Confirming Target Engagement of Reversible Inhibitors In Vivo by Kinetically Tuned Activity-Based Probes

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SUPPLEMENTARY INFORMATION

SUPPLEMENTARY METHODS

Recombinant protein expression and purification. Human LYPLA1 and LYPLA2 genes were amplified from Jurkat cell cDNA, then sub-cloned into the pTrcHis2A bacterial expression vector using BamHI and EcoRI restriction sites for the in-frame fusion to a C-terminal poly-histidine tag. Each gene was truncated to eliminate the first 5 residues to begin translation at the second methionine. After reaching an optical density of 0.5 at 600 nm, cultures of BL21 (DE3) *E. coli* cells were induced for 4 hours with 1 mM IPTG. Cell pellets were resuspended in RIPA buffer supplemented with 1 mg/mL lysozyme and 1 mg/mL DNase I. After incubation on ice for 1 hour, the suspension was sonicated, followed by centrifugation at 10,000 x g for 15 minutes. The supernatant was incubated with Talon cobalt affinity beads (Invitrogen), washed with 50 mM Tris pH 8 with 100 mM NaCl (Assay Buffer), and eluted in 2 steps with 10 mM imidazole, followed by 50 mM imidiazole. The eluted protein was then concentrated using a 10 KD cutoff Amicon centrifugal unit for three cycles of buffer exchange with assay buffer to remove residual imidazole. Following quantification using the Bio-Rad DC Protein Assay kit, the protein was diluted to 50 μM in assay buffer supplemented with 10% glycerol and stored at -80° C. Each protein was expressed at 1 - 2 milligrams per liter of bacteria. Catalytic dead mutants LYPLA1 (S119A) and LYPLA2 (S112A) were expressed and purified using the methods described above with similar yields.

Fluopol-ABPP assays. LYPLA1 and LYPLA2 were screened separately against a compound library of 18,974 molecules as previously described.[1] This library includes 16,000 compounds (10 μM) from the Maybridge Hitfinder™ Collection, 974 compounds (10 μM) from a hydrolase-directed inhibitor library synthesized by the Boger group at TSRI, and 2,000 compounds (5 μM) from a validation fraction of the National Institutes of Health Molecular Libraries Small Molecule Repository. Separate fluopol-ABPP assays were performed for both LYPLA1 and LYPLA2 in a 384-well format. Briefly, 10 μL of recombinant LYPLA1 (5 nM) or LYPLA2 (20 nM) in assay buffer (50 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.01% Pluronic F-127 (Invitrogen)) was added to test compound and negative control wells, and 10 μL assay buffer alone was added to positive control wells. Compounds were then added to test compound wells and DMSO to control wells, and plates were then incubated at 25 °C for 30 min. FP-rhodamine (1.1 μL; 750 nM in assay buffer; 75 nM final concentration in fluorescence polarization assay) was added to all wells. The plates were incubated for an additional 10 min at 25 °C and then read on an Envision plate reader (Perkin Elmer).

HTS screen. The National Institutes of Health Molecular Libraries Small Molecule Repository containing 315004 compounds were separately screened against LYPLA1 and LYPLA2. Prior to the start of the assay, 4.0 μL of Assay Buffer containing 6.25 nM LYPLA1 or 9.38 nM of LYPLA2 protein was dispensed into 1536 microtiter plates. Next, 30 nL of test compound in DMSO or DMSO alone (0.59% final concentration) were added to the appropriate wells at a final concentration of 5.9 μM and incubated for 30 minutes at 25 °C. The assay was started by dispensing 1.0 μL of 375 nM FP-Rh probe in Assay Buffer to all wells. Plates were centrifuged and after 10 minutes of incubation at 25 °C, fluorescence polarization was read on a Viewlux microplate reader (PerkinElmer, Turku, Finland) using a BODIPY TMR FP filter set and a BODIPY dichroic mirror (excitation = 525nm, emission = 598nm). Fluorescence polarization was read for 15 seconds for each polarization plane (parallel and perpendicular). The well fluorescence polarization value (mP) was obtained via the PerkinElmer Viewlux software. Compounds were classified as active the percent inhibition was greater than the sum of the average percent inhibition of all compounds tested plus three times the standard deviation. 499 (0.156%) compounds were found to inhibitor LYPLA1. 1197 (0.380%) compounds were shown to inhibit LYPLA2 by this measure. These compounds were re-tested using the same protocol as a confirmatory screen, which validated 331 of 478 LYPLA1 inhibitors and 790 of 1199 LYPLA2 inhibitors. We then manually filtered these compounds for those with the highest inhibition,

while excluding non-selective compounds identified in RBBP9 and GSTO inhibitor screens. Screening plates containing 10 μL of 10 mM stocks of 91 LYPLA1, 61 LYPLA2, and 95 dual LYPLA1/LYPLA2 inhibitors were sent for gel-based confirmatory analysis.

Kinetic evaluation of reversible LYPLA1 and LYPLA2 inhibitors. Resorufin acetate (Sigma) was dissolved in DMSO. Assays were performed in PBS adjusted to pH 6.5 with sodium acetate and 0.2% pluronic F127. In a black bottom half area 96 well plate, 5 μL of substrate was aliquoted at varying concentrations. The assay was initiated with 95 μL of 10 nM enzyme using a multi-channel pipette, and then quickly mixed by pipetting up and down several times. The plate was then measured on a Tecan F500 plate reader at room temperature every 30 seconds using a 525/35 nM excitation filter, a 600/10 nM emission filter, and a 560 LP dichroic filter. Reactions were run for 20 minutes. Each concentration was performed as 4 separate replicates, run simultaneously with 4 replicates performed using the catalytic dead enzyme (LYPLA1-S119A and LYPLA2-S122A, respectively). Furthermore, data from two separate experiments were combined for each enzyme. Initial velocities were calculated using only the first 6 -7 minutes. The data was analyzed using standard Michaelis-Menten kinetics in GraphPad Prism to derive the V_{max} and K_m values. To calculate the Ki values for each reversible inhibitor, 10 nM enzyme was incubated with varying inhibitor concentrations for 15 minutes, and then aliquoted to 96-well plate wells containing a fixed concentration of substrate (50 μM and 30 μM resorufin acetate for LYPLA1 and LYPLA2, respectively). After subtraction of the catalytic dead enzyme activity, the initial velocities were plotted and fit to derive the IC_{50}. The K_i for **Compound 1** and **Compound 21** were derived using the Cheng-Prusoff equation.

Validation of inhibitors by gel-based ABPP. Soluble proteomes of HEK293T cells were incubated with inhibitors (1 μM) for 30 minutes at 37 °C. FP-peg-Rh (5 μM) was added and the lysates were incubated for additional 30 minutes at room temperature. Reducing SDS loading buffer was added and the proteomes were separated by SDS-PAGE. Fluorescently labeled serine hydrolases were imaged using a Hitachi FMBio II fluorescence flatbed scanner.

Gel-filtration experiments to assess the reversibility of inhibitors. These experiments were performed as described previously.[2]

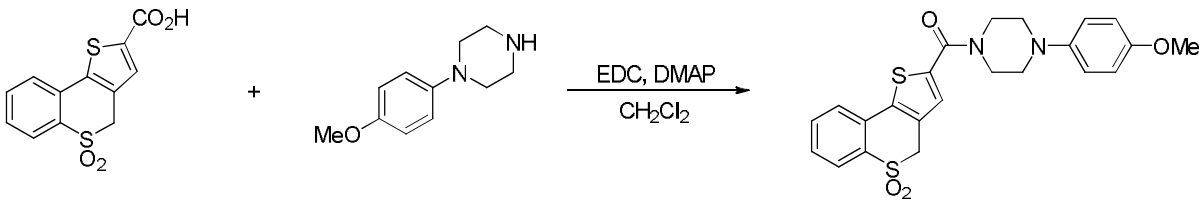
In situ treatment of living cells with inhibitors and proteome preparation for ABPP experiments. BW5147-derived murine T-cell hybridoma cells were grown in RPMI-1640 medium with 10% FCS and 1x Penicillin, Streptomycin, Glutamine solution (Invitrogen) at 37°C and 5% CO_2. HEK293T cells were grown in DMEM with 10% FCS and 1x Penicillin, Streptomycin, Glutamine solution (Invitrogen) at 37°C and 5% CO_2. Inhibitors **1** and **21** (both 5 μM) were directly added to the cell culture media. After 3 h, probe **35** (50 μM) was added and the cells were incubated for an additional hour. The cells were harvested and the pellets were isolated by centrifugation at 1,400 x g for 3 min, re-suspended in 4:1:3 volumes of methanol /chloroform/dH_2O and vortexed. After centrifugation at 13,000 x g for 3 min, upper layer was removed and 3 vol. of methanol were added and vortexed. Protein was pelleted by centrifugation at 13,000 x g for 6 min. The supernatant was removed and the pellet was air-dried and re-suspended in PBS by sonication. For subsequent ABPP experiments, rhodamine or biotin azide (100 μM), TCEP (0.5 μM), ligand (100 μM), and $CuSO_4$ (1 mM) were added. The reactions were incubated at room temperature for 1 h.

In vivo studies with inhibitors 1 and 21 and proteome preparation for ABPP experiments. Inhibitors **1** and **21** were prepared as a homogeneous PEG solution by vortexing and sonicating neat compound directly into PEG300 (Fluka). Mice (<6 months old, 20–28 g) were i.p. administered with **1** and **21** (50 mg/kg; 4 μL/g) and, after 3 h, with probe **35** (PEG solution; 100 mg/kg). After 1 h, mice were sacrificed, and tissues

were removed and Dounce-homogenized directly in 4:1:3 volumes of methanol /chloroform/dH_2O. After centrifugation at 13,000 x g for 3 min, upper layer was removed and 3 vol. of methanol were added and vortexed. Protein was pelleted by centrifugation at 13,000 x g for 6 min. The supernatant was removed and the pellet was air-dried and re-suspended in PBS by sonication for subsequent ABPP analyses.

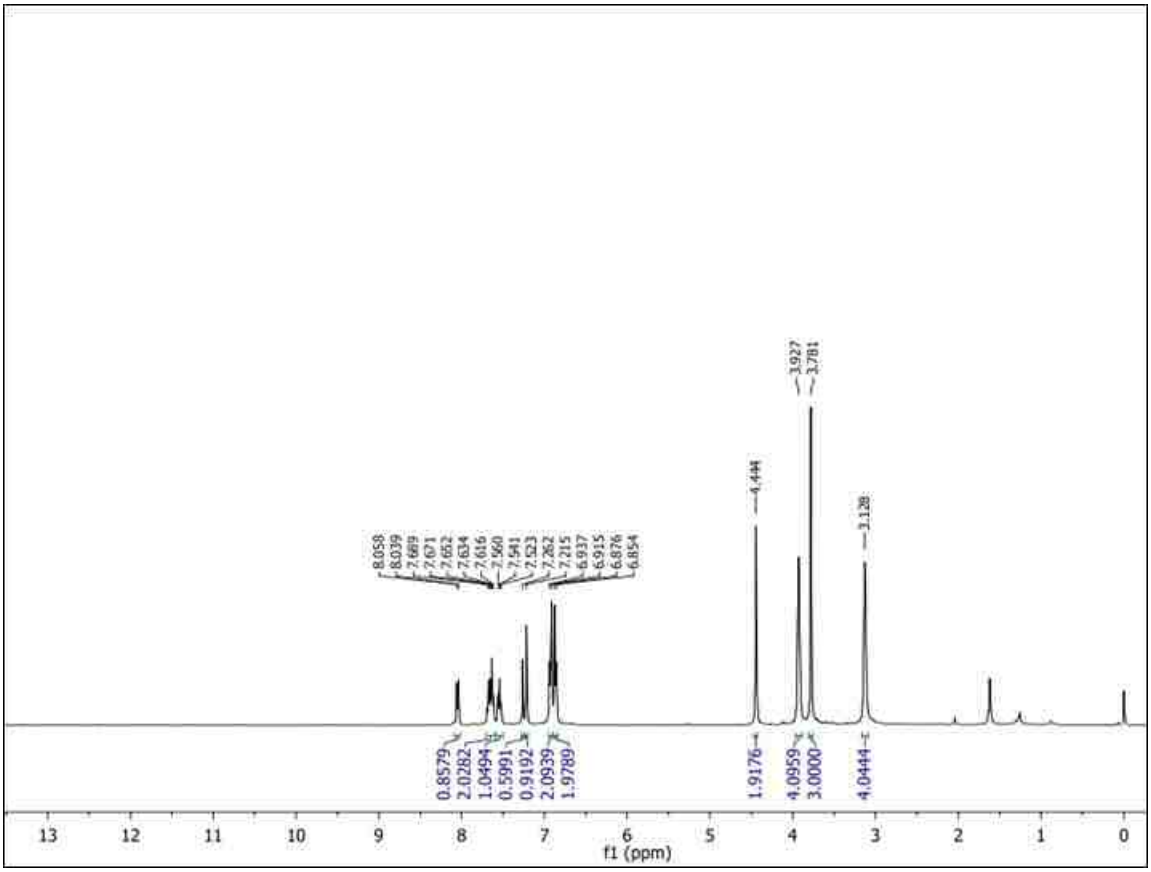
ABPP-SILAC experiments. ABPP-SILAC experiments were performed and analyzed as described previously.[3]

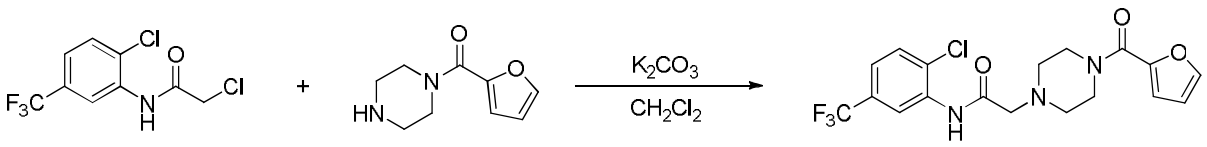
General synthetic methods. All reagents were purchased from Sigma-Aldrich, Acros, Fisher, Fluka, ChemDiv, or Aurora and used without further purification, except where noted. Dry solvents were obtained by passing commercially available pre-dried, oxygen-free formulations through activated alumina columns. All reactions were carried out under a nitrogen atmosphere using oven-dried glassware unless otherwise noted. Flash chromatography was performed using 230-400 mesh silica gel. NMR spectra were recorded in $CDCl_3$ on a Varian Inova-400 spectrometer and were referenced to trimethylsilane (TMS) or the residual solvent peak. Chemical shifts are reported in ppm relative to TMS and J values are reported in Hz. High resolution mass spectrometry (HRMS) experiments were performed at The Scripps Research Institute Mass Spectrometry Core on an Agilent mass spectrometer using electrospray ionization-time of flight (ESI-TOF).



(5,5-Dioxido-4H-thieno[3,2-c]thiochromen-2-yl)(4-(4-methoxyphenyl)piperazin-1-yl)methanone (1)

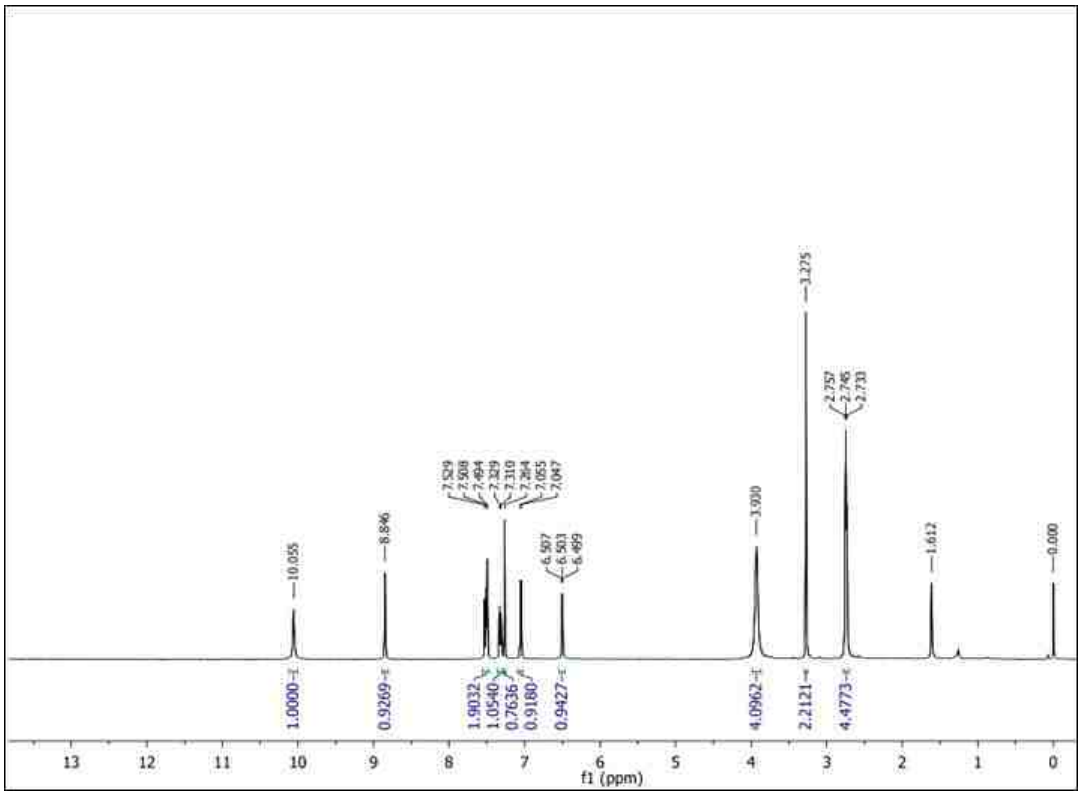
To a solution of 4H-thieno[3,2-c]thiochromene-2-carboxylic acid 5,5-dioxide (25 mg, 0.089 mmol) in dichloromethane (1.0 mL) were added 1-(4-methoxyphenyl)piperazine (17 mg, 0.089 mmol), EDC (51 mg, 0.267 mmol) and 4-DMAP (catalyst) at room temperature. After stirring for 12 hours at room temperature, the reaction was quenched with 3.0 mL of saturated aqueous $NaHCO_3$ solution. The organic layer was separated and the aqueous layer was extracted with dichloromethane. The combined organic extracts were washed with brine, dried over $MgSO_4$, filtered and concentrated. The crude product was purified by flash column chromatography using a 97:3 v/v dichloromethane:methanol as solvent to afford the desired compound (35 mg, 87 % yield) as a yellow solid. ^{1}H NMR 400 MHz ($CDCl_3$): δ 8.05 - 8.03 (*d*, *J* = 7.6 Hz, 1H), 7.68 - 7.61 (*m*, 2H), 7.56 - 7.52 (*t*, *J* = 7.6 Hz, 1H), 7.21 (*s*, 1H), 6.93 - 6.91 (*m*, 2H), 6.87- 6.85 (*m*, 2H), 4.44 (*s*, 2H), 3.93 (*m*, 4H), 3.78 (*s*, 3H), 3.13 (*m*, 4H); ^{13}C NMR 133 MHz ($CDCl_3$): δ 162.5, 154.9, 145.4, 137.7, 136.6, 134.3, 133.9, 130.4, 130.2, 129.6, 128.2, 126.2, 124.6, 119.4, 114.9, 77.6, 77.1, 55.9, 51.6; HRMS (m/z): $[M+H]^+$calculated for $C_{23}H_{22}N_2O_4S_2$, 455.1094; found, 455.1095.





N-(2-chloro-5-(trifluoromethyl)phenyl)-2-(4-(furan-2-carbonyl)piperazin-1-yl)acetamide (21)

To a solution of furan-2-yl(piperazin-1-yl)methanone (20 mg, 0.110 mmol) in dichloromethane (1.0 mL) were added 2-chloro-N-(2-chloro-5-(trifluoromethyl)phenyl)- acetamide (20 mg, 0.073 mmol) and K_2CO_3 (31 mg, 0.220 mmol) at room temperature. After stirring for 12 hours at 60 °C, the reaction was quenched with 3.0 mL of sat. aq. $NaHCO_3$ solution. The organic layer was separated and the aqueous layer was extracted with dichloromethane. The combined organic extracts were washed with brine, dried over $MgSO_4$, filtered and concentrated. The crude product was purified by flash column chromatography using a 98:2 v/v dichloromethane: methanol as solvent to afford the desired compound (28 mg, 93 % yield) as a yellow solid. ^{1}H NMR 400 MHz ($CDCl_3$): δ 10.05 (*s*, 1H), 8.85 (*s*, 1H), 7.53 - 7.49 (*m*, 2H), 7.31 (*d*, *J* = 7.6 Hz, 1H), 7.05 (*d*, *J* = 3.2 Hz, 1H), 6.51- 6.49 (*m*, 1H), 3.93 (*m*, 4H), 3.27 (*s*, 2H), 2.76 - 2.73 (*m*, 4H); ^{13}C NMR: 133 MHz ($CDCl_3$) δ 168.5, 159.4, 148.0, 144.1, 135.2, 129.8, 126.1, 121.4, 121.3, 118.0, 117.9, 117.2, 111.7, 62.2, 53.8, 53.7; HRMS (m/z): $[M+H]^+$calculated for $C_{18}H_{17}ClF_3N_3O_3$, 416.0983; found, 416.0983.



SUPPLEMENTARY FIGURES

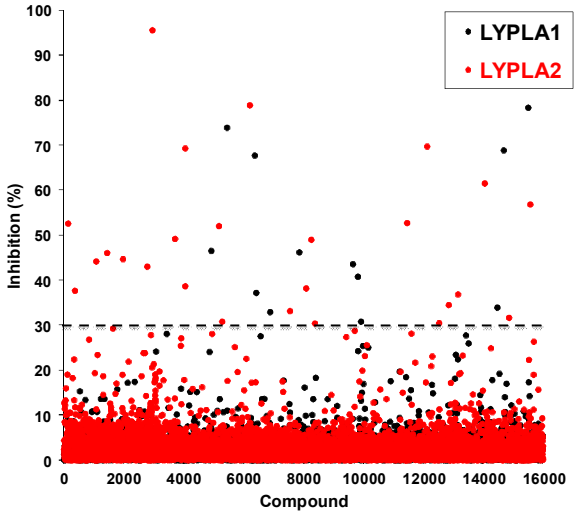


Figure S1. Fluopol-ABPP screening results for representative 16,000 compounds of the NIH small-molecule library.

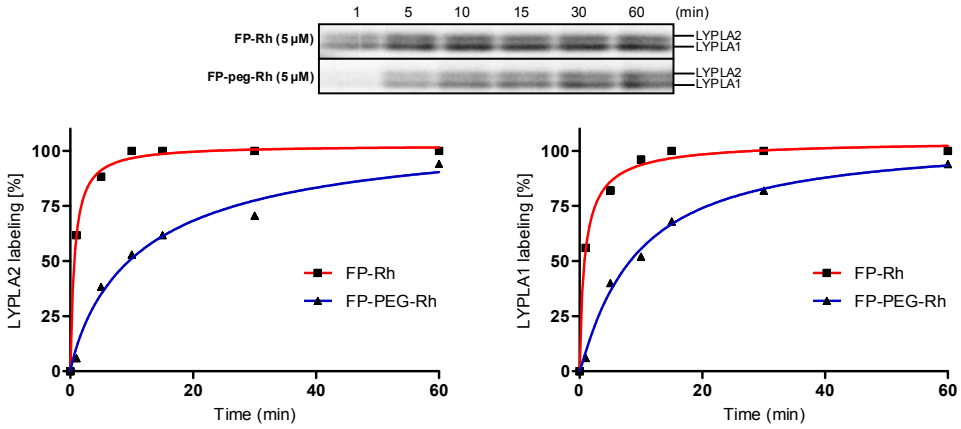


Figure S2. Time-dependent labeling of LYPLA1 and LYPLA2 by SH-directed probes FP-Rh and FP-peg-Rh (both tested at 5 μM). Note that both LYPLA1 and LYPLA2 showed time-dependent increases in labeling with FP-peg-Rh throughout the 1 hr time course, while enzyme labeling was complete by 10 min with the FP-Rh probe.

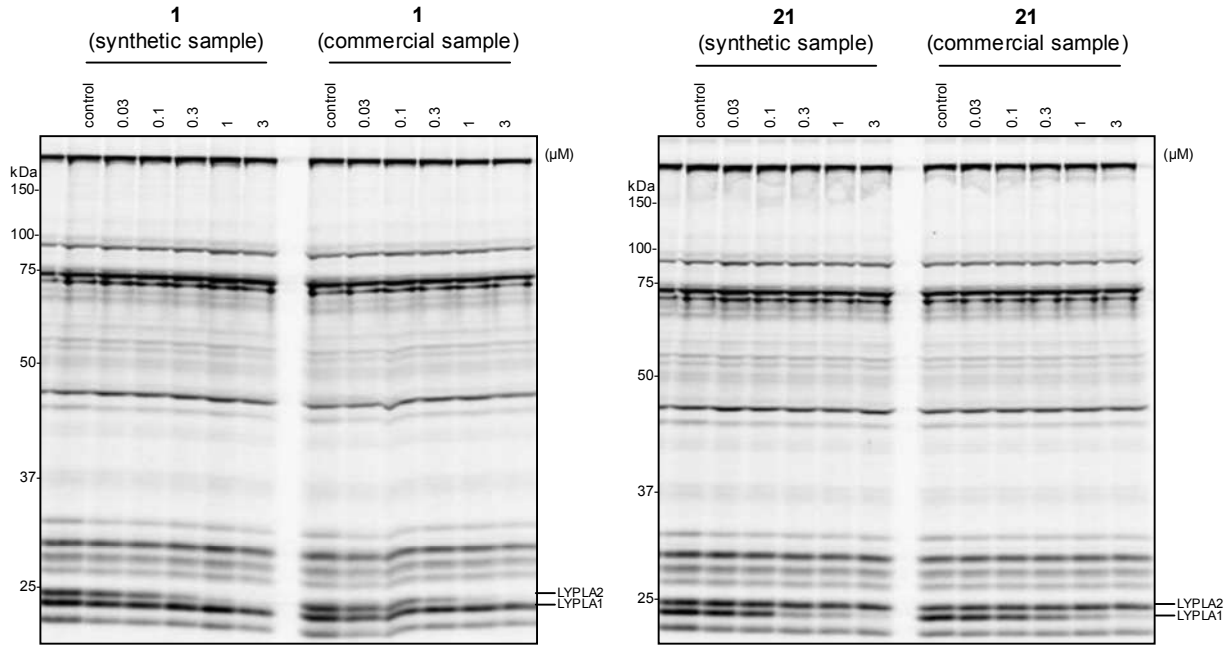


Figure S3. Concentration-dependent blockade of FP-peg-Rh labeling of LYPLA2 and LYPLA1 by synthetic and commercial samples of inhibitors **1** and **21**, respectively, in the soluble proteome of HEK 293T cells.

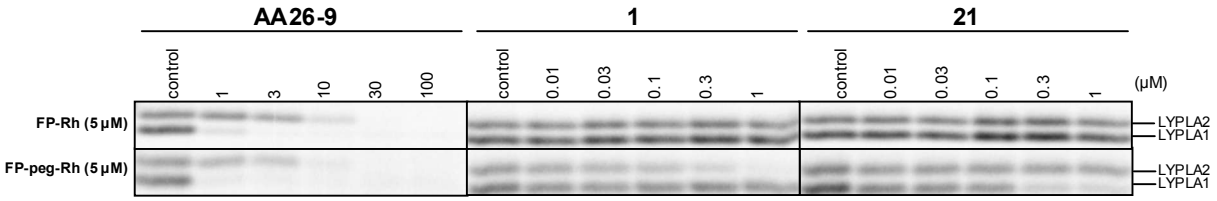


Figure S4. Concentration-dependent blockade of FP-Rh and FP-peg-Rh labeling of LYPLA2 and LYPLA1 by inhibitors **1** and **21**, respectively, in the soluble proteome of HEK 293T cells. Lysates were treated with inhibitors (30 min at 37 °C) following by FP-Rh or FP-peg-Rh (30 min at r.t.).

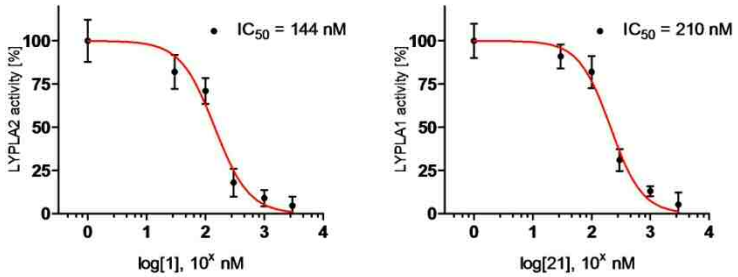


Figure S5. Concentration-dependent inhibition curves for **1** and **21** against LYPLA2 and LYPLA1, respectively, calculated from data shown in **Figure 2C**. Calculated values represent means ± s.e.m for three independent experiments.

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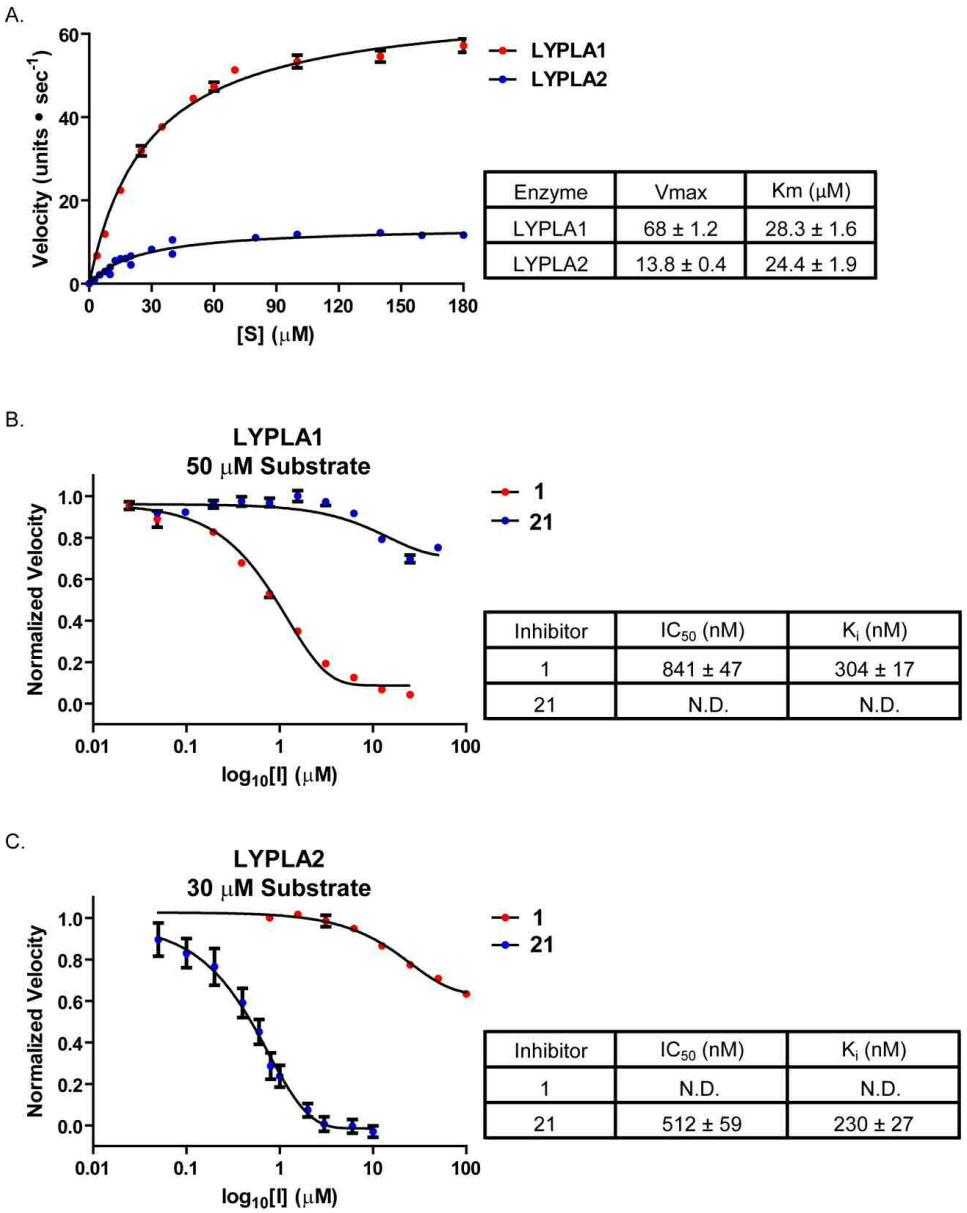

Enzyme	Vmax	Km (μM)
LYPLA1	68 ± 1.2	28.3 ± 1.6
LYPLA2	13.8 ± 0.4	24.4 ± 1.9

Inhibitor	IC_{50} (nM)	K_i (nM)
1	841 ± 47	304 ± 17
21	N.D.	N.D.

Inhibitor	IC_{50} (nM)	K_i (nM)
1	N.D.	N.D.
21	512 ± 59	230 ± 27

Figure S6. Kinetic evaluation of inhibitors **1** and **21** using a fluorogenic resorufin acetate substrate hydrolysis assay with 10 nM purified human LYPLA1 and LYPLA2. **(A)** Michaelis-Menten kinetic analysis of LYPLA1 and LYPLA2. K_m and V_{max} values are reported for each enzyme. **(B)** and **(C)** Inhibition of substrate turnover by LYPLA1 **(B)** and LYPLA2 **(C)**. The Ki values for **1** and **21** were derived using Cheng-Prusoff equation. Calculated values represent means ± s.e.m for four independent experiments.

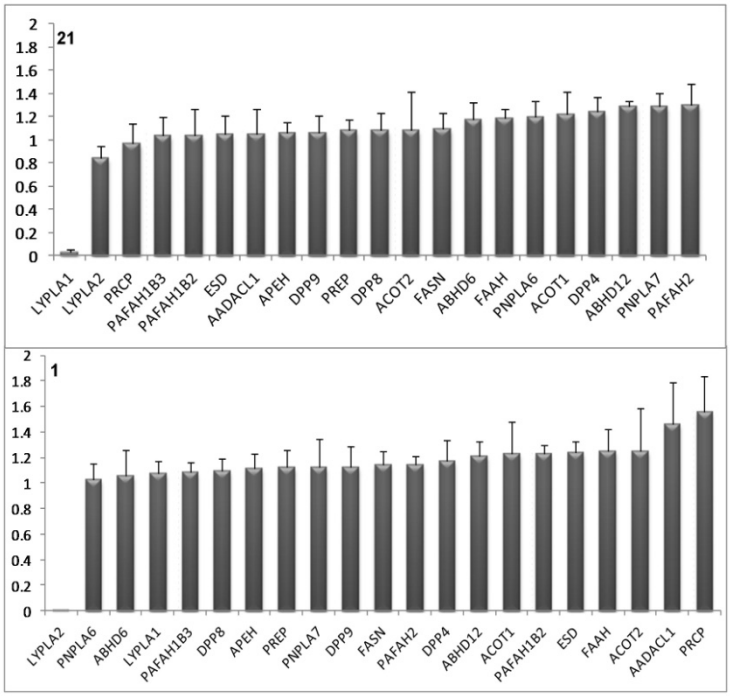

Figure S7. ABPP-SILAC analysis of serine hydrolase activities from inhibitor-treated mouse T-cell lysates. Bars represent means ± s.d. of light/heavy ratios for the multiple peptides observed for each enzyme; data are derived from two independent biological replicates.

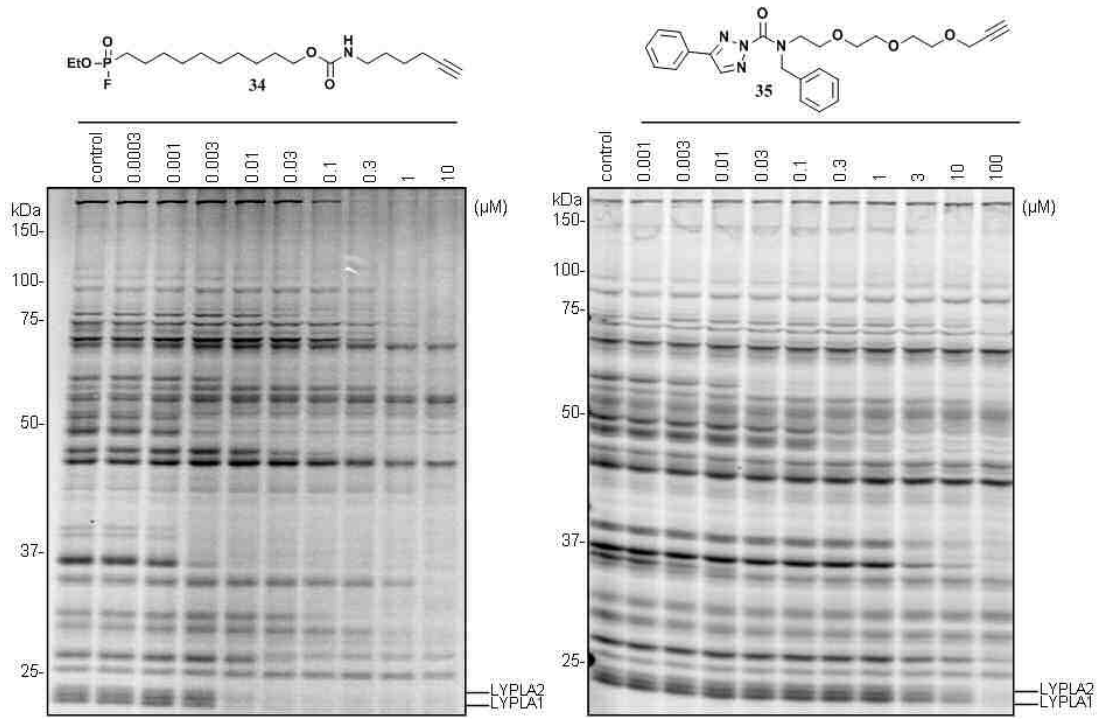

Figure S8. Concentration-dependent blockade of FP-Rh labeling by FP-alkyne **34** and triazole urea probe **35** in mouse brain membrane proteome.

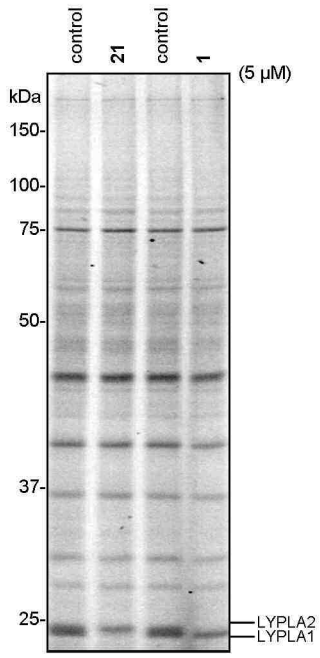

Figure S9. Gel-based competitive ABPP analysis of mouse T-cells treated with **21** and **1** (5 μM, 3 h) following by probe **35** (50 μM, 1 h) to measure *in situ* target engagement with LYPLA1 and LYPLA2, respectively.

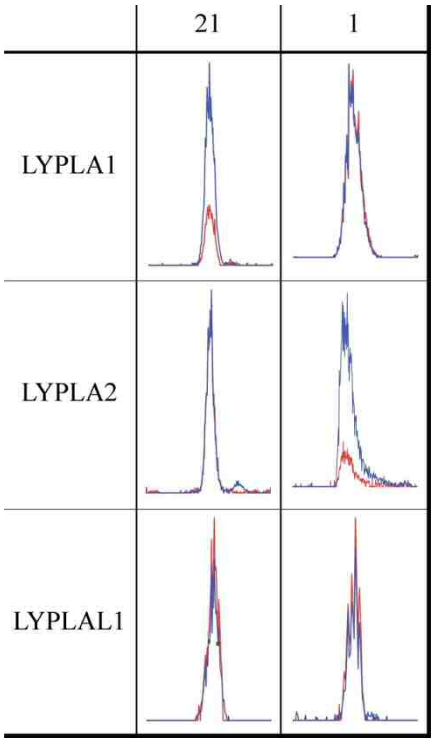

Figure S10. Orthogonal selectivity of inhibitors **21** and **1**, shown by heavy (blue, control) and light (red, inhibitor treated) MS1 peak pairs for representative tryptic peptides from highly homologous enzymes LYPLA1, LYPLA2, and LYPLAL1.

Table S1. Proteomic data sets for experiments described in Figures **3c**, **S7**, and **S10**. Listed are data on peptide and protein identifications, as well as mass, charge state, and quantitative ratio for each individual peptide. Calculated values represent means ± s.d. for at least two biological replicates. The data sets are organized within the Excel spreadsheet as tabs with names that relate the results to the corresponding figures.

See accompanying Excel spreadsheet.

REFERENCES

(1) Bachovchin, D. A.; Brown, S. J.; Rosen, H.; Cravatt, B. F. *Nat Biotechnol* **2009**, *27*, 387.
(2) Bachovchin, D. A.; Mohr, J. T.; Speers, A. E.; Wang, C.; Berlin, J. M.; Spicer, T. P.; Fernandez-Vega, V.; Chase, P.; Hodder, P. S.; Schurer, S. C.; Nomura, D. K.; Rosen, H.; Fu, G. C.; Cravatt, B. F. *Proc. Natl. Acad. Sci. USA* **2011**, *108*, 6811.
(3) Adibekian, A.; Martin, B. R.; Wang, C.; Hsu, K. L.; Bachovchin, D. A.; Niessen, S.; Hoover, H.; Cravatt, B. F. *Nat Chem Biol* **2011**, *7*, 469.