Supplementary Materials

Combined delivery of sorafenib and a MEK inhibitor using CXCR4-targeted nanoparticles reduces hepatic fibrosis and prevents tumor development

Yun-Chieh Sung[1,2,†], Ya-Chi Liu[1,†], Po-Han Chao[1,†], Chih-Chun Chang[1], Peiru Jin[1], Ts-Ting Lin[1], Ja-An Lin[3], Hui-Teng Cheng[4,5], Jane Wang[2], Charles P. Lai[6], Ling-Hsuan Chen[1,4], Anthony Y. Wu[1,6], Ting-Lun Ho[1], Tsaiyu Chiang[1], Dong-Yu Gao[1], Dan G. Duda[7], Yunching Chen[1,]*

[1] Institute of Biomedical Engineering, National Tsing Hua University, Hsinchu 30013, Taiwan

[2] Department of Chemical Engineering, National Tsing Hua University, Hsinchu 30013, Taiwan

[3] Department of Biostatistics, University of North Caroline at Chapel Hill

[4] Department of Internal Medicine, National Taiwan University Hospital Hsin-Chu Branch, Hsin Chu City 30059, Taiwan

[5] Research Center for Developmental Biology and Regenerative Medicine, National Taiwan University, Taipei, Taiwan

[6] Institute of Atomic and Molecular Sciences (IAMS), Academia Sinica, National Taiwan University, Taipei, Taiwan

[7] Steele Laboratories for Tumor Biology, Department of Radiation Oncology, Massachusetts General Hospital and Harvard Medical School, Boston, MA 02114, USA

[†] These authors contributed equally to this work.

* Corresponding author: Yunching Chen, PhD, Institute of Biomedical Engineering, National Tsing Hua University, Hsinchu 30013, Taiwan, ROC; phone: 886-3-571-5131; ext: 35503; email: yunching@mx.nthu.edu.tw

Supplementary Materials and methods

In vitro cytotoxicity

Cells were seeded in 96-well plates overnight and then treated with different concentrations of sorafenib or AZD6244 loaded in different formulations. After two days, 15 µl of MTT (5 mg/ml, dissolved in PBS) was added into each well and incubated for 3 hours at 37 °C. The medium was removed, and 50 µl of DMSO was added into each well. The absorbance was measured using a Multiskan (Thermo, USA) at 570 nm. All data were normalized based on the background intensity.

In vitro cellular uptake

Coumarin 6 was used as a tracer molecule formulated in the NPs. HSCs were seeded in a 12-well plate (Costar, IL, USA) and incubated overnight. The cells were then treated with Coumarin 6 in different formulations at 37 °C for 4 hours. The cells were washed with PBS, fixed with 4% paraformaldehyde (PFA) for 10 minutes and counterstained with mounting solution (4',6-diamidino-2-phenylindole or DAPI, Vector Laboratories, Burlingame, CA). The cellular uptake of Coumarin 6 was examined and quantified using a confocal microscope (LSM-780, Carl Zeiss, Germany).

To perform a peptide competitive cellular uptake assay, cells were prepared as previously described. Before treatment with Coumarin 6 loaded in CTCE9908-NPs, the cells were pre-treated with free CTCE9908 peptides at final concentrations of 0, 50, and 100 µg/ml for 30 minutes. The cellular uptake of Coumarin 6 was examined and quantified using a confocal microscope, as described in the previous paragraph.

Tissue distribution and fibrotic liver uptake

Mice with CCl4-induced liver fibrosis were intravenously injected with Coumarin 6-loaded NPs modified by CTCE9908 or scramble peptides (Coumarin 6=0.5 mg/kg). Four hours after the administration of Coumarin 6-loaded NPs, mice were sacrificed and tissues were collected and homogenized in RIPA lysis buffer before they were incubated on ice for 30 minutes. The tissue lysate was transferred to a black 96-well plate (Corning). The fluorescence intensity of the sample was measured using a plate reader (Fluoroskan Ascent FL, Thermo Scientific) at an excitation wavelength of 485 nm and an emission wavelength of 538 nm. The Coumarin 6 concentration in each sample was calculated from a standard curve.

The cellular uptake of Coumarin 6-loaded NPs in the fibrotic livers was further examined by confocal microscopy. Mice were sacrificed 4 hours after intravenous administration of Coumarin 6-loaded NPs. Liver tissues were collected and fixed in 4% paraformaldehyde. Frozen tissue sections (10 μm in thickness) were cut, stained with DAPI and imaged using a laser scanning confocal microscope (LSM 780, Zeiss, Germany).

Immunofluorescence

Frozen sections (10 μm thick) were fixed in acetone at -20 °C for 10 minutes and washed with PBS. The sections were then blocked with 5% bovine serum albumin (BSA, in PBS) for 1 hour and incubated overnight with primary antibodies against von Willebrand Factor (Dako, Denmark), α-SMA (Abcam Inc., Cambridge, MA), collagen I and collagen III (Abcam Inc., Cambridge, MA) at 4 °C. After washing with PBST (0.1% Tween 20 in 1x PBS), the sections were further incubated with Alexa Fluor® 488 secondary anti-rabbit IgG antibodies (Life Technologies, Grand Island, NY) for 1 hour. Unbound secondary antibodies were washed away with PBST, and the sections were counterstained with DAPI (Vector Laboratories, Burlingame, CA) and imaged using a confocal microscope (LSM 780, Zeiss, Germany).

The expression level was quantified by measuring the area occupied by the staining area of interest normalized by the area of DAPI-stained nuclei as the ratio of green/blue (Alexa Fluor 488/DAPI) relative fluorescence units. The fluorescence intensity was analyzed using Image J. Identical analysis settings and thresholds were applied for all liver sections. Vessel diameters were measured with confocal line scans across the blood vessels.

Masson's trichrome staining

The liver tissue was collected and fixed in 4% paraformaldehyde (PFA, in PBS) overnight before it was embedded in paraffin wax. The sections were then stained with Masson's trichrome according to the standard procedure; afterward, they were observed using a Nikon microscope (Eclipse E800, Tallahassee, FL).

Western blot analysis

Cells were lysed in lysis buffer RIPA for 30 min on ice and the supernatant was collected after centrifugation at 12,000 rcf. Cell lysate were separated on a 10% acrylamide gel and transferred to a PVDF membrane. Membranes were blocked for 1hr in 5% skim milk and then incubated overnight with polyclonal antibodies against collagen, αSMA, p-ERK, ERK, p-AKT, AKT, p-IkB and β-actin (from Cell Signaling, Danvers, MA). PDGFR inhibitor CP673451 was purchased from Selleck Chemicals (Houston, TX).

***In vivo* chemically induced HCC mice models**

C3H mice were administered diethylnitrosamine (DEN; single ip injection of 1mg/kg at 21 days of age). Later, CCl_4 (100μL of 16% (v/v), 3/week ip starting at 4 weeks of age) was administered for 17 weeks to develop advanced liver fibrosis and chemical-induced, fibrosis-promoted HCC. Treatment of sorafenib and AZD6244 loaded in CXCR4-targeted NPs was initiated after 9 weeks of CCl_4 administration, and spontaneous HCC formation and alterations in the hepatic microenvironment were examined after 9 weeks of treatment.

qRT-PCR array assay

HSCs were seeded in 12-well transparent plates (Costar, IL, USA) overnight and then treated with different doses of sorafenib and AZD6244 in CTCE9908-NPs and cultured in a CO_2 incubator for 24 h. Cells were washed once with PBS, and RNA was isolated from the cells using the RNeasy Mini Kit (Qiagen, Valencia, CA). RT was performed using the RT^2 First Strand cDNA Synthesis kit (QIAGEN). We determined the relative level of gene expression by using Real-Time SBYR Green PCR master mix (Applied Biosystems) on the ABI Prism 7500 Detection system. For analysis, the expression level for each gene of interest was calculated by normalization to the house keeping genes. The primer pairs used were *TGFB1 F (5'-CCCAGCATCTGCAAAGCTC-3') and TGFB1 R (5'-GTCAATGTACAGCTGCCGCA-3'), IL-1B F (5'-AATCTGTACCTGTCCTGCGTGTT-3') and IL-1B R (5'-TGGGTAATTTTTGGGATCTACACTCT-3'), VEGFA F (5'-TCTTCAAGCCATCCTGTGTG-3') and VEGFA R (5'- ATCCGCATAATCTGCATGGT -3'), PPARγ F (5'-CGTGGCCGCAGATTTGAA-3') and PPARγ R (5'-CTTCCATTACGGAGAGATCCAC-3').*

Sorafenib and AZD6244 (total daily dose: 5 mg/kg, two doses per week) loaded in the CXCR4 NPs were intravenously administered to mice with CCl_4-induced liver fibrosis beginning 4 weeks after the start of CCl_4 administration. The mRNA expressions of the fibrotic and inflammation markers were evaluated after 4 weeks of treatment. The primer pairs used were *TNF-a F (5'-CCGATGGGTTGTACCTTGT-3') and TNF-a R (5'-CGGACTCCGCAAAGTCTAAG-3'), IL-1B F (5'-TGCCACCTTTTGACAGTGAT-3') and IL-1B R (5'-TGTCCTCATCCTGGAAGGTC-3'), IL-6 F (5'-TGATGCACTTGCAGAAAACA-3') and IL-6 R (5'-ACCAGAGGAAATTTTCAATAGGC-3'), ACTA2 F (5'-CGCTTCCGCTGCCCAGAGACT-3') and ACTA2 R (5'-TATAGGTGGTTTCGTGGATGCCCGCT-3'), PPARγ F (5'-ACTATGGAGTTCATGCTTGTG-3') and PPARγ R (5'-CCTGATGGCATTGTGAGAC-3'), Col1A1 F (5'-CATGTTCAGCTTTGTGGACCT-3') and Col1A1 R (5'-GCAGCTGACTTCAGGGATGT-3').*

Toxicity study. Sera of C3H mice were collected 24 hours after IV injections of sorafenib and AZD6244-loaded in CTCE-9908 NPs (5 mg/kg) for evaluation of liver enzyme levels such as ALT, AST, ALP, and γ-GT and kidney injury markers such as BUN and CREA. Data are shown as mean values ± S.E.M..

Assessment of Apoptosis by TUNEL Staining. Frozen sections of livers were stained by using TACS^{TM} TdT Kit (R&D Systems, Minneapolis, MN) according to the manufacturer's recommendations. The apoptotic cells were counted in four randomly selected visual fields for each sample. The apoptotic index was calculated as the fraction of apoptotic nuclei.

Flow cytometry. Mice were anesthetized, perfused using intracardiac injection of PBS and sacrificed. Liver tissues were harvested in HBSS 1X buffer (Corning Cellgro, Manassas, VA), minced and digested into a single cell suspension in a solution containing collagenase type 1A (1.5 mg/ml), hyaluronidase (1.5 mg/ml), and DNase (2 mg/ml) in DMEM for 1 hr at 37° C. The suspension was filtered through a 70-µm cell strainer and then it was washed 3 times and then resuspended in cold flow buffer (1% BSA, 0.1% NaN$_3$ in PBS). Single-cell suspensions were incubated with rat anti-mouse CD16/CD32 mAb for FcR-blockade (to avoid non-specific staining), and then immunostained, washed and re-suspended in cold flow buffer. Flow cytometry data were acquired on a BD FACSAria III flow cytometer (Becton Dickinson) and analyzed with FACSDiva^{TM} software. We used the following monoclonal anti-mouse antibodies: CD45-FITC, CD11b-APC-Cy7 (BD Biosciences), F4/80-PE (eBioscience), and 7-AAD as previously described [1].

Supplementary Fig. 1. Effect of combined PDGFR inhibitor and MEK inhibitor on activation of the signal transduction pathways and myofibroblast differentiation in HSCs. A-B, Short-term treatment of PDGFR inhibitor (CP673451) for 2 hours suppressed AKT activation (**A**). Both pERK (**A**) and pIkB-α (**B**) remained unchanged after treatment of PDGFR inhibitor. **C,** Combination of MEK inhibitor AZD6244 and PDGFR inhibitor decreased p-IkB-**α** and **α**-SMA expression in HSCs 24 hours after treatment.
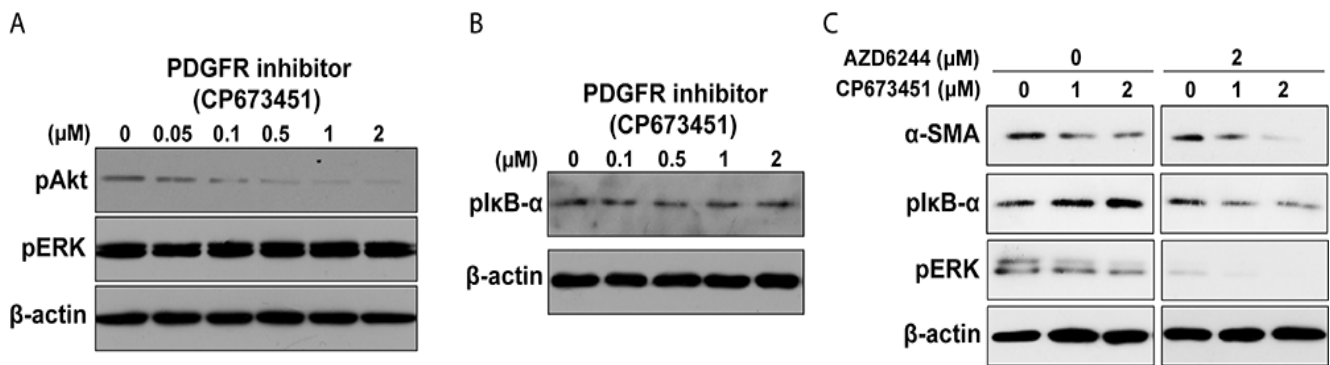
Supplementary Fig. 2. The uptake of CTCE9908-NPs in activated HSCs was competitively inhibited by addition of free CTCE-9908 peptides in a dose-dependent manner. HSCs were treated with CTCE9908 peptides prior to CTCE9908-NPs and analyzed for fluorescence signal by the confocal microscopy. Scale bar=100 μm.
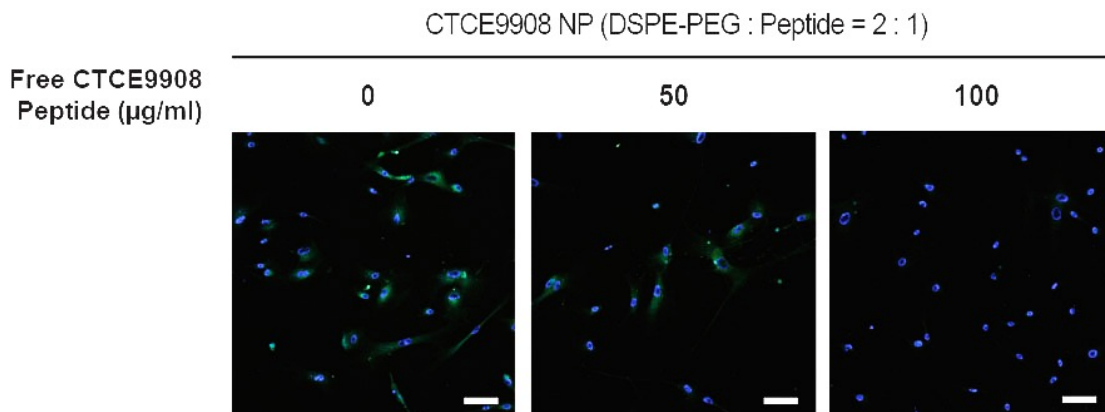
Supplementary Fig. 3. Sorafenib and AZD6244 loaded in CXCR4-targeted NPs suppressed mRNA expression of inflammatory markers such as TGF-β1, IL-1b, VEGF, PPARγ in activated HSCs in a dose-dependent manner (normalized by β-actin) (n=3). The data are the mean values ± the S.E.M. *p<0.05, **p<0.01.
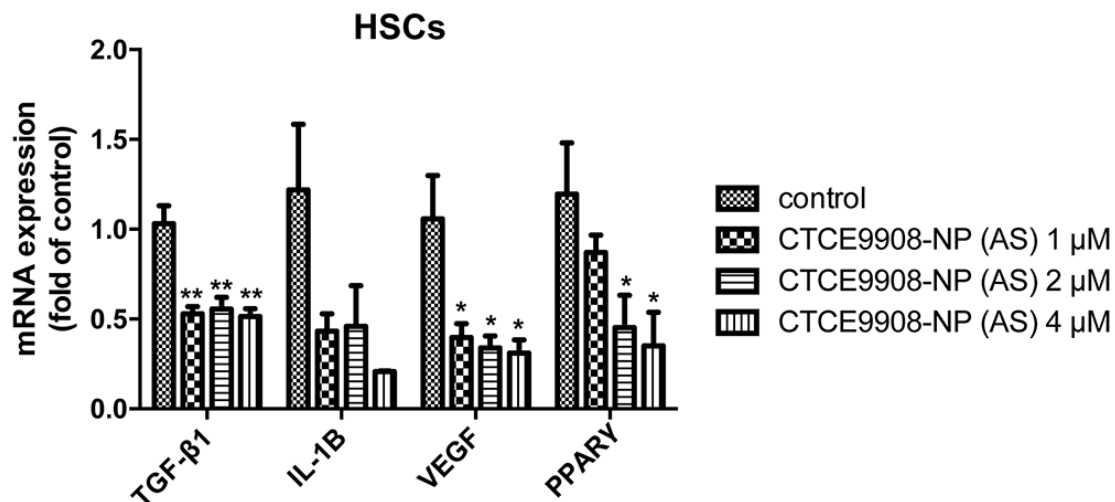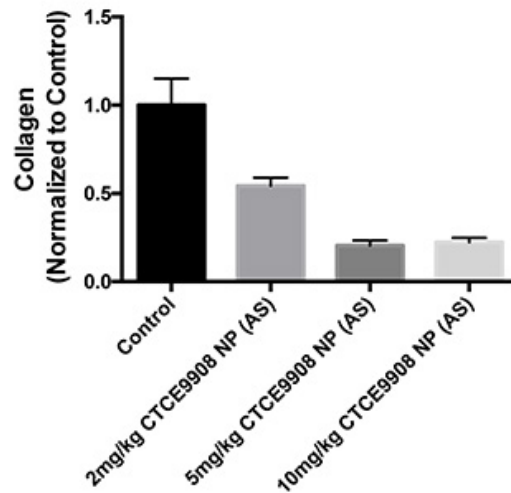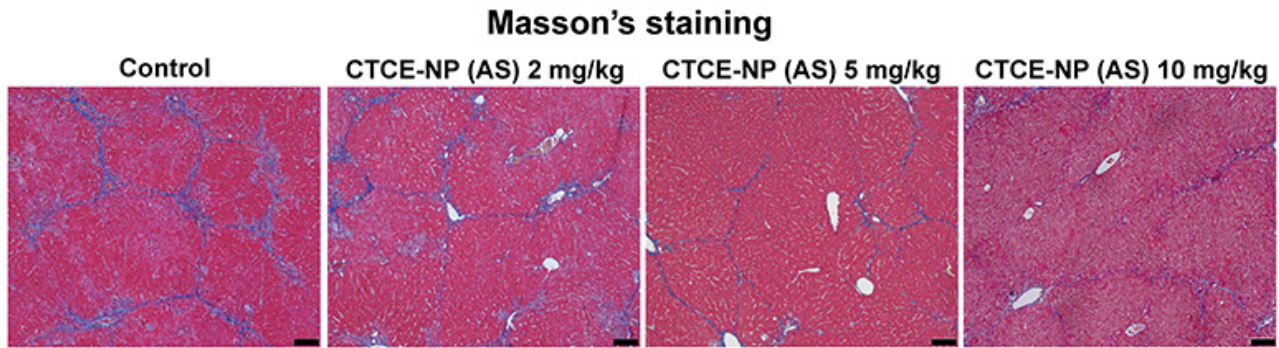

Supplementary Fig. 4. Sorafenib and AZD6244 in CXCR4 NPs (5 mg/kg) significantly decreased ECM deposition in the fibrotic livers of CCl4-treated mice, as indicated by Masson's trichrome staining. Masson's trichrome staining was analyzed by ImageJ (n = 5). The data are given as the mean values ± S.E.M., ***p<0.001.
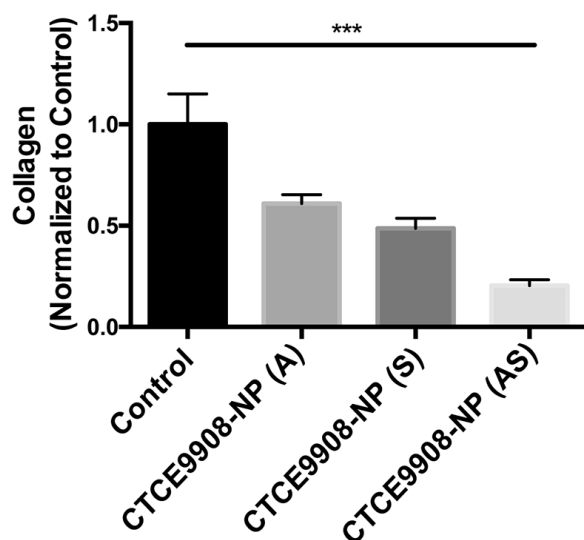
Supplementary Fig. 5. Sorafenib and the MEK inhibitor AZD6244 co-formulated in CTCE9908-NPs decreased liver fibrosis in the CCl₄-induced mouse model of liver fibrosis. Sorafenib and AZD6244 loaded in different formulations reduced liver fibrosis in a dose-dependent manner, as indicated by decrease in α-SMA and collagen III expression in the fibrotic livers (n=6). Scale bar=100 μm. The data are presented as mean values ± S.E.M., ***p<0.001.
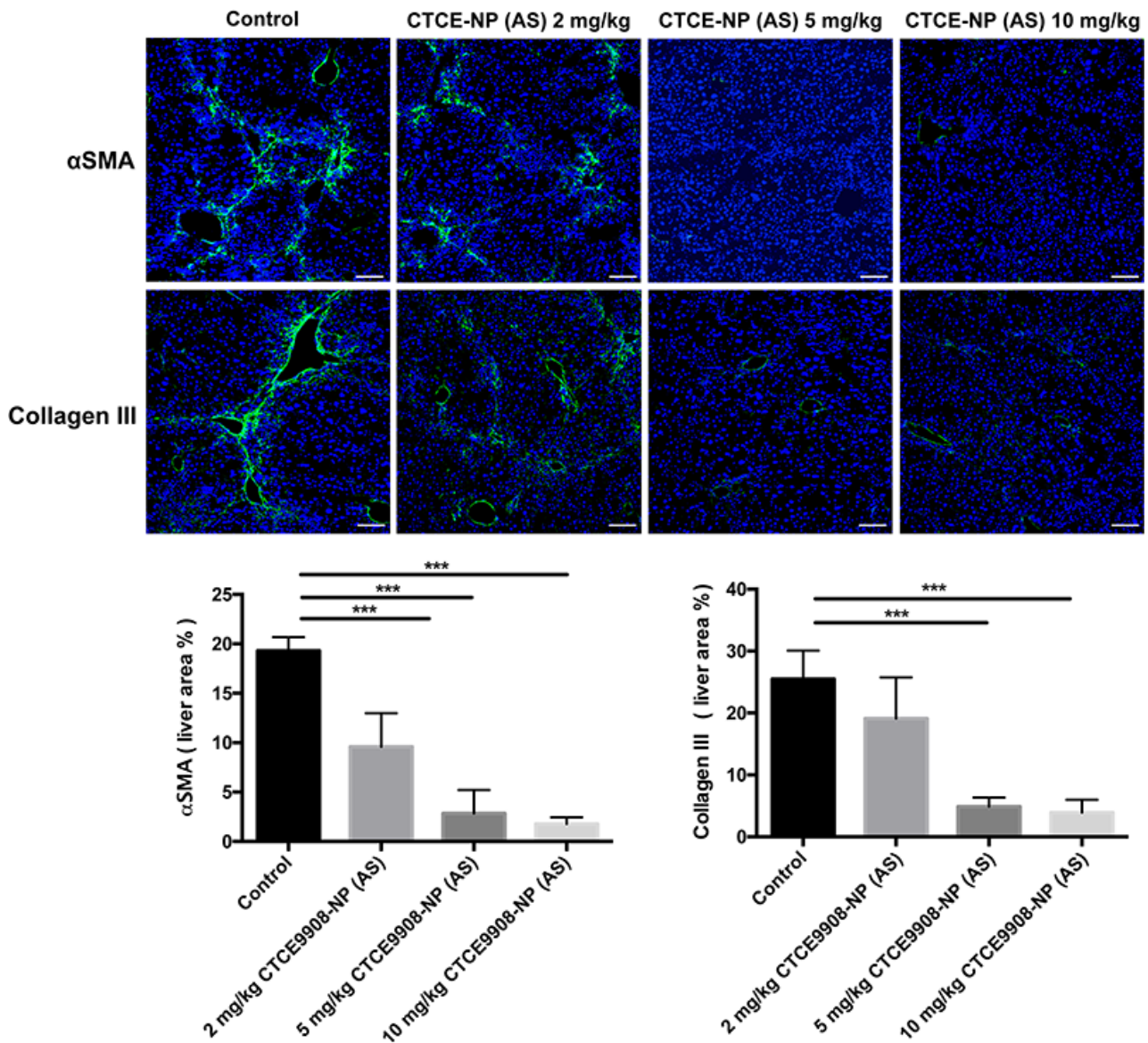
Supplementary Fig. 6. Sorafenib and the MEK inhibitor AZD6244 co-formulated in CTCE9908-NPs decreased liver fibrosis in the CCl4-induced mouse model of liver fibrosis in a dose-dependent manner, indicated by Masson's trichrome staining (n=5-7). The data are presented as mean values ± S.E.M.. Scale bar=100 μm.

Supplementary Fig. 7. Sorafenib and the MEK inhibitor AZD6244 co-formulated in CTCE9908-NPs reduced liver damage markers and facilitated liver repair in the CCl4-induced mouse model of liver fibrosis. **A**, Treatment schedule of sorafenib and AZD6244 in the CCl4-induced murine model of liver damage. **B,** Administration of CCl4 significantly increased the concentration of AST and ALT in serum compared with the levels in the control group. Sorafenib and the MEK inhibitor AZD6244 in CTCE9908-NPs dramatically reversed serum levels of ALT and AST (n=5). The data are presented as mean values ± S.E.M., *$p<0.05$, **$p<0.01$.
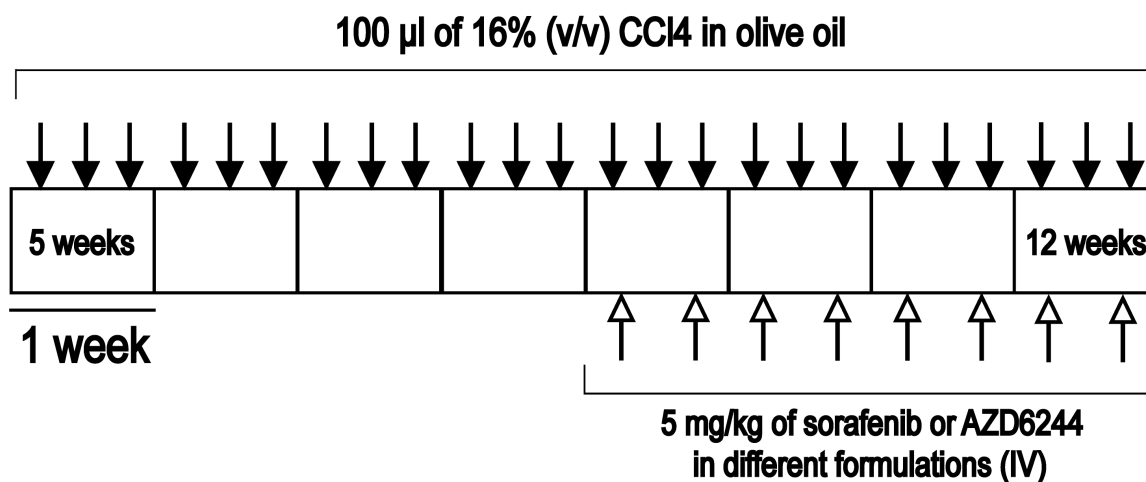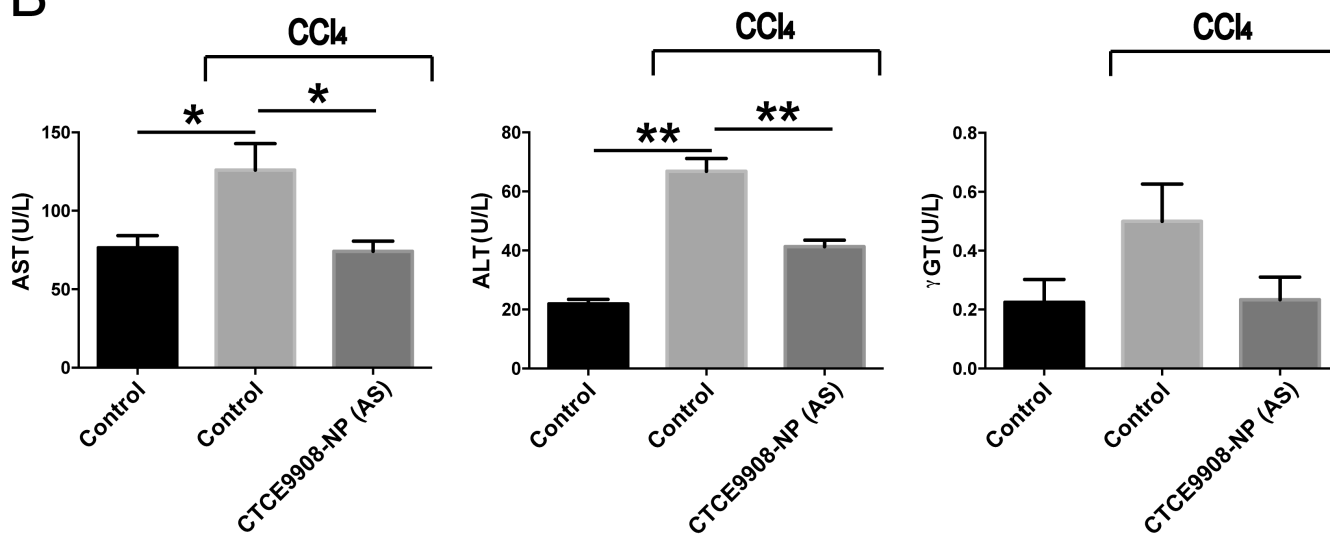
Supplementary Fig. 8. Increased apoptosis induction in tumors of CCl₄-induced spontaneous HCC models treated with Sorafenib and AZD6244 co-formulated in CTCE9908-NPs. A, CTCE9908-NPs containing sorafenib and AZD6244 significantly increased the induction of apoptosis in the tumor tissues. **B,** Representative TUNEL staining in the tumor tissues of CCl₄-induced spontaneous HCC models (n=6). Scale bar=50 μm.   ***$p<0.001$.
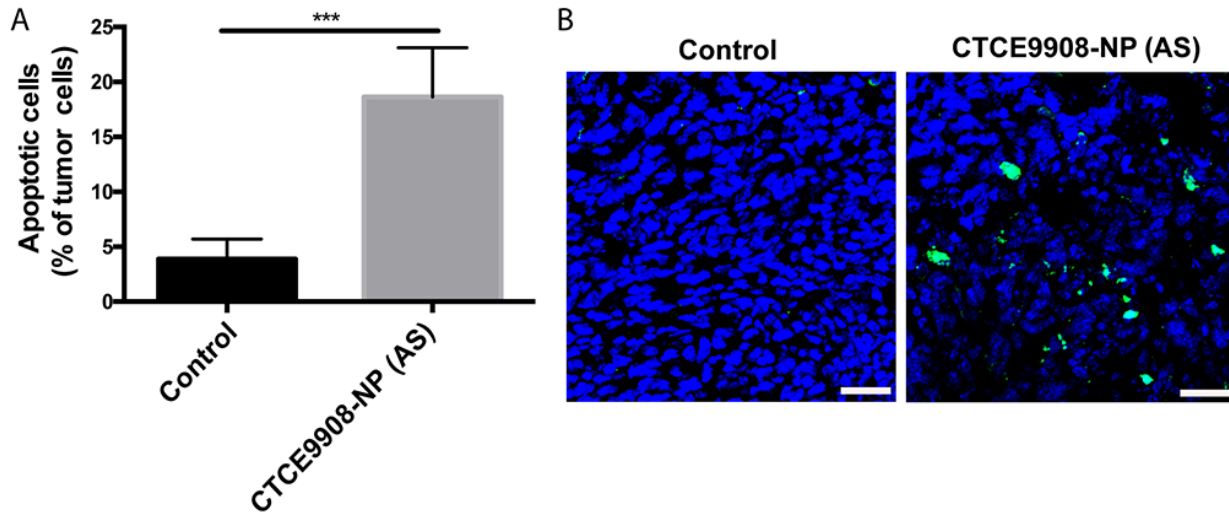
Supplementary Fig. 9. Sorafenib and AZD6244 (5 mg/kg) loaded in CXCR4-targeted NPs reduced the expression of collagen I in livers of syngeneic orthotopic PDAC murine models (n=5-8). Scale bar=100 μm. **p<0.01.
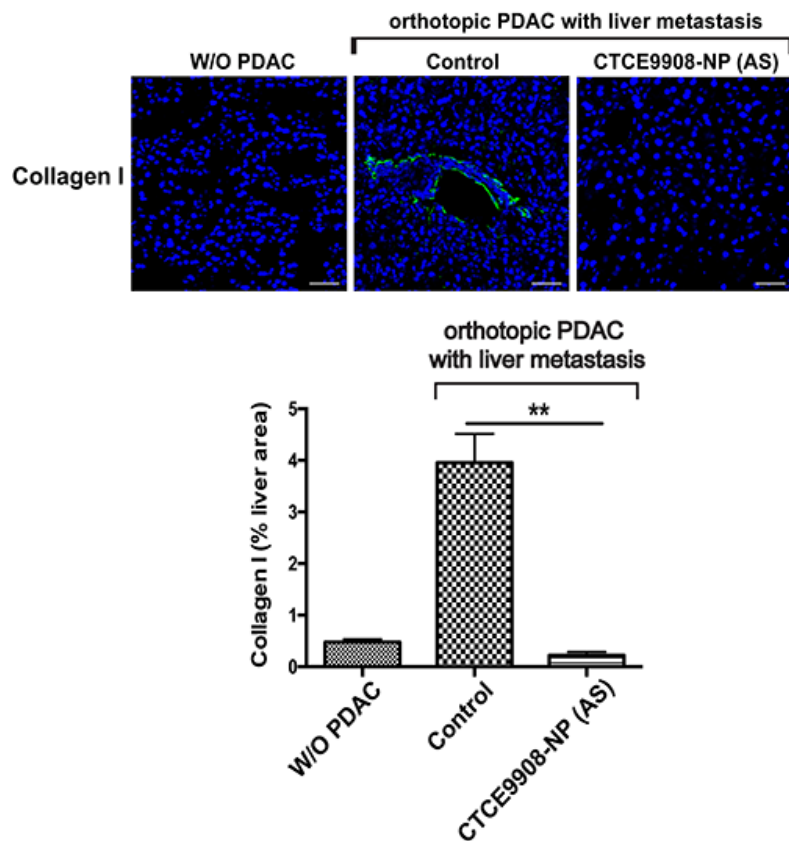
Supplementary Fig. 10. Sorafenib and the MEK inhibitor AZD6244 co-formulated in CTCE9908-NPs reduced the infiltration of F4/80+ macrophage in livers in the orthotopic PDAC (AK4.4) models (n=4-5). The data are presented as mean values ± S.E.M., *p<0.05.
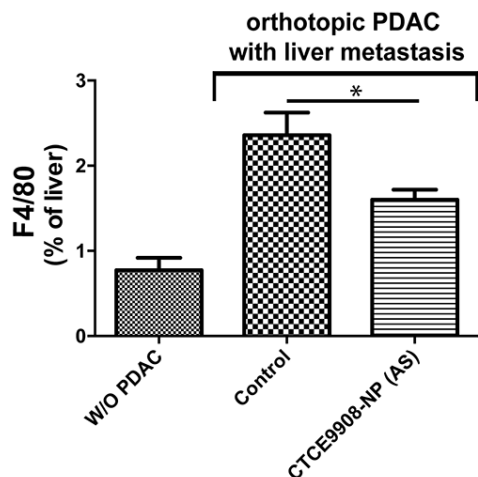
Reference:

1.   Huang Y, Yuan J, Righi E, Kamoun WS, Ancukiewicz M, Nezivar J, et al. Vascular normalizing doses of antiangiogenic treatment reprogram the immunosuppressive tumor microenvironment and enhance immunotherapy. Proc Natl Acad Sci U S A. 2012; 109: 17561-6.