Alpha-linolenic acid-valproic acid conjugates: toward single-molecule polypharmacology for multiple sclerosis

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Supporting Information

Contents

Figure S1. Structure and *in vivo* hydrolysis of codrug sultamicillin

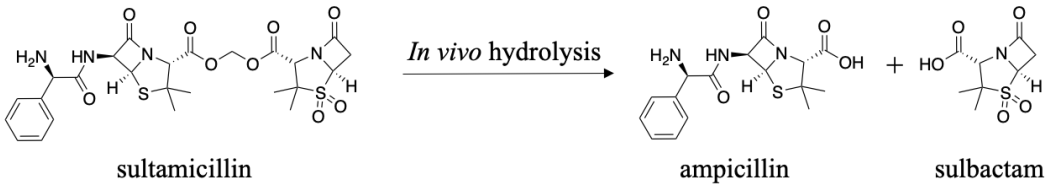


Table S1. Effect of **1-4** and parent compounds VPA and ALA on cell survival/death in HepG2 cells, as determined by MTT assay after 24 h treatment

	% Mean ± SE[a]				
Compound	**0 [µM]**	**5 [µM]**	**10 [µM]**	**25 [µM]**	**50 [µM]**
VPA	100 ± 5	108 ± 15	95 ± 4	88 ± 10	105 ± 14
ALA	100 ± 5	109 ± 17	90 ± 5	91 ± 13	92 ± 9
1	100 ± 5	87 ± 13	93 ± 5	81 ± 10	64 ± 8*
2	100 ± 5	102 ± 10	104 ± 4	84 ± 6	80 ± 9
3	100 ± 5	86 ± 10	92 ± 3	84 ± 10	75 ± 13
4	100 ± 5	95 ± 14	99 ± 5	75 ± 12	74 ± 12

[a]Results are expressed as percentages of controls and are the mean ± SE of at least 3 indepedent experiments, each run in triplicate. *p<0.0 compared to control conditions (0 µM), one way-ANOVA followed by Bonferroni's post-hoc test.

Experimental procedures

Chemistry

All the commercially available reagents and solvents were purchased from Sigma Aldrich (Italy) and Nu-Chek Prep (USA) and used without further purification. Reactions were routinely monitored by thin-layer chromatography (TLC) in silica gel (F254 Silicycle plates) and the products visualized with iodine or ultraviolet lamp (254 and 365 nm). For normal pressure and flash column chromatography purifications, Silicycle silica gel type 60 (size 70-230 and 230-400 mesh, respectively) was used. Nuclear magnetic resonance (NMR) experiments were run on a Varian VXR 400 instrument. ^{1}H and ^{13}C NMR spectra were acquired at 300 K using deuterated chloroform ($CDCl_3$) as solvent. Chemical shifts (δ) are expressed in parts per million (ppm) relative to internal tetramethylsilane and coupling constants (*J*) are reported in hertz (Hz). The spin multiplicities are reported as s (singlet), br s (broad singlet), d (doublet), t (triplet), q (quartet), and m (multiplet). Full scan MS spectra were recorded by using LC-DAD-MS/MS apparatus. Compounds were named following IUPAC rules as applied by ChemBioDraw Ultra (version 14.0). All of the final compounds showed ≥90% purity by analytical HPLC.

General procedure for the preparation of O-TBS linkers (5, 6)

To a solution of ethylene glycol or ethanolamine (1 mmol) in DCM (10 ml), imidazole (1.2 mmol) and tert-butyldimethylsilyl chloride (TBSCl, 0.2 mmol or 1.1 mmol, respectively) were added and the resulting mixture was stirred at room temperature for 16 hours. The solvent was evaporated in vacuo and the crude was purified by flash chromatography on silica gel to afford title compounds **5** or **6**.

2-((tert-butyldimethylsilyl)oxy)ethan-1-ol (5)

The resulting residue was purified by using flash chromatography (9/1 DCM/MeOH) affording **5** as pale-white oil. Yield 92%.

^{1}H-NMR ($CDCl_3$, 400 MHz) δ (ppm): 0.07 (t, 6H, J = 4 Hz), 0.88-0.90 (m, 9H), 2.09 (bs, 1H), 3.61-3.41 (m, 2H), 3.69-3.71 (m, 2H). ^{13}C-NMR ($CDCl_3$, 100 MHz) δ (ppm): -5.4, 18.3, 25.9, 63.6, 64.1.

2-((tert-butyldimethylsilyl)oxy)ethan-1-amine (6)

The resulting residue was purified by using flash chromatography (9/1/0.1 DCM/MeOH/33% aq NH_3) affording **6** as a pale-yellow oil. Yield 87%.

^{1}H-NMR ($CDCl_3$, 400 MHz) δ (ppm): 0.02 (t, 6H, J = 4 Hz), 0.87 (s, 9H), 1.72 (s, 2H), 2.73 (t, 2H, J = 4 Hz), 3.58 (t, 2H, J = 8 Hz). ^{13}C-NMR ($CDCl_3$, 100 MHz) δ (ppm): -5.4, 18.2, 25.8, 44.2, 65.2.

General procedure for the preparation of N-Boc linkers (7, 8)

To a solution of ethylenediamine or ethanolamine (1 mmol) in DCM (10 ml), tert-butoxycarbonyl anhydride (Boc_2O, 0.2 mmol or 1.1 mmol, respectively) was added and the resulting mixture was stirred at room temperature for 16 hours. The solvent was evaporated in vacuo and the crude was purified by flash chromatography on silica gel by using different eluent mixtures to give the title compounds **7**, **8**.

Tert-butyl (2-aminoethyl)carbamate (7)

The resulting residue was purified by flash chromatography (9/1/0.1 DCM/ MeOH/33% aq NH_3) affording **7** as pale-yellow oil. Yield 98%.

^{1}H-NMR ($CDCl_3$, 400 MHz) δ (ppm): 1.41 (s, 11H), 2.77 (t, 2H, J = 4 Hz), 3.14 (m, 2H), 5.01 (bs, 1H). ^{13}C-NMR ($CDCl_3$, 100 MHz) δ (ppm): 28.4, 41.8, 43.3, 79.7, 156.2.

Tert-butyl (2-hydroxyethyl)carbamate (8)

The resulting residue was purified by flash chromatography (9/1/0.1 DCM/ MeOH/33% aq NH_3) affording **8** as pale-yellow oil. Yield 97%.

^{1}H-NMR ($CDCl_3$, 400 MHz) δ (ppm): 1.43 (s, 9H), 2.87 (bs, 1H), 3.24-3.28 (m, 2H), 3.65-3.69 (m, 2H), 5.04 (bs, 1H). ^{13}C-NMR ($CDCl_3$, 100 MHz) δ (ppm): 28.3, 43.0, 62.5, 79.7, 156.8.

General procedure for the preparation compounds (9-12)

To a solution of valproic acid (VPA, 1.2 mmol) in dry DCM (3 ml) under nitrogen condition, was added at 0°C, 1-etil-3 (3-dimetilaminopropil) carbodiimide chloride (EDC, 1.2 mmol) and the activation was stirred at room temperature for 1 hour. Successively, a solution contained (**5-8**) (1 mmol) and 4-dimethylaminopyridine (DMAP, 10% mmol) in dry DCM (1 ml) were added in the activated solution. The resulting mixture was stirred at room temperature for 8 hours. The solvent was evaporated in vacuo and the crude was purified by using flash chromatography on silica gel using different eluent mixtures to give the title compounds **9-12**.

Tert-butyl (2-(2-propylpentanamido)ethyl)carbamate (9)

The resulting residue was purified by flash chromatography (9/1 DCM/ MeOH) affording **9** as pale-yellow oil. Yield 57%.

^{1}H-NMR ($CDCl_3$, 400 MHz) δ (ppm): 0.86-0.90 (m, 6H), 1.23-1.43 (m, 15H), 1.54-160 (m, 2H), 2.01-2.06 (m, 1H), 3.24-3-29 (m, 2H), 3.34-3.38 (m, 2H), 4.97 (bs, 1H), 6.18 (bs, 1H). ^{13}C-NMR ($CDCl_3$, 100 MHz) δ (ppm): 14.0, 20.7, 28.3, 35.1, 40.3, 40.4, 47.5, 79.5, 156.9, 176.9.

N-(2-((tert-butyldimethylsilyl)oxy)ethyl)-2-propylpentanamide (10)

The resulting residue was purified by flash chromatography (9/1 DCM/ MeOH) affording **10** as pale-yellow oil. Yield 68%.

^{1}H-NMR ($CDCl_3$, 400 MHz) δ (ppm): 0.03 (s, 6H), 0.84-0.88 (m, 15H), 1.20-1.37 (m, 6H), 1.53-1.59 (m, 2H), 2.01 (q, 1H, J = 4 Hz), 3.30-3.37 (m, 2H), 3.64 (t, 2H, J = 4 Hz), 5.84 (bs, 1H). ^{13}C-NMR ($CDCl_3$, 100 MHz) δ (ppm): -5.5, 14.0, 18.2, 20.8, 25.8, 35.2, 41.3, 47.7, 61.9, 175.9.

2-((tert-butoxycarbonyl)amino)ethyl 2-propylpentanoate (11)

The resulting residue was purified by flash chromatography (9/1 DCM/ MeOH) affording **11** as pale-yellow oil. Yield 63%.

^{1}H-NMR ($CDCl_3$, 400 MHz) δ (ppm): 0.84 (t, 6H, J = 8 Hz), 1.21-1.26 (m, 2H), 1.39 (s, 10H), 1.49-1.57 (m, 2H), 2.31 (q, 1H, J = 4 Hz), 3.31-3.35 (m, 2H), 4.08 (t, 2H, J = 8 Hz), 4.80 (bs, 1H). ^{13}C-NMR ($CDCl_3$, 100 MHz) δ (ppm): 13.9, 20.5, 28.3, 34.5, 39.7, 45.1, 62.9, 79.3, 155.7, 176.4.

2-((tert-butyldimethylsilyl)oxy)ethyl 2-propylpentanoate (12)

The resulting residue was purified by flash chromatography (9.5/0.5 DCM/ MeOH) affording **12** as pale-white oil. Yield 42%.

^{1}H-NMR ($CDCl_3$, 400 MHz) δ (ppm): 0.06 (m, 6H), 0.89 (m, 15H), 1.25-1.42 (m, 6H), 1.56-1.62 (m, 2H), 2.36 (q, 1H, J = 4 Hz), 3.77-3.80 (m, 2H), 4.10-4.13 (m, 2H). ^{13}C-NMR ($CDCl_3$, 100 MHz) δ (ppm): -5.4, 13.9, 18.2, 20.6, 25.8, 34.6, 45.3, 61.2, 65.4, 176.5.

General procedure for N-Boc deprotected intermediates (13, **15)**

To a cold solution of **9** or **11** (1 mmol) in dry DCM (5 ml), was added dropwise trifluoroacetic acid (TFA, 20 mmol) under anhydrous condition. The resulting mixture was stirred at room temperature till the complete disappearance of the starting material (1.5-2.5 hours). The solvent was evaporated in vacuo and the crude was purified by flash chromatography on silica gel using different eluent mixtures, to give the title compounds **13** or **15**.

N-(2-aminoethyl)-2-propylpentanamide (13)

The resulting residue was purified by using flash chromatography (9/1/0.1 DCM/MeOH/33% aq NH_3) affording **13** as pale-yellow oil. Yield 98%.

^{1}H-NMR ($CDCl_3$, 400 MHz) δ (ppm): 0.86 (t, 6H, J = 8 Hz), 1.21-1.31 (m, 9H), 1.55 (m, 2H), 2.02 (q, 1H, J = 4 Hz), 2.79 (t, 2H, J = 4 Hz), 3.25-3.30 (m, 2H), 6.06 (bs, 1H). ^{13}C-NMR ($CDCl_3$, 100 MHz) δ (ppm): 14.1, 20.8, 29.6, 35.2, 41.3, 41.6, 47.7, 176.3.

2-aminoethyl 2-propylpentanoate (15)

The resulting residue was purified by using flash chromatography (9/1/0.1 DCM/MeOH/33% aq NH_3) affording **15** as pale-yellow oil. Yield 95%.

^{1}H-NMR ($CDCl_3$, 400 MHz) δ (ppm): 0.85 (t, 6H, J = 8 Hz), 1.21-1.37 (m, 6H), 1.51-156 (m, 2H), 2.07 (q, 1H, J = 4 Hz), 3.36-3.39 (t, 2H, J = 4 Hz), 3.66 (t, 2H, J = 4 Hz), 6.06 (bs, 1H). ^{13}C-NMR ($CDCl_3$, 100 MHz) δ (ppm): 13.6, 20.3, 34.0, 39.6, 44.8, 60.3, 177.4.

General procedure for O-TBS deprotected intermediates (14, 16)

To a cold solution of **10** or **12** (1 mmol) in dry DCM (5 ml), was added dropwise tetrabutylammonium fluoride (TBAF, 5 mmol) under anhydrous condition. The resulting mixture was stirred at room temperature till the complete disappearance of the starting material (around 12-15 hours). The solvent was evaporated in vacuo and the crude was purified by flash chromatography on silica gel using different eluent mixtures, to give the title compounds **14-16**.

N-(2-hydroxyethyl)-2-propylpentanamide (14)

The resulting residue was purified by using flash chromatography (9/1 DCM/MeOH) affording **14** as pale-yellow oil. Yield 89%.

^{1}H-NMR ($CDCl_3$, 400 MHz) δ (ppm): 0.7 (m, 6H), 1.23-1.38 (m, 6H), 1.52-1.58 (m, 2H), 2.08 (q, 1H), 3.37-3.41 (m, 3H), 3.67 (t, 2H, J = 4 Hz), 6.42 (bs, 1H). ^{13}C-NMR ($CDCl_3$, 100 MHz) δ (ppm): 14.1, 20.7, 35.2, 42.3, 47.4, 62.4, 177.6.

2-hydroxyethyl 2-propylpentanoate (16)

The resulting residue was purified by using flash chromatography (9/1 DCM/MeOH) affording **16** as pale-white oil. Yield 92%.

^{1}H-NMR ($CDCl_3$, 400 MHz) δ (ppm): 0.85 (t, 6H, J = 4 Hz), 1.22-1.27 (m, 4H), 1.37-1.40 (m, 2H), 1.53-1.58 (m, 2H), 2.36 (q, 1H), 2.58 (s, 1H), 3.74-3.77 (m, 2H), 4.14-4.17 (m, 2H). ^{13}C-NMR ($CDCl_3$, 100 MHz) δ (ppm): 13.9, 20.5, 34.5, 45.2, 61.1, 65.6, 177.1.

General procedure for the preparation of valproic acid-alpha linolenic acid conjugate (1-4)

To a solution of alpha-linolenic acid (ALA, 1.2 mmol) in dry DCM (3 ml) under nitrogen condition, was added at 0°C, EDC (1.2 mmol) and the activation was stirred at room temperature for 1 hour in the dark. Successively, a solution contained (**13-16**, 1 mmol) and DMAP (10% mmol) in dry DCM (1 ml) was added to the activated solution. The resulting mixture was stirred at room temperature around 4-6 hours. The solvent was evaporated in vacuo and the crude was purified by flash chromatography on silica gel using different eluent mixtures to give the title compounds **1-4**.

(9*Z*,12*Z*,15*Z*)-*N*-(2-(2-propylpentanamido)ethyl)octadeca-9,12,15-trienamide (1)

The resulting residue was purified by using flash chromatography (9.5/0.5 DCM/MeOH) affording **1** as pale-yellow oil. Yield 32%.

^{1}H-NMR ($CDCl_3$, 400 MHz) δ (ppm): 0.87 (t, 6H, J = 8 Hz), 0.96 (t, 3H, J = 8 Hz), 1.23-1.35 (m, 14H), 1.53-1-58 (m, 4H), 2.03-2.08 (m, 5H), 2.15 (t, 2H, J = 8 Hz), 2.78-2.80 (m, 4H), 3.37 (s, 4H), 5.27-5.41 (m, 6H), 6.43 (bs, 1H), 6.52 (bs, 1H).^{13}C-NMR ($CDCl_3$, 100 MHz) δ (ppm): 14.2, 14.4, 20.7, 20.9, 25.6, 25.7, 25.8, 27.3, 29.3, 29.41, 29.44, 29.7, 35.3, 36.8, 40.0, 40.5, 47.6, 127.2, 127.8, 128.37, 128.41, 130.4, 132.1, 174.6, 177.7. LRMS calcd for $C_{28}H_{50}N_2O_2$ m/z: 446.71, found 446.86.

2-((9*Z*,12*Z*,15*Z*)-octadeca-9,12,15-trienamido)ethyl 2-propylpentanoate (2)

The resulting residue was purified by using flash chromatography (9.5/0.5 DCM/MeOH) affording **2** as pale-yellow oil. Yield 37%.

^{1}H-NMR ($CDCl_3$, 400 MHz) δ (ppm): 0.89 (t, 6H, J = 8 Hz), 0.94 (t, 3H, J = 8 Hz), 1.24-1.45 (m, 14H), 1.53-1-63 (m, 4H), 2.01-2.08 (m, 4H), 2.16 (t, 2H, J = 8 Hz), 2.35-2.39 (m, 1H), 2.78-2.81 (m, 4H), 3.49-3.53 (m, 2H), 4.17 (m, 2H, J = 4 Hz), 5.27-5.41 (m, 6H), 5.77 (bs, 1H). ^{13}C-NMR ($CDCl_3$, 100 MHz) δ (ppm): 14,1, 14.4, 20.6, 20.8, 25.6, 25.71, 25.75, 27.3, 29.2, 29.4, 29.7, 34.7, 36.8, 39.0, 45.30 62.9, 127.2, 127.8, 128.3, 128.4, 130.3, 132.0, 173.3, 176.9. LRMS calcd for $C_{28}H_{49}NO_3$ m/z: 447.69, found 447.94.

2-(2-propylpentanamido)ethyl (9*Z*,12*Z*,15*Z*)-octadeca-9,12,15-trienoate (3)

The resulting residue was purified by using flash chromatography (9.5/0.5 DCM/MeOH) affording **3** as pale-yellow oil. Yield 28%.

^{1}H-NMR ($CDCl_3$, 400 MHz) δ (ppm): 0.88 (t, 6H, J = 4 Hz), 0.96 (t, 3H, J = 8 Hz), 1.24-1.35 (m, 15H), 1.56-1.62 (m, 4H), 2.03-2.06 (m, 5H), 2.30 (t, 2H, J = 8 Hz), 2.79 (s, 4H), 3.51-3.5 (m, 2H), 4.15 (t, 3H, J = 8 Hz), 5.29-5.40 (m, 6H), 5.76 (bs, 1H). ^{13}C-NMR ($CDCl_3$, 100 MHz) δ (ppm): 14.1,14.2, 20.5, 20.7, 24.8, 25.5, 25.6, 27.1, 29.06, 29.09, 29.14, 29.5, 34.1, 35.2, 38.6, 47.6, 63.0,

127.1, 127.7, 128.2, 128.3, 130.2, 131.9, 173.9, 176.1. LRMS calcd for $C_{28}H_{49}NO_3$ m/z: 447.69, found 447.82.

2-((2-propylpentanoyl)oxy)ethyl (9*Z*,12*Z*,15*Z*)-octadeca-9,12,15-trienoate (4)

The resulting residue was purified by using flash chromatography (9.8/0.2 DCM/MeOH) affording **4** as pale-white oil. Yield 25%.

^{1}H-NMR ($CDCl_3$, 400 MHz) δ (ppm): 0.89 (t, 6H, J = 8 Hz), 0.97 (t, 3H, J = 8 Hz), 1.26-1.45 (m, 15H), 1.55-1.64 (m, 4H), 2.04-2.09 (m, 4H), 2.31 (t, 2H, J = 8 Hz), 2.39 (q, 1H, J = 4 Hz) , 2.79-2.80 (s, 4H), 4.27 (s, 4H), 5.29-5.43 (m, 6H). ^{13}C-NMR ($CDCl_3$, 100 MHz) δ (ppm): 14.0, 14.2, 20.5, 20.6, 24.8, 25.5, 25.6, 27.2, 29.08, 29.14, 29.5, 34.1, 34.6, 45.1, 61.7, 62.0, 127.1, 127.7, 128.2, 128.3, 130.2, 131.9, 173.5, 176.3. LRMS calcd for $C_{28}H_{48}O_4$ m/z: 448.68, found 448.76.

LC-DAD-MS/MS analysis

Chemicals and instrumentation

MS-grade acetonitrile (ACN), methanol (MeOH) and analytical-grade formic acid (FA) were purchased from Sigma Aldrich, while ultrapure water (18.2 MΩ·cm) was obtained by means of a Milli-Q system from Millipore (Burlington, MA, USA). Stock solutions of compounds 1 and 2 (1 mg/mL) were prepared in MeOH and stored at -20 °C, working solutions were prepared daily by dilution in mobile phase and stored protected from light in amber glass vials from Waters Corporation (Milford, MA, USA).

LC-DAD-MS/MS analysis was performed on a Waters Alliance e2695 chromatographic system with autosampler coupled to a Waters 2998 photo diode array detector and a Waters Micromass Quattro Micro triple-quadrupole mass spectrometer, equipped with an electrospray ion source working in positive ion mode (ESI+). Data processing was performed using Waters MassLynx 4.1 software. A

Velp (Usmate, Italy) RX3 vortex mixer and an Elma (Singen, Germany) Sonic T310 Trans ultrasonic bath were also used.

Analytical Conditions

Chromatographic runs were obtained on an Agilent (Palo Alto, Ca, US) ZORBAX SB-C18 column (50 × 2.1 mm I.D., 3.5 μm particles), maintained at room temperature and equipped with a guard column. The mobile phase was a mixture of 0.1% aqueous formic acid (A) and acetonitrile (B), flowing at a constant rate of 0.3 mL/min. The mobile phase was set in isocratic mode with A:B ratio of (65:35, V:V); the two components of the mobile phase were filtered through Sartorius (Göttingen, Germany) membrane filters (47 mm diameter, polyamide, 0.2 μm pore size) and degassed by an ultrasonic bath. The injection volume was 10 μL. For compound purity, Total Ion Current (TIC) mode was used and DAD chromatograms were checked at 200 nm and 540 nm. For compound stability and hydrolysis studies, multiple reaction monitoring (MRM) acquisition mode was set up for both compounds 1 and 2, by flow-injection analysis directly in the MS source, in order to optimize ionization parameters and to select two exclusive MRM *m/z* transitions for each analyte, exploited for quantitative purposes and for qualitative confirmation, respectively.

Sample analysis and method validation

For compound purity assessment, stock solutions of compounds **1** and **2** (1 mg/mL) were prepared in MeOH, while working solutions were prepared by suitably diluting stock solutions with mobile phase into amber glass vials. LC-DAD-MS/MS analysis were performed under the previously described conditions by exploiting TIC acquisition mode and by checking DAD chromatograms for possible impurities. Analysis were performed in triplicate, compound purity and standard deviation (SD) were calculated and the results are reported in Table S1. Representative TIC LC-MS chromatograms obtained from working solutions of **1** and **2** are reported in Fig S1a and S1b, respectively.

For compound stability and hydrolysis studies, after setting up MRM acquisition mode for both **1** and **2** as described previously, the LC-MS/MS method was coupled to an optimized sample preparation protocol to be applied to biological matrices, namely rat plasma and rat brain homogenate. Briefly, for both matrices, 1-mL sample was brought to 37±0.5°C by means of a thermostatted water bath. Then, 50µL of analyte working solution was added to the matrix sample in order to obtain a final concentration of 1 µg/mL. The sample was gently mixed and t0 was noted for both stability study (in plasma) and hydrolysis (in brain homogenate). Then, at regular intervals (5, 10, 15, 20, 25, 30, 45, 60 min) a 50-µL aliquot was sampled from the vial, added with 50 µL of ice-cold ACN and centrifuged for 5 min at 4500 rpm at 5°C. The resulting supernatant was subjected to an original miniaturized extraction procedure, based on microextraction by packed sorbent (MEPS), in order to purify the samples from potential interfering signals deriving from the matrices. The solution obtained after MEPS procedure was directly injected into the LC-MS/MS system under the optimized conditions previously described. Compound quantitation was obtained by integrating peak areas obtained from sample analysis and by interpolation on the linearity curve of each analyte. All analyses were carried out in triplicate and results were then expressed as % of remaining compound with respect to that assessed at t0 (100%), plotted against the defined time points.

In order to be applied for stability and hydrolysis studies, the MEPS-LC-MS/MS method was fully validated on both plasma and brain homogenate samples fortified with known amounts of compounds **1** and **2**, pretreated and analyzed right after the addition of analyte working solutions. Method validation was performed according to the main international guidelines [ICH Harmonised Tripartite Guideline. Validation of Analytical Procedures: Text and Methodology Q2(R1); International Conference on Harmonisation of Technical] in terms of linearity (including limit of detection, LOD and limit of quantitation, LOQ), precision, accuracy, extraction yield and matrix effect.

BBB permeation studies.

For the prediction of tested compounds to passively penetrate the BBB PAMPA modified protocol was used.[1] Briefly, the filter membrane of the donor plate was coated with PBL (Polar Brain Lipid, Avanti, USA) in dodecane (4 µl of 20 mg/ml PBL in dodecane) and the acceptor well was filled with 300 µl of PBS pH 7.4 buffer (VD). Tested compounds were dissolved first in DMSO and diluted with PBS pH 7.4 to reach the final concentration 100 µM in the donor well. Concentration of DMSO did not exceed 0.5% (V/V) in the donor solution. Next, 300 µl of the donor solution was added to the donor wells (VA) and the donor filter plate was carefully put on the acceptor plate so that coated membrane was "in touch" with both donor solution and acceptor buffer. Test compound diffused from the donor well through the lipid membrane (Area=0.28cm^2) to the acceptor well. The concentration of the drug in both donor and the acceptor wells was assessed after 3, 4, 5 and 6 h of incubation in quadruplicate using the UV plate reader Synergy HT (Biotek, USA) at the maximum absorption wavelength of each compound. Besides that, solution of theoretical compound concentration, simulating the equilibrium state established if the membrane were ideally permeable, was prepared and assessed as well. Concentration of the compounds in the donor and acceptor well and equilibrium concentration were calculated from the standard curve and expressed as the permeability (Pe) according the equation:

$$P_e = C \times ln\left(1 - \frac{[drug]\,acceptor}{[drug]\,equilibrium}\right)$$

$$where\ C = \frac{(V_D \times V_A)}{(V_D \times V_A)Area \times Time}$$

Data obtained for the new compounds are correlated to standard drugs, where CNS availability is known.

Biology

Cellular HDAC inhibition assay

DAOY cells (a human cerebellar medulloblastoma cell line, purchased from American Type Culture Collection, ATCC, Rockville, MD, USA) were routinely grown in Dulbecco's Modified Eagle Medium, (DMEM, Life Technologies, Carlsbad, CA, USA) supplemented with 10% fetal bovine

serum (FBS, Life Technologies), nonessential aminoacids and sodium pyruvate. Cells were kept at 37 °C under 5% CO_2 humidified atmosphere. To evaluate acetylation levels of histone H3, DAOY cells were treated with conjugates **1**, **2** (both 0.5 mM, dissolved in methanol/vehicle) or VPA (0.1 – 1.5 mM) for 48 h; then cells were detached using a scraper and centrifuged at 2000 rpm for 2 minutes. Histones extraction was performed using Histone Extraction Kit (AbCam, Cambridge, United Kingdom). Pellets were resuspended in 1X Pre-Lysis Buffer at the concentration of 10^7 cells/mL, transferred into 1.5 mL tubes and incubated for 10 minutes on ice with gently shaking. At the end of the incubation, the samples were centrifuged at 1000 rpm for 1 minute at 4°C. Supernatant was discarded and cell pellets were resuspended with 3x-volume of Lysis Buffer, incubated for 30 minutes on ice and samples were centrifuged at 12000 rpm for 5 minutes at 4°C. The supernatant containing soluble basic proteins such as histones was transferred in a new tube; then, 0.3 volumes of Balance-DTT Buffer were added immediately to the supernatant and samples were store at -80°C until further use; protein concentration was determined by BCA assay (Pierce, Rockford, IL, USA). Basic protein extracts were denatured at 95 °C for 3 min before loading and separation by 15% SDS-PAGE. The membranes were blocked in 5% BSA in TBS+0.05% Tween-20 for 1 h and incubated with anti-acetyl-H3 (1:2000; #06-599, Millipore) or anti-total H3 antibodies (1:5000; #05-928, Millipore) overnight at 4 °C. Thereafter, the membranes were incubated with peroxidase-conjugated secondary antibodies at a 1:8000 dilution (Santa Cruz Biotechnology). Digital images were acquired and analysed according to a previously reported method.[2] Experiments were replicated independently at least three times. Data are presented as mean ± SD and were analyzed by one-way ANOVA followed by Newman–Keuls test. The GraphPad Prism, version 6.0 (Graph-Pad Software, Inc.) was used, and P values <0.05 were considered significant.

Neurotoxicity assay

Primary cultures of CGNs were prepared from 7 day-old pups of the same rat strain, as previously described.[3, 4] All animal experiments were authorized by the University of Bologna bioethical

committee (Protocol n° 17-72-1212) and performed according to Italian and European Community laws on the use of animals for experimental purposes. For cerebellar granule cultures, cells were dissociated from cerebella and plated on 96 well plates, previously coated with 10 μg/mL poly-L-lysine, at a density of 1.2×10^5 cells/0.2 mL medium/well in BME supplemented with 100 mL/L heat-inactivated FBS (Aurogene), 2 mM glutamine, 100 μM gentamicin sulphate and 25 mM KCl (all from Sigma-Aldrich). 16 h later, 10 μM cytosine arabino-furanoside (Sigma-Aldrich) was added to avoid glial proliferation. After 7 days in vitro, differentiated neurons were shifted to serum free BME medium containing 25 mM KCl and different treatments were performed. The viability of CGNs exposed to increasing concentrations of the studied compounds (0, 5, 10 and 25 μM) for 24 h was evaluated through the MTT assay. Briefly, thiazolyl blue was added to the culture medium at a final concentration of 0.1 mg/mL. Following a 20 min incubation at 37 °C in the dark, the MTT precipitate was dissolved in 0.1 M Tris-HCl pH 7.5 buffer containing 5% Triton X-100 (all from Sigma-Aldrich) and absorbance was read at 570 nm in a multiplate spectophotometric reader (Bio-Rad). To test neuroprotection, glutamate toxicity was induced on differentiated CGNs after 8 days in vitro. CGNs cultures were switched to serum-free BME medium in presence or absence of 100 μM glutamate/10 μM glycine (Sigma-Aldrich). Increasing concentrations (0, 5, 10 or 25 μM) of selected compounds were tested with both a 6 hours pre-treatment and a 24 hours co-treatment with the excitotoxic stimulus. After 24 hours, neuronal survival has been evaluated through MTT assay.

Hepatotoxicity assay

Human hepatoma cells (HepG2) were cultured in Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% heat inactivated Fetal Bovine Serum (FBS), 1% Penicillin/Streptomycin and 2mM glutamine (all reagents were from Aurogene, beside glutamine, which was from Sigma-Aldrich) at 37^o C in a humidified atmosphere containing 5% CO_2. Cells were plated on 96 well plates, previously coated with 10 μg/mL poly-L-lysine (Sigma-Aldrich) at the density of 2.5 x 10^4/0.2 mL

medium/well and treated with increasing concentrations of the compounds (0.5, 1, 5 and 10 μM), in serum-free DMEM for 24 hours. After 24 hours, the viability was evaluated through the MTT assay.

Immunomodulation assays

Mouse N9-microglial cells were cultured in Dulbecco Modified Eagle Medium (DMEM) supplemented with 10% heat inactivated Fetal Bovine Serum (FBS), 1% Penicillin/Streptomycin and 2mM Glutamine (all cell cultures' reagents were from Aurogene). At confluence, after a short wash with sterile PBS, microglia were trypsinized for 5 minutes at 37 ° C and trypsin was inactivated with complete DMEM medium. Detached cells were than collected, centrifuged for 5 minutes at 300xg and resuspended to be counted. For the experiments, microglial cells were plated at the density of $2.5x10^5$ in 35 mm dish and exposed to 100ng/ml lipopolysaccharide (LPS), in presence or absence of increasing concentrations of the compounds to be tested (0.5, 1, 5 and 10 μM for immunomodulation). After pre-treatment for 6 hours and administration for further 24 h, microglial cells were lysed in ice-cold lysis buffer (50 mM Tris-HCl pH 7.4, 1% SDS, 0.05% protease inhibitor cocktail; all from Sigma-Aldrich), protein content was determined by using the Lowry method and samples were loaded for western blot analysis of iNOS (M1 microglia marker), TREM2 (M2 microglia marker) and GAPDH (loading control) expression.

Nitrites Measurement

Nitrite accumulation in cell media was assayed by the Griess reaction. A nitrate standard curve was performed with $NaNO_2$ (Sigma-Aldrich) at known concentrations. 1 mM sulfanilamide, 0.1N HCl and 0.4 mM NEDA (all from Sigma-Aldrich) were added to the culture medium and the standard curve. Absorbance was read at 540 nm using a plate reader after 15 min of incubation at room temperature in the dark.

Oli-Neu cells toxicity assay

Oli-Neu cells were grown in SATO medium (DMEM medium, 2 mM glutamine, 10 μg/ml insulin, 5.5 μg/ml transferrin, 38.72 nM sodium selenite, 100 μM putrescine, 520 nM L-thyroxine (T4), 500 nM triiodo-L-thyronine (T3), 200 nM progesterone, 25 μg/ml of gentamycin) supplemented with 1% heat-inactivated horse serum (HS) in a humidified, 5% CO2 incubator at 37°C. Cell culture medium and all chemicals were from Sigma-Aldrich, except for insulin-transferrin-sodium selenite 100X supplement (Life Technologies Corporation). Cells were plated on 96 well plates, previously coated with 10 μg/mL poly-L-lysine (Sigma-Aldrich; 10 μg/ml) at the density of 2.5 x 10^4/0.2 mL medium/well and treated with increasing concentrations of the compounds (0.5, 1, 5 and 10 μM). After 24 hours, the viability was evaluated through the MTT assay.

Oli-Neu cells proliferation and differentiation assay

1 x 10^5 Oli-Neu cells were plated in 35-mm Petri dish. and exposed to 5 μM of the compounds. After 48 hours, for cell counting and measure of filaments, 5 random images for each condition were acquired with an Eclipse TS100 (Nikon) microscope using a 10X objective. Cells number and filament length were measured with Fiji ImageJ2 software. After 48 hours for proliferation and 72 hours of treatment for differentiation analysis, cells were lysed in ice-cold lysis buffer (50 mM Tris-HCl pH 7.4, 1% SDS, 0.05% protease inhibitor cocktail; all from Sigma-Aldrich) and protein content was determined by using the Lowry method.

Western Blot analysis

30 μg of protein extract were resuspended in loading buffer (0.05 M Tris-HCl pH 6.8; 40 g/L sodium dodecyl sulfate; 20 mL/L glycerol; 2 g/L bromophenol blue and 0.02 M dithiothreitol; all from Sigma-Aldrich) and loaded onto 12.5% sodium dodecyl sulfate-polyacrylamide gels (SDS-PAGE; Bio-Rad Laboratories SrL, Segrate, MI, IT). After electrophoresis and transfer onto nitrocellulose membranes (GE Healthcare Europe GmbH, Milano, MI, IT), membranes were blocked for 1 h in 5% non-fat milk /0.1% Tween-20 in PBS (Sigma-Aldrich), pH 7.4, and incubated overnight at 4°C with primary

antibodies (for immunomodulation rabbit polyclonal anti-iNOS from Cell Signalling, rabbit polyclonal anti-TREM2 from Millipore, for proliferation/differentiation assay rabbit polyclonal anti-Olig2 from Santa Cruz Biotechnology, rabbit polyclonal anti- CNPase from Cell Signaling and mouse monoclonal anti-GAPDH from Santa Cruz Biotechnology, all 1:1000 except for GAPDH 1:20000) in 0.1% Tween-20/PBS. Membranes were then incubated with an anti-rabbit or anti-mouse secondary antibody conjugated to horseradish peroxidase (1:2000; both from Santa Cruz Biotechnology), for 90 min at RT in 0.1% Tween-20/PBS. Labeled proteins were detected by using the enhanced chemiluminescence method (ECL; BioRAD) with a Chemidoc (BioRad) chemiluminiscence detector. Densitometric analysis was performed by using Biorad Image Lab software.

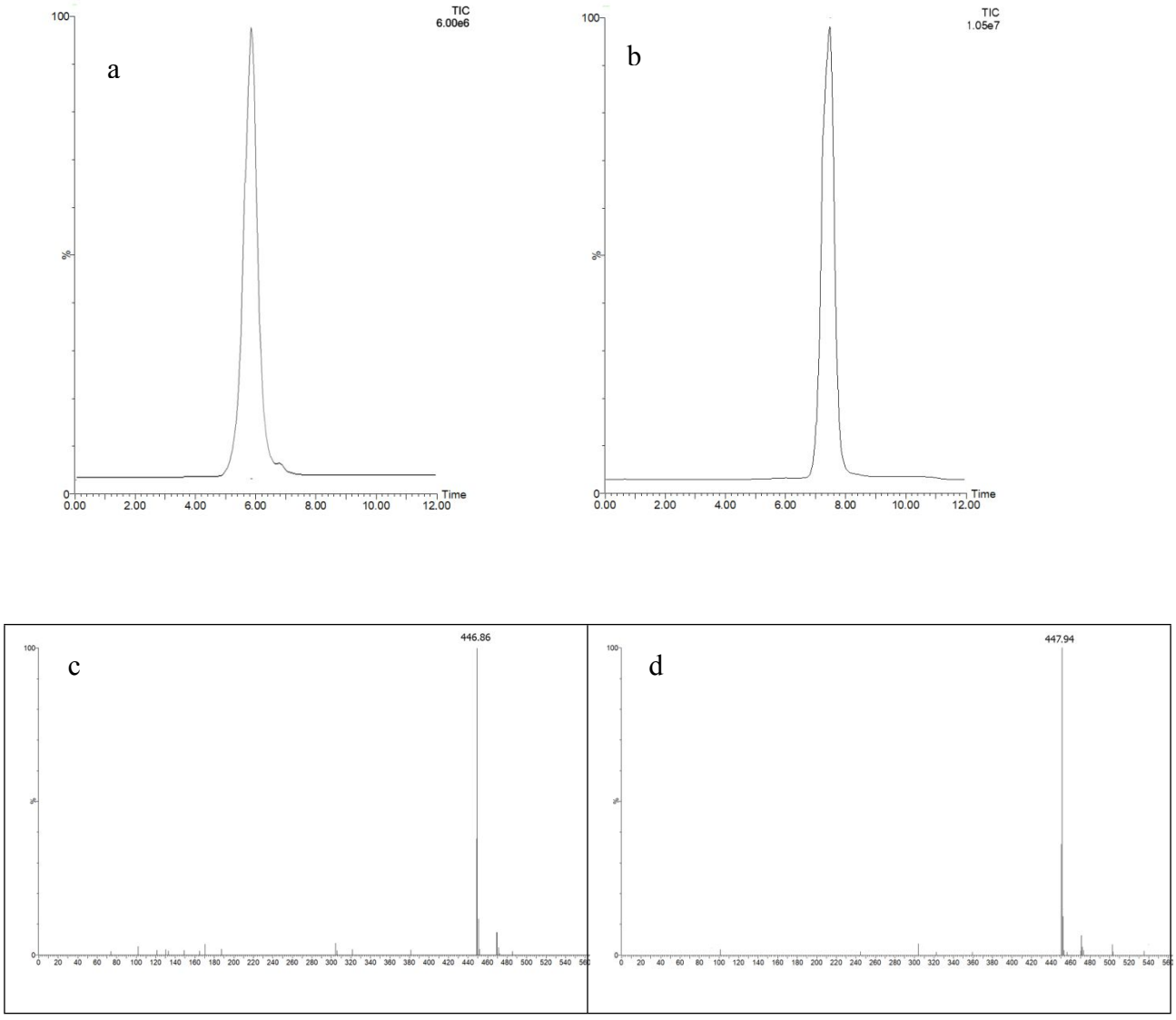
Statistical Analysis

All results were subject to statistical analysis with Student's T-test or one-way ANOVA followed by Bonferroni's post-hoc comparison test by using Graph Pad Prism 4 software. P-values less than 0.05 were considered statistically significant.

Table S2. Compound purity assessed by LC-MS/MS

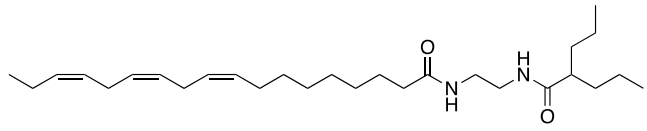
Compound	Purity (%)
1	95.8
2	97.2

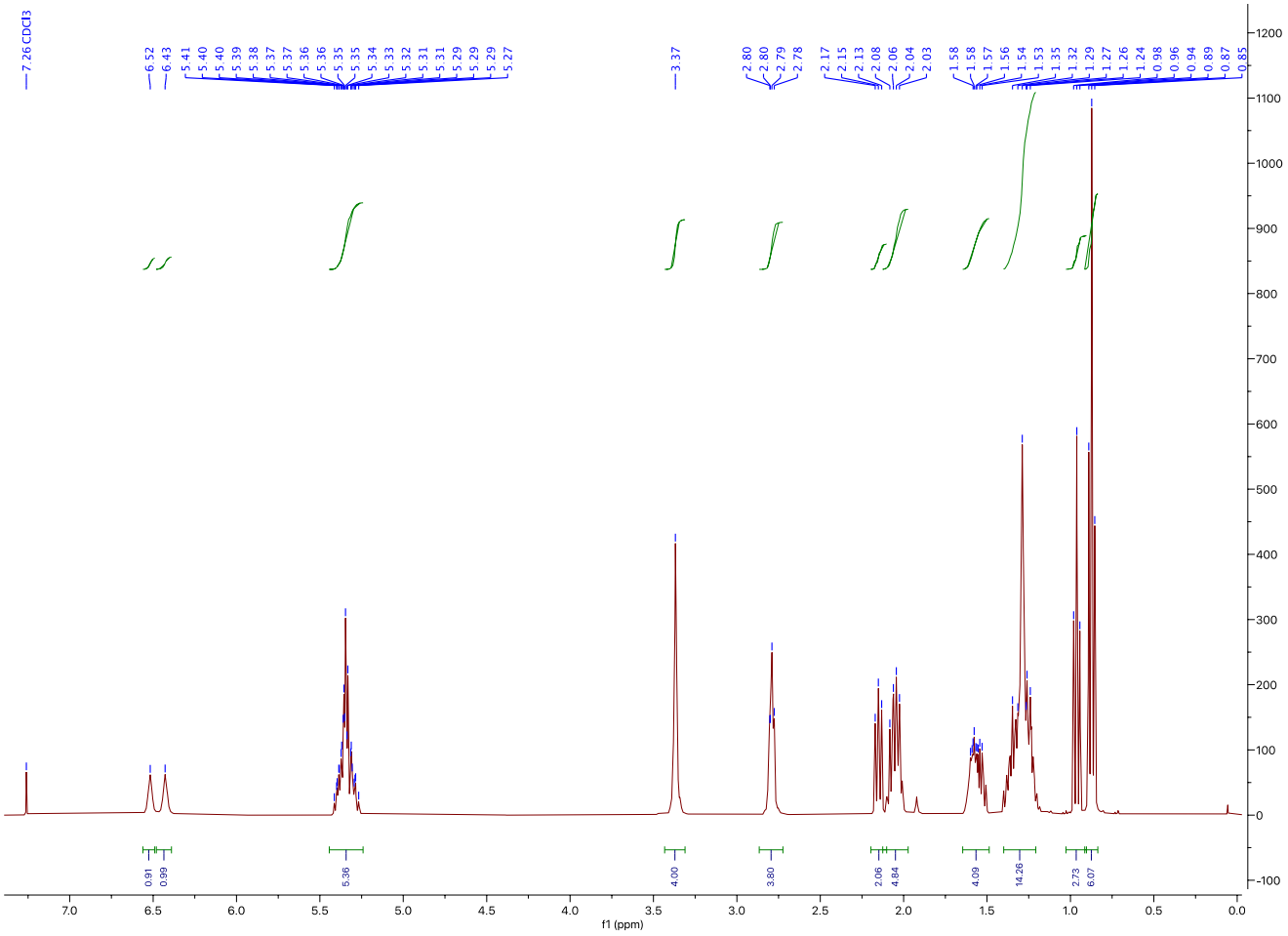
Figure S2. LC-MS/MS chromatograms and full scan ESI^+ MS spectra of **1** (a, c) and **2** (b, d)



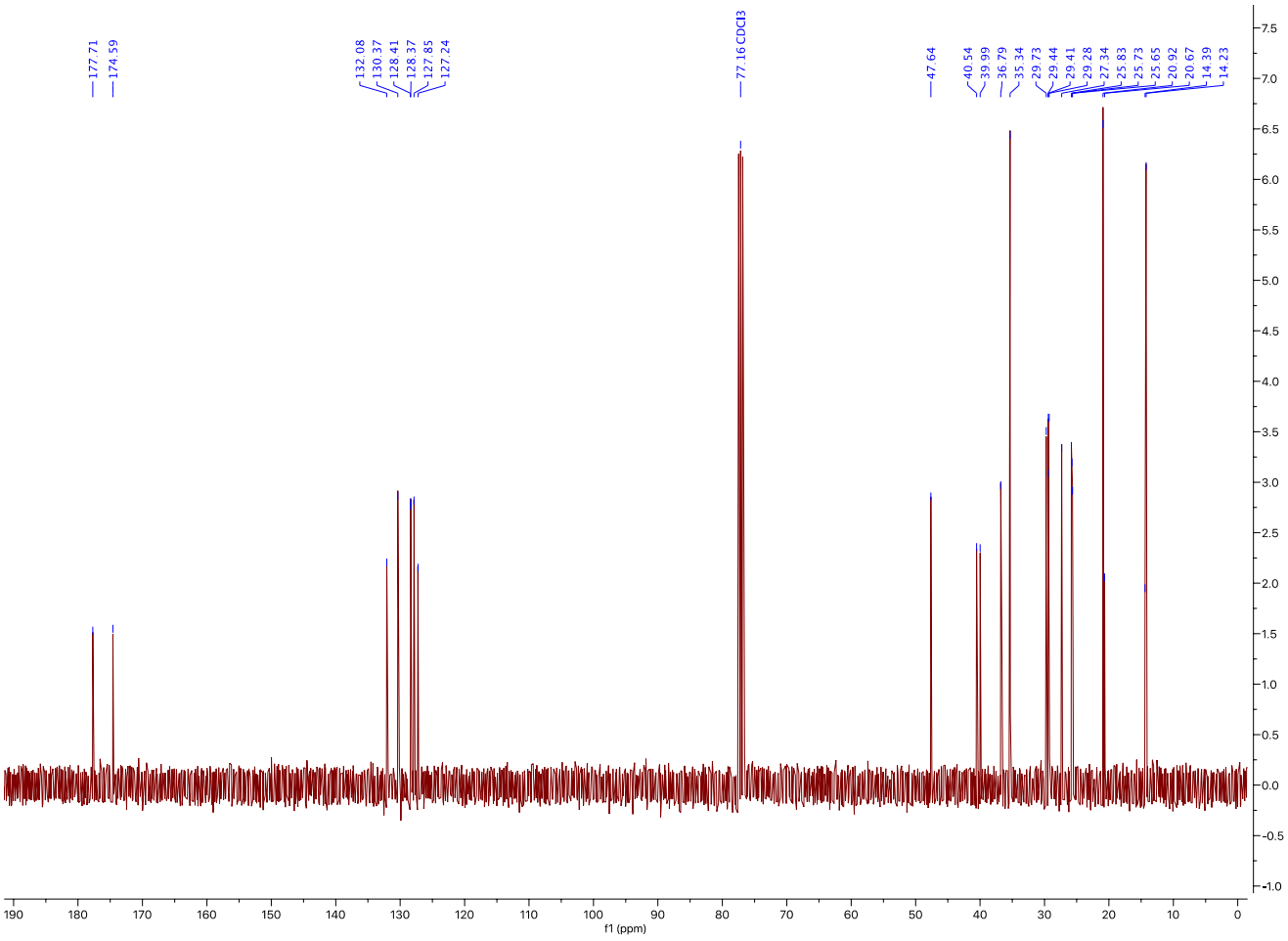
Spectral copies of ^{1}H NMR and ^{13}C NMR of ALA-VPA conjugates 1 and 2.

ALA-VPA conjugate **1**: ^{1}H-NMR (400 MHz, $CDCl_3$)

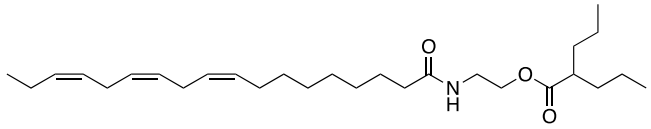


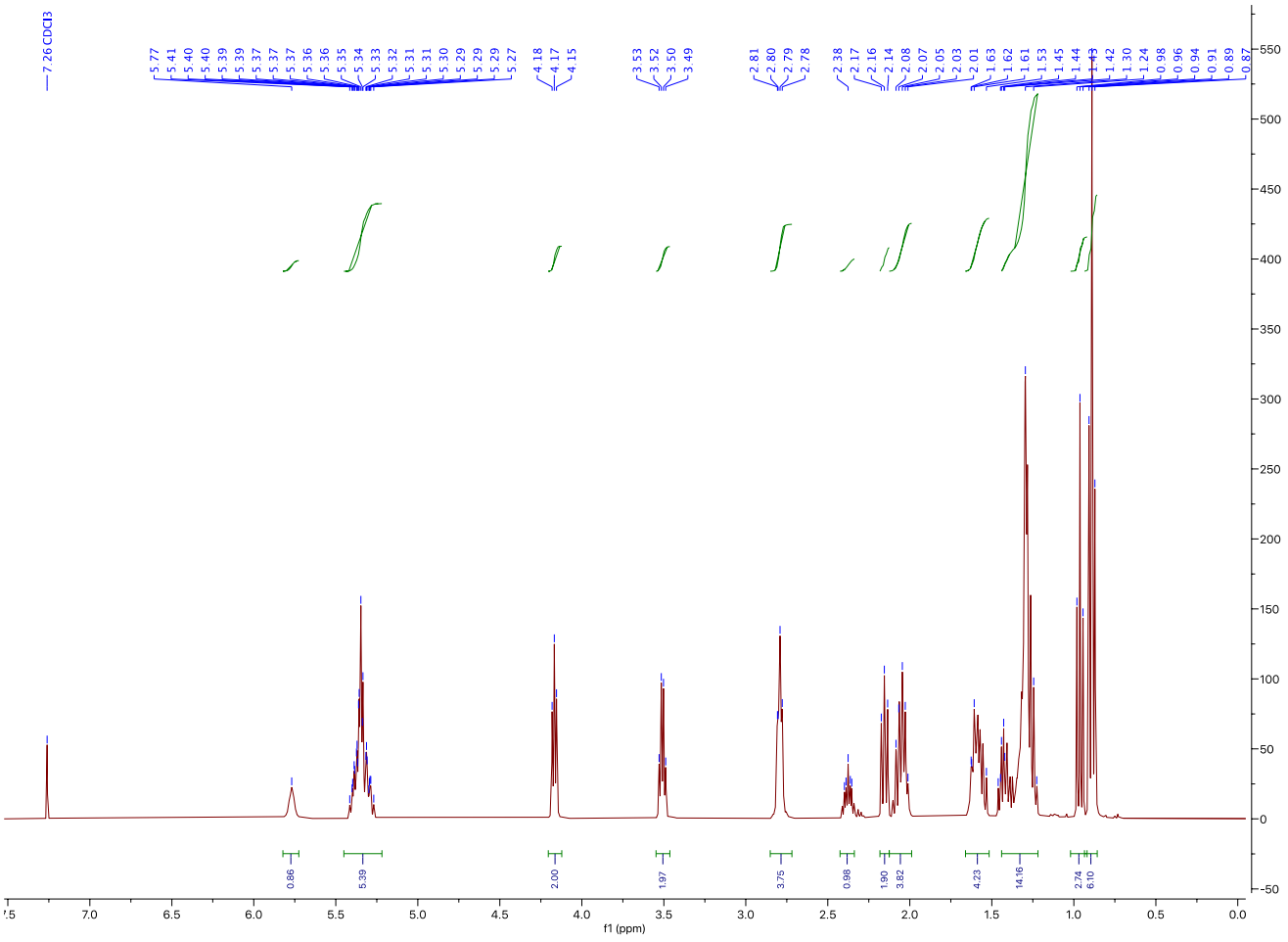


ALA-VPA conjugate **1**: ^{13}C-NMR (100 MHz, $CDCl_3$)



ALA-VPA conjugate **2**: ^{1}H-NMR (400 MHz, $CDCl_3$)





ALA-VPA conjugate **2**: ^{13}C-NMR (100 MHz, $CDCl_3$)

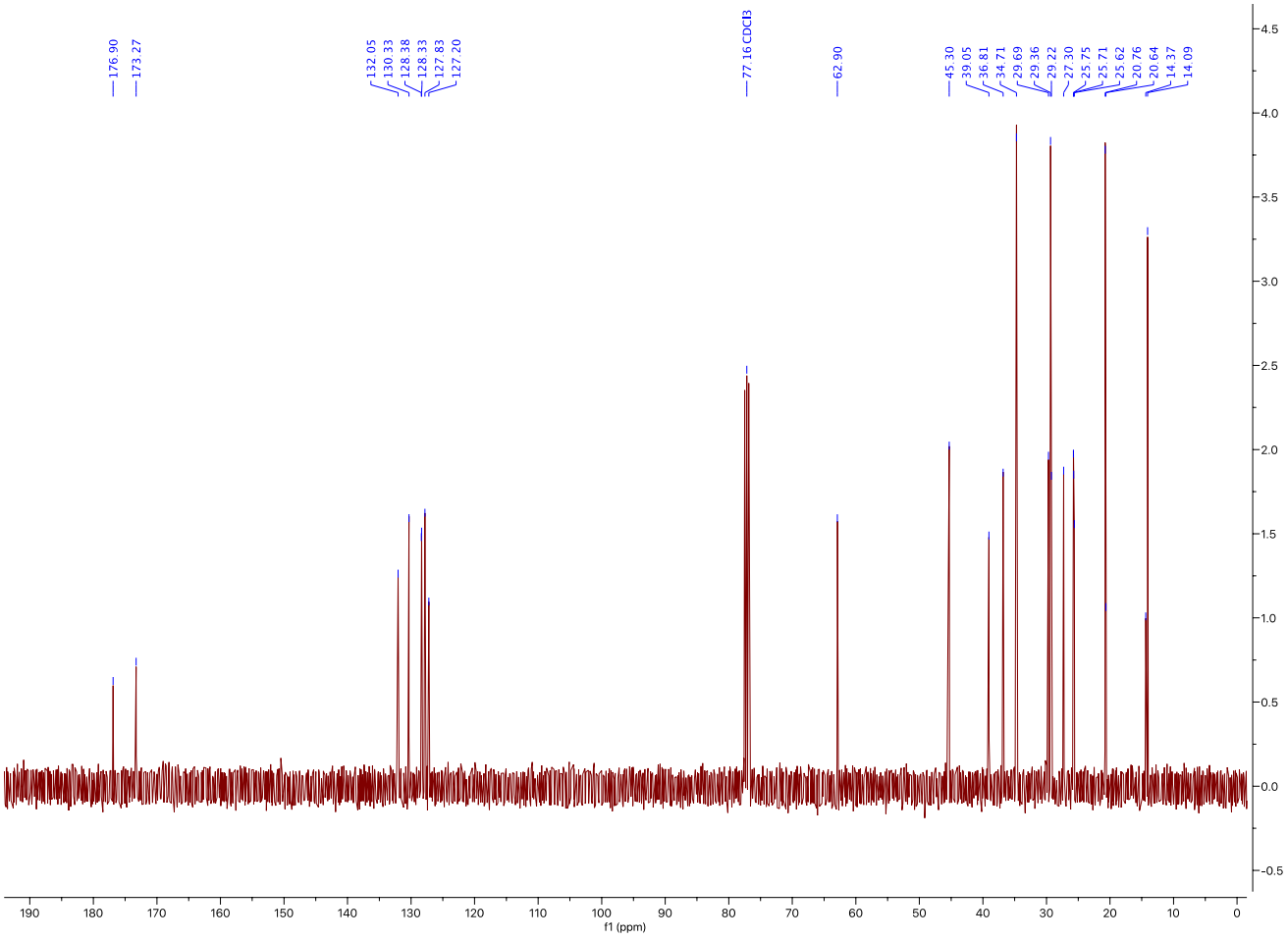


Figure S3: CGNs cells toxicity data

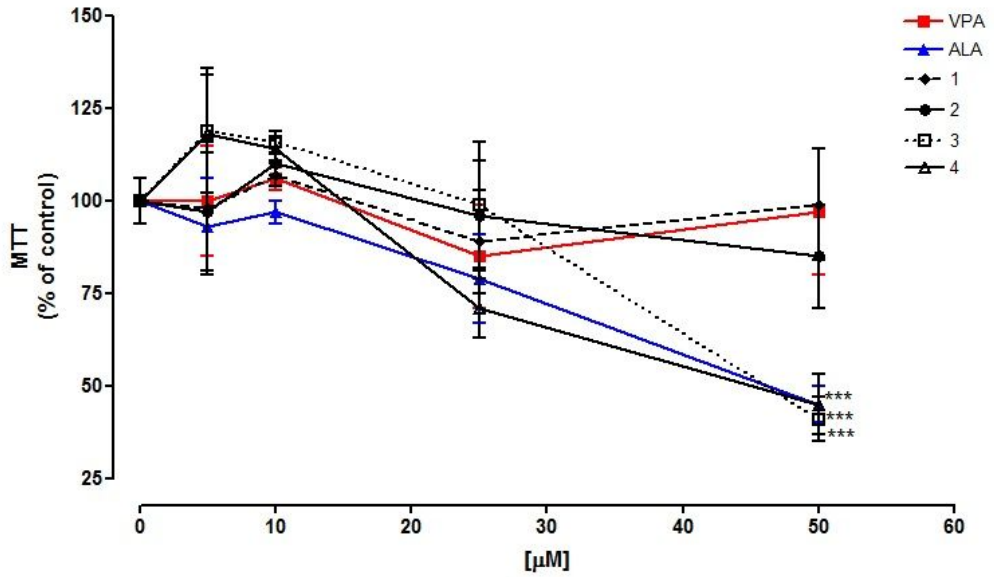


Figure S4: HepG2 cells toxicity data

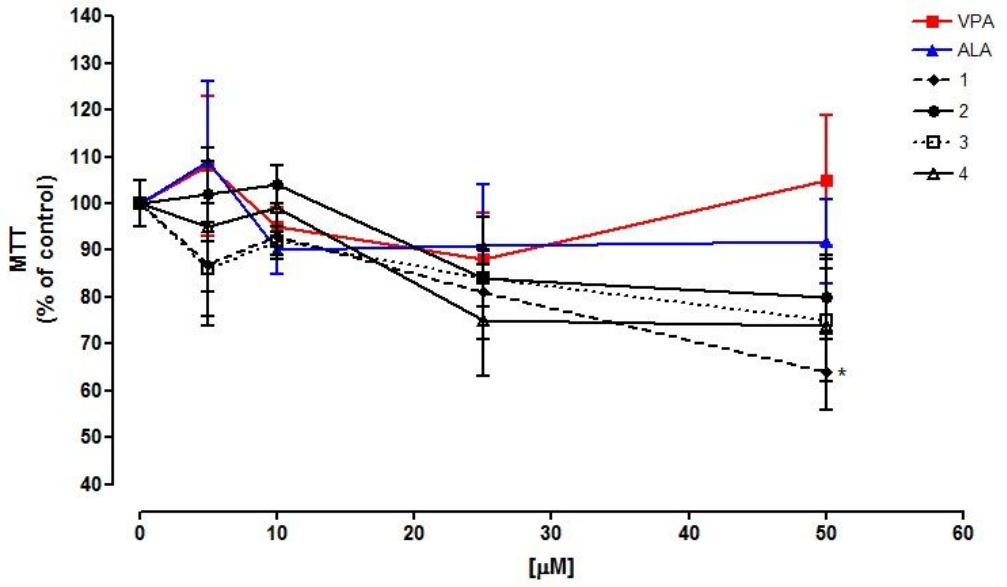
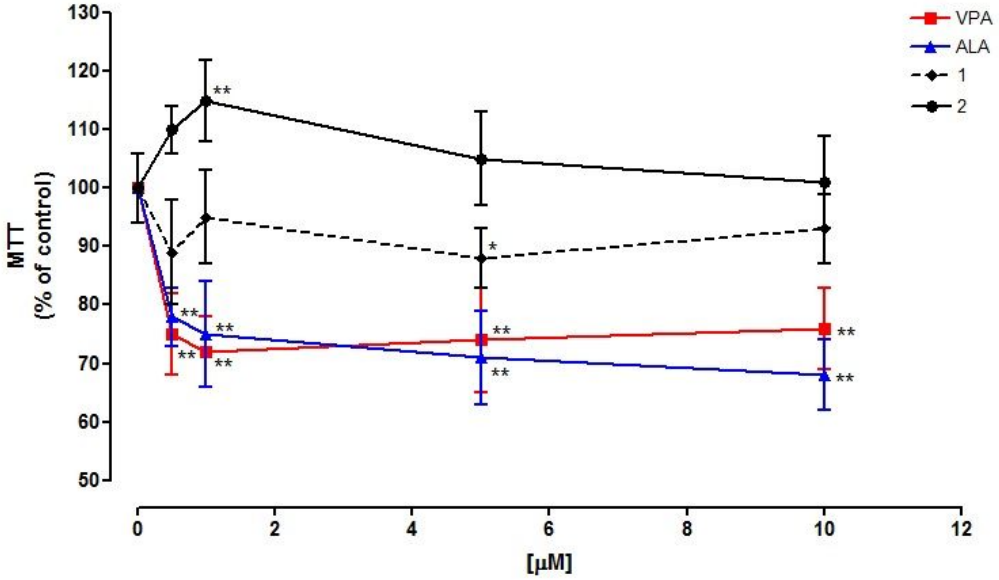


Figure S5: Oli-Neu cells toxicity data


VPA
ALA
1
2
MTT
(% of control)
130
120
110
100
90
80
70
60
50
0
2
4
6
8
10
12
[μM]
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