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Targeted Delivery of Doxorubicin to Mitochondria

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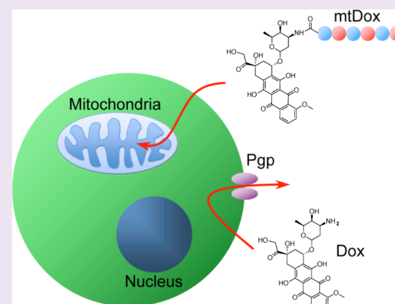
Supporting Information

ABSTRACT: Several families of highly effective anticancer drugs are selectively toxic to cancer cells because they disrupt nucleic acid synthesis in the nucleus. Much less is known, however, about whether interfering with nucleic acid synthesis in the mitochondria would have significant cellular effects. In this study, we explore this with a mitochondrially targeted form of the anticancer drug doxorubicin, which inhibits DNA topoisomerase II, an enzyme that is both in mitochondria and nuclei of human cells. When doxorubicin is attached to a peptide that targets mitochondria, it exhibits significant toxicity. However, when challenged with a cell line that overexpresses a common efflux pump, it does not exhibit the reduced activity of the nuclear-localized parent drug and resists being removed from the cell. These results indicate that targeting drugs to the mitochondria provides a means to limit drug efflux and provide evidence that a mitochondrially targeted DNA topoisomerase poison is active within the organelle.



Disruption of nucleic acid synthesis in the nuclei of human cells is one of the most powerful ways that rapidly dividing cancer cells can be selectively eliminated.[1−3] The drugs that target nuclear DNA are effective in many tumor types but are often made ineffective because drug-resistant cancer cells can readily generate efflux pumps that reside in the plasma and nuclear membranes.[4] While the clinical relevance of this phenomenon is debated,[5] it is clear that many of the most effective drugs are potential substrates for efflux.[6]

Many of the same processes involving nucleic acids in the nucleus also take place within the mitochondria of human cells. Much less is known, however, about whether these processes represent druggable cancer targets because it is difficult to selectively target drugs to this highly impermeable organelle. Developing molecules that disrupt DNA or RNA synthesis in mitochondria could provide a significant advantage, however, because most efflux pumps cannot readily access molecules in this organelle.

In order to explore whether this advantage could be realized, we characterized the activity of a mitochondrially targeted inhibitor of DNA topoisomerase II (TopoII), doxorubicin (Dox). Dox is used in the treatment of a wide range of cancers, and its chief mechanism of action is the generation of TopoII-mediated lesions in nuclear DNA leading to apoptosis.[7] The clinical utility of Dox may be compromised by its susceptibility to various multidrug-resistance mechanisms, most notably because it is an excellent substrate for P-glycoprotein (Pgp) efflux pumps.[4] The same TopoII that is active within nuclei is also targeted to the mitochondria,[8] and therefore, Dox is an ideal molecule to probe the effects of interfering with nucleic acid integrity in an organelle where this type of drug efflux should not be effective.

Our laboratory recently developed a synthetic, peptide-based carrier that can impart mitochondrial localization to a wide variety of molecules.[9−11] Previous work with this vector yielded the successful delivery of another anticancer drug, chlorambucil, to the mitochondria.[12] This drug was shown to exhibit significant levels of toxicity and caused a variety of types of biomolecular damage within mitochondria.[13] Here, we explore whether a molecule that interferes with a specific aspect of DNA synthesis could be effective. While the mitochondrial effects of having Dox within the cell have been contemplated,[14] this is the first form of Dox that is specifically targeted to mitochondria and localized only to this site within the cell.

In order to retarget Dox to the mitochondria, we used a peptide previously tested in our lab that is protease resistant and nontoxic.[9] Our mitochondria-penetrating peptides (MPPs) are short cationic sequences that can deliver cargoes into the mitochondrial matrix because of their alternating cationic and hydrophobic residues.[10,11] A mitochondrially targeted version of Dox (mtDox) was synthesized by coupling the primary amine of the sugar motif to a succinic anhydride conjugated to the N-terminus of the MPP (Figure 1).

We investigated the intracellular distribution of mtDox compared to the Dox parent compound (Figure 1). HeLa cells, which possess large, well-defined mitochondria that are straightforward to image, exhibited strong nuclear localization of Dox, as determined using the intrinsic Dox fluorescence. MtDox showed a distinctive mitochondrial distribution of the drug, confirmed by monitoring colocalization with a commercially available mitochondria-specific dye, Mitotracker.

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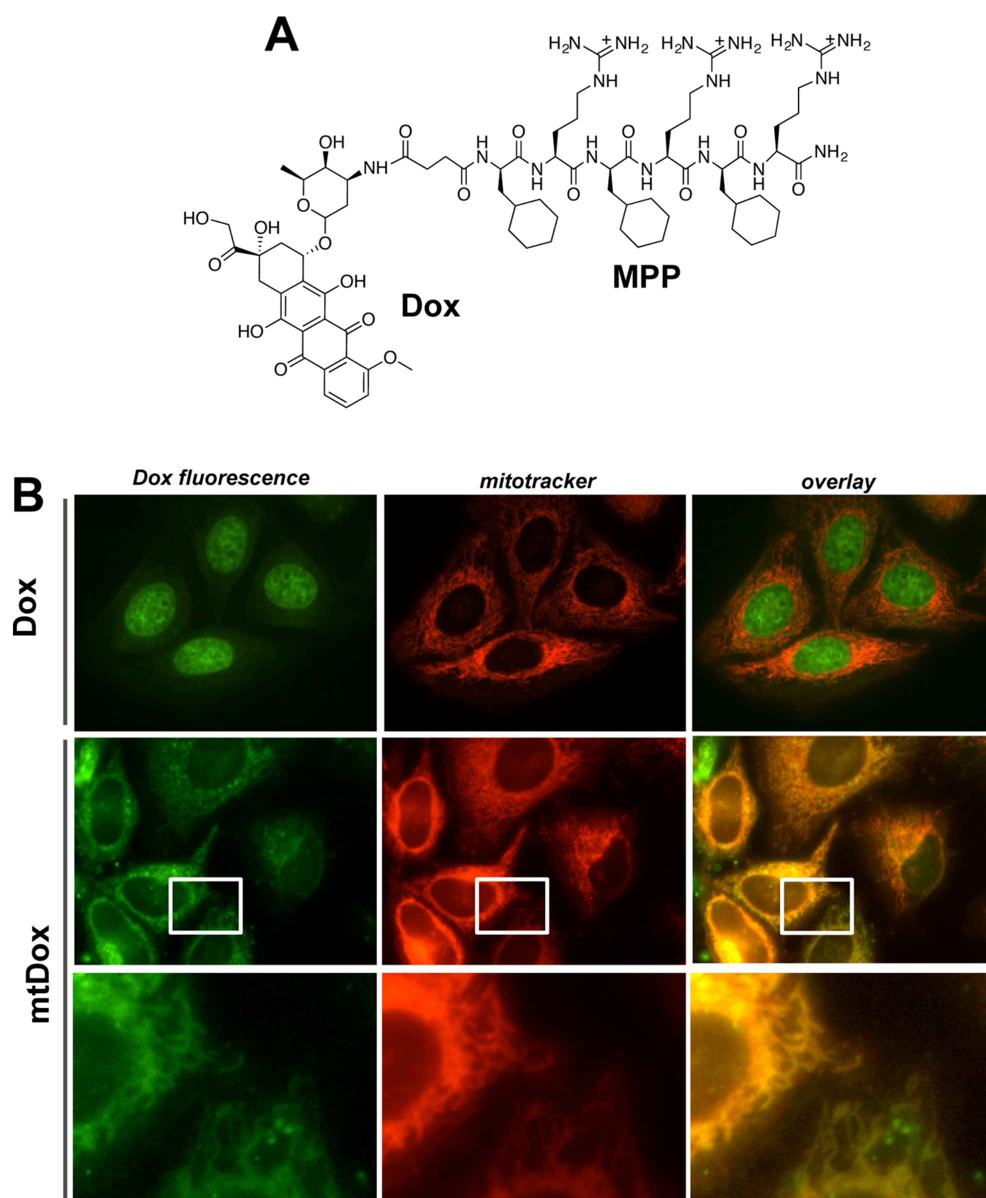

Figure 1. Structure and subcellular localization of mtDox. (A) The DNA topoisomerase II inhibitor doxorubicin (Dox) was conjugated to the N-terminus of a mitochondria-penetrating peptide (MPP). (B) Subcellular localization of mtDox. (top row) Dox (green channel) demonstrates strong nuclear staining as monitored using its intrinsic fluorescence and no colocalization with Mitotracker 633 (red channel) as shown in overlay image (right). (middle row) mtDox (red channel) shows a high level of mitochondrial accumulation with a staining pattern that matches Mitotracker 633 (green channel). The high degree of colocalization can be visualized in the bottom row closeup images.

These results indicate that Dox was successfully delivered into the mitochondria through conjugation to the MPP.

The primary mechanism of action of Dox is the generation of double-stranded breaks (DSBs) in DNA following intercalation into DNA and the stabilization of the TopoII ternary complex.[7] We first explored *in vitro* whether this activity is preserved for mtDox using two different assays that test intercalation and inhibition of TopoII activity. To assess intercalation into DNA, we assessed the quenching of mtDox versus Dox fluorescence in the presence of DNA.[15,16] MtDox demonstrated equivalent quenching to Dox, suggesting that both forms of the drug intercalated effectively (Figure 2A). In order to assess the inhibition of TopoII by mtDox, we used a decatenation assay using kinetoplast DNA (kDNA). KDNA consists of interlocking minicircles of DNA that can be decatenated using TopoII, which then demonstrate increased mobility on an agarose gel. Both Dox and mtDox demonstrated full inhibition of the decatenation reaction at doses of 10 and 15 μM, respectively (Figure 2B). These data confirm that mtDox maintains reasonable equivalency to Dox in its ability to intercalate with DNA and inhibit TopoII function.

We tested whether mtDox could also cause DSBs intracellularly with a PCR-based assay. This approach allows DNA damage to be detected in both nuclear DNA (nDNA) and mitochondrial DNA (mtDNA).[17] As shown in Figure 2C, while Dox caused damage within nuclear DNA, mtDox generated damage only in the mtDNA, supporting the notion that the activity of the drug is maintained while altering its distribution within the cell. Together with the above data, this strongly suggests that mtDox maintains its activity compared to Dox and that differences observed are attributable to the change in localization from the nucleus to the mitochondria.

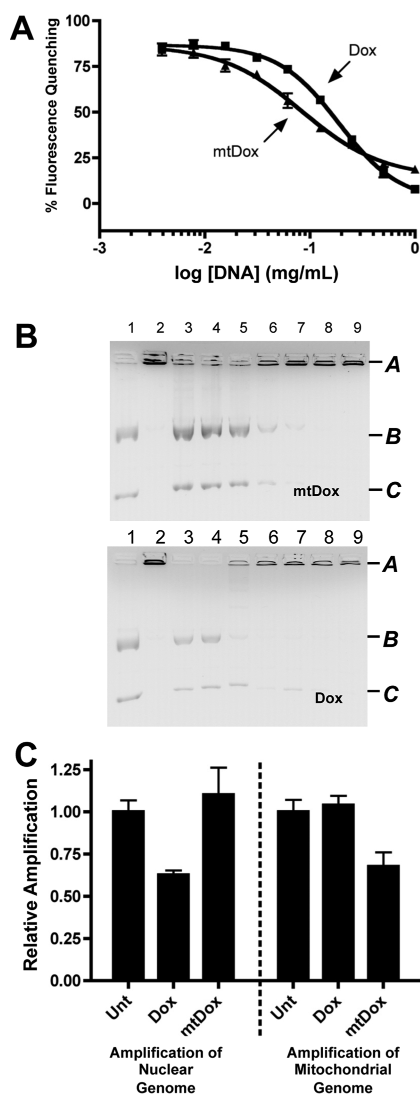

Figure 2. mtDox maintains function and selectively damages mtDNA. (A) Intercalation of Dox and mtDox into plasmid DNA as monitored via fluorescence quenching. Dox and mtDox demonstrated increased quenching of their intrinsic fluorescence with increased concentration of plasmid DNA demonstrating intercalation into DNA. (B) Inhibitory effect of Dox and mtDox on the decatenation of kinetoplast DNA (kDNA) by topoisomerase II: 1, decatenated kDNA marker; 2, control kDNA; 3, kDNA incubated with topoisomerase II in the absence of drug; 4−9, kDNA incubated with 1, 5, 10, 15, 20, and 25 μM drug, respectively. Part A corresponds to the band containing catenated DNA, part B corresponds to the band containing open circular DNA, and part C corresponds to the band containing relaxed DNA. (C) Assessment of DNA strand breaks in cellulo by measuring relative amplification of nuclear and mitochondrial DNA segments. Cells were treated with Dox (64 μM, 6 h) or mtDox (8 μM, 2 h) before isolation of DNA and PCR analysis. The concentrations selected for these experiments were not intended to reflect relative LD50 values but rather were what was needed to obtain reproducible lesion levels.

The intracellular activity of mtDox and the ability of the drug to cause cell death was examined in a human ovarian cancer cell line (A2780) and a Dox-resistant subline (A2780ADR) to determine the effect of P-glycoprotein (also known as ABCB1 or MDR1)[18] mediated resistance. In the sensitive A2780 line, Dox demonstrated higher potency than mtDox (Figure 3A). In the Pgp-expressing resistant line, however, Dox demonstrated a highly attenuated cytotoxicity, whereas mtDox maintained its efficacy (Figure 3B). It is noteworthy that the concentrations where mtDox exhibits toxicity in both cell lines are well below concentrations where any toxicity is observed for the peptide alone (>500 μM),[9] and the activity of the drug is clearly linked to the presence of the Dox pharmacophore.

We probed the mechanism of cell death caused by mtDox in A2780 cells. Using flow cytometry, a significant population of early apoptotic cells was visualized 24 h after treatments with mtDox (Figure 3C). Shorter incubation intervals (data not shown) did not yield any apoptotic or necrotic populations, indicating that gradual triggering of apoptosis underlies the toxicity of mtDox. This type of cell death mirrors what is observed with the parent compound,[19] indicating that, despite the different sites of action for the TopoII poisons, the overall effects are similar.

In order to confirm that the resistance trends for Dox compared to mtDox were attributable to Pgp pumps, the Pgp inhibitor cyclosporine A (CsA) was incubated with cells and both forms of the drug. A partial recovery of the activity of Dox was observed, whereas no change to mtDox (Figure 4A) activity occurred. A resistance factor (RF) can be used to quantify the effects of the resistance mechanism on the cytoxicity of the drug by comparing the LD_{50} in a sensitive line to the LD_{50} in a resistant line. In Pgp-expressing cells, Dox exhibited an RF of over 280, where mtDox showed an RF of less than 1.5 (Figure 4B). This indicates that mtDox remains unaffected by Pgp pumps, whereas the cytotoxicity of Dox is highly susceptible to efflux.

To elucidate the mechanism by which mtDox overcomes Pgp-mediated resistance, we first examined the localization of the drugs in sensitive and resistant cells. Using fluorescence microscopy, nuclear staining was observed for Dox incubated with sensitive A2780 cells (Supplementary Figure S1). In resistant A2780ADR cells, however, Dox demonstrated membrane staining, which indicates efflux of the drug (Supplementary Figure S1). MtDox, however, demonstrated a predominantly mitochondrial staining in both sensitive and resistant cells (Supplementary Figure S2).

Measuring cellular uptake of the drugs corroborated the imaging data suggesting efflux of Dox but not mtDox. Dox demonstrated decreased uptake in resistant cells that could be partially rescued using CsA (Figure 4C). MtDox, however, demonstrated no difference in uptake in either resistant or sensitive cells (Figure 4D). This pattern of results suggest that the attenuation of Dox's toxicity in resistant cells is best explained by decreased accumulation of drug and that mtDox overcomes this resistance mechanism.

There are three hypotheses that could explain the ability of mtDox to overcome this Pgp-mediated resistance. First, the structure of the drug with the MPP could cause the drug to inhibit Pgp.[20] Second, the structure of mtDox could be sufficiently different from Dox that it is no longer a substrate for Pgp pumps given the importance of the primary amine for Dox efflux.[21] Third, mtDox could be sequestered in the mitochondria and therefore unavailable for efflux by Pgp since no such pumps are found in the mitochondrial membrane.[22]

To test the first hypothesis, we examined the uptake of a known Pgp substrate, Calcein AM, which fluoresces upon hydrolysis within the cell. The Pgp inhibitor, CsA, caused a large increase in the fluorescence of Calcein, but neither Dox nor mtDox increased the fluorescence intensity (Figure 4F). These data demonstrate that the effects of mtDox are

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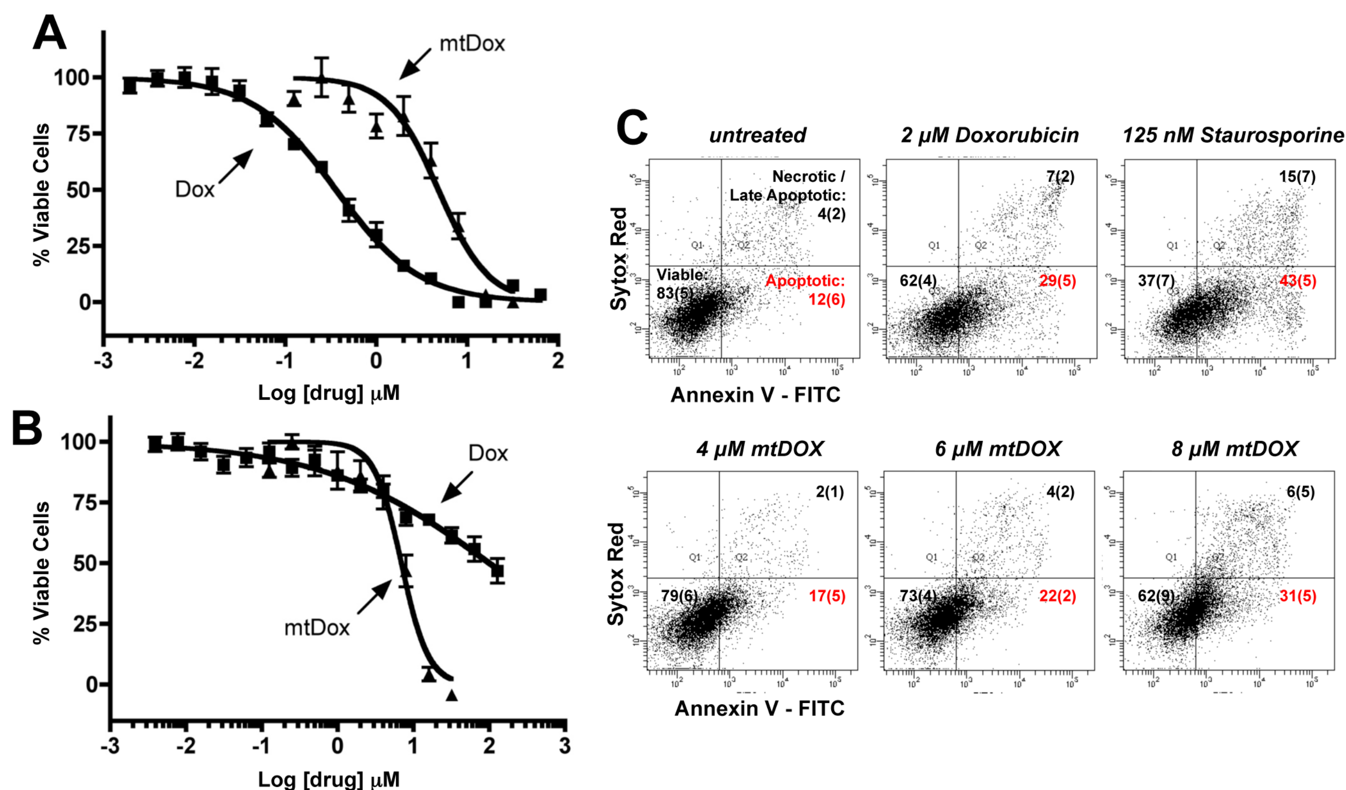

Figure 3. Toxicity and cell death mechanism for Dox and mtDox. (A) mtDox and Dox are both toxic to sensitive A2780 cells. (B) The toxicity of Dox is highly attenuated in A2780ADR Pgp-expressing cells. The toxicity of mtDox is unchanged in A2780ADR resistant cell. (C) Flow cytometry analysis of A2780 Annexin V-FITC (A-FITC)/SYTOX Red (SR) cell staining following 24 h treatment of staurosporin, Dox, or mtDOX. Shown are representative plots for each treatment condition (top right quadrant, A-FITC+/SR+, necrotic/late apoptotic cells; bottom left, A-FITC-/SR-, viable cells; bottom right, A-FITC+/SR-, early apoptotic cells). Also shown for each treatment condition is the percentage of each of these cell populations averaged over three independent experiments (standard deviations are shown in brackets).

independent of any inhibitory properties intrinsic to the drug with the delivery vector.

To assess the capacity for mtDox to act as a substrate for Pgp pumps outside of cells, we used a luciferase-based assay that allowed quantification of Pgp activity exposed to a substrate. The observed increase in activity of Pgp pumps exposed to mtDox was comparable to both Dox and the established control Verapamil (Figure 4E), demonstrating that mtDox acts as an equally good substrate as Dox despite not being effluxed. These results, taken together, clearly point to the lack of efflux for mtDox being derived from its sequestration in an impermeable organelle. Future studies will assess whether this is a general phenomenon across all cell types that overexpress efflux pumps. It has been suggested that mitochondrial localization of some transporters may occur, although this appears to be cell-type dependent.[22,23]

In summary, we have engineered the first mitochondrially targeted version of Dox. This compound maintains the ability to inhibit TopoII and damage mtDNA selectively. It is noteworthy that the potency of mtDox is diminished somewhat from the parent drug in sensitive cells. This finding may indicate that TopoII is not as essential in mitochondria as in the nucleus or that the DSB when generated is not monitored as rigorously. MtDox may provide a useful tool for the study of mt TopoII, an enzyme about which little is known. However, mtDox demonstrated a clear ability to overcome multidrug resistance mechanisms such as Pgp-mediated efflux. We have shown that, by retargeting existing drugs to the mitochondria where there are homologous targets, they can be sequestered therein and evade efflux. This work complements that done previously in our laboratory showing that DNA alkylators can also avoid drug resistance caused by overexpression of antiapoptotic factors or chemical deactivation factors.[12]

METHODS

General Cell Culture Conditions. HeLa cells were cultured in MEM-α (Invitrogen, Carlsbads CA) supplemented with 10% (v/v) FBS at 37 °C with 5% CO_2. A2780 wild-type and Dox-resistant lines and HL60 wild-type were cultured in RPMI 1640 supplemented with 10% (v/v) FBS at 37 °C with 5% CO_2. The A2780 Dox-resistant line was treated with 200 nM Dox once a week to maintain resistance.

Peptide and Drug Conjugate Synthesis and Characterization. Solid-phase synthesis was performed on Rink amid MBHA resin (0.7 mmol/g, 100−200 mesh) (NovaBiochem) using a Prelude Protein Technologies peptide synthesizer as described previously.[9] Doxorubicin was coupled on the peptide N-terminus using procedures adapted from those previously described.[24,25] Briefly, succinic anhydride was coupled on resin to the peptide using DIPEA (6 eq; Sigma-Aldrich, St. Louis, DIPEA = *N*,*N*-diisopropylethylamine) in *N*,*N*-dimethyl formamide (DMF) for 2 h. Peptides were deprotected and cleaved from the resin using trifluoroacetic acid (TFA)/triisopropylsilane/H_2O (95:2.5:2.5) and precipitated in cold ether. Peptides were purified to >95% purity by RP-HPLC on a C18 column with an H_2O/MeCN gradient in 0.1% TFA. Doxorubicin (LC Laboratories, Woburn, MA) was coupled to the peptide using HBTU (3 equiv; Protein Technologies, Tucson, Arizona, HBTU = *O*-(benzotriazol-l-yl)-*N*,*N*,*N*′,*N*′-tetramethyl-uronium hexafluorophosphate) and DIPEA (6 equiv) in DMF overnight in the dark. The conjugate was purified to >95% purity by RP-HPLC on a C18 column with an H_2O·MeCN gradient in 0.1% TFA, and identity was confirmed using electrospray ionization spectroscopy. Conjugates

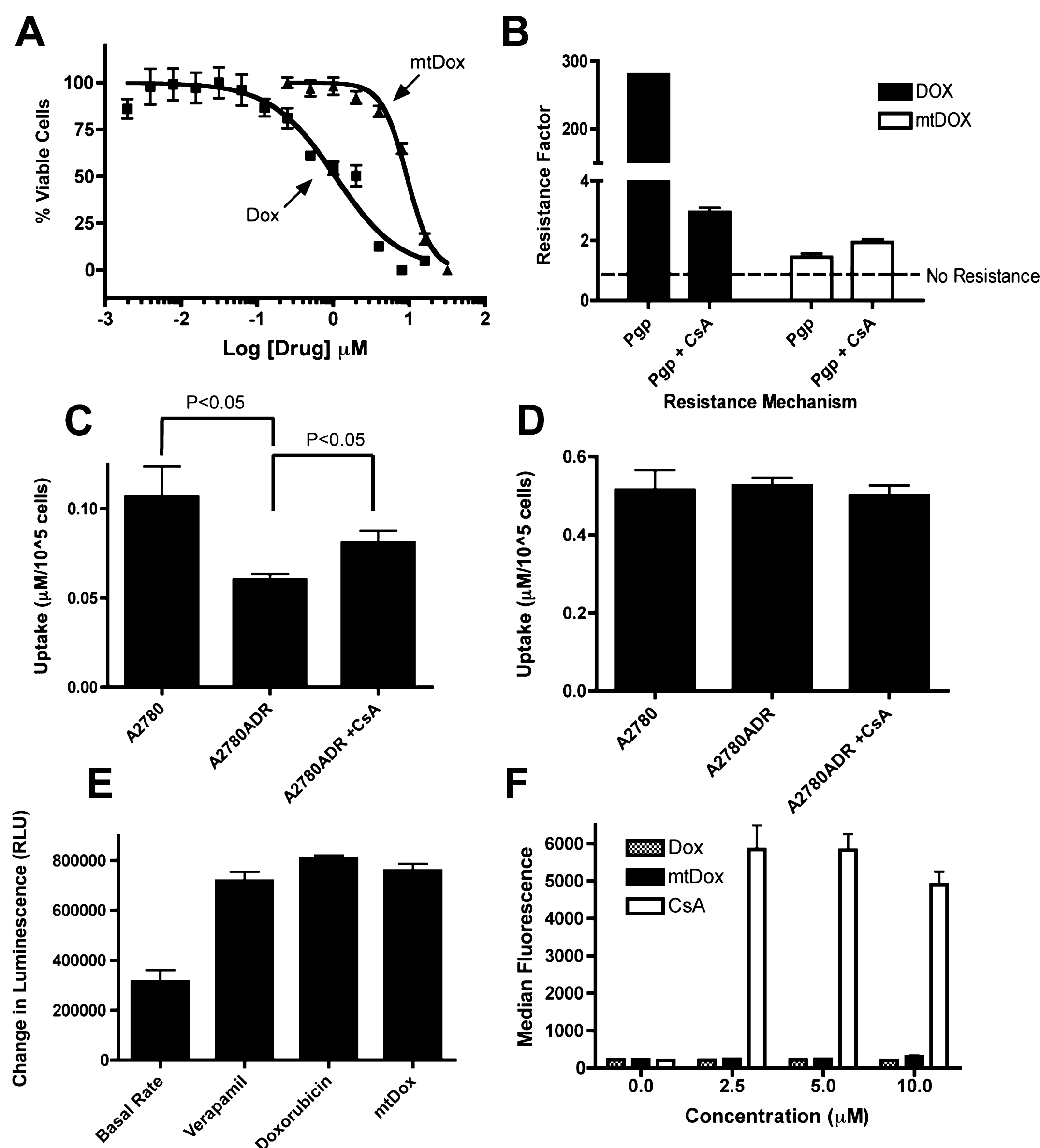

Figure 4. Evaluation of mtDox interactions with Pgp. (A) Preincubation of A2780ADR Pgp-expressing cells with CsA (5 μM) partially rescued the toxicity of Dox but had no effect on mtDox. (B) The resistance factor for the Dox and mtDox was calculated as a ratio of the LD_{50} of the sensitive line compared to a resistant line. Dox was found to be much more sensitive to Pgp-mediated resistance than mtDox. (C) Uptake of Dox is decreased in the A2780ADR resistant line, which can be overcome through Pgp inhibition by CsA (5 μM). (D) mtDox uptake is unchanged in sensitive and resistant cells. This is unaffected by Pgp inhibition by CsA (5 μM). (E) mtDox stimulates Pgp ATPase activity to a level similar to that seen for known substrates Dox and Verapamil. (F) mtDox shows no activity as an inhibitor of Pgp-mediated efflux. A2780ADR cells showed no uptake of Calcein AM with coincubation of either Dox or mtDox.

were quantified by absorbance at 488 nm in H_2O using the doxorubicin extinction coefficient of 11 500 M^{-1} cm^{-1}.

Microscopy. Cells were seeded in 8-well μ-slides (iBidi, Germany) at a density of 25 000 cells per well one day prior to experiments. Peptide incubations (10 μM for Dox and 8 μM for mtDox) were performed for 60 min in OPTI-MEM (Invitrogen). Mitotracker 633 (150 nM) (Invitrogen) and/or Hoechst 33342 (Invitrogen) (500 nM) were added for the last 30 min of the incubation. Cells were then washed twice with PBS and imaged using an inverted Zeiss Observer.Z1 microscope.

Analysis of Toxicity. A2780 wild-type and A2780-ADR cell lines were seeded in 96-well flat bottom tissue culture plates (Starsted, NC) at a density of 20 000 cells per well one day prior to experiments. The culture media was removed, and cells were washed with PBS. Drug incubations were performed in OPTI-MEM media. Cellular viability was analyzed after an overnight incubation at 37 °C with 5% CO_2 using the CCK-8 viability dye (Dojindo, Rockville, MD) at an absorbance of 450 nm.

DNA Intercalation. Dox or mtDox (100 nM) were diluted into Tris-EDTA buffer (pH 8) with increasing concentrations of pCA24N DNA and incubated for 5 min at RT. Fluorescence was excited at 490 nm and detected at 590 nm. Quenching of fluorescence was measured as a percentage of the fluorescence of drug incubated in the absence of DNA.

Topoisomerase II Decatenation Assay. The assay was performed according to the protocol of TopoGen, Inc. (Port Orange, Florida). The total reaction volume was held at 20 μL in assay buffer (120 mM KCl, 50 mM Tris-HCl, 10 mM $MgCl_2$, 0.5 mM dithiothreitol, 0.5 mM ATP, and 30 μg/mL BSA) and 120 ng of catenated kinetoplast DNA (kDNA). One unit of TopoIIα in the presence or absence of drug was added to initiate the reaction and incubated for 30 min at 37 °C. The reaction was stopped by the

addition of 5 μL of stop buffer (5% sarkosyl, 0.025% bromophenol blue, and 50% glycerol). The samples were then analyzed using electrophoresis at 100 V using a 1% agarose gel in Tris-borate-EDTA buffer with 0.5 μg/mL ethidium bromide.

Determination of DNA Lesion Frequency by Quantitative PCR. A total of 5×10^6 HL60 cells were treated with Dox (64 μM) or mtDox (8 μM) for 6 or 2 h, respectively. DNA was isolated from flash frozen cell pellets using the QIAGEN Genomic Tip and Genomic DNA Buffer Set Kit (QIAGEN) and quantified using the Picogreen dye (Invitrogen). Quantitative amplification of the 8.9 kb mitochondrial segment and the 17.7 kb β-globin target sequence used the GeneAmp XL PCR kit (Perkin-Elmer) as described previously.[16]

Annexin V Apoptosis Assay. A2780 wild-type cells were seeded in 6-well flat bottom tissue culture plates (Corning, NY), at a density of 100 000 cells per well one day prior to experiments. The culture media was removed; cells were washed with PBS and incubated with the indicated concentrations of mtDOX, DOX, or staurosporine (Sigma-Aldrich) in serum free media for 24 h at 37 °C with 5% CO_2. Cells were harvested, washed once with PBS and once with Annexin V binding buffer (50 mM HEPES, 700 mM NaCl, 12.5 mM $CaCl_2$, pH 7.4), and then stained with Annexin V-FITC (Cell Signaling Technology, MA) and SYTOX Red (Life Technologies) according to manufacturers' protocols. Analysis of staining was performed using a FACSCanto flow cytometer (BD Biosciences), using a minimum of 10 000 cells for each treatment. Singly stained, unstained, and nontreated samples were analyzed in parallel and used to define fluorescence compensation parameters in order to account for the intrinsic fluorescence of doxorubicin. For each treatment condition, experiments were performed in triplicate.

Measurement of Dox and mtDox Uptake. Cells were seeded in 96-well plates at a density of 12 000 cells per week one day prior to experiments. Cells were treated with 8 μM of Dox or mtDox for 2 h in Opti-MEM at 37 °C. For cells coincubated with cyclosporine A, this was added to cells 30 min before the drugs were added. Treated cells were washed 3× with PBS, lysed in 1% TritonX-100, and left on ice for 30 min. One hundred microliters of the sample was transferred to a 96-well plate, and fluorescence was measured (ex, 485 nm; em, 590 nm). Concentrations of Dox and mtDox were determined using a standard curve with known concentrations of the drugs.

P-gp ATPAse Assay. Activity of P-gp ATPase in the presence of Dox and mtDox was determined using the Pgp-Glo assay system (Promega, Madison, WI) using the protocol provided by the supplier. In an untreated 96-well plate (Corning, Tewksbury, MA), recombinant P-gp (25 μg) was incubated with assay buffer (20 μL), verapamil (200 μM), sodium orthovanadate (100 μM), Dox (10 μM), or mtDox (10 μM). The reaction was initiated by the addition of MgATP (10 mM) and incubated for 40 min at 37 °C. The reaction was stopped by the addition of 50 μL of firefly luciferase and incubated for 40 min at RT. P-gp ATPase activity was measured as a reduction in the luminescence signal.

■ ASSOCIATED CONTENT

Ⓢ Supporting Information

Analysis of subcellular Dox and mtDox distribution in sensitive and resistant cells. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

The authors declare no competing financial interest.

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■ ABBREVIATIONS

CsA, cyclosporin A; Dox, doxorubicin; DSBs, double-stranded breaks; kDNA, kinetoplast DNA; MPPs, mitochondria-penetrating peptides; mtDox, mitochondrially targeted doxorubicin; mtTopoII, mitochondrial topoisomerase II; Pgp, P-glycoprotein; TopoII, topoisomerase II

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