Materials and Methods

L-Ascorbic acid (Honeywell Riedel-de-Haën, Seelze, Germany); CaCl2 (Sigma Aldrich, Munich, Germany); CdCl2·H2O (Honeywell Fluka™, Seelze, Germany); Chelex® 100 Resin (Bio-Rad, Hercules, CA, USA); CoCl2·6H2O (Honeywell Riedel-de-Haën, Seelze, Germany); CuSO4 (Sigma Aldrich, Munich, Germany); Dulbecco’s modified Eagles medium (DMEM) (PAN-Biotech, Aidenbach, Germany); EGTA (Honeywell Fluka™, Seelze, Germany); FeSO4·7H2O (Merck-Millipore, Darmstadt, Germany); Fe2(SO4)3 (Sigma Aldrich, Munich, Germany); fetal calf serum (FCS) (CCPro, Oberdorla, Germany); HgCl2 (Merck-Millipore, Darmstadt, Germany); MgCl2·6H2O (Honeywell Riedel-de-Haën, Seelze, Germany); MnSO4·H2O (Carl Roth, Karlsruhe; Germany); Na2EDTA∙2H2O (Carl Roth, Karlsruhe; Germany); Pb(NO3)2 (Sigma Aldrich, Munich, Germany); Penicillin-Streptomycin solution (Sigma Aldrich, Munich, Germany); ZnSO4·7H2O (Sigma Aldrich, Munich, Germany)

Synthesis of phosphonate-functionalized porphyrins

The phosphonate-functionalized porphyrins were synthesized as metal-free variants employing our recent research methodologies. The synthesis of 5,10,15,20-tetrakis[p-(diisopropoxyphosphoryl)phenyl]porphyrin (p-H8TPPA-iPr8) and 5,10,15,20-Tetrakis[p-phenylphosphonic acid] porphyrin (p-H8TPPA) is described in Maares et al. (2019) [1]. 5,10,15,20-Tetrakis[m-phenylphosphonic acid] porphyrin (m-H8TPPA) was synthesized in a Pd-catalyzed Arbuzov reaction according to Yücesan et al. (2020) [2]. All compounds were separately dissolved as 10 mM stock solutions in DMSO.

General measurements of absorption and fluorescence spectroscopy

Absorption spectra were recorded with a Tecan Infinite M200 Reader (Tecan Austria GmbH, Grödig/Salzburg, Switzerland) from 350 to 500 nm. The same device was used in fluorescence mode to measure fluorescence excitation (350-500 nm; λ Em. 650 nm) and fluorescence emission (550-850 nm; λ Exc. 415 nm) spectra. 3D scanning was performed on a Spark® multimode microplate reader (Tecan Austria GmbH, Grödig/Salzburg, Switzerland) from 350 to 450 nm (Exc.) and 555 to 850 nm (Em.) with a 5 nm step size, excitation and emission bandwidths of 5 nm, and a detector gain of 100. A typical reaction mix was made by combining equal volumes of 20 μM phosphonate porphyrins diluted in assay-buffer (50 mM HEPES in bidistilled water adjusted to pH 6.5 with sodium hydroxide solution; depleted of multivalent cations with Chelex® 100 Resin pretreatment [3]) and a double-concentrated metal cation solution. Following 30 min incubations at 37°C absorption or fluorescence measurements were done. For redox conversion tests the CuSO4 solutions were pretreated with 500 µM of L-ascorbic acid before admixing into the phosphonate porphyrin dilutions. To evaluate the effect of metal chelators, the metal cation-treated incubation mixtures were posttreated with 50 equivalents of EDTA or EGTA for 15 min before fluorescence emission readout.

Time-correlated single photon counting (TCSPC)

Fluorescence decay curves were recorded on samples of p-H8TPPA and m-H8TPPA at a final concentration of 10 μM in assay-buffer solution in standard 1x1 cm glass cuvettes while admixing amounts of 100 μM and 1 mM CuSO4 to achieve the actual cupric concentration. After addition the sample was gently mixed and incubated for 1 minute. Measurements were performed employing a Hamamatsu R5900 16-channel multi-anode photomultiplier tube (PMT) with 16 separate output (anode) elements and a common cathode and dynode system (PML-16C, Becker&Hickl, Berlin, Germany) as described in Schmitt et al. (2019) [4]. A 405 nm pulsed laser diode (LDH-405, Picoquant, Berlin) delivering 80 ps FWHM pulses, driven at a repetition rate of 20 MHz, was used for excitation. The fluorescence was observed via a 430 nm longpass filter (FF01-430/LP-25, AHF Analysentechnik, Tübingen, Germany). The decay curves were fitted employing a Levenberg-Marquardt algorithm for the minimization of the χ2 with Origin® (Origin Inc. Illinois, USA) [4].
Cell culture
The human intestinal cell line Caco-2 (European Collection of Cell Cultures, Porton Down, UK) were routinely cultured at 37°C, 5% CO₂ and humidified atmosphere in Dulbecco’s Modified Eagles Medium (DMEM), containing 10% fetal calf serum (FCS) 100 U/mL penicillin and 100 µg/ml streptomycin. For cellular phosphonate porphyrins experiments, Caco-2 cells were transferred into 96 wells (initially seeding 5000 cells per well) and cultured for 14 days for differentiation into an enterocyte-like monolayer \[5,6\]. For phosphonate labeling, differentiated cells were treated with either \(m\)-H₈TPPA, \(p\)-H₈TPPA or \(p\)-H₈TPPA-iPr₈ in a HEPES-based incubation buffer (10 mM HEPES, pH 7.35, 120 mM NaCl, 5.4 mM KCl, 5 mM glucose, 1.3 mM CaCl₂, 1 mM MgCl₂, 1 mM Na₂HPO₄, 0.3 % bovine serum albumin) for 30 min. Excess fluorescent dye was removed by multiple washing steps before fluorescence emission scanning using 416 nm excitation (Tecan Infinite M200 reader; Tecan, Grödig/Salzburg, Germany). To analyze metal responsiveness of the intracellular phosphonate porphyrins, cells were post-treated with 50 µM of different metal solutions at 37°C in an albumin-free incubation buffer in a time-course experiment.

Confocal imaging
Fluorescence images of phosphonate porphyrin-labeled Caco-2 enterocytes were acquired on a Leica TCS SP8 laser scanning confocal microscope equipped with LAS X 3.5.5.19976 software platform, using a HC PL APO CS2 63x/1.20 water objective. The filters settings were \(\lambda_{\text{Exc}}\) 488 nm/ \(\lambda_{\text{Em}}\) 580 nm; pinhole 111.5 µm, pinhole size 1 AU.
Suppl. Fig. 1 Metal-dependent changes of m-H₈TPPA absorbance and fluorescence properties.
10 μM m-H₈TPPA dilutions were treated with various metal solutions (final concentration 40 μM) before recording (A) absorbance, (B) fluorescence excitation (λₘₐₓ 650 nm) and (C) fluorescence emission (λₑₓc 415 nm) spectra. Data are representative for three independent experiments.

Suppl. Fig. 2 Caco-2 fluorescence labeling with Phenylphosphonate-substituted porphyrines
Caco-2 cells were incubated with increasing concentrations of p-H₈TPPA or m-H₈TPPA followed by fluorescence measurement (λₑₓc 416 nm/650 nm). Data are means ± S.E.M. of n=3 independent experiments.
A) p-H$_2$THPPA

Buffer

\begin{array}{c}
\text{Excitation (nm)} \\
\text{Fluorescence (a.u.)} \\
\text{Emission (nm)}
\end{array}

Co$^{2+}$

\begin{array}{c}
\text{Excitation (nm)} \\
\text{Fluorescence (a.u.)} \\
\text{Emission (nm)}
\end{array}

Cd$^{2+}$

\begin{array}{c}
\text{Excitation (nm)} \\
\text{Fluorescence (a.u.)} \\
\text{Emission (nm)}
\end{array}

Hg$^{2+}$

\begin{array}{c}
\text{Excitation (nm)} \\
\text{Fluorescence (a.u.)} \\
\text{Emission (nm)}
\end{array}
Suppl. Fig. 3 3D fluorescence spectra of the phosphorylphenyl substituted porphyrins after metal cation treatment

10 µM p-H8TPPA (A) or m-H8TPPA (B) dilutions were treated with either buffer or 40 µM CoCl2, CdSO4 or HgCl2. A 3D scan was performed from 350 to 450 nm (Exc.) and 555 to 850 nm (Em.), with a 5 nm step size and excitation and emission bandwidths of 5 nm and a detector gain of 100.
Suppl. Fig. 4 Fluorescence titration of p-H_8TPPA and m-H_8TPPA upon addition of metal cations

10 µM p-H_8TPPA or p-H_8TPPA solutions were treated with increasing quantities of metal cations followed by detection of fluorescence emission (\( \lambda_{\text{Exc}}/\lambda_{\text{Em}} \), 416 nm/650 nm). Data are means ± S.E.M. of at least n=3 independent experiments. Sigmoidal dose–response curves were fitted by non-linear regression and the resulting EC_{50} values are indicated.
Suppl. Fig. 5 Effect of Cu-redox conversion on p-H₈TPPA and m-H₈TPPA fluorescence properties

10 μM CuSO₄ solutions pretreated +/- 500 μM of l-ascorbic acid (15 min) were mixed with p-H₈TPPA or m-H₈TPPA followed by detection of fluorescence emission (λ_{ex/em} 416 nm/650 nm). Data are means ± S.E.M. of n=3 independent experiments. Statistically significant differences from control ***p<0.001; two-way ANOVA/ Tukey post hoc test) or from Cu²⁺ alone (#p< 0.05; two-way ANOVA/ Tukey post hoc test) are indicated.

Suppl. Fig. 6 Summary of cellular p-H₈TPPA and m-H₈TPPA metal responsiveness

Data were taken from the last fluorescence readout shown in Fig. 6. Data are means ± S.E.M. of n=3 independent experiments. Significant differences from buffer incubation ***p<0.001; **p<0.01; *p<0.05; one-way ANOVA/ Dunnett's multiple comparison test.
References:

[1] M. Maares, M. M. Ayhan, K. B. Yu, A. O. Yazaydin, K. Harmandar, H. Haase, J. Beckmann, Y. Zorlu, G. Yücesan, Chem. – A Eur. J. 2019, 25, 11214 –11217.

[2] G. Yücesan, Y. Zorlu, C. Brown, C. Keil, M. M. Ayhan, H. Haase, R. B. Thompson, I. Lengyel, Chem. – A Eur. J. 2020, chem.202001613.

[3] W. Alker, T. Schwerdtle, L. Schomburg, H. Haase, Int. J. Mol. Sci. 2019, 20, 4006.

[4] F.-J. Schmitt, · Züleyha, Y. Campbell, · Mai, V. Bui, A. Hüls, T. Tomo, · Min Chen, E. G. Maksimov, · Suleyman, I. Allakhverdiev, T. Friedrich, Photosynth. Res. 2019, 139, 185–201.

[5] M. Pinto, S. Robine-Leon, M.-D. Appay, M. Kedinger, N. Triadou, E. Dussaulx, B. Lacroix, P. Simon-assmann, K. Haffen, J. Fogh, A. Zweibaum, Biol. Cell 1983, 47, 323–330.

[6] A. Ferraretto, M. Bottani, P. De Luca, L. Cornaghi, F. Arnaboldi, M. Maggioni, A. Fiorilli, E. Donetti, Biosci. Rep. 2018, 38, 1–16.