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Emerging approaches to study cell-cell interactions

Poulcharidis, D.

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Author: Poulcharidis, D.

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***In vivo* and *in vitro* evaluation of a phospholipid ether analogue for the detection of tumours**

6.1 Introduction

A primary goal in cancer research is the specific targeting and delivery of therapeutic agents or diagnostic imaging tools to tumours overpassing healthy tissues. Methods to achieve this goal are focusing on agents and tools already in development or approved, which target cell membrane. A wide variety of tumour-targeting methods has been developed based on nanoparticles, peptides, antibodies and viruses to deliver different cargos to cancer cells, for example imaging agents, radioactive isotopes and drugs.¹⁻⁶ To date, the available tools for cancer therapy can be placed in two categories: on the one hand are aspecific tools available for a wide variety of cancer types; i.e. radiation, surgery or chemotherapeutic drugs. On the other hand are cancer-specific tools based on the expression of genetic drug-targets in cancer cells

compared to healthy cells. Examples of the latter approach are targeting the epidermal growth factor receptor (EGFR) or the Philadelphia chromosome (BCR-ABL fusion).^{7,8} In the clinic, many cancers reappear despite the advancement of novel treatments. Recent studies have shown that the cancer reappearance most likely occurs due to the cancer-cell resistance to the known therapies.⁹⁻¹⁴ In addition, an ideal therapeutic model should eliminate the side effects on the healthy cells and incorporate cancer selectivity with an extensive anticancer mechanism.

In the late 1960s Snyder *et al.* performed an evaluation of the lipid composition of normal and neoplastic tissues.^{15,16} These studies revealed that large quantities of ether lipids were found to be present in both animal and human tumour tissues. In the early 1980s, follow-up studies showed that cancer tissues expressed lower amounts of the O-alkyl glycerol monooxygenase enzyme AGMO (EC 1.14.16.5),¹⁷ which could result in the accumulation of ether lipids in the membrane of hepatomas cells.¹⁸ Subsequent studies in the early 2000s have shown that specific phospholipid ether (PLE) analogues which are not substrates for AGMO, also accumulate in breast, colon and pancreas tumours.¹⁹⁻²¹

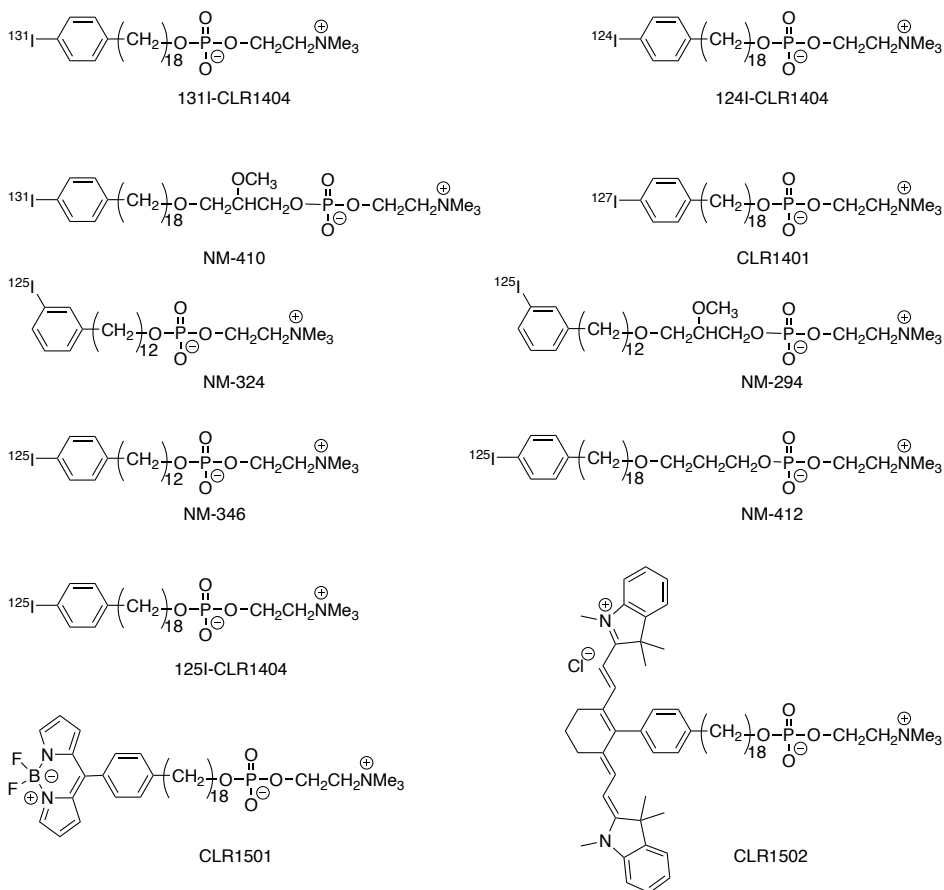


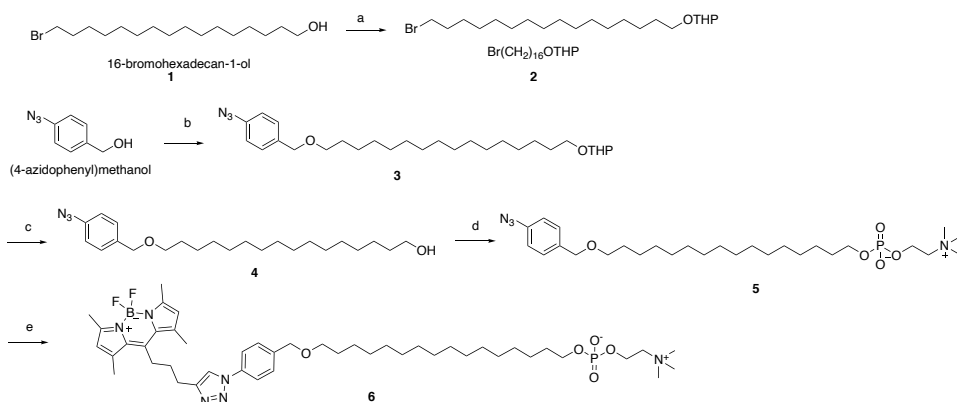
Figure 1 Structures of phospholipid ether analogues (alkylphosphocholines; APC). A novel therapeutic platform is based on phospholipid ether analogues. Cellectar Biosciences has developed a phospholipid ether analogue-based platform that is currently being investigated for diagnostic and therapeutic oncologic applications. CLR1404 as a radioactive compound (^{125}I , ^{124}I , or ^{131}I), as a fluorescent analog (CLR1501), or as a near-infrared analog (CLR1502). ^{125}I -CLR1404 is currently under clinical investigation as a positron emission tomography (PET) tracer. CLR1502 is a near-infrared fluorescent labelled phospholipid ether analogue being investigated for regional imaging applications. [Structures reproduced from references: D. A. Deming, M. E. Maher, A. A. Leystra, J. P. Grudzinski, L. Clipson, D. M. Albrecht, M. K. Washington, K. A. Matkowskyj, L. T. Hall, S. J. Lubner, J. P. Weichert and R. B. Halberg, *PLoS One*, 2014, **9**, e109668; and A. N. Pinchuk, M. A. Rampy, M. A. Longino, R. W. S. Skinner, M. D. Gross, J. P. Weichert and R. E. Counsell, *J. Med. Chem.*, 2006, **49**, 2155–2165.]

Due to the avidity of these phospholipid ether analogues to accumulate in different tumours, they are being studied as therapeutic and diagnostic tools (Figure 1).^{17,18,22–25} Recent studies have shown that the length of the phospholipid ethers plays an essential role in tumour docking in rat models. It has been reported by Pinchuk *et al.* that the alkyl chain of the PLE should have more than 11 methylene groups, and analogues with alkyl chain length between C15–C18 have the optimum tumour uptake in rat prostate cancer models.^{26–28} Additionally, previous studies have shown that although the meta-iodinated alkylphosphocholine NM-324 (Figure 1) was more stable and giving high tumour uptake, it accumulated in nontarget tissues including bladder, kidneys and liver.²⁹ Therefore, the para-isomer NM-346 was used instead, which also displayed significant tumour uptake *in vivo*. Moreover, the PLE chain can be labelled with different therapeutic moieties or imaging markers.^{29–31} The remarkable ability of specific radioiodinated PLE analogues to be visualised in different mammalian tumour cells using gamma camera (γ -camera) scintigraphy has been extensively described. For example, the radioisotope-labelled PLE analogue ¹²⁴I-18-(*p*-iodophenyl)octadecyl phosphocholine (CLR1404) is being used as a positron emission tomography (PET) tracer in patients with metastatic colon cancer.^{27,29,32} Moreover, the PLE analogue CLR1502 has the same structure as CLR1404 but is labelled with a near-infrared fluorescent marker and is under investigation for oncologic imaging application, whereas the PLE analogue CLR1501 is labelled with a bodipy fluorescent marker (Figure 1). To date, the reason for the retention of PLE and analogues in cancer cells remains unknown, and the strategy for designing agents with such properties has been mostly empirical.^{32,33} The hypothesis is that these PLE analogues have an affinity for tumour cell membranes because of their inability to be metabolised from cancer cells whereas

they are metabolised in healthy tissues.^{34,35} These PLE analogues take advantage of the lipid composition of cell membrane as ports of entry into tumours.³⁶ This chapter reports on the synthesis of a modified fluorescent alkyl phospholipid ether analogue and its avidity to localise in different tumour cell lines *in vitro*. Moreover, the chapter describes – for the first time – the *in vivo* imaging testing of the modified fluorescent alkyl phospholipid in a zebrafish prostate cancer model.

6.2 Results and discussion

The synthetic approach taken here for the synthesis of the bodipy-labelled phospholipid ether analogue was based on the published procedure of Pinchuk *et al.*²⁹ and the copper(I)-catalysed alkyne-azide cycloaddition (CuAAC). To improve solubility and due to synthetic accessibility, the route was modified to include an alkoxy-group in C17 (Scheme 1). The building blocks were chosen based on their commercial availability of starting materials. Initially, the synthesis was started by the THP protection of 16-bromohexadecanol **1** to the 2-((16-bromohexadecyl)oxy)tetrahydropyran **2** as shown in Scheme 1. Next, the THP-protected halide **2** was further coupled with (4-azidophenyl) methanol in a Williamson ether reaction. The 16-((4-azidobenzyl)oxy) alcohol **4** obtained after THP-deprotection. This alcohol was converted into the corresponding azido-benzyloxy-alkylphosphocholine **5** according to published procedures.^{26,29,37} Fluorescent coupling with bodipy-488 was accomplished by a copper-catalysed cycloaddition method routinely employed in our laboratory resulting in the bodipy-488-labelled alkylphosphocholine **6**.³⁸



Scheme 1 Reagents and conditions: (a) 3,4-dihydro-2H-pyran, PPTS, CH₂Cl₂, rt, 5h; (b) i) NaH, Dry THF, 0 °C, 30 mins; ii) Br(CH₂)₁₆OTHP (2), TBAL, 0 °C, 5 h ; (c) PPTS, EtOH, 40 °C; (d) i) 2-chloro-1,3,2-dioxaphospholane 2-oxide, Et₃N, Benzene, rt, 24 h; ii) Me₃N, sealed bottle, 80 °C, 24 h (e) bodipy-alkyne, CuSO₄, NaAsc, tBuOH: ACN: Water (1/1/1), 60 °C, 24 h.

Following synthesis and purification, the accumulation of the fluorescently labelled phospholipid 6 was tested *in vitro*. Weichert *et.al* have reported the optimum localisation of CLR1501, a fluorescently labelled CLR1404 analog in different cancer cell lines (renal, colorectal, glioma, prostate), obtained after 24 h.²⁷ Therefore, three different cancer cell lines including cervical cancer (HeLa), prostate cancer (PC-3) and lung cancer (A459) as well as healthy primary dendritic cells (BMDC) were treated with bodipy-PLE 6 (5 or 10 μ m final concentration). After 24 hours, confocal microscopy and flow cytometry results showed specific targeting of cancer cell lines and exceptionally high accumulation at PC-3 prostate cancer cells in plasma and organelle membranes (Figure 2).

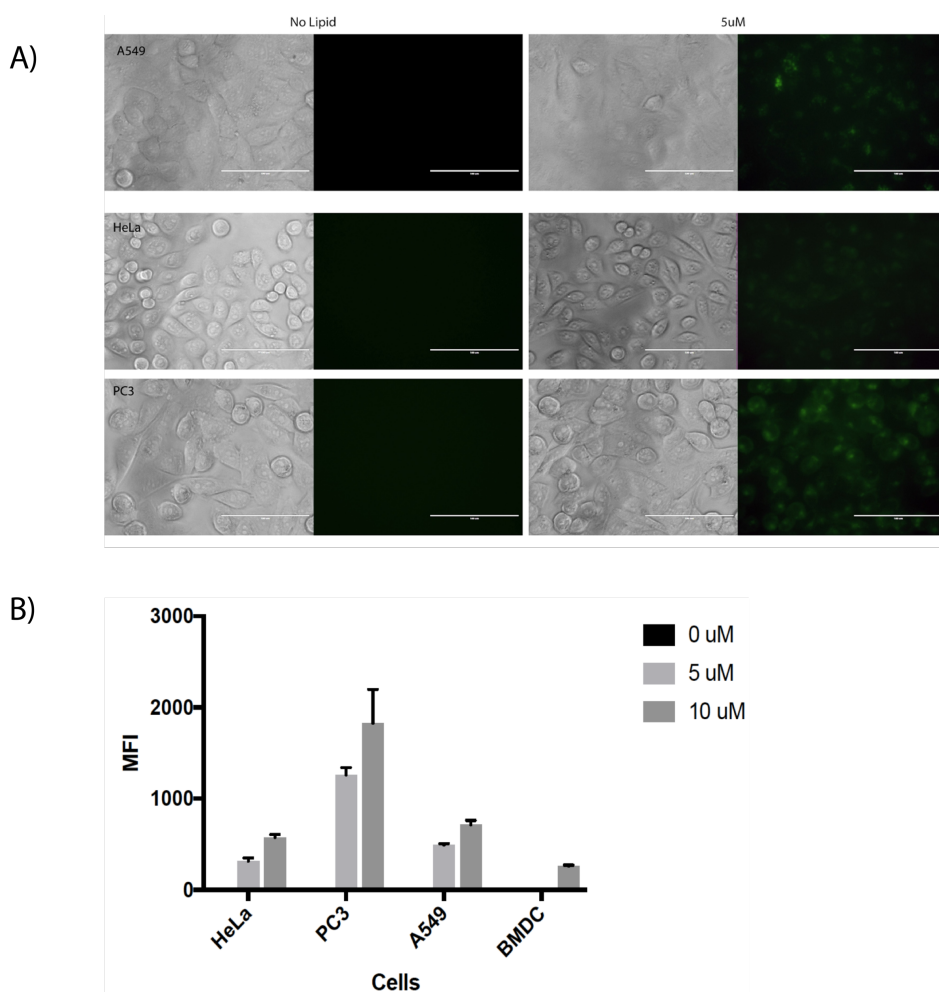


Figure 2 Bodipy-labelled alkylphosphocholine 6 accumulates in specific cell line deposits. Lung (A549), cervix (HeLa), prostate (PC-3) human cell lines and bone marrow DC were treated with 5 or 10 μ M final concentration of bodipy-PLE (6) at 37 °C. (A) Confocal microscopy images (fluorescence, bright field) of different cell lines. Scale bar: 10 μ m. (B) Flow cytometry indicates a cell-line dependent docking. The assay was set up in triplicate. Data represented as median $\pm$ SD. Error bars SD.

This cell-type dependent accumulation could be explained either due to different metabolism rates or the specific membrane affinity of the PLEs in cancer cells. Additionally, the accumulation of the similar structured ^{124}I -18-(p-iodophenyl)octadecyl phosphocholine (CLR1404) in mice bearing PC-3 prostate cancer xenografts was recently reported.²⁹ Next, the phospholipid ether analogue **6** was tested *in vivo* – for the first time – in a zebrafish model (*Danio rerio*). The zebrafish model has been studied as a powerful method for research in cancer biology.^{39–43} A wide variety of cancers, for example, breast, colon and prostate cancer, has been studied using zebrafish embryo xenograft models.^{39–43} An important advantage of zebrafish is their optical transparency. In this study a prostate cancer PC-3 model provided by L. Chen *et al.* was used.^{39,42} This embryonic zebrafish cancer model represents a human tumour cell line that was used to determine the target (tumour cells) to nontarget ratio of PLE analogue **6**. These xenografts zebrafish were selected randomly. Based on prior studies, most of the zebrafish were expected to possess prostate tumour. The median age was two days old. All zebrafish were injected with **6** (1 nl) based on injected methods routinely employed at our laboratory.⁴¹ Zebrafish tolerated this agent well without acute changes in activity or baseline weight. After one hour, zebrafish were prepared and imaged using confocal microscopy.

All zebrafish used contained a prostate cancer tumour. The bodipy-labelled alkylphosphocholine **6** is fluorescent in the FITC range (excitation 488 nm and emission 525 nm) and accumulation was quantified. At first, the circulation of the bodipy-lipid was tested in a wild-type zebrafish (Figure 3). The distribution of phospholipid ether analogue **6** in the wild-type fish (Figure 3) was examined and one hour post injection lipid **6** was cleared from circulation, most likely by

scavenger receptor stabilin-2. Besides, accumulation was observed in the sinusoidal endothelial cells (SECs) of zebrafish, which are homologous to mammalian liver endothelial cells.⁴¹ Therefore, whether the phospholipid ether analogue **6** targets tumours was tested in the cancer model.⁴¹

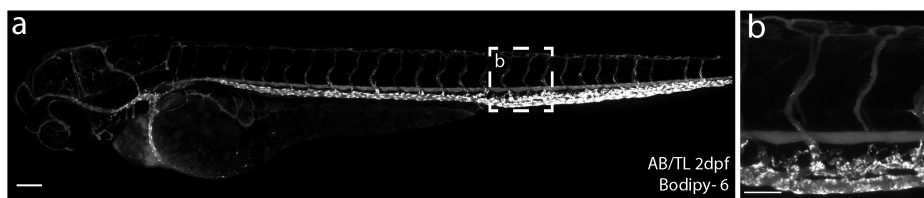


Figure 3 In vivo distribution of bodipy-labelled alkylphosphocholine **6**. (a) AB/TL (wild-type) zebrafish 2 days post fertilization (dpf) 1 h post-injection of the bodipy-labelled alkylphosphocholine **6**. Scale bar 100 μm . (b) Caudal vein region showing uptake of bodipy-labelled alkylphosphocholine **6** by sinusoidal endothelial cells (SECs). Scale bar 50 μm .

In a following experiment, alkylphosphocholine **6** was injected with prior administration of dextran sulphate (20 mg/ml) to competitively inhibit the uptake/clearance of stabilin-2-receptor(s).⁴¹ As a result, the circulation lifetime of the alkylphosphocholine **6** was increased (Figure 4). In this study circulation of **6** was observed without significant accumulation in surrounding malignant or healthy tissues, therefore no co-localization between the prostate cancer cells PC-3 (magenta) and the lipid (green) was observed (Figure 4).

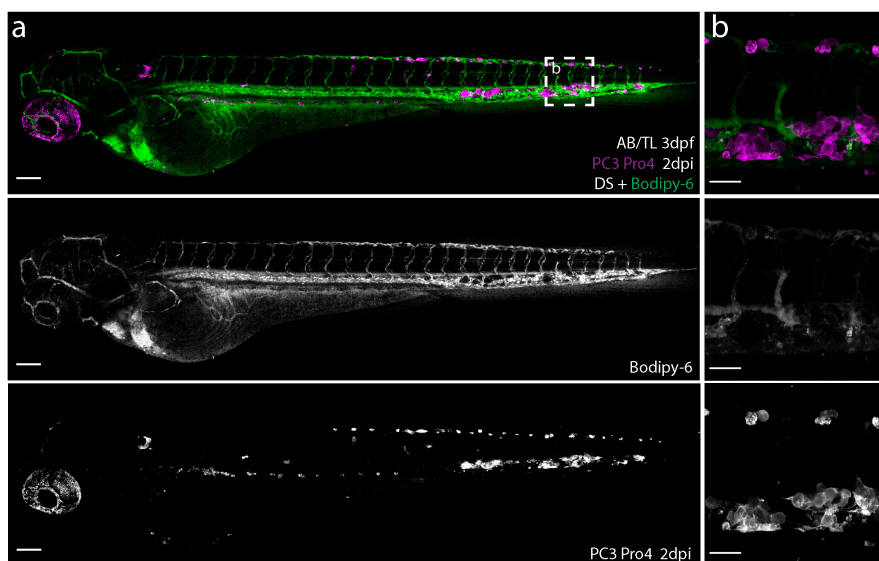


Figure 4 In vivo distribution of bodipy-labelled alkylphosphocholine **6** in a xenograft zebrafish model after inhibition of SECs. (a) AB/TL zebrafish (3 dpf) 2 days post injected (dpi) with PC-3 Pro4 prostate malignant cells, 1 h after injection of dextran sulphate (DS 20mg/ml) followed by bodipy-labelled-**6**. Scale bar 100 um. (b) Caudal vein region showing the tumour cells (magenta) and the circulating bodipy-labelled **6** in green. Autofluorescence present in the eye region. Scale bar 50um.

6.3 Conclusions

An initial study was performed to study whether a modified phospholipid ether analogue (**6**) is a potential tool for targeting prostate cancer membranes *in vitro* and in a zebrafish xenograft model *in vivo*. This phospholipid can be used with current fluorescence imaging devices to visualise prostate cancer *in vitro*. For *in vivo* studies, a zebrafish xenografts model was used. Moreover, zebrafish xenografts models are suitable for live cell imaging allowing the examination of circulation and distribution of labelled compounds in real time.^{41–46} Nonetheless, zebrafish xenografts are considered complementary to murine models and have physiological and genetic similarities with humans.^{41–46} A statistically significant accumulation of **6** was not noted in the invasive carcinomas as compared with the

healthy tissues of the zebrafish model. However, further studies should be made in different cancer models. Moreover, kinetic experiments and the use of agents that disrupt membranes could potentially shed some light on the docking mechanism of this ether phospholipid.

6.4 Experimental methods

Method and materials

All commercially available reagents and solvents were used without further purification. For “dry” solvents, solvents were dried accordingly before use over 4Å or 3Å molecular sieves. Column chromatography was performed using Merck silica gel 60 (0.040-0.063 mm). ¹H and ¹³C NMR spectra were recorded using a Brüker AV-300 (300/75 MHz), AV- 400 (400/101 MHz), AV-500 (500/126 MHz) or AV-850 (850/214). Recorded data were interpreted and analysed using MestReNova 14 software. Chemical shifts are reported in ppm (δ). Synthesis of compounds was performed according to literature.²⁹

2-((16-bromohexadecyl)oxy)tetrahydro-2Hpyran (2) was synthesised in full accordance with the reported procedure.²⁹ A solution of 16-bromohexadecan-1-ol (500 mg, 1,556 mmol) and 3,4-dihydro-2H-pyran (1,420 ml, 15,56 mmol) in dry dichloromethane (5 ml) containing pyridin-1-ium 4-methylbenzenesulfonate (39,1 mg, 0,156 mmol) was stirred at room temperature for five hours. Then, hexane was added and the solution washed with water, and dried over Na₂SO₄. Flash chromatography in hexanes-ether (30:1) afforded the product 2-((16 bromohexadecyl) oxy) tetrahydro-2H-pyran (555 mg, 1,369 mmol, 88 % yield) as a white wax. ¹H NMR (400 MHz,

Chloroform- d) δ 4.57 (t, J = 3.5 Hz, 1H), 3.87 (s, 1H), 3.73 (d, J = 9.4 Hz, 1H), 3.50 (dt, J = 10.6, 5.0 Hz, 1H), 3.40 (td, J = 6.9, 1.7 Hz, 3H), 1.85 (dd, J = 10.8, 4.4 Hz, 3H), 1.71 (d, J = 3.0 Hz, 1H), 1.56 (ddd, J = 22.0, 10.7, 6.7 Hz, 5H), 1.42 (t, J = 7.5 Hz, 2H), 1.34 – 1.24 (m, 23H).

2-((16-((4-azidobenzyl)oxy)hexadecyl)oxy)tetrahydro-2H-pyran (3)

was synthesised in full accordance with the reported procedure.²⁹ Sodium hydride (57,3 mg, 1,433 mmol) under argon at 0 °C was added to a solution of (4-azidophenyl) methanol (285 mg, 1,911 mmol) in dry THF (5 ml). Following stirring for 30 minutes, a solution of 2-((16-bromohexadecyl) oxy) tetrahydro-2H-pyran (387 mg, 0,955 mmol) in dry THF (5,00 ml) was cannulated, followed by the addition of tetrabutylammonium iodide (176 mg, 0,478 mmol). The reaction mixture was stirred for five hours, before saturated aqueous NH_4Cl solution was added. The mixture was then extracted with EtOAc and the combined layer was washed with water and brine, dried over Na_2SO_4 , filtered, and concentrated in vacuo. The residue was purified by flash chromatography (PS: Et₂O 150:0, 150:1, 150:3) to afford 440 mg of yellow oil (a mixture of product and unreacted THP-alcohol).

16-((4-azidobenzyl)oxy)hexadecan-1-ol (4) was synthesised in full accordance with the reported procedure.²⁹ In a 25 mL round-bottomed flask was added 2-((16-((4azidobenzyl)oxy)hexadecyl)oxy) tetrahydro-2H-pyran (100 mg, 0,211 mmol) and pyridin-1-ium 4 methylbenzenesulfonate (5,31 mg, 0,021 mmol) in ethanol (5 ml) and the reaction mixture refluxed for 24 hours until TLC showed no starting material. The reaction was diluted with water (10,00 ml). The aq layer was extracted with ethyl acetate (10,00 ml) (3 x 10 mL). The organic layers were combined and washed with brine (10,00 ml) (2 x 10 mL). The organic layer was dried, filtrated and concentrated. Flash

chromatography in hexane-ethyl acetate (90:10-80:20) afforded the product (16-((4-azidobenzyl)oxy)hexadecan-1-ol (44 mg, 0,113 mmol, 53,5 % yield) as a white wax. ¹H NMR (400 MHz, Chloroform-d) δ 7.26 (d, J = 8.4 Hz, 2H), 6.93 (d, J = 8.5 Hz, 2H), 4.39 (s, 2H), 3.57 (s, 2H), 3.38 (s, 2H), 1.98 (s, 1H), 1.52 (s, 4H), 1.25 – 1.13 (m, 24H).

16-((4-azidobenzyl)oxy)hexadecyl (2-(trimethylammonium)ethyl phosphate) (5) was synthesised in full accordance with the reported procedure.²⁹ 2-chloro-1,3,2-dioxaphospholane 2-oxide (53,0 μ l, 0,577 mmol) was added to the stirred solution of 16-((4-azidobenzyl)oxy)hexadecan-1-ol (125 mg, 0,321 mmol) in benzene (10 ml) containing triethylamine (67,0 μ l, 0,480 mmol). This was followed by stirring overnight. The triethylamine hydrochloride salt was then filtrated, and the solvent evaporated in vacuo. The residue was transferred into a pressure bottle. A solution of 5 ml trimethylamine (3813 μ l, 16,02 mmol) in ethanol was added. The bottle was sealed and heated at 80 °C for 24 hours. The ethanol was then removed, and flash chromatography in DCM-methanol (gradient from 10:0 to 5:5) afforded the product (127 mg, 0,229 mmol, 71,5 % yield) as white solid. ¹H NMR (300 MHz, Chloroform-d) δ 7.32 (d, J = 8.1 Hz, 2H), 7.04 – 6.94 (m, 2H), 4.45 (s, 2H), 4.35 (d, J = 10.1 Hz, 2H), 3.89 (s, 2H), 3.78 (d, J = 7.0 Hz, 2H), 3.19 – 3.01 (m, 2H), 2.85 (s, 2H), 2.05 (s, 9H), 1.37 – 1.23 (m, 26H).

16-((4-(4-(3-(5,5-difluoro-1,3,7,9-tetramethyl-5H-4l4,5l4-dipyrrolo[1,2-c:2',1'-f][1,3,2]diazaborinin-10-yl)propyl)-1H-1,2,3-triazol-1yl)benzyl)oxy)hexadecyl (2-(trimethylammonium)ethyl phosphate) (6). Fluorescent analogue bodipy-alkyne and 16-((4-azidobenzyl)oxy)hexadecyl (2-(trimethylammonium)ethyl)phosphate (40 mg, 0,072 mmol) were dissolved in a mixture of tBuOH: ACN:

water (1/1/1, 2mL), before an aqueous solution of sodium ascorbate (200 uL, 0.10 mM, 0.15 eq) and an aqueous solution of CuSO₄ (200uL 0.1 mM, 0.10 eq) were added. The reaction was mixture stirred at 60 °C for 12 hours, before being concentrated in vacuo. Flash chromatography in 5% MeOH in DCM afforded the compound as an orange solid (51 mg, 0,059 mmol, 82%). ¹H NMR (850 MHz, Methanol-d₄) δ 7.70 (d, J = 7.8 Hz, 1H), 7.44 (d, J = 7.9 Hz, 1H), 7.26 (d, J = 7.9 Hz, 1H), 6.94 (d, J = 7.9 Hz, 1H), 4.47 (s, 1H), 4.37 (s, 1H), 3.81 (dt, J = 37.0, 5.7 Hz, 4H), 3.60 (d, J = 5.4 Hz, 2H), 3.48 – 3.32 (m, 2H), 3.21 (t, J = 2.7 Hz, 19H), 3.03 – 2.97 (m, 1H), 2.78 (s, 1H), 2.33 (d, J = 11.5 Hz, 5H), 1.88 (p, J = 7.6 Hz, 1H), 1.66 (hept, J = 8.6, 8.2 Hz, 1H), 1.51 (dt, J = 19.9, 6.7 Hz, 5H), 1.24 – 1.13 (m, 27H).). ¹³C NMR (151 MHz, Methanol-d₄) δ 183.54, 176.44, 170.78, 169.71, 166.27, 165.50, 161.19, 159.12, 156.57, 155.27, 154.10, 151.94, 151.20, 149.89, 148.50, 101.74, 101.49, 100.26, 100.00, 96.78, 95.55, 91.45, 78.47, 78.01, 77.87, 77.73, 77.59, 77.44, 77.30, 77.16, 60.95, 60.39, 60.35, 59.39, 59.38, 59.35, 59.31, 59.09, 59.06, 57.65, 55.85, 55.83, 55.50, 54.56, 45.10, 43.01.

Zebrafish intravenous injections

An embryonic zebrafish xenograft model containing prostate human cancer was developed and provided by L. Chen (Leiden University).³⁹ PC-3M-Pro4-mCherry prostate cancer cells (ATCC; RRID, CVCL_D579) were incubated at 33 °C for 24 hours prior to injection into zebrafish embryos. Zebrafish embryos at two days post-fertilisation (dpf) were immobilised using 1.2 mM tricaine methanesulfonate. 250 DiI-labelled prostate cancer cells in a volume of 5 nl were injected into the vein of Cuvier. Following injection, fish were carefully removed from the agarose/tricaine solution and transferred individually into 96-well plate imaging chambers created from 1% agarose using 3D-printed pins.⁴⁷

The bodipy-labelled alkylphosphocholine **6** was injected into two- to three-day-old zebrafish embryos using a modified micrography protocol.⁴⁸ Embryos were anaesthetised in 0.01% tricaine and embedded in 0.4% agarose containing tricaine before injection. 1 nl of the lipid was injected in the common cardinal vein or duct of Cuvier.

Zebrafish imaging

Confocal z-stacks were captured on a Leica TCS SPE confocal microscope, using a 10× air objective (HCX PL FLUOTAR) or a 40× water-immersion objective (HCX APO L). Images were processed using the ImageJ.^{49,50}

Confocal microscopy imaging

Cells were seeded in an 8-well slide (μ -Slide 8 well; Ibidi, Munich, Germany) at a density of 1×10^4 cells per well in complete media without phenol red. Cells were treated with different concentrations (0, 5 and 10 μ M) of bodipy-labelled alkylphosphocholine **6** overnight. Before confocal microscopy, cells were washed three times with PBS and fresh media. The fluorescent images were achieved using a Leica TCS SPE confocal laser scanning microscope (Leica Microsystems, Wetzlar, Germany) and analysed using the ImageJ software (National Institutes of Health, Bethesda, MD, USA). The wavelength settings for bodipy-488 Ex/Em: 488/510nm (Ex laser: 488 nm).

Mammalian cell culture

HeLa (cervix)⁵¹, A549 (lung)^{52,53} and PC-3 (prostate)⁵⁴ cells were cultivated in Dulbecco's Modified Eagle's Medium (DMEM), supplemented with 10% foetal calf serum, 2 mM L-glutamine, 1% penicillin and 1% streptomycin. Cells were cultured in an atmosphere of 7% CO₂ at 37 °C. The medium was refreshed every two days and cells passaged at 70%

confluence by treatment with trypsin-EDTA (0.05% trypsin). Cells were cultured in 25 cm² flasks and split at 70-80% confluence (three times per week). The flasks were incubated at 37 °C at 7.0% CO₂. The medium was refreshed three times a week. Cells used in all biological experiments were cultured for a maximum of 8 weeks. Adherent cell cultures with a maximum confluence of 70-80% were trypsinised and centrifuged [1.5 mins, 2000/4000rcf (live/fixed cells)], and the cells were re-suspended using fresh media. 10 μ L of cell suspension and 10 μ L of trypan blue were mixed and pipetted into a cell counting slide, and cells were counted using a BioRad TC10 automated cell counter. The cell suspension was diluted to the appropriate seeding density. BMDC cells were cultured in IMDM in non-adhesive petri dishes at 37 °C, 5% CO₂, under humidified air supplemented with 8% heat-inactivated foetal calf serum, 2 mM Glutamax, 20 μ M β -Mercaptoethanol, 50 IU/mL penicillin and 50 μ g/mL streptomycin, and recombinant GM-CSF (20 ng/mL, Peprotech, ref# 315-03) to a concentration of 0.5 x 10⁶ cells/mL.

Flow cytometry

Cells were seeded in a 35 mm dish at a density of 5*10⁵ cells per well in complete media without phenol red. Cells were treated with different concentrations (0, 5 and 10 μ M) of bodipy-labelled alkylphosphocholine **6** overnight. Before flow cytometry, cells were washed three times with PBS and fresh media. Flow cytometry assays were performed using the Merck Guava® easyCyte 12HT Benchtop Flow Cytometer, and all data were analysed using FlowJo™ v10.1 (FlowJo, LLC). Counting and characterization were performed by measuring 30,000 events in triplicate and concatenation of this data. For manual gating, the outermost ring of the dot plot was selected. Quadrants were manually selected to illustrate fluorescence plots. No compensation was required.

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