PROTEIN KINASE C-ACTIVATING ISOPHTHALATE DERIVATIVES MITIGATE ALZHEIMER’S DISEASE-RELATED CELLULAR ALTERATIONS

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ABSTRACT

Abnormal protein kinase C (PKC) function contributes to many pathophysiological processes relevant for Alzheimer’s disease (AD), such as amyloid precursor protein (APP) processing. Phorbol esters and other PKC activators have been demonstrated to enhance the secretion of soluble APPα (sAPPα), reduce the levels of β-amyloid (Aβ), induce synaptogenesis, and promote neuroprotection. We have previously described isophthalate derivatives as a structurally simple family of PKC activators. Here, we characterized the effects of isophthalate derivatives HMI-1a3 and HMI-1b11 on neuronal viability, neuroinflammatory response, processing of APP and dendritic spine density and morphology in in vitro. HMI-1a3 increased the viability of embryonic primary cortical neurons and decreased the production of the pro-inflammatory mediator TNFα, but not that of nitric oxide, in mouse neuron-BV2 microglia co-cultures upon LPS- and IFN-γ-induced neuroinflammation. Furthermore, both HMI-1a3 and HMI-1b11 increased the levels of sAPPα relative to total sAPP and the ratio of Aβ42/Aβ40 in human SH-SY5Y neuroblastoma cells. Finally, bryostatin-1, but not HMI-1a3, increased the number of mushroom spines in proportion to total spine density in mature mouse hippocampal neuron cultures. These results suggest that the PKC activator HMI-1a3 exerts neuroprotective functions in the in vitro models relevant for AD by reducing the production of TNFα and increasing the secretion of neuroprotective sAPPα.

Keywords: Protein kinase C, Alzheimer’s disease, APP-processing, Neuroinflammation, Neuroprotection, Isophthalate derivates
Alzheimer’s disease (AD) is the most common cause of dementia in the aging population without existing intervention approaches to halt or even slow down the disease progression. A central pathological feature of AD includes accumulation of toxic and aggregation-prone β-amyloid (Aβ) peptides. Aβ causes synaptic dysfunction, activation of microglia and astrocytes, oxidative and inflammatory stress, and formation of intraneuronal neurofibrillary tangles (NFTs) consisting of hyperphosphorylated tau (Hardy, 2002). Aβ is generated from amyloid precursor protein (APP) as a result of sequential cleavages by β-site-APP-cleaving-enzyme-1 (BACE1) and γ-secretase (De Strooper and Annaert, 2000). Depending on the site of the γ-secretase cleavage, Aβ peptides of different lengths are generated and particularly Aβ42 is prone to aggregate (Selkoe, 1994). In contrast, α-secretase (ADAM10 and ADAM17) cleavage preceding the γ-secretase cleavage of APP leads to the extracellular release of soluble APPα (sAPPα) and a C-terminal fragment (C83) on the membrane, thus precluding the generation of intact Aβ peptide (De Strooper and Annaert, 2000; Haass et al., 1993). Increased production of sAPPα has been demonstrated to have many beneficial effects, including stimulation of neurite outgrowth, cell proliferation, synaptic density, memory retention, and neuroprotection (reviewed in (Müller et al., 2017)). The hyperphosphorylation of tau protein leads to accumulation of intracellular NFTs and neuronal dysfunction (Grundke-Iqbal et al., 1986a, 1986b).

Several signaling pathways are known to regulate the pathophysiological processes involved in the development or progress of AD, one of them being protein kinase C (PKC) (Choi et al., 2006; Crews and Masliah, 2010; Godoy et al., 2014; Lucke-Wold et al., 2015). PKC is a family of serine/threonine kinases with at least 10 isoforms found in mammals (Newton, 2003). These isoforms are divided into three classes based on their activators. Classical PKCs require both Ca²⁺ and diacylglycerol (DAG) for activation whereas novel PKCs
are independent of Ca\textsuperscript{2+}, but they require DAG. On the other hand, atypical PKCs are activated by mechanisms independent of DAG and Ca\textsuperscript{2+}. PKC has been called a “memory kinase” and it has been widely studied in relation to its central role in memory formation in both normal and pathological settings, such as AD models (Lucke-Wold et al., 2015). As PKC regulates several processes linked to AD pathophysiology, its pharmacological activation is considered a potential therapeutic strategy for treating AD (Alkon et al., 2007; Sun and Alkon, 2012, 2010; Talman et al., 2016). A number of studies have shown that PKC activation directs APP processing to the non-pathogenic α-secretase pathway and thereby increases the production of neuroprotective sAPP\textalpha and reduces the production of neurotoxic Aβ species both \textit{in vitro} and \textit{in vivo} (Alkon et al., 2007; Buxbaum et al., 1990; Etcheberrigaray et al., 2004; Jacobsen et al., 1994; Kozikowski et al., 2003). Activation of PKC has also been suggested to inhibit Aβ accumulation by increasing its degradation through upregulation of gelsolin and activation of endothelin-converting enzyme (Choi et al., 2006; Ji et al., 2010). Additionally, PKC activation has been reported to protect neurons from Aβ cytotoxicity (Garrido et al., 2002; Han et al., 2004). Besides the β-amyloid pathology, activated PKC also inhibits tau hyperphosphorylation by phosphorylating and inactivating glycogen synthase kinase 3β (GSK3β), a central kinase phosphorylating tau (Isagawa et al., 2000). Furthermore, PKC activation has been shown to induce neurite elongation (Shirai et al., 2008; Yang et al., 2010) and synaptogenesis (Sen et al., 2016) and to restore mushroom spine synapses (Hongpaisan et al., 2013), indicating that PKC activation might even exhibit neurorestorative potential.

Pharmacological PKC activation can be achieved by targeting its regulatory C1 domain, which is also the binding site for the physiological activator DAG. Several families of C1 domain-targeting PKC agonists, such as phorbol esters, bryostatins, DAG lactones and benzolactams, have been described and investigated in the \textit{in vitro} and \textit{in vivo} models of AD (Boije af Gennäs et al., 2011; Talman et al., 2016). Most PKC activators are however scarce in
natural sources and highly complex in their chemical structure, making them expensive to isolate in sufficient quantities and difficult to synthesize. Furthermore, in cellular context prolonged activation of PKC with ultrapotent agonists, such as phorbol esters, leads to dephosphorylation and subsequent degradation of PKC protein, thus eventually resulting in diminished PKC activity (Newton, 2003). We have previously developed and reported a novel group of C1 domain-targeted PKC modulators, derivatives of 5-(hydroxymethyl)isophthalic acid, which are easy to synthesize from commercially available starting material (Boije af Gennäs et al., 2009). The best-characterised derivatives, namely HMI-1a3 and HMI-1b11, promoted neurite outgrowth in SH-SY5Y neuroblastoma cells and HMI-1b11 was shown to induce PKC-dependent upregulation of the neuronal differentiation marker GAP-43 (Talman et al., 2013). Instead of testing HMI-1a3 and HMI-1b11 directly in the in vivo models relevant for AD, we first wanted to elucidate the effects of these isophthalate derivatives on neuronal viability, neuroinflammatory response, APP processing, and spine morphology using in vitro models of neuroinflammation and AD.
2. Material and methods

2.1 Isophthalic acid derivatives

Isophthalic acid derivatives HMI-1a3, HMI-1b11 (which both bind to PKC C1-domain), and NI-15e (HMI-1a3 analog, which does not bind to C1 domain and was used as a negative control) were synthesized at the Division of Pharmaceutical Chemistry and Technology, Faculty of Pharmacy, University of Helsinki (Helsinki, Finland) as described earlier (Boije af Gennäs et al., 2009).

2.2 Mouse embryonic primary cortical neuron and BV2 microglial cell co-cultures and treatments

Co-cultures were prepared as described before (Gresa-Arribas et al., 2012; Natunen et al., 2016). Shortly, neuronal cells were isolated from the cortices of embryonic stage 18 (E18) JAXC57BL/6J-mouse embryos and plated in Neurobasal medium supplemented with B27 (Gibco), penicillin, streptomycin and L-glutamine on poly-D-lysine-coated (PDL; Sigma) 48-well plates at the density of $20 \times 10^4$ cells/well. Fresh medium was changed on 5 days in vitro (DIV5) to feed the cells. Mouse microglial BV2 cells were cultured in RPMI-1640 medium (Sigma) containing 10% FBS, 2 mM L-glutamine and 100 U/ml penicillin and 100 μg/ml streptomycin for two passages. RPMI-medium was replaced with Neurobasal medium and BV2 cells were gently detached with a cell scraper. BV2 cells were added to primary cortical neuron cultures in one-to-five ratio (BV2:neurons) at DIV5. Furthermore, all treatments were implemented for samples without BV2-microglia (neurons only samples). After one hour, the co-cultured cells were treated with vehicle (0.1% DMSO), anti-inflammatory cytokine IL-10 (50 ng/ml; PeproTech), nitric oxide synthase inhibitor 1400W (20 μM; Tocris), PKC activator phorbol-12-myristate-13-acetate (PMA; 10 and 100 nM; Sigma-Aldrich), bryostatin-1 (10 and 100 nM; Sigma-Aldrich), pan-PKC inhibitor Gö6983 (1 μM; Sigma-Aldrich), and isophthalate derivatives (each with concentrations of 1, 4, 10 and 20...
µM), and let settle for 1 h. Finally, neuroinflammation was induced by treating co-cultures with 200 ng/ml of lipopolysaccharide (LPS) and 20 ng/ml of interferon-γ (IFN-γ) (both from Sigma-Aldrich). Co-culture media was collected 48 hours after LPS/IFN-γ treatment and assayed for TNFα, NO, Aβ 1-40, and Aβ 1-42 levels.

2.3 Neuronal viability assay
The MAP2-ABTS assay for neuronal viability was performed as described previously (Martiskainen et al., 2017; Natunen et al., 2016). Shortly, primary cortical neuron-BV2 microglia co-cultures were fixed in 4% paraformaldehyde (PFA) immediately after removal of cell media, permeabilized in methanol containing 0.3% H₂O₂, and subsequently blocked with blocking solution (PBS, 1% BSA and 10% horse serum; Vector Labs) for 20 min. Neuronal cells were stained with anti-MAP2 primary antibody (1:2000; Sigma, M9942) overnight at +4°C. Next day, cells were incubated with biotinylated horse anti-mouse secondary antibody (1:200; Vector Labs) and ExtrAvidin-HRP tertiary antibody staining (1:500; Sigma) for 1 hour. After tertiary antibody incubation, cells were washed with PBS and incubated with the ABTS peroxidase substrate (Vector Labs, #SK-4500) giving the color reaction according to the manufacturer's instructions. From each well, 150 µl of substrate solution was transferred to a fresh 96-well plate and absorbance was measured at 405 nm with ELISA microplate reader (Infinite® M200, Tecan).

2.4 TNFα, NO, and Aβ measurements in primary cortical neuron and BV2 microglial cell co-cultures
Conditioned media from the cell samples were centrifuged at 10000 × g for 10 min. The levels of secreted TNFα, Aβ x-40, and Aβ x-42 in the medium was measured using ELISA kits (Ready-SET-Go mouse-TNFα ELISA kit, eBioscience; Human/Rat β Amyloid 40 and 42, Wako, Cat no 294-64701/Cat no 292-64501, respectively), following manufacturer’s
instructions. Aβ results were normalized to neuronal viability as shown previously (Martiskainen et al., 2017). NO production was assessed using the Griess Reagent Kit for Nitrite Determination (G-7921, Molecular Probes) following manufacturer’s instructions. Briefly, co-culture supernatants were collected 48 h after LPS/IFN-γ treatment and incubated with Griess reagent for 30 min at RT. Optical density at 540 nm was measured using a microplate reader (Infinite® M200, Tecan). Nitrite concentration was calculated from a sodium nitrite standard curve.

2.5 SH-SY5Y-APP751 cell culture and treatments

SH-SY5Y human neuroblastoma cells overexpressing human APP751 (SH-SY5Y-APP751) were grown on 6-well plates (400 000 cells/well) as described previously (Sarajärvi et al., 2009). The next day cells were exposed to HMI-1a3 and HMI-1b11 (both 20 µM), PMA (10 nM), γ-secretase inhibitor N-[N-(3,5 Difluorophenacetyl) L-alanyl]-S-phenylglycine t-butyl ester (DAPT; Sigma-Aldrich, 10 µM) and bryostatin-1 (10 and 100 nM) in FBS free medium. Exposure times were 4, 24 and 48 h, after which the media were collected, detached cells spun down (2000 × g, 2 min at +4°C) and the supernatant collected. The media samples from 4-h and 24-h time points were used for analyzing secreted amyloid precursor proteins (sAPPs) with Western blotting and from 48-h time point for determining the Aβ40 and Aβ42 levels with ELISA (Human/Rat β Amyloid 40 and 42, Wako, Cat no 294-64701/Cat no 292-64501, respectively). Aβ levels were normalized to total protein levels in the corresponding cell lysates. After collecting the media, the plates were put on ice, cells washed once with cold PBS followed by scraping and lysing with a lysis buffer containing 10 mM TRIS-HCl pH 6.8, 1 mM EDTA, 150 mM NaCl, 0.25% Nonidet P-40 and 1% Triton X-100 complemented with PHOStop protein phosphatase inhibitor and Complete protease inhibitor cocktails (Roche). Cell homogenates were centrifuged (16000 × g, 4 min at +4°C) and supernatants collected. To determine the effect of isophthalates on PKC protein levels, SH-SY5Y naïve cells were used.
The cells were exposed to isophthalates (HMI-1a3 and HMI-1b11) or PMA (100 nM) for 24 hours in serum-supplemented medium. Cell homogenates were prepared by lysing the cells in 1% SDS (in 50 mM Tris-HCl, pH 7.5) and processed for Western blotting as described below.

2.6 Western blot analysis

Protein concentrations were measured using Bicinchoninic acid (BCA) protein assay kit (ThermoFisher Scientific) and equal amounts of protein (20-30 µg) were resolved in 4-12% gradient Bis-Tris gels (Criterion-XT, Bio-rad) under reducing conditions. Media samples were resolved under similar conditions, but due to their low protein content, a maximal volume of media (33 µl) was taken. Samples for PKC level determination were boiled for 5 min with Laemmli sample buffer (#161-0747, Bio-rad) containing 10 % β-mercaptoethanol (Sigma) and resolved in 10 % polyacrylamide gels. After transfer to polyvinylidene difluoride (PVDF) membranes (Trans-Blot Turbo Midi PVDF transfer pack, Bio-rad) or nitrocellulose membranes (PKC level determination), the membranes were blocked with either 5% milk or 5% BSA (depending on the antibody) and then incubated overnight with the primary antibodies at +4°C in a shaker. On the following day, the blots were incubated with secondary antibodies (goat anti-rabbit, #170-6515, Bio-rad and anti-mouse IgG #7076S, Cell Signaling Technology), which were subsequently detected with chemiluminescent substrate (SuperSignal West Pico, #34080, or Supersignal West Femto, #34095, ThermoFisher Scientific) utilizing LAS 3000 Imaging System (Fujifilm) or exposed to film (PKC level determination). Optical densities (OD) of the bands were measured using ImageJ software (https://imagej.net/Downloads). Cell lysates from primary cortical neuron-BV2 microglia co-cultures were probed with phospho-S536 NF-κB p65 antibody (ab28856, anti-pNF-κB p65 (S536) rabbit polyclonal antibody 1:2000, Abcam), total NF-κB p65 antibody (ab16502, anti-NF-κB p65, rabbit polyclonal antibody 1:2000, Abcam), and normalized with β-actin (ab8226, anti-β-actin, mouse
monoclonal antibody 1:1000, Abcam). The blots from cell lysates were probed with APP C-terminus binding antibody (A8717, rabbit anti-APP C-terminus, 1:2000, Sigma). The OD of these bands were normalized with the OD of GAPDH bands from the same samples (sc47724, anti-GAPDH, 1:2000, Santa Cruz Biotechnology). The blots from the cell media, were probed with N-terminus binding antibodies (6E10, mouse anti-Aβ1-16, 1:1000, Biosite, and 22c11, mouse anti-APP N-terminus, 1:1000, Merck) § For determining the PKC protein levels, all primary antibodies were from Santa Cruz Biotechnology PKCa (#8393), PKCβI (#8049), PKCδ (#937) except for PKCe (BD Biosciences, #610085) and were used at 1:1000 dilution. The experiments were repeated 3 times with 2 parallels.

2.7 Mouse primary hippocampal neuron culture, transient transfection, and spine morphology analysis

Primary hippocampal neuronal cultures were prepared from 18-day-old mouse JAXC57BL/6J embryos according to the protocol previously described (Kurkinen et al., 2016). Briefly, single-cell solution (240 000 cells/cm²) was plated on 4-well chamber slides (LabTek) coated with poly-D-lysine and 30 µg/ml laminin in feeding media composed of Neurobasal medium supplemented with 2% B27, 0.5 mM L-glutamine, 100 U/ml penicillin and 100 µg/ml streptomycin. Hippocampal neurons were grown in a cell culture incubator at 37°C in 5% CO₂. Half of the culture media was replaced with fresh feeding media after every 5 days. On DIV19, mature hippocampal neurons in 4-well chamber slides were transiently transfected with a mixture containing 2 µl of Lipofectamine 2000 (Invitrogen) and 0.8 µg of enhanced green fluorescent protein (pEGFP). On DIV20 the cells were treated with 10 µM HMI-1a3, 100 nM bryostatin-1 (Sigma Aldrich), or 0.1% DMSO (vehicle control) for 2 hours, after which the hippocampal neurons were fixed in 4% PFA (24 hours after pEGFP transfection). Hippocampal dendritic spines from GFP-positive neurons were imaged with a Zeiss Axio Observer.Z1 inverted microscope (63 x NA 1.4 oil objective) equipped with Zeiss LSM 800 confocal
module (Carl Zeiss Microimaging GmbH, Jena, Germany). Serial Z-stacks of optical sections from dendritic segments were captured for spine analysis performed with NeuronStudio software (Rodriguez et al., 2008) as described previously (Bertling et al., 2016).

2.8 Statistical analyses
Statistical analyses were performed using the SPSS (version 21.0) or GraphPad Prism (version 5.02) software. A comparison of three or more groups was performed using one-way ANOVA followed by the Fisher’s least significant difference (LSD) post-hoc test or Dunnett’s test. Statistical significance between two groups was tested using the independent sample t-test. All values are reported as mean ± standard error of mean (SEM). The level of statistical significance was defined as $p<0.05$. 
3. Results

3.1 HMI-1a3 reduces neuronal loss after LPS+IFN-γ-induced neuroinflammation in mouse primary neuron-BV2 microglia co-cultures

Mouse primary neuron-BV2 microglia co-cultures have been previously used to study the effects of LPS+IFN-γ-induced neuroinflammation (Martiskainen et al., 2017). Similarly, we observed a significant reduction in the neuronal viability after LPS+IFN-γ treatment as compared to vehicle treated co-cultures at DIV7 (Fig. 1a). The reduction in neuronal viability was accompanied with significantly increased levels of the proinflammatory cytokine TNFα and nitric oxide (NO) measured from the co-culture medium 48 hours after the initiation of LPS+IFN-γ treatment. Pre-treatment of co-cultures with the specific iNOS inhibitor 1400W resulted in an average of 85% reduction in NO levels and reduced neuronal loss, without affecting the levels of TNFα (Fig. 1a). However, pre-treatment of co-cultures with IL-10 significantly decreased the TNFα levels in LPS+IFN-γ-treated cells by 60%, without effects on neuronal viability or the levels of NO (Fig. 1a). LPS+IFN-γ treatment of mouse primary cortical neuronal cultures without addition of BV2 microglial cells did not affect the production of TNFα or NO, or neuronal viability as compared to vehicle treated samples (data not shown).

Collectively, these results are consistent with the previous findings in the neuron-BV2 microglia co-cultures upon neuroinflammation (Martiskainen et al., 2017) and thus corroborate the usage of this co-culture system as a feasible model to investigate protein kinase C-activating isophthalate derivatives in the cellular processes relevant for AD, such as neuroinflammation.

To investigate whether HMI-1a3 and HMI-1b11 exert neuroprotective effects, we administered the test compounds with or without the pan-PKC inhibitor Gö6983 into the co-culture medium 1 h after the addition of BV2 microglial cells into neuronal cultures. NI-15e, which is a structural analogue of HMI-1a3 without significant affinity to the C1 domain, was used as a negative control as well as PMA and bryostatin-1 as positive controls for PKC...
activation. Neuroinflammation was induced 1 h later with LPS+IFN-γ. HMI-1b11 had no effect on neuronal viability (data not shown), whereas HMI-1a3 increased neuronal viability upon LPS+IFN-γ treatment, especially at 20 µM concentration as compared to vehicle treated cells (Fig. 1b). Interestingly, the PKC inhibitor Gö6983 alone (1 µM) did not affect neuronal viability, but neuronal loss was observed, when it was administered in combination with any of the HMI-1a3 concentrations studied (Fig. 1b). Unexpectedly, the negative control NI-15e induced approximately 20% neuronal loss at all concentrations as compared to the vehicle control. Similarly, the potent PKC activator PMA reduced neuronal viability significantly at both 10 nM and 100 nM concentrations, and this effect was not affected by simultaneous PKC inhibition with Gö6983 (Fig. 1c). Bryostatin-1, another PKC activator, had no effect on neuronal viability alone, while in combination with Gö6983, it increased the neuronal viability (Supplementary Fig. S1a). Taken together, these results suggest that HMI-1a3 exhibits neuroprotective activity in neuron-BV2 microglial co-cultures subjected to LPS+IFN-γ-induced neuroinflammation.

3.2 HMI-1a3 treatment decreases the levels of TNFα but does not affect NO production in mouse primary neuron-BV2 microglia co-cultures subjected to neuroinflammation

It is well-established that several microglia-activating cascades, including Aβ deposition, initiate neuroinflammation in brain. Activated microglia play an important role in the brain tissue by inducing the expression of pro-inflammatory cytokines, such as interleukins and TNFα, which in turn trigger the expression of inducible nitric oxide synthase (iNOS) and the production of NO (Wang et al., 2015). In neurons, TNFα and NO act as important mediators with both pro-inflammatory and destructive effects. HMI-1a3 decreased TNFα levels in a concentration-dependent manner as compared to vehicle treated cells (Fig. 2a). However, HMI-1a3 had no effect on TNFα production in the presence of 1µM Gö6983, indicating that the HMI-1a3-induced decrease in TNFα secretion was mediated by PKC. As with HMI-1a3,
bryostatin-1 alone decreased TNFα levels, while this effect was not reversed in the presence of Gö6983 (Supplementary Fig. S1b). As expected, NI-15e had no effect on the levels of TNFα upon LPS+IFN-γ-induced neuroinflammation (Fig. 2a). In contrast with the effects of HMI-1a3, the treatment of co-cultures with 100 nM PMA increased the levels of TNFα both alone and in the presence of Gö6983 (Fig. 2b), which is in line with its PKC-independent neurotoxic effects in the ABTS assay. Neither HMI-1a3 nor NI-15e had any effect on the production of NO in LPS+IFN-γ-treated co-cultures (Fig. 2c). Similarly, PMA alone had no effect on production of NO, but when co-administered with 1µM Gö6983, it increased the NO production at both 10 nM and 100 nM concentrations (Fig. 2d). Conversely, bryostatin-1 increased the production of NO in a PKC activation-dependent manner (Supplementary Fig. S1c). In summary, HMI-1a3 decreased the levels of TNFα through activation of PKC, but did not affect the production of NO.

### 3.3 HMI-1a3 does not affect the S536 phosphorylation of NFκB p65 in the primary neuron-BV2 microglia co-cultures upon neuroinflammation

Since nuclear factor kappa B (NFκB) is the key transcriptional regulator of genes that control inflammation, immune regulation, proliferation and cell death (Christian et al., 2016), we next assessed the phosphorylation status and the total levels of NFκB p65 subunit in the primary neuron-BV2 microglia co-cultures treated with HMI-1a3 or bryostatin-1 upon neuroinflammation (Fig. 3). The S536 phosphorylation site in NFκB p65 subunit was selected as it is one of the best-understood phosphorylation targets in the transactivation domain (Christian et al., 2016). Primary neuron-BV2 microglia co-cultures were treated with 20 µM HMI-1a3, which in the previous experiments affected significantly neuronal viability and the levels of TNFα upon LPS+IFN-γ-induced neuroinflammation (Fig. 1b and Fig. 2a). Bryostatin-1 (10 and 100 nM) was used as a control and all the LPS+IFN-γ-treated samples showed the
expected increase in the levels of NO, confirming the induction of neuroinflammation in the co-cultures (data not shown). Western blot analysis of total protein lysates of HMI-1a3- or bryostatin-1-treated samples upon LPS+IFN-γ-induced neuroinflammation did not reveal statistically significant changes in the S536 phosphorylation status of NFκB p65 when normalized to the total levels of NFκB p65 (Fig. 3a and Fig. 3b). HMI-1a3, but not bryostatin-1, significantly increased the total levels of NFκB p65 on average by 20% (Fig. 3a and Fig. 3b). These results suggest that HMI-1a3 does not affect the S536 phosphorylation status in the transactivation domain of NFκB p65 but instead increases moderately the levels of total NFκB p65 in the primary neuron-BV2 microglia co-cultures upon neuroinflammation.

3.4 HMI-1a3 and HMI-1b11 do not significantly affect the levels of Aβ40 or Aβ42 in primary neuron-BV2 microglia co-cultures upon neuroinflammation or in neuroblastoma cells

We next elucidated whether the isophthalate derivatives affect the levels of Aβ40 and Aβ42, or the ratio of these Aβ species in the primary neuron-BV2 microglia co-culture media upon neuroinflammation. HMI-1a3 did not significantly affect the levels of Aβ40 (Fig. 4a) or Aβ42 (Fig. 4b), nor the ratio of Aβ42/Aβ40 (Fig. 4c) as compared to vehicle-treated cells. However, there was a trend towards increased levels of Aβ40 and Aβ42, which prompted us to investigate whether the isophthalate derivatives affect APP processing in human SH-SY5Y neuroblastoma cells overexpressing APP751 (SH-SY5Y-APP751). In our previous study with naïve SH-SY5Y cells, both HMI-1a3 and HMI-1b11 were shown to promote neurite growth, while HMI-1b11 was better tolerated (Talman et al., 2013). Upon treatment with HMI-1a3 or HMI-1b11 for 48 h, both Aβ40 and Aβ42 levels in the cell culture medium showed a moderate, but non-significant increase when compared to vehicle-treated control cells (Fig. 4d). In addition, the ratio of Aβ42/Aβ40 was moderately, but not statistically significantly decreased (Fig. 4f). However, 10 nM PMA, which was used as a positive control, caused a significant increase in
the levels of Aβ40 (Fig. 4d), without any effect on the levels of Aβ42 (Fig. 4e), leading to a 
~70% decrease in the ratio of Aβ42/Aβ40 as compared to a vehicle-treated cells (Fig. 4f). This 
is in line with numerous previous studies showing increased α-secretase-mediated APP 
cleavage in response to PKC activation with phorbol esters (Skovronsky et al., 2000; Zhu et 
al., 2001). 10 nM bryostatin-1 did not affect the levels of Aβ40, but it decreased the levels of 
Aβ42, thereby significantly decreasing the ratio of Aβ42/Aβ40 (Supplementary Fig. S2). No 
effects were observed with the treatment of 100 nM bryostatin-1 (Supplementary Fig. S2). 
Although we observed a trend towards a decreased ratio of Aβ42/Aβ40 in cells treated with the 
isophthalate derivatives, these results suggest that the isophthalates are not as effective as PMA 
or bryostatin-1 in modulating the APP processing towards the non-amyloidogenic pathway.

3.5 HMI-1a3 affects the levels of APP C83 and the maturation of APP in neuroblastoma 
cells

Next, we explored the effects of these PKC activators on the levels and maturation of APP and 
APP C-terminal fragment (APP C83) in SH-SY5Y-APP751 cells after 4- (Fig. 5a-b) and 24- 
hour (Fig. 5c-d) treatments. Additionally, the γ-secretase inhibitor DAPT (10 µM) was used as 
a positive control in combination with PMA (10 nM) to induce accumulation of C-terminal 
fragments, especially APP C83 (indicated as PMA+DAPT in Fig. 5). A 4-h treatment with 
HMI-1a3 decreased, while a 24-h treatment with HMI-1a3, HMI-1b11, and PMA increased the 
levels of APP C83 significantly in SH-SY5Y-APP751 cells as compared to vehicle-treated 
cells. Importantly, the increase in APP C83 in 24-h treatment samples coincided with the 
increased levels of total APP (APPtot) with all treatments (Fig. 5d). A similar increase in the 
levels of APPtot was not observed in the 4-h treated samples, except with PMA+DAPT (Fig. 
5b). A statistically significant increase in the levels of immature APP (APPim) and a concurrent 
decrease in the levels of mature APP (APPm) was observed after a 4-h treatment with HMI-1a3 
(Fig. 5b). The PKC activators HMI-1a3, HMI-1b11 and PMA induced statistically significant
decreases in the ratio of APPm/APPim after both 4-h and 24-h treatments (Figs. 5b and 5d, respectively), suggesting that treatment with PKC activators inhibited the maturation of APP. Bryostatin-1 did not induce statistically significant changes in the maturation of APP after 4-h or 24-h treatments (Supplementary Fig. S4). Collectively, these results suggest that both isophthalate derivatives decrease the maturation of APP in neuroblastoma cells.

3.6 HMI-1a3 and HMI-1b11 increase the levels of secreted sAPPα relative to total sAPP in neuroblastoma cells

As we observed significant changes in the processing and maturation of APP in SH-SY5Y-APP751 cells treated with HMI-1a3 and HMI-1b11, we next assessed whether these compounds affect the soluble APPα (sAPPα) and the total soluble APP (sAPPtot) levels in the cell culture medium (Fig. 6a-b and Fig. 6c-d). After a 24-h treatment, a statistically significant increase in sAPPα levels was observed with all compounds as compared to vehicle-treated cells (Fig. 6d). A similar trend towards increased levels of sAPPα was observed after a 4-h treatment with all compounds except with HMI-1a3. In contrast to sAPPα levels, approximately 40% decrease in the levels of sAPPtot was observed after a 4-h treatment with both HMI-1a3 (p=0.06) and HMI-1b11 (p<0.05) as compared to vehicle-treated cells (Fig. 6b). Due to the fact that the levels of APPtot were affected, particularly after a 24-hour treatment (Fig. 5d), we quantified the ratio of sAPPα and sAPPtot (sAPPα/sAPPtot). Both HMI-1a3 and HMI-1b11 increased the ratio of sAPPα/sAPPtot after 4-h and 24-h treatments as compared to the vehicle-treated cells. PMA alone and in combination with DAPT (PMA+DAPT) increased the ratio of sAPPα/sAPPtot significantly after 4 h, but not after 24 h. A similar trend was seen with bryostatin-1, although the results were not statistically significant (Supplementary Fig. S4). This may relate to the fact that PMA and PMA+DAPT have a more robust effect on the levels of sAPPtot as compared to HMI-1a3 and HMI-1b11. This difference between isophthalates and PMA may be due to the down-regulation of PKC after the 24-h PMA exposure, a phenomenon
which is not seen in response to isophthalate treatment (Supplementary Fig. S5). Collectively, these findings suggest that HMI-1a3 and HMI-1b11 promote non-amyloidogenic APP processing and the secretion sAPPα relative to the levels of sAPPot.

3.7 The PKC activator bryostatin-1 increases the number of mushroom spines in mouse mature hippocampal neuron cultures

PKC isoforms are located within hippocampal dendritic spines and they are known to play a role in the modulation of dendritic spine morphology (Alkon et al., 2007; Calabrese and Halpain, 2005). Therefore, the role of PKC activators HMI-1a3 (10 µM) and bryostatin-1 (100 nM) were investigated in mature primary mouse hippocampal neurons (DIV20) in a short-term 2-h treatment. As a result, total spine density, stubby spine density, and thin spine density decreased significantly with bryostatin-1 as compared to vehicle treated cells (Fig. 7a). In contrast, a moderate non-significant increase in total, mushroom, stubby, and thin spine densities was observed after HMI-1a3 treatment (Fig. 7a). Analysis of spine head morphology revealed that the 2-h exposure of hippocampal neurons to HMI-1a3 was not able to modify the shape of the spine head, while bryostatin-1 significantly increased the diameter of mushroom and stubby spine heads and decreased the diameter of thin spine heads (Fig. 7b). Overall, HMI-1a3 did not significantly modify the number of mushroom, stubby, or thin spine head morphology, whereas bryostatin-1 treatment increased the mushroom spine head density and decreased the thin spine head density in ratio to total number of spine heads (Fig. 7c). Collectively, these data suggest that bryostatin-1 supports stabilization of spine heads towards the mature, active mushroom-like shape.
4. Discussion

Dysregulation in PKC activation has been shown to associate with many pathophysiological features of AD, including memory loss, increased levels of Aβ and β-amyloid plaques, neurofibrillary tangles as well as neuroinflammation, and PKC activators have therefore been suggested to represent promising drug candidates for the treatment of AD (Alkon et al., 2007; Sun and Alkon, 2012). Furthermore, several PKC activators, such as DAG-lactones and bryostatins, have been shown to alleviate pathophysiological hallmarks of AD in both in vitro and in vivo models (reviewed in (Talman et al., 2016)). Here, we report for the first time the effects of PKC C1 domain ligands, 5-(hydroxymethyl) isophthalate derivatives HMI-1a3 and HMI-1b11 in different in vitro models of relevant for AD. The key findings of the study are that HMI-1a3 enhances neuronal viability and reduces the production of TNFα in the neuron-BV2 microglia co-cultures upon LPS- and IFN-γ-induced neuroinflammation. These changes were not associated with significant alterations in the S536 phosphorylation status in the transactivation domain of NFκB p65 subunit. Instead, the total levels NFκB p65 were moderately increased owing to HMI-1a3 treatment in the neuron-BV2 microglia co-cultures upon neuroinflammation. Furthermore, both HMI-1a3 and HMI-1b11 altered processing and maturation of APP as well as the levels of soluble APPα in SH-SY5Y neuroblastoma cells. Finally, we demonstrate that bryostatin-1, but not HMI-1a3, increases the number of mushroom spines in mature mouse hippocampal primary neuronal cultures upon basal conditions. These data suggest that the isophthalate derivatives can reduce neuroinflammation and promote neuronal survival to some extent in in vitro. Therefore, it is justified to continue the assessment of potential neuroprotective effects as well as the underlying molecular mechanisms of these derivatives e.g. in the in vivo models relevant for AD in the future.

Insufficient PKC activation has been associated with abnormalities in neuroinflammatory signaling (Alkon et al., 2007), a phenomenon characteristic in the early pathophysiology of AD.
Furthermore, the levels and activity of PKC isoforms is attenuated in the brain of AD patients (Lucke-Wold et al., 2015; Wang et al., 1994). In line with these observations, encouraging results on the effects of PKC activators in both in vitro and in vivo models of AD have been reported (Talman et al., 2016). Many of the positive effects induced by PKC activators have been attributed to the activation of isoforms ε and γ (Lucke-Wold et al., 2015), which has evoked ideas about specifically targeting these isoforms. However, most of the studies on PKC activators have been done with activators that are not isoform specific, such as bryostatin-1. Similar to bryostatin-1, HMI-1α3 binds to the C1-domain of both novel and classical isoforms of PKC and is therefore not isoform-specific. Furthermore, as Gö6983 inhibits several PKC isoforms (Gschwendt et al., 1996), identification the exact isoform(s) responsible for HMI-1α3-induced effects reported here, requires further investigations.

PKC activation has been shown to direct APP processing towards the production of neuroprotective sAPPα in several cell-based models and restoration of synapses has been reported in both cells as well as in rodents with various PKC activators (Talman et al., 2016). The PKC activator bryostatin-1 has been shown to improve memory in behavioral tests in several rodent models and has progressed to clinical trials as the first PKC-targeted therapy for neurodegenerative diseases (www.clinicaltrials.gov) (Talman et al., 2016). Although PKC activators have been traditionally considered as tumor promoters, this view has been challenged by a recent comprehensive study, which showed that the majority of cancer-associated PKC mutations were loss-of-function mutations and none were activating (Antal et al., 2015). The tumor-promoting properties of some PKC activators, such as the phorbol esters, may thus in fact be due to PKC down-regulation and not increased activity per se. Therefore, it appears that the loss of PKC activity might lead to tumor promotion, which makes the development drugs that are partial agonists even more appealing.
In the present study, we found that HMI-1a3 promoted to some extent neuronal survival effects in the primary neuron-BV2 microglial cell co-cultures upon LPS-IFN-γ-induced neuroinflammation. In the presence of PKC inhibitor Gö6983 (1µM), the neuroprotective effect of HMI-1a3 was abolished implicating that the effect was PKC-dependent. Surprisingly, viability of neurons decreased when cells were treated with both compounds, whereas Gö6983 alone had no effect on neuronal viability. This may relate to fact that in addition to PKC, six other protein families, such as Munc13, protein Kinase D (PKD), RasGRP, chimaerins, diacylglycerol kinases (DAGKs) and myotonic dystrophy kinase-related Cdc42-binding kinase (MRCK), contain analogous C1 domains and consequently HMI-1a3 has been shown to bind to several of these (Talman et al., 2014). Therefore, it is possible that HMI-1a3 could modulate some C1 domain-dependent pathways that counteract PKC signalling, which would then lead to compromised neuronal viability when PKC is inhibited by Gö6983. Furthermore, Gö6983 binds to the ATP binding site of PKC (Wu-Zhang and Newton, 2013) and not to the C1 domain, and therefore does not affect the activity of other C1 domain containing proteins. The potent PKC activator PMA reduced neuronal viability significantly at both 10 nM and 100 nM concentrations, and this was not affected by the pan-PKC inhibitor Gö6983. PMA, like isophthalates, also have other targets besides PKC and the observed reduction in neuronal viability with PMA in combination with Gö6983, could be due to activation of these other target proteins. The observation that both HMI-1a3 and PMA caused a decrease in neuronal viability when used in combination with Gö6983 supports the notion that other C1-domain containing proteins are likely to be responsible for this outcome. Furthermore, the difference in the response to these compounds alone could be explained by the level of PKC activation they induce. Too robust and prolonged activation by PMA leads to the downregulation of PKC protein levels and a decrease in neuronal viability, whereas HMI-1a3 induces PKC activation
without causing downregulation. Thus, the results suggest that the activation of PKC without
downregulation is neuroprotective upon LPS-IFN-γ-induced neuroinflammation.

The effect of HMI-1a3 on neuronal viability could be explained by its effect on TNFα.
HMI-1a3 concentration-dependently reduced the levels of TNFα and this effect was abolished
with Gö6983, suggesting that the effect was mediated by PKC. PMA had no effect on the TNFα
levels at 10 nM concentration but induced an increase at 100 nM. At 100 nM concentration and
after a 48-h exposure PMA already downregulates PKC, while still activating other targets
containing a phorbol-responsive C1 domain. For example, activation of Munc-13 has been
shown to positively regulate TNFα release in macrophages (Mori et al., 2011). When PMA
was combined with Gö6983, the increase in the levels of TNFα could be detected already at
the 10 nM PMA concentration. It is possible that PKC activation with 10 nM PMA
concentration counteracts the PKC-independent effects of PMA and when PKC activation is
inhibited with Gö6983, this PKC-independent effect becomes dominant. Slightly in contrast
with these PMA results, but in line with the effect of HMI-1a3, bryostatin-1 decreased the
levels of TNFα. However, this effect was not abolished, but instead was potentiated by PKC
inhibition, indicating that also other targets of bryostatin-1 may contribute to the observed
decrease in the production of TNFα. To elucidate the potential molecular mechanism
underlying the increased neuronal viability and the reduced levels of TNFα owing to HMI-1a3,
we determined the S536 phosphorylation status in the transactivation domain of NFκB p65
subunit (Christian et al., 2016), and the levels of total NFκB p65 in the primary neuron-BV2
microglia co-cultures upon neuroinflammation. It is a well-established observation that the
increased S536 phosphorylation leads to the enhanced transactivation of NFκB via the
increased binding of CREB-binding protein/p300 and acetylation at K310 of NFκB p65 (Chen
et al., 2005). Consequently, the activated nuclear NFκB mediates gene transcription of certain
chemokines and interleukins, such as TNFα, IFNγ and IL6, known to play a central role in the
immune response, proliferation, and cell death. However, we did not find any significant changes in the phosphorylation status of S536 in NFκB p65, but instead we observed a moderate, but statistically significant increase in the total levels of NFκB p65 in the co-cultures treated with HMI-1a3 upon neuroinflammation. Interestingly, bryostatin-1 did not have any significant effects on phosphorylation status or total levels of NFκB p65 upon neuroinflammation. The relevance of the increased levels of NFκB p65 subunit (~20%) owing to HMI-1a3 treatment is not clear and thus further studies are needed to elucidate whether this relatively small increase would play a prominent role in terms of neuroprotection. Conversely, it should be noted that the increased levels of NFκB p65 did not lead to the augmentation of S536 phosphorylation in NFκB p65 subunit, suggesting that enhanced transactivation of NFκB did not take place after HMI-1a3 treatment upon neuroinflammation. Although the S536 phosphorylation site in NFκB p65 is one of the best-understood phosphorylation targets in the transactivation domain of NFκB p65 (Christian et al., 2016), we cannot rule out the possibility that some other phosphorylation target in NFκB known to control NFκB-directed transactivation could be affected by HMI-1a3 or bryostatin-1 upon neuroinflammation. Despite the observed compound-induced changes in the levels of TNFα upon neuroinflammation, we did not observe major effects on the production of NO. The observation that PMA increases the production of NO only in conditions where PKC is inhibited, may suggest that the effect is again mediated by another C1 domain-containing protein, which under normal conditions is suppressed by PKC activity. For example, PKD has been reported to directly activate neuronal NO synthase (Sánchez-Ruiloba et al., 2014). Collectively, these results suggest that HMI-1a3 may exert neuroprotection by activating PKC, and this is probably mediated by alleviating the production of proinflammatory cytokine TNFα upon LPS+IFN-γ-induced neuroinflammation in neuron-BV2 cell co-cultures. As neuroinflammation is considered a central detrimental
phenomenon in AD and other neurodegenerative diseases, this anti-inflammatory and neuroprotective effect of HMI-1a3 is very encouraging.

PKC activators, such as phorbol esters and bryostatin-1, have previously been shown to reduce Aβ40 and Aβ42 levels in in vivo models of AD (Etcheberrigaray et al., 2004; Savage et al., 1998). In contrary to this, we did not observe significant effect on Aβ40 or Aβ42 levels in neuron-BV2 co-cultures when treated with HMI-1a3 or HMI-1b11. However, both isophthalate derivatives induced a moderate, but non-significant decrease in Aβ42/Aβ40 ratio in SH-SY5Y-APP751 cells. This was confirmed to be a consistent outcome as we observed a significant increase in the levels of APP C83, decrease in APPm/APPim ratio, and an increase in sAPPα/sAPPtot ratio in SH-SY5Y-APP751 cells when the cells were treated with HMI-1a3 or HMI1b11 for 24 h. Additionally, as both APPm and APPim levels were increased after isophthalate treatment for 24 h, it can be speculated that isophthalate-induced PKC activation might enhance post-translational modification of APP, such as glycosylation, sulfation, and phosphorylation during transit through the intracellular protein secretory pathway (da Cruz e Silva et al., 2009). Indeed, PKC has been reported to not only directly phosphorylate APP, but also regulate its trafficking by controlling the formation of APP bearing vesicles in the Trans-Golgi network (TGN) (Gandy et al., 1988; Xu et al., 1995). In addition, PKC activation has been shown to increase the transcription of APP already after 3 h, and induce a 4-fold increase in the APP mRNA levels after a 24-h exposure to PMA (100 nM) (Trejo et al., 1994), which is in line with our finding that total APP levels were increased at 24 h in response to the isophthalates, PMA and also to some extent bryostatin-1. Previous studies have indicated that the cellular responses to PKC-activating compounds are dependent on the exposure time. Acute PKC activation has been reported to increase the release of sAPPα by activating α-secretase, while chronic exposure to PKC activators has also increased the expression levels of APP (da Cruz e Silva et al., 2009; Trejo et al., 1994). Furthermore, da Cruz de Silva et al (2009) reported
that a 24-h exposure to phorbol ester increased total APP production, decreased sAPPα, increased Aβ levels and caused accumulation of APP in TGN. However, most of these phenomena were explained by PKC downregulation. The isophthalate derivatives do not induce PKC down-regulation within 24 h in SHSY5Y cells, and although the accumulation of APP in TGN was visible after 24-h exposure, the sAPPα/sAPPtot ratio was still increased with isophthalates, unlike with PMA. Notably, the increased levels of both APP C83 and sAPPα, but not that of sAPPtot suggest that the exposure to different PKC agonist might specifically promote the accumulation of APP at the cell surface and influence the recycling of re-internalized APP back to the plasma membrane, where majority of the non-amyloidogenic α-cleavage takes place. Our findings support the idea that activation of PKC may increase the levels of sAPPα by mechanisms involving the formation and release of secretory vesicles from the TGN, thus enhancing APP trafficking to the cell surface (Thinakaran and Koo, 2008; Xu et al., 1995). Since sAPPα is neuroprotective, the sustained increase in its production (sAPPα/sAPPtot ratio) achieved with isophthalates, but not with PMA, further supports the idea that activating PKC, without inducing its downregulation, may represent a promising therapeutic strategy for AD.

We also detected increased mushroom spine density relative to total spine density in the mature mouse hippocampal neurons upon basal growth conditions after a 2-h treatment with PKC activator bryostatin-1, suggesting that PKC activation increases the number of functional excitatory post-synaptic terminals. To support this, we also observed a significant increase in mushroom spine head diameter in bryostatin-1-treated hippocampal neurons, indicating that enlargement of mushroom spine head through activation of PKC signaling cascade may lead to formation of a postsynaptic density in dendritic mushroom spine as previously reported (Calabrese and Halpain, 2005; Sen et al., 2016; Yoshihara et al., 2009). In this study, HMI-1a3 did not affect spine morphology. This could be due to the relatively short treatment time, which
is probably enough to induce changes with a very potent PKC activator, such as bryostatin-1, but might not be long enough for less potent activators, such as the isophthalates. Whether longer isophthalate treatments would affect spine morphology remains to be determined. Moreover, these experiments were carried out in basal conditions without induction of neuroinflammation. Isophthalates appear to promote neuroprotection by alleviating inflammation and therefore it would be interesting to see, if they could affect the morphology of the spines or protect them upon neuroinflammation. However, the changes observed with bryostatin-1 by us and others (Hongpaisan et al., 2013; Sen et al., 2016) reinforce the need of further investigation of C1-domain binding PKC activators and suggest that activation of PKC could be a promising strategy for treating neurodegenerative diseases, such as AD.

Taken together, these studies conducted in several different in vitro cellular models and conditions, suggest that C1 domain-binding PKC activators affect various molecular processes related to neuronal survival and processing and maturation of APP, which are all known to play a role in the pathophysiology of AD. However, further research is required to determine the in vivo efficacy of isophthalate derivatives in AD-related neurodegeneration.

5. Conclusions

In summary, our data suggest that the PKC-activating isophthalate derivatives show neuroprotective activity against neuroinflammation-induced toxicity in vitro, possibly through the attenuation of cytotoxic microglia-derived production of TNFα in the neuron-microglia co-cultures. Furthermore, the isophthalate derivatives promoted non-amyloidogenic processing and maturation of APP by enhancing its post-translational modification at the secretory pathway and by increasing the α-cleavage of APP in SH-SY5Y-APP751 cells. Together with previous reports, our findings suggest that the synthetic isophthalate derivatives and other PKC agonists, such as bryostatin-1, may exert neuroprotective effects in various stress conditions.
associated with AD and other neurodegenerative diseases. Thus, the isophthalate derivatives may be considered potential candidates in the search of new therapeutic strategies against synaptic dysfunction and neurodegeneration known to take place in AD.

Conflicts of interest
The authors declare that they have no conflicts of interests.

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Ethics approval and consent to participate
The licenses for preparation of primary neuronal cultures from E18 mouse embryo and primary astrocyte and microglia cultures from P1-3 neonatal mouse are granted by the Lab Animal Center, UEF (approval of EKS-008-2016).
References


https://doi.org/10.1038/sj.bjp.0705688


https://doi.org/10.1126/science.1072994


https://doi.org/10.1021/jm020350r


https://doi.org/10.1074/jbc.M116.730440


https://doi.org/10.1074/jbc.275.4.2568


https://doi.org/10.1016/B978-0-12-394816-8.00008-8


https://doi.org/10.1016/j.pharmthera.2010.03.001


https://doi.org/10.1016/j.ejps.2014.01.002

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Figure legends

**Figure 1.** HMI-1a3 reduces neuronal loss upon LPS+IFN-γ-induced neuroinflammation in mouse primary neuron-BV2 microglia co-cultures. (A) Characterization of the mouse primary cortical neuron and BV2 microglial cell co-cultures and effects upon LPS+IFN-γ-induced neuroinflammation. (B) Effects of HMI-1a3 alone and in combination with 1 µM Gö6983 on neuronal viability upon LPS+IFN-γ-induced neuroinflammation. NI-15e was used as a negative control for HMI-1a3. (C) Effects of PMA alone and in combination with Gö6983 on neuronal viability. (*)-sign indicates the statistical difference compared between same concentration of HMI-1a3 and HMI-1a3 + 1 µM Gö6983 samples, and (#)-sign indicates the statistical difference compared between the treated sample and a vehicle treated (0 µM) sample within the group. One-way ANOVA and independent samples t-test, *#p<0.05, **##p<0.01, ***###p<0.001, mean±SEM, n=3-12; 0 µM = Vehicle (0.1% DMSO) + LPS+IFN-γ (± 1 µM Gö6983).

**Figure 2.** HMI-1a3 decreases the levels of TNFα but does not affect production of NO in mouse primary neuron-BV2 microglia co-cultures subjected to neuroinflammation. (A) Effects of HMI-1a3 alone and in combination with 1 µM Gö6983 on TNFα levels upon LPS+IFN-γ-induced neuroinflammation. NI-15e was used as a negative control for HMI-1a3. (B) Effects of PMA on the levels of TNFα alone and in combination with 1 µM Gö6983. (C) Effects of HMI-1a3 alone and in combination with 1 µM Gö6983 on the production of NO upon LPS+IFN-γ-induced neuroinflammation. NI-15e was used as a negative control for HMI-1a3. (D) Effects of PMA on the production of NO alone and in combination with 1 µM Gö6983. (*)-sign indicates the statistical difference compared between same concentration of HMI-1a3 and HMI-1a3 + 1 µM Gö6983 samples, and (#)-sign indicates the statistical difference compared between the treated sample and a vehicle sample within the group. One-way
ANOVA and independent samples t-test, LSD, */p < 0.05, **/#/p < 0.01, ***p < 0.001, mean+SEM, n=4-16; 0 µM = Vehicle (0.1% DMSO) + LPS+IFN-γ (± 1 µM Gö6983).

Figure 3. HMI-1a3 increases the levels of total NFκB p65 in primary neuron-BV2 microglia co-cultures upon neuroinflammation. Western blotting was used to analyse the levels of S536 phosphorylated NFκB p65 (p-NFκB p65) and total NFκB p65 (Tot-NFκB p65).

(A) Representative blots and (B) quantification of the ratio of S536 phosphorylated NFκB p65 to total NFκB p65 as well as the levels of total NFκB p65 normalized to β-actin. Vehicle-treated control sample is also shown from the same Western blot. One-way ANOVA followed by Dunnet’s test, *p < 0.05, mean+SEM, n=4; Vehicle = 0.1% DMSO.

Figure 4. HMI-1a3 and HMI-1b11 do not significantly affect Aβ40 and Aβ42 levels in primary neuron-BV2 co-cultures upon neuroinflammation or in SH-SY5Y neuroblastoma cells. (A-C), The effects of HMI-1a3 on the levels of Aβ40 (A), Aβ42 (B) and the ratio of Aβ42/Aβ40 (C) in LPS+IFN-γ-treated neuron-BV2 co-cultures after 48 hours. (D-F), The effects of 48-h treatments with HMI-1a3, HMI-1b11 and PMA on the levels of Aβ40 (D) and Aβ42 (E) and the ratio of Aβ42/Aβ40 (F) in SH-SY5Y-APP751 cells. Data are represented as mean+SEM (One-way ANOVA followed by Dunnett’s test and independent samples t-test, *p < 0.05, mean+SEM, n=3-8; Vehicle = 0.1% DMSO; 0 µM = Vehicle + LPS+IFN-γ).

Figure 5. HMI-1a3 and HMI-1b11 inhibit the maturation of APP in SH-SY5Y neuroblastoma cells. Representative Western blot images (A and C) and quantifications (B and D) indicating the effects of HMI-1b11, HMI-1a3, PMA, and co-treatment of PMA (10 nM) and γ-secretase inhibitor DAPT (10 µM) (PMA+DAPT) on APP C83, mature APP (APPm), immature APP (APPim), total APP (APPm+APPim) levels and APPm/APPim ratios in SH-SY5Y-APP751 cells after 4-hour (A-B) and 24-hour (C-D) treatment. The representative...
Western blot image for the APP C83 fragment from 24-hour treatment (C) is presented as two
images from the same blot, with and without PMA+DAPT treated samples. To make the bands
of interest more visible, contrast and intensity were adjusted in the image omitting the PMA-
DAPT-treated samples. The quantification values represent optical densities normalized to the
GAPDH levels and are shown as % of vehicle control (0.1% DMSO). The data are presented as
mean+SEM (One-way ANOVA followed by Dunnett’s test and independent samples t-test,
*p < 0.05, **p < 0.01, ***p < 0.001, n=4; N.S. = not statistically significant).

Figure 6. HMI-1a3 and HMI-1b11 increase the levels of sAPPα relative to total sAPP in
the cell culture medium of SH-SY5Y neuroblastoma cells. Representative Western blot
images (A and C) and quantifications (B and D) indicating the effects of HMI-1a3, HMI-1b11,
and PMA, or co-treatment of PMA (10 nM) and γ-secretase inhibitor DAPT (10 μM)
(PMA+DAPT) on sAPPα and total sAPP (sAPPtot) levels in SH-SY5Y-APP751 cell culture
medium after 4-hour (A-B) and 24-hour (C-D) treatment. The values are optical densities
normalized to the total APP (APPm+APPim from the corresponding cell lysates, which is
normalised to GAPDH) and are shown as % of vehicle control (0.1% DMSO). The data are
presented as mean+SEM (n=4). One-way ANOVA followed by Dunnett’s test and independent
samples t-test, **p < 0.01).

Figure 7. The PKC activator Bryostatin-1 increases the number of mushroom spines after
2-hour treatment in mature mouse hippocampal neuron cultures at DIV20. (A) The effects
of 2-h treatments of bryostatin-1 (100 nM) or HMI-1a3 (10 μM) on total, stubby, and thin spine
densities. (B) The effects of 2-h treatments with bryostatin-1 (100 nM) or HMI-1a3 (10 μM)
on spine head morphology (width). (C) The effects of 2-h treatments with bryostatin-1 (100
nM) or HMI-1a3 (10 μM) on mushroom and thin spine density ratio to total spine density. One-
way ANOVA and independent samples t-test, *p < 0.05, **p < 0.01, ***p < 0.001, mean+SEM, n=14-33; Vehicle = 0.1% DMSO.

Supplementary material

Figure S1. Bryostatin-1 decreases TNFα levels but has no effect on neuronal viability in mouse primary neuron-BV2 microglia co-cultures upon LPS+IFN-γ-induced neuroinflammation. Effects of bryostatin-1 alone and in combination with 1 µM Gö6983 on neuronal viability upon LPS+IFN-γ-induced neuroinflammation (A), the levels of TNFα (B) and the production of NO (C) in mouse primary neuron-BV2 microglia co-cultures. One-way ANOVA followed by Dunnett’s test, *p < 0.05, **p < 0.01, ***p < 0.001, mean+SEM, n=6; 0 µM = Vehicle (0.1% DMSO) + LPS+IFN-γ.

Figure S2. PKC activation with bryostatin-1 decreases Aβ42 levels and Aβ42/Aβ40 ratio in mouse primary neuron-BV2 microglia co-cultures upon neuroinflammation. Effects of bryostatin-1 on Aβ40 and Aβ42 levels, and on the ratio of Aβ42/Aβ40 in mouse primary neuron-BV2 microglia co-cultures. One-way ANOVA followed by Dunnett’s test, *p < 0.05, mean+SEM, n=6; 0 µM = Vehicle (0.1% DMSO) + LPS+IFN-γ.

Figure S3. Bryostatin-1 does not exhibit a significant effect on the maturation of APP in SH-SY5Y cells overexpressing APP751. Representative Western blot images (A and C) and quantifications (B and D) indicating the effects of bryostatin-1 on APP C83, mature APP (APPm), immature APP (APPim), and total APP (APPm+APPim) levels as well as APPm/APPim ratio in SH-SY5Y-APP751 cells after 4- (A and B) and 24-hour (C and D) treatments. The quantification values represent optical densities normalized to GAPDH and are
shown as % of vehicle control (0.1 % DMSO). The data are presented as mean+SEM from
three independent experiments (One-way ANOVA followed by Dunnett’s test).

Figure S4. Bryostatin-1 does not induce significant changes in the levels of sAPPα in SH-
SY5Y cells overexpressing APP751. Representative Western blot images (A and C) and
quantifications (B and D), showing the effects of bryostatin-1 on sAPPα and total sAPP
(sAPPtot) levels in SH-SY5Y-APP751 cell culture medium after 4-hour (A-B) and 24-hour (C-
D) treatments. The values are optical densities normalized to the total APP (APPm+APPim
from the corresponding cell lysate, which is normalised to GAPDH) and are presented as % of
vehicle control (0.1% DMSO) treatment. The data are presented as mean+SEM from three
independent experiments (One-way ANOVA followed by Dunnett’s test).

Figure S5. The levels of PKC isoforms α, βI, δ, and ε in SH-SY5Y neuroblastoma cells
after 24-hour treatment with 100 nM PMA, 10 µM HMI-1a3 and 10 µM HMI-1b11.
Densitometric analysis of Western blots (n=3). Results presented as mean+SEM. One-way
ANOVA followed by Dunnett’s test and independent samples t-test (*p < 0.05, **p < 0.01,
***p < 0.001).