Fluorogenic probes to monitor cytosolic phospholipase A2 activity†

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Arachidonic acid derivatives equipped with either one or two fluorescent groups attached to the tip of the alkyl chains were synthesized and shown to function as inhibitor and substrate probes of cPLA2. The inhibitor probe was demonstrated to perform dual functions of inhibition and imaging while the substrate probe could be used for activity assay.

Arachidonic acid (AA) is a precursor of a family of lipid mediators, including prostaglandins, that regulates a wide variety of physiological responses and disease pathogenesis.1 The biosynthesis of AA occurs mainly through the activation of phospholipase A2 (PLA2) which catalyzes the hydrolysis of the sn-2 ester bond of glycerophospholipids to release AA. Mammalian cells have diverse forms of PLA2 including the secreted small molecular weight sPLA2, the larger cytosolic Ca2+-dependent cPLA2 and Ca2+-independent iPLA2. Although sPLA2 could participate in the release of arachidonate during injury, the main gatekeeper for the enzymatic conversion of arachidonate is cPLA2.2 Given that cPLA2 is an ubiquitous enzyme which is highly selective for glycerophospholipids containing AA, there has been much interest to design and identify cPLA2 inhibitors to better understand the molecular mechanism regulating this enzyme and to develop efficacious therapeutics for the treatment of cPLA2-upregulated diseases.3–6 To this end, we are interested to identify novel chemical tools, particularly fluorescent probes which could provide a means to monitor cPLA2 activity. To our knowledge, Chiorazzo et al have recently reported a cPLA2 activatable fluorophore for imaging cPLA2 at cellular level and for the study of cPLA2 inhibition.7 Herein, we present our work on fluorescent inhibitor and substrate probes for the direct detection of cPLA2 in breast cancer cells.7 Herein, we present our work on fluorescent inhibitor and substrate probes for the direct detection of cPLA2 at cellular level and for the study of cPLA2 inhibition.

Arachidonyl trifluoromethyl ketone (AACOCF3), a potent inhibitor of cPLA2 has been shown to cause a reduction in neuronal losses through decreased oxidative stress and inflammation upon inhibiting cPLA2 in mouse model of spinal cord injury.8 This suggests its ability to penetrate the blood–brain barrier and was thus chosen as the scaffold for our cPLA2 inhibitor probe. In the design of our fluorogenic inhibitor probe (Fig. 1), the arachidonyl functionality was used to promote enzyme recognition, a trifluoromethylketone moiety was incorporated to inhibit the active site of cPLA2 and a fluorogenic group was attached to the distal end of the alkyl chain so that it does not hinder interactions with the enzyme’s active site. A representative number of inhibitor probes were synthesized and screened for their inhibitory activities. Briefly, the chemical synthesis of these compounds (Scheme S1, ESI†) involved a series of copper-catalyzed coupling and bromination of the alkyne building blocks to achieve a quadruple alkyne intermediate which was subsequently hydrogenated with Brown’s catalyst to form the alkene. The trifluoromethyl ketone moiety was then introduced by reacting an intermediate carboxylic acid with trifluoroacetic anhydride in the presence of pyridine. The alkyl tail end of the alkene was then functionalized with either a Br or NH2 group which could be readily coupled to a fluorophore via Williamson-ether synthesis or an amide coupling reaction. Seven fluorogenic inhibitors were synthesized and their inhibitory...
activities were tested with recombinant cPLA$_2$ via a PLA$_2$ assay. 7OHCou-AACF3 (Table S1, ESI†) yielded the lowest enzyme activity of 6.2 ± 0.8% at a concentration of 10 μM and has an IC$_{50}$ of 12.5 ± 1.0 μM (Fig. S1, ESI†). This is comparable to AACOCF$_3$ which has an IC$_{50}$ of 16.5 ± 3.0 μM. Selectivity test with recombinant sPLA$_2$ at 10 μM showed that 7OHCou-AACF3 did not give an observable bioactivity with sPLA$_2$ (Fig. S2, ESI†).

Next, we evaluated the inhibitory property of 7OHCou-AACF3 on cPLA$_2$ in lipopolysaccharide (LPS) stimulated mouse microglial BV-2 cells. Earlier study have shown that BV-2 cells upon LPS insult resulted in an increase in inducible nitric oxide synthase (iNOS) which produces excess nitric oxide (NO) causing neurotoxicity.$^9$ Upon application of a cPLA$_2$ specific inhibitor, the protein level of iNOS was found to decrease, demonstrating its ability to mediate the effects of NO. Similarly, when 7OHCou-AACF3 (10 μM) was incubated with BV-2 cells in the presence of LPS, the level of iNOS decreased to less than 20% (Fig. 2) showing its potential neuroprotective ability through the inhibition of cPLA$_2$. (This is further supported by cell viability assay [vide infra]).

To explore the use of 7OHCou-AACF3 as an imaging tool, two cell lines, BV-2 and SHSY5Y, were treated with different stimulants and stained with 7OHCou-AACF3 (10 μM) (Fig. 3). The cPLA$_2$ levels were found to be not statistically different in the control (untreated BV-2 cells), the LPS-stimulated and the 7OHCou-AACF3-treated LPS-stimulated BV-2 cells whilst in the SHSY5Y cells, the TSA-treated cells were stained more significantly than the non-TSA-treated cells (Fig. 3B and C). These results corroborated with the western blot data (Fig. 3A(i) and (ii)) and are in agreement with earlier study which showed that stimulating BV-2 microglial cells with LPS induces cPLA$_2$ phosphorylation but does not increase the cPLA$_2$ level$^9$ whilst treating SHSY5Y with histone deacetylase inhibitor, trichloroacetic acid A (TSA) results in an overexpression of cPLA$_2$. 10

Encouraged by the results, we decided to expand our studies by developing a substrate probe to measure cPLA$_2$ activity. The conventional assay of cPLA$_2$ activity uses 1-palmitoyl-2-[14C]-arachidonoyl phosphatidylcholine as a substrate. The radio-labeled arachidonate released during the reaction was recovered through aqueous extraction and the released radioactivity was determined by liquid scintillation counting.$^{11,12}$ However the use of radiometric assays is highly undesirable due to the hazards surrounding radioactive materials. It would thus be desirable to develop a simpler assay which does not use radioisotopes for screening cPLA$_2$ activities. Various colorimetric and fluorogenic assay kits for the measurement of cPLA$_2$ activity are now commercially available. However these assays...
are not selective to cPLA₂ and thus there is a need to develop an alternative probe. 13

Since 7OHCou-AACF₃ has the highest potency amongst the fluorogenic inhibitor probes synthesized, we hypothesized that having the arachidonyl and 7-hydroxycoumarin moieties in the substrate could potentially lead to stronger binding with cPLA₂. Hence we designed the substrate probe Flu7OHCou (Scheme 1) to comprise of a glycerylphosphorylcholine (GPC) scaffold attached to an arachionyl moiety to confer selectivity to cPLA₂. To enable ratiometric measurement, two compatible fluorogenic groups were placed at the alkyl chain end of the fatty acids esterified to the GPC moiety. Since 7OHCou-AACF₃ possesses an emission maximum at 447 nm, fluorescein was chosen as the other fluorophore since its absorption maximum is at 458 nm, making it suitable for Förster resonance energy transfer (FRET). Flu7OHCou was prepared by hydrolyzing 1,2-diheptanoyl-sn-glycero-3-phosphocholine 1 to glycerylphosphorylcholine 2 with methanolic sodium hydroxide. Esterification of the hydroxyl groups on 2 with fluorescein 3 gave predominantly 4 and some di-esterified product. Treating the mixture of 4 and di-esterified product with commercial bee venom PLA₂ selectively hydrolyzed the ester on the secondary alcohol, thus converting the di-esterified product to 4. Esterification of 4 with 5 provided Flu7OHCou in 30% overall yield.

7OHCou-AACF₃ and Flu7OHCou were evaluated for their cytotoxicities against the BV-2 cells after 48 and 72 h of treatment. Results showed that more than 80% of the cells survived after both the time-points (Fig. S5, ESIF), indicating that both compounds are non-cytotoxic. Next, the photophysical property of Flu7OHCou was examined by constituting it in a 20 μM Tris–HCl (50 mM Tris–HCl, 100 mM NaCl, 20 mM CaCl₂) liposomal system containing 1% Triton X-100 at pH 7.3 with 1% Triton X-100. Donor peak increased significantly as time increased from 0 min to 60 min while acceptor peak decreased with time. (B) Addition of recombinant cPLA₂ (0.05 unit per mL) to 20 μM Flu7OHCou constituted in a liposome formed by vortexing DOPG and DOPC in a Tris–HCl buffer, pH 7.3 with 1% Triton X-100. (C) Addition of recombinant cPLA₂ and sPLA₂ (0.05 unit per mL) to 20 μM Flu7OHCou constituted in a liposome formed by vortexing DOPG and DOPC in a Tris–HCl buffer, pH 8.9 (i) without Triton X-100 (ii) with 1% Triton X-100.

Fig. 4

Flu7OHCou substrate assay (A) Addition of bee venom (5 units per mL) to 20 μM Flu7OHCou constituted in a liposome formed by vortexing DOPG and DOPC in a Tris–HCl buffer, pH 7.3 with 1% Triton X-100. (B) Addition of recombinant cPLA₂ (0.05 unit per mL) to 20 μM Flu7OHCou constituted in a liposome formed by vortexing DOPG and DOPC in a Tris–HCl buffer, pH 7.3 with 1% Triton X-100. (C) Addition of recombinant cPLA₂ and sPLA₂ (0.05 unit per mL) to 20 μM Flu7OHCou constituted in a liposome formed by vortexing DOPG and DOPC in a Tris–HCl buffer, pH 8.9 (i) without Triton X-100 (ii) with 1% Triton X-100.

an appreciable blue fluorescence was detected from the donor 7-hydroxycoumarin while the rest of the emission came from the green fluorescein acceptor when irradiated at 410 nm (Fig. 4A and Fig. S8, ESIF). These results are congruent with earlier observations which showed that a membrane-like environment
is essential for cPLA₂ to recognize the substrate, highlighting the need to convert the substrate into liposomes.¹¹,¹⁴ The addition of Triton X-100 enables the formation of smaller liposomes which facilitate FRET between the 2 fluorophores. When PLA₂-rich bee venom (5 units per mL) was added to the liposomal system containing Flu7OHCou, the fluorescence intensity changed from a quantum yield of 0.04 to 0.08. The reaction was largely completed within 45 min and no further changes were observed in the emission spectra. This demonstrates the potential of Flu7OHCou to be used as a PLA₂ substrate probe.

To investigate if Flu7OHCou is a substrate to cPLA₂, the same assay was treated with 0.05 unit per mL of recombinant cPLA₂. No activity was detected even when the pH of the buffer was increased to 8.9 to encourage higher PLA₂ activity (Fig. 4B(i)).¹⁵ This loss of activity was attributed to Triton X-100 which has been shown previously to abolish cPLA₂ activity.¹⁶ To confirm this hypothesis, a 20 µM liposomal Tris–HCl (50 mM Tris–HCl, 100 mM NaCl, 20 mM CaCl₂, pH 8.9) buffer solution of Flu7OHCou without Triton X-100 was prepared and added to 0.05 unit per mL of recombinant cPLA₂. This led to a significant fluorescence increase from 331 to 554 RFU, confirming that Triton X-100 had resulted in the loss of cPLA₂ activity (Fig. 4B(ii)). These results also suggest the possibility of developing an assay with Flu7OHCou that uses Triton X-100 to isolate out the cPLA₂'s activity without the need of an inhibitor.

To investigate the PLA₂-selectivity of Flu7OHCou, 0.05 unit per mL of cPLA₂ and sPLA₂ were added to the liposomal substrate without Triton X-100. A large increase in fluorescence intensity was observed from the liposomal solution containing cPLA₂ while the same solution containing sPLA₂ exhibited only a smaller increase in fluorescence intensity as time increases (Fig. 4C(i)). The opposite result was observed in the presence of 1% Triton X-100, where a larger increase in fluorescence intensity for the solution containing sPLA₂ was detected (Fig. 4C(ii)). These results indicate that Flu7OHCou has a higher selectivity to cPLA₂ than sPLA₂ in the absence of Triton X-100 while the same result could be reversed in 1% Triton X-100. This also suggests the potential of differentiating cPLA₂ and sPLA₂ via an assay with the use of Triton X-100. To further examine the possible use of Flu7OHCou for inhibitor screening experiments, we proceeded to determine the IC₅₀ of AACOCF₃ by incubating Flu7OHCou in a liposomal solution with recombinant cPLA₂ and varying concentrations of AACOCF₃. By plotting a dose–response curve, the IC₅₀ of AACOCF₃ was found to be 19.0 ± 1.5 µM (Fig. S9, ES†) which is in good agreement with our experimental data of 16.5 ± 3.0 µM determined via the commercial PLA₂ assay.

In conclusion, 7OHCou-AACF₃, a fluorogenic inhibitor of cPLA₂ with dual functions of imaging and inhibition has been developed. 7OHCou-AACF₃ has an IC₅₀ of 12.5 ± 1.0 µM which is comparable with AACOCF₃. It is able to quench iNOS production via its role as an inhibitor and is capable of imaging the increase in cPLA₂ protein levels. We have also developed Flu7OHCou, a cPLA₂-selective fluorogenic substrate and demonstrated its use for inhibitor screening assays.

The authors thank the Ministry of Education (Singapore) (MoE Tier 2: R-143-000-589-112; YL) and NMRC (1222-2009; CML) for the financial support and the National University of Singapore for a PhD scholarship to C. Y. N.

References