Exploring the Synergistic Behaviour of Paclitaxel and Vorinostat upon Co-loading in Albumin Nanoparticles for Breast Cancer Management

SUPPLEMENTARY

1. Method:

1.1 Shape and Surface Morphology

The shape and surface morphology of PTX-VOR-BSA-NPs were analyzed by Transmission emission microscopy (TEM, FEI Tecnai G2F20, Netherlands). The PTX-VOR-BSA-NPs were fixed on a stub by a double-sided tape with subsequent gold-palladium alloy coating (10kV) by sputtering technique (E-1010, Hitachi, Japan) to minimize the surface charging. For TEM analysis, a drop of the sample (diluted 50x with HPLC grade water) was mounted on the carbon-coated copper grid, dried, and stained with phosphotungstic acid (2% w/v). The grid was then scanned using TEM and observed under the microscope at an accelerating voltage of 80 kV.

1.2 *In vitro* drug release analysis

The drug release study was carried out by dialysis bag method (Sigma, dialysis membrane MWCO 12000). Lyophilized BSA-NPs was suspended in release media to form a nanoparticle suspension containing 1 mg of PTX and 1 mg of VOR. The resulted suspension was taken inside of dialysis bag which was then sealed at both ends and placed in 25 ml of release media (PBS, pH 7.4) with 0.5 % tween 80 to solubilize the released drug) to maintain sink conditions. The whole setup was placed in shaker bath at 37 ^{o}C with 50 rpm and samples (1 ml) were withdrawn from the receptor compartment at predetermined time intervals of 0.5, 1, 2, 4, 6, 8,10, 12, 24 h with the replacement of same volume with release media. Same procedure was followed to test the release profiles of free drugs (PTX and VOR). Then released drugs were quantified using validated HPLC method.

1.3 *In vitro* hemolysis test

Blood was freshly withdrawn from male Sprague Dawley (SD) rats in heparinized microcentrifuge tubes and centrifuged at 3000 rpm for 5 min to separate red blood cells (RBCs). The supernatant was discarded and RBCs were washed thrice with physiological saline solution. The stock of 1% v/v RBCs was prepared in PBS. 1 ml of this stock was mixed with 100 µl of PTX and VOR loaded

and PTX loaded albumin nanoparticles solution equivalent to 1 mg/ml of PTX and VOR. Also, equivalent free PTX and free VOR was taken. RBCs mixed with Triton X-100 solution and PBS were employed as positive and negative control, respectively. The samples were incubated at 37ºC for 1 hour and then centrifuged at 2500 rpm for 5 min to remove non-lysed RBCs. The supernatants were collected which was allowed to stand at RT for 30 min to oxidize hemoglobin (Hb). The absorbance of oxygenated hemoglobin (Oxy-Hb) was measured by spectrophotometric determination at 540 nm, and % hemolysis was calculated by following equation,

$$\% \text{ Hemolysis} = (ABs/AB100) \text{ X } 100$$

Where, Abs is the absorbance of the sample and AB100 is the absorbance of the control.

The RBC pellet thus obtained was incubated with 0.5% glutaraldehyde and fixed. They were then observed under Scanning Electron Microscope (SEM) (S-3400, Hitachi Ltd., Japan) for morphological changes.

1.4 *In vitro* analysis on cell-Lines

1.4.1 Cell lines

MCF-7 and MDA-MB-231 (ATCC, Manassas, VA, USA) were cultured and maintained as earlier reports. In brief, MCF-7 and MDA-MB-231 cells were grown in tissue culture flasks and maintained under 5% CO_2 atmosphere at 37ºC. The cultured cells were trypsinized once 90% confluent with 0.25% trypsin-EDTA solution (Sigma, USA). MCF-7 cells were seeded at a density of 10,000 cells/well in 96 well plate for MTT assay. For uptake and apoptosis study MDA-MB-231 (50,000 cells/well) cells were seeded in 6-well culture plate (Costars, Corning Inc., NY, USA).

1.4.2 Qualitative Cellular Uptake Study

For qualitative cell uptake studies coumarin-6 (C-6) loaded nanoparticles were prepared by following the same method except using C-6 in place of drugs. The cells were incubated with the BSA-NPs (equivalent to 1 µg/mL of free C-6) for 4 h. After 4 h media was aspirated and cells were washed 3 times using buffer (7.4 pH). Further, the cells were fixed with glutaraldehyde (2.5% v/v) (Sigma, USA) and visualized under confocal laser microscope (CLSM) (Olympus FV1000).

1.4.3 Cell cytotoxicity evaluation

To determine anti-tumor efficacy of the PTX-VOR-BSA-NPs MTT assay was performed. MTT

assay was performed in MCF-7 cell lines. For *in vitro* cytotoxicity assay, cells were seeded in 96-well and kept overnight for attachment. Then cells were incubated for 24 h with PTX (1, 5, 10, 20 µg/ml), VOR (1, 10, 20, 30 µg/ml) and PTX-VOR-BSA-NPs (1, 2.5, 5 and 10 µg/ml) respectively. After completion of the incubation period, medium containing formulation was removed and subsequently, treated with MTT solution (500 µg/mL in PBS) for 3 h. The excess solution was then removed carefully, and MTT formazan crystals were dissolved in 200 µL of DMSO. The optical density of the resultant solution was then measured at 550 nm using an ELISA plate reader. PTX concentrations required to inhibit growth by 50% (IC_{50}) were determined from concentration dependent cell viability curves.

$$\text{Cell viability (\%)} = (Abs_S/Abs_{ctrl})\ 100$$

Where Abs_S is the absorbance of cells tested with various formulations, Abs_{ctrl} is the absorbance of control cells.

1.4.4 Annexin-V apoptosis assay

The cell cytotoxicity potential of the formulations was further assessed as a function of their capability to induce apoptosis in MDA-MB-231 cell lines. Cells were seeded at a density of 50,000 cells/well in 6-well culture plate (Costars, Corning Inc., NY, USA) and allowed to attach overnight at 37ºC and 5% CO_2. They were further incubated with different formulations equivalent to 10 µg/mL of free PTX and VOR for 6 h. Post incubation, cells were washed three times with DPBS (pH 7.4) and stained with Annexin V-Cy3.18 conjugate (AnnCy3) and 6-carboxyfluorescein diacetate (6-CFDA) as per the manufacturer's protocol (Annexin V-Cy3™ Apoptosis Detection Kit, Sigma, USA) and observed through CLSM (Olympus, FV1000) under red and green channels for AnnCy3 and 6-CFDA, respectively. The cells were visualized under CLSM under green (for 6-CFDA) and red (for AnnCy3) channels. The cells stained with only green fluorescence were considered as live; while cells stained only with red were considered as necrotic. Apoptosis index, ratio of the fluorescence intensity from the red to green fluorescence was also calculated for the developed formulations. The fluorescence intensity was quantified by Image J software (U.S. National Institutes of Health, Maryland, USA).

1.5 *In vivo* Studies

1.5.1 *In vivo* pharmacokinetics study

For pharmacokinetics studies Female Sprague Dawley (SD) rats (150-200 g) were used. The animals were randomly distributed into 4 groups each containing five animals. Intaxel®, PTX BSA-NPS and PTX-VOR-BSA-NPs were administered to each group respectively via intravenous administration at a dose of 1 mg/kg of PTX. The blood samples (approximately 0.2 ml) were collected from tail vein into the heparinized micro-centrifuge tubes at different time points of 0.5, 1, 2, 4, 8, 12, 24 and 48 h. Subsequently, plasma was separated from the blood by centrifuging the samples at a speed of 10,000 rpm for 10 min at 4°C and the drug was extracted from the plasma. The extraction procedure involved the precipitation of plasma proteins by acetonitrile and methanol followed by centrifugation to settle out the precipitated protein. Methanol was added during the precipitation step to dissolve the drug and the clear supernatant obtained after centrifugation was analyzed for PTX by validated HPLC bioanalytical method. The pharmacokinetic analysis of plasma drug concentration-time data was analyzed. Required pharmaco-kinetics parameters like total area under the curve (AUC)0-∞, terminal phase half-life ($t_{1/2}$) were determined.

1.5.2 *In vivo* antitumor activity of PTX-VOR-BSA-NPs

The anticancer efficacy of the various BSA-NPs was determined in 4T1 cell line-induced breast tumor model. Subcutaneous injection of 1×10^5 4T1 cells was given to the mammary fat pad of female BALB/c mice for tumor induction. After attaining an average tumor volume of 100 Cu.mm, the animals were divided into four groups (control, Intaxel, PTX BSA-NPs and PTX-VOR-BSA-NPs) and each group was treated 5 mg/kg PTX intravenously at interval of 5 days (three dosing, 21 days study protocol). Groups that received no treatment were kept as control. Tumor volume regression was evaluated for obtaining the antitumor efficacy of the developed formulation.

1.5.3 *In vivo* toxicity analysis

For analyzing the systemic toxicity of PTX-VOR-BSA-NPs, the biochemical parameters were determined in healthy female Swiss albino mice (weight 20−25 g). The animals were divided into three groups (Intaxel, PTX BSA-NPs and PTX-VOR-BSA-NPs) and each group received intravenous dose of 5 mg/kg equivalent of PTX. The animals were sacrificed 7 days post treatment

and the plasma from the collected blood was analyzed for various toxicity markers of blood [alanine aminotransferase (ALT), aspartate aminotransferase (AST), for blood urea nitrogen (BUN), and creatinine] using commercial diagnostic kits (AccuRex Biomedical Pvt. Ltd, India).

2. Results and discussion

2.1 Preparation and optimization of PTX-VOR-BSA-NPs formulations

The optimization of PTX-VOR-BSA-NPs based on pH of albumin solution, volume of glutaraldehyde and drug loading is provided in Table S1.

Table S1. Optimization of various critical parameters for PTX-VOR-BSA-NPs development

Optimization parameter	**Size (nm)**	**PDI**	**% EE**	
			PTX	**VOR**
pH of albumin solution				
6.0	267.73±16.99	0.177±0.01	66.79±4.03	60.77±4.65
6.5	198.1±11.90	0.159±0.05	74.72±3.9	66.04±6.04
7.0	138.06±11.10	0.171±0.02	80.61±4.0	69.66±5.12
7.5	179.6±13.00	0.299±0.06	76.30±3.83	62.08±5.29
8.0	236.7±12.80	0.264±0.013	72.83±5.83	58.54±7.14
Volume of glutaraldehyde (µl)				
25	240.73±14.69	0.196±0.01	63.19±5.10	57.76±5.51
30	204.66±13.52	0.224±0.01	67.0±4.53	59.07±3.93
35	185.20±11.59	0.209±0.01	75.14±5.95	64.35±5.12
40	147.76±12.60	0.176±0.01	79.8±3.68	68.26±4.79
45	285.16±15.44	0.225±0.04	74.71±6.49	65.96±4.68
50	335.23±14.40	0.413±0.04	71.66±6.8	62.29±5.02
55	450.50±18.95	0.841±0.10	68.11±7.54	60.68±7.09
Drug loading (%)				
7.5	206.23±10.03	0.216±0.01	82.1±5.18	78.87±4.97
10.0	143.33±12.39	0.184±0.01	77.37±5.03	66.27±5.14

12.5	166.80±10.06	0.207±0.04	61.59±5.03	60.15±8.44

2.2 Lyophilization of PTX-VOR-BSA-NPs

The optimization of various types and concentrations of cryoprotectants based on particle size, PDI and %EE of PTX-VOR-BSA-NPs pH is shown in Table S2.

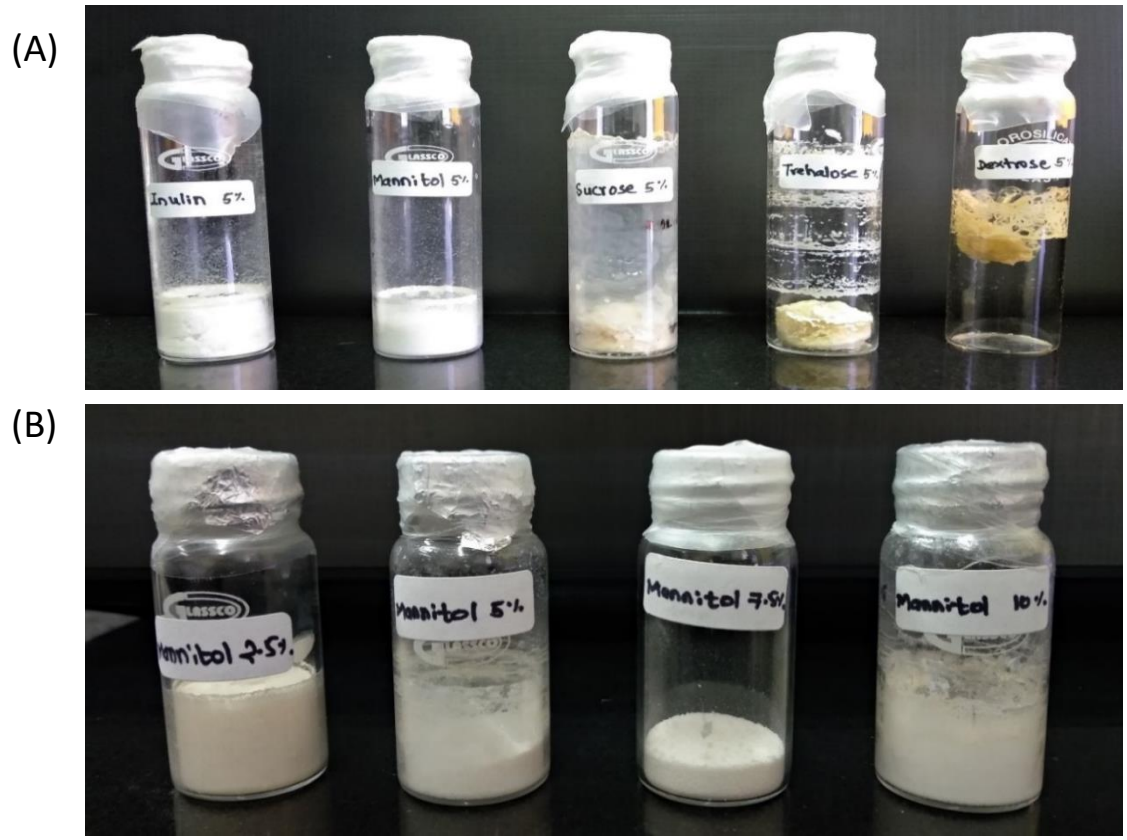

Figure S1. Lyophilization of PTX-VOR-BSA-NPs using (A) various cryoprotectants and (B) different concentrations of mannitol

Table S2. Screening of different concentrations of mannitol for lyophilization

Mannitol	Before freeze drying				After freeze drying				Ratio (Sf/Si)	R. score
	Particle Size (nm)	PDI	%EE (PTX)	%EE (VOR)	Particle size (nm)	PDI	%EE (PTX)	%EE (VOR)		
2.5 %	**140.5± 9.35**	**0.18 ± 0.02**	**78.74± 5.49**	**68.46 ± 4.71**	237.8±26.74	0.312±0.06	53.91±4.48	52.74±6.70	1.63	**
5.0 %					201.9±16.30	0.265±0.04	70.83±4.05	59.86±4.91	1.38	***
7.5 %					**166.7±13.25**	**0.215±0.02**	**73.59±3.83**	**62.54±5.03**	**1.14**	***
10.0 %					194.6±15.81	0.239±0.04	69.38±4.17	60.54±4.69	1.33	***

Values are mean ±SD, n=3

Sf/Si ratio is the ratio of particle size post lyophilization to particle size before lyophilization.

*** Lyophilized cake was easily redispersed in 0.5 ml water within 20 sec by simple shaking.

** Cake was easily redispersed in 0.5 ml water within 45 sec by vortexing.

2.3 Storage stability Assessment

The lyophilized formulation showed no change in terms of shrinkage of cake or any other change in physical appearance. Upon resuspension, there was no significant difference found in the particle size and PDI after 3 months of testing period (Table S3). Thus, the lyophilized formulation was stable for an extended period of time.

Table S3. Stability data of PTX-VOR-BSA-NPs at different temperature conditions

Temperature conditions	Sampling (Days)	Particle size (nm)	PDI	% EE	
				PTX	VOR
Initial	**0**	**169.1±5.10**	**0.175±0.01**	**73.17±3.57**	**62.23±1.93**

Room temperature	30	175.86±8.68	0.191±0.01	71.03±2.54	59.92±3.01
	60	189.5±4.43	0.215±0.02	68.92±3.84	58.09±3.62
	90	193.46±4.93	0.210±0.01	63.44±2.92	54.60±3.49
2-8 ^{o}C	30	172.93±5.93	0.185±0.03	72.76±3.10	60.95±2.33
	30	177.2±8.79	0.201±0.01	69.06±3.70	58.83±2.50
	90	185.3±10.98	0.218±0.01	66.53±5.04	55.01±3.19

Values are mean ±SD, n=3