Electronic Supplementary Material (ESI) for RSC Medicinal Chemistry.

Boron-based hybrids as novel scaffolds for the development of drugs with neuroprotective properties

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SUPPORTING INFORMATION

Experimental protocol and additional biological data

Experimental Section

Materials. All the reagents, unless otherwise stated, were from Sigma Aldrich Co. (St. Louis, MO, USA). Chromatographic purifications were performed on silica gel using column chromatography (Merck 60, 70-230 mesh ASTM silica gel), and compounds were detected with UV light (λ = 254 nm). Before performing biological studies, chemical structures, and purities (> 98%) of boronated compounds were confirmed by ^{1}H-,^{13}C-NMR, MS spectra. NMR spectra were recorded with a Varian VXR-300 spectrometer (300 MHz). MS spectra were recorded using Mass Spectrometer (Thermo Finnigan LCQ Deca XP Plus) and a Phenomenex C18 150 x 2.1 mm, 5 μm column. The capillary temperature was set at 300 °C. Microanalysis was performed on a Carlo Erba instrument model E1110 and revealed that all compounds showed a purity major 95%; all the results agreed within ± 0.4% of the theoretical values.
SH-SY5Y (CRL-2266™) cell line was provided from ATCC (Rockville, MD, USA). Amyloid β protein fragments 1-42 were purchased from Sigma (Saint Louis, USA) and penicillin/streptomycin (10,000 U/mL) from Thermo Fisher® (Waltham, MA USA). Dulbecco's Modified Eagle's Medium/Nutrient Mixture F-12 Ham (1:1 mixture), Fetal Bovine Serum (FBS), Trypsin-EDTA solution, All-trans-Retinoic acid, and BDNF (Brain Derived Neurotrophic Factor, Sigma Aldrich®) were also provided from Sigma Aldrich®. ADP and Collagen for platelet aggregation were purchased from Hart Biologicals (Hartlepool, UK).

Synthesis of (3aR)-2-(azidomethyl)-3a,5,5-trimethylhexahydro-4,6-methanobenzo[d][1,3,2]dioxaborole (2). A solution of **1** (2.10 g, 8.1 mmol) in dichloromethane (21 mL) was added dropwise to a stirred mixture of sodium azide (4.9 g, 73.5 mmol) and tetrabutylammonium bromide (130 mg, 4 mmol) in a mixture dichloromethane/water (149 mL/31.5 mL). The organic phase was separated, and the water phase was treated with dichloromethane. The residue was evaporated and dried to give compound **2** (1.4 g, yield 73%). ^{1}H-NMR ($CDCl_3$) δ: 0.83 (C*H*$_3$, 3H, s), 1.07-1.15 (C*H*, 1H, d, J = 11.1 Hz), 1.28 (C*H*$_3$, 3H, s), 1.41 (C*H*$_3$, 3H, s), 1.86-1.94 (C*H*$_2$, 2H, m), 2.07 (C*H*, 1H, t), 2.23-2.33 (C*H*$_2$, 2H, m), 3.10 (C*H*, 1H, s), 4.33 (C*H*, 0.5 H, d, J = 1.8 Hz), 4.36 (C*H*, 0.5H, d, J = 1.8 Hz). Calcd for $C_{11}H_{18}BN_3O_2$: C, 56.20; H, 7.72; B, 4.60; N, 17.87; O, 13.61; Found: C, 56.26; H, 7.70; B, 4.57; N, 17.88; O, 13.59.

Synthesis of ((3aR)-3a,5,5-trimethylhexahydro-4,6-methanobenzo[d][1,3,2]dioxaborol-2-yl)methanaminium chloride (3). Lithium aluminum hydride (240 mL, 5.68 mmol) was added to a solution of β-azidoboronate **2** (1.12 g, 4.72 mmol) in THF cooled at -78 °C. After 24 h at room temperature, water was added, the precipitate was filtered and washed with ether. The organic layers were dried and dissolved in pentane (8 mL). HCl 1.25 M in ethanol (8 mL) was added at 0 °C to the solution of the amine. After one night under stirring at room temperature solvents were evaporated and compound **3** was obtained with a yield of 49% (600 mg). ^{1}H-NMR ($CDCl_3$) δ: 0.82 (C*H*$_3$, 3H, s), 1.10 (C*H*, 1H, d, J = 11.1), 1.27 (C*H*$_3$, 3H, s), 1.41 (C*H*$_3$, 3H, s), 1.89-1.91 (CH_2, 2H, m), 2.06-2.08 (C*H*, 1H, t), 2.25-2.41 (C*H*$_2$, 2H, m), 3.08 (C*H*$_2$, 2H, s), 4.32-4.33 (C*H*, 0.5 H, d, J = 1.2 Hz), 4.36-4.35 (C*H*, 0.5H, d, J =1.2 Hz). Calcd for $C_{11}H_{21}BClNO_2$: C, 53.80; H, 8.62; B, 4.40; Cl, 14.44; N, 5.70; O, 13.03. Found: C, 53.77; H, 8.66; B, 4.38; Cl, 14.46; N, 5.69; O, 13.04.

Synthesis of 5-((S)-1,2-dithiolan-3-yl)-N-(((3aR)-3a,5,5-trimethylhexahydro-4,6-methanobenzo[d][1,3,2]dioxaborol-2-yl)methyl)pentanamide (BLA). To a stirred solution of aminoboronate·HCl (**3**) (550 mg, 2.11 mmol) in dry dichloromethane (4 mL) triethylamine (0.73 mL, 5.27 mmol) and then LA (435 mg, 2.11 mmol) were added at room temperature under stirring. After 10 minutes, Benzotriazol-1-yloxy)tris(dimethylamino)phosphonium hexafluorophosphate (BOP) (1.212 g, 2.74 mmol) in dry dichloromethane (2 mL) was added. After 3.5 h at room temperature, the reaction mixture was removed under vacuum. The residue was taken up in dichloromethane and washed with $KHSO_4$ 1N, $NaHCO_3$, and brine; the organic layer was dried over anhydrous Na_2SO_4, and the solvent was removed under vacuum. Chromatography was performed on silica gel with CH_2Cl_2:AcOEt (9:1) as eluant to give 376 mg of 5-((S)-1,2-dithiolan-3-yl)-N-(((3aR)-3a,5,5-trimethylhexahydro-4,6-methanobenzo[d][1,3,2]dioxaborol-2-yl)methyl)pentanamide (**BLA**). Yield: 44.8%; Rf = 0.23, $CHCl_3$; ^{1}H-NMR ($CDCl_3$) δ: 0.77 (C*H*$_3$, 3H, s), 1.10 (C*H*, 1H, d, J = 11.1), 1.25-1.40 (C*H*$_2$, 2H, m), 1.26 (C*H*$_3$, 3H, s), 1.39 (C*H*$_3$, 3H, s), 1.60-1.71 (3 x C*H*$_2$, 6H, m), 1.80-2.10 (2 x C*H*$_2$, 4H, m), 2.20-2.55 (2 x C*H*$_2$, 4H, m), 3.15-3.22 (C*H* and C*H*$_2$, 3H, m), 3.55 (C*H*, 1H, m), 4.35 (C*H*, 1H, m), 7.25 (N*H*, 1H); ^{13}C-NMR ($CDCl_3$) δ: 23.95 (*C*H$_3$), 24.58 (*C*H$_3$), 26.29 (*C*H$_3$), 26.43 (*C*H$_2$), 26.96 (*C*H$_2$), 28.42 (*C*H$_2$), 28.76 (*C*H$_2$), 34.58 (*C*H$_2$), 35.59 (*C*H$_2$), 38.33 (*C*H$_2$), 39.33 (*C*), 40.20 (*C*H$_2$), 50.98 (*C*H$_2$), 51.38 (*C*H), 56.31 (*C*H), 84.73 (*C*), 86.96 (*C*H), 181.69 (*C*O); MS (ESI) m/z 398.19 $[M + H]^+$. Calcd for $C_{19}H_{32}BNO_3S_2$: C, 57.42; H, 8.12; B, 2.72; N, 3.52; O, 12.08; S, 16.14. Found: C, 57.40; H, 8.15; B, 2.69; N, 3.55; O, 12.10; S, 16.11.

Synthesis of (S)-((5-(1,2-dithiolan-3-yl)- pentanamido)methyl)boronic acid (BLA1). Phenylboronic acid (273 mg, 2.24 mmol) was added to a solution of BLA (230 mg, 0.56 mmol) in diethyl ether/water (1:1) (14 mL) and the reaction mixture was stirred for 12 h. The aqueous phase was filtered, washed with diethyl ether, and concentrated under vacuum to give the pure product. Yield: 98%. Rf = 0.1, $CHCl_3$; ^{1}H-NMR (d_6-DMSO) δ: 1.30-1.42 (C*H*$_2$, 2H, m), 1.45-1.61 (C*H*$_2$, 2H, m), 1.63-1.65 (1/2 C*H*$_2$, 1H, m), 1.80-1.89 (1/2 C*H*$_2$, 1H, m), 2.15-2.17 (C*H*$_2$, 2H, t), 2.35-2.48 (C*H*$_2$, 2H, m), 3.04-3.19 (C*H*$_2$, 2H, m), 3.10-3.55 (C*H*$_2$ and 2 x O*H*, 4H, br), 3.53-3.60 (C*H*, 1H, m); ^{13}C-NMR (d_6-DMSO) δ: 25.26 (*C*H$_2$), 28.63 (*C*H$_2$), 34.43 (*C*H$_2$), 35.79 (*C*H$_2$), 38.46 (*C*H$_2$), 40.21 (*C*H$_2$), 41.47 (*C*H$_2$), 56.31 (*C*H), 172.59 (CO); MS (ESI) m/z 264.08 $[M + H]^+$. Calcd for $C_9H_{18}BNO_3S_2$: C, 41.07; H, 6.89; B, 4.11; N, 5.32; O, 18.24; S, 24.37; Found: C, 41.04; H, 6.90; B, 4.09; N, 5.32; O, 18.29; S, 24.36.

ADMET properties. ADMET properties of **BLA** and **BLA1** were determined on preADMET online server at the address http://preadmet.bmdrc.org/. The structures of boron-based hybrids were converted into molfile (*.mol). The program automatically calculated the predictive absorption for Caco-2 cell, MDCK cell, HIA (Human Intestinal Absorption), BBB penetration, and distribution for plasma protein binding.

Cell cultures and differentiation. SH-SY5Y cell line were growth in DMEM:F12 (1:1) media including 10% FBS and 1% penicillin/streptomycin at 37 °C and 5% CO_2 until cells reach confluence. Then, cells harvested via using trypsin/EDTA solution and transferred to 48 well plates. For differentiation, media were changed to supplemented media with 10 µg/mL retinoic acid and incubated for 5 days. Finally, media were changed into serum free media supplementing with both 10 µM retinoic acid and 25 ng/mL BDNF. Differentiated cells were determined under invert microscope and the cells were used within 10 days.[1,2]

Treatments. Cells at 70–80% confluence were treated with concentrations of BLA, MEM, LA (0.1, 1, 10, 25, 50, and 100 µM) and Aβ1-42 peptide (20 µM) for 24 h. The stock solutions of BLA and LA were freshly dissolved in DMSO and consequently diluted in FBS. At the final dilutions, the obtained concentration of DMSO never exceeded 0.1%. MEM and Aβ1-42 peptides were dissolved in sterile double-distilled water. Cells (n = 6) treated with vehicl (0.1% DMSO) were used as a negative control. 1% Triton X, ascorbic acid (10 µM) and hydrogen peroxide (H_2O_2, 25 µM) were used as positive controls for cell viability, TAC and TOS analysis, respectively.

MTT assay. According to the manufacturer's instructions (Cayman Chemical Company®, Ann Arbor, MI, USA), 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) solution was applied to cell cultures. As summary, 20 µL of MTT was added and incubated in the cell cultures for 3 h at 37°C and after incubation dimethyl sulfoxide (DMSO) was used to solve formazan crystals, and a plate reader was used to analyze cultures at 570 nm wavelength.[3]

LDH assay. For LDH assay application, LDH cytotoxicity assay kit (Cayman Chemical Company®, Ann Arbor, MI, USA) was used according to the manufacturer's recommendations. The cells were transferred to 48-well plates and different concentrations of drugs were applied for 24 h. After that, 100 µL supernatant and 100 µL of reaction mixture were transferred to a fresh 48-well plate and incubated for 30 min at room temperature. Released LDH catalysed the oxidation of lactate to pyruvate alongside the reduction of NAD+ to NADH. The amount of NAD+ reduction was determined via considering increase in observed absorbance values. Finally, the absorbance of the cultures was analyzed at 490 nm using a microplate reader as reported by Turkez *et al*.[4]

Determination of AChE activity. The activity of AChE within the cellular Alzheimer Disease's model was measured by the Acetylcholinesterase Assay Kit (Colorimetric) from Abcam(R) (Cambridge, MA, USA) according to the manufacturer's recommended protocol. In brief, the medium was removed from the wells and, 100 µL lysis buffer was added into each well at room temperature for 15 min, protected from light. The cells were centrifuged at 1500 rpm for 5 min and the obtained supernatant was used for the assay. The absorbance increases were measured using an absorbance microplate reader at 410 nm.

TAC and TOS analysis. In TAC method, the total amount of antioxidants in samples reduce the dark bluish green colored 2,2′-azino-bis (3-ethylbenzothiazoline-6-sulfonic acid) diammonium salt (ABTS) to a reduced form of ABTS exhibiting colorless nature. The measured alteration of absorbances recorded at 660 nm is correlated with the amount of total antioxidant capacity in tested samples. TAC method was calibrated by using a vitamin E analogue, known as Trolox equivalent. Likewise, In TOS method, the available all kind of oxidants in tested samples oxidize the ferrous ion–chelator complex to ferric ion, impetuously. The exurgent ferric ion generates a colored complex with chromogen. The spectrophotometrically determined color intensity is correlated with the amount of the total oxidant molecules in the sample. Since the TOS method was calibrated using H_2O_2, the results were presented as µM H_2O_2 equivalent/L. The automated TAC and TOS assays were carried out by commercially available kits (Rel Assay Diagnostics, Gaziantep, Turkey) on samples from the cultures as previously reported.[5]

Apoptosis detection by Hoechst 33258 staining. Hoechst 33258 staining was used to detect apoptotic nuclei as previously reported.[6] The positive control (only Aβ1-42, 20 µM), negative control and BLA/MEM (25 and 50 µM) with β-amyloid cultures were incubated for 24 h to analyze nuclei morphology. Then, cells fixed with 4% paraformaldehyde in phosphate buffered saline at 4 °C for 30 min. After washing cells with PBS, the nuclear DNAs were incubated with 1 mM Hoechst 33258 fluorescent dye for 5 min at room temperature. Cells were observed and photographed under a fluorescence microscopy (Leica® DM IL LED).

Apoptosis-Necrosis assay. The frequencies of viable, apoptotic, and necrotic cells were detected with the Annexin V-FITC apoptosis detection Kit I (BD Pharmingen, USA). Briefly, 5 x 10^4 cells were collected by centrifugation and cells resuspended in 500 µL of 1X binding buffer. Then, 5 µL of Annexin V-FITC and 5 µl of propidium iodide (PI 50 µg/ml) were added to the cultures and incubated 5 min in the dark. After that, cells fixed with 4% paraformaldehyde in phosphate buffered saline at 4 °C for 30 min. Cells from each well were stained according to the manufacturer's instructions and analyzed using a flow cytometer (CyFlow Cube 6, Partec, Germany). A computer system was used for data acquisition and analysis. Four different populations of cells are detected with the Annexin V-FITC kit: viable cells that are annexin negative and PI negative and express no fluorescence, early apoptotic cells that are annexin

positive and PI negative and that express green fluorescence, late apoptotic/necrotic cells that are annexin positive and PI positive and that express green and orange fluorescence, necrotic cells that are annexin negative and PI positive and that express orange fluorescence.[7]

Toxicological evaluation. For evaluating biosafety of formulations, we used cultured human peripheral blood cells purchased by 3H Biomedical ab (Uppsala, Sweden). The blood cultures were set up according to a slight modification of the protocol described by Evans and O'Riordan.[8] The heparinized blood (0.5 mL-1) was cultured in 6.0 mL-1 of culture medium (PB-MAX) Karyotyping Medium Gibco, Spain) with 5.0 mg/mL-1 of phytohemagglutinin (Sigma Aldrich(R), Steinheim, Germany). Different concentrations of BLA, LA, and MEM were added to the cultures just before the incubation. For assessing cytotoxicity and genotoxicity potentials of the drugs, MTT, LDH, Sister chromatid exchange (SCE) and 8-hydroxy-2′-deoxyguanosine assays were performed.

SCE assay. For scoring SCEs, 5-bromo-2-deoxyuridine (BrdU) was added at culture cells and exactly 70 h and 30 min after beginning of the incubations demecolcine (N-Diacetyl-N-methyl colchicine) was added into the culture tubes as previously reported by Turkez *et al*.[9]After hypotonic treatment (as 0.075 M KCl), followed by fixation in methanol/acetic acid solution (3:1, v/v), centrifugation and re-suspension, the obtained cell suspension was dropped onto chilled, icy, and grease-free microscopic slides. Then the slides were air-dried, aged for three days, and then differentially stained for the inspection of the SCE rate according to the fluorescence plus Giemsa (FPG) procedure For each treatment condition, well-spread twenty-five second division metaphases containing 42-46 chromosomes per cell were scored and the values obtained were presented as SCEs/cell.

Nucleic acid oxidation. 8-hydroxy-2′-deoxyguanosine assay kits (Cayman Chemical®) was used for determining 8- OH-dG levels in the cultures. It is a competitive assay that can be used for the quantification of 8-OHdG in homogenates and recognizes both free 8-OHdG and DNA-incorporated 8-OH-dG. This assay depends on the competition between 8-OH-dG and 8-OH-dGacetylcholinesterase (AChE) conjugate for a limited amount of 8-OH-dG monoclonal antibody.[10] The development of plates were carried out in accordance with the provider's manual and the plates were read at a wavelength between 405 and 420 nm.

Effects of $A\beta_{1-42}$ peptide treatments on the viability of differentiated SH-SY5Y cells

SH-SY5Y human neuroblastoma cells were differentiated to a neuronal-like state *in vitro* using a combination of retinoic acid (RA) and brain-derived neurotrophic factor (BDNF). For establishing the effective concentration of $A\beta_{1-42}$ fragments on the viability of differentiated SH-SY5Y cells as an experimental model for Alzheimer's, a wide range of $A\beta_{1-42}$ concentrations (0, 1.25, 2.5, 5, 10, 20, 40, 80, and 160 μM) were added into the cell cultures for 24 h. SH-SY5Y cells with differentiation were exposed to $A\beta_{1-42}$ for 24 h and then assessed for cell viability by MTT assay. The results of MTT analysis indicated that $A\beta$ toxicity was in a clear concentrations dependent manner. 52.1% and 48% of the SH-SY5Y cells showed decreased levels of cell viability after the 24h treatment with 20 and 40μM $A\beta_{1-42}$ (Figure 1S). The protocol using the 24 h treatment of 20 μM $A\beta_{1-42}$ was selected for further experiments of this investigation.

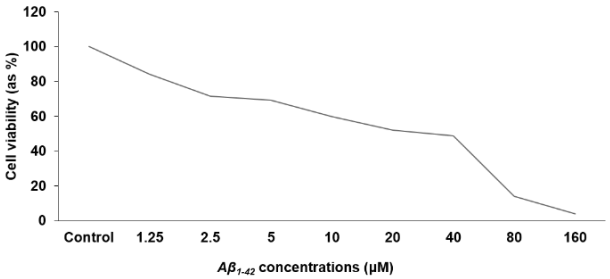

Figure 1S. In vitro evaluation of cytotoxicity of Ab1-42 peptide at different concentrations (0-160 mM) in differentiated SH-SY5Y human neuroblastoma cells.

Antitumor activity of BLA and MEM

Antitumor activity of BLA and MEM was tested on SHSY-5Y human neuroblastoma cell line using MTT and LDH assays. Cells were grown to near-confluency before treatment. Growth of the cell line was inhibited by both drug application in a dose-dependent manner. BLA and MEM showed moderate antitumor effects. However, the lower concentrations (< 50 µM) of BLA was found more effective than MEM. Results proved that the activity of BLA against tumor cells was greater than MEM (Figures 2S and 3S).

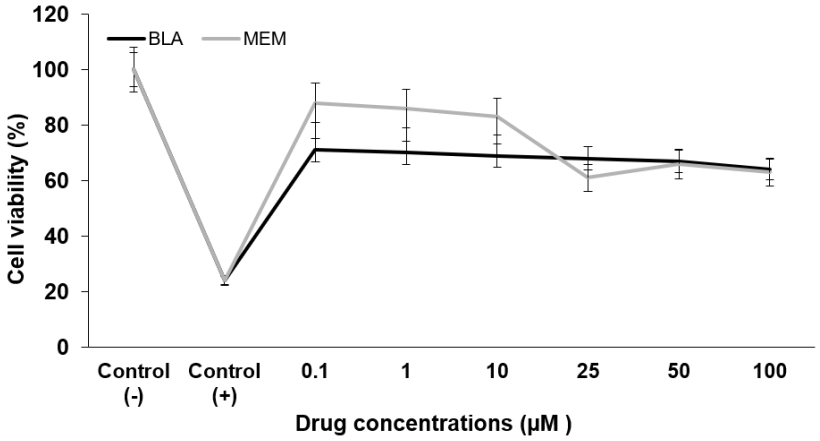

Figure 2S. Results of the cytotoxicity of BLA and MEM at different concentrations (0.1-100 mM) in SH-SY5Y tumor cell line (MTT assay).

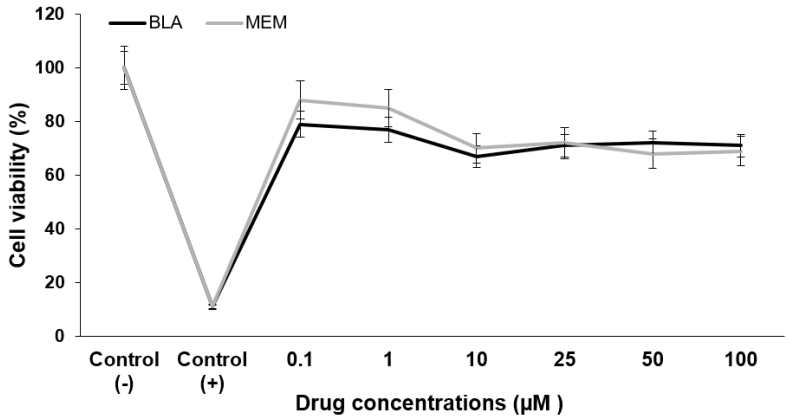

Figure 3S. Results of the cytotoxicity of BLA and MEM at different concentrations (0.1-100 mM) in SH-SY5Y tumor cell line (LDH assay).

Effects of BLA and MEM on apoptosis and necrosis in cellular model of AD

Staining with Hoechst 33258 executed that a co-treatment with BLA, LA or MEM (25 and 50 µM) protected nuclear integrity and inhibited apoptosis induced by Aβ1-42. Likewise, the observations by using apoptosis-necrosis assay indicated that Aβ toxicity occurred via a necrotic rather than an apoptotic pathway. This assay demonstrated that BLA, LA or MEM protected from Aβ1-42-induced necrosis in a dose-dependent manner (Figure 4S). Flow cytometric analysis of apoptosis and necrosis showed that Aβ1-42 application caused a significant ($P<0.05$) cell death rate (66.09%) via necrosis. On the contrary, BLA, LA and MEM decreased the necrotic cell percentage by Aβ1-42 in the rates of %68.38, %61.67 and %65.97, respectively. Apoptosis was not observed in flow cytometric investigations and so, it could be concluded that Aβ1-42 caused cell death through necrotic pathways. In brief, BLA was considered as more able to protect the human neuron-like cells from Aβ1-42-induced necrosis and late apoptosis than LA or MEM (Figure 5S).

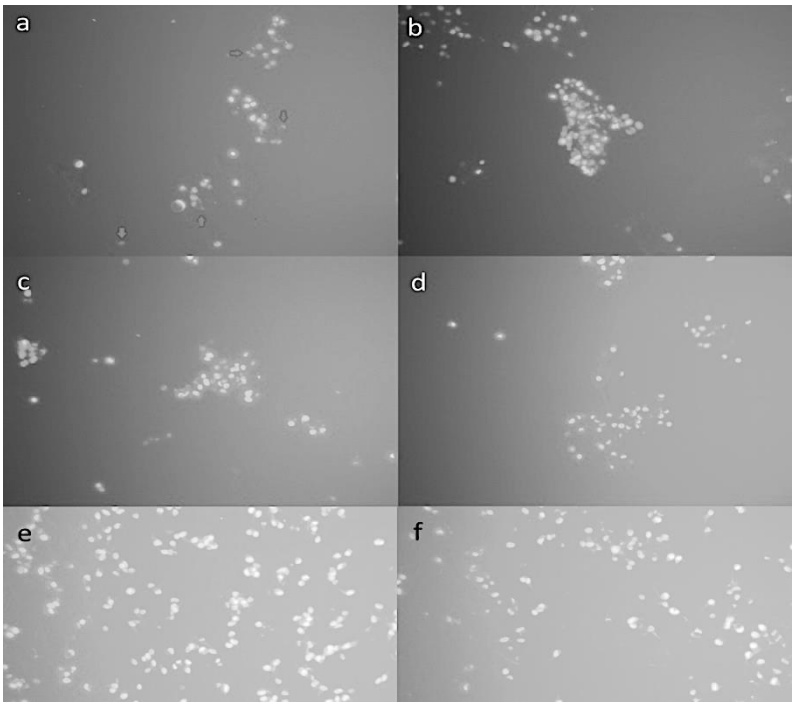

Figure 4S. Effects of BLA, MEM and LA on apoptosis and necrosis in cellular model of Alzheimer's Disease (Hoechst 33258), A) Aβ1-42, B) BLA (50 µM) + Aβ1-42, C) LA (50 µM) + Aβ1-42, D) MEM (50 µM) + Aβ1-42, E-F) Negative controls. Red arrows indicate necrotic cells with damaged chromosomal structure.

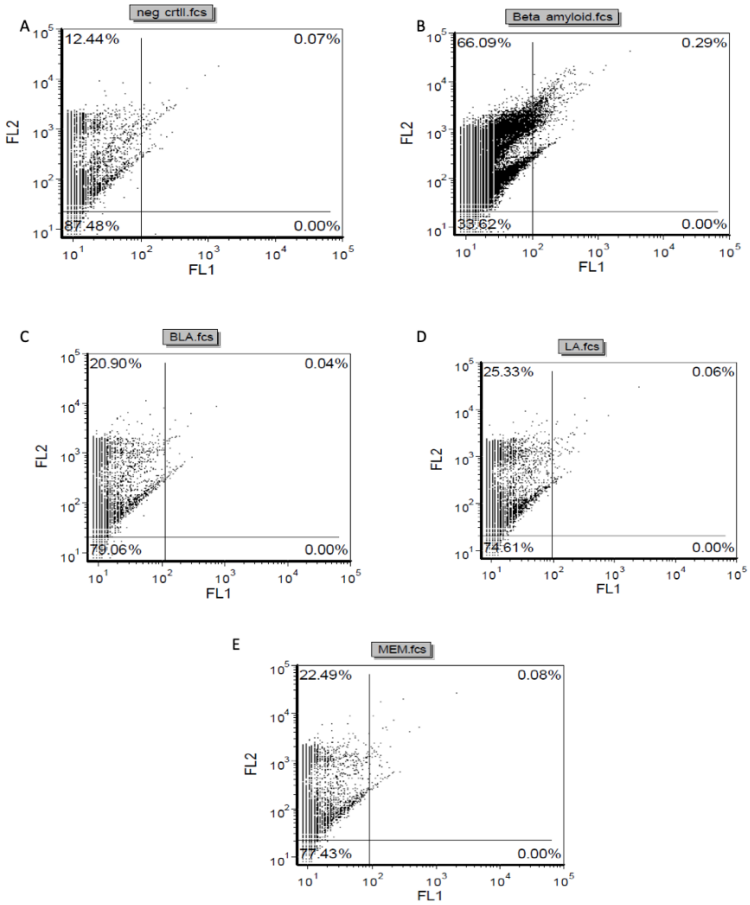

Figure 5S. Flow cytometric analysis of Annexin V-FITC/PI double labeled cells in cellular model of Alzheimer's Disease, A) negative control, B) Aβ1-42, C) BLA (50 µM) + Aβ1-42, D) LA (50 µM) + Aβ1-42, E) MEM (50 µM) + Aβ1-42.

Cytotoxicity assay

MTT and LDH assays were performed to measure cell death in response to different concentrations of BLA, MEM, and LA. The cultured peripheral human whole blood (PHWB) cells were exposed to 0.1 to 100 µM of BLA, MEM and LA. Both BLA and LA did not show any significant changes in cell viability during 24 h, as determined by MTT and LDH assays (Figures 4S and 5S). Likewise, the results of both cytotoxicity assays showed that up to 50 µM of MEM did not cause any significant changes in cell viability, but the application with 100 µM of MEM caused a slight toxicity ($p > 0.05$, $p < 0.1$, MTT assay) compared to the untreated group (Figures 6S and 7S).

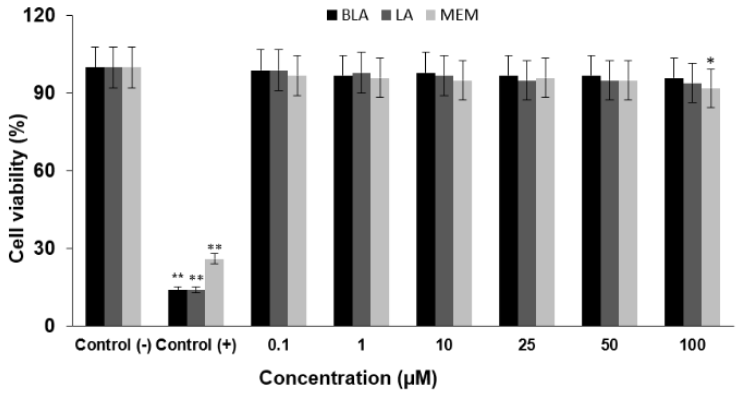

Figure 6S. Results of the vitality of PHWB cells after 24 h exposure to different BLA, LA, and MEM concentrations (0.1-100 mM); results are expressed as percentage of the control group (n = 6); data are expressed as the mean ± SD. ** $p < 0.05$, * $p < 0.1$.

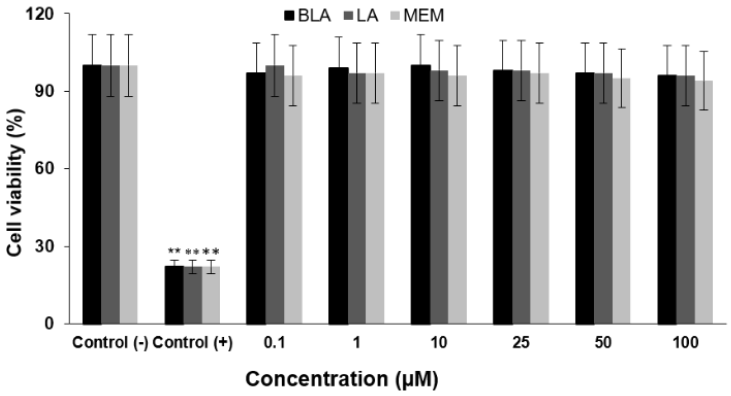

Figure 7S. Results of the extracellular LDH levels in cultured PHWB cells in the presence of different BLA, LA, and MEM concentrations (0.1-100 mM); ** $p < 0.05$, * $p < 0.1$.

Statistical analysis. The results were expressed by mean ± S.D. from at least six independent experiments. For statistical comparisons, quantitative data was analysed by one-way analysis of variance (ANOVA) followed by Duncan's test according to the statistical program SPSS software (version 20.0, SPSS, Chicago, IL, USA). A p-value less than 0.05 was considered as significant.

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