# Reversal of multidrug resistance by magnetic $Fe_3O_4$ nanoparticle copolymerizating daunorubicin and 5-bromotetrandrine in xenograft nude-mice

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**Abstract:** In this paper we establish the xenograft leukemia model with stable multidrug resistance in nude mice and to investigate the reversal effect of 5-bromotetrandrine (5-BrTet) and magnetic nanoparticle of $Fe_3O_4$ (MNP-$Fe_3O_4$) combined with daunorubicin (DNR) *in vivo*. Two subclones of K562 and K562/A02 cells were inoculated subcutaneously into the back of athymic nude mice ($1 \times 10^7$ cells/each) respectively to establish leukemia xenograft models. Drug-resistant and sensitive tumor-bearing nude mice were assigned randomly into five groups which were treated with normal saline; DNR; NP-$Fe_3O_4$ combined with DNR; 5-BrTet combined with DNR; 5-BrTet and MNP-$Fe_3O_4$ combined with DNR, respectively. The incidence of formation, growth characteristics, weight, and volume of tumors were observed. The histopathologic examination of tumors and organs were detected. For resistant tumors, the protein levels of Bcl-2, and BAX were detected by Western blot. Bcl-2, BAX, and caspase-3 genes were also detected. For K562/A02 cells xenograft tumors, 5-BrTet and MNP-$Fe_3O_4$ combined with DNR significantly suppressed growth of tumor. A histopathologic examination of tumors clearly showed necrosis of the tumors. Application of 5-BrTet and MNP-$Fe_3O_4$ inhibited the expression of Bcl-2 protein and upregulated the expression of BAX and caspase-3 proteins in K562/A02 cells xenograft tumor. It is concluded that 5-BrTet and MNP-$Fe_3O_4$ combined with DNR had a significant tumor-suppressing effect on a MDR leukemia cells xenograft model.

**Keywords:** 5-bromotetrandrine, magnetic nanoparticle of $Fe_3O_4$, multidrug-resistance, xenograft model

Multidrug resistance (MDR) is a well-defined phenomenon of cross-resistance of mammalian cells to a number of anticancer agents following exposure to one such drug. It is a major obstacle to successful chemotherapy in leukemia.[1] More than 90% of patients with malignant tumor die of MDR. A diverse range of agents involved in MDR include alkaloid compounds and bacterial and fungal antibiotics such as anthracyclines and etoposide. An accepted mechanism of MDR is a reduced cellular accumulation and an altered subcellular distribution of cytotoxic drugs. In many instances, this is mediated by increased expression at the cell surface of the MDR1 gene product, P-glycoprotein (P-gp), a 170-kD energy-dependent efflux pump. Different chemotherapeutic agents have been shown to induce the death of susceptible cells by apoptosis, and this can be inhibited by proteins such as Bcl-2[47] and Bcl-xL. The purpose of this paper is to find a low mammalian toxicity, high-efficiency, high-selectivity modulator.

Close attention has been paid to current nanoparticle techniques. The magnetic nanoparticle of $Fe_3O_4$ (MNP-$Fe_3O_4$)[2,3] that we invented has good biocompatibility and no cytotoxicity. MNP-$Fe_3O_4$ can increase the intracellular effective concentration of chemotherapeutic drugs *in vitro* in order to reverse MDR.[4,5] 5-Bromotetrandrine

(5-BrTet), when highlighted by isoquinoline alkaloid, is a hypotoxic calcium channel blocker.[6] 5-BrTet inhibits the function of P-gp in order to accumulate anticancer drugs in the body.[7] Most research on the reversal of MDR was on the basis of experiments *in vitro*.

This paper was to establish the xenograft leukemia model with stable MDR in nude mice; to investigate the reversal effect of 5-BrTet and MNP-$Fe_3O_4$ combined with daunorubicin (DNR) *in vivo* and to search for possible reversal mechanisms in order to provide theoretic evidence for the clinical applications.

## Material and method
### Cell lines and cell culture
K562 cell was a cell line cloned from human chronic myelogenous leukemia by repeated passage in nude mice and in culture alternatively. The MDR leukemia cell K562/A02 was established by stepwise selection with an increasing concentration of adriamycin (ADM). K562 cells were maintained in RPMI 1640 medium with 10% newborn bovine serum at 37 °C in a humidified 5% $CO_2$ atmosphere. K562/A02 was cultured in a medium containing 1 μg/ml ADM for maintaining MDR phenotype.

### Experimental agents
Adriamycin (Hisun Phamaceutical Co., Zhejiang, China) and DNR (Main Luck Pharmaceuticals Inc., Shenzheng, China) 2 mg/ml stock solution were prepared with 0.01 mol/l phosphate buffer saline (PBS) (pH 7.4). Nutritional medium: RPMI1640 (Gibco Chemical Co., Carlsbad, CA, USA), calf serum (Gibco Chemical Co); MNP-$Fe_3O_4$ was provided by the Biological Science College at Southeast University, Nanjing; DNR conjugated with MNP-$Fe_3O_4$ colloidal suspension were prepared by mechanical absorption polymerization at 4 °C for 48 h as reported previously. 5-BrTet (Kanghong Chemical Co, Chengdou, China) was diluted with 0.01 mol/l PBS (pH 7.4).

### Experimental animals
Female BALB/*c* nude mice which were age-matched (four weeks of age) and weight-matched (16–22 g) were purchased from Shanghai National Center for Laboratory Animals. They were maintained in specific pathogen-free (SPF) facilities and fed with irradiated food. Indoor temperature was maintained at 22 °C and humidity was controlled at 45 ± 5%.

### Xenograft leukemia model in nude mice
The two subclones of K562/A02 and K562 cells were inoculated subcutaneously into the back of athymic nude mice ($1 \times 10^7$ cells/0.2 ml/each) respectively to establish the human leukemia xenograft models: the drug-resistant xenograft nude-mice models and the sensitive tumor-bearing xenograft nude-mice models.

## Using animal ultrasonic equipment to evaluate tumor formation
Six days after cell inoculation, pink neoplasm had emerged in the inoculated part. We used animal ultrasonic equipment to detect the neoplasm.

## Tumor-bearing nude mice divided into groups. The effect of reversal of MDR in tumor-bearing nude mice determined
When the tumor size reached to 75–150 mm³, the drug-resistant and sensitive tumor-bearing nude mice were both assigned randomly to five groups. Each group contained eight nude mice. The drugs were administered by intraperitoneal injection except for MNP-$Fe_3O_4$ copolymerizing DNR delivered by vena caudalis injection. In group A, the control group was treated with normal saline (NS) 0.2 ml/each day; in group B, DNR 1 mg/(kg·day); in group C, 5-BrTet 2.5 mg/(kg·day) combined with DNR 1 mg/(kg·day), 5-BrTet was administered one hour before DNR; in group D, NP-$Fe_3O_4$ combined with DNR 0.63 mg/(kg·day); in group E, 5-BrTet 2.5 mg/(kg·day) and MNP-$Fe_3O_4$ combined with DNR 0.63 mg/(kg·day), 5-BrTet was administered one hour before MNP-$Fe_3O_4$. Each group was treated every day for 10 days. The incidence of formation, growth characteristics, weight, and volume of tumor were observed for each group, respectively. All the tumor-bearing nude mice were killed on the eleventh day to detect tumors and gains.

### Tumor volume
Tumor dimensions were measured with digital calipers or animal ultrasonic inspection to obtain two diameters of the tumor formed. Tumor size was calculated by using the formula as follows: Tumor volume = $1/2 \times a \times b^2$, where a is the longest diameter and b is the shortest. The inhibition rate (IR) (%) = (one-experimental group relative tumor volume [RTV]/control group RTV)·100%. RTV = Vx/V1, Vx and V1 represent tumor volume on the X day and the first day after treatment. Tumor weight was calculated by torsion balance and growth-inhibiting rat.

### Histopathologic examination of tumors and organs
After the nude mice are killed, the tumors and organs must be fixed in formalin immediately, hematoxylin and eosin stained, and observed by 400x light microscope.

## Apoptosis-related proteins detected by Western blot for resistant tumors

Cellular nucleic protein was obtained by grinding the tumors as discussed previously. Protein concentration (Bcl-2, BAX, Caspase-3) was settled to 2 µg/µl. Each lane contained 40 µg proteins. The proteins were resolved by 10% SDS/PAGE for one hour, and then transferred to nitrocellulose membrane. After being blocked in Tris-buffered saline/Tween-20 (TBST) with 5% skim milk overnight, the membrane was incubated with primary polyclonal antibody (1:500; Biolegend, San Diego, CA, USA) for one hour, and subsequently incubated with horseradish peroxidase-linked goat anti-rabbit secondary antibody (1:7500; Pierce, Rockford, IL, USA) for one hour. The protein bands were visualized by enhanced chemiluminescence regents (Santa Cruz Biotechnology, Inc., Santa Cruz, CA, USA).

## Quantitative real-time PCR analysis

After killing the nude mice, total RNA of tumor 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 (Bcl-2, forward: 5'-GGGAGAACAGGGTAC-GATAA-3'; reverse: 5'-CCACCGAACTCAAAGAAGG-3'. BAX, forward: 5'-TTTTGCTTCAGGGTTTCATC-3'; reverse: 5'-GACACTCGCTCAGCTTCTTG -3'. Caspase-3, forward: 5'-GCTATTGTGAGGCGGTTGT-3'; reverse: 5'-TGTTTCCCTGAGGTTTGC-3'. GAPDH, forward: 5'-CGGATTTGGTCGTATTG-3'; reverse: 5'-GAA-GATGGTGATGGGATT-3'). Quantitative real-time PCR (QPCR) was performed by real-time monitoring of 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.

## Statistical analysis

The data was analyzed with SPSS v. 11.5 (SPSS Inc., Chicago, IL, USA) software. The significance test used factor variance analysis and the difference had statistical significance, $p < 0.05$.

# Results

## The animal model of hematologic disease was established successfully

The median latency of K562 cells was six days when it formed neoplasm, which was shorter than that of K562/A02 cells at eight days. We used animal ultrasonic inspection to confirm the presence of tumor. Figure 1 displays the tumor formation incidences of K562 and K562/A02 cells at 100%.

## The relative tumor volume and inhibition rate of five experimental groups for K562 sensitive cells xenograft tumor

Six days after K562 cell inoculation, the tumors reached a volume of more than 100 mm$^3$. The mice were divided into five groups and subject to 10 days of treatment. When the K562 cell tumor-bearing nude mice were killed, the relative tumor volumes of the five experimental groups were as follows: the control group A was 15.24 ± 0.10; groups B, C, D, and E were 5.79 ± 0.15, 5.77 ± 0.15, 5.78 ± 0.08, and 5.66 ± 0.06, respectively ($p = 0.743$). The inhibition rates of groups B, C, D, and E compared with the control group were 62.01 ± 0.31%, 62.12 ± 0.11%, 62.11 ± 0.22%, and 62.82 ± 0.18%, respectively ($p = 0.678$). The tumor weight was measured and the inhibition rate of tumor growth groups B, C, D, and E was calculated at 59.05 ± 0.14%, 59.64 ± 0.31%, 59.52 ± 0.37%, and 60.52 ± 0.44%, respectively ($p = 0.748$).

## The relative tumor volume and inhibition rate of five experimental groups for K562/A02 resistant cells xenograft tumor

Eight days after K562/A02 cells inoculation, the tumors reached a volume of more than 100 mm$^3$. The mice were



**Figure 1** Animal ultrasonic inspection of pink neoplasm: tumor formation.

divided into five groups and subject to 10 days of treatment. When K562/A02 cell tumor-bearing nude mice were killed, the relative tumor volumes of the five experimental groups were as follows: the control group A was $12.94 \pm 0.14$; groups B, C, D, E were $12.47 \pm 0.30$, $9.30 \pm 0.21$, $7.26 \pm 0.34$, and $4.89 \pm 0.24$, respectively ($p = 0.024$; Figure 2). The inhibition rates of groups B, C, D, and E compared with the control group were $3.68 \pm 0.17\%$, $28.24 \pm 0.32\%$, $40.80 \pm 0.16\%$, $62.76 \pm 0.42\%$, respectively. There were significant differences in groups B, C, D, and E ($p = 0.015$). The tumor weight was measured and the inhibition rates of tumor growth of groups B, C, D, and E were calculated at $1.34 \pm 0.43\%$, $28.08 \pm 0.41\%$, $46.40 \pm 0.22\%$, $59.75 \pm 0.16\%$, respectively ($p = 0.021$).

## Histopathologic examination of K562/A02 resistant tumors and organs

In K562/A02-resistant tumors, the pathology of tumors from control group A and the DNR-alone group B showed more heterogeneity of neoplastic cells, karyomegaly, more mitotic figures, and vasiformation. Tumors from groups C and D showed a few necroses. The pathology of tumors from control group E showed obvious necrosis. There were no apparent histopathologic damage to organs such as the heart, liver, kidney, etc.

Western blot analysis of apoptosis-related protein expression in K562/A02 resistant tumors was performed. Application of 5-BrTet and MNP-Fe$_3$O$_4$ downregulated the expression of Bcl-2 protein compared with the control group and upregulated the expression of BAX and caspase-3 protein (Figure 3).

## Expressions of Bcl-2, BAX, and caspase-3 genes in K562/A02-resistant tumors and K562 tumors

The expression levels of Bcl-2, BAX, and caspase-3 genes were detected by PCR. Compared with the control group, both BAX and caspase-3 mRNA expression increased markedly in in K562 tumors in group E. The expression of Bcl-2 was significantly suppressed by application of 5-BrTet and MNP-Fe$_3$O$_4$ (Table 1).

## Discussion

Chemotherapy is the main method of leukemia therapy. The clinical therapeutic effect is not as good as imagined, although more and more new chemotherapeutic drugs are emerging. One of the most important reasons for this clinical effect is MDR. The common cause of MDR is believed to be the enhanced expression of a transmembrane glycoprotein termed P-glycoprotein (P-gp) coded by the mdr1 gene in cancer cells.[8,9] Some of the drug molecules that diffuse into cells are removed by P-gp. The industry development of MDR modulators is extremely fast, but their weak activity and toxic reaction limit clinical and therapeutic applications. Recently, exploratory research in nanoparticle medicine showed a reversal in MDR by the application of
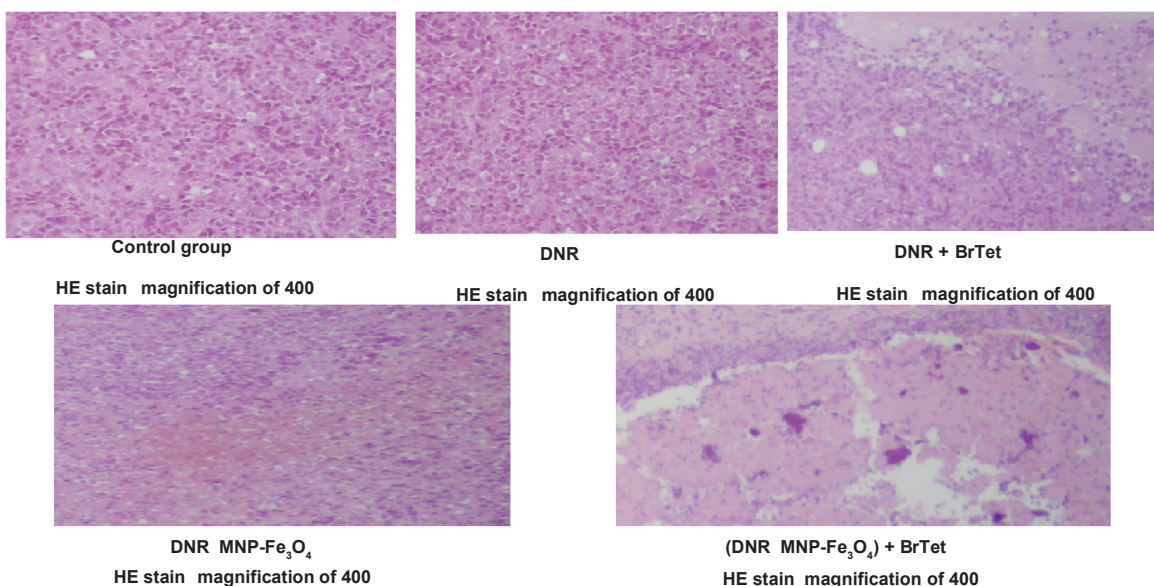


Control group

HE stain  magnification of 400

DNR

HE stain  magnification of 400

DNR + BrTet

HE stain  magnification of 400

DNR  MNP-Fe$_3$O$_4$

HE stain  magnification of 400

(DNR  MNP-Fe$_3$O$_4$) + BrTet

HE stain  magnification of 400

**Figure 2** Histopathologic examination of K562/A02 resistant tumors of different groups (hematoxylin and eosin stain, 400 × light microscope).
**Abbreviations:** BrTet, 5-bromotetrandrine; DNR, daunorubicin; HE, hematoxylin and eosin; MNP-Fe$_3$O$_4$, magnetic nanoparticle of Fe$_3$O$_4$.
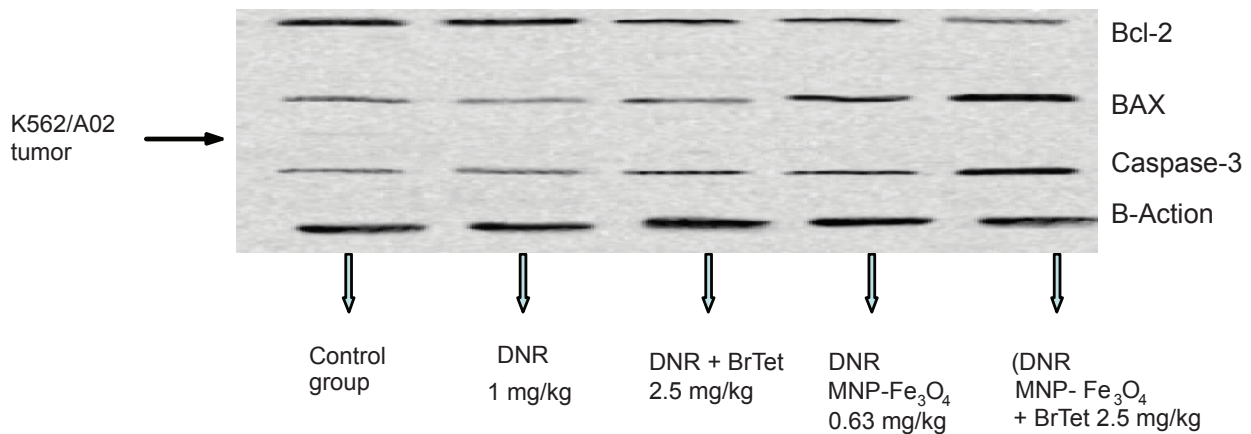
**Figure 3** Western blot of apoptosis-related protein expression in K562/A02-resistant tumors.
**Abbreviations:** BrTet, 5-bromotetrandrine; DNR, daunorubicin; MNP-$Fe_3O_4$, magnetic nanoparticle of $Fe_3O_4$.

magnetic nanoparticles loaded with chemotherapeutics.[10–12] MNP-$Fe_3O_4$ has been considered as a potential MDR cancer reversal agent, although its involved molecular mechanism has not been well elucidated. The diameter of MNP-$Fe_3O_4$ manufactured by the Biological Science College at Southeast University was 20–30 nm, which suits reversal of MDR.[13] Some research reports that the fraction of nanoparticle-loaded doxorubicin (Dox), which enters cells by endocytosis, may be associated with solid lipids and is not easily cleared from the cell by P-gp drug efflux. Nanoparticle-loaded Dox may inhibit P-gp function. 5-BrTet could step down the toxicity of chemotherapeutics and may not impact the pharmacokinetics of chemotherapeutics *in vivo*.[14]

This experiment shows that the leukemia xenograft nude-mice model maintains MDR. 5-BrTet and MNP-$Fe_3O_4$ combined with DNR had a significant tumor-suppressing effect on the MDR leukemia cells xenograft model. This activity may enhance apoptosis. We propose further studies to determine whether 5-BrTet and MNP-$Fe_3O_4$ may suppress tumor cell proliferation and induce apoptosis by blocking multiple pathways. Our results indicated that application of 5-BrTet and MNP-$Fe_3O_4$ had a better effect in reversal MDR than DNR, 5-BrTet combined with DNR, or MNP-$Fe_3O_4$ combined with DNR. 5-BrTet and MNP-$Fe_3O_4$ may be the most promising MDR modulators for eventual assessment in the clinic. 5-BrTet and MNP-$Fe_3O_4$ combined with DNR had a significant tumor-suppressing effect on the MDR leukemia cells xenograft model.

## Acknowledgments

**Table 1** Expressions of Bcl-2, BAX, and caspase-3 genes in different groups ($\bar{x} \pm$ SD)

| Gene expressions | k562/A02 control group | k562/A02 DNR 1 mg/kg | k562/A02 DNR MNP-$Fe_3O_4$ 0.63 mg/kg | k562/A02 DNR 1 mg/kg + 2-BrTet 5 mg/kg | k562/A02 (DNR MNP-$Fe_3O_4$) 0.63 mg/kg + 5-BrTet 2.5 mg/kg | k562 control group |
|---|---|---|---|---|---|---|
| Bcl-2 | 9.364 ± 0.010 | 6.248 ± 0.120 | 4.012 ± 0.200 | 3.679 ± 0.138 | 2.004 ± 0.025 | 8.567 ± 0.225 |
| BAX | 2.013 ± 0.085 | 1.968 ± 0.038 | 6.025 ± 0.106 | 5.994 ± 0.173 | 11.256 ± 0.166 | 5.689 ± 0.281 |
| Caspase-3 | 2.063 ± 0.047 | 1.958 ± 0.057 | 3.668 ± 0.205 | 4.025 ± 0.193 | 12.334 ± 0.187 | 3.665 ± 0.043 |

**Abbreviations:** BrTet, 5-bromotetrandrine; DNR, daunorubicin; MNP-$Fe_3O_4$, magnetic nanoparticle of $Fe_3O_4$.

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