Supporting Information

A Multifunctional Lipid-based Nanodevice for the Highly-specific Co-delivery of Sorafenib and Midkine siRNA to Hepatic Cancer Cells

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MATERIALS. SOR was obtained from AstaTech Inc., USA. MK-siRNA and control siRNA (siGL4) were synthesized by Hokkaido System Science, Japan and their sequences are listed in Table S1. Oligonucleotide primers used in qRT-PCR were supplied by Sigma genosys (Japan) and their sequences are listed in Table S2. Cysteine-terminated SP94 peptide was synthesized by SMC Co., Japan with the following aminoacid sequence: SFSIIHTPILPL-Cys. YSK05 lipid was synthesized in our laboratory. N- [1- (2,3- dioleoyloxy) propyl] - N, N, N- trimethylammonium chloride (DOTAP), 1,2-dioleoyl-sn-glycero-3-phosphoethanolamine (DOPE), 1,2-dioleoyl-sn-glycero-3-phosphocholine (DOPC) and 1,2-distearoyl-sn-glycero-3-phosphocholine (DSPC) were obtained from Avanti Polar Lipids, USA. 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphoethanolamine (POPE), egg L-α-phosphatidylcholine (EPC) and 1,2-distearoyl-sn-glycero-3-phosphoethanolamine-N-[maleimide(polyethylene glycol)-2000] (DSPE-PEG_{2k}-MA) were obtained from the NOF corporation, Japan. Cholesterol, Triton X100, the Tri reagent and sinapic acid were obtained from Sigma-Aldrich, USA. Polyethylenimine (PEI)-branched type (average molecular weight = 10 KDa), Acetonitrile, Ethanol, Dextran Sulphate, Chloroform and Isopropanol were obtained from Wako, Japan. Stearylated octaarginine peptide (STR-R8) was synthesized by Kurabo, Japan. Trifluoroacetic acid (TFA) was obtained from Tokyo Chemical Industry Co. (TCI, Japan). HEPES buffer powder was obtained from Dojindo, Japan. Phosphate Buffer Saline (PBS) buffer tablets were obtained from Takara, Japan. Ribogreen was obtained from Invitrogen, USA. 3-(4,5-Dimethylthiazol-2-yl)-2,5-Diphenyltetrazolium Bromide (MTT) cell proliferation assay kit was obtained from Abcam, UK. ReverTra Ace qPCR RT Master Mix kit with gDNA Remover and Thunderbird SYBR qPCR Mix were obtained from TOYOBO, Japan. Cell culture plates; 2.5 × 150 mm, 6-well plates and 24-well plates were obtained from Corning, USA. 35-mm glass-base dishes were obtained from Iwaki Co., Japan.

Nuclease-free water, 1,1'-Dioctadecyl-3,3,3',3'-Tetramethylindodicarbocyanine 4-Chlorobenzenesulfonate Salt (DiD) and 2'-(4-ethoxyphenyl)-5-(4-methylpiperazin-1-yl)-2,5'-bibenzimidazole (Hoechst 33342) were obtained from Thermo Fisher Scientific, USA. Cy3-labeled siRNA was obtained from Qiagen, USA. Dialysis membranes with cut-off molecular weight of 3.5 KDa (Spectra/Por 6) were obtained from Spectrum Labs, USA.

CELL LINES. The human HCC cell line, HepG2, the human cervical adenocarcinoma cell line, HeLa, the mouse hepatocellular carcinoma cell line, Hepa 1-6, and the mouse normal hepatocytes cell line, FL83B, were obtained from the American Type Culture Collection (ATCC, USA). Each cell line was cultured and maintained according to manufacturer's guidelines. HepG2 and Hepa 1-6 cells were cultured in Dulbecco's Modified Eagle Medium (DMEM) containing high proportion of glucose (4.5 g/L) (Nacalai tesque, Japan) while HeLa cells were cultured in DMEM containing low proportion of glucose (1 g/L) (Sigma Aldrich, USA). FL83B cells were cultured in F-12K medium (ATCC, USA). All media were supplemented with 10% v/v heat-inactivated fetal bovine serum (FBS) (Biosera, USA) and antibiotic solution (Thermo Fisher Scientific, USA) containing 100 unit/mL penicillin and 100 µg/mL streptomycin to prevent microbial contamination. All cells were kept in an incubator (Thermo Electron Corporation, USA) at 37 °C, 5% CO2 and 95% humidity.

SUPPLEMENTARY METHODS

Synthesis of SP94-modified DSPE-PEG. Cysteine-terminated SP94 peptide was simply conjugated to DSPE-PEG_{2k}-MA via Micheal reaction schemed in Fig. S4. Individual ethanolic or aqueous solution of each reactant was prepared in concentration of 2 mM, then equal volumes of the two solutions were mixed and incubated under shaking at 900 rpm, 30 °C for 24 hours to obtain the conjugate with a final concentration of 1 mM. Matrix-assisted Laser Desorption

Ionization-Time of Flight Mass Spectrometry (MALDI-TOF-MS) was used to confirm the completion of reaction and the molecular weight of the conjugate.

MALDI-TOF-MS analysis. MALDI-TOF-MS was used after conjugation reaction to confirm molecular weight of reactants and product. Aqueous solution of acetonitrile (30%) containing 0.1% trifluoracetic acid (TFA) and 1% sinapic acid was used as a matrix solution. Molecular weight of each sample was determined using MALDI-TOF Ultraflex II equipment (Bruker Daltonics, USA).

Decoration of LNPs with SP94-modified DSPE-PEG. SP94-modified DSPE-PEG was inserted into the LNPs either by a pre-insertion or a post-insertion method. In the pre-insertion method, an ethanolic solution of SP94-modified DSPE-PEG was directly added in the desired amount to an ethanolic solution of the other lipids before the evaporation and formation of the thin lipid film. In the post-insertion method, the desired amount of an aqueous solution of SP94-modified DSPE-PEG was added to the aqueous solution of the LNPs after their formation and the LNPs then were incubated at room temperature or 60 °C for 30 minutes to allow the nanoparticles to stabilize.

Examination of the morphology of SP94-NPs. A Transmission Electron Microscope (TEM) (Hitachi HD2000, Japan) was used to visualize the morphology of the optimized SP94-NPs as reported previously.[1]

***In vitro* drug release.** *In vitro* drug release profile from LNPs was determined at two different pH values; 7.4, which represented the physiologic pH, and 5.5, representing endosomal pH. To ensure sink condition, amount of LNPs equivalent to SOR dose 17.43 μg was diluted to total volume 500 μL with HEPES buffer (pH 7.4), introduced into pre-hydrated dialysis membrane

(cut-off molecular weight = 3.5 KDa) and dialyzed against 2 L of HEPES buffer (pH 7.4 or 5.5) over a magnetic stirrer (300 rpm, 37 °C) for 48 hours. At different time intervals, samples of 10 µL of LNPs were diluted with ethanol and assayed spectrophotometrically for the remaining SOR content. The amount of SOR released was determined by subtracting the remaining SOR content in LNPs from the initial SOR content at zero time and expressed as percentage of SOR dose released against time.

Kinetic analysis of drug release from LNPs. The mechanism of drug release from LNPs was determined by linear regression analysis according to zero-order, first-order and Higuchi-diffusion models which are the most common models for controlled-release delivery systems. The correlation coefficient (r) value was calculated for each model. The highest value of the calculated correlation coefficients assigned the kinetic model of the drug release. The drug release data were fitted to the following equations:

Zero-order model: $M_t / M_\infty = K_0 t$. *(Equation S1)*

First-order model: $M_t / M_\infty = e^{-K1t}$ *(Equation S2)*

Higuchi-diffusion model : $M_t / M_\infty = k_H t^{½}$. *(Equation S3)*

Where (M_t / M_∞) is the fractional release of the drug at time t, k_0 = Zero-order rate constant, k_1 = First-order rate constant, k_H = Higuchi rate constant and t = time of release.

Then, the release data were analyzed using the equation proposed by Ritger and Peppas:

$M_t / M_\infty = Kt^n$ *(Equation S4)*

Where M_t / M_∞ is the fractional release of the drug at time t, K is the release rate constant and n is the diffusional exponent that characterizes the type of release mechanism during the

dissolution process. In case of LNPs (spherical sample) , n= 0.43 for Fickian diffusion; while in case of non-Fickian release, the value of (n) falls between 0.43 and 0.85; for zero order release (case II transport), n= 0.85 and for supercase II transport, n > 0.85.

Competitive inhibition of the uptake of SP94-NPs. HepG2 and Hepa 1-6 cells, which showed the maximum uptake for SP94-NPs, were selected for this experiment, while FL83B cells, which showed negligible uptake of these LNPs, were excluded as they were likely lacking specific receptors for SP94 peptide. The cells were incubated with increasing concentrations of free SP94 peptide in serum-free medium at 37 °C for 1 hour before the experiment to saturate its specific receptors. Then, the medium was replaced with fresh serum-free medium and the cells were co-incubated with fixed concentration of SP94-NPs (equivalent to 10 μM SOR dose) and increasing concentrations of free SP94 for additional 4 hours. The cells were harvested and the cellular uptake was measured by FACS as described previously. Mean fluorescence intensity (MFI) of each sample was normalized to MFI of cells that received SP94-NPs without incubation with free SP94 peptide, which was considered as 100% cellular uptake.

Long-term stability testing of LNPs. The optimum LNPs were stored in refrigerator (4 °C) for two months. Average particle size was measured weekly to investigate nanoparticles liability for aggregation upon long-term storage.

SUPPLEMENTARY FIGURES

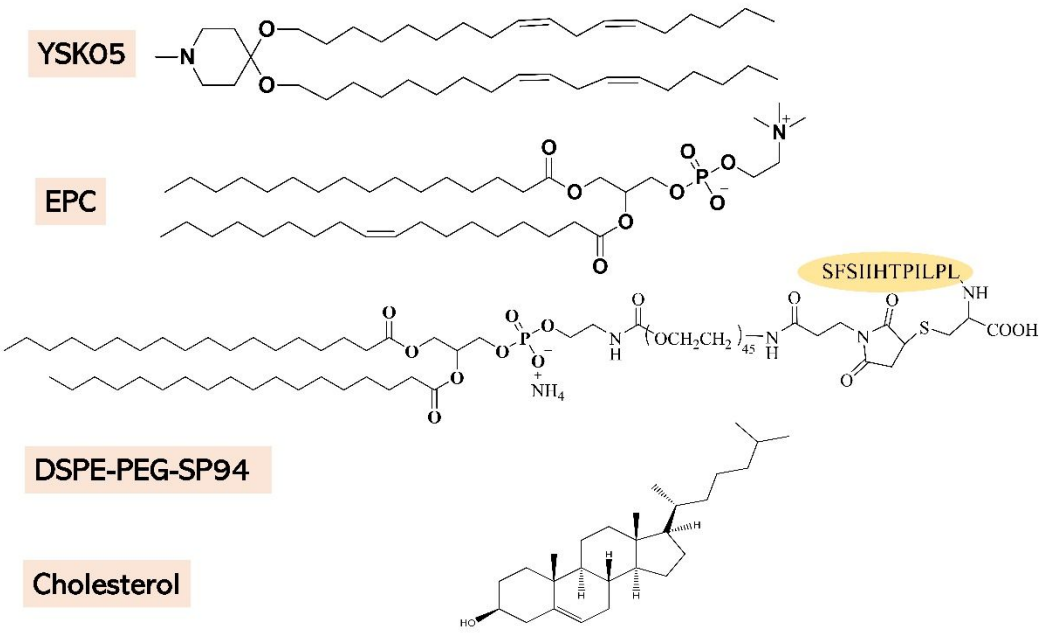

Fig. S1. Chemical structures of lipids used in the optimized LNPs (SP94-NPs).

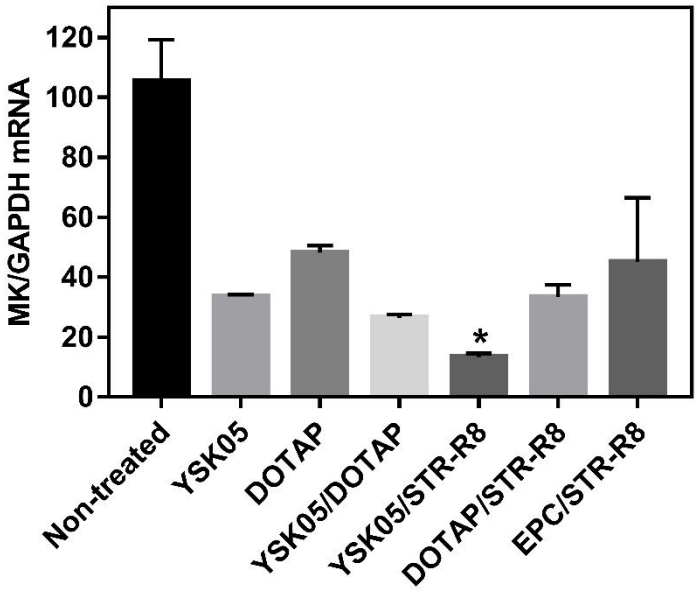

Fig. S2. Effect of LNPs based on different main lipids encapsulating MK-siRNA on MK gene expression in HepG2 cells at fixed siRNA dose (*P<0.05 versus the other LNPs). MK-siRNA concentration=100 nM. The average of three independent experiments was considered and expressed ± standard deviation.

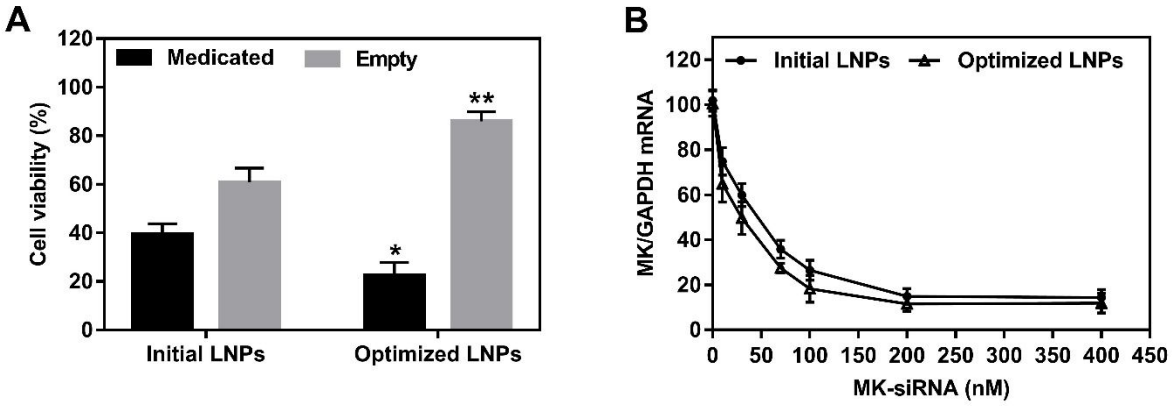

Fig. S3. Comparison between the performance of the initial and optimized STR-R8 NPs in HepG2 cells. A) Evaluation of cytotoxic effect (*P<0.05 versus medicated initial LNPs, **P<0.001 versus empty initial LNPs). SOR concentration= 10 μM, MK-siRNA concentration=400 nM. B) Evaluation of MK gene knockdown activity at different MK-siRNA doses. The average of three independent experiments was considered and expressed ± standard deviation.

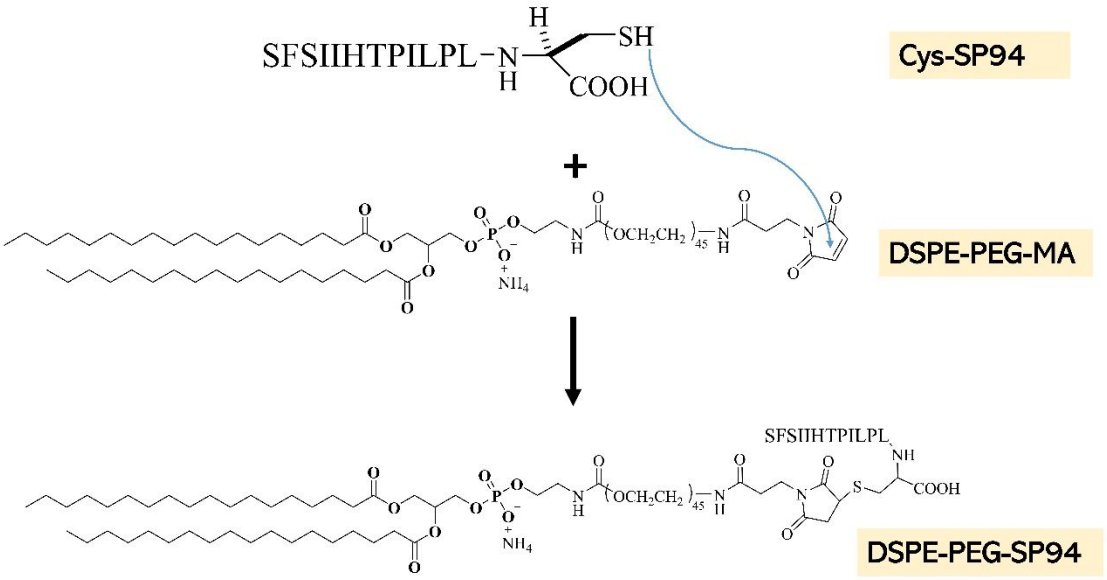

Fig. S4. Schematic representation for the synthesis of SP94-modified DSPE-PEG.

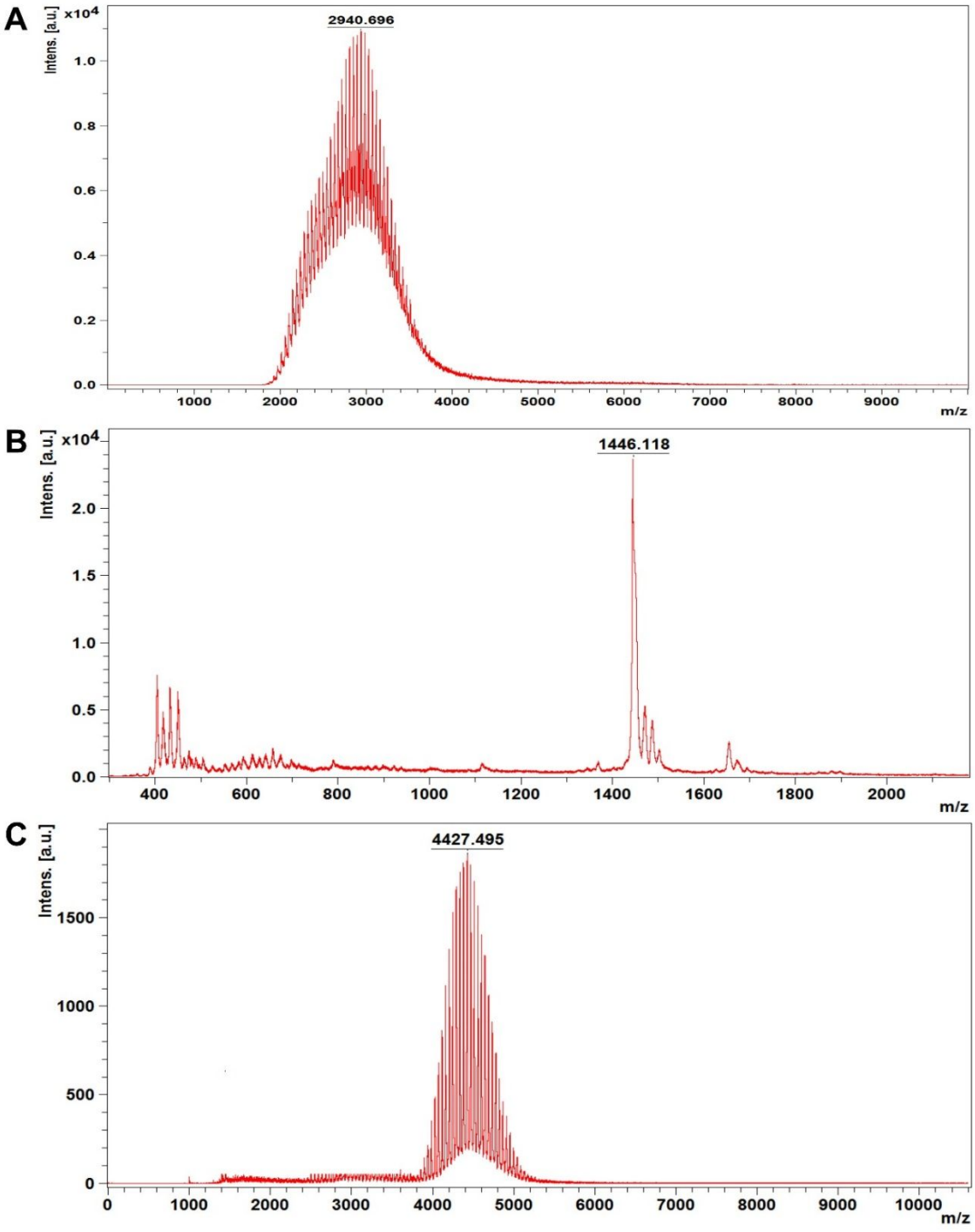

Fig. S5. MALDI-TOF-MS spectra for DSPE-PEG_{2K}-MA (A), SP94 peptide (B) and SP94-modified DSPE-PEG (C).

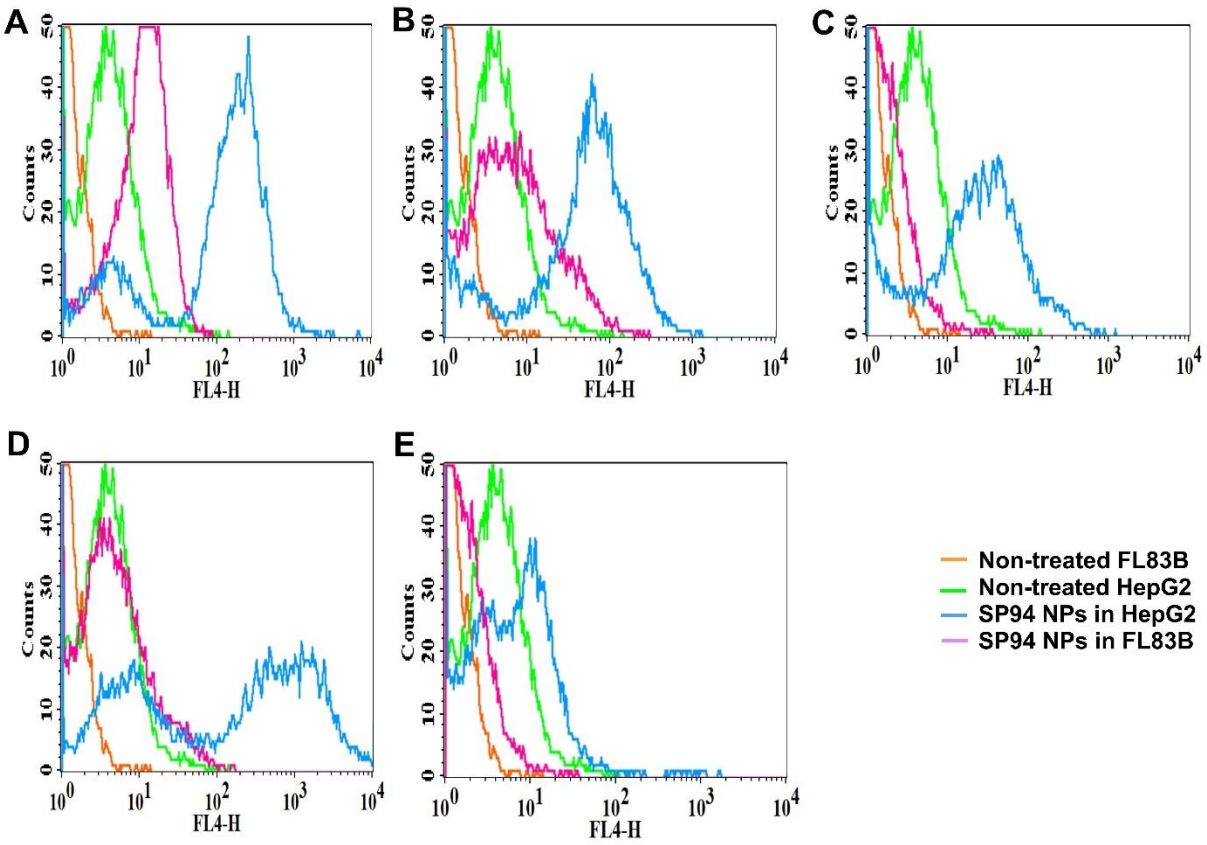

Fig. S6. FACS histogram plots for the uptake of LNPs modified with different concentrations of DSPE-PEG-SP94 in HepG2 and FL83B cells. A) 0 mol%. B) 1.25 mol%. C) 2.5 mol%. D) 5 mol%. E) 10 mol%. SOR concentration= 10 μM, MK-siRNA concentration=400 nM.

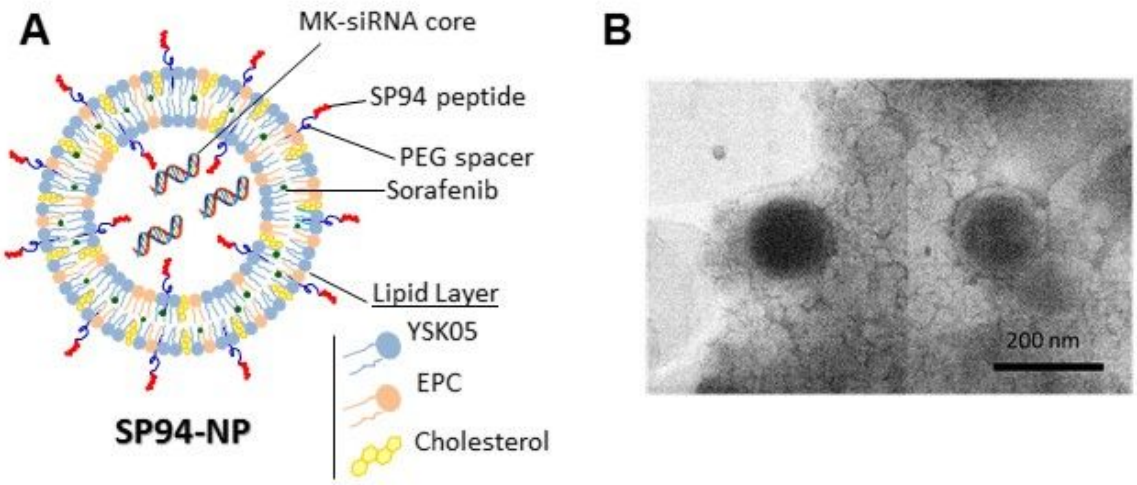

Fig. S7. Structure of the optimized SP94 NPs. A) A schematic representation for the composition of SP94 NPs. B) TEM image for the optimized SP94 NP.

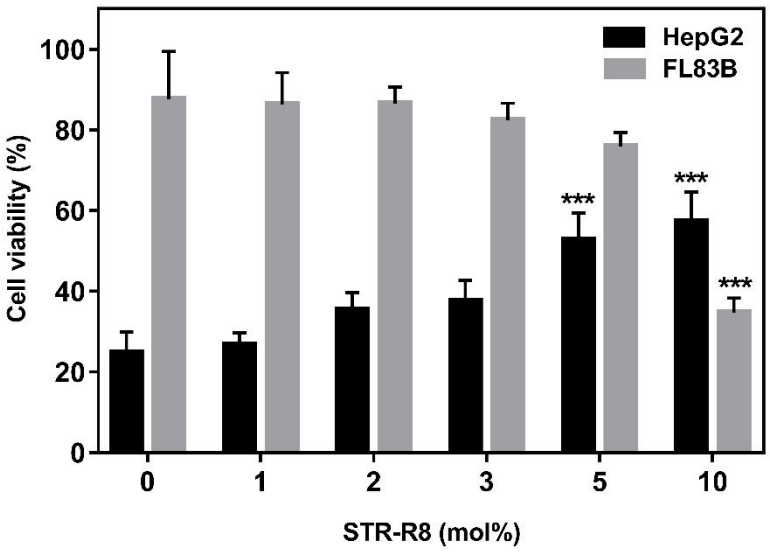

Fig. S8. Effect of LNPs modified with 5 mol% PEG-SP94 and different concentrations of STR-R8 on the cell viability of HepG2 and FL83B cells (***P<0.0001 versus 0-3 mol% in HepG2 cells, ***P<0.0001 versus 1-5 mol% in FL83B cells). SOR concentration= 10 μM, MK-siRNA concentration=400 nM. The average of three independent experiments was considered and expressed ± standard deviation.

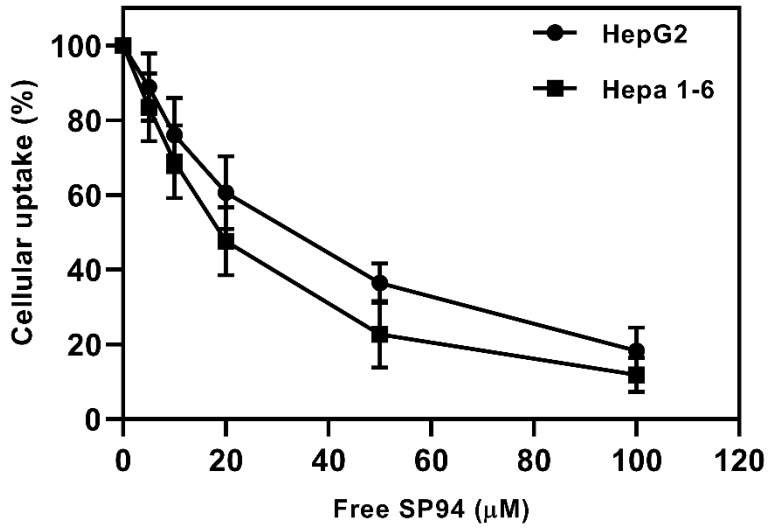

Fig. S9. Competitive inhibition of SP94-NPs uptake in HepG2 and Hepa 1-6 cells by co-incubation with increasing concentrations of free SP94 peptide. SOR concentration= 10 μM, MK-siRNA concentration=400 nM. MFI of each sample was normalized to MFI of cells that received SP94-NPs without incubation with free SP94 peptide, which was considered as 100% cellular uptake. The average of three independent experiments was considered and expressed ± standard deviation.

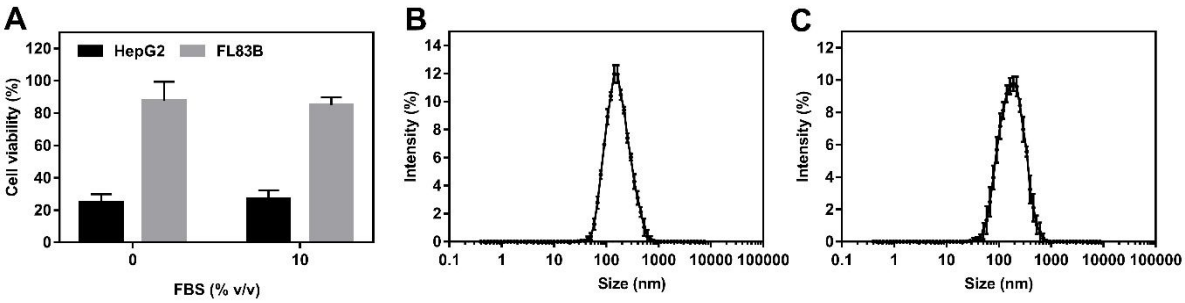

Fig. S10. Serum compatibility and long-term stability of SP94-NPs. A) Effect of FBS on efficiency of SP94-NPs in HepG2 cells (SOR concentration= 10 μM, MK-siRNA concentration=400 nM). The average of three independent experiments was considered and expressed ± standard deviation. B) Frequency particle size distribution curve for SP94-NPs at zero-time. C) Frequency particle size distribution curve for SP94-NPs after 2-month storage at 4 °C (average ±standard deviation, n=6).

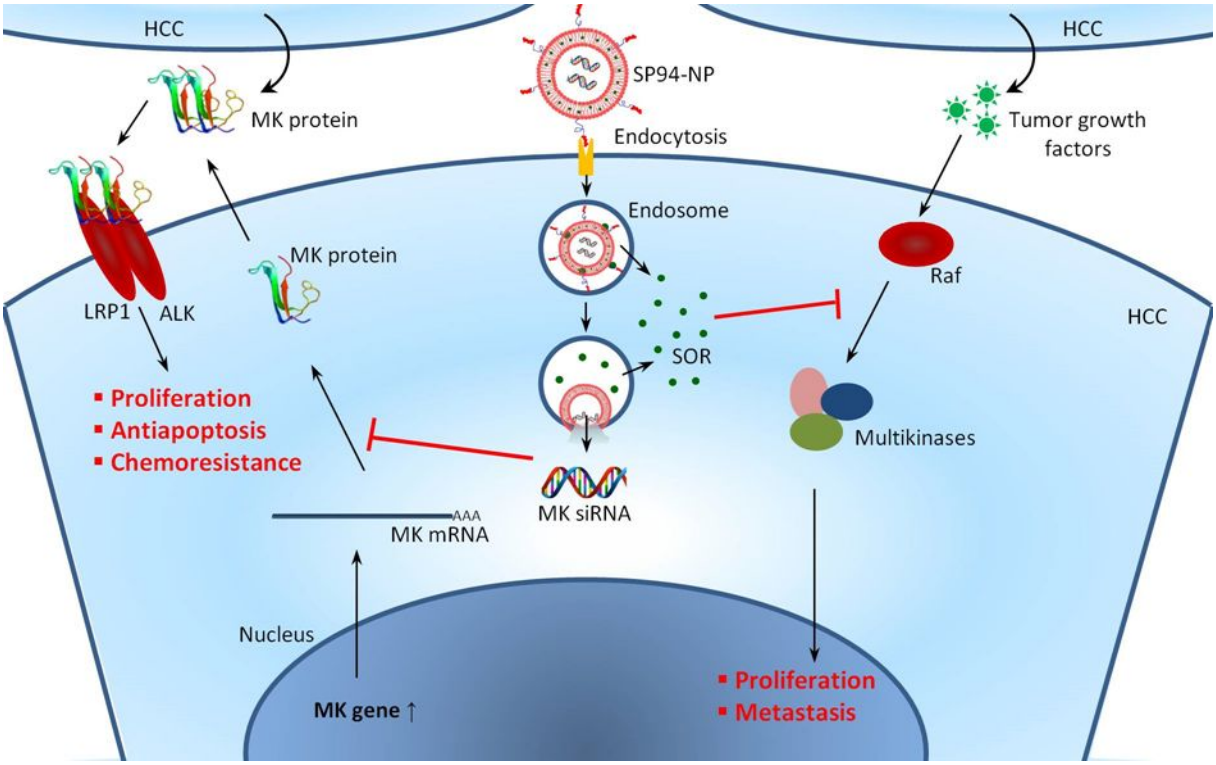

Fig. S11. A schematic illustration for the proposed mechanism of performance of SP94-NPs and the role of its different components. SP94-NPs are internalized into HCCs via specific SP94 receptors. The fusogenic lipid, YSK05, mediates a strong endosomal escape to release the payload into the cytosol. SOR inhibits Raf signaling and the action of other multikinases leading to apoptosis, while MK-siRNA prevents the translation of MK-mRNA into MK protein eliminating its proliferative, anti-apoptotic and chemoresistant functions which consequently, increases the sensitivity of HCCs to SOR. Abbreviations: HCC, Hepatic cancer cell; MK, Midkine; LRP1, LDL-receptor-related protein 1; ALK, anaplastic lymphoma kinase; mRNA, messenger RNA; siRNA, small interfering RNA; Raf, rapidly accelerated fibrosarcoma tyrosine kinase effector; SOR, sorafenib; NP, nanoparticle.

SUPPLEMENTARY TABLES

Table S1. Sequences of siRNAs used in this study

		sequence (5'→3')
MK-siRNA	Sense	GGA UUG CGG CGU GGG UUU Ctt
	Anti-sense	GAA ACC CAC GCC GCA AUC Ctt
Control siRNA (siGL4)	Sense	GCG CUG CUG GUG CCA ACC CdTdT
	Anti-sense	GGG UUG GCA CCA GCA GCG CdTdT

Table S2. Sequences of primers used in qRT-PCR in this study

Gene		sequence (5'→3')
Human MK	Forward	AGA TGC AGC ACC GAG GCT
	Reverse	CTT TCT TTT TGG CGA CCG
Human GAPDH	Forward	CCT CTG ACT TCA ACA GCG AC
	Reverse	CGT TGT CAT ACC AGG AAA TGA G
Mouse MK	Forward	GTC AAT CAC GCC TGT CCT CT
	Reverse	CAA GTA TCA GGG TGG GGA GA
Mouse GAPDH	Forward	AGC AAG GAC ACT GAG CAA G
	Reverse	TAG GCC CCT CCT GTT ATT ATG

Table S3. Effect of drug loading relative to total amount of lipids on SOR encapsulation efficiency in the prepared LNPs (average ± standard deviation, n=6, total lipid amount=1500 nmole)

SOR amount (nmole)	SOR loading (mol%)	SOR EE (%)
75	5	91.5±9.8
112.5	7.5	70.4±5.5
150	10	50±7.2

Table S4. Composition of the investigated SOR-loaded LNPs[a] based on different main lipids with/without CPP (STR-R8) and their corresponding IC_{50} values measured after 24 hour-incubation with HepG2 cells.

Formulation	Lipid composition and their molar ratios	IC_{50} (μM)[b]
Free SOR	-	14±2.80
YSK	YSK05/Cholesterol 7:3	12±2
DOTAP	DOTAP/DOPE/Cholesterol 4:3:3	18±4.75
YSK/DOTAP	DOTAP/YSK05/Cholesterol 4:3:3	14.64±2.50
YSK/STR-R8	YSK05/Cholesterol/STR-R8 6:3:1	9±1.70
DOTAP/STR-R8	DOTAP/DOPE/Cholesterol/STR-R8 4:3:3:1	13±3.40
EPC/STR-R8	EPC/Cholesterol/STR-R8 6:3:1	17±4.10

[a]SOR loading was 5 mol% of the total lipid amount (75 nmole SOR/1500 nmole lipids).

[b]The average of three independent experiments was considered and expressed ± standard deviation.

Table S5. Kinetic analysis of the release of SOR from SP94-NPs at different pH values (the average cumulative percentage of SOR dose released at different time points for three independent experiments was used in the calculations)

pH	Correlation coefficient (r)			$t_{50\%}$[a] (hr)	K_{rel}[b]
	Zero-order model	First-order model	Higuchi-diffusion model		
5.5	0.9809	0.1749	0.9934	24	10.2062
7.4	0.9739	0.3559	0.9925	> 48	N/A

[a]$t_{50\%}$: the time required for the release of 50% of SOR dose.

[b]$K_{rel.}$: drug release rate constant calculated based on the best-fit kinetic model.

Table S6. Analysis of SOR release data from SP94-NPs according to the Ritger and Peppas equation (the average cumulative percentage of SOR dose released at different time points for three independent experiments was used in the calculations)

pH	n[a]	K[a]	r[b]	Mechanism
5.5	0.777605	4.572692	0.9836	non-fickian
7.4	0.742614	2.103373	0.9896	non-fickian

[a]n and K are the parameters of Ritger and Peppas equation defined in supplementary methods section (Equation S4).

[b]r is the correlation coefficient calculated by linear regression analysis of the Logarithm of cumulative percentage of drug released (Log M_t / M_∞) versus the Logarithm of the corresponding different time points (Log t).

REFERENCES FOR SUPPORTING INFORMATION:

(1) Yamada, Y.; Burger, L.; Kawamura, E.; Harashima, H., Packaging of the Coenzyme Q10 into a Liposome for Mitochondrial Delivery and the Intracellular Observation in Patient Derived Mitochondrial Disease Cells. *Biological and Pharmaceutical Bulletin* **2017**, *40* (12), 2183-2190.