

Paclitaxel-Fe₃O₄ nanoparticles inhibit growth of CD138⁺ CD34⁺ tumor stem-like cells in multiple myeloma-bearing mice

Cuiping Yang^{1,3,*}Jing Wang^{2,*}Dengyu Chen^{1,*}Junsong Chen¹Fei Xiong⁴Hongyi Zhang¹Yunxia Zhang²Ning Gu⁴Jun Dou¹¹Department of Pathogenic Biology and Immunology, Medical School,²Department of Gynecology and Obstetrics, Zhongda Hospital, Southeast University, Nanjing,³Department of Pathogenic Biology and Immunology, School of Basic Medicine, Jiangxi University of Traditional Chinese Medicine, Nanchang,⁴School of Biological Science and Medical Engineering, Southeast University, Nanjing, People's Republic of China

*These authors contributed equally to this work

Background: There is growing evidence that CD138⁺ CD34⁺ cells may actually be tumor stem cells responsible for initiation and relapse of multiple myeloma. However, effective drugs targeted at CD138⁺ CD34⁺ tumor stem cells are yet to be developed. The purpose of this study was to investigate the inhibitory effect of paclitaxel-loaded Fe₃O₄ nanoparticles (PTX-NPs) on CD138⁺ CD34⁺ tumor stem cells in multiple myeloma-bearing mice.

Methods: CD138⁺ CD34⁺ cells were isolated from a human U266 multiple myeloma cell line using an immune magnetic bead sorting method and then subcutaneously injected into mice with nonobese diabetic/severe combined immunodeficiency to develop a multiple myeloma-bearing mouse model. The mice were treated with Fe₃O₄ nanoparticles 2 mg/kg, paclitaxel 4.8 mg/kg, and PTX-NPs 0.64 mg/kg for 2 weeks. Tumor growth, pathological changes, serum and urinary interleukin-6 levels, and molecular expression of caspase-3, caspase-8, and caspase-9 were evaluated.

Results: CD138⁺ CD34⁺ cells were found to have tumor stem cell characteristics. All the mice developed tumors in 40 days after injection of 1×10^6 CD138⁺ CD34⁺ tumor stem cells. Tumor growth in mice treated with PTX-NPs was significantly inhibited compared with the controls ($P < 0.005$), and the groups that received nanoparticles alone ($P < 0.005$) or paclitaxel alone ($P < 0.05$). In addition, the PTX-NPs markedly inhibited interleukin-6 secretion, increased caspase-8, caspase-9, and caspase-3 expression, and induced apoptosis of tumor cells in the treated mice.

Conclusion: PTX-NPs proved to be a potent anticancer treatment strategy that may contribute to targeted therapy for multiple myeloma tumor stem cells in future clinical trials.

Keywords: multiple myeloma, tumor stem cells, Fe₃O₄ nanoparticles, paclitaxel

Introduction

Understanding the cellular origins of cancer can help improve the prevention and treatment available for the disease. Multiple myeloma is a bone marrow cancer arising from either differentiated B cells or plasma cells, and interactions between clonal plasma cells and bone marrow microenvironments may lead to tumor-initiating cells. Although conventional and new therapies have improved patient survival, multiple myeloma remains incurable for the majority of patients due to relapses and disease progression.^{1,2} Currently, although many available anti-multiple myeloma strategies have been effective in targeting the bulk of cancer cells, it has been postulated that a tumor-initiating population or tumor stem cells persists in patients with multiple myeloma, which may be responsible for eventual relapses in these patients.^{2,3} Research has demonstrated that CD138⁺ CD34⁺ cells constitute a multiple myeloma cell

Correspondence: Jun Dou
Department of Pathogenic Biology and Immunology, Medical School, Southeast University, 87 Ding Jiaqiao Rd, Nanjing 210009, People's Republic of China
Tel +86 25 8327 2454
Fax +86 25 8327 2295
Email njdoujun@yahoo.com.cn

subpopulation which is a rich source of tumor-initiating cells with the properties of stem cells. This cell subpopulation has the capacity for high proliferation, self-renewal, colony formation, drug resistance, and strong tumorigenesis.⁴

Immunomodulatory drugs, including thalidomide and lenalidomide, the proteasome inhibitor, bortezomib, and other therapeutics are currently being assessed.⁵ However, the therapeutic effects of drugs in patients are associated with dose-dependent and duration-dependent side effects. To control hematological malignancy, further investigation of the molecular mechanisms of relapse is needed, along with development of targeted therapeutic drugs for treating CD138⁺ CD34⁺ tumor stem cells in patients with multiple myeloma. Rapid developments in nanoscience and nanotechnology have seen many studies of nanoparticles for drug delivery performed to enhance drug accumulation and to improve safety in cancer chemotherapy.⁶ In addition, chemotherapeutic agents loaded into nanoparticles have shown increased solubility and decreased toxicity, as well as an ability to protect against chemical and biological degradation.^{7–9} Because of these advantages, drugs loaded into nanoparticles can be considered potential agents for treating relapsed and refractory tumors.

Paclitaxel, extracted from the bark of the Pacific yew tree, has been one of the natural effective antineoplastic drugs identified in recent decades.⁹ Paclitaxel has been shown to be effective against a wide spectrum of malignancies, including ovarian cancer, breast cancer, glioma, and multiple myeloma.^{10–12} However, clinical utilization of paclitaxel has been difficult due to its poor solubility in water and in most pharmaceutical reagents. Cremophor® EL (BASF Corporation, Ludwigschafen, Germany) has been used as an adjuvant for paclitaxel in the clinic, but has been found to be responsible for many serious side effects when used in patients. Fortunately, Abraxane® (Celgene, Summit, NJ, USA), an albumin-paclitaxel nanoparticle, is an advanced formulation in which a lipophilic drug and human serum albumin are passed through a jet under high pressure to form nanoparticle albumin-bound paclitaxel-loaded Fe₃O₄ nanoparticles (PTX-NPs). Abraxane is water-soluble and was initially approved by the US Food and Drug Administration for the treatment of metastatic breast cancer, with indications now being extended to other tumors including pancreatic cancer, non-small cell lung carcinoma, melanoma, and head and neck cancer, often in combination with conventional chemotherapy.¹³

Nanoparticles may provide an alternative solution to this problem by providing controlled and targeted delivery of paclitaxel with better efficacy and fewer side effects.^{10,14–16}

In this study, we developed PTX-NPs to improve the therapeutic effect of paclitaxel on multiple myeloma CD138⁺ CD34⁺ tumor stem cells in mice with nonobese diabetic/severe combined immunodeficiency (NOD/SCID). Our novel agent, PTX-NPs, when used in the treatment of multiple myeloma tumor stem cells, resulted in significant inhibition of tumor growth and obvious tumor cell apoptosis in multiple myeloma-bearing mice. The results were attributable to the enhanced permeation and retention effect of paclitaxel. Our current study also demonstrates that PTX-NPs are an effective strategy for the treatment of cancer that should be explored further for treating multiple myeloma in the clinic.

Materials and methods

Cell line

A human U266 multiple myeloma cell line was purchased from the Cell Institute in Beijing, People's Republic of China. The cells were cultured in complete medium consisting of RPMI 1640, 2 mM L-glutamine, 50 U/mL penicillin, 50 µg/mL streptomycin, and 10% fetal bovine serum.

Animals

NOD/SCID mice aged 5 weeks and weighing 16 ± 2 g were purchased from the Shanghai Laboratory Animal Center, People's Republic of China. All the mice were maintained in a specific pathogen-free facility on a 12-hour light/dark cycle and were fed sterile food. The environmental temperature was maintained at 22°C with a 40%–50% relative humidity level. The animal experiments were performed in compliance with the Guidelines of the Animal Research Ethics Board of Southeast University. Full details of approval of the study can be found in the approval ID: 20080925.

Preparation of PTX-NPs

To develop the PTX-NPs, we synthesized oleic acid-coated iron oxide nanoparticles (Fe₃O₄@OA nanoparticles) using a two-step method reported elsewhere.¹⁷ Briefly, the Fe₃O₄@OA nanoparticles were dissolved in tetrahydrofuran at a ratio of 1:5 (w/w). The mixture was then added slowly into a 1% polyoxyethylene-polyoxypropylene copolymer solution under sonication; the resulting suspension was first stirred to evaporate tetrahydrofuran, and then filtered and lyophilized. The drug loaded into the PTX-NPs was 10.71 wt%. The morphology of the PTX-NPs after rehydration was examined using a transmission electron microscope (JEM-2000EX, JEOL Ltd, Tokyo, Japan). The hydrodynamic diameter, size distribution, and zeta potential of the PTX-NPs were measured by dynamic light scattering (Zetasizer 3000, Malvern Instruments, Worcestershire, UK).

In vitro drug release from PTX-NPs

We studied the kinetics of paclitaxel release from the PTX-NPs as follows: 1 mL of a PTX-NP sample was put into a dialysis bag (8000–10,000 molecular weight cutoff, Sigma, St Louis, MO, USA) and immersed in 100 mL of pH 7.4 phosphate buffer with 0.5% Tween-80; the mixture was kept at 37°C and stirred for 96 hours. Subsequently, at 2, 6, 12, 24, 48, and 96 hours, a 1 mL aliquot of dialysis medium was removed and replaced with the same volume of fresh buffer. The concentration of paclitaxel in the sample withdrawn was determined by high-pressure liquid chromatography.

Stability of PTX-NPs

The stability of the rehydrated PTX-NPs was evaluated by the change in particle size in solution. At 0, 1, 4, 8, 12, and 24 hours after being rehydrated with water, the particle size of the PTX-NP in solution was measured in triplicate with a photon correlation light scattering particle sizer (3000HS, Malvern Instruments, Worcestershire, UK).

Isolation of CD138⁺ CD34⁺ multiple myeloma cells

A subpopulation of CD138⁺ CD34⁺ cells was isolated from the human U266 multiple myeloma cell line using a magnetic activated cell sorting method (Miltenyi Biotec, Gladbach, Germany). The CD138⁺ subpopulation was first isolated using the mouse antihuman CD138 antibody coupled with magnetic microbeads (Miltenyi Biotec); this was followed by magnetic column selection or depletion, and the resulting cells were depleted of CD34⁺ subsets using the mouse antihuman CD34 antibody coupled with magnetic microbeads (Miltenyi Biotec). We named CD138⁺CD34⁺ cells for the multiple myeloma stem-like cells (CD138⁺CD34⁺ cells).^{4,18}

Proliferative and clonal assays of CD138⁺ CD34⁺ cells

Next, 2×10^3 CD138⁺ CD34⁺ cell suspensions were seeded into 96-well plates and were assayed for proliferative activity in triplicate wells. To test for cell viability, the CD138⁺ CD34⁺ cell suspensions were mixed with 0.4% Trypan blue (Sigma) after 1, 2, 3, 4, 5, and 6 days of incubation; mean values of the viable counts were obtained by a hemocytometer chamber.¹⁹ In a colony-forming assay in soft agar medium, we followed the protocol described in our previous report.¹⁸ Colony efficiency was determined as the number of colonies formed

divided by the total number of cells plated. Non-CD138⁺ CD34⁺ cells were used in the assays as controls.

Drug resistance assay

We then seeded 1×10^5 CD138⁺ CD34⁺ or non-CD138⁺ CD34⁺ cells into 96-well plates with 5 µg of vincristine (100 µL/well) for 24 hours, and this resistance assay was repeated three times.

Migration and invasion assays

We resuspended 2×10^5 multiple myeloma tumor stem cells or non-CD138⁺ CD34⁺ cells in complete medium and seeded them into the upper compartments of polycarbonate Transwell® two-chamber migration plates (Costar®, Corning Inc, Corning, NY, USA). The total medium volume in the lower compartments in a six-well plate was 600 µL. After 24 hours of incubation, the number of cells that had migrated through the membrane to the lower chamber was determined using a blood cell counting board.^{20,21} A cell invasion assay was performed for both the CD138⁺ CD34⁺ and non-CD138⁺ CD34⁺ cells using a chamber with monolayer-coated polyethylene terephthalate membranes (six-well insert, pore size 8 µm, Becton Dickinson, Franklin Lakes, NJ, USA) and matrigel-coated membrane inserts (BD Biosciences, Bedford, MA, USA). Detailed procedures for this can be found in an already published paper.²²

Murine multiple myeloma xenograft model and treatment protocol

The NOD/SCID mice were inoculated subcutaneously in the flank with the 1×10^6 CD138⁺ CD34⁺ multiple myeloma cells. About 40 days after inoculation, the tumors were felt by touch in the mice. Twelve tumor-bearing mice were randomly divided into four groups, with three mice per group. The treatment protocol was as follows: the control mice were treated with normal saline; the group of mice receiving nanoparticles alone were treated with nanoparticles at a dose of 2 mg/kg; the group of mice receiving paclitaxel alone were treated with paclitaxel 4.8 mg/kg; the group of mice receiving PTX-NPs were treated with PTX-NPs 0.64 mg/kg on a once-weekly schedule, with 0.2 mL administered on each occasion for 2 weeks. The tumor volume was assessed twice a week for each mouse using the following formula²³ before the mouse was sacrificed:

$$\text{Volume} = (1/2) \times (\text{length}) \times (\text{width})^2$$

Whole blood and xenograft tumors were harvested and processed for serum interleukin-6 detection, histopathologic analysis, and determination of caspase protein expression.

Histopathologic analysis of tumors

Hematoxylin and eosin staining

After the xenograft tumors were harvested, the tumors were fixed immediately in 4% paraformaldehyde, dehydrated in a graded series of alcohol, and then embedded in paraffin. Tumor sections 0.5 mm thick were cut and stained with hematoxylin and eosin for microscopic examination. All sections were observed in a blinded fashion, and were photographed using a 400× normal light microscope.²⁴

Immunohistochemistry

The immunohistochemistry assay was done using the standard avidin-biotin complex peroxidase method on formalin-fixed, paraffin-embedded sections of tumor tissue excised from xenografts after treatment. The tumor sections were subjected to terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling (TUNEL, Roche) staining or were stained with Bcl-2 (Bio-World, Dublin, OH, USA) and Bax (Bio-World), and viewed under a light microscope. A brown color indicated apoptotic nuclei, and were visualized using a 3,3'-diaminobenzidine substrate. Apoptotic cells were counted under a light microscope (400× magnification) in randomly chosen fields.²⁵

Enzyme-linked immunosorbent assay of serum interleukin-6

The serum and urine interleukin-6 levels in the treated multiple myeloma-bearing mice were measured with a human interleukin-6 precoated enzyme-linked immunosorbent assay kit (Dakewe Biotech, Shenzhen, People's Republic of China) following the manufacturer's protocol. The detection was performed in triplicate.²⁶

Western blot assays

Samples of tumor tissue weighing 0.1 g were collected from each mouse and homogenized for detection of caspase-3, caspase-8, and caspase-9. Briefly, the tissues were pulped with liquid nitrogen and lysed in a protein extraction buffer (Novagen, Madison, WI, USA) according to the manufacturer's protocol; the proteins were separated using sodium dodecyl sulfate-polyacrylamide gel electrophoresis. The membrane was blocked with the buffer containing 10% fat-free dry milk; a rabbit antihuman GAPDH antibody (1:5000), a rabbit antihuman caspase-3 antibody (1:500), a rabbit antihuman caspase-8 antibody (1:500), or a rabbit antihuman caspase-9 antibody (1:500) was used as the primary antibody overnight, and was subsequently incubated with a secondary horseradish peroxidase-conjugated

antirabbit antibody (1:1000) for one hour at room temperature. All the antibodies used in the study were ordered from Bio-World. The bands were detected using an enhanced chemiluminescence system.^{26,27}

Statistical analysis

All the experimental data were expressed as the mean ± standard deviation and analyzed using GraphPad prism software (GraphPad Software Inc, San Diego, CA, USA). $P < 0.05$ was considered to be statistically significant.

Results

Identification of CD138⁺ CD34⁺ tumor stem cells in human U266 cell line

It is known that cell proliferative activity and clone formation ability may reflect a self-renewal capacity, which is a characteristic of tumor stem cells.^{3,28} CD138⁺ CD34⁺ cells isolated from the human U266 cell line demonstrated higher proliferation ability after 6 days of culture ($18,964 \pm 786$ cells versus $12,102 \pm 723$ cells, $P < 0.01$, Figure 1A) and stronger clone formation potency after 14 days of culture ($26.5\% \pm 4.03\%$ versus $7.2\% \pm 3.86\%$, $P < 0.01$, Figure 1B and C) than the non-CD138⁺ CD34⁺ cells. After 72 hours of exposure to 5 μg of vincristine, the CD138⁺ CD34⁺ cells showed a maximum survival rate of $80.2\% \pm 3.22\%$ as well as significantly higher drug resistance than the non-CD138⁺ CD34⁺ cells ($33.2\% \pm 4.25\%$, $P < 0.01$), as shown in Figure 1D. The CD138⁺ CD34⁺ cells also exhibited greater migration and invasion activity (Figure 1E and F) as well as stronger tumorigenicity in NOD/SCID mice (Figure 1G) than the non-CD138⁺ CD34⁺ cells. The differences were statistically significant ($P < 0.01$). These findings suggest that the U266 cell line contained tumor stem-like cells, and that the U266 multiple myeloma cell phenotypes lacked molecular expression of CD138 and CD34 on their surface.

Physicochemical properties of PTX-NPs

Transmission electron microscopic images showed that the PTX-NPs were about 7.02 nm in size (Figure 2A). Figure 2B shows the hydrodynamic diameter of the PTX-NPs to be 65.1 nm, as detected by dynamic light scattering. Sustained drug-release behavior is shown in Figure 2C, with only $50.93\% \pm 2.02\%$ of paclitaxel being released from PTX-NPs at 96 hours. In addition, the lyophilized PTX-NPs powder was stable for over 2 years. After rehydration, the change in size of the PTX-NPs over 24 hours was very slight (from 64.0 ± 2.16 nm to 72.5 ± 2.9 nm, Figure 2D), indicating

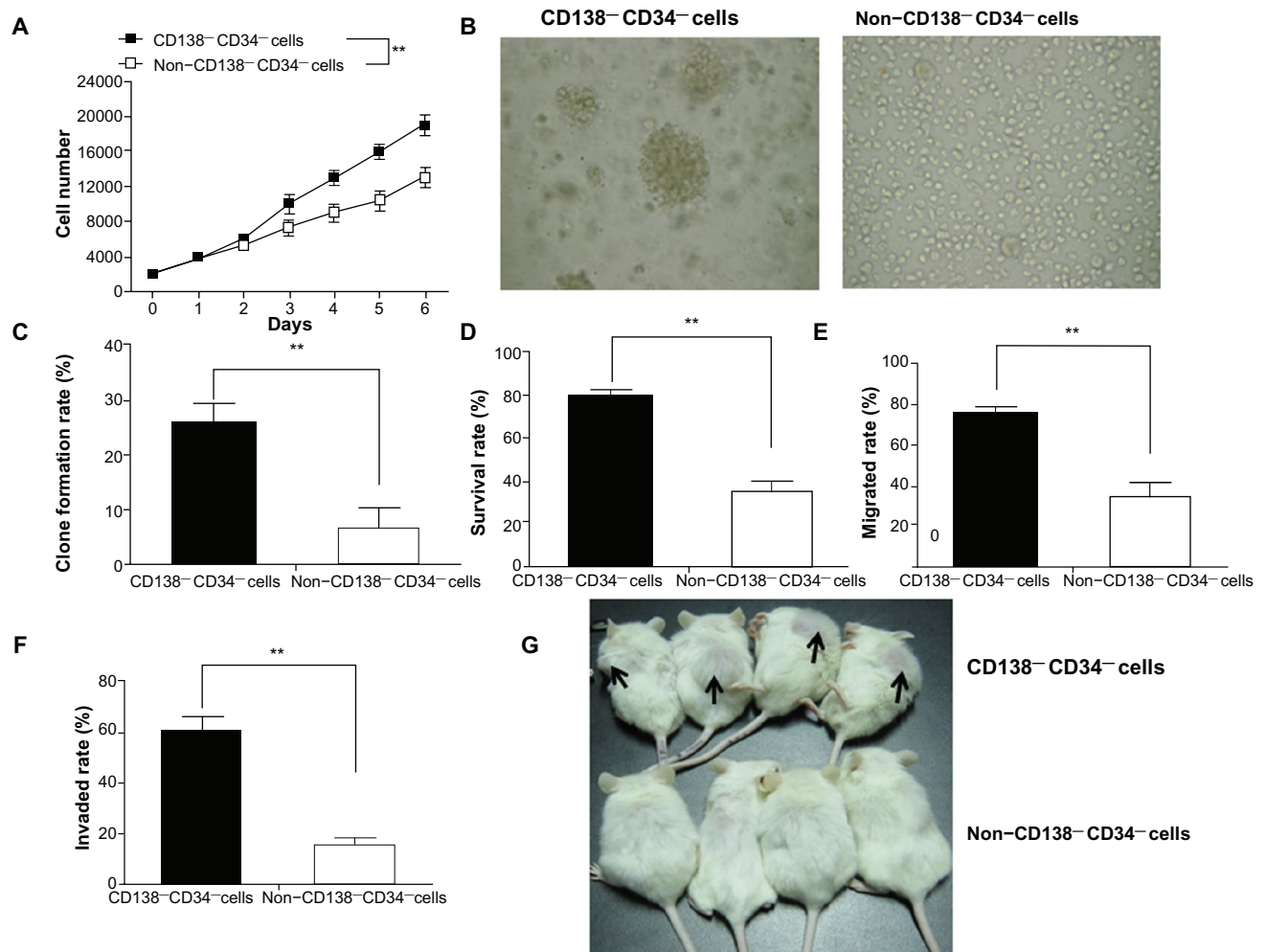


Figure 1 CD138⁺ CD34⁻ cell characteristics. **(A)** Proliferation potency in common medium. **(B)** Clonogenicity capability in soft agar medium with 15 day culture. **(C)** Clone formation rate. **(D)** Survival rate after culture of cells with vincristine 5 μ g in 96-well plate for 72 hours. **(E)** Cell migration rate. **(F)** Cell invasion rate. **(G)** Images (arrows) of tumors in NOD/SCID mice injected with 1×10^6 human U266 CD138⁺ CD34⁻ multiple myeloma cells and non-CD138⁺ CD34⁻ cells after 70 days.

Note: ** $P < 0.01$.

Abbreviations: NPs, Fe₃O₄ nanoparticles; PTX, paclitaxel; PTX-NPs, paclitaxel-loaded Fe₃O₄ nanoparticles.

excellent stability and suitability for clinical application. Figure 2E shows a schematic representation of PTX-NPs.

PTX-NPs inhibited tumor stem cell growth in multiple myeloma-bearing mice

All mice inoculated with 1×10^6 CD138⁺ CD34⁻ multiple myeloma cells developed tumors that were palpable in around 40 days. Figure 3A shows the dynamic state of palpable tumor growth in mice treated with various agents and observed for up to 90 days. The control mice, the mice receiving nanoparticles alone, and the mice receiving paclitaxel alone were sacrificed after 50 days, 55 days, and 75 days, respectively, due to excessive tumor growth. Figure 3B shows the sizes of the tumors excised from the sacrificed mice treated with the different agents. The tumor sizes were significantly smaller in the PTX-NP group than in the control group ($P < 0.005$),

the group receiving nanoparticles alone ($P < 0.01$), and the group receiving paclitaxel alone ($P < 0.05$, Figure 3B). These results suggest that PTX-NPs had obvious inhibitory effects on tumor growth in the CD138⁺ CD34⁻ multiple myeloma-bearing mouse model.

Mechanism for inhibition of CD138⁺ CD34⁻ tumor stem cell growth

To clarify the functional mechanism of action of PTX-NPs on CD138⁺ CD34⁻ tumor stem cells in this multiple myeloma-bearing mouse model, we performed a series of assays. In view of the critical role of interleukin-6 in the growth and survival of multiple myeloma cells,^{29–31} we first examined the effects of nanoparticles alone, paclitaxel alone, and PTX-NPs, respectively, on the secretion of interleukin-6 in mouse serum and urine. Figure 4 shows that the group given

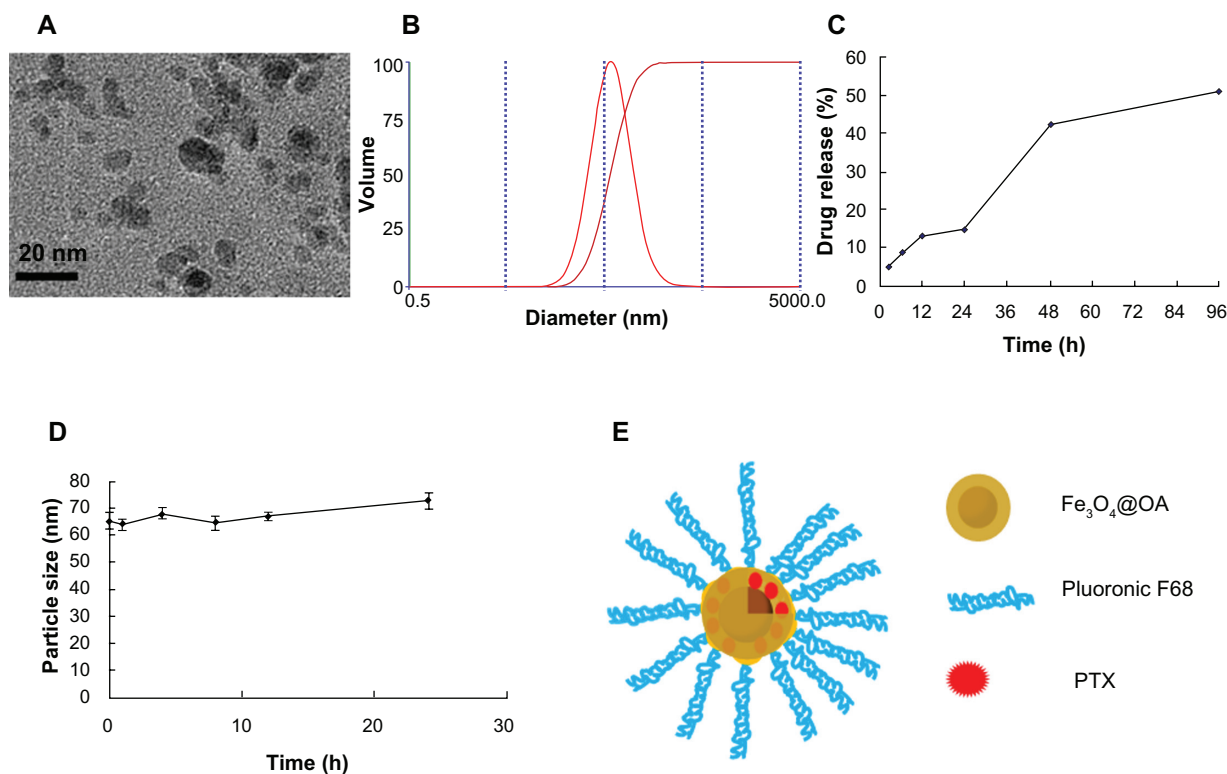


Figure 2 Transmission electron microscopic images of PTX-NPs and paclitaxel release from these particles. **(A)** Nanoparticles in the transmission electron microscopic images were 7.02 nm in size. **(B)** Mean hydrodynamic diameter of 65.1 nm for reconstituted PTX-NPs measured by dynamic light scattering. **(C)** Kinetics of paclitaxel release from PTX-NPs. **(D)** Stability of drug-loaded nanoparticles. **(E)** Schematic representation of PTX-NPs.

Abbreviation: PTX-NPs, paclitaxel-loaded Fe_3O_4 nanoparticles.

PTX-NPs and the group given paclitaxel alone had obviously suppressed secretion of interleukin-6 in comparison with the control group and the group that received nanoparticles alone. The group given PTX-NPs showed the strongest inhibition of interleukin-6 secretion in serum and urine.

We next analyzed the effect of PTX-NPs on apoptosis of CD138⁺ CD34⁺ multiple myeloma stem cells in vivo by hematoxylin and eosin and immunohistochemistry staining. Figure 5A shows histopathological images of cells in tumor tissue with nuclear damage, an incomplete cell membrane, and pink cytoplasm (indicated by dark arrows). Changes in karyopyknosis and karyorrhexis could be found in the apoptotic cells, particularly in tumor tissue from mice treated with the PTX-NPs; few apoptotic cells were observed in the controls and the group receiving nanoparticles only.³² Furthermore, the immunohistochemistry staining results indicated that paclitaxel and the PTX-NPs significantly increased TUNEL-positive cells, representing apoptotic tumor cells (Figure 5B), downregulated expression of Bcl-2, an apoptosis-suppressing molecule (Figure 5C), and upregulated expression of Bax, an apoptosis-promoting molecule (Figure 5D). This efficacy was markedly apparent in the PTX-NP group, as shown in the histograms in Figure 5.

Western blotting results in Figure 6 further confirm that the PTX-NPs were more potent for inducing tumor cell apoptosis in multiple myeloma-bearing mice than the nanoparticles or paclitaxel alone. This was because the PTX-NPs enhanced expression of the caspase-8 and caspase-9 initiators as well as expression of the caspase-3 executioner, and led to apoptosis (Figure 6A). The relative intensity of expression of these proteins is shown in Figure 6B.

Discussion

Studies of multiple myeloma have improved our understanding of human carcinogenesis and pioneered clinic applications accordingly. Because of the potential role of tumor stem cells in the pathogenesis of multiple myeloma, scientists have strived to find new anti-multiple myeloma agents targeting tumor stem cells.³³ Given the ability of tumor stem cells to initiate malignancy through self-renewal, identifying multiple myeloma stem cells and designing a therapy directly targeted to tumor stem cells is an important strategy for successful treatment of multiple myeloma.^{4,33} To this end, we focused on multiple myeloma stem cells in our current study by identifying tumor stem-like cells, called tumor stem cells, in a cultured human U266 cell line, and by carrying out

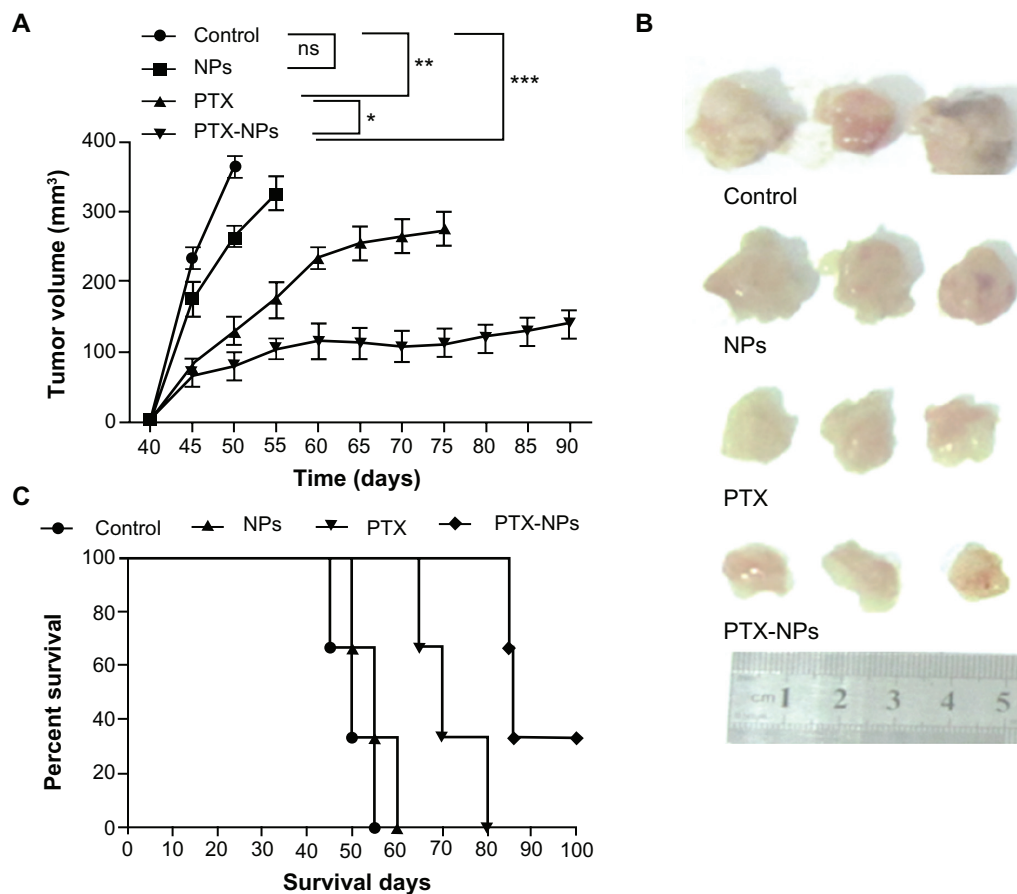


Figure 3 Relative tumor volumes and tumor images. (A) In around 40 days, palpable tumors in the NOD/SCID mice inoculated with 1×10^6 U266 CD138⁺ CD34⁺ cells were treated with normal saline, Fe₃O₄ nanoparticles alone (2 mg/kg), paclitaxel alone (4.8 mg/kg), and PTX-NPs 0.64 mg/kg, respectively, according to a schedule of once a week, 0.2 mL on each occasion for 2 weeks. (B) Tumor sizes for mice treated with various agents. (C) The PTX-NPs group mice had a longer survival time compared with the other groups. **Notes:** * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$.

Abbreviations: ns, no statistical significance; NPs, Fe₃O₄ nanoparticles; PTX, paclitaxel; PTX-NPs, paclitaxel-loaded Fe₃O₄ nanoparticles.

therapeutic experiments to assess the effect of PTX-NPs in inhibiting growth of CD138⁺ CD34⁺ tumor stem cells in a multiple myeloma-bearing mouse model.

The encouraging findings of our study demonstrate that CD138⁺ CD34⁺ cells are the multiple myeloma tumor

stem-like cells in the human U266 cell line. Compared with the non-CD138⁺ CD34⁺ cells, the CD138⁺ CD34⁺ cells exhibited such characteristics as stronger proliferation and colony formation ability, more migration and invasion activity, stronger drug resistance to the anticancer agent

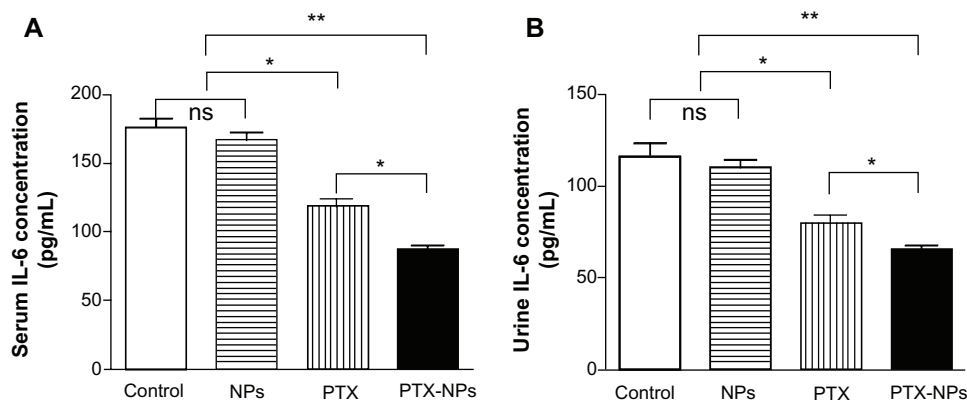


Figure 4 IL-6 concentration detected by enzyme-linked immunosorbent assay. (A) Serum IL-6 concentration. (B) Urinary IL-6 concentration.

Note: * $P < 0.05$; ** $P < 0.01$.

Abbreviations: IL-6, interleukin-6; NPs, Fe₃O₄ nanoparticles; PTX, paclitaxel; PTX-NPs, paclitaxel-loaded Fe₃O₄ nanoparticles.

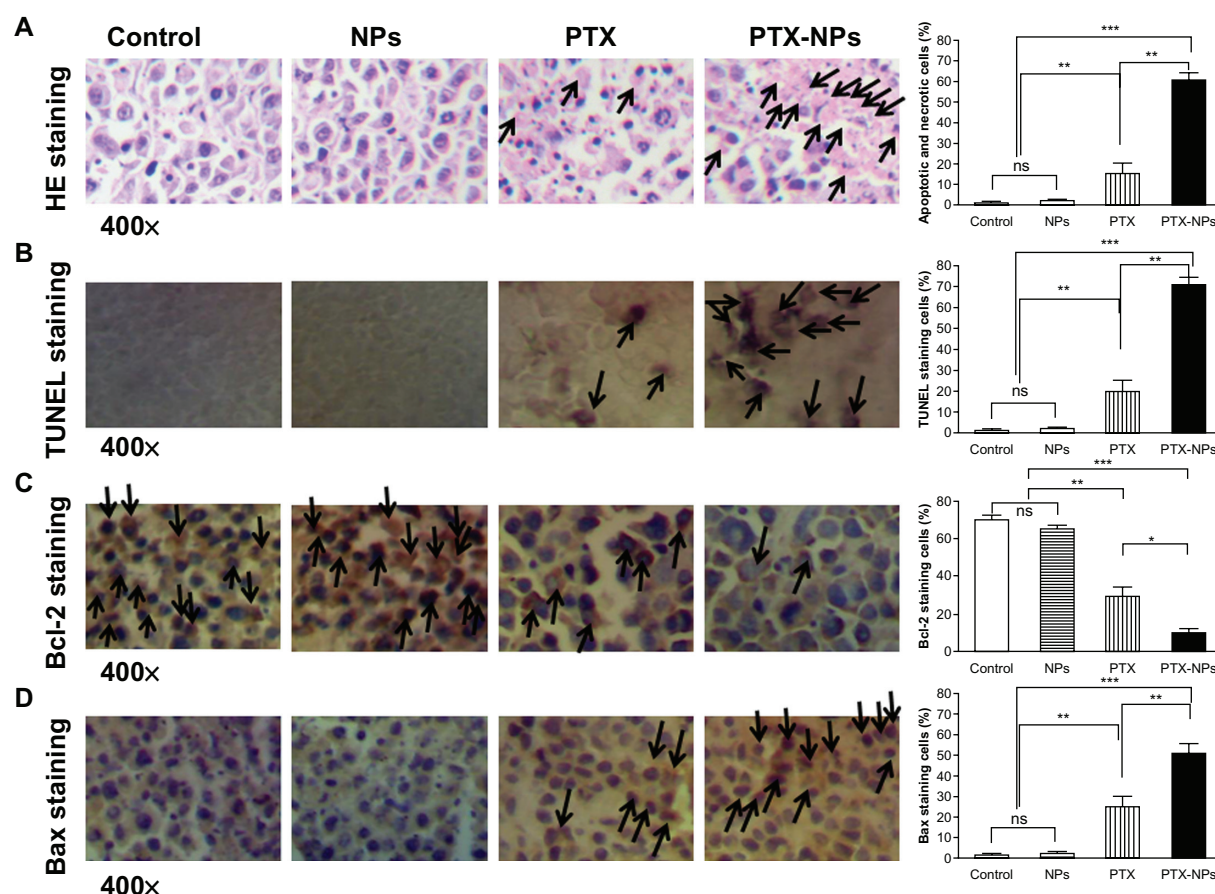


Figure 5 Representative histopathologic changes in tumor tissue in mice after treatment (400x). (A) Hematoxylin and eosin staining, (B) TUNEL staining by immunohistochemistry, (C) Bcl-2 staining by immunohistochemistry, and (D) Bax staining by immunohistochemistry.

Notes: * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$.

Abbreviations: ns, no statistical significance; NPs, Fe_3O_4 nanoparticles; PTX, paclitaxel; PTX-NPs, paclitaxel-loaded Fe_3O_4 nanoparticles; HE, hematoxylin and eosin; TUNEL, terminal deoxynucleotidyl transferase-mediated dUTP nick end labeling.

vincristine in vitro, and more powerful tumorigenic potential in NOD/SCID mice.

The therapeutic results in Figure 3 show a markedly smaller tumor cell volume in mice treated with PTX-NPs than in the control mice or those treated with nanoparticles alone or paclitaxel alone. Regarding the therapeutic efficacy of PTX-NPs in the multiple myeloma bearing-mouse model, we wanted to know their mechanism of action in vivo. Human U266 multiple myeloma cells produce interleukin-6, which acts as a growth factor and an antiapoptotic factor in multiple myeloma.^{29,34} Therefore, suppression of interleukin-6 secretion is a possible mechanism for the inhibition of multiple myeloma growth in mice. We found that interleukin-6 secretion was indeed inhibited in mice treated with PTX-NPs, and this was reflected in serum and urinary levels of interleukin-6. In addition, although the dose of paclitaxel in the PTX-NP group was only 1.3% of the dose used for the group receiving paclitaxel alone, the PTX-NPs clearly showed

a greater capacity to inhibit IL-6 secretion than paclitaxel alone ($P < 0.05$). Immunohistochemistry staining cells from mice in the PTX-NP group showed more Bax and TUNEL-staining cells but fewer Bcl-2 staining cells than did the controls or mice receiving paclitaxel alone or nanoparticles alone, which is consistent with our histopathological results. The PTX-NPs were found to be capable of downregulating Bax and Bcl-2 expression and improving tumor cell sensitivity to paclitaxel therapy to speed up tumor cell apoptosis in the treated mice. These results also suggest that Fe_3O_4 nanoparticles do not have a direct biological effect on $\text{CD}138^+ \text{CD}34^-$ tumor stem cell growth or apoptosis; instead, Fe_3O_4 nanoparticles enabled adequate drug delivery to improve chemotherapeutic efficacy and reduce drug toxicity.

It has been demonstrated that apoptotic cell death can occur via the mitochondrial and death receptor pathways, and that an apoptotic death stimulus results in activation of caspases, with caspase-3, caspase-8, and caspase-9 being

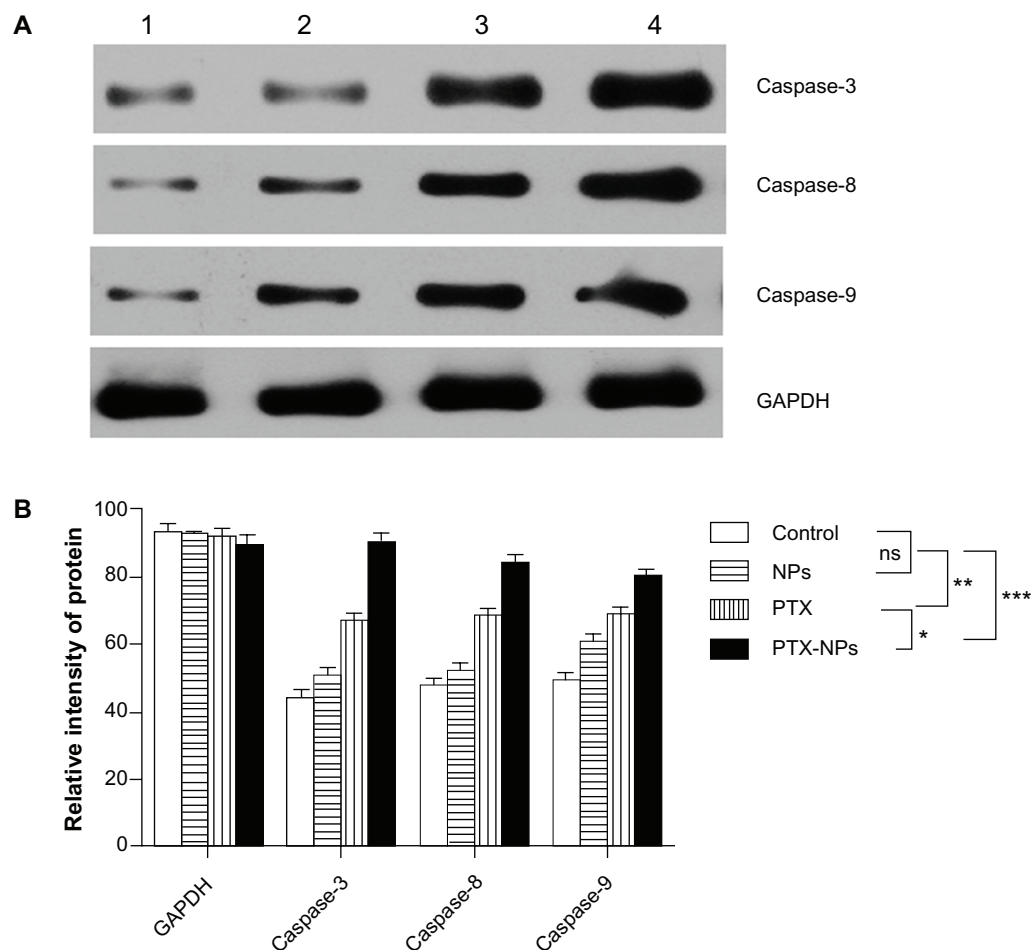


Figure 6 Expression of apoptosis-related proteins in multiple myeloma-bearing mouse model after treatment. **(A)** After treatment, mouse tumor tissues were homogenized and analyzed for expressions of caspase-8, caspase-9, and caspase-3, by Western blotting. GAPDH served as a loading control. **(B)** Relative intensity of protein expression. **Notes:** * $P < 0.05$; ** $P < 0.01$; *** $P < 0.005$.

Abbreviation: ns, no statistical significance.

situated at pivotal junctions in apoptotic pathways.^{35,36} Therefore, in this study, we detected caspase-3, caspase-8, and caspase-9 protein expression levels by Western blotting (Figure 6). We did not find a significant difference in the relative intensity of protein expression between the control group and the group that received nanoparticles alone; however, caspase-3, caspase-8, and caspase-9 protein expression was found to be markedly increased in the groups receiving paclitaxel only and PTX-NP, and especially in the PTX-NP group when compared with the control group and the group that received nanoparticles alone. It is known that caspase-8 and caspase-9 are activated via the death receptor and mitochondrial signaling pathways during apoptosis, whereas caspase-3 is a common effector of both signaling pathways.^{37,38} Therefore, we speculate that PTX-NPs might induce apoptosis of CD138⁺ CD34⁺ tumor stem cells via both caspase-dependent receptor and mitochondrial apoptotic pathways.

Conclusion

In this study, we demonstrated the promising activity of PTX-NPs as a novel treatment strategy for multiple myeloma stem cells in a mouse model. The strong contribution of PTX-NPs to the therapeutic efficacy is seen in the finding that PTX-NPs induced CD138⁺ CD34⁺ tumor stem cell apoptosis, thereby inhibiting multiple myeloma stem cell growth in mice treated with PTX-NPs. Fe₃O₄ nanoparticles might extend drug release and enhance the effect of paclitaxel on CD138⁺ CD34⁺ multiple myeloma stem cells in vivo. This study provides experimental data which are relevant for future clinical studies of PTX-NPs in the treatment of multiple myeloma.

Acknowledgments

This work was supported by the 973 National Nature Science Foundation of People's Republic of China (2011CB933500), the Graduate Student Research and Innovation Program of

Jiangsu Province (CXZZ0174), and the Scientific Research Foundation of Graduate School of Southeast University (YBJJ1222).

Disclosure

The authors report no potential conflicts of interest in this work.

References

- Agarwal JR, Matsui W. Multiple myeloma: a paradigm for translation of the cancer stem cell hypothesis. *Anticancer Agents Med Chem*. 2010;10:116–120.
- Vicente-Dueñas C, Romero-Camarero I, González-Herrero I, et al. A novel molecular mechanism involved in multiple myeloma development revealed by targeting MafB to haematopoietic progenitors. *EMBO J*. 2012;31:3704–3717.
- Jakubikova J, Adamia S, Kost-Alimova M, et al. Lenalidomide targets clonogenic side population in multiple myeloma: pathophysiologic and clinical implications. *Blood*. 2011;117:4409–4419.
- Matsui W, Huff CA, Wang Q, et al. Characterization of clonogenic multiple myeloma cells. *Blood*. 2004;103:2332–2336.
- Laubach J, Richardson P, Anderson K. Multiple myeloma. *Annu Rev Med*. 2011;62:249–264.
- Martin W. Natural and synthetic polymers as inhibitors of drug efflux pumps. *Pharm Res*. 2008;25:500–511.
- Gindy ME, Prud'homme RK. Multifunctional nanoparticles for imaging, delivery and targeting in cancer therapy. *Expert Opin Drug Deliv*. 2009;6:865–878.
- Haley B, Frenkel E. Nanoparticles for drug delivery in cancer treatment. *Urol Oncol*. 2008;26:57–64.
- Peer D, Karp JM, Hong S, Farokhzad OC, Margalit R, Langer R. Nanocarriers as an emerging platform for cancer therapy. *Nat Nanotechnol*. 2007;2:751–760.
- Seo K, Chung SW, Byun Y, Kim D. Paclitaxel loaded nano-aggregates based on pH sensitive polyaspartamide amphiphilic graft copolymers. *Int J Pharm*. 2012;424:26–32.
- Weeden C, Hartlieb KJ, Lim LY. Preparation and physicochemical characterization of a novel paclitaxel-loaded amphiphilic aminocalixarene nanoparticle platform for anticancer chemotherapy. *J Pharm Pharmacol*. 2012;64:1403–1411.
- Letourneau I, Quinn MC, Wang LL, et al. Derivation and characterization of matched cell lines from primary and recurrent serous ovarian cancer. *BMC Cancer*. 2012;12:379.
- Elsadek B, Kratz F. Impact of albumin on drug delivery – new applications on the horizon. *J Control Release*. 2012;157:4–28.
- Qiu B, Sun X, Zhang D, Wang Y, Tao J, Ou S. TRAIL and paclitaxel synergize to kill U87 cells and U87-derived stem-like cells in vitro. *Int J Mol Sci*. 2012;13:9142–9156.
- Feng SS, Mu L, Win KY, Huang G. Nanoparticles of biodegradable polymers for clinical administration of paclitaxel. *Curr Med Chem*. 2004;11:413–424.
- Oh KS, Lee S, Na JH, et al. Blood-pool multifunctional nanoparticles formed by temperature-induced phase transition for cancer-targeting therapy and molecular imaging. *Int J Pharm*. 2012;437:192–202.
- Guan N, Liu C, Sun D, Xu J. A facile method to synthesize carboxyl-functionalized magnetic polystyrene nanospheres. *Colloids Surf A*. 2009;335:174–180.
- Dou J, Pan M, Wen P, et al. Isolation and identification of cancer stem like cells from murine melanoma cell lines. *Cell Mol Immunol*. 2007;4:467–472.
- Marañón C, Planelles L, Alonso C, López MC. HSP70 from *Trypanosoma cruzi* is endowed with specific cell proliferation potential leading to apoptosis. *Int Immunol*. 2000;12:1685–1693.
- Holt RU, Fagerli UM, Baykov V, et al. Hepatocyte growth factor promotes migration of human myeloma cells. *Haematologica*. 2008;93:619–622.
- Cirstea D, Hideshima T, Rodig S, et al. Dual inhibition of Akt/mammalian target of rapamycin pathway by nanoparticle albumin-bound rapamycin and perifosine induces antitumor activity in multiple myeloma. *Mol Cancer Ther*. 2010;9:963–975.
- Ponnusamy MP, Lakshmanan I, Jain M, et al. MUC4 mucin-induced epithelial to mesenchymal transition: a novel mechanism for metastasis of human ovarian cancer cells. *Oncogene*. 2010;29:5741–5754.
- Hu W, Wang J, Dou J, et al. Augmenting therapy of ovarian cancer efficacy by secreting IL-21 human umbilical cord blood stem cells in nude mice. *Cell Transplant*. 2011;20:669–680.
- Yu F, Wang J, Dou J, et al. Nanoparticle-based adjuvant for enhanced protective efficacy of DNA vaccine Ag85A-ESAT-6-IL-21 against *Mycobacterium tuberculosis* infection. *Nanomedicine*. 2012;8:1337–1344.
- Brown CO, Salem K, Wagner BA, et al. Interleukin-6 counteracts therapy-induced cellular oxidative stress in multiple myeloma by up-regulating manganese superoxide dismutase. *Biochem J*. 2012;444:515–527.
- Hu K, Dou J, Yu F, et al. An ocular mucosal administration of nanoparticles containing DNA vaccine pRSC-gD-IL-21 confers protection against mucosal challenge with herpes simplex virus type 1 in mice. *Vaccine*. 2011;29:1455–1462.
- He X, Wang J, Zhao F, et al. Antitumor efficacy of viable tumor vaccine modified by heterogenous ESAT-6 antigen and cytokine IL-21 in melanomatous mouse. *Immunol Res*. 2012;52:240–249.
- Gou S, Liu T, Wang C, et al. Establishment of clonal colony-forming assay for propagation of pancreatic cancer cells with stem cell properties. *Pancreas*. 2007;34:429–435.
- Stephens OW, Zhang Q, Qu P, et al. An intermediate-risk multiple myeloma subgroup is defined by siL-6r: levels synergistically increase with incidence of SNP rs2228145 and 1q21 amplification. *Blood*. 2012;119:503–512.
- Lin L, Benson DM Jr, DeAngelis S, et al. A small molecule, LLL12 inhibits constitutive STAT3 and IL-6-induced STAT3 signaling and exhibits potent growth suppressive activity in human multiple myeloma cells. *Int J Cancer*. 2012;130:1459–1469.
- Guo Y, Xu F, Lu T, Duan Z, Zhang Z. Interleukin-6 signaling pathway in targeted therapy for cancer. *Cancer Treat Rev*. 2012;38:904–910.
- Ren Y, Zhang H, Chen B, et al. Multifunctional magnetic Fe₃O₄ nanoparticles combined with chemotherapy and hyperthermia to overcome multidrug resistance. *Int J Nanomedicine*. 2012;7:2261–2269.
- Matsui W. Perspective: a model disease. *Nature*. 2011;480:S58.
- Bommert K, Bargou R, Stuhmer T. Signaling and survival pathways in multiple myeloma. *Eur J Cancer*. 2006;42:1574–1580.
- Reed JC, Pelliccia M. Apoptosis-based therapies for hematologic malignancies. *Blood*. 2005;106:408–418.
- Gogvadze V, Orrenius S. Mitochondrial regulation of apoptotic cell death. *Chem Biol Interact*. 2006;163:4–14.
- Tinel A, Tschoep J. The PIDDosome, a protein complex implicated in activation of caspase-2 in response to genotoxic stress. *Science*. 2004;304:843–846.
- Kasibhatla S, Jessen KA, Maliartchouk S, et al. A role for transferrin receptor in triggering apoptosis when targeted with gambogic acid. *Proc Natl Acad Sci U S A*. 2005;102:12095–12100.

International Journal of Nanomedicine**Dovepress****Publish your work in this journal**

The International Journal of Nanomedicine is an international, peer-reviewed journal focusing on the application of nanotechnology in diagnostics, therapeutics, and drug delivery systems throughout the biomedical field. This journal is indexed on PubMed Central, MedLine, CAS, SciSearch®, Current Contents®/Clinical Medicine,

Journal Citation Reports/Science Edition, EMBase, Scopus and the Elsevier Bibliographic databases. The manuscript management system is completely online and includes a very quick and fair peer-review system, which is all easy to use. Visit <http://www.dovepress.com/testimonials.php> to read real quotes from published authors.

Submit your manuscript here: <http://www.dovepress.com/international-journal-of-nanomedicine-journal>