Daunorubicin-loaded magnetic nanoparticles of Fe$_3$O$_4$ overcome multidrug resistance and induce apoptosis of K562-n/VCR cells in vivo

Abstract: Multidrug resistance (MDR) is a major obstacle to cancer chemotherapy. We evaluated the effect of daunorubicin (DNR)-loaded magnetic nanoparticles of Fe$_3$O$_4$ (MNPs-Fe$_3$O$_4$) on K562-n/VCR cells in vivo. K562-n and its MDR counterpart K562-n/VCR cell were inoculated into nude mice subcutaneously. The mice were randomly divided into four groups: group A received normal saline, group B received DNR, group C received MNPs-Fe$_3$O$_4$, and group D received DNR-loaded MNPs-Fe$_3$O$_4$. For K562-n/VCR tumor, the weight was markedly lower in group D than that in groups A, B, and C. The transcriptions of Mdr-1 and Bcl-2 gene were significantly lower in group D than those in groups A, B, and C. The expression of Bcl-2 was lower in group D than those in groups A, B, and C, but there was no difference in the expression of P-glycoprotein. The transcriptions and expressions of Bax and caspase-3 in group D were increased significantly when compared with groups A, B, and C. In conclusion, DNR-loaded MNPs-Fe$_3$O$_4$ can overcome MDR in vivo.

Keywords: multidrug-resistance reversal, leukemia, magnetic nanoparticles of Fe$_3$O$_4$, in vivo

Introduction

The development of new cytotoxic drugs and treatment strategies has resulted in improved response rates for patients with hematological malignancies. However, multidrug resistance (MDR) is the primary cause for almost 90% of cancer treatment failures. Cancer cells are becoming resistant to the cytotoxic effects of a wide range of structurally and mechanistically unrelated anticancer drugs. It has been proved that various molecular mechanisms, including the activation of detoxifying systems of the glutathione-S-transferase gene, DNA repair, and alteration in drug-induced apoptosis of genes in the Bcl-2 pathway, contribute to the complex story of cancer drug resistance. However, the most commonly encountered effect of MDR in the laboratory is the decreased intracellular drug accumulation caused by enhanced drug efflux from tumor cells. This is related to the overexpression of a family of energy-dependent transporters, known as ATP-binding cassette (ABC) transporters such as P-glycoprotein (P-gp) and MDR-associated protein. Among these ABC transporters, P-gp confers cancer cells the strongest resistance to the widest variety of compounds. P-gp, a 170-kDa membrane-associated glycoprotein, transports a broad class of hydrophobic cytotoxic drugs that are central to most chemotherapeutic regimens from cell cytoplasm to outside the plasma membrane. Despite the wealth of information collected about the biochemistry and substrate specificity of P-gp and other types of ABC transporters, translation of this knowledge from the bench to the bedside has proved to be unexpectedly difficult. Because cytotoxic drugs typically
carry numerous dose-limiting normal tissue side effects, it is generally impractical to overcome this form of drug resistance simply by increasing the drug dose. Extensive efforts have been made to design P-gp modulators (chemosensitizers). Unfortunately, modulation of P-gp activity by current chemosensitizers to inhibit ABC transporters is limited for several reasons, such as low efficiency, strong adverse effects, and pharmacokinetic interactions that limit anticancer drug clearance and metabolism.

Nanotechnology is no stranger to oncology. A wide variety of nanovectors have been used for the delivery of anticancer drugs to overcome drug resistance as well as to improve the effectiveness and safety of cancer chemotherapy, such as the use of drug-loaded liposomes, stealth liposomes, polymeric nanospheres with equivalent stealth properties, polymeric nanocapsules, and solid lipid nanoparticles. The major advantages sought by the use of nanovectors over simple drugs are as follow: the specific delivery of large amounts of therapeutic agents using biorecognition targets, protection of the drug from premature degradation and interaction with the biological environment; enhanced absorption of the drugs into a selected tissue through enhanced permeability and retention (EPR) effect; controlling the pharmacokinetic and drug tissue distribution profile; and improvement of intracellular penetration, which ameliorates the therapeutic index.

In our previous research, we have developed tetraheptylammonium-capped MNPs-Fe₃O₄, which could facilitate the drug accumulation of daunorubicin (DNR) inside MDR leukemia K562/A02 cells and enhance the response of DNR in MDR leukemia K562/A02 cells in vitro. It has been demonstrated that DNR polymerized with MNPs-Fe₃O₄ have shown more chemosensitizing activities than those of DNR alone. The cytotoxicity test in vitro revealed that MNPs-Fe₃O₄ exhibit excellent biocompatibility. Furthermore, no carcinogenic effects have been observed for MNPs-Fe₃O₄. Its effect on MDR leukemic cells in vivo remains unknown.

In this work, we demonstrate that DNR-loaded MNPs-Fe₃O₄ are able to reverse MDR leukemic K562-n/VCR cells in vitro by inducing apoptosis. However, DNR-loaded MNPs-Fe₃O₄ fails to enhance cytotoxicity response in sensitive leukemic K562-n cells in vivo.

**Materials and methods**

**Preparation of DNR-loaded MNPs-Fe₃O₄**

MNPs-Fe₃O₄ were produced by electrochemical deposition under oxidizing conditions in a 0.1 mol/L tetraheptylammonium 2-propanol solution, where the magnetization and the size of MNPs-Fe₃O₄ was found to be $25.6 \times 10^{-3}$ emu/mg and 30 nm, respectively. The deposited clusters were capped with tetraheptylammonium, which acts as a stabilizer of the colloidal nanocrystallites. Before being applied in this experiment, the magnetized nanoparticles of Fe₃O₄ were well distributed in 0.9% NaCl solution by using ultrasound treatment in order to obtain colloidal suspension of MNPs-Fe₃O₄. 0.2 g/L DNR (Sigma Aldrich, St. Louis, MO, USA) conjugated with 0.116 g/L MNPs-Fe₃O₄ were prepared by mechanical absorption polymerization as previously reported.

**Cell lines and cell culture**

K562-n, a cell strain with high tumorigenicity in nude mice, was derived from K562 cells, which were cloned from a patient with human chronic myelogenous leukemia by repeated passage in nude mice and in culture alternatively. MDR leukemia cell K562-n/VCR, which expresses MDR gene (mdr-1), was established by a long-term, intermittent, low-dose, and gradually escalated vincristine (VCR) added into the culture medium. K562-n/VCR has been proved to be resistant to some extent to many cytotoxins including VCR, DNR, and has high tumorigenicity in nude mice. It has been proved that K562-n/VCR cells in the xenograft model had a comparatively stable MDR phenotype to the corresponding cells. K562-n and K562-n/VCR cells (gifted from the department of hematology, Shanghai Hospital, Second Military Medical University, Shanghai, China) were maintained in RPMI 1640 medium (Gibco, Carlsbad, CA, USA) with 10% fetal bovine serum (Sijiqing, Hang Zhou, China), penicillin G (100 IU/ml), and streptomycin (100 µg/mL) at 37°C in a humidified 5% CO₂ atmosphere. 0.64 µg/ml VCR was added regularly in K562-n/VCR culture medium to maintain drug resistance. K562-n/VCR cells were incubated in VCR-free medium for over two weeks before the planned experiment.

**Animals and establishment of the xenograft leukemia model in nude mice**

Female BALB/c-nu/nu mice were purchased from Beijing National Center for Laboratory Animals, the Chinese Academy of Medical Sciences, and were four weeks old at the beginning of our experiments. They were maintained in specific pathogen-free (SPF) facilities in our college and fed with irradiated chow. The two subclones of cells, K562-n and K562-n/VCR, were inoculated subcutaneously into each side of the back of athymic nude mice ($5 \times 10^6$ cells/each) simultaneously, resulting in the formation of two tumors per mouse. The mice were assigned randomly to five groups...
after inoculation for 10 days when both tumors formed. Mice in the control group were treated with normal saline 0.2 ml (group A). Nude mice in group B were treated with MNPs-Fe₃O₄ (0.58 mg/kg). Group C were treated with DNR (1 mg/kg). Group D were treated with MNPs-Fe₃O₄ (0.58 mg/kg) loaded with DNR (1 mg/kg) every other day for 20 days by vena caudalis injection, respectively.

**Tumor assessment**

After the planned treatment all animals were killed with ether anesthesia. Tumors were isolated for weight detection and then for quantitative real-time reverse transcription–polymerase chain reaction and western blotting.

**Quantitative real-time reverse transcription–polymerase chain reaction**

A total RNA of K562-n/VCR tumors from all groups and K562-n tumor of group A were isolated by using Trizol reagent (Invitrogen Life Technologies, Carlsbad, CA, USA) according to the manufacturer’s protocol. One microgram of total RNA was used to generate cDNA by using SuperScript II Reverse Transcriptase (Invitrogen Life Technologies). PCR primers were designed to amplify products within target and control sequences (primer sequences for Mdr-1 (427 bp) forward, 5'-TGTTTGTGACGATGGTGGG-3' and reverse, 5'-AGATCAGCAAGAAGCAGCCTA-3'; Bcl-2 (452bp) forward, 5'-GGGAGAAGAGGTACGATAA-3' and reverse, 5'-CCACCGAATCAAAAGAAG-3'; Bax (114bp) forward, 5'-TTTGTGCTCGGTTTATCAT-3' and reverse, 5'-GACACTGCTCAGCTTCTG-3'; caspase-3 (445 bp) forward, 5'-CACATAGCCAACCATCAG-3' and reverse, 5'-GGACATACAGTGTCGTTTCA-3'; GAPDH (205bp) forward, 5'-CGGATTGTGTGTGCATTG-3' and reverse, 5'-GAAGATGGTGATGGGATT-3'). Quantitative PCR was performed by monitoring in real-time the increase in fluorescence of SYBR green I dye (Takara, Shiga, Japan) with Rotor-Gene 3000 (Corbett Research, Sydney, Australia). Each experiment was undertaken in triplicate. The relative gene copy number was calculated by the concentration-CT method and normalized using the average expression of GAPDH.

**Western blot analysis**

Western blotting was used for the detection of P-gp. After treatment for 20 days, 0.1 g K562-n/VCR tumor tissue was collected from each mouse and homogenized for P-gp detection, tumor tissue from K562n is used as negative control. Tissues were extracted with 1 g/L Triton X-100 and protein concentration was determined with a Bio-Rad protein assay kit (Bio-Rad Laboratories, Hercules, CA, USA) and standardized with bovineserum albumin. A 50 μg sample of protein was separated on electrophoresed SDS-PAGE and electroblotted onto PVDF membrane (BioRad Laboratories). The membrane was blocked with buffer containing 10% fat free dry milk. Mouse anti-Bcl-2 antibody (1:500), mouse anti-Bax antibody (1:500), mouse anti-Caspase-3 antibody (1:500), mouse anti-P-gp antibody (1:500) and mouse anti-β-actin antibody (1:1000) were used respectively as the primary antibody. Horseradish peroxidase-conjugated anti-rabbit antibody (1:1000) was the secondary antibody. The band was detected by using an enhanced chemiluminescence (ECL) detection system.

**Statistical analysis**

For tumor diameter assessment and pathological examinations, experiments were repeated at least three times and evaluated by two independent researchers when indicated. Reported values represent the means ± SD. The significance of differences between experimental variables was determined using parametric Student’s t-test.

**Results**

DNR-loaded MNPs-Fe₃O₄ could greatly suppress the growth of K562n/VCR tumor, but failed to further inhibit the proliferation of sensitive K562-n tumor in vivo.

All models of nude mice with transplanted leukemic K562-n cell and its MDR counterpart K562n/VCR were established after subcutaneous injection for 10 days, with an average volume of 100 mm³. The rate of subcutaneous tumor formation was 100%. For MDR-bearing K562-n/VCR tumors, there was no significant difference in the average tumor weight between groups A and B, the average tumor volume of group C seemed smaller but without statistic difference. The average tumor weight was less in group D than that in groups A, B, and C (group D vs groups A, B, C) (Figure 1A). For sensitive K562-n tumors, there was no significant difference in the average tumor weight between groups A and B. The average tumor weight was less in groups C and D than that in groups A and B (P < 0.05). But the average tumor weight showed no difference in groups C and D (Figure 1B).

**Transcription of Mdr-1, Bcl-2, Bax, and caspase-3 in K562-n/VCR tumor by RT-PCR**

The transcription of Mdr-1 and Bcl-2 gene was significantly lower in group D than those in groups A, B, and C (group D vs groups A, B, C; P < 0.05). The transcription of Bax and caspase-3...
Discussion

In the present study, DNR-loaded MNPs-Fe₃O₄ could selectivity inhibit the growth of MDR leukemic K562-n/VCR cells that transplanted in nude mice when compared with DNR alone according to the average tumor weight (P < 0.05), while MNPs-Fe₃O₄ didn’t show any antitumor activity without DNR.

Many chemotherapeutic agents target intracellular organelles or molecules to achieve their anticancer activities. Effective chemotherapy thus requires an effectively high level of drug molecules to accumulate within the cancer cells. In addition, the effectiveness of chemotherapy is also correlated with drug exposure time. In P-gp overexpressing cells, it becomes a difficult task to maintain a high intracellular drug level for a reasonable length of time. Experimental studies have shown that the main antitumor effect of anthracyclines is correlated to the induction of apoptotic cells. Escape from apoptotic signals often accompanies MDR and tumor progression. In general, apoptosis may occur through specific apoptosis signaling pathways such as death receptors and mitochondria. However, the mitochondrial pathway plays a crucial role in anthracycline-related apoptosis, which is regulated by the Bcl-2 protein family. Bcl-2 family proteins consist of antiapoptotic and pro-apoptotic members. Previous reports have also documented that the ratio of antiapoptotic Bcl-2 to proapoptotic Bax protein determined, at least in part, the susceptibility of cells to a death signal, and are used as a predictive marker for therapeutic response to radiotherapy. It is well known that caspase-3 plays the central role in the initiation of apoptosis. Our study showed that DNR-loaded MNPs-Fe₃O₄ could decrease the expression of Bcl-2 and increase the expression of Bax and caspase-3 in K562-n/VCR tumors (P < 0.05), indicating that this therapy can overturn poor response and induce apoptosis of MDR K562-n/VCR cells to anticancer drugs in vivo. Our outcomes clearly indicate that a MNPs-Fe₃O₄ drug delivery system can facilitate DNR accumulation in MDR cells and enhance apoptosis in MDR cells.

It has been reported that DNR can interact with DNA in the presence of Fe (III) ions which hampers the recognition of DNA by transcription factors and then inhibits transcription. We also found that DNR-loaded MNPs-Fe₃O₄ could downregulate the transcription of mdr-1 gene. The downregulation of the transcription of mdr-1 gene may attribute to the relatively high DNR concentration in K562-n/VCR, since MNPs-Fe₃O₄ alone is not able to inhibit the mdr-1 gene transcription. On the contrary, there is no difference in the quantity of P-gp on K562-n/VCR tumors among the four groups. Therefore functionalized MNPs-Fe₃O₄ can enhance DNR accumulation inside MDR cells without interfering with the quantity of P-gp, a mechanism which differs from many types of P-gp modulators such as tetrandrine, PC833,
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and so on. Quantity or content, but not function, of P-gp can be detected by western blot. Studies have shown that some of the lipids and surfactants used in detergent-based formulations including Pluronic block copolymers and solid lipid nanoparticles possess intrinsic P-gp inhibitory activities. Looking at structural features, Pluronic block copolymers, solid lipid nanoparticles, and tetraheptylammonium-capped MNPs-Fe₃O₄ are similar in having hydrophobic segments. The literature suggests that nonionic surfactants may inhibit drug efflux transport through increased membrane fluidization, which induces changes in the conformation of P-gp and ATPase activity. It is reasonable to infer from enhanced DNR accumulation inside the MDR cells that hydrophobic tetraheptyl segments capped outside MNPs-Fe₃O₄ can incorporate DNR in the lipid membrane and induce changes in membrane structure. The interrelationship between the membrane fluidization and the suppression of P-gp ATPase activity can be better understood in view of the current picture of P-gp structure describing P-gp that contains two transmembrane domains (TMDs) and two nucleotide ATP-binding domains (NBDs). Structures of P-gp protein suggest that the two NBDs form a common binding site where the energy of ATP is harvested to promote efflux through a pore that is delineated by transmembrane helices. Proper interaction of these two ATP-binding sites is crucial for the proper functioning of P-gp. Therefore, the structural perturbations in the lipid membranes induced by MNPs-Fe₃O₄ may decrease the affinity of ATP to its binding site and interfere with the ATPase activity. Not only have MNPs-Fe₃O₄ the ability to block P-gp function, but they also have the potential for aggregation and drug capsulation. Nanoparticles loaded with anticancer drugs could readily approach the cell membrane, leading to drug concentrations at the cell surface higher than those obtained with the same amount of free drug solution, which leads in turn to higher intracellular drug concentrations. It has been reported that it is more difficult for P-gp to remove the drug molecules from the cells when these molecules are associated with nanoparticles. DNR-loaded MNPs-Fe₃O₄ complexes are likely to be too large to be handled by P-gp and are consequently “trapped” within the cancer cells once they gain entrance into the targeted cells. This is another possible mechanism responsible for enhanced cellular drug retention. These mechanisms may be the reason that MNPs-Fe₃O₄, which has no cytotoxicity to cells, is able to reverse MDR. Another important observation of the present work is that DNR-loaded MNPs-Fe₃O₄ fail to further suppress the growth of K562-n tumor cells. The advantages of nanoparticles over solution appear to be augmented by the increases in P-gp levels of the treated cells. This phenomenon is consistent with recent studies of polymer-lipid hybrid nanoparticle systems and Pluronic block copolymers. However, these mechanisms are not able to explain the remarkable selectivity of DNR-loaded MNPs-Fe₃O₄ complexes with respect to MDR cells. Since MNPs-Fe₃O₄ may not completely inhibit the P-gp function, DNR-loaded MNPs-Fe₃O₄ complexes are able to enhance

Figure 2 The transcription of Mdr-1, Bcl-2, Bax, and caspase-3 gene after treatment for 20 days detected by real time RT-PCR. A) negative control; B) MNPs-Fe₃O₄; C) DNR; D) MNPs-Fe₃O₄ + DNR. Notes: *P < 0.05, the transcription of mdr-1 was significantly lower in group D than that in groups A, B, and C; **P < 0.05, the transcription of Bcl-2 gene was significantly lower in group D than that in group A, B and C; ***P < 0.05, the transcription of caspase-3 was increased significantly in group D than that in group A, B and C.

Abbreviations: DNR, daunorubicin; MNPs-Fe₃O₄, magnetic nanoparticles of Fe₃O₄.
Our histological data show that the application of MNPs-Fe₃O₄ was well tolerated by nude mice. In conclusion, our results support the proposition that MNPs-Fe₃O₄ offers an attractive means of delivering DNR into MDR tumor cells and enhances apoptosis in the MDR cells without reducing P-gp protein in the cell membrane. DNR-loaded MNPs-Fe₃O₄ complex is insensitive to P-gp-mediated drug efflux and is more effective than free DNR in MDR cells. In any case, the DNR-loaded MNPs-Fe₃O₄ complex may target the Achilles' heel of MDR cells.

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