A water soluble cuprizone derivative: synthesis, characterization and in vitro studies.

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Cuprizone (1).

A 250mL flask was charged with oxalyldihydrazide (600 mg, 5.08 mmol, 1 eq). 150mL of ethanol was added to the reagent as well as cyclohexanone (1.08 mL, 10.42 mmol, 2.05 eq) and 30mg of p-TsOH as a catalysator. The mixture was refluxed for 5 hours and then cooled for 30 minutes in an ice bath to ensure full precipitation. The mixture was filtered, washed with ethanol (3x 15mL) and dried to obtain the product as a white solid (1.25 g, 4.49 mmol, 88% yield).

^{1}H NMR (600 MHz, Chloroform-d): δ = 10.03 (s, 2H), 2.48 – 2.41 (m, 4H), 2.39 (t, *J* = 6.4 Hz, 4H), 1.75 (m, 8H), 1.66 (m, 4H).

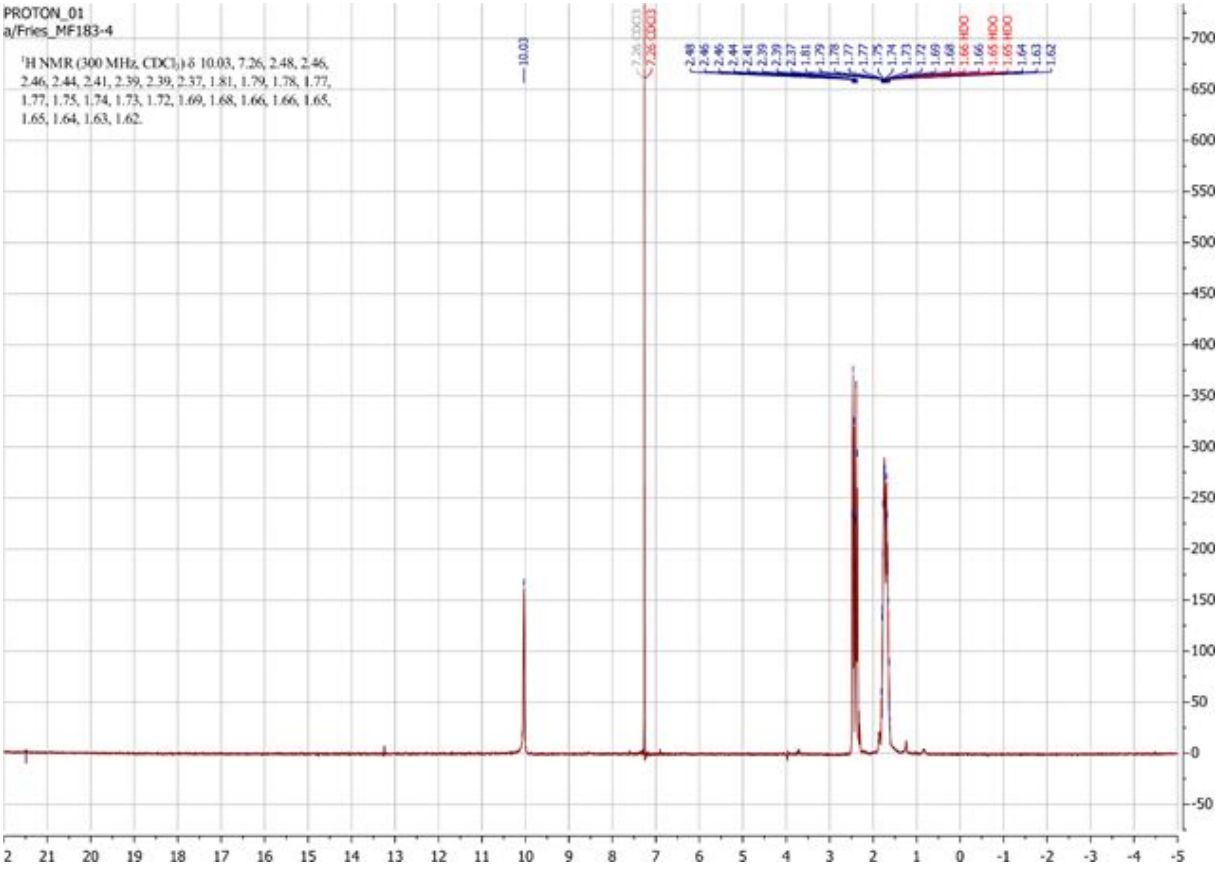

*Figure S1: ^{1}H Spectrum of Cuprizone (**1**).*

^{13}C NMR (151 MHz, Chloroform-d): δ = 166.06, 155.49, 35.72, 27.19, 26.96, 26.10, 25.46. Elemental analysis for $C_{14}H_{22}N_4O_2$ (301.1635): calcd. C 60.41, H 7.97, N 20.13; found C 60.20, H 7.75, N 20.36. IR (KBr): □ = 728, 876, 1211, 1439, 1738, 2938, 3226 cm^{-1}. ESI: calcd. for $[C_{14}H_{22}N_4NaO_2]^+$ $[M+Na]^+$ 301.1635, found 301.1625.

N,N-Dimethylated cuprizone 2 and cyclization product 2'.

Cyclohexylidene methylhydrazine (535 mg, 4.2 mmol, 1.2 eq.) were dissolved in 25mL of anhydrous THF and at 0°C triethylamine (1.3 mL, 9.4 mmol, 2.5 eq.) was added. Oxalyldichloride (0.4 mL, 3.5 mmol, 1 eq.) in 5mL of anhydrous THF was added within 1 h. The mixture was stirred at 0°C for 1 hour and then for 20 hours at room temperature. The white precipitate was filtered off and was extracted with THF (twice, 2 mL) to obtain **2** as a orange oil (859 mg, 48%) as three isomers E/E, E/Z and Z/Z.

^{1}H NMR (400 MHz, Chloroform-d): δ = 3.23 (s, CH_3), 3.17 (s, CH_3), 3.11 (s, CH_3), 3.02 (s, CH_3), 2.45 – 2.14 (m, CH_2), 1.83 – 1.57 (m, CH_2). IR (KBr): $\tilde{\nu}$ = 3519, 3285, 2932, 2859, 2667, 2329, 2092, 1918, 1643, 1446, 1348, 1248, 1191, 1130, 1053, 993, 912, 858, 763, 663 cm^{-1}. ESI: calcd. for $[C_{16}H_{27}N_4O_2]^+$: 307.272, found: 307.212.

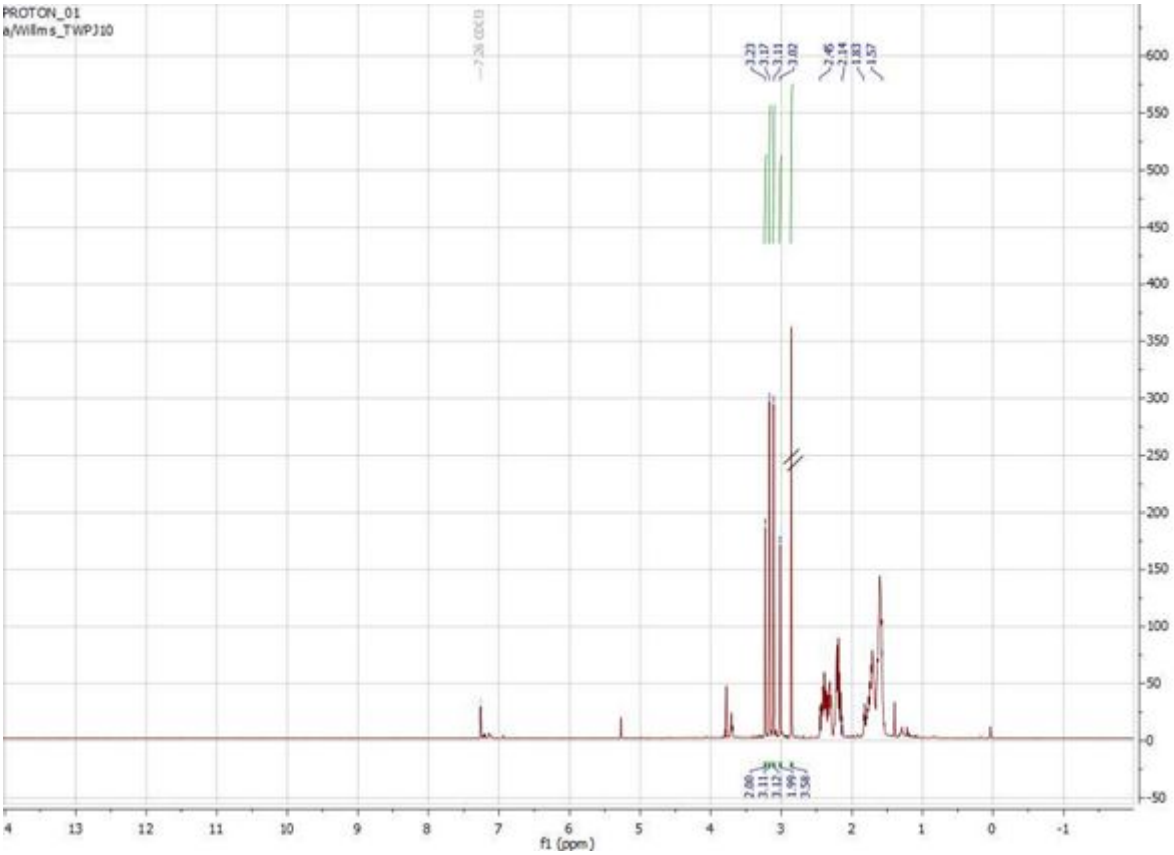

*Figure S2: ^{1}H Spectrum of compound **2**.*

The oil was dissolved in methanol (10 mL) and solvent was removed. The remaining orange solid was dissolved in ethylacetate / hexane (1:3). After filtration the solution was kept at 6 °C for several days to obtain **2'** (90 mg) as orange needles.

^{1}H NMR (600 MHz, Chloroform-d): δ = 4.88(s, 2H) 3.15 (s, 6H), 1.68-1.47 (m, 10 H).

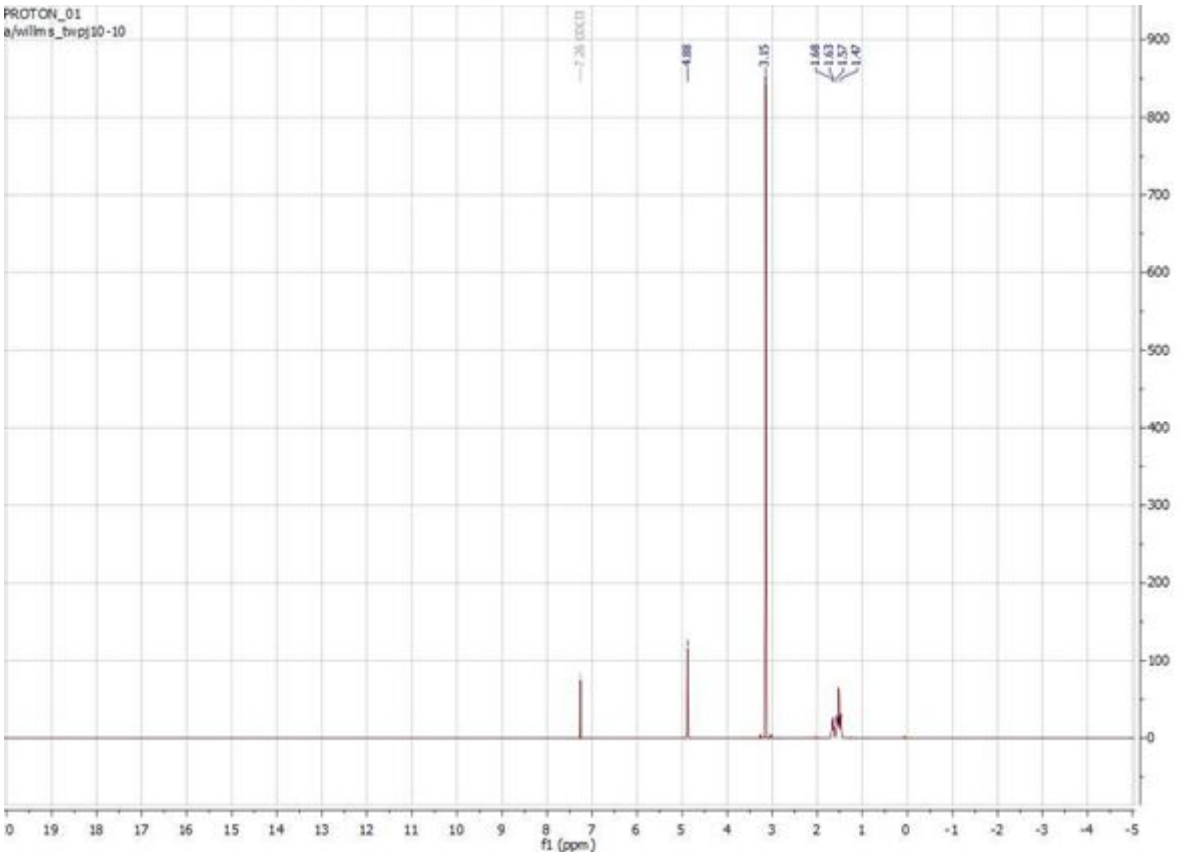

*Figure S3: [1]H Spectrum of compound **2'**.*

[13]C NMR (151 MHz, Chloroform-d): δ = 166.47, 78.67, 38.19, 32.59, 25.74, 21.86. Elemental analysis for $C_{10}H_{18}N_4O_2$ • ¼ H_2O (232.7): calcd. C 52.04, H 8.08, N 24.28; found C 52.15, H 7.81, N 24.43. IR (KBr): $\tilde{\nu}$ = 3830, 3273, 2928, 2328, 2183, 2064, 1823, 1657, 1396, 1271, 1098, 939, 799 cm^{-1}. EI MS (70 eV): m/z = 227.2 (100, $[M-H]^+$).

Cycloheptylderivative of cuprizone (3).

A 250mL flask was charged with oxalylhydrazide (590 mg, 5 mmol, 1 eq). 150mL of ethanol was added to the reagent as well as cycloheptanone (1.77 mL, 15 mmol, 3.00 eq) and 30 mg of p-TsOH as a catalyst. The mixture was refluxed for 5 hours and then cooled for 30 minutes in an ice bath to ensure full precipitation. The mixture was filtered, washed with ethanol (3x 15mL) and dried to obtain the product as a white solid (1.09 g, 3.56 mmol, 71 % yield).

[1]H NMR (600 MHz, Chloroform-*d*): δ = 9.95 (s, 2H), 2.64 – 2.62 (m, 4H), 2.52 – 2.50 (m, 4H), 1.80 (dt, *J* = 10.0, 6.3 Hz, 4H), 1.71 – 1.66 (m, 4H), 1.65 – 1.61 (m, 8H).

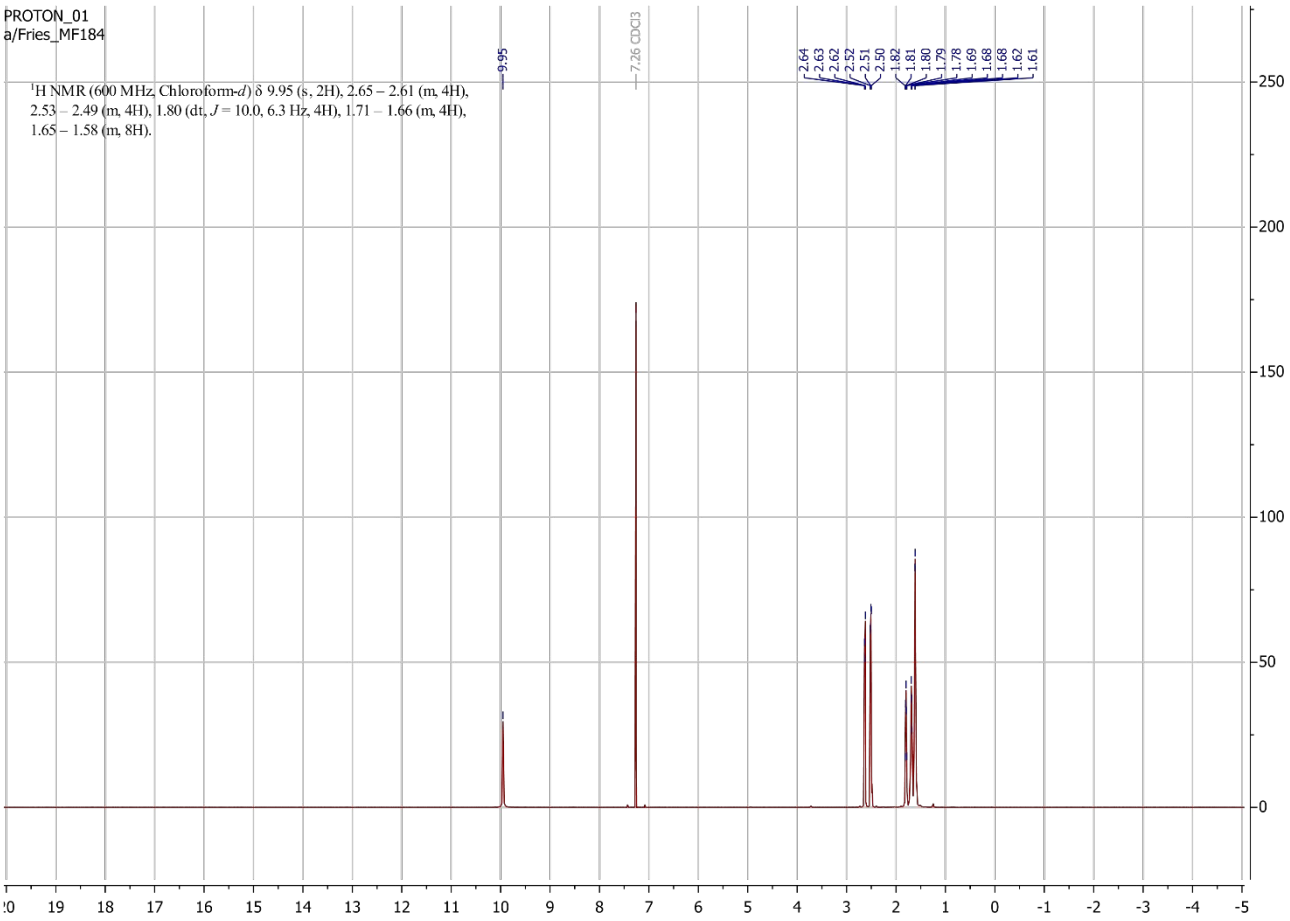

*Figure S4: ^{1}H Spectrum of compound **3**.*

^{13}C NMR (151 MHz, Chloroform-*d*): δ = 168.13, 155.49, 37.35, 30.55, 30.36, 30.24, 27.46, 24.35. Elemental analysis for $C_{16}H_{26}N_4O_2$ (306.2056): calcd. C 62.72, H 8.55, N 18.29, found C 62.72, H 8.68, N 18.33. IR (KBr): $\tilde{\nu}$ = 684, 1487, 1656 cm^{-1}. ESI: calcd. for $[C_{16}H_{26}NaN_4O_2]^+$ $[M+Na]^+$ 329.1948, found 329.1947.

BiMPi (4).

A 250 mL flask was charged with oxalylhydrazide (1.6 g, 13.55 mmol, 1 eq). 150mL of ethanol was added to the reagent as well as N-methyl-4-piperidone **(**3.23 mL, 27.77 mmol, 2.05 eq) and 30mg of p-TsOH as catalyst. The mixture was refluxed for 5 hours and then cooled for 30 minutes in an ice bath to ensure full precipitation. The mixture was filtered, washed with ethanol (3x 15mL) and dried to obtain the product as a white solid (3.58 g, 11.61 mmol, 86% yield).

^{1}H NMR (600 MHz, Chloroform-*d*): δ = 10.02 (s, 2H), 2.62 (s, 8H), 2.58 – 2.55 (m, 4H), 2.55 (d, *J* = 4.7 Hz, 4H), 2.36 (s, 6H).

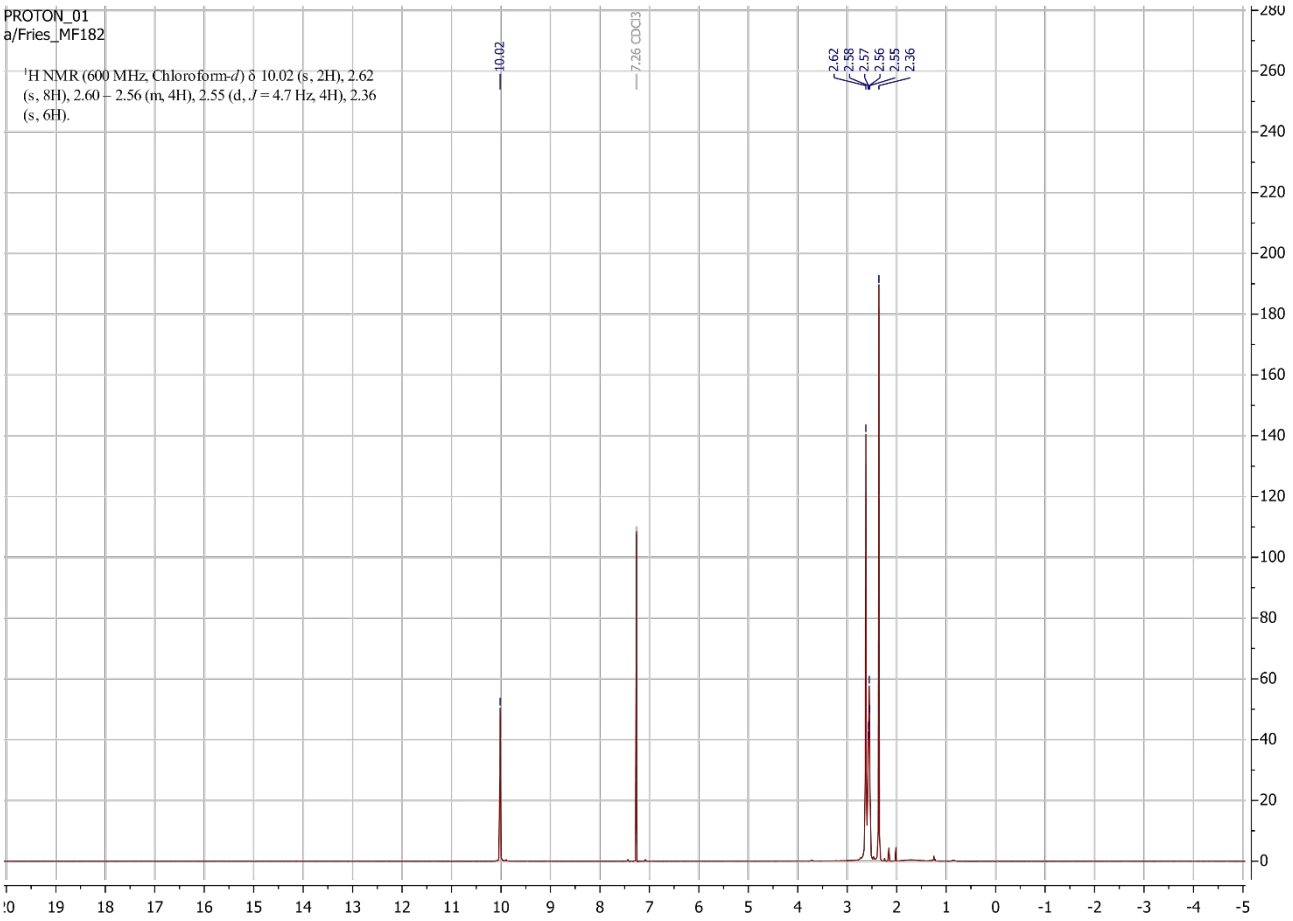

*Figure S5: 1H Spectrum of BiMPi (**4**).*

^{13}C NMR (151 MHz, Chloroform-*d*): δ = 162.52, 155.51, 55.74, 54.38, 45.74, 34.93, 27.23. Elemental analysis for $C_{14}H_{24}N_6O_2$ (308.1961): calcd. C 54.53, H 7.84, N 27.25; found C 54.39, H 7.76, N 27.25. IR(KBr): $\tilde{\nu}$ = 697, 865, 1030, 1489, 1651, 3223 cm^{-1}. ESI: calcd. for $[C_{14}H_{25}N_6O_2]^+$ $[M+H]^+$ 309.2034, found 309.2026, calcd. for $[C_{14}H_{23}N_6O_2]^-$ $[M-H]^-$ 307.1888, found 307.1909.

Complexation of compound 1 and 4 with $CuCl_2$.

Copper(II) chloride was added to BiMPi **4** as well as cuprizone **1** in water and the complexation has been followed by UV-Vis.

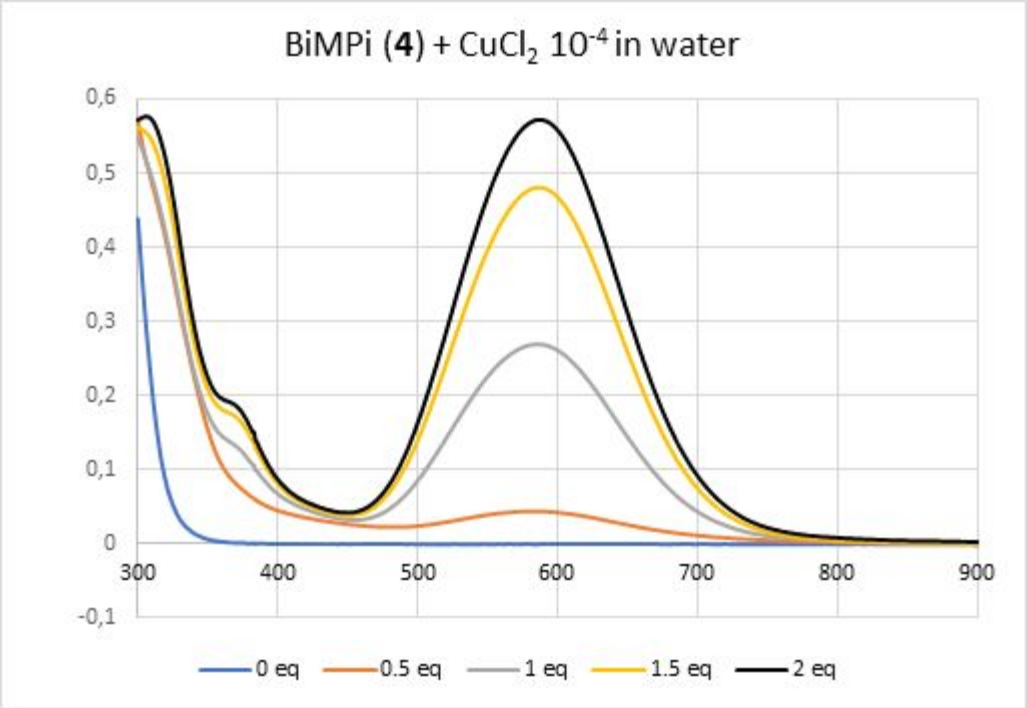

*Figure S6: UV-Vis spectrum of BiMPi (**4**) with different equivalents of $CuCl_2$.*

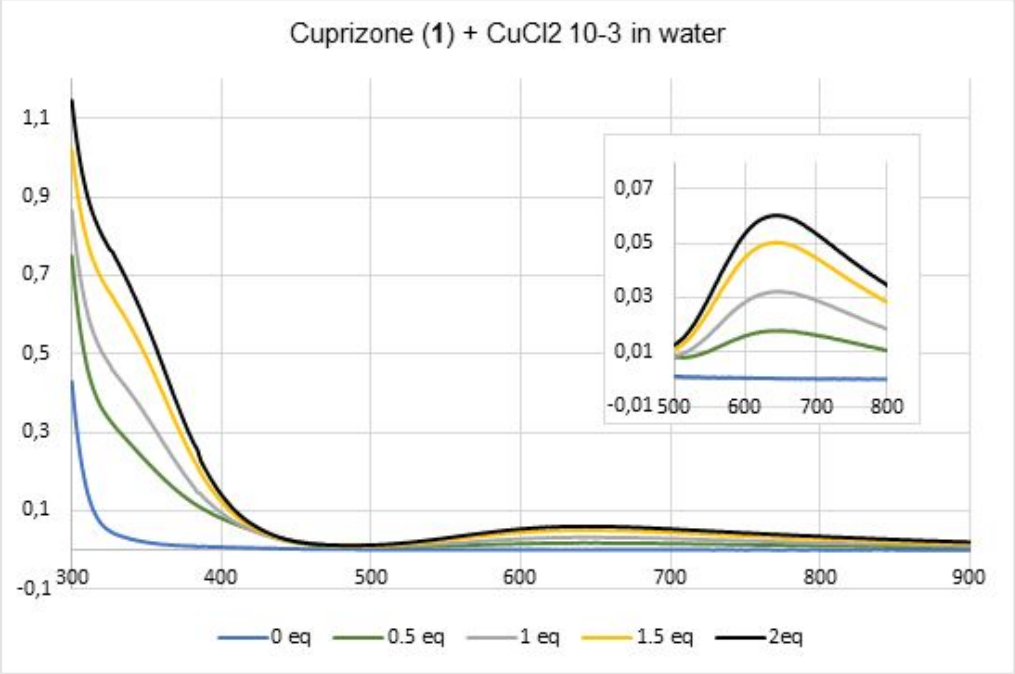

*Figure S7: UV-Vis spectrum of Cuprizone (**1**) with different equivalents of $CuCl_2$. (Differences in the UV spectrum to the one reported in the literature[S1] are due to the use of a copper(II) chloride instead acetate in non-buffered solution. Adjustment of the pH results in a spectrum similar to the one reported).*

X-ray experimental

Single crystals were obtained from ethyl acetate/hexane (**2'**) or methanol (**3** and **4**).

Single crystal X-ray data were collected at 170(1) or 173 (1) K using a Bruker-Nonius KappaCCD diffractometer with an APEX-II detector and utilizing monochromatized Mo-Kα (λ = 0.71073 Å) radiation. COLLECT[S2] software was used for the data collection (θ and ω scans) and DENZO-SMN[S3] for the processing. The absorption effects were corrected with multi-scan method by SADABS.[S4] The structures were solved by intrinsic phasing method with SHELXT[S5] and refined by full-matrix least-squares methods with SHELXL-2018/1.[S6] Anisotropic refinement was utilized for all non-hydrogen atoms. All C–H hydrogen positions were calculated and refined as a riding atom model with 1.2 or 1.5 times the thermal parameter of the C atoms. H-atoms bonded to N atoms were also found from the electron density maps, restrained (s = 0.02) to a distance of 0.91 Å from N atoms and their thermal parameters set to values of 1.2 times the N atom parameters.

Crystal Data

2': $C_{10}H_{18}N_4O_2$, F_w = 226.28, monoclinic, $P2_1/c$, a = 10.0843(5), b = 6.9309(4), c = 16.8152(6) Å, β = 103.215(3)°, V = 1144.2(1) Å^3, Z = 4, ρ_{calc} = 1.314 Mg/m^3, μ = 0.094 mm^{-1}, F_{000} = 588, crystal size 0.10 x 0.17 x 0.45 mm, θ range = 2.83 - 25.242°, 4748 reflections collected of which 2839 are independent, R_{int} = 0.0291, data completeness 99.7 %, 2 restraints, 153 parameters, GoF = 1.038, final R indices [I>2σ(I)] R1 = 0.0567 and wR2 = 0.1276, R indices (all data) R1 = 0.0894 and wR2 = 0.1429, largest diff. peak and hole 0.263 and -0.275 e.Å^{-3}. CCDC deposition number: CCDC 1846185.

3: $C_{16}H_{26}N_4O_2$, F_w = 306.41, monoclinic, $C2/c$, a = 17.4632(6), b = 11.1189(3), c = 10.0126(3) Å, β = 123.357(1)°, V = 1623.88(9) Å^3, Z = 4, ρ_{calc} = 1.253 Mg/m^3, μ = 0.085 mm^{-1}, F_{000} = 664, crystal size 0.16 x 0.27 x 0.38 mm, θ range = 2.33 - 25.242°, 4081 reflections collected of which 2125 are independent, R_{int} = 0.020, data completeness 99.6 %, 1 restraints, 103 parameters, GoF = 1.045, final R indices [I>2σ(I)] R1 = 0.0396 and wR2 = 0.0923, R indices (all data) R1 = 0.0542 and wR2 = 0.0990, largest diff. peak and hole 0.282 and -0.152 e.Å^{-3}. CCDC deposition number: CCDC 1846186.

4: $C_{14}H_{24}N_6O_8$, F_w = 308.39, triclinic, P-1, a = 5.0087(4), b = 9.2461(6), c = 9.6238(7) Å, α = 113.873(4), β = 95.049(4), γ = 97.513(4) °, V = 399.15(5) Å^3, Z = 1, ρ_{calc} = 1.283 Mg/m^3, μ = 0.090 mm^{-1}, F_{000} = 166, crystal size 0.05 x 0.06 x 0.32 mm, θ range = 2.34 - 25.242°, 3683 reflections collected of which 2063 are independent, R_{int} = 0.0435, data completeness 99.8 %, 1 restraints, 103 parameters, GoF = 1.032, final R indices [I>2σ(I)] R1 = 0.0625 and wR2 = 0.1103, R indices (all data) R1 = 0.1175 and wR2 = 0.1295, largest diff. peak and hole 0.222 and -0.242 e.Å^{-3}. CCDC deposition number: CCDC 1846187.

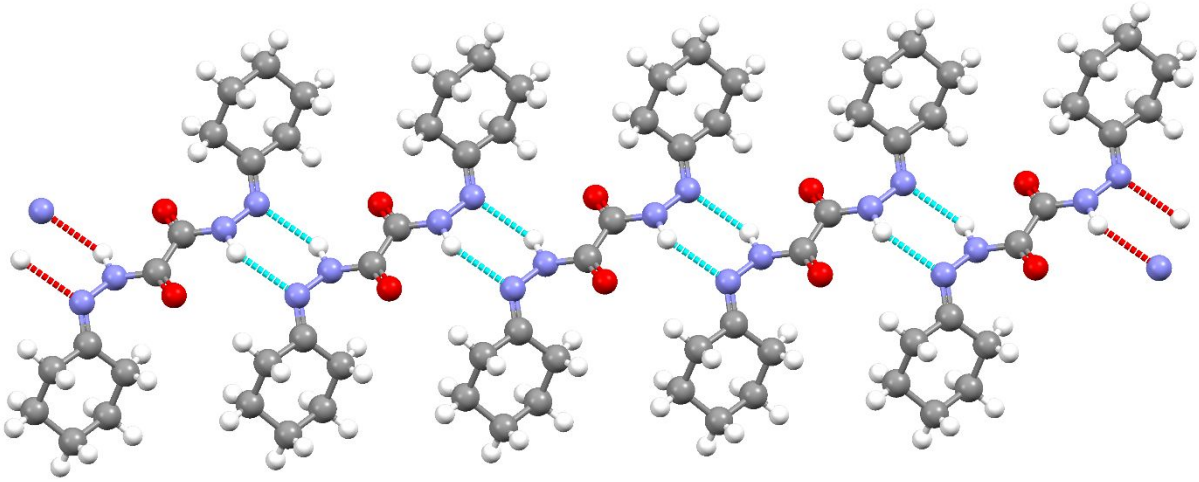

*Figure S8: Hydrogen bond motif along crystallographic b-axis in **1** (CSD: NIZTUM).*[S7]

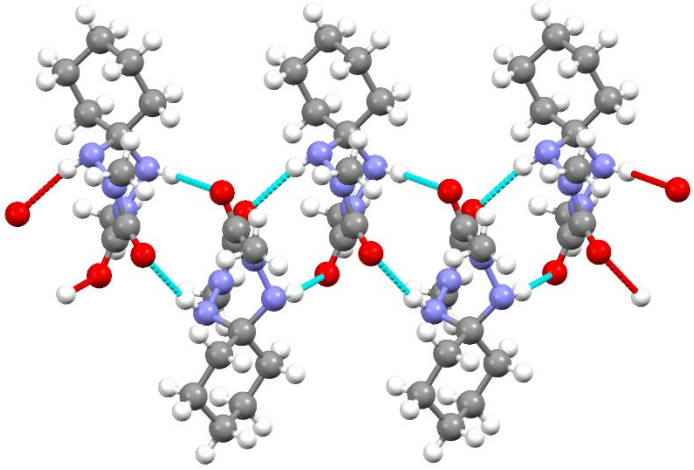

*Figure S9: Hydrogen bond motif along crystallographic b-axis in **2'**.*

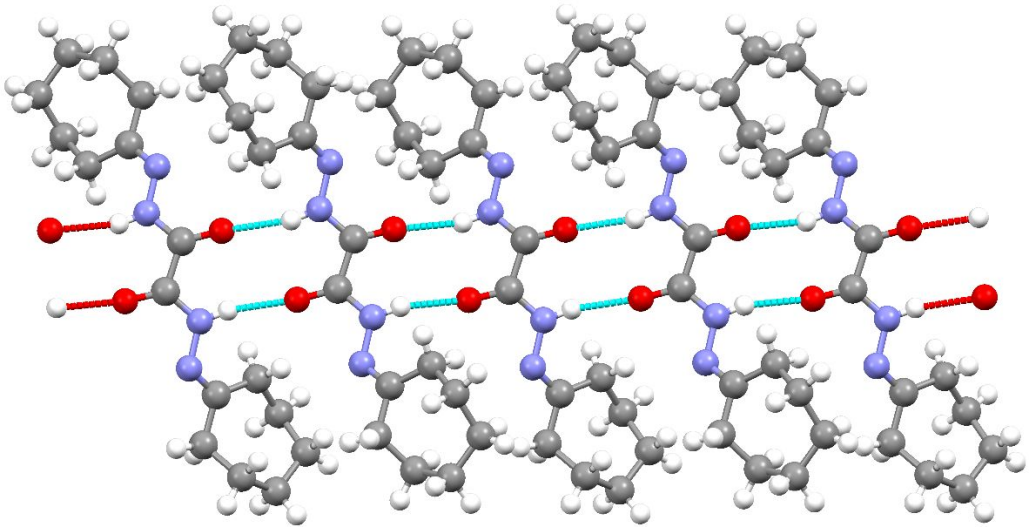

*Figure S10: Hydrogen bond motif along crystallographic c-axis in **3**.*

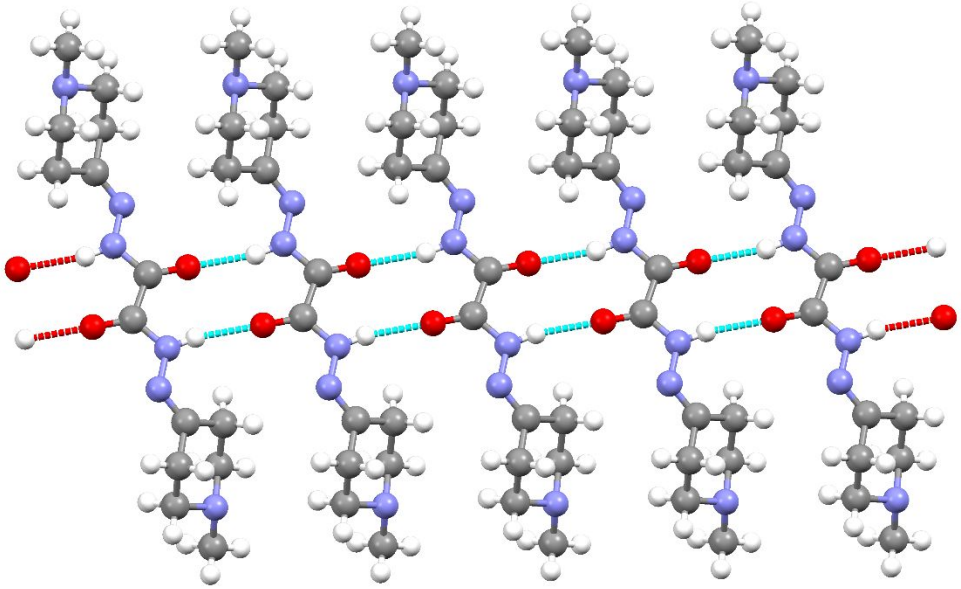

*Figure S11: Hydrogen bond motif along crystallographic a-axis in **1**.*

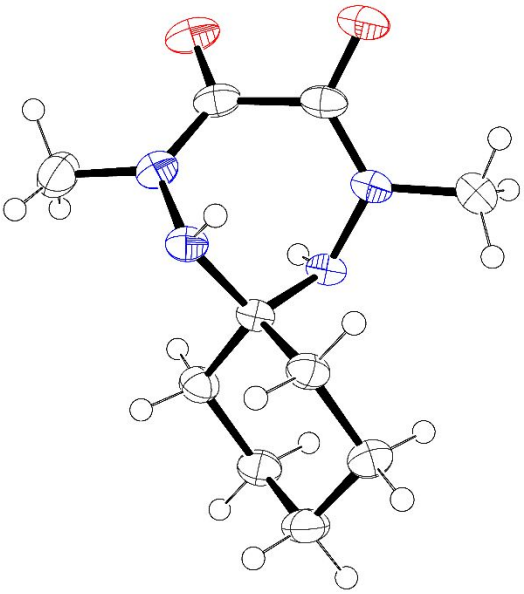

*Figure S12: Ortep diagram of **2'** with 50 % ellipsoid probability level (color codes: C = gray, H = black circles, N = blue and O = red).*

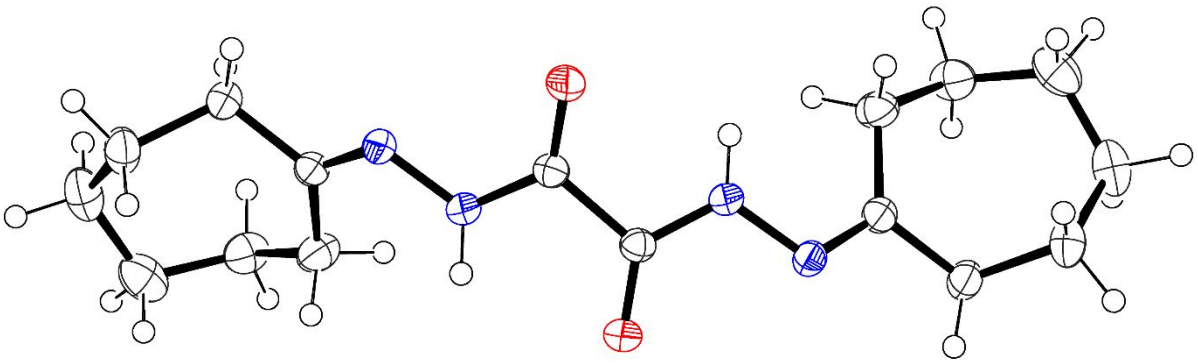

*Figure S13: Ortep diagram of **3** with 50 % ellipsoid probability level (color codes: C = gray, H = black circles, N = blue and O = red).*

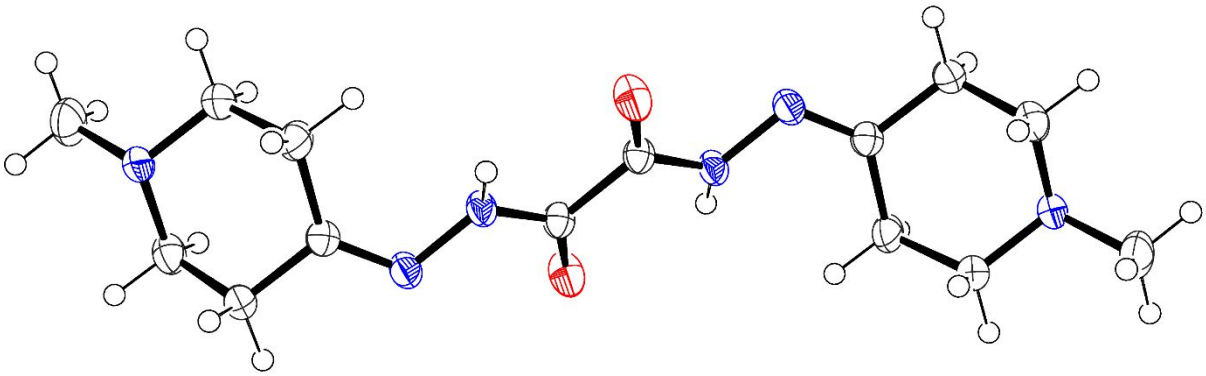

*Figure S14: Ortep diagram of **4** with 50 % ellipsoid probability level (color codes: C = gray, H = black circles, N = blue and O = red).*

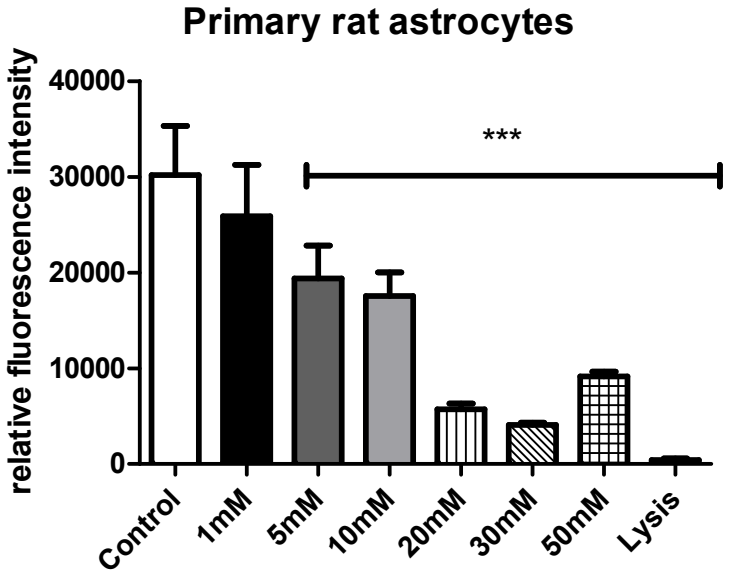

Figure S15: Dose-dependent toxicity of BiMPi in primary rat astrocyte cultures

Cell culture

The murine oligodendroglial cell line OliNeu was cultured in SATO medium containing 2% FCS. SATO is composed of Dulbecco's modified Eagle's medium (DMEM; Gibco Life Technologies, #41966-029) with 2% bovine serum albumin (BSA, Carl ROTH, #CP84.2), 1% N2 Supplement (Gibco Life Technologies, #17502-048), 1% Penicillin/Streptomycin 100 x 10.000 U/ml (Gibco Life Technologies, #15140-122), 0.1% N-Acetylcystein (Sigma-Aldrich, #9165), 0.002% Biotin (Sigma-Aldrich, #B4639).

For gene expression measurements, OliNeu cells were seeded on poly-D-lysine (PDL, Sigma #P6407) coated (10 µg/mL) 6-well plates at a density of 3 x 10^5 cells/well and exposed to starving conditions (SATO containing 0.5% FCS) for 48 h before treatment. After the experiment the cells were washed once with 1x PBS and lysed in PeqGold (peqlab, #30-1010) for RNA isolation and subsequent qRT-PCR. OLN93 cell line was received from Dr C. Richter-Landsberg (RRID:CVCL_5850; Oldenburg, Germany) (Richter-Landsberg and Heinrich 1996). For propagation, cells were grown in DMEM with 4.5 g/L D-Glucose, sodium pyruvate and L-glutamine (DMEM, Gibco life technologies # 41966-029), and were supplemented with 1% penicillin/streptomycin (Gibco Life Technologies, #15140-122), and 10% fetal bovine serum (FBS, Gibco Life Technologies, #10500-064).

For experiments, cells were seeded in plastic culture dishes pre-coated with poly-D-lysine (PDL, Sigma #P6407; 10 µg/mL) in modified SATO medium. Modified SATO is composed of DMEM with 1% N2 supplement (Gibco Life Technologies, #17502-048), 0.1% Tri-Iodo-thyronine (Sigma-Aldrich, #T6397) and 0.016% L-thyroxin (Sigma-Aldrich,# T1775). Both cell lines were cultured at 37 °C in a humidified, 5% CO_2 atmosphere with medium replenishment every 2–3 days during cell maintenance. Metabolic activity of both cell lines was determined via CellTiter-Blue Cell Viability Assay (Promega, #G8080) according to the manufacturer´s instruction. For these experiments, cells were plated in 96-well plates at a density of 1.5 x 10^4 cells/well and grown in phenol red-free SATO. Cells were treated with different concentrations in a range from 1 mM to 100 mM of BiMPi or 100 mM sodium azide (SA) for up to 24 h. After this, the fluorescence (CTB) of the supernatant was measured using the Infinite M200 microplate reader (Tecan, Switzerland). Lysed cells served as negative control (dead cells).

For primary cell cultures, rat pubs were decapitated at the age of 2-4 days, cerebral cortices were dissected, meninges removed and tissue dispersed in PBS containing 1% trypsin and 0.02% ethylenediaminetetraacetic acid (EDTA). Thereafter, cells were filtered through a 50-µm nylon mesh, and centrifuged at 400 x g for 5 min. Cells were re-suspended in DMEM (Gibco Life Technologies, #41966-029) containing 10% FCS.[S11-S13]

Primary rat astrocytes were isolated from these mixed glia cell cultures after 10–12 days in culture by first detaching microglia by shaking for 2 hours at 130 rpm at 37°C (pre-shaking). Thereafter oligodendrocyte precursors were removed from the culture by shaking for additional 18 hours at 230 rpm at 37°C. The remaining adhesive astrocytes were propagated in a 1:3 dilution to 10 cm tissue culture dishes and further cultured in DMEM supplemented with 1% penicillin/streptomycin (Gibco Life Technologies, #15140-122), and 10% fetal bovine serum (FBS, Gibco Life Technologies, #10500-064). Astrocytic cells were used for experiments after two passages and seeded into 6-well plates at a density of 2 x 10^5 cells/well. Before treatment with BiMPi, cells were exposed to starving conditions for 24 hours (DMEM containing 0.5% FCS) to induce a non-proliferating, “resting” phenotype.

SOD activity measures

For SOD measurements in animal tissue, mice (n=4) were fed with 0.2% cuprizone as published previously.[S9] Mice receiving normal chow served as controls. Thereafter, mice were sacrificed and perfused with 30 ml ice cold 1 x PBS, brains quickly dissected and snap frozen in liquid nitrogen. 50 mg of frozen tissue (telencepahlic cortex and adjacent corpus callosum) was homogenized in 1.5 ml sucrose buffer (17,114 g sucrose; 36,434 g D-Mannit in 1L Tris/EDTA buffer) using a glass homogenizer. Samples were centrifuged at 10,000 x g at 4°C, the supernatant collected and further used for protein concentration measurements

using Pierce™ BCA Protein Assay Kit (Thermo Fisher Scientific #23225). SOD activity (see below) was normalized to total protein levels. SOD activity was measured with the Superoxide Dismutase Colorimetric Activity Kit (Thermo Scientific # EIASODC) according to the manufacturers instructions.

Mitochondrial membrane potential measures

Mitochondrial membrane potential was quantified using the Muse™ MitoPotential Kit (Merck, Germany, #MCH100110). To this end, cells were seeded onto 9.6 cm^2 (~5 x 10^5 cells/well) plastic culture dishes pre-coated with 10 µg/mL poly-D-lysin in modified SATO medium. After 47 h, cells were exposed to BiMPi for another 60 min and then harvested using TrypLE™ (Thermo Fisher Scientific, Germany) Express enzyme solution (Gibco Life Technologies, #12604039). Thereafter, cells were stained and flow cytometry analyses were performed according to the manufacturers instruction. To avoid any bias, gating of the different cell populations was performed in a blinded manner. Experiments were performed with three biological and one technical replicate.

Gene expression analysis

For gene expression analyses, cells were treated for 24 hours with 1 and 5 mM BiMPi in modified SATO medium. Gene expression levels were measured using reverse transcription real time-PCR technology (RT rt-PCR; Bio-Rad, Germany), SensiMix Plus SYBR plus Fluorescein (Quantace, Bioline, #QT615-05), and a standardized protocol as described previously by our group.[S10] Experiments were performed with at least two biological and three technical replicates. Primer sequences and individual annealing temperatures are as follows:

HPRT: CACAGGACTAGAACACCTGC (forward) and GCTGGTGAAAAGGACCTCT (reverse) at 62°C annealing temperature (AT).

GAPDH: TGTGTCCGTCGTGGATCTGA (forward) and CCTGCTTCACCACCTTCTTGA (reverse) at 65°C AT.

HO1: AAGCCGAGAATGCTGAGTTCA (forward) and GCCGTGTAGATATGGTACAAG (reverse) at 62°C AT.

NQ1: CTACCCCCAGTGGTGATAGAAA (forward) and AGAGAGTGCTCGTAGCAGGAT (reverse) at 60°C AT.

Olig2: GGCGGTGGCTTCAAGTCATC (forward) and TAGTTTCGCGCCAGCAGCAG (reverse) at 61°C AT.

PLP1: TGGCGACTACAAGACCACCA (forward) and GACACACCCGCTCCAAAGAA (reverse) at 56°C AT.

Relative quantification was performed using the delta-delta Ct method which results in ratios between target genes and the reference genes (HPRT and GAPDH). Melting curves and gel electrophoresis of the PCR products were routinely performed to determine the specificity of the PCR reaction.

Statistics

Statistical analyses were performed using Prism 5 (GraphPad Software Inc., San Diego, CA). All data are given as arithmetic means +/- SEMs. If not stated otherwise, at least two experimental settings were performed. Equal variances and normal distribution were checked by the Bartlett test and the Shapiro-Wilk test respectively. Intergroup differences of parametric data were tested ANOVA, followed by Tukey's or Bonferroni post hoc tests using GraphPad Prism 5 (GraphPad Software). All data are given as arithmetic means ± SEM. The *p* values are indicated as $*p \leq 0.05$, $**p \leq 0.01$, $***p \leq 0.001$. For further information, see figure legends.

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