Electronic Supplementary Material (ESI) for ChemComm.

Supporting Information

Silica particles with a Quercetin-R5 peptide conjugate are taken up into HT-29 cells and translocate into the nucleus

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Synthesis of quercetin derivative

2-(3,4-diacetoxyphenyl)-4-oxo-4H-chromene-3,5,7-triyl triacetate (2)

20 mg (66.2 µmol, 1 eq.) quercetin were dissolved in 1 mL of anhydrous pyridine at room temperature. 62.2 µL (67.6 mg, 662 µmol, 10 eq.) acetic anhydride were added to the yellow solution. It was then heated to 70°C and stirred for 6 h. The solvent was removed to obtain a slightly brownish, crystalline solid, which was then recrystallized from a minimal amount of acetone. Yield: 80 %.

HR-MS (pos.):

$[M+H]^+$ m/z calc. 513.1028 obs. 513.1034

$[M+Na]^+$ m/z calc. 535.0847 obs. 535.0853

^{1}H-NMR (600.25 MHz, $CDCl_3$): δ = 2.32 (s, 6H), 2.33 (s, 3H), 2.34 (s, 3H), 2.43 (s, 3H), 6.87 (d, J = 2.2 Hz, 1H), 7.33 (d, J = 2.2 Hz, 1H), 7.34 (d, J = 8.5 Hz, 1H), 7.69 (d, J = 2.1 Hz, 1H), 7.71 (dd, J = 8.5 Hz, 2.1 Hz, 1H) ppm.

^{13}C-NMR (600.25 MHz, $CDCl_3$): δ = 20.63, 20.78 (2C), 21.15, 21.29, 109.11, 114.01, 114.87, 123.95, 124.05, 126.54, 127.86, 134.16, 142.31, 144.50, 150.50, 153.88, 154.38, 156.96, 167.87, 167.93, 167.95, 167.98, 169.36, 170.14 ppm.

4-(3,5-diacetoxy-7-hydroxy-4-oxo-4H-chromen-2-yl)-1,2-phenylene diacetate (3)

2.87 g (5.60 mmol, 1 eq.) quercetin penta-acetate (**2**) were dissolved in a solution of 351 mg (5.15 mmol, 0.92 eq.) imidazole in 50 mL N-methyl-2pyrrolidone (NMP) using the sonicator. The solution was cooled to 0°C in a salt-and-ice bath before adding 540 µL (5.32 mmol, 0.95 eq.) thiophenol. The mixture was stirred for 1.5 h while warming to room temperature. Afterwards it was acidified with 50 mL of 2M HCl and extracted three times with 100 mL ethyl acetate. The unified extracts were dried at the rotavap. The crude product was purified by silica flash column chromatography (petroleum ether/EtOAc = 1:2). The product was obtained in 21 % yield.

HR-MS (pos.):

$[M+H]^+$ m/z calc. 471.0922, obs. 471.0917

$[M+Na]^+$ m/z calc. 493.0741, obs. 493.0735

$[2M+Na]^+$ m/z calc. 963.1590, obs. 963.1581

^{1}H-NMR (600.25 MHz, *d_6*-DMSO): δ = 2.29 (s, 3H), 2.30 (s, 3H), 2.33 (s, 3H), 2.33 (s, 3H), 6.64 (d, J = 2.3 Hz, 1H), 6.93 (d, J = 2.3 Hz, 1H), 7.51 (d, J = 8.5 Hz, 1H), 7,80 - 7.82 (m, 2H), 11.33 (s, 1H) ppm.

^{13}C-NMR (600.25 MHz, *d_6*-DMSO): δ = 20.69, 20.84, 20.91, 21.34, 101.45, 109.50, 109.78, 124.01, 124.91, 126.97, 127.90, 133.22, 142.60, 144.66, 150.66, 152.86, 158.02, 163.29, 168.37, 168.50, 168.67, 169.21, 169.41 ppm.

4-(3,5-diacetoxy-7-(2-ethoxy-2-oxoethoxy)-4-oxo-4H-chromen-2-yl)-1,2-phenylene diacetate (4)

327.4 mg (696 µmol, 1 eq.) quercetin tetra-acetate (**3**) were dissolved in 20 mL dry DMF with 4 Å molecular sieves under argon atmosphere. 108 µL (195 mg, 912 µmol, 1.31 eq.) ethyl iodoacetate were added and the mixture was cooled to 0°C in an ice bath. While stirring at 0 °C, 270 mg (828 µmol, 1.19 eq.) Cs_2CO_3 were added to the solution. The mixture was allowed to warm to room temperature and stirred for 1.5 h in the dark at rt. Afterwards it was titrated to pH 6 using HCl. The solvent was removed and the residue purified by silica flash column chromatography (petroleum ether/EtOAc = 1:1.5). A slightly beige solid was obtained in 75 % yield (289 mg, 519 µmol).

HR-MS (pos.):

$[M+Na]^+$ m/z calc. 579.1109 obs. 579.1115

$[2M+Na]^+$ m/z calc. 1135.2326 obs. 1135.2323

^{1}H-NMR (600.25 MHz, $CDCl_3$): δ = 1.29 (t, J = 7.1 Hz, 3H), 2.310 (s, 3H), 2.313 (s, 6H), 2.41 (s, 3H), 4.28 (q, J = 7.1 Hz, 2H), 4.69 (s, 2H), 6.67 (d, J = 2.5 Hz, 1H), 6.78 (d, J = 2.5 Hz, 1H), 7.32 (d, J = 8.5 Hz, 1H), 7.64 (d, J = 2.1 Hz, 1H), 7.68 (dd, J = 8.5 Hz, 2.1 Hz, 1H) ppm.

^{13}C-NMR (600.25 MHz, $CDCl_3$): δ = 14.15, 20.55, 20.69 (2C), 21.11, 61.92, 65.61, 99.76, 109.04, 111.84, 123.76, 123.89, 126.41, 127.99, 133.93, 142.19, 144.24, 150.94, 153.35, 157.89, 161.89, 167.45, 167.79, 167.86, 168.02, 169.40, 169.94 ppm.

2-((2-(3,4-dihydroxyphenyl)-3,5-dihydroxy-4-oxo-4H-chromen-7-yl)oxy)acetic acid (5)

300 mg (539 µmol) **4** were dissolved in 20 mL acetone. 10 mL of 6M hydrochloric acid were added. The colorless mixture was refluxed for 1.5 h. During reaction, it turned yellow. After cooling down, 100 mL water, 100 mL ethyl acetate and 1 mL brine were added. The phases were separated and the aqueous phase extracted two more times with 100 mL ethyl acetate each. The unified organic extracts were washed with 3 M HCl (3 x 100 mL). The solvent was then removed to yield 168 mg (90%) of product.

MS (pos.):

$[M+H]^+$ m/z calc. 361.06 obs. 360.57

MS (neg.):

$[M-H]^-$ m/z calc. 359.04 obs. 359.02

^{1}H-NMR (600.25 MHz, d_6-DMSO): δ = 4.84 (s, 2H), 6.36 (dd, J = 7.8 Hz, 2.2 Hz, 1H), 6.70 (dd, J = 11.4 Hz, 2.2 Hz, 1H), 6.89 (d, J = 8.5 Hz, 1H), 7.60 (ddd, J = 30.1 Hz, 8.6 Hz, 2.2 Hz, 1H), 7.73 (dd, J = 10.1 Hz, 2.2 Hz, 1 H), 9.31 (s, 1H), 9.51 (s, 1H), 9.63 (s, 1H), 12.43 (s, 1H), 12.49 (s, 1H) ppm.

^{13}C-NMR (600.25 MHz, d_6-DMSO): δ = 64.86, 92.55, 97.82, 104.27, 115.20, 115.58, 120.06, 121.82, 136.07, 145.09, 147.38, 147.88, 155.85, 160.36, 163.29, 169.56, 175.94 ppm.

Coupling to the R5 peptide

The 20 amino acid CR5 peptide was synthesized on Wang resin using Fmoc-based SPPS. It was either done manually or on a CEM Liberty Blue peptide synthesizer (Matthews, NC). Coupling of the modified quercetin (2.5 equivalents) was performed on resin using 1.97 equivalents of HOBt and 1.95 equivalents of DIC for activation. The reaction was allowed to proceed overnight in the absence of light. The resin-bound product was dried before cleaving it with a standard cleavage cocktail (92.5 % TFA, 2.5 % H2O, 5 % Tis). The cleaved conjugate was then purified by RP-HPLC using a C18 semiprep column. Purity of the conjugate was confirmed by analytical RP-HPLC and ESI-MS.

Quercetin-R5 spectra

Spectra of the conjugate were recorded at 1 mg/mL concentration in 50 mM potassium phosphate buffer (pH 7). The most efficient fluorescence is observed when exciting at 440 nm and detecting at 540 nm wavelength. Absorbance was measured using a Thermo Scientific NanoDrop 2000c spectrophotometer. Excitation and Emission spectra were collected using a Horiba Scientific FluoroMax-4 spectrofluorometer with 5 nm slit width.

Silica particle generation

The nanoparticles were prepared from a 1 mg/mL solution of either the CR5 peptide or the conjugate in 50 mM potassium phosphate buffer (pH 7) which was incubated overnight at room temperature. To 90 µL of the solution, 10 µL of silicic acid (freshly prepared from 960 µL of 1 mM hydrochloric acid and 40 µL of TMOS) were added. The mixture was vortexed and the precipitation allowed to proceed for 30 min, before separating the particles by centrifugation in a table-top centrifuge at 17,000 g for 5 min.

To create fluorescein-labeled silica particles, a solution of 0.1 mg triethoxysilyl fluorescein (figure S7) in 250 µL 50 mM potassium phosphate buffer (pH 7) was incubated for 3 h. 10 µL of this solution were then added to the peptide solution in addition to the silicic acid before precipitation of the particles.

Particles were washed three times with 100 µl water (Milli-Q) before further use.

Scanning Electron Microscopy

For SEM imaging, the silica particles precipitated from 100 µL reaction volume were resuspended in 500 µL of water. 5 µL of the resuspended particles were spotted onto a cell culture coverslip, dried and sputtered with 5 nm of gold. Samples were then imaged on a Zeiss Supra 55 VP electron microscope using 5 kV accelerating voltage.

Scanning Transmission Electron Microscopy

Ultrathin sections of HT-29 colon carcinoma cells incubated with SiPs were prepared for STEM by the following procedure. After incubation, the cells were washed with PBS and fixed with Karnovskys Fixants (0.5 ml 6.6 M paraformaldehyde, 2.5 mL 2.5 M glutaraldehyde solution, 40 mL 0.2 M sodium-cacodylic buffer pH 7.4, 7 mL bidestilled water) for 15 min. Thereafter, cells on the transwell-membrane were treated with osmium tetroxide (1% v/v) for 7 min at 4 °C to enhance the contrast of cellular membranes in electron microscopy. Subsequently, the transwell-membrane was treated with an ethanol gradient to a final concentration of 100% of ethanol (specially dried). After this, membranes were immediately transferred in 100% propylene oxide for ethanol exchange followed by embedding in Epon resin 812. Afterwards 50 nm thin sections cut with an ultramicrotome (Leica EM UC6) were deposited on bare copper TEM grids.

High-angle annular dark-field (HAADF)-STEM imaging was performed at 15 keV in a Strata 400S scanning electron microscope from Thermo Fisher Scientific (previously FEI) which is equipped with a STEM detector. HAADF-STEM yields strong material contrast with an intensity that is approximately proportional to Z^2 (Z: atomic number). This leads to strong bright contrast of SiPs in Figure 2A,B.

HT-29 cells cultivation and toxicological characterization

Human colon adenocarcinoma cells HT-29 (ATCC) were cultivated and used according to the specification of the supplier. Accordingly, cells were cultivated in humidified incubators at 37 °C and 5 % CO_2 in DMEM medium supplemented with 10 % fetal bovine serum (FBS) and 1 % penicillin/streptomycin (P/S, 50 U/ml). Cell culture media and supplements were purchased from GIBCO Invitrogen (Karlsruhe, Germany), Sigma-Aldrich Chemie GmbH (Munich, Germany) and disposables from Sarstedt AG&Co (Nuembrecht, Germany) and Ibidi (Martinsried, Germany). Cell viability presented in Figure S2 was measured with the Cell Proliferation Reagent WST-1 for mitochondrial activity (Roche Diagnostics GmbH, Mannheim, Germany; abs. 450 nm and reference wavelength 650 nm) or with sulforhodamine B assay for protein content (absorbance 570 nm) with a Victor^{3}V 1420 Multilabel Counter Plate Reader (Perkin Elmer, Waltham, USA). Assays were performed according to the specification of the supplier as previously described in detail.[1, 2] Genotoxic potential of quercetin-R5 SiPs was assessed by single-cell gel electrophoresis ("comet") assay as previously reported.[3, 4]

Live cell imaging

In order to verify the kinetic of the uptake of the quercetin-R5 SiPs in HT-29 cells, as well as if the uptake was an active process or consequence of an alteration of the permeability of the cell membrane, detailed live cell imaging experiments were performed. To this aim cells were stained with CellMask™ Deep Red Plasma Membrane Stain (1:1000 dilution, white) and LysoTracker® Red DND-99 (1:1000 dilution, red). Imaging was performed in Live Cell Imaging Solution (all from Molecular Probes, Life Technologies, Thermo Fisher Scientific, Waltham, USA). For the live cell imaging experiments a confocal LSM microscope Zeiss 710 equipped with ELYRA PS. 1 was used equipped with a Plan Apochromat 63X/1.4 oil or with Water Plan-Apochromat 63x/1.0 objective.

Figures S1-S11

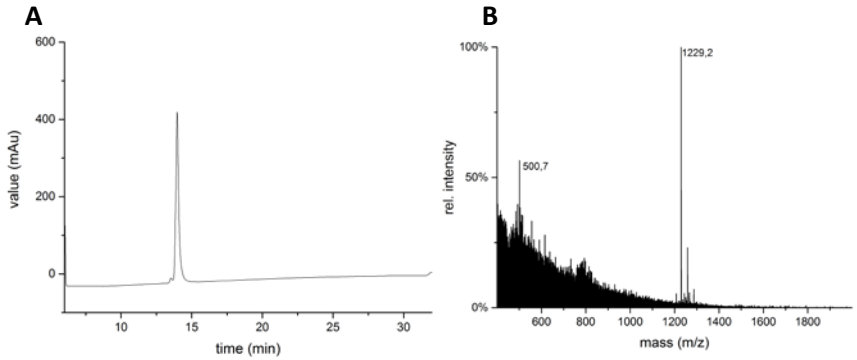

Figure S1: HPLC-MS analysis of conjugate released from the silica particles - A: chromatogram, B: mass spectrum (calc. M([M+2H]$^{2+}$ m/z 1230.1).

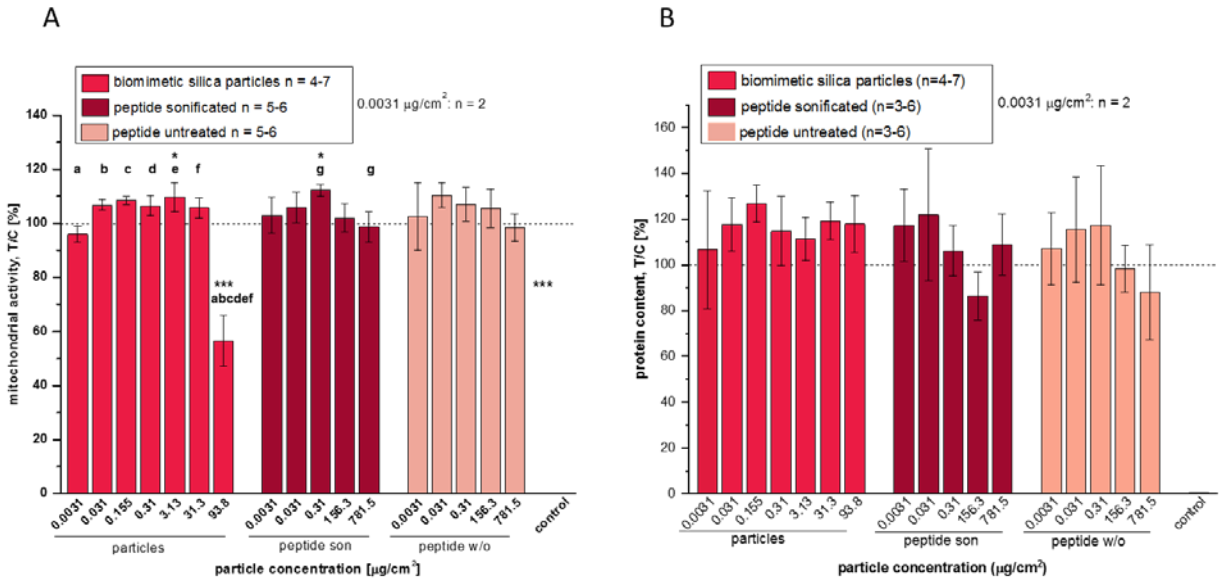

Figure S2. Characterization of the cytotoxic potential of the R5 SiPs (particles) of the R5 peptide after sonication or suspension. A. WST-1 measurement of the mitochondrial activity[1]. B. SRB measurements of the total cell protein content.

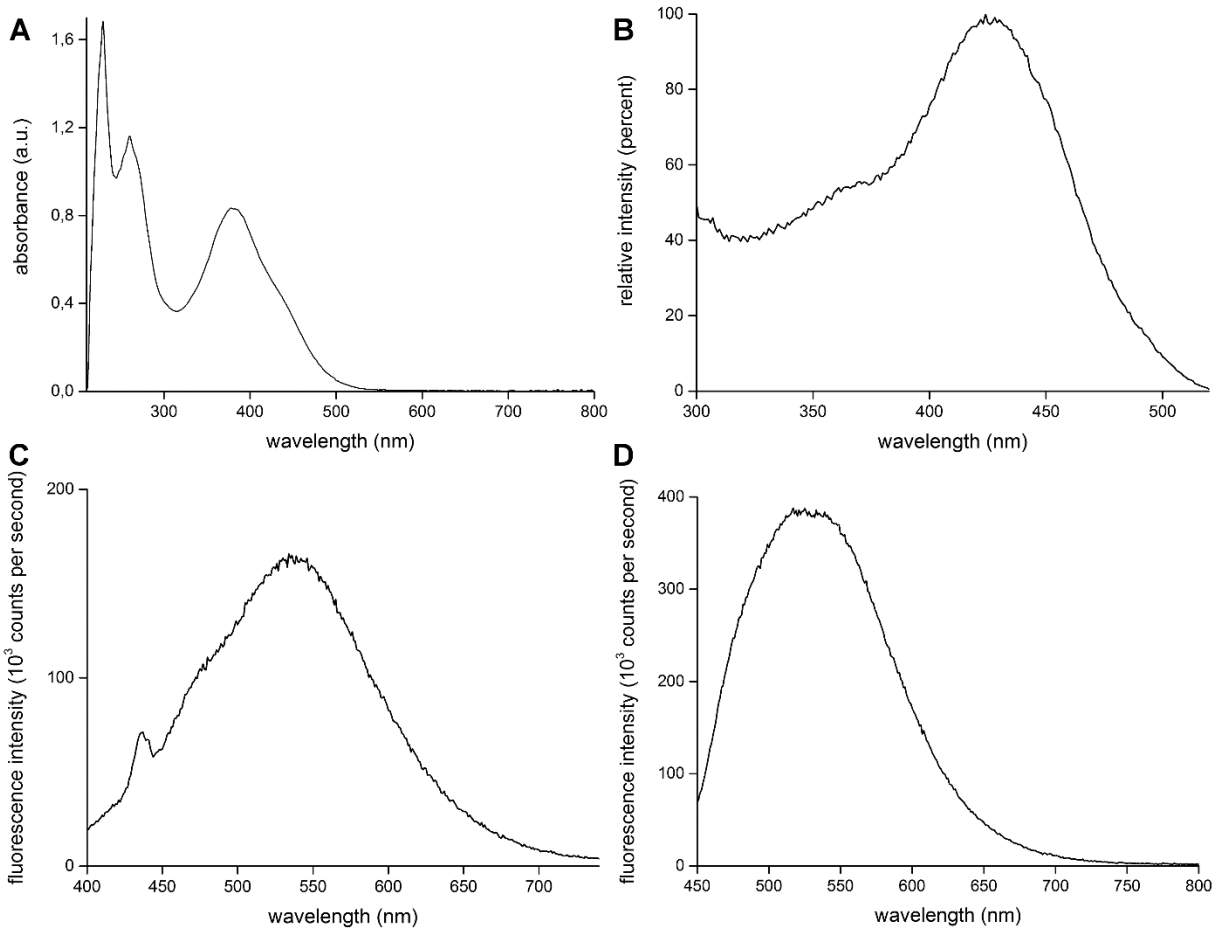

Figure S3: Spectra of quercetin-R5 conjugate A – absorbance, B – excitation spectrum (540 nm emission), C – emission spectrum (380 nm excitation), D – emission spectrum (440 nm excitation).

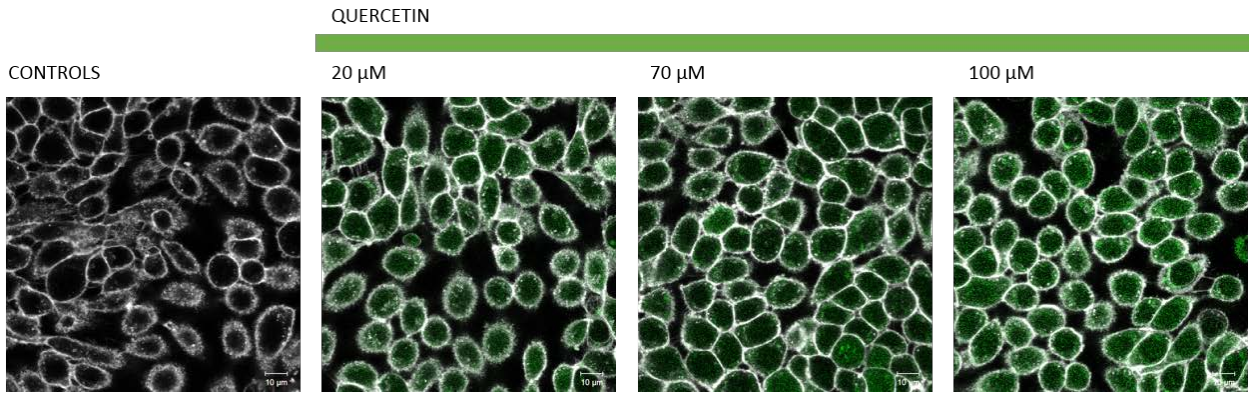

Figure S4: Concentration-dependent quercetin uptake (green) by live cell imaging in HT-29 cells (cell membrane in white with Cell Mask).

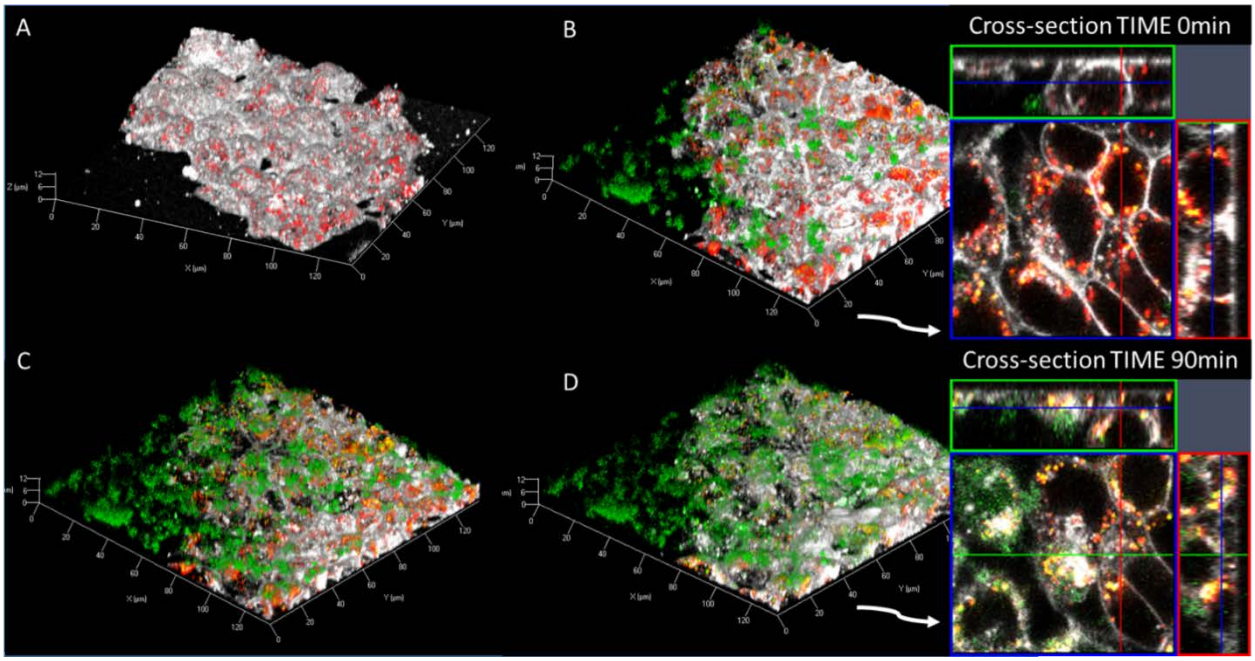

Figure S5: Live cell imaging of HT-29 cells incubated with quercetin-R5 SiPs (green). A. Pre incubation. B. Application of quercetin-R5 SiPs at 70 µM (time 0). C. 30 min incubation. D. 90 min incubation. Lysosomes are marked in red with LysoTracker; cell membranes in white with Cell Mask.

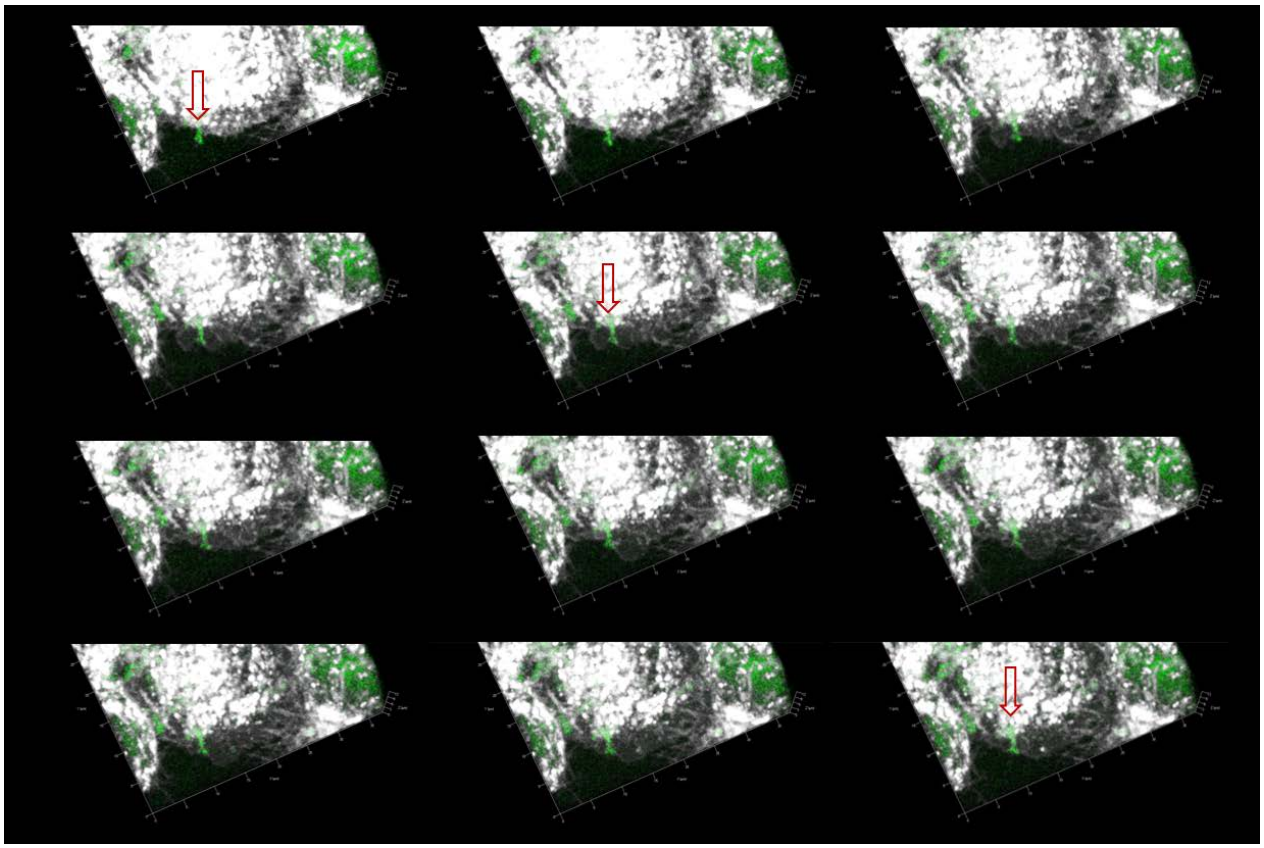

Figure S6: Live cell imaging of HT-29 cells in presence of quercetin-R5 SiPs (green). Red arrows indicate the site of active engulfment through the formation of membrane blebs (white).

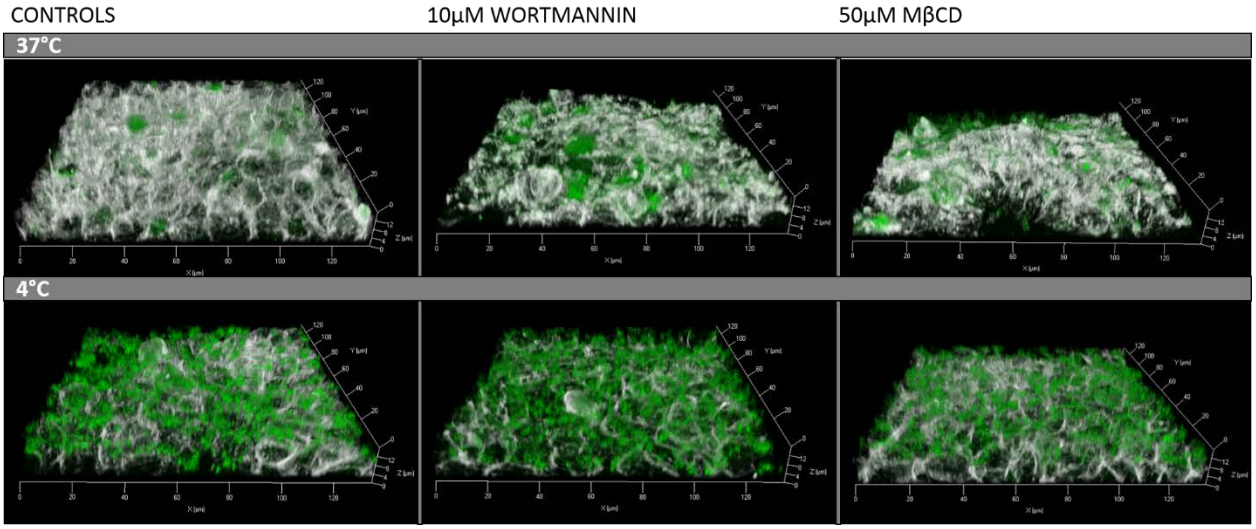

Figure S7: Uptake of quercetin-R5-SiPs in HT-29 cells (cell membrane depicted in white) imaged after 3 h. Incubation with quercetin-R5-SiPs (green) at 37 or 4°C. Phosphatidylinositol-3-kinase inhibitor wortmannin (10 µM, inhibitor of phagocytosis/ micropinocytosis[5]) and cholesterol-complexing agent MβCD (50 µM, alteration of membrane organization and fluidity[6]) were used to test for specific uptake mechanisms.

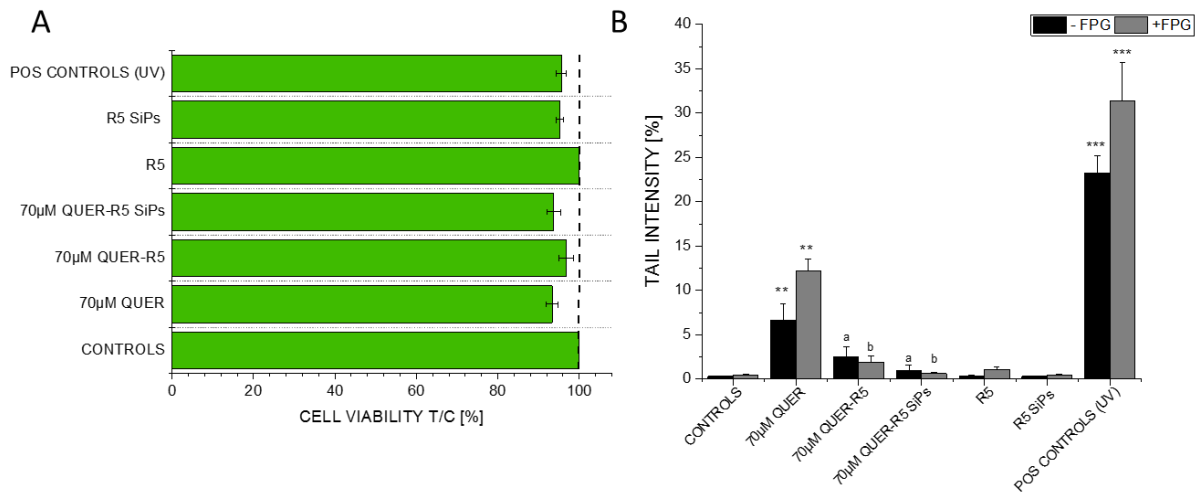

Figure S8: Impact of quercetin-R5 SiPs on DNA integrity in HT-29 cells. A. Control of the cell viability performed before the comet assay with the Trypan Blue exclusion test. B. Tail intensity [%] measured during comet assay (image analysis with Comet Assay IV System, Perceptive Instruments, Suffolk, Great Britain) in presence (gray) or absence (black) of the formamidopyrimidine DNA-glycosylase (FPG) for the detection of oxidatively damaged DNA bases. Data are expressed as mean ±S.E. of n > 3 independent experiments measured in technical duplicates. * indicates significant difference in comparison to controls (**p < 0.01 and ***p < 0.001). Letters indicate significant differences in comparison to quercetin alone (a: incubation without FPG; b: incubation with FPG p < 0.05, one way ANOVA with Fisher test). POS. CONTROLS results from the exposure with UV-B light (1 min. λ = 312 nm; energy: 1 J)

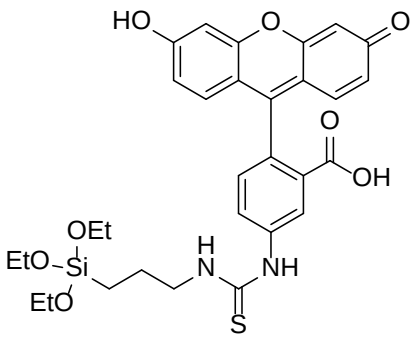

Figure S9: Structure of triethoxysilyl fluorescein. The compound is used to create fluorescent silica particles as described by Ciccione et al.[7]

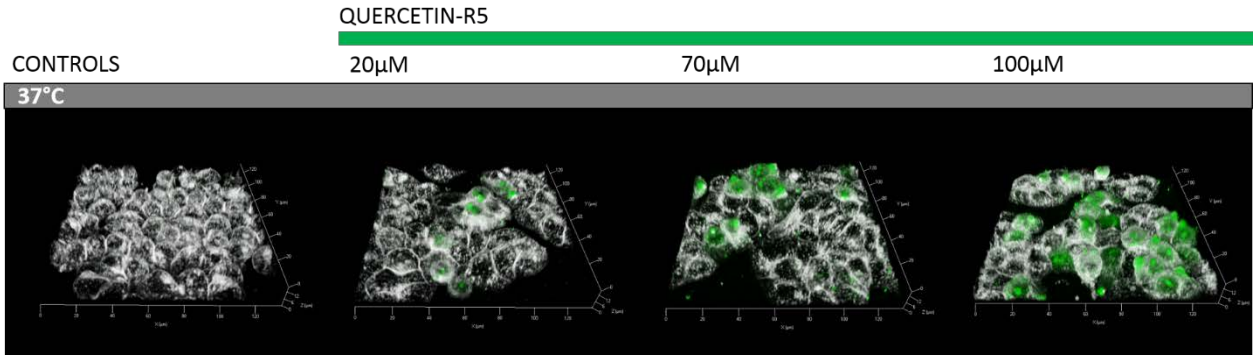

Figure S10: Concentration dependent uptake of quercetin-R5. HT-29 cells (cell membrane depicted in white) were imaged after 3 h incubation with quercetin-R5 (green, 20, 70, 100 µM) at 37°C.

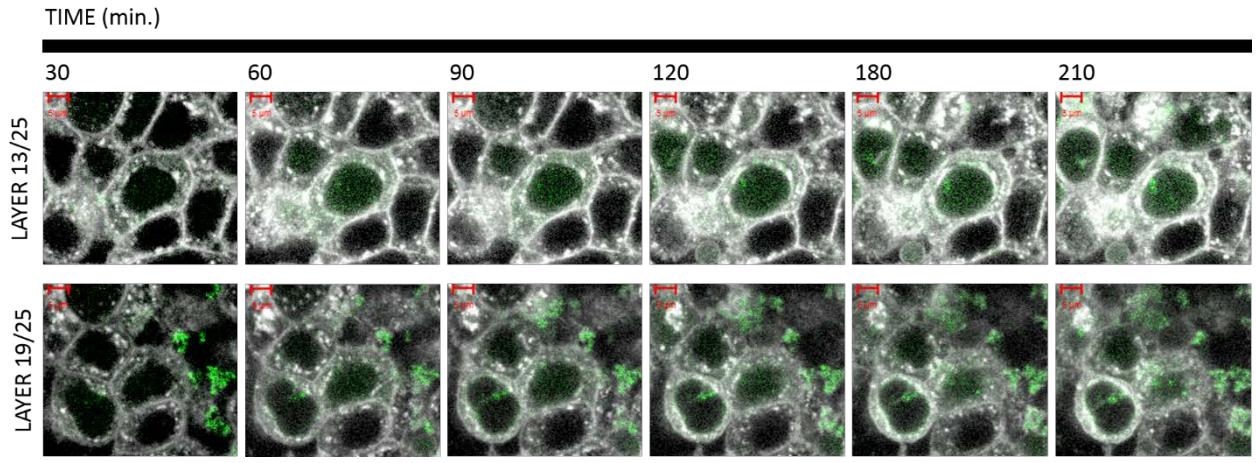

Figure S11: Time dependent uptake of quercetin-R5 SiPs (green) in HT-29 cells (cell membrane depicted in white). 2D images were extracted from 3D reconstruction in figure 3 of the manuscript and represent layers 13 and 19 of the z-stack (25 layers in total). Scale bars 5µm.

References

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