Self-assembled Human Serum Protein Core-shell Nanoparticles inhibit key oncogenic pathways in Drug-Resistant Leukaemia

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Supplemental information

Methods

Development of K562R cell line

Imatinib mesylate and Dasatinib were purchased from Selleck Chemicals USA and stocks were prepared in DMSO and stored at -20^{0}C. Cells were cultured in RPMI-1640 (Sigma Aldrich, USA) supplemented with 10% FBS (Gibco, USA), 1% L-glutamine (Sigma Aldrich, USA), and 1% penicillin/streptomycin (Gibco, USA). The cells were exposed gradually to increasing concentrations of Imatinib (rate of 0.1μmol/L) every 10 days and cultured for three months. Further live cells were subcultured and characterized for imatinib resistance and hereafter termed K562R cells.[1]

Isolation and characterization of patient-derived leukemic mononuclear cells

Blood/bone marrow samples were collected from CML patients after their informed consent and approval from the institutional ethics committee of Amrita Institute of Medical Sciences and Research Centre. The mononuclear cells were isolated using Ficoll-histopaque density gradient centrifugation. The cells were washed and diluted in RPMI1640 medium supplemented with 10% FBS, 1% L-glutamine, and 1% penicillin/streptomycin. FISH analysis, TfR1 expression, gene expression of BCR-ABL and STAT5, IM resistance mutation analysis (IRMA) were performed as described in our earlier report. [1] Mutation analysis was performed at Oncquest laboratories using RT-qPCR. 2 ml whole blood was collected from patients after their informed consent in an EDTA vacutainer and sent to the laboratory at Trivandrum under refrigerated conditions for analysis. The method used at the laboratory is a combination of RTPCR and double-pass sequencing. As reported previously, the sequence

results were analyzed using BLAST software from NCBI and GRAPH ANALYSIS software from applied biosystems.[2]

In silico design of Tf-soraf nano-shell

From the protein data bank, the X-ray crystallographic structure of sorafenib (PDB ID: 3HEG) was retrieved, gasteiger partial charges were added, molecular geometry and energy optimization were effected using MM+ force field. Following that X-ray crystallography structures of albumin (1AO6) and transferrin (2HAU) were retrieved from PDB, and the 3D structures were individually curated by adding polar H-atoms and merging non-polar H-atoms, and energy was minimized using Amber force field. Using the AutoGrid module, a simulation box with grid map (60 x 60 x 60 Å) enclosing the active site was created for both the proteins. Bonding atoms of ligands (sorafenib) were assigned as flexible, and the active sites of the proteins were assigned as rigid. All other essential solvation parameters of AutoDock were kept as default. Finally, the docking simulation between the curated structures of sorafenib-transferrin was utilized and drug-protein docking simulations were performed using AuoDock 4.2 molecular docking software. Amber force field embedded in AutoDock was used for binding-free energy calculations. An atomic distance of < 3.5 Å between the interacting ligand and protein was considered for analyzing the binding energy interactions.

Sorafenib encapsulation efficiency and *in vitro* release

The encapsulation efficiency of sorafenib in Tf-Soraf nano-shell was studied using high-performance liquid chromatography LC-2010A HT HPLC system (Schimadzu, Japan) and Qualisil Gold c18 column (4.6x250mm, 5µm, LCGC chromatographic solutions Pvt Ltd). Sorafenib was eluted using a low-pressure gradient at a flow rate of 1ml/min. The mobile phase consisted of 35% 25mM KH_2PO_4, pH 6.3 (MERCK, USA), and 65% Acetonitrile (MERCK, USA). The eluent was monitored at a wavelength of 260 nm. The data was analyzed using Shimadzu LC solutions.

Intracellular concentrations of sorafenib versus Tf-Soraf

Intracellular concentration of sorafenib at different time points was analyzed using HPLC. ~ 2×10^5 cells were treated with free sorafenib or Tf-Soraf nano-shell nanoparticles for 30min, 1h, 2h, 4h and 6h. ~ 12µg of free or Tf-bound sorafenib (~ 10µM) per well was used. Later cells were collected by low-speed centrifugation at 1500rpm for 5 min, the supernatant was separated, and cell lysis was done by sonicating in 200µl 0.85% ice-cold saline for 1h. The

supernatant was centrifuged at high speed (15000rpm for 15min) under refrigerated conditions to pellet the nanoparticles, which were not taken up by the cells and cell debris. The final supernatant was collected separately. All the samples were analyzed in HPLC according to the protocol discussed above.

Preparation and characterization of (PS-siRNA)-(Tf-Soraf) core-shell particles (CS-NP1)

To prepare BCR-ABL siRNA loaded protamine nano-core, protamine sulphate (Sigma Aldrich, USA) abbreviated as PS was used. Twenty-one nucleotide double stranded siRNAs (Dharmacon, USA) sequences were selected from earlier reports. [3] siRNA stock solutions (375nM) and 1mg/ml protamine sulphate stock solutions were prepared in RNase-free water. For preparing the nano-core, aqueous solution PS was added slowly to siRNA. Amine to phosphate (N/P) ratio of PS to siRNA was kept at 10. The protamine-siRNA nanoconjugates thus formed are hereafter referred to as PS-siRNA. Serum stability of siRNA in the core-shell nanoparticles versus aqueous solution was characterized using agarose gel electrophoresis. Samples of siRNA, either aqueous solution of free siRNA or PS-siRNA nanocore was mixed in a 1:1 ratio with fresh serum to give 50% serum concentration and incubated at 37°C for 24h. Aliquots from each sample were loaded into 2% agarose gel and electrophoresis was performed to visualize intact siRNA. To form the core-shell system, PS-siRNA nano-core (1mg/ml) was suspended in Au nanocluster doped Tf in RNase-free water. The synthesis of Tf-Soraf was carried out in two steps. In the first step, Au nanocluster doped Tf was synthesized following the protocol from our earlier report. [1] In a typical synthesis procedure, 5ml of 10mM $HAuCl_4$ was added drop-wise into 50mg/ml of aqueous solution of human serum transferrin that was kept under stirring at 37°C. 500µl of 1M NaOH (Sigma Aldrich, USA) was added drop-wise and incubated under stirring for 24h at 37°C. The growth of Au nanoclusters in Tf makes it fluorescent with red-NIR emission, which is used for cell imaging. The pH was adjusted to neutral using 1.2N HCl and purified using Zeba spin column (Thermo Scientific, USA). The core-shell nanoparticles were formed by spontaneous nanoprecipitation at room temperature upon the addition of sorafenib. Au nanocluster doped Tf-Soraf formed a nano-shell on the surface of PS-siRNA nanoconjugates to generate fluorescently labelled core-shell nanoparticles having the self-tracking capability. The morphology, size, and size distribution were analyzed.

Encapsulation efficiency and *in vitro* release of siRNA

The encapsulation efficiency and *in vitro* release kinetics of siRNA were studied using Nanodrop spectrophotometer Nd-1000 (Thermo Scientific, USA) respectively. The core-shell nanoparticles containing 0.6 µg siRNA were incubated in PBS pH 7.4 at 37°C in a dialysis bag. At regular time intervals, the samples were collected and stored at -20°C. For estimating siRNA, absorption at 260nm was recorded in the spectrophotometer. The absorbances at different time points were extrapolated from the standard graph to obtain the concentrations of siRNA released at different time points.

Preparation and characterization of (Alb-Dasat) -(Tf-Soraf) core-shell particle (CS-NP2)

Human serum albumin (HSA) was used for synthesizing Albumin-Dasatinib nano-core (abbreviated as Alb-Dasat). DS solubilized in DMSO, was added dropwise to aqueous solution of 1mg/ml albumin and kept under stirring. The final concentration of DS was adjusted to 20µM. Albumin and DS were allowed to interact for ~ 1 h at room temperature, followed by the addition of 2mg EDC and 4mg NHS for cross-linking albumin. After 1h, the solution became cloudy indicating the formation of nanoconjugates of DS encapsulated in albumin. Free albumin and DS were removed by passing through Slide A Lyzer dialysis cassette (Thermo Scientific, USA). To prepare CS-NPs, the Alb-Dasat nanocore (2mg/ml) was suspended in Au nanocluster doped Tf and allowed to interact for 1h at room temperature. To this suspension, sorafenib (10mg/ml) was added dropwise to obtain a final concentration of 500µM sorafenib. The solution was incubated at room temperature under continuous stirring at 1500 rpm for 1h at room temperature and on ice for 15 minutes to ensure homogenous complexation of sorafenib with Tf. Free sorafenib was removed by centrifugation at 10000rpm for 15 minutes. The size distribution was done using dynamic light scattering (DLS-ZP/Particle Sizer Nicomp^{TM} 380 ZLS, USA). The morphology and size were analyzed using atomic force microscopy (AFM, JEOL JSPM-5200, Japan) and scanning electron microscopy (SEM, JEOL JSM-6490LA, Japan).

Cellular uptake

To examine the binding and internalization efficiency of the core-shell nanoparticles, ~1×10^5 cells were incubated with 1mg/ml of CS-NP1 & 2 doped with red-NIR emitting Au nanoclusters for 2h under culture conditions. The controls were untreated cells and nano-core alone. To confirm the targeted uptake of nanoparticles, the cells were pre-treated with 1mg/ml of free Tf for 15 min under culture conditions before the addition of core-shell

nanoparticles. To confirm whether the core-shell nanoparticles were taken up by the cells by receptor mediated endocytosis, the cells were treated with the core-shell nanoparticles at 4°C. Later, cells were collected and resuspended in 500µl PBS, pH 7.4 and analysed using flow cytometry. Confocal laser scanning microscope (Leica, USA) was used for visualizing the cells. Au nanocluster fluorescence was recorded using 543 nm He–Ne laser excitation.

Cell viability and apoptosis assay

K562R cells (~ 1×10^4 cells) were cultured in the presence or absence of nano-core or nano-shell or CS-NP1 & 2 for 48h under culture conditions. The concentrations of siRNA in the nano-core were varied from 1, 2, 5, and 10nM and dasatinib in the nanocore were 10, 20, 30, 40, 50, 60, 70, 80, 90, and 100µM. The concentrations of sorafenib in the nano-shell were varied from 2.5, 5, 10, 15 and 20µM. Two types of core-shell nanoparticles were used; CS-NP1 carrying siNA and sorafenib, CS-NP2 carrying dasatinib and sorafenib. The nanocore was prepared using protamine for CS-NP1 for siRNA complexation, whereas in the case of CS-NP2 the nanocore was prepared using albumin. The nanoshell was prepared using Tf in both cases. CS-NP1a had 2nM siRNA in the nanocore and CS-NP1b had 5nM siRNA in the nanocore. Sorafenib concentration remained the same (10µM) in the Tf-Soraf nanoshell for both CS-NP1a and CS-NP1b. CS-NP2 had 20µM dasatinib in the nanocore and 10µM sorafenib in the nanoshell. The cell viability after 48h was analyzed, using the MTT cell viability assay as described earlier. [2] Apoptosis of cells was determined using Annexin V/PI apoptosis detection kit according to the manufacturer's instructions. The cells were analyzed in the flow cytometer (FITC excitation: 488nm, PI excitation: 543nm). For visualizing the apoptotic cells in confocal microscopy, the cells were transferred to a glass petri dish and observed under confocal laser scanning microscopy (Leica, TCSSP52, UK) with a laser excitation of 488nm for annexin V-FITC and 543nm for PI.

Gene expression studies using RT-PCR

The cells were treated with PS-siRNA nano-core or Tf-Soraf nano-shell as described above. Total RNA extraction and cDNA synthesis were carried out as reported earlier (Ref). **[1]** The gene expression profile of *BCR-ABL* and *Mcl-1* were analyzed keeping GAPDH as the endogenous control. OneStep RT-PCR kit from Qiagen Inc. was used for RT-PCR following the manufacturer's instructions. The primers reported in previously published studies were used for abl and bcr-abl **[3]** as follows: abl-FP (24mer), 5′-CTC ATA TCA ACC CGA GTG TCT CTT-3′; abl-RP (24mer), 5′-TGC TAC CTC TGC ACT ATG TCC ATG-3′; bcr-abl fusion-FP (26mer), 5′-GAA GAA GTG TTT CAG AAG CTT CTC CC-3′; and bcr-abl

fusion-RP (25mer), 5′-GAC CCG GAG CTT TTC ACC TTT AGT T-3′. For Mcl-1, the following primers were used, 5′-TGC TGG AGT TGG TCG GGG AA-3′ (forward) and 5′-TCG TAA GGT CTC CAG CGC CT-3′ (reverse)[4]. The reverse transcriptase reaction, HotStar Taq DNA polymerase activation, denaturation, annealing, and activation followed the conditions reported earlier[3]. The fold change of *BCR-ABL* and *Mcl-1* was converted to percentage expression and plotted. As dasatinib is a known BCR-ABL inhibitor we did not perform the gene expression studies.

Cell viability assay of cells isolated from CML patients treated with core-shell nanoparticles

Mononuclear cells from patient samples were resuspended in a Stemspan culture medium, supplemented with 20% autologous serum. ~ 1×10^4 cells were treated with PS-siRNA/Alb-Dasat nano-core, Tf-Soraf nano-shell, or CS-NP1 &2 for 48h as mentioned above. The cell viability was analyzed using MTT cell viability assay as described earlier.[1]

Hemocompatibility Analysis

For hemolysis assay, the optical absorption of plasma derived from CS-NP treated or untreated whole blood was recorded at 380, 415, and 450 nm. The plasma absorption was calculated using the Soret band method.[1] For studying the inflammatory response of healthy peripheral blood mononuclear cells (PBMCs) to the core-shell nanoparticles, PBMCs were isolated from the whole blood of healthy volunteers using density gradient centrifugation, and ~ 2×10^5 cells were cultured in the presence or absence of core-shell nanoparticles for 24h. 1mg/ml bacterial lipopolysaccharide (LPS) treated cells were used as the positive control. The quantitative expression of IL8, IL1β, IL6, IL10, and TNF-alpha was quantified in the culture supernatant using a CBA bead array (BD Biosciences, USA). The cell viability of healthy human PBMCs treated with the nano-core, nano-shell, or CS-NPs was also done using MTT assay as described earlier.[2]

The effect of core-shell nanoparticles on RBC, WBC platelet and CD34+ve healthy mouse bone marrow progenitor cell count

Healthy Swiss albino mice (6-8 weeks old) were used for the study. The effect of CS-NP1 & 2 on RBC, WBC platelet and CD34+ve healthy mouse bone marrow progenitor cell count was studied by administering 10mg/kg of CS-NPs by tail vein injection. The controls were saline-injected mice. After a period of 48h, the animals were euthanized and whole blood was drawn by cardiac puncture. RBC, WBC, and platelet count were done using microscopic

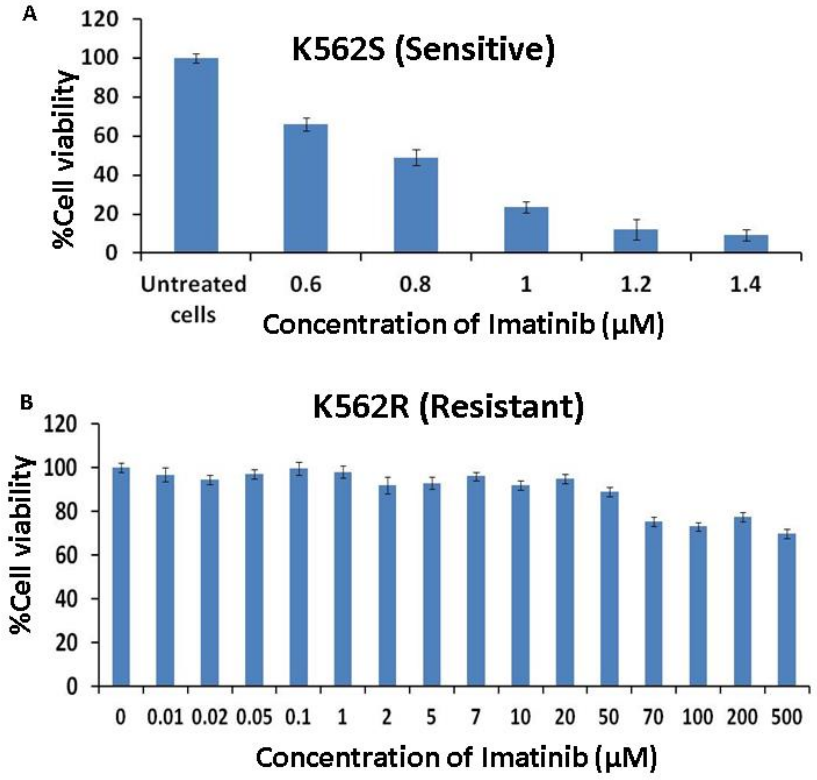
examination. Bone marrow cells were isolated and ~ 5×10^5 cells were incubated with FITC conjugated rabbit monoclonal antibody against mouse CD34 (BD Pharmingen USA), according to manufacturer's instructions. Later CD34+ve cells were enumerated using flow cytometry.

In vivo anti-tumor activity of Core-shell nanoparticles in subcutaneous CML xenograft model.

The subcutaneous CML xenograft model was developed in Swiss albino nu/nu mice after the approval from the institution's animal ethics committee. ~10^7 K562 cells resuspended in a 1:1 ratio of matrigel: basal media were injected subcutaneously into the nude mice. After the 14^{th} day of cell injection when the tumor volume reached approximately 0.5-0.6cm^3, the core shell nanoparticles were injected intra-tumorally for 5 days (n=9). In the case of siRNA nanocore, the dosage of nano-core (PS-siRNA) was 1mg/kg, for bare sorafenib as well as for nanoshell (Tf-Soraf) dosage was 20mg/kg and for the CS-NP1 a combined dose of nano-core (PS-siRNA) of 1mg/kg and nano-shell (Tf-Soraf) of 20mg/kg was given. In the case of Alb-Dasat nano-core, the dosage for nano-core was 10mg/kg of dasatinib along with 10mg/kg of sorafenib whereas the dose for CS-NP2 (Alb-Dasat)-(Tf-Soraf) is 10mg/kg of Sorafenib along with 10mg/kg of Dasatinib. The tumor was measured with vernier using the formula $\pi/6xLxW^2$.

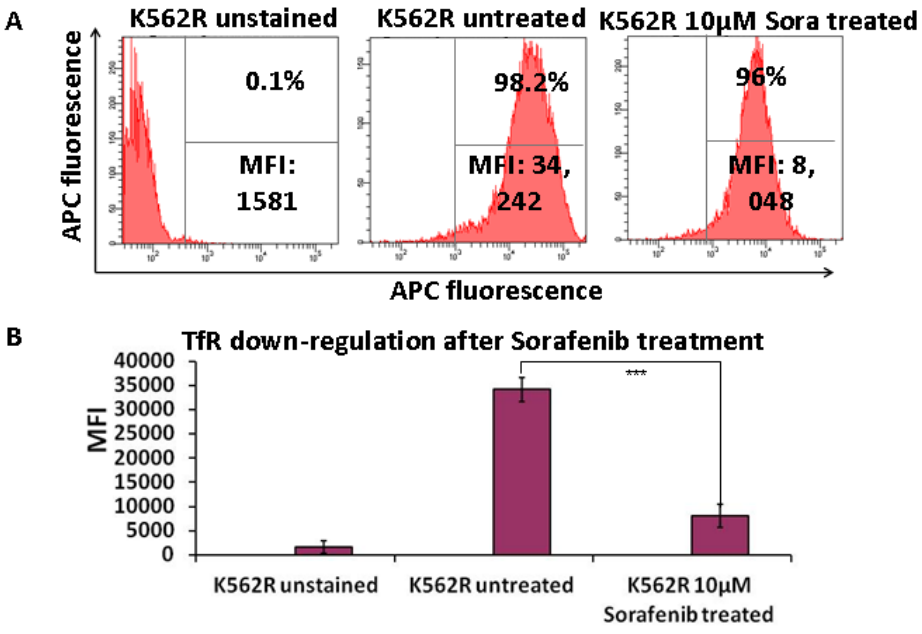
Supplemental figures

Supplemental Figure 1.



IM sensitivity analysis. (A) K562S. (B) K562R.

Supplemental Figure 2.



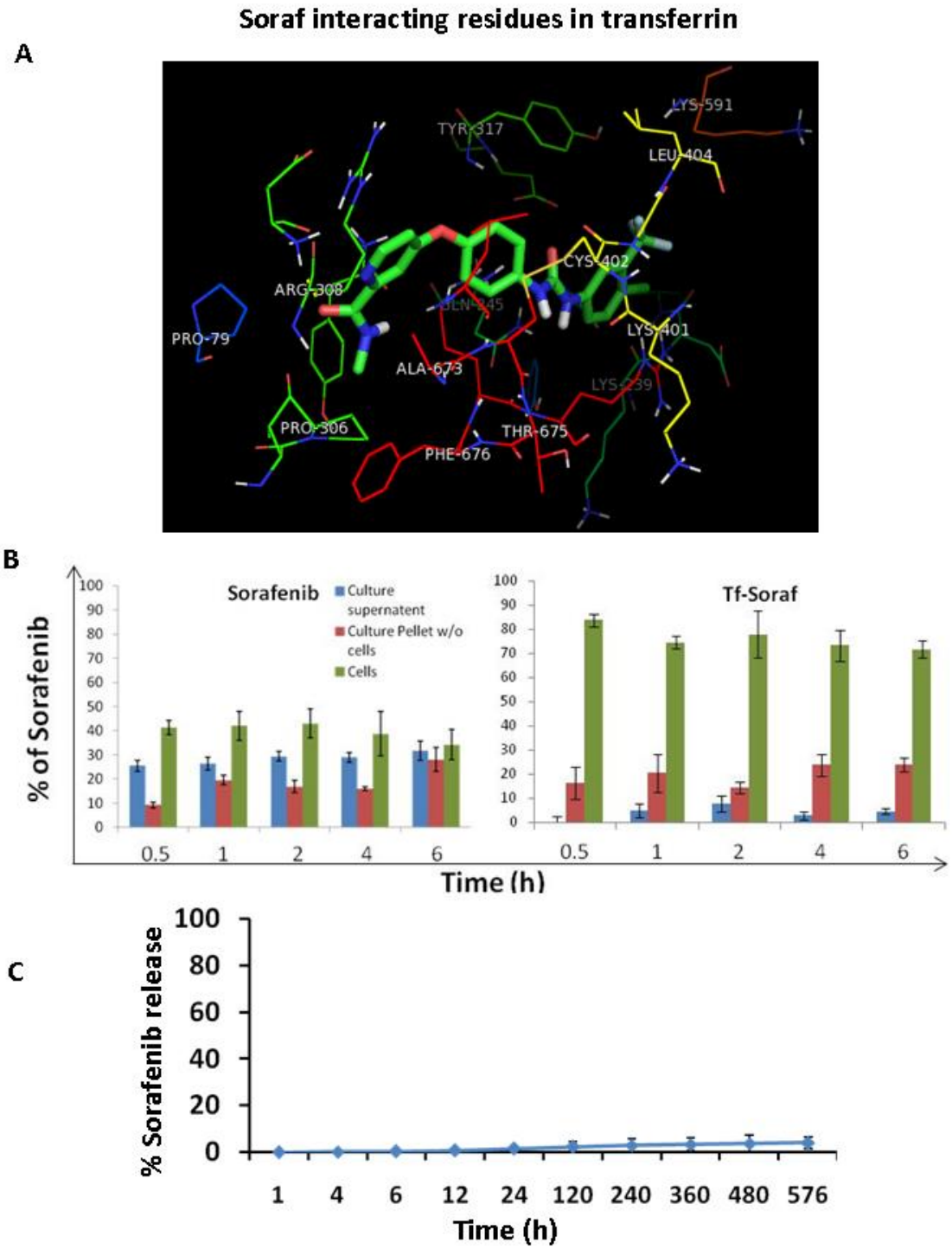
(A) Flow cytogram showing TfR1 down-regulation in K562R cells treated with 10µM Soraf. (B) Graphical representation of TfR1 down-regulation.

Supplemental Table 1:

Patient	Age	Sex	Clinical status at the time of diagnosis	Clinical status at the time of sampling	Q-PCR(BCR-ABL/ABL ratio) (at the time of sampling)	Mutation status	Count (K/uL) (at the time of sampling)
P1	43	F	CP	CP On IM,	3.589%	Nil	5.17
P2	30	F	CP	CP	-	Nil	71.4
P3	27	F	CP	CP	-	Nil	116.0
P4	69	M	CP	CP On IM Loss of response	25.975%	Nil	18.7
P5	58	M	CP	CP On IM Loss of response	-	Nil	4.24
P6	60	F	CP	CP On IM Loss of response	30.778%	Nil	6.14
P7	56	M	CP	CP On IM Loss of response	-	F311I	5.5
P8	54	F	CP	CP IM resistance	100%	T315I	20.1
P9	65	M	CP	AP IM resistance On DS	-	Nil	60.2
P10	35	M	CP	Relapsed CML IM resistance	6.25%	Nil	16.4
P11	26	M	CP	Myeloid BC IM resistance	-	Nil	69.4
P12	15	M	CP	Lymphoid BC IM resistance	2.368%	Nil	1.62
P13	62	M	CP	Myeloid BC IM resistance On DS	-	G250E	62.
P14	70	F	BC	Myeloid BC IM resistance	-	Nil	2.59

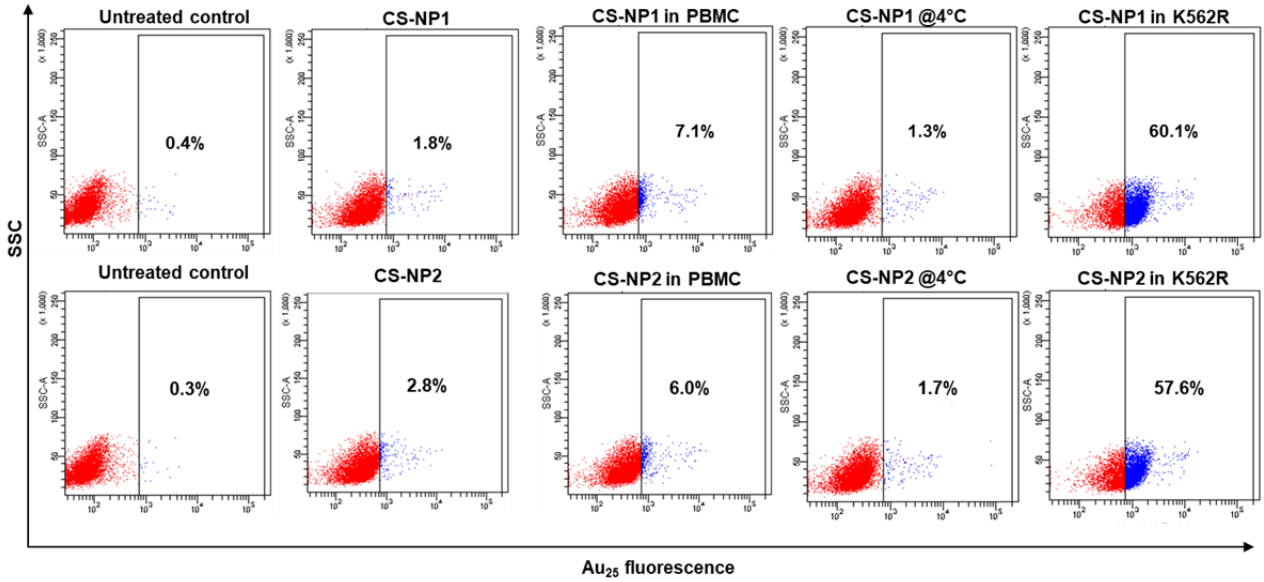
Patient Characteristics - CP: Chronic phase, BC: Blast crisis, F: Female, M: Male

Supplemental Figure 3.



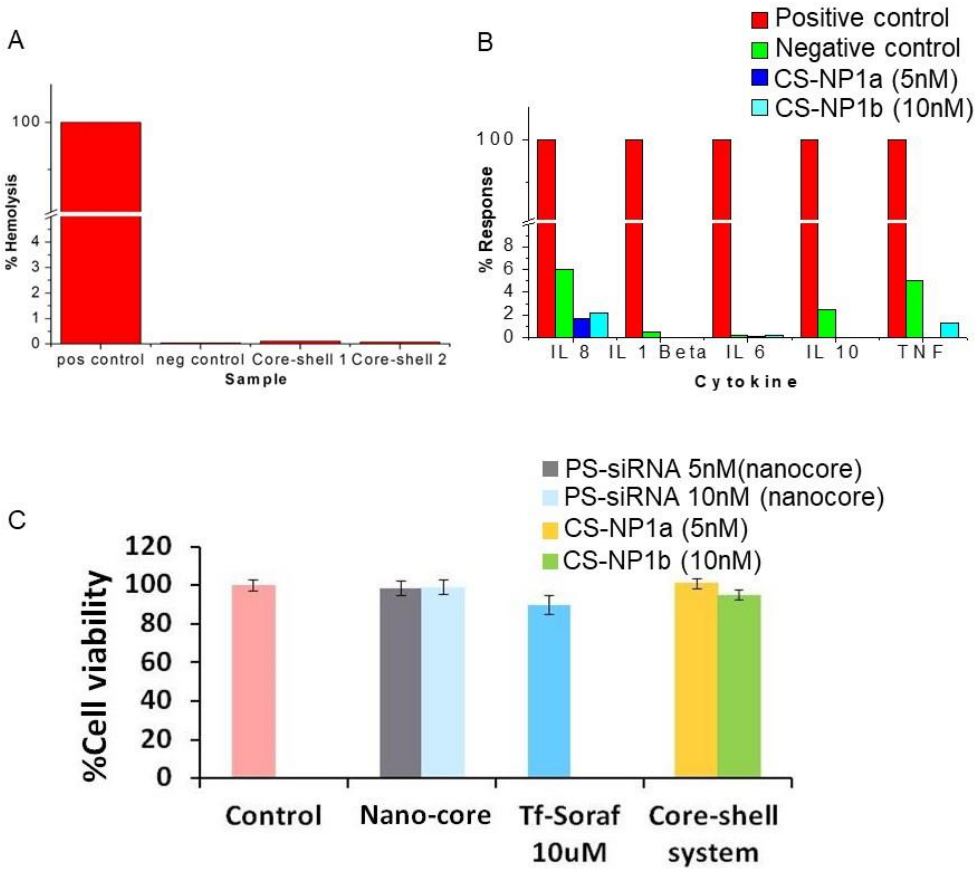
(A) *In silico* studies showing sorafenib interacting residues in transferrin. (B) Cellular kinetics of Sorafenib Vs Tf-Soraf in K562R cells for a period of 6h under culture conditions. After the specific time intervals, cell lysates, culture pellet without live cells and culture supernatant were analysed for sorafenib and quantified using HPLC. (C) *In vitro* drug release of sorafenib in the presence of serum from the Tf-Soraf nano-shell for a period of 24 days (576h). Sorafenib was quantified using HPLC.

Supplemental Figure 4.



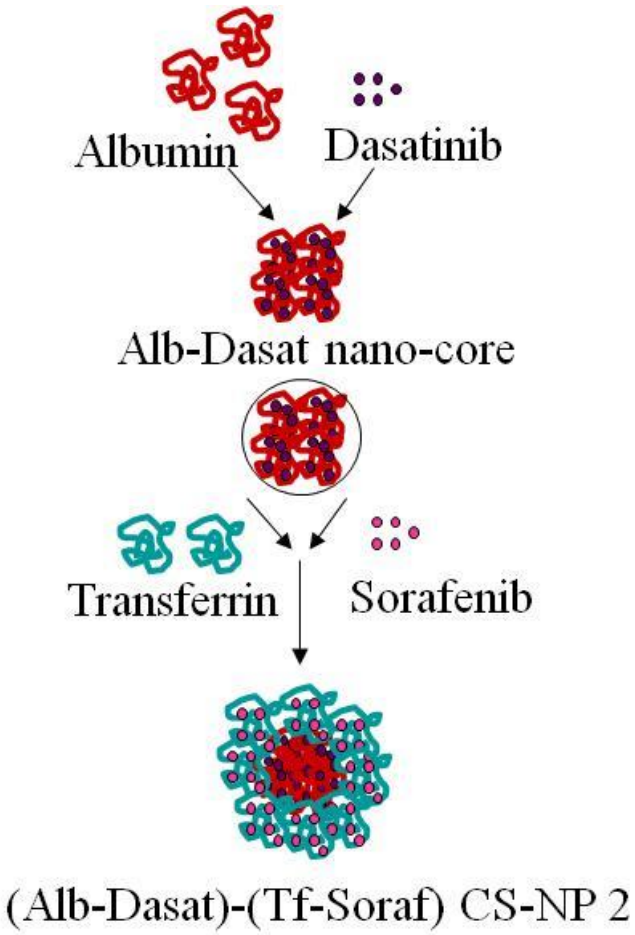
Cellular uptake of CS-NPs over a period of 4h.

Supplemental Figure 5.



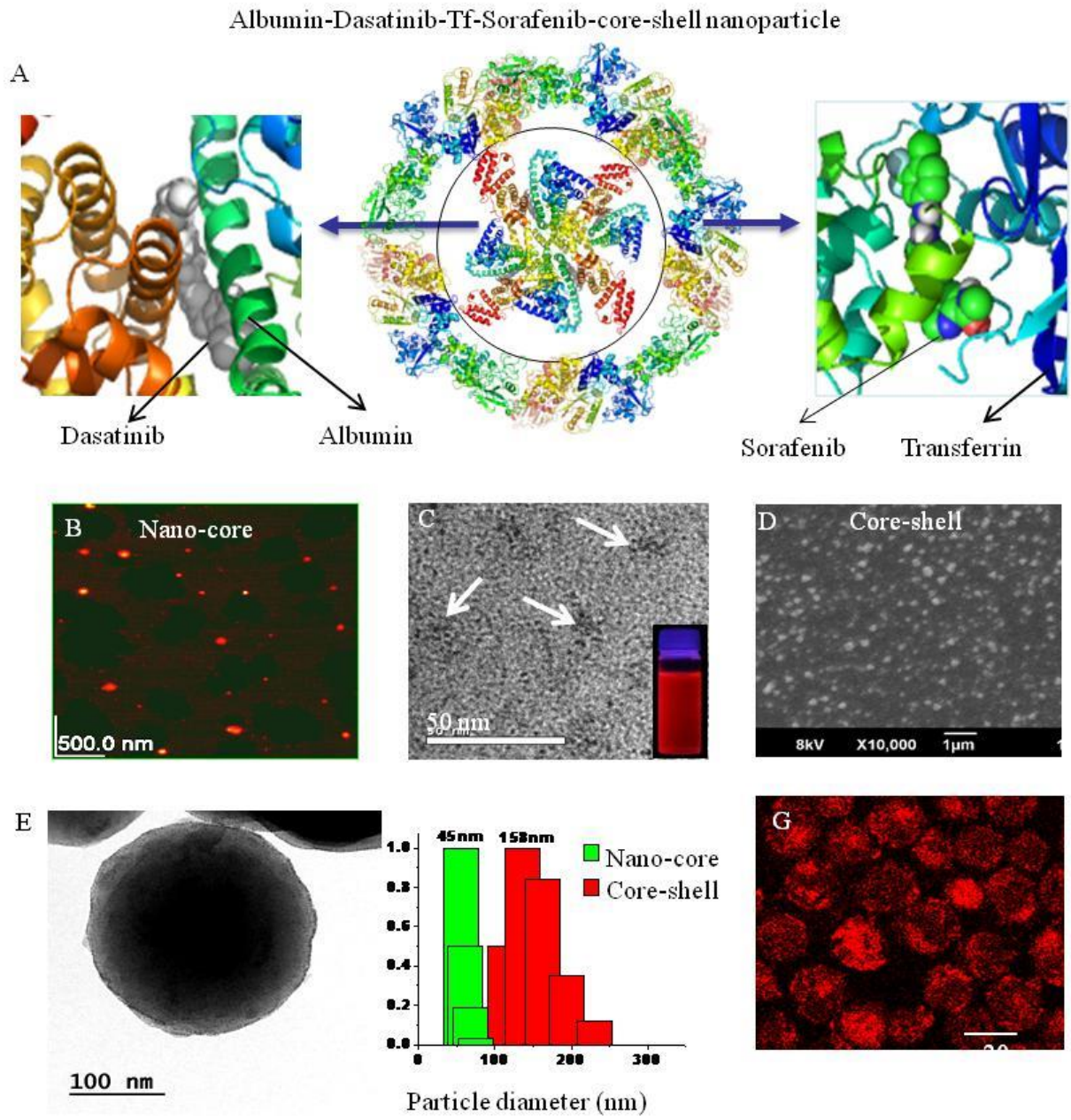
***In vitro* and *in vivo* toxicity analysis of core-shell nanoparticles.** (A) Hemolysis assay using of healthy whole blood treated with core-shell nanoparticles for a period of 1h at 37°C. Positive control was 1% Triton treated whole blood and negative control was untreated whole blood. The core-shell nanoparticles did not induce significant haemolytic response. (B) Inflammatory response of core-shell nanoparticles in healthy human PBMCs. Positive control was 1mg/ml bacterial lipopolysaccharide. Negative control was untreated cells. The quantitative expression of IL8, IL1β, IL6, IL10 and TNF-alpha were quantified in the culture supernatant using CBA bead array in the flow cytometer. (C) Cell viability of healthy human PBMCs treated with core-shell nanoparticles over a period of 48h. Untreated cells were used as the control. MTT assay was used for analyzing the cell viability which showed no significant deleterious effects on the viability of normal healthy cells.

Supplemental Figure 6.



Schematic diagram showing the preparation of (Alb-Dasat)-(Tf-Soraf) core-shell nanoparticles (CS-NP2).

Supplemental Figure 7.



Design, characterization and optimization of (Alb-Dasat)-(Tf-Soraf) core-shell nanoparticles (CS-NP2). (A) Schematic of core-shell nanomedicine comprising of Alb-Dasat nano-core and Tf-Soraf nano-shell. (B) AFM image of Alb-Dasat nano-core. The particle suspensions were dried on a mica substrate. All measurements were recorded in both height and amplitude modes. Height images are presented here. (C) TEM image of metallic nanoclusters of Au present in the nano-shell. (D) SEM image and (E) TEM image showing CS-NP2, inset shows the bright red emission of Au nanocluster doped CS-NP2. (F) DLS showing size-distribution of Alb-Dasat nano-core and CS-NP2. (G) Cellular uptake of Au nanocluster doped CS-NP2 showing bright red fluorescence emerging from the nanoparticles in the cytosol.

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