

Magnetic nanoparticle of Fe_3O_4 and 5-bromotetrandrin interact synergistically to induce apoptosis by daunorubicin in leukemia cells

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Abstract: Apoptosis is a common pathway that finally mediated the killing functions of anticancer drugs, which is an important cause of multidrug resistance (MDR). The aim of this study was to investigate the potential benefit of combination therapy with magnetic nanoparticle of Fe_3O_4 (MNP(Fe_3O_4)) and 5-bromotetrandrin (BrTet). Analysis of the apoptosis percentage showed that combination of daunorubicin (DNR) with either MNP(Fe_3O_4) or BrTet exerted a potent cytotoxic effect on K562/A02 cells, while MNP(Fe_3O_4) and BrTet cotreatment can synergistically enhance DNR-induced apoptosis. Importantly, we confirmed that the distinct synergism effect of that composite on reverse multidrug resistance may owe to the regulation of various proliferative and antiapoptotic gene products, including P53 and caspase-3. Thus our *in vitro* data strongly suggests a potential clinical application of MNP(Fe_3O_4) and BrTet combination on CML.

Keywords: K562/A02 leukemic cells, multidrug resistance, magnetic nanoparticle of Fe_3O_4 , 5-bromotetrandrin, apoptosis, P53, caspase-3

Introduction

The primary cause of treatment failures in leukemia is the emergence of multidrug resistance (MDR).^{1,2} Most chemotherapy agents are used in the treatment of hematologic malignancies cells by inducing apoptosis; the resistance of leukemic cells to chemotherapy-induced apoptosis remains the most significant problem in the treatment of leukemia.^{3,4} To reverse the resistance mechanism and reduce side effects during chemotherapy, a promising approach is to combine a conventional chemotherapy with new strategies inducing leukemia cell apoptosis. The possible solutions may include using drug delivery systems to enhance the related drug delivery efficiency to cancer cells and chemosensitizers to inhibit ATP-binding cassette (ABC) transporters.⁵ In order to achieve this goal, we chose the application of magnetic nanoparticle of Fe_3O_4 (MNP(Fe_3O_4)) and 5-bromotetrandrin (BrTet) to sensitize MDR cells in acute leukemia.

Traditional strategies towards the tissue-specific drug delivery utilize cytotoxic drugs attached to targeting moieties.⁶ Recently, many polymer nanospheres and nanoparticles have been introduced as drug delivery systems to enhance the related drug delivery efficiency to cancer cells based on the ability to target specific locations in the body.⁷ Perhaps the most promising materials related to diagnosis and treatment of cancer are magnetic nanoparticles. Their magnetic properties enable these particles to be used in numerous applications⁸ such as being guided or held in place by means of a magnetic field and heated in a magnetic field to trigger drug release or to produce hyperthermia of tissue. Fe_3O_4 magnetic nanoparticles, a kind of biocompatible nanomaterial that is feasible to produce, characterize, and easily functionalize, are

widely used for targeted-drug carriers with target-orientation and sustained-release properties because of their chemical stability, low toxicity, and superparamagnetic qualities.⁹ Some reports have demonstrated the synergistic effect of MNP(Fe_3O_4) with the anticancer drug daunorubicin (DNR) on the drug accumulation of leukemia cells.¹⁰ The diameter of MNP(Fe_3O_4) manufactured by Biological Science College of Southeast University is 20–30 nm, which is appropriate for reversal of MDR.¹¹

Tetrandrine (Tet), a bisbenzylisoquinoline alkaloid isolated from the Chinese herb “Hanfangji” (*Stephania tetrandra* Radix), holds great promise as a MDR modulator for the treatment of P-glycoprotein (P-gp)-mediated MDR cancers¹² which have no apparent effect on doxorubicin pharmacokinetics in mice.¹³ Moreover, it is relatively well tolerated in these patients with poor risk acute myeloid leukemia (AML).¹⁴ 5-Bromotetrandrine (BrTet), a bromized derivative of tetrandrine (Tet), shows more significant MDR reversal activity *in vitro* and *in vivo* related to the inhibition of P-gp overexpression and the increase in intracellular accumulation of anticancer drugs.^{15,16}

The present studies were undertaken to evaluate the ability of MNP(Fe_3O_4) and BrTet to sensitize MDR cells to anticancer agents *in vitro* and determine their combination effects on apoptosis induced by combining DNR in leukemic cells. Flow cytometry studies demonstrated that MNP(Fe_3O_4) and/or BrTet could enhance DNR-induced apoptosis in K562/A02 cells. Moreover, DNA gel electrophoresis revealed that the effects of MNP(Fe_3O_4) and BrTet combining with small dose chemotherapeutic agents were different from the effects of large dose chemotherapeutic agents on K562/A02 cells which induced necrosis. Importantly, by measuring the expressions of apoptosis-related gene and proteins, we found a distinct synergistic effect of that composite on reverse MDR may owe to the regulation of various proliferative and antiapoptotic gene products, including P53 and caspase-3, thus demonstrating the potential for using MNP(Fe_3O_4) and BrTet as a more sufficient and less toxic anti-MDR method.

Materials and methods

Main reagents

Adriamycin (ADM; Hisun Pharmaceutical Co., Zhejiang, China) and DNR (Main Luck Pharmaceuticals Inc., Shenzheng, China) stock solution 2 mg/ml were prepared with 0.01 mol/l phosphate buffer saline (PBS) (PH 7.4). BrTet (Kanghong, Chengdou, China) was diluted with 0.01 mol/l PBS (PH 7.4). Fe_3O_4 nanoparticles (State Key Lab of

Bioelectronics, Chien-Shiung Wu Laboratory, Southeast University, Nanjing, China) were well distributed in RPMI 1640 medium (Gibco/BRL, Carlsbad, CA, USA) containing 10% (v/v) heat-inactivated new-born calf serum (Sijiqing, Hangzhou, China) by using ultrasound treatment in order to obtain MNP(Fe_3O_4) colloidal suspension. DNR conjugated with MNP(Fe_3O_4) were prepared by mechanical absorption polymerization at 4 °C for 48 h as previously reported.¹¹

Cell lines and culture conditions

Human leukemia cell line K562 and its adriamycin-selected Pgp-overexpressing subline K562/A02 were obtained from the Institute of Hematology, Chinese Academy of Medical Sciences (Tianjin, China). Both cells were cultured in RPMI 1640 medium (Gibco/BRL) containing 10%(v/v) heat-inactivated new-born calf serum (Sijiqing), 100 U/ml penicillin and 100 µg/ml streptomycin at 37 °C in a humidified 5% CO_2 incubator. K562/A02 was cultured in the medium containing 1 µg/ml adriamycin for maintaining MDR phenotype, and maintained in drug-free medium for at least seven days before used.

Flow cytometric apoptosis assay

The apoptosis rates were measured using flow cytometric assay.¹⁷ Cell labeling by Annexin-V conjugated to fluorescein isothiocyanate (FITC; Trevigen, Gaithersburg, MD, USA), which binds to phosphatidylserine exposed on the surface membrane of cells undergoing apoptosis. After the 48 h incubation in the medium containing different drugs at 37 °C, cell suspensions were washed twice with PBS and centrifugation at 1000r/min for 5 min. Then the cells were suspended in 500 µl of binding buffer (KenGen, Nanjing, China) and 5 µl of Annexin V-FITC (KenGen, Nanjing, China), incubated at room temperature for 15 min in the dark. The samples were measured on a flow cytometry with CellQuest software (Becton Dickinson).

DNA agarose gel electrophoresis

The typical biochemistry feature of apoptotic was detected by DNA gel electrophoresis.⁸ After treatment with the drugs, we used Apoptosis DNA Ladder Detection Kit (KenGen, China) to extract DNA and then analyze DNA by electrophoresis in 1.5% agarose gels, stained with 0.5 µg/ml ethidium bromide (Tiangen Biotech, Beijing, China), photographed on an ultraviolet transilluminator. Standard molecular-weight markers (DNA Marker DL2000; Dongsheng, Guangzhou, China) were electrophoresed on each gel.

Quantitative real-time PCR (QPCR) analysis

After treatment with drugs, total RNA was isolated 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 using SuperScript™ II reverse transcriptase (Invitrogen Life Technologies). PCR primers were designed to amplify products within target and control sequences. (Primer sequences: P53(422bp):forward:5'-ACCCAGGTCCAGATGAAG-3'; reverse:5'-CACTCGGATAAGATGCTGA-3'. Caspase-3(270bp):forward:5'-GCTATTGTGAGGCGGTTGT-3';reverse:5'-TGTTTCCCTGAGGTTTGC-3'. GAPDH(205bp): forward:5'-CGGATTTGGTCGTATTG-3';reverse:5'-GAAGATGGTGATGGGATT-3') QPCR 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 done in triplicate. The relative gene copy number was calculated by the concentration-CT standard curve method and normalized using the average expression of GAPDH.¹⁹

Western blot analysis

After drug treatment, total protein was isolated and subjected to sodium dodecyl sulfate PAGE, and transferred to a polyvinylidene difluoride membrane. The blots were

stained with primary antibodies (1:1000–1200) overnight at 4 °C, and then with horseradish peroxidase conjugated goat antirabbit or mouse secondary antibody (1:5000) for 1 h at room temperature. The signal was detected with an ECL Western Blotting Detection Kit (ZhongShan Co., Beijing, China). After normalization by the corresponding expression of β -actin, protein expression levels were determined by densitometry scans.¹⁹

Statistical analysis

All experiments were repeated at least three times, data were expressed as mean $\pm$ SD and analyzed with the Statistical Package for Social Science (SPSS Release 11.5; SPSS Inc., Chicago, IL, USA). Differences were evaluated using one-way ANOVA. A P value of < 0.05 was considered to be statistically significant.

Results

Enhancement of DNR-induced apoptosis in K562/A02 cells

The apoptotic percentage of K562/A02 cells induced by DNR with MNP(Fe_3O_4) (50 μM) or BrTet (0.5 μM) increased by $15.60\% \pm 0.76\%$ and $19.53\% \pm 0.67\%$, respectively, compared with DNR alone ($8.30\% \pm 0.40\%$) ($p < 0.05$), while co-treatment of DNR with MNP(Fe_3O_4) (50 μM) and BrTet (0.5 μM) significantly increased Annexin-V staining at $40.20\% \pm 1.49\%$ ($p < 0.05$) (Figure 1).

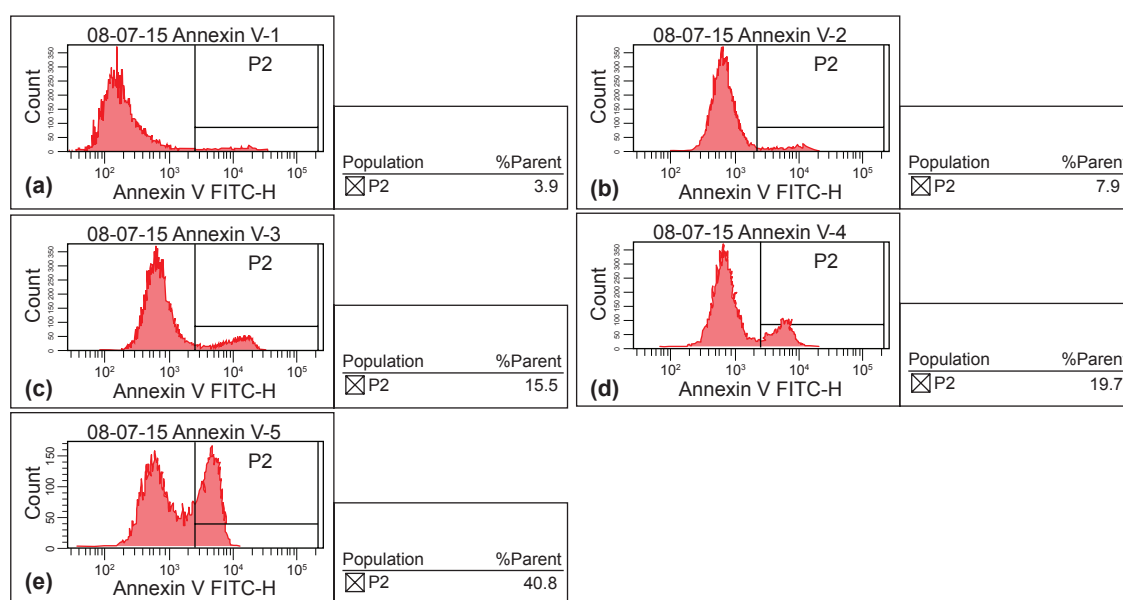


Figure 1 Effects of MNP(Fe_3O_4) and BrTet on DNR-induced apoptosis in K562/A02 cells for 48 h. (a) control; (b) DNR 1.0 $\mu\text{g/ml}$; (c) DNR 1.0 $\mu\text{g/ml}$ + MNP(Fe_3O_4) 50 μM ; (d) DNR 1.0 $\mu\text{g/ml}$ + BrTet 0.5 μM ; (e) DNR 1.0 $\mu\text{g/ml}$ + MNP(Fe_3O_4) 50 μM + BrTet 0.5 μM .

Abbreviations: BrTet, 5-bromotetrandrin; DNR, daunorubicin; MNP(Fe_3O_4), magnetic nanoparticle of Fe_3O_4 .

DNA agarose gel electrophoresis demonstrated apoptosis

After co-treatment of DNR (1.0 $\mu\text{g/ml}$) with MNP(Fe_3O_4) (50 μM) and BrTet (0.5 μM), K562/A02 cells appeared on the DNA ladder; while a large dose of the chemotherapeutic agent, DNR (20.0 $\mu\text{g/ml}$), was incubated with K562/A02 cells for 48 h, DNA agarose gel electrophoresis showed smear (Figure 2).

Effects of MNP(Fe_3O_4) and/or BrTet on P53 mRNA and its protein

The expression of p53 level was higher in K562/A02 cells than in the parental sensitive K562 cells ($p < 0.05$), whereas 50 μM MNP(Fe_3O_4), 0.5 μM BrTet or 1.0 $\mu\text{g/ml}$ DNR did not influence its expression in K562/A02 cells ($p > 0.05$) (Figures 3A, 3B). The level of p53 protein whose gene was upregulated by MNP(Fe_3O_4) or BrTet with DNR in K562/A02 cells also elevated ($p < 0.05$). Furthermore, the results suggest that MNP(Fe_3O_4) and BrTet with DNR together increased the expression of p53 protein more effectively than MNP(Fe_3O_4) or BrTet with DNR alone ($p < 0.05$).

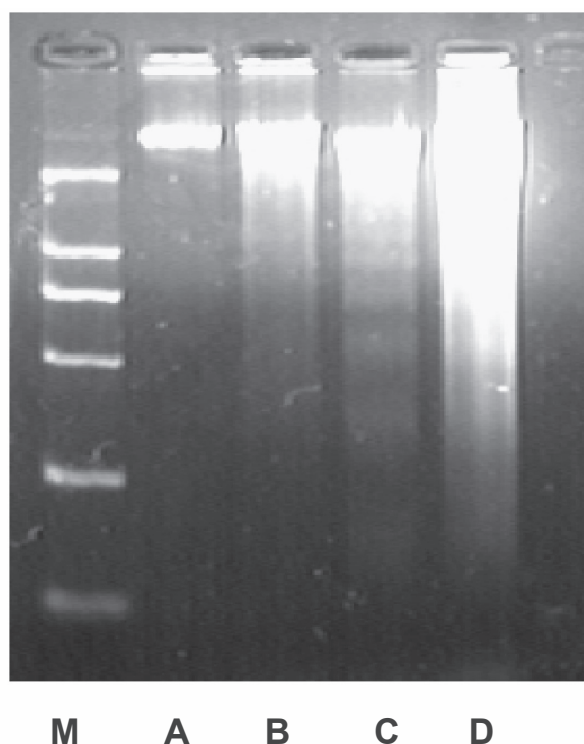


Figure 2 DNA agarose gel electrophoresis of K562/A02 incubated with different drugs for 48 h.

Notes: M, Marker; A, control; B, DNR 1.0 $\mu\text{g/ml}$; C, DNR 1.0 $\mu\text{g/ml}$ + MNP(Fe_3O_4) 50 μM + BrTet 0.5 μM ; D, DNR 20.0 $\mu\text{g/ml}$.

Abbreviations: BrTet, 5-bromotetrandrin; DNR, daunorubicin; MNP(Fe_3O_4), magnetic nanoparticle of Fe_3O_4 .

Effects of MNP(Fe_3O_4) and/or BrTet on caspase-3 mRNA and its protein

As shown in Figures 4A and 4B, caspase-3 was inhibited in Pgp-overexpressed cells after treatment with DNR. Nevertheless, when cells were treated with MNP(Fe_3O_4) or BrTet alone, the K562/A02 cells expressing caspase-3 showed significant resistance to these agents ($p > 0.05$). However, the combination of DNR and MNP(Fe_3O_4) or BrTet yielded much more significant activation of caspase-3, and the expression of caspase-3 mRNA and protein were increased in response to application of MNP(Fe_3O_4) and BrTet with DNR together ($p < 0.05$).

Discussion

Most cytotoxic agents exert their anticancer effects by inducing apoptotic-programmed cell death.²⁰ The resistance of leukemic cells to chemotherapy-induced apoptosis remains the most significant problem in the development of drug resistance and treatment failure in leukemia.³ Defects in the receptor- or mitochondrial-mediated apoptosis signaling pathway are implicated in the development of drug resistance and treatment failure in leukemia.^{21,22} In order to reverse the resistance mechanism, we chose the application of MNP(Fe_3O_4) and BrTet to enhance inducing leukemia cells apoptosis. MNP(Fe_3O_4) are widely used for targeted-drug carriers with target-orientation and sustained-release properties.⁹ In previous studies have demonstrated the synergistic effect of MNP(Fe_3O_4) with anticancer drug on the drug accumulation of leukemia K562 cells may help it to inhibit the relative drug resistance.¹⁰ We previously reported that BrTet could effectively reverse P-gp-mediated MDR,¹⁶ and MNP(Fe_3O_4)s loaded with chemotherapeutic agents and chemosensitizers have a synergetic effect on reversal in MDR.²³

In order to investigate the cytotoxicity of MNP(Fe_3O_4) and BrTet on K562/A02 cells *in vitro*, we investigated the combined effects of DNR with either MNP(Fe_3O_4) or BrTet on the apoptosis of K562/A02 cell lines (Figure 1). The combination of DNR with either MNP(Fe_3O_4) or BrTet exerted a potent cytotoxic effect in K562/A02 cells, and co-treatment of MNP(Fe_3O_4) and BrTet had a distinct synergism on the enhancement of DNR-induced apoptosis in K562/A02 cells. This result showed that MNP(Fe_3O_4) or BrTet could enhance DNR-induced apoptosis in K562/A02 cells, and when used together, they have distinct synergism.

To check whether the effects of MNP(Fe_3O_4) and BrTet combining with a small dose chemotherapeutic agent was different from the effects of a large dose chemotherapeutic agent on K562/A02 cells, we detected the typical

biochemistry feature of apoptotic by DNA gel electrophoresis. As shown in Figure 2, the typical apoptotic features appeared after co-treatment of DNR (1.0 $\mu\text{g}/\text{ml}$) with $\text{MNP}(\text{Fe}_3\text{O}_4)$ and BrTet for 48 h, while most cells showed necrosis after incubated with DNR (20.0 $\mu\text{g}/\text{ml}$) for 48 h. This result showed that $\text{MNP}(\text{Fe}_3\text{O}_4)$ or BrTet enhanced DNR-induced apoptosis in K562/A02 cells, while 20.0 $\mu\text{g}/\text{ml}$ DNR led cells to necrosis, which supports our assumption that the effects of $\text{MNP}(\text{Fe}_3\text{O}_4)$ and BrTet combining with a small dose chemotherapeutic agent was different from the effects of a large dose chemotherapeutic agent on K562/A02 cell. This suggests that a combination of $\text{MNP}(\text{Fe}_3\text{O}_4)$ and BrTet could be a highly feasible candidate for the development of a new MDR modulator.

Apoptosis in response to most DNA-damaging agents usually requires p53 function, which engages primarily the intrinsic pathway.²⁴ Tumors that have lost p53 function might help circumvent resistance to conventional therapies.²⁵ Therefore, further analysis of p53 status is required in cancer therapies. To explore the involvement of P53 in the resistance to antitumor agent-induced apoptosis, we determined the level of P53 by real-time PCR assay and Western blot. Our results revealed that p53 defects are linked

to reduced chemosensitivity. Whereas $\text{MNP}(\text{Fe}_3\text{O}_4)$, BrTet, or DNR did not change the expression of p53 mRNA in K562/A02 cells, $\text{MNP}(\text{Fe}_3\text{O}_4)$ and BrTet with DNR together increased the expression of p53 protein more effectively than $\text{MNP}(\text{Fe}_3\text{O}_4)$ or BrTet with DNR alone (Figure 3). These results suggest that the augmentation of P53 expression by DNR with $\text{MNP}(\text{Fe}_3\text{O}_4)$ and BrTet possibly contributes to its apoptotic induction in this system. Since p53 defects are a major contributing factor in drug resistance and have been recently linked to reduced chemosensitivity, our results may have very important clinical implications.²⁶

The initiation of a cascade of cysteine proteases of the ICE/ced3 family (caspases) plays a pivotal role in apoptosis.²⁷ Caspase-3 inhibition is essential for caspase activation in apoptosis. Our findings demonstrated that caspase-3 was the most likely caspase to mediate the composite-induced apoptosis, as shown in Figures 4A, 4B.

Conclusion

Our study demonstrates for the first time that $\text{MNP}(\text{Fe}_3\text{O}_4)$ and BrTet at subcytotoxic doses significantly reversed MDR by synergistically enhancing DNR-induced apoptosis, and the distinct synergistic effect of that composite on reverse

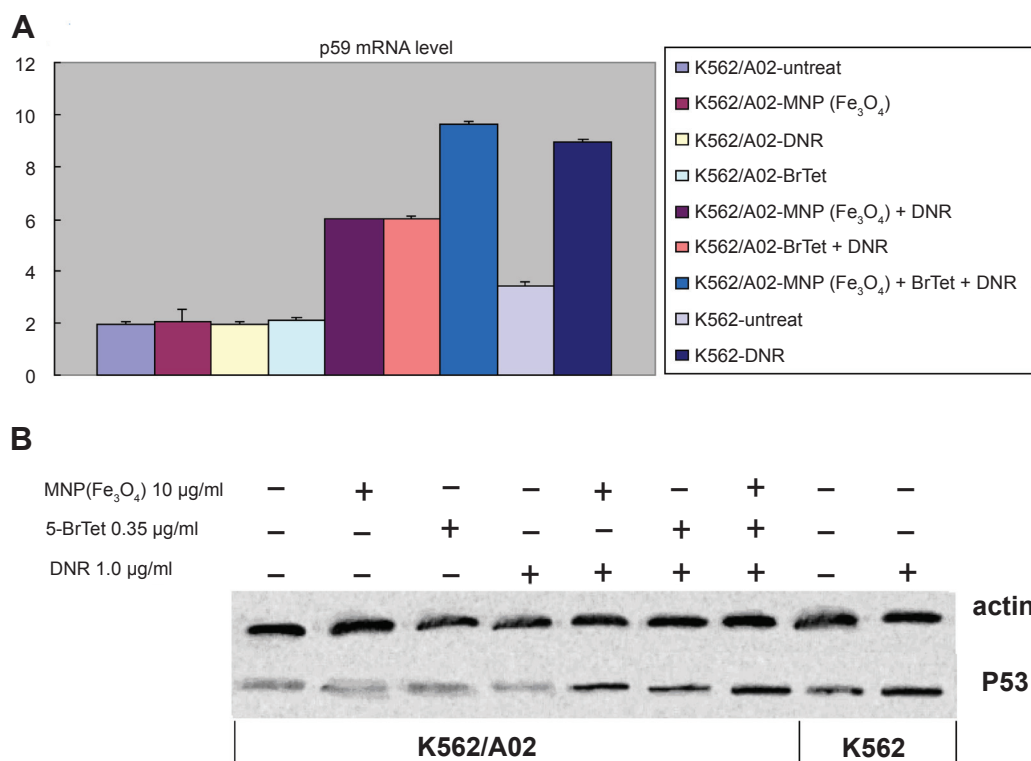


Figure 3 Effects of $\text{MNP}(\text{Fe}_3\text{O}_4)$ and/or BrTet on P53 mRNA and its protein in K562 cells and K562/A02 cells. **A**) Real-time PCR analysis of P53 of K562 cells and K562/A02 cells after 48 h of treatment. **B**) Western blot analysis of P53 of K562 cells and K562/A02 cells after 48 h of treatment.

Abbreviations: BrTet, 5-bromotetrandrin; $\text{MNP}(\text{Fe}_3\text{O}_4)$, magnetic nanoparticle of Fe_3O_4 ; PCR, polymerase chain reaction.

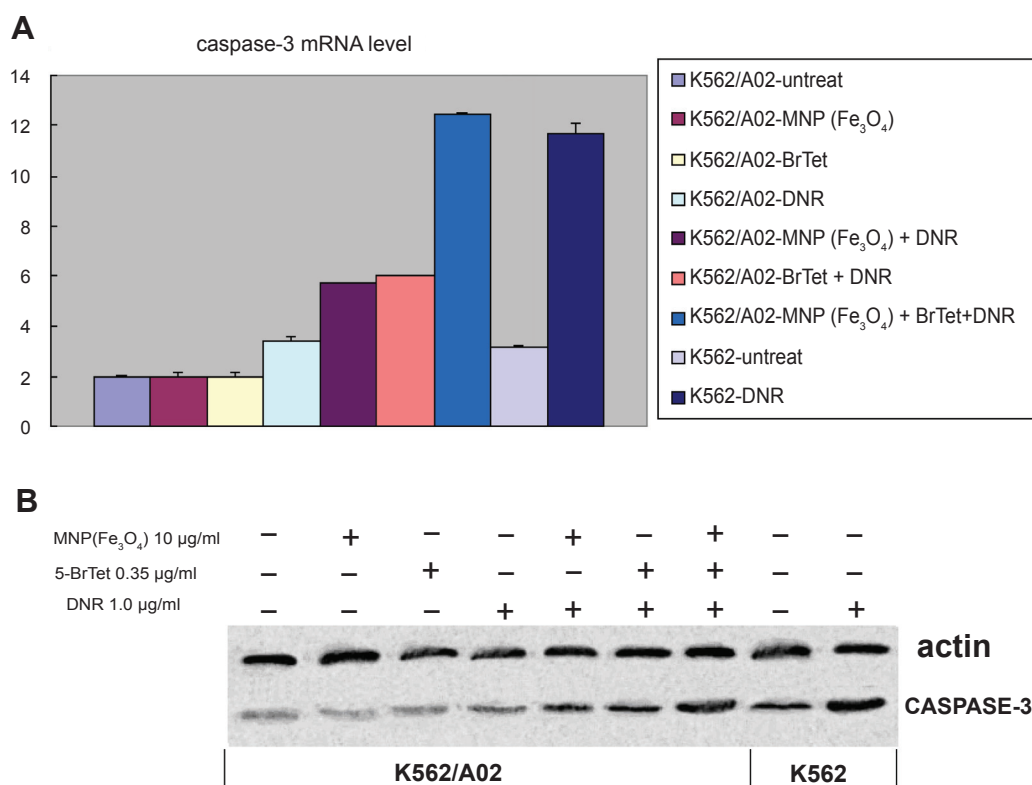


Figure 4 Effects of MNP(Fe_3O_4) and/or BrTet on caspase-3 mRNA and its protein in K562 cells and K562/A02 cells. **A**) Real-time PCR analysis of caspase-3 of K562 cells and K562/A02 cells after 48 h of treatment. **B**) Western blot analysis of caspase-3 of K562 cells and K562/A02 cells after 48 h of treatment.

Abbreviations: BrTet, 5-bromotetrandrin; MNP(Fe_3O_4), magnetic nanoparticle of Fe_3O_4 ; PCR, polymerase chain reaction.

MDR may owe to the regulation of various proliferative and antiapoptotic gene products, including P53 and caspase-3. Thus, it may be possible that a combination of MNP(Fe_3O_4) and BrTet may be a more sufficient and less toxic anti-MDR method.

Acknowledgments

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