Enhanced cytotoxicity of doxorubicin conjugated to ultrasmall Au nanoparticles

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ABSTRACT

In this study we find that ultra-small (mean diameter, 2.8 nm) gold nanoparticles conjugated to doxorubicin (Au-Dox) are up to five-fold more cytotoxic to B16 melanoma cells than the equivalent concentration of doxorubicin alone. The Au particles themselves are non-toxic to cells although they are endocytosed. The particles enter the cell cytoplasm and nuclei, as observed by fluorescence confocal and transmission electron microscopy. At the concentrations at which the greatest enhancement effect of the Au particles is seen, cell death with doxorubicin alone is apoptotic, whereas with Au-Dox, death is primarily necrotic. Transfection of cells with Bcl-2 protects against doxorubicin alone but not against Au-Dox. These results have implications for the design of chemotherapeutic nanoparticles.

Keywords: Au nanoparticle, doxorubicin, apoptosis, necrosis, cancer

1 INTRODUCTION

Doxorubicin (Dox), an anthracycline antibiotic, is among the most widely used anticancer drugs for chemotherapy treatments[1]. It is a cytostatic chemotherapeutic agent with multiple modes of action. Dox can inhibit RNA synthesis by binding to RNA polymerase II and topoisomerase II[2]; it may generate reactive oxygen species (ROS) that can cause cell death either by disturbing the cellular redox equilibrium or by damaging the DNA[3]. The major side effects of using doxorubicin in cancer treatment are cell chemoresistance[4] and cardiotoxicity [1, 5]. Some studies have shown that Dox is more effective when conjugated to hydrophobic nanoparticles (NPs) that penetrate more deeply into the cell than the drug alone[4, 6-9]. Gold nanoparticles have proven to be nontoxic to human cells[10] and gold is FDA approved, constituting an alternative to heavy-metal-based nanoparticles. Functionalized gold NPs have attracted great interest during the past decade due to their potential biomedical applications[11-13]. Conjugates of gold NPs to Dox have been suggested by a few studies. Asadishad B. *et al.* published the synthesis of gold nanoparticles functionalized with folic acid and doxorubicin for targeted cancer therapy[14]. You. J *et al.* demonstrated the use of hollow gold nanoparticles as a doxorubicin carrier with increased Dox activity compared to Dox alone[15]. Aryal *et al.* created a thiol-stabilized Au NP conjugated to Dox via a hydrazone bond that can be broken upon intracellular pH change, leading to a lower toxicity[16]. Park *et al.* deposited an Au film on doxorubicin-loaded biocompatible and biodegradable poly(lactic-*co*-glycolic acid) NPs, which released the drug upon degradation under near infrared (NIR) irradiation resulting in higher therapeutic efficiency and shorter treatment time[17]. In an earlier study, we showed that gold Nps (2-5 nm) in core size were taken up by cultured HEK 293 cells when conjugated to Dox. Although the particles did not inter the nucleus, toxicity of the conjugates was approximately equivalent to that of free Dox[18].

It is important to determine the mechanism of actionof any Dox-based therapy to maximize its therapeutic effects as well as to minimize the severe dose-dependent cytotoxic side effects seen with this drug. Here we investigate the mechanisms of action of Dox alone or conjugated to ultra-small gold nanoparticles (mean diameter, 2.8 nm). Unlike larger particles, these nanoparticles enter the cell nucleus, as observed by fluorescence confocal and transmission electron microscopy. Measurement of cell proliferation indicates that Au-Dox is up to five-fold more effective against B16 melanoma cells than the equivalent concentrations of Dox alone. The anti-apoptotic gene Bcl-2 will rescue cells exposed to Dox alone but not those exposed to Au-Dox, and staining indicates that the mechanism of action of Au-Dox is more necrotic than apoptotic, with damage to cell membranes.

These results have implications for the design of chemotherapeutic nanoparticles. They indicate that very small particles can enter cell nuclei and possibly react with DNA, and that doxorubicin remains highly active when conjugated to Au, without the need for cleavable linkers to the particle. It is expected that these particles will accumulate in tumors without further targeting, although future animal studies will be necessary to measure this. Results will be immediately translatable to clinical studies as all the elements of the formulation are FDA approved.

2 MATERIALS AND METHODS

2.1 Particle synthesis and conjugation

Synthesis of tiopronin-capped gold nanoparticles was according to published methods [19]. The particles were lyophilized, weighed, and characterized by TEM, zeta-potential, infrared, UV-Vis and fluorescence. Particle concentration was estimated from a solution of known w/v by using the average radius measured by TEM to calculate a mean molecular weight per particle. Conjugation to Dox

was carried out in 1mL PBS containing Au NPs (2 mM), Dox (50 mM), and 1-ethyl-3-(3dimethylaminopropyl)-carbodiimide (EDC) (10 mM). Conjugation was performed in the dark at room temperature for 1 h followed by dialysis against dH_2O for 3 hr. The amount of bound Dox per particle was measured using UV-Vis.

2.2 Cell culture and toxicity assays

B16 melanoma and HeLa cells were cultured in high-glucose DMEM supplemented with L-glutamine (0.2 mM), penicillin (100 U/mL), streptomycin (100 μg/mL), and FBS (10%) in a 5% CO_2 atmosphere. The HeLa cells were stably transfected with either the Bcl-2 gene or a mock vector, both selected with G418. The SRB assay was performed on cells that had been passaged at 5 x 10^3 cells per well in 96 well culture plates ~24 h before use. Once at 60 % confluency, they were washed with PBS, incubated with Au alone, Dox alone or Au-Dox at various concentrations in serum-free medium for 30 min, washed with PBS, and incubated in 200 μL of supplemented DMEM. After 24h or 48h, the cells were fixed with trichloroacetic acid for 2 h, washed 5x with distilled water, air-dried overnight and stained with SRB reagent (sulforhodamine) (50 μL) for 30 min. Unbound SRB was removed with acetic acid 1%, bound SRB was dissolved in Tris and absorbance was read at 500 nm. For fluorescence microscopy, cells were prepared in 35 mm glass bottom dishes and incubated with selected concentrations of Dox, Au-Dox, or Au alone for 1-2 h. They were then rinsed in PBS and imaged in PBS. When ethidium bromide/acridine orange were used, they were added during the last 30 min of incubation. KCN was used as a positive control for necrosis. For TEM of cells, cells were prepared on 10 cm plates and incubated with selected concentrations for 1 hr, then trypsinized, pelleted, and fixed in glutaraldehyde prior to embedding and thin-sectioning. Confocal and TEM were as described [20] [21].

3 RESULTS

Confocal fluorescence imaging was used to observe Dox and Au-Dox in cells. The Au particles were too weakly fluorescent to be observed, so their uptake could not be assessed in this fashion (Fig. 1A). Dox alone was almost entirely restricted to the cell nucleus (Fig. 1B), whereas Au-Dox showed labeling throughout the cytoplasm as well as the nucleus (Fig. 1C). Overall labeling was brighter with Au-Dox than with Dox alone (Fig. 1D).

TEM was able to demonstrate endosomal uptake of both Au and Au-Dox, although the uptake of Au alone was rather limited (not shown). The Au particles could be observed in unstained specimens; in this case their actual size could be appreciated, although it was difficult to distinguish organelles under these conditions (not shown). When the sections were stained with osmium tetrachloride/uranyl acetate, clusters of Au particles appeared as large bright areas, primarily endosomal (Fig. 2 A). Some Au could also be observed associated with the nuclear membrane and inside the nucleus (Fig. 2 B).

Ethidium bromide/acridine orange was used to distinguish apoptosis from necrosis. After 1 hr of incubation, Dox-only cells showed signs of apoptosis, namely green staining with evidence of condensed chromatin (Fig. 3A). KCN-exposed (Fig. 3B) and Au-Dox (Fig. 3C) cells showed necrosis, whereas cells with Au only were unharmed (Fig. 3D).

B16 cells were very sensitive to Au-Dox, with significant increases in cell death seen over Dox alone at Dox concentrations as low as 0.8 μM, and very marked between 1 and 20 μM (Fig. 4A). HeLa cells stably transfected with Bcl-2 were used to test the mechanism of cell death. Bcl-2 is an anti-apoptotic factor frequently upregulated in cancer, and its overexpression prevents against apoptotic cell death but not necrosis [22]. Bcl-2 protected these cells against Dox alone, with significantly enhanced survival at all concentrations (Fig. 4 B). However, Bcl-2 expressing cells were killed just as effectively as wild-type cells by Au-Dox, which retained its overall greater effectiveness (Fig. 4C).

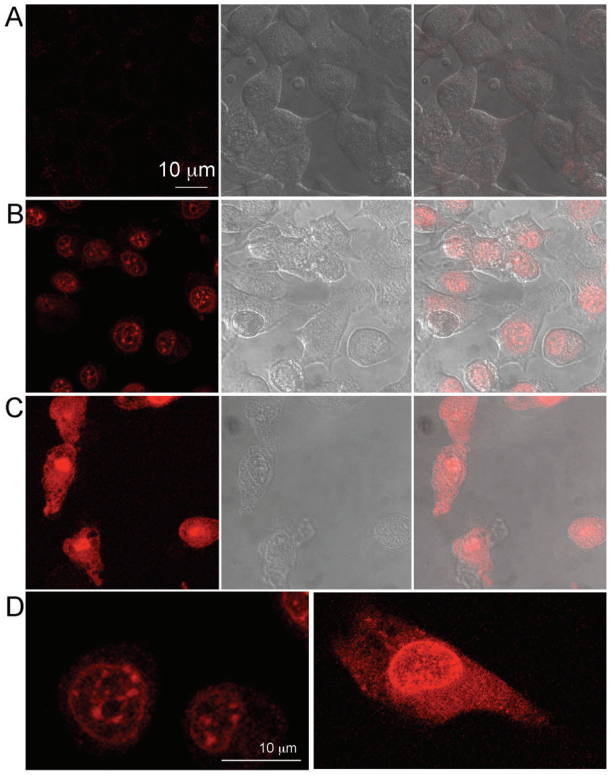

Figure 1. Fluorescence confocal and differential interference contrast (DIC) images of labeled cells excited with 488 nm. (B) Cells with Au particles only show no significant signal. (C) Cells with Dox alone show red fluorescence primarily in the nucleus. (D) Cells with Au-Dox show fluorescence throughout the cell, concentrated in the nucleus. (E) High resolution comparison of Dox alone (left) and Au-Dox (right) showing enhanced overall fluorescence in Au-Dox cell with more cytoplasmic labeling than with Dox alone.

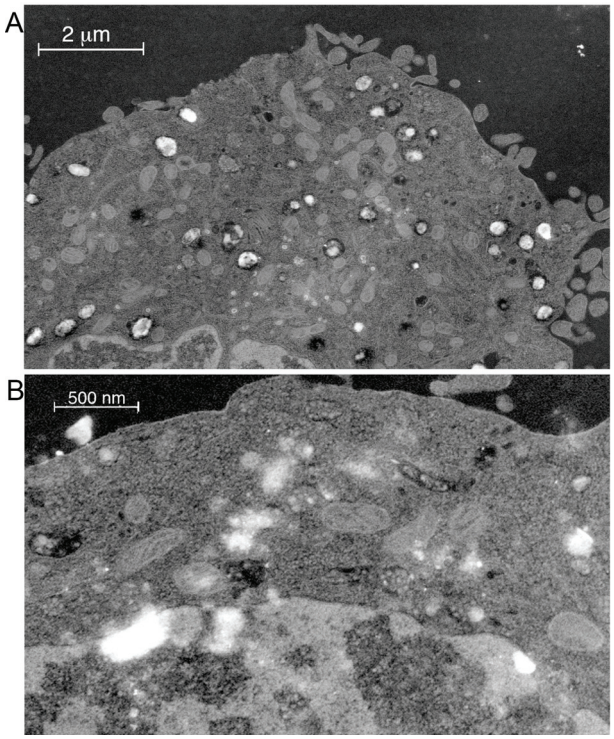

Figure 2. TEM images of thin sections of B16 cells with Au-Dox (stained with osmium tetrachloride/uranyl acetate). The bright regions are Au. (A) Image showing endosomal uptake. (B) Higher resolution image showing Au in the nucleus.

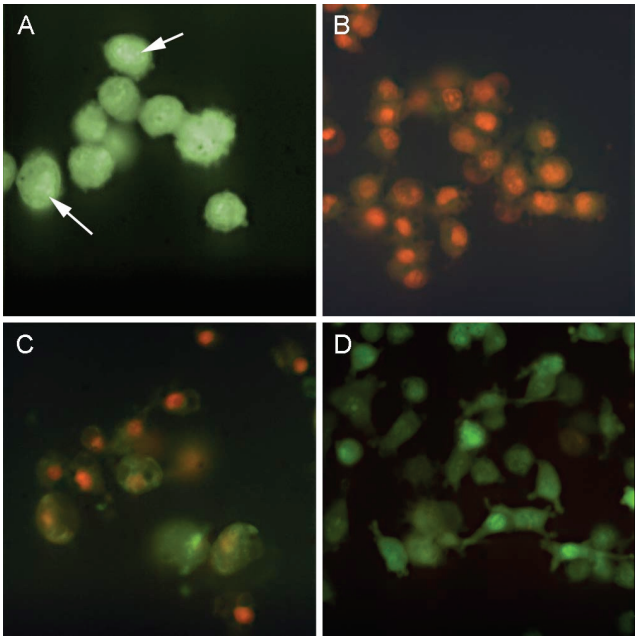

Figure 3. Acridine orange-ethidium bromide (AcOr/EtBr) staining of B16 cells stained with Dox alone (A), KCN as a positive control for necrosis (B), Au-Dox (C), and Au alone (D). Live and early apoptotic cells are green and cells with membrane compromise (necrotic or late apoptotic) show red nuclei.

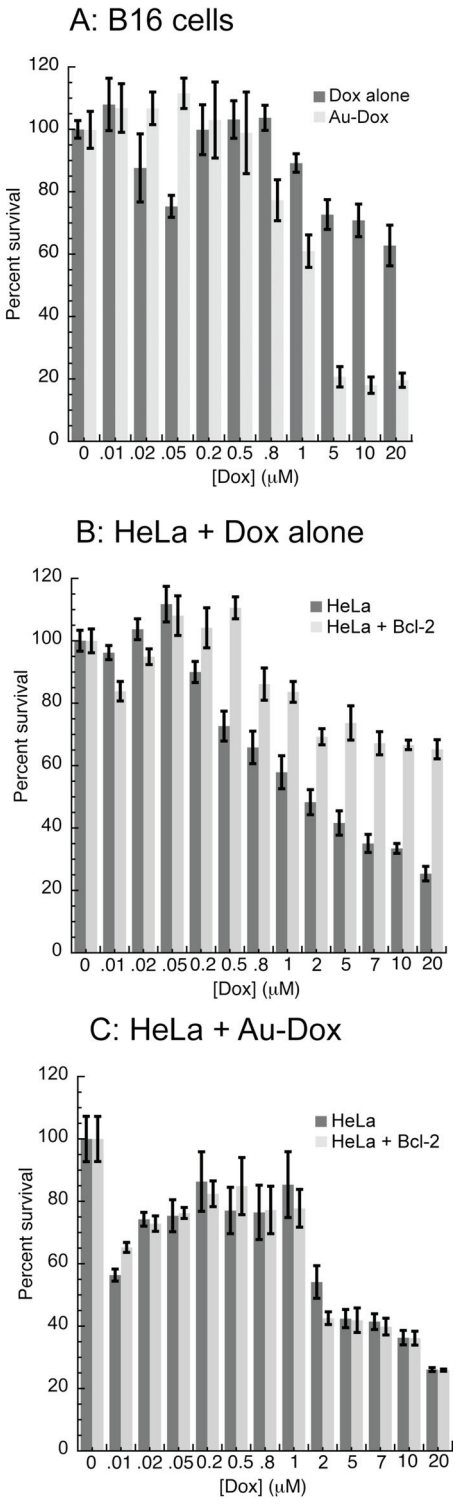

Figure 4. Survival with Dox vs. Au-Dox measured by the SRB assay. (A) B16 melanoma cells with Au-Dox show significant inhibition relative to Dox alone beginning at concentrations of 0.8 μM. (B) Stable transfection with Bcl-2 significantly reduces Dox effectiveness. (C) There is no effect of Bcl-2 transfection on cell survival with Au-Dox.

4 DISCUSSION

The preliminary results are intriguing and encouraging. Ultrasmall Au nanoparticles, which cause no harm to cells on their own, are taken up efficiently by cancer cells when conjugated to Dox and show faster, more efficient cell killing than the drug alone. Overexpression of Bcl-2 does not prevent cell death, making this promising for targeting cancers in which the apoptotic machinery is impaired.

Further investigation is needed to establish the exact molecular mechanisms of cell death with Au-Dox, and to determine whether these are clinically useful. The generation of reactive oxygen species in solution and in cells should be evaluated, and oxidation of lipids (peroxidation) and DNA (formation of 8-hydroxydeoxyguanosine) should be compared for Dox alone and Au-Dox at both comparable concentrations and concentrations at which Dox alone is effective against 80-90% of cells. Caspase-3 activation should be evaluated and a cell cycle analysis done.

The tracking of these particles to tumors and their rate of clearance from the bloodstream should also be measured in mouse models. Because of the particles' small size, they might be expected to be cleared by the kidneys within several hours [23]. The enhanced penetration and retention effect may serve to target the particles to tumors without need for specific targeting [24]. However, targeting might be further improved by the addition of small targeting peptides along with Dox to the particle surface.

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