Supporting Information

Polyethylene Glycol-Encapsulated Histone Deacetylase Inhibitor Drug-Composite Nanoparticles for Combination Therapy with Artesunate

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**Materials:** Chitosan (degree of deacetylation >75%), HAuCl₄ (Au, 17 wt % in dilute HCl; 99.99%), mercapto propionic acid (MPA), sodium butyrate (NaB), (3-(4, 5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide) (MTT), sinapinic acid, propium iodide, (5,5’,6,6’-tetrachloro-1,1’,3,3’- tetraethylbenzimidazolylcarbocyanine iodide (JC-1), chloropromazine methyl-β-cyclodextrin and HDAC Activity Assay Kit were obtained from Sigma-Aldrich, U.S.A. Artesunate was procured from commercial source. AnnexinV-7AAD kit used for apoptosis and APO-DIRECT™ Kit i.e., for TUNEL assay were acquired from BD lifescience. For lysosomal staining, cytopainter green was used, which was purchased from Abcam. For all experiments, Milli-Q grade water (18.2 MΩ cm) was used. HeLa (human cervical carcinoma) cells were procured from National Centre for Cell Sciences, India (NCCS) Pune.

**Quantum Yield Measurements:** The fluorescence quantum yield of as-synthesized PEG-Au NC-NAB-NPs was determined with quinine sulphate as reference sample where quinine sulphate was dissolved in 0.1 M H₂SO₄. To calculate the quantum yield of PEG-AuNC-NAB-NPs the following equation has been used:

\[
QY_S = QY_R \times \frac{A_S}{A_R} \times \frac{Abs_R}{Abs_S} \times \frac{\eta_S^2}{\eta_R^2}
\]

Where,

- \(QY_S\) = quantum yield of as synthesized PEG-AuNC-NAB-NPs (sample)
- \(QY_R\) = quantum yield of quinine sulphate, which was dissolved in 0.1 M H₂SO₄ (reference).
- The standard quantum yield of the reference (\(QY_R\)) is 0.54.
- \(A_S\) = area under the emission spectrum of PEG-AuNC-NaB-NPs obtained at \(\lambda_{ex} = 320\) nm at slit-width = 2 nm
- \(A_R\) = area under the emission spectrum of reference
- \(Abs_R\) = absorbance of the reference
- \(Abs_S\) = absorbance of the sample
- \(\eta_S\) = refractive index of solvent of sample
- \(\eta_R^2\) = refractive index of solvent of reference

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**In Vitro** FACS based Experiments

**Determination of Reactive Oxygen Species Generation with FACS**

The reactive oxygen species generated on treatment with Au NCs, NaB, PEG Au NC-NaB-NPs, ART and PEG-AuNC-NaB-NPs-ART were investigated in CytoFLEX flow cytometer (Beckman Coulter) by staining the samples with 2,7-dichlorofluoresceindiacetate (DCFH-DA; Sigma-Aldrich, USA). For this $10 \times 10^3$ cells/well were seeded in 6 well plates and were allowed to grow overnight. After acquiring required confluency, the cells were treated with IC$_{50}$ dose of Au NCs, NaB, PEG Au NC-NaB-NPs, ART and PEG-AuNC-NaB-NPs-ART for 3 h. Thereafter, the respective wells along with the control were incubated with 10 μM of DCFH-DA at 37 °C for 30 min. DCFH-DA, which is a flurogenic dye diffuses into the cell through plasma membrane and is converted to a non-fluorescent compound DCFH by cellular esterases. The DCFH in presence of ROS generated by the cell is oxidized into highly fluorescent compound 2’, 7’ –dichlorofluorescein (DCF) having green fluorescence ($\lambda_{ex}$=488 nm and $\lambda_{em}$=530 nm). Thus the obtained green fluorescence was analyzed in FITC channel (530/30nm), which corresponds to green emission. For this the cells were collected after 30 min treatment and were analyzed using CytoFLEX flow cytometer (Beckman Coulter).

**Cell Cycle Analysis**

Propidium iodide (PI) based cell cycle analysis for DNA content measurement was carried out in CytoFLEX flow cytometer (Beckman Coulter), which distinguishes cells in different phases of the cell cycle. Here PI stains DNA quantitatively after permeabilisation. Cells ($10 \times 10^3$ cells / well) were seeded in a 6 well plate and treatment with IC$_{50}$ dose of Au NCs, NaB, PEG Au NC-NaB-NPs, ART and PEG-AuNC-NaB-NPs-ART were given for 48 h. Thereafter cells were collected and fixed with ice cold (1 mL) 70% ethanol. Ethanol was be added slowly while vortexing and cells were then stored at -20 °C for 1 h. Then 70% ethanol was removed by centrifugation by discarding the supernatant and the pellet was redispersed in PBS followed by incubation in 0.4 mg/mL RNase solution for 1 h at 37 °C. At last cells were incubated with PI (10 μg/mL) for 30 min under dark condition and thereafter analysis was carried in PE-A channel (band-pass filter, 585/42 nm) of CytoFLEX flow cytometer (Beckman Coulter) using an excitation wavelength of 488 nm. The ModFit LT software 5.0 was used for data analysis.
**PE Annexin V- 7-AAD Apoptosis Detection Assay**

For detection of early and late apoptotic cells, PE Annexin V detection kit (BD Biosciences) in conjunction with 7-amino-actinomycin (7-AAD) dye was used. In a similar way, 10×10³ cells were seeded in 6 well plates and were allowed to grow for 24 h. This was followed by treatment with Au NCs, Sodium Butyrate, PEG Au NC-NaB-NPs, ART and PEG-AuNC-NaB-NPs-ART for 48 h. In dead cells, because of membrane damage, 7-AAD is permeable as opposed to intact membrane in viable cells. Thus viable cells are both PE Annexin V and 7-AAD negative; early apoptotic cells are PE Annexin V positive and 7-AAD negative and dead cells are both PE Annexin V and 7-AAD positive. The samples were prepared based on the protocol provided by the company (BD Lifescience) and were analysed in CytoFLEX flow cytometer (Beckman Coulter).

![Figure S1](image_url)

**Figure S1.** (a) TEM image of Au NCs (scale bar 20 nm) and (b) particle size distribution of Au NCs.
Figure S2. UV-vis spectrum of as-synthesized chitosan stabilized Au NCs.

Figure S3. TEM image of PEG-Au NC-NaB-NPs (scale bar 100 nm) with the portion magnified is marked in yellow.
Figure S4. Zeta potential of (a) Au NCs, (b) NaB and (c) PEG-Au NC-NaB-NPs.

Figure S5. DLS results representing the hydrodynamic size of as-synthesized PEG-Au NC-NaB-NPs.

Figure S6. Binding efficiency of drug (NaB) with its varied concentration (10 - 60 mM). For this fixed concentration of Au NCs with the respective drug concentrations were used for the synthesis of composite nanoparticles. Maximum binding of 65% was achieved at 50 mM concentration, after which saturation was attained.
Figure S7. (a) Emission spectrum of PEG-Au NC-NaB-NPs recorded under confocal microscope in live Hela cell. (b) Corresponding cells showing red luminescence.

Figure S8. Confocal laser scanning microscopy images of control HeLa cells (without any treatment). (a) Bright field image. (b) Red channel showing no luminescence
Figure S9. Confocal laser scanning microscopy images of HeLa cells after 4 h of treatment with PEG-Au NC-NaB-NPs and with nucleus staining marker DAPI (10 min). (a) Luminescence of PEG-Au NC-NaB-NPs as observed in red channel ($\lambda_{\text{ex}} = 405$ nm and $\lambda_{\text{em}} = 610$ nm), (b) luminescence of cells labelled in nuclear marker DAPI ($\lambda_{\text{ex}} = 355$ nm and $\lambda_{\text{em}} = 460$ nm), (c) bright field image and (d) merged image of a, b and c.

![Figure S9](image.png)

Figure S10. Cell viability based on MTT assay after 48 h of treatment with only Au NCs.

![Figure S10](image.png)

Figure S11. Cell viability assay of combination therapy of PEG-Au NC-NaB-NPs –ART on normal L132 cells.

![Figure S11](image.png)

Table S1. Table showing CI (combination index) values for combination therapy of PEG-AuNC-NaB-NPs (1.8 mM) with varied concentration of ART (100 $\mu$M) on Hela cells. The CI values were found to be <1 for all concentrations and hence synergism was considered to have been present.
| PEG-Au NC-NaB-NPs (mM) | ART (µM) | Effect (%) | CI value < 1 (Synergism) |
|------------------------|----------|------------|--------------------------|
| 1.8                    | 4.4      | 0.84       | 0.63                     |
| 1.8                    | 8.8      | 0.72       | 0.61                     |
| 1.8                    | 13.3     | 0.59       | 0.58                     |
| 1.8                    | 17.7     | 0.47       | 0.51                     |
| 1.8                    | 22.2     | 0.35       | 0.44                     |

**Figure S12.** FACS analysis of ROS production evaluated by DCF fluorescence in HeLa cells investigated in FITC-H channel, which corresponds to the green emission of the DCF. Cells were treated with IC$_{50}$ dosage of Au NCs, NaB, ART, PEG-AuNC-NaB-NPs and PEG-AuNC-NaB-NPs-ART, which exhibited prominent shifts as compared to that of control cells.
Figure S13. Flurometry based HDAC activity of cell lysates of HeLa cells, following treatments with NaB (10.52 mM), PEG-AuNC-NaB-NPs (8.69 mM) and PEG-AuNC-NaB-NP-ART (1.82 mM with respect to NaB and 17.7 µM ART)

Figure S14. FACS based cell cycle analysis of HeLa cells treated with NaB, ART, PEG-AuNC-NaB-NPs and PEG-AuNC-NaB-NPs-ART along with its control.

Figure S15. Flow cytometry histograms of TUNEL assay showing the shifting of population towards FL1-H green channel, which is due to DNA fragmentation. The luminescence intensity after
treatment: (a) control (without treatment), (b) sample treated with PEG-Au NC-NaB-NPs (8.69 mM) and (c) treated with PEG-Au NC-NaB-NPs-ART (1.8 mM NaB and 17.7 μM ART).

**In Vivo Experiments**

**Acute toxicity studies:** Following the guidelines of Organization for Economic Co-operation and Development (OECD) acute toxicity studies were conducted to test the chemicals on mice. Swiss albino mice of either sex (n=6) were chosen for the experiments to perform. Before administration of test drugs the selected mice were fasted overnight and were given only water. To check the mortality a single dose of PEG-Au NC-NaB-NPs-ART at 50 mg/kg was administered separately to three mice each. Following treatment if mortality was observed in 2 out of 3 mice then the given dose was regarded as toxic and the experiment was repeated. Even if mortality was observed in 1 mice then also the experiments were repeated where treatments were given with lower doses (20, 10, 5 & 2 mg/kg body weight). Thus, based on the result of mortality doses were decided. Irwin scale parameters - like presence or absence of lethality, convulsions, straub tail, sedation, excitation, jumps, loss of balance, abnormal writhes, piloerection, stereotypies (sniffing, chewing or head movements), head twitches, scratching, abnormal respiration, aggressiveness towards the experimenter, loss of righting reflex, loss of corneal reflex, defecation, salivation and lacrimation were followed for initial assessment. Thereafter to check the mortality, mice were kept under observation for 14 days in order.

**Trypan Blue Test:** The cell viability assays following treatment were analysed using trypan blue. Trypan blue is a diazo dye used to stain compromised or dead cells and is not taken by live cells because of their intact cell membrane. For this, briefly, 0.1 mL of DLA cells were mixed with 0.1 mL of trypan blue (0.4 %) solution and were counted using cell counter (Countess II FL, Life Technologies, USA). The (%) of cell cytotoxicity was calculated by the following formula:
% of cell cytotoxicity = \left[ \frac{100 - \frac{\text{No of viable cells in the treated group}}{\text{No of viable cells in the untreated control group}}}{\text{No of viable cells in the untreated control group}} \right] \times 100

**Haematological profiling:** Following treatment the blood from various treatment groups were taken and were stored in vials coated with ethylenediamine tetra acetic acid (EDTA). For haematological profiling the levels of blood components like red blood cells (RBC), white blood cells (WBC) and haemoglobin (Hb) were analyzed using haematology analysers (Sysmex, Japan).

**Biochemical Parameters:** To find out the serum levels of aspartate transaminase (AST), alanine transaminase (ALT) and alkaline phosphatase (ALP) a part of blood was collected in non-anticoagulant vials after completing the drug treatment period. The collected bloods were centrifuged at 1500 rpm for 10 min at 4 °C and from which supernatant (serum) was taken and experiments were performed using biochemical kits obtained from Accurex, India following the instructions given by the manufacturer.

**Histopathological Analysis:** At the end of drug treatment period i.e., on 17th day five animals from each group had been sacrificed to collect blood, liver and kidney to conduct biochemical and histopathological analyses. For histopathology analysis, livers and kidney from all the treatment groups had been collected in 10 % buffered formaldehyde and stored for at least 24 h before analysis. Further tissue samples were dehydrated through alcohol (70-100%), cleared in xylene and embedded in paraffin blocks. Thin tissue sections (5 µm) were prepared through microtome and then stained with hematoxylin and eosin. The pathological changes were observed under light microscope and pictures were captured at 10X magnification.
Measurement of Mean Survival Time (MST) & Increase in Life Span % (ILS):

To determine the effect of drug treatment on mortality rate of the cancer (DLA) mice, five mice from each treated groups were monitored for 50 days. Kaplan-Meier curve was established to represent the survivability of mice following drug treatment. MST and % ILS determined with the following formulas:

\[
Mean \text{ survival time} = \frac{[first \text{ death} + last \text{ death}]}{2}
\]

\[
Increase \text{ in life span (ILS) \%} = \left[ \frac{Mean \text{ survival time of treated group}}{Mean \text{ survival time of untreated group}} - 1 \right] \times 100
\]

**Figure S16.** Confocal microscopic fluorescence images of DLA cells after 4 h of incubation demonstrating the uptake of Peg-Au NC-NaB-NPs. The excitation wavelength was 405 nm.

**Table S2.** Effect of drug treatment on tumor volume:
All the results were expressed in mean ± S.D. * p < 0.05 in comparison of drug treated groups with untreated group.

| S. No | Treatment                          | Weight change (gm) | Viable cell count (10^5 cells/ml) |
|-------|-----------------------------------|--------------------|-----------------------------------|
| 1     | DLA + Saline                      | 11.28 ± 1.62       | 418.4 ± 19.7                      |
| 2     | DLA + AuNC                        | 10.52 ± 1.52       | 402.26 ± 17.8                     |
| 3     | DLA + NaB                         | 3.37 ± 0.52*       | 9.47 ± 1.7*                       |
| 4     | DLA + PEG-AuNC-NaB-NPs           | 2.44 ± 0.36*       | 4.97 ± 1.02*                      |
| 5     | DLA + ART                         | 4.87 ± 0.48*       | 10.35 ± 1.5*                      |
| 6     | DLA + AuNC-NaB-NPs-Art           | 1.85 ± 0.31*       | 2.51 ± 0.78*                      |

Figure S17. Photographs of DLA induced mice: (a) control i.e., without treatment (belly swollen) taken on 14th day and those treated with (b) Au NC on 14th day, (c) NaB taken on 25th day; and (d) PEG-AuNC-NaB-NPs taken on 30th day, (e) ART taken on 30th day and (f) PEG-AuNC-NaB-NPs-Art taken on 30th day.

Table S3. Effect of drug treatment on hematological parameters:
All the results were expressed in mean ± S.D. $^{5} p< 0.05$ in comparison of saline treated DLA animals with normal animals. * $p < 0.05$ in comparison of drug treated DLA animals with untreated group DLA animals.

**Table S4.** Effect of drug treatment on serum biochemical enzymes:

| S.No | Treatment                                      | SGOT     | SGPT      | ALP         |
|------|-----------------------------------------------|----------|-----------|-------------|
| 1    | Normal animals                                | 46.3 ± 2.74 | 38.2 ± 2.43 | 114.6 ± 5.26 |
| 2    | DLA + Saline                                  | 92.8 ± 4.26$^{5}$ | 68.7 ± 3.64$^{5}$ | 226.7 ± 9.42$^{5}$ |
| 3    | DLA + Au NC                                   | 87.6 ± 5.26 | 63.8 ± 4.18 | 219.6 ± 11.53 |
| 4    | DLA + NaB                                     | 63.4 ± 3.67* | 55.2 ± 2.84* | 151.4 ± 7.35* |
| 5    | DLA + PEG-AuNC-NaB-NPs                       | 55.7 ± 3.48* | 46.5 ± 3.22* | 134.8 ± 6.71* |

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|   | Treatment                  | Value 1        | Value 2        | Value 3        |
|---|----------------------------|----------------|----------------|----------------|
| 6 | DLA + ART                  | $67.2 \pm 4.14^*$ | $59.1 \pm 3.36^*$ | $157.2 \pm 8.27^*$ |
| 7 | DLA + PEG-AuNC-NaB-NPs-Art | $51.2 \pm 3.21^*$ | $40.4 \pm 2.91^*$ | $122.5 \pm 7.14^*$ |

All the results were expressed in mean ± S.D. $^\$ p< 0.05 in comparison of saline treated DLA animals with normal animals. * p < 0.05 in comparison of drug treated DLA animals with untreated group DLA animals.