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Enhanced Antiproliferative Activity of Transferrin-Conjugated Paclitaxel-Loaded Nanoparticles Is Mediated via Sustained Intracellular Drug Retention

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Abstract: We studied the molecular mechanism of greater efficacy of paclitaxel-loaded nanoparticles (Tx-NPs) following conjugation to transferrin (Tf) ligand in breast cancer cell line. NPs were formulated using biodegradable polymer, poly(lactic-*co*-glycolide) (PLGA), with encapsulated Tx and conjugated to Tf ligand via an epoxy linker. Tf-conjugated NPs demonstrated greater and sustained antiproliferative activity of the drug in dose- and time-dependent studies compared to that with drug in solution or unconjugated NPs in MCF-7 and MCF-7/Adr cells. The mechanism of greater antiproliferative activity of the drug with conjugated NPs was determined to be due to their greater cellular uptake and reduced exocytosis compared to that of unconjugated NPs, thus leading to higher and sustained intracellular drug levels. The increase in antiproliferative activity of the drug with incubation time in MCF-7/Adr cells with Tf-conjugated NPs suggests that the drug resistance can be overcome by sustaining intracellular drug retention. The intracellular disposition characteristics of Tf-conjugated NPs following their cellular uptake via Tf receptors could have been different from that of unconjugated NPs via nonspecific endocytic pathway, thus influencing the NP uptake, their intracellular retention, and hence the therapeutic efficacy of the encapsulated drug.

Keywords: Cancer therapy; drug resistance; sustained action; intracellular uptake and sorting; exocytosis and endocytosis

Introduction

The primary function of transferrin (Tf), a glycoprotein found abundantly in the blood, is to transport iron through the blood to cells through the transferrin receptors (TfR).[1,2] Since TfR are overexpressed in malignant tissues compared to normal tissues, Tf is also being extensively investigated as a ligand for drug targeting.[3,4] Further, TfR are overexpressed in certain body tissue such as in the liver, epidermis, intestinal epithelium, vascular endothelium of the brain capillary, and certain populations of blood cells in the bone marrow.[5,6] Therefore, it is also used as a ligand for targeting of drugs to these tissues. This approach has been specifically investigated for the delivery of therapeutic agents to the brain

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because Tf ligand can facilitate the transcytosis of the conjugated drug carrier systems across the blood−brain barrier (BBB).[7,8] Another motivation for using Tf as a ligand is its potential to overcome drug resistance due to the membrane-associated drug resistant (MDR) proteins such as p-glycoprotein (P-gp).[9,10] These proteins are overexpressed on tumor cell membrane, and therefore limit the intracellular uptake of antineoplastic agents and their therapeutic efficacy in cancer therapy.[11,12]

Targeting approaches via the Tf ligand usually involve conjugating drug molecules themselves or drug-loaded nanosystems such as liposomes, micelles, or drug−polymer complexes.[13−15] Our interest is in investigating biodegradable nanoparticles (NPs) as a drug delivery system for antineoplastic agents. The advantage of these NPs is their sustained release property, and since the drug is encapsulated, it is not exposed to the cell membrane associated efflux transporters.[16−18] This is expected to bypass the efflux action of these transporters and thus can result in greater cellular drug uptake than that with drug in solution. Further, the slow intracellular release of the drug from the NPs localized inside the cells is expected to sustain the drug effect, and hence can increase its overall therapeutic efficacy.[17,19]

However, recently we have shown that a major fraction of the internalized NPs undergoes rapid exocytosis.[20] This seems to occur because of their inefficient escape from the endosomal compartment to the cytoplasmic compartment during their transit through the cell. We hypothesized that Tf-conjugated NPs could have a different intracellular sorting pathway following their uptake via TfR than that of unconjugated NPs via nonspecific endocytosis. This difference in the uptake and sorting pathways of conjugated and unconjugated NPs could influence the intracellular retention of NPs as well as the therapeutic efficacy of the encapsulated agent. The objective of the present study was to determine the relative efficacy of the drug in NPs following their conjugation to the Tf ligand and to investigate the mechanism of their efficacy. Paclitaxel (Tx) was used as a model antineoplastic agent, and the efficacy of the drug-loaded NPs was tested in both normal (MCF-7) and drug resistant (MCF-7/Adr) breast cancer cell lines.

Experimental Section

Materials. Poly(D,L-lactide-*co*-glycolide) (PLGA, MW 23 000 Da, copolymer ratio 50:50) was purchased from Birmingham Polymers, Inc. (Birmingham, AL). Zinc tetrafluoroborate hydrate, holo-transferrin, poly(vinyl alcohol) (PVA, average MW 30 000−70 000 Da), and Tween 20 were purchased from Sigma Chemical Co. (St. Louis, MO). Paclitaxel was purchased from Hauser Chemical Company (Boulder, CO) whereas tritium-labeled paclitaxel was purchased from Moravek Biochemicals (Bera California, CA). Texas Red conjugated transferrin and Oregon Green 488 labeled paclitaxel were purchased from Molecular Probes (Eugene, OR). Denacol EX-521 (MW 742, Pentaepoxy) was a gift from Nagase Chemicals Ltd (Tokyo, Japan). 6-Coumarin was purchased from Polyscience Inc. (Warrington, PA). All salts used in the preparation of buffers were from Fisher Scientific (Pittsburgh, PA). All aqueous solutions were prepared with distilled and deionized water (Water pro plus, Labconco, Kansas City, MO).

Formulation of NPs. NPs containing Tx were formulated using an emulsion−solvent evaporation technique. In brief, a solution of 90 mg of PLGA polymer and 6 mg of Tx in 3 mL of chloroform was emulsified into 12 mL of 5% w/v aqueous solution of PVA to form an oil-in-water emulsion. The emulsification was carried out using a microtip probe sonicator set at 55 W of energy output (XL 2015 Sonicator ultrasonic processor, Misonix Inc., Farmingdale, NY) for 2

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min over an ice bath. The emulsion thus formed was stirred for ~18 h at room temperature on a magnetic stir plate to allow the evaporation of chloroform. NPs thus formed were recovered by ultracentrifugation at 110000*g* for 20 min at 4 °C (Beckman Optima LE-80K, Beckman Instruments, Inc., Palo Alto, CA), washed twice with water to remove PVA and the unencapsulated drug, and then lyophilized for 2 days (VirTis Company, Inc. Freeze Dryer, Gardiner, NY). To determine cellular uptake, NPs containing a fluorescent dye, 6-coumarin, were prepared using the identical procedure except that 50 μg of the dye was added to the polymer solution prior to emulsification. The incorporated dye acts as a probe for NPs and offers a sensitive method to quantitatively determine their intracellular uptake and retention.[21] The dye remains associated with NPs even after their incubation in PBS buffer in the presence of an oil phase[22] or in an acidic pH buffer (endosomal pH ~4).[20,23] To determine intracellular retention of the drug, NPs containing tritium-labeled or fluorescent-labeled Tx were used.

Tf Conjugation to NPs. Tf was conjugated to NPs in two steps.[24] In the first step, NPs were activated by epoxy compound, and in the second step, the activated NPs were conjugated to Tf.

Step 1. Typically, to a suspension of NPs (5 mg/mL, 5 mL in borate buffer, 50 mM, pH 5.0) prepared by sonication using a microtip probe sonicator as above for 30 s over an ice bath, 6 mg of zinc tetrafluoroborate hydrate was added followed by a solution of Denacol (10 mg in 2 mL of borate buffer) while the reaction mixture was stirred on a magnetic stir plate at 37 °C. After 30 min of stirring, the particles were separated by ultracentrifugation at 110000*g* for 20 min at 4 °C and were washed three times with borate buffer to remove unreacted Denacol.

Step 2. To a suspension of epoxy-activated NPs (4 mg/mL, 5 mL in borate buffer) was added a solution of Tf in borate buffer (10 mg/mL, 1 mL), and the reaction was carried out at 37 °C for 2 h with stirring on a magnetic stir plate. Unreacted Tf was removed, first by ultracentrifugation as above followed by overnight dialysis against 1 L of water (Spectropore, mol wt cutoff 100 kDa). The suspension of NPs from the bag was collected, frozen at −70 °C, and then lyophilized for 48 h.

Physical Characterization of NPs. The ^{1}H NMR spectrum of Tf-conjugated NPs was recorded on a Bruker AMX 500 spectrophotometer in D_2O. Particle size and size distribution were determined by photon correlation spectroscopy (PCS). A dilute suspension of NPs (100 μg/mL) was prepared in double distilled water and sonicated as above on an ice bath for 30 s. The sample was subjected to particle size analysis in a ZetaPlus particle size analyzer (Brookhaven Instrument Corp, Holtsville, NY). NPs were also evaluated for size by a transmission electron microscope (TEM, Philips/FEI, Briarcliff Manor, NY). For this purpose, a sample of NPs (0.5 mg/mL) was suspended in water and particles were visualized after negative staining with 2% w/v uranyl acetate (Electron Microscopy Services, Ft. Washington, PA). To measure ζ potential, a suspension of NPs was prepared in 0.001 M HEPES buffer (pH 7.4) and analyzed immediately using a ZetaPlus ζ potential analyzer. The amount of PVA associated with NPs was determined using a colorimetric method as described in our previous study.[25] Tx loading in NPs was determined by extracting the drug from NPs by shaking a sample (5 mg) with 2 mL of methanol at 37 °C for 48 h at 150 rpm using an Environ orbital shaker (Lab Line, Melrose Park, IL). The sample was centrifuged at 14 000 rpm for 10 min in a microcentrifuge; 100 μL of the supernatant was diluted to 500 μL with methanol and analyzed for drug levels using the HPLC method described previously.[24] This method resulted in more than 95% drug recovery from NPs. The drug release from NPs was carried out in double diffusion chambers in PBS (154 mM, pH 7.4) containing 0.1% Tween 20 which was added to maintain a sink condition. Drug levels in the released samples were quantitated using HPLC.[24] The amount of Tf conjugated to NPs was determined by using Texas Red conjugated Tf.[24] Tf that was not conjugated to NPs was determined by analyzing the fluorescence of the washings at $\lambda_{ex} = 595$ nm and $\lambda_{em} = 615$ nm (Varian, Cary Eclipse, Walnut Creek, CA) collected from the conjugation reaction. The amount of Tf conjugated to NPs was determined by subtracting the unconjugated Tf from the total amount added during the reaction.

DSC Analysis of Tx-Loaded NPs. The physical state of the Tx encapsulated in NPs was characterized using a differential scanning calorimetric (DSC) thermogram analysis (Shimadzu, DSC-50, fitted with a Shimadzu TA-50 data processor, Columbia, MD). Each sample (8 mg, Tx, placebo NPs and Tx-loaded NPs) was sealed separately in a standard aluminum pan, the samples were purged in DSC with pure dry nitrogen set at a flow rate of 20 mL/min, the temperature ramp speed was set at 10 °C/min, and the heat flow was recorded from 0 to 350 °C.

Cell Culture. MCF-7 cells were obtained from American Type Culture Collection (ATCC, Manassas, VA) and were grown in RPMI 1640 supplemented with 10% fetal bovine serum and 100 μg/mL penicillin G and 100 μg/mL strepto-

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mycin (Gibco BRL, Grand Island, NY) at 37 °C in a humidified, 5% CO_2 atmosphere. The MCF-7/Adr cell line was a gift from Dr. U. S. Rao's laboratory, Department of Biochemistry and Molecular Biology, University of Nebraska Medical Center. These cells were maintained in DMEM medium supplemented with 10% Cosmic calf serum (Hyclone, Logan, UT) and 100 μg/mL penicillin G and 100 μg/mL streptomycin (Gibco BRL, Grand Island, NY) at 37 °C in a humidified, 5% CO_2 atmosphere.

Intracellular Uptake of NPs. Twenty-four-well plates were seeded with MCF-7 cells at 50 000 per well density, and the cells were allowed to attach for 24 h. The medium in each well was replaced with 1 mL of freshly prepared suspension of NPs in medium (100 μg/well), and the plates were incubated for 1 h. To examine the specificity of Tf-receptor-mediated uptake of the Tf-conjugated NPs, cells were incubated with an excess dose of free Tf (50 μg) for 1 h prior to incubating with NPs. Cells were then washed three times with PBS to remove uninternalized NPs and then were incubated with 0.1 mL of 1X cell culture lysis reagent (Promega, Madison, WI) for 30 min at 37 °C. A 5 μL aliquot of each cell lysate was used for cell protein determination using Bradford protein assay (Bio-Rad, Hercules, CA), and the remaining portion was lyophilized. The dye from NPs in the lyophilized samples was extracted by shaking each sample with 1 mL of methanol at 37 °C for 48 h at 150 rpm using an Environ orbital shaker (Lab Line, Melrose Park, IL). The samples were centrifuged at 14 000 rpm for 10 min in a microcentrifuge (Eppendorf 5417R, Brinkmann Instruments, Westbury, NY) to remove cell debris. The supernatant was analyzed for 6-coumarin levels using HPLC as per our previously described procedure.[25] A standard plot with different concentration of NPs was constructed simultaneously under similar conditions to determine the amount of NPs in the cell lysate.

Exocytosis of NPs. Exocytosis of NPs was carried out as per our previously described protocol.[20] In brief, 24-well plates were seeded with MCF-7 cells at 50 000 per well density and the cells were allowed to attach for 24 h. The medium in each well was replaced with 1 mL of freshly prepared NP suspension in medium (100 μg/well), and the plates were incubated for 1 h. For this experiment, the formulation of NPs loaded with 6-coumarin dye was used. The cells were washed three times with PBS to remove uninternalized NPs. The intracellular NP levels after the cells were washed were taken as the uptake. The cells in other wells were incubated with fresh medium; and at different time points, the medium was removed and cells were washed three times with PBS and lysed with 0.1 mL of 1X cell culture lysis reagent (Promega, Madison, WI) to determine NP levels.[20]

Mitogenic Assay. MCF-7 or MCF-7/Adr cells were seeded at 4000 per well density in 96-well plates, and cells were allowed to attach for 24 h. A stock solution of Tx was prepared in ethanol (1 mg/mL) and stored at −70 °C. Different aliquots of the above stock Tx solution following suitable dilution in medium were added to the wells. Concentration of ethanol in the medium was kept <0.1% so that it has no effect on cell proliferation. Drug either as a solution or encapsulated in NPs (unconjugated or Tf-conjugated) at different concentrations (1−1000 ng/mL) was added to separate wells. Medium and unconjugated or Tf-conjugated NPs (without drug) served as the respective controls. Medium was changed on day 2 after the treatment and then on every alternate day thereafter, and no further dose of the drug was added. In a dose−response study, the cell viability was determined at 5 days following the treatment, whereas, in an experiment to study the effect of incubation time on antiproliferative effect of the drug, the cell viability was determined at 2, 5, and 8 days following the treatment. A standard 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTS) based colorimetric assay (CellTiter 96 AQ_{ueous}, Promega, Madison, WI) was used to determine cell viability. Reagents were mixed and added to each well (20 μL/well), plates were incubated for 3 h at 37 °C in a cell culture incubator, and the color intensity was measured at 490 nm using a microplate reader (BT 2000 Microkinetices Reader, BioTek Instruments, Inc., Winooski, VT). The antiproliferative effect of different treatments was calculated as a percentage of cell growth with respect to the respective controls.

Confocal Microscopic Studies. MCF-7 cells were seeded in Bioptechs plates (Bioptechs, Butler, PA) at 50 000 cells/plate in 1 mL of growth medium 24 h prior to the experiment. To study the intracellular retention of drug, cells were treated with either drug in solution, drug-loaded NPs, or Tf-conjugated drug-loaded NPs (10 ng/mL). Fluorescently labeled Tx was used for this study. Untreated cells were used as a control to account for the autofluorescence, if any. The medium was changed on day 2 after the treatment and on every alternate day thereafter, and no further dose of the drug was added. At different time points, the cells were washed three times with PBS before their visualization using a confocal microscope that is equipped with an argon−krypton laser (LSM410, Carl Zeiss Microimaging, Thornwood, NY) with an excitation wavelength set at 488 nm and a long-pass filter of 505 nm.[26]

Intracellular Drug Levels. MCF-7 cells were plated at a density of 100 000 cells per well per 2 mL in six-well plates, and the cells were allowed to attach overnight. The cells were treated either with 10 ng/mL of tritiated drug-loaded NPs, with Tf-conjugated NPs, or as a solution. The medium was changed on day 2 and on every other day thereafter, and no further dose of the drug was added. At different time intervals, the cells were washed with PBS three times to remove uninternalized NPs or free drug, and then lysed by being incubated with 100 μL of 1X cell culture lysis reagent (Promega) for 30 min at 37 °C. A fraction of each cell lysate (5 μL) was used for cell protein determination using a Bradford protein assay (Bio-Rad, Hercules, CA), and the

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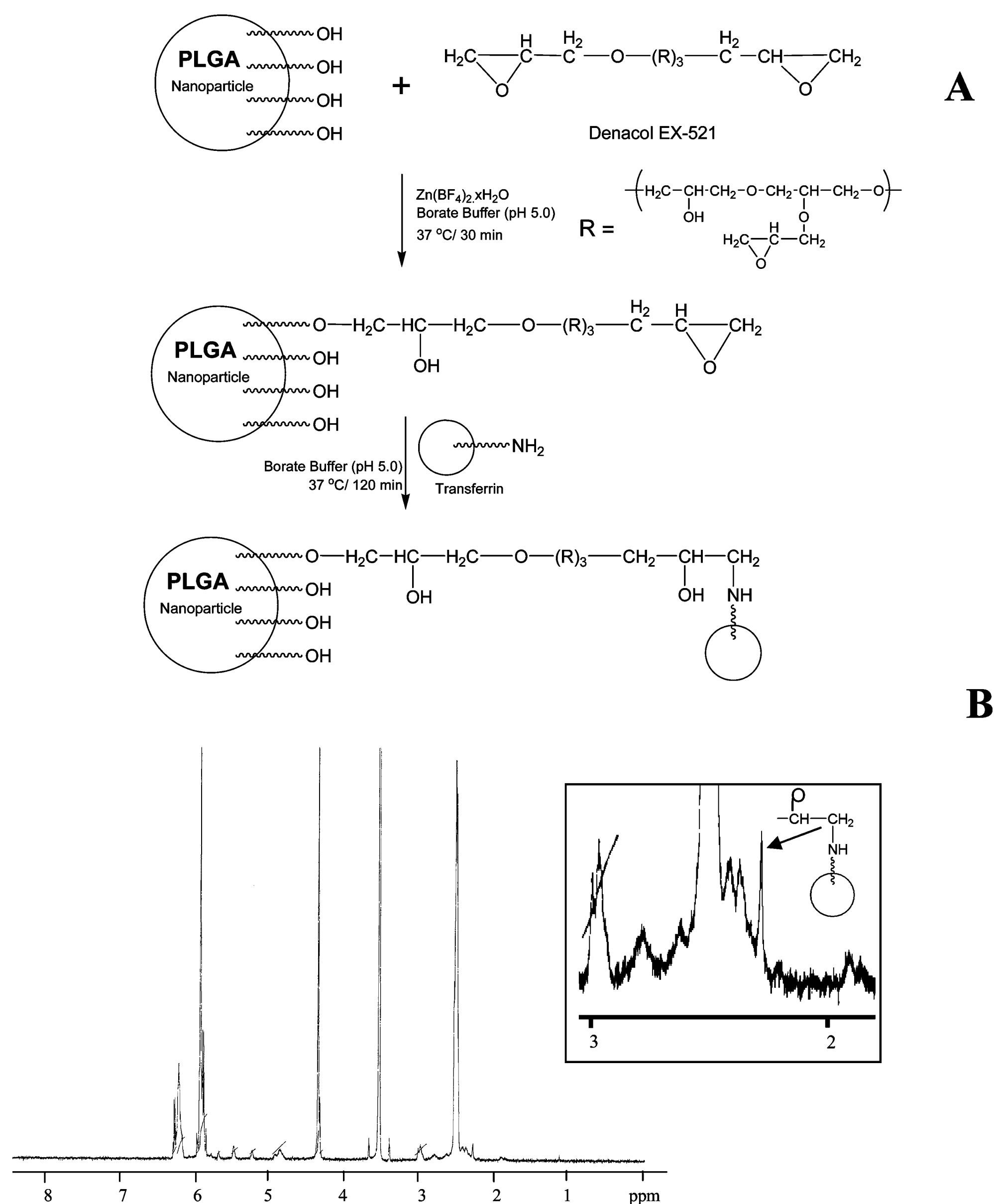

Figure 1. (A) Chemical scheme for the conjugation of Tf to NPs. (B) ^{1}H NMR spectra of Tf-conjugated NPs demonstrating a peak at 2.2 ppm that represents conjugation between amino group of Tf and methylene group of epoxy compound (see inset).

remaining portion was lyophilized. The drug from each lyophilized cell lysate was extracted with dimethyl sulfoxide for 48 h, and the drug level in each extract was measured using a liquid scintillation counter (Packard, Downers Grove, IL). A standard plot was prepared using the identical protocol.

Statistical Analysis. Statistical analyses were performed using a Student's t test. The differences were considered significant for p values of <0.05.

Results

Characterization of NPs. Tf was chemically coupled to the hydroxyl groups of the PVA associated with the NP surface (5 ± 1.2% w/w) through a multifunctional epoxy compound, Denacol EX-521. It has five epoxy groups; at least one of its epoxy groups may have been conjugated to the hydroxyl group of the PVA associated with NPs, and the other epoxy groups to the amine group of Tf (Figure 1A). Previously, we have shown that PVA forms an interconnected network at the NP interface and remains bound to the particle surface despite repeated washing, and thus can be used for surface modification of NPs.[25] ^{1}H NMR spectroscopy to determine the chemical composition of Tf-conjugated NPs demonstrated a peak at 2.2 ppm, confirming that the amino group of Tf has conjugated to epoxy groups (Figure 1B). The amount of Tf conjugated to NPs was 2.9% w/w, which represents approximately 440 Tf molecules per NP.[24]

NPs demonstrated a mean hydrodynamic diameter of 216 nm with a polydispersity index of 0.12, suggesting a uniform particle size distribution. Conjugation reaction slightly increased the mean hydrodynamic diameter of particles (~6

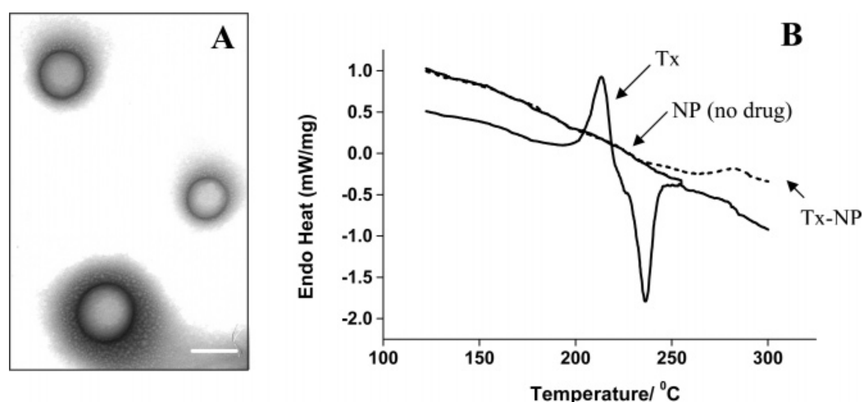

Figure 2. Characterization of Tx-loaded NPs by (A) transmission electron microscopy of Tx-loaded NPs (bar = 100 nm) and (B) differential scanning calorimetry for encapsulation of Tx in NPs.

nm). The mean particle size of NPs with TEM was 110 nm ± 6 (mean ± SD, particles counted from 8 different TEM fields) (Figure 2A). The difference in particle size measured by laser light scattering and by TEM has been reported in our previous studies.[27] The laser light scattering measures hydrodynamic diameter, and hydration of the surface associated PVA probably contributes toward the hydrodynamic diameter of NPs. Tx loading in NPs was 5.4% w/w with an encapsulation efficiency of 86% (i.e., 86% of the drug added in formulation was entrapped in NPs). The ζ potential of the conjugated NPs was slightly more negative than that of unconjugated NPs (−8.12 ± 2.8 mV vs −9.34 ± 2.6 mV). NPs demonstrated a sustained release of the encapsulated drug, with ~30% cumulative drug release occurring in 1 week and ~60% in 2 months.[24] The preliminary assessment of the solid-state solubility of Tx in PLGA and PLA polymers of different composition (ratio of lactide to glycolide) and molecular weight was determined as per our earlier procedure to identify the polymer in which the drug has greater solid-state solubility.[28] It was shown in our studies that PLGA/PLA polymers in which drug has greater solid-state solubility form NPs with greater drug encapsulation efficiency.[28] On the basis of the results of this study, PLGA polymer (50:50) of 23 kDa was chosen for the formulation of Tx-loaded NPs. DSC thermograms demonstrated that only pure Tx had an endothermic peak of melting at 215−217 °C whereas control or drug-loaded NPs had no such peak in the range 150−250 °C (Figure 2B). The results thus indicate that Tx encapsulated in NPs is in the amorphous or disordered-crystalline phase or in the solid-state solubilized form in the polymer matrix of NPs.[29]

Cellular Uptake and Retention of NPs. Cellular uptake of Tf-conjugated NPs was about 2-fold greater than that of unconjugated NPs (Figure 3A). The specificity of Tf-mediated binding of conjugated NPs was evident from the reduced uptake of Tf-conjugated NPs in the presence of free Tf. Further, Tf-conjugated NPs demonstrated reduced exocytosis compared to unconjugated NPs. More than 75% of the internalized unconjugated NPs underwent exocytosis during the first 2 h compared to 50% of the Tf-conjugated NPs (Figure 3B). In our previous studies, we have shown that the major fraction of NPs (over 90%) associated with cells is internalized, and this was confirmed following cell treatment with trypsin to remove surface associated NPs.[30] Further, in several of our previous studies, we have demonstrated intracellular uptake of NPs using confocal and transmission electron microscopy.[17,20,23] Hence, the higher cellular uptake observed with conjugated NPs is due to their greater intracellular delivery.

Antiproliferative Activity of Tx-Loaded NPs. Tf-conjugated drug-loaded NPs at the lowest dose of the drug studied (1 ng/mL) demonstrated greater antiproliferative activity than drug in solution or unconjugated drug-loaded NPs (Figure 4A). At higher doses of the drug, all the treatments demonstrated almost similar inhibitory effect. However, the important observation was the increase in the antiproliferative activity of the drug with incubation time when cells were treated at the lowest dose of the drug using Tf-conjugated drug-loaded NPs (Tx dose =1 ng/mL). Although unconjugated drug-loaded NPs demonstrated greater antiproliferative activity of the drug than drug in solution; no significant change in the antiproliferative effect was observed with incubation time in these groups. This means that the drug-loaded NPs or drug in solution suppressed the cell growth whereas Tf-conjugated NPs demonstrated an

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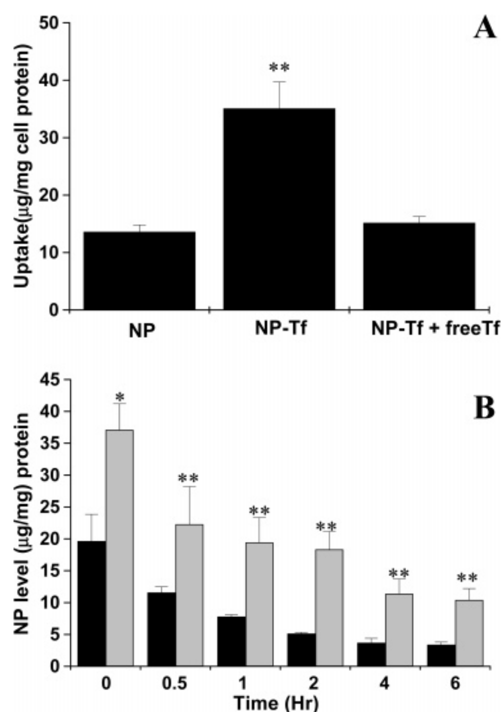

Figure 3. Cellular uptake and exocytosis of Tf-conjugated and unconjugated NPs. (A) Uptake of Tf-conjugated NPs (NPs-Tf) and unconjugated NPs (NPs) in MCF-7 cells. A suspension of NPs (100 μg/mL) was incubated with MCF-7 (5×10^4 cells) for 1 h, cells were washed, and NP levels in cells were determined by HPLC. To determine the competitive inhibition of uptake of Tf-conjugated NPs, an excess dose of free Tf (50 μg) was added to the medium prior to incubating cells with Tf-conjugated NPs. Data as mean $\pm$ SEM ($n = 6$), (*) $p < 0.05$ NPs-Tf + free Tf versus NPs. (**) $p < 0.005$ NPs-Tf versus NPs. (B) Exocytosis of Tf-conjugated and unconjugated NPs in MCF-7 cells. Cells were incubated with Tf-conjugated NPs (gray) and unconjugated NPs (black) at 100 μg/mL concentration for 1 h, cells were washed, and then cells were incubated with fresh medium. This NP level was taken as the cellular uptake (0 h time point). In other wells, the cells were washed and incubated with medium, and were processed as above at different time points to determine intracellular retention of NPs.

increase in the antiproliferative effect of the drug with incubation time (Figure 4B).

The studies in MCF-7/Adr cells demonstrated that the drug does not show antiproliferative activity up to 100 ng/mL concentration; however, at a higher dose (1000 ng/mL), Tf-conjugated NPs demonstrated a relatively greater antiproliferative effect of the drug compared to its solution or unconjugated NPs (~70% vs 20% for Tx-Sol and 55% for Tx-NPs) (Figure 4C). Interestingly, Tf-conjugated NPs demonstrated an increase in the antiproliferative effect of the drug with incubation time whereas the cells treated with drug in solution demonstrated a transient effect, and regained growth 5 days after the treatment. In the case of unconjugated NPs, the antiproliferative effect was greater and more sustained than that with drug in solution, but it was lower than that with Tf-conjugated NPs (Figure 4D). Although the drug doses used in two cell lines (normal and resistant) were different (1 vs 100 ng/mL), Tf-conjugated NPs seem to have a greater effect of the drug in the resistant cell line than in the sensitive cell line, especially with incubation time (Figure 4B vs Figure 4D). Since control NPs (without drug), either conjugated or unconjugated, demonstrated growth curves similar to that of the medium control, the higher antiproliferative effect seen with Tf-conjugated NPs is due to the loaded drug.

Intracellular Drug Retention. Confocal laser scanning microscopy of MCF-7 cells treated with fluorescent dye labeled Tx in solution demonstrated drug internalization within 4 h of incubation; however, its fluorescence intensity was reduced slowly with incubation time and was insignificant at 5 days following the treatment. On the other hand, the fluorescence intensity increased with incubation time in the cells treated with drug-loaded NPs or Tf-conjugated NPs. Intracellular drug retention with conjugated NPs was more sustained than that with unconjugated NPs, and this difference was clearly evident at 8 days following the treatment. The increase in fluorescence intensity in cells treated with NPs seems to be due to slow intracellular release of the encapsulated drug from the NPs which are localized inside the cells (Figure 5). Untreated cells did not show any autofluorescence. The difference in drug levels between different treatment groups was apparent when their levels were quantified using tritium-labeled Tx. The drug levels were more sustained in the cells treated with Tf-conjugated NPs than in those treated with drug in solution or unconjugated NPs (Figure 6), thus substantiating the above confocal microscopic observation.

Discussion

Endocytoplasmic transport of nonviral gene expression vectors has been extensively studied for gene therapy applications.[17,31,32] This is one of the cellular processes that can influence the efficiency of gene expression since nucleic acids are susceptible to rapid degradation by the enzymes of the endosomal vesicles. Similarly, for certain drugs, whose targets are cytoplasmic or other intracellular compartments such as the nucleus or mitochondria, it is necessary that the drug carrier system escape the endosomal compartment so that the drug can exert its pharmacologic effect in the cytoplasmic compartment.[33] For example, Tx, a potent antineoplastic agent, which shows its antiproliferative action

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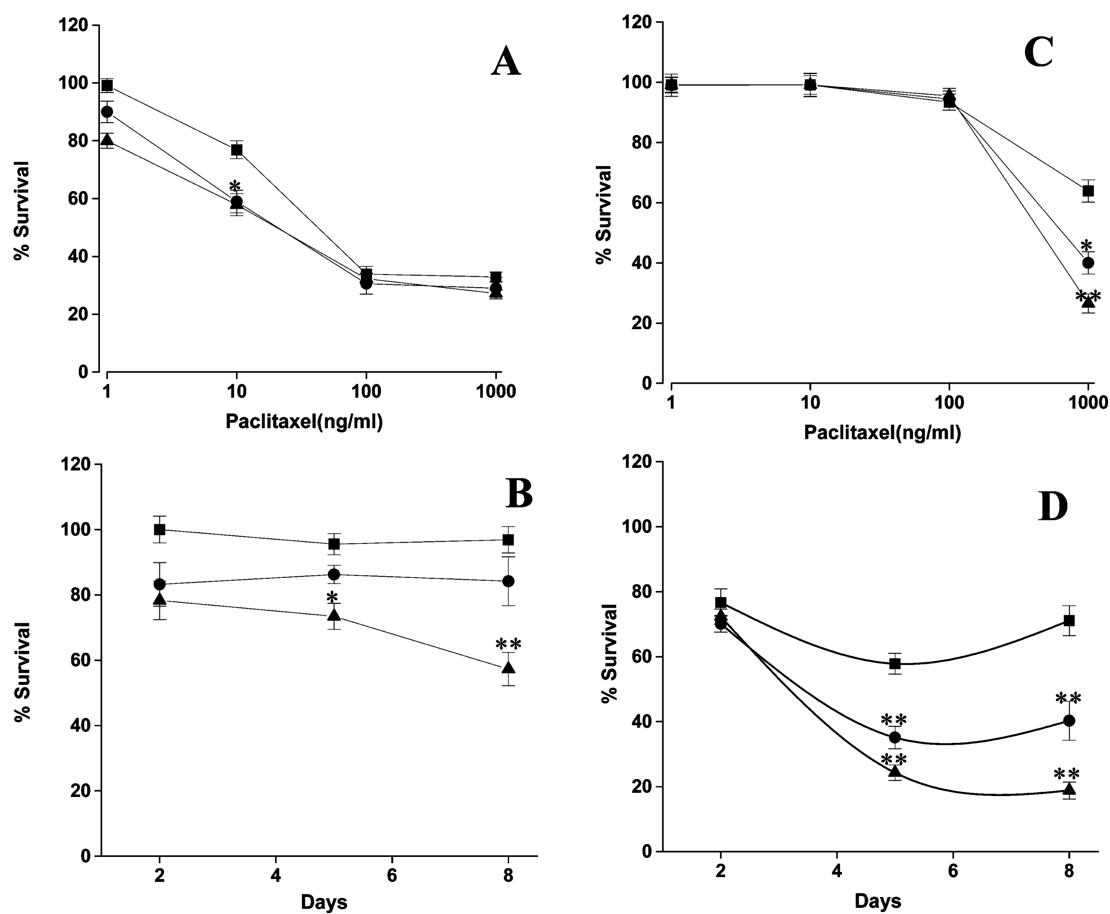

Figure 4. Dose-and time-dependent cytotoxicity of Tx in MCF-7 (A and B) and in MCF-7/Adr (C and D) cells. Different concentrations of Tx either as solution (Tx-Sol, ■) or encapsulated in NPs (Tx-NPs, ●) or Tf-conjugated NPs (Tx-NPs-Tf, ▲) were added to wells with medium or Tf-conjugated or unconjugated NPs (without drug) acting as respective controls. Medium was changed at 2 days and then on every alternate day thereafter with no further dose of the drug added. The extent of growth inhibition was measured at 5 days (A and C) using MTS assay. The extent of growth inhibition with incubation time was measured at 1 ng/mL in MCF-7 (C) and at 1000 ng/mL in MCF-7/Adr cells (D) at 2, 5, and 8 days following treatment. The inhibition was calculated with respect to respective controls. Data as mean ± SEM, $n = 6$, (*) $p < 0.005$ Tx-NPs-Tf versus Tx-Sol and Tx-NPs.

by binding to microtubules,[34] needs to be in the cytoplasmic compartment. Doxorubicin is another antineoplastic agent which acts by intercalation with the nuclear DNA, and therefore, it should escape the endosomal vesicles to the cytosol for its subsequent diffusion into the nucleus. Apart from the effect of the membrane associate efflux transporter proteins (e.g., P-gp), drug resistance in cancer cells has been attributed to sequestering of antineoplastic agents into these acidic endosomal vesicles, thus reducing their levels in the cytosolic compartment.[16] Thus, there are a multitude of reasons for which drug delivery systems should escape the endosomal vesicles.

In addition to drug disposition to the right intracellular target compartment, the duration of drug retention at the target site could be a critical factor in certain disease conditions to achieve the desired therapeutic outcome. For example, sustained inhibition of vascular smooth muscle proliferation is necessary to prevent hyperplasia in the injured artery following balloon angioplasty or stenting.[35] Similarly, cancer is a disease condition that requires chronic drug therapy to completely regress the tumor growth and to avoid its relapse.[36] Therefore, in addition to targeting of antineoplastic agents to cancer cells, their retention at therapeutic doses in the target tissue is equally important.

Recently, we have demonstrated that NPs rapidly escape the endosomal vesicles following their cellular uptake;[19] however, in our subsequent studies, we have seen that a major fraction of it undergoes exocytosis (~85%).[18] This could be because of their inefficient escape from the endosomes to the cytoplasmic compartment during their transit through the cell. The fraction of NPs that escape into the cytosolic compartment have been shown to remain inside

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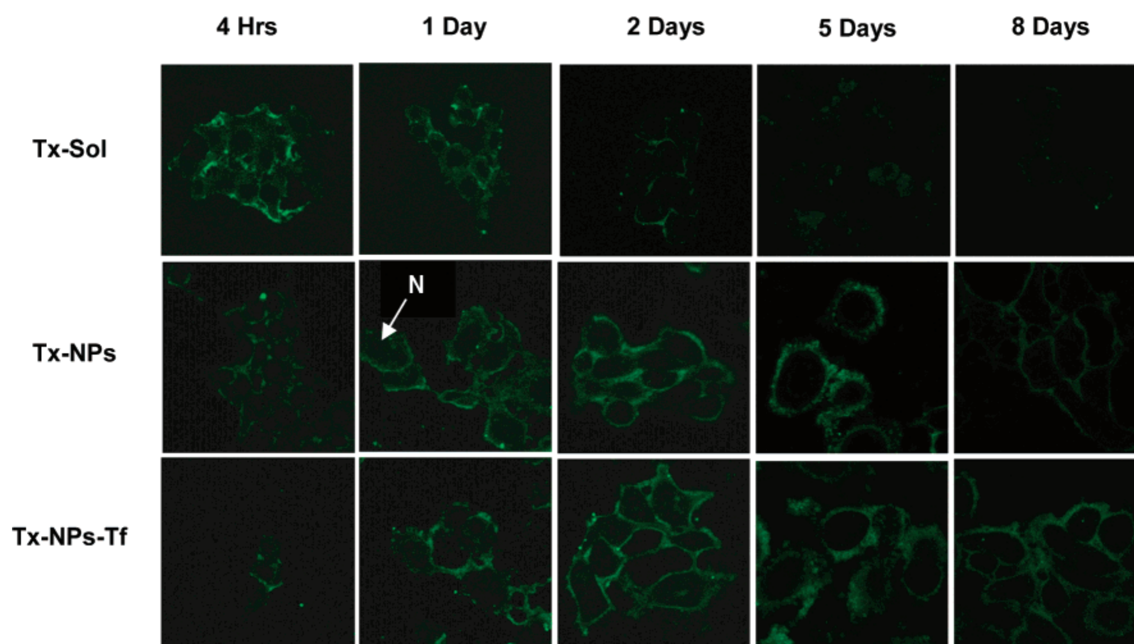

Figure 5. Time course study of intracellular retention of fluorescent-labeled Tx in MCF-7 cells. Cells were treated with drug in solution (Tx-Sol) or unconjugated drug-loaded NPs (Tx-NPs) or Tf-conjugated NPs (Tx-NPs-Tf) (dose = 10 ng/mL) in the growth medium. Cells treated with Tx-Sol showed a decrease in green fluorescence intensity of the drug with incubation time whereas Tx-NPs and Tx-NPs-Tf demonstrated an increase, with Tf-conjugated NPs demonstrating the fluorescence of the drug lasting up to 8 days. N = nucleus.

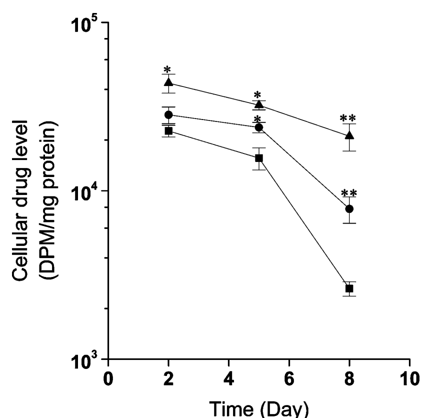

Figure 6. Intracellular Tx levels following treatment with tritiated Tx either as solution (Tx-Sol, ■) or encapsulated in NPs (Tx-NPs, ●) or Tf-conjugated NPs (Tx-NPs-Tf, ▲) at Tx concentration (10 ng/mL). The medium was changed at 2 days and then on every alternate day thereafter with no further dose of the drug added. At different time points, the radioactivity in cell lysates was measured. One nanogram of Tx = 13 645 dpm. Data are means ± SEM ($n = 3$), (*) $p < 0.05$ Tx-NPs-Tf or Tx-NPs versus Tx-Sol, (**) $p < 0.005$ Tx-NPs-Tf or Tx-NPs versus Tx-Sol.

the cells for a sustained period of time and release the encapsulated drug slowly. Previously, we have shown that the pharmacological effect (magnitude and duration) of dexamethasone, a glucocorticoid, as represented by the inhibition of vascular smooth muscle cell proliferation, depends on the dose and the duration of drug release from the NPs inside the cells.[37] The above studies thus clearly demonstrate that the dose of the drug available from the carrier system in the intracellular target compartment influences its therapeutic efficacy. Hence, it is anticipated that the formulation of NPs that results in greater cytoplasmic localization would enhance the therapeutic efficacy of those drugs which require cytoplasmic delivery for pharmacological action.

In this study, we envisioned that the uptake of Tf-conjugated NPs via TfR-mediated endocytosis could have an intracellular disposition pathway different from that of unconjugated NPs. This could influence the intracellular retention of NPs and hence the therapeutic efficacy of the encapsulated drug. The basic feature of the TfR-mediated endocytic pathway has been extensively studied and reviewed.[4] Although the phenomenon of greater cellular uptake of drug or drug carrier systems following conjugation to Tf ligand has been known,[3,4,24] the effect of such conjugation on duration of drug retention inside the cells and its influence on therapeutic efficacy have not been investigated.

The interesting observation in this study was the greater antiproliferative effect of the drug with Tf-conjugated NPs with incubation time in MCF-7/Adr than in MCF-7 cells. In MCF-7 cells, the difference in the antiproliferative effect of the drug with Tf-conjugated NPs compared to that with drug in solution or unconjugated drug-loaded NPs was evident only at 8 days following the treatment (Figure 4B). However, in MCF-7/Adr cells, Tf-conjugated NPs demonstrated a

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steady increase in the antiproliferative effect of the drug with incubation time, which, with solution, was transient, and cells began to proliferate 5 days after the treatment (Figure 4D). The confocal microscopy and quantitative determinations demonstrated intracellular retention of the drug up to 5 days in cells treated with drug in solution (Figures 5 and 6) whereas that with unconjugated NPs or Tf-conjugated NPs was more sustained (Figures 5 and 6). Tf-conjugated NPs demonstrated more sustained drug retention than unconjugated NPs (Figure 6). Thus, the antiproliferative effect of the drug very well correlated with the duration of its intracellular retention.

The effect of drug retention on antiproliferative activity was greater in the resistant cell line than in the nonresistant cell line. In the resistant cell line, Tf-conjugated drug-loaded NPs demonstrated about 80% inhibition in cell growth at 8 days following treatment whereas unconjugated drug-loaded NPs demonstrated 40% and drug in solution only demonstrated 20% inhibition compared to untreated cells (Figure 4B vs Figure 4D). On the basis of studies by others in MCF-7/Adr cells, it would require about 8-fold higher drug concentration than that used in Tf-conjugated NPs (8500 ng/mL vs 1000 ng/mL) to achieve 80% inhibition in cell proliferation.[38] Thus, Tf-conjugated NPs significantly enhanced the overall antiproliferative activity of the drug by sustaining its intracellular retention, especially in the resistant cell line. A relatively greater drug effect following Tf conjugation in resistant cells than in sensitive cells has been demonstrated with doxorubicin. For example, Singh et al. have shown that Tf-conjugated doxorubicin is 5−10 times more effective than unconjugated drug in killing doxorubicin-resistant cell lines whereas it is only 4−5 times more effective in a sensitive cell line.[39] The enhanced effect of Tf conjugation becomes even more significant in the highly resistant KB cell line. Tf-doxorubicin exhibited an IC_{50} value as low as 0.025 mM in this cell line while doxorubicin demonstrated no cytotoxic effects at as high as 1 mM concentration.[40]

Lopes et al.[41] have demonstrated that both the incubation time and drug concentration play a major role in cytotoxicity of Tx and demonstrated that a long period of exposure to a particular dose is necessary to kill a higher percentage of cells. They have demonstrated that exposure of a lower dose of Tx (0.8 μg/mL) to CHO cells for a longer duration (24 h) was effective in killing 99% of cells whereas a 6-fold higher dose of the drug (5 μg/mL) exposed for a shorter duration (6 h) was effective in killing only 30% of cells. The longer exposure time allows more cells to enter into the mitosis phase.[41] This explains the higher efficacy of Tf-conjugated NPs than unconjugated NPs or drug in solution observed in our studies.

The exact mechanism of Tf-based enhancement of drug effect is still to be elucidated completely,[40] but it is generally suggested to be due to enhanced cellular drug uptake and the effect on efflux transporters (P-gp).[42,43] Wang et al. have shown that doxorubicin mainly accumulates in the cytoplasm around the nucleus in MCF-7/Adr cells; however, the Tf conjugate of doxorubicin-gallium accumulates in both the cytoplasm and nucleus of both the MCF-7/Adr and MCF-7 cells.[9] Since doxorubicin works by intercalating nuclear DNA, its localization in the nucleus is necessary for therapeutic efficacy. Similarly, St'astny et al. have speculated that biodegradable *N*-(2-hydroxypropyl)methacrylamide (HPMA) copolymer carrier containing doxorubicin and conjugated to Tf has different intracellular trafficking (in membrane-limited organelles) in contrast to free diffusion of drug alone, and therefore the conjugated carrier was shown to be partially effective in overcoming P-gp-mediated drug resistance.[44] In yet another approach, Huwyler et al. using immunoliposomes conjugated to anti-transferrin receptor monoclonal antibody (OX26 mAb) have demonstrated cellular uptake of digoxin by a factor of 25 in immortalized RBE4 rat brain capillary endothelial cells.[45] Similarly, the greater efficacy of Tf-conjugated gene expression vectors has been generally attributed to their greater intracellular uptake than unconjugated vector.[46−48] This Tf conjugation to drug or drug delivery systems facilitates the intracellular uptake. However, we speculate, on the basis of our observation with Tf-conjugated NPs, that the greater efficacy of Tf-conjugated drugs or gene expression vectors may, in part,

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be also related to their sustained intracellular retention compared to that of unconjugated counterparts.

This difference in retention could be because of the difference in their cellular uptake mechanisms. The uptake of Tf-conjugated systems occurs via a TfR-mediated process as opposed to a nonspecific endocytic pathway of unconjugated systems or diffusion of drug molecules across the cell membrane when used as a solution. This difference in the uptake pathways probably influences the intracellular distribution and retention of the drug. Lemieux and Page have shown that the pretreatment of MCF-7/Adr cells with verapamil, a P-gp inhibitor, influences the uptake of free doxorubicin but not that of conjugated doxorubicin, thus showing a different mechanism of entry for conjugated drug.[10] Although we have not shown directly the difference in the intracellular distribution of Tf-conjugated and unconjugated NPs, the evidence that the drug is retained inside the cells for a longer period of time with Tf-conjugated NPs and demonstrates a greater antiproliferative effect in a dose− and time−response study than that with unconjugated NPs or drug in solution supports the above mechanism. The drug enhancement effect observed in this study following Tf conjugation to NPs very well correlates with the efficacy of these NPs in vivo in a prostate tumor model.[24] A single-dose intratumoral injection of Tf-conjugated Tx-loaded NPs completely regressed the tumor whereas unconjugated NPs or drug in Cremophor EL demonstrated tumor relapse after an initial inhibition in growth.

While developing drug or gene delivery systems, oftentimes the emphasis is placed on the efficiency of uptake. Although the uptake is an important factor, the effect of retention time of a carrier system on therapeutic efficacy has been not been considered. In our previous studies, we have demonstrated that sustained NP-mediated *wt*-p53 gene delivery was more effective in inducing antiproliferative effect in tumor cells *in vitro* than that with a lipid-mediated higher but transient gene expression.[23] These studies clearly demonstrate the significance of drug retention or duration of gene expression on therapeutic efficacy. Therefore, a drug delivery strategy that is focused not only on enhancing the cellular drug uptake but also on sustaining its intracellular retention could be more effective in tumor therapy. The dose and duration of drug retention required for optimal therapeutic efficacy, however, would depend on several factors including the therapeutic agent, its mechanism of action, and cell line.

Conclusions

We have demonstrated that Tf conjugation to NPs results in greater cellular drug uptake, sustains intracellular drug retention, and enhances the antiproliferative effect of the encapsulated antineoplastic agent in cancer cells. The data with Tf-conjugated NPs in a drug resistant cell line suggest that the drug resistance can be overcome by sustaining intracellular drug retention. It is possible that the drug release rate from NPs could further influence the antiproliferative effect of the formulation, and needs to be optimized. It would be of interest to determine how other ligands, such as folic acid or epidermal growth factor, for which receptors are overexpressed in most cancer cells, following their conjugation to NPs influence intracellular drug retention and its therapeutic efficacy.

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