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Synthesis and Evaluation of Cytosolic Phospholipase A₂ Activatable Fluorophores for Cancer Imaging

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Abstract

Activatable fluorophores selective to cytosolic phospholipase A₂ (cPLA₂) were synthesized and evaluated for their ability to image triple negative breast cancer cells. The activatable constructs were synthesized by esterification of a small molecule fluorophore with a fatty acid resulting in ablated fluorescence. Selectivity for cPLA₂ was generated through the choice of fluorophore and fatty acid. Esterification with arachidonic acid was sufficient to impart specificity to cPLA₂ when compared to esterification with palmitic acid. *In vitro* analysis of probes incorporated into phosphatidylcholine liposomes demonstrated that a non-selective phospholipase (sPLA₂ group IB) was able to hydrolyze both arachidonate and palmitate coupled fluorophores resulting in the generation of fluorescence. Of the four fluorophores tested, DDAO (7-hydroxy-9H-(1,3-dichloro-9,9-dimethylacridin-2-one)) was observed to perform optimally *in vitro* and was analyzed further in 4175-Luc+ cells, a metastatic triple negative human breast cancer cell line expressing high levels of cPLA₂. In contrast to the *in vitro* analysis, DDAO arachidonate was shown to activate selectively in 4175-Luc+ cells compared to the control DDAO palmitate as measured by fluorescence microscopy and quantitated with fluorescence spectroscopy. The addition of two agents known to activate cPLA₂ enhanced DDAO arachidonate fluorescence without inducing any change to DDAO palmitate. Inhibition of cPLA₂ resulted in reduced fluorescence of DDAO arachidonate but not DDAO palmitate. Together, we report the synthesis of a cPLA₂ selective activatable fluorophore capable of detecting cPLA₂ in triple negative breast cancer cells.

Keywords

Phospholipase A₂; cPLA₂; sPLA₂; Fluorescence optical imaging; NIR fluorescence imaging; Breast cancer; Activatable fluorescence

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SUPPORTING INFORMATION

¹H NMR spectra for resorufin arachidonate (S1), resorufin palmitate (S2), DDAO arachidonate (S3), DDAO palmitate (S4), and naphthofluorescein diarachidonate (S5).

INTRODUCTION

Molecular imaging of cancer offers the potential to provide phenotypic and genotypic information in addition to anatomical tumor location. Real-time knowledge of the molecular characteristics of tumors allows for the determination, monitoring, and possible alteration of treatment protocols.¹ In addition, tools that can assess cancer signatures in *in vivo* models can fuel anti-cancer drug design and evaluation.² Fluorescence imaging allows for the development of activatable fluorophores, which are quenched until acted upon, commonly through enzymatic cleavage, resulting in the release of an active fluorophore.³⁻⁷ In addition to providing a high degree of functional information enzymatic activation can cleave multiple substrate molecules, allowing for amplification of signal through increased accumulation of fluorescent product, decreasing background signal and thus increasing detection sensitivity.⁸

Phospholipases, along with other enzymes involved in lipid metabolism, have been shown to have increased activity in cancers and function as potential biomarkers.^{7, 9-15} Increased lipid metabolism in tumors allows for higher membrane turnover associated with an increased rate of proliferation along with generating signaling molecules, which can influence cancer growth.^{16, 17} Cytosolic phospholipase A₂ (cPLA₂) is an active signaling phospholipase responsible for the release of arachidonic acid from peri-nuclear membranes.¹⁸⁻²⁰

Arachidonic acid can be metabolized by several enzymes including cyclooxygenase 2 (COX2), which is overexpressed in numerous cancers.²¹⁻²⁵ COX2 activity promotes cancer progression, largely through the production of the downstream metabolite prostaglandin E2 (PGE2), which has been reported to increase proliferation, evade apoptosis, sustain angiogenesis, and promote metastasis.^{26, 27} cPLA₂ activity itself has also been found to play a role in a number of cancers, which may be attributed to its role in PGE2 synthesis.^{18, 28-30}

In breast cancer, cPLA₂ expression has been shown to vary based on the genetic profile of the cells. Estrogen/progesterone receptor positive (luminal-like) tumors have significantly lower cPLA₂ activity compared with the more aggressive and therapy resistant HER2/*neu* and triple negative (basal-like) tumors.¹² Furthermore, cPLA₂ activation has been suggested to play a role in developing resistance to endocrine therapy.^{31, 32} Therefore, a means of monitoring cPLA₂ activity could offer a powerful tool for breast cancer diagnosis and treatment evaluation.

Near infrared phospholipase activatable fluorophores have been previously synthesized with selectivity for phospholipase C and A₂.^{7, 33, 34} These probes consisted of a modified phospholipid in which the head group was linked to a dark quencher and the *sn*-2 fatty acid on the glycerol backbone was replaced with a fluorophore. Hydrolysis by phospholipase A₂ or C removes the fluorophore or the quencher respectively from the molecule and the separation of the two moieties results in fluorescence generation. Specificity for phospholipase type was controlled by altering the length of the *sn*-2 linker, with longer linkers being more amenable to cleavage by PLA₂ due to reduced steric hindrance. In designing a cPLA₂ activatable fluorophore, arachidonic acid at the *sn*-2 position is necessary, since cPLA₂ is the only phospholipase with absolute specificity for arachidonic acid at this position.³⁵ Furthermore, crystallographic analysis of cPLA₂ identifies a small

pocket where the phospholipid head group lies during lipolysis.³⁶ The presence of a large quencher at this position would most likely decrease activity. Therefore a simpler probe design is needed.

Previous studies have identified a small arachidonic acid caged fluorophore, 7-hydroxycoumarin arachidonate (7-HCA) which when incubated with purified cytosolic phospholipase A₂ results in hydrolysis and release of 7-hydroxycoumarin (7-HC) and arachidonic acid.^{37, 38} This reaction occurred at an identical rate to enzymatic cleavage of phospholipids, suggesting that a small fluorophore esterified with arachidonic acid could be substituted for the endogenous substrate of cPLA₂ and that this conjugate can function as a cPLA₂ activatable probe. Though promising, the low emission wavelength of 7-HC (460 nm) leads to significant absorption by biological tissue, thus limiting the *in vivo* and clinical potential of 7-HCA.³⁹ Several red-shifted small fluorophores contain similar resonance active hydroxyl groups that can be coupled to arachidonic acid to generate similar activatable probes that may have utility for *in vivo* detection of cPLA₂ activity.

In this study, we developed a series of cPLA₂ activatable imaging agents using arachidonic acid coupled to fluorophores. By using red-shifted fluorophores, we can minimize the effects of light absorption by hemoglobin, thereby increasing tissue penetration and the potential *in vivo* utility. We first sought to address whether arachidonic acid coupled to these fluorophores are cleavable by phospholipases. Secondly, we determined whether arachidonic acid confers selectivity towards cPLA₂. Finally, we assessed the ability for optimized probes to image cPLA₂ activity in 4175-Luc+ triple negative breast cancer cells.

RESULTS AND DISCUSSION

Synthesis of Fatty Acid Coupled Caged Fluorophores

cPLA₂ activatable probes were synthesized by esterifying a fatty acid to a fluorophore through a resonant active hydroxyl group resulting in caged fluorescence. Fluorophores with red-shifted excitation/emission profiles are ideal, allowing for the potential of *in vivo* imaging. Three red-shifted fluorophores were chosen as candidates for the synthesis of a cPLA₂ activatable probe (Table 1).

Resorufin (ex/em: 572/585 nm) is a small tricyclic fluorophore with one available hydroxyl group. Resorufin has been shown to have ablated fluorescence upon conjugation to a photoremovable protecting group.⁴⁰ Naphthofluorescein (ex/em: 595/660 nm) contains two resonant active hydroxyl groups and has been shown previously to have reduced fluorescence upon conjugation.⁴¹ These two hydroxyls are available for esterification, allowing for the synthesis of a diarachidonate probe. 7-Hydroxy-9H-(1,3-dichloro-9,9-dimethylacridin-2-one) (DDAO) (ex/em: 600/660 nm) is an *ortho*-dichlorinated resorufin derivative that results in red-shifted fluorescence. DDAO has also been conjugated to form activatable fluorophores through conjugation of its hydroxyl group resulting in photoactivatable and sulfatase activatable probes.^{42, 43}

Five potential phospholipase sensitive probes were synthesized, 3 containing arachidonate: naphthofluorescein diarachidonate (98% yield), resorufin arachidonate (19.9% yield), and

DDAO arachidonate (65% yield). In order to control for enzymatic selectivity, resorufin and DDAO were also coupled to palmitic acid, a 16-carbon saturated fatty acid not expected to be cleavable by cPLA₂. These syntheses resulted in resorufin palmitate (14.3% yield), and DDAO palmitate (33% yield). (Figure 1) Esterification of the hydroxyl groups with fatty acyl chlorides was carried out in pyridine, which we found to be the best solvent for this reaction type.⁴⁴

Reaction progress could be visually monitored by the characteristic shifts in color following esterification. In the unesterified state, dissolved fluorophores resulted in a dark red solution. Following esterification, the solution became a light yellow. Individual probes were purified with column chromatography and structures were confirmed with mass spectrometry and ¹H Nuclear Magnetic Resonance (¹H NMR). Due to the asymmetry present in the fluorophore DDAO, two tautomeric forms arising from proton transfer between the hydroxyl and ketone groups are possible. However, despite this tautomerization, ¹H NMR analysis did not show a mixture of isomers indicating only one product was obtained. Previous literature indicated that substitution of the hydroxyl on the chlorinated ring occurred to a much higher extent.^{42, 45} The acylation on the dichloro side of DDAO was supported by analysis of the ¹H NMR spectra. In the NMR spectrum of resorufin arachidonate the protons on the alpha and beta methylene groups of arachidonate O-C(O)-CH₂-CH₂- had a different chemical shift indicating that these methylene groups are pro-*RS*-stereogenic (diastereotopic)⁴⁶⁻⁴⁸ and are in different environments (see NMR data in Materials and Methods section and Supporting Information). Modeling of resorufin arachidonate with Chem3D Pro, version 13.0.3015, revealed that the first two methylene fragments of the arachidonoyl moiety form a plane that is *not* perpendicular to the aromatic ring of resorufin. A similar tilted structure was observed when modeling the DDAO arachidonate isomer substituted at the non-chlorinated side. In contrast, when the isomer substituted at the dichloro side was modeled, the O-C(O)-CH₂-CH₂- plane was orthogonal to the DDAO aromatic ring. In this case, the isomer environment is the same for the methylene protons and they would not be distinguishable in the proton NMR spectrum, consistent with our experimental observations (see NMR data).

Characterization of Spectral Properties

For all experiments, probes were packaged into egg-phosphatidylcholine liposomes at a mole fraction of 0.1. Synthesized probes were characterized for their absorbance and fluorescence properties compared to free fluorophore. Naphthofluorescein displayed a maximum absorbance peak at 600 nm (Table 1). Following conjugation, this absorbance was lost. When conjugated, resorufin underwent a shift from absorbance at 572 nm to absorbance at 450 nm. Similarly, DDAO conjugation resulted in a shift from a broad absorbance peak between 600 and 650 nm, to an absorbance peak between 400 and 450 nm. The loss of absorbance ablates fluorescence, creating a mechanism for activatable fluorescence following hydrolysis. This is shown in Figure 2 for DDAO arachidonate. To determine the theoretical extent of quenching achieved through this mechanism for DDAO, we analyzed the ionized fluorophore at pH 9 versus the protonated fluorophore at pH 3.25 for fluorescence (ex 600, em 660). The fluorescence of the protonated fluorophore was 1.4%

of that of ionized fluorophore, indicating a very high degree of quenching when DDAO is locked in a protonated or esterified state.

Probe Activation

To test for sensitivity to phospholipase cleavage, 1 nmol of probe was incubated with porcine secretory phospholipase A₂ group IB (sPLA₂ GIB) and fluorescence generation monitored as a function of time (Figure 3). sPLA₂ GIB is a pancreatic phospholipase with no selectivity for arachidonic acid. Naphthofluorescein diarachidonate displayed a low initial fluorescence (Figure 3A). Addition of sPLA₂ resulted in increased fluorescence generation that positively correlated with enzyme concentration. However the relative fluorescence units (R.F.U.) were low, remaining under 20 R.F.U. even for the maximum concentration of enzyme added. Naphthofluorescein diarachidonate was the largest fluorophore synthesized, and it is possible that steric hindrance contributed to poor enzymatic cleavage. In addition, naphthofluorescein has a pK_a of 7.6, which may contribute to the poor relative fluorescence observed at pH 7.4. At a pH of 8.0 it was possible to increase relative fluorescence generation nine fold (data not shown). Because of these observed limitations, a naphthofluorescein dipalmitate control probe was not synthesized. Resorufin arachidonate and resorufin palmitate both resulted in increased fluorescence with time, which was positively correlated to the concentration of sPLA₂ GIB (Figure 3B,C). However, a high initial fluorescence was observed indicating either poor intramolecular quenching or rapid enzymatic hydrolysis. Even in controls conducted without the addition of enzyme, initial fluorescence was fairly high and increased over time. This would indicate that the resorufin-conjugated fatty acids may be susceptible to non-specific aqueous hydrolysis in addition to enzymatic cleavage. DDAO arachidonate and palmitate both showed activation in the presence of sPLA₂ GIB that positively correlated with enzymatic concentration (Figure 3D,E). Initial fluorescence of both DDAO arachidonate and DDAO palmitate was low (less than 100 R.F.U.) and a significant ($p = 5.7 \times 10^{-26}$ and $p = 2.8 \times 10^{-14}$ respectively for 100 units sPLA₂ at 720 min) time-dependent increase in fluorescence was observed in the presence of sPLA₂. The large difference in initial fluorescence observed between DDAO and resorufin conjugates may be due to steric or electrostatic protection by the two chloride groups at two *ortho*- positions to the ester bond in DDAO. This is consistent with previous literature showing 2,6-dichlorophenyl acetate has a hydrolysis constant that is 1.5 times lower than phenol acetate.⁴⁹ For DDAO arachidonate, a more rapid initial rate of fluorescence activation was observed compared to the palmitate probe, suggesting arachidonate conjugation results in a more readily hydrolyzable fluorophore. Because of DDAO's high initial quenching and significant increase in fluorescence versus other fluorophores, it was chosen as our candidate cPLA₂ probe for further studies.

In order to confirm that DDAO arachidonate and palmitate fluorescence activation directly results from phospholipase activity, experiments were performed using an sPLA₂ inhibitor, LY311727 (Figure 4). In the presence of LY311727, fluorescence generation was significantly reduced for both probes (Figure 4A,B). Quantitation of the final fluorescence intensity at 720 minutes (Figure 4C) showed that both DDAO arachidonate and DDAO palmitate fluorescence was significantly ($p = 1.4 \times 10^{-11}$ and $p = 3.2 \times 10^{-7}$ respectively) reduced in the presence of LY311727. The final fluorescence intensity of DDAO

arachidonate was not significantly different from that of DDAO palmitate. This suggests both DDAO arachidonate and DDAO palmitate are being fully hydrolyzed directly through the actions of sPLA₂ GIB, albeit at different rates. As expected, because sPLA₂ GIB has no selectivity for arachidonic acid, no difference in final fluorescence was observed. However, DDAO arachidonate in the absence of enzyme did give significantly ($p = 3.1 \times 10^{-11}$) higher fluorescence than that of DDAO palmitate indicating DDAO arachidonate may be more sensitive to aqueous hydrolysis. This may be an additional factor contributing to the differing rates of enzymatic hydrolysis observed.

Using DDAO Arachidonate to Image Cancer Cells

DDAO arachidonate was assessed for its ability to measure cPLA₂ activity in the 4175-Luc+ cell line. 4175-Luc+ is a triple negative breast cancer cell line derived from a murine lung metastasis of the MDA-MB-231 line and engineered to express luciferase.⁵⁰ MDA-MB-231 cells have previously been shown to have increased cPLA₂ activity consistent with observations of increased cPLA₂ expression in triple negative basal-like breast cancer.³² 4175-Luc+ cells were incubated with DDAO arachidonate or DDAO palmitate for up to 3 hours and co-stained with the nuclear stain Syto 9. The cells were analyzed with wide field fluorescent microscopy (Figure 5A). At 30 min weak intracellular fluorescence from DDAO arachidonate was observable with no fluorescence visible from DDAO palmitate. At 3 hours, strong intracellular fluorescence was seen from DDAO arachidonate, however no fluorescence was present in cells incubated with DDAO palmitate. DDAO arachidonate fluorescence was dispersed throughout the cytoplasm but with high perinuclear fluorescence, consistent with the subcellular location of cPLA activity.^{51,52} In order to quantitate fluorescence activation, the experiment was repeated in a clear-bottom 96 well black wall plate and fluorimetry was used to measure fluorescence (ex/em: 600/660 nm) with time (Figure 5B). Quantitation showed that DDAO arachidonate gave a strong time-dependent increase in fluorescence whereas little change was observed in DDAO palmitate incubated cells. Unlike the non-selective sPLA₂ GIB cleavage studies shown in Figure 3, DDAO arachidonate displayed a significantly ($p = 1.3 \times 10^{-6}$) higher final fluorescence intensity in cells compared to DDAO palmitate. The final fluorescence intensity was also noted to be lower than the relative fluorescence achieved through *in vitro* studies. This may be attributed to a combination of increased scattering and absorption of light by cells, differences in enzyme concentration, along with variations in cellular uptake. However, these results indicate DDAO arachidonate is specifically activated in 4175-Luc+ breast cancer cells whereas DDAO palmitate is not.

To determine whether the observed differences in activation were the result of cPLA₂, two agents which induce cPLA₂ activity were employed: ionomycin and hydrogen peroxide. Ionomycin is a calcium ionophore, which when incubated with cells leads to calcium release from intracellular stores into the cytosol.⁵³ cPLA₂, which resides in the cytosol, requires calcium for translocation to membranes where it can act on membrane localized phospholipids.^{54, 55} Previous work has indicated hydrogen peroxide as a potent activator of cPLA₂ activity, partly due to phosphorylation through the MAPK pathway.⁵⁶

Incubation of 4175-Luc+ cells with either ionomycin (2 μ M) or hydrogen peroxide (300 μ M) in the presence of DDAO arachidonate resulted in a significant ($p = 1.9 \times 10^{-7}$ and $p = 2.9 \times 10^{-6}$ respectively) increase in the final relative fluorescence (Figure 6A,B). However, neither ionomycin nor hydrogen peroxide had a significant effect on DDAO palmitate fluorescence. It was noted that at 3 hours, cells treated with ionomycin began showing signs of stress, most notably rounding of the cells and detachment from the plate to a small extent. It is possible that the slight non-significant increase in DDAO palmitate fluorescence could be attributed to increased light penetration resulting from rounded up cells.

Phospholipase specificity was further evaluated by monitoring fluorescence following incubation with several phospholipase inhibitors (Figure 6C). Inhibitors were added to 4175-Luc+ cells for four hours at a concentration of 10 μ M, cells were then washed in PBS and fresh media containing either DDAO arachidonate or DDAO palmitate was added. Fluorescence (ex 600, em 660) was measured following 30 min of incubation. Arachidonyl trifluoromethyl ketone (AACOCF₃), a cPLA₂ and iPLA₂ selective inhibitor, resulted in a significant reduction in DDAO arachidonate fluorescence compared to untreated controls ($p = 0.003$). Bromoenol lactone (BEL), a iPLA₂ selective inhibitor resulted in no significant change in fluorescence. These data indicate that the fluorescence inhibition caused by AACOCF₃ is a direct result of cPLA₂ activity and not iPLA₂ activity. *In vitro* studies (Figures 3 and 4) indicated that DDAO arachidonate is also susceptible to cleavage by sPLA₂. Therefore to control for sPLA₂ selective activation, the sPLA₂ inhibitor LY311727 was used. A small decrease in fluorescence was witnessed in the presence of LY311727, however this was not significant. DDAO palmitate gave no significant changes in fluorescence in the presence of any of the inhibitors. We conclude that although DDAO arachidonate is susceptible to sPLA₂ mediated cleavage *in vitro*, the fluorescence observed in cancer cells directly results from cPLA₂ activity.

In summary, our results demonstrate that both DDAO arachidonate and DDAO palmitate were activated to the same degree by a non-selective phospholipase. The incorporation of arachidonic acid conferred selectivity for cPLA₂ and upon incubation with triple negative breast cancer cells, only DDAO arachidonate resulted in fluorescence generation. Enhancing cPLA₂ activity through inducing agents led to increased fluorescence of DDAO arachidonate but not DDAO palmitate. Inhibition of cPLA₂ resulted in decreased fluorescence of DDAO arachidonate but not DDAO palmitate. Inhibitors of iPLA₂ and sPLA₂ had no significant effects on fluorescence of DDAO arachidonate. Furthermore, DDAO arachidonate was taken up into cells and was activated on the peri-nuclear membrane giving further indication that cPLA₂ is directly responsible for cleavage. DDAO palmitate, being activated by sPLA₂ to the same extent as DDAO arachidonate, but not by cPLA₂, can serve as a useful control for non-selective cleavage when assaying cPLA₂ activity.

Together, we present evidence for the first red-emitting cPLA₂ activatable fluorophore. We demonstrated this probe's ability to activate in cancer cell lines expressing cPLA₂, specifically triple negative breast cancer. The red fluorescence of this probe lies at the edge of the near infrared window, a region of wavelengths that are ideal for *in vivo* imaging, and therefore may allow for the detection of cPLA₂ in tumors in addition to cells. Finally, we

believe this probe may offer the potential to image other disease models that have overactive cPLA₂ such as inflammation and atherosclerosis.

EXPERIMENTAL PROCEDURES

Materials

Resorufin, resorufin sodium salt, naphthofluorescein, palmitoyl chloride, phospholipase A₂ from porcine pancreas, arachidonyl trifluoromethyl ketone, bromoenol lactone, ionomycin, and H₂O₂ 30% (w/w) were purchased from Sigma-Aldrich. DDAO, arachidonoyl chloride, and LY311727 were purchased from Santa Cruz Biotechnology. 7-HCA was purchased from Cayman Chemical. Syto 9 was purchased from Life Technologies. Egg phosphatidylcholine was purchased from Avanti Polar Lipids, Inc.

¹H NMR and Mass Spectrometry

¹H NMR spectra was obtained in CDCl₃ on a Bruker DMX 360. Spectra were analyzed with MestReNova. Matrix-assisted laser desorption/ionization time of flight (MALDI-TOF) was performed on a Bruker MALDI-TOF. Samples were prepared in saturated solutions of either 2-(4'-hydroxybenzeneazo)benzoic acid or α-cyano-4-hydroxycinnamic acid as a 50:50 mixture (acetonitrile/H₂O) with 0.1% trifluoroacetic acid. Electro spray ionization (ESI) mass spectrometry was performed on a Waters SQD equipped with an Acquity UPLC. Samples were passed through an acetonitrile gradient in water of 80 to 20%.

Chemical Synthesis

Naphthofluorescein diarachidonate—Naphthofluorescein (0.0775 mmol, 33.5 mg) was dissolved in 30 mL pyridine at room temperature. Arachidonoyl chloride (100 mg, 0.31 mmol) was added to the solution. The flask was flushed with argon and left to stir at room temperature for 24 hours. Pyridine was removed through rotary evaporation and the resulting solid was dissolved in hexanes and filtered through a porous cotton plug to remove pyridinium salts. The solution was purified with flash column chromatography (silica gel, under N₂) in 25 mL gradients beginning with ethyl acetate : dichloromethane (DCM) (2:8, v/v), followed by ethyl acetate : DCM (7:3, v/v), ethyl acetate (pure), and methanol : ethyl acetate (3:7, v/v). The product was isolated as a dark solid (76.4 mg, 98% yield). ¹H NMR (360 MHz, CDCl₃, δ ppm) 8.51 (d, 2H, J = 9.0 Hz, -CH- (Ar)) 7.97 (m, 1H, -CH- (Ar)) 7.55 (m, 2H, -CH- (Ar)) 7.22 (dd, 4H, J = 9.0, 2.63 Hz, -CH- (Ar)), 7.05 (d, 3H, J = 2.4 Hz, -CH- (Ar)), 6.60 (d, 2H, J = 8.8 Hz, -CH- (Ar)) 5.25 (m, 16H, -CH=), 2.70 (m, 12H, =CH-CH₂-CH=), 2.20 (t, 4H, J = 8.0 Hz, -CH₂C(O)), 1.97 (m, 8H, -CH₂-CH₂-CH₂C(O)), 1.58 (q, 4H, Bu-CH₂-CH=), 1.19 (m, 12H, CH₃-CH₂-CH₂-CH₂-), 0.77 (t, 6H, J = 6.7 Hz, CH₃-). EI-MS (m/z): 1004.51 (M)⁺.

Resorufin arachidonate—Resorufin (0.0775 mmol, 16.5 mg) was dissolved in 50 mL of pyridine and slightly cooled with liquid N₂. Resorufin was not completely soluble in solution. Arachidonoyl chloride (0.31 mmol, 100 mg) was added to the solution. The flask was flushed with argon and left to stir at room temperature for 24 hours. Following the reaction, the solution was clear with no solids present. Pyridine was removed through rotary evaporation and the solid was dissolved in hexanes: DCM (1:1, v/v) and filtered through a

porous cotton plug to remove pyridinium salts. The solution was purified with flash column chromatography (silica gel, under N₂) in 25 mL gradients beginning with hexanes : DCM (1:1, v/v), followed by hexanes : DCM (9:1, v/v), hexanes : DCM (8:2, v/v), hexanes : DCM (7:3, v/v), hexanes : DCM (6:4, v/v), hexanes : DCM (1:1, v/v), hexanes : DCM (3:7, v/v), hexanes : DCM (1:9, v/v) DCM (pure), DCM : ethyl acetate (19:1, v/v). The product was isolated as a yellow solid (7.7 mg, 19.9% yield). ¹H NMR (360 MHz, CDCl₃, δ ppm) 7.80 (d, 1H, J = 8.7 Hz, -CH- (Ar)), 7.44 (d, 1H, J = 7.4 Hz, -CH- (Ar)), 7.15 (d, 1H, J = 2.2 Hz, -CH- (Ar)) 7.12 (dd, 1H, J = 8.6, 2.5 Hz, -CH- (Ar)), 6.86 (dd, 1H, J = 9.8, 2.0 Hz, -CH- (Ar)), 6.33 (d, 1H, 2.0 Hz, -CH- (Ar)), 5.38 (m, 8H, -CH=), 2.83 (m, 6H, =CH-CH₂-CH=), 2.62 (t, 1H, J = 7.5 Hz, pro-*RS*-stereogenic -CHHC(O)), 2.37 (t, 1H, J = 7.5 Hz, pro-*RS*-stereogenic -CHHC(O)), 2.23 (q, 2H, J = 7.2 Hz, -CH₂-CH₂-CH₂C(O)) 2.14 (q, 2H, J = 7.2 Hz, Bu-CH₂-CH=), 1.86 (quintet, 1H, J = 7.5 Hz, pro-*RS*-stereogenic -CH₂-CHH-CH₂C(O)) 1.73 (quintet, 1H, J = 7.5 Hz, pro-*RS*-stereogenic -CH₂-CHH-CH₂C(O)) 1.30 (m, 6H, CH₃-CH₂-CH₂-, 0.89 (t, 3H, J = 6.9 Hz, CH₃-). EI-MS (m/z): 500.45 (M + 1)⁺.

DDAO (7-Hydroxy-9H-(1,3-Dichloro-9,9-Dimethylacridin-2-One)) arachidonate

—DDAO (0.0325 mmol, 10 mg) was dissolved in 50 mL pyridine. Arachidonoyl chloride (0.065 mmol, 27 μL) was added to the solution and the reaction was carried out as described above. The solid product was dissolved in a hexane : DCM (1:1, v/v), filtered and purified with flash column chromatography using 25 mL gradients of hexanes : DCM (3:7, 2:8, 1:9, v/v), DCM (pure), DCM : ethyl acetate (19:1, 17:3, 7:3, 4:6, and 2:8, v/v). The product was isolated as a yellow solid (12.9 mg, 65% yield). ¹H NMR (360 MHz, CDCl₃, δ ppm) 7.64 (d, 1H, J = 8.6 Hz, -CH- (Ar)), 7.62 (s, 1H, -CH- (Ar)), 7.21 (d, 1H, J = 2.4 Hz, -CH- (Ar)), 7.11 (dd, 1H, J = 8.6, 2.4 Hz, -CH- (Ar)), 5.37 (m, 8H, -CH=), 2.81 (m, 6H, =CH-CH₂-CH=), 2.59 (t, 2H, J = 7.5 Hz, -CH₂C(O)), 2.21 (q, 2H, J = 7.2 Hz, -CH₂-CH₂-CH₂C(O)), 2.03 (q, 2H, J = 6.8 Hz, Bu-CH₂-CH=), 1.86 (s, 6H, C(CH₃)₂), 1.27 (m, 8H, CH₃-CH₂-CH₂-CH₂- and -CH₂-CH₂C(O)), 0.87 (t, 3H, J = 7.2 Hz, -CH₂-CH₃). MALDI-MS (m/z): 593.94 (M)⁺.

DDAO (7-Hydroxy-9H-(1,3-Dichloro-9,9-Dimethylacridin-2-One)) palmitate—

DDAO (0.0325 mmol, 10 mg) was dissolved in 50 mL pyridine. Palmitoyl chloride (0.065 mmol, 20 μL) was added to the solution. The reaction proceeded as described above and the resulting solid was dissolved in a hexane : DCM (1:1, v/v), filtered and purified with flash column chromatography using 25 mL gradients of DCM (pure), DCM : ethyl acetate (19:1, 9:1, 8:2, and 1:1, v/v). The product was isolated as a yellow solid (5.9 mg, 33% yield). ¹H NMR (360 MHz, CDCl₃, δ ppm) 7.65 (d, 1H, J = 8.5 Hz, -CH- (Ar)), 7.62 (s, 1H, -CH- (Ar)), 7.21 (d, 1H, J = 2.4 Hz, -CH- (Ar)), 7.11 (dd, 1H, J = 8.6, 2.4 Hz, -CH- (Ar)), 2.57 (t, 2H, J = 7.6 Hz, -CH₂C(O)), 1.86 (s, 6H, C(CH₃)₂), 1.75 (quintet, 2H, J = 7.5 Hz, -CH₂-CH₂C(O)), 1.24 (m, 24H, CH₃-CH₂-CH₂-CH₂-CH₂-), 0.86 (t, 3H, J = 6.9 Hz, -CH₂-CH₃). MALDI-MS (m/z): 567.24 (M + 1 + Na)⁺.

Resorufin palmitate—Resorufin sodium salt (0.0774 mmol, 18.2 mg) was dissolved in 80 mL of pyridine and slightly cooled with liquid N₂. Resorufin was not completely soluble in solution. Palmitoyl chloride (0.31 mmol, 95 μL) was added to the solution. The reaction proceeded as described above and the resulting solid was dissolved in hexanes, filtered and

purified with flash column chromatography using 25 mL gradients of hexanes:DCM (9:1, 8:2, 6:4, 2:8, v/v), DCM (pure), DCM:ethyl acetate (9:1, v/v), ethyl acetate (pure). The product was isolated as a yellow solid (5.0 mg, 14.3 %). ¹H NMR (360 MHz, CDCl₃, δ ppm) 7.80 (d, 1H, J = 8.8 Hz, -CH- (Ar)), 7.43 (d, 1H, J = 10.1 Hz, -CH- (Ar)), 7.15 (d, 1H, J = 2.2 Hz, -CH- (Ar)), 7.12 (dd, 1H, J = 8.6, 2.5 Hz, -CH- (Ar)), 6.86 (dd, 1H, J = 9.8, 2.0 Hz, -CH- (Ar)), 6.33 (d, 1H, J = 1.8 Hz, -CH- (Ar)), 2.60 (t, 2H, J = 7.6 Hz, -CH₂C(O)), 1.77 (quintet, 2H, J = 7.7 Hz, -CH₂-CH₂C(O)), 1.26 (m, 24H, CH₃-CH₂-CH₂-CH₂-CH₂-CH₂-), 0.88 (t, 3H, J = 7.0 Hz, CH₃-). EI-MS (m/z): 452.21 (M + 1)⁺.

Liposome Preparation—0.1 μmol of probe was mixed with 0.9 μmol of egg-phosphatidylcholine (Avanti Polar Lipids, Inc.) in chloroform. Chloroform was removed under a stream of nitrogen gas. 1 mL of 10 mM HEPES (pH 7.0) with 0.1 μM EDTA and 1.1 mM CaCl₂ was added to the dried lipid cake. The lipid cake was rehydrated in a water bath sonicator (4 °C) until clear.

sPLA₂ Cleavage Assay—In a 96 well plate, 80 μL of 50 mM Tris (pH 7.4) with 1.1 mM CaCl₂ was mixed with 0-100 Units of sPLA₂ Group IB (Sigma) in the presence or absence of 100 μM LY311727 (Santa Cruz Biotech) in DMSO resulting in a total volume of 90 μL per well. To initiate the reaction, 10 μL of liposomes containing the probe were added to each well and fluorescence was measured as a function of time on a Spectra Max M5 plate reader (top read; ex/em: 600/660 nm DDAO, 595/660 nm naphthofluorescein, 572/585 nm resorufin).

Cell Culture—4175-Luc+ cells, which are positive for luciferase and green fluorescent protein, were obtained from Dr. Andy Minn of the Abramson Family Cancer Research Center, University of Pennsylvania in August of 2013 with permission from Dr. Joan Massagué of Memorial Sloan Kettering Cancer Center, and used for all experiments. These cells are a mouse-adapted derivative of the human triple negative line MDA-MB-231 harvested from a lung metastasis.⁵⁰

The cells were maintained at 37°C and 5% CO₂ in DMEM supplemented with 10% fetal bovine serum, 1% L-glutamine, and 1% penicillin-streptomycin. Selection with 5 mg/mL blasticidin containing media was performed prior to experiments. Cells were passaged by washing 2 times with 4°C PBS, suspending in 1 mL of 0.25% trypsin, and diluting into 37°C supplemented DMEM. Cells were used up to 20 passages and then discarded. For experiments, cells were suspended in trypsin containing 0.25% EDTA, counted, and plated in 96 or 24 well plates at 2.5 × 10⁵ cells/mL.

Cellular Fluorescence Assay—4175-Luc+ cells were seeded at 2.5 × 10⁵ cells / ml in 0.1 mL and cultured for 24 hours in a 96 well black-wall, clear bottom plate. The media was aspirated and cells were washed 1x in 0.1 mL PBS (4°C, without Ca²⁺ and Mg²⁺) and 90 μL of phenol red free DMEM containing drug treatments was added to each well. For phospholipase inhibition experiments, 10 μM of inhibitor was incubated with cells for four hours, at which point cells were washed in PBS and fresh phenol red free DMEM was added. Assays were initiated by adding 10 μL of liposomes containing DDAO fluorescent

probes to the wells. Fluorescence was monitored as a function of time on a Spectra Max plate reader at 37 °C (bottom read, ex/em: 600/660 nm).

Fluorescent Microscopy—4175-Luc+ cells were seeded at 2.5×10^5 cells / mL in 0.5 mL and cultured overnight in a 24 well plate. The media was aspirated and the cells were washed 2x in PBS. Phenol red free DMEM (450 μ L) with 1 μ M Syto 9 (Invitrogen) was added to each well and cells were incubated three hours prior to analysis. Liposomes were added either one or three hours before analysis. Immediately before analysis DMEM was aspirated, cells were washed 1x in PBS, and fresh phenol red free DMEM was added. Wide field fluorescence microscopy was performed on an EVOS FL Auto Cell Imaging System (Life Technologies). An LED light cube for Cy5 was used to excite DDAO fluorescence and a GFP light cube was used to excite Syto9 fluorescence. Images were acquired at 40x magnification and analyzed with Image J.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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cPLA₂ Probes: **Control Probes:** **Figure 1. Scheme for probe synthesis**

Free fluorophore was coupled with a fatty acyl chloride through a one-step reaction in pyridine. Arachidonoyl chloride was used to generate cPLA₂ selective fluorophores. Palmitoyl chloride was used to generate control probes.

Figure 2. Mechanism of activation shown for DDAO arachidonate

Absorbance in the esterified quenched state is shown in solid blue. Absorbance in the unesterified state is shown in red. Cleavage of the esterified probe (10 μ M) generates absorbance at 600-660 nm allowing for fluorescence (dotted red line) at 660 nm.

Figure 3. Cleavage and fluorescence generation of candidate probes by a non-selective phospholipase (sPLA₂ GIB)

Fluorescence generation is shown with time in the presence of increasing concentrations of sPLA₂ GIB for naphthofluorescein arachidonate (A), resorufin arachidonate (B), resorufin palmitate (C), DDAO arachidonate (D), and DDAO palmitate (E). One unit is equivalent to the amount of enzyme producing 1 μmol of free fatty acid per minute at pH 8 and at 40 °C.

Figure 4. DDAO fluorescence generation is attenuated by the sPLA₂ inhibitor LY311727

The fluorescence generated by 100 Units sPLA₂ was inhibited by the addition of 100 μ M of the sPLA₂ inhibitor LY311727 for both DDAO arachidonate (A) and DDAO palmitate (B). C) Quantifying the fluorescence at 720 min shows that LY311727 significantly inhibits the final fluorescence intensity of DDAO arachidonate ($p=1.4 \times 10^{-11}$) and DDAO palmitate ($p=3.2 \times 10^{-7}$). In the absence of enzyme, DDAO arachidonate gives significantly higher fluorescence ($p=3.1 \times 10^{-11}$) than DDAO palmitate. No significant difference exists in the final fluorescence between DDAO arachidonate and DDAO palmitate in the presence of 100 Units sPLA₂.

Figure 5. Cellular activation of DDAO arachidonate and DDAO palmitate

A) DDAO arachidonate and DDAO palmitate were incubated with 4175-Luc+ cells for 30 minutes and for 3 hours. Wide field fluorescent microscopy was performed to visualize free DDAO fluorescence. Nuclei were counterstained with Syto 9. Separate and merged fluorescence images are shown with yellow indicating overlap of fluorescence in the merged image. B) Fluorimetry was used to quantify DDAO fluorescence as a function of time and demonstrated significantly increased fluorescence at 3 h from DDAO arachidonate relative to DDAO palmitate ($p=1.3 \times 10^{-6}$).

Figure 6. cPLA₂ enzymatic induction and inhibition

cPLA₂ activity was induced through either H₂O₂ (A) or Ionomycin (B). Fluorescence generation was monitored with time. DDAO arachidonate showed significant enhancement of fluorescence in the presence of H₂O₂ ($p=2.9 \times 10^{-6}$) and ionomycin ($p=1.9 \times 10^{-7}$) whereas DDAO palmitate did not show any significant changes in fluorescence. Phospholipase inhibitors (C) were incubated with cells for four hours at which point cells were washed in PBS and fresh media containing either DDAO arachidonate or DDAO palmitate was added. Fluorescence was recorded following 30 minutes of incubation. AACOCF3 results in a significant ($p=0.003$) reduction of fluorescence relative to control. BEL and LY311727 did not alter fluorescence significantly. No significant changes in fluorescence were recorded in the presence of DDAO palmitate.

Table 1
Fluorophores used for fatty acid conjugation

Resorufin, naphthofluorescein, and DDAO were conjugated to fatty acid(s) through esterification of their free hydroxyl group. The structure of 7-hydroxycoumarin is also shown, identified in the literature to be cleavable by cPLA₂ when esterified

Fluorophore	Structure	Excitation (nm)	Emission (nm)
7-Hydroxycoumarin		360	460
Resorufin		572	585
Naphthofluorescein		595	660
DDAO		600	660