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Nanocarriers Enhance Doxorubicin Uptake in Drug-Resistant Ovarian Cancer Cells

Hans C. Arora, Mark P. Jensen, Ye Yuan, Aiguo Wu, Stefan Vogt, Tatjana Paunesku, and Gayle E. Woloschak

Abstract

Resistance to anthracyclines and other chemotherapeutics due to P-glycoprotein (pgp)-mediated export is a frequent problem in cancer treatment. Here, we report that iron oxide–titanium dioxide core-shell nanocomposites can serve as efficient carriers for doxorubicin to overcome this common mechanism of drug resistance in cancer cells. Doxorubicin nanocarriers (DNC) increased effective drug uptake in drug-resistant ovarian cells. Mechanistically, doxorubicin bound to the TiO2 surface by a labile bond that was severed upon acidification within cell endosomes. Upon its release, doxorubicin traversed the intracellular milieu and entered the cell nucleus by a route that evaded pgp-mediated drug export. Confocal and X-ray fluorescence microscopy and flow cytometry were used to show the ability of DNCs to modulate transferrin uptake and distribution in cells. Increased transferrin uptake occurred through clathrin-mediated endocytosis, indicating that nanocomposites and DNCs may both interfere with removal of transferrin from cells. Together, our findings show that DNCs not only provide an alternative route of delivery of doxorubicin to pgp-overexpressing cancer cells but also may boost the uptake of transferrin-tagged therapeutic agents. Cancer Res; 72(3); 769–78. ©2011 AACR

Introduction

Doxorubicin is one of the most widely used chemotherapy agents. Although its normal tissue toxicity may complicate its therapeutic use, the most serious problem with doxorubicin is the development of drug resistance, especially in ovarian, colon, and breast cancers (1–3). Resistance is typically mediated by overexpression of P-glycoprotein (pgp), a membrane transporter that actively pumps doxorubicin out of the cell (4). Different doxorubicin formulations and modifications that allow it to evade membrane transporters have been the subject of many new doxorubicin-containing drug formulations, many of them using nanotechnology (5, 6).

Ovarian carcinoma cell lines A2780 (doxorubicin sensitive) and A2780/AD (doxorubicin resistant) used in this study were derived from the same tumor but are not isogenic (7, 8) and have different pgp status and endocytic potential. The goal of this work was to enable accumulation of doxorubicin in pgp-overexpressing A2780/AD. Pharmacologic inhibition of pgp can be achieved with calcium antagonists such as verapamil, which has been shown to improve efficacy of doxorubicin in ovarian cancer cells by a dose-modifying factor of 3 to 12 in resistant cells. Nevertheless, verapamil causes alterations in calcium metabolism which affect many additional cellular processes (7). The use of an alternative route for doxorubicin delivery may not only boost accumulation of doxorubicin in A2780/AD cells but also help us study the mechanisms of their doxorubicin resistance, irrespective of pgp overexpression and provides new ways to overcome them. The approach we present here introduces doxorubicin into A2780/AD cells through a 2-step process—doxorubicin enters the cells attached to nanocomposites, detaches from the nanocomposite surface following natural acidification of endosomes, and permeates through the intracellular milieu ultimately reaching the cell nucleus.

Doxorubicin nanocarriers (DNC) used in this work were iron oxide–titanium dioxide core-shell nanocomposites with a 2 to 3 nm core diameter and 6 to 8 nm nanocomposite diameter. These nanocomposites were selected as a vector for doxorubicin with the intention to develop a theranostic agent for subsequent in vivo experiments. The core of these nanocomposites is superparamagnetic; the presence of the shell does not alter their capacity for use as contrast agents for MRI (9). Below 20 nm, the physical strain on the titanium-oxygen bonds on the nanoparticle surface causes high reactivity; these bonds can be broken easily to form stable polar covalent bonds with hydroxyl groups of catechol ligands such as dopamine and Alizarin Red S (10–14). At the same time, surface TiO2 molecules form less stable bonds with other hydroxyl group containing molecules (15–20). We found that the interaction...
between the TiO₂ surface and doxorubicin is relatively labile and is pH-dependent and show several lines of evidence for doxorubicin dissociation from nanocomposites inside cells. Despite expression of transferrin (Tf) receptors 1 and 2 (TfR1 and 2) by both cell lines, A2780 and A2780/AD cells have different potential for transferrin uptake by clathrin-mediated endocytosis (CME). Similarly, active uptake of DNCs is unequal as well. Surprisingly, while DNCs provide an alternative route of delivery of doxorubicin to pgp-overexpressing cells, they also modulate delivery of transferrin, providing a new route to boost uptake of transferrin-tagged therapeutics.

Materials and Methods

Nanoconjugate preparation

Core-shell nanoparticles (referred to as "nanocomposites") were prepared using a low-temperature alkaline hydrolysis method adapted from several procedures (9, 21, 22). Briefly, 2 to 3 nm Fe₃O₄ nanoparticles were prepared and used as cores for the addition of a TiO₂ shell, for a final diameter of 6 to 8 nm. Sizing was done by atomic force microscopy on a Multimode V AFM (Veeco). Images were analyzed using Nanoscope Analysis version 1.2 software (Veeco). Concentrations of Fe and Ti were determined on an X-series 2 ICP-MS (Thermo-Fisher Scientific). Nanocomposite Concentrations was 30 μmol/L; the average concentration of surface Ti atoms and potential surface-binding sites was 24 mmol/L (calculations in Supplementary Data). Nanocomposites were dialyzed and stored at 4°C in 10 mmol/L Na₂HPO₄ buffer, pH 6.0.

Doxorubicin hydrochloride (Sigma-Aldrich) was prepared as a 10 mg/mL solution in water and mixed overnight with nanocomposites at a stoichiometric ratio of doxorubicin:nanocomposite surface sites of 0.35:1. UV-visible light spectroscopy was carried out on a NanoDrop 1000 UV-Vis Spectrophotometer (Thermo Fisher Scientific). Zeta potential was measured on a Zetasizer Nano ZS Particle Size Analyzer (Malvern Instruments). DNCs were stored at 4°C and used within 1 week after preparation. DNC concentrations are expressed throughout the text as conjoined concentrations of doxorubicin and nanocomposite components.

Cell culture conditions

All cell culture reagents were purchased from Mediatech Inc., unless otherwise specified. A2780 and A2780/AD human ovarian carcinoma cells were grown in RPMI-1640 medium supplemented with 10% FBS, 2 mmol/L l-glutamine, 100 U/mL penicillin, 100 μg/mL streptomycin, 0.25 μg/mL amphotericin.
B, and 0.25 U/mL insulin (Sigma-Aldrich) at 37°C in a 5% CO₂ atmosphere. Cells were refreshed every 3 months from frozen stocks prepared at the time of receipt. A2780 and A2780/AD cells were last authenticated in May 2011 using microsatellite marker analysis with Coriel standard markers on an Agilent system in the Molecular Genetics Laboratory at Fox Chase Cancer Center. Before treatment, cells were washed with PBS and placed in serum-free RPMI-1640 medium for 1 hour at 37°C and treated for 2 hours, unless otherwise specified. Endocytosis conditions were variable, as indicated throughout the text. Transferrin-AlexaFluor488 or 647 were obtained from Invitrogen. After treatment, cells were washed with acidic glycine solution (200 mmol/L glycine and 150 mmol/L NaCl; Sigma-Aldrich; pH = 3) and PBS.

Temperature inhibition of endocytosis
After 1-hour incubation under serum-free conditions, cells were moved to 4°C for an additional hour or allowed to remain in a 37°C incubator as a control. Media were replaced with precooled treatment-containing serum-free media for 2 hours at 4°C or at 37°C before preparation for flow cytometry. This approach is based on our previous work (11).

Inhibition of CME
Cells were washed with hypertonic media containing 0.45 mol/L sucrose at room temperature and then incubated for 10 minutes in hypertonic media at 37°C in a 5% CO₂ atmosphere. Fresh hypertonic media with transferrin and/or DNCs or nanocomposites were added and cells were incubated for 2 hours at 37°C. This work followed an approach outlined by Heuser and Anderson (23).

Flow cytometry
Cells were collected and fixed in a 1.85 wt% solution of formaldehyde (diluted from 37 wt% solution, Sigma-Aldrich) and stored in PBS at 4°C up to 24 hours prior to analysis. Flow cytometry was carried out on a CyAn ADP LX Analyzer (Beckman Coulter, Inc.). Respective excitation and emission wavelengths (nm) used for doxorubicin, AlexaFluor488, and AlexaFluor647 were as follows: 488, 575/25; 488, 530/40; and 635, 665/20. Data analysis was conducted using FCS Express V3 software (De Novo Software).

Confocal microscopy
After treatment, cells were fixed in 3.7 wt% formaldehyde (Sigma-Aldrich). Nuclear DNA was stained with 4 μg/mL Hoechst 33342, trihydrochloride, trihydrate (Invitrogen). Imaging was carried out on a Zeiss UV LSM 510 META (Carl Zeiss, Inc.) laser scanning confocal microscope. Excitation and emission wavelengths (nm) used for Hoechst 33342, doxorubicin, and AlexaFluor647 were as follows: 405, 420; 488, 560 to 615; and 633, 657 to 679.

X-ray fluorescence microscopy
Cells were grown and treated on silicon nitride (Si₃N₄) windows. Detailed scans were done with 0.3 × 0.3 μm steps and elemental distribution data were recorded, extracted, and analyzed as described previously (10, 12, 14).

Results
To modulate the route of doxorubicin uptake by ovarian cancer cells, doxorubicin was bound to the TiO₂ surface of...
Fe₃O₄@TiO₂ core-shell nanocomposites. Although catechol molecules attach very strongly by bidentate polar covalent bonds, other hydroxyl groups attach to the TiO₂ surface by looser bidentate or monodentate bonds (15–20, 24). The doxorubicin molecule falls into the latter category. In an initial set of experiments, we found that doxorubicin nanocomposite binding is pH-dependent (Fig. 1A; Supplementary Fig. S1A). IR spectroscopy indicates that absorptive binding occurred without the β-hydroxyquinone moieties in the anthraquinone core but rather involves other hydroxyl groups (Fig. 1B, Supplementary Table S1; refs. 25–29). Mild shifts in a few IR spectral peaks and the lack of new peaks formed upon doxorubicin adsorption indicate that no significant structural changes occurred to either doxorubicin or the nanocomposite (Fig. 1C, Supplementary Table S1). Binding with doxorubicin also causes a shift in the zeta potential (Supplementary Fig. S1B). Doxorubicin separates from the nanocomposite as pH decreases (Fig. 1A). Similarly, inside cells, as endosomes become acidified, interaction between doxorubicin and the TiO₂ surface weakens, allowing doxorubicin to be displaced by other ligands within the endosome.

We treated ovarian cancer cell lines with doxorubicin alone, doxorubicin and nanocomposites added to the cell culture media separately but simultaneously, or as DNCs, and imaged the cells at 2 hours posttreatment by confocal fluorescent microscopy (Fig. 2A and B). The distribution of doxorubicin showed a pattern that differed for cells exposed to free doxorubicin or DNCs. In the doxorubicin-sensitive cell line A2780 (Fig. 2A), free doxorubicin showed an exclusively nuclear staining, whereas doxorubicin-treated A2780/AD showed no doxorubicin staining at all (Fig. 2B). However, doxorubicin fluorescence signal after the DNC treatment showed a similar pattern in both cell lines (Fig. 2A and B), although not the same signal intensity. In both cases, doxorubicin fluorescence was...
more intense in the cytoplasm than the nucleus; with a nevertheless prominent nuclear staining.

To investigate whether whole DNCs or doxorubicin alone migrated to the nucleus, we imaged similarly prepared cells by X-ray fluorescence microscopy (XFM; Fig. 3A and B). This technique allows 2-dimensional elemental mapping of cells for all elements between P and Zn in the periodic table (10, 12, 14). Although doxorubicin is not visible by XFM, nanocomposite components Ti and Fe can be identified and quantified (Supplementary Fig. S2). To make the analysis easier to interpret, we separated each image into 3 regions of interest: cell nucleus (pink), cytoplasm (yellow), and the empty area next to the cell (light blue). This technique showed that cells treated with DNCs for 30 minutes or 2 hours retain the nanocomposite component of DNCs in the cytoplasm only, despite a strong doxorubicin presence in cell nuclei.

To further investigate uptake of doxorubicin delivered via DNCs, we used increasing concentrations of doxorubicin or DNCs (Fig. 4A). In A2780 cells, DNCs improved doxorubicin accumulation only slightly; however, the difference in accumulation in the resistant A2780/AD cell line was 2.5- to 6.4-fold depending on the concentration administered (Supplementary Table S2).

To ensure that uptake of DNCs was driven by active endocytosis, we treated cells with doxorubicin or DNCs under a nonphysiologic temperature of 4°C and evaluated doxorubicin accumulation by flow cytometry (Fig. 4B, Supplementary Table S3). We found that uptake of doxorubicin alone or on DNCs was inhibited under these conditions.

Our previous work suggested that cellular uptake of nanoparticles progresses by any one of the major endocytic mechanisms; of these, CME was a leading mechanism of uptake for untargeted nanoparticles without any surface modifications (11). In A2780 and A2780/AD cells, CME is the most prominent endocytic mechanism (30). To investigate whether DNC uptake was CME mediated, we exposed cells to hypertonic AB160140120100806040200/0

0/0 0.83/3 25/90 83/300

Figure 4. Conjugation to nanocomposites increases doxorubicin (DOX) uptake in A2780 and A2780/AD cells. A, A2780 (light gray) and A2780/AD (dark gray) cells were treated with increasing concentrations of doxorubicin (△, triangles) or DNCs (○, circles) before analysis by flow cytometry. X-axis shows concentration of DNCs as conjoined concentrations of doxorubicin and nanocomposite components, or in the case of doxorubicin alone the first number gives the doxorubicin concentration. Y-axis is expressed in thousands (Supplementary Table S2). B, A2780 (light gray) and A2780/AD (dark gray) cells were exposed to increasing concentrations of DNCs at 37°C (○, circles) and 4°C (□, squares) temperatures. Y-axis is expressed in thousands (Supplementary Table S3). C, A2780 (light gray) and A2780/AD (dark gray) cells were treated under isotonic (○, circles) or hypertonic (△, triangles) incubation with increasing concentrations of DNCs and a constant concentration of 5 µg/mL transferrin-AlexaFluor647 before analysis by flow cytometry. An increase in DNC uptake was observed concurrent with increases in treatment concentration. Hypertonic treatment caused a decline in uptake, although not by more than 30% to 40% of the overall uptake indicating that CME is only one of the possible endocytic mechanisms by which A2780 and A2780/AD cells take up DNCs. Y-axis is expressed in thousands (Supplementary Table S4). Error bars represent SD (n = 3). Error bars are presented for each data point; however, visual representation for small margins of error may be obscured by data markers.
conditions (Fig. 4C, Supplementary Table S4). Decrease of doxorubicin signal was significant in both cell lines, although hypertonic treatment did not completely abolish DNC uptake. CME uptake of DNCs cannot be ascribed to any particular nanocomposite–receptor interaction but rather to a process whereby uptake is facilitated by cell membrane remodeling that takes place in any endocytic process (11). To investigate whether we could boost the DNC uptake by cotreatment with CME triggering ligands, we used transferrin, a ligand for receptors processed via CME (30).

Abundant and biphasic uptake of transferrin is found in cell lines expressing both TIR1 and 2 as is the case with A2780 and A2780/AD cells (31, 32). Although both cell types took up high levels of transferrin, overall higher uptake was found in the A2780/AD cell line (Supplementary Fig. S3 and Table S5; ref. 31). This uptake seemed nonsaturable, consistent with the literature for other TfR2-expressing cells (31). We proceeded to image transferrin uptake by confocal microscopy (Supplementary Fig. S4) in the absence (Supplementary Fig. S4A) or presence of nanocomposites (Supplementary Fig. S4B), doxorubicin (Supplementary Fig. S4C), or DNCs (Supplementary Fig. S4D). The appearance of the transferrin distribution pattern at 2 hours was the same regardless of the cotreatment with any of the other reagents.

At this point, we focused on cotreatment of ovarian cancer cells with DNCs and transferrin. An inspection of endocytic vesicles carrying DNCs (Fig. 5A and B) at 30 minutes post-treatment showed partial overlap of doxorubicin and AlexaFluor647 (transferrin) fluorescence signals. However, although most of the transferrin signal overlaps with doxorubicin, the opposite is not true; moreover, doxorubicin distribution is more diffuse, showing a patternless punctuate-vesicular than that of transferrin. This difference is even more apparent at 2 hours. These findings are consistent with the dissociation of doxorubicin from nanocomposites. Free doxorubicin as a small molecule distributes through the cell and stains cell nuclei in a pattern overlapping with Hoechst 33342 at 2 hours in A2780 cells. In A2780/AD cells, doxorubicin in the nucleus showed a slightly more diffuse pattern and a more prominent presence of doxorubicin-rich vesicle-like structures in nuclei and cytoplasm. The origin and significance of these structures remain to be elucidated.

**Figure 5.** Intracellular distribution of transferrin and doxorubicin delivered via DNCs. Cells were treated with 50 μg/mL transferrin-AlexaFluor647 and 10 μmol/L/36 nmol/L DNC for 30 minutes or 2 hours. Doxorubicin and AlexaFluor647 signals increased in both (A) A2780 and (B) A2780/AD cells with incubation time. Similarly to A2780 cells, AlexaFluor647 signals in A2780/AD cells show complete overlap with doxorubicin, although the vesicle distribution pattern seems different than in A2780 cells. In both types of cells, all AlexaFluor647 signal is colocalized with doxorubicin, indicating that DNCs are not only taken up in the same endocytic vesicles as transferrin but remain in them at this later time point. Doxorubicin released from nanocomposites follows the same distribution pattern as in A2780 cells: staining is mostly nuclear, with occasional doxorubicin-rich “blebs” in the nucleus and cytoplasm.
To test whether we could improve the uptake of DNCs through coendocytosis with transferrin, we cotreated cells with a steady level of one agent and an increasing concentration of the other. Exposure to a constant concentration of DNCs and increasing concentrations of transferrin (Fig. 6A and B, Supplementary Table S6) showed that DNC uptake was constant and not assisted by transferrin treatment. However, in all experiments with the reverse experimental setup, the opposite was true. When a constant concentration of transferrin was administered in the presence of nanocomposites (Supplementary Fig. S5A and Table S7), uptake of transferrin increased; this uptake was much affected by treatment temperature. To differentiate between effects of nanocomposites from effects of DNCs or doxorubicin alone, this experiment was repeated (Supplementary Fig. S5B and Table S8) with the same concentrations of nanocomposites, DNCs, or doxorubicin alone. There were no significant differences in transferrin uptake between cells treated with transferrin alone or with transferrin and doxorubicin, whereas cells treated with either nanocomposites or DNCs showed a similar increase of transferrin uptake. Uptake differences between these 2 pairs of samples were statistically significant.

To investigate whether transferrin uptake at constant transferrin concentration and increasing DNC concentration remains CME dependent, cells were treated under isotonic or hypertonic conditions (Fig. 6C, Supplementary Table S9). Hypertonic treatment significantly inhibited transferrin uptake to the same background level, regardless of increasing DNC concentration. Isotonic treatment again led to increased transferrin uptake with increasing DNC concentrations.

Discussion

Resistance to anthracycline antibiotics and other chemotherapy through cellular export is a frequent problem in cancer treatment and numerous approaches have been developed to overcome it. Several different variations on "stealth" liposomal delivery were developed as well as approaches for binding doxorubicin to copolymers or polymeric micelles (6). In some cases, these constructs deliver doxorubicin to pgp-overexpressing cells in a 2-step fashion, similar to the DNCs reported in this work. Nevertheless, DNCs described here combine several unique advantages as doxorubicin delivery vectors: (i) superparamagnetic cores allow nanocomposites to be used as MRI contrast agents; (ii) delivery of doxorubicin into cells bypassing the pgp transporter followed by doxorubicin detachment and migration to cell nuclei; (iii) capacity for permanent functionalization by catechol-modified molecules, for example, nucleic acids, peptides, or targeting small molecules, which allows targeting or retention of the nanocomposites in different subcellular compartments, different cell types, or intercellular space; (iv) functionalization with catechol-conjugated contrast agents such as gadolinium-based MRI contrast or PET agents; finally, work shown here suggests that (v) DNCs stimulate uptake of transferrin and may be a convenient cotreatment with transferrin-targeted nanotherapeutics. Although many of these characteristics of nanocarriers were used in proof-of-principle work.
between TiO2 and doxorubicin destabilize and the doxorubicin pump in the endosomal membrane, changing pH from 5 to 6 and A2780/AD cells show some differences, with both cell in vivo is normally found only in the liver and intestine, but it is with non-therapeutics (43). Increased TfR1 expression also correlates with non-pgp-mediated resistance to doxorubicin, and a drug can be replaced by other molecules with plentiful hydroxyl groups. We also found that cotreatment of ovarian cancer cells with a combination of DNCs (or doxorubicin-free nanocomposites) and fluorescent transferrin increased transferrin uptake but not nanocarrier uptake.

In addition to its role in trafficking and homeostasis of iron, transferrin and its receptors (TfR) play an important role in cancer progression. TfR and TfR precursor expression were increased transferrin uptake by nanocomposite-treated cells in this study. When cotreatment was done, a significant overlap in the doxorubicin and fluorescently labeled transferrin signals was found in cytoplasm, in endocytic vesicles as well as in MVBs and trans-Golgi apparatus. Nevertheless, we show that this enhanced transferrin uptake remains completely dependent on CME. Transferin uptake by either cell line was completely abolished by hypertonic treatment and this response was preserved in nanocomposite-treated cells. Increased uptake of transferrin at 2 hours implies that this change is not dependent on changes in TfR expression but rather on modulation of CME and/or trans-Golgi activity. There have been several findings in the recent literature suggesting that nanoparticle uptake can affect the cytoskeleton and focal adhesion points (49, 50). Such cytoskeleton modulation could lead to decreased exocytosis or otherwise alter vesicular trafficking.

There is always the possibility to use nanoparticles to increase the uptake of other chemotherapeutics that rely on endocytosis. More specifically, delivery of nano-therapeutics targeted by transferrin (43) could be increased by cotreatment with DNCs.

**Disclosure of Potential Conflicts of Interest**

No potential conflicts of interest were disclosed.

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