Supporting Information

Multifunctional hybrid nanoconstructs facilitates intracellular localization of Doxorubicin and Genistein to enhance apoptotic and anti-angiogenic efficacy in breast adenocarcinoma

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1.1 Experimental design

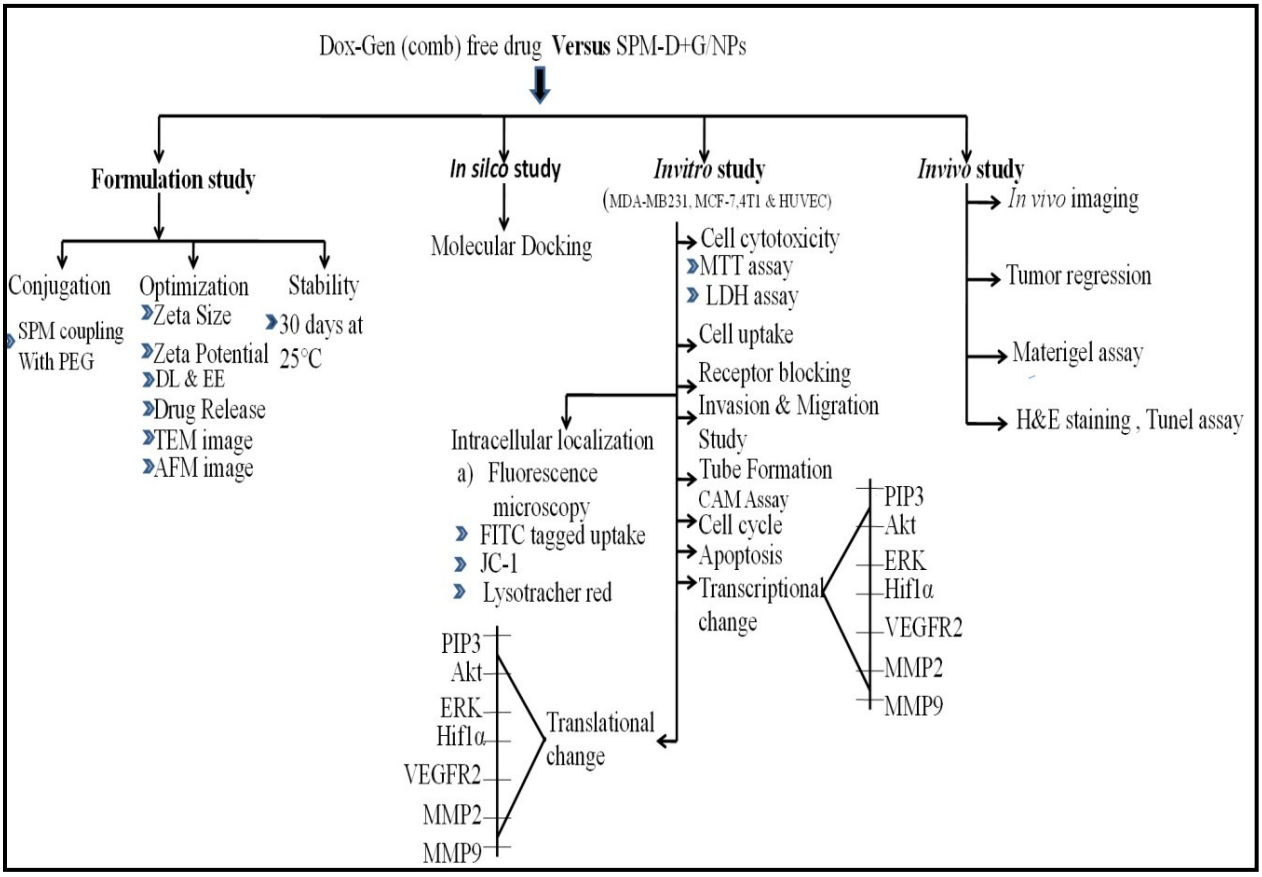


Figure S1. Schematic presentation of experimental design proclaiming the experimental groups, synthesis protocol, endpoint studied under *in vitro* (MDA-MB-231,4T1 & HUVEC cells) *in silico* molecular docking, *in ovo* and *in vivo* studies.

1.2 Stability study

Results of lipid-polymersome stability studies at 25°C for 30 days. The comparative stability was evaluated as a function of particle size, polydispersity index (PDI) between targeted (SPM-D+G/NPs) and nontargeted (D+G/NPs) nanoparticles.

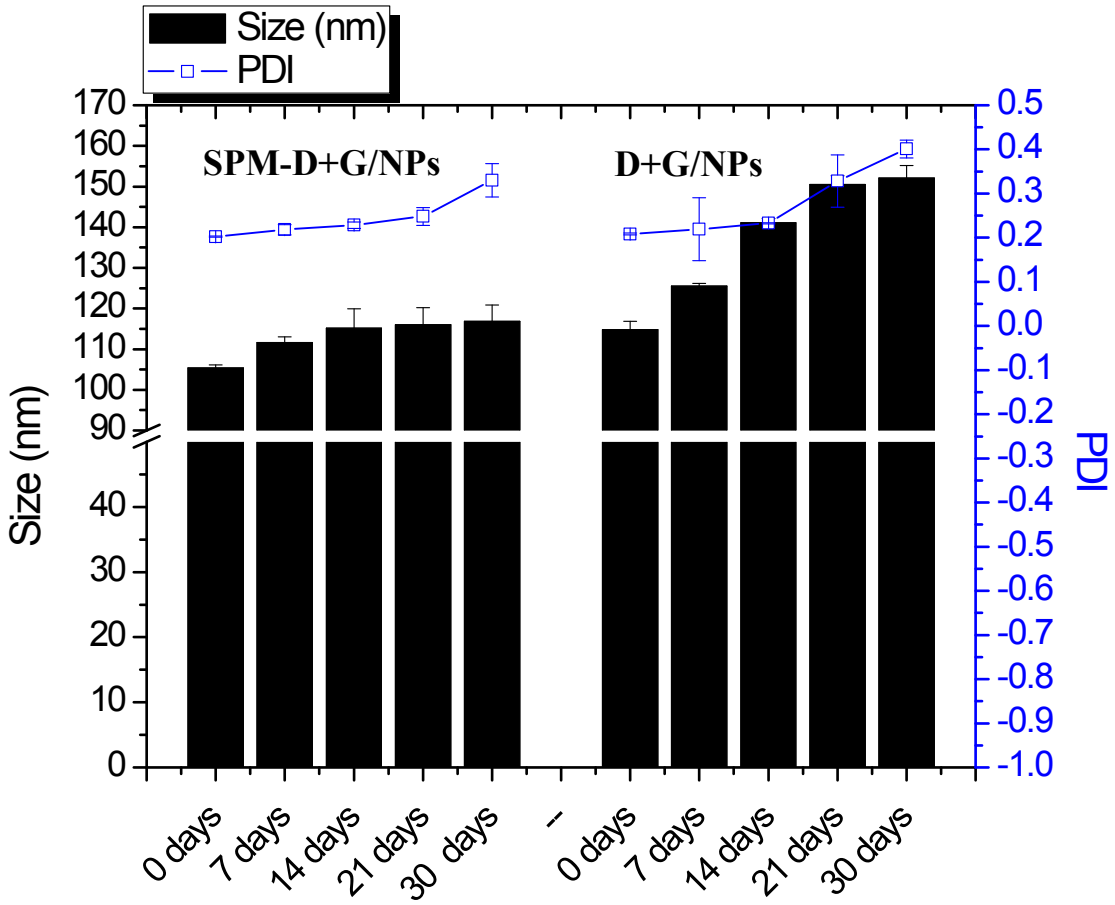


Figure S.2. The change in particle size and PDI for a period of 30days.

1.3 Gene Expression Analysis by qRT-PCR

In order to measure the expression profile of genes involved in synergistic cytotoxicity and the anti-angiogenic pathway, the qRT-PCR analysis was performed. Briefly, the total RNA was isolated from tumor mass of a controlled and treated group of Balb/C modelled mice using trizol reagent. Genomic DNA was removed utilizing RNase free water (Ambion- USA). RNA pellet was re-suspended in DEPC- treated water (Ambion). From the total RNA equal amount of RNA were reverse transcribed by first-strand cDNA synthesis kit with oligodT primer (Invitrogen, USA) and was diluted up to final conc. 10ng/µL by nuclease free water. Housekeeping gene actin was used as control to the normalized value. The qRT PCR was detected and quantified by utilizing ABI Prism 7900 sequence detector system (PE Applied Biosystems; Foster City,CA,

USA) and SYBR green dye (G-Biosciences, USA). Gene relative expression was calculated with the ΔΔct method. The primer sequences are presented in supplementary data.

1.4 List of RT-PCR Primer sequences used for quantitative investigation

Gene Forward primer Reverse primer

Akt F-5'AGCGACGTGGCTATTGTGAAG 3' R-5'GCCATCATTCTTGAGGAGGAAGT 3'

PIP3 F-5'AGAGCACTTGGTAATCGGAGG 3' R-5'CTTCCCCGGCAGTATGCTTC 3'

ERK F-5'TCACACAGGGTTCCTGACAGA 3' R-5'ATGCAGCCTACAGACCAAATATC3'

Hif1α F-5'GAACGTCGAAAAGAAAAGTCTCG 3'R-5'CCTTATCAAGATGCGAACTCACA 3'

VEGFR2 F-5'GGCCCAATAATCAGAGTGGCA 3'R-5' CCAGTGTCATTTCCGATCACTTT 3'

MMP2 F-5'CCCACTGCGGTTTTCTCGAAT 3R-5' CAAAGGGGTATCCATCGCCAT 3'

MMP9F-5'TGTACCGCTATGGTTACACTGC 3'R-5' GGCAGGGACAGTTGCTTCT 3'

β-actinF-5′-ACTACCTCATGAAGATCCTC-3′R-5'CTAGAAGCATTTGCGGTCGACGATGG 3'

1.5 Molecular Docking

To investigate molecular aspect of synergistic outcome, we performed the analysis of compound binding position in protein binding pocket and comparison of its binding energy in several different proteins of BAC, we used molecular docking method to calculatebinding energy performed by AutoDock v4.2 (http://autodock.scripps.edu/).

a) Ligand selection and preparation

Compound (CID) three-dimensional structure downloaded from PubChem library (https://pubchem.ncbi.nlm.nih.gov/) database. The structure was prepared by defining root and appropriate torsion angles. Refined structure saved into PDBQT(Protein Databank partial charges ('Q') and AutoDock4 (AD4) atom types ('T')) format for docking calculations.

b) Receptor structure preparation

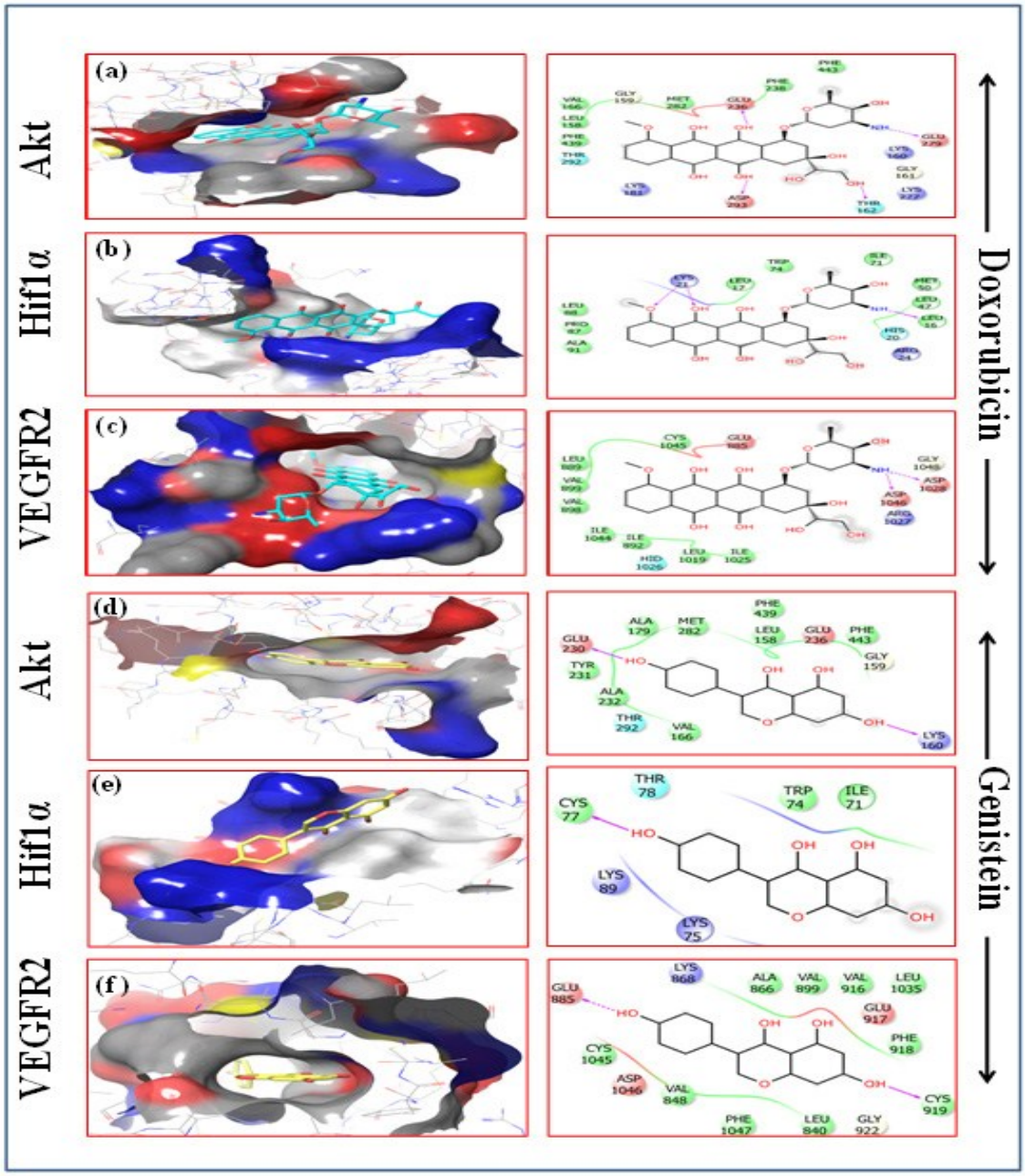
Protein crystal structures (protein name with PDB ID) were downloaded from protein databank (https://www.rcsb.org/). Crystal structures have some extra hetero and water atoms that were removed. Protein structure prepared by adding polar hydrogen atoms with Gasteiger and Kollman charges and finally added AD4 atom types and saved into PDBQT format. Grid was

prepared by defining active site of each protein and set grid box area into 50x50x50 for xyz dimensions. Search parameters of ligand conformation generation applied Genetic algorithm using default parameters. To the selection of top conformation out of 10 were based on lowest binding energy using a Lamarckian-Genetic algorithm with default parameters.

c) Visualization

Protein-ligand complexes were visualized by Schrodinger-Maestro2017 software (https://www.schrodinger.com/).

A



B

Protein	Compound	Binding Energy	Inhibition Constant (Ki)	Final Intermolecular Energy
Akt	Dox	-6.23	27.28uM	-09.51
	Gen	-6.36	21.63uM	-07.56
Hif1α	Dox	-4.00	01.17uM	-07.28
	Gen	-3.08	05.25uM	-06.36
VEGFR2	Dox	-6.83	09.83uM	-10.11
	Gen	-7.62	02.59uM	-08.73

Figure S3. Molecular docking of a profile of protein Akt, Hif1α and VEGFR2 with Gen and Dox. (A) (a-c) Akt, Hif1α and VEGFR2 with ligand Gen and (d-f) Akt, Hif1α and VEGFR2 with ligand Dox. (B) Represents a series of docking outcome.

1.6 Cell culture and cytotoxicity studies

Human breast cancer cell line MDA-MB-231 was purchased from American Type Culture Collection (ATCC, Rockville, MD), Human Umbilical Vein Endothelial cells(HUVEC) were purchased from Thermo fisher scientific whereas 4T1 cell line was obtained from (National centre for cell sciences NCCS, Pune, India). The cells MDA-MB-231 &4T1 were cultured in Roswell Park Memorial Institute medium-1640 (RPMI-1640), $NaHCO_3$, with 10% (v/v)FBS along with 100µg/mL streptomycin, 100 U/mL penicillin. HUVEC cells were cultured with Medium-200 and large vessel endothelial supplement (LVES) obtained from Invitrogen, (Thermo Fisher Scientific USA) under standards condition 37°C temperature, 90% of the humidified environment of 5% CO_2.

In vitro cytotoxicity of free Dox& Gen, D+G(comb), D+G/NPs, SPM-D+G/NPs and Blank NPs were assessed by using MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay on MDA-MB-231, 4T1 and HUVEC cells lines separately. In brief MDA-MB-231, 4T1 and HUVEC cells were seeded in 96-well plates separately (3 x 10^3 cells/well) in 100 µL of RPMI medium containing 10% FBS for MDA-MB-231 and 4T1 whereas HUVEC with M-200 and LVES supplement and kept in CO_2 incubator at 37^{o}C. Experiments were carried out when cells had reached 90% of confluency stage.

Prior to formulation development, the synergy between Dox and Gen was screened by regular using MTT assay. To distinguish synergy different concentration ranges of Dox(0.0039µM-1.0µM) and Gen(0.059µM-15µM) combination were treated and the results were evaluated with compusyn version 1.0 software. Following the results, the free drug, D+G(comb) and synergistically loaded different formulation were screened to the cells in Medium of RPMI and 10% of FBS for 48hr at 37^{o}C. After 48 hr media was removed and cells were treated with MTT reagent 10µl from 4mg /ml stock for 3.5 hrs. Then 100µl of DMSO biological grade was added to dissolve formazan crystals. The colour developed was measured on an automated microplate reader at 570 nm; its absorbance is proportional to the number of viable cells. Those untreated

cells were taken as a control in comparison to the treated cells and the results were presented as the percentage of cell viability. All experiments were carried out in triplicate (n=3) and data were expressed as mean ± standard deviation (SD).

1.7 Synergy identification between Gen and Dox in MDA-MB-231

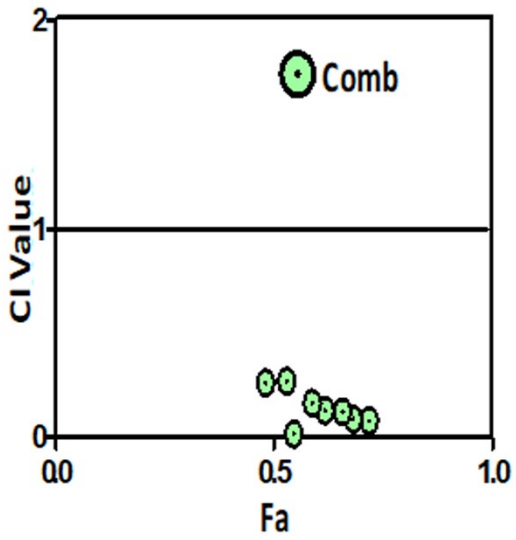


Figure S4. Combination index (CI) of expected synergy combinations

1.8 Cellular localization of nanoparticles

From the literature, It has been proved that Dox acts in nucleus. So, its successful delivery in nucleus is crucial. To confirm this, a subcellular compartment labeling method was applied to examine the distribution of NPs inside the cell. To determine this, a subcellular compartment labeling method was used to observe the distribution of NPs inside the cell. The intracellular distribution of SPM-D+G/NPs was evaluated by florescence microscope using FITC as fluorescent dye. Lysotracker red was taken as a probe to stain the acidic organelles inside MDA-MB-231 cells (figure S5). After 1hr, the FITC tagged NPs trapping was visible in the endosome and move to yellow in appearance, resulting from the red (Lysotracker) and green (FITC) spots overlapping. Above data confirmed that SPM-D+G/NPs efficiently releasing drug in cytosol and travers to nucleus.

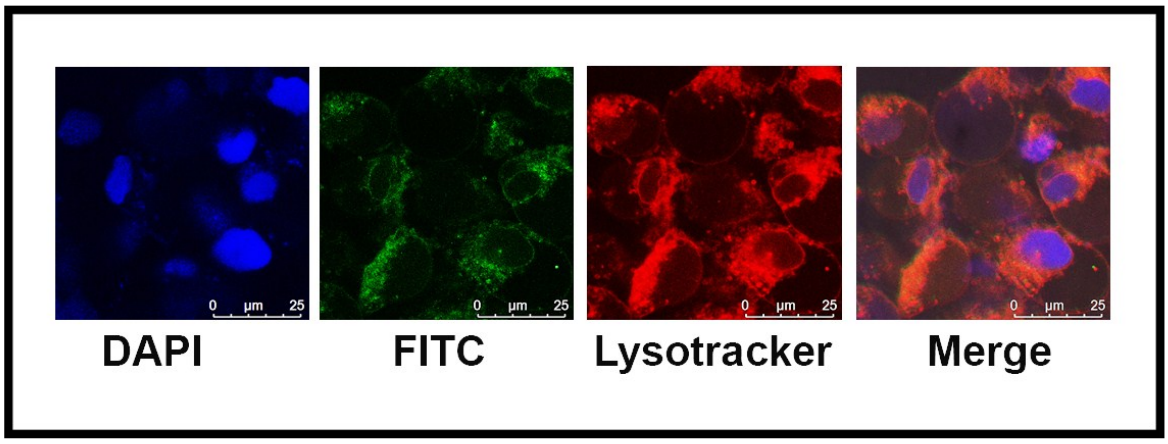


Figure S5. Confocal images of live MDA-MB-231 treated with FITC tagged nanoparticles for 3 hr. Lysotracker (red) was used to stain the acidic organelles of the cell. Scale bar 25µm

1.8 Cell migration and invasion assay

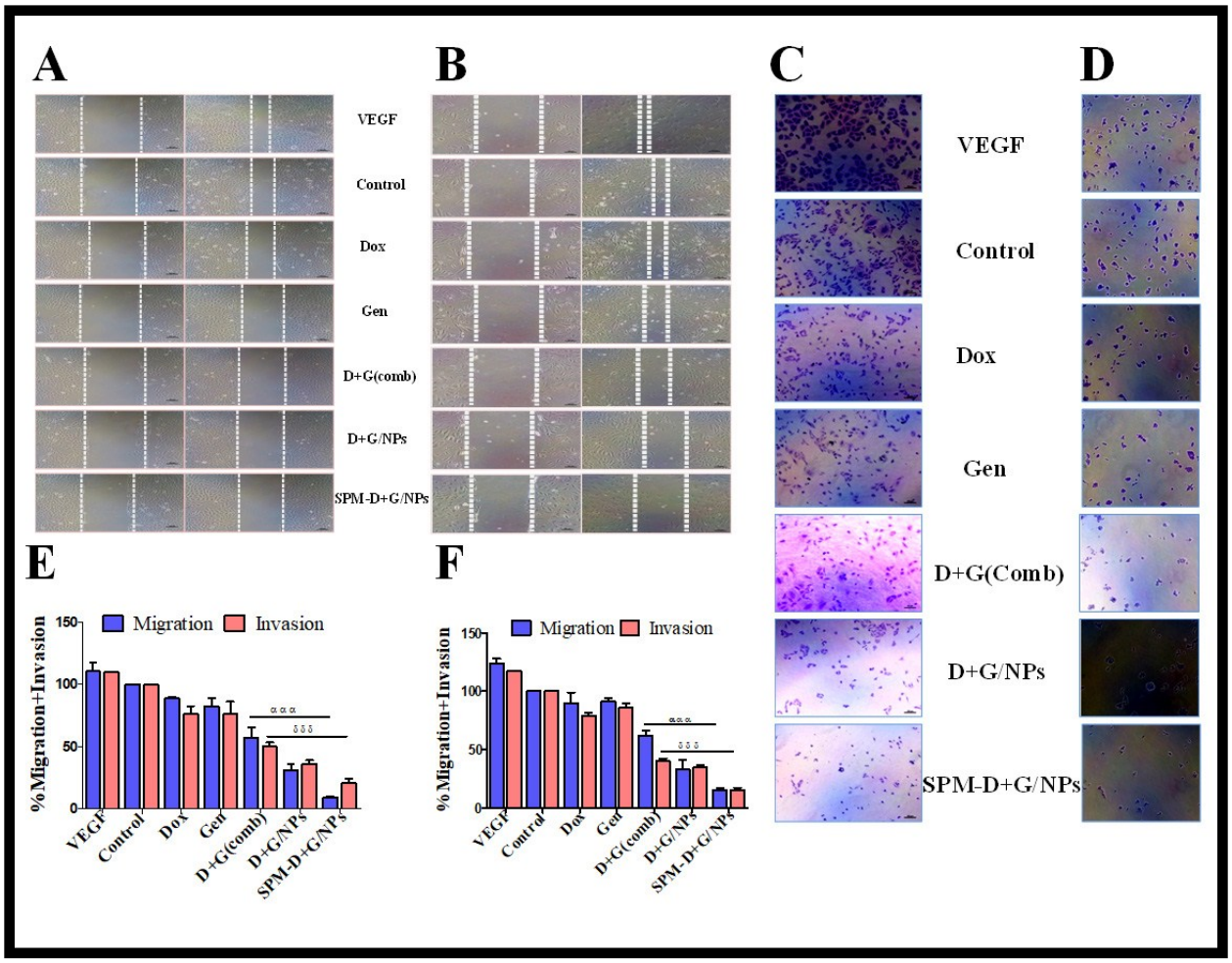


Figure S6. *In vitro* cell migration and invasion potential of developed formulation againstMDA-MB-231 and HUVEC cell line. (A-B) The scratch based wound healing assay showing metastasis inhibiting potential of the SPM-D+G/NPs formulation on MDA-MB-231 (upper left panel) and HUVEC (upper right panel) cells after 24 hr. (C-D) Cell invasion assay on MDA-MB-231 (left panel) and HUVECs (right panel) cells via matrigel pre-coated cell culture inserts after 24 hr of the treatment. Invaded cells on the lower surface of the membrane were captured with a microscope and were counted in 10 different microscopic fields with image j software. (E-F) bar diagram represents percentage cell migration and cell invasion on MDA-MB-231 and HUVEC cells respectively. All the experiments were done n=3 and results were obtained from three independent experiments, mean ± SEM. *$p < 0.05$ versus control.

1.9 Quantification of PEG-SPM conjugation by 1H-NMR

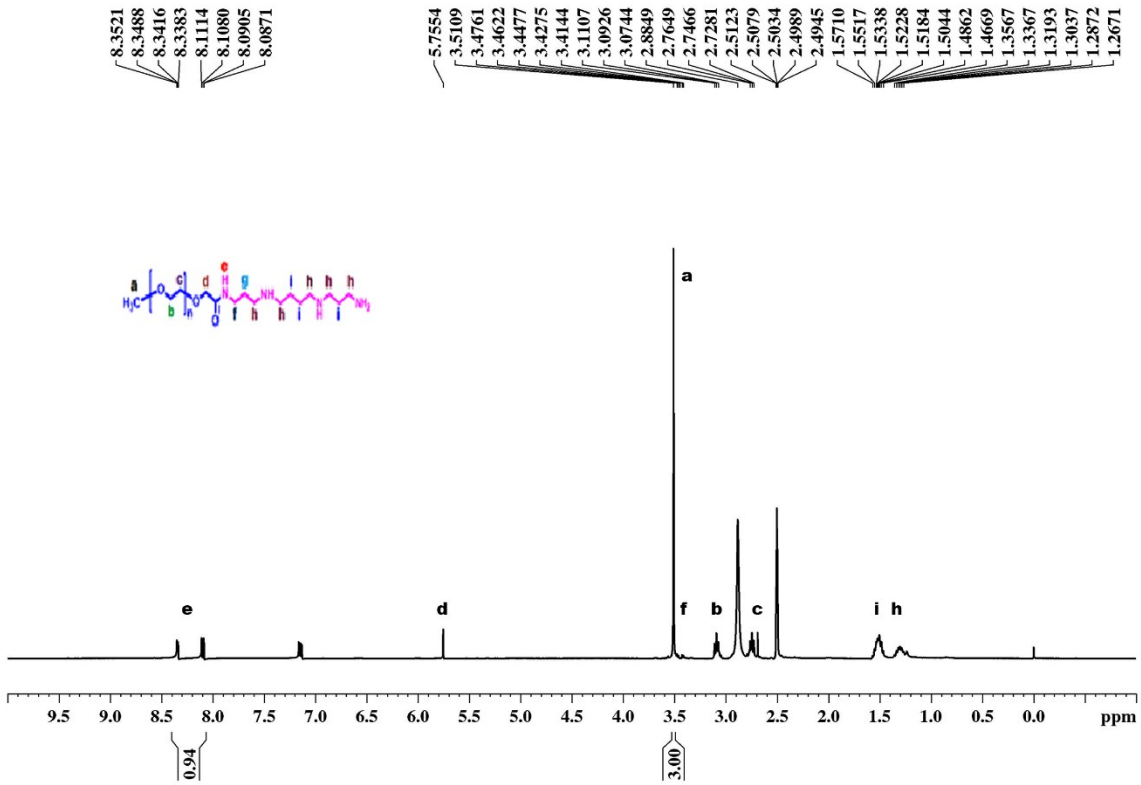


Figure S7. 1 H NMR spectra of (A) PEG-SPM in DMSOd6