Supporting Information

Polymer mediated transport of the Hsp90 inhibitor LB76, a polar cyclic peptide, produces an Hsp90 cellular phenotype.

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Abbreviations

$CDCl_3$	Deuterated chloroform
DMAEMA	dimethyl amino ethyl methacryle
DMSO	dimethylsulfoxide
EDTA	ethylenediaminetetraacetic acid
ESI	electrospray ionisation
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HPLC	high performance liquid chromatography
iPrOH	isopropanol, propan-2-ol
LC/MS	liquid chromatography/mass spectrometry
MeOH	methanol
MES	2-(N-morpholino)ethanesulfonic acid
N_2	nitrogen
NMR	nuclear magnetic resonance
PBS	Phosphate buffered saline
PBS	phosphate buffered saline
PEGMEMA	poly(ethylene glycol) methyl ether methacrylate
PSA	polar surface area
PTFE	Polytetrafluoroethylene
RAFT	Reversible addition-fragmentation chain transfer
SEC	Size exclusion chromatography
tBuMA	*tert*-butyl methacrylate
TFA	trifluoroacetic acid
TFE	trifluoroethanol

Experimental Procedures

Synthetic Remarks and Procedures

Synthesis of 4-Cyano-4-[(propylsulfanylthiocarbonyl)sulfanyl]pentanoic acid

The RAFT agent was synthesized following a previously described method.[1, 2] Propanethiol (0.3 g, 3.9 mmol) was dissolved with potassium hydroxide (0.44 g, 7.79 mmol) in a 1:1 mixture of acetone and water (20 mL). After cooling in an ice bath, carbon disulphide (285 µL, 4.73 mmol) was added dropwise and the reaction was stirred for 2 h at room temperature. Tosyl chloride (0.90 g, 4.73 mmol) was then added dropwise in acetone (5 mL), and the reaction was stirred for a further 1 h, acidified to pH 2 and extracted with ethyl acetate (2 x 50 mL). The solution was then concentrated to 25 mL by rotary evaporation, 4,4-azobis(4-cyanovaleric acid) (VA501, 2.2 g, 7.9 mmol) was added, and the mixture was stirred under reflux at 80°C overnight. The crude was purified by column chromatography without workup, over silica, taking ethyl acetate / hexane (1:3) + 1% (v/v) acetic acid as an eluent to yield the product as a yellow oil (0.98 g, 90%). ^{1}H-NMR ($CDCl_3$): δ ppm = 1.03 (3H, t, *J* = 7.5 Hz, $C\underline{H}_3CH_2$), 1.72 (2H, sext., *J* = 5.25, $CH_3C\underline{H}_2$), 1.88 (3H, s, $SC(C\underline{H}_3)$), 2.50 (2H, m, $C\underline{H}_2CH_2CO_2H$), 2.71 (2H, m, $C\underline{H}_2CO_2H$), 3.33 (2H, t, *J* = 7.5 Hz, $SC\underline{H}_2$). Mass spectrum (ESI, +ve) [M+H]+ for $C_{10}H_{15}NO_2S_3$ calcd. 278.03, found 278.0.

General procedure for RAFT polymerisations

RAFT agent (or macroRAFT agent for block extension polymerisations), monomer, and azobisisobutyronitrile (AIBN) were dissolved in toluene and degassed by sparging with N_2 for 20 min. The ratio of AIBN to RAFT was held at 0.1 in all experiments and the other experimental details are outlined in table S1. The PEGMEMA and tBuMA polymerisations were conducted at 65ºC for 18h, and the DMAEMA polymerisation at 70ºC for 5h. After polymerization the conversion was determined by ^{1}H-NMR. The polymer was precipitated twice from either cyclohexane (P1), or hexane (P2-4), dried, and characterized by size exclusion chromatography (SEC).

Size exclusion chromatography

Size exclusion chromatography (SEC) was performed using *N,N*-dimethylformamide (DMF) + 0.01 % (w/v) LiBr as the eluent on a Shimadzu modular system comprising an auto injector and a differential refractive index detector. Three Phenomenex 5.0 µm bead-size columns (10^5, 10^4 and 10^3 Å) were used for separation, and all samples were filtered (0.45µm PTFE) prior to injection. Molecular weights were estimated relative to narrow molecular weight distribution poly(methyl methacrylate) (100 to 1 x 10^6 g.mol^{-1}) calibration standards without Mark-Houwink correction.

Experimental details

Table S1. Experimental details and characterization data for the polymers used

#	CTA	Monomer	[M] (mol.L^{-1})	[M] / [CTA]	X	DP_{theo}	$M_{n,\,theo}$	$M_{n,\,GPC}$	*Đ*	*HLB*
P1	RAFT	PEGMEMA	1	15	92%	13.6	4,357	7,400	1.08	-
P2	**P1**	DMAEMA	0.5	50	53%	34.5	9,780	9,500	1.06	-
P3	**P2**	*t*BuMA	0.5	29	75%	18	13,050	11,300	1.06	16.7
P4	**P3**	*t*BuMA	0.5	48	85%	32	14,300	11,900	1.09	13.6

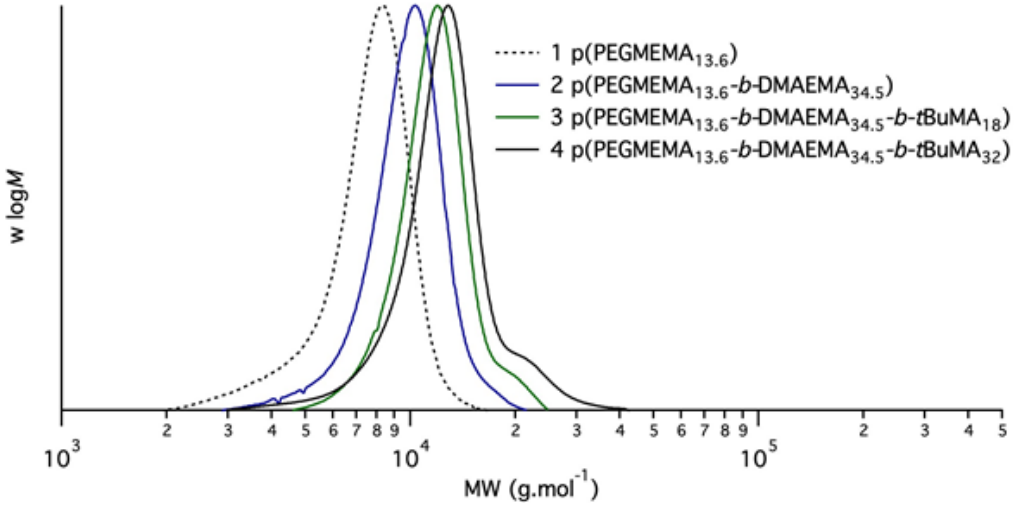


Figure S1. Size exclusion chromatography (SEC) traces of the four polymers detailed in **Table S1**. Molecular weights reported relative to pMMA standards without correction.

Synthesis of nanoparticles

The polymer (50 mg, 1 eq.) was dissolved in milliQ-water (10 mL) with the aid of mild sonication and tBuMA (760 eq.), ethylene glycol dimethacrylate (76 eq.), dodecane (2 or 20% w/w relative to oil phase) and AIBN (0.5 eq.) such that the ratio of oil to water phase was approximately 10% (w/w). The mixture was emulsified using a Branson sonifier with a microtip for 10 min @ 70% amplitude. A sample of this mixture was taken for characterization by dynamic light scattering (DLS), and the remainder was degassed by sparging with N2 for 20 min and polymerized at 65ºC for 16h. After poylmerisation the conversion was determined by ^{1}H-NMR to be ~90-95% in all cases and the mixture was dialysed (12-14kDa cellulose membrane) against ethanol / water (4:1 v/v) for 5h. The purified suspension of particles was then concentrated to ~2 mL, and diluted with trifluoroacetic acid (4 mL) and left for 6h to remove the *tert*-butyl groups. The particles were then diluted with water and again dialysed against EtOH / water (4:1 v/v), basic water (pH >10) and neutral water before freeze drying to a powder. The powder was resuspended in the appropriate buffer prior to loading with the peptide, with the aid of mild sonication.

Experimental details

Table S2. Dynamic light scattering (DLS) data from the nanoparticles

#	Polymer	[Dodecane]	DLS before polymerisation		DLS after polymerisation	
		(wt%)	Z-ave D_h (nm)	PDI	Z-ave D_h (nm)	PDI
NP1	**P3**	20	340 ± 4	0.222 ± 0.009	254 ± 9	0.202 ± 0.022
NP2	**P4**	20	221 ± 2	0.17 ± 0.002	238 ± 2	0.209 ± 0.005
NP3	**P4**	2	230 ± 3	0.183 ± 0.024	248 ± 1	0.21 ± 0.014

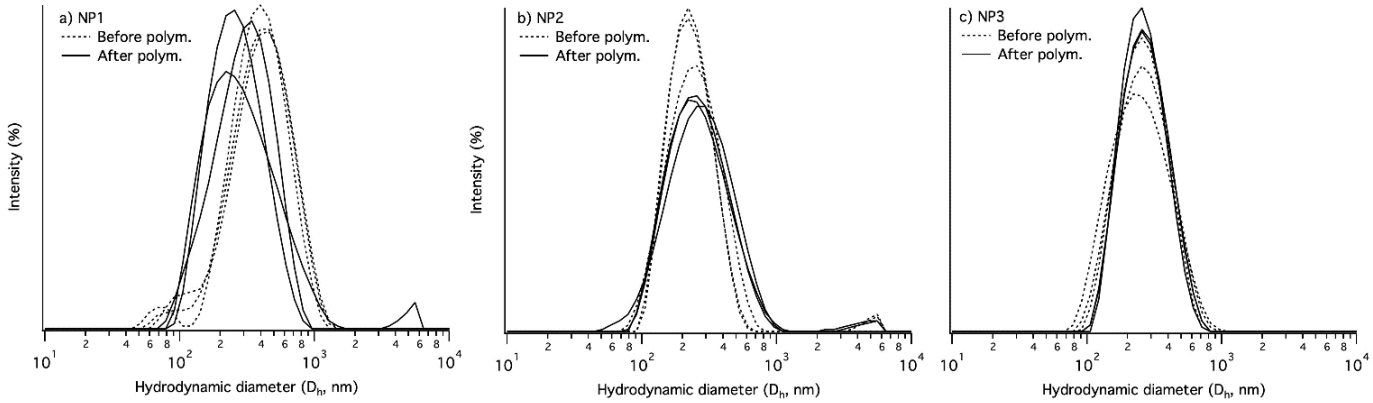


Figure S2. Dynamic light scattering (DLS) intensity distributions for the three nanoparticles a) NP1 made using polymer P3, b) NP2 made using polymer P4, and c) NP3 made using P4, before and after polymerization. Measurements taken in triplicate in PBS.

Table S3. Dynamic light scattering (DLS) data from NP2 after purification in PBS and after addition of a concentrated citrate buffer (pH5.5, 200mM), showing the shrinkage at pH 5.5

Buffer	Solution pH	Z-ave D_h (nm)	Int-ave D_h (nm)	PDI
PBS	7.4	316 ±69	262 ± 9	0.334 ± 0.015
PBS / Citrate (200mM) (4:1 v/v)	5.5	168 ±1	147 ± 1	0.119 ± 0.006

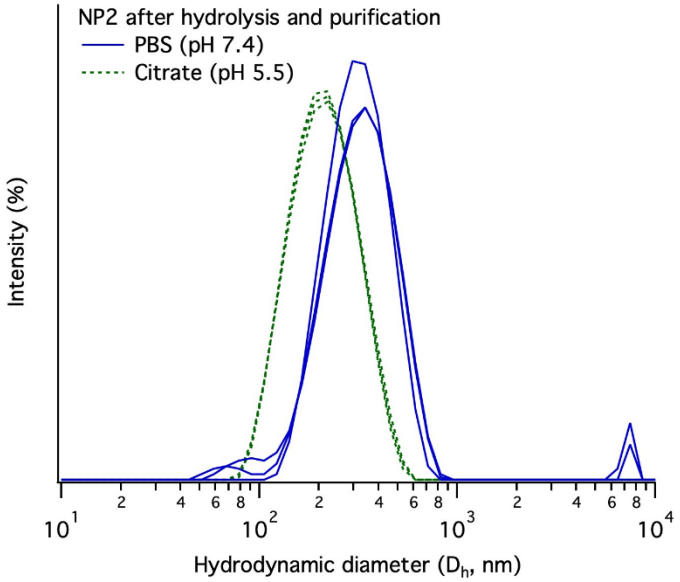


Figure S3. Dynamic light scattering (DLS) intensity distributions for NP2 after purification at pH 7.4 and pH 5.5 as shown in Table S3. Measurements taken in triplicate.

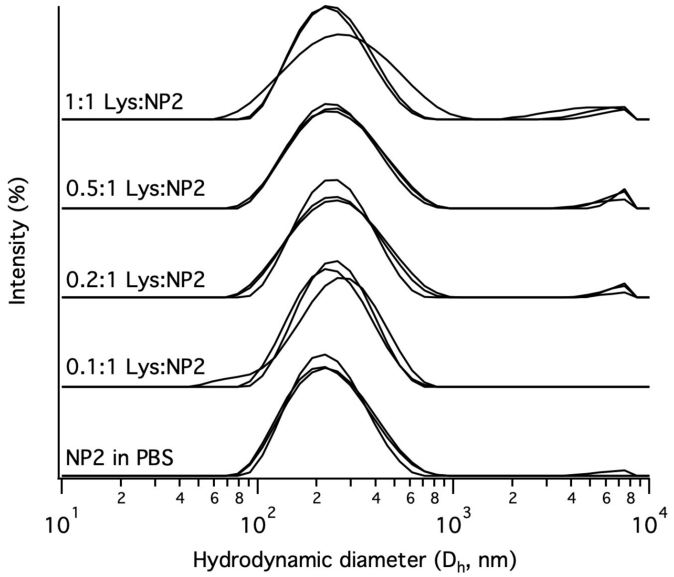


Figure S4. Particle size as measured by DLS of a 1mg/ml solution of NP2 incubated with varying concentrations of lysine (0.1 eq by weight to 1 eq by weight). No change in particle size was observed.

Materials and Methods

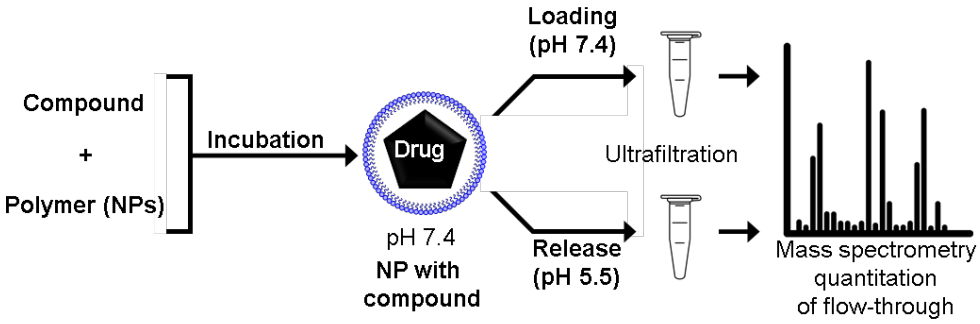


Figure S5: Diagram of compound loading and release using NP2

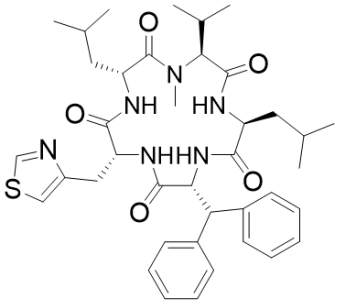
Micelle loading and stability assays *via* mass spectrometry

Synthesis of compounds used in biological assays has been reported elsewhere.[3, 4] Compounds **SM253**, **LB71** and **LB76** were added to NP2 in PBS buffer (pH 7.4) to a total volume of 500µL. NP2 concentration was kept constant at 1000µg/mL and drug concentrations of 125µg/mL, 250µg/mL, 500µg/mL and 1000µg/mL were tested to generate ratios of 1:8, 1:4, 1:2 and 1:1 peptide: polymer. 100µL aliquots of micelles were taken at 1h, 2h, 6h and 24h timepoints after addition of peptide and polymer. These aliquots were filtered using 10K centrifugal filters (UFC501096, Merck Millipore) using a 40º fixed angle rotor at 14,000 x g, room temperature for 10 minutes. The filtrate was diluted x20 or x50 in ultrapure water to a final volume of 1000µL in preparation for mass spectrometry. A standard curve was generated using 0.5µg/mL, 1µg/mL, 2µg/mL, 4µg/mL and 8µg/mL of compound. Samples were analysed using a Phenomenex Aeris XB-C18 column (3.6 µm, 2.1 x 100 mm) on a Shimadzu LCMS 8030. The mobile phase consisted of milli-Q water with 0.1% (v/v) formic acid (Mobile Phase A), and HPLC grade acetonitrile with 0.1% (v/v) formic acid (Mobile Phase B). Samples were processed at a flow rate of 0.2 mL/min, starting at 95% Mobile Phase A and 5% Mobile Phase B. Compounds were analysed using a selected ion monitoring (SIM) scan to detect specific mass of compound.

Raw loading data for SM253

Table S4: Raw data for loading of **SM253** into polymeric NPs in different conditions

Ratio (peptide: polymer)	Peptide (µg/mL)	Polymer (µg/mL)	Loading monitoring (hours)	Loading from Shimadzu (µg/mL)	Dilution factor	Loading (µg/mL)	Loading % (w/w)	Loading efficiency (%)
1:1	1000	1000	1h	9.280	20	185.602	18.56%	18.56%
			2h	10.197	20	203.947	20.39%	20.39%
			6h	12.478	20	249.567	24.96%	24.96%
			24h	10.477	20	209.533	20.95%	20.95%
1:2	500	1000	1h	3.889	20	77.776	7.78%	15.56%
			2h	4.323	20	86.467	8.65%	17.29%
			6h	4.895	20	97.898	9.79%	19.58%
			24h	4.303	20	86.070	8.61%	17.21%
1:4	250	1000	1h	3.547	20	70.940	7.09%	28.38%
			2h	4.420	20	88.392	8.84%	35.36%
			6h	4.039	20	80.773	8.08%	32.31%
			24h	3.911	20	78.223	7.82%	31.29%
1:8	125	1000	1h	2.376	20	47.523	4.75%	38.02%
			2h	3.339	20	66.781	6.68%	53.42%
			6h	3.037	20	60.730	6.07%	48.58%
			24h	2.592	20	51.850	5.18%	41.48%



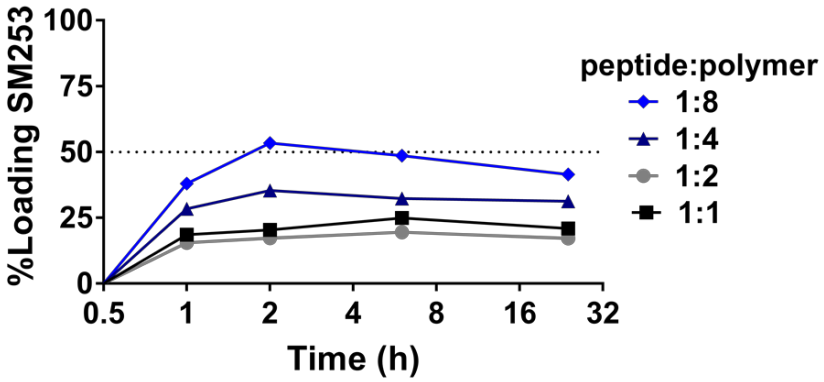


Figure S6: Loading efficiency and stability of compound **SM253** loaded into NP2 over 24 hours.

Raw loading data for LB71

Table S5: Raw data for loading of **LB71** into polymeric NPs in different conditions

Ratio (peptide: polymer)	Peptide (µg/mL)	Polymer (µg/mL)	Loading monitoring (hours)	Loading from Shimadzu (µg/mL)	Dilution factor	Loading (µg/mL)	Loading % (w/w)	Loading efficiency (%)
1:1	1000	1000	1h	8.434	50	421.720	42.17%	42.17%
			2h	9.673	50	483.640	48.36%	48.36%
			6h	10.695	50	534.730	53.47%	53.47%
			24h	10.787	50	539.360	53.94%	53.94%
1:2	500	1000	1h	4.683	50	234.160	23.42%	46.83%
			2h	5.426	50	271.305	27.13%	54.26%
			6h	6.080	50	303.990	30.40%	60.80%
			24h	6.126	50	306.310	30.63%	61.26%
1:4	250	1000	1h	4.271	50	213.553	21.36%	85.42%
			2h	4.463	50	223.160	22.32%	89.26%
			6h	4.658	50	232.913	23.29%	93.17%
			24h	4.799	50	239.955	24.00%	95.98%
1:8	125	1000	1h	2.282	50	114.098	11.41%	91.28%
			2h	2.334	50	116.705	11.67%	93.36%
			6h	2.456	50	122.801	12.28%	98.24%
			24h	2.456	50	122.824	12.28%	98.26%

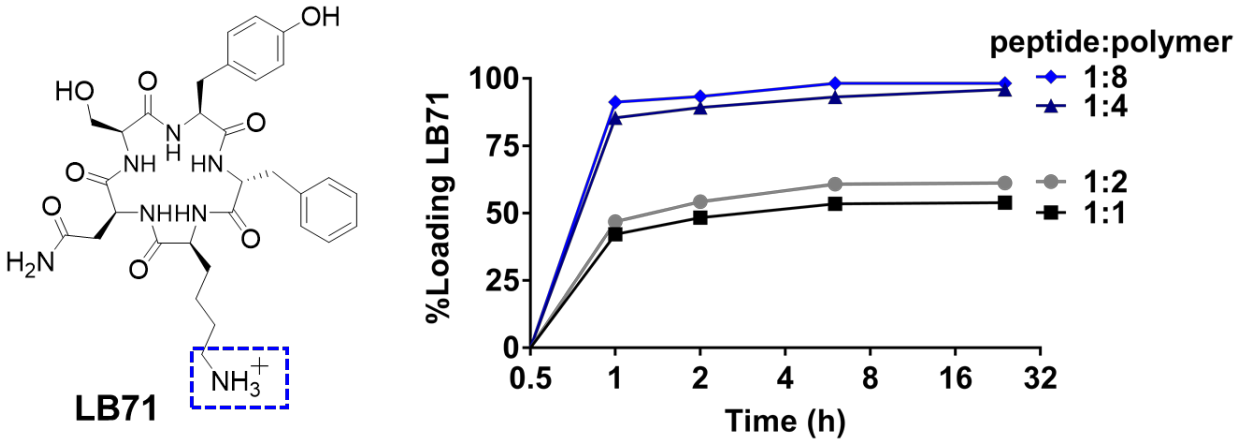
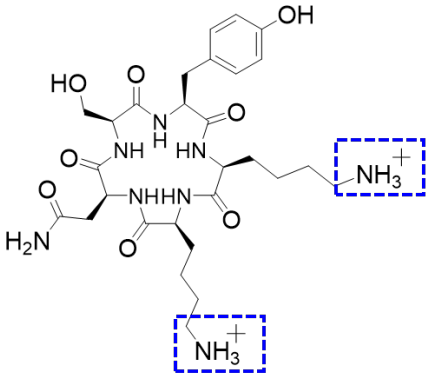


Figure S7: Loading efficiency and stability of compound **LB71** loaded into NP2 over 24 hours.

Raw loading data for LB76

Table S6: Raw data for loading of **LB76** into polymeric NPs in different conditions

Ratio (peptide: polymer)	Peptide (µg/mL)	Polymer (µg/mL)	Loading monitoring (hours)	Loading from Shimadzu (µg/mL)	Dilution factor	Loading (µg/mL)	Loading % (w/w)	Loading efficiency (%)
1:1	1000	1000	1h	12.015	50	600.728	60.07%	60.07%
			2h	12.244	50	612.183	61.22%	61.22%
			6h	12.597	50	629.863	62.99%	62.99%
			24h	12.639	50	631.927	63.19%	63.19%
1:2	500	1000	1h	6.304	50	315.193	31.52%	63.04%
			2h	6.805	50	340.246	34.02%	68.05%
			6h	7.104	50	355.182	35.52%	71.04%
			24h	7.109	50	355.426	35.54%	71.09%
1:4	250	1000	1h	4.317	50	215.837	21.58%	86.33%
			2h	4.703	50	235.153	23.52%	94.06%
			6h	4.750	50	237.518	23.75%	95.01%
			24h	4.751	50	237.563	23.76%	95.03%
1:8	125	1000	1h	2.425	50	121.252	12.13%	97.00%
			2h	2.425	50	121.257	12.13%	97.01%
			6h	2.425	50	121.257	12.13%	97.01%
			24h	2.493	50	124.627	12.46%	99.70%



LB76

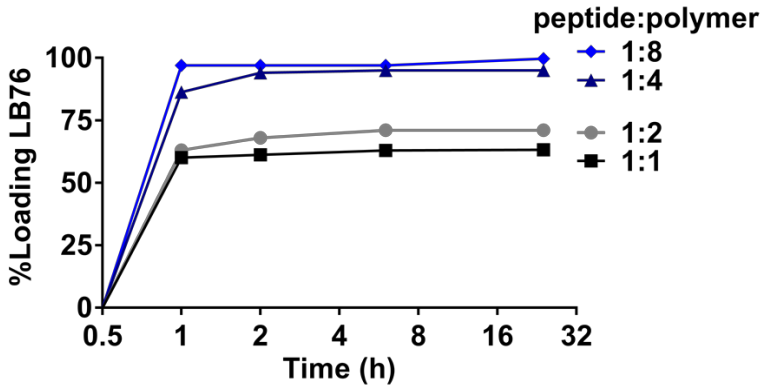


Figure S8: Loading efficiency and stability of compound **LB76** loaded into NP2 over 24 hours.

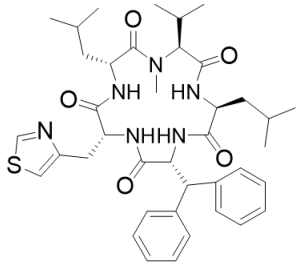
Micelle release assays *via* mass spectrometry

Compounds **SM253**, **LB71** and **LB76** were added to NP2 in MES buffer (100mM, pH 7.4) to a concentration of 125µg/mL **SM253**, 250µg/mL **LB71** and **LB76** and 1000µg/mL NPs over 16 hours. The total volume of mixtures was made up to 100µL. Loaded NPs were centrifuged using 10K centrifugal filters (UFC501096, Merck Millipore) using a 40º fixed angle rotor at 14,000 x g, room temperature for 10 minutes to remove unloaded peptides. Micelles were resuspended in 100µL MES buffer (100mM, pH 7.4). 4 x 25µL of micelles were diluted in 4 x 75µL MES buffer (100mM, pH 5.5). Micelles were filtered using 10K centrifugal filters (UFC501096, Merck Millipore) using a 40º fixed angle rotor at 14,000 x g for 10 minutes at 1h, 2h, 6h, and 24h after pH 5.5 treatment. The filtrate was diluted x20 in ultrapure water to a final volume of 1000µL in preparation for mass spectrometry. A standard curve was generated using 0.5µg/mL, 1µg/mL, 2µg/mL, 4µg/mL and 8µg/mL of **SM253** and 0.5µg/mL, 1µg/mL, 2µg/mL, 4µg/mL, 8µg/mL, and 16µg/mL of **LB76** and **LB71**. Samples were analysed using a Phenomenex Aeris XB-C18 column (3.6 µm, 2.1 x 100 mm) on a Shimadzu LCMS 8030. The mobile phase consisted of milli-Q water with 0.1% (v/v) formic acid (Mobile Phase A), and HPLC grade acetonitrile with 0.1% (v/v) formic acid (Mobile Phase B). The samples were run at a flow rate of 0.2 mL/min, starting at 95% Mobile Phase A and 5% Mobile Phase B. Compounds were analysed using a selected ion monitoring (SIM) scan to detect specific mass of compound.

Raw release data for SM253

Table S7: Raw data for release of **SM253** from polymeric NPs at 1:8 peptide:polymer ratio. * Assuming 100% loading of compound

Ratio (peptide: polymer)	Peptide (µg/mL)	Polymer (µg/mL)	Release monitoring (hours)	Release from Shimadzu (µg/mL)	Dilution factor	Release (µg/mL)	Release %*
1:8	125	1000	1h	0.342	20	6.848	5.48%
			2h	0.902	20	18.040	14.43%
			6h	1.276	20	25.524	20.42%
			24h	1.340	20	26.792	21.43%



SM253

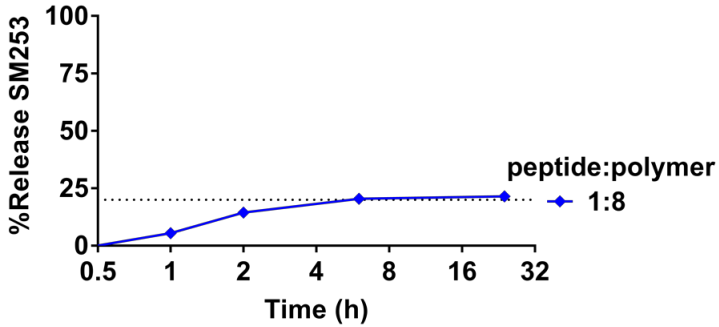


Figure S9: Release efficiency of compound **SM253** from NP2 over 24 hours.

Raw release data for LB71

Table S8: Raw data for release of **LB71** from polymeric NPs at 1:4 peptide:polymer ratio. * Assuming 100% loading of compound

Ratio (peptide: polymer)	Peptide (µg/mL)	Polymer (µg/mL)	Release monitoring (hours)	Release from Shimadzu (µg/mL)	Dilution factor	Release (µg/mL)	Release %*
1:4	250	1000	1h	1.007	50	50.353	20.14%
			2h	1.441	50	72.025	28.81%
			6h	1.837	50	91.858	36.74%
			24h	1.965	50	98.233	39.29%

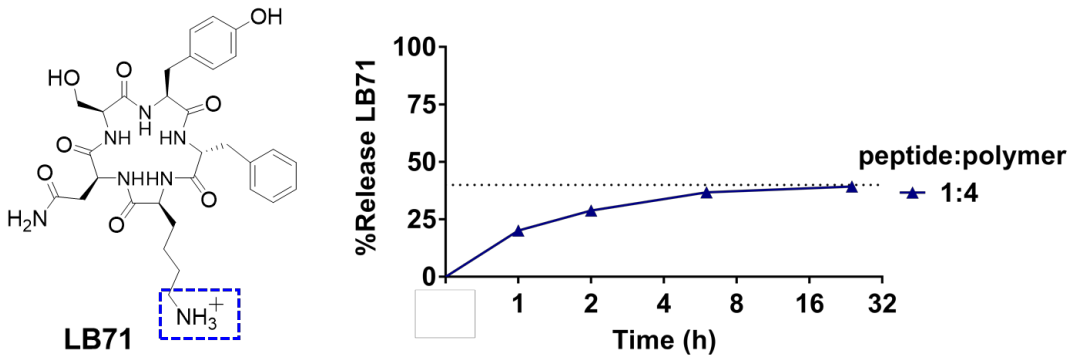
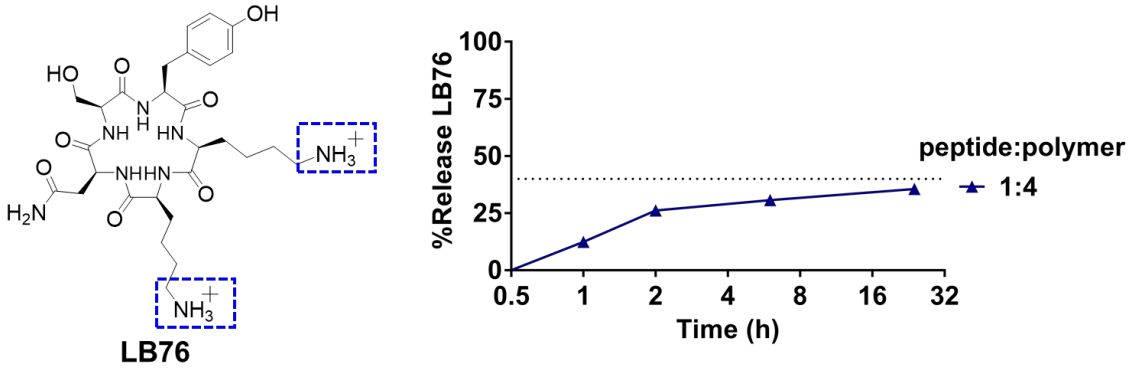


Figure S10: Release efficiency of compound **LB71** from NP2 over 24 hours.

Raw release data for LB76

Table S9: Raw data for release of **LB76** from polymeric NPs at 1:4 peptide:polymer ratio. * Assuming 100% loading of compound

Ratio (peptide: polymer)	Peptide (µg/mL)	Polymer (µg/mL)	Release monitoring (hours)	Release from Shimadzu (µg/mL)	Dilution factor	Release (µg/mL)	Release %*
1:4	250	1000	1h	0.620	50	31.018	12.41%
			2h	1.307	50	65.335	26.13%
			6h	1.532	50	76.615	30.65%
			24h	1.777	50	88.853	35.54%



. **Figure S11**: Release efficiency of compound **LB76** from NP2 over 24 hours.

Cytotoxicity assay

Cytotoxicity of the compounds and micelles against human colon cancer cell line HCT-116 cells was determined using Cell Counting Kit-8 [2-(2-methoxy-4-nitrophenyl)-3-(4-nitrophenyl)-5-(2,4-disulfophenyl)-2H-tetrazolium, monosodium salt] reduction assay. The cells were maintained in Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin (Invitrogen/Life Technologies). HCT-116 cells were seeded in 96-well dishes (2 x 10^3 cells per well) and allowed to adhere to the dish for 24 hours. Compounds were diluted in DMSO and NP2-compounds were prepared using protocol described above (Micelle loading and stability assays *via* mass spectrometry). Once prepared, the buffer for micelles were exchanged for Dulbecco's Modified Eagle Medium (DMEM), supplemented with 10% fetal bovine serum (FBS) and 1% penicillin/streptomycin and 10% DMSO. This prepared micelles for use in cell-based assays. For each assay (100 µl reaction volume), cells were incubated in the presence of control compounds or test compounds in complete growth medium (DMEM with 10% FBS). Each compound and NP-compound was tested at a range of concentrations to determine the GI_{50}. Controls for the assay were media alone (no cells; background control), DMSO (1%; negative control), and micelles only (in 1% DMSO). Cells were incubated in the presence of compound for 72 hours at 37ºC with 5% CO_2.Proliferation of HCT-116 cells was measured using a Cell Counting Kit-8 assay (CCK-8; Dojindo Molecular Technologies, Rockville, Maryland, USA), following the manufacturer's instructions. Reduction of the formazan dye was measured using a ChroMate 4300 microplate reader (450 nm; Awareness Technology Inc.). The absorbance values for compound-treated samples were compared to the DMSO control, and the average percent growth inhibition was determined for each compound tested. GI_{50} values were determined by plotting the percent growth inhibition versus the concentration of compound, and analysis was performed using GraphPad Prism software (GraphPad Software, Inc., La Jolla, California, USA). Assays were performed in at least triplicate. GI_{50} values is an average of cytotoxicity assays with standard error of mean represent error.

Protein expression assay

HCT116 cells were seeded in 6-well plates (3 × 10^5 cells per well) and incubated for 24 h before treatments. Cells were treated with indicated drugs and NP-compounds for 24 h and then lysed in lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.5% sodium deoxycholate and 0.5% NP40) supplemented with cocktail protease inhibitors (Roche) for a further 4 h. The total protein concentrations of lysates were determined by the bicinchoninic acid (BCA) method with the BCA kit (Pierce) following the manufacturer's instructions. 50 µg of total protein were separated by 8% Tris-Glycine gel and transferred to a PVDF membrane (Thermo Fisher Scientific). Membranes were blocked with 5% non-fat milk in TBS-T (Tris-buffered saline containing 0.1% Tween-20) for 1 h and incubated with respective primary antibodies in 2.5% non-fat milk (in TBS-T) at 4 °C overnight. After wash with cold TBS-T, membranes were incubated with respective HRP-conjugated secondary antibodies at 4 °C for 30 min, following by three-time wash with cold TBS-T and one wash with cold TBS (Tris-buffered saline). Immunoblotting was performed using chemiluminescent substrates (Thermo scientific) and the images were captured by ImageQuant LAS 4010 digital imaging system (GE Healthcare).

Protein levels of Hsp70 following treatment with free drug and micelles

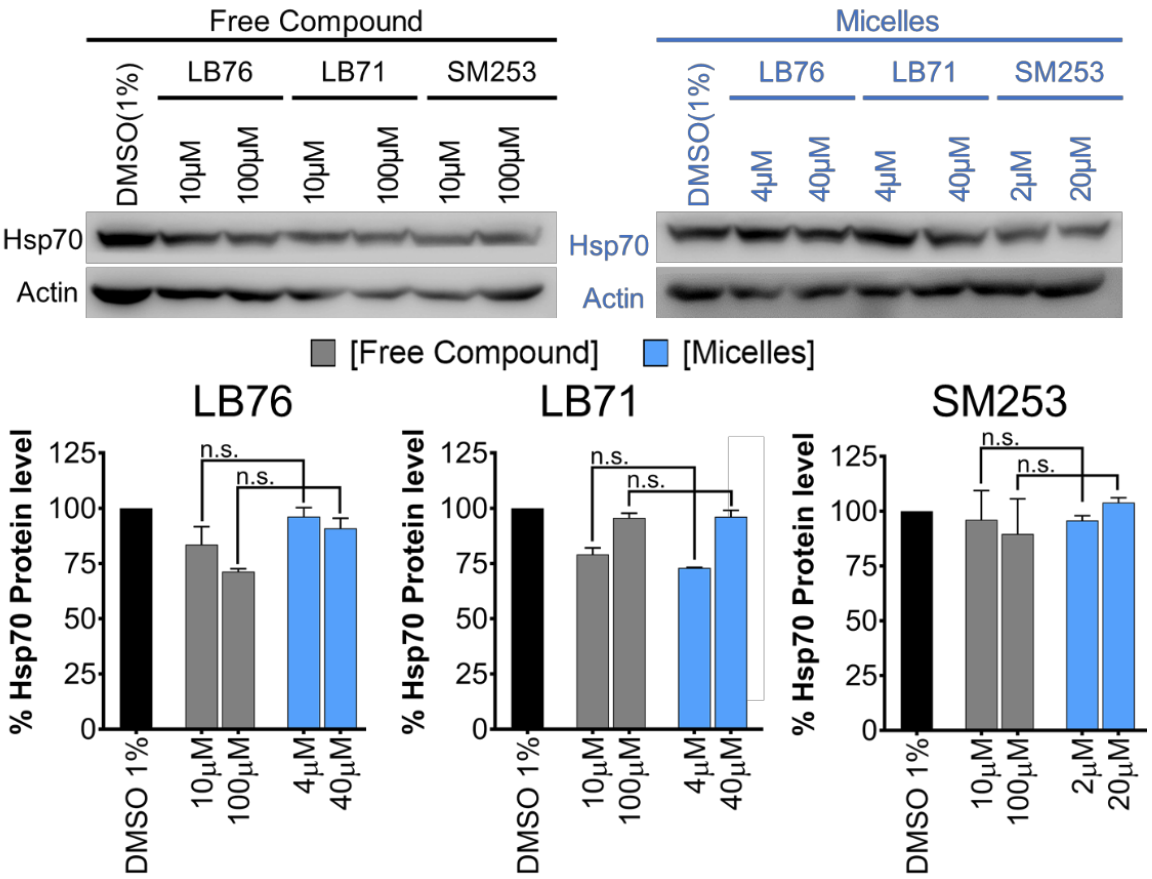
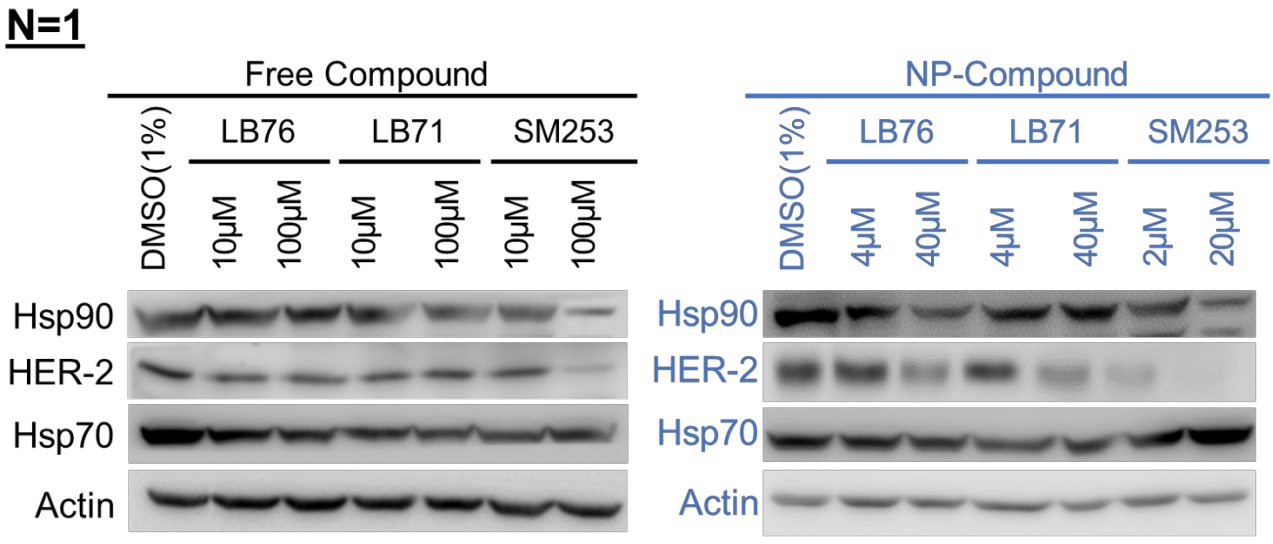
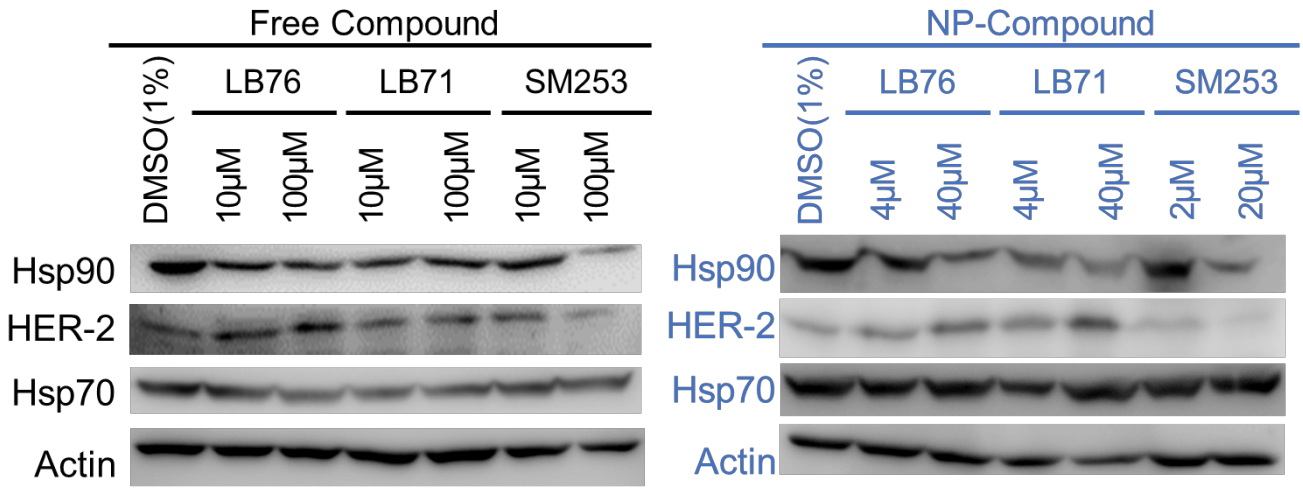


Figure S12: Impact of free compounds and NP-drugs on expression levels of Hsp70. All experiments repeated (n= ≥ 3) and normalized against respective actin band. *Estimated compound concentration based on release studies.

Replicates of protein expression



N=2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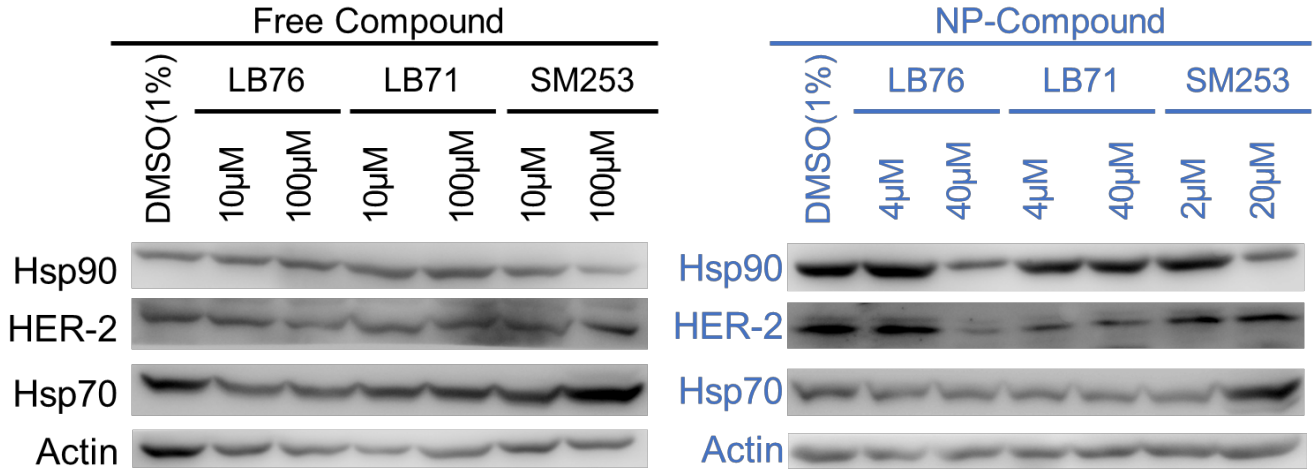
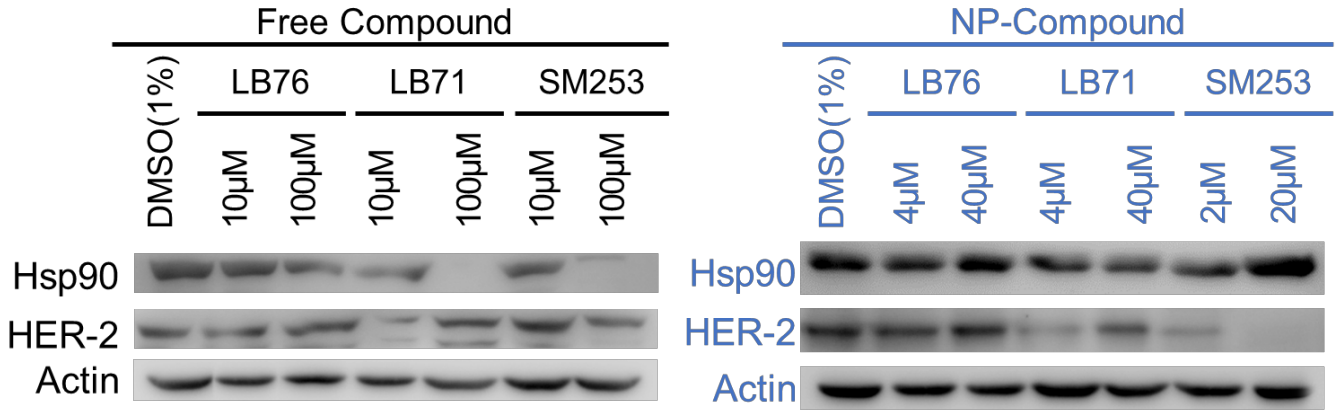
LB76
LB71
SM253
10μM
100μM
10μM
100μM
10μM
100μM
Hsp90
HER-2
Hsp70
Actin
NP-Compound
DMSO(1%)
LB76
LB71
SM253
4μM
40μM
4μM
40μM
2μM
20μM
Hsp90
HER-2
Hsp70
Actin

N=3


Free Compound
DMSO(1%)
LB76
LB71
SM253
10μM
100μM
10μM
100μM
10μM
100μM
Hsp90
HER-2
Hsp70
Actin
NP-Compound
DMSO(1%)
LB76
LB71
SM253
4μM
40μM
4μM
40μM
2μM
20μM
Hsp90
HER-2
Hsp70
Actin

N=4


Free Compound
DMSO(1%)
LB76
LB71
SM253
10μM
100μM
10μM
100μM
10μM
100μM
Hsp90
HER-2
Actin
NP-Compound
DMSO(1%)
LB76
LB71
SM253
4μM
40μM
4μM
40μM
2μM
20μM
Hsp90
HER-2
Actin

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