ORIGINAL RESEARCH

Effect of magnetic Fe_3O_4 nanoparticles with 2-methoxyestradiol on the cell-cycle progression and apoptosis of myelodysplastic syndrome cells

Guohua Xia[1,2]
Baoan Chen[1,2]
Jiahua Ding[1,2]
Chong Gao[1,2]
Huixia Lu[1,2]
Zeye Shao[1,2]
Feng Gao[1,2]
Xuemei Wang[2,3]

[1]Department of Hematology, Zhongda Hospital, Medical School, Southeast University, Nanjing, People's Republic of China; [2]Faculty of Oncology, Medical School, Southeast University, Nanjing, People's Republic of China; [3]National Key Laboratory of Bioelectronics (Chien-Shiung Wu Laboratory), Southeast University, Nanjing, People's Republic of China

Correspondence: Baoan Chen
Department of Hematology, Zhongda Hospital, Medical School, Southeast University, Nanjing 210009, People's Republic of China
Tel +86 25 8327 2006
Fax +86 25 8327 2011
Email cba8888@hotmail.com

Abstract: This study aims to evaluate the potential benefit of combination therapy of 2-methoxyestradiol (2ME) and magnetic nanoparticles of Fe_3O_4 (MNPs-Fe_3O_4) on myelodysplastic syndrome (MDS) SKM-1 cells and its underlying mechanisms. The effect of the unique properties of tetraheptylammonium-capped MNPs-Fe_3O_4 with 2ME on cytotoxicity was tested by 3-(4,5-dimethylthiazol-2-yl)-2,5- diphenyltetrazolium bromide assay. Cell-cycle distribution and apoptosis were assessed by flow cytometry. The expression of cell-cycle marker protein was measured by Western blotting. Growth inhibition rate of SKM-1 cells treated with the 2ME-loaded MNPs-Fe_3O_4 was enhanced when compared with 2ME alone. 2ME led to an increase of caspase-3 expression, followed by apoptosis, which was significantly increased when combined with an MNPs-Fe_3O_4 carrier. Moreover, the copolymer of 2ME with MNPs-Fe_3O_4 blocked a nearly two-fold increase in SKM-1 cells located in G_2/M phase than in 2ME alone, which may be associated with an accompanying increase of p21 as well as a decrease in cyclin B1 and cdc2 expression, but there was no obvious difference between the MNPs-Fe_3O_4 and control group. These findings suggest that the unique properties of MNPs-Fe_3O_4 as a carrier for 2ME, a new anticancer agent currently in clinical trials, may be a logical strategy to enhance the therapeutic activity of MDS.

Keywords: MDS, MNPs-Fe_3O_4, SKM-1 cell, cell cycle

Introduction

Myelodysplastic syndromes (MDS) are a group of clonal hematopoietic stem-cell disorders characterized by ineffective hematopoiesis, peripheral blood cytopenias, and a propensity to transform to acute myeloid leukemia.[1] Although new treatments for MDS are emerging, allogeneic stem-cell transplantation offers the only possible cure.[2] Thus, there is an overwhelming need for new agents with more benign toxicity profiles and improved activity for the treatment of MDS.

The estrogen metabolite 2-methoxyestradiol (2ME) is a highly potent anticancer agent that effectively induces apoptosis in several cell lines, and several different proposed mechanisms have been investigated in vitro and in vivo.[3–9] Notably, 2ME is not dependent on estrogen receptor binding, and has been found to be more toxic to leukemia cells than to their normal hematopoietic counterparts.[10,11] However, up to now, 2ME-triggered cellular events are still not fully understood.

Nowadays, cancer therapy, particularly with respect to drug delivery, has evolved from traditional methodology.[12] Nanotechnologies and nanoparticles are becoming a focus for human medical application for their unique properties.[13] The diagnostic and therapeutic applications of magnetic iron oxide nanoparticles such as drug delivery,

magnetic resonance imaging, hyperthermia techniques, cell separation, and tissue repair have expanded enormously in recent decades.[14] The application of drug-coated polymer nanospheres and nanoparticles to inhibit the related drug resistance has attracted much attention. The authors' earlier studies as well as the studies of others have shown that magnetic nanoparticles of Fe_3O_4 (MNPs-Fe_3O_4), as an anticancer drug deliverer,[15] could enhance the sensitivity of anticancer drugs and reverse the drug resistance of tumor cells.[16] Given that 2ME targets tumor cells specifically and possesses promising clinical activity, albeit with low bioavailability, the authors of this present paper have explored a novel strategy to inhibit MDS cells by combining the unique properties of tetraheptylammonium-capped MNPs-Fe_3O_4 with 2ME.

Materials and methods
Main materials
Iron chloride hexagydrate, iron sulfate heptahydrate, and ammonium hydroxide were supplied by Lingfeng Chemical Reagent Co Ltd (Shanghai, China); MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) and 2ME, by Sigma (St Louis, MO); Annexin V-FITC apoptosis detection kit, by Becton Dickinson (Franklin Lakes, NJ); BCA protein assay kit, by Biosynthesis Biotechnology Co Ltd (Beijing, China); anti-cyclin B1, anti-cdc2, anti-active caspase-3, and anti-p21, by Cell Signaling Technology Inc (Danvers, MA); anti-β-actin, by Santa Cruz Biotechnology (Santa Cruz, CA); RPMI 1640, by Gibco BRL (Gaithersburg, MD); and fetal bovine serum (FBS), by Sijiqing Co (Hangzhou, China).

Preparation of 2ME-loaded MNPs-Fe_3O_4
MNPs-Fe_3O_4 was produced by electrochemical deposition under oxidizing conditions in a 0.1 M tetraheptylammonium 2-propanol solution. The deposited clusters were capped with tetraheptylammonium, which acts as a stabilizer of the colloidal nanocrystallites.[17] 2ME was dissolved in absolute ethanol to give a 20 mM solution, and the final concentration of ethanol in the medium of 2ME-treated cells was adjusted to 0.1% (v/v).[18] Before applied in this experiment, the prepared MNPs-Fe_3O_4 were well distributed in RPMI 1640 medium containing 10% (v/v) heat-inactivated FBS by using ultrasound treatment in order to obtain an MNPs-Fe_3O_4 colloidal suspension. 2ME was conjugated with MNPs-Fe_3O_4 at the molar ratio of 100:1 by mechanical absorption polymerization at 4°C for 12 hours, as previously reported.[16]

Characteristic of MNPs-Fe_3O_4
To observe the particle size and morphology of MNPs-Fe_3O_4, the sample was dispersed in deionized water and measured using a JEM-2100 transmission electron microscope (JEOL, Tokyo, Japan).

Cell line and cell culture
SKM-1, an MDS cell line, was from the Japanese Collection of Research Bioresources Cell Bank.[19] The cells were cultured in RPMI-1640 containing 10% FBS, 100 U/mL penicillin and 100 µg/mL streptomycin in an environment of saturated humidity, 5% CO_2, and 37°C. The cells in the logarithmic growth phase were used in all experiments.

Cell proliferation assay
As described previously,[20] 1×10^5 SKM-1 cells per well were incubated with either 2ME alone or loaded with MNPs-Fe_3O_4. After incubation for 12, 24, 48, and 72 hours, a 20 µL MTT solution (5 mg/mL) was added to each well at 37°C in the dark for at least 4 hours. Thereafter, the formazan crystals were solubilized in 200 µL dimethyl sulfoxide in every well, and the reduction of MTT was quantified using a Model 550 microplate reader (Bio-Rad Laboratories, Tokyo, Japan). The inhibition ratio of cells was determined as follows $(1 - A_{test}/A_{control}) \times 100\%$.

Annexin V-PI assays for apoptosis
Cells were stained and evaluated for apoptosis by flow cytometry. After treatment for 24 hours as described above, SKM-1 cells were collected and washed twice with PBS, suspended in 200 µL binding buffer and 10 µL Annexin V-FITC for 20 minutes in the dark, and thereafter, 300 µL binding buffer and 5 µL propidium iodide (PI) were added to each sample. The apoptotic cells were determined using a flow cytometer (Becton Dickinson) with CellQuest (Becton Dickinson) software.

Analysis of cell cycle distribution
As described before, cells were harvested, washed with ice-cold PBS, and stained with 50 µg/mL PI and 250 µg/mL RNase for 30 minutes. The percentage of cells in each phase of the cell cycle was determined with a computer-programmed ModFit LT2.0 DNA assay (Becton Dickinson) using a flow cytometer.

Western blot analysis
Western blot assay was performed as previously described.[16] Briefly, total protein was isolated from the harvested cells

and measured using BCA protein assay kit. Samples containing 25 μg proteins were subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis and transferred to a nitrocellulose membrane. Nonspecific binding sites were blocked for 1 hour with 5% nonfat milk. The blots were stained with anti-p21, cyclin B1, cdc2 (1:200), or caspase-3 (1:400) antibodies overnight at 4°C, and then with a secondary horseradish peroxidase-conjugated goat antimouse antibody (1:5000) for 1 hour at room temperature. The blots were visualized by enhanced chemiluminescence (ECL system, Amersham, UK), and β-actin was used as the internal control.

Statistical analysis

All data were presented as mean ± standard deviation in triplicate, and all analyses were performed with SPSS software (v 11.5; SPSS Inc, Chicago, IL). Statistical significance and differences observed between experimental groups were determined using Student's t-test. $P < 0.05$ was considered significant.

Results

Characteristics of MNPs-Fe₃O₄

The majority of MNPs-Fe₃O₄ were spherical, and particle sizes were 15.9 ± 4.6 nm (Figure 1).

Inhibition of cell proliferation

2ME significantly inhibited the growth of SKM-1 cells in a time- and dose-dependent manner (Figure 2). Notably, when 2ME was loaded with MNPs-Fe₃O₄, the cell inhibition rate was increased ($P < 0.05$); however, MNPs-Fe₃O₄ alone
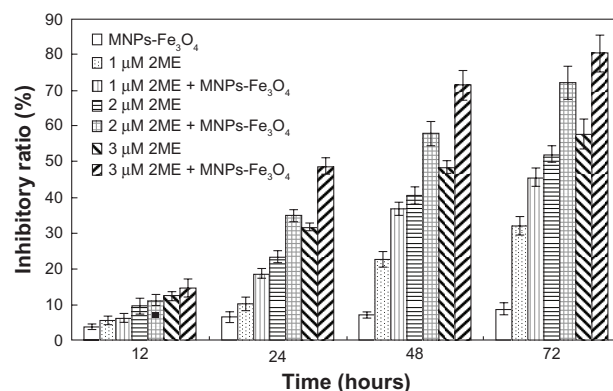
Figure 2 Proliferation inhibitory ratios of SKM-1 cells incubated with either 2ME alone or loaded with MNPs-Fe₃O₄ (100:1) for 12, 24, 48, and 72 hours.
Abbreviations: MNPs-Fe₃O₄, magnetic nanoparticles of Fe₃O₄; 2ME, 2-methoxyestradiol.

did not generate significant cytotoxicity compared with the control group.

Induction of apoptosis

The percentage of apoptotic cells treated with 2ME alone or loaded with MNPs-Fe₃O₄ at 24 hours were higher than that in the control group. Notably, the apoptotic rate of the copolymer was two-fold compared with 2ME alone ($P < 0.05$); however, there was no significant difference between the MNPs-Fe₃O₄ and control groups ($P > 0.05$) (Figure 3).

Distribution of cell cycle

Treatment of SKM-1 cells with 2ME alone for 24 hours resulted in a shift of cell distribution into the G₂/M phase compared with the control group; interestingly, when the 2ME was loaded with MNPs-Fe₃O₄, the number of cells
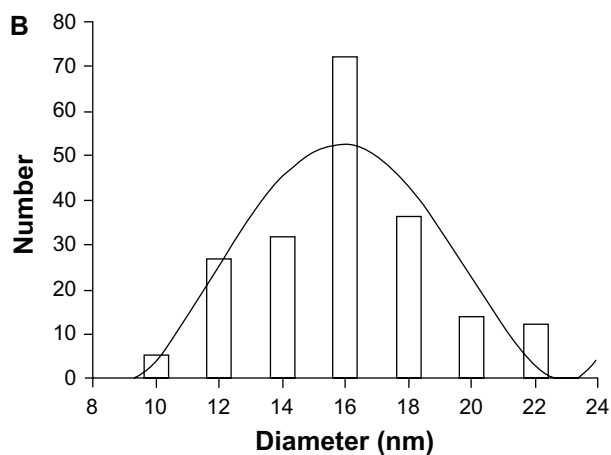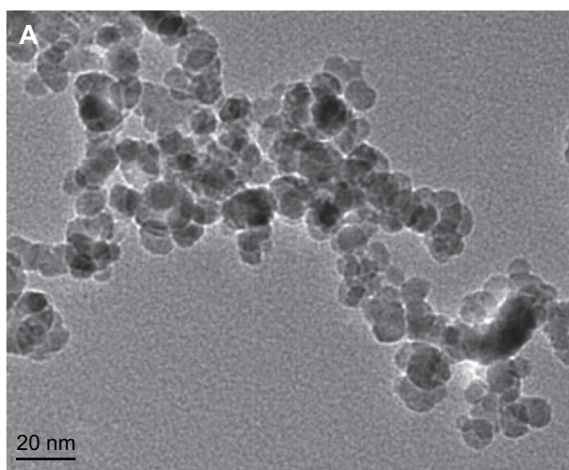
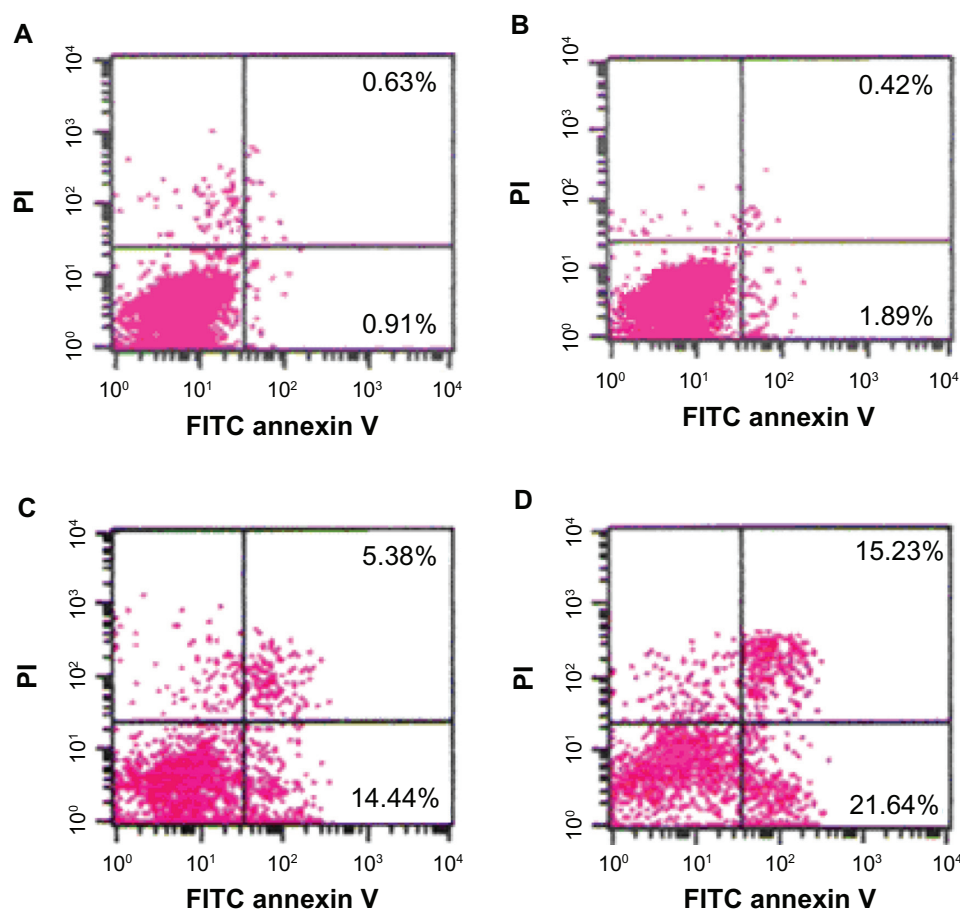**Figure 1** Image (**A**) and mean size (**B**) of magnetic nanoparticles of Fe₃O₄.

Figure 3 Apoptosis of SKM-1 cells treated with either 2ME alone or loaded with MNPs-Fe$_3$O$_4$ (100:1) for 24 hours. (**A**) Control. (**B**) MNPs-Fe$_3$O$_4$. (**C**) 2 μM 2ME. (**D**) Copolymer of 2 μM 2ME with MNPs-Fe$_3$O$_4$ (100:1).
Abbreviations: MNPs-Fe$_3$O$_4$, magnetic nanoparticles of Fe$_3$O$_4$; 2ME, 2-methoxyestradiol; PI, propidium iodide; FITC, fluorescein isothiocyanate.

in the G$_2$/M phase was increased from 34.9% ± 2.8% and 49.3% ± 3.1% to 70.8% ± 4.8% and 79.2% ± 5.1% for 1 and 2 μM 2ME, respectively, and in the G$_1$ phase was decreased from 32.6% ± 2.5% and 21.0% ± 1.7% to 12.2% ± 1.1% and 6.3% ± 1.5% for 1 and 2 μM 2ME, respectively, but there was no significant difference between MNPs-Fe$_3$O$_4$ and control group ($P > 0.05$; Figure 4).

Expression of cell cycle proteins

The expressions of cdc2 and cyclin B1 in SKM-1 cells treated with 2ME for 24 hours were slightly downregulated compared with the control group, and the decrease was even more apparent when combined with MNPs-Fe$_3$O$_4$ ($P < 0.05$). In addition, the level of p21 was consistently increased after treatment with 2ME, and the increase was further augmented by addition of MNPs-Fe$_3$O$_4$ ($P < 0.05$). Similar results for caspase-3 were observed; conversely, there was no obvious difference between MNPs-Fe$_3$O$_4$ and the control group ($P > 0.05$) (Figure 5).

Discussion

Recent exciting data suggest that nanomaterials have been successfully manipulated to create a new drug-delivery system that can not only solve the problem of poor water solubility of most promising currently available anticancer drugs but also reduce toxic side effects and, thereby, increase their effectiveness. MNPs-Fe$_3$O$_4$, one of the most promising biocompatible materials, is feasible to produce, easy to functionalize, and not only shows satisfactory water solubilization and degradation in vivo but also improves the sensitivity of anticancer drugs.[16,21] In our study, the majority of synthesized MNPs-Fe$_3$O$_4$ were spherical and the average diameter size was 15.9 ± 4.6 nm, which is suitable for biological applications. Wang et al[17] demonstrate that the unique magnetic nanoparticles have a higher surface-to-volume ratio and a relatively smaller size, which could allow faster movement and easier entry into cells. Therefore, MNPs-Fe$_3$O$_4$ may offer an exciting nanomaterial toward developing an effective drug-delivery system for solving the solubility of 2ME.
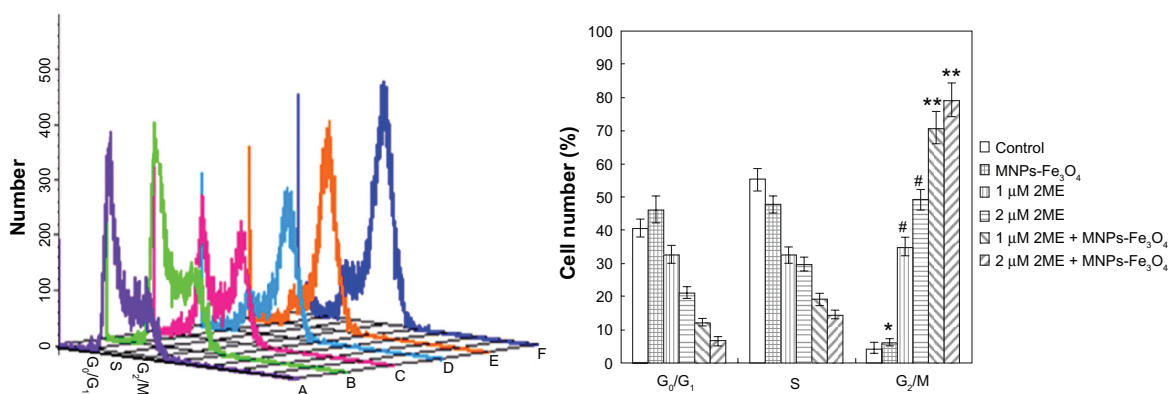
Figure 4 Cell-cycle distributions after treatment with either 2ME alone or loaded with MNPs-Fe$_3$O$_4$ (100:1) for 24 hours. (**A**) Control group. (**B**) MNPs-Fe$_3$O$_4$ alone. (**C**) 1 µM 2ME. (**D**) 2 µM 2ME. (**E**) Copolymer of 1 µM 2ME with MNPs-Fe$_3$O$_4$ (100:1). (**F**) Copolymer of 2 µM 2ME with MNPs-Fe$_3$O$_4$ (100:1).
Notes: *$P > 0.05$, when compared with the control group; #$P < 0.05$, when compared with MNPs-Fe$_3$O$_4$ and control groups; **$P < 0.05$, when compared with 2ME alone.
Abbreviations: MNPs-Fe$_3$O$_4$, magnetic nanoparticles of Fe$_3$O$_4$; 2ME, 2-methoxyestradiol.

It is noteworthy that 2ME is considered to be a promising anticancer agent with several advantages in chemoprevention as well as therapy.[9,10,22] As we know, after the adsorption of the nanoparticles on the cellular membrane, the uptake occurs via several possible mechanisms such as pinocytosis, nonspecific or receptor-mediated endocytosis, or phagocytosis.[23] In our study, 2ME inhibited cell growth significantly in a dose- and time-dependent manner, which is consistent with studies in multiple myeloma.[24] Interestingly, the growth inhibition rate was augmented by the MNPs-Fe$_3$O$_4$, while there was no significant influence on SKM-1 cells by MNPs-Fe$_3$O$_4$ alone. Moreover, literature reports that the chemotherapeutic drug-coated poly (methacrylate) nanosphere composite could be internalized by an endocytotic process, and this process can cause higher intracellular

drug accumulation.[17] Thus, we infer that MNPs-Fe$_3$O$_4$ can increase the sensitivity of SKM-1 cells to 2ME via altering the pharmacokinetics of 2ME.

Cell growth is tightly regulated by cell-cycle checkpoints. It is worth noting that 2ME has been shown to block many human hematologic cell lines[25] and other cells[3] in the G$_2$/M phase in vitro. In our study, 2ME resulted in a shift of cell distribution into the G$_2$/M phase, and this shift was enhanced by MNPs-Fe$_3$O$_4$, while some other researchers reported that 2ME arrested cells in G$_1$/S,[9] or both G$_1$/S and G$_2$/M.[5] These opposing results suggest that there may be fundamental differences between cell types. Our data indicated that the copolymer of 2ME with MNPs-Fe$_3$O$_4$ results in cell-cycle block in the G$_2$/M phase, thereby decelerating proliferation; further studies should be investigated to establish whether the 2ME-induced accumulation of SKM-1 cells is due to a specific block in G$_2$ or in the M phase.

Evidence in the literature suggests that the progression through the cell cycle is regulated by different types of cyclins and several different cyclin-dependent kinases (CDKs),[26] and the activities of CDKs are responsible for phosphorylation of downstream targets, while the cyclin-dependent kinase inhibitors are the key players in the negative regulation of the cell cycle in response to a wide variety of antiproliferative signals. Among several CDK inhibitors, p21 is one of the important inhibitory proteins in the G$_2$/M checkpoint, which can inactivate the cdc2–cyclinB1 complex, thus evoking cell cycle arrest.[27] The results of this present study showed that 2ME decreased the expression of cyclin B1 and cdc2, whereas it induced an increase in expression of p21, and this effect was augmented by the carrier of MNPs-Fe$_3$O$_4$. These data and other studies incuding Barboule et al[3] suggest that p21 is
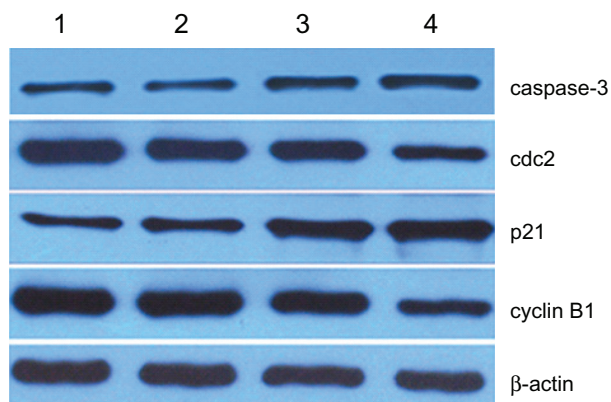

Figure 5 Expression of cell-cycle marker protein in SKM-1 cells treated with 2ME alone or loaded with MNPs-Fe$_3$O$_4$ (100:1) for 24 hours.
Notes: Column 1, control; column 2, MNPs-Fe$_3$O$_4$; column 3, 2 µM 2ME; column 4, the copolymer of 2 µM 2ME with MNPs-Fe$_3$O$_4$ (100:1).
Abbreviations: MNPs-Fe$_3$O$_4$, magnetic nanoparticles of Fe$_3$O$_4$; 2ME, 2-methoxyestradiol.

involved in the cyclin–cdc machinery during the G_2/M-phase cell-cycle arrest in response to the copolymer of 2ME with MNPs-Fe_3O_4 in SKM-1 cells.

The authors of this paper are investigating the molecular mechanisms involved in mediating 2ME-induced cell apoptosis. The results showed that SKM-1 cells underwent typical apoptotic changes as indicated by flow cytometry, which is consistent with previous trials.[4–8] Caspase-3 is a critical mediator of apoptosis, which is responsible for the proteolytic cleavage of many key proteins such as the nuclear enzyme poly (ADP- ribose) polymerase (PARP).[28] 2ME in this study increased the expression of caspase-3, and the increase was further augmented by MNPs-Fe_3O_4, implicating the role of active caspase-3 in SKM-1 cells. It is well established that p21 is not only involved in cell-cycle arrest, but also involved in the induction of apoptosis.[3,6,29] 2ME induced the increase of p21 in this study, which is inconsistent with the results of Zhou et al,[4] suggesting that it depends on the cellular context whether p21 participates in regulating apoptotic function in response to 2ME. Therefore, we cannot exclude the possibility that some other mechanism participates in the 2ME-induced apoptosis. Overall, the results of this study revealed that the copolymer of 2ME with MNPs-Fe_3O_4 which prolongs cell-cycle arrest, can lead to cell death, mediated by the activation of the intrinsic pathway of apoptosis.

Conclusion

These results indicate that the unique properties of MNPs-Fe_3O_4 as a carrier for 2ME, a new anticancer agent currently in clinical trials, may be a logical strategy to enhance therapeutic activity of MDS.

Acknowledgments

Disclosure

The authors have no conflicts of interest to report in this work.

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