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Selective inhibitors and tailored activity probes for lipoprotein-associated phospholipase A_2

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ABSTRACT

Lipoprotein-associated phospholipase A_2 (Lp-PLA_2 or PLA_2G7) binds to low-density lipoprotein (LDL) particles, where it is thought to hydrolyze oxidatively truncated phospholipids. Lp-PLA_2 has also been implicated as a pro-tumorigenic enzyme in human prostate cancer. Several inhibitors of Lp-PLA_2 have been described, including darapladib, which is currently in phase 3 clinical development for the treatment of atherosclerosis. The selectivity that darapladib and other Lp-PLA_2 inhibitors display across the larger serine hydrolase family has not, however, been reported. Here, we describe the use of both general and tailored activity-based probes for profiling Lp-PLA_2 and inhibitors of this enzyme in native biological systems. We show that both darapladib and a novel class of structurally distinct carbamate inhibitors inactivate Lp-PLA_2 in mouse tissues and human cell lines with high selectivity. Our findings thus identify both inhibitors and chemoproteomic probes that are suitable for investigating Lp-PLA_2 function in biological systems.

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Lipoprotein-associated phospholipase A_2 (Lp-PLA_2 or PLA_2G7) is a secreted serine hydrolase that is found on low-density lipoprotein (LDL) particles and, in response to oxidation of LDL particles (ox-LDL), is able to hydrolyze oxidized phosphatidylcholine into lysophosphatidylcholine (LPC) and oxidized fatty acids.[1] The released LPC and oxidized fatty acids are thought to promote inflammation,[2,3] thus designating Lp-PLA_2 as a potential risk factor in the development of atherosclerosis. This premise is also supported by a positive correlation between Lp-PLA_2 levels and the incidence of coronary heart disease.[4] Genetic studies have further shown that a small percentage of the Asian population carries an inactivating mutation of a conserved valine (V279F) in Lp-PLA_2,[5] and both positive and negative correlations between this mutation and cardiovascular disease have been reported.[6–8] Based on the connection between Lp-PLA_2 and cardiovascular disease, as well as the genetic evidence that inactivation of Lp-PLA_2 may protect against coronary heart disease, this enzyme is considered a potential biomarker and therapeutic target for the treatment of atherosclerosis. Several studies have also connected elevated levels of Lp-PLA_2 expression to the progression of prostate cancer.[9–11] Interestingly, siRNA-induced Lp-PLA_2 inactivation in the VCaP human prostate cancer cell line has been shown to induce apoptosis and sensitize the cells to oxidative stress.[9] Thus, Lp-PLA_2 may also serve as a biomarker and therapeutic target for prostate cancer.

Due to the interest in Lp-PLA_2 as a therapeutic target, several inhibitors of this enzyme have been reported. These include β-lactams,[12] amides of xanthurenic acid,[13] and the compound darapladib,[14] which is in Phase III clinical trials for the treatment of atherosclerosis. Darapladib has been shown to significantly decrease plasma Lp-PLA_2 activity and atherosclerotic plaque formation in ApoE-deficient mice,[15] in diabetic and hypercholesterolemic swine,[16] and in human patients with coronary heart disease.[17,18]

Despite the generation of several classes of Lp-PLA_2 inhibitors, including a compound in late-stage clinical development, we are not aware of any detailed reports on the selectivity of these agents. This question is especially pertinent for inhibitors of serine hydrolases, like Lp-PLA_2, given the huge number of enzymes from this class in humans.[19] Lp-PLA_2 also lacks precise assays that report on its activity in complex biological systems. Measurements of

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Lp-PLA$_2$ activity are typically performed using either the artificial fluorescent substrate 1-decanoyl-2-(4-nitrophenylglutaryl)phosphatidylcholine (DNPG)[20] or radioactive substrate [^{3}H]-PAF,[21] neither of which can readily distinguish Lp-PLA$_2$ from several other PAF hydrolases that exist in mammalian proteomes.[22,23]

With these considerations in mind, we set out to develop and apply activity-based protein profiling (ABPP) methods[24,25] for characterizing Lp-PLA$_2$ and its inhibitors, including: (1) performing a high-throughput screen for novel classes of inhibitors, (2) identifying tailored activity-based probes for detecting Lp-PLA$_2$ activity in cell and tissue proteomes, and (3) evaluating established and newly discovered Lp-PLA$_2$ inhibitors for target engagement and selectivity in human cells. The high-throughput screen was performed using purified human Lp-PLA$_2$ protein[26,27] and a recently described fluorescence polarization-ABPP (FluoPol-ABPP) platform[28] in collaboration with the Molecular Libraries Screening Center Network at The Scripps Research Institute. FluoPol-ABPP assays showed time-dependent increases in fluorescence polarization of Lp-PLA$_2$ labeled with the serine hydrolase-directed activity-based probe fluorophosphonate-rhodamine (FP-Rh), while catalytically inactive enzyme, in which the serine nucleophile S273 was mutated to alanine (Lp-PLA$_2$SA), and no-enzyme control samples showed no increase in fluorescence polarization (Fig. 1). From a library of 326,141 compounds screened at 3.39 μM (PubChem AID: 463082), we identified 4934 compounds (1.5%) that reduced the fluorescence polarization (>24%) of Lp-PLA$_2$ labeling by the FP-Rh probe. A confirmation screen (AID 463230) was performed on 2500 compounds at the same concentration in triplicate and 1675 (71.6%) of the compounds were confirmed as active. From these results, we chose 153 compounds based on their high (>60%) inhibition of Lp-PLA$_2$, selectivity for Lp-PLA$_2$ compared to other screened enzymes and bioassays listed in PubChem, lack of undesired functional groups, and medicinal chemistry potential. We rescreened these 153 compounds versus recombinant mouse Lp-PLA$_2$ by gel-based competitive ABPP.[29] Briefly, lysates from HEK-293T cells transfected with a mouse Lp-PLA$_2$ cDNA were incubated with each test compound (5 μM) for 30 min at 37 °C and then reacted with FP-Rh (1 μM, 30 min, 25 °C). Proteomic reactions were run on SDS–PAGE and visualized by in-gel fluorescence scanning. Twelve compounds were confirmed to inhibit Lp-PLA$_2$ by >75% at 5 μM, and eight of these compounds were carbamates,

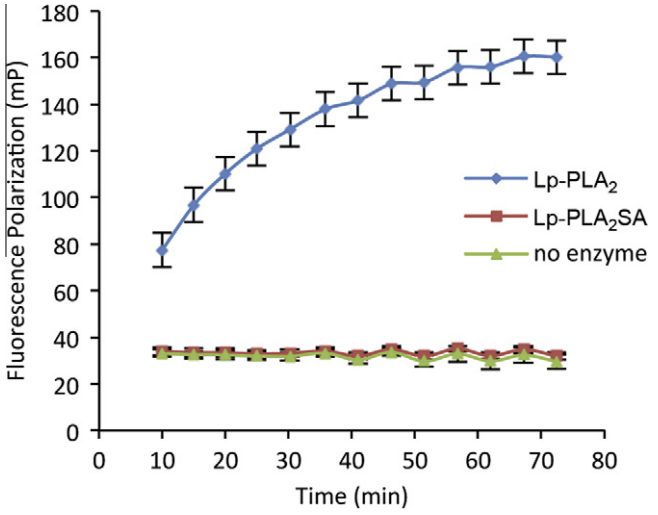

Figure 1. Lp-PLA$_2$ FluoPol-ABPP assay. A 10 nM sample of purified human Lp-PLA$_2$, catalytically inactive S273A mutant (Lp-PLA$_2$ SA), or no enzyme was incubated with 75 nM FP-Rh at room temperature. Fluorescence polarization was measured as a function of time. $Z' = 0.82$ at $t = 15$ min.

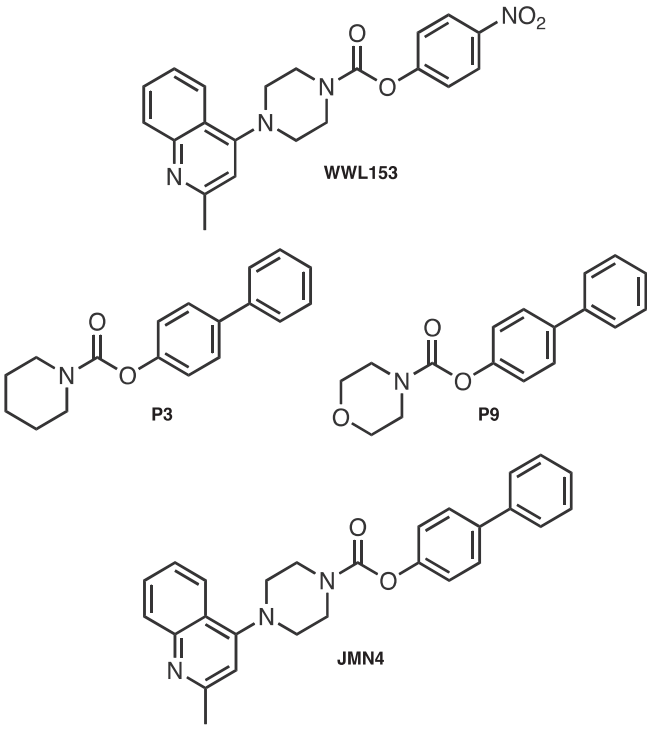

Figure 2. Structures of lead Lp-PLA$_2$ inhibitors WWL153, P3, P9, and of the optimized inhibitor JMN4.

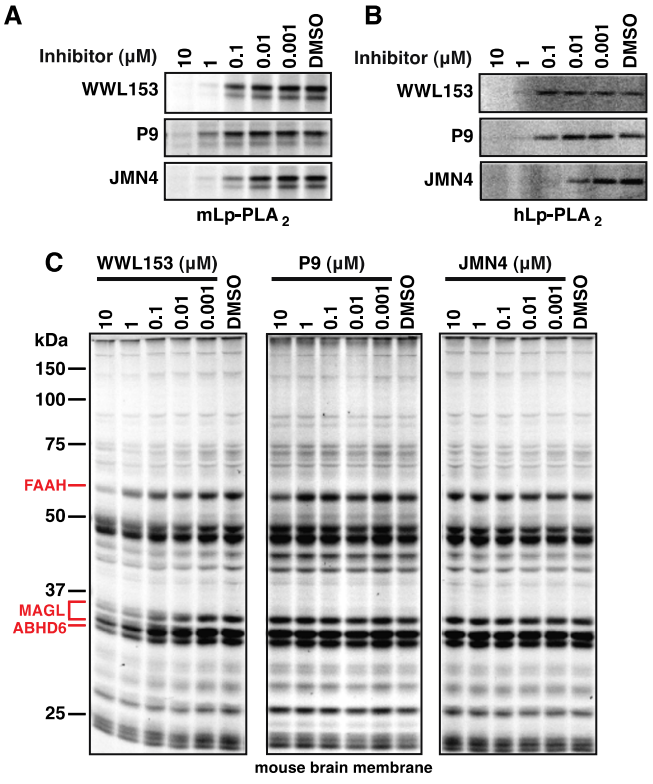

Figure 3. In vitro potency and selectivity profiles of Lp-PLA$_2$ inhibitors WWL153, P9, and JMN4. Evaluation of Lp-PLA$_2$ inhibitors by competitive ABPP with the FP-Rh probe and (A) recombinant mouse (mLp-PLA$_2$) and (B) human (hLp-PLA$_2$) Lp-PLA$_2$ enzymes at indicated concentrations. (C) Evaluation of the selectivity of Lp-PLA$_2$ inhibitors at indicated concentrations by competitive ABPP in the mouse brain membrane proteome. Fluorescent gels are shown in gray scale.

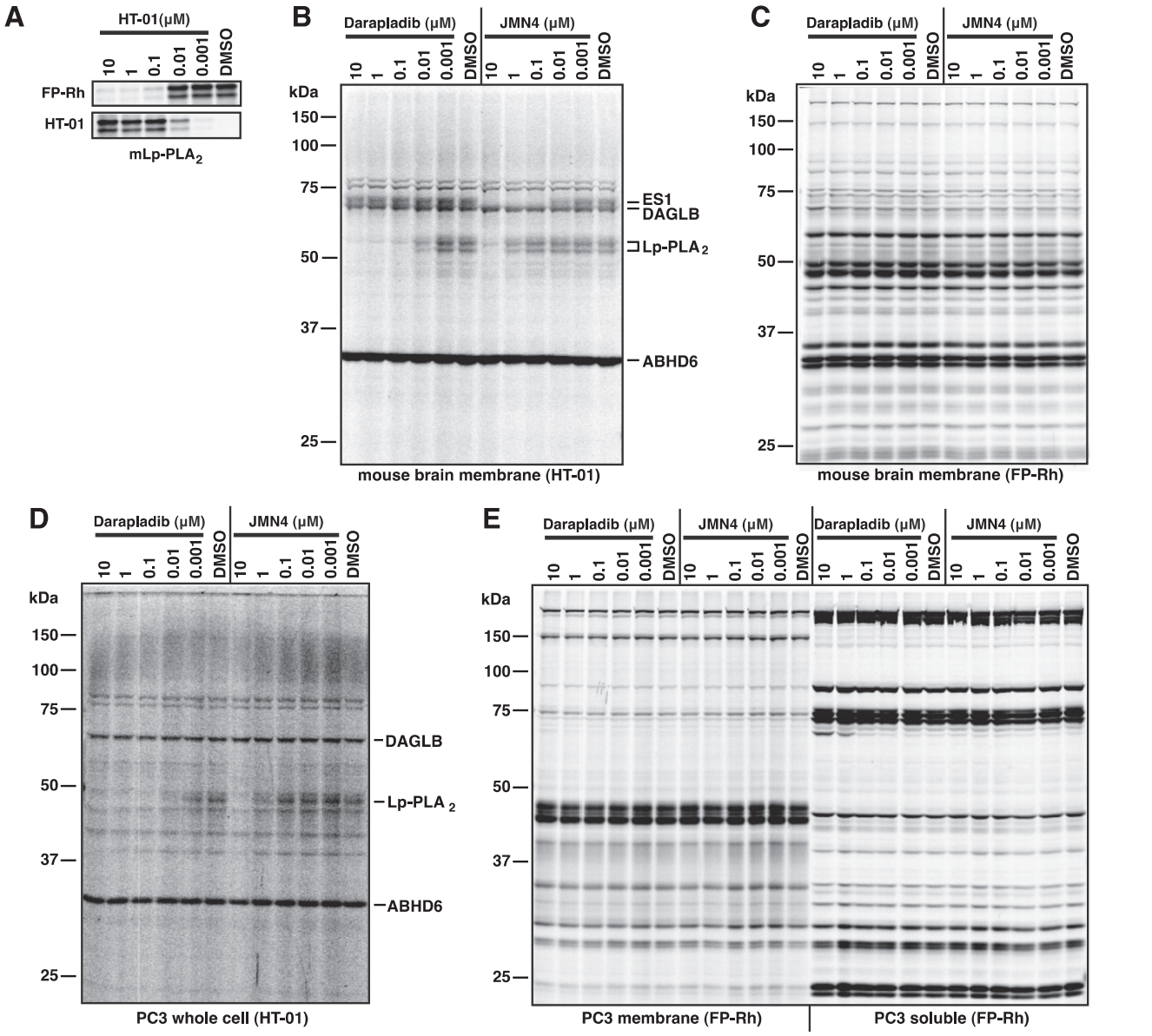

Figure 4. In vitro potency and selectivity profiles of darapladib and JMN4 in mouse brain membrane and PC3 prostate cancer cells. (A) Inhibition and labeling of recombinant mouse Lp-PLA_2 with HT-01. Inhibition was measured by competitive ABPP with the FP-Rh probe and direct detection of labeling by HT-01, which contains a BODIPY fluorophore. (B) Evaluation of darapladib and JMN4 inhibitory activity with mouse brain membranes probed with (B) HT-01 and (C) FP-Rh. (D) Evaluation of darapladib and JMN4 inhibitory activity in PC3 cell proteomes probed with (D) HT-01 and (E) FP-Rh.

two of which contained a biphenyl moiety (P3 and P9, Fig. 2). A previous study[30] from our lab identified a structurally distinct carbamate WWL153 (Fig. 2) as a lead inhibitor for Lp-PLA_2 that displayed good potency (IC_{50} = 290 nM), but sub-optimal selectivity (see below). We therefore combined the 2-methyl-4-piperazinoquinoline portion of WWL153 with the biphenyl moiety of P3 and P9 to create the compound JMN4 (Fig. 2).

Additional competitive gel-based ABPP assays revealed that JMN4 exhibited superior potency for inhibiting recombinant mouse (IC_{50} = 90 nM) and human (IC_{50} = 5.9 nM) Lp-PLA_2 compared to either WWL153 (IC_{50} = 290 nM for mouse Lp-PLA_2; IC_{50} = 250 nM for human Lp-PLA_2) or P9 (IC_{50} = 470 nM for mouse Lp-PLA_2; IC_{50} = 100 nM for human Lp-PLA_2) (Fig. 3A, B). Competitive ABPP experiments in the mouse brain proteome also revealed that JMN4 cross-reacted with fewer serine hydrolases than WWL153 and P9, both which inhibited FAAH at high concentrations (Fig. 3C). WWL153 also inhibited MAGL and ABHD6 (Fig. 3C). These data, taken together, designated JMN4 as a potent and selective inhibitor of mouse and human Lp-PLA_2.

It is important to note that, although Lp-PLA_2 is expressed in the mouse brain,[30] it is not visualized by gel-based ABPP using FP-Rh, presumably due to the presence of more abundant, co-migrating serine hydrolases. We reasoned that a more selective activity-based probe could facilitate the detection of endogenous Lp-PLA_2 in complex proteomes such as mouse brain. Recently, we reported a 1,2,3-triazole urea activity-based probe termed HT-01 and its use to visualize the low-abundance serine hydrolase DAGLβ in cell and tissue proteomes.[31] Examination of the HT-01 labeling profile of mouse brain membrane proteome revealed the presence of an additional tetrahydrolipstatin (THL)-sensitive serine hydrolase activity that exhibited a molecular weight (~55 kDa doublet) consistent with glycosylated Lp-PLA_2. We had previously shown that mouse Lp-PLA_2 was inhibited by THL[32] and various 1,2,3-triazole ureas[31] and found here that this enzyme was also inhibited by

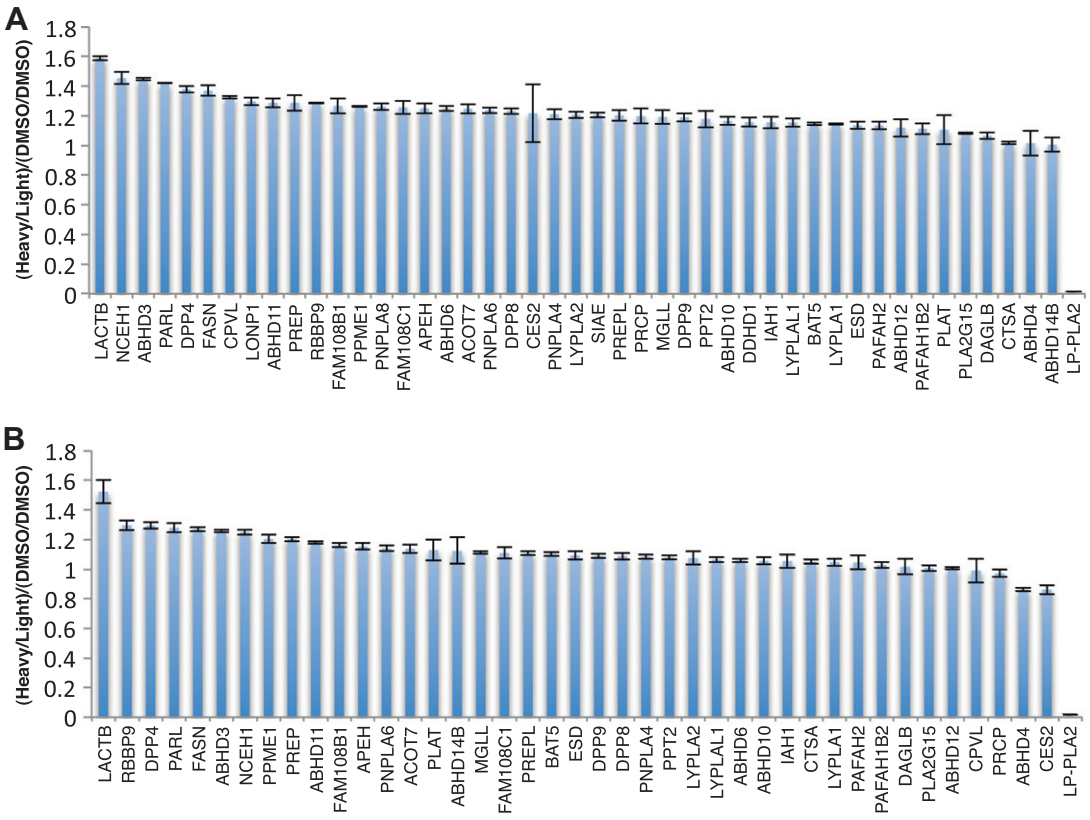

Fig. 5. ABPP-SILAC analysis of inhibitor-treated PC3 cells. Heavy-labeled cells were treated in situ for 4 h with 100 nM darapladib (A) or JMN4 (B); light-labeled cells were treated with DMSO. Data are reported as mean values ± SEM of all peptides quantified for each serine hydrolase. SILAC ratios were normalized to a control experiment where both heavy and light cells were treated with DMSO.

HT-01 with an IC_{50} of 39 nM (Fig. 4A). Based on these results, we tested both darapladib and JMN4 in gel-based competitive ABPP experiments with HT-01 and found that these compounds blocked HT-01 labeling of the 55 kDa mouse membrane enzyme, supporting its designation as endogenous Lp-PLA_2 (Fig. 4B). JMN4 showed lower potency against mouse brain Lp-PLA_2 (IC_{50} ~1 μM) compared to recombinant mouse Lp-PLA_2, which we speculate may be due to the greater amount of total proteome needed in the assay to detect endogenous Lp-PLA_2 in mouse brain (1.0 mg brain protein/mL vs 0.25 mg transfected cell protein/mL). HT-01 also identified a ~70 kDa protein that was inhibited by JMN4, but not darapladib (Fig. 4B). This enzyme is likely the blood-derived serine hydrolase carboxylesterase ES1, which has been a common off-target for many carbamate inhibitors.[30] Finally, we performed a competitive gel-based ABPP analysis with FP-Rh, which showed that neither darapladib nor JMN4 cross-reacted with other serine hydrolases detected in mouse brain at concentrations up to 10 μM (Fig. 4C).

The aforementioned studies indicated that JMN4 and darapladib were selective inhibitors of mouse Lp-PLA_2 and that HT-01 served as a tailored activity-based probe for detection of this enzyme in native proteomes. We next investigated the performance of these inhibitors/probes in human proteomes. As previously mentioned, human prostate cancer cells have been found to express Lp-PLA_2.[9–11] Consistent with these past studies, we detected an ~50 kDa protein in the PC3 human prostate cancer cell proteome that exhibited darapladib- and JMN4-sensitive labeling by the HT-01 probe (Fig. 4D). These features indicated that the 50 kDa protein was human Lp-PLA_2. Interestingly, as we previously observed in the mouse brain proteome (Fig. 4C), human Lp-PLA_2 could not be detected by the general serine hydrolase probe FP-Rh in the PC3 cell proteome (Fig. 4E), presumably due to overlapping signals from more abundant, co-migrating serine hydrolases. These FP-Rh profiles did, however, provide evidence that darapladib and JMN4 exhibited good selectivity for Lp-PLA_2 in that other detected serine hydrolase activities in the PC3 cell proteome were not inhibited by these compounds (Fig. 4E).

We next asked whether darapladib and JMN4 could inhibit human Lp-PLA_2 in situ by treating PC3 cells with these compounds and then analyzing the serine hydrolase activity profiles by the liquid chromatography–mass spectrometry (LC–MS) platform ABPP-SILAC.[33–36] PC3 cells were cultured in isotopically heavy or light media and treated with 100 nM darapladib or JMN4 (heavy cells) or DMSO (light cells) for 4 h. Cells were harvested, lysed, and labeled with the activity-based probe FP-biotin.[29] Light and heavy fractions were mixed in a 1:1 ratio, enriched with avidin-conjugated beads, digested on-bead with trypsin and analyzed by liquid chromatography–tandem MS (LC–MS/MS) using an LTQ-Velos Orbitrap instrument. SILAC ratios for each peptide were generated using the CIMAGE software[37] to quantify light and heavy signals from the parent ion (MS1) peaks. Protein identities were determined from product ion peptide profiles (MS2) using the ProLuCID search algorithm.[38] We also performed a control experiment where both heavy and light cells were treated with DMSO to control for the effects of isotopic growth media on protein expression and activity. This analysis showed that both darapladib (Fig. 5A) and JMN4 (Fig. 5B) completely inhibited Lp-PLA_2 in human prostate cancer cells, while not affecting any of the other ~40 serine hydrolases detected in these cells.

In conclusion, we have shown that both darapladib and the newly developed compound JMN4 are potent and selective inhibitors of Lp-PLA_2 in both mouse tissues and human cell lines, indicating that these compounds can be used as a structurally and mechanistically[39] distinct pair of probes to study Lp-PLA_2 function in biological systems. We also believe that the tailored probe HT-01 should prove useful for profiling Lp-PLA_2 activity in native proteomes, which is otherwise difficult to detect by gel-based ABPP assays using more general serine hydrolase-directed probes. We speculate, for instance, that HT-01 could form the basis for an ex vivo target engagement assay to confirm Lp-PLA_2 inhibition in human clinical studies, as has recently been performed for proteasome inhibitors using FP-biotin.[40]

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Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.bmcl.2012.11.061.

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