Supplementary materials and methods

Tumor specific delivery of siRNA coupled superparamagnetic iron-oxide-nanoparticles, targeted against PLK1, stops progression of pancreatic cancer

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siRNAs

Sequence of PLK1 siRNA duplex, directed against sense: GCCUGAUUCUCUACAAUGAdGdT, and antisense: {Bi}-CAUUGUAGAGAAUCAGGCdGdt with biotin at 5 prime of the antisense strand was designed and synthesized to target *in vivo* PLK1 by Dharmacon (Thermo Scientific, MA, USA). siRNA for mismatch control with sense sequence: UUCUCCGAAGCUGUCACGUdGdT, and antisense: {Bi}-ACGUGACACGUUCGGAGAAdGdT) was designed and synthesized analogous to siPLK1. Myristoylated-poly-arginine-peptide, MPAP and underglycosylated non-immunogenic MUC1 specific peptide, EPPT1 peptides were designed and synthesized by Genscript Corporation, NJ, USA. All reagents were purchased from Sigma-Aldrich unless otherwise mentioned.

Animal strains

All animals' studies were approved by local institutional animal care committee of the Ernst-Moritz-Arndt University, Greifswald and complied with the NIH guidelines on handling of experimental animals. C57BL/6N mice were purchased from Charles-River Inc. Sulzfeld, Germany and housed under standard conditions. After one week of adjustment to the local conditions, the cells from the syngenic 6606PDA cell line were implanted in animals by orthotopic injection in the head of the pancreas [1]. 14 days after tumor implantation animals were randomized to three groups to receive 3qD intravenous injections (i.v.) of siPLK1 (100nM/kg), siControl-StAv-SPIONs (5 mg/kg of iron) or siPLK1-StAv-SPIONs (5 mg/kg of iron) respectively. The treatment schedule was maintained for four weeks and on completion of treatment, animals were euthanized and tissue harvested for further investigation.

The LSL-Kras^{G12D}, LSL-Trp53^{R172H}, Pdx-1-Cre (KPC) strains of mice have been generated as previously reported [2]. 16-20 weeks old mice were randomized into three groups. Animals were treated in a randomized double blind study for two weeks to receive 3qD intravenous injections (i.v.) of StAv-SPIONs (5 mg/kg of iron), siControl-StAv-SPIONs (5 mg/kg of iron) or siPLK1-StAv-SPIONs (5 mg/kg of iron) respectively. Tumor growth was monitored by ultrasound

imaging as previously described [3]. Stably transfected pancreatic ductal adenocarcinoma cells, 6606PDA were maintained in culture as described previously [1]. For all the *in vitro* cell-culture experiments, we used 10nM of siPLK1 (control), 50µM of iron in siPLK1-StAv-SPIONs whereas for all *in vivo* studies, we used 5mg/kg BW of iron in siPLK1-StAv-SPIONs. 100nM/kg naked siPLK1 served as control for siPLK1 in *in vivo* studies. 50µM of iron in siPLK1-StAv-SPIONs corresponds to 5nM of siPLK1 whereas 5mg/kg BW of iron corresponds to 90nM/kg BW of siPLK1. For all the studies, StAv-SPIONs and siPLK1-StAv-SPIONs were dose-matched.

Detail probe synthesis

Synthesis of dextran coated magnetic nanoparticles (SPIONs) was performed from the method adopted and modified from US patent 5262176 [4]. Briefly, nitrogen purged cooled solution of Dextran T10 (Pharmacosmos, Denmark) and ferric chloride were added to freshly prepared ferrous chloride solution with vigorous stirring on ice. The resultant solution was rapidly neutralized by addition of ammonium hydroxide solution to get green slurry. This green slurry with nitrogen purging was heated for one hour in water bath gradually increasing the temperature from 75^0C to 85^0C and further heated for another hour at 85^0C. On cooling, this magnetic slurry was purified using magnetic-activated cell sorting (MACS) LS columns (Miltenyi Biotech) followed by concentration using ultrafilteration (Millipore). Concentrated SPIONs were characterized to measure hydrodynamic diameter, iron content and zeta potential.

Aliquots of SPIONs were suspended in phosphate citrate buffer (50mM, pH 5.0) and allowed to react with 1 mg/mg sodium periodate equivalent to iron at 4^0C overnight. The resultant solution was diluted with phosphate buffer (10 mM, pH 7.4) and excess of sodium periodate was washed in PD10 desalting columns (GE Healthcare, Buckinghamshire, UK) equilibrated with phosphate buffer 10mM pH 7.4. These columns were eluted with phosphate buffer 10 mM pH 7.4 in a tube containing 2 mg streptavidin (iBA GmbH). Streptavidin conjugated solution was incubated at 4^0C overnight with gentle shaking. Conjugation reaction was stopped by addition of 140 mM borandimethylamine and incubated at 4^0C for 6-8 hours. The excess of aldehyde formed during

reaction was neutralized using 0.5M ethanolamine at 4^0C overnight. The resultant particles (StAv-SPIONs) were purified in magnetic-activated cell sorting (MACS) LS columns and resuspended in ultrapure nuclease free water.

The StAv-SPIONs were conjugated with 10 µM biotinylated siRNA duplex with biotin coupled to 5' end of antisense strand at 4^0C for 1 hour. Further, 10µg/µl of MPAP peptide ({Myr}ARRRRRRC-{Bi-Lys}) and 10µg/µl of underglycosylated nonimunogenic peptide, EPPT1 (C{AHA}Y{Cys(ACM)}AREPPTRTFAYWG-{Bi}) were conjugated with StAv-SPIONs at 4^0C for 1 hour to get siPLK1-StAv-SPIONs. siPLK1-StAv-SPIONs were purified to remove unconjugated siRNA duplex and peptide ligands using magnetic-activated cell sorting (MACS) LS columns and resuspended in ultrapure nuclease free water.

Characterization of SPIONs

The shape, size, composition and lattice structure of the siPLK1-StAv-SPIONs were investigated with a transmission electron microscope (TEM) with a FEI Tecnai G220 S-Twin TEM and Philips CM200/FEG high-resolution TEM (HRTEM) operated at 200 kV which was equipped with an energy dispersive X-ray detector (EDX). The TEM grids were prepared for imaging by placing a small drop of the specimen solution on a copper grid having an amorphous carbon film less than 20 nm thick and allowing it to dry completely in air at ambient temperature. Considering magnetite SAED diffraction pattern (JCPDS card 19-0629) as standards, SAED diffraction patterns of siPLK1-StAv-SPIONs were evaluated using Diffraction-ring profiler software.

To characterize the structural features of the nanoparticles in air, AFM imaging was performed using a BioScope II scanning probe microscope from Digital Instruments Inc. (Santa Barbara, CA). Nanoparticle solutions were placed on freshly cleaved mica, glued to a glass slide. After 10 seconds of incubation, samples were washed with ultrapure water and imaged in tapping mode at room temperature, using a silicon tip from Veeco Probes (Camarillo, CA, drive frequency of 320 kHz, radius 8 nm, TESP). Images (10 x 10 μm^2) were analyzed using NanoScope 7.3

software. In house script in MATLAB (7.11, MathWorks, Natick, MA) was used to analyze the height distribution of the features imaged with AFM tapping mode as previously described [5]. Briefly, the script scans over the images looking for the highest point within a moving 17x17 points rectangle. To avoid crosstalk with measurement noise, a 0.9 nm threshold was used in this study. The grains found in this process were saved with their position and height. The height values were then merged and plotted into frequency distributions.

The hydrodynamic diameter and effective surface charges (ζ-potential) of siPLK1-StAv-SPIONs were measured with samples thermostated at 25^0C using Malvern Instruments Zetasizer, Worcestershire, UK.

To evaluate binding efficiency of streptavidin, StAv-SPIONs were treated with biotinylated BSA (Pierce, Thermo Scientific) (range: 100ng to 500ng) and change in hydrodynamic diameter was measured over a period of 1 hour with samples thermostated at 25^0C using Malvern Instruments Zetasizer, Worcestershire, UK.

The quantification of siRNA binding was performed using 2% agarose gel electrophoresis using Tris-borate-EDTA buffer after pretreatment of the nanoparticles solutions with 1% SDS with 5 min boiling to release siRNA duplex from siPLK1-StAv-SPIONs. The band thus obtained was quantified using ImageJ software. For quantification of bound siRNA in nanoparticle solution, Ribogreen RNA quantification kit (Molecular probes) was used as prescribed in manufacturer's instruction.

Predicted protein structures of Streptavidin for PyMOL analysis were retrieved from the protein data bank and were used for molecular docking with biotinylated siRNA duplex in their 3D conformation using PyMOL (DeLano Scientific) software. We used SwissDock online docking portal using StAv (PDB: 1MM9) with biotinylated siRNA duplex to evaluate influence of biotinylated siPLK1 binding to StAv.

Measurement of iron content

Quantitative determination of siPLK1-StAv-SPION probes uptake was performed after 2 and 4 hours of incubation with 50µM of iron siPLK1-StAv-SPIONs followed by extraction and measurement of iron calorimetrically as described previously [6]. Briefly, aliquots of cell lysates were mixed with 10mM HCl and iron releasing reagent (a freshly mixed solution of equal volumes of 1.4M HCl and 4.5%w/v $KMnO_4$). After incubation for 2 hours at 60°C a mixture of iron detection reagent (6.5mM ferrozine, 6.5mM neocuproine, 2.5M ammonium acetate, and 1M ascorbic acid) was added and after 30 min, absorbance was measured at 550nm. The iron content of the sample was calculated by comparing its absorbance to that of a range of standard iron concentrations. The intracellular iron concentration determined for each well of cell culture was normalized against protein content.

Cell cycle analysis

For cell cycle analysis, cells were treated for 18 hours with siControl-StAv-SPIONs, 5FU (10µM), gemcitabine (Actavis, Dublin, Ireland, 10µM), nocodazole (30µM), BI6727 (Boehringer Ingelheim, Ingelheim am Rhein, Germany, 30nM) and siPLK1-StAv-SPIONs. siControl and siPLK1 transfection served as further controls and controlled transfection efficiency. Cell cycle analysis was performed as described previously [7]. Briefly, 1×10^6 cells were trypsinized, washed twice with PBS, and fixed in 70% ice-cold ethanol. Cell pellet was resuspended in 1ml of propidium iodide (PI) staining solution I and incubated for 30 min at room temperature. Cell-cycle distributions were determined using BD LSR II system (Becton Dickinson, NJ, USA). The fluorescence signal was detected through the FL2 channel and the percentage of cells in G_0/G_1, S or G_2/M phase was analyzed by FlowJo.

Proliferation assay

To assess the influence on proliferation, 6606PDA cells were treated with different subsets of treatment regimens and cell proliferation ELISA. BrdU (colorimetric) (Roche Diagnostics, IN, USA) were performed as per manufacturer's instruction at 0, 6 18, 24, 36, 48, 72 and 96 hours.

siPLK1 transfection by means of electroporation (Amexa biosystem) using Amexa cell line nucleofactor kit V (Lonza, Basel, Switzerland) served as control for transfection efficiency.

Live cell imaging and uptake measurement

For fluorescent live cell imaging siPLK1-StAv-SPIONs were further conjugated with biotinylated Cy5 (Nanoc Inc, NY, USA). Cells were treated with 50µM of siPLK1-StAv-SPIONs and live cell confocal laser scanning microscopy was carried out for 30 min at 37^0C under controlled atmosphere on Olympus FLUOVIEW FV1000 microscope. To determine uptake mechanism, cells were preincubated for 30 min with Anti-MUC1 antibody (Cell Signaling, Danvers, USA) and 30 min with Dynasore respectively prior to 30 min treatment of Cy5 coupled siPLK1-StAV-SPIONs. Image processing and analysis were performed with CellSens (Olympus, Tokyo, Japan) software.

Transmission electron microscopy

For transmission electron microscopy, cells were grown on polycarbonate mesh and at 0 min, 5 min and 30 min treatment with siPLK1-StAv-SPIONs the membrane was immersed in iced 2% gluteraldehyde/ 2% formaldehyde solution at pH 7.4 with 0.1 M cacodylate buffer. Blocks were post-fixed in 1% osmium tetroxide and embedded in Glycidether 100 (formerly called Epon 812), cut with diamond knifes with a Leica ultratome to 500 and 750 nm thick semi-thin slides and stained according to Richardson. Ultrathin sections of 70-90 nm were stained with uranyl acetate and lead citrate and examined with a Libra 120 electron microscope from Carl Zeiss (Jena, Germany) [8].

Magnetic resonance imaging

In vivo 7 Tesla small animal MRI imaging were performed in tumor bearing mice before and 6 hours after injections of 5 mg/kg of iron of siPLK1-StAv-SPIONs using Bruker, ClinScan,7.0 Tesla, 290 mTesla/m gradient strength (Bruker, Ettlingen, Germany) as described previously [1]. For in-situ tumor volume analysis, MRI imaging was performed at 14, 21, 28 and 40 days after tumor implantation analogous to single measurement. Generated images were analyzed

employing AidScans (Anyintelli Inc.) All image slices of the tumors were marked with regions of interest (ROI) which facilitated the calculation of the tumor volume. Quantitative determination of iron overload in tumor calculated using R2* fat corrected single peak echos collected during MRI imaging using Osirix Diacom viewer software. The color contrast images were processed using SPIN, Detroit, MI, USA. For checking the influence of different ligands on tumor specificity, we injected MPAP(-)EPPT1(-)siPLK1-StAv-SPIONs, MPAP(-)EPPT1(+)siPLK1-StAv-SPIONs, MPAP(+)EPPT1(-)siPLK1-StAv-SPIONs and MPAP(+)EPPT(+)siPLK1-StAv-SPIONs 2 hours prior to MRI. Quantitative determination of iron overload in tumor calculated using R2* fat corrected single peak echos collected during MRI imaging using Osirix Diacom viewer software.

Intracellular Iron concentration

Quantitative determination of influence of MPAP and EPPT1 on intracellular uptake of tumor was performed 6 hours after injection of siPLK1-StAv-SPIONs with and without MPAP and EPPT1, followed by extraction of tumor and measurement of iron calorimetrically as described previously [6]. Aliquots of cell lysates were mixed with 10mM HCl and iron releasing reagent (a freshly mixed solution of equal volumes of 1.4M HCl and 4.5%w/v $KMnO_4$). On 2 hours at 60 °C incubation of this mixture, iron detection reagent (6.5mM ferrozine, 6.5mM neocuproine, 2.5M ammonium acetate, and 1M ascorbic acid) was added and after 30 minutes incubation, absorbance was measured at 550nm. The iron content of the sample was calculated by comparing its absorbance to that of a range of standard iron concentrations. The intracellular iron concentration determined for each well of a cell culture was normalized against the protein content.

Iron staining in tumor section (Perl's staining)

6606PDA cells, 12 hours after siPLK1-StAv-SPIONs treatment, were fixed with fixed with 4% paraformaldehyde in PBS at 4^0C for 30 minutes. Fixed cells were permeabilized using 0.01% Triton-X100 for 5 minutes and washed with PBS, followed by iron staining using Accustain iron staining kit (Sigma-Aldrich) as per manufacturer's instructions. Mice were treated with siPLK1-

StAv-SPIONs and after 6 hours of treatment, tissues were harvested, Tissues were fixed in 4% neutral buffered formalin, dehydrated and embedded in paraffin. In KPC mice, paraffin section from tumor harvested after completion of treatment, were used. The deparaffinized sections were stained for iron staining using Accustain iron staining kit (Sigma-Aldrich) as per manufacturer's instructions. Quantitation of iron uptake was performed using Aperio Imagescope software (Leica Biosystems, Germany) using Positive cell count v9 algorithm with set parameters as Hue value 0.66, Hue width 0.01,Saturation 0.08. The obtained results were analysed for negative, weak positive, positive and strong positive pixels.

Histology and immunohistochemistry

Stromal staining was performed as described previously [9]. For immunohistochemistry staining, the deparaffinized sections were incubated with 10mM antigen retrieval buffer (Dako) for 30 min in pressure cooker. The sections then treated with 0.3% H_2O_2 for 20min and then blocked with 1% Aurion BSA for 1 hour. The section were immunolabeled using anti-Ki67, (Bethyl laboratories Inc., 1:200), anti-PLK1 (Thermo Scientific, 1:100), pHH3(santacruz, 1:100), pericentrin (Santacruz, 1:100), cdc25 (santacruz, 1:100), CDK1/2 (santacruz, 1:100), p53 (santacruz, 1:100), MUC1 (cell signaling, 1:100), Orc2 (santacruz, 1:100) and anti-CD31 (Abcam, 1:500) overnight at 4^0C. The immunobound antibodies were detected using Envision+system HRP labeled polymer secondary antibodies(Dako) for 1 hour and sections were developed using 3,3'-diaminobenzadine (DAB) staining using Vector peroxidase substrate kit (Vector laboratories) and section were further counterstained with haematoxylin. The number of DAB+ nuclei were analysed from 8-10 visual fields from each sections and quantitatively analysed using NIH ImageJ analysis software.

The apoptotic cells were detected from deparaffinized sections by Tunel assay using *in-situ* cell detection kit, TMR red (Roche Diagnostics) as per manufacturer's instruction.

BrdU positive staining were performed on deparaffinized sections using APO-BrdU-IHC™ Immunohistochemistry Kit (AbD Serotec, UK) as per manufacturer’s instruction.

Immunoblotting

6606PDA cell-lines 12, 18,24 and 36 hours after siPLK1-StAv-SPIONs and corresponding control treatment were lysed on ice in lysis buffer (25mM HEPES pH 7.5, 75mM NaCl, 0.5% Triton X-100, 5% glycerol, 1mM EDTA, 10mM NaF, and 5mM $Na_4P_2O_7$, and 1mM PMSF and 1 mg/ml aprotinin. Protein content was determined by Bradford assay. In all, 50 µg samples of total protein were loaded on 12.5% SDS-polyacrylamide gels and transferred to nitrocellulose membranes for immunoblotting as previously described [10]. Anti-PLK1 (cell signaling, 1:1000), were used for the immunoblotting and anti-GAPDH (Meridian Biosciences, 1:1000) was used for loading control. Tissue samples for immunoblotting were homogenized on ice in lysis buffer (25mM HEPES pH 7.5, 75mM NaCl, 0.5% Triton X-100, 5% glycerol, 1mM EDTA, 10mM NaF, and 5mM $Na_4P_2O_7$, and 1mM PMSF and 1 mg/ml aprotinin. Protein content was determined by Bradford assay. In all, 100 mg samples of total protein were loaded on 12.5% SDS-polyacrylamide gels and transferred to nitrocellulose membranes for immunoblotting as previously described[10]. Anti-PLK1 (cell signaling, 1:1000) were used for the immunoblotting and anti-GAPDH (Meridian, 1:1000) was used for loading control.

Survival analysis

Tumor bearing mice were enrolled 14 days after syngenic orthotopic tumor implantation. Animals were randomized into three groups to receive siControl-StAv-SPIONs (mismatch control), BI6727 or siPLK1-StAv-SPIONs respectively. BI6727 was administered intraperitoneally (i.p.) weekly at a dosage of 25mg/kg BW. siControl-StAv-SPIONs and siPLK1-StAv-SPIONs were administered intravenously (i.v.) twice weekly at a concentration of 5mg/kg of iron. Treatment schedule was maintained for maximum 12 weeks after initiation of the treatment. Objective response was monitored daily by visual examination of the animals and endpoint criteria were defined as general morbidity, lethargy or lack of social interaction.

In solution stability of siPLK1-StAv-SPIONs

To determine the in solution stability, we incubated siPLK1-StAv-SPIONs in saline and in 5%FCS at 37^0C for duration ranging from 0 to 14 days and checked for siPLK1 efficiency using FACS cell cycle analysis. Cell cycle analysis was performed as described [7]. Percent of the cells in G2/M phase of cell cycle where analyzed as efficiency parameter.

***In vitro* toxicity**

To determine generation of reactive oxidative species (ROS), approx. 1×10^6 cells were incubated with siPLK1-StAv-SPIONs for 2 and 4 hours followed by 1 hour of 50µM of cell permeable dye 2',7'-dichlorofluorescein diacetate (DCF-DA) which convert to flourogenic 2', 7'-dichlorofluorescin, when oxidized by ROS. Cells were repeatedly washed with PBS and cell lysates were prepared. The Fluorescence of cell lysates thus obtained, were measured in FluorStar Optima flourometer (BMG Biotech) and corrected to the protein content of the lysates.

Fluorescence Immunostaining

Cells were treated either with anti-MUC1 antibody (Abcam, concentration range 0.5µg/ml to 2µg/ml), Dynasore (concentration range 10-50µM) or MDC (concentration range 10-50µM) 30 minutes prior to biotinylated Cy5 coupled siPLK1-StAv-SPIONs treatment treated and after treatment regimen, cells fixed with 4% paraformaldehyde in PBS at 4^0C for 30 minutes. Fixed cells were permeabilized using 0.01% Triton-X100 for 5 minutes and washed with immunofluorescence buffer (0.2%BSA, 0.05%Saponin, 0.01%Triton-X100 in PBS, incubated 5 minutes with DAPI (Fluka) and Oregon Green 488 (Life Technologies) to counterstain nuclei and plasma membrane, respectively. Immunostaining of MUC1 antibody was performed with anti mouse-Alexa flour 488 (Life Technologies) as secondary antibody. Images were captured on Olympus FLUOVIEW FV1000 microscope and images were processed on CellSens (Olympus) software. For quantification of Cy5 approximately 100 random cells were selected to

calculate mean fluorescent intensity of Cy5. Region of interest were selected according to the plasma membrane staining.

For colocalization analysis of Cy5 coupled-siPLK1-StAv-SPIONs with endosomal marker EEA1, 6606PDA cells were incubated with Cy5 coupled-siPLK1-StAv-SPIONs for period ranging from 0 to 30 min and slides were fixed using 4% paraformaldehyde in PBS at 4^0C for 30 minutes. Fixed cells were permeabilized using 0.01% Triton-X100 for 5 minutes and incubated 1 hour with immunofluorescence buffer (0.2%BSA, 0.05%Saponin, 0.01%Triton-X100 in PBS and incubated overnight with anti-Rabbit EEA1 (Cell signaling) overnight at 4^0C. Slides were washed three times with immunofluorescence buffer and incubated with FITC coupled anti rabbit secondary antibody (Jackson Laboratories) for 1 hour at room temperature. Slides were washed again three times with immunofluorescence buffer. Slides were counterstained with DAPI (Fluka). Images were captured on Olympus FLUOVIEW FV1000 microscope and processed on CellSens (Olympus) software. Quantification of colocalization was performed using colocalization plugin of ImageJ software using stacked sections for each time point.

Flow cytometric analysis

To analyze uptake of siPLK1-StAv-SPIONs in 6606PDA cells, 1×10^6 cells were treated for 30 minutes with different subsets of biotinylated Cy5 conjugated nanoparticles with or without peptide ligands such as MPAP(-)EEPT1(-)siPLK1-StAv-SPIONs, MPAP(-)EEPT1(+)siPLK1-StAv-SPIONs, MPAP(+)EEPT1(-)siPLK1-StAv-SPIONs and MPAP(+)EEPT(+)siPLK1-StAv-SPIONs for 30 minutes. After incubation, cells were washed, trypsinized and pellets were resuspended in 1 ml of FACS buffer. Cells were permeabilized using permeabilisation buffer (Miltenyi Biotech, USA) for 30 minutes as per manufacturer's instructions.

To analyze uptake mechanism, cells were treated with MUC1 antibody (Abcam, concentration range 0.5-2µg/ml) and in another set with Dynasore (concentration range 10-100µM) 30 minutes prior to siPLK1-StAv-SPIONs treatment and cells were processed analogous to the uptake experiments.

Cells immunolabeled with Cy5 conjugated nanoparticles were recorded using the BD LSR II system (Becton Dickinson, USA). The results were analysed using FlowJo software.

Pharmacokinetics

The pharmacokinetic profiling of siPLK1-StAv-SPIONs was carried out after single intravenous injection in mice and harvesting serum at 0, 3, 5, 10, 30, 60, 240,480 min. These serum samples were analysed for iron using Atomic absorbance spectroscopy (contrAA 700, ASpect CS 2.0.1.0). Control mice bleeding at time equivalents were considered as black for respective groups. The results were analyzed using PKSolver 2.0 [11]. Non-compartmental analysis of plasma data after intravenous bolus input was considered for model approximation.

IC_{50} determination and Scratch assay

For determination of IC50 of BI6727, we treated 6606PDA, 6606 liver metastatic and 7265PDA cell-lines with increase concentration ranging from 0 to 1µM of BI6727 for 24 hours. On completion of incubation, we performed MTT proliferation assay as descried previously [12]. To determine influence of BI6727 (0.1 µM), siCotrol-StAv-SPIONs (50µM of iron) and siPLK1-StAv-SPIONs (50µM of iron) on migratory potential of cancer cells, we performed scratch assay on 24 hours after treatment over a period of 48 hours in Ibidi scratch assay chambers (Ibidi, Germany) as per manufacturer's instruction. The treatment schedule was followed by 2mg/ml Mitomycin C treatment and we evaluate influence on migration over a period of 48 hours after completion of treatment. We analyzed sractch area using TScratch software (CSElab, ETH Zurich).

Quantitative real-time PCR analysis

Total RNA was isolated from tumors using a Qiagen Mini RNA extraction kit (Qiagen) as per the manufacturer's instructions. After quantification, RNA quality was assessed using agarose gels. From isolated RNA, cDNA synthesis was performed using Quantitect reverse transcription kit (Qiagen). Real time PCR (TaqMan) were carried out for *plk1* and *rpl32* using SYBRGreen PCR master mix (Applied Biosystem) in 7500 real time PCR system (Applied Biosystem). Gene expression was standardized using *rpl32* as a candidate control. Data is expressed as fold

changes with respective to control in mean ± SD. All the primers were designed and synthesized by Invitrogen.

plk1	Sense:	TAATGACTCAACACGCCTGATT
	Antisense:	AGCTCAGCAGCTTGTCTACCAT
rpl32	Sense:	AACCCAGAGGCATTGACAAC
	Antisense:	CACCTCCAGCTCCTTGACAT

Caspase 3, 8 and 9 activity measurements

Tumors tissues were homogenized in PBS pH 7.0 and a caspase 3 activity was measured by fluorometric enzymatic kinetics over a period of 30 minutes at 37^0C using z-DEVD-R110 (Invitrogen, Germany) as substrate. Similarly, Caspase 8 and 9 activities were measured using flourogenic substrates AMC-IETD (AAT Bioquest, CA, USA) and R110-LETD(AAT Bioquest, CA, USA) respectively. The caspase activities of samples were corrected by protein content.

Clinical chemistry to determine toxicity

For Haematological analysis blood samples were collected in by retro-orbital bleeding in EDTA containing tubes (Kabe Labortechnik, Germany). For other experiments, blood samples were collected in BD Vacutainer SSTII advance (BD biosciences, UK) during euthanization by retro-orbital bleeding. Blood samples were centrifuged at 4^0C (3000g, 10 minutes) and serum was stored at −20°C for further studies. Whole blood count, serum glucose, bilirubin, blood urea nitrogen, creatinine, aspartate and alanine aminotransaminase were measured using automated blood analyzer at the Institute of clinical chemistry and laboratory medicine, Medical University of Greifswald, Germany.

Health status

Health status scoring was performed by visual examination of indivisual mice as described previously [13]. Body condition scoring (BCS) were evaluated as per the criteria described earlier [14].

Stoichiometric calculations

The number of peptides/SPIONs was calculated using fluorometric measurement. For determination of EPPT1 we used Cy3 conjugated EPPT1 and for determination of MPAP we used Cy5.5 conjugated MPAP. Cy3 labeling was performed using Amersham Cy3 Dye Antibody Labeling Kits (GE healthcare, Freiburg, Germany) as per manufacturer's instruction. Cy5.5 labeling to MPAP were performed using Cy5.5-NHS ester (Amersham, GE healthcare, Freiburg; Germany) as per manufacturer's instruction.EPPT1 and MPAP per SPIONs were determined using modified method described earlier by Moore *et al* [15]. Briefly Fluorometric measurement were performed at ex:.485nm, em: 520 for Cy3 and ex: 544nm, em: 620nm for Cy5.5. The peptide to particles ratio was obtained from concentration of peptide and iron, assuming that each SPIONs in this study have a similar crystallographic structure, a mean 10 nm core diameter SPIONs would contain 15907 iron atoms. For determination of siPLK1/SPIONs, we used Ribogreen RNA quantification kit (Molecular probes) as described in manufacturer's instruction followed by calculation for number of SPIONs/ml.

In vivo fluorescent imaging

For in vivo fluorescent measurement were performed after narcosis in fluorescent imaging cabinet (Lightools research) siGFP-StAv-SPIONs were injected in dosage as described in earlier section. For in vivo and in vitro experiments, calculation of GFP fluorescent intensity was determined using Leica MM AF (Leica, Germany) software. siGFP sequence: Sense: GCAAGCUGACCCUGAAGUUCUU; Antisense: Biotin-GAACUUCAGGGUCAGCUUGCUU by Dharmacon (Thermo Scientific, MA, USA).

Statistical analyses

Data are expressed as mean ± SD unless otherwise stated and analysed using GraphPad (Prism 5.0, CA, USA). Normally distributed two-grouped data were analysed using Mann-Whitney U test. Multigroup data were analysed by Kruskal-Wallis test followed by Dunn's multiple comparison post-hoc test. Kaplan-Meier survival data was analysed using a log rank

test. Statistical correlations were analysed using Pearson test. $P<0.05$ was considered statistically significant.

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