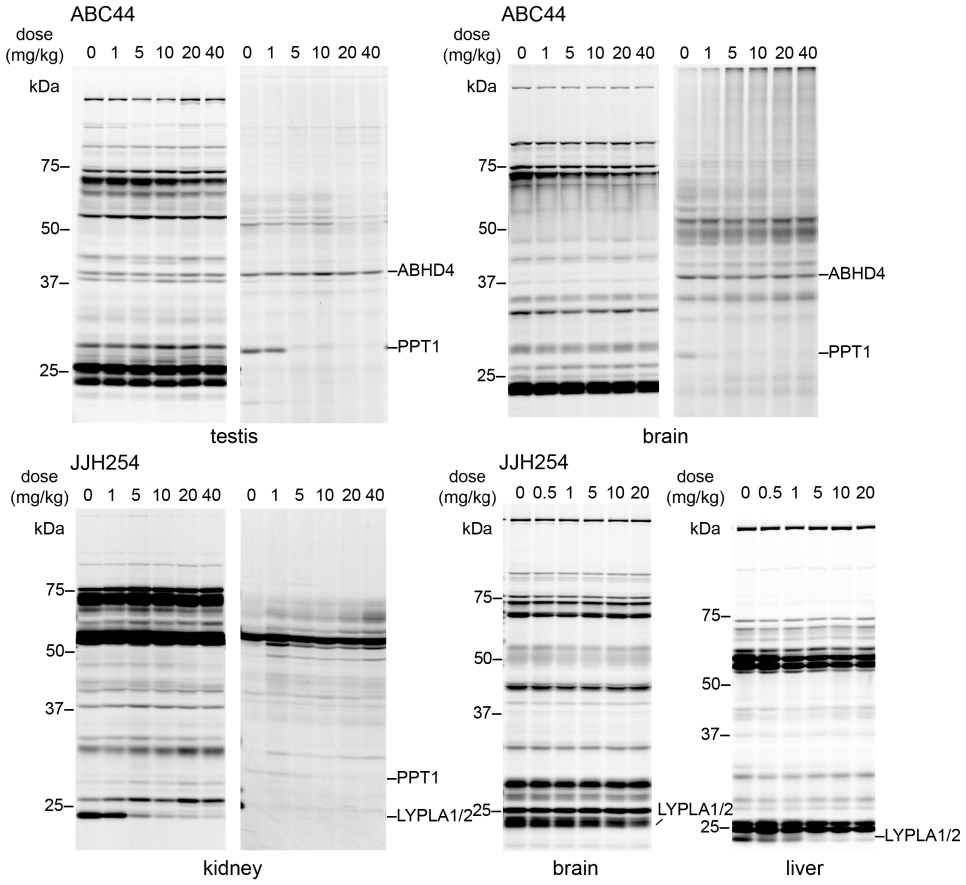


Figure S4. NHH carbamates inhibit the activity of PPT1 and ABHD4 (related to **Figure 4**). (A) Concentration-dependent *in situ* inhibition of PPT1 by NHH carbamates ABC44 (**17**), ABC51 (**14**), and JJH254 (**7**). Data represent competitive gel-based ABPP signals for recombinant PPT1 expressed by transient transfection in HEK293T cells. See **Figure 4A** for representative competitive gel-based ABPP data and **Table 1** for calculated IC_{50} values. Data represent average values ± S.E. for three independent experiments. (B) ABHD4-transfected HEK293T cell lysates show much greater NAPE lipase activity

compared to mock-transfected HEK239T cell lysates (left) and this activity is inhibited by NHH carbamates (right). NAPE hydrolysis was measured by following the release of oleic acid from the NAPE substrate (1,2-dioleoyl-sn-glycero-3-phospho (N-arachidonoyl) ethanolamine), as previously described (Lee et al., 2015). Bar graph data represent the mean ± S.D. of three independent experiments; IC_{50} data represent the mean ± S.E. for three independent experiments. (C) ABPP-SILAC analysis showing *in situ* SH inhibition profiles for ABC44 (**17**) (1 μM, 4 h) in PC3 cells where SH enrichment and inhibition were measured with ABC45 in ABC44- versus DMSO-treated cells. Data represent average values ± S.D. for two independent experiments.

A


ABC44
dose (mg/kg) 0 1 5 10 20 40
kDa
75–
50–
37–
25–
ABHD4
PPT1
testis
ABC44
dose (mg/kg) 0 1 5 10 20 40
ABHD4
PPT1
brain
JJH254
dose (mg/kg) 0 1 5 10 20 40
PPT1
LYPLA1/2
kidney
JJH254
dose (mg/kg) 0 0.5 1 5 10 20
LYPLA1/2
brain
dose (mg/kg) 0 0.5 1 5 10 20
LYPLA1/2
liver

B

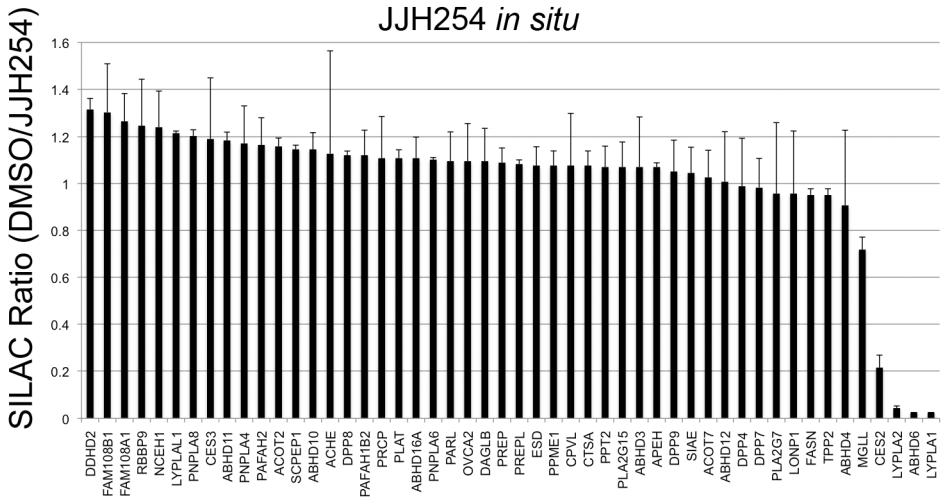

JJH254 *in situ*
SILAC Ratio (DMSO/JJH254)
1.6
1.4
1.2
1
0.8
0.6
0.4
0.2
0
DDHD2
FAM108B1
FAM108A1
RBBP9
NCEH1
LYPLAL1
PNPLA8
CES3
ABHD11
PNPLA4
PAFAH2
ACOT2
SCPEP1
ABHD10
ACHE
DPP8
PAFAH1B2
PRCP
PLAT
ABHD16A
PNPLA6
PARL
OVCA2
DAGLB
PREP
PREPL
ESD
PPME1
CPVL
CTSA
PPT2
PLA2G15
ABHD3
APEH
DPP9
SIAE
ACOT7
ABHD12
DPP4
DPP7
PLA2G7
LONP1
FASN
TPP2
ABHD4
MGLL
CES2
LYPLA2
ABHD6
LYPLA1

Figure S5. Analysis of SH reactivity profiles of NHH carbamates *in vivo* and *in situ*. (A) Full gels showing competitive ABPP results for tissues from mice treated with the indicated NHH carbamates *in vivo* (related to **Figure 5**). The left and right gels in each pair represent tissues treated with the FP-Rh (1 μM, 30 min) and ABC45 (2 μM, 60 min), respectively. Cropped gels containing key extracted information are shown in **Figure 5**. (B) ABPP-SILAC analysis showing *in situ* SH inhibition profiles for JJH254 (**7**; 1 μM, 4 h) in PC3 cells where SH enrichment and inhibition were measured with the FP-biotin probe in JJH254- versus DMSO-treated cells. Data represent average values ± S.D. for two independent experiments.

SUPPLEMENTAL EXPERIMENTAL PROCEDURES

Biological Methods

Competitive Gel-Based ABPP

In vitro and *in situ* competitive gel-based ABPP was performed as described previously (Chang et al., 2013; Hsu et al., 2012). Briefly, after *in vitro* (30 min, 37 °C) or *in situ* (described above) inhibitor or DMSO treatment, cell lysates were subjected to gel-based ABPP analysis.

In Vivo Administration of Inhibitors

Mice were injected with ABC44, ABC51, JJH254, or vehicle, as described previously (Chang et al., 2013; Hsu et al., 2012). Briefly, C57Bl/6 mice were injected intraperitoneally with an 18:1:1 (v/v/v) solution of saline/ethanol/emulphor containing the indicated concentration of compounds. After 4 h, mice were anesthetized with isoflurane, euthanized by cervical dislocation, and harvested tissues were prepared for gel-based ABPP analysis. The studies were performed with the approval of the Institutional Animal Care and Use Committee at The Scripps Research Institute in accordance with the Guide for the Care and Use of Laboratory Animals.

IsoTOP Site-of-Labeling of PPT1 by ABC45

Whole cell lysates (500 µL, 1.5 mg/mL) of hPPT1 transfected HEK239Ts were processed for MS analysis using the previously described isoTOP-ABPP protocol (Weerapana et al., 2010). In brief, for analysis of ABC45-modified peptides, proteomes were split into two fractions, to which ABC45 (5 µM, 60 min) or DMSO was added. The lysates were then subjected to Click Chemistry-ABPP conditions with either light (ABC45 treated samples) or heavy (DMSO treated samples) isotopically labeled TEV-tags. Light

and heavy-tagged proteomes were then combined, and, following enrichment of probe-labeled targets using streptavidin beads, proteins were digested on-bead with trypsin and the remaining immobilized peptides were subsequently released with TEV protease. The resulting probe-modified peptides were pressure loaded onto a 100 μm (inner diameter) fused silica capillary column with a 5 μm tip containing 10 cm C18 resin (5 μm, Phenomenex), eluted with a 180 min gradient from 0% to 100% Buffer B (Buffer A: 5% acetonitrile, 95% water, 0.1% formic acid; Buffer B: 80% acetonitrile, 20% water, 0.1% formic acid), and collected for MS analysis on an LTQ-Orbitrap. Samples were then searched as described above. The mass of the modification used to search for probe-modified peptides was +626.3289 m/z for the probe adduct plus the light TEV-tag and +632.3427 m/z for the heavy counterpart. MS1 peaks for probe-labeled quantified peptides were extracted with Xcalibur Qual Browser 2.2 (Thermo Scientific).

Transient Overexpression of Proteins

HEK293T cells were seeded at 2 x 10^5 per well on 6 well plates and grown for 48 h. They were then transfected with mouse *ABHD3* (pCMV-Sport6), human *ABHD4* (pcDNA3.1/myc-his), human *PPT1* (pCMV6-XL5), or human *PPT1*-S115A (pCMV6-XL5) cDNA-containing vectors, by incubating 0.5 μg of DNA with 3 μL of PEI MAX (1 mg/mL, Polysciences, Inc.) in serum-free DMEM for 30 min, then adding them to cells. After 48 h cells were either treated with compound *in situ*, as described above, or harvested for *in vitro* studies. The S110A PPT1 mutation was introduced using QuikChange II Site Directed Mutagenesis Kit (Agilent) with the following primers:

hPPT1 S->A QC Forward: aattggcctccctgggcgaatcccatagcattg

hPPT1 S->A QC Reverse: caatgctatgggattcgcccagggaggccaatt

Western Blotting

Following SDS-PAGE, PPT1 samples were transferred to nitrocellulose for 2 h at 50 V. Transfers were then washed, blocked with 5% milk, and incubated overnight with an anti-PPT1 antibody (Abcam ab89022, 1:2000 in TBS-T with 5% milk). Transfers were then washed with TBS-T (3x) and incubated with a secondary antibody (Odyssey 926-23310, 1:5000 in TBS-T with 5% milk) for 2 h. Transfers were then washed again (3x, TBS-T) and imaged on a Li-cor Odyssey (Model 9120).

PPT1 Substrate Assay

PPT1 activity was assessed *in vitro* using a substrate assay adapted from previous studies (van Diggelen et al., 1999). Lysates from HEK293T cells transfected with an empty vector, or human PPT1 under a CMV promoter were adjusted to 1 mg/mL in PBS (25 µl), and then combined 1:1 with McIlvain's phosphate/citrate buffer containing 0.375% (v/v) Triton-X 100 and 15 mM DTT (pH 5.0). This sample was pre-treated with inhibitor or DMSO for 30 min at 37 ^{o}C, and 10 µl (5 µg protein) was used in the substrate assay as follows: Samples were diluted in a black 96-well plate (half-area) with 20 µl McIlvain's phosphate/citrate buffer (pH 5.0) containing 0.375% TX-100 and 15 mM DTT, as well as 0.64 mM MU-6S-Palm-bD-Glc and 0.1 U sweet almond glucosidase (Sigma). The reactions were incubated at 37 ^{o}C for 1h, quenched with 120 µl sodium bicarbonate buffer (pH 10.0), and hydrolysis by 4-MU fluorescence measured by excitement at 380 nM and emission intensity at 460 nM. 16 µg protein was used for analysis of native PC3 cells.

Cell Viability Assay

The cytotoxicity of ABC44 and ABC51 against HeLa cells was assayed as previously described (Hulce et al., 2014). Briefly, HeLa cells were grown to 80% confluency in DMEM medium supplemented with 10% FCS and 1X Pen-Strep-Glutamine in a 96-well

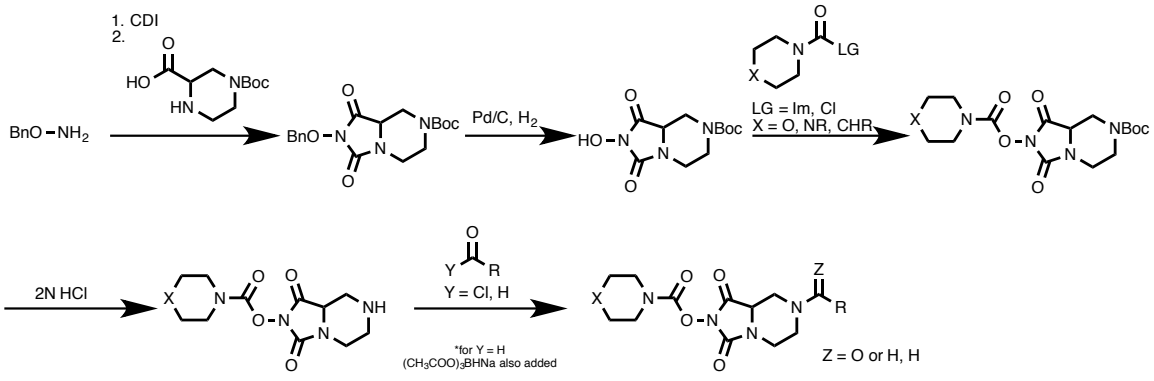
plate. Media was then removed and either DMSO (2 µL) or inhibitors (10, 100, 1000, 10000, or 100000 nM final concentration, 2 µL in DMSO) were incubated with the cells for 48 hours, in both serum-containing and serum-free DMEM (100 µL). Cell viability was then determined using the WST-1 assay (Roche).

Chemical Synthesis

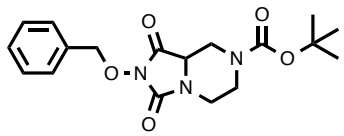
Materials

Wherever available, chemical building blocks such as acyl imidazoles and acyl chlorides were purchased from reputable chemical vendors. The 2-carboxy-4-boc-piperazine used for the generation of the core bicycle common to all eNHH carbamates examined except MJN193 was obtained from Santa Cruz Biotechnology. Carbonyl diimidazole and O-benzylhydroxylamine were obtained from VWR. ^{1}H NMR Spectra were obtained on a Varian 400 mHz or a Bruker 500 mHz. Coupling constants (*J*) are reported in hertz. HRMS service was performed by the TSRI Center for Mass Spectrometry. All compounds were prepared as racemates.

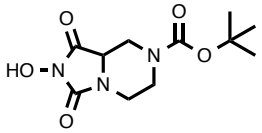
General Synthetic Scheme



Syntheses

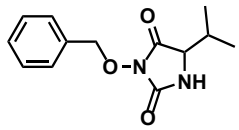


tert-butyl 2-(benzyloxy)-1,3-dioxohexahydroimidazo[1,5-a]pyrazine-7(1H)-carboxylate: To a stirred 0 ^{o}C solution of O-benzylhydroxylamine (1.73 g, 10.8 mmol, 1 eq) and N-methylmorpholine (1.31 mL, 1.1 eq) in CH_2Cl_2 (55 mL) was added carbonyldiimidazole (1.76 g, 1 eq). The resulting solution was stirred to room temperature for 2 h. At this time, 4-Boc-piperazine-2-carboxylic acid (2.0 g, 8.67 mmol, 0.8 eq) was added all at once, followed by additional NMM (1.91 mL, 1.6 eq). The resulting suspension was stirred for 24 h at room temperature, at which time moisture was notably generated in the reaction vessel; sodium sulfate was added, followed by additional CH_2Cl_2 (110 mL), and the mixture was stirred for an additional 24 h. The suspension was then filtered, and concentrated, and the residue separated by flash chromatography on SiO_2 to yield the bicyclic protected hydantoin (2.08 g, 66%) as a white solid. ^{1}H NMR (CDCl3, 400 MHz) δ 7.50 (m, 2H), 7.39 (m, 3H), 5.17 (s, 2H), 4.43 (m, 1H), 4.14 (m, 1H), 4.01 (dd, *J* = 13.6 Hz, 3.6 Hz, 1H), 3.86 (m, 1H), 2.96 (m, 1H), 2.64 (m, 1H), 2.47 (m, 1H), 1.46 (s, 9H). HRMS calculated for $C_{18}H_{23}N_3O_5Na$ $[M+Na]^+$ 384.1530, found 384.1534.



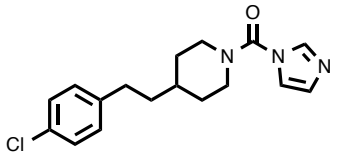
tert-butyl 2-hydroxy-1,3-dioxohexahydroimidazo[1,5-a]pyrazine-7(1H)-carboxylate: The bis-protected N-hydroxyhydantoin (300 mg, 0.831 mmol, 1 eq) was dissolved in EtOAc (4 mL), and palladium on carbon (10% w/w) (85 mg, ~10 mol%) was added. The

suspension was sparged with hydrogen gas, and then stirred under an atmosphere of hydrogen at ambient pressure overnight. At this time, the mixture was filtered over celite and concentrated *in vacuo*, quantitatively yielding the free extended N-hydroxyhydantoin as a crystalline white solid that was used without further purification. ^{1}H NMR (CDCl3, 400 MHz) δ 4.56 (m, 1H), 4.13 (m, 2H), 4.04 (m, 2H), 3.06 (m, 1H), 2.78 (m, 2H), 1.46 (s, 9H). HRMS calculated for $C_{11}H_{17}N_3O_5Na$ $[M+Na]^+$ 294.1060, found 294.1064.

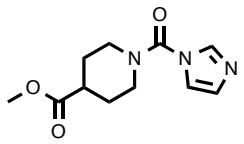


3-(benzyloxy)-5-isopropylimidazolidine-2,4-dione: To a stirring solution of *N*-Boc-L-valine (337 mg, 1.55 mmol, 1 eq), *O*-benzylhydroxylamine hydrochloride (272 mg, 1.1 eq) and *N*-methylmorpholine (171 µL, 1 eq) in anhydrous CH_2Cl_2 (10 mL) under nitrogen was added EDCI (327 mg, 1.1 eq) at 0 ºC. After stirring for 1 h, the reaction was allowed to warm to room temperature and stirred for an additional 4 h. The reaction was quenched with ice cold 5% aqueous HCl (10 mL) and the product was extracted with CH_2Cl_2 (3 x 25 mL). The combined organic layers were washed with brine (2 x 25 mL), dried over anhydrous Na_2SO_4 and concentrated under reduced pressure to provide the crude product which was deprotected upon addition of HCl (5 mL, 4 N in 1,4-dioxane). After 1 h at room temperature, the deprotected amino amide was concentrated under a stream of N_2 and then under reduced pressure. The remaining residue was dissolved in anhydrous CH_2Cl_2 (50 mL) and to this solution was added NMM (341 µL, 2.0 eq) followed by 1,1'-carbonyldiimidazole (277 mg, 1.1 eq) at room temperature. After stirring the reaction mixture overnight, the reaction was poured into a separatory funnel containing ice cold 5% aqueous HCl (50 mL) and the product was extracted with CH_2Cl_2 (3 x 50 mL). The combined organic layers dried over anhydrous Na_2SO_4 and

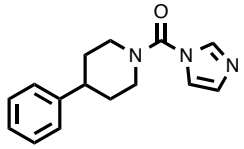
concentrated under reduced pressure. The remaining residue was purified by SiO_2 flash chromatography (30% EtOAc/hexanes) to provide the title compound (327 mg, 85%) as a white solid: ^{1}H NMR (600 MHz, $CDCl_3$) δ 7.50 (m, 2H), 7.39 (m, 3H), 6.87 (s, 1H), 5.15 (m, 2H), 3.85 (m, 1H), 2.20 (m, 1H), 0.98 (d, *J* = 6.99 Hz, 3H), 0.82 (d, *J* = 6.85 Hz, 3H); ^{13}C NMR (151 MHz, $CDCl_3$) δ 168.71, 155.62, 134.26, 130.75, 130.22, 129.36, 80.13, 61.23, 30.94, 19.17, 16.77; MS calculated for $[M+H]^+$ $C_{13}H_{17}N_2O_3$: 249.1, found 249.5.



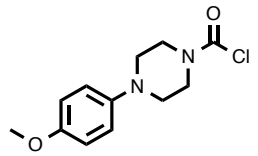
(4-(4-chlorophenethyl)piperidin-1-yl)(1*H*-imidazol-1-yl)methanone: 4-chlorophenethylpiperidine hydrochloride (520 mg, 2 mmol, 1 eq) was suspended in 10 mL methylene chloride, triethylamine (404 mg, ~2 eq) was added, and the resulting solution was cooled to 0 ^{o}C. At this time, carbonyldiimidazole (389 mg, 1.2 eq) was added all at once. The resulting mixture was stirred to room temperature over night. At this time, the mixture was concentrated to a residue, dissolved in EtOAc (with 5% MeOH v/v) and transferred to a separatory funnel, washed successively with water, 0.2 N aq. LiOH, and water again, dried (Na_2SO_4) and concentrated *in vacuo* to yield the imidazole urea as an off-white solid (439 mg, 69%) that was used without further purification.^{1}H NMR (CDCl3, 400 MHz) δ 7.85 (s, 1H), 7.25 (d, *J* = 8 Hz, 2H), 7.19 (s, 1H), 7.10 (d, *J* = 8 Hz, 2H), 7.09 (s, 1H), 4.11 (d, *J* = 13.2 Hz, 2H), 3.00 (m, 2H), 2.63 (t, *J* = 7.6 Hz, 2H), 1.86 (d, *J* = 12.4 Hz, 2H), 1.61 (m, 2H), 1.58 (m, 1H), 1.28 (m, 2H). HRMS calculated for $C_{17}H_{20}ClN_3O$ $[M+H]^+$ 318.1368, found 318.1368.



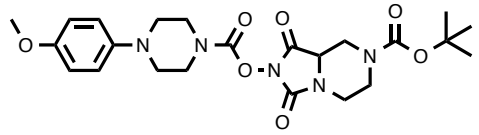
methyl 1-(1H-imidazole-1-carbonyl)piperidine-4-carboxylate: 4-carboxymethyl piperidine hydrochloride (358 mg, 2 mmol, 1 eq) was suspended in 8 mL methylene chloride, triethylamine was added (606 mg, 6 mmol, 3 eq), and the solution was cooled to 0 ^{o}C. Carbonyldiimidazole (405 mg, 1.25 eq) was added all at once, and the solution was stirred to room temperature overnight. The resulting mixture was concentrated under reduced pressure, and separated by flash chromatography over SiO_2 in 50% acetone/hexanes to yield the imidazole urea as an off-white solid (255 mg, 54%). ^{1}H NMR (CDCl3, 400 MHz) δ 7.89 (s, 1H), 7.20 (s, 1H), 7.11 (s, 1H), 4.04 (m, 2H), 3.99 (s, 3H), 3.24 (m, 2H), 2.66 (m, 1H), 2.04 (m, 2H), 1.84 (m, 2H). HRMS calculated for $C_{11}H_{16}N_3O_3$ $[M+H]^+$ 238.1186, found 238.1185.



(1H-imidazol-1-yl)(4-phenylpiperidin-1-yl)methanone: 4-phenylpiperidine (50 mg, 0.31 mmol, 1 eq) was dissolved in 1.56 mL THF, and triethylamine (63 mg, 2 eq) and carbonyldiimidazole (60 mg, 1.2 eq) added in order at room temperature. The resulting solution was stirred at room temperature overnight, and concentrated to a residue that was separated by prep-TLC (60% acetone/hexanes) to yield the imidazole urea as a crystalline white solid (19 mg, 24%). ^{1}H NMR (CDCl3, 500 MHz) δ 7.93 (s, 1H), 7.36 (m, 2H), 7.29 (s, 1H), 7.26 (m, 3H), 7.13 (s, 1H), 4.30 (m, 2H), 3.18 (m, 2H), 2.84 (m, 2H), 2.00 (m, 2H), 1.82 (m, 2H). HRMS calculated for $C_{15}H_{18}N_3O$ $[M+H]^+$ 256.1444, found 256.1445.

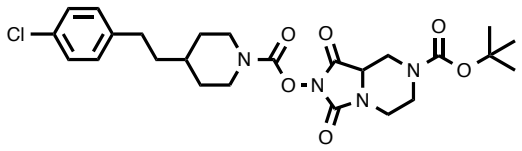


4-(4-methoxyphenyl)piperazine-1-carbonyl chloride: 4-(4-MeO-Ph)-piperazine (14.3 g, 74.1 mmol, 1 eq) was dissolved in 120 mL dry THF, and pyridine (30 mL, ~5 eq) was added, and the resulting solution was cooled to 0 ^{o}C. At this time, triphosgene (11 g, 0.5 eq) was added in small portions. The resulting mixture was allowed to warm to room temperature and was stirred overnight. The mixture was filtered, concentrated, and the residue was separated by flash chromatography over SiO2 to yield the carbamyl chloride (1.0 g, 5%) as a pale yellow solid. ^{1}H NMR (CDCl3, 400 MHz) δ 6.88 (m, 4H), 3.90 (m, 2H), 3.81 (m, 2H), 3.78 (s, 3H), 3.11 (m, 4H). HRMS calculated for $C_{12}H_{16}ClN_2O_2$ $[M+H]^+$ 255.0895, found 255.0895.

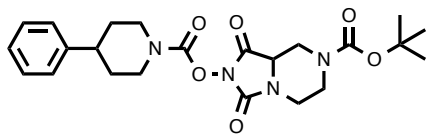


tert-butyl 2-((4-(4-methoxyphenyl)piperazine-1-carbonyl)oxy)-1,3-dioxohexahydroimidazo[1,5-a]pyrazine-7(1H)-carboxylate (MJN202; Compound 3): The free *N*-Boc-NHH (200 mg, 0.737 mmol, 1 eq) was dissolved in 3.5 mL dry THF, and triethylamine (225 mg, 3 eq) and a catalytic amount of 4-DMAP, followed by 4-MeO-Ph-piperazine carbamyl chloride (188 mg, 1 eq) at room temperature. The solution was heated at 60 ^{o}C for 2 h, filtered, and concentrated to a residue *in vacuo* that was Boc-deprotected without further purification (267 mg, 93% over two steps). A sample was separated by prep-TLC (60% EtOAc/hexanes) for analysis to yield an off-white solid. ^{1}H NMR (CDCl3, 500 MHz) δ 6.90 (m, 2H), 6.84 (m, 2H), 4.55 (m, 1H), 4.18 (m, 1H), 4.08

(m, 2H), 3.80 (m, 2H), 3.77 (s, 3H), 3.67 (m, 2H), 3.07 (m, 4H), 3.02 (m, 1H), 2.84 (m, 2H), 1.46 (s, 9H). HRMS calculated for $C_{23}H_{32}N_5O_7$ $[M+H]^+$ 490.2296, found 490.2296.

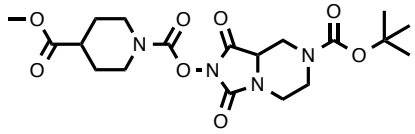


tert-butyl 2-((4-(4-chlorophenethyl)piperidine-1-carbonyl)oxy)-1,3-dioxohexahydroimidazo[1,5-a]pyrazine-7(1H)-carboxylate (JJH221; Compound 4): A vial was charged with the free *N*-Boc-NHH (22 mg, 0.081 mmol, 1 eq), *p*-chlorophenethyl-4-piperidine imidazole urea (25 mg, 1 eq), triethylamine (40 mg, 5 eq), and a catalytic amount of 4-DMAP in 0.4 mL dry THF. The mixture was heated at 70 ^oC for 6 h, cooled to room temperature and concentrated to a residue, which was separated by prep-TLC (50% EtOAc/hexanes) to yield JJH221 as a white solid (33.7 mg, 80%). ^1H NMR (CDCl3, 400 MHz) δ 7.26 (d, *J* = 8 Hz, 2H), 7.10 (d, *J* = 8 Hz, 2H), 4.56 (bs, 1H), 4.22 (m, 2H), 4.06 (m, 3H), 3.04 (m, 2H), 2.86 (m, 3H), 2.61 (t, *J* = 7.6 Hz, 2H), 1.77 (d, *J* = 12.8 Hz, 2H), 1.59 (m, 2H), 1.51 (m, 1H), 1.46 (s, 9H), 1.30 (m, 2H). HRMS calculated for $C_{25}H_{33}ClN_4O_6Na$ $[M+Na]^+$ 543.1981, found 543.1984.

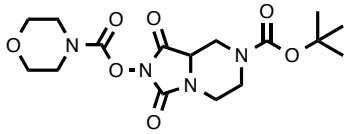


tert-butyl 1,3-dioxo-2-((4-phenylpiperidine-1-carbonyl)oxy) hexahydroimidazo[1,5-a]pyrazine-7(1H)-carboxylate (JJH321): The Boc-NHH (20 mg, 0.0737 mmol, 1 eq) was dissolved in 0.4 mL dry THF, and triethylamine (38 mg, 5 eq) and a catalytic amount of 4-DMAP were added, followed by 4-phenylpiperidine imidazole urea (19 mg, 1 eq) at room temperature. The solution was heated at 70 ^oC for 5 h, and

concentrated to a residue that was separated by prep-TLC (50% EtOAc/hexanes) to yield JJH321 as a white solid (27.2 mg, 81%). ^{1}H NMR (CDCl3, 400 MHz) δ 7.34 (m, 2H), 7.25 (m, 1H), 7.22 (m, 2H), 4.57 (m, 1H), 4.34 (m, 2H), 4.22 (m, 1H), 4.11 (m, 2H), 3.14 (m, 1H), 3.08 (m, 1H), 3.02 (m, 1H), 2.86 (m, 2H), 2.72 (m, 1H), 1.94 (m, 2H), 1.82 (m, 2H), 1.49 (s, 9H). HRMS calculated for $C_{23}H_{30}N_4O_6Na$ $[M+Na]^+$ 481.2057, found 481.2056.

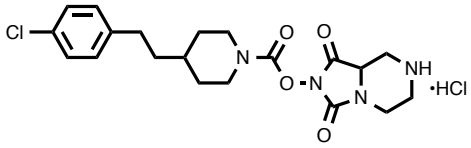


1-(7-(tert-butoxycarbonyl)-1,3-dioxohexahydroimidazo[1,5-a]pyrazin-2(3H)-yl) 4-methyl piperidine-1,4-dicarboxylate (JJH238): The Boc-NHH (7 mg, 0.026 mmol, 1.1 eq) was dissolved in 0.24 mL dry THF, and triethylamine (12 mg, 5 eq) and a catalytic amount of 4-DMAP were added, followed by 4-carboxymethylpiperidine imidazole urea (5.4 mg, 1 eq) at room temperature. The solution was heated at 70 ^{o}C overnight, and concentrated to a residue that was separated by prep-TLC (75% EtOAc/hexanes) to yield JJH238 as a white solid (8.3 mg, 73%). ^{1}H NMR (CDCl3, 400 MHz) δ 4.56 (m, 1H), 4.17 (m, 1H), 4.07 (m, 3H), 3.98 (m, 1H), 3.71 (s, 3H), 3.25 (m, 1H), 3.08 (m, 2 H), 2.84 (m, 2H), 2.54 (m, 1H), 2.01 (m, 2H), 1.82 (m, 2H), 1.49 (s, 9H). HRMS calculated for $C_{19}H_{28}N_4O_8Na$ $[M+Na]^+$ 463.1799, found 463.1799.

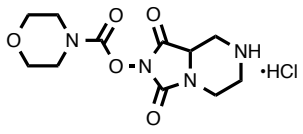


7**-(tert-butoxycarbonyl)-1,3-dioxohexahydroimidazo[1,5-a]pyrazin-2(3H)-yl morpholine-4-carboxylate (JJH253):** The Boc-NHH (29.4 mg, 0.108 mmol, 1 eq) was

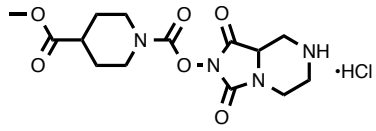
dissolved in 0.5 mL dry methylene chloride, and diisopropylethylamine (45 µL, 3 eq) and a catalytic amount of 4-DMAP were added, followed by 4-morpholine carbamyl chloride (30 µl, ~2 eq). The resulting solution was sealed and heated at 50 °C for 1 h, concentrated to a residue, and separated by prep-TLC (80% EtOAc/hexanes) to yield JJH253 as a white solid (33 mg, 79%). ^{1}H NMR (CDCl3, 500 MHz) δ 4.54 (m, 1H), 4.17 (m, 1H), 4.07 (m, 2H), 3.74 (m, 4H), 3.65 (m, 2H), 3.51 (m, 2H), 3.04 (m, 1H), 2.83 (m, 2H), 1.48 (s, 9H). HRMS calculated for $C_{16}H_{25}N_4O_7$ $[M+H]^+$ 385.1718, found 385.1710.



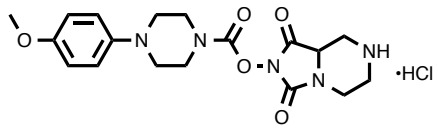
1,3-dioxohexahydroimidazo[1,5-a]pyrazin-2(3H)-yl 4-(4-chloro-phenethyl)piperidine-1-carboxylate hydrochloride (JJH222): The Boc-protected NHH-carbamate JJH221 was dissolved in 2 N methanolic HCl (10 mL) at 0 °C, and stirred to room temperature for 30 minutes. The solvent was removed *in vacuo*, and the resulting hydrochloride salt was used without further purification. An analytical sample was prepared by recrystallization of the salt from methanol and ether, yielding white needles. ^{1}H NMR (CDCl3, 400 MHz) δ 7.25 (d, *J* = 8 Hz, 2H), 7.09 (d, *J* = 8 Hz, 2H), 4.85 (d, *J* = 10.8 Hz, 1H), 4.28 (d, *J* = 14.4 Hz, 1H), 4.18 (d, *J* = 13.6 Hz, 1H), 4.05 (m, 2H), 3.68 (m, 2H), 3.02 (m, 3 H), 2.63 (m, 1H) 1.45-1.81 (m, 9H), 1.33 (m, 1H). HRMS calculated for $C_{20}H_{26}ClN_4O_4$ $[M+H]^+$ 421.1637, found 421.1640.



1,3-dioxohexahydroimidazo[1,5-a]pyrazin-2(3H)-yl morpholine-4-carboxylate hydrochloride (JJH256): JJH253 was Boc-deprotected using methanolic HCl as described for JJH222, yielding a white solid, which was used without further purification. ^{1}H NMR (D_2O, 500 MHz) δ 4.85 (m, 1H), 4.36 (m, 1H), 3.98 (m, 1H), 3.85 (m, 4H), 3.73 (m, 2H), 3.65 (m, 1H), 3.58 (m, 2H), 3.52 (m, 1H), 3.36 (m, 1H), 3.25 (m, 1H). HRMS calculated for $C_{11}H_{17}N_4O_5$ $[M+H]^+$ 285.1193, found 285.1193.

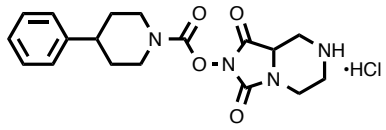


1-(1,3-dioxohexahydroimidazo[1,5-a]pyrazin-2(3H)-yl) 4-methyl piperidine-1,4-dicarboxylate hydrochloride (JJH257): JJH238 was Boc deprotected using methanolic HCl as described for JJH222, yielding a white solid, which was used without further purification. ^{1}H NMR (CDCl3, 400 MHz) δ 4.94 (m, 1H), 4.28 (m, 1H), 4.06 (m, 2H), 3.93 (m, 2H), 3.71 (s, 3H), 3.22 (m, 2 H), 3.10 (m, 2H), 2.56 (m, 2H), 1.99 (m, 2H), 1.81 (m, 2H). HRMS calculated for $C_{14}H_{21}N_4O_6$ $[M+H]^+$ 341.1456, found 341.1455.

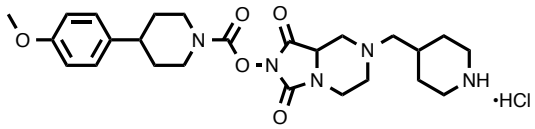


1,3-dioxohexahydroimidazo[1,5-a]pyrazin-2(3H)-yl 4-(4-methoxyphenyl)piperazine-1-carboxylate hydrochloride (JJH331): MJN202 was Boc-deprotected using methanolic HCl as described for JJH222, yielding a white solid, after recrystallization from MeOH/ether (267 mg, 93%). ^{1}H NMR (D_2O, 400 MHz) δ 7.47 (d, *J* = 6.8 Hz, 2H), 7.14 (d, *J* = 6.8 Hz, 2H), 4.85 (m, 1H), 4.37 (m, 1H), 4.09 (m, 2H), 3.98 (m, 1H), 3.94 (m,

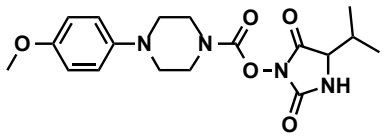
2H), 3.88 (s, 3H), 3.68 (m, 1H), 3.66 (m, 4H), 3.56 (m, 1H), 3.41 (m, 1H), 3.29 (m, 1H). HRMS calculated for $C_{18}H_{24}N_5O_5$ $[M+H]^+$ 390.1772, found 390.1772.



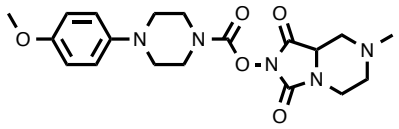
1,3-dioxohexahydroimidazo[1,5-a]pyrazin-2(3H)-yl 4-phenylpiperidine-1-carboxylate hydrochloride (JJH322): JJH321 was Boc-deprotected using methanolic HCl as described for JJH222, yielding a white solid, which was used without further purification. ^{1}H NMR (CDCl3, 400 MHz) δ 7.32 (m, 2H), 7.25 (m, 1H), 7.22 (m, 2H), 4.89 (m, 1H), 4.33 (m, 2H), 4.21 (m, 1H), 3.67 (m, 1H), 3.13 (m, 2H), 3.01 (m, 2H), 2.72 (m, 1H), 1.94 (m, 2H), 1.79 (m, 2H), 1.64 (m, 2H). HRMS calculated for $C_{18}H_{23}N_4O_4$ $[M+H]^+$ 359.1714, found 359.1715.



1,3-dioxo-7-(piperidin-4-ylmethyl)hexahydroimidazo[1,5-a]pyrazin-2(3H)-yl 4-(4-methoxyphenyl)piperidine-1-carboxylate hydrochloride (ABC38; Compound 15): ABC37 was Boc-deprotected using methanolic HCl as described for JJH222, yielding a yellow solid, which was used without further purification. ^{1}H NMR (CD_3OD, 500 MHz) δ 7.80 (m, 2 H), 7.22 (m, 2H), 5.15 (m, 1H), 4.41 (m, 1H), 4.28 (m, 2H), 4.19 (m, 3H), 3.94 (s, 3H), 3.90 (m, 4H), 3.86 (m, 2H), 3.54 (m, 2H), 3.38 (m, 3H), 3.14 (m, 3H), 2.48 (m, 1H), 2.33 (m, 2H), 1.66 (m, 2H). HRMS calculated for $C_{24}H_{35}N_6O_5$ $[M+H]^+$ 487.2663, found 487.2664.

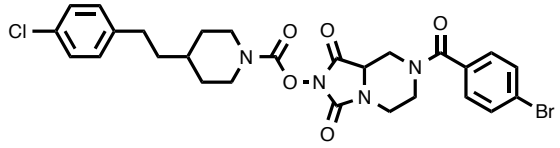


4-isopropyl-2,5-dioxoimidazolidin-1-yl-4-(4-methoxyphenyl) piperazine-1-carboxylate (MJN193; Compound 1): In a vial fitted with a rubber septa, 3-(benzyloxy)-5-isopropylimidazolidine-2,4-dione (99 mg, 0.40 mmol, 2 eq) was dissolved in a 1:4 mixture of EtOAc:MeOH (5 mL) under N_2. To this solution was added 10% Pd/C (10 mg) and the vial was purged with H_2. The reaction mixture was stirred under H_2 (1 atm, balloon) until the starting material had been completely consumed as judged by TLC (~ 2 h). The reaction vial was then purged with N_2, and the reaction mixture was filtered through Celite and concentrated under reduced pressure. The remaining residue was redissolved in anhydrous CH_2Cl_2 (5 mL) and to this solution was added 4-(4-methoxyphenyl)piperazine-1-carbonyl chloride (50 mg, 1 eq) and catalytic DMAP. The reaction was stirred overnight at room temperature and then concentrated under a stream of N_2 to a residue that was separated by prep-TLC (50% EtOAc/hexanes) yielding MJN193 as a white solid (45 mg, 60%). ^{1}H NMR (600 MHz, $CDCl_3$) δ 6.91 (m, 2H), 6.86 (m, 2H), 6.58 (s, 1H), 4.06 (m, 1H), 3.79 (m, 2H), 3.77 (s, 3H), 3.67 (m, 2H), 3.10 (m, 4H), 2.28 (s, 1H), 1.08 (d, *J* = 6.95 Hz, 3H), 1.02 (d, *J* = 5.37 Hz, 3H); HRMS calculated for $C_{18}H_{25}N_4O_5$ $[M+H]^+$ 377.1819, found 377.1838.

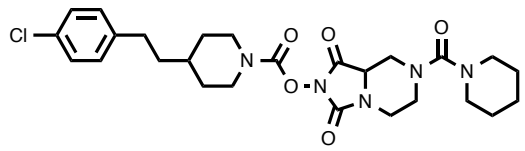


7-methyl-1,3-dioxohexahydroimidazo[1,5-a]pyrazin-2(3H)-yl 4-(4-methoxyphenyl)piperazine-1-carboxylate (MJN200; Compound 2): JJH331 (hydrochloride; 20 mg, 0.047 mmol, 1 eq) was suspended in 0.2 ml dry THF, and

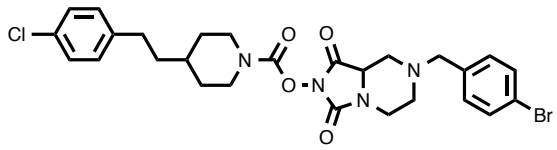
formaldehyde (6 μl, 37% wt. in water, 1.5 eq), acetic acid (2.8 μl, 1eq) and sodium triacetoxyborohydride (11 mg, 1.1 eq) were added at room temperature. The resulting suspension was stirred at room temperature overnight, concentrated to a residue, and separated by prep-TLC (EtOAc + 1% Et_3N) to yield MJN200 as an off-white solid (12 mg, 63%). ^{1}H NMR (CDCl3, 400 MHz) δ 6.90 (m, 2H), 6.85 (m, 2H), 4.18 (m, 1H), 4.06 (m, 1H), 3.80 (m, 2H), 3.77 (s, 3H), 3.67 (m, 2H), 3.23 (m, 1H), 3.17 (m, 1H), 3.14 (m, 4H), 2.80 (m, 1H), 2.37 (s, 3H), 2.08 (m, 2H). HRMS calculated for $C_{19}H_{26}N_5O_5$ $[M+H]^+$ 404.1928, found 404.1930.



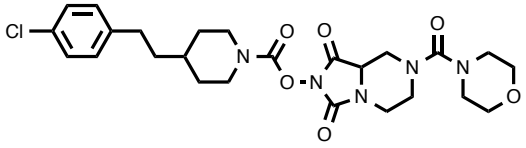
7-(4-bromobenzoyl)-1,3-dioxohexahydroimidazo[1,5-a]pyrazin-2(3H)-yl 4-(4-chlorophenethyl)piperidine-1-carboxylate (JJH248; Compound 5): JJH222 (hydrochloride; 20 mg, 0.042 mmol, 1 eq) was suspended in 0.3 mL dry THF, and triethylamine (13 mg, 3 eq), 4-DMAP (catalytic) and 4-bromo-benzoyl chloride (11 mg, 1.2 eq) were added at room temperature. The mixture was stirred at 60 ^{o}C for 2 h, cooled to room temperature, filtered, concentrated, and separated by prep-TLC (25 mg, 99%). ^{1}H NMR (CDCl3, 400 MHz) δ 7.62 (d, *J* = 8 Hz, 2H), 7.32 (d, *J* = 8 Hz, 2H), 7.24 (d, *J* = 8 Hz, 2H), 7.10 (d, *J* = 8 Hz, 2H), 4.15 (m, 5H), 2.95 (m, 6H), 2.61 (t, *J* = 8 Hz, 2H), 1.78 (d, 12 Hz, 2H), 1.59 (m, 2H), 1.32 (m, 1H), 1.26 (m, 2H). HRMS calculated for $C_{27}H_{28}BrClN_4O_5Na$ $[M+Na]^+$ 625.0824, found 625.0827.



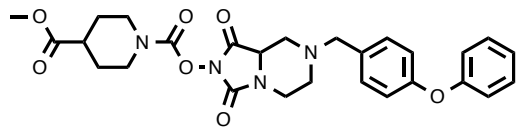
1,3-dioxo-7-(piperidine-1-carbonyl)hexahydroimidazo[1,5-a]pyrazin-2(3H)-yl 4-(4-chlorophenethyl)piperidine-1-carboxylate (JJH250; Compound 6): The title compound was prepared as described for JJH248, using piperidine carbamyl chloride in place of the benzoyl chloride providing a white solid (19 mg, 85%). ^{1}H NMR (CDCl3, 400 MHz) δ 7.26 (d, *J* = 8 Hz, 2H), 7.10 (d, *J* = 8 Hz, 2H), 4.25 (m, 2H), 4.06 (m, 3H), 3.65 (m, 1H), 3.24 (m, 4H), 3.14 (m, 1H), 2.98 (m, 4H), 2.63 (t, *J* = 8 Hz, 2H), 1.80 (m, 2H), 1.60 (m, 8H), 1.50 (m, 1H), 1.32 (m, 2H). HRMS calculated for $C_{26}H_{35}ClN_5O_5$ $[M+H]^+$ 532.2321, found 532.2322.



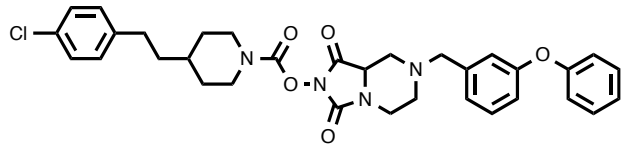
7-(4-bromobenzyl)-1,3-dioxohexahydroimidazo[1,5-a]pyrazin-2(3H)-yl 4-(4-chlorophenethyl)piperidine-1-carboxylate (JJH251; Compound 8): The title compound was prepared as described for MJN200, using JJH222 in place of JJH331, and 4-bromobenzaldehyde in place of formaldehyde providing a white solid (14 mg, 57%). ^{1}H NMR (CDCl3, 400 MHz) δ 7.62 (d, *J* = 8 Hz, 2H), 7.54 (d, *J* = 8 Hz, 2H), 7.24 (d, *J* = 8 Hz, 2H), 7.10 (d, *J* = 8 Hz, 2H), 5.14 (m, 1H), 4.23 (m, 2H), 4.11 (s, 2H), 4.05 (m, 2H), 3.72 (m, 1H), 3.41 (m, 1H), 2.95 (m, 1H), 2.87 (m, 1H), 2.72 (m, 2H), 2.61 (t, *J* = 8 Hz, 2H), 1.81 (m, 2H), 1.61 (m, 2H), 1.56 (m, 1H), 1.26 (m, 2H). HRMS calculated for $C_{27}H_{31}BrClN_4O_4$ $[M+H]^+$ 589.1212, found 589.1213.



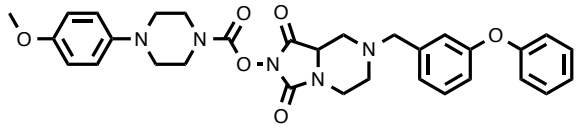
7-(morpholine-4-carbonyl)-1,3-dioxohexahydroimidazo[1,5-a]pyrazin-2(3H)-yl 4-(4-chlorophenethyl)piperidine-1-carboxylate (JJH254; Compound 7): The title compound was prepared as described for JJH248, using 4-morpholine carbamyl chloride in place of the benzoyl chloride to provide JJH254 (20 mg, 89%). ^{1}H NMR (CDCl3, 400 MHz) δ 7.25 (d, *J* = 8.4 Hz, 2H), 7.09 (d, *J* = 8.4 Hz, 2H), 4.22 (m, 2 H), 4.09 (m, 2H) (4.06, s, 1H), 3.70 (m, 4H), 3.68 (s, 1H), 3.32 (m, 4H), 3.12 (m, 1H), 2.95 (m, 2H), 2.87 (m, 1H), 2.61 (m, 2H), 1.79 (d, *J* = 13.2 Hz, 2H), 1.25-1.59 (m, 6H). HRMS calculated for $C_{25}H_{33}ClN_5O_6$ $[M+H]^+$ 534.2114, found 534.2116.



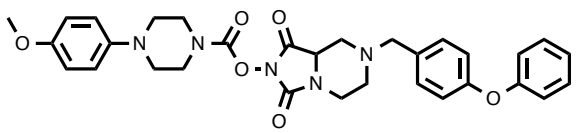
1-(1,3-dioxo-7-(4-phenoxybenzyl)hexahydroimidazo[1,5-a]pyrazin-2(3H)-yl) 4-methyl piperidine-1,4-dicarboxylate (ABC5; Compound 9): The title compound was prepared as described for MJN200, using JJH257 in place of JJH331, and 3-phenoxybenzaldehyde in place of formaldehyde, to yield ABC5 (6.8 mg, 87%) ^{1}H NMR (CDCl3, 500 MHz) δ 7.36 (m, 2H), 7.28 (m, 1H), 7.11 (m, 1H), 7.01 (m, 4H), 6.92 (m, 1H), 4.12 (m, 2H), 4.02 (m, 1H), 3.96 (m, 1H), 3.70 (s, 3H), 3.61 (d, *J* = 15 Hz, 1H), 3.53 (d, *J* = 15 Hz, 1H), 3.25 (m, 2H), 3.12 (m, 2H), 2.84 (m, 1H), 2.52 (m, 1H), 2.15 (m, 2H), 1.99 (m, 2H), 1.81 (m, 2H). HRMS calculated for $C_{27}H_{31}N_4O_7$ $[M+H]^+$ 523.2187, found 523.2187.



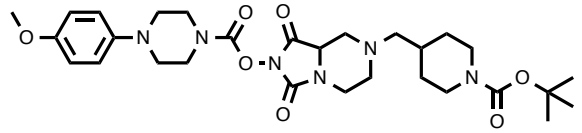
1,3-dioxo-7-(3-phenoxybenzyl)hexahydroimidazo[1,5-a]pyrazin-2(3H)-yl 4-(4-chlorophenethyl)piperidine-1-carboxylate (ABC16; Compound 11): The title compound was prepared as described for JJH251, using 3-phenoxybenzaldehyde in place of formaldehyde to yield ABC16 (22 mg, 69%). ^{1}H NMR (CDCl3, 500 MHz) δ 7.36 (m, 2H), 7.28 (m, 3H), 7.11 (m, 3H), 7.00 (m, 4H), 6.92 (m, 1H), 4.21 (m, 1H), 4.12 (m, 2H), 4.02 (m, 1H), 3.61 (d, *J* = 15 Hz, 1H), 3.53 (d, *J* = 15 Hz, 1H), 3.26 (m, 1H), 3.11 (m, 1H), 2.99 (m, 2H), 2.86 (m, 2H), 2.60 (t, *J* = 10 Hz, 2H), 2.14 (m, 1H), 1.76 (m, 2H), 1.58 (m, 2H), 1.48 (m, 1H), 1.34 (m, 2H). HRMS calculated for $C_{33}H_{36}ClN_4O_5$ $[M+H]^+$ 603.2369, found 603.2370.



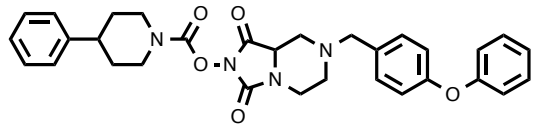
1,3-dioxo-7-(3-phenoxybenzyl)hexahydroimidazo[1,5-a]pyrazin-2(3H)-yl 4-(4-methoxyphenyl)piperazine-1-carboxylate (ABC23; Compound 12): The title compound was prepared as described for MJN200, using 3-phenoxybenzaldehyde in place of formaldehyde to yield ABC23 (4.6 mg, 17%). ^{1}H NMR (CDCl3, 500 MHz) δ 7.36 (m, 2H), 7.33 (m, 1H), 7.13 (m, 1H), 7.02 (m, 4 H), 6.92 (m, 3H), 6.86 (d, *J* = 10 Hz, 2H), 4.14 (m, 1H), 4.05 (m, 1H), 3.79 (m, 2H), 3.77 (s, 3H), 3.68 (m, 2H), 3.62 (d, *J* = 15 Hz, 1H), 3.54 (d, *J* = 15 Hz, 1H), 3.28 (m, 1H), 3.15 (m, 1H), 3.10 (m, 4H), 2.87 (m, 1H), 2.14 (m, 2H). HRMS calculated for $C_{31}H_{34}N_5O_6$ $[M+H]^+$ 572.2503, found 572.2504.



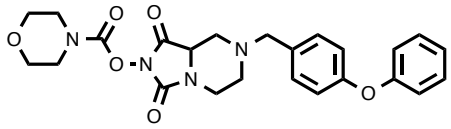
1,3-dioxo-7-(4-phenoxybenzyl)hexahydroimidazo[1,5-a]pyrazin-2(3H)-yl 4-(4-methoxyphenyl)piperazine-1-carboxylate (ABC34; Compound 13): The title compound was prepared as described for MJN200 (0.5X eq scale), using 4-phenoxybenzaldehyde in place of formaldehyde to yield ABC34 (6.1 mg, 45%). ^{1}H NMR (CDCl3, 500 MHz) δ 7.36 (m, 2H), 7.24 (d, *J* = 10 Hz, 2H), 7.12 (m, 1H), 7.04 (d, *J* = 10 Hz, 2H), 6.97 (d, *J* = 10 Hz, 2H), 6.90 (d, *J* = 10 Hz, 2H), 6.86 (d, *J* = 8 Hz, 2H), 4.16 (m, 1 H), 4.06 (m, 1H), 3.80 (m, 2H), 3.78 (s, 3H), 3.68 (m, 2H), 3.62 (d, *J* = 15 Hz, 1H), 3.53 (d, *J* = 15 Hz, 1H), 3.30 (m, 1H), 3.18 (m, 1H), 3.11 (m, 4H), 2.89 (m, 1H), 2.17 (m, 2H). HRMS calculated for $C_{31}H_{34}N_5O_6$ $[M+H]^+$ 572.2503, found 572.2504.



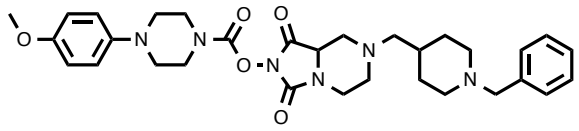
7-((1-(tert-butoxycarbonyl)piperidin-4-yl)methyl)-1,3-dioxohexahydroimidazo[1,5-a]pyrazin-2(3H)-yl 4-(4-methoxyphenyl)piperazine-1-carboxylate (ABC37; Compound 16): The title compound was prepared as described for MJN200 (2X eq scale), using N-Boc-4-piperidine carboxaldehyde in place of formaldehyde to yield ABC37 (36.4 mg, 66%). ^{1}H NMR (CDCl3, 500 MHz) δ 6.90 (d, *J* = 10 Hz, 2H), 6.85 (d, *J* = 10 Hz, 2H), 4.15 (m, 2H), 4.04 (m, 2H), 3.77 (s, 3H), 3.67 (m, 2H), 3.26 (m, 1H), 3.14 (m, 1H), 3.11 (m, 4H), 2.80 (m, 1H), 2.69 (m, 2H), 2.27 (m, 2H), 2.12 (m, 2H), 1.68 (m, 5H), 1.46 (s, 9H), 1.10 (m, 2H). HRMS calculated for $C_{29}H_{43}N_6O_7$ $[M+H]^+$ 587.3188, found 587.3188.



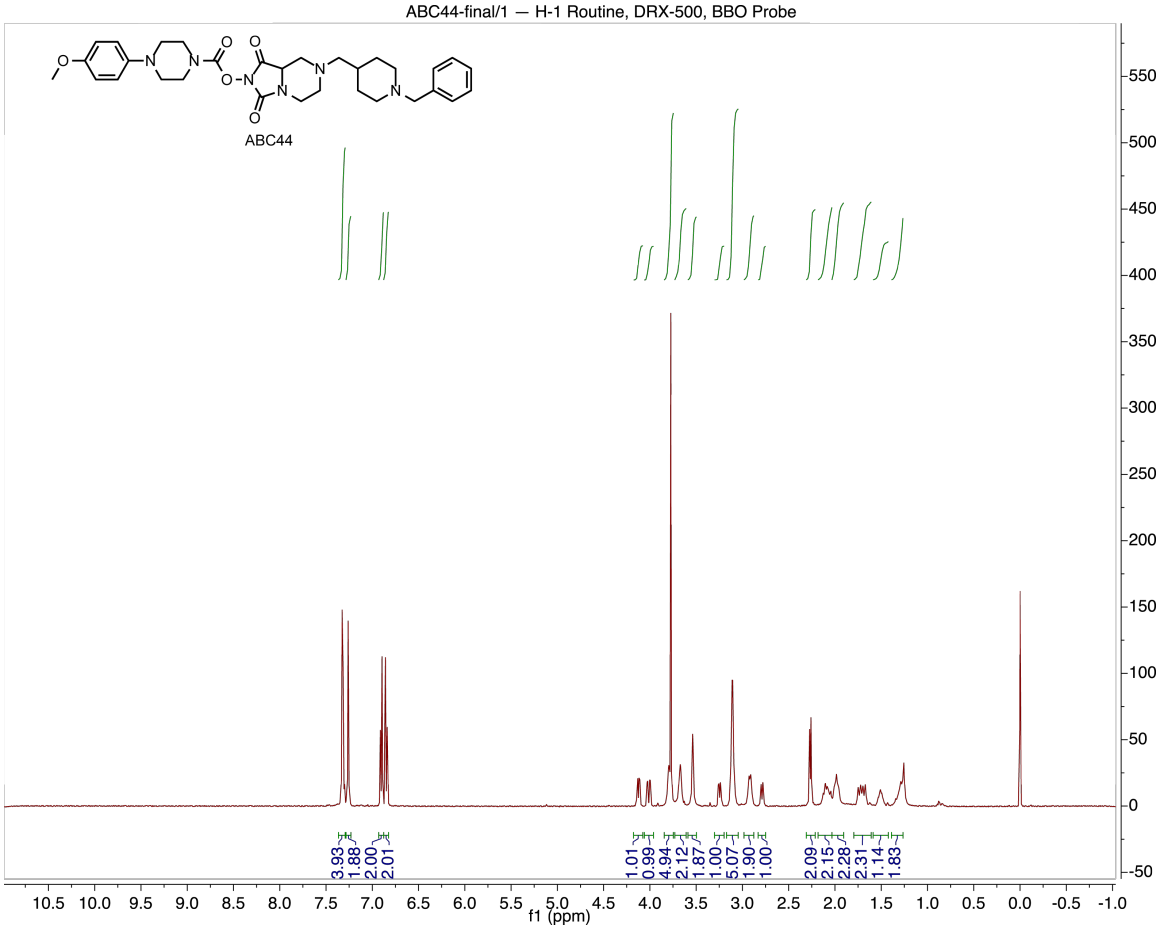
1,3-dioxo-7-(4-phenoxybenzyl)hexahydroimidazo[1,5-a]pyrazin-2(3H)-yl 4-phenylpiperidine-1-carboxylate (ABC47; Compound 10): The title compound was prepared as described for MJN200, using JJH322 in place of JJH331, and 4-phenoxybenzaldehyde in place of formaldehyde to yield ABC47 (19 mg, 62%). ^{1}H NMR (CDCl3, 500 MHz) δ 7.36 (m, 4H), 7.28 (m, 5H), 7.11 (m, 1H), 7.04 (m, 2H), 6.97 (m, 2H), 4.36 (m, 1H), 4.28 (m, 1H), 4.17 (m, 1H), 4.08 (m, 1H), 3.63 (d, *J* = 15 Hz, 1H), 3.54 (d, *J* = 15 Hz, 1H), 3.31 (m, 1H), 3.16 (m, 2H), 3.01 (m, 1H), 2.90 (m, 1H), 2.74 (m, 1H), 2.18 (m, 2H), 1.91 (m, 2H), 1.80 (m, 2H). HRMS calculated for $C_{31}H_{33}N_4O_5$ $[M+H]^+$ 541.2445, found 541.2448.



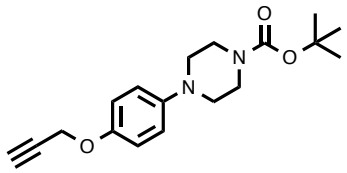
1,3-dioxo-7-(4-phenoxybenzyl)hexahydroimidazo[1,5-a]pyrazin-2(3H)-yl morpholine-4-carboxylate (ABC51; Compound 14): The title compound was prepared as described for MJN200, using JJH256 in place of JJH331, and 4-phenoxybenzaldehyde in place of formaldehyde to yield ABC51 (18 mg, 64%). ^{1}H NMR (CDCl3, 400 MHz) δ 7.37 (m, 2H), 7.27 (m, 2H), 7.13 (m, 1H), 6.98 (m, 2H), 6.96 (m, 2H), 4.16 (m, 1H), 4.06 (m, 1H), 3.75 (m, 4H), 3.66 (m, 2H), 3.59 (m, 1H), 3.51 (m, 2H), 3.50 (m, 1H), 3.30 (m, 1H), 3.15 (m, 2H), 2.89 (m, 1H), 2.10 (m, 1H). HRMS calculated for $C_{24}H_{27}N_4O_6$ $[M+H]^+$ 467.1925, found 467.1927.



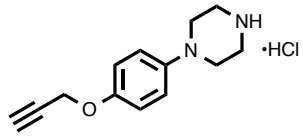
7-((1-benzylpiperidin-4-yl)methyl)-1,3-dioxohexahydroimidazo[1,5-a]pyrazin-2(3H)-yl 4-(4-methoxyphenyl)piperazine-1-carboxylate (ABC44; Compound 17): Was prepared as described for MJN200 (~10X eq scale), using *N*-benzyl-4-piperidine carboxaldehyde in place of formaldehyde to yield ABC44 (97 mg, 34%). ^{1}H NMR (CDCl3, 500 MHz) δ 7.40 (m, 5H), 6.98 (m, 2H), 6.92 (m, 2H), 4.22 (m, 1H), 4.10 (m, 1H), 3.88 (m, 2H), 3.85 (s, 3H), 3.75 (m, 2H), 3.62 (s, 2H), 3.33 (m, 1H), 3.19 (m, 1H), 3.18 (m, 4H), 2.99 (m, 2H), 2.88 (m, 1H), 2.34 (m, 2H), 2.19 (m, 2H), 2.07 (m, 2H), 1.80 (m, 2H), 1.58 (m, 1H), 1.37 (m, 2H). HRMS calculated for $C_{31}H_{41}N_6O_5$ $[M+H]^+$ 577.3133, found 577.3133.



Synthesis of ABC45 (Probe 1)

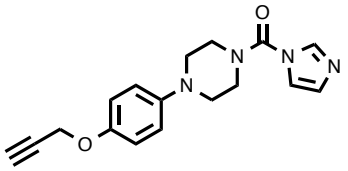


tert-butyl 4-(4-(prop-2-yn-1-yloxy)phenyl)piperazine-1-carboxylate: Tert-butyl 4-(4-hydroxyphenyl)piperazine-1-carboxylate (100 mg, 360 µmol, 1 eq) was suspended in 0.75 mL DMF, and propargyl bromide (80% by weight in toluene, 67 µL, 440 µmol, 1.25 eq) and potassium carbonate (75 mg, 540 µmol, 1.5 eq) were added. The resulting mixture was stirred at 60 °C for 18 h, cooled to room temperature, concentrated under a stream of nitrogen, and separated by prep-TLC (25% EtOAc/hexanes) to yield an off-white solid (62 mg, 54%). 1H NMR (CDCl3, 500 MHz) δ 6.91 (m, 4H), 4.64 (d, J = 2.4 Hz, 2H), 3.57 (t, J = 5.0 Hz, 4H), 3.02 (t, J = 5.0 Hz, 4H), 2.50 (t, J = 2.4 Hz, 1H), 1.48 (s, 9H). HRMS calculated for $C_{18}H_{25}N_2O_3$ $[M+H]^+$ 317.1860, found 317.1858.



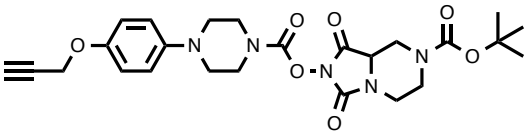
1-(4-(prop-2-yn-1-yloxy)phenyl)piperazine hydrochloride:

Tert-butyl 4-(4-(prop-2-yn-1-yloxy)phenyl)piperazine-1-carboxylate was Boc deprotected using methanolic HCl as described for JJH222, yielding an off-white solid that was used without further purification. 1H NMR (D2O, 500 MHz) δ 7.43 (m, 2H), 7.18 (m, 2H), 4.83 (d, J = 2.3 Hz, 2H), 3.76 (m, 4H), 3.67 (m, 4H), 2.98 (t, J = 2.4 Hz, 1H). HRMS calculated for $C_{13}H_{17}N_2O$ $[M+H]^+$ 217.1335, found 217.1335.



(1*H*-imidazol-1-yl)(4-(4-(prop-2-yn-1-yloxy)phenyl)piperazin-1-yl)methanone:

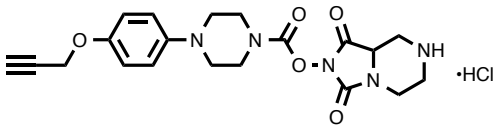
1-(4-(prop-2-yn-1-yloxy)phenyl)piperazine hydrochloride (49 mg, 196 µmol, 1 eq) was taken up in 2 mL methylene chloride, and triethylamine (27 µL, 196 µmol, 1 eq) and carbonyldiimidazole (48 mg, 294 µmol, 1.5 eq) were added. The resulting mixture was stirred at room temperature over night, concentrated and separated by flash chromatography over SiO_2 in 60% EtOAc/hexanes to yield the corresponding imidazole urea (47 mg, 77%). 1H NMR (CDCl3, 500 MHz) δ 7.92 (s, 1H), 7.23 (t, J = 1.4 Hz, 1H), 7.13 (br m, 1H), 6.93 (m, 4H), 4.65 (d, J = 2.4 Hz, 2H), 3.77 (m, 4H), 3.15 (m, 4H), 2.51 (t, J = 2.3 Hz, 1H). HRMS calculated for $C_{17}H_{19}N_4O_2$ $[M+H]^+$ 311.1502, found 311.1504.



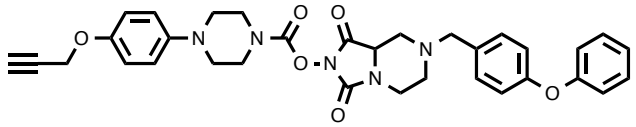
tert-butyl 1,3-dioxo-2-((4-(4-(prop-2-yn-1-yloxy)phenyl)piperazine-1-carbonyl)oxy)hexahydroimidazo[1,5-a]pyrazine-7(1H)-carboxylate:

The free N-Boc-NHH (9.5 mg, 35.2 µmol, 1.1 eq) was dissolved in 0.35 mL THF, and triethylamine (13 µl, 96 µmol, 3 eq) was added, followed by (1*H*-imidazol-1-yl)(4-(4-(prop-2-yn-1-yloxy)phenyl)piperazin-1-yl)methanone (10 mg, 32 µmol, 1 eq) at room temperature. The solution was stirred and heated at 70 ^oC for 2 h, concentrated to a residue, and separated by prep-TLC (25% EtOAc/hexanes) to yield an off-white solid (14 mg, 85%). 1H NMR (CDCl3, 500 MHz) δ 6.92 (m, 4H), 4.65 (d, J = 2.3 Hz, 2H), 4.56 (s, 1H), 4.19 (s, 1H), 4.08 (m, 2H), 3.80 (s, 2H), 3.67 (s, 2H), 3.13 (s, 4H), 3.05 (t, J = 12.0

Hz, 1H), 2.85 (s, 2H), 2.51 (t, J = 2.4 Hz, 1H), 1.49 (s, 9H). HRMS calculated for $C_{25}H_{32}N_5O_7$ $[M+H]^+$ 514.2296, found 514.2296.



1,3-dioxohexahydroimidazo[1,5-*a*]pyrazin-2(3*H*)-yl 4-(4-(prop-2-yn-1-yloxy)phenyl)piperazine-1-carboxylate hydrochloride: Tert-butyl 1,3-dioxo-2-((4-(4-(prop-2-yn-1-yloxy)phenyl)piperazine-1-carbonyl)oxy)hexahydroimidazo[1,5-a]pyrazine-7(1H)-carboxylate was Boc deprotected using methanolic HCl as described for JJH222, yielding an off-white solid that was used without further purification. 1H NMR (D2O, 500 MHz) δ 7.58 (m, 2H), 7.25 (m, 2H), 4.87 (m, 3H), 4.36 (m, 1H), 4.16 (s, 2H), 3.99 (m, 3H), 3.80 (br s, 4H), 3.64 (dd, J = 13.3, 3.5 Hz, 1H), 3.54 (ddd, J = 15.0, 12.8, 3.7 Hz, 1H), 3.39 (t, J = 12.5 Hz, 1H), 3.28 (td, J = 12.9, 4.5 Hz, 1H), 3.00 (t, J = 2.4 Hz, 1H). HRMS calculated for $C_{20}H_{24}N_5O_5$ $[M+H]^+$ 414.1772, found 414.1771.



1,3-dioxo-7-(4-phenoxybenzyl)hexahydroimidazo[1,5-*a*]pyrazin-2(3*H*)-yl 4-(4-(prop-2-yn-1-yloxy)phenyl)piperazine-1-carboxylate (ABC45; Probe 1): 1,3-dioxohexahydroimidazo[1,5-*a*]pyrazin-2(3*H*)-yl 4-(4-(prop-2-yn-1-yloxy)phenyl)piperazine-1-carboxylate hydrochloride (12 mg, 27 μmol, 1 eq) was suspended in 270 μL methylene chloride, and 4-phenoxy benzaldehyde (22 mg,109 μmol, 4 eq) was added followed by sodium triacetoxyborohydride (12.2 mg, 54 μmol, 2 eq). The resulting mixture was stirred at room temperature over night, concentrated and separated by prep-TLC (50% EtOAc/hexanes) to yield ABC45 as a clear oil (11.6 mg,

72%). 1H NMR (CDCl3, 500 MHz) δ 7.35 (m, 2H), 7.24 (m, 3H), 7.12 (t, J = 7.3 Hz, 1H), 7.03 (m, 2H), 6.96 (m, 2H), 6.92 (m, 3H), 4.65 (br s, J = 2.7, 1.5 Hz, 2H), 4.15 (d, J = 10.5 Hz, 1H), 4.05 (d, J = 13.3 Hz, 1H), 3.79 (s, 2H), 3.67 (s, 2H), 3.60 (d, J = 13.0 Hz, 1H), 3.51 (d, J = 12.7 Hz, 1H), 3.29 (d, J = 9.5 Hz, 1H), 3.13 (s, 5H), 2.88 (d, J = 11.1 Hz, 1H), 2.51 (s, 1H), 2.14 (br s, 2H). HRMS calculated for $C_{33}H_{34}N_5O_6$ $[M+H]^+$ 596.2503, found 596.2507.

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