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Design and Synthesis of Phospholipase C and A_2-Activatable Near Infrared Fluorescent Smart Probes

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Abstract

The primary focus of this work was to develop activatable probes suitable for *in vivo* detection of phospholipase activity. Phospholipases (PLs) are ubiquitous enzymes that perform a number of critical regulatory functions. They catalyze phospholipid breakdown and are categorized as A_1, A_2 (PLA_2), C (PLC) and D (PLD) based on their site of action. Here we report the design, synthesis and characterization of self-quenching reporter probes that release fluorescent moieties upon cleavage with PLA_2 or PLC. A series of phospholipids were synthesized bearing the NIR fluorophore Pyropheophorbide *a* (Pyro) at the *sn*-2 position. Fluorescence quenching was achieved by attachment of either a positively charged Black Hole Quencher-3 (BHQ-3) to the phospholipid head group or another neutral Pyro moiety at the *sn*-1 position. The specificity to different phospholipases was modulated by insertion of spacers (C_6, C_{12}) between Pyro and the lipid backbone. The specificity of the quenched fluorescent phospholipids were assayed on a plate reader against a number of phospholipases and compared with two commercial probes bearing the visible fluorophore BODIPY. While $PyroC_6$-$PyroC_6$-PtdCho revealed significant background fluorescence, and a 10% fluorescence increase under the action of PLA_2, Pyro-PtdEtn-BHQ demonstrated high selective sensitivity to PLC, particularly to the PC-PLC isoform, and its sensitivity to PLA_2 was negligible due to steric hindrance at the *sn*-2 position. In contrast, the C_{12}-spacered $PyroC_{12}$-PtdEtn-BHQ demonstrated a remarkable selectivity for PLA_2 and the best relative PLA_2/PLC sensitivity, significantly outperforming previously known probes. These results open an avenue for future *in vivo* experiments and for new probes to detect PL activity.

Phospholipases (PLs) are ubiquitous enzymes that perform a number of critical regulatory functions. They catalyze phospholipid breakdown and are categorized as A_1, A_2 (PLA_2), C (PLC) and D (PLD) based on their site of action (1,2) (Figure 1).

Since PLA_2 is elevated in a number of diseases (e.g. prostate and breast cancer, rheumatoid arthritis, etc) (3–5) we have chosen to develop probes that distinguish PLA_2 from other PLs. In addition, since increased PLC is detected in melanoma, ovarian and breast cancers and PLC has been implicated in maintaining the high levels of phosphocholine (PC) and phosphoethanolamine (PE) characteristic of many human tumors (6–10), we are also developing probes to specifically detect the actions of this enzyme.

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The primary focus of this work is to develop methods to investigate the expression and activity of PLs *in vivo* in mammals. The above diseases can be detected, monitored and in some cases treated using soft tissue penetrating near infrared (NIR) light (11,12). As a result we are employing optical imaging using NIR fluorophores (NIRF).

Our approach is to design self-quenching reporter probes that release fluorescent moieties only upon cleavage with PLA_2 or PLC. The structures of these smart probes are depicted in Figure 2. The hypothesis is that a bulky porphyrin moiety attached at the *sn*-2 position without a spacer would sterically hinder PLA_2 activity while leaving open the PLC cleavage site, whereas incorporation of spacers of different length will make the C-O bond at the *sn*-2-position accessible for PLA_2 mediated hydrolysis. We have chosen Pyropheophorbide *a* (Pyro, **1**, λ_{abs} = 675 nm, λ_{em} = 725 nm) (13,14) as a NIRF. For quenchers, we employ either the positively charged Black Hole Quencher-3 (BHQ-3) (13,14) attached to the head group of the phospholipid or another neutral Pyro moiety at the *sn*-1 position.

Scheme 1 demonstrates the synthesis of three self-quenched Pyro-bearing probes **4**, **7** and **10**. NIRF acid **1** was coupled with *N*-BOC *Lyso*-phosphatidylethanolamine (*Lyso*-PtdEtn, **2**) under basic conditions in order to conserve the steric configuration at the *sn*-2 site and avoid racemization (15,16). Next TFA-mediated BOC-deprotection resulted in the permanently fluorescent Pyro-PtdEtn (**3**). In the final step BHQ-3 was attached to the amino group of lipid **3** resulting in a self-quenching Pyro-PtdEtn-BHQ (**4**). To incorporate spacers we have synthesized $PyroC_{12}$- (**5**) and $PyroC_6$- (**8**) acids. Acid **5** was used as described above with acid **1** for production of the permanent lipid fluorophore $PyroC_{12}$-PtdEtn (**6**), which was then converted into its self-quenched derivative (**7**). Two molecules of acid **8** were conjugated with *sn*-glycero-3-phosphocholine (**9**) to give rise to $PyroC_6$-$PyroC_6$-PtdCho (**10**) where self-quenching is achieved by interactions between the two NIRFs.

Farber *et al.* had demonstrated that PLA_2 activity can be detected by visual fluorescence in transparent zebra fish larvae (17,18). These and other probes are now available commercially (Scheme 1, compounds **11**, **12**). The sensitivity of those phospholipids **11**, **12** to PLs other than PLA_2 was not tested at that time. As seen from Scheme 1 compounds **4**, **7** and **11** have a fluorophore attached at the *sn*-2 position and a quencher bound to the nitrogen atom of the PE head. Compounds **10** and **12** exploit self-quenching of two of the same fluorophores attached to the *sn*-1 and *sn*-2 positions.

We have tested our three self-quenched probes **4**, **7** and **10** as well as commercial probes **11** and **12** as substrates for different phospholipases. The results are presented in Figure 3. Probe **4** revealed high selective sensitivity to PLC, particularly to the PC-PLC isoform (Fig. 3a), while sensitivity to other tested phospholipases including PLA_2 is negligible. NIRF compounds **10** and **7** were tested as potential PLA_2-sensitive probes in comparison with their visible counterparts **12** and **11**, respectively. PtdCho **10** revealed significant background fluorescence (Fig. 3b) because both substituents at *sn*-1 and *sn*-2 positions are self-fluorescent and their quenching efficiency is not high. Only a modest (10%) fluorescence increase was detected under the action of PLA_2, similar to those observed for the visible analog **12** (Fig. 3f). In contrast the C_{12}-spacered PtdEtn **7** demonstrated a remarkable selectivity for PLA_2 (Fig. 3c) and the best relative PLA_2/PLC sensitivity, significantly outperforming the magnitude of the best previously known probe **11** (Fig. 3e). Moreover, this construct exhibited significant PLA_2 isoform specificity with Porcine $sPLA_2$ IB demonstrating the highest activity (Fig. 3d).

PC-PLC was able to cleave Pyro-PtdEtn-BHQ **4** and PED-6 **11** even though relatively bulky quencher moieties were attached to the head group. PC-PLC, in contrast to its name, has a relatively broad range of head group specificities. It has been shown to be effective at

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hydrolyzing phospholipids other than PtdCho, including PtdEtn and PtdSer with relative specificities of 10:7:1 (19–22). PyroC12-PtdEtn-BHQ **7** and PED-6 **11** on the other hand were both effectively cleaved by porcine $sPLA_2$ IB. For $sPLA_2$s, cleavage specificities are primarily due to differences in binding to the vesicle surface. $sPLA_2$s, including $sPLA_2$ IB bind tightly and act preferentially on anionic phospholipid vesicles (PtdGro, PtdEtn and PtdSer) compared to charge-neutral PtdCho vesicles. The isoforms of $sPLA_2$ III and V employed on the other hand, will hydrolyze both anionic phospholipids and PtdCho. (23–26). Their inactivity to PyroC12-PtdEtn-BHQ **7** may be a result of the bulky BHQ head group, but variations in chain length may also play a role as discussed below. SMase and PI-PLC are highly head-group specific to their substrates (27–30). Table 1 in the Supporting Information contains additional information on the enzymes employed here and their substrate specificities.

Our results showed that increasing the chain length in the *sn*-2 position conferred increased sensitivity to $sPLA_2$ IB but decreased sensitivity to PC-PLC. Although fatty acid composition is relatively unimportant in determining $sPLA_2$ specificity (24), the presence of bulky substituents in the *sn*-1 position will lessen the enzymatic activity due to steric hindrance (31). It has also been shown $sPLA_2$ activity is dependent on the *sn*-2 fatty acid chain length, which must be 7 carbons or greater, with optimal activity obtained with chain lengths between 16 and 21 carbons (32–34). The chain length requirements are not so important for PC-PLC, which contains a shallow cleft for phospholipid docking (32–35).

The phospholipase-sensitive probes described here are highly lipophilic and we have employed egg-yolk PtdCho vesicles to solubilize them (see Supporting Information). For *in vivo* experiments, similar techniques will be required either using micelles or liposomes for delivery. Liposomal formulations for drug delivery are well established (36) and more lipophilic molecules are often processed and excreted through the liver and stool.

In conclusion, we have designed, synthesized and tested for the first time near-infrared self-quenched phospholipid probes that reveal high sensitivity to PLC and PLA_2. These results open an avenue for *in vivo* experiments with mammals and for the development of sensitive new probes to detect PL activity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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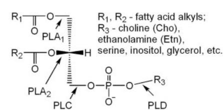

Figure 1.
Phospholipase-mediated cleavage sites.

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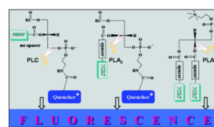

Figure 2.
Design of NIRF-bearing PLC- and PLA2-cleavable self quenched phospholipids.

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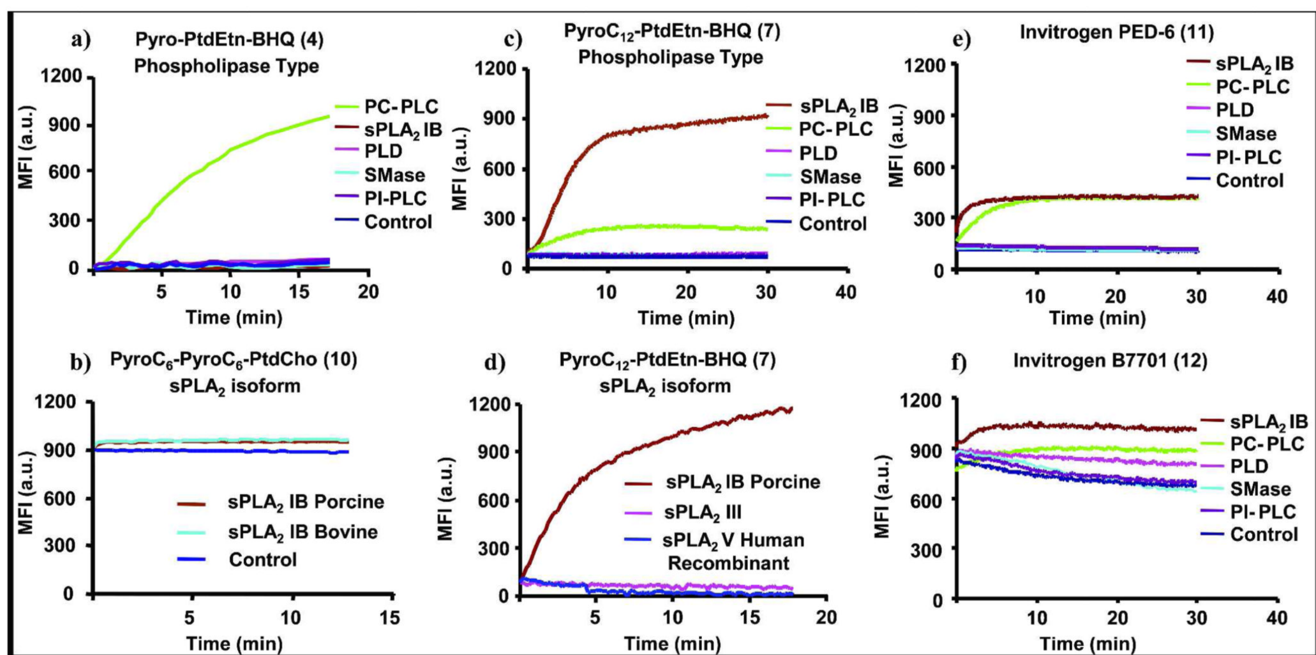

Figure 3.

Sensitivity of self-quenched fluorescent probes to different phospholipase isoforms as measured by fluorescence release on a plate reader (See Supporting Information).

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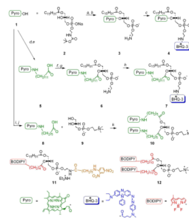

Scheme 1.

Synthesis of Pyro-PtdEtn-BHQ (4), PyroC12-PtdEtn-BHQ (7) and PyroC6-PyroC6-PtdCho (10) and structures for comparison of commercially available self-quenched PLA2-sensitive green fluorescent phospholipid probes PED-6 (11) and B7701 (12).

* Attachment sites for fluorophores/quencher.

Reagents, conditions (all reactions were performed under Ar in dark) and isolated yields (relative to starting NIRF 1, one arrow indicates one-pot reaction): (a) EDC, DMAP, DCM, rt, 72 h; (b) TFA, DCM, 0 °C, 4 h, 20%; (c) BHQ-3+SU PF6-, TEA, DCM, rt, 12 h, 15%; (d) NHS, EDC, DMAP, DCM, rt, 3 h; (e) H2N(CH2)11CO2H, TEA, DCM, rt, 72 h, 85% (f) 2, EDC, DMAP, DCM, rt, 72 h; (g) TFA, DCM, 0 °C, 4 h, 15%; (h) BHQ-3+SU PF6-, TEA, DCM, rt 12 h, 10%; (i) NHS, EDC, DMAP, DCM, rt, 3 h; (j) H2N(CH2)5CO2H, TEA, DCM, rt, 72 h, 75%; (k) EDC, DMAP, DCM, 40 °C, 85 h, 8%