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Efficacy of decitabine-loaded nanogels in overcoming cancer drug resistance is mediated via sustained DNA methyltransferase 1 (DNMT1) depletion

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Abstract

DNA Methyltransferase 1 (DNMT1) promotes DNA methylation to maintain cancer drug resistance. The epigenetic drug, decitabine (DAC) is a potent hypomethylating agent, but its effect is transient because of its instability. We tested the efficacy of DAC-loaded nanogels in doxorubicin-resistant breast cancer cells, DAC-resistant melanoma cells, and leukemia cells. DAC in nanogel sustained DNMT1 depletion, prolonged cell arrest in the G2/M cell-cycle phase, and significantly enhanced antiproliferative effect of DAC. The efficacy of DAC-loaded nanogels was more significant in resistant than sensitive cells. Our data suggest that effective delivery of DAC and prolonged DNMT1 depletion are critical to overcoming drug resistance.

Keywords

Hypermethylation; Epigenetic drugs; Nanoparticles; Cell-cycle analysis; Drug delivery; Cancer therapy

1. Introduction

Aberrant expression of DNA methyltransferases (DNMTs), which promote methylation of DNA, is recognized as a key factor in the onset of cancer and drug resistance [1, 2]. DNMT1 methylates deoxycytidylate-phosphate-deoxyguanylate (CpG) islands in the promoter region of genes [3] to transcriptionally silence tumor suppressor genes [4] that are involved in various cellular mechanisms, such as the cell cycle itself, apoptosis, and DNA damage response and repair [5]. This transcriptional gene silencing favors tumor progression and drug resistance [6, 7]. High levels of DNMT1 expression have been reported in cancer patients who are not responsive to cancer chemotherapy [1].

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Conflict of Interest Statement None

The heritable nature of DNA hypermethylation in cancer depends on continued expression of DNMT1 [8, 9]. Depletion of this enzyme using pharmacologic inhibitors has been shown to result in gene hypomethylation and consequent reactivation of the silenced genes [10, 11]. The nucleoside deoxycytidine analog 5-aza-2'-deoxycytidine (decitabine), the most widely investigated demethylating agent [12], is approved by the US FDA for use as a monotherapeutic agent against hematological cancers [13]. The major disadvantage of DAC is its instability, both *in vitro* (half-life in aqueous solution is ~4 h [14], in cell culture medium ~17 h [15]) and *in vivo* (half-life 10-35 min [16]). The main mechanism of action of DAC is via depletion of DNMT1 [17]. Because of its transient effect, DAC has not been effective in sustaining DNMT1 depletion; hence its efficacy in treating solid tumors has been limited [18].

Since DNMT1 promotes methylation of DNA and is a key factor in maintaining drug resistance [7], we hypothesized that a nanogel formulation would be able to protect DAC and deliver it efficiently intracellularly while sustaining DNMT1 depletion to overcome drug resistance. In this study, we demonstrated the efficacy of a DAC-loaded nanogel formulation to sustain DNMT1 depletion and the increased antiproliferative effect of DAC in three cell types: doxorubicin-resistant breast cancer cell, DAC-resistant melanoma cells, and leukemia cells.

2. Materials and Methods

N-isopropylacrylamide (NIPAM), n-hexane, benzene, vinyl pyrrolidone (VP), sodium dodecylsulfate (SDS), sodium acrylate, N,N'-cystamine bisacrylamide (S-S crosslinker), ammonium persulfate (APS), maleic anhydride, dimethyl sulfoxide and 5-aza-2'-deoxycytidine (decitabine) were purchased from Sigma-Aldrich Chemical Company (St. Louis, MO). Poly(ethylene glycol) (PEG, M.W. ~5000) was purchased from Polysciences, Inc. (Warrington, PA). Cell culture media, Dulbecco's phosphate-buffered saline (DPBS), penicillin and streptomycin were purchased from the Central Cell Services Media Laboratory of our institution. MTS reagent was purchased from Promega (Madison, WI).

2.1 Nanogel synthesis

Nanogels were synthesized by surfactant polymerization of NIPAM in the presence of disulfide crosslinker and ammonium persulfate (APS) initiator. NIPAM was used after recrystallization from *n*-hexane: benzene (1:3 v/v). VP was distilled just before polymerization. All other chemicals such as sodium dodecyl sulfate (SDS), sodium acrylate, and APS were used without further purification. PEG-Maleic anhydride was prepared by reacting an equimolar ratio of PEG and maleic anhydride at an elevated temperature, as reported elsewhere [19]. Nanogel polymerization was conducted in a three-necked flask with nitrogen inlet and outlet at 70 °C for 6 h. Four formulations (NG-70, NG-80, NG-85 and NG-100) were synthesized with different ratios of NIPAM, VP and PEG-maleic anhydride (Table 1). NG-70 was prepared by dissolving 700 mg NIPAM, 200 mg SDS, 200 mg VP and 100 mg PEG-maleic anhydride in 100 mL of Milli-Q water and stirring the solution under nitrogen for 30 min at room temperature. The reaction temperature of the polymer solution was raised to 70 °C to which 60 mg of S-S cross linker dissolved in 2 mL methanol followed by 80 mg of APS dissolved in 5 mL were added to initiate the reaction. The reaction was continued for 6 h at this temperature. The obtained nanogel suspension was dialyzed against Milli-Q water (2 L) using a Spectra/Por® dialysis bag (MWCO [molecular weight cutoff] 12-kD, Spectrum® Laboratories, Inc., Rancho Dominguez, CA) for 2 weeks to remove the unreacted monomer, surfactant and electrolytes by changing water every day. The suspension of nanogel collected from the dialysis tubing was lyophilized for 48 h at -48 °C, 3.5 Pa, using FreeZone 4.5 (Labconco Corp., Kansas City, MO) to obtain a dry powder. All other nanogel formulations were synthesized using the

same protocol with the exception of NG-100, which was prepared without VP and PEGMA but with 200 mg of SA added just prior to the addition of APS initiator.

2.2 DAC loading in nanogels

Nanogel suspension for DAC loading was prepared by adding 6 mL Milli-Q water to 30 mg lyophilized nanogel weighed in a 14.8 mL clean glass vial (Fisher Scientific, Pittsburgh, PA). The nanogel was stirred slowly at room temperature until completely dispersed. Prior to drug loading, nanogel size and polydispersity index were measured by dynamic light scattering (DLS) to ensure uniform dispersion. DAC dissolved in dimethyl sulfoxide (300 μ L, 8.1 mg/mL) was then added dropwise to the nanogel suspension and stirred for 3 h on a magnetic stirrer in a cold room at 4°C. Drug-loaded nanogel suspension was dialyzed against Milli-Q water in a dialysis bag (MWCO 12-kD, Spectrum®) for 30 min in a cold room to remove the untrapped drug. We determined that 30 min dialysis time is sufficient to remove free drug since there was no further diffusion of DAC even if the dialysis was carried out over 2 h. The drug-loaded nanogel suspension was lyophilized as above and stored at -20 °C until used. The above protocol was optimized to maximize DAC loading in nanogels.

2.3 Determination of DAC loading in nanogel formulations

DAC loading in nanogels was determined by extracting the drug from each formulation of nanogels. Briefly, to each 1 mg of DAC-loaded nanogel, 2mL of methanol was added and the suspension was stirred overnight over a magnetic stirrer at 100 rpm for 12 h in cold room. One mL of sample was drawn into a 1.5 mL Eppendorf tube and centrifuged at 14,000 rpm (Eppendorf 5417 R, Hauppauge, NY) for 10 min at 4 °C. DAC concentration in the supernatant was determined using HPLC (Shimadzu Scientific Instruments, Inc., Columbia, MD). A standard plot of DAC (0-200 μ g/mL) was prepared under identical conditions. Further, we determined the recovery of DAC from nanogel to ensure that the loading determined as above is accurate. For this, known amount of DAC in solution (0, 50, 100 or 200 μ g/mL) was added to void nanogel suspension in Milli Q water (1mg/mL), stirred for 3 h on a magnetic stirrer in cold room at 4 °C, lyophilized, and processed as above for DAC extraction, with DAC alone without nanogel acting as a positive control.

HPLC conditions: *Stationary phase*: C18 reversed phase column (Atlantis T3 -4.6 $\times$ 250 mm - 5 μ m); *Mobile phase*: Sterile degassed methanol:water (60:40 v/v); Injection volume: 25 μ L; Flow rate: 1.2 mL/min, isocratic mode for 6 min wavelength -228 nm, UV detector.

2.4 Physical characterization of nanogels

2.4.1 Particle size and zeta potential—The mean hydrodynamic particle size of nanogels before and after DAC loading was determined in water by dynamic light scattering at a scattering angle of 90° at 25 °C using NICOMP™380 ZLS (Particle Sizing Systems, Santa Barbara, CA). For this step, 5 mg/mL nanogel formulation was dispersed in Milli-Q water as described above. The above nanogel suspension was diluted (1:100 v/v) in Milli-Q water prior to size determination. The effective mean hydrodynamic diameters of nanogels were obtained from 2 runs of each sample (each run; 5 min). The undiluted nanogel suspension was used to measure the zeta potential in the phase analysis mode and the current mode at a scattering angle of -14°.

2.4.2 Transmission electron microscopy—Nanogels were also characterized for size using a transmission electron microscope (TEM; Philips 201 TEM, Philips/FEI Inc., Briarcliff Manor, NY) operating at 200 kV. For TEM measurements, a drop of nanogel suspension (500 μ g/ml) prepared in Milli-Q water was placed on 200 mesh Formvar-coated copper TEM grid (grid size: 97 μ m) (TED PELLA, Redding, CA) to which 2% w/v of

uranyl acetate solution (negative stain) was added. The excess solution was removed using a piece of filter paper, and the samples were air dried for 5 h prior to imaging. From TEM images, the sizes of the nanogels were measured using Image J software. On average, 50 particles were counted to determine the mean size for each formulation using the above protocol.

2.5 Cell lines and culture conditions—Doxorubicin-resistant breast cancer (MCF-7/Adr) cells were grown in Dulbecco's modified Eagle's (DMEM) supplemented with 15 % fetal bovine serum and 100 µg/mL penicillin G and 100 µg/mL streptomycin at 37 °C in a humidified and 5% CO₂ atmosphere. DAC-resistant melanoma (B16 res) and -sensitive (B16 sen) cells were grown in DMEM supplemented with 10% fetal bovine serum and antibiotics at 37 °C in CO₂ incubator. The same conditions were used for culturing leukemia (THP1) cells. To maintain doxorubicin resistance, MCF-7/Adr cells were cultured in a medium containing 100 ng/mL of doxorubicin. MCF-7/Adr cells were maintained in drug-free media for the last two passages prior to any experiment. Doxorubicin-sensitive human breast cancer (MCF-7) cells were maintained in drug-free DMEM supplemented with 10% fetal bovine serum and antibiotics. Conditions for culturing MCF-7/Adr and MCF-7 were optimized. Human vascular smooth muscle cells (HVSMDs), used for testing cytocompatibility of the void nanogel formulation, were purchased from Cascade Biologics (Portland, OR) and were maintained in medium 231 supplemented with smooth muscle growth supplement (Cascade Biologics) at 37 °C in a humidified, 5% CO₂ atmosphere.

2.6 Cytocompatibility of nanogel formulations

HVSMDs at passage 5 were seeded at a density of 5,000 cells/0.1 mL/well in 96-well plates (Microtest, Becton Dickinson Labware, Franklin Lakes, NJ). At 24 h post seeding, different doses of void nanogels (0-500 µg/mL), dispersed in cell culture media by vortexing, were added and incubated for 72 h. Medium in the wells was changed after 72 h and on every alternate day thereafter with no further addition of nanogels. Cell viability was determined on day 8 using an MTS assay. An aliquot of 20 µL MTS reagent was added to each well, the plates were incubated for 2 h at 37 °C, and color intensity was measured at 490 nm using a plate reader (Bio-Tek Instruments, Inc., Winooski, VT). The cytocompatibility of void nanogels was calculated as the percentage of cell growth occurring in the presence vs. absence of nanogels.

2.7 Antiproliferative activity of DAC in doxorubicin-resistant and -sensitive breast cancer cells

The antiproliferative effect of DAC in solution and of the drug loaded in different nanogel formulations was determined in both doxorubicin-sensitive and -resistant breast cancer cells at different time points following treatment. DAC solution stock was prepared in dimethyl sulfoxide (8.1 mg/mL), and then diluted in cell culture medium to obtain the desired concentrations. This DAC nanogel stock suspension was prepared in cell culture media (typically 5 mg/mL) by pipetting up and down nanogel in culture media and then making appropriate dilutions to obtain nanogel suspension with desired DAC concentration.

In a typical experiment, cells were seeded at 3,000 cells/well in 96-well plates for 5 days, whereas the cell density was 1,500 cells/well in 96-well plates for the 10- and 12-day time points. Cell densities were optimized for the long-term antiproliferative effect of DAC-nanogel formulations to avoid cells in the control group becoming overconfluent. We confirmed that cell density does not significantly influence antiproliferative effect of DAC. At 24 h post seeding, 0.1 mL cell culture medium containing different concentrations of DAC solution or DAC nanogel was added and incubated for 3 d. The cells were then washed with 1x-DPBS and the medium in the plates was replaced with drug-free medium every 48 h

until the endpoint. No further doses of the drug were added. Cell viability was measured at each time point using the MTS assay as described above. The effect of DAC on cell proliferation was calculated as the percentage cell growth vs. growth of control cells that received no drug for each time point. Since void nanogels (without drug) showed no cytotoxicity, culture medium was used as a control in all the experiments. The IC₅₀ values for each treatment and its time points were calculated using the following equation:

$$y = \frac{A_1 - A_2}{1 + (x/x_o)^p} + A_2 \quad \text{Eq. 1}$$

where x = DAC concentration, y = % cell growth as determined by MTS assay, A_1 = % growth on the top plateau region of the growth curve, A_2 = % growth at the bottom plateau region of the curve, x_o = inflection point of the curve, and p = slope. The data points were fit to this equation using OriginPro 8 (OriginLab Corporation, Northampton, MA). IC₅₀ was determined by using $y = 50$ in the above equation and calculating x using the parameters obtained after curve fitting. The mean of six replications for each set of experiments was used to calculate IC₅₀. The IC₅₀ values were plotted against the treatment time for cells treated with DAC solution or DAC nanogels.

2.8 Antiproliferative activity of DAC nanogels in DAC-resistant and -sensitive melanoma cells and in leukemia cells

The efficacy of DAC nanogel in inhibiting cell growth was also tested in DAC-resistant melanoma (B16 res) and – sensitive melanoma (B16), as well in leukemia cell (THP1) lines. In a typical experiment, cells were seeded at 2,000 cells/well/0.1 mL in 96-well plates. Following 24 h incubation, cells were incubated for 3 d in a medium containing different concentrations of DAC in solution or nanogels. Cells were washed with DPBS, and cell viability was determined by MTS assay. In the experiment involving 6-d treatment, cells were incubated with DAC solution or DAC nanogels for 3 d as above and then, with the culture medium replaced without any DAC, cells were incubated for an additional 3 d prior to the MTS assay as above.

2.9 Effect of DAC on DNMT1 depletion

Cells were seeded (5×10^6 cells/dish) in 100-mm cell culture dishes (BD Biosciences) and incubated for 24 h in a CO₂ incubator for attachment. Cells were treated with DAC solution or DAC nanogel. Cellular extracts generated at 1, 3, or 5 d post treatment were assessed by western blot using an anti-DNMT1 monoclonal antibody (Abcam, Cambridge, MA) or an anti-actin monoclonal antibody (loading control; Sigma-Aldrich). Cell lysates were prepared by lysing 1×10^6 treated or untreated cells using RIPA buffer (Sigma-Aldrich) containing 1x protease inhibitor cocktail (Calbiochem, Gibbstown, NJ). Lysates were collected by centrifugation at 14,000 rpm for 15 min at 4 °C in a refrigerated microcentrifuge. The protein concentration of each lysate was determined using a bicinchoninic acid assay kit (Pierce, Rockford, IL). Proteins (50-100 µg) from cell lysates were electrophoresed through 4-15 % linear pre-cast polyacrylamide gradient gel (Bio-Rad Laboratories, Hercules, CA) and transferred to polyvinylidene difluoride membranes (GE Healthcare Bio-sciences, Corp., Piscataway, NJ). The blots were probed for mouse monoclonal DNMT1 (Abcam Inc., Cambridge, MA), and mouse monoclonal anti-actin (Sigma-Aldrich). To detect any bound antibody, a polyvinylidene difluoride membrane was incubated with horseradish peroxidase-tagged goat anti-mouse antibody. After incubation, the membrane was washed with Tris-buffered saline with 0.5% Tween 20, and stained with an enhanced chemiluminescence reagent alone or an enhanced chemiluminescence plus reagent (GE Healthcare) according to the manufacturer's protocol. Change in DNMT1 levels in cells treated with void nanogels was also determined to ensure that the effect seen is because of the drug loaded in nanogels.

2.10 Cell-cycle analysis

The effect of DAC solution vs. DAC nanogel on cell-cycle analysis was studied in breast cancer resistant cells. Briefly, 1×10^6 cells were seeded in a 100-mm cell culture dish and allowed to attach for 24 h. For 8- and 10-d treatments, the initial cell seeding was 500,000 cells. Cells were treated with a single dose of DAC solution (50 ng/mL) or DAC nanogel (equivalent drug dose) and incubated for 5, 8 or 10 d without further addition of DAC. Cells were trypsinized at different time points and centrifuged at 1,300 rpm for 3 min at 4 °C (Sorvall Legend RT centrifuge, Thermo Electron Corp., Waltham, MA). The cell pellet was washed twice with ice-cold 1x DPBS (pH 7.4), and cells were resuspended in propidium iodide solution (12.5 mg propidium iodide, 250 mg sodium citrate and 250 μ L Triton X-100 in 250 mL of water). Cells were then incubated in an ice bucket and kept in a cold room for 2 h; the DNA content was then determined by flow cytometry using a FACScan flow cytometer (Becton Dickinson, San Jose, CA). ModFit LT software (Verity Software House, Inc., Topsham, ME) was used for data analysis.

2.11 Statistical Analysis

Data are expressed as mean $\pm$ standard error of mean (s.e.m.). Statistical analyses were performed using Student's *t* test. Differences were considered significant for *p* $\leq$ 0.05.

3. Results

3.1 Physical characterization of nanogels

The hydrodynamic diameter of the nanogels varied with their composition: NG-80, NG-85 and NG-100 nanogels were smaller in diameter (range 90-135 nm) than NG-70 nanogel (233 nm). A polydispersity index <0.1 for most formulations indicates the uniform nature of the nanogel formulations (Table 2). The size of nanogels measured using TEM in a dry state was in the range of 50-80 nm (Fig. 1). DAC loading in nanogels did not significantly change the particle size. DAC loading in different nanogel formulations ranged from 6% to 7% w/w. Recovery of decitabine from nanogel was above 99%. All nanogel formulations showed negative zeta potentials, with loaded DAC having an insignificant effect on surface charge (Table 2).

3.2 Cytocompatibility of nanogels

Void nanogels (without DAC) showed negligible cytotoxicity in HVSMCs even at the highest dose of nanogel tested (500 μ g/mL), with the exception of NG-85, which showed ~8% cell death at 500 μ g/mL. The concentration of nanogel used for efficacy studies was significantly lower (<100 μ g/mL) than the maximum concentration used for the cytotoxicity study above. No effect of void nanogels was observed at the doses used for efficacy studies with DAC-loaded nanogels. Similarly, void nanogels did not show any cytotoxic effect in MCF-7 and MCF-7/Adr cells.

3.3 Antiproliferative activity of DAC nanogels vs. DAC solution in doxorubicin-resistant and -sensitive breast cancer cells

All the DAC nanogel formulations demonstrated a greater and longer-term antiproliferative effect than DAC solution in both doxorubicin-resistant (MCF-7Adr) and -sensitive (MCF-7) cells; however, the NG-70 nanogel formulation demonstrated a more sustained antiproliferative effect than other nanogel formulations (Fig. 2). For example, in MCF-7/Adr cells, the IC₅₀ was 334-fold lower with the DAC-loaded NG-70 formulation at 12 d post-treatment than with DAC in solution (1.3 ng vs. 434 ng/mL), whereas these values were 82- and 68-fold lower with NG-80 and NG-85 nanogel formulations, respectively, as compared with DAC solution.

Although the DAC solution demonstrated a greater antiproliferative effect than DAC nanogels at 5 d post treatment in resistant breast cancer (MCF-7/Adr) cells, the effect of DAC solution was transient, and cells began proliferating after the initial effect. In contrast, DAC nanogel showed a lower antiproliferative effect in the beginning than DAC solution, but the antiproliferative effect of DAC nanogel increased with treatment time (Fig. 2b). However, unlike in resistant cells, which showed greater efficacy with DAC nanogels at later time points (10- and 12-d post treatment) than DAC solution, sensitive cells demonstrated greater efficacy with DAC nanogels at early and later time points (Fig. 2b vs. 2e). The other interesting observation was significantly greater sensitivity of DAC nanogel in MCF-7/Adr than in MCF-7 cells, which is evident from the dose-response study with the cells treated with DAC solution vs. DAC nanogel (Fig. 2c vs. 2f).

3.4 Efficacy of DAC nanogels in DAC-resistant and -sensitive melanoma cells and in leukemia cells

DAC in nanogel formulations demonstrated a greater and more sustained antiproliferative effect than DAC in solution in both DAC-resistant melanoma (B16 resistant) and -sensitive (B16-sensitive) cells. However, the effect of the nanogel formulation was significantly greater in B16-resistant than in B16-sensitive cells. At 6 d post treatment, the IC₅₀ of DAC in B16-resistant cells treated with DAC-loaded nanogel (NG-70) was 24-fold lower than for the cells treated with DAC in solution. However, this difference was 8-fold for B16-sensitive cells (Fig. 3). These studies also demonstrated that the DAC-loaded NG-70 nanogel formulation showed a better antiproliferative effect than other formulations of nanogels. DAC-loaded NG-70 nanogels demonstrated similar greater efficacy in THP1 cells than DAC in solution (Fig. 4).

3.5 DNMT1 depletion

Resistant cells treated with DAC nanogel showed a greater and more sustained DNMT1 depletion than did cells treated with DAC solution (Fig. 5). For example, in breast cancer resistant cells, DNMT1 depletion in cells treated with DAC solution lasted only 1 d following treatment, whereas cells treated with DAC nanogel continued to show DNMT1 depletion beyond the 5-d post-treatment point (Fig. 5a,b). A similar trend in DNMT1 depletion was seen in B16-resistant cells (Fig 5c,d) and THP1 cells (data not shown). Cells treated with void nanogels did not show any effect on depletion of DNMT1 and the results were same as untreated cells.

3.6 Cell-cycle analysis

Resistant breast cancer cells treated with DAC nanogel (NG-70) showed nearly the same number of cells in G2/M cell-cycle arrest phase at 5 d post treatment as did cells treated with DAC solution. However, at 8 and 10 d post treatment, the percentage of cells in G2/M cell-cycle arrest phase in the DAC solution-treated group reached the same level as that of the untreated group, whereas the cells treated with DAC nanogel demonstrated a sustained effect, maintaining the cells in the G2/M cell-cycle arrest phase (Fig. 6).

4. Discussion

Our data demonstrate that DAC nanogel sustains the DAC effect that leads to prolonged depletion of DNMT1 and greater and sustained antiproliferative effect than with DAC solution. As shown with doxorubicin resistant breast cancer cells, sustained DNMT1 depletion with DAC nanogel maintains cells in a cell-cycle arrest phase, thus significantly enhancing the long-term antiproliferative effect of DAC. Furthermore, DAC delivery using nanogels is shown in our study to be effective in DAC-resistant cells. In general, sustained

depletion of DNMT1 with nanogel formulations significantly enhances the antiproliferative effect of DAC and overcomes drug resistance in cells that are resistant to DAC.

Despite having a relatively larger hydrodynamic diameter than other nanogel formulations (Table 2), the NG-70 nanogel formulation was more effective in sustaining the DAC effect than other nanogel formulations. This is evident from the antiproliferation data, particularly with time in both the resistant and sensitive cells (Figs. 2 and 3). This NG-70 formulation has a relatively higher proportion of VP than do other formulations. Because VP is hydrophilic in nature, it causes nanogels to swell more in an aqueous environment [20, 21]. However, in the dry state, different nanogel formulations have similar particle diameters. The balance of NIPAM and VP in the nanogel formulation is also essential to maintaining their lower critical solution temperature (LCST) above the physiological temperature of 37 °C [21, 22]. For example, the nanogel formulated with NIPAM alone has a LCST temperature near 32 °C [23], which at the physiological temperature of 37 °C shows phase transition, causing the nanogel to collapse, with unintended rapid drug release [22, 24]. The addition of VP increases the LCST temperature, thus preventing the collapse of the gel at physiological temperature [22]. The sustained DAC effect with the NG-70 nanogel formulation could be partly due to its higher LCST and also VP sustaining DAC release due to its interaction with VP through hydrogen bonding. Since DAC is unstable at 37 °C in an aqueous medium, it was not feasible to carry out its release from different formulations of nanogels *in vitro*, but the more sustained DAC effect seen with the NG-70 formulation compared with other nanogel formulations indirectly validates the above point. Previously we have shown release of rapamycin from similar nanogel formulation over 28 days under *in vitro* conditions [25].

The efficacy of nanogels in DAC-resistant cells could be due to their ability to bypass the nucleoside transporters via which DAC is taken up by cells. The human equilibrative nucleoside transporter is responsible for the transport of DAC across the cell membrane [26, 27]. Damaraju et al. [25] have shown that DAC sensitivity depends on the expression of human equilibrative nucleoside transporters in the membrane, and the leukemia cells lacking nucleoside transport activity exhibit resistance to DAC [27]. Our data in B16-resistant cells demonstrate that DAC in nanogel formulation overcomes drug resistance, which could be due to effective delivery of DAC in nanogels via an endocytic mechanism rather than the DAC transport via the membrane transporter in the case of cells treated with DAC solution (Fig. 3). Similar efficacy is seen with DAC nanogel in the THP1 cells (Fig. 4).

The effect of DAC in nanogel was found to be more significant in resistant cells than in the respective sensitive cells. This efficacy is evident from the dose-response study, which demonstrated a significantly lower IC₅₀ with DAC nanogel than DAC solution in resistant cells compared with sensitive cells (Fig. 2c vs. 2f). Similarly, in B16 cells, the difference in the IC₅₀ between resistant cells treated with DAC nanogel vs. DAC solution was 24-fold vs. only 7-fold in sensitive cells at the 6-day time point (Fig. 3).

One interesting point to note is that the antiproliferative efficacy of DAC nanogel in the resistant breast cancer cells was seen following a prolonged drug effect (beyond 5 d post treatment), whereas in sensitive cells, there was no such a lag phase (Fig. 2b vs. e). The results thus suggest that a sustained DAC effect and prolonged depletion of DNMT1 are critical to overcoming drug resistance in MCF-7/Adr cells. Similar levels of antiproliferative efficacy of the DAC nanogel were seen in DAC-resistant melanoma and leukemia cells (Figs. 3 and 4). However, there was no significant increase in antiproliferative effect with treatment time, suggesting that effective DAC delivery that is achieved with nanogels is critical to overcoming DAC resistance in these cells. In resistant breast cancer cells, in

addition to effective delivery, a sustained DAC effect appears critical to overcoming drug resistance.

Intracellularly pro-drug DAC is converted to its active form DAC triphosphate and subsequently incorporated into DNA during the S phase of the cell cycle [28, 29]. DAC incorporated into DNA covalently binds to DNMT1, leading to the reduction of available DNMT1 protein in cells, which in turn results in DNA demethylation and expression of methylation-silenced genes [29, 30]. The duration of DNMT1 depletion depends on the availability of DAC to cells. Since DAC is highly unstable in aqueous solutions [14], it cannot sustain DNMT1 depletion, and hence cells treated with DAC in solution show a transient inhibition of DNMT1 compared with DAC in nanogel formulation (Fig. 5). It would be interesting to determine the kinetics and duration of conversion of DAC to DAC triphosphate in cells treated with DAC solution vs. those treated with DAC nanogel to complement the DNMT1 depletion data [31]. The data from such studies may provide further insight in correlating DAC cellular levels with the agent's antiproliferative effect and in understanding the mechanism of efficacy in overcoming drug resistance in drug- and DAC-resistant cell lines.

Cell-cycle analysis data in resistant breast cancer cells showed that the majority of cells treated with DAC nanogel remained in the G2/M cell-cycle arrest phase, whereas cells treated with DAC solution remain in cell-cycle arrest phase for a transient period of time (Fig. 6). In our previous studies, we demonstrated upregulation of the p21^{WAF1/CIP1} (cell cycle regulator) gene in DAC-treated resistant breast cancer cells [32]. The increased expression of p21 is known to suppress DNMT1 transcription [33], thereby it could synergistically favor global genome hypomethylation. Although we have not investigated the other mechanisms of inhibition of cell growth, it is known that cells cannot remain in cycle arrest phase for an indefinite period of time; either they follow the apoptotic path or re-enter the cell cycle and begin to grow [34]. In DAC solution-treated cells, since the drug effect is transient, cells undergo mitotic slippage to re-enter the cell cycle, whereas the cells treated with DAC nanogel could follow the apoptotic pathway by remaining in cell cycle arrest for a long time.

Despite the success of DAC in clinical trials against hematologic malignancies and its antiproliferative effect *in vitro*, its efficacy in solid tumors has been disappointing [35]. This is due to the difference in the pharmacokinetics and pharmacodynamics of DAC between solid and hematologic malignancies. DAC has a very high clearance rate *in vivo* (half life = 10-35 min) due to its low plasma protein binding and rapid inactivation by cytidine deaminase, found principally in the liver, as well as in granulocytes, intestinal epithelium and plasma [28]. In addition, DAC rapidly degrades in acidic conditions, which most solid tumors exhibit [28, 36]. Since DAC targets rapidly dividing cells such as bone marrow stem cells [37], its repeated dosing or higher doses to achieve therapeutic dose in solid tumors was found to have serious side effects, such as myelosuppression and leukopenia [38]. The toxicity of DAC at higher doses is due to its DNA-damaging activity rather than DNA demethylation activity [16].

In addition to the above issues, the discrepancy between the *in vitro* efficacy of epigenetic drugs, including DAC, and their *in vivo* efficacy in solid tumor has been attributed to the slowly dividing nature of cancer cells *in vivo* vs. *in vitro* [38, 39]. Since DAC is S-phase specific, its activity in the tumor may not last long enough for all the tumor cells to pass through the S-phase and thus for DAC to be effective. It is estimated that it takes approximately 5-15 d, depending on the tumor type and growth rate, for all the cancer cells in solid tumors to pass through S-phase [39-41]. Since our nanogel formulation sustains the DAC effect over several days, we anticipate that it could be effective *in vivo* in treating solid

tumors. In addition to changing the composition, one could tailor the drug release rate and duration of release by controlling the concentration of cross-linker used. More cross-lined nanogels could prolong the release of drug. Nanogels are small in size and their surface is associated with PEG, which could prolong their circulation time for tumor accumulation via the enhanced permeation retention effect. Nanogels could be functionalized with targeting ligands for active targeting to tumor cells. Further studies investigating the pharmacokinetics and pharmacodynamics of DAC in nanogels would set a rationale for evaluating their efficacy in treating solid tumors.

5. Conclusions

Our data show that nanogels are effective in sustaining the DAC effect than DAC in solution. Sustained depletion of DNMT1 using the DAC nanogel formulation was shown to inhibit cell proliferation via cell-cycle arrest and is effective in overcoming drug resistance, even in cancer cells that are resistant to DAC. Nanogels with epigenetic drugs could potentially be explored for treating solid tumors.

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ABBREVIATIONS

APS	Ammonium persulfate
DAC	5-aza- 2'-deoxycytidine (decitabine)
Dox	Doxorubicin
DMEM	Dulbecco's Modified Eagle's Medium
DNMT	DNA Methyltransferase
DPBS	Dulbecco's phosphate-buffered saline
HVSMCs	Human vascular smooth muscle cells
IC₅₀	Inhibitory concentration required for 50% cell death
LCST	Lower critical solution temperature
NIPAM	N-Isopropylacrylamide
NG	Nanogel
SDS	Sodium dodecyl sulfate
TEM	Transmission electron microscope
VP	Vinyl pyrrolidone

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Fig. 1. Hydrodynamic diameter and particle size distribution, and transmission electron microscopic analysis of the DAC-loaded NG-70 nanogel formulation.

Fig. 2.

Antiproliferative effect of DAC nanogel in doxorubicin-resistant (MCF-7/Adr) and doxorubicin-sensitive (MCF-7) breast cancer cells. **a)** Comparison of IC_{50} of DAC in solution vs. in different nanogel formulations over time. Cells treated with DAC-nanogel formulations demonstrated sustained antiproliferative effect compared with cells treated with DAC solution. Efficacy of DAC nanogel depends on the nanogel composition, with NG-70 showing a more sustained antiproliferative effect than other formulations of nanogels. **b)** Comparison of IC_{50} of DAC solution vs. DAC nanogel (NG-70), depicting the transient antiproliferative effect of DAC in solution vs. the sustained effect with DAC nanogel. **c)** Dose-response curves showing the difference in efficacy of DAC in solution and DAC in nanogel (NG-70) at 12 d post treatment. **d) to f)** Efficacy of DAC solution vs. DAC nanogel formulations in MCF-7 cells. IC_{50} values were calculated from the dose response curve for each treatment group and treatment time using the formula mentioned in the method section. Data are expressed as mean $\pm$ s.e.m, $n = 6$ * $p < 0.0005$, # $p < 0.005$ DAC solution vs. DAC nanogel.

Fig. 3.

Antiproliferative effect of DAC nanogel vs. DAC solution in DAC-resistant and -sensitive melanoma cells. The data show the relative efficacy of different nanogel formulations with treatment time. IC_{50} values were calculated from the dose response curve for each treatment group and treatment time. Data are expressed as mean $\pm$ s.e.m, $n = 6$ * $p < 0.0005$, # $p < 0.005$ DAC solution vs. DAC nanogel.

Fig. 4. Antiproliferative effect of DAC nanogel vs. DAC solution in DAC-resistant leukemia cells with treatment time. The efficacy was tested with the NG-70 nanogel formulation. IC₅₀ values were calculated from the dose response curve for each treatment group and treatment time. Data are expressed as mean $\pm$ s.e.m, $n = 6$ * $p < 0.0005$, # $p < 0.005$ DAC solution vs. DAC nanogel.

Fig. 5.

Analysis of DNMT1 depletion in resistant cells treated with DAC solution or DAC nanogel (NG-70). Actin was used as the loading control and the ratio of DNMT1/actin is plotted. Shown are western blots and quantification of the data using Image J analysis software for a) doxorubicin-resistant breast cancer cells (MDR-7/Adr) and b) DAC-resistant B16 melanoma cells. Two independent experiments showed similar results.

Fig. 6. Cell-cycle analysis of doxorubicin-resistant breast cancer cells treated with DAC in solution or in nanogel for different time points. Cell-cycle analysis data at **a)** 5 days **b)** 8 days and **c)** 10 days post treatment. **d)** Quantification of number of cells in G2/M arrest phase treated with DAC solution or DAC nanogel over time. Representative histograms are from two independent experiments.

Table 1

Nanogel composition

Nanogel code	NIPAM (mg)	VP (mg)	PEG-MA _y (mg)
NG-70	700	200	100
NG-80	800	100	100
NG-85	850	100	50
NG-100 [*]	1000	-	-

^{*} NG-100 contains sodium acrylate. NIPAM, N-isopropylacrylamide; VP, vinyl pyrrolidone; PEG-MA, PEG-maleic anhydride; NG, nanogel.

Table 2

Physical characterization of nanogel formulations.

Nanogel formulation	Efficiency of Loading (%)	Loading (%)	Mean diameter (nm)	PI	TEM diameter	Zeta potential (mV)
NG-70	—	—	233	0.06	75 ± 8	-25 ± 4.0
	80 ± 5	6.4 ± 0.4	244	0.11	85 ± 9	-19 ± 1.0
NG-80	—	—	129	0.05	59 ± 5	-20 ± 1.0
	73*	6	126	0.01	61 ± 6	-20 ± 0.1
NG-85	—	—	132	0.05	65 ± 6	-17 ± 0.8
	70*	6	134	0.02	66 ± 5	-17 ± 2.0
NG-100	—	—	99	0.08	53 ± 2	-14 ± 0.3
	69 ± 8	5.4 ± 0.8	116	0.06	61 ± 2	-16 ± 0.3

Drug loading is expressed as mean ± s.e.m., n = 4; *Data from a single experiment.

PI, polydispersity index; TEM, transmission electron microscopy; NG, nanogel.