Supporting Information

Development of Putrescince anchored nano-crystalsomes bearing doxorubicin and Oleanolic acid- Deciphering its role in inhibiting metastatic breast cancer

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Methods :1.1 Receptor blocking study in cancer

To investigate the gp-1 receptor mediated uptake of Put-D+O@NCs a receptor blocking study was carried out in gp-1 positive MDA-MB-231 and low expression in MCF-7 cells[1]. In order to facilitate cell adhesion, the cells were seeded in six plates and incubated for 24 h. Afterwards, the cells were washed with PBS pH 7.4 and incubated with serum-free media with or without 100uM of Putrescine (wt: 88.15 g mol^{-1}) for 2 h in a CO_2 incubator. Further, the cells were washed again with PBS, followed by treatment with fresh medium containing FITC -labelled D+O@NCs and Put-D+O@NCs crystalsomes and incubated for 6 h. Then, cells were subsequently harvested, washed twice for removal of unwanted dye, resuspended in 400 µL of PBS and investigated by flow cytometry.

1.2 Matrigel transwell invasion assay

In brief, the transwell invasion assay was conducted on the Matrigel-coated transwell chamber (BD, Biosciences) emanating from the manufacturer protocol. In the upper chamber, $2x10^4$cells were seeded and in the lower chamber, a corresponding medium was added including 20ng/ml VEGF.The cells were treated with free Dox, OA, D+O(comb), D+O@NCs and Put-D+O@NCs having a different concentration of OA (equivalent to 2.5µM) Dox (equivalent to 0.25µM) and incubated in a CO_2 incubator at 37°C for 24 h. Then the non-invasive cells were softly wiped out from the upper surface by a cotton swab. The invasive lower surface cells were fixed with 4 % paraformaldehyde and labelled with 0.1 % crystal violet dye (Sigma-Aldrich) for 20 min, and then the images were collected using an inverted microscope and the invasive cells were quantified using Image J software.Results were framed as an invasion percentage in comparison to the respective controls.

Results

2.1 Stability study

Results of HSPG targeted crystalsomes stability studies at 25°C for 30 days. Comparative stability was measured as a characteristic of particle size, polydispersity index (PDI) between targeted (Put-D+O@NCs) and non-targeted (D+O@NCs) nanoparticles (figure S1).

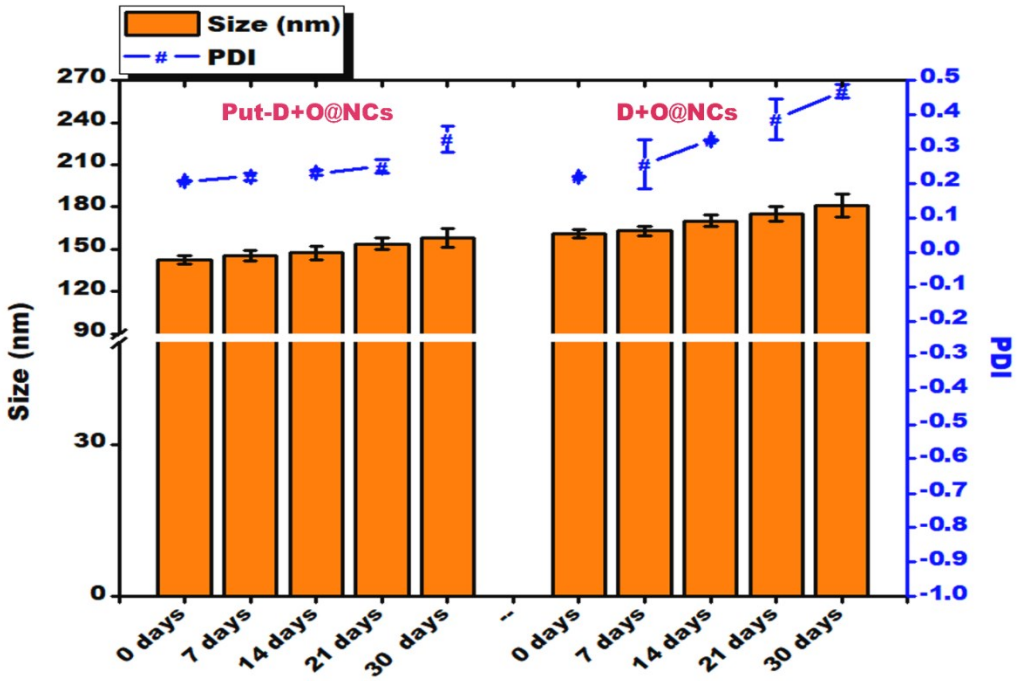

Figure S1. The resulted change in particle size and PDI for a period of 30days.

2.2 Receptor blocking study

The receptor blocking was performed in MDA-MB-231 and MCF-7 cell lines to study the glypican-1 mediated cellular internalization mechanism. For this purpose , both MDA-MB-231 and MCF-7 cells were pre-incubated with 100 µM free Put for 2 h to saturate receptors and then both non-modified (D+O@NCs) and surface-modified (Put-D+O@NCs) were treated for 6 h, subsequently observed that both formulations had a similar fluorescence mean intensity (figure S2 A & B). Thus this study supports a correlation between Put and gp-1 receptors and

encourages the role of gp-1 reactors to enhance cellular uptake and to use the treatment of metastatic breast cancer.

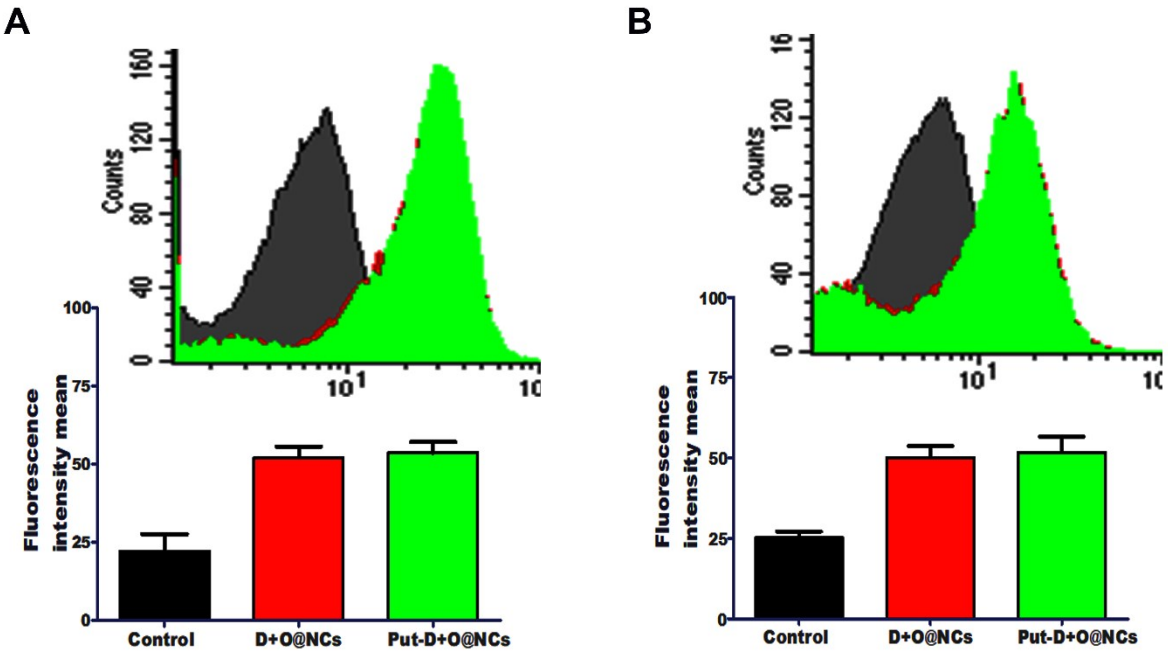

Figure S2. Receptor blocking experiment analyzed with FACS in MDA-MB231 and MCF7 cell line followed by 6 h treatment with FITC-control (black colour) , D+O@NCs (red colour) and Put-D+O@NCs (green colour). (A&B) fluorescence intensity and bar diagram of MDA-MB-231 and MCF7 cell line respectively.

2.3. List of RT-PCR Primer sequences used for quantitative investigation

Gene Forward Primer and Reverse Primer

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

MMP9 F-5'TGTACCGCTATGGTTACACTGC 3' R-5' GGCAGGGACAGTTGCTTCT 3'

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

β-actin F-5′-ACTACCTCATGAAGATCCTC-3′ R-5’CTAGAAGCATTTGCGGTCGACGATGG 3’

2.4 Cell migration and invasion assay

The cell migration and invasion is a key aspect in tumorigenesis[2]. So that the effect of Put-D+O@NCs and other treatment groups on migratory ability has also been studied on MDA-MB-231 cell line. In which the wound healing assay was performed to determine the effects of treated samples on breast cancer cell migration. The findings suggested that Put-D+O@NCs shown dose-dependent migration inhibition after 24 h of treatment (figure. S2A& C). Likewise, Put-D+O@NCs inhibited significantly the invasiveness in MDA-MB-231 cells when it was cultured in matrigel-coated cell culture inserts (figure. S3 A&B). Put-D+O@NCs also inhibits significantly VEGF-induced migration and invasion, whose findings indicate that Put-D+O@NCs has anti-migratory and anti-invasive ability at sub-toxic doses.

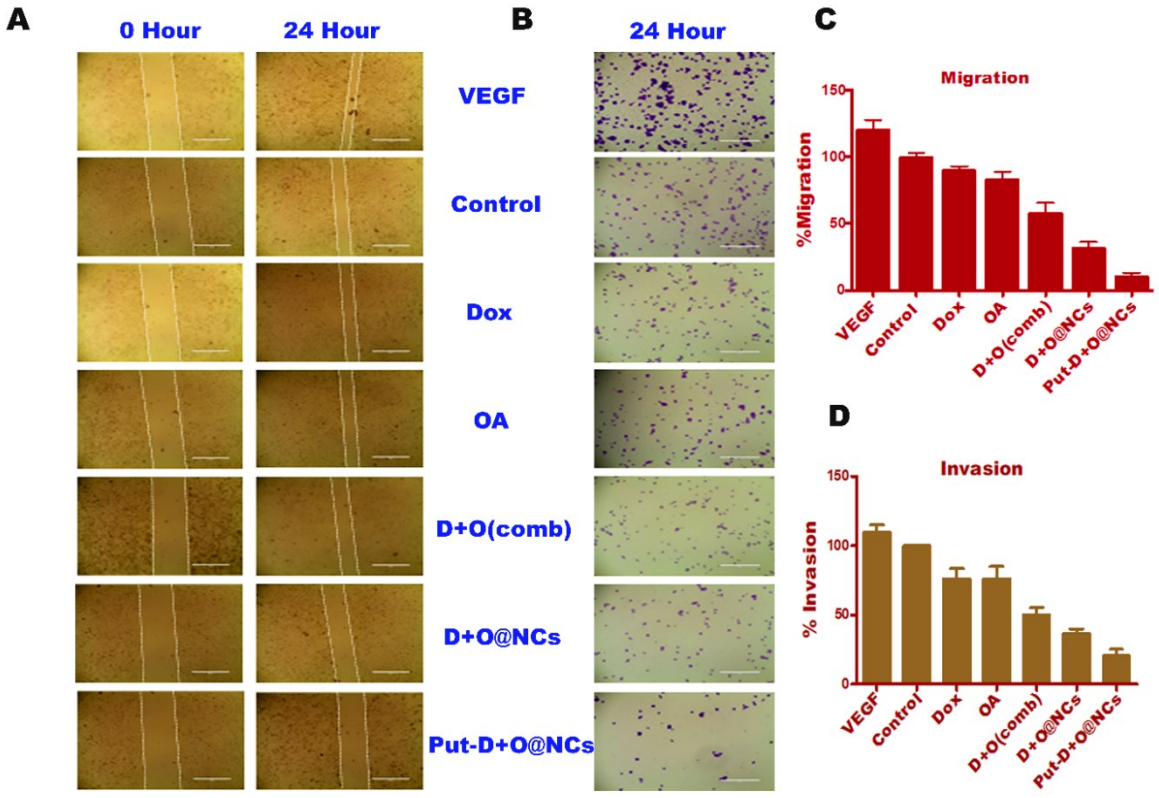

Figure S3. The cell migration and invasion evolution of developed formulation in MDA-MB-231 cell line. (A) The wound healing assay displaying anti metastasis potential of the Put-D+O@NCs crystalsomes on MDA-MB-231 after 24 h. (B) Cell invasion assay on MDA-MB-231 cells via matrigel pre-coated cell culture inserts after 24 h 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. (C-D) 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 ± SD.

2.5 Chick Chorioallantoic Membrane Assay (CAM assay)

After acquiring very significant in vitro results, we conducted an *in ovo* CAM assay to determine the impact of treatment on pre-existing vasculature. However, during the CAM study, high vasculatrisation in the control group was observed as compared to Put-D+O@NCs, D+O@NCs, D+O(comb), Dox and OA treated groups (figure S3 A-B). Quantitevely, among them Put-D+O@NCs had shown the highest degree of depletion in vessel sprouting compared with other treatment groups. The above findings also have comprehenced the *in vitro* outcome.

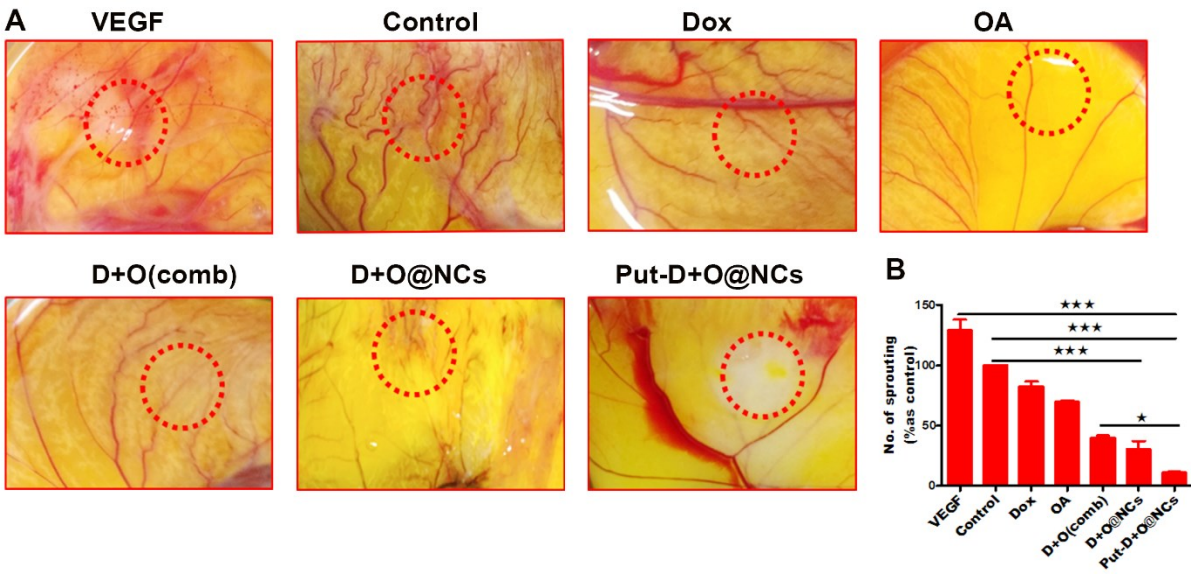

Figure S4. The effect of various samples on neovasculature growth in vessels sprouting *in ovo* by CAM assay.Samples including VEGF were loaded into gelatin sponge, inserted through the CAM eggshell window, and pictured by stereozoom microscopy and sprouting vessels were counted (the circled area is representing gelatin sponge position inside the CAM).

Table S1. Pharmacokinetic parameters of Dox and OA in mice plasma after single intravenous administration of crystalsomes formulations at a dosage of 1mg/kg Dox and 8.4 mg/kg OA. All data are expressed as mean ± SD (n=3).

Parameter	**OA**			**Dox**		
	D+O(comb)	**D+O@NCs**	**Put-D+O@NCs**	**D+O(comb)**	**D+O@NCs**	**Put-D+O@NCs**
C_{max}(µg/ml)	79.076 ± 5.21	75.58±3.41	79.854±8.11	6.35±0.21	7.026±1.11	7.583±0.12
$t_{1/2}$(h)	6.981 ± 1.24	17.065±2.11	39.547±4.01	3.518±0.11	15.429±1.23	20.206±2.17
$AUC_{0-\alpha}$(µgh/ml)	583.630±30.34	1751.058±150.12	3663.567±142.21	29.654±2.23	152.836±12.63	205.2623±12.37
Vss(ml)	0.1442±0.21	0.1416±0.041	0.14444±0.11	1.547±0.11	1.351±0.21	1.3223±0.81
Cl(ml h^{-1})	0.0161±0.051	0.00536±0.01	0.00256±0.01	0.3169±0.04	0.06150±0.05	0.0457±0.061
MRT(h)	8.954±1.32	26.39555±2.12	56.2954±2.4	4.883±1.01	21.970±2.12	28.875±1.21

References :

1. S. Urandur, 2018, **10**, 12960-12974.
2. P. Chowdhury, P. K. B. Nagesh, E. Hatami, S. Wagh, N. Dan, M. K. Tripathi, S. Khan, B. B. Hafeez, B. Meibohm, S. C. Chauhan, M. Jaggi and M. M. Yallapu, *Journal of colloid and interface science*, 2019, **535**, 133-148.