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## Protein kinase C-activating isophthalate derivatives mitigate Alzheimer's disease-related cellular alterations

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1 **PROTEIN KINASE C -ACTIVATING ISOPHTHALATE DERIVATIVES MITIGATE**  
2 **ALZHEIMER'S DISEASE-RELATED CELLULAR ALTERATIONS**

3

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25 **ABSTRACT**

26 Abnormal protein kinase C (PKC) function contributes to many pathophysiological processes  
27 relevant for Alzheimer's disease (AD), such as amyloid precursor protein (APP) processing.  
28 Phorbol esters and other PKC activators have been demonstrated to enhance the secretion of  
29 soluble APP $\alpha$  (sAPP $\alpha$ ), reduce the levels of  $\beta$ -amyloid (A $\beta$ ), induce synaptogenesis, and  
30 promote neuroprotection. We have previously described isophthalate derivatives as a  
31 structurally simple family of PKC activators. Here, we characterized the effects of isophthalate  
32 derivatives HMI-1a3 and HMI-1b11 on neuronal viability, neuroinflammatory response,  
33 processing of APP and dendritic spine density and morphology in *in vitro*. HMI-1a3 increased  
34 the viability of embryonic primary cortical neurons and decreased the production of the  
35 pro-inflammatory mediator TNF $\alpha$ , but not that of nitric oxide, in mouse neuron-BV2 microglia  
36 co-cultures upon LPS- and IFN- $\gamma$ -induced neuroinflammation. Furthermore, both HMI-1a3  
37 and HMI-1b11 increased the levels of sAPP $\alpha$  relative to total sAPP and the ratio of A $\beta$ 42/A $\beta$ 40  
38 in human SH-SY5Y neuroblastoma cells. Finally, bryostatin-1, but not HMI-1a3, increased the  
39 number of mushroom spines in proportion to total spine density in mature mouse hippocampal  
40 neuron cultures. These results suggest that the PKC activator HMI-1a3 exerts neuroprotective  
41 functions in the *in vitro* models relevant for AD by reducing the production of TNF $\alpha$  and  
42 increasing the secretion of neuroprotective sAPP $\alpha$ .

43

44 **Keywords:** Protein kinase C, Alzheimer's disease, APP-processing, Neuroinflammation,  
45 Neuroprotection, Isophthalate derivatives

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## 48 **1. Introduction**

49 Alzheimer's disease (AD) is the most common cause of dementia in the aging population  
50 without existing intervention approaches to halt or even slow down the disease progression. A  
51 central pathological feature of AD includes accumulation of toxic and aggregation-prone  $\beta$ -  
52 amyloid ( $A\beta$ ) peptides.  $A\beta$  causes synaptic dysfunction, activation of microglia and astrocytes,  
53 oxidative and inflammatory stress, and formation of intraneuronal neurofibrillary tangles  
54 (NFTs) consisting of hyperphosphorylated tau (Hardy, 2002).  $A\beta$  is generated from amyloid  
55 precursor protein (APP) as a result of sequential cleavages by  $\beta$ -site-APP-cleaving-enzyme-1  
56 (BACE1) and  $\gamma$ -secretase (De Strooper and Annaert, 2000). Depending on the site of the  $\gamma$ -  
57 secretase cleavage,  $A\beta$  peptides of different lengths are generated and particularly  $A\beta_{42}$  is  
58 prone to aggregate (Selkoe, 1994). In contrast,  $\alpha$ -secretase (ADAM10 and ADAM17) cleavage  
59 preceding the  $\gamma$ -secretase cleavage of APP leads to the extracellular release of soluble  $APP\alpha$   
60 ( $sAPP\alpha$ ) and a C-terminal fragment (C83) on the membrane, thus precluding the generation of  
61 intact  $A\beta$  peptide (De Strooper and Annaert, 2000; Haass et al., 1993). Increased production of  
62  $sAPP\alpha$  has been demonstrated to have many beneficial effects, including stimulation of neurite  
63 outgrowth, cell proliferation, synaptic density, memory retention, and neuroprotection  
64 (reviewed in (Müller et al., 2017)). The hyperphosphorylation of tau protein leads to  
65 accumulation of intracellular NFTs and neuronal dysfunction (Grundke-Iqbal et al., 1986a,  
66 1986b).

67 Several signaling pathways are known to regulate the pathophysiological processes  
68 involved in the development or progress of AD, one of them being protein kinase C (PKC)  
69 (Choi et al., 2006; Crews and Masliah, 2010; Godoy et al., 2014; Lucke-Wold et al., 2015).  
70 PKC is a family of serine/threonine kinases with at least 10 isoforms found in mammals  
71 (Newton, 2003). These isoforms are divided into three classes based on their activators.  
72 Classical PKCs require both  $Ca^{2+}$  and diacylglycerol (DAG) for activation whereas novel PKCs

73 are independent of  $\text{Ca}^{2+}$ , but they require DAG. On the other hand, atypical PKCs are activated  
74 by mechanisms independent of DAG and  $\text{Ca}^{2+}$ . PKC has been called a “memory kinase” and it  
75 has been widely studied in relation to its central role in memory formation in both normal and  
76 pathological settings, such as AD models (Lucke-Wold et al., 2015). As PKC regulates several  
77 processes linked to AD pathophysiology, its pharmacological activation is considered a  
78 potential therapeutic strategy for treating AD (Alkon et al., 2007; Sun and Alkon, 2012, 2010;  
79 Talman et al., 2016). A number of studies have shown that PKC activation directs APP  
80 processing to the non-pathogenic  $\alpha$ -secretase pathway and thereby increases the production of  
81 neuroprotective sAPP $\alpha$  and reduces the production of neurotoxic A $\beta$  species both *in vitro* and  
82 *in vivo* (Alkon et al., 2007; Buxbaum et al., 1990; Etcheberrigaray et al., 2004; Jacobsen et al.,  
83 1994; Kozikowski et al., 2003). Activation of PKC has also been suggested to inhibit A $\beta$   
84 accumulation by increasing its degradation through upregulation of gelsolin and activation of  
85 endothelin-converting enzyme (Choi et al., 2006; Ji et al., 2010). Additionally, PKC activation  
86 has been reported to protect neurons from A $\beta$  cytotoxicity (Garrido et al., 2002; Han et al.,  
87 2004). Besides the  $\beta$ -amyloid pathology, activated PKC also inhibits tau hyperphosphorylation  
88 by phosphorylating and inactivating glycogen synthase kinase 3 $\beta$  (GSK3 $\beta$ ), a central kinase  
89 phosphorylating tau (Isagawa et al., 2000). Furthermore, PKC activation has been shown to  
90 induce neurite elongation (Shirai et al., 2008; Yang et al., 2010) and synaptogenesis (Sen et al.,  
91 2016) and to restore mushroom spine synapses (Hongpaisan et al., 2013), indicating that PKC  
92 activation might even exhibit neurorestorative potential.

93       Pharmacological PKC activation can be achieved by targeting its regulatory C1 domain,  
94 which is also the binding site for the physiological activator DAG. Several families of C1  
95 domain-targeting PKC agonists, such as phorbol esters, bryostatins, DAG lactones and  
96 benzolactams, have been described and investigated in the *in vitro* and *in vivo* models of AD  
97 (Boije af Gennäs et al., 2011; Talman et al., 2016). Most PKC activators are however scarce in

98 natural sources and highly complex in their chemical structure, making them expensive to  
99 isolate in sufficient quantities and difficult to synthesize. Furthermore, in cellular context  
100 prolonged activation of PKC with ultrapotent agonists, such as phorbol esters, leads to  
101 dephosphorylation and subsequent degradation of PKC protein, thus eventually resulting in  
102 diminished PKC activity (Newton, 2003). We have previously developed and reported a novel  
103 group of C1 domain-targeted PKC modulators, derivatives of 5-(hydroxymethyl)isophthalic  
104 acid, which are easy to synthesize from commercially available starting material (Boije af  
105 Gennäs et al., 2009). The best-characterised derivatives, namely HMI-1a3 and HMI-1b11,  
106 promoted neurite outgrowth in SH-SY5Y neuroblastoma cells and HMI-1b11 was shown to  
107 induce PKC-dependent upregulation of the neuronal differentiation marker GAP-43 (Talman  
108 et al., 2013). Instead of testing HMI-1a3 and HMI-1b11 directly in the *in vivo* models relevant  
109 for AD, we first wanted to elucidate the effects of these isophthalate derivatives on neuronal  
110 viability, neuroinflammatory response, APP processing, and spine morphology using *in vitro*  
111 models of neuroinflammation and AD.

112 **2. Material and methods**

113 **2.1 Isophthalic acid derivatives**

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

119 **2.2 Mouse embryonic primary cortical neuron and BV2 microglial cell co-cultures and**  
120 **treatments**

121 Co-cultures were prepared as described before (Gresa-Arribas et al., 2012; Natunen et al.,  
122 2016). Shortly, neuronal cells were isolated from the cortices of embryonic stage 18 (E18)  
123 JAXC57BL/6J-mouse embryos and plated in Neurobasal medium supplemented with B27  
124 (Gibco), penicillin, streptomycin and L-glutamine on poly-D-lysine-coated (PDL; Sigma)  
125 48-well plates at the density of  $20 \times 10^4$  cells/well. Fresh medium was changed on 5 days *in*  
126 *vitro* (DIV5) to feed the cells. Mouse microglial BV2 cells were cultured in RPMI-1640  
127 medium (Sigma) containing 10% FBS, 2 mM L-glutamine and 100 U/ml penicillin and  
128 100 µg/ml streptomycin for two passages. RPMI-medium was replaced with Neurobasal  
129 medium and BV2 cells were gently detached with a cell scraper. BV2 cells were added to  
130 primary cortical neuron cultures in one-to-five ratio (BV2:neurons) at DIV5. Furthermore, all  
131 treatments were implemented for samples without BV2-microglia (neurons only samples).  
132 After one hour, the co-cultured cells were treated with vehicle (0.1% DMSO), anti-  
133 inflammatory cytokine IL-10 (50 ng/ml; PeproTech), nitric oxide synthase inhibitor 1400W  
134 (20 µM; Tocris), PKC activator phorbol-12-myristate-13-acetate (PMA; 10 and 100 nM;  
135 Sigma-Aldrich), bryostatin-1 (10 and 100 nM; Sigma-Aldrich), pan-PKC inhibitor Gö6983 (1  
136 µM; Sigma-Aldrich), and isophthalate derivatives (each with concentrations of 1, 4, 10 and 20

137  $\mu\text{M}$ ), and let settle for 1 h. Finally, neuroinflammation was induced by treating co-cultures with  
138 200 ng/ml of lipopolysaccharide (LPS) and 20 ng/ml of interferon- $\gamma$  (IFN- $\gamma$ ) (both from  
139 Sigma-Aldrich). Co-culture media was collected 48 hours after LPS/IFN- $\gamma$  treatment and  
140 assayed for TNF $\alpha$ , NO, A $\beta$  1-40, and A $\beta$  1-42 levels.

### 141 ***2.3 Neuronal viability assay***

142 The MAP2-ABTS assay for neuronal viability was performed as described previously  
143 (Martiskainen et al., 2017; Natunen et al., 2016). Shortly, primary cortical neuron-BV2  
144 microglia co-cultures were fixed in 4% paraformaldehyde (PFA) immediately after removal of  
145 cell media, permeabilized in methanol containing 0.3% H<sub>2</sub>O<sub>2</sub>, and subsequently blocked with  
146 blocking solution (PBS, 1% BSA and 10% horse serum; Vector Labs) for 20 min. Neuronal  
147 cells were stained with anti-MAP2 primary antibody (1:2000; Sigma, M9942) overnight at  
148 +4°C. Next day, cells were incubated with biotinylated horse anti-mouse secondary antibody  
149 (1:200; Vector Labs) and ExtrAvidin-HRP tertiary antibody staining (1:500; Sigma) for 1 hour.  
150 After tertiary antibody incubation, cells were washed with PBS and incubated with the ABTS  
151 peroxidase substrate (Vector Labs, #SK-4500) giving the color reaction according to the  
152 manufacturer's instructions. From each well, 150  $\mu\text{l}$  of substrate solution was transferred to a  
153 fresh 96-well plate and absorbance was measured at 405 nm with ELISA microplate reader  
154 (Infinite<sup>®</sup> M200, Tecan).

### 155 ***2.4 TNF $\alpha$ , NO, and A $\beta$ measurements in primary cortical neuron and BV2 microglial cell*** 156 ***co-cultures***

157 Conditioned media from the cell samples were centrifuged at 10000  $\times$  g for 10 min. The levels  
158 of secreted TNF $\alpha$ , A $\beta$  x-40, and A $\beta$  x-42 in the medium was measured using ELISA kits  
159 (Ready-SET-Go mouse-TNF $\alpha$  ELISA kit, eBioscience; Human/Rat  $\beta$  Amyloid 40 and 42,  
160 Wako, Cat no 294-64701/Cat no 292-64501, respectively), following manufacturer's



161 instructions. A $\beta$  results were normalized to neuronal viability as shown previously  
162 (Martiskainen et al., 2017). NO production was assessed using the Griess Reagent Kit for  
163 Nitrite Determination (G-7921, Molecular Probes) following manufacturer's instructions.  
164 Briefly, co-culture supernatants were collected 48 h after LPS/IFN- $\gamma$  treatment and incubated  
165 with Griess reagent for 30 min at RT. Optical density at 540 nm was measured using a  
166 microplate reader (Infinite<sup>®</sup> M200, Tecan). Nitrite concentration was calculated from a sodium  
167 nitrite standard curve.

### 168 ***2.5 SH-SY5Y-APP751 cell culture and treatments***

169 SH-SY5Y human neuroblastoma cells overexpressing human APP751 (SH-SY5Y-APP751)  
170 were grown on 6-well plates (400 000 cells/well) as described previously (Sarajärvi et al.,  
171 2009). The next day cells were exposed to HMI-1a3 and HMI-1b11 (both 20  $\mu$ M), PMA (10  
172 nM),  $\gamma$ -secretase inhibitor N-[N-(3,5 Difluorophenacetyl) L-alanyl]-S-phenylglycine t-butyl  
173 ester (DAPT; Sigma-Aldrich, 10  $\mu$ M) and bryostatin-1 (10 and 100 nM) in FBS free medium.  
174 Exposure times were 4, 24 and 48 h, after which the media were collected, detached cells spun  
175 down (2000  $\times$  g, 2 min at +4°C) and the supernatant collected. The media samples from 4-h  
176 and 24-h time points were used for analyzing secreted amyloid precursor proteins (sAPPs) with  
177 Western blotting and from 48-h time point for determining the A $\beta$ 40 and A $\beta$ 42 levels with  
178 ELISA (Human/Rat  $\beta$  Amyloid 40 and 42, Wako, Cat no 294-64701/Cat no 292-64501,  
179 respectively). A $\beta$  levels were normalized to total protein levels in the corresponding cell  
180 lysates. After collecting the media, the plates were put on ice, cells washed once with cold PBS  
181 followed by scraping and lysing with a lysis buffer containing 10 mM TRIS-HCl pH 6.8, 1 mM  
182 EDTA, 150 mM NaCl, 0.25% Nonidet P-40 and 1% Triton X-100 complemented with  
183 PHOStop protein phosphatase inhibitor and Complete protease inhibitor cocktails (Roche).  
184 Cell homogenates were centrifuged (16000  $\times$  g, 4 min at +4°C) and supernatants collected. To  
185 determine the effect of isophthalates on PKC protein levels, SH-SY5Y naïve cells were used.

186 The cells were exposed to isophthalates (HMI-1a3 and HMI-1b11) or PMA (100 nM) for 24  
187 hours in serum-supplemented medium. Cell homogenates were prepared by lysing the cells in  
188 1% SDS (in 50 mM Tris-HCl, pH 7.5) and processed for Western blotting as described below.

189

## 190 ***2.6 Western blot analysis***

191 Protein concentrations were measured using Bicinchononic acid (BCA) protein assay kit  
192 (ThermoFisher Scientific) and equal amounts of protein (20-30  $\mu$ g) were resolved in 4-12%  
193 gradient Bis-Tris gels (Criterion-XT, Bio-rad) under reducing conditions. Media samples were  
194 resolved under similar conditions, but due to their low protein content, a maximal volume of  
195 media (33  $\mu$ l) was taken. Samples for PKC level determination were boiled for 5 min with  
196 Laemmli sample buffer (#161-0747, Bio-rad) containing 10 %  $\beta$ -mercaptoethanol (Sigma) and  
197 resolved in 10 % polyacrylamide gels. After transfer to polyvinylidene difluoride (PVDF)  
198 membranes (Trans-Blot Turbo Midi PVDF transfer pack, Bio-rad) or nitrocellulose membranes  
199 (PKC level determination), the membranes were blocked with either 5% milk or 5% BSA  
200 (depending on the antibody) and then incubated overnight with the primary antibodies at +4°C  
201 in a shaker. On the following day, the blots were incubated with secondary antibodies (goat  
202 anti-rabbit, #170-6515, Bio-rad and anti-mouse IgG #7076S, Cell Signaling Technology),  
203 which were subsequently detected with chemiluminescent substrate (SuperSignal West Pico,  
204 #34080, or Supersignal West Femto, #34095, ThermoFisher Scientific) utilizing LAS 3000  
205 Imaging System (Fujifilm) or exposed to film (PKC level determination). Optical densities  
206 (OD) of the bands were measured using ImageJ software (<https://imagej.net/Downloads>). Cell  
207 lysates from primary cortical neuron-BV2 microglia co-cultures were probed with phospho-  
208 S536 NF- $\kappa$ B p65 antibody (ab28856, anti-pNF- $\kappa$ B p65 (S536) rabbit polyclonal antibody  
209 1:2000, Abcam), total NF- $\kappa$ B p65 antibody (ab16502, anti-NF- $\kappa$ B p65, rabbit polyclonal  
210 antibody 1:2000, Abcam), and normalized with  $\beta$ -actin (ab8226, anti- $\beta$ -actin, mouse

211 monoclonal antibody 1:1000, Abcam). The blots from cell lysates were probed with APP C-  
212 terminus binding antibody (A8717, rabbit anti-APP C-terminus, 1:2000, Sigma). The OD of  
213 these bands were normalized with the OD of GAPDH bands from the same samples (sc47724,  
214 anti-GAPDH, 1:2000, Santa Cruz Biotechnology). The blots from the cell media, were probed  
215 with N-terminus binding antibodies (6E10, mouse anti-A $\beta$ <sub>1-16</sub>, 1:1000, Biosite, and 22c11,  
216 mouse anti-APP N-terminus, 1:1000, Merck) § For determining the PKC protein levels, all  
217 primary antibodies were from Santa Cruz Biotechnology PKC $\alpha$  (#8393), PKC $\beta$ I (#8049),  
218 PKC $\delta$  (#937) except for PKC $\epsilon$  (BD Biosciences, #610085) and were used at 1:1000 dilution.  
219 The experiments were repeated 3 times with 2 parallels.

### 220 *2.7 Mouse primary hippocampal neuron culture, transient transfection, and spine* 221 *morphology analysis*

222 Primary hippocampal neuronal cultures were prepared from 18-day-old mouse JAXC57BL/6J  
223 embryos according to the protocol previously described (Kurkinen et al., 2016). Briefly, single-  
224 cell solution (240 000 cells/cm<sup>2</sup>) was plated on 4-well chamber slides (LabTek) coated with  
225 poly-D-lysine and 30  $\mu$ g/ml laminin in feeding media composed of Neurobasal medium  
226 supplemented with 2% B27, 0.5 mM L-glutamine, 100 U/ml penicillin and 100  $\mu$ g/ml  
227 streptomycin. Hippocampal neurons were grown in a cell culture incubator at 37°C in 5% CO<sub>2</sub>.  
228 Half of the culture media was replaced with fresh feeding media after every 5 days. On DIV19,  
229 mature hippocampal neurons in 4-well chamber slides were transiently transfected with a  
230 mixture containing 2  $\mu$ l of Lipofectamine 2000 (Invitrogen) and 0.8  $\mu$ g of enhanced green  
231 fluorescent protein (pEGFP). On DIV20 the cells were treated with 10  $\mu$ M HMI-1a3, 100 nM  
232 bryostatin-1 (Sigma Aldrich), or 0.1% DMSO (vehicle control) for 2 hours, after which the  
233 hippocampal neurons were fixed in 4% PFA (24 hours after pEGFP transfection). Hippocampal  
234 dendritic spines from GFP-positive neurons were imaged with a Zeiss Axio Observer.Z1  
235 inverted microscope (63 x NA 1.4 oil objective) equipped with Zeiss LSM 800 confocal

236 module (Carl Zeiss Microimaging GmbH, Jena, Germany). Serial Z-stacks of optical sections  
237 from dendritic segments were captured for spine analysis performed with NeuronStudio  
238 software (Rodriguez et al., 2008) as described previously (Bertling et al., 2016).

## 239 **2.8 Statistical analyses**

240 Statistical analyses were performed using the SPSS (version 21.0) or GraphPad Prism (version  
241 5.02) software. A comparison of three or more groups was performed using one-way ANOVA  
242 followed by the Fisher's least significant difference (LSD) post-hoc test or Dunnett's test.  
243 Statistical significance between two groups was tested using the independent sample t-test. All  
244 values are reported as mean + standard error of mean (SEM). The level of statistical  
245 significance was defined as  $p < 0.05$ .

246

247 **3. Results**

248 ***3.1 HMI-1a3 reduces neuronal loss after LPS+IFN- $\gamma$ -induced neuroinflammation in mouse***  
249 ***primary neuron-BV2 microglia co-cultures***

250 Mouse primary neuron-BV2 microglia co-cultures have been previously used to study the  
251 effects of LPS+IFN- $\gamma$ -induced neuroinflammation (Martiskainen et al., 2017). Similarly, we  
252 observed a significant reduction in the neuronal viability after LPS+IFN- $\gamma$  treatment as  
253 compared to vehicle treated co-cultures at DIV7 (Fig. 1a). The reduction in neuronal viability  
254 was accompanied with significantly increased levels of the proinflammatory cytokine TNF $\alpha$   
255 and nitric oxide (NO) measured from the co-culture medium 48 hours after the initiation of  
256 LPS+IFN- $\gamma$  treatment. Pre-treatment of co-cultures with the specific iNOS inhibitor 1400W  
257 resulted in an average of 85% reduction in NO levels and reduced neuronal loss, without  
258 affecting the levels of TNF $\alpha$  (Fig. 1a). However, pre-treatment of co-cultures with IL-10  
259 significantly decreased the TNF $\alpha$  levels in LPS+IFN- $\gamma$ -treated cells by 60%, without effects on  
260 neuronal viability or the levels of NO (Fig. 1a). LPS+IFN- $\gamma$  treatment of mouse primary  
261 cortical neuronal cultures without addition of BV2 microglial cells did not affect the production  
262 of TNF $\alpha$  or NO, or neuronal viability as compared to vehicle treated samples (data not shown).  
263 Collectively, these results are consistent with the previous findings in the neuron-BV2  
264 microglia co-cultures upon neuroinflammation (Martiskainen et al., 2017) and thus corroborate  
265 the usage of this co-culture system as a feasible model to investigate protein kinase C-activating  
266 isophthalate derivatives in the cellular processes relevant for AD, such as neuroinflammation.

267 To investigate whether HMI-1a3 and HMI-1b11 exert neuroprotective effects, we  
268 administered the test compounds with or without the pan-PKC inhibitor Gö6983 into the co-  
269 culture medium 1 h after the addition of BV2 microglial cells into neuronal cultures. NI-15e,  
270 which is a structural analogue of HMI-1a3 without significant affinity to the C1 domain, was  
271 used as a negative control as well as PMA and bryostatin-1 as positive controls for PKC

272 activation. Neuroinflammation was induced 1 h later with LPS+IFN- $\gamma$ . HMI-1b11 had no effect  
273 on neuronal viability (data not shown), whereas HMI-1a3 increased neuronal viability upon  
274 LPS+IFN- $\gamma$  treatment, especially at 20  $\mu$ M concentration as compared to vehicle treated cells  
275 (Fig. 1b). Interestingly, the PKC inhibitor Gö6983 alone (1  $\mu$ M) did not affect neuronal  
276 viability, but neuronal loss was observed, when it was administered in combination with any  
277 of the HMI-1a3 concentrations studied (Fig. 1b). Unexpectedly, the negative control NI-15e  
278 induced approximately 20% neuronal loss at all concentrations as compared to the vehicle  
279 control. Similarly, the potent PKC activator PMA reduced neuronal viability significantly at  
280 both 10 nM and 100 nM concentrations, and this effect was not affected by simultaneous PKC  
281 inhibition with Gö6983 (Fig. 1c). Bryostatin-1, another PKC activator, had no effect on  
282 neuronal viability alone, while in combination with Gö6983, it increased the neuronal viability  
283 (Supplementary Fig. S1a). Taken together, these results suggest that HMI-1a3 exhibits  
284 neuroprotective activity in neuron-BV2 microglial co-cultures subjected to LPS+IFN- $\gamma$ -  
285 induced neuroinflammation.

### 286 ***3.2 HMI-1a3 treatment decreases the levels of TNF $\alpha$ but does not affect NO production in*** 287 ***mouse primary neuron-BV2 microglia co-cultures subjected to neuroinflammation***

288 It is well-established that several microglia-activating cascades, including A $\beta$  deposition,  
289 initiate neuroinflammation in brain. Activated microglia play an important role in the brain  
290 tissue by inducing the expression of pro-inflammatory cytokines, such as interleukins and  
291 TNF $\alpha$ , which in turn trigger the expression of inducible nitric oxide synthase (iNOS) and the  
292 production of NO (Wang et al., 2015). In neurons, TNF $\alpha$  and NO act as important mediators  
293 with both pro-inflammatory and destructive effects. HMI-1a3 decreased TNF $\alpha$  levels in a  
294 concentration-dependent manner as compared to vehicle treated cells (Fig. 2a). However, HMI-  
295 1a3 had no effect on TNF $\alpha$  production in the presence of 1 $\mu$ M Gö6983, indicating that the  
296 HMI-1a3-induced decrease in TNF $\alpha$  secretion was mediated by PKC. As with HMI-1a3,

297 bryostatin-1 alone decreased TNF $\alpha$  levels, while this effect was not reversed in the presence of  
298 Gö6983 (Supplementary Fig. S1b). As expected, NI-15e had no effect on the levels of TNF $\alpha$   
299 upon LPS+IFN- $\gamma$ -induced neuroinflammation (Fig. 2a). In contrast with the effects of HMI-  
300 1a3, the treatment of co-cultures with 100 nM PMA increased the levels of TNF $\alpha$  both alone  
301 and in the presence of Gö6983 (Fig. 2b), which is in line with its PKC-independent neurotoxic  
302 effects in the ABTS assay. Neither HMI-1a3 nor NI-15e had any effect on the production of  
303 NO in LPS+IFN- $\gamma$ -treated co-cultures (Fig. 2c). Similarly, PMA alone had no effect on  
304 production of NO, but when co-administered with 1 $\mu$ M Gö6983, it increased the NO  
305 production at both 10 nM and 100 nM concentrations (Fig. 2d). Conversely, bryostatin-1  
306 increased the production of NO in a PKC activation-dependent manner (Supplementary Fig.  
307 S1c). In summary, HMI-1a3 decreased the levels of TNF $\alpha$  through activation of PKC, but did  
308 not affect the production of NO.

309

### 310 ***3.3 HMI-1a3 does not affect the S536 phosphorylation of NF $\kappa$ B p65 in the primary neuron-*** 311 ***BV2 microglia co-cultures upon neuroinflammation***

312 Since nuclear factor kappa B (NF $\kappa$ B) is the key transcriptional regulator of genes that control  
313 inflammation, immune regulation, proliferation and cell death (Christian et al., 2016), we next  
314 assessed the phosphorylation status and the total levels of NF $\kappa$ B p65 subunit in the primary  
315 neuron-BV2 microglia co-cultures treated with HMI-1a3 or bryostatin-1 upon  
316 neuroinflammation (Fig. 3). The S536 phosphorylation site in NF $\kappa$ B p65 subunit was selected  
317 as it is one of the best-understood phosphorylation targets in the transactivation domain  
318 (Christian et al., 2016). Primary neuron-BV2 microglia co-cultures were treated with 20  $\mu$ M  
319 HMI-1a3, which in the previous experiments affected significantly neuronal viability and the  
320 levels of TNF $\alpha$  upon LPS+IFN- $\gamma$ -induced neuroinflammation (Fig. 1b and Fig. 2a). Bryostatin-  
321 1 (10 and 100 nM) was used as a control and all the LPS+IFN- $\gamma$ -treated samples showed the

322 expected increase in the levels of NO, confirming the induction of neuroinflammation in the  
323 co-cultures (data not shown). Western blot analysis of total protein lysates of HMI-1a3- or  
324 bryostatin-1-treated samples upon LPS+IFN- $\gamma$ -induced neuroinflammation did not reveal  
325 statistically significant changes in the S536 phosphorylation status of NF $\kappa$ B p65 when  
326 normalized to the total levels of NF $\kappa$ B p65 (Fig. 3a and Fig. 3b). HMI-1a3, but not bryostatin-  
327 1, significantly increased the total levels of NF $\kappa$ B p65 on average by 20% (Fig. 3a and Fig.  
328 3b). These results suggest that HMI-1a3 does not affect the S536 phosphorylation status in the  
329 transactivation domain of NF $\kappa$ B p65 but instead increases moderately the levels of total NF $\kappa$ B  
330 p65 in the primary neuron-BV2 microglia co-cultures upon neuroinflammation.

#### 331 ***3.4 HMI-1a3 and HMI-1b11 do not significantly affect the levels of A $\beta$ 40 or A $\beta$ 42 in primary*** 332 ***neuron-BV2 microglia co-cultures upon neuroinflammation or in neuroblastoma cells***

333 We next elucidated whether the isophthalate derivatives affect the levels of A $\beta$ 40 and A $\beta$ 42,  
334 or the ratio of these A $\beta$  species in the primary neuron-BV2 microglia co-culture media upon  
335 neuroinflammation. HMI-1a3 did not significantly affect the levels of A $\beta$ 40 (Fig. 4a) or A $\beta$ 42  
336 (Fig. 4b), nor the ratio of A $\beta$ 42/A $\beta$ 40 (Fig. 4c) as compared to vehicle-treated cells. However,  
337 there was a trend towards increased levels of A $\beta$ 40 and A $\beta$ 42, which prompted us to investigate  
338 whether the isophthalate derivatives affect APP processing in human SH-SY5Y neuroblastoma  
339 cells overexpressing APP751 (SH-SY5Y-APP751). In our previous study with naïve SH-  
340 SY5Y cells, both HMI-1a3 and HMI-1b11 were shown to promote neurite growth, while HMI-  
341 1b11 was better tolerated (Talman et al., 2013). Upon treatment with HMI-1a3 or HMI-1b11  
342 for 48 h, both A $\beta$ 40 and A $\beta$ 42 levels in the cell culture medium showed a moderate, but non-  
343 significant increase when compared to vehicle-treated control cells (Fig. 4d). In addition, the  
344 ratio of A $\beta$ 42/A $\beta$ 40 was moderately, but not statistically significantly decreased (Fig. 4f).  
345 However, 10 nM PMA, which was used as a positive control, caused a significant increase in



346 the levels of A $\beta$ 40 (Fig. 4d), without any effect on the levels of A $\beta$ 42 (Fig. 4e), leading to a  
347 ~70% decrease in the ratio of A $\beta$ 42/A $\beta$ 40 as compared to a vehicle-treated cells (Fig. 4f). This  
348 is in line with numerous previous studies showing increased  $\alpha$ -secretase-mediated APP  
349 cleavage in response to PKC activation with phorbol esters (Skovronsky et al., 2000; Zhu et  
350 al., 2001). 10 nM bryostatin-1 did not affect the levels of A $\beta$ 40, but it decreased the levels of  
351 A $\beta$ 42, thereby significantly decreasing the ratio of A $\beta$ 42/A $\beta$ 40 (Supplementary Fig. S2). No  
352 effects were observed with the treatment of 100 nM bryostatin-1 (Supplementary Fig. S2).  
353 Although we observed a trend towards a decreased ratio of A $\beta$ 42/A $\beta$ 40 in cells treated with the  
354 isophthalate derivatives, these results suggest that the isophthalates are not as effective as PMA  
355 or bryostatin-1 in modulating the APP processing towards the non-amyloidogenic pathway.

### 356 ***3.5 HMI-1a3 affects the levels of APP C83 and the maturation of APP in neuroblastoma*** 357 ***cells***

358 Next, we explored the effects of these PKC activators on the levels and maturation of APP and  
359 APP C-terminal fragment (APP C83) in SH-SY5Y-APP751 cells after 4- (Fig. 5a-b) and 24-  
360 hour (Fig. 5c-d) treatments. Additionally, the  $\gamma$ -secretase inhibitor DAPT (10  $\mu$ M) was used as  
361 a positive control in combination with PMA (10 nM) to induce accumulation of C-terminal  
362 fragments, especially APP C83 (indicated as PMA+DAPT in Fig. 5). A 4-h treatment with  
363 HMI-1a3 decreased, while a 24-h treatment with HMI-1a3, HMI-1b11, and PMA increased the  
364 levels of APP C83 significantly in SH-SY5Y-APP751 cells as compared to vehicle-treated  
365 cells. Importantly, the increase in APP C83 in 24-h treatment samples coincided with the  
366 increased levels of total APP (APP<sub>tot</sub>) with all treatments (Fig. 5d). A similar increase in the  
367 levels of APP<sub>tot</sub> was not observed in the 4-h treated samples, except with PMA+DAPT (Fig.  
368 5b). A statistically significant increase in the levels of immature APP (APP<sub>im</sub>) and a concurrent  
369 decrease in the levels of mature APP (APP<sub>m</sub>) was observed after a 4-h treatment with HMI-1a3  
370 (Fig. 5b). The PKC activators HMI-1a3, HMI-1b11 and PMA induced statistically significant

371 decreases in the ratio of APP<sub>m</sub>/APP<sub>im</sub> after both 4-h and 24-h treatments (Figs. 5b and 5d,  
372 respectively), suggesting that treatment with PKC activators inhibited the maturation of APP.  
373 Bryostatin-1 did not induce statistically significant changes in the maturation of APP after 4-h  
374 or 24-h treatments (Supplementary Fig. S4). Collectively, these results suggest that both  
375 isophthalate derivatives decrease the maturation of APP in neuroblastoma cells.

### 376 ***3.6 HMI-1a3 and HMI-1b11 increase the levels of secreted sAPP $\alpha$ relative to total sAPP in*** 377 ***neuroblastoma cells***

378 As we observed significant changes in the processing and maturation of APP in SH-SY5Y-  
379 APP751 cells treated with HMI-1a3 and HMI-1b11, we next assessed whether these  
380 compounds affect the soluble APP $\alpha$  (sAPP $\alpha$ ) and the total soluble APP (sAPP<sub>tot</sub>) levels in the  
381 cell culture medium (Fig. 6a-b and Fig. 6c-d). After a 24-h treatment, a statistically significant  
382 increase in sAPP $\alpha$  levels was observed with all compounds as compared to vehicle-treated cells  
383 (Fig. 6d). A similar trend towards increased levels of sAPP $\alpha$  was observed after a 4-h treatment  
384 with all compounds except with HMI-1a3. In contrast to sAPP $\alpha$  levels, approximately 40%  
385 decrease in the levels of sAPP<sub>tot</sub> was observed after a 4-h treatment with both HMI-1a3  
386 ( $p=0.06$ ) and HMI-1b11 ( $p<0.05$ ) as compared to vehicle-treated cells (Fig. 6b). Due to the fact  
387 that the levels of APP<sub>tot</sub> were affected, particularly after a 24-hour treatment (Fig. 5d), we  
388 quantified the ratio of sAPP $\alpha$  and sAPP<sub>tot</sub> (sAPP $\alpha$ /sAPP<sub>tot</sub>). Both HMI-1a3 and HMI-1b11  
389 increased the ratio of sAPP $\alpha$ /sAPP<sub>tot</sub> after 4-h and 24-h treatments as compared to the vehicle-  
390 treated cells. PMA alone and in combination with DAPT (PMA+DAPT) increased the ratio of  
391 sAPP $\alpha$ /sAPP<sub>tot</sub> significantly after 4 h, but not after 24 h. A similar trend was seen with  
392 bryostatin-1, although the results were not statistically significant (Supplementary Fig. S4).  
393 This may relate to the fact that PMA and PMA+DAPT have a more robust effect on the levels  
394 of sAPP<sub>tot</sub> as compared to HMI-1a3 and HMI-1b11. This difference between isophthalates and  
395 PMA may be due to the down-regulation of PKC after the 24-h PMA exposure, a phenomenon

396 which is not seen in response to isophthalate treatment (Supplementary Fig. S5). Collectively,  
397 these findings suggest that HMI-1a3 and HMI-1b11 promote non-amyloidogenic APP  
398 processing and the secretion sAPP $\alpha$  relative to the levels of sAPP $\text{tot}$ .

### 399 ***3.7 The PKC activator bryostatin-1 increases the number of mushroom spines in mouse*** 400 ***mature hippocampal neuron cultures***

401 PKC isoforms are located within hippocampal dendritic spines and they are known to play a  
402 role in the modulation of dendritic spine morphology (Alkon et al., 2007; Calabrese and  
403 Halpain, 2005). Therefore, the role of PKC activators HMI-1a3 (10  $\mu$ M) and bryostatin-1 (100  
404 nM) were investigated in mature primary mouse hippocampal neurons (DIV20) in a short-term  
405 2-h treatment. As a result, total spine density, stubby spine density, and thin spine density  
406 decreased significantly with bryostatin-1 as compared to vehicle treated cells (Fig. 7a). In  
407 contrast, a moderate non-significant increase in total, mushroom, stubby, and thin spine  
408 densities was observed after HMI-1a3 treatment (Fig. 7a). Analysis of spine head morphology  
409 revealed that the 2-h exposure of hippocampal neurons to HMI-1a3 was not able to modify the  
410 shape of the spine head, while bryostatin-1 significantly increased the diameter of mushroom  
411 and stubby spine heads and decreased the diameter of thin spine heads (Fig. 7b). Overall,  
412 HMI-1a3 did not significantly modify the number of mushroom, stubby, or thin spine head  
413 morphology, whereas bryostatin-1 treatment increased the mushroom spine head density and  
414 decreased the thin spine head density in ratio to total number of spine heads (Fig. 7c).  
415 Collectively, these data suggest that bryostatin-1 supports stabilization of spine heads towards  
416 the mature, active mushroom-like shape.

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#### 421 **4. Discussion**

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

444 Insufficient PKC activation has been associated with abnormalities in neuroinflammatory  
445 signaling (Alkon et al., 2007), a phenomenon characteristic in the early pathophysiology of AD

446 (Tarkowski et al., 2003). Furthermore, the levels and activity of PKC isoforms is attenuated in  
447 the brain of AD patients (Lucke-Wold et al., 2015; Wang et al., 1994). In line with these  
448 observations, encouraging results on the effects of PKC activators in both *in vitro* and *in vivo*  
449 models of AD have been reported (Talman et al., 2016). Many of the positive effects induced  
450 by PKC activators have been attributed to the activation of isoforms  $\epsilon$  and  $\gamma$  (Lucke-Wold et  
451 al., 2015), which has evoked ideas about specifically targeting these isoforms. However, most  
452 of the studies on PKC activators have been done with activators that are not isoform specific,  
453 such as bryostatin-1. Similar to bryostatin-1, HMI-1a3 binds to the C1-domain of both novel  
454 and classical isoforms of PKC and is therefore not isoform-specific. Furthermore, as Gö6983  
455 inhibits several PKC isoforms (Gschwendt et al., 1996), identification the exact isoform(s)  
456 responsible for HMI-1a3-induced effects reported here, requires further investigations.

457 PKC activation has been shown to direct APP processing towards the production of  
458 neuroprotective sAPP $\alpha$  in several cell-based models and restoration of synapses has been  
459 reported in both cells as well as in rodents with various PKC activators (Talman et al., 2016).  
460 The PKC activator bryostatin-1 has been shown to improve memory in behavioral tests in  
461 several rodent models and has progressed to clinical trials as the first PKC-targeted therapy for  
462 neurodegenerative diseases ([www.clinicaltrials.gov](http://www.clinicaltrials.gov)) (Talman et al., 2016). Although PKC  
463 activators have been traditionally considered as tumor promoters, this view has been challenged  
464 by a recent comprehensive study, which showed that the majority of cancer-associated PKC  
465 mutations were loss-of-function mutations and none were activating (Antal et al., 2015). The  
466 tumor-promoting properties of some PKC activators, such as the phorbol esters, may thus in  
467 fact be due to PKC down-regulation and not increased activity *per se*. Therefore, it appears that  
468 the loss of PKC activity might lead to tumor promotion, which makes the development drugs  
469 that are partial agonists even more appealing.

470 In the present study, we found that HMI-1a3 promoted to some extent neuronal survival  
471 effects in the primary neuron-BV2 microglial cell co-cultures upon LPS-IFN- $\gamma$ -induced  
472 neuroinflammation. In the presence of PKC inhibitor Gö6983 (1 $\mu$ M), the neuroprotective  
473 effect of HMI-1a3 was abolished implicating that the effect was PKC-dependent. Surprisingly,  
474 viability of neurons decreased when cells were treated with both compounds, whereas Gö6983  
475 alone had no effect on neuronal viability. This may relate to fact that in addition to PKC, six  
476 other protein families, such as Munc13, protein Kinase D (PKD), RasGRP, chimaerins,  
477 diacylglycerol kinases (DAGKs) and myotonic dystrophy kinase-related Cdc42-binding kinase  
478 (MRCK), contain analogous C1 domains and consequently HMI-1a3 has been shown to bind  
479 to several of these (Talman et al., 2014). Therefore, it is possible that HMI-1a3 could modulate  
480 some C1 domain-dependent pathways that counteract PKC signalling, which would then lead  
481 to compromised neuronal viability when PKC is inhibited by Gö6983. Furthermore, Gö6983  
482 binds to the ATP binding site of PKC (Wu-Zhang and Newton, 2013) and not to the C1 domain,  
483 and therefore does not affect the activity of other C1 domain containing proteins. The potent  
484 PKC activator PMA reduced neuronal viability significantly at both 10 nM and 100 nM  
485 concentrations, and this was not affected by the pan-PKC inhibitor Gö6983. PMA, like  
486 isophthalates, also have other targets besides PKC and the observed reduction in neuronal  
487 viability with PMA in combination with Gö6983, could be due to activation of these other  
488 target proteins. The observation that both HMI-1a3 and PMA caused a decrease in neuronal  
489 viability when used in combination with Gö6983 supports the notion that other C1-domain  
490 containing proteins are likely to be responsible for this outcome. Furthermore, the difference  
491 in the response to these compounds alone could be explained by the level of PKC activation  
492 they induce. Too robust and prolonged activation by PMA leads to the downregulation of PKC  
493 protein levels and a decrease in neuronal viability, whereas HMI-1a3 induces PKC activation

494 without causing downregulation. Thus, the results suggest that the activation of PKC without  
495 downregulation is neuroprotective upon LPS-IFN- $\gamma$ -induced neuroinflammation.

496 The effect of HMI-1a3 on neuronal viability could be explained by its effect on TNF $\alpha$ .  
497 HMI-1a3 concentration-dependently reduced the levels of TNF $\alpha$  and this effect was abolished  
498 with Gö6983, suggesting that the effect was mediated by PKC. PMA had no effect on the TNF $\alpha$   
499 levels at 10 nM concentration but induced an increase at 100 nM. At 100 nM concentration and  
500 after a 48-h exposure PMA already downregulates PKC, while still activating other targets  
501 containing a phorbol-responsive C1 domain. For example, activation of Munc-13 has been  
502 shown to positively regulate TNF $\alpha$  release in macrophages (Mori et al., 2011). When PMA  
503 was combined with Gö6983, the increase in the levels of TNF $\alpha$  could be detected already at  
504 the 10 nM PMA concentration. It is possible that PKC activation with 10 nM PMA  
505 concentration counteracts the PKC-independent effects of PMA and when PKC activation is  
506 inhibited with Gö6983, this PKC-independent effect becomes dominant. Slightly in contrast  
507 with these PMA results, but in line with the effect of HMI-1a3, bryostatin-1 decreased the  
508 levels of TNF $\alpha$ . However, this effect was not abolished, but instead was potentiated by PKC  
509 inhibition, indicating that also other targets of bryostatin-1 may contribute to the observed  
510 decrease in the production of TNF $\alpha$ . To elucidate the potential molecular mechanism  
511 underlying the increased neuronal viability and the reduced levels of TNF $\alpha$  owing to HMI-1a3,  
512 we determined the S536 phosphorylation status in the transactivation domain of NF $\kappa$ B p65  
513 subunit (Christian et al., 2016), and the levels of total NF $\kappa$ B p65 in the primary neuron-BV2  
514 microglia co-cultures upon neuroinflammation. It is a well-established observation that the  
515 increased S536 phosphorylation leads to the enhanced transactivation of NF $\kappa$ B via the  
516 increased binding of CREB-binding protein/p300 and acetylation at K310 of NF $\kappa$ B p65 (Chen  
517 et al., 2005). Consequently, the activated nuclear NF $\kappa$ B mediates gene transcription of certain  
518 chemokines and interleukins, such as TNF $\alpha$ , IFN $\gamma$  and IL6, known to play a central role in the

519 immune response, proliferation, and cell death. However, we did not find any significant  
520 changes in the phosphorylation status of S536 in NFκB p65, but instead we observed a  
521 moderate, but statistically significant increase in the total levels of NFκB p65 in the co-cultures  
522 treated with HMI-1a3 upon neuroinflammation. Interestingly, bryostatin-1 did not have any  
523 significant effects on phosphorylation status or total levels of NFκB p65 upon  
524 neuroinflammation. The relevance of the increased levels of NFκB p65 subunit (~20%) owing  
525 to HMI-1a3 treatment is not clear and thus further studies are needed to elucidate whether this  
526 relatively small increase would play a prominent role in terms of neuroprotection. Conversely,  
527 it should be noted that the increased levels of NFκB p65 did not lead to the augmentation of  
528 S536 phosphorylation in NFκB p65 subunit, suggesting that enhanced transactivation of NFκB  
529 did not take place after HMI-1a3 treatment upon neuroinflammation. Although the S536  
530 phosphorylation site in NFκB p65 is one of the best-understood phosphorylation targets in the  
531 transactivation domain of NFκB p65 (Christian et al., 2016), we cannot rule out the possibility  
532 that some other phosphorylation target in NFκB known to control NFκB-directed  
533 transactivation could be affected by HMI-1a3 or bryostatin-1 upon neuroinflammation. Despite  
534 the observed compound-induced changes in the levels of TNFα upon neuroinflammation, we  
535 did not observe major effects on the production of NO. The observation that PMA increases  
536 the production of NO only in conditions where PKC is inhibited, may suggest that the effect is  
537 again mediated by another C1 domain-containing protein, which under normal conditions is  
538 suppressed by PKC activity. For example, PKD has been reported to directly activate neuronal  
539 NO synthase (Sánchez-Ruiloba et al., 2014). Collectively, these results suggest that HMI-1a3  
540 may exert neuroprotection by activating PKC, and this is probably mediated by alleviating the  
541 production of proinflammatory cytokine TNFα upon LPS+IFN-γ-induced neuroinflammation  
542 in neuron-BV2 cell co-cultures. As neuroinflammation is considered a central detrimental



543 phenomenon in AD and other neurodegenerative diseases, this anti-inflammatory and  
544 neuroprotective effect of HMI-1a3 is very encouraging.

545 PKC activators, such as phorbol esters and bryostatin-1, have previously been shown to  
546 reduce A $\beta$ 40 and A $\beta$ 42 levels in *in vivo* models of AD (Etcheberrigaray et al., 2004; Savage et  
547 al., 1998). In contrary to this, we did not observe significant effect on A $\beta$ 40 or A $\beta$ 42 levels in  
548 neuron-BV2 co-cultures when treated with HMI-1a3 or HMI-1b11. However, both isophthalate  
549 derivatives induced a moderate, but non-significant decrease in A $\beta$ 42/A $\beta$ 40 ratio in SH-SY5Y-  
550 APP751 cells. This was confirmed to be a consistent outcome as we observed a significant  
551 increase in the levels of APP C83, decrease in APP<sub>m</sub>/APP<sub>im</sub> ratio, and an increase in  
552 sAPP $\alpha$ /sAPP<sub>tot</sub> ratio in SH-SY5Y-APP751 cells when the cells were treated with HMI-1a3 or  
553 HMI1b11 for 24 h. Additionally, as both APP<sub>m</sub> and APP<sub>im</sub> levels were increased after  
554 isophthalate treatment for 24 h, it can be speculated that isophthalate-induced PKC activation  
555 might enhance post-translational modification of APP, such as glycosylation, sulfation, and  
556 phosphorylation during transit through the intracellular protein secretory pathway (da Cruz e  
557 Silva et al., 2009). Indeed, PKC has been reported to not only directly phosphorylate APP, but  
558 also regulate its trafficking by controlling the formation of APP bearing vesicles in the Trans-  
559 Golgi network (TGN) (Gandy et al., 1988; Xu et al., 1995). In addition, PKC activation has  
560 been shown to increase the transcription of APP already after 3 h, and induce a 4-fold increase  
561 in the APP mRNA levels after a 24-h exposure to PMA (100 nM) (Trejo et al., 1994), which is  
562 in line with our finding that total APP levels were increased at 24 h in response to the  
563 isophthalates, PMA and also to some extent bryostatin-1. Previous studies have indicated that  
564 the cellular responses to PKC-activating compounds are dependent on the exposure time. Acute  
565 PKC activation has been reported to increase the release of sAPP $\alpha$  by activating  $\alpha$ -secretase,  
566 while chronic exposure to PKC activators has also increased the expression levels of APP (da  
567 Cruz e Silva et al., 2009; Trejo et al., 1994). Furthermore, da Cruz de Silva *et al* (2009) reported

568 that a 24-h exposure to phorbol ester increased total APP production, decreased sAPP $\alpha$ ,  
569 increased A $\beta$  levels and caused accumulation of APP in TGN. However, most of these  
570 phenomena were explained by PKC downregulation. The isophthalate derivatives do not  
571 induce PKC down-regulation within 24 h in SHSY5Y cells, and although the accumulation of  
572 APP in TGN was visible after 24-h exposure, the sAPP $\alpha$ /sAPP $_{tot}$  ratio was still increased with  
573 isophthalates, unlike with PMA. Notably, the increased levels of both APP C83 and sAPP $\alpha$ ,  
574 but not that of sAPP $_{tot}$  suggest that the exposure to different PKC agonist might specifically  
575 promote the accumulation of APP at the cell surface and influence the recycling of re-  
576 internalized APP back to the plasma membrane, where majority of the non-amyloidogenic  $\alpha$ -  
577 cleavage takes place. Our findings support the idea that activation of PKC may increase the  
578 levels of sAPP $\alpha$  by mechanisms involving the formation and release of secretory vesicles from  
579 the TGN, thus enhancing APP trafficking to the cell surface (Thinakaran and Koo, 2008; Xu  
580 et al., 1995). Since sAPP $\alpha$  is neuroprotective, the sustained increase in its production  
581 (sAPP $\alpha$ /sAPP $_{tot}$  ratio) achieved with isophthalates, but not with PMA, further supports the  
582 idea that activating PKC, without inducing its downregulation, may represent a promising  
583 therapeutic strategy for AD.

584 We also detected increased mushroom spine density relative to total spine density in the  
585 mature mouse hippocampal neurons upon basal growth conditions after a 2-h treatment with  
586 PKC activator bryostatin-1, suggesting that PKC activation increases the number of functional  
587 excitatory post-synaptic terminals. To support this, we also observed a significant increase in  
588 mushroom spine head diameter in bryostatin-1-treated hippocampal neurons, indicating that  
589 enlargement of mushroom spine head through activation of PKC signaling cascade may lead  
590 to formation of a postsynaptic density in dendritic mushroom spine as previously reported  
591 (Calabrese and Halpain, 2005; Sen et al., 2016; Yoshihara et al., 2009). In this study, HMI-1a3  
592 did not affect spine morphology. This could be due to the relatively short treatment time, which

593 is probably enough to induce changes with a very potent PKC activator, such as bryostatin-1,  
594 but might not be long enough for less potent activators, such as the isophthalates. Whether  
595 longer isophthalate treatments would affect spine morphology remains to be determined.  
596 Moreover, these experiments were carried out in basal conditions without induction of  
597 neuroinflammation. Isophthalates appear to promote neuroprotection by alleviating  
598 inflammation and therefore it would be interesting to see, if they could affect the morphology  
599 of the spines or protect them upon neuroinflammation. However, the changes observed with  
600 bryostatin-1 by us and others (Hongpaisan et al., 2013; Sen et al., 2016) reinforce the need of  
601 further investigation of C1-domain binding PKC activators and suggest that activation of PKC  
602 could be a promising strategy for treating neurodegenerative diseases, such as AD.

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

608

## 609 **5. Conclusions**

610 In summary, our data suggest that the PKC-activating isophthalate derivatives show  
611 neuroprotective activity against neuroinflammation-induced toxicity *in vitro*, possibly through  
612 the attenuation of cytotoxic microglia-derived production of TNF $\alpha$  in the neuron-microglia co-  
613 cultures. Furthermore, the isophthalate derivatives promoted non-amyloidogenic processing  
614 and maturation of APP by enhancing its post-translational modification at the secretory  
615 pathway and by increasing the  $\alpha$ -cleavage of APP in SH-SY5Y-APP751 cells. Together with  
616 previous reports, our findings suggest that the synthetic isophthalate derivatives and other PKC  
617 agonists, such as bryostatin-1, may exert neuroprotective effects in various stress conditions

618 associated with AD and other neurodegenerative diseases. Thus, the isophthalate derivatives  
619 may be considered potential candidates in the search of new therapeutic strategies against  
620 synaptic dysfunction and neurodegeneration known to take place in AD.

621

#### 622 **Conflicts of interest**

623 The authors declare that they have no conflicts of interests.

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#### 634 **Ethics approval and consent to participate**

635 The licenses for preparation of primary neuronal cultures from E18 mouse embryo and primary  
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842

843 **Figure legends**

844 **Figure 1. HMI-1a3 reduces neuronal loss upon LPS+IFN- $\gamma$ -induced neuroinflammation**  
845 **in mouse primary neuron-BV2 microglia co-cultures.** (A) Characterization of the mouse  
846 primary cortical neuron and BV2 microglial cell co-cultures and effects upon LPS+IFN- $\gamma$ -  
847 induced neuroinflammation. (B) Effects of HMI-1a3 alone and in combination with 1  $\mu$ M  
848 Gö6983 on neuronal viability upon LPS+IFN- $\gamma$ -induced neuroinflammation. NI-15e was used  
849 as a negative control for HMI-1a3. (C) Effects of PMA alone and in combination with Gö6983  
850 on neuronal viability. (#)-sign indicates the statistical difference compared between same  
851 concentration of HMI-1a3 and HMI-1a3 + 1  $\mu$ M Gö6983 samples, and (\*)-sign indicates the  
852 statistical difference compared between the treated sample and a vehicle treated (0  $\mu$ M) sample  
853 within the group. One-way ANOVA and independent samples t-test,  $^{*/\#}p<0.05$ ,  $^{**/\#\#}p<0.01$ ,  
854  $^{***/\#\#\#}p<0.001$ , mean+SEM,  $n=3-12$ ; 0  $\mu$ M = Vehicle (0.1% DMSO) + LPS+IFN- $\gamma$  ( $\pm$   
855 1  $\mu$ M Gö6983).

856 **Figure 2. HMI-1a3 decreases the levels of TNF $\alpha$  but does not affect production of NO in**  
857 **mouse primary neuron-BV2 microglia co-cultures subjected to neuroinflammation.** (A)  
858 Effects of HMI-1a3 alone and in combination with 1  $\mu$ M Gö6983 on TNF $\alpha$  levels upon  
859 LPS+IFN- $\gamma$ -induced neuroinflammation. NI-15e was used as a negative control for HMI-1a3.  
860 (B) Effects of PMA on the levels of TNF $\alpha$  alone and in combination with 1  $\mu$ M Gö6983. (C)  
861 Effects of HMI-1a3 alone and in combination with 1  $\mu$ M Gö6983 on the production of NO  
862 upon LPS+IFN- $\gamma$ -induced neuroinflammation. NI-15e was used as a negative control for HMI-  
863 1a3. (D) Effects of PMA on the production of NO alone and in combination with 1  $\mu$ M Gö6983.  
864 (#)-sign indicates the statistical difference compared between same concentration of HMI-1a3  
865 and HMI-1a3 + 1  $\mu$ M Gö6983 samples, and (\*)-sign indicates the statistical difference  
866 compared between the treated sample and a vehicle sample within the group. One-way

867 ANOVA and independent samples t-test, LSD,  $^{*/\#}p < 0.05$ ,  $^{**/\#\#}p < 0.01$ ,  $^{***}p < 0.001$ ,  
868 mean+SEM,  $n=4-16$ ;  $0 \mu\text{M} = \text{Vehicle (0.1\% DMSO) + LPS+IFN-}\gamma (\pm 1 \mu\text{M G}\ddot{0}6983)$ .

869 **Figure 3. HMI-1a3 increases the levels of total NF $\kappa$ B p65 in primary neuron-BV2**  
870 **microglia co-cultures upon neuroinflammation.** Western blotting was used to analyse the  
871 levels of S536 phosphorylated NF $\kappa$ B p65 (p-NF $\kappa$ B p65) and total NF $\kappa$ B p65 (Tot-NF $\kappa$ B p65).  
872 (A) Representative blots and (B) quantification of the ratio of S536 phosphorylated NF $\kappa$ B p65  
873 to total NF $\kappa$ B p65 as well as the levels of total NF $\kappa$ B p65 normalized to  $\beta$ -actin. Vehicle-  
874 treated control sample is also shown from the same Western blot. One-way ANOVA followed  
875 by Dunnet's test,  $^*p < 0.05$ , mean+SEM,  $n=4$ ; Vehicle = 0.1% DMSO.

876 **Figure 4. HMI-1a3 and HMI-1b11 do not significantly affect A $\beta$ 40 and A $\beta$ 40 levels in**  
877 **primary neuron-BV2 co-cultures upon neuroinflammation or in SH-SY5Y**  
878 **neuroblastoma cells.** (A-C), The effects of HMI-1a3 on the levels of A $\beta$ 40 (A), A $\beta$ 42 (B) and  
879 the ratio of A $\beta$ 42/A $\beta$ 40 (C) in LPS+IFN- $\gamma$ -treated neuron-BV2 co-cultures after 48 hours. (D-  
880 F), The effects of 48-h treatments with HMI-1a3, HMI-1b11 and PMA on the levels of A $\beta$ 40  
881 (D) and A $\beta$ 42 (E) and the ratio of A $\beta$ 42/A $\beta$ 40 (F) in SH-SY5Y-APP751 cells. Data are  
882 represented as mean+SEM (One-way ANOVA followed by Dunnett's test and independent  
883 samples t-test, LSD,  $^*p < 0.05$ , mean+SEM,  $n=3-8$ ; Vehicle = 0.1% DMSO;  $0 \mu\text{M} = \text{Vehicle}$   
884  $+ \text{LPS+IFN-}\gamma$ ).

885 **Figure 5. HMI-1a3 and HMI-1b11 inhibit the maturation of APP in SH-SY5Y**  
886 **neuroblastoma cells.** Representative Western blot images (A and C) and quantifications (B  
887 and D) indicating the effects of HMI-1b11, HMI-1a3, PMA, and co-treatment of PMA (10 nM)  
888 and  $\gamma$ -secretase inhibitor DAPT (10  $\mu\text{M}$ ) (PMA+DAPT) on APP C83, mature APP (APP<sub>m</sub>),  
889 immature APP (APP<sub>im</sub>), total APP (APP<sub>m</sub>+APP<sub>im</sub>) levels and APP<sub>m</sub>/APP<sub>im</sub> ratios in SH-  
890 SY5Y-APP751 cells after 4-hour (A-B) and 24-hour (C-D) treatment. The representative

891 Western blot image for the APP C83 fragment from 24-hour treatment (C) is presented as two  
892 images from the same blot, with and without PMA+DAPT treated samples. To make the bands  
893 of interest more visible, contrast and intensity were adjusted in the image omitting the PMA-  
894 DAPT-treated samples. The quantification values represent optical densities normalized to the  
895 GAPDH levels and are shown as % of vehicle control (0.1% DMSO). The data are presented as  
896 mean+SEM (One-way ANOVA followed by Dunnett's test and independent samples t-test,  
897 \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\* $p < 0.001$ ,  $n=4$ ; *N.S.* = not statistically significant).

898 **Figure 6. HMI-1a3 and HMI-1b11 increase the levels of sAPP $\alpha$  relative to total sAPP in**  
899 **the cell culture medium of SH-SY5Y neuroblastoma cells.** Representative Western blot  
900 images (A and C) and quantifications (B and D) indicating the effects of HMI-1a3, HMI-1b11,  
901 and PMA, or co-treatment of PMA (10 nM) and  $\gamma$ -secretase inhibitor DAPT (10  $\mu$ M)  
902 (PMA+DAPT) on sAPP $\alpha$  and total sAPP (sAPP<sub>tot</sub>) levels in SH-SY5Y-APP751 cell culture  
903 medium after 4-hour (A-B) and 24-hour (C-D) treatment. The values are optical densities  
904 normalized to the total APP (APP<sub>m</sub>+APP<sub>im</sub> from the corresponding cell lysates, which is  
905 normalised to GAPDH) and are shown as % of vehicle control (0.1% DMSO). The data are  
906 presented as mean+SEM ( $n=4$ ). One-way ANOVA followed by Dunnett's test and independent  
907 samples t-test, \*\* $p < 0.01$ ).

908 **Figure 7. The PKC activator Bryostatin-1 increases the number of mushroom spines after**  
909 **2-hour treatment in mature mouse hippocampal neuron cultures at DIV20.** (A) The effects  
910 of 2-h treatments of bryostatin-1 (100 nM) or HMI-1a3 (10  $\mu$ M) on total, stubby, and thin spine  
911 densities. (B) The effects of 2-h treatments with bryostatin-1 (100 nM) or HMI-1a3 (10  $\mu$ M)  
912 on spine head morphology (width). (C) The effects of 2-h treatments with bryostatin-1 (100  
913 nM) or HMI-1a3 (10  $\mu$ M) on mushroom and thin spine density ratio to total spine density. One-



914 way ANOVA and independent samples t-test,  $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ ,  
915 mean+SEM,  $n=14-33$ ; Vehicle = 0.1% DMSO.

916

## 917 **Supplementary material**

918

919 **Figure S1. Bryostatin-1 decreases TNF $\alpha$  levels but has no effect on neuronal viability in**  
920 **mouse primary neuron-BV2 microglia co-cultures upon LPS+IFN- $\gamma$ -induced**  
921 **neuroinflammation.** Effects of bryostatin-1 alone and in combination with 1  $\mu$ M Gö6983 on  
922 neuronal viability upon LPS+IFN- $\gamma$ -induced neuroinflammation (A), the levels of TNF $\alpha$  (B)  
923 and the production of NO (C) in mouse primary neuron-BV2 microglia co-cultures. One-way  
924 ANOVA followed by Dunnett's test,  $*p < 0.05$ ,  $**p < 0.01$ ,  $***p < 0.001$ , mean+SEM,  $n=6$ ;  
925 0  $\mu$ M = Vehicle (0.1% DMSO) + LPS+IFN- $\gamma$ .

926

927 **Figure S2. PKC activation with bryostatin-1 decreases A $\beta$ 42 levels and A $\beta$ 42/A $\beta$ 40 ratio**  
928 **in mouse primary neuron-BV2 microglia co-cultures upon neuroinflammation.** Effects of  
929 bryostatin-1 on A $\beta$ 40 and A $\beta$ 42 levels, and on the ratio of A $\beta$ 42/A $\beta$ 40 in mouse primary  
930 neuron-BV2 microglia co-cultures. One-way ANOVA followed by Dunnett's test,  $*p < 0.05$ ,  
931 mean+SEM,  $n=6$ ; 0  $\mu$ M = Vehicle (0.1% DMSO) + LPS+IFN- $\gamma$ .

932

933 **Figure S3. Bryostatin-1 does not exhibit a significant effect on the maturation of APP in**  
934 **SH-SY5Y cells overexpressing APP751.** Representative Western blot images (A and C) and  
935 quantifications (B and D) indicating the effects of bryostatin-1 on APP C83, mature APP  
936 (APP<sub>m</sub>), immature APP (APP<sub>im</sub>), and total APP (APP<sub>m</sub>+APP<sub>im</sub>) levels as well as  
937 APP<sub>m</sub>/APP<sub>im</sub> ratio in SH-SY5Y-APP751 cells after 4- (A and B) and 24-hour (C and D)  
938 treatments. The quantification values represent optical densities normalized to GAPDH and are

939 shown as % of vehicle control (0.1 % DMSO). The data are presented as mean+SEM from  
940 three independent experiments (One-way ANOVA followed by Dunnett's test).

941

942 **Figure S4. Bryostatin-1 does not induce significant changes in the levels of sAPP $\alpha$  in SH-**  
943 **SY5Y cells overexpressing APP751.** Representative Western blot images (A and C) and  
944 quantifications (B and D), showing the effects of bryostatin-1 on sAPP $\alpha$  and total sAPP  
945 (sAPP $_{tot}$ ) levels in SH-SY5Y-APP751 cell culture medium after 4-hour (A-B) and 24-hour (C-  
946 D) treatments. The values are optical densities normalized to the total APP (APP $_{m}$ +APP $_{im}$ )  
947 from the corresponding cell lysate, which is normalised to GAPDH) and are presented as % of  
948 vehicle control (0.1% DMSO) treatment. The data are presented as mean+SEM from three  
949 independent experiments (One-way ANOVA followed by Dunnett's test).

950

951 **Figure S5. The levels of PKC isoforms  $\alpha$ ,  $\beta I$ ,  $\delta$ , and  $\epsilon$  in SH-SY5Y neuroblastoma cells**  
952 **after 24-hour treatment with 100 nM PMA, 10  $\mu$ M HMI-1a3 and 10  $\mu$ M HMI-1b11.**  
953 Densitometric analysis of Western blots ( $n=3$ ). Results presented as mean+SEM. One-way  
954 ANOVA followed by Dunnett's test and independent samples t-test ( $*p < 0.05$ ,  $**p < 0.01$ ,  
955  $***p < 0.001$ ).