Anionic porphyrin as a new powerful cell death inducer of Tobacco Bright Yellow-2 cells.

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ELECTRONIC SUPPLEMENTARY INFORMATION

Materials and Methods

General

Meso-tetra (N-methyl-4-pyridyl) porphyrin tetrachloride (**CP**) and Zn(II) *meso*-tetra (N-methyl-4-pyridyl) porphine tetrachloride (**CP-Zn**) were purchased from Frontier Scientific Inc. (Logan, UT, USA). *Meso*-tetra (p-sulfonatophenyl) porphyrin tetrasodium salt (**AP**) was purchased from Sigma-Aldrich (St Louis, MO) as all other compounds and reagents, except for **AP-Zn** which was obtained by metallation of **AP** with zinc acetate. All solvents were purchased from Carlo Erba Reactifs (Nanterre,France). ^{1}H NMR spectroscopy was performed with a Bruker DPX 400 spectrometer. Chemical shifts are reported as δ (parts per million), downfield from internal TMS.

Synthesis of Zinc (II) *Meso*-tetra (p-sulfonatophenyl)porphyrin tetrasodium salt (AP-Zn) :

AP (60.3 mg, 62 µmol) and zinc(II) acetate (4.25 equiv, 48.3 mg, 0.264 mmol) were dissolved in 17 mL of water. The mixture was stirred for 3 h at reflux and the progress of the reaction was monitored by UV-vis absorption spectroscopy. Thus, crude was purified by dialysis (1000 Da membrane) during 24 hours. **AP-Zn**, after lyophilisation, was obtained as a purple solid with a quantitative yield. ^{1}H NMR (DMSO d_6, 400 MHz) δ ppm 8.78 (s, 8H), 8.13 (d, J=7.96 Hz, 8H), 8.00 (d, J=7.96 Hz, 8H); UV-vis (H_20) λmax nm (ε, L.mol^{-1}.cm^{-1}) 422 (434700), 557 (13700), 596 (5500).

UV-visible and fluorescence spectroscopy

UV–vis spectra were so recorded on a Specord 210 (Analytik Jena) double-beam spectrophotometer using 10 mm quartz cells. Fluorescence spectra were recorded in non-deoxygenated solvents at 20 □C with a QM-4/QuantaMaster fluorometer from PTI equipped with rapid monochannel detection and continuous excitation source. Quantum yields were determined using cresyl violet as a standard reference (Φf) 0.54 at 20 □C in MeOH.

Electron Paramagnetic Resonance

The samples were exposed to a 20 W halogen lamp. The intensity of illumination was measured by a luxmeter (Digital Lux Tester YF-1065). EPR spectra were recorded with a Bruker Model ESP300E spectrometer operating at room temperature.

Singlet oxygen detection. To 50 µL of fresh TEMP solution (25 mM in 0.01M phosphate buffer, pH 7.4) were added 50 µL of fresh porphyrin solution (80 µM in 0.01M phosphate

buffer, pH 7.4). The solution obtained was then immediately transferred into quartz capillaries (100 µL) and placed at 20 cm from the source of illumination with a light intensity of 270 $\mu E.s^{-1}.m^{-2}$. EPR spectra were performed under the following conditions: modulation frequency, 100 kHz; microwave frequency, 9.78 GHz; microwave power, 4 mW; modulation amplitude, 0.987 G; time constant, 10.24 ms; scans number 2.

Superoxide anion detection. To 50 µL of fresh DMPO solution (225 mM in DMSO) were added 50 µL of fresh porphyrin solution (different concentration in DMSO- water 90-10 v/v solution). The solution obtained was then immediately transferred into quartz capillaries (100 µL) and placed at 40 cm from the source of illumination with light intensity of 68 $\mu E.s^{-1}.m^{-2}$. The ER conditions were the same as above except: microwave power 20 mW.

Biological experiments

Plant cell suspensions. Tobacco cell (Bright Yellow-2) suspensions cultures were maintained in the dark under continuous agitation (140 rpm) at 22 °C. TBY-2 cell suspensions was growing into liquid modified Murashige and Skoog medium supplemented by 0.27 $mg.L^{-1}$ 2,4-dichlorophenoxyacetic acid and 10 $mg.mL^{-1}$ thiamine solutions. The suspensions were subcultured every 7 days by transferring 3 mL aliquot into 40 mL of fresh medium. Exponential growth phase cells (3 or 4 days after dilution) were used for all the experiences.

Experimental protocol. 10 mL of cells were incubated with porphyrins (final concentration: 3.5 $\mu mol.L^{-1}$) for 3 hours under dark and agitation (140 rpm). Porphyrins (1 mM stock solution) were dissolved in water, conserved at room temperature under dark and used for the experiments. After 3 h incubation with the porphyrins under dark conditions, cells were centrifugated at 3000 rpm for 3 min at room temperature. Supernatant was discarded. 20 mL of TBY-2 medium was added to the pellet and cells were centrifugated to throw away the excess of porphyrins. Supernatant was disrupted and 20 mL of new TBY-2 medium was

finally added to cells. Cell suspensions were then placed for 5 h at 95 µE. $s^{-1}.m^{-2}$ (Neon tubes 36W 860, ClaudLux Germany) or in the dark (control cells). After illumination, cells were put back to dark for 18 h. Cell death percentage was determined by Trypan blue staining (0.05 % w/v) and counting (> 1500 cells per condition) under photonic microscope. 10 µL of 3,3'-diaminobenzidine tetrahydrochloride (DAB) solution (DAB liquid substrate dropper system, Sigma St Louis, MO.) was diluted into 1 mL aliquot of TBY-2 cell suspensions. After 1 hour incubation under dark at 22 °C, cells were counting and percentage of DAB stained cells, corresponding to H_2O_2 producing cell percentage, was evaluated.

DNA extraction. 500 µl of treated or control TBY-2 cells were ground twice using beads (2 mm diameter) and a RETSCH grounder (GmbH, Germany) for 30 s at maximal frequency. 750 µL of extraction buffer, pre-warmed at 65°C (100 mM Tris-HCl pH 8, 700 mM NaCl, 50 mM EDTA, 140 mM β-mercaptoethanol and 1 % (w/v) CTAB) were added to cells. After a 30 min at 65 °C incubation, 40 µL of proteinase K (20 mg/ml) were added to tubes that were kept at 37 °C for 1h. 250 µL of aqueous NaCl (5 $mol.L^{-1}$) was then added and a centrifugation at 13000 rpm for 10 min at room temperature was performed. 750 µL of isopropanol was added to 750 µL of supernatant. After centrifugation, the pellet was rinsed twice with 70 % ethanol, dried, resuspended in 80 µL of H_2O and kept overnight at 4°C. 40 µL of aqueous DNA was tested on 1.8 % agarose gel (50 V, 8 h migration).

Statistical analysis. R package (ver. 2.13.1, R Development Core Team 2012, Vienna Austria) was used for statistical analyses. Percentages were compared by using the chi-square test of independence.

UV-visible absorption and fluorescence emission spectra in water