A New Generation of Arachidonic Acid Analogues as Potential Neurological Agent Targeting Cytosolic Phospholipase A\textsubscript{2}

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Cytosolic phospholipase A\textsubscript{2} (cPLA\textsubscript{2}) is an enzyme that releases arachidonic acid (AA) for the synthesis of eicosanoids and lysophospholipids which play critical roles in the initiation and modulation of oxidative stress and neuroinflammation. In the central nervous system, cPLA\textsubscript{2} activation is implicated in the pathogenesis of various neurodegenerative diseases that involves neuroinflammation, thus making it an important pharmacological target. In this paper, a new class of arachidonic acid (AA) analogues was synthesized and evaluated for their ability to inhibit cPLA\textsubscript{2}. Several compounds were found to inhibit cPLA\textsubscript{2} more strongly than arachidonoyl trifluoromethyl ketone (AACOCF\textsubscript{3}), an inhibitor that is commonly used in the study of cPLA\textsubscript{2}-related neurodegenerative diseases. Subsequent experiments concluded that one of the inhibitors was found to be cPLA\textsubscript{2}-selective, non-cytotoxic, cell and brain penetrant and capable of reducing reactive oxygen species (ROS) and nitric oxide (NO) production in stimulated microglial cells. Computational studies were employed to understand how the compound interacts with cPLA\textsubscript{2}.

Phospholipases A\textsubscript{2} (PLA\textsubscript{2}s) are a superfamily of enzymes characterized by their ability to hydrolyze the ester bond at the sn-2 position of glycerophospholipids to yield polyunsaturated fatty acids (PUFAs) and a lysophospholipid\textsuperscript{1}. The mammalian PLA\textsubscript{2} family comprises six main types of enzymes, namely the secreted small molecular weight sPLA\textsubscript{2}, the larger cytosolic Ca\textsuperscript{2+}-dependent cPLA\textsubscript{2}, the Ca\textsuperscript{2+}-independent iPLA\textsubscript{2}, the platelet-activating factor acetylhydrolases PAF-AH and the lysosomal PLA\textsubscript{2}. Of these PLA\textsubscript{2} enzymes, the ubiquitous cPLA\textsubscript{2} is found to be responsible for the release of arachidonic acid (AA), an important PUA which serves as the precursor for the synthesis of eicosanoids and prostanoids that mediate a wide variety of inflammatory responses\textsuperscript{2}. In the central nervous system, cPLA\textsubscript{2} activation has been implicated in the pathogenesis of a number of neurodegenerative (Alzheimer’s disease, Parkinson’s disease and prion diseases) and neuropsychiatric (schizophrenia and depressive disorder) diseases\textsuperscript{3–7}. Effects of neuroinflammation and its involvement in nitrosative/oxidative signaling pathways for example have been frequently cited as a pathogenetic link to A\textsubscript{β} amyloid production due to the actions of cPLA\textsubscript{2}\textsuperscript{8}. A direct effect of sustained and aberrant cPLA\textsubscript{2} activation could result in enhanced neural membrane destruction eventually compromising functions on membrane ion receptors, leading to cell death\textsuperscript{3–7}. Thus cPLA\textsubscript{2} is an important target for drug discovery, and for the development of new therapeutics to treat neurodegenerative and neuropsychiatric disorders. An ideal cPLA\textsubscript{2} inhibitor should not only reach the site where inflammatory processes are taking place (this may be achieved through improved drug delivery systems), they should also inhibit inflammation, block oxidative stress and cross the blood-brain barrier (BBB). To-date, various compounds have been shown to inhibit cPLA\textsubscript{2} selectively\textsuperscript{9–16}. However many of these compounds have not yet been shown

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to possess brain penetrability\textsuperscript{17,18}. Thus as part of our efforts to develop cPLA\textsubscript{2} inhibitors as potential drug candidates for the treatment of neurological disorders, we have synthesized a new series of AA analogues 1–2 and evaluated them for their PLA\textsubscript{2} inhibitory activities and ability to cross the BBB. In our inhibitor design, we have focused on the arachidonyl scaffold as earlier studies have shown that this pharmacophore is strongly recognized by cPLA\textsubscript{2}\textsuperscript{19–23}. We herein present the synthesis of AA analogues 1–2 and the investigation of these compounds for their (i) inhibition of cPLA\textsubscript{2}, (ii) cytotoxicity, (iii) selectivity and anti-neuroinflammatory properties and (iv) ability to cross the blood-brain barrier; finally computational studies were performed to understand how the compounds bind to cPLA\textsubscript{2}.

Results and Discussion

Chemistry. Using AA as a scaffold, our inhibitor design involved modification at different selected positions of the compound (Fig. 1A). In particular, the carboxylic acid functionality could be modified to a trifluoromethylketone group to form the well-known cPLA\textsubscript{2} inhibitor, AACOCF\textsubscript{3}. Being cell penetrant, AACOCF\textsubscript{3} has been effective in reducing the undesirable effects of dysregulated cPLA\textsubscript{2} systems, including those found in neurological diseases\textsuperscript{9,24–26}. A series of compound with the CF\textsubscript{3} moiety was synthesized and presented as compound 1a.

Compound 1a was initially hydrogenated with Brown's P2 nickel catalyst in ethanol according to a published procedure\textsuperscript{27}. However this did not provide the desired compound 9a. Optimization of the hydrogenation reaction by varying the equivalent of the catalyst and reaction conditions eventually provided 9a in 68% yield (Supplementary Table S1). Initial attempts to trifluoromethylate 9a by stirring TMSCF\textsubscript{3} and CsF as a TMS activator with the ester in CHCl\textsubscript{3} at room temperature did not yield 1a (AACOCF\textsubscript{3})\textsuperscript{31}. Replacing CsF with TBAF also failed to provide the desired compound\textsuperscript{32}. We attempted to convert 9a to the corresponding carboxylic acid and then treating it with LDA and Et\textsubscript{2}O-CF\textsubscript{3} \textsuperscript{33}. However this too did not yield 1a. Finally we trifluoromethylated the intermediate carboxylic

Figure 1. (A) Functionalities on AA that could be varied. (B) Reagent and conditions: (a) 4-Chloro-2-butyn-1-ol, Cul, NaI, K\textsubscript{2}CO\textsubscript{3}, DMF, rt, overnight (b) CBr\textsubscript{4}, PPh\textsubscript{3}, CH\textsubscript{2}Cl\textsubscript{2}, −40 °C to −20 °C, 1 h (c) Propargyl alcohol, Cul, NaI, K\textsubscript{2}CO\textsubscript{3}, DMF, rt, overnight (d) CBr\textsubscript{4}, PPh\textsubscript{3}, CH\textsubscript{2}Cl\textsubscript{2}, −40 °C to −20 °C, 1 h (e) Methyl 6-hexynoate, Cul, NaI, K\textsubscript{2}CO\textsubscript{3}, DMF, rt, overnight (f) Propyl 5-pentynoate, Cul, NaI, K\textsubscript{2}CO\textsubscript{3}, DMF, rt, overnight (g) H\textsubscript{2}, Ni(OAc)\textsubscript{2}, 4H\textsubscript{2}O, NaBH\textsubscript{4}, en, 95% EtOH, rt, 2 h (h) NaOH, MW 120 °C, 1 h (i) Trifluoroacetic anhydride, Pyridine, CH\textsubscript{2}Cl\textsubscript{2}, rt, 2 h (j) Chlorodifluoroacetic anhydride, Pyridine, CH\textsubscript{2}Cl\textsubscript{2}, rt, 2 h (k) Difluoroacetic anhydride, Pyridine, CH\textsubscript{2}Cl\textsubscript{2}, rt, 2 h (l) Methyl 6-hexynoate, Cul, NaI, K\textsubscript{2}CO\textsubscript{3}, DMF, rt, overnight.
acid with pyridine and TFAA\textsuperscript{34,35} and gratifyingly this gave 1\textit{a} in 48\% yield (over 2 steps). Analogues 1\textit{b}–1\textit{h} were synthesized in the same manner by varying alkyne 3 in the first step of the reaction (Supplementary Scheme S1).

In the synthesis of 1\textit{i}–1\textit{l}, the precursor intermediates 13\textit{a}–d are not commercially available and were synthesized by treating alkyl halides 12\textit{a}–d with propargyl alcohol in the presence of butyllithium and an additive as a solvating agent. Initially \textit{N}, \textit{N}′-dimethylpropyleneurea (DMPU) was used as the additive but it gave 13\textit{b} in only 20\% yield. Hence we tried hexamethylphosphoramide (HMPA) which provided 13\textit{b} in 86\% yield. This may be attributed to the increased polarity of the P=O bond in HMPA as compared to the C=O in DMPU which stabilizes the acetylide to a greater extent. HMPA was thus used as an additive for the synthesis of intermediates 13\textit{a}–\textit{d}.

To form the primary amide 1\textit{m}, tetraene 9\textit{a} was amidated by heating it overnight at 80 °C in a solution of ammonia dissolved in methanol and Mg(OMe)\textsubscript{2}. The chlorodifluoroketo and difluoroketo functionalities in 1\textit{n} and 1\textit{o} were respectively synthesized by hydrolyzing the ester 9\textit{a} to the corresponding carboxylic acid followed by a reaction with the respective anhydrides in pyridine and dichloromethane at room temperature (Fig. 1C).

Compound 2 differs from 1 as it contains an extension from the carbonyl amide. 2 was synthesized by first coupling the skipped dyne 7\textit{a} and 7\textit{b} with the respective alkynes to form 8\textit{a} and 17\textit{a}–\textit{c} (Fig. 2). Subsequent hydrogenation and ester hydrolysis gave the corresponding carboxylic acids 19\textit{a}–\textit{d} which were then coupled to the appropriate amines using EDC.HCl, HOBt, and TEA to give 20\textit{a}–20\textit{k}, 2\textit{l}–2\textit{m}. Coupling of 19\textit{a} with 3-aminobenzenesulfonamide give 2\textit{p} under EDC.HCl and TEA coupling condition (Fig. 2B). To synthesize 2\textit{n} and 2\textit{o}, the acid 19\textit{a} had to be converted into an acid chloride with oxalyl chloride before reacting it with the respective amines to form 20\textit{n} and 20\textit{o}. This was attributed to the conjugative electron-withdrawing effects from the methyl ester on the benzene ring, resulting in a decrease in electron density of the amine which in turn affected its reactivity. As a result, a harsher amidation condition was required. 2\textit{n} and 2\textit{o} were then hydrolyzed to the free acid 2\textit{n} and 2\textit{o} in a similar manner (Fig. 2B). 2\textit{q} was synthesized by coupling 7\textit{a} with 22 followed by hydrogenation and hydrolysis (Fig. 2C).
### Testing of inhibitor compounds using cPLA₂ assay.

Table 1 shows the inhibitory activities of 1 and 2 on cPLA₂. The compounds were evaluated with a fluorogenic PLA₂ assay kit (EnzChek® Phospholipase A₂ Assay Kit, Catalog No: E10217) at a standard 10 μM concentration. Potential inhibitors were determined by their ability to lower cPLA₂ activity to a greater extent than 1a.

### Modification of the tail length.

From Table 1 (Cpd 1a–1c and 1i–1l), varying the tail length did not provide a significant improvement in cPLA₂ inhibitory activity as compared to AACOCF₃. In fact, increasing the alkyl chain length from C₅ (1i) to C₁₃ (1l) resulted in a significant decrease in cPLA₂ inhibition. Replacing the alkyl tail with a phenyl moiety provided 1d which showed the highest cPLA₂ inhibitory activity in the trifluoromethylketone series of compounds. This could be attributed to the additional interactions with cPLA₂ (vide infra).

### Table 1. Inhibitory activities of 1 and 2 on cPLA₂ at 10 μM and their physiochemical properties.

| Cpd | R² | m | n | R¹ | % cPLA₂ Activity | % Inhibition | PSA⁺ | Cpd | R² | q | R¹ | % cPLA₂ Activity | % Inhibition | PSA⁺ |
|-----|----|---|---|----|-----------------|-------------|------|-----|----|---|----|-----------------|-------------|------|
| 1a  | CF₃ | 2 | 4 | C₅H₁₁ | 40.0 ± 0.9 | 60.0 | 17.1 | 2a  | C₅H₁₁ | 39.7 ± 2.6 | 60.3 | 66.4 |
| 1b  | CF₃ | 2 | 4 | C₅H₁₁ | 37.0 ± 3.9 | 63.0 | 17.1 | 2b  | C₅H₁₁ | 26.7 ± 0.1 | 73.3 | 66.4 |
| 1c  | CF₃ | 2 | 4 | C₅H₁₁ | 45.0 ± 3.5 | 55.0 | 17.1 | 2c  | C₅H₁₁ | 40.9 ± 1.1 | 59.1 | 66.4 |
| 1d  | CF₃ | 2 | 4 | Ph   | 31.8 ± 2.0 | 68.2 | 17.1 | 2d  | C₅H₁₁ | 13.2 ± 1.2 | 86.8 | 66.4 |
| 1e  | CF₃ | 1 | 4 | C₇H₁₇ | 40.5 ± 3.6 | 59.5 | 17.1 | 2e  | C₅H₁₁ | 25.8 ± 0.1 | 74.2 | 66.4 |
| 1f  | CF₃ | 2 | 3 | C₅H₁₁ | 33.8 ± 2.2 | 66.2 | 17.1 | 2f  | C₅H₁₁ | 35.6 ± 2.1 | 64.4 | 66.4 |
| 1g  | CF₃ | 2 | 3 | C₅H₁₁ | 42.5 ± 0.3 | 57.5 | 17.1 | 2g  | C₅H₁₁ | 15.5 ± 0.3 | 84.5 | 66.4 |
| 1h  | CF₃ | 2 | 3 | C₅H₁₁ | 45.6 ± 2.8 | 54.4 | 17.1 | 2h  | C₅H₁₁ | 43.6 ± 0.2 | 56.4 | 66.4 |
| 1i  | CF₃ | 2 | 2 | C₅H₁₁ | 36.4 ± 3.3 | 63.6 | 17.1 | 2i  | C₅H₁₁ | 11.5 ± 1.0 | 88.5 | 66.4 |
| 1j  | CF₃ | 2 | 2 | C₅H₁₁ | 33.9 ± 4.8 | 66.1 | 17.1 | 2j  | C₅H₁₁ | 16.5 ± 1.2 | 83.5 | 66.4 |
| 1k  | CF₃ | 2 | 2 | C₈H₁₇ | 54.0 ± 6.9 | 46.0 | 17.1 | 2k  | C₅H₁₁ | 37.7 ± 1.5 | 62.3 | 66.4 |
| 1l  | CF₃ | 2 | 2 | C₅H₁₁ | 72.4 ± 12.9 | 27.6 | 17.1 | 2l  | C₅H₁₁ | 67.4 ± 5.7 | 32.6 | 72.2 |
| 1m  | NH₂ | 2 | 4 | C₅H₁₁ | 60.7 ± 1.9 | 39.3 | 43.1 | 2m  | C₅H₁₁ | 57.5 ± 1.4 | 42.5 | 72.2 |
| 1n  | CClF₂ | 2 | 4 | C₅H₁₁ | 49.2 ± 4.5 | 50.8 | 17.1 | 2n  | C₅H₁₁ | 33.3 ± 0.9 | 66.7 | 66.4 |
| 1o  | CHF₂ | 2 | 4 | C₅H₁₁ | 40.2 ± 0.5 | 59.8 | 17.1 | 2o  | C₅H₁₁ | 65.5 ± 4.0 | 34.5 | 66.4 |
| 1p  | Me  | 2 | 4 | C₅H₁₁ | 53.9 ± 2.7 | 46.1 | 89.3 | 2q  | C₅H₁₁ | 56.6 ± 4.9 | 43.4 | 66.4 |
| 1r  | Me  | 2 | 4 | C₅H₁₁ | 84.8 ± 1.0 | 15.2 | 55.4 | 20c | C₅H₁₁ | 84.8 ± 1.0 | 15.2 | 55.4 |

Values were obtained from ChemBioDraw Ultra 12.
Modification of number of double bonds. The skipped alkene functionality in the arachidonyl backbone causes 1a to be light and oxygen sensitive. We hypothesized that reducing the number of skipped alkenes on the backbone could improve the compound’s stability. Hence analogues with a reduced number of double bonds (Table 1 Cpd 1f–I) were synthesized (Fig. 1B,C). However the potencies of these compounds were found to be lower than that of 1a, indicating that it was more favourable for analogues with the same number of carbons to contain more double bonds (Table 1, Cpd 1a, 1h and 1k). A plausible explanation could be due to the increased rigidity of the compound with more double bonds, making them less rotatable and hence improving their affinity for the enzyme pocket.

Modification of the number of methylene groups between the first double bond and the carbonyl group. Earlier studies have shown that the number of methylene groups between the carbonyl carbon and the first double bond (Fig. 1A) is important for enzyme recognition. To better understand the effects of these methylene groups on the inhibitory properties of the compound, 1e with two methylene groups was synthesized. Results obtained showed that 1e had the same cPLA2 inhibitory activity as 1a (Table 1) suggesting that the position of the double bonds from the carbonyl for enzyme recognition may not be as important as it was previously postulated.

Modification of the functionality on the carbonyl group. Earlier studies have shown that the presence of a trifluoromethylketo moiety confers a very high electrophilicity on AACOCF3. This can result in the keto existing largely in the hydrated form, thus reducing the concentration of the active specie. To vary the electrophilicity of the carbonyl group, we modulated the number of fluorine atoms or replaced the trifluoro moiety with other functionalities (Table 1, Cpd 1m–1o). However, reducing the electrophilicity of the carbonyl group by reducing the number of fluorine atoms on the trifluoromethyl moiety did not result in a more potent cPLA2 inhibitor (Table 1, Cpd 1n–1o). Similarly a decrease in cPLA2 inhibitory activity was observed in 1m when the carbonyl was converted into a primary amide. However all the compounds in Table 1 have the same polar surface area (17.1 Å²) except 1m (43.1 Å²). Since the polar surface area measures the compound’s ability to permeate cells, we decided to explore other analogues of 1m by functionalizing the amide moiety.

Pickard et al. have shown that site-directed mutagenesis of Arg200 in cPLA2 severely affected its catalytic property while molecular docking and crystallography studies by other groups have indicated that Arg200 plays a stabilizing role by interacting with the phosphate group on phosphatidylcholine or the carboxylate groups on cPLA2 inhibitors. Through a combination of deuterium exchange mass spectrometry and modeling, Burke and co-workers have also reported the interaction of Arg200 with cPLA2 inhibitors containing carboxyl groups. We thus postulated that functionalizing the amide moiety of 1m with a negatively charged moiety that interacts with Arg200 could improve the compound’s cPLA2 inhibitory activity. Hence various analogues containing an alkyl extension ending with either a carboxylic acid, ester, sulfonamide or amide functionality were synthesized (Fig. 2).

We started the investigation by synthesizing 2a–2c which contained different number of methylene groups between the amide and carboxylic acid functionalities. 2b was found to possess the best cPLA2 inhibitory activity amongst the three compounds (Table 1 Cpd 2a–2c), suggesting that four methylene groups were optimal between the amide and carbonyl groups. To determine if the interaction between the carboxylic acid functionality and Arg200 was responsible for increasing the compound’s cPLA2 inhibitory activity, 2c containing an ester instead of a carboxylic acid was also prepared. A significant decrease in activity was observed in 2c, thus confirming the importance of the carboxylic acid moiety.

Next, we directed our efforts to determine the effect of the number of methylene groups between the first double bond and the carbonyl group. Although it was observed in our earlier studies of compound 1 that this effect is insignificant, the presence of a carboxyl-containing extension in 2 would result in a longer molecule than 1, which could alter the interactions between 2 and the putative binding pocket in cPLA2. Comparison of the activities of 2b, 2g, 2k and 2q (Table 1) showed that cPLA2 inhibition improved when the number of methylene groups was increased, with four methylene groups (2g) between the first double bond and the carbonyl group being the optimal number.

We have also explored rigidifying the compound by replacing the alkyl groups between the amide and carboxylic acid with a phenyl moiety. Three analogues containing a meta- (2d), para- (2n) and ortho- (2o) carboxylic acid were synthesized. 2d exhibited the best cPLA2 inhibition, providing a 2.5-fold increase in activity. When the carboxylic acid moiety was replaced with an amide or sulfonamide (Table 1), lower cPLA2 inhibitory activities were observed.

Varying the alkyl tail of 2d provided 2i and 2j, 2i exhibited 88.5% cPLA2 inhibition and is the most potent compound found in this study. Hence from our structure-activity studies, three compounds, 2d, 2g and 2i which significantly decreased the cPLA2 activity were identified. Since 2d and 2i are structurally analogous, only the stronger inhibitor 2i was selected for further studies. The IC50 values of 2g and 2i were determined to be 5.3 ± 0.3 and 2.9 ± 0.2 μM respectively, indicating that these compounds display 3-fold and 5.5-fold stronger cPLA2 inhibition than 1a (IC50 = 16.5 ± 3.0 μM).

Cell survival test. To explore whether the inhibitors are cytotoxic, MTS assay were conducted on BV-2 cells, a murine microglial cell line derived from post-natal mouse cerebella, and HEK293T cells that are derived from human embryonic kidney. Both cell types were treated with 10 μM of 1a, 2g and 2i for 48 h and 72 h (Fig. 3A,B). Dimethyl sulfoxide (DMSO) vehicle control was also included as the compounds were dissolved in DMSO. The amount of DMSO used in the assay was limited to 1% of the total volume to ensure that it does not result in cytotoxicity of cells. Both 2g and 2i showed no significant toxic effects on BV-2 and HEK293T cells as compared to vehicle control.
**sPLA<sub>2</sub> assay.** To further explore their selectivity towards cPLA<sub>2</sub>, 10 μM of 2g and 2i were evaluated for their inhibition against recombinant sPLA<sub>2</sub> using the fluorogenic PLA<sub>2</sub> assay (Fig. 3C). Thioetheramide-PC, a specific inhibitor of sPLA<sub>2</sub> (IC<sub>50</sub> = 2 μM) was used as a reference and produced a 62% reduction of sPLA<sub>2</sub> activity. 2i did not provide a significant change in sPLA<sub>2</sub> activity, indicating that it could not inhibit sPLA<sub>2</sub>. However, 2g showed a significant increase of sPLA<sub>2</sub> activity to 184%, suggesting that it could possibly be a sPLA<sub>2</sub> activator, thereby disqualifying it as a selective cPLA<sub>2</sub> inhibitor. With the elimination of 2g, only 2i was assessed for its ability to oppose microglia activation via cPLA<sub>2</sub> inhibition (there is a possibility of 2i inhibiting iPLA<sub>2</sub>. However the purified enzymatically active form of iPLA<sub>2</sub> is not commercially available. Hence in this study, we did not evaluate 2i for its inhibitory activity against iPLA<sub>2</sub>. Such testing would be important in the future e.g. through the purification of iPLA<sub>2</sub> from rat brain lysates to obtain active iPLA<sub>2</sub>.

**LPS stimulation of BV-2 cells triggers iNOS production.** It is well-established that reactive oxygen species (ROS) and nitric oxide (NO), produced during microglia activation, contribute to inflammatory response...
and cytotoxic damage to the surrounding neurons and neighbouring cells. Recent studies have shown that BV-2 microglial cell could serve as an in vitro model to mimic such neuroinflammatory states when stimulated with lipopolysaccharide (LPS). LPS activates BV-2 cells by triggering a cascade of inflammatory events which includes the production of NO. This event is characterized by the generation of the biomarker, inducible nitric oxide synthase (iNOS), as well as ROS. Chuang et al. have also shown that cPLA₂ is a major contributor in this pathway and inhibiting this enzyme reduces the production of the neurotoxic mediators and provide neuroprotective effects. Therefore to investigate the ability of 2i in inhibiting cPLA₂, in a neuroinflammatory model, BV-2 cells were treated with LPS (2 μg/mL), incubated for different time points, collected and lysed for protein extraction. Western blots conducted showed that iNOS level was highest between 8–14 h (Fig. 3D). Densitometry analyses showed that there were no statistical differences in the iNOS levels between the 8 and 14 h time points (Fig. 3E). The cPLA₂ level was also determined and found to be largely constant throughout the entire activation process with a slight drop at the 24 h timepoint (Fig. 3F). Next, 2i (1, 10 and 20 μM) were incubated together with LPS in the BV-2 cells for 14 h (Fig. 4A, B). The LPS-induced iNOS expression was significantly reduced by 50% when treated with 20 μM of compound 2i. Varying the concentration of 2i from 1–20 μM showed a dose-dependent decrease in the iNOS level, indicating that 2i was capable of reducing iNOS production.

**ROS production.** CM-H2DCFDA was used as the main detection reagent for ROS for this study. Being a chloromethyl derivative of the conventional H2DCFDA, it is better retained in the cells due to the reaction of its chloromethyl group with intracellular thiol species. In addition, esterase present in the cells would convert the acetate group found in CM-H2DCFDA into carboxylic acid, further retaining it in the cell. To investigate the ROS production by BV-2 cell stimulated with LPS, intracellular ROS was measured 14 h post-stimulation with CM-H2DCFDA fluorescence. A 2-fold increase in ROS was detected as compared to the control. When 2i (2, 10 and 20 μM) were incubated with the BV-2 cells in the presence of 2 μg/mL LPS for 14 h, a dose-dependent decrease in ROS was observed (Fig. 4C), indicating 2i could reduce ROS production under neurotoxic conditions. However, residual ROS were observed for samples treated with 2i. This could be because the activation of ROS production might not be solely due to the inflammatory pathway mechanism involving cPLA₂. It was reported in previous studies that a multitude of several other processes producing ROS may be at work.

**In vitro evaluation of the blood-brain-barrier (BBB) permeation.** A major requirement for the development of a successful drug for the treatment of central nervous system (CNS) disorder is its ability to pass through the BBB to reach the therapeutic target. Hence screening for its ability to penetrate the BBB is of great importance.

Earlier studies have demonstrated that the parallel artificial membrane permeation assay (PAMPA) assay provides good prediction of in vivo BBB permeability and is a useful tool to screen compounds for brain penetration. Thus to explore whether 2i is able to penetrate into the brain, we used PAMPA with porcine brain lipids as the lipid barrier. Commercially available and highly potent cPLA₂ inhibitors, CDIBA (an analogue of efipladib) and pyrrophenone, were also evaluated for their ability to penetrate the BBB. The effective permeability (Pₑ) of 2i, CDIBA and pyrrophenone were found to be 12.34 ± 1.46 × 10⁻⁶, 3.98 ± 0.24 × 10⁻⁶ and 2.00 ± 0.05 × 10⁻⁶ cm/s (16 h, 25°C). Pₑ values of reference compounds determined under similar conditions were of the order propranolol > carbamazepine > quinidine > caffeine > dopamine, which agreed with reported literature. A minimum Pₑ of 7 × 10⁻⁶ cm/s has been cited as the threshold for permeability across the blood brain barrier. As the Pₑ of 2i exceeded this value, we are optimistic that 2i has the potential to transverse the BBB. We also found good aqueous....

**Figure 4.** (A, B) Changes in iNOS protein expression in BV-2 cells after 14 h treatment with 2 μg/mL of LPS and different concentrations of 2i. BV-2 cells were collected, lysed and western blot was performed. (A) Representative western blot out of 3 independent experiments showing protein bands of iNOS and GAPDH. (B) Densitometry analysis of iNOS after normalization with GAPDH. Data analysed by one-way ANOVA with Dunnett’s Multiple Comparison Test indicates significant difference between LPS treated versus LPS treated with 20 μM of 2i (*P < 0.05). (C) 2i reduces ROS production in BV-2 cells after a 14 h 2 μg/mL LPS insult. Different concentrations of 2i were dissolved with LPS in culture medium and treated to the cells for 14 h. Five independent biological repeat of cells were conducted. Data analysed by one-way ANOVA with Bonferroni’s Multiple Comparison Test indicates significant difference when comparing between LPS-treated versus LPS-treated with different concentrations of 2i (***P < 0.01, *P < 0.05). Uncropped images of blots can be found in Supplementary information Fig. S3.
In-silico docking analysis. A docking study was performed to rationalize the inhibitory activities and to identify the possible binding sites of 2g and 2i on the cPLA₂ enzyme. The crystal structure of cPLA₂ in its apo form (PDB ID 1CJY, resolution 2.5 Å) and with a few missing regions was obtained from the protein data bank. The missing regions were modelled and the complete structure was subjected to molecular dynamics (MD) simulations (as outlined in Methods). The complete model of cPLA₂ remained stable during the simulation. The conformations sampled during the last 50 ns of the MD simulations were clustered into conformational sub-states using the Kclust program from the MMTSB tool set, with an rmsd of 2 Å set as cutoff. The cluster centroids of the top 5 most populated clusters were used for docking calculations.

Docking calculations identified a cPLA₂ binding site around the catalytically important residue Ser228. This binding site was shown to be highly negatively charged on one end and slightly positively charged on the other end. Both these charged ends are connected by a 22 Å long, narrow tunnel that is made up of hydrophobic amino acids (Fig. 5A). The cPLA₂ binding site was calculated to have a total volume of ~205 Å³. Since there are no co-crystal structures of cPLA₂-inhibitor available, various analogues of 1 and 2 were docked around this region with different conformations of cPLA₂ identified through clustering the MD simulation trajectories. Ten poses for each compound were computed and the best docking pose for each compound was chosen by ranking the computed binding energy (docking score). Analysis of the top scored solutions (lowest energy solutions) showed that all the compounds bind similarly in the active sites of cPLA₂ with (i) the electrophilic carbon of the substituted ketone in 1 or amide in 2 being in close proximity to Ser228, and (ii) Gly197/Gly198 interacting with the carbonyl oxygen of 1 or the amide oxygen of 2. In addition, docking analysis of 2 shows the carboxylic acid moiety interacting with the side chains of Arg200. For the aminobenzoic acid analogues 2d and 2n, the carboxylic acid functionality is in closer proximity to Arg200 when it is in the meta position than the para position (Fig. 5D,F). This indicates a stronger attractive interaction which could contribute to the stronger inhibitory property of 2d. Furthermore, the interactions between the carboxylic acid moiety and Arg200 provide a tighter binding for 2g and 2i than 1a (Fig. 5D,F). 2i which has its carboxylic acid moiety closest to Arg200, has the most favorable interaction with cPLA₂. These results corroborated with our experimental cPLA₂ inhibition assay data which show 2i as the strongest cPLA₂ inhibitor.
Conclusion
cPLA₂ activation has been shown to be critically important in the regulation of homeostatic processes and disease pathogenesis. Therefore, cPLA₂ represents a potential novel therapeutic target to treat a wide range of diseases from cancer to neurodegenerative diseases such as Alzheimer’s disease and multiple sclerosis. In our efforts to develop potential drug candidates to treat neurological disorders, we have synthesized a new class of AA analogues and identified one compound, 2i that is a non-cytotoxic, cPLA₂-selective inhibitor which inhibits the enzyme more potently than AACOCF₃. Computational modelling and extensive simulations support the ability of 2i to dock into the active site of cPLA₂. 2i is predicted to be brain penetrant through our PAMPA assay and is able to reduce ROS and NO production in LPS-stimulated BV-2 microglial cells. Further studies are presently ongoing to develop the compound as a lead compound. Understanding its in vivo effect will be crucial in evaluating its clinical potential.

Methods
Synthesis of 1 and 2. Detailed experimental procedures and compound characterization data can be found in the supplementary information, available in the online version of the paper.

Enzchek phospholipase A₂ fluorogenic assay (% activity). The assay was conducted according to the manufacturer’s instruction (E10217, Molecular Probes) with slight modification. Briefly, substrate solution was separately prepared by mixing 5 μL of DOPG, 5 μL of DOPC and 5 μL of substrate respectively in 1.5 mL of 1X phospholipase A₂ reaction buffer. The mixture was vortexed vigorously for 3 min before allowing to stand at room temperature for 1 h. 48 μL of 1X phospholipase A₂ reaction buffer were added into each well of a 96-wells plate. This was followed by addition of 1 μL of recombinant cPLA₂ (P4074-03R, US Biological) and 1 μL of 1 mM of the respective compound dissolved in DMSO. The final concentration of the test compound achieved is 10 μM. The reaction was initiated by adding 50 μL of the substrate solution and incubating at room temperature for 1 h. Fluorescence was measured using a Varioskan plate reader (Thermo Scientific, MA, USA) by exciting at 485 nm, while fluorescence emission was detected at 515 nm. Positive control was performed when 1 μL of test compound was replaced by vehicle DMSO. % activity was expressed as the mean value of duplicate wells from three experiments. % activity was calculated by the following formula:

\[
\% \text{activity} = \frac{\text{fluorescence of tested well} - \text{background}}{\text{fluorescence of positive control} - \text{background}}
\]

Enzchek phospholipase A₂ fluorogenic assay (IC₅₀). The assay was conducted as described above. Instead of adding a fixed concentration of the test compound, various concentrations of compound were reconstituted in DMSO. 48 μL of 1X phospholipase A₂ reaction buffer were added into each well of a 96-wells plate. This was followed by addition of 1 μL of recombinant cPLA₂ (P4074-03R, US Biological) and 1 μL of the respective concentration of the test compound dissolved in DMSO. The reaction was initiated by adding 50 μL of the substrate solution and incubating at room temperature for 1 h. Fluorescence was measured using a Varioskan plate reader by exciting at 485 nm, while fluorescence emission was detected at 515 nm. Positive control was performed when 1 μL of test compound was replaced by vehicle DMSO. IC₅₀ was expressed as the mean value of duplicate wells from three experiments.

Enzchek phospholipase A₂ fluorogenic assay (selectivity). The assay was conducted as described above. Instead of adding cPLA₂, it was replaced by sPLA₂. 48 μL of 1X phospholipase A₂ reaction buffer was added into each well of a 96-wells plate. This was followed by addition of 1 μL of sPLA₂, human recombinant type V (10095963, Cayman Chemical) and 1 μL of 1 mM of the respective compound or thioetheramide-PC (62750, Cayman Chemical) dissolved in DMSO. The final concentration of the test compound achieved is 10 μM. The reaction was initiated by adding 50 μL of the substrate solution and incubating at room temperature for 1 h. Fluorescence was measured using a Varioskan plate reader by exciting at 485 nm, while fluorescence emission was detected at 515 nm. Positive control was performed when 1 μL of test compound was replaced by vehicle DMSO. % activity was expressed as the mean value of duplicate wells from four experiments. % activity was calculated by the following formula:

\[
\% \text{activity} = \frac{\text{fluorescence of tested well} - \text{background}}{\text{fluorescence of positive control} - \text{background}}
\]

Cell-culture and LPS treatment. Murine BV-2 cells were cultured in DMEM (11965092, ThermoFisher Scientific) supplemented with 10% fetal bovine serum, 100 μg/mL streptomycin and 100 units/mL penicillin. Cells were maintained in an incubator at 37 °C, under a 5% CO₂ and in a water saturated environment. When performing LPS treatment, BV-2 cells were plated on a 10 cm dish in 10 mL culture medium at a density of 1 x 10⁶ cells per dish one day prior to the experiment. After observing that cells were at 90% confluency, lipopolysaccharide (L6529, Sigma) pre-dissolved in culture medium to achieve a concentration of 2 μg/mL was added to the dish. Different concentrations of test compounds in DMSO were added to the LPS containing medium which was then transferred to the 10 cm dish. DMSO was used as a vehicle control and added to the non-LPS treated control. The cells were incubated at 37 °C, under a 5% CO₂ environment. After the respective timepoints, cells were washed once with 1xPBS (10 mL) and scrapped on ice with 8 mL 1xPBS. Collected cells were centrifuged at 800 rpm for 3 min at 4°C, before discarding the supernatant. Cells were lysed by RIPA buffer supplemented with 1 x protease inhibitor (Roche) and 1 mM PMSF and incubated under ice for 30 min. This was followed by pelleting the cell
debris at 4 °C for 30 min at 13200 rpm before transferring the supernatant to a clean tube and storing the sample at −20 °C. Three independent biological repeats were performed.

**MCTS cell proliferation assay.** Cells were plated on a 96-wells plate and grown for 24 h to a density of 8000 cells per well for both HEK293T and BV-2 cells. Cells were cultured at 37°C under a 5% CO2 environment in 100μL of culture medium. Thereafter, the compound of interest dissolved in culture medium at a concentration of 10μM was replaced into each well. DMSO was added as vehicle in the control group. 3 biological replicates were performed. The plates were then incubated at 37°C with 5% CO2, for 48 h and 72 h. At each individual time point, 20μL of CellTiter 96® AQueous One Solution (MTS reagent) was added into each well and re-incubated at 37°C with 5% CO2 without light for 3h. Colorimetric readings were taken using a Varioskan plate reader at 490 nm and analyzed. Three independent biological repeats with four technical replicates were performed.

**BCA Assay.** BCA assay (23225, Pierce BCA Protein Assay Kit) was performed to quantify the concentration of proteins in the cell lysate. 10μL of test samples (1μL with 9μL of lysis buffer) and BSA standards (range from 1.25 to 50μg/mL) were mixed in a 96 well-plate and incubated with 190μL of working reagent mixture (Ratio of Reagent A:B used was 50:1) at 37°C for 30 min. The colour intensity that was developed was read at 562 nm using a spectrophotometer. A standard curve of different concentrations of BSA was plotted using the average blank-corrected readings for each standard vs. its respective concentration (μg/mL). This was then used to determine the protein concentration for unknown samples.

**Western Blot.** 25μg of individual protein samples were mixed with an equal volume of 2x Laemmli buffer containing DTT and denatured at 90°C for 10 min. The lysates were ran in a 10% tris-glycine SDS-PAGE gel at 100 V for 1.5 h in 1x tris-glycine running buffer. The protein samples on the gel were then transferred onto a PVDF membrane. The membranes were then blocked for 1 h at room temperature in 5% skim milk in 1x TBS-0.1% Tween 20 (TBST). Secondary detection were conducted with a secondary horse-radish peroxidase-conjugated antibodies (Santa Cruz, 1:10000). Enhanced chemiluminescence (ECL) detection of protein bands was performed using ECL Plus Western Blotting detecting system (GE Healthcare). Antibodies used in western blot include rabbit polyclonal anti-NOS2 (Santa-Cruz, sc-456, 1:100), mouse monoclonal anti-cPLA2; primary antibody (Santa Cruz, sc-456, 1:200), mouse monoclonal anti-GAPDH (Merck, 1:10000), with secondary horse-radish peroxidase-conjugated antibodies (Santa Cruz, 1:10000).

**ROS assay.** 20,000 BV-2 cells were seeded into a 96-well plate in 100μL of culture medium and allowed to grow overnight. When a 90% cell confluency was observed, lipopolysaccharide pre-dissolved in culture medium to achieve a concentration of 2μg/mL was added to each well of the 96 well. Different concentrations of test compounds in DMSO were added to the LPS containing medium and was then transferred to the cells. DMSO was used as vehicle control and added to the non-LPS treated control. The cells were incubated for 14 h at 37°C, under a 5% CO2 environment. Medium were removed and washed once with 1x HBSS. 100μL of 10μM CM-H2DCFDA (C6827, Molecular Probes) in 1x HBSS solution was added into each well and incubated at 37°C for 1 h. Fluorescence detection was done by exciting the dye at 485 nm and detection of emission at 525 nm. Five independent biological repeats with six technical replicates were conducted.

**Solubility determinations.** Solubility determinations were carried out on Multiscreen® Solubility filter plates (Millipore-MSSLBP019) from Millipore Corporation (MA, USA) following the protocol (PC2445EN00) from the manufacturer.

**Determination of permeability.** The Parallel Artificial Membrane Permeability Assay (PAMPA) was used to determine the effective permeability (P<sub>e</sub>) of 21. The method reported by Di et al. was followed with modifications. Determinations were carried out on Multiscreen-IP PAMPA assay (donor) plates (MAIPNTR10) and MultiScreen Receiver Plates (MATRNPS050) from Millipore Corporation (USA) with porcine brain lipids (Avanti Polar Lipids Inc, Alabaster, AL) as the lipid barrier.

An aliquot (5μL) of 2% porcine brain lipids in dodecane (ReagentPlus®, Sigma Aldrich, USA) was dispensed into the donor well. A stock solution (5 mM) of 21 prepared in DMSO was diluted with 1x phosphate buffer (PBS, pH 7.4) to give a 50 μM solution. An aliquot (300μL) of the solution was dispensed into the donor well and an equal volume of buffer (1xPBS with 1% DMSO) was added to the corresponding acceptor well. The donor and acceptor plates were assembled, the unit was placed in a humidified box and gently agitated on a mini shaker at room temperature (25°C) for 16 h. After this time, aliquots (200μL) were withdrawn from the donor and acceptor wells, diluted separately to 1 mL with PBS, and quantified at λmax of 290 nm on a Shimadzu UV-1800 Spectrophotometer.

The PAMPA permeabilities of standards (caffeine, quinidine, carbamazepine, dopamine and propanolol) were determined under similar conditions. 300μL aliquots of stock solutions (500μM, 1xPBS, 0.1% DMSO) were dispensed to donor wells as described earlier. Quantification was by UV at λmax of 272 nm (caffeine), 284 nm (carbamazepine), 330 nm (quinidine), 280 nm (dopamine) and 288 nm (propanolol). Calibration plots of standards were determined under similar analytical conditions. The effective permeability (Pe) of these compounds have been reported to vary in the sequence propanolol (most permeable) > carbamazepine > quinidine > caffeine > dopamine (least permeable) and was verified experimentally in our hand. P<sub>e</sub> was obtained from (3):

\[
Pe = -2.303 \times \frac{V_d V_p}{[V_a + V_d]} \times A \times t \times \log[1 - \frac{[V_a + V_d]/(V_d + S)}{C_{A0}/C_{D0}}]
\]

(3)
where $V_A$ and $V_D$ are the volumes of acceptor (cm$^3$) and donor (cm$^3$) wells respectively, $A$ is the area of the surface area of the membrane (0.24 cm$^2$), $t$ is the permeation time (s). The $P_e$ of each compound was obtained from at least 3 separate experiments using 2 different stock solutions. For each independent determination, triplicates (3 wells) were run for each compound.

**In silico docking analysis.** Details on protein modeling, ligand preparation and ligand docking can be found in the supplementary information, available in the online version of the paper.

**Data Availability.** All data generated or analysed during this study are included in this published article (and its Supplementary Information files).

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Author Contributions

C.Y.N. synthesized and characterized all the compounds with the assistance of W.Y.O. C.Y.N. evaluated the compounds using cPLA2 and sPLA2 assays and conducted the cell survival test and iNOS and ROS production analyses with the assistance of F.C.K.T. S.K. performed the in-silico docking analysis. Y.J.C. and M.L.G. performed the BBB permeation analysis. C.Y.N., S. K., F.C.K.T., M.L.G., C.S.V., C.-M.L. and Y.L. analyzed the results and wrote the manuscript.

Additional Information

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