

# Magnetic Fe<sub>3</sub>O<sub>4</sub> nanoparticles and chemotherapy agents interact synergistically to induce apoptosis in lymphoma cells

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**Abstract:** The purpose of this study was to investigate the potential effects of combination therapy using magnetic nanoparticles of Fe<sub>3</sub>O<sub>4</sub> (MNP-Fe<sub>3</sub>O<sub>4</sub>) and chemotherapeutic drugs on lymphoma cells. Proliferation, inhibition, and viability of Raji cells were detected by MTT and trypan blue exclusion. The percentage of cells undergoing apoptosis was detected by flow cytometry using fluorescein isothiocyanate-annexin V and propidium iodide staining. p53 and nuclear factor-κB (NF-κB) protein levels were measured by Western blot. The results showed that proliferation of Raji cells was inhibited by adriamycin or daunorubicin in a dose- and time-dependent manner. Cell sensitivity was improved and the 50% inhibitory concentrations of adriamycin and daunorubicin decreased when combined with a MNP-Fe<sub>3</sub>O<sub>4</sub> carrier. Interestingly, increased apoptosis in Raji lymphoma cells was accompanied by upregulation of p53 protein and downregulation of NF-κB protein. Furthermore, the combination of MNP-Fe<sub>3</sub>O<sub>4</sub> with adriamycin or daunorubicin increased p53 protein levels and decreased NF-κB protein levels more than adriamycin or daunorubicin alone, indicating that MNP-Fe<sub>3</sub>O<sub>4</sub> could enhance the effect of chemotherapeutic drugs on p53 and NF-κB. Similar results for cell apoptosis and protein expression were not observed for the groups treated with dexamethasone ± MNP-Fe<sub>3</sub>O<sub>4</sub> ( $P > 0.05$ ). These findings suggest a potential clinical application for MNP-Fe<sub>3</sub>O<sub>4</sub> in combination with daunorubicin or adriamycin in the treatment of lymphoma.

**Keywords:** magnetic nanoparticles, Raji cells, apoptosis, p53, NF-κB

## Introduction

Treatment of aggressive lymphoma is difficult, and the standard of care involves multiagent chemotherapeutic protocols incorporating adriamycin, an anthracycline antibiotic. Adriamycin and a similar compound, daunorubicin, originally isolated from streptomycetes, are widely used and highly effective chemotherapeutic agents.<sup>1</sup> It has recently been reported that glucocorticoids such as dexamethasone induce apoptosis in lymphoid cells and therefore are commonly used in chemotherapy protocols for lymphoid malignancies.<sup>2,3</sup> However, this effect is of questionable clinical relevance because the drug doses required for significant intercalation to occur are associated with unacceptable side effects.<sup>4</sup> To minimize dose-related side effects during chemotherapy, a promising approach is to combine conventional chemotherapy with new strategies to maximize the efficacy of chemotherapy by enhancing drug delivery and increasing the drug concentration reaching tumor cells.<sup>5</sup>

In recent years, nanomaterials, defined as particles with diameters of less than 100 nm, have been widely applied in the fields of technology and medicine due to their unique physiochemical properties and tunable characteristics.<sup>6,7</sup> Especially, it

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has been incorporated into clinical application in therapy and diagnosis owing to it both improves the traditional drug delivery route and resists the initiative excretion of drugs in tumor cells.<sup>7,8</sup> Nowadays, the most promising biocompatible materials are magnetic nanoparticles of iron oxide (MNP-Fe<sub>3</sub>O<sub>4</sub>), which are feasible to produce, easy to functionalize, and show satisfactory water solubilization and degradation *in vivo*.<sup>8</sup> It has also been reported that MNP-Fe<sub>3</sub>O<sub>4</sub> can improve the sensitivity of anticancer drugs and reverse multidrug resistance, so could be used for targeted drug carriers with target orientation.<sup>8,9</sup>

Apoptosis is the main mechanism of cell death, and is mediated by a cell-intrinsic suicide program, with the relative balance of pro- and antiapoptotic signaling pathways determining the fate of the cell.<sup>10</sup> Nuclear factor- $\kappa$ B (NF- $\kappa$ B) transcription factors play a central role as mediators of immune and inflammatory responses, as well as in the control of cell proliferation and apoptosis.<sup>11</sup> In addition to the well known function of p53 as a cell cycle regulator, the regulation of cell proliferation and apoptosis are also closely associated with regulation of normal development.<sup>5,12,13</sup>

The combination of MNP-Fe<sub>3</sub>O<sub>4</sub> with different chemotherapeutics may provide new clinical options in the treatment of lymphoma. However, few *in vitro* data are available for the combination of MNP-Fe<sub>3</sub>O<sub>4</sub> with other compounds, eg, adriamycin, daunorubicin, and dexamethasone, all of which are currently used in the treatment of lymphoma. The present study was designed to identify the cytotoxicity of this treatment strategy in a human Burkitt's lymphoma cell line, also known as Raji cells, and to investigate possible underlying mechanisms by specifying a group of genes that can be altered significantly by the above mentioned agents.

## Materials and methods

### Materials

The main materials used in this study were daunorubicin (Pfizer, New York, NY), adriamycin (Hisun Pharmaceutical Co, Zhejiang, China), dexamethasone (Xianju Pharmaceutical Co, Zhejiang, China), MTT (Sigma-Aldrich Co, St. Louis, MO), and the Annexin-V-FITC Apoptosis Detection Kit (Peking University Center for Human Disease Genomics, Beijing, China). MNP-Fe<sub>3</sub>O<sub>4</sub> obtained from the State Key Laboratory of Bioelectronics, Southeast University, Nanjing, China, were well distributed in RPMI 1640 medium (Invitrogen, Carlsbad, CA) containing 10% (v/v) heat-inactivated fetal bovine serum (HyClone, Logan, UT) by using ultrasound treatment to obtain an MNP-Fe<sub>3</sub>O<sub>4</sub> colloidal suspension. Different concentrations of adriamycin, daunorubicin, and

dexamethasone conjugated with 50  $\mu$ M MNP-Fe<sub>3</sub>O<sub>4</sub> were prepared by mechanical absorption polymerization at 4°C for 48 hours, as previously reported.<sup>14</sup>

### Cell lines and culture

The Raji lymphoma cell line was obtained from the Center for Human Disease Genomics of Peking University. The cells were cultured in RPMI 1640 medium containing 10% (v/v) heat-inactivated fetal bovine serum, 100 U/mL penicillin, and 100  $\mu$ g/mL streptomycin at 37°C in a humidified 5% CO<sub>2</sub> incubator, and passaged once every 2–3 days.

### MTT assays

To measure the cell inhibition rate,  $2 \times 10^5$ /mL Raji cells were incubated in 96-well flat-bottomed plates (CoStar, Charlotte, NC). Different concentrations of adriamycin, daunorubicin, dexamethasone, MNP-Fe<sub>3</sub>O<sub>4</sub>, adriamycin + MNP-Fe<sub>3</sub>O<sub>4</sub>, daunorubicin + MNP-Fe<sub>3</sub>O<sub>4</sub>, dexamethasone + MNP-Fe<sub>3</sub>O<sub>4</sub>, and RPMI 1640 alone were added to these cells and cultured at 37°C for 6, 12, 24, 48, and 72 hours, respectively. After cells were added to each well with 5  $\mu$ L MTT (5 mg/mL) and incubated at 37°C for four hours, dimethyl sulfoxide 100  $\mu$ L was added to each well and the plates were gently shaken for 10 minutes. Reduction of MTT was quantified by optical densities (OD) at a measurement wavelength of 570 nm and a reference wavelength of 630 nm using a Thermo Scientific Varioskan Flash (Thermo Fisher Scientific, Rockford, IL). The cell inhibition ratio was calculated as (1-OD treated group/OD control group)  $\times$  100%. IC<sub>50</sub> was defined as the concentration required for 50% inhibition of cell growth.

### Trypan blue staining

Cell viability was tested using trypan blue staining. Briefly, adriamycin 0.1  $\mu$ g/mL, daunorubicin 0.1  $\mu$ g/mL, and dexamethasone 100  $\mu$ g/mL alone and in combination with MNP-Fe<sub>3</sub>O<sub>4</sub> 50  $\mu$ mol/L were incubated with Raji cells in 24-well plates for different lengths of time. Cells were obtained and stained with trypan blue solution 0.4%. Finally, at least 200 cells were counted under the microscope.

### Apoptosis assay by flow cytometric assay

Apoptosis rates were measured by flow cytometric assay.<sup>15</sup> After incubation in MTT assays, cells were collected at 48 hours, washed twice with phosphate-buffered saline, suspended in 200  $\mu$ L of binding buffer and 10  $\mu$ L of Annexin V-FITC for 20 minutes in the dark, and thereafter 300  $\mu$ L of binding buffer and 5  $\mu$ L of propidium iodide were added to each sample. Analyses were done using FACSCalibur® flow

cytometry (Becton Dickinson, San Antonio, TX) with Cell Quest software.

## Western blot analysis

After the experimental treatment, total protein was isolated and subjected to sodium dodecyl sulfate polyacrylamide gel electrophoresis, and then transferred to a nitrocellulose membrane. The blots were stained with either p53 or NF- $\kappa$ B antibodies (1:10000) overnight at 4°C, and then with horseradish peroxidase-conjugated goat antirabbit or mouse secondary antibody (1:5000) for one hour at room temperature. The signal was detected using the Odyssey® Infrared Imaging System (LI-COR Biosciences, Lincoln, NE), the signal received was analyzed by the Odyssey software, and  $\beta$ -actin was used as the internal control.<sup>16</sup>

## Statistical analysis

All experiments were repeated three times. Data were expressed as mean  $\pm$  standard deviation and analyzed using the Statistical Package for Social Science (SPSS Release 17.0; SPSS Inc, Chicago, IL).  $P < 0.05$  was considered to indicate a statistically significant difference.

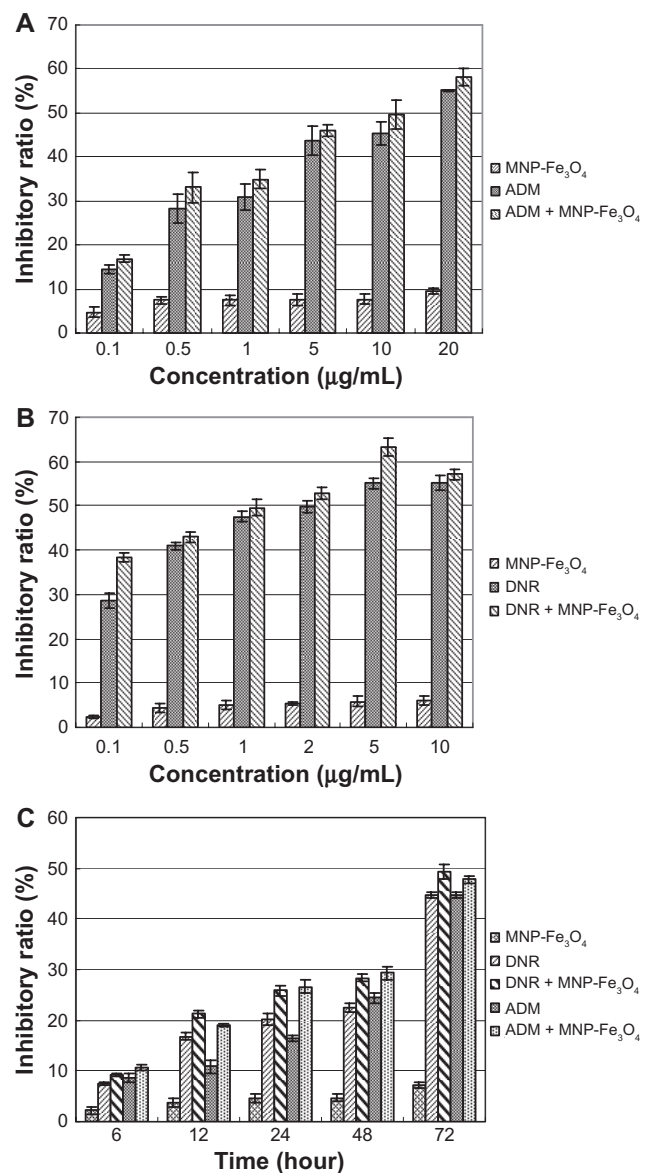
## Results

### Cell growth and inhibition

Inhibition of proliferation rate in Raji cells was enhanced by an increase in concentration and incubation time. After being coincubated with MNP-Fe<sub>3</sub>O<sub>4</sub> for 48 hours, Raji cell proliferation was markedly inhibited. The IC<sub>50</sub> of adriamycin decreased from 12.273  $\mu$ g/mL to 8.529  $\mu$ g/mL and that of daunorubicin from 2.695  $\mu$ g/mL to 1.105  $\mu$ g/mL. There were significant differences between the adriamycin + MNP-Fe<sub>3</sub>O<sub>4</sub>-treated group and the adriamycin-treated group ( $P < 0.05$ ), and between the (daunorubicin + MNP-Fe<sub>3</sub>O<sub>4</sub>)-treated group and daunorubicin-treated group (Figure 1), but there was no obvious enhancement in the dexamethasone + MNP-Fe<sub>3</sub>O<sub>4</sub>-treated group (data not shown). Similar results were found on trypan blue staining (see Table 1).

### Enhancement of apoptosis by adriamycin and daunorubicin

After being incubated with MNP-Fe<sub>3</sub>O<sub>4</sub> for 48 hours, the proportion of apoptosis in Raji cells induced by the combination of MNP-Fe<sub>3</sub>O<sub>4</sub> with adriamycin and with daunorubicin was 69.04  $\pm$  0.38% and 55.36  $\pm$  0.71%, respectively, and was increased significantly ( $P < 0.05$ ) when compared with either adriamycin (38.37  $\pm$  0.46%) or daunorubicin (39.49  $\pm$  0.61%) used alone. However, there was no significant difference in



**Figure 1** Proliferation inhibitory ratio of Raji cells incubated with different drugs with or without MNP-Fe<sub>3</sub>O<sub>4</sub>. **A)** ADM incubated with Raji cells for 48 hours. **B)** DNR incubated with Raji cells for 48 hours. **C)** Either 0.1  $\mu$ g/mL ADM or 0.1  $\mu$ g/mL DNR  $\pm$  50  $\mu$ mol/L MNP-Fe<sub>3</sub>O<sub>4</sub> incubated with Raji cells for different lengths of time. **Abbreviations:** MNP-Fe<sub>3</sub>O<sub>4</sub>, magnetic nanoparticles of Fe<sub>3</sub>O<sub>4</sub>; ADM, adriamycin; DNR, daunorubicin.

the relative proportion of apoptotic cells between cells treated with dexamethasone + MNP-Fe<sub>3</sub>O<sub>4</sub> and cells treated with dexamethasone alone (34.24  $\pm$  0.88% versus 27.41  $\pm$  0.23%,  $P > 0.05$ , Figure 2).

### Expression of p53 and NF- $\kappa$ B

Western blot assay revealed the p53 protein levels for the different treatment groups (Figure 3). Combination of MNP-Fe<sub>3</sub>O<sub>4</sub> with either adriamycin or daunorubicin increased the expression of p53 protein more effectively than

**Table 1** Cell viability of different drugs with or without MNP-Fe<sub>3</sub>O<sub>4</sub> for different times (% , mean ± SD)

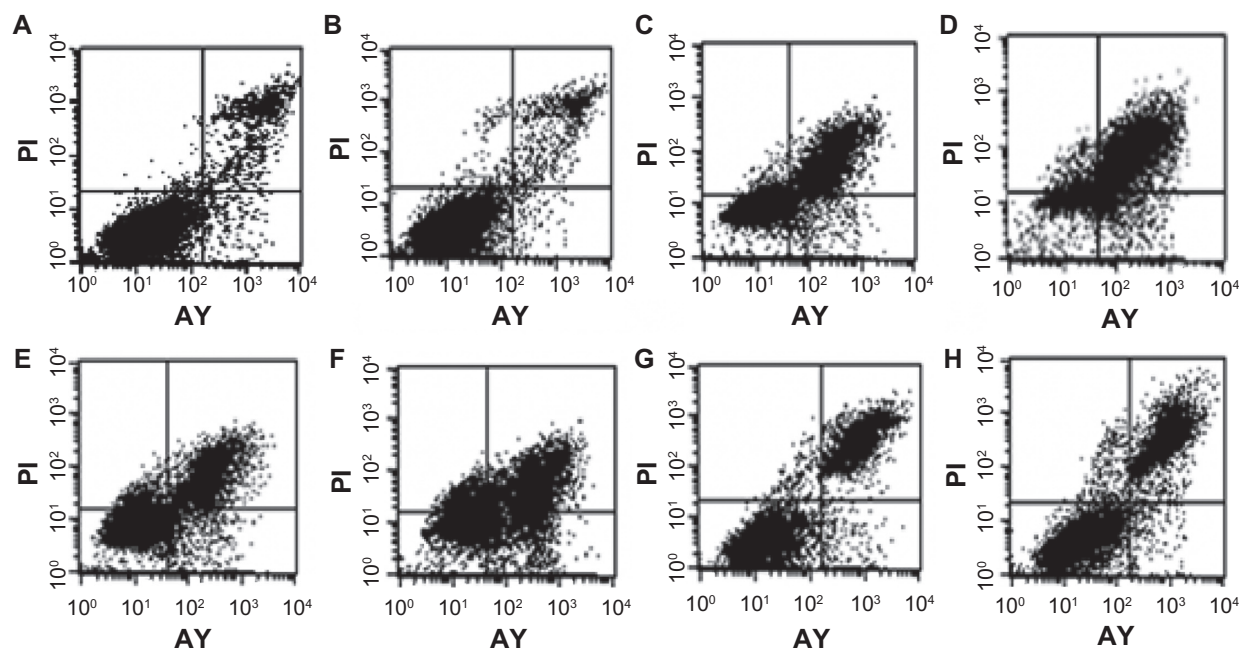
Group	6 hours	12 hours	24 hours	48 hours	72 hours
0.1 µg/mL ADM	92 ± 0.3	88.3 ± 0.6	82.2 ± 0.6	78.3 ± 0.6	70.8 ± 0.5
0.1 µg/mL ADM + 50 µmol/L MNP-Fe <sub>3</sub> O <sub>4</sub>	91.4 ± 0.7	86.4 ± 0.7	80.3 ± 0.9	76.4 ± 0.5	66.7 ± 1.1
0.1 µg/mL DNR	92.6 ± 0.4	87.3 ± 0.6	81.1 ± 0.2	79.1 ± 0.7	69.6 ± 1.0
0.1 µg/mL DNR +50 µmol/LMNP-Fe <sub>3</sub> O <sub>4</sub>	91.6 ± 0.3	85.2 ± 0.5	80.0 ± 0.7	77.4 ± 0.9	64.5 ± 2.3
100 µg/mL DEX	93.0 ± 0.5	90.1 ± 0.4	84.5 ± 0.9	79.8 ± 0.8	73.1 ± 0.8
100 µg/mL DEX +50 µmol/L MNP-Fe <sub>3</sub> O <sub>4</sub>	92.4 ± 0.3	89.6 ± 0.8	85.7 ± 0.7	80.9 ± 1.0	71.5 ± 1.2

**Abbreviations:** MNP-Fe<sub>3</sub>O<sub>4</sub>, magnetic nanoparticles of Fe<sub>3</sub>O<sub>4</sub>; ADM, adriamycin; DNR, daunorubicin; DEX, dexamethasone.

adriamycin or daunorubicin alone. However, there was no significant difference in expression of p53 protein between cells treated with dexamethasone + MNP-Fe<sub>3</sub>O<sub>4</sub> and cells treated with dexamethasone alone ( $P > 0.05$ ). NF-κB protein levels were also influenced by adriamycin + MNP-Fe<sub>3</sub>O<sub>4</sub> and daunorubicin + MNP-Fe<sub>3</sub>O<sub>4</sub>. Compared with adriamycin treatment alone, MNP-Fe<sub>3</sub>O<sub>4</sub> + adriamycin decreased NF-κB protein levels more effectively ( $P < 0.05$ ), as did MNP-Fe<sub>3</sub>O<sub>4</sub> + daunorubicin when compared with daunorubicin alone, suggesting that MNP-Fe<sub>3</sub>O<sub>4</sub> can enhance the effects of both adriamycin and daunorubicin. However, p53 protein expression in Raji cells was not significantly different ( $P > 0.05$ ) between cells treated with dexamethasone ± MNP-Fe<sub>3</sub>O<sub>4</sub>.

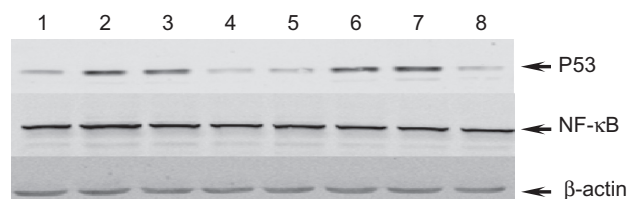
## Discussion

Resistance of lymphoma cells to chemotherapy-induced apoptosis remains the most significant contributor to the development of multidrug resistance and treatment failure. Moreover, the toxicity of high-dose anticancer drugs limits the efficacy of many chemotherapeutic agents.<sup>17</sup> Strategies to reverse the resistance mechanism have been a hot topic in tumor therapy. The potential medical applications of MNP-Fe<sub>3</sub>O<sub>4</sub> as a targeted-drug carrier with target orientation and sustained-release properties have been widely studied. It has been previously reported that MNP-Fe<sub>3</sub>O<sub>4</sub> in combination with anticancer drugs could improve the efficacy of chemotherapeutic agents which has a synergistic effect in multidrug resistance.<sup>18–21</sup>



**Figure 2** Apoptosis of Raji cells incubated with different drugs for 48 hours. **A)** control, **B)** MNP-Fe<sub>3</sub>O<sub>4</sub>, **C)** ADM, **D)** ADM + MNP-Fe<sub>3</sub>O<sub>4</sub>, **E)** DNR, **F)** DNR + MNP-Fe<sub>3</sub>O<sub>4</sub>, **G)** DEX, **H)** DEX + MNP-Fe<sub>3</sub>O<sub>4</sub>.

**Abbreviations:** MNP-Fe<sub>3</sub>O<sub>4</sub>, magnetic nanoparticles of Fe<sub>3</sub>O<sub>4</sub>; ADM, adriamycin; DNR, daunorubicin; DEX, dexamethasone.



**Figure 3** Expressions of P53 and NF- $\kappa$ B protein in Raji cells. Lane 1) control; Lane 2) 0.1  $\mu$ g/mL ADM; Lane 3) 0.1  $\mu$ g/mL DNR; Lane 4) 100  $\mu$ g/mL DEX; Lane 5) 50  $\mu$ mol/L MNP- $\text{Fe}_3\text{O}_4$ ; Lane 6) 0.1  $\mu$ g/mL ADM+ 50  $\mu$ mol/L MNP- $\text{Fe}_3\text{O}_4$ ; Lane 7) 0.1  $\mu$ g/mL DNR+ 50  $\mu$ mol/L MNP- $\text{Fe}_3\text{O}_4$ ; Lane 8) 100  $\mu$ g/mL DEX+ 50  $\mu$ mol/L MNP- $\text{Fe}_3\text{O}_4$ . **Abbreviations:** MNPs( $\text{Fe}_3\text{O}_4$ ), magnetic nanoparticles of  $\text{Fe}_3\text{O}_4$ ; ADM, adriamycin; DNR, daunorubicin; DEX, dexamethasone.

To determine if the effects of MNP- $\text{Fe}_3\text{O}_4$  combined with low-dose chemotherapy are different from those of high-dose chemotherapy, we investigated the effects of a combination of chemotherapeutic agents (adriamycin, daunorubicin, dexamethasone) with MNP- $\text{Fe}_3\text{O}_4$  on proliferation and viability of Raji cells in vitro. Our results show that both adriamycin and daunorubicin can inhibit Raji cell proliferation effectively in a dose- and time-dependent manner. Furthermore, the effect on cell sensitivity may be further improved, and the  $\text{IC}_{50}$  values for adriamycin and daunorubicin were decreased when these agents were combined with (MNP- $\text{Fe}_3\text{O}_4$ ) as a carrier, so it may be inferred that MNP- $\text{Fe}_3\text{O}_4$  has synergism with adriamycin and daunorubicin.

Apoptosis is the consequence of a series of precisely regulated cellular events and is an important metabolic step in regulating cell numbers and growth. If apoptosis is blocked, cell metabolism becomes disordered, predisposing to development and growth of tumors.<sup>22</sup> We have investigated the cytotoxicity of MNP- $\text{Fe}_3\text{O}_4$  and chemotherapeutic agents to Raji cells in vitro by flow cytometry. The combination of adriamycin or daunorubicin with MNP- $\text{Fe}_3\text{O}_4$  exerted a potent cytotoxic effect on Raji cells and induced apoptosis, suggesting that MNP- $\text{Fe}_3\text{O}_4$  synergistically enhances the apoptosis induced by adriamycin or daunorubicin. However, similar results were not observed for dexamethasone.

It is well known that p53 is an important tumor suppressor leading to apoptosis in response to most DNA-damaging agents involving primarily the intrinsic pathway,<sup>23</sup> and that NF- $\kappa$ B also plays an important role. The NF- $\kappa$ B family of transcription factors, which regulate the expression of several genes, plays a critical role in numerous cellular processes and in tumor progression via activation of the NF- $\kappa$ B signal way.<sup>24</sup> p53 defects and NF- $\kappa$ B overexpression are major contributing factors to drug resistance, and have recently been linked to reduced chemosensitivity.<sup>25</sup> In most patients with lymphoma who have loss of p53 function and

overexpression of NF- $\kappa$ B might help circumvent resistance to conventional therapies. Our results showed that either adriamycin or daunorubicin increased apoptosis of Raji cells and that this was accompanied by upregulation of p53 protein and downregulation of NF- $\kappa$ B protein. Interestingly, in the present study, MNP- $\text{Fe}_3\text{O}_4$  combined with adriamycin or daunorubicin increased upregulation of p53 protein and downregulation of NF- $\kappa$ B protein, but similar results were not found with dexamethasone. Our findings for NF- $\kappa$ B were similar to those reported by Ottonello et al, inhibition of NF- $\kappa$ B activation decreased the production of XIAP, an antiapoptotic molecule, leading to uncontrolled activity of regulators of apoptosis (eg, caspase 3).<sup>26</sup> Dexamethasone-induced apoptosis in human follicular lymphoma cells with Bcl-XL and dominant negative caspase 9 overexpression has been reported,<sup>3</sup> but this was not found for the Raji cells used in our study, and was not improved by combination with (MNP- $\text{Fe}_3\text{O}_4$ ), which needs to be explored further in the future. Overall, our results indicate that increased p53 expression and reduction of NF- $\kappa$ B induced by different activators contribute to inhibition of cell proliferation and induction of apoptosis in this system.

## Conclusion

Our study demonstrates that concomitant treatment with adriamycin or daunorubicin and MNP- $\text{Fe}_3\text{O}_4$  could enhance proliferation and inhibition, as well as increase adriamycin- and daunorubicin-induced cell apoptosis in vitro, as a result of regulation of various proliferative and antiapoptotic gene products, including p53 and NF- $\kappa$ B. This would promote a potential clinical application for MNP- $\text{Fe}_3\text{O}_4$  in combination with daunorubicin or adriamycin against lymphoma. However, the effect and safety of such combinations need to be investigated further in the clinical setting.

## Disclosure

The authors report no conflicts of interest in this work.

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