Enhanced Chemotherapy of Cancer Using pH-Sensitive Mesoporous Silica Nanoparticles to Antagonize P-Glycoprotein-Mediated Drug Resistance

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Key Words: mesoporous silica nanoparticles (MSNs), Multidrug resistance (MDR), P-glycoprotein (PGP), pH-sensitive, drug delivery

Abbreviations list:

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<th>Abbreviation</th>
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<tr>
<td>ABC</td>
<td>ATP-Binding Cassette</td>
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<tr>
<td>BCRC</td>
<td>Bioresource Collection and Research Center</td>
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<td>DDS</td>
<td>drug delivery systems</td>
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<tr>
<td>Dox</td>
<td>doxorubicin</td>
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<td>HMW</td>
<td>high molecular weight</td>
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<tr>
<td>i.t.</td>
<td>intratumoral</td>
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<td>MDR</td>
<td>Multidrug resistance</td>
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<td>MRP</td>
<td>multidrug-resistance-associated protein</td>
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<td>MSNs</td>
<td>mesoporous silica nanoparticles</td>
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<tr>
<td>NIR</td>
<td>near-infrared</td>
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<tr>
<td>PBS</td>
<td>phosphate-buffered saline</td>
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<tr>
<td>PDT</td>
<td>photodynamic therapy</td>
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<td>PEI</td>
<td>polyethylenimine</td>
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<td>PGP</td>
<td>P-glycoprotein</td>
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<td>POD</td>
<td>peroxidase</td>
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siRNA | small interfering RNA
---|---
TA | trimethylammonium
TEM | Transmission electron microscopy
TUNEL | Terminal deoxynucleotidyl transferase dUTP Nick End Labeling
VRP | verapamil

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Abstract

Multidrug resistance (MDR) is the major clinical obstacle in management of cancer by chemotherapy. Overexpression of ATP-dependent efflux transporter P-glycoprotein (PGP) is a key factor contributing to multidrug resistance of cancer cells. The purpose of the present study was to use the endosomal pH-sensitive MSN (MSN-Hydrazone-Dox) for controlled release of doxorubicin (Dox) in an attempt to overcome the PGP-mediated MDR. In vitro cell culture studies indicate that uptake of MSN-Hydrazone-Dox by the human uterine sarcoma MES-SA/Dox-resistant tumor (MES-SA/Dx-5) cell occurs through endocytosis thus bypassing the efflux pump resistance. This improves the efficacy of the drug and leads to significant cytotoxicity and DNA fragmentation evidenced by TUNEL and DNA laddering assays. In vivo studies show that the intratumor injection of MSN-Hydrazone-Dox induces significant apoptosis of MES-SA/Dox-resistant cancer cells. This is validated by active caspase-3 immunohistochemical analysis. However, MSN-Hydrazone, without Dox-conjugation, cannot induce apoptosis in vitro and in vivo. In conclusion, both in vitro and in vivo studies show that MSN could serve as an efficient nanocarrier entering cell avidly via endocytosis, thus bypassing the PGP efflux pump to compromise the PGP-mediated MDR. MSN-Hydrazone-Dox could further respond to endosomal acidic pH to release Dox in a sustained manner. Besides the cell study, this
is the first report that successfully demonstrates the therapeutic efficacy of using MSN against MDR cancer in vivo.
Introduction

Drug resistance is the major obstacle in management of cancer using chemotherapy. Multidrug resistance (MDR) is a term used to describe the ability of cancer cells to develop resistance to a broad range of structurally and functionally unrelated chemotherapeutic agents. It is estimated that approximately 500,000 new cases of cancer each year will soon eventually exhibit multidrug resistant phenotype (1). MDR is a complex phenomenon that can result from numerous mechanisms, including overexpressed ATP-dependent efflux pumps, which can diminish intracellular drug concentrations (such as P-glycoprotein (PGP) and multidrug-resistance-associated protein (MRP)), altered activity of specific enzyme systems which can diminish the chemosensitivity of the drug resistant cells (such as glutathione-S-transferase and topoisomerase), altered apoptosis regulation via loss of genes (such as p53) demanded for cell death or overexpression of genes (such as bcl-2) that block cell death, reduced drug uptake via alteration of folate carriers, increased DNA repair capacity and others (2-4). Among these mechanisms, alteration of cell membrane transport (mediated by PGP) is the best studied phenomenon clinically (1). PGP - a 170 kDa transmembrane protein encoded by the *MDR1* (or *ABCB1*) gene belongs to the ABC (ATP-Binding Cassette) transporter family. PGP is a wide-range multidrug efflux pump that contains 12 transmembrane regions and 2 ATP-binding sites. It acts as an energy-dependent
drug efflux pump that actively pumps the drug out of cells leading to reduced intracellular drug concentrations and decreased therapeutic efficacy. Some examples include doxorubicin, anthracyclines, vinca alkaloids, epipodophyllotoxins, and taxanes (3, 5, 6).

To overcome the MDR, several generations of PGP inhibitors have been developed but their clinical applicability is limited. There is high inherent toxicity of PGP inhibitors and altered pharmacokinetics and biodistribution of anticancer drugs when coadministered with PGP inhibitors (4, 7). Not until recently a number of nanoparticle-based drug delivery systems (DDS) such as polymer-drug conjugates, polymeric-micelles and liposomes have been developed to overcome MDR (8).

Among others, mesoporous silica nanoparticles (MSNs) possess several advantages for use as efficient DDS. MSNs have large surface areas and porous interiors that enable large doses of drugs. In addition, the silica framework lets MSNs resist hydrolysis and enzymatic degradation. Additionally the MSN’s nanochannels we have used for this study provide a physical barrier to enzyme entry because most enzymes are bigger than the MSN’s pores (~5 nm). The drugs are also conjugated in the inner walls of the MSN’s nanochannels, which protects the drugs from in vivo hydrolysis and premature release. The advantage of MSNs is that they can enhance the oral bioavailability of drugs by reducing the “first pass effect”. The “first pass effect”
involves the liver metabolizing these drugs before they move into systemic circulation.

The reason for this is the liver has a high concentration of metabolizing enzymes (9).

Moreover, cell uptake of MSNs can be increased through easily modified surface charge of MSNs (10, 11).

Recently, MSNs have been used in numerous biological fields, such as fluorescent markers in vitro and in vivo, clinical diagnoses, drug and gene delivery, and MRI contrast agents (12). In previously reported work, we developed the tri-functionalized MSNs for use as theranostic compounds that orchestrate the triad of imaging, target, and therapy in a single particle. The MSNs are functionalized in sequence with (A) a near-infrared (NIR) fluorescent contrast agent that is incorporated into the silica framework of MSNs for traceable imaging of particle targeting, (B) drug payloads in the MSN’s nanochannels for therapeutic intervention and, (C) biomolecular ligands tiling the outermost surfaces of MSNs for highly-targeted particle delivery (13).

We reported in our earlier study the use of positive-charge functionalized MSNs for controllable release of anionic drug molecules and to develop a pH-triggered drug release system. The MSN-TA can hold the drug molecules in acidic condition and protect them from degradation in the stomach. Therefore this nanoparticle could also be applied to oral drug delivery systems (14). In addition, we also used MSNs with a covalently incorporated photosensitizer (PdTPP) for cancer photodynamic therapy
(PDT) in MDA-MB-231 human breast cancer cells (15). Chen et al. recently reported that dendrimer-modified MSNs co-deliver doxorubicin and Bcl-2 siRNA to drug resistant human ovarian cancer cells. Bcl-2 siRNA can effectively silence the Bcl-2 mRNA and significantly repress the pump-independent resistance thereby enhancing anticancer efficiency (10). Recently Meng et al. reported their work using polyethylenimine(PEI)-modified MSNs to transflect PGP siRNA judiciously and deliver Dox which would be electrostatically absorbed against multidrug resistant KB-V1 human cervix carcinoma cells (16). It is known that high molecular weight (HMW) PEI has high cytotoxicity but is effective at transfecting nucleotides. Thus, selection of the appropriate PEI polymer size and dose of treatment is important to prevent particle toxicity.

However, our MSNs possess large pore diameters (~5.0 nm) and well-ordered pore structures. Although enzymes cannot easily enter MSN nanochannels, the protons found within acidic endosomes and lysosomes can; thereby providing a means of cleaving the labile hydrazone bonds that link drugs to the nanochannel walls. Thus we could conjugate doxorubicin with a pH sensitive linker (hydrazone bonds) and design sustained and proportionate release of doxorubicin. We could also increase cellular uptake of the drug through easily modified surface charges (17).

In this study, we used MSN-Hydrazone-Dox nanoparticles, where the doxorubicin
was covalently conjugated onto MSN’s nanochannel via pH-sensitive linkers. Such a design, as evident from both \textit{in vitro} and \textit{in vivo} studies, can overcome PGP-mediated MDR bypassing of the PGP efflux pump and allow sustained release of doxorubicin in a well-controlled manner.

\section*{Materials and Methods}

\subsection*{Experimental reagents}

Doxorubicin hydrochloride (DOX HCl), bovine serum albumin (BSA), Adipic acid dihydrazide, H$_2$O$_2$, phosphate buffer saline (PBS, 0.1 M, pH7.4), paraformaldehyde, verapamil and other chemicals were purchased from Sigma-Aldrich Chemical Co. (St. Louis, MO, USA). MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) was purchased from Chemicon International, Inc. McCoy’s 5A medium, fetal bovine serum (FBS), Trypsin-2.5\% (W/V) EDTA solution (10X) were purchased from Gibco Co. BD Matrigel$^{\text{TM}}$ Basement Membrane Matrix was purchased from BD Biosciences. MSN-Hydrazone-Dox nanoparticles were prepared and characterized as previously described (17). The loading percentage of doxorubicin within MSNs was 1.09\% (wt\%) and determined by measuring changes in optical absorption of the solution phase at 490 nm. A concentration of 50 $\mu$g/ml MSN-Hydrazone-Dox contains a doxorubicin equivalent dose at 0.55 $\mu$g/ml and according to the loading percentage (1.09\%).
Cell Line

Human uterine sarcoma MES-SA/Dox-resistant tumor (MES-SA/Dx-5) cell line was obtained from the Bioresource Collection and Research Center (BCRC), Taiwan. MES-SA/Dx-5 obtained from BCRC were immediately expanded and frozen down such that all cell lines could be restarted every 2 to 3 months from a frozen vial of the same batch of cells. The cell line was shown to have an MDR phenotype by its elevated expression of P-glycoprotein, wide cross-resistance and defective intracellular drug accumulation. The MDR cell line was maintained in McCoy's 5A medium containing 1.5 mM L-glutamine supplemented with 10% FBS in a humidified incubator at 37 °C in an atmosphere of 5% CO₂.

Cellular Retention of Doxorubicin

Doxorubicin cellular retention assay was measured by FACS analysis (18). 1 × 10⁵ MES-SA/Dx5 cells were seeded onto 12-well flat-bottom plates and incubated in 5% CO₂, at 37 °C overnight. The cells were treated with 0.55 μg/ml doxorubicin and 50 μg/ml MSN-Hydrazone-Dox in a serum-free medium for 2 h, at 37 °C, in total darkness. The cells were then washed three times with PBS. Later, fresh culture medium (with or without verapamil), was added to the cells, which were incubated for 0 to 6 and 24 h at 37 °C. Following incubation, the cells were collected and resuspended in 1 ml PBS. Flow cytometric analysis was performed by FACS.
Calibur™ flow cytometer with excitation wavelength of 488 nm and emission wavelength of 585 nm (FL2). To quantify the cellular retention of doxorubicin, the percentage of cells was determined as the fraction (%) of cell counts retained in the area of the marker bar at different times (19).

**Confocal Microscopy**

To visualize the intracellular uptake and localization of Atto647-MSN-Hydrazone-Dox on MES-SA/Dx5 cells, 5 × 10^4 MES-SA/Dx5 cells were seeded onto 35 mm ibidi dishes and incubated overnight in 5% CO₂, at 37 °C. The cells were treated with 50 μg/ml MSN-Hydrazone-Dox in a serum-free medium for 2 h, at 37 °C, in total darkness. The cells were washed twice with PBS and stained with 1 μM LysoTracker Green DND-26 and Hoechst 33342, for 5 to 10 min before being washed three times with PBS, and changed to serum free medium. Images of the cells were taken on a LEICA TCS SPE confocal imaging system with fluorescence observed at excitation wavelengths of 350, 488, 504 and 647 nm.

**In Vitro Cytotoxicity**

The MES-SA/Dx5 cells (1.5 × 10^4 cells/well) were treated with 5, 0.55 μg/ml free Dox and 50 μg/ml MSN-Hydrazone-Dox (containing doxorubicin equivalent dose at 0.55 μg/ml), for 24 h. The concentration of each drug was treated in triplicate. Cytotoxicity was determined by MTT assay, in which 10 μl of MTT (5 mg/ml)
solution was added to each well, and incubated for an additional 4 h, prior to the
addition of 100 µl of color development solution. Absorbance was read on an ELISA
plate reader with a test wavelength of 570 nm and a reference wavelength of 630 nm.

**DNA Fragmentation**

1 × 10^6 MES-SA/Dx5 cells were seeded onto 10 cm dish and incubated overnight
in 5% CO₂, at 37 °C. The cells were treated with 0.55 µg/ml free Dox and 50 µg/ml
MSN-Hydrazone-Dox (containing doxorubicin equivalent dose at 0.55 µg/ml), for 24
and 48 h, at 37 °C. Cells were collected and resuspended in 200 µl PBS. The
procedures followed the commercial genomic DNA extraction system from
VIOGENE, wherein 1 µg DNA of each sample was loaded into 1% agarose gel and
electrophoresis was performed at 50V, in a TBE buffer (90 mM Tris/HCl, 90 mM
boric acid, 2 mM EDTA, pH 8.0), using 1kb DNA ladder molecular weight markers
(Fermentas). The DNA was stained with 1.5 µg/ml ethidium bromide in TBE buffer
and visualized under ultraviolet transillumination.

**The TUNEL assay**

Terminal deoxynucleotidyl transferase dUTP Nick End labeling (TUNEL) assay
was used for the detection of apoptosis. Apoptotic cell death was detected using an in
situ cell death detection kit, POD (Roche Inc.) 1 × 10^5 MES-SA/Dx5 cells/ml were
seeded onto 8-well chamber slides (BD Falcon™) and incubated overnight in 5% CO₂,
at 37 °C. The cells were treated with 0.55 µg/ml free Dox and 50 µg/ml MSN-Hydrazone-Dox (containing doxorubicin equivalent dose at 0.55 µg/ml), for 24 and 48 h, at 37 °C. The slides were washed three times with PBS and fixed with freshly prepared 4% paraformaldehyde in PBS, for 1 h, at room temperature. The labeling and signal conversion procedures followed the protocol of this kit. The slides were developed with 3,3'-diaminobenzidine (DAB) and counterstained with hematoxylin. The slides were then analyzed by light microscopy.

**Animal model**

Four- to six-week-old male nude mice (nu/nu) were purchased from BioLASCO Taiwan Co. The mice were housed in a controlled environment, with food and water provided ad libitum. MES-SA/Dx5 cells were collected and suspended at a concentration of $5 \times 10^6/100 \mu l$ in PBS, and mixed with the same volume BD Matrigel™. The cell mixture was subcutaneously injected into the right and left flanks of the mice. When the tumor volume had reached 50-100 mm$^3$, the mice were randomly assigned to three different treatment groups and treated with intratumoral injection, including a control (treated with PBS), vehicle-control (treated with vehicle only, MSN-Hydrazone 50 µg/ml) and MSN-Hydrazone-Dox 50 µg/ml. Two days after the treatment, the mice were sacrificed. Tumors were collected, and embedded in OCT compound for immunostaining.
TEM Imaging of tumor tissue

For electron microscopy, tumor tissue specimens were fixed overnight in glutaraldehyde, buffered (2.5%) with PBS. The tissue was washed with 3 changes of PBS, and postfixed for 1 h in a solution containing OsO4 buffered (2%) with PBS, whereupon it was washed in 3 changes of dH2O and dehydrated stepwise in ethanol. The tissue was polymerized using Spurr resin at 68 °C, for 15 h. The embedded specimens were thin sectioned at 70 nm and viewed on a Hitachi H-7650 TEM operating at 80 kV.

Immunohistochemistry

The apoptotic effects of MSN-Hydrazone-Dox were determined by active caspase-3 immunostaining. The sections of tumor tissue (4 μm thick) were washed three times with PBS for 5 min, and fixed with freshly prepared 4% paraformaldehyde in PBS, for 15 min, at room temperature. The sections were probed overnight with a polyclonal rabbit anti-mouse active Caspase-3 antibody (ABcam Inc.) at 4 °C, followed by incubation with peroxidase-conjugated polyclonal goat anti-rabbit antibodies (ABcam Inc.) for 2 h, at room temperature. A positive reaction was demonstrated using 3,3'-diaminobenzidine (DAB) as chromagen. The sections were counterstained with hematoxylin and mounted with glass coverslips. The tissue sections were then analyzed by light microscopy.
Results

**MSN-Hydrazone-Dox enhances cellular retention of doxorubicin.** As illustrated in Figure 1, our MSNs delivered doxorubicin to the MDR tumor cells. Uptake of MSN-Hydrazone-Dox by the MDR cells occurred through endocytosis. Therefore, it was possible to bypass efflux pump resistance, thereby improving drug efficacy. When local pH values were between 4 and 6 (corresponding to those found within endosomes and lysosomes), doxorubicin was released from the MSN-Hydrazone-Dox. To confirm the bypassing of the efflux pump resistance, doxorubicin cellular retention assays were measured by FACS analysis. MES-SA/Dx5 cancer cells were treated with 0.55 µg/ml of free doxorubicin and 50 µg/ml of MSN-Hydrazone-Dox (containing an equivalent doxorubicin dose of 0.55 µg/ml), for 2 h. The retention of doxorubicin in the cells was examined in MES-SA/Dx5 cancer cells in the presence or the absence of P-gp inhibitors at various times (Fig. 2). After uptake for 2 h, doxorubicin cellular accumulation in cells treated with MSN-Hydrazone-Dox had led to approximately a 2-fold increase, compared to treatment with free doxorubicin. The doxorubicin cellular retention in MSN-Hydrazone-Dox group [with or without P-gp inhibitor (100 µM Verapamil)], was above 90% at different times, even after 24 h. However, the doxorubicin cellular retention in free doxorubicin group without P-gp inhibitor decreased with time, and the cellular retention of doxorubicin was 1.2% at 24 h,
which was nearly the same as the control cells (indicated by gray filled histograms); with P-gp inhibitor, the cellular retention of doxorubicin gradually decreased; and the cellular retention of doxorubicin was 27.1%, at 24 h. The result showed that the uptake of doxorubicin in MSN-Hydrazone-Dox group was greater than in the free doxorubicin group and 77 times as much doxorubicin was retained in the cells compared with the free doxorubicin group at 24 h.

**MSN-Hydrazone-Dox is uptaken by the MES-SA/Dx5 cancer cell through endocytosis.** To verify that MSN-Hydrazone-Dox had been able to enter the MES-SA/Dx5 cancer cells through endocytosis and accumulate within the endosomes and lysosomes, we used confocal microscopy to characterize cellular uptake of Atto-647-labeled MSN-Hydrazone-Dox (Fig. 3). In the treatment of Atto-647-MSN-Hydrazone-Dox to MES-SA/Dx5 cancer cells, endosomes and lysosomes were labeled with green fluorescent Lysotracker (Fig. 3B). After 2 h of incubation, the red fluorescence of Atto-647-MSN-Hydrazone-Dox could be observed within MES-SA/Dx5 cancer cells (red in Fig. 3A), and co-localized with Lysotracker green fluorescence (green in Fig. 3B, yellow in Fig. 3C), indicating that the Atto-647-MSN-Hydrazone-Dox had become highly concentrated within the MES-SA/Dx5 the endosomes and lysosomes of the cancer cells.

**MSN-Hydrazone-Dox enhances cytotoxicity of the MES-SA/Dx5 cancer cells.**
To assess the release of doxorubicin from the MSN, the drug’s endosomal escape, bypassing the efflux pump resistance, and its effect on cell viability, we employed 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assays to detect cell viability. MES-SA/Dx5 cancer cells were incubated for 24 h with free doxorubicin, MSN-Hydrazone and MSN-Hydrazone-Dox. MTT analysis showed (Fig. 4) that MSN-Hydrazone-Dox possessed greater anticancer activity than free doxorubicin at equivalent doses. However, MSN-Hydrazone did not lead to cytotoxicity. Therefore, we inferred that doxorubicin had been released from MSN-Hydrazone-Dox and escaped from the endosomes prior to entering the nuclei and leading to cell apoptosis.

MSN-Hydrazone-Dox but not MSN-Hydrazone induces and enhances apoptosis in MES-SA/Dx5 cells. The anticancer activity of free and MSN-conjugated doxorubicin was also confirmed by DNA fragmentation and TUNEL (Terminal deoxynucleotidyl transferase dUTP Nick End Labeling) assays. To study DNA fragmentation, MES-SA/Dx5 cancer cells were incubated with MSN, 0.55 μg/mL free and MSN-conjugated doxorubicin for 24 and 48 h. DNA ladders were applied to 1.5% agarose gels for electrophoretic comparison of doxorubicin-induced DNA fragmentation (Fig. 5A). As can be seen from lanes 3, 6 and lanes 4, 7 of Fig. 5A, MSN-Hydrazone-conjugated doxorubicin had greater anticancer activity than free
doxorubicin, when containing equivalent doses of doxorubicin. However, MSN-Hydrazone itself did not induce cell apoptosis. Thus, the in vitro performance of our MSN platform was more efficient than using the free drug alone. We employed TUNEL assays to detect breaks in the DNA strands (Fig. 5B). By labeling the terminal ends of nucleic acids, TUNEL assays enable highly efficient detection of DNA fragments resulting from cell apoptosis. TUNEL-positive cells displayed brown nuclei and TUNEL-negative cells displayed blue nuclei. MES-SA/Dx5 cancer cells were incubated with MSN, free and MSN-conjugated doxorubicin for 24 and 48 h. As shown in Fig. 5B, MSN-Hydrazone-Dox was able to induce cell apoptosis but not MSN-Hydrazone. These results are in agreement with the above-mentioned data.

**MSN-Hydrazone-Dox is uptaken and induces apoptosis of tumor cells in vivo.**

In order to perform in vivo investigation of the anticancer activity of MSN-Hydrazone-Dox, we established human uterine sarcoma MES-SA/Dox-resistant cancer xenografts in nude mice. Confirmation of tumor uptake was provided by TEM imaging of frozen tumor cross-sections harvested 6 h post i.t. injection of MSN-Hydrazone-Dox. The MSN-Hydrazone-Dox was found concentrated in the intracellular vesicles of MES-SA/Dox-resistant cancer cells, indicated by arrows (Fig. 6A). We employed immunohistochemical analysis to detect active caspase-3, which is a signature protease activated during the apoptosis. At the 2 day mark, an i.t. injection
of 50 μg/mL MSN-Hydrazone-Dox, active caspase-3-positive tumor cells (brown in cytoplasm; Fig. 6B-a) were detected. However, they were not in free doxorubicin (same doxorubicin dose, 0.55 μg/ml), MSN-Hydrazone, or control (Fig. 6B-b, c, d).

Discussion

Multidrug resistance of cancer cells is a major cause of failure of anti-cancer chemotherapy in the treatment of cancer patients. One of the mechanisms of resistance is the prevention of the intracellular accumulation of anti-cancer drugs by overexpression of ATP-dependent transport proteins, such as P-glycoprotein (PGP), which pumps out the drugs from cells (20, 21). The human uterine sarcoma MES-SA/Dox-resistant tumor (MES-SA/Dx-5) cells overexpress P-glycoprotein (PGP) when compared with native MES-SA tumor cells (Fig. S1). Thus in these cells, PGP pumps out doxorubicin from cells making it ineffective. Verapamil (VRP) is a calcium channel blocker that enhances intracellular accumulation of many anticancer drugs at very high concentrations, including doxorubicin in various cancer cell lines (22-24). In present study, 100 μM VRP slightly increased intracellular accumulation of doxorubicin (Fig. 2A). However VRP enhanced cytotoxicity in normal cells only at high concentration (25). The MSN-Hydrazone-Dox nanoparticle on the other hand improved cell uptake and intracellular doxorubicin retention without PGP inhibitor in MES-SA/Dx5 cancer cells (Fig. 2B).
MSNs are less than 300 nm in diameter and suitable for biomedical applications. Several previous studies have demonstrated that MSNs are able to undergo cellular uptake by endocytosis in various cancer cells without inducing cytotoxicity. The nanoparticles are mainly located in the acidic organelles such as endosomes and lysosomes when taken up by cells (11, 12, 26-32). Our results are consistent with previous studies. As shown in Figure 3, Atto-647-labeled MSN-Hydrazone-Dox was taken up by the cells and it accumulated within endosomes and lysosomes, which were labeled with green fluorescent Lysotracker within MES-SA/Dx5 cancer cells. They were concentrated in the peripheral region of the nucleus and doxorubicin was released from MSNs and entered the nucleus easily. Similar results were observed in TEM images of the MES-SA/Dx5 tumor tissue section (Fig. 6A). Furthermore, MSN-Hydrazone (without doxorubicin conjugation) did not lead to cytotoxicity (Fig. 4). Additionally, we used confocal microscopy to analyze cellular uptake of free doxorubicin. The MES-SA/Dx5 cells (1.5 × 10^4 cells/well) were treated with 5 and 0.55 µg/ml free doxorubicin (50 µg/ml MSN-Hydrazone-Dox containing doxorubicin equivalent dose at 0.55 µg/ml), for 2 h. As shown in Figure S2, the red fluorescence of free doxorubicin could not be observed within MES-SA/Dx5 cancer cells that were treated with 0.55 µg/ml of free doxorubicin (red in Fig. S2A). The free doxorubicin had entered the cell through diffusion and was pumped out by PGP of MES-SA/Dx5.
cells. Simultaneously, the MES-SA/Dx5 cells were treated with a high dose of free doxorubicin (5 μg/ml). The red fluorescence of free doxorubicin could be observed within MES-SA/Dx5 cancer cells that were treated with 5 μg/ml of free doxorubicin (red in Fig. S2C). The MES-SA/Dx5 cells were treated with additional P-gp inhibitor (100 μM Verapamil); the red fluorescence of free doxorubicin was higher than the absence of P-gp inhibitors within MES-SA/Dx5 cancer cells (red in Fig. S2B and D).

Figure S2 shows that the red fluorescence of free doxorubicin had not co-localized with Lysotracker green fluorescence after 2 hours of incubation. It implicated that MES-SA/Dx5 cells do not sequester doxorubicin in intracellular vesicles such as endosomes or lysosomes.

Doxorubicin is a common chemotherapeutic agent in clinical practice. Doxorubicin interacts with DNA and topoisomerase II in the nucleus thereby inducing protein-associated DNA strand breaks and leads to cell death (33-36). We employed DNA fragmentation and TUNEL assays to detect DNA strand breaks. As shown in Figure 5, MSN-conjugated doxorubicin has higher anticancer activity than free doxorubicin at equivalent doxorubicin dosage.

These data suggest that MSNs can be an appropriate carrier of doxorubicin via endocytosis and bypass efflux pump related resistance thereby raising cellular accumulation of doxorubicin. Another advantage of these particles is that doxorubicin
is released continuously from the MSN-Hydrazone-Dox nanoparticles due to decreasing pH in endosomes and lysosomes. \textit{In vivo} data also reveals that MSN-Hydrazone-Dox nanoparticles induce significant cell apoptosis in MES-SA/Dox-resistant tumor (MES-SA/Dx-5).

Meng \textit{et al.} showed that Doxorubicin loaded in the porous interior by an electrostatic binding interaction in the PEI-modified MSNs could be released about 60\% after 12 h in acidic environment. They also observed apoptosis of cells at 72 h via Annexin V-SYTOX and TUNEL staining (16). However, for our study we conjugated doxorubicin with pH sensitive linker so that the release of the drug could be sustained around 60\% after 60 h (17). We also observed apoptosis of cells at 24 h via DNA fragmentation and TUNEL assays and additionally the tumor at 2 days via active caspase-3 immunostaining. In addition, our study used the sequential functionalization of nanoparticle domains that can enable traceable imaging of thranostic targeting by NIR fluorescent contrast agent (ATTO 647N) along with drug payload for therapeutic intervention by doxorubicin.

In conclusion, MSNs as nanocarriers can effectively deliver PGP substrates, such as doxorubicin, into MDR cells. They can bypass the PGP efflux pump to antagonize PGP-mediated MDR and allow for sustained release of doxorubicin. As shown the first time for the \textit{in vivo} study, this therapeutic strategy to enhance chemotherapeutic
efficacy and overcome PGP-mediated multidrug resistance may have significant clinical potential in the future.

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**Figure 1.** Schematic diagram of pH-sensitive MSNs drug delivery system to MES-SA/Dx5 human uterine sarcoma cancer cells for enhanced chemotherapy efficacy. Non-specific uptake of MSN-Hydrazone-Dox by the uterine sarcoma cancer cells occurs through endocytosis. Hydrolysis of pH-sensitive linker’s hydrazone bond in presence of acidic environment of endosomes/lysosomes allows the release of doxorubicin intracellularly from the MSN’s nanochannels.

**Figure 2.** Drug cellular retention assays of Dox (A) and MSN-Hydrazone-Dox (B) in MES-SA/Dx5 cancer cells in the presence or the absence of P-gp inhibitor (100 μM Verapamil) was determined by flow cytometry. MES-SA/Dx5 cancer cells were treated with 0.55 μg/ml of free Doxorubicin (red and orange lines) and 50 μg/ml of MSN-Hydrazone-Dox (blue and purple lines) for 2 h, respectively. Cells were washed and incubated in fresh culture medium with (orange and purple lines) or without (red and blue lines) verapamil for 0 to 6 and 24 h at 37 °C. Control cell was incubated in culture medium (gray filled histograms). After 24 h, MSN-Hydrazone-Dox was retained in cells with or without verapamil.

**Figure 3.** Confocal microscopy of the cellular uptake behavior of Atto-647-MSN-Hydrazone-Dox on MES-SA/Dx5 cells. (A) MES-SA/Dx5 cells were
treated with Atto-647-MSN-Hydrazone-Dox at 37 °C for 2 h (MSN denoted via red fluorescence of Atto-647, and nuclei denoted via blue fluorescence of Hoechst 33342 staining). (B) MES-SA/Dx5 cells were labeled with Lysotracker to mark endosomes and lysosomes (green). (C) Merging images A and B. Scale bar: 5 µm.

**Figure 4.** Cell viability of MES-SA/Dx5 human uterine sarcoma cancer cells incubated for 24 h with free doxorubicin, MSN-Hydrazone and MSN-Hydrazone-Dox. 50 µg/ml MSN-Hydrazone-Dox containing doxorubicin dose equivalent of 0.55 µg/ml. After 24 h, the concentration of doxorubicin released form MSN-Hydrazone-Dox was approximately 0.22 µg/ml.

**Figure 5.** MSN-Hydrazone-Dox and free Doxorubicin induced DNA fragmentation and apoptosis in MES-SA/Dx5 human uterine sarcoma cancer cells. (A) MES-SA/Dx5 cells were incubated with different treatments for 24 and 48 h. DNA was extracted and electrophoresed on 1% agarose gel with 1µg DNA loaded in each lane. MW: DNA molecular weight marker. Lane 1: doxorubicin-free control, Lanes 2, 5: 50 µg/ml of MSN-Hydrazone, Lanes 3, 6: 0.55 µg/ml of free doxorubicin, Lanes 4, 7: 50 µg/ml of MSN-Hydrazone-Dox containing doxorubicin dose equivalent of 0.55 µg/ml. (B) Apoptotic cell death was observed using a TUNEL (DNA fragment) assay.
MES-SA/Dx5 cells were treated with 0.55 and 5 µg/ml of free doxorubicin, 50 µg/ml of MSN-Hydrazone (MSN) and 50 µg/ml of MSN-Hydrazone-Dox containing doxorubicin dose equivalent of 0.55 µg/ml for 24 and 48 h. Slides were developed with 3,3'-diaminobenzidine (DAB) and counterstained with hematoxylin. Scale bar: 100 µm.

**Figure 6.** (A) TEM image of the 70 nm thick MES-SA/Dx5 tumor tissue section. Mice were treated with MSN-Hydrazone-Dox for 6 h by intratumoral injection. MSN-Hydrazone-Dox nanoparticles are indicated by arrows. N - Cell nucleus. Scale bar: 1 µm. (B) The apoptotic effects of MSN-Hydrazone-Dox were determined by active caspase-3 immunostaining. MES-SA/Dx5 cells were subcutaneously injected in right and left flanks of nude mice. Mice were treated with MSN-Hydrazone-Dox containing a doxorubicin dose equivalent of 0.55 µg/ml (a), 0.55 µg/ml of free doxorubicin (b), PBS as control, (c) and 50 µg/ml of MSN-Hydrazone (d) once for 2 days by intratumoral injection. Mice were sacrificed for immunohistochemistry after 2 days. Tumor tissue sections were immunostained for the presence of active caspase-3. Tumor tissue sections were developed with 3,3'-diaminobenzidine (DAB) and counterstained with hematoxylin. Scale bar: 100 µm.
Figure 1.
Figure 2.
Figure 3.
Figure 4.
Figure 5.

A

B
Figure 6.

A

B

(a)  
(b)

(c)  
(d)

37
Bypass of efflux pump

Endosome/lysosome pH 5~6

Nucleus

: P-glycoprotein

: Doxorubicin

: MSN-Hydrazone-Dox

ATP → ADP

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I-Ping Huang, Shu-Pin Sun, Shih-Hsun Cheng, et al.

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