Supporting Information

Mutually Exclusive Cellular Uptake of Combinatorial Supramolecular Copolymers

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Materials:
All solvents were purchased from Biosolve unless stated otherwise. O-Benzotriazolyl-\(N,N,N',N'\)-tetramethyl-uronium-hexafluoro-phosphate (HBTU) and \(N,N'\)-diisopropylethylamine (DIPEA), Fmoc protected amino acids, pre-loaded resins and trifluoroacetic acid (TFA) were purchased from Biosolve. (Benzotriazol-1-yloxy)tripyrrolidinophosphonium hexafluorophosphate (PyBOP), \(\text{Pd(PPh}_3\text{)}_4\), barbituric acid, succinic anhydride, trimethylamine (TEA) and \(1\H\)-Pyrazole-1-carboxamidine hydrochloride were purchased from Sigma-Aldrich. \(N\)-1-trityl-deamino-histidine was purchased from Iris-biotech GMBH. 2,2,2-trifluoroethanol (TFE) was purchased from Acros. Water was demineralized before use. Thin layer chromatography (TLC) was carried out using Merck pre-coated silica gel plates using light with a wavelength of 254 or 365 nm. Manual column chromatography was carried out using Merck 60 Å pore size silica gel (particle size: 63-200 \(\mu\)m). Manual size-exclusion chromatography was performed on BIO RAD BioBeads S-X1 (200-400 mesh) in a long glass column at atmospheric pressure in dichloromethane (DCM). Reversed-phase high pressure liquid chromatography (RP-HPLC) was performed on a Shimadzu LC-8A JPLC system by using a Gemini 5u C18. A gradient of water and acetonitrile (ACN) with 0.1% TFA was used as eluent. Detection was performed by a Shimadzu SPD-10AV UV-detector (\(\lambda =240\) nm). LC-MS samples were characterized using a Shimadzu SCL-10 AD VP series HPLC coupled to a diode array detector (Finnigan Surveyor PDA Plus detector, Thermo Electron Corporation) and an Ion-Trap (LCQ Fleet, Thermo Scientific). Analyses were performed using a reversed phase HPLC column (GraceSmart PP18, 50 mm x 2.1 mm, 3 \(\mu\)m), using an injection volume of 1-4 \(\mu\)L, a flow rate of 0.20 mL / min and typically a gradient (5% to 100% in 10 minutes, held at 100% for 1 more minute) of acetonitrile in water (both containing 0.1% formic acid) at 298K. MALDI-TOF-MS was measured on a PerSeptive Biosystems Voyager-DE PRO spectrometer with a Biospectrometry workstation using 2:([2E]-3-(4-tert-butylphenyl)-2-methylprop-2-enylidene)malononitrile (DCTB) and \(\alpha\)-Cyano-4-hydroxycinnamic acid (CHCA) as matrix material and chloroform or methanol as solvent. \(^1\)H-NMR spectra were measured using a Varian Mercury Vx 400 MHz NMR spectrometer at 298 K. Chemical shifts are given in ppm and the spectra are calibrated to residual solvent signals of CDCl\(\text{3}\) (7.26 ppm). Splitting patterns are labeled as s, singlet; d, doublet; dd, double doublet; t, triplet and m, multiplet.
8-well culture slides were purchased from DB Biosciences. Dulbecco's Modified Eagle Medium (DMEM) Fetal bovine serum (FBS), Penicillin-Streptomycin solution (Pen/Strep), RPMI 1640, DMEM and DMEM phenol red free cell culture media were purchased from Invitrogen. SYTO59 was purchased from life technologies. Hela cells were obtained from M. de Liefde. Primary mouse embryonic cells (3T3) were obtained from Michael Sonntag. Breast cancer cells (MCF-7) cells and melanoma (MDA-MB-435) cells were obtained from DSMZ and ATCC respectively.
Synthesis:
Inert-disc,[1] 1NH2-disc,[2] 3NH2-disc,[3] 1cRGD-disc,[4] 1Fe-disc,[4] and 1F-disc[5] were synthesized as previously reported.

Peptide synthesis
c(RADfK) peptide was synthesized in a similar way as previously reported for the c(RGDfK)[4] following the below mentioned approach.

Supporting Scheme 1. Overview of the synthetic route of an c(RADfK)-peptide. Reagents and conditions: (a) PyBOP, DIPEA, DMF, DCM, 12 h, RT, quant.; (b) barbituric acid, Pd(PPh3)4, DMF, DCM, 2 h, RT, quant.; (c) TEA, succinic anhydride, DMF, 2 h, RT, 21%.

Fmoc peptide synthesis (1): The linear peptide sequence H-Asp(OtBu)-D-Phe-Lys(Alloc)-Arg(Pbf)-Ala-OH, was manually synthesized via Fmoc solid-phase peptide synthesis (SPPS) on an alanine pre-loaded 2-chlorotrityl chloride resin (Iris Biotech: 0.53 mmol/g), cleaved from the resin under mild conditions with 10 mL TFE/DCM (1:4 v/v) and purified by preparative RP-HPLC yielding 1 (48%). LC-MS: Mw 1027.21; found 1028.67 [M+H]+.

Cyclization (2): Subsequently, the peptide was cyclized under dilute conditions. The linear peptide 1 (98.7 mg, 96.1 μmol, 1 eq.) was dissolved in 3 mL dry DMF and added dropwise using a syringe pump over a period of 2 hours to a solution of PyBOP (110 mg, 0.21 mmol, 2.2 eq.) and DIPEA (144 μL, 0.83 mmol, 8.6 eq.) in a mixture of dry DCM (11 mL) and dry DMF (28 mL) yielding 2 (quant. conversion). LC-MS: Mw 1010.21; found 1010.67 [M+H]+.

Lysine deprotection (3): After evaporating the solvent, the crude cyclic peptide 2 (96.1 μmol, 1 eq.) was dissolved in a mixture of 6 mL dry DCM and 2 mL dry DMF where after Pd(PPh3)4 (55 mg, 47 μmol, 2 eq.) and barbituric acid (75 mg, 481 μmol, 5 eq.) were added. After 2 hours at room temperature the solvent mixture was evaporated and LC-MS confirmed complete lysine deprotection yielding 3 (quant.). LC-MS: Mw 926.13; found 926.50 [M+H]+.

Introduction reactive handle (4): The crude peptide 3 (96.1 μmol, 1 eq.) was dissolved in 5 mL dry DCM and 60 μL TEA (0.4 mmol, 4.2 eq.) and succinic anhydride (57.6 mg, 0.58 mmol, 6 eq.) were added. After 2 hours at room temperature LC-MS confirmed complete conversion. The crude peptide mixture was purified via reverse phase chromatography (a gradient of acetonitrile in water, 5% to 100% in 10 minutes and held at 100% for 1 more minute, containing 0.1% TFA was used to elute products) and column chromatography (Silica, 5%-10% MeOH and 0.1% formic acid (FA) in DCM), yielding pure 4 (21%). LC-MS: Mw 1026.21; found 1026.83 [M+H]+.
Disc synthesis

Supporting Scheme 2. Synthesis of peptide and guanidine functionalized discotics. a) 1H-Pyrazole-1-carboxamidine-hydrochloride, DIPEA, RT, 12 h, 80%; b) 4, HBTU, DIPEA, DMF, RT, 12 h, 78%; (c) TFA, DCM, RT, 3 h, quant.; d) 5, HBTU, DIPEA, DMF, RT, 12 h, 60%; e) TFA, DCM, RT, 3 h, quant.; f) 1H-Pyrazole-1-carboxamidine-hydrochloride, DIPEA, RT, 12 h, 43%.

peptide-disc (1cRAD-disc, 1cRGD-disc: 8 & 9)
1NH2-disc (5.9 mg, 1.8 μmol, 1 eq.) was dissolved in dry DMF (0.5 mL) with DIPEA (0.6 μL, 3.6 μmol, 2 eq) under dry conditions. Cyclic peptide (4 & 5) (3.7 mg, 3.6 μmol, 2 eq.) was preactivated separately for 20 minutes at room temperature with PyBOP (4.0 mg, 7.8 μmol, 2 eq.) and DIPEA (2.7 μL, 15.6 μmol, 4 eq.) in dry DMF (0.5 mL). This solution was dropwise added to the solution with the 1NH2-disc and stirred for 24 hours under argon at room temperature (RT). Completion of the reaction was monitored with TLC (10% MeOH in DCM, silica, Rf = 0.2). The crude was purified using silica gel chromatography (silica, 5-15% MeOH in DCM) yielding pure 6 and 7 (78%, 60%). MALDI-ToF-MS 6: Mw 4316.9; found 4339.5 [M+Na]+. MALDI-ToF-MS 7: Mw 4302.9; found 4325.7 [M+Na]+. Protected peptide-disc (6,7) (6.0 mg, 1.4 μmol, 1 eq.) was dissolved in DCM (0.5 mL) with TIS (40 μL, 195 μmol, 139 eq.). Subsequently, TFA was added (1.5 mL) dropwise and stirred for 3 hours at RT. TFA was removed under vacuum by co-evaporating with toluene (3x 2 mL) yielding pure 1cRAD- 8 and 1cRGD-disc 9 (both quant.).
MALDI-ToF-MS (8): Mw 4008.5; found; 4009.0 [M+H]^+ (Figure S1). \(^1\)H NMR (399 MHz, Chloroform-d) \(\delta\) 15.54 (s, 2H), 14.50 (s, 3H), 9.60 (d, \(J = 9.1\) Hz, 3H), 9.38 (d, \(J = 8.8\) Hz, 3H), 9.29 (s, 2H), 9.06 (s, 3H), 8.50 (d, \(J = 17.6\) Hz, 3H), 7.54 (d, \(J = 16.6\) Hz, 6H), 7.34 (d, \(J = 9.3\) Hz, 6H), 4.28 (s, 18H), 3.91 (s, 12H), 3.84 (s, 7H), 3.74 (s, 28H), 3.65 (m, 111H), 3.53 (s, 18H), 3.35 (s, 24H), 2.16 - 0.69 (m, 102H) (Figure S2).

MALDI-ToF-MS (9): Mw 3994.5; found; 3995.0 [M+H]^+ (Figure S1). \(^1\)H NMR (399 MHz, Chloroform-d) \(\delta\) 15.53 (s, 3H), 14.49 (s, 3H), 9.60 (d, \(J = 8.8\) Hz, 3H), 9.40 (d, \(J = 8.5\) Hz, 3H), 9.29 (s, 3H), 9.06 (d, \(J = 4.3\) Hz, 3H), 8.53 (d, \(J = 4.2\) Hz, 3H), 7.57 (dd, \(J = 8.5, 4.3\) Hz, 6H), 7.36 (s, 6H), 7.19 (dd, \(J = 14.3, 8.1\) Hz, 5H), 4.28 (q, \(J = 4.9\) Hz, 19H), 3.91 (t, \(J = 4.4\) Hz, 12H), 3.84 (t, \(J = 4.5\) Hz, 12H), 3.75 (q, \(J = 3.8\) Hz, 19H), 3.56 - 3.50 (m, 112H), 3.38 (s, 6H), 3.36 (d, \(J = 9.1\) Hz, 12H) (Figure S2).

1G-disc (10) A solution of 1H-Pyrazole-1-carboxamidine-hydrochloride (3.3 mg, 22.5 \(\mu\)mol, 15 eq.) with DIPEA (3.9 \(\mu\)L, 22.5 \(\mu\)mol, 15 eq.) in 0.15 mL DMF was dropwise added to a solution of 1NH2-disc (5.2 mg, 1.5 \(\mu\)mol, 1 eq.) in DMF (0.5 mL) and stirred for 2 days under argon at RT. The crude was purified via size exclusion chromatography yielding pure 1G-disc (80%). MALDI-ToF-MS: Mw 3350.8; found 3351.6 [M+H]^+ (Figure S1). \(^1\)H NMR (399 MHz, Chloroform-d) \(\delta\) 15.52 (s, 3H), 14.50 (d, \(J = 4.9\) Hz, 3H), 9.60 (d, \(J = 8.0\) Hz, 3H), 9.39 (d, \(J = 8.7\) Hz, 3H), 9.27 (s, 3H), 9.05 (d, \(J = 3.6\) Hz, 3H), 8.52 (d, \(J = 4.0\) Hz, 3H), 7.57 (dd, \(J = 8.1, 4.2\) Hz, 6H), 7.35 (s, 6H), 4.28 (dd, \(J = 4.4\) Hz, 18H), 3.91 (t, \(J = 6.0\) Hz, 12H), 3.84 (t, \(J = 4.0\) Hz, 6H), 3.74 (q, \(J = 4.8\) Hz, 12H), 3.70 - 3.60 (m, 112H), 3.54 (q, \(J = 5.5\) Hz, 18H), 3.38 (s, 6H), 3.36 (s, 18H) (Figure S2).

3G-disc (11) A solution of 1H-Pyrazole-1-carboxamidine-hydrochloride (9.9 mg, 67.5 \(\mu\)mol, 45 eq.) with DIPEA (11.7 \(\mu\)L, 67.5 \(\mu\)mol, 45 eq.) in 0.4 mL DMF was dropwise added to a solution of 3NH2-disc (5 mg, 1.5 \(\mu\)mol, 1 eq.) in DMF (0.5 mL) and stirred for 2 days under argon at RT. The crude was purified via size exclusion chromatography yielding pure 3G-disc (43%). MALDI-ToF-MS: Mw 3492.9; found 3494.0 [M+H]^+ (Figure S1). \(^1\)H NMR (399 MHz, Chloroform-d) \(\delta\) 15.52 (d, \(J = 9.3\) Hz, 3H), 14.49 (d, \(J = 8.5\) Hz, 3H), 9.60 (d, \(J = 9.5\) Hz, 3H), 9.38 (d, \(J = 8.1\) Hz, 3H), 9.27 (s, 3H), 9.06 (d, \(J = 3.2\) Hz, 3H), 8.54 (d, \(J = 2.9\) Hz, 3H), 7.57 (dd, \(J = 8.8, 4.3\) Hz, 6H), 7.34 (s, 6H), 4.29 (dd, \(J = 4.4, 6.0\) Hz, 18H), 3.93 (t, \(J = 4.4\) Hz, 12H), 3.85 (t, \(J = 4.8\) Hz, 6H), 3.75 (q, \(J = 3.6\) Hz, 19H), 3.71 - 3.58 (m, 132H), 3.53 (dd, \(J = 5.6, 3.5\) Hz, 18H), 3.40 (s, 6H), 3.36 (s, 18H) (Figure S2).
Supporting Figure 1. MALDI-TOF spectra of 1cRAD-disc (8), 1cRGD-disc (9), 1G-disc (10) and 3G-disc (11).
Supporting Figure 2. $^1$H-NMR spectra of 1cRAD-disc (8), 1cRGD-disc (9), 1G-disc (10) and 3G-disc (11).
Self-assembly:

**UV/vis and fluorescence spectroscopy**

UV/vis spectra were measured on a Jasco V-650 spectrophotometer equipped with a Perkin-Elmer PTP-1 Peltier temperature control system. Fluorescence spectra were recorded on a Varian Cary Eclipse fluorescence spectrophotometer equipped with a Perkin-Elmer PTP-1 Peltier temperature control system. All measurements were performed in quartz cuvettes with a 10 mm light path (Hellma) and 1800 μL minimal volume. The excitation and emission slits were both set to 5 nm. Discs were measured in their assembled state at a 5 μM stock solutions in sodium phosphate buffer (pH 7.3, 5 M NaCl) (PBS) or dichloromethane (DCM) in which the disc is molecular dissolved. Between measurements the cuvettes were washed with acetone and incubated for an hour in helifax at 37 °C.

Discs self-assemble into columnar stacks when diluted in polar-protic solvents driven by hydrophobic interactions, π-π stacking between the aromatic cores and hydrogen bond formation. These hydrogen bonds are formed intra-molecular between the amide N-H groups and their neighboring bi-pyridine nitrogen atoms[5] thereby arranging the disc into a propeller like conformation enabling the organized stacking of the disc via arene-arene interactions. The self-assembly can be visualized using UV/Vis and fluorescence spectroscopy by the typical redshift in absorbance and increase in fluorescence upon self-assembly.[1]

[Supporting Figure 3. A) Characteristic absorption (dotted line) and emission spectra (solid line) of 1cRGD- and 1cRAD-disc self-assembled into columnar stacks in PBS or molecularly dissolved in DCM (5 μM, λex = 340 nm, T = 20°C ) as observed by the red-shift in the UV/Vis spectra and an increase in fluorescence. B) Characteristic absorption (dotted line) and emission spectra (solid line) of 3NH2-, 3G- and 1F-Disc self-assembled into columnar stacks in PBS (5 μM, λex = 360 nm, T = 20°C ) as observed by the red-shift in the UV/Vis spectra and an increase in fluorescence.]}
Nanoparticle tracking analysis

Nanoparticle tracking analysis was performed with the Malvern Pananalytical NanoSight and used to evaluate the dispersity of formed assemblies. Solutions of 3G-disc or 1cRGD-disc (100 μM, milliQ) were filtered (PVDF, 200 μm) prior measurement.

Supporting Figure 4. Nanosight size distribution of the disc assemblies if assumed to be spherical for the 1cRGD-disc and 3G-disc (100 μM) in milliQ after filtration (PVDF, 200 μm). Both polymers showed two different populations. Error bars are given by purple and red shadows respectively and indicate 1 standard error of the mean.

Transmission Electron Microscopy (TEM):

Supporting Figure 5. A) TEM image of 1cRGD-disc copolymer with 1Fe-disc, (9:1) with a high (left) and low (right) magnification. B) TEM image of 3G-disc copolymer with 1Fe-disc (9:1) at a low magnification. Scale bar represents 50 nm.
Charge mediated uptake:

Supporting Figure 6. Multiphoton microscopy images of Hela cells incubated with inert-disc, 1NH2-disc or 1G-disc (5 μM) after 2 hours. Scale bar represents 25 μm. No cellular uptake of the inert-disc nor of the single charged 1NH2-disc or 1G-disc is observed.

Supporting Figure 7. Multiphoton microscopy images of Hela cells incubated with a 5 μM mixture of 3NH2-disc for 5-50 minutes. Scale bar represents 50 μm.
Supporting Figure 8. Multiphoton microscopy images of Hela cells incubated with a 5 μM mixture of 3G-disc for 5-50 minutes. Scale bar represents 25 μm. Within 15 minutes the 3G-disc binds to the cellular membrane. From 30 minutes, 3G-disc is also internalized progressively in a similar way as previously reported for the 3NH2-disc.[5]

Uptake efficiency disc with confocal microscopy

The average intensity per cell was determined via confocal microscopy. Three images with a total of 350 cells per condition were selected. The raw intensity density (the sum of the values of the pixels in the image or selection) and area were calculated with ImageJ. The average background signal was determined by selecting 4 different areas within a single picture without cells. To correct for background noise, the signal was subtracted from the total raw intensity density of the cells and divided by the number of cells to gain the average intensity per cell.

Supporting Figure 9. A) Confocal microscopy images of the cellular uptake of 5 μM solutions of 3G- and 3NH2-disc in culture medium by Hela cells after a 16 hour incubation, additionally cell nuclei were stained with CYTO59 prior imaging. Scale bar represent 25 μm. B) Average fluorescence per cell after a 16 hour incubation of 5 μM solutions of 3G- and 3NH2-Dics, Fluorescence of over 350 cells per condition were measured at 3 different places within the culture well.
Cell-viability (MTT assay)

Hela cells were counted and seeded in 96 well plates (7500 cells per well) using DMEM supplemented with 1% Pen/Strep and 10% FBS and incubated overnight at 37 °C, 5% CO₂. 10x stock solution of disc were prepared in PBS, diluted down in medium to a concentration between 2.5 and 7.5 μM and added to the cells. After an overnight incubation at 37 °C, 5% CO₂ the cells were washed once with PBS and incubated with 0.83 mg/mL MTT in medium without phenol red at 37 °C, 5% CO₂ for 3.5 hours. Subsequently, the medium was removed and the MTT crystals were dissolved in 150 μL DMSO for 15 minutes on a shaker. The absorbance was read out at 595 nm with a plate reader. The absorbance was normalized using the absorbance of PBS treated cells.

Supporting Figure 10. Metabolic activity of Hela cells after a 17 hour incubation with 5 μM 3G- or 3NH₂-disc at 37 °C, 5% CO₂. For the whole concentration range the 3G-disc is slightly cytotoxic in contrast to the 3NH₂-disc.
Supporting Figure 11. Multiphoton microscopy images of Hela cells incubated with a 5 μM mixture of 3G-disc (A,B) or 3NH2-disc (C,D) with 1F-disc 9:1 (A,C) or 8:2 (B,D) after 2 hours. Scale bar represents 10 μm. Images in figure B are identical to Figure 3C in the main manuscript. Addition of 10-20% 1F-disc hampers the uptake of mixtures with the 3NH2-disc. On the contrary, 3G-disc act as a carriers for the cell impermeable 1F-disc.
Supporting Figure 12. Multiphoton microscopy images of separate fluorescent channels, complementing figure 3D in the main manuscript. Hela cells incubated with a 5 μM mixture of 3G-disc with 1F-disc (9:1) for 10-140 minutes. 3G- and 1F-disc were intermixed 2 hours before addition to the cells. Scale bar represents 25 μm. The 9:1 mixture of 3G-disc with 1F-disc shows a similar uptake profile as the 3G-disc. Only the internalization is less progressive as an effect of the incorporation of 10% cell impermeable 1F-disc.
Supporting Figure 13. Flow cytometry analysis of Hela cells treated with a 5 μM solution of 3G-disc (A) or 3NH2-disc (B) with 1F-disc (9:1). Cells were incubated with the disc solution between 10 to 120 minutes at 37 °C, 5% CO₂. For both analysis an increase in uptake is observed when the incubation time is increased. Mixtures with the 3G-disc improve the uptake rate as well as the uptake efficiency in comparison to the mixtures with 3NH2-disc.

Supporting Figure 14. (a) Flow cytometry analysis of Hela cells after an overnight incubation with non-cell permeable 5 μM Inert-disc/1F-disc (9:1), 1F-disc or PBS and (b) after an overnight incubation with non-fluorescein containing solutions of 5 μM 3NH2- or 3G-disc at 37 °C, 5% CO₂. Cell impermeable discs are not taken up by the cell and discs and there is no spectral interference between the fluorescein readout and disc.
**Integrin mediated uptake:**

Supporting Figure 15. Average fluorescence per cell after overnight incubation of 5 μM cRGD-disc, Fluorescence of 10 cells per condition were measured within a single culture well.
Supporting Figure 16. Bright field images and corresponding multiphoton microscopy images of (A) MDA-MB-435S, (B) 3T3 and (C) MCF-7 cells after an overnight incubation with 5 μM 3G-disc. Scale bar bright field and multiphoton images represents 50 μm. No intrinsic difference in charge mediated uptake between these different cell strains is observed. Additionally, all discs have a similar punctuated distribution throughout the cell indicating entrapment in vesicles.
Supporting Figure 17. Bright field images and corresponding multiphoton microscopy images of (A) MDA-MB-435S, (B) 3T3 and (C) MCF-7 cells with increasing expression levels of αvβ3 integrin after an overnight incubation with a 5 μM 1cRAD-disc. Scale bar represents 25 μm. No uptake of the 1cRAD-disc for any cell strain was detected thus supporting an interaction governed by the cRGD functionalization of the disc.
Supporting Figure 18. Flow cytometry analysis of MDA-MB-435S and (a) MCF-7 (b) cells followed over time at 37 °C, 5% CO₂ after the addition of 5 μM mixtures of 1cRGD-disc (left) or 1RAD-disc (right) with 1F-disc (9:1). Uptake kinetics for the integrin mediated uptake are rather slow in comparison to the charge mediated uptake induced by cationic discs. For all above combinations the uptake is maximized after incubation times between 7-17 hours. The total uptake of cRGD-disc for the integrin rich MDA-MB-435S strain is the largest while only minor changes in uptake are observed for the cell trains incubated with cRAD-disc.
Dual-molecular targeting:

Supporting Figure 19. Flow cytometry analysis of 1cRAD-disc (A) or 1cRGD-disc (B) copolymers (5 μM) with 1F-disc (10%) and 3G-disc (0%-90%) after a 7 hour incubation on MCF-7 cells. Similar trends were observed for the MDA-MB-435S strain with a reduced uptake correspondingly seen for the 1cRGD-copolymer studies.

Supporting Figure 20. (a) Flow cytometry analysis of MDA-MB-435S (A) and MCF-7 (B) cells after a 7 hour incubation with PBS, PBS with propidium iodide (PI) or a mixture of Inert-disc and 1F-disc (9:1). Cell impermeable discs are not taken up by the cell and show a similar uptake pattern as mixture with the 1cRAD-disc.
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