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Research paper

Development, synthesis and biological investigation of a novel class of potent PC-PLC inhibitors

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ABSTRACT

Phospholipases are enzymes that are involved in the hydrolysis of acyl and phosphate esters of phospholipids, generating secondary messengers that have implications in various cellular processes including proliferation, differentiation and motility. As such inhibitors of phospholipases have been widely studied for their use as anti-cancer therapeutics. Phosphatidylcholine-specific phospholipase C (PC-PLC) is implicated in the progression of a number of cancer cell lines including aggressing triple-negative breast cancers. Most current studies on PC-PLC have utilised D609 as the standard inhibitor however it is known to have multiple failings, including poor stability in aqueous media. 2-Morpholinobenzoic acids were recently identified using vHTS as a potential class of lead compounds, with improvements over D609. In this work 129 analogues in this class were prepared and their PC-PLC inhibitory activity was assessed. It was found that the majority of these novel compounds had improved activity when compared to D609 with the most potent inhibitors completely inhibiting enzyme activity. It was determined that the best compound/s contained a morpholino and 2-substituted *N*-benzyl moieties with these findings explained using molecular modelling. The compounds reported here will allow for improved study of PC-PLC activity.

1. Introduction

Phospholipases are enzymes that are involved in the hydrolysis of acyl and phosphate esters of phospholipids, generating secondary messengers that are capable of signal transduction with implications in various cellular processes including proliferation, differentiation, motility, apoptosis and gene expression [1]. Phospholipases can be classified into four different classes (A-D), categorised according to their site of cleavage on the phospholipid substrate - the phospholipase C (PLC) family of enzymes catalyses the hydrolysis of glycerophosphate bonds to form diacylglycerol (DAG) [2]. PLC enzymes can be further classified as either phosphatidylinositol-specific phospholipase C (PI-PLC) and phosphatidylcholine-specific phospholipase C (PC-PLC), depending on the substrate they target [3]. PC-PLC hydrolyses the phospholipid, phosphatidylcholine (PC) to produce DAG and phosphocholine [1b,4].

Both breakdown products of phosphatidylcholine catabolism, phosphocholine and DAG, act through kinases and transcription factors to indirectly activate cell proliferation and differentiation, although DAG holds the most significance due to its prevalence and action in a variety of responses [4c,5]. DAG has an important involvement in the signal transduction cascade to activate protein kinase C enzymes (PKC) to subsequently phosphorylate downstream proteins and induce an array of signalling events [5] and upregulation of these events can lead to the development and growth of cancer. Furthermore, DAG is additionally able to activate acidic sphingomyelinases (SMase) linked to various mitogenic

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processes [1b,4c].

As a consequence of its role in cell signalling, PC-PLC shows promising potential as a therapeutic target in anticancer therapies and there is an abundance of evidence that supports the idea that PC-PLC is involved in cancer development. Such evidence includes the increased expression of PC-PLC in ovarian tumour cells [6] and highly metastatic triple-negative MDA-MB-231 breast cancer cell line [7]. Furthermore, a downregulation of the HER2 oncogene has been observed upon PC-PLC inhibition [8], and there is evidence of an association between PC-PLC expression and leukaemia and hepatocarcinoma progression [9].

While the structure of eukaryotic PC-PLC has not been obtained, the structure of prokaryotic PC-PLC enzymes have been characterised and the most-commonly studied PC-PLC structure is that isolated from *Bacillus cereus*, first solved in 1989 [10]. Recently, we have reported the use of virtual high throughput screening (vHTS) to discover hit compounds against human PC-PLC for potential anticancer drug development using PC-PLC$_{Bc}$ as a model for mammalian PC-PLC [11]. This approach was justified, as it has been reported that PC-PLC$_{Bc}$ has antigenic similarity to mammalian PC-PLC and can mimic similar responses to mammalian PLC, *e.g.*, enhancement of prostaglandin biosynthesis [12]. Prior to this vHTS, a small number of PC-PLC inhibitors were known (2-aminohydroxamic acids, univalent anions, *N,N′*-dihydroxyureas, phospholipid analogues and the acclaimed xanthate, tricyclodecan-9-yl-xanthogenate, D609; Fig. 1) but unfortunately none of these compounds could be considered to be *drug-like* [13]. Of these inhibitors, D609 has been considered to be the most effective and has been the literature standard for PC-PLC inhibition. D609 competes with phosphatidylcholine and is widely recognised as a competitive inhibitor of PC-PLC with a Ki of 6.4 μM. *In vitro*, D609 demonstrated ability to block proliferation in EOC (epithelial ovarian cancer) cells and in MDA-MB-231 cells with 60–80% inhibition using 50 μg/mL, however *in vivo* studies required the use of very high doses of the compound in order to achieve the 60–80% inhibition previously outlined [6]. This may be due to its lack of *drug-like* properties (unstable and short half-life in aqueous media) that makes it unsuitable for distribution in the body and limits its therapeutic use. Furthermore, D609 has 8 possible stereoisomers (with different activities) and no selective synthesis of these isomers – this fact, coupled with the knowledge that different commercial sources give different isomeric ratios render D609 an unsuitable, but currently the best option, standard molecule for PC-PLC inhibition [14].

Due to the lack of PC-PLC inhibitors with *drug-like* properties and with tractable synthetic routes for further development, the aforementioned vHTS study was carried out, which identified four distinct structural classes as potential hits, namely benzenesulphonamides, pyrido[3,4-*b*]indoles, morpholinobenzoic acids and benzamidobenzoic acids (Fig. 2) [11]. Upon assessing the PC-PLC inhibitory activity of various compounds belonging to these structural classes, it was discovered that members of the pyrido[3,4-*b*]indole and morpholinobenzoic acid classes were very active against PC-PLC, with IC_{50} values of 3–4 μM. Pleasingly, it was shown that the tested compounds were more active against PC-PLC than the much-lauded established inhibitor D609 (IC_{50} 8.1 μM), establishing these classes, and morpholinobenzoic acids in particular, as *drug-like*, hit compound classes with a new standard of potency against PC-PLC [11].

This recently published work details the vHTS process and establishment of the morpholinobenzoic acid class of compounds as potent PC-PLC inhibitors, yet it only reports the testing of a small number of commercially-available analogues. Consequently, it was decided to design and implement a synthetic procedure that would allow access to previously-undiscovered analogues of these morpholinobenzoic acids in an attempt to establish a comprehensive structure-activity relationship (SAR) and discover analogues more potent against PC-PLC than those already identified.

2. Results and discussion

The main priority when designing a synthetic route for the morpholinobenzoic acids was to create a methodology that would be easily adapted to the synthesis of a large number of analogues exhibiting various different aspects of structural variation from the hit compound, **1** (IC_{50} 3.69 ± 0.23 μM against PC-PLC, Fig. 3). The compounds previously tested [11] were determined by their availability, this set was neither systematic nor comprehensive and only allowed a rudimentary SAR to be developed to date. As such, in the first series of this study, it was decided to synthesise a range of analogues to complement this non-systematic set of compounds. This would fully explore the *N*-benzyl moiety and determine the effect of electron-withdrawing and electron-donating substituents and their relative substitution positions, on the activity of the resulting morpholinobenzoic acid (purple, Fig. 3). As part of this first series, it was also decided to synthesise the corresponding esters of each of the compounds. In molecular modelling of **1** and its analogues, it was shown that the carboxylic acid moiety coordinates to the Zn atoms present at the PC-PLC binding site. If this were an accurate representation of the actual binding mode

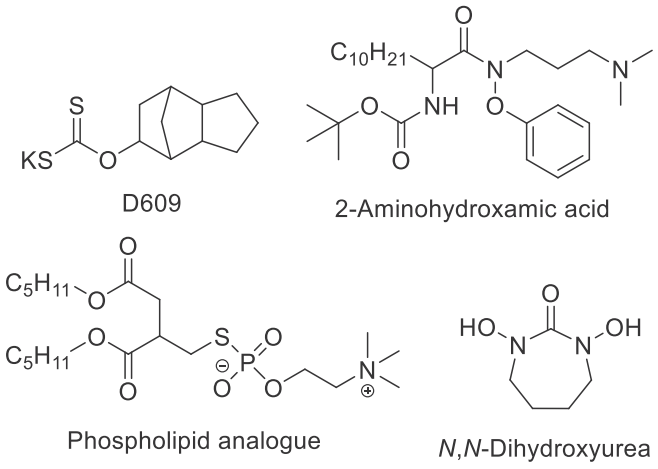


Fig. 1. Examples of known PC-PLC inhibitors.

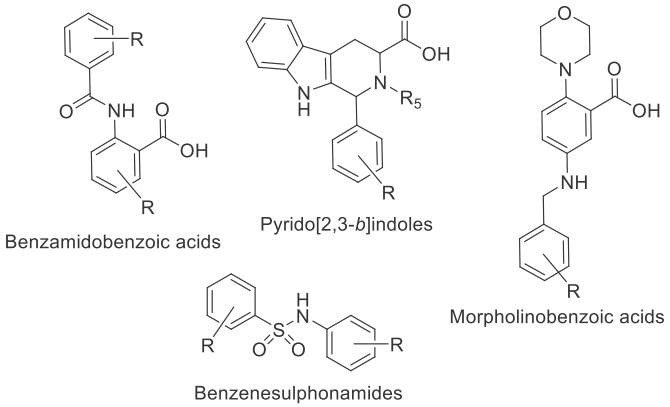
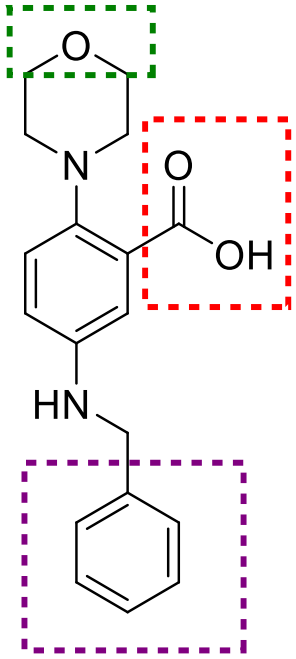


Fig. 2. The four classes of compounds identified as potential hits against PC-PLC; benzenesulphonamides, pyrido[3,4-*b*]indoles, morpholinobenzoic acids and benzamidobenzoic acids.



1a

Fig. 3. Structure of hit compound **1a** and points of variation explored in this study; Series 1 (red and purple), Series 2 (green and purple). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

then conversion of the carboxylic acid to an ester would be expected to be detrimental to the PC-PLC inhibitory activity.

It was decided to begin the synthesis from commercially-available 2-chloro-5-nitrobenzoic acid **2**, the carboxylic acid functionality of which was esterified using standard conditions [15], providing ester **3** in 96% yield. Once formed, **3** underwent nucleophilic aromatic substitution of the chlorine with morpholine, providing morpholinobenzoic acid **4**, the nitro group of which was then reduced to give aniline **5**. With **5** in hand, a panel of benzaldehydes **6a-y** with a range of electron-donating (i.e. -Me, -OMe, −OH) and electron-withdrawing (i.e. halogens, $-CF_3$) at *ortho*-, *meta*- and *para*-positions were condensed to form an imine, which was immediately reduced to the secondary amines present in the desired esters **7a-y**. These esters **7a-y** were then hydrolysed to give the corresponding carboxylic acids **1a-y** (see Scheme 1**).**

In addition to esters **7a-y** and carboxylic acids **1a-y**, it was decided to also synthesise derivatives with no functionality at this position. This required the preparation of a new amine, **8**, which was achieved in two steps — substitution to install the morpholine moiety and reduction of the nitro group - from 1-chloro-4-nitrobenzene **9** (Scheme 2). Once formed, amine **8** was reacted with a range of benzaldehydes **6a-y** and the resulting imines were reduced, providing the desired final products **10a-y**.

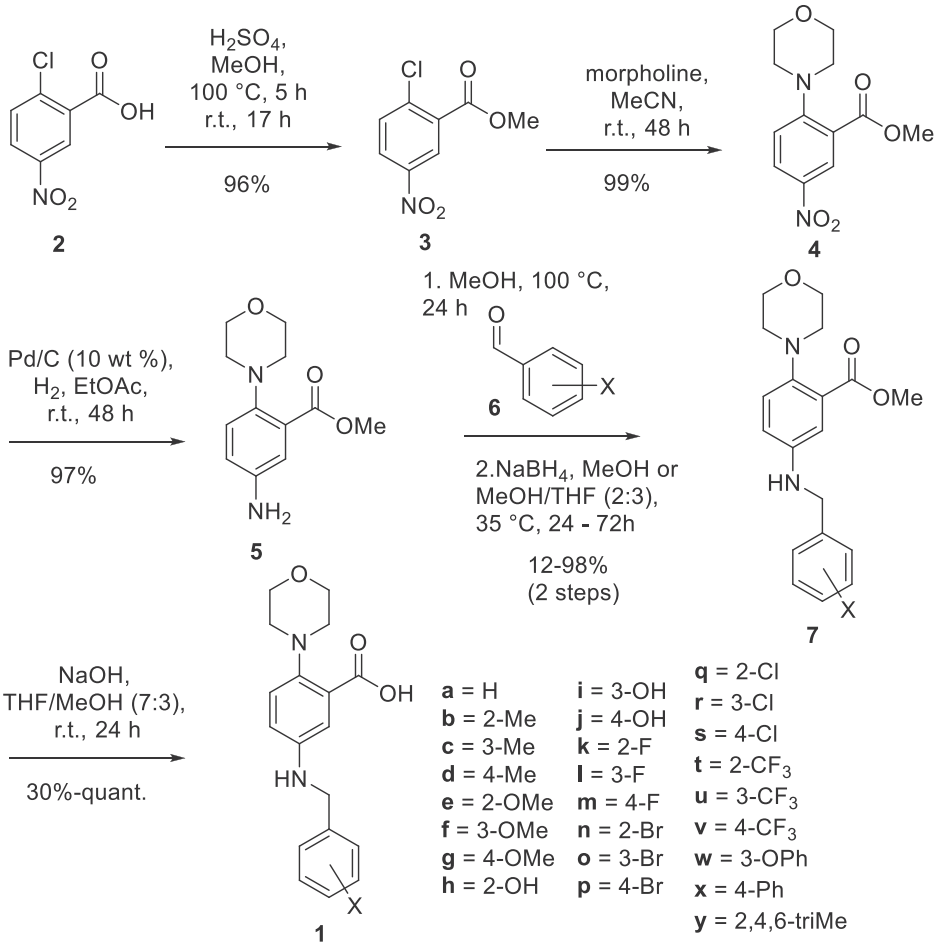
With the successful synthesis of analogues **1**, **7** and **10**, constituting Series 1 of this study, it was decided to assess the PC-PLC inhibitory activity of these 73 compounds. The PC-PLC inhibition was measured using a well-established, commercial Amplex Red Assay and the results for Series 1 are summarised in Table 1 (D609 = 59.7% inhibition, i.e. 40.3% relative PC-PLC enzyme activity).

As can be seen, the carboxylic acids **1** were, in all cases more active that the respective esters **7** (except for **h** with a 2-OH substitution on the *N*-benzyl ring where they not significantly different) as demonstrated by the lower measured PC-PLC activity in the presence of these compounds. As mentioned previously, this observation was expected based on the premise that the carboxylic acid should aid binding to the PC-PLC enzyme, through coordination to the Zn atoms present at the binding pocket. Surprisingly, when the analogues **10** with no ester or carboxylic acid functionality were tested, it was found that in all cases the inhibitory activity of these compounds far exceeded that of both the esters **7** and carboxylic acids **1**. It should be noted that every analogue with no ester or acid functionality, **10**, and carboxylic acid **1**, had greater activity against PC-PLC than the literature standard, D609, confirming this class of compounds to be potent PC-PLC inhibitors and far more effectual than the previously-reported best inhibitor. Assessing the effect of the *N*-benzyl substituent, it was immediately clear that the larger substituents (3-OPh **w**, 4-Ph **x** and 2,4,6-triMe **y**) were not well-tolerated, with these analogues generally exhibiting the lowest activities in each of the analogue series. It could also be seen that the CF_3-substituted analogues **t**, **u** and **v**, were less active. While there was no apparent trend in the effect of the *N*-benzyl substituent position for the carboxylic acids **1** and esters **7**, for analogues **10** it was apparent that the *ortho*-substituted analogues were more active than the *meta*- and *para*-substituted compounds which had comparable activity. It could be seen that compounds with both electron-donating and electron-withdrawing substituents exhibited very good activities, with no clear observable trend. The results from this screen of PC-PLC inhibitory activity of the derivatives at 10 μM was further investigated through the measuring of IC_{50} values of D609 (8.08 ± 0.19 μM), the best-performing acid/ester **1r** (3.60 ± 0.19 μM) and one of the three most active compounds that completely inhibited PC-PLC activity at 10 μM, **10k** (1.06 ± 0.05 μM). These IC_{50} values show that the best-performing inhibitors were ~8 times more effective than the previous literature standard D609.

Following on from the biological assessment of the first series of compounds, it was decided to also investigate the impact of exchanging the morpholine moiety (green in Fig. 3) with other cyclic amines, i.e. piperidine **11** and (methyl **12**, *N*-Boc **13** and non-functionalised **14**) piperazine variants, comprising a set of Series 2 analogues. The previously-described method utilised to synthesise Series 1 compounds was able to be implemented for Series 2 (Scheme 3). As for analogues **10**, 1-chloro-4-nitrobenzene **9** was the starting material for all Series 2 compounds — chloride **9** was reacted with piperidine, 1-methylpiperazine and 1-Boc-piperazine to give substituted products **15**, **16** and **17**, respectively. Nitroaromatics **15**–**17** were then reduced using standard reductive procedures (Pd/C under a H_2 atmosphere) to provide their respective amines **18**–**20**. For Series 2, it was decided to reduce the number of benzaldehydes **6**, to focus on those that generally exhibited better activities, i.e. all *ortho*-substituted variants, in addition to benzaldehydes with hydroxyl, fluoro and chloro substituents at all positions. As performed in the synthesis of Series 1 analogues, amines **18**–**20** were each condensed with 14 different benzaldehydes **6**, giving the corresponding imine which, upon isolation, was immediately reduced to provide the desired final compounds **11**–**13**. Finally, to obtain the non-*N*-substituted piperazine analogues **14**, the Boc-protected compounds **13** were treated with TFA in CH_2Cl_2.

As for Series 1, after Series 2 was successfully synthesised, the generated analogues **11**–**14** were tested for their PC-PLC inhibitory activity using the Amplex Red Assay (Table 2).

Looking at the results from the biological assays for Series 2 compounds, it was apparent that for most of the variations investigated, the piperidine-substituted compounds **11** were the most active than the piperazine **14** and piperazine-derived **12** and **13** analogues. However, interestingly the trend of the most active



Scheme 1. Synthesis of Series 1 analogues, altering substitution (substituent and position) on the *N*-benzyl ring, synthesising both carboxylic acids and their corresponding esters.

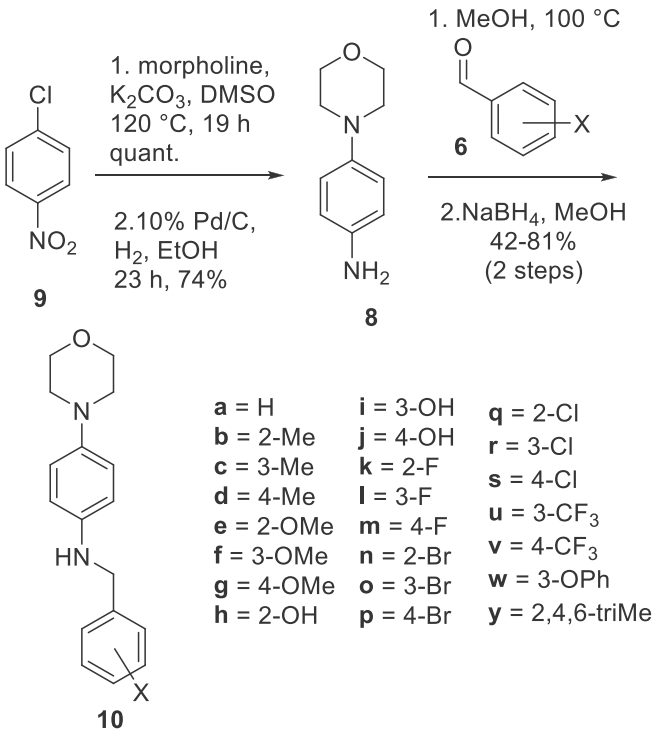
compounds being the *ortho*-*N*-benzyl-substituted compounds did not hold for this series. Most importantly, it was immediately clear that the morpholine-substituted derivatives **10** exhibited greater PC-PLC inhibitory action than their piperidine **11** and (methyl **12**, *N*-Boc **13** and non-functionalised **14**) piperazine counterparts.

With the identification of analogues **10** with no carboxylic acid or ester groups and possessing a morpholine moiety as having the most potent activities against PC-PLC, particularly **10a, 10h** and **10l** with no, *ortho*-OH and *ortho*-F substituents on the *N*-benzyl ring, respectively, we wished to further investigate their potency and how they bind through molecular modelling and docking at the PC-PLC active site. We also wished to explore if molecular docking could account for the differences in activity seen between the acids **1**, esters **7** and the most active analogues **10**, as well as the greater activity exhibited by morpholine-containing compounds, i.e. piperidines **11**.

Comparing the binding modes of acid **1a**, ester **7a** and analogue **10a**, it was immediately clear that they all display similar binding modes within the PC-PLC binding pocket (Fig. 4). The morpholine moiety occupies the negatively-charged space to the right of the binding pocket, while the *N*-benzyl group is situated in the lipophilic area to the left. It can also be seen that the secondary amine linking the aromatic rings is situated in close proximity to the partially-positive cleft near the centre of the binding cavity. Both acid **1a** and ester **7a** are positioned with these titular functionalities buried deep within the binding site, however the absence of these moieties in **10a** does not greatly affect its positioning. Furthermore, comparing **10a** with no substitution on the *N*-benzyl ring and **10k**, with an *ortho*-fluoro substituent, it appears that small substituents to not greatly impact on the positioning of the structure within the site. Interestingly, the effect of the morpholine group is apparent when looking at the binding mode of the piperidine derivative, **11a,** which shows the structure to be completely flipped in the binding pocket, potentially offering justification for its reduced activity – while the oxygen in the morpholine moiety coordinates to the amino acid residues to the right of the pocket, coaxing it into the depicted position, the absence of this functionality as an anchor-point leads to a significant change in binding mode which seems to be responsible for a significant loss in inhibitory activity.

3. Conclusion

PC-PLC is implicated in the progression of a number of cancer cell lines and the current inhibitor of choice for study of this enzyme, D609, is known to have multiple failings, including poor stability in aqueous media. Our recent study identified 2-morpholinobenzoic acids as a potential class of lead compounds which could have greater activity than D609, whilst also having more *drug-like* characteristics. In this work 129 analogues in this class were prepared and their PC-PLC inhibitory activity was assessed. It was found that the majority of these novel compounds had improved activity compared to D609 and the most potent inhibitors completely inhibited enzyme activity. Unlike D609 which is difficult to prepare and is only available commercially as an undefined mixture of stereoisomers, the compounds introduced here are easily prepared at scale and are achiral. It was determined that



Scheme 2. Synthesis of non-ester/carboxylic Series 1 analogues, altering substitution (substituent and position) on the *N*-benzyl ring.

the best compound/s contained a morpholino and 2-substituted *N*-benzyl moieties, with these findings explained using molecular modelling. The compounds reported here will allow for improved study of PC-PLC activity and samples of these inhibitors are easily prepared using the reported methods or can be supplied upon request.

4. Experimental procedures and methodology

4.1. General experimental details

NMR spectra were recorded on a 400 MHz spectrometer. Chemical shifts are reported relative to the solvent peak of chloroform (δ 7.26 for ^{1}H and δ 77.0 for ^{13}C) or DMSO-d_6 (δ 2.50 for ^{1}H and δ 39.5 for ^{13}C). ^{1}H NMR data is reported as position (δ), relative integral, multiplicity (s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; br, broad peak; qd, quartet of doublets), coupling constant (J, Hz), and the assignment of the atom. Broadband proton-decoupled ^{13}C NMR data are reported as position (δ) and assignment of the atom. NMR assignments were performed using HSQC and HMBC experiments. High-resolution mass spectroscopy (HRMS) was carried out by electrospray ionization (ESI) on a MicroTOF-Q mass spectrometer. Unless noted, chemical reagents were used as purchased.

4.2. Synthesis of compounds

General procedures can be found in the Supporting Information.

Methyl 2-chloro-5-nitrobenzoate ***3;*** To a solution of 2-chloro-5-nitrobenzoic acid **2** (7.50 g, 0.037 mol) in MeOH (30 mL) was added concentrated H_2SO_4 (1.35 mL) slowly. The reaction was then heated at reflux for 24 h. The reaction was cooled and the solvent was then removed *in vacuo* and ice-water (30 mL) added to the residue, followed by diethyl ether (30 mL). The organic layer was separated and the aqueous layer further extracted with diethyl ether (2 × 30 mL). The combined organic extracts were washed with water (25 mL), 1 M NaOH (2 × 25 mL) and water (25 mL), dried ($MgSO_4$) and the solvent removed *in vacuo* to give the *title product* **3** (7.68 g, 96%) as a white solid. m.p.: 69–71 °C (Lit [16]. 68–69 °C). R_F: 0.87 (9:1 CH_2Cl_2–MeOH). δ_H (400 MHz; $CDCl_3$; Me_4Si) 4.00 (3H, s, OCH_3), 7.66 (1H, d, J = 8.0 Hz, H-3), 8.27 (1H, dd, J = 2.0, 8.0 Hz, H-4), 8.71 (1H, d, J = 2.0 Hz, H-6). δ_C (100 MHz; $CDCl_3$) 53.1 (OCH_3), 126.7 (C-6), 126.8 (C-4), 131.0 (C-1), 132.3 (C-3), 140.8 (C-2), 146.1 (C-5), 164.0 (C=O). The ^{1}H NMR data was consistent with literature values [15].

Methyl 2-morpholino-5-nitrobenzoate ***4***; To a solution of methyl 2-chloro-5-nitrobenzoate **3** (7.60 g, 0.035 mol) in MeCN (50 mL) was added morpholine (9.13 mL, 0.106 mol) and the mixture stirred under an atmosphere of nitrogen for 24 h. The mixture was then combined with ice-cold water (75 mL) and extracted with diethyl ether (3 × 75 mL). The combined organic extracts were washed with brine (100 mL), dried ($MgSO_4$) and the solvent removed *in vacuo* to give the *title product* **4** (9.37 g, 99%) as a bright yellow solid. m.p.: 117–118 °C (Lit [17]. 110 °C). R_F: 0.63 (1:1 petroleum ether-ethyl acetate). δ_H (400 MHz; $CDCl_3$; Me_4Si) 3.25 (4H, t, J = 4.0 Hz, N(CH_2CH_2)$_2$O), 3.87 (4H, t, J = 4.0 Hz, N(CH_2CH_2)$_2$O), 3.93 (3H, s, OCH_3), 6.99 (1H, d, J = 8.0 Hz, H-3), 8.23 (1H, dd, J = 2.0, 8.0 Hz, H-4), 8.64 (1H, d, J = 2.0 Hz, H-6). δ_C (100 MHz; $CDCl_3$) 51.7 (N(CH_2CH_2)$_2$O), 52.5 (OCH_3), 66.4 (N(CH_2CH_2)$_2$O), 117.5 (C-3), 120.4 (C-1), 128.0 (C-4), 128.7 (C-6), 139.7 (C-5), 156.1 (C-2), 166.3 (C=O). IR: ν_{max}(ATR)/cm-1; 2986 (CH aromatic), 2870, 2849 (CH alkane), 1722 (C=O ester), 1600, 1571, 1503 (C=C aromatic), 1489, 1326 (CH alkane), 1226, 1105 (C–O ether), 926, 822, 714 (CH aromatic). *m/z* (ESI+): 289 (MNa^+, 100%). HRMS (ESI+) Found (MNa^+): 289.0805$C_{12}H_{14}N_2NaO_5$ requires 289.0795.

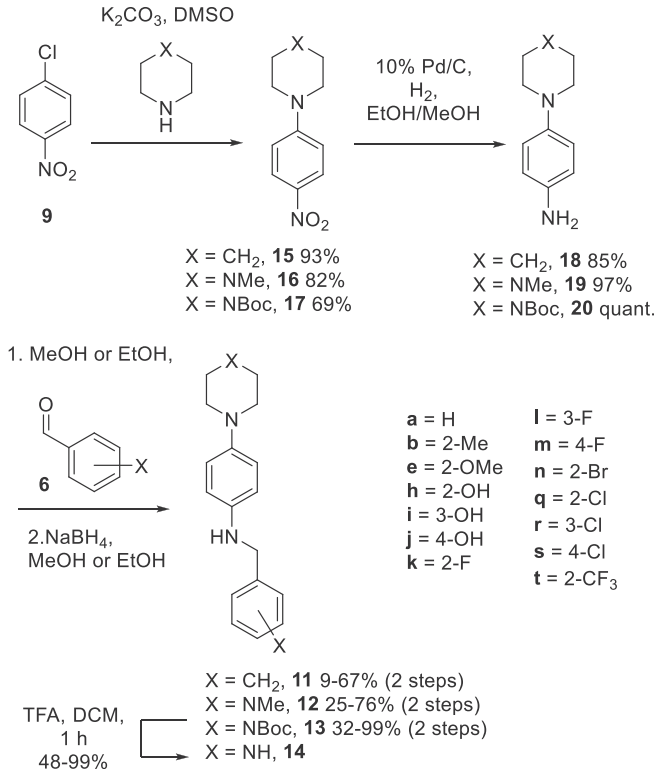
Methyl 5-amino-2-morpholinobenzoate ***5;*** To a solution of methyl 2-morpholino-5-nitrobenzoate **4** (9.30 g, 0.035 mol) in EtOH (50 mL) was added Pd on carbon (10 wt %, 0.93 g) and the reaction stirred under an atmosphere of hydrogen for 48 h. The reaction mixture was then filtered through a plug of Celite and the solvent removed *in vacuo* to give the *title product* **5** (8.04 g, 97%) as a pale yellow solid. m.p.: 119–121 °C (Lit [18]. 121–122 °C). R_F: 0.39 (1:1 petroleum ether-ethyl acetate). δ_H (400 MHz; $CDCl_3$; Me_4Si) 2.93 (4H, t, J = 4.0 Hz, N(CH_2CH_2)$_2$O), 3.59 (2H, br s, NH_2), 3.82 (4H, t, J = 4.0 Hz, N(CH_2CH_2)$_2$O), 3.87 (3H, s, OCH_3), 6.77 (1H, dd, J = 2.0, 8.0 Hz, H-4), 6.95 (1H, d, J = 8.0 Hz, H-3), 7.04 (1H, d, J = 2.0 Hz, H-6). δ_C (100 MHz; $CDCl_3$) 52.0 (OCH_3), 53.6 (N(CH_2CH_2)$_2$O), 67.3 (N(CH_2CH_2)$_2$O), 117.5 (C-6), 119.4 (C-4), 121.1 (C-3), 127.4 (C-1), 141.4 (C-5), 144.2 (C-2), 168.1 (C=O). IR: ν_{max}(ATR)/cm^{-1}; 3452, 3365 (NH), 2960 (CH aromatic), 2842 (CH alkane), 1700 (C=O ester), 1628, 1498 (C=C aromatic), 1445, 1324 (CH alkane), 1243, 1112 (C–O ether), 935, 825 (CH aromatic). *m/z* (ESI+): 259 (MNa^+, 10%), 237 (MH^+, 50), 205 (100). HRMS (ESI+) Found (MNa^+): 259.1051$C_{12}H_{16}N_2NaO_3$ requires 259.1053. Found (MH^+): 237.1235$C_{12}H_{17}N_2O_3$ requires 237.1234. The ^{1}H NMR data was consistent literature values [18].

Methyl 5-(benzylamino)-2-morpholinobenzoate ***7a;*** Formation of the crude imine was carried out according to General Procedure A-1, using methyl 5-amino-2-morpholinobenzoate **5** (0.2 g, 0.85 mmol) and benzaldehyde **6a** (0.31 g, 1.69 mmol) in MeOH (15 mL) to give methyl (*E*)-5-(benzylideneamino)-2-morpholinobenzoate (0.33 g, 97%) as a bright yellow solid that was used in the next reaction without further purification. R_F: 0.70 (1:1 petroleum ether-ethyl acetate). Reduction of the crude imine was carried out according to General Procedure B-1, using methyl (*E*)-5-(benzylideneamino)-2-morpholinobenzoate (0.28 g, 0.85 mmol) in THF (8 mL) and $NaBH_4$ (2 × 96 mg, 2.5 mmol) in THF/MeOH (2:3, 5 mL) for 20 h, followed by an additional 24 h after the second addition of $NaBH_4$. The crude product was purified using

Table 1
PC-PLC activity upon treatment by Series 1 compounds using an Amplex Red Assay. Testing was performed in triplicate. Results shaded light grade exhibit 80–90% inhibition whilst results shaded dark grey exhibit >90% inhibition.

N-Benzyl substitution	Relative PC-PLC activity (% ± s.e.) at 10 μM		
	Carboxylic acid, **1**	Ester, **7**	No carboxylic acid/ester, **10**
No substitution/H, **a**	32.5 ± 0.6	57.7 ± 1.9	0 ± 0.5
2-Me, **b**	35.0 ± 1.0	58.4 ± 0.7	2.2 ± 0.4
3-Me, **c**	23.7 ± 2.1	54.0 ± 1.7	8.3 ± 1.4
4-Me, **d**	33.4 ± 4.9	54.3 ± 1.8	5.4 ± 0.6
2-OMe, **e**	32.5 ± 0.7	43.7 ± 5.3	1.2 ± 0.4
3-OMe, **f**	28.1 ± 2.4	64.6 ± 11.0	5.6 ± 0.1
4-OMe, **g**	32.3 ± 0.8	58.9 ± 8.0	3.2 ± 0.3
2-OH, **h**	40.0 ± 3.8	38.6 ± 3.7	0 ± 0.1
3-OH, **i**	41.6 ± 2.9	52.8 ± 0.7	3.4 ± 0.5
4-OH, **j**	43.8 ± 2.1	48.3 ± 1.1	6.5 ± 0.4
2-F, **k**	29.9 ± 0.8	56.8 ± 0.3	0 ± 0.2
3-F, **l**	49.5 ± 7.6	64.5 ± 1.6	6.1 ± 0.9
4-F, **m**	30.3 ± 0.1	48.9 ± 0.8	1.8 ± 0.2
2-Br, **n**	40.4 ± 3.5	56.7 ± 0.03	14.1 ± 0.05
3-Br, **o**	20.4 ± 9.0	60.9 ± 8.4	11.6 ± 1.8
4-Br, **p**	28.7 ± 1.0	77.3 ± 2.3	18.1 ± 1.3
2-Cl, **q**	11.5 ± 8.0	73.1 ± 5.4	7.9 ± 0.3
3-Cl, **r**	10.7 ± 1.5	65.6 ± 2.7	9.1 ± 0.9
4-Cl, **s**	26.7 ± 1.0	87.5 ± 0.9	12.9 ± 0.3
2-CF_3, **t**	34.1 ± 1.6	83.0 ± 21.0	-
3-CF_3, **u**	44.6 ± 7.0	64.1 ± 2.7	23.4 ± 1.4
4-CF_3, **v**	45.5 ± 8.5	78.6 ± 0.8	27.6 ± 0.1
3-OPh, **w**	47.5 ± 8.5	130.3 ± 26.9	39.3 ± 0.5
4-Ph, **x**	40.1 ± 4.4	100.4 ± 16.4	-
2,4,6-triMe, **y**	45.3 ± 6.5	96.8 ± 20.6	28.9 ± 1.0
D609		40.3 ± 3.2	

flash chromatography (2:1 petroleum ether-ethyl acetate) to give the *title product* **7a** (0.22 g, 81% over two steps) as a light yellow solid. m.p.: 105–108 °C. R_F: 0.77 (2:1 petroleum ether-ethyl acetate). δ_H (400 MHz; $(CD_3)_2SO$; Me_4Si) 2.76 (4H, br s, $N(CH_2CH_2)_2O$), 3.63 (4H, br s, $N(CH_2CH_2)_2O$), 3.87 (3H, s, $COOCH_3$), 4.24 (2H, d, J = 6.0 Hz, CH_2), 6.25 (1H, t, J = 6.0 Hz, NH), 6.67 (1H, dd, J = 3.0, 9.0 Hz, H-4), 6.79 (1H, d, J = 3.0 Hz, H-6), 6.96 (1H, d, J = 9.0 Hz, H-3), 7.22 (1H, m, H-4′), 7.29–7.34 (4H, m, H-2′, H-3′). δ_C (100 MHz; $(CD_3)_2SO$) 46.6 (CH_2), 51.7 ($COOCH_3$), 53.3 ($N(CH_2CH_2)_2O$), 66.6 ($N(CH_2CH_2)_2O$), 113.1 (C-6), 115.3 (C-4), 121.3 (C-3), 126.6 (C-4′), 127.1 (C-2′), 128.1 (C-1), 128.3 (C-3′), 140.0 (C-1′), 140.9 (C-2), 144.5 (C-5), 168.3 (C=O). IR: ν_{max}(ATR)/cm^{-1}; 3304 (NH), 2955 (CH



Scheme 3. Synthesis of Series 2 analogues, varying at the saturated cyclic ring, substituting morpholine with piperidine **11** and (methyl **12**, *N*-Boc **13** and non-functionalised **14**) piperazine variants and altering the altering substitution (substituent and position) on the *N*-benzyl ring.

aromatic), 2803 (CH alkane), 1717 (C=O ester), 1612, 1568 (C=C aromatic), 1496, 1450 (CH alkane), 1309, 1202, 1069 (C–O ether), 926, 817, 743 (CH aromatic). *m/z* (ESI+): 327 (MH^+, 100%), 295 (75). HRMS (ESI+) Found (MNa^+): 349.1531 $C_{19}H_{19}N_2NaO_3$ requires 349.1523. Found (MH^+): 327.1711 $C_{19}H_{23}N_2O_3$ requires 327.1703.

5-(Benzylamino)-2-morpholinobenzoic acid ***1a;*** The reaction was carried out following General Procedure C using methyl 5-(benzylamino)-2-morpholinobenzoate **7a** (0.10 g, 0.3 mmol) and 1M NaOH (4.4 mL) in MeOH/THF (2:3, 10 mL) for 24 h, to give the *title product* **1a** (0.07 g, 70%) as a white solid. m.p.: >230 °C. R_F: 0.09 (1:1 petroleum ether-ethyl acetate). δ_H (400 MHz; $(CD_3)_2SO$) 2.96 (4H, br s, $N(CH_2CH_2)_2O$), 3.76 (4H, br s, $N(CH_2CH_2)_2O$), 4.29 (2H, br s, CH_2), 6.71 (1H, br s, NH), 6.81 (1H, dd, $J = 3.0, 9.0$ Hz, H-4), 7.23 (1H, m, H-4′), 7.25 (1H, d, $J = 3.0$ Hz, H-6), 7.31–7.34 (4H, m, H-2′, H-3′), 7.43 (1H, d, $J = 9.0$ Hz, H-3). δ_C (100 MHz; $(CD_3)_2SO$) 46.0 (CH_2), 52.9 ($N(CH_2CH_2)_2O$), 66.3 ($N(CH_2CH_2)_2O$), 112.9 (C-6), 116.8 (C-4), 123.9 (C-3), 125.2 (C-1), 126.7 (C-4′), 127.0 (C-2′), 128.3 (C-3′), 138.3 (C-2), 139.6 (C-1′), 147.6 (C-5), 166.8 (C=O). IR: ν_{max}(ATR)/cm^{-1}; 3322 (OH, NH), 2975 (CH aromatic), 2861 (CH alkane), 1689 (C=O carboxylic acid), 1607, 1514 (C=C aromatic), 1493, 1454 (CH alkane), 1331, 1260, 1067 (C–O ether), 976, 895, 654 (CH aromatic). *m/z* (ESI+): 355 (MNa^+, 100%), 313 (MH^+, 10). HRMS (ESI+) Found (MNa^+): 335.1372 $C_{18}H_{20}N_2NaO_3$ requires 355.1366. Found (MH^+): 313.1542 $C_{18}H_{21}N_2O_3$ requires 313.1542.

4′-(4-Nitrophenyl)morpholine; A mixture of morpholine (6.6 mL, 0.075 mol), 4-chloronitrobenzene **9** (11.82 g, 0.075 mol) and K_2CO_3 (10.35 g, 0.075 mol) in DMSO (50 mL) was heated at 120 °C for 19 h with stirring. The reaction was cooled to r.t., then 1:1 EtOH:H_2O (200 mL) added. The resulting precipitate was collected by filtration and dried to give the *title compound* (15.62 g, quant.) as an orange solid. m.p. 147–149 °C (Lit [19]. 147–149 °C). δ_H (400 MHz; $CDCl_3$) 3.37 (4H, t, $J = 5.0$ Hz, H-3′), 3.86 (4H, t, $J = 5.0$ Hz, H-2′), 6.82 (2H, d, $J = 9.4$ Hz, H-3), 8.13 (2H, d, $J = 9.4$ Hz, H-2). The ^{1}H NMR data were in agreement with literature values [20].

4-Morpholinoaniline ***8***; To a solution of 4-(4-nitrophenyl)morpholine (8.45 g, 0.041 mol) in EtOH (250 mL) was added 10% Pd/C (0.843 g, 10% w/w). The resulting mixture was stirred at room temperature under an atmosphere of hydrogen for 23 h. After completion, the mixture was filtered through Celite and washed with EtOH. The solvent was removed *in vacuo* to give the *title compound* **8** (5.25 g, 74%) as a pink solid which was used without further purification. m.p. 129–130 °C (Lit [21]. 129.5–130.5 °C). δ_H (400 MHz; $CDCl_3$) 3.02 (4H, t, $J = 4.7$ Hz, H-3′), 3.44 (2H, s, NH_2), 3.85, (4H, t, $J = 4.7$ Hz, H-2′), 6.66, (2H, d, $J = 8.8$ Hz, H-3), 6.80 (2H, d, $J = 8.8$ Hz, H-2). δ_C (100 MHz; $CDCl_3$) 51.1 (C-3′), 67.0 (C-2′), 116.2 (C-2), 118.1 (C-3), 140.3 (C-1), 144.4 (C-4); IR ν_{max}(ATR)/cm^{-1}: 3393, 2855, 1641, 1411, 1259, 1107, 825; *m/z* (ESI+): 227 (17%), 179 (MH^+, 100%); HRMS (ESI+) found (MH^+): 179.1178, $C_{10}H_{15}N_2O$ requires 179.1179. The ^{1}H NMR data were in agreement with literature values [22].

N-Benzyl-4-morpholinoaniline ***10a;*** The reaction was carried out according to General Procedure A-2 with benzaldehyde **6a** (0.20 mL, 1.96 mmol) and 4-morpholinoaniline **8** (0.200 g, 0.96 mmol) for 2 h to afford the crude imine (0.185 g, 0.695 mmol) as a yellow solid that was reduced according to General Procedure B-2 with $NaBH_4$ (0.085 g, 2.25 mol) for 4 h to give the *title compound* **10a** (0.130 g, 50%) as a white solid. m.p. 72–74 °C. δ_H (400 MHz; $CDCl_3$) 3.01 (4H, t, $J = 4.7$ Hz, H-3′), 3.80 (1H, s, NH), 3.85 (4H, t, $J = 4.7$ Hz, H-2′), 4.30 (2H, s, CH_2), 6.63 (2H, d, $J = 8.9$ Hz, H-2), 6.83, (2H, d, $J = 8.9$ Hz. H-3) 7.27–7.39 (5H, m, H-2″, H-3″, H-4″, H-5″, H-6″); δ_C (100 MHz; $CDCl_3$) 49.2 (CH_2), 51.4 (C-3′), 67.3 (C-2′), 114.1 (C-2), 118.5 (C-3), 127.3 (C-4″), 127.7 (C-2″), 128.7 (C-3″), 139.8 (C-1″), 142.9 (C-1), 143.8 (C-4); IR ν_{max}(ATR)/cm^{-1}: 3309, 2923, 2853, 1613, 1518, 1295, 1107; *m/z* (ESI+): 269 (MH^+, 47%), 178 (M-$CH_2(C_6H_4)$, 100%); HRMS (ESI+) found (MH^+): 269.1643, $C_{17}H_{21}N_2O_2$ requires 269.1648. The ^{1}H and ^{13}C NMR data were in agreement with literature values [23].

1-(4-Nitrophenyl)piperidine ***15;*** A mixture of piperidine (7.42 mL, 0.075 mol), 4-chloronitrobenzene **9** (11.82 g, 0.075 mol) and K_2CO_3 (10.37 g, 0.075 mol) in DMSO (50 mL) was heated at 120 °C for 23 h with stirring. The reaction was cooled to r.t., then 1:1 EtOH:H_2O (200 mL) added. The resulting precipitate was collected by filtration and dried to give the *title compound* **15** (14.45 g, 93%) as an orange solid which was used without further purification. m.p. 99–101 °C (Lit [24]. 101–102.5 °C) The ^{1}H NMR data were in agreement with literature values [25]. δ_H (400 MHz; $CDCl_3$) 1.67–1.70 (6H, m, H-3′, H-4′), 3.43 (4H, s, H-2′), 6.79 (2H, d, $J = 9.5$ Hz, H-2), 8.10 (2H, d, $J = 9.5$ Hz, H-3).

4-(Piperidin-1′-yl)aniline ***18***; To a solution of 1-(4-nitrophenyl) piperidine **15** (5.67 g, 0.028 mol) in EtOH (150 mL) was added 10% Pd/C (0.586 g, 10% w/w). The resulting mixture was stirred at room temperature under an atmosphere of hydrogen for 23 h. After completion, the mixture was filtered through Celite and washed with EtOH. The solvent was removed *in vacuo* to afford a red oil which was extracted with DCM (3 × 10 mL) and washed with brine. The combined organic extracts were dried ($MgSO_4$) and the solvent was removed *in vacuo* to give the *title compound* **18** (4.12 g, 85%) as a red oil which was used without further purification. δ_H (400 MHz; $CDCl_3$) 1.51–1.57 (2H, m, H-4′), 1.70–1.75 (4H, m, H-3′), 3.00 (4H, t, $J = 5.5$ Hz, H-2′), 3.41 (2H, s, NH_2), 6.63 (2H, d, $J = 8.8$ Hz, H-2), 6.83 (2H, d, $J = 8.8$ Hz, H-3). δ_C (100 MHz; $CDCl_3$) 24.2 (C-4′), 26.2 (C-3′), 52.6 (C-2′), 116.2 (C-2), 119.1 (C-3), 139.8 (C-1), 145.8 (C-4); IR ν_{max}(ATR)/cm^{-1}: 3336, 2930, 2851, 1620, 1509, 1229, 819; *m/z* (ESI+): 259 (71%), 177 (100%); HRMS (ESI+) found (MH^+): 177.1380, $C_{11}H_{17}N_2$ requires 177.1386. The ^{1}H NMR data were in agreement with literature values [26].

Table 2
PC-PLC activity upon treatment by Series 2 compounds using an Amplex Red Assay. Testing was performed in triplicate. Results shaded light grade exhibit 80–90% inhibition whilst results shaded dark grey exhibit >90% inhibition.

N-Benzyl substitution	Relative PC-PLC activity (% ± s.e.) at 10 μM			
	Piperidine **11**	*N*-Methyl piperazine **12**	*N*-Boc piperazine **13**	Piperazine **14**
No substitution/H, **a**	8.1 ± 1.3	31.5 ± 7.1	42.3 ± 16.3	26.3 ± 2.9
2-Me, **b**	14.7 ± 0.9	40.9 ± 9.7	32.2 ± 4.8	29.3 ± 4.3
2-OMe, **e**	9.9 ± 0.9	29.3 ± 4.3	19.9 ± 0.9	27.7 ± 0.6
2-OH, **h**	14.8 ± 0.5	13.4 ± 6.4	26.5 ± 7.5	37.9 ± 10.6
3-OH, **i**	7.6 ± 0.8	12.7 ± 6.2	18.4 ± 6.3	30.9 ± 4.8
4-OH, **j**	11.0 ± 0.1	9.4 ± 4.6	23.9 ± 8.6	51.3 ± 3.7
2-F, **k**	9.0 ± 1.9	42.7 ± 11.5	29.4 ± 4.2	24.1 ± 1.4
3-F, **l**	10.7 ± 2.0	44.2 ± 12.8	33.5 ± 6.5	26.0 ± 4.9
4-F, **m**	6.6 ± 2.8	23.9 ± 2.6	68.3 ± 15.9	21.1 ± 3.3
2-Br, **n**	28.0 ± 0.4	20.0 ± 7.5	35.6 ± 3.6	26.8 ± 0.8
2-Cl, **q**	22.6 ± 2.6	29.7 ± 4.6	43.2 ± 6.7	31.0 ± 4.3
3-Cl, **r**	24.7 ± 5.1	19.5 ± 5.9	36.8 ± 1.4	27.4 ± 0.4
4-Cl, **s**	23.0 ± 1.8	16.6 ± 2.9	53.3 ± 16.1	31.3 ± 6.3
2-CF_3, **t**	49.3 ± 10.6	25.4 ± 7.9	41.3 ± 3.4	30.4 ± 4.2
D609	40.3 ± 3.2			

N-Benzyl-4-(piperidin-1′-yl)aniline ***11a;*** The reaction was carried out according to General Procedure A-3, with benzaldehyde **6a** (0.27 mL, 2.65 mmol) and 4-(piperidin-1′-yl)aniline **15** (0.2332 g, 1.32 mmol) for 7 d to afford the crude imine (0.073 g, 0.276 mmol) as a yellow solid that was reduced according to General Procedure B-3 with $NaBH_4$ (0.038 g, 0.991 mmol) for 1 h to give the *title compound* **11a** (0.073 g, 21%) as a white solid. m.p. 59–61 °C. δ_H (400 MHz; $CDCl_3$) 1.50–1.56 (2H, m, H-4′), 1.69–1.74 (4H, m, H-3′), 2.98 (4H, t, $J = 5.4$ Hz, H-2′), 3.75 (1H, s, NH), 4.29 (2H, d, $J = 5.5$ Hz, CH_2), 6.61 (2H, d, $J = 8.8$ Hz, H-2), 6.86 (2H, d, $J = 8.8$ Hz, H-3), 7.25–7.39 (5H, m, H-2″, H-3″, H-4″); δ_C (100 MHz; $CDCl_3$) 24.4 (C-4′), 26.4 (C-3′), 49.3 (CH_2), 52.8 (C-2′), 114.0 (C-2), 119.4 (C-3), 127.3 (C-4″), 127.8 (C-2″), 128.7 (C-3″), 140.0 (C-1″), 142.5 (C-1), 145.3 (C-4); IR ν_{max}(ATR)/cm^{-1}: 3396, 2929, 1613, 1512, 1268, 818; *m/z* (ESI^+): 267 (MH^+, 100%); HRMS (ESI^+) found (MH^+): 267.1859, $C_{18}H_{23}N_2$ requires 267.1856.

1-Methyl-4-(4′-nitrophenyl)piperazine ***16;*** A mixture of 1-chloro-4-nitrobenzene **9** (5.0 g, 31.7 mmol) and K_2CO_3 (5.26 g, 38.1 mmol) in DMSO (10 mL) was stirred at r.t. for 30 min before 1-methylpiperazine (3.52 mL, 31.7 mmol) was added dropwise. The mixture was then heated to 90 °C and stirred for 24 h before being poured into cold water and the resultant solid filtered and collected. The solid was dissolved in EtOAc and washed with acid (aq. 2M HCl, 3 × 10 mL). The combined aqueous layers were then neutralised with base (1M NaOH) and extracted with EtOAc (3 × 10 mL). The solvent was removed *in vacuo* to give the *title compound* **16** (5.75 g, 82%) as an orange solid. m.p. 104–106 °C. δ_H (400 MHz, $CDCl_3$) 2.35 (s, 3H, CH_3), 2.55 (4H, t, $J = 5.2$ Hz, H-2, H-6), 3.43 (4H, t, $J = 5.2$ Hz, H-3, H-5), 6.82 (2H, d, $J = 9.6$ Hz, H-2′, H-6′), 8.11 (2H, d, $J = 9.6$ Hz, H-3′, H-5′). The spectroscopic values were in agreement with literature [27].

4-(4′-Methylpiperazin-1′-yl)aniline ***19;*** To a stirred solution of 1′-methyl-4′-(4-nitrophenyl)piperazine (5.75 g, 26.0 mmol) in 1:1 MeOH:EtOAc (60 mL) was added 10% palladium on carbon (0.058 g, 10% w/w) slowly. The mixture was then stirred under an atmosphere of hydrogen for 24 h before being filtered through Celite and washed with further MeOH. The solvent was removed *in vacuo* to give the *title compound* **19** (4.84 g, 97%) as a maroon solid. m.p. 85–87 °C. δ_H (400 MHz, $CDCl_3$) 2.33 (3H, s, CH_3), 2.57 (4H, t, $J = 4.8$ Hz, H-3′, H-5′), 3.06 (4H, t, $J = 4.8$ Hz, H-2′, H-6′), 3.41 (2H, br s, NH_2), 6.64 (2H, d, $J = 8.6$ Hz, H-2, H-6), 6.81 (2H, d, $J = 8.6$ Hz, H-3, H-5). The spectroscopic values were in agreement with literature [27].

tert-Butyl 4-(4′-nitrophenyl)piperazine-1-carboxylate ***17;*** A mixture of 1-chloro-4-nitrobenzene **9** (5.0 g, 31.7 mmol) and K_2CO_3 (5.26 g, 38.1 mmol) in DMSO (10 mL) was stirred at r.t. for 30 min before *tert*-butyl piperazine-1-carboxylate (5.91 g, 31.7 mmol) was added dropwise. The mixture was then heated to 90 °C and stirred for 24 h before being poured into cold water and the resultant solid filtered and collected. The solid was recrystallized from petroleum ether, filtered while hot and precipitated to give the *title compound* **17** (6.74 g, 69%) as a yellow solid. m.p. 144–145 °C. δ_H (400 MHz, $CDCl_3$) 1.49 (9H, s, *t*-Bu), 3.42 (4H, t, $J = 5.6$ Hz, H-3, H-5), 3.60 (4H, t, $J = 5.6$ Hz, H-2, H-6), 6.82 (2H, d, $J = 9.4$ Hz, H-2′, H-6′), 8.14 (2H, d,

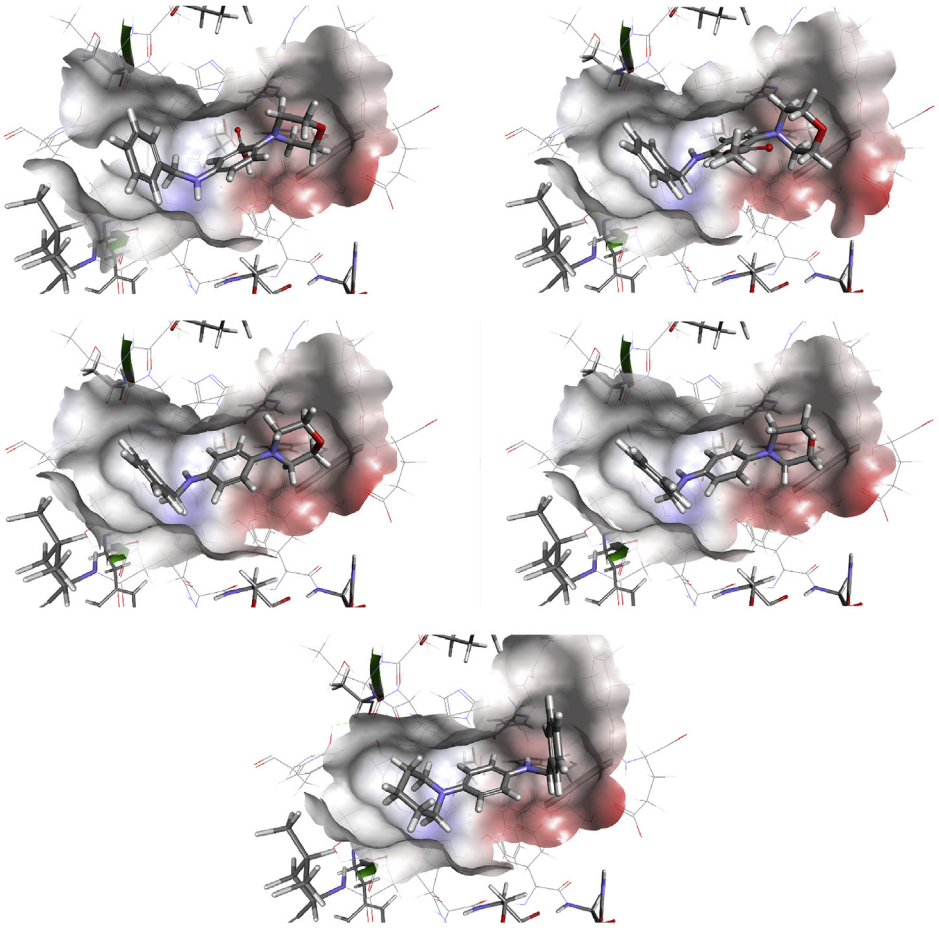


Fig. 4. The docked configuration of acid **1a** (top left), ester **7a** (top right), analogues **10a** (middle left) and **10k** (middle right) and piperidine **11a** (bottom middle) in the PC-PLCBc binding site. The protein surface is rendered. Red and blue surfaces depicts negative and positive partial charge whereas grey shows neutral lipophilic areas. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

J = 9.4 Hz, H-3′, H-5′). δ_C (100 MHz, $CDCl_3$) 28.4 (CH_3), 46.9 (C-2, C-3, C-5, C-6), 80.4 (*t*-Bu), 112.9 (C-2′, C-6′), 126.0 (C-3′, C-5′), 138.8 (C-4′), 154.55 (C═O), 154.63 (C-1′). IR ν_{max}(ATR)/cm^{-1} 2971, 1672, 1585, 1319, 1239, 830. *m/z* (ESI^+): 330 (MNa^+, 100%); HRMS (ESI^+) found (MNa^+): 330.1428, $C_{15}H_{21}N_3NaO_4$ requires 330.1424. The ^1H NMR data was in accordance with literature [28].

tert-Butyl 4-(4′-aminophenyl)piperazine-1-carboxylate ***20;*** *To* a stirred solution of *tert*-butyl 4-(4-nitrophenyl)piperazine-1-carboxylate (3.00 g, 9.76 mmol) in MeOH (30 mL) was added 10% palladium on carbon (0.30 g, 10% w/w) slowly. The mixture was then stirred under an atmosphere of hydrogen for 24 h before being filtered through Celite and washed with further MeOH. The solvent was removed *in vacuo* to give the *title compound* **20** (2.85 g, quant.) as a purple solid. m.p. 89–91 °C. δ_H (400 MHz, $CDCl_3$) 1.48 (9H, s, *t*-Bu), 2.96 (4H, t, J = 4.8 Hz, H-3, H-5), 3.45 (2H, br s, NH_2), 3.56 (4H, t, J = 4.8 Hz, H-2, H-6), 6.65 (2H, d, J = 8.6 Hz, H-3′, H-5′), 6.80 (2H, d, J = 8.6 Hz, H-2′, H-6′). δ_C (100 MHz, $CDCl_3$) 28.5 (CH_3), 51.2 (C-2, C-3, C-5, C-6), 79.8 (*t*-Bu), 116.2 (C-2′), 119.2 (C-3′), 140.7 (C-4′), 144.5 (C-1′), 154.8 (C═O). IR ν_{max}(ATR)/cm^{-1} 3446, 3345, 2965, 1673, 1627, 1512, 1432, 1128, 819. *m/z* (ESI^+): 300 (MNa^+, 24%), 222 ($C_{11}H_{16}N_3O_2^+$, 48%), 178 ($C_{10}H_{16}N_3^+$, 100%); HRMS (ESI^+) found (MNa^+): 300.1678, $C_{15}H_{23}N_3NaO_2$ requires 300.1682. The ^1H NMR data was in accordance with literature [29].

N-Benzyl-4-(4′-methylpiperazin-1′-yl)aniline ***12a;*** The reaction was carried out according to General Procedure AB-4, with 4-(4′-methylpiperazin-1′-yl)aniline **19** (0.20 g, 1.05 mmol) and benzaldehyde **6a** (0.10 mL, 1.05 mmol). The crude imine was triturated from petroleum ether. Reduction with $NaBH_4$ afforded the *title compound* as a beige solid without further purification (0.22 g, 75%). m.p. 117–119 °C. δ_H (400 MHz, $CDCl_3$) 2.37 (3H, s, CH_3), 2.62 (4H, t, J = 4.8 Hz, H-3′, H-5′), 3.09 (4H, t, J = 4.8 Hz, H-2′, H-6′), 4.29 (2H, s, CH_2), 6.61 (2H, d, J = 8.8 Hz, H-2, H-6), 6.85 (2H, d, J = 8.8 Hz, H-3, H-5), 7.22–7.30 (1H, m, H-4″), 7.30–7.40 (4H, m, H-2″, H-3″, H-5″, H-6″). δ_C (100 MHz, $CDCl_3$) 45.8 (CH_3), 49.0 (CH_2), 50.7 (C-2′, C-6′), 55.2 (C-3′, C-6′), 113.8 (C-2, C-6), 118.9 (C-3, C-5), 127.1 (C-4″), 127.5 (C-2″, C-6″), 128.6 (C-3″, C-5″), 139.7 (C-1″), 142.8 (C-1), 143.5 (C-4). IR ν_{max}(ATR)/cm^{-1} 3235, 2932, 2802, 1619, 1517, 1450, 1231, 816. *m/z* (ESI+): 282 (100), 223 (7), 197 (2), 191 (5), 176 (1), 145 (2), 132 (3); HRMS (ESI+) found (MH+): 282.1963, $C_{18}H_{24}N_3$+ requires 282.1965.

tert-Butyl 4-(4′-(benzylamino)phenyl)piperazine-1-carboxylate ***13a;*** The reaction was carried out according to General Procedure AB-4, with *tert*-butyl 4-(4-aminophenyl)piperazine-1-carboxylate **20** (0.20 g, 0.72 mmol) and benzaldehyde **6a** (73 μL, 0.72 mmol). The crude imine was filtered and washed with MeOH. Reduction with $NaBH_4$ afforded the *title compound* **13a** as a light-tan solid without further purification (0.16 g, 56%). m.p. 148–150 °C. δ_H (400 MHz, $CDCl_3$) 1.48 (9H, s, *t*-Bu), 2.95–2.97 (4H, m, H-2′, H-6′), 3.55–37 (4H, m, H-3′, H-5′), 4.29 (2H, s, CH_2), 6.61 (2H, d, J = 8.8 Hz, H-3, H-5), 6.84 (2H, d, J = 8.8 Hz, H-2, H-6), 7.24–7.29 (1H, m, H-4″), 7.31–7.39 (4H, m, H-2″, H-3″, H-5″, H-6″). δ_C (100 MHz, $CDCl_3$) 28.4 (CH_3), 43.4 (C-2, C-6), 48.9 (CH_2), 51.8 (C-3, C-5), 79.9 (*t*-Bu), 113.9 (C-3, C-5), 119.7 (C-2, C-6), 127.3 (C-4″), 127.6 (C-2″, C-6″), 128.6 (C-3″, C-5″), 139.3 (C-1″), 144.4 (C-4′), 154.7 (C═O). C-1′ not observed.

IR ν_{max}(ATR)/cm^{-1}: 3369, 2971, 2856, 1684, 1516, 1415, 1225, 1156, 815; *m/z* (ESI+): 368 (100), 312 (45), 277 (36), 268 (23), 221 (18); HRMS (ESI+) found (MH+): 368.2331, $C_{22}H_{30}N_3O_2$+ requires 368.2333.

N-Benzyl-4-(piperazin-1′-yl)aniline ***14a;*** The reaction was carried out according to General Procedure D, with *tert*-butyl 4-(4'-(benzylamino)phenyl)piperazine-1-carboxylate **13a** (0.1 g, 0.27 mmol) to afford the *title compound* **14a** as a green solid (48 mg, 66%). m.p. 100–102 °C. δ_H (500 MHz, $CDCl_3$) 2.96–3.05 (8H, m, H-2′, H-3′, H-5′, H-6′), 4.28 (2H, s, CH_2), 6.61 (2H, d, J = 8.7 Hz, H-2, H-6), 6.84 (2H, d, J = 8.7 Hz, H-3, H-5), 7.24–7.28 (1H, m, H-4″), 7.30–7.38 (4H, m, H-2″, H-3″, H-5″, H-6″). δ_C (100 MHz, $CDCl_3$) 46.2 (C-3′, C-5′), 49.0 (CH_2), 52.3 (C-2′, C-6′), 113.8 (C-2), 118.8 (C-3), 127.1 (C-4″), 127.5 (C-2″, C-6″), 128.5 (C-3″, C-5″), 139.7 (C-1″), 142.7 (C-1), 144.3 (C-4). IR ν_{max}(ATR)/cm^{-1}: 3293, 3209, 2939, 2795, 1616, 1509, 1452, 1227, 812; $^{m/z}$ (ESI+): 268 (20), 237 (15), 223 (100), 180 (73), 123 (23); HRMS (ESI+) found (MH+): 268.1800, $C_{17}H_{22}N_3$+ requires 268.1808.

4.3. Molecular modelling

The crystal structure was obtained from Protein Data Bank (PDB) [30] ID: 1AH7[10] with resolution 1.50 Å. The Scigress v2.6 program [31] was used to prepare the crystal structure for docking, *i.e.*, hydrogen atoms were added and crystallographic water molecules removed. The centre of PC-PLC$_{Bc}$ binding pocket was defined as the position of Zn^{2+} ion (x = 42.4820, y = 22.996, z = 8.556) with 10 Å radius. The basic amino acids lysine and arginine were defined as protonated. Furthermore, aspartic and glutamic acids were assumed to be deprotonated. The GoldScore (GS) [32], ChemScore (CS) [33,34], Piecewise Linear Potential (ChemPLP) [35] and Astex Statistical Potential (ASP) [36] scoring functions were implemented to validate the predicted binding modes and relative energies of the ligands using the Genetic Optimisation for Ligand Docking software package (GOLD) version 5.4.

4.4. Biological testing

Relative changes of PC-PLC activities were determined using the Amplex Red assay kit (Molecular Probe, Inc.), as described by the manufacturer. Briefly, PC-PLC activity was determined by adding the 0.01U PC-PLC protein to a reaction mixture containing 0.4 mM Amplex Red, 1 unit/mL horseradish peroxidase, 4 unit/mL alkaline phosphatase, 0.1 unit/mL choline oxidase, and 0.5 mM phosphotidylcholine (PtdCho) in 1X Reaction Buffer (50 mM Tris-HCl, pH 7.4, 0.14 M NaCl, 10 mM dimethylglutarate, 2 mM $CaCl_2$). Phosphocholine released from PtdCho by PC-PLC was converted to choline by alkaline phosphatase, which choline was oxidized to form H_2O_2. In the presence of horseradish peroxidase, the H_2O_2 reacts with Amplex Red to generate the fluorescent product, resorufin, which was detected using λ_{ex} = 560 nm and λ_{em} = 590 nm on the EnSpire multimode plate reader. Inhibition concentration at 50% (IC_{50}) are the averages of five replicates using the Amplex Red biochemical assay. Six different concentrations were used to derive the IC_{50} values: 0, 0.1, 0.4, 1.1, 3.3 and 10.0 μM.

Declaration of competing interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ejmech.2020.112162.

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