



# Dual drug-loaded PLA nanoparticles bypassing drug resistance for improved leukemia therapy

Liuliu Zhang · Huayun Zhu · Yu Gu · Xiaohua Wang · Pingping Wu

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**Abstract** Drug resistance is still a bottle-neck hindering successful chemotherapy in leukemia treatment. Nanocarriers have emerged as promising candidates to circumvent drug resistance and discover potent drug combinations. Here, we showed that co-encapsulation of daunorubicin (DNR) and glycyrrhizic acid (GA) in polylactic acid (PLA) nanoparticles effectively bypassed drug resistance and remarkably inhibited the growth of drug-resistant leukemia cells. The quantification of intracellular drug found that the encapsulation effectively increased drug uptake in the resistant K562/A02 cells. Modification of P-glycoprotein antibody on

nanoparticles further enhanced drug accumulation in the leukemia cells, which was also confirmed by fluorescent microscopy imaging. More importantly, loaded in PLA nanoparticles, DNR and GA exerted an excellent synergistic effect, leading to significantly improved cell inhibition compared to the treatments without nanocarriers and those with a single drug. Correlative mechanism study revealed that the drug delivery system drastically enhanced cell apoptosis by regulating apoptotic genes but did not influence MDR1 expression. The present study proposes a versatile strategy to improve the therapeutic efficacy against drug-resistant leukemia and inspires the discovery of more potent drug combinations for enhanced leukemia therapy.

Liuliu Zhang and Huayun Zhu contributed equally to this work.

L. Zhang · H. Zhu · X. Wang · P. Wu (✉)  
Department of Medical Oncology, Jiangsu Cancer Hospital & Jiangsu Institute of Cancer Research & The Affiliated Cancer Hospital of Nanjing Medical University, Nanjing 210009, China  
e-mail: wupingpingnano@sina.com

L. Zhang  
e-mail: zhangliuliu212@163.com

H. Zhu  
e-mail: 304311140@qq.com

X. Wang  
e-mail: wangxiaohua80@hotmail.com

Y. Gu  
Department of General Medicine, Jiangsu Province Traditional Chinese Medicine Hospital, Nanjing 210029, China  
e-mail: guyugg@hotmail.com

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## Introduction

Leukemia is a life-threatening cancer featured by fast proliferation of abnormal white blood cells in the bone marrow, which interferes with the production of normal blood cells and deteriorates rapidly (Kihara et al. 2014; Picaud et al. 2015; Nishida et al. 2015). Although clinical strategies against leukemia have gained rapid development in recent years, multidrug resistance still hinders effective therapy, especially for drug-resistant cancer.

Prolonged drug exposure or larger doses are often used to combat resistant cancer cells, but subsequently developed leukemia may be induced, which is a detrimental consequence after chemotherapy (Whitlock et al. 1991; Bishop 1997). Moreover, due to multidrug resistance, the patients achieving partial or complete remission after conventional treatments often suffer from tumor recurrence with low disease-free survival rate (Holohan et al. 2013; Li et al. 2014; Zhang et al. 2014, 2015a, b). On the other hand, conventional chemotherapy often cause various unpleasant side effects due to non-specific drug distribution that poses unavoidable toxicity to normal tissues (Döhner et al. 2015; Fox et al. 2017; Stauss and Morris 2013). Therefore, it is particularly attractive to precisely deliver drugs to cancer cells, overcome drug resistance, and relieve the toxicity of chemotherapy.

Nanocarriers with distinct advantages and multiple functionalities can serve as potential candidates to address the above issues (Chen et al. 2017; Zhang et al. 2015a, b). The encapsulation of chemotherapeutic agents, such as doxorubicin and daunorubicin, in nanocarriers facilitates the bypass of efflux-pump in resistant cells (Meng et al. 2013; Zhu et al. 2014; Kirtane et al. 2013; Loos et al. 2014). In addition, nanocarriers have been proved effective to reduce side effects by improving the pharmacokinetic property and increasing drug concentration in tumor (Wang et al. 2013; Rochani et al. 2016; Zhao and Zhu 2016; Singh et al. 2010). However, few of the reported nanosystems have demonstrated a potent effect in drug-resistant myeloid leukemia that responds poorly to first-line chemotherapy. To further improve the therapeutic efficacy of nano-drugs in leukemia, the co-encapsulation of different chemo-drugs is particularly appealing.

As a broad-spectrum anticancer drug, daunorubicin (DNR) is remarkably effective in both solid tumors and leukemia by disrupting DNA replication. However, drug resistance and its cardiotoxicity have substantially limited its clinical application (Sheng et al. 2015; Halley et al. 2016). Glycyrrhizic acid (GA) is a natural product extracted from the root of the plant *Glycyrrhiza glabra* (licorice) with multiple therapeutic effects (Csuk 2014; Cai et al. 2016). It was found to induce apoptosis in different types of promyelotic leukemia (Hibasami et al. 2005; Thirugnanam et al. 2008). Different from DNR, GA suppresses the proliferation of leukemia cells mainly by interfering with the AKT signaling pathway (He et al. 2015). Therefore, a synergistic effect may be achieved through the combination of DNR with GA.

Nonetheless, to the best of our knowledge, the combination of DNR and GA in one nanocarrier remains barely explored in leukemia therapy.

Among the numerous categories of nanocarriers for drug delivery, including liposomes, polymeric micelles, dendrimers, and inorganic nanoparticles (Mura et al. 2013), polylactic acid (PLA) has attracted much attention due to its chemical versatility, biodegradability, and biocompatibility, which are important properties for further development (Danafar et al. 2014; Cui et al. 2013). For the delivery of specific drugs, it is also necessary to compare the drug loading properties of different nanoparticles to select the optimal carrier. In this study, PLA/polyvinyl alcohol (PVA) nanoparticles were encapsulated with DNR and GA and further functionalized with P-glycoprotein (P-gp) antibody for targeted drug delivery in resistant leukemia cells. The nanosystem was constructed and optimized with detailed evaluation of encapsulation efficiency (EE) and drug loading capacity (DL). The comparison of EE and DL with chitosan and albumin nanoparticles was also performed. Then, circumvent of efflux-pump and the improved drug uptake by antibody modification were proved by fluorescence microscopy and HPLC quantification. Afterwards, we evaluated if the drug combination could substantially improve the therapeutic efficacy against MDR leukemia cells. Further, we revealed the underlying molecular mechanism by which the nanosystem circumvented drug resistance. To provide basis for future in vivo study, the toxicity of the nanoconstruct was evaluated on healthy mice. Altogether, this study has provided solid evidence for the combined application of DNR and GA in one simple nanosystem, inspiring the development of nano-medicine for more effective therapy against drug-resistant leukemia.

## Materials and methods

### Preparation of drug-carried nanocapsules

The solvent evaporation method was used to prepare the nanocapsules, which carried both GA and DNR. The total mass of nanocapsules were fixed to 1 g. Briefly, PLA (350 mg) and PVA (150 mg) were dissolved in chloroform by 5 min ultrasound. GA (250 mg) and DNR (250 mg) were then added into the solution for another 5 min ultrasound. Afterwards, the suspension was slowly dropped into the PVA aqueous solution and

stirred constantly for 2 h, of which the DNR and GA were saturated. Third, the milky white product, “water in oil in water” (W/O/W) compound emulsion, was formed after 10,000 rpm centrifugation for 1 h. Rotary evaporator was then used to vaporize the chloroform completely. Finally, the microcapsules were washed with distilled water for 4–5 times at room temperature, and dried with a vacuum dryer for 24 h. To conjugate P-g Ab on the nanoparticles, 25% glutaraldehyde solution was added into the microcapsules at room temperature. After 10 min cross-linking, the excessive glutaraldehyde was removed with physiological saline by dialysis for 4 h. Five micrograms P-g Ab was then added into the solution with magnetic stirring and reacted overnight. A laser diffraction micrometer particle size measuring instrument (Master Sizer) was adapted to measure the nanoparticle diameter with the function of the Spherical multimodal model, and the structure of nanocapsules was observed by transmission electron microscope.

For the preparation of chitosan nanoparticles, chitosan (1.0 g, 50 kDa) was dissolved and then dispersed in methanol with fierce stirring. Succinic anhydride acetone ( $0.18 \text{ g mL}^{-1}$ ) was reacted with chitosan for 36 h and the mixture was neutralized by 1 M NaOH. After lyophilization, the products were re-dispersed in 1% acetic acid and reacted with 0.5 g octaldehyde for 6 h. Then, 0.2 g/mL  $\text{NaBH}_4$  was slowly added and reacted overnight. The reaction was stopped by 1 M NaOH, and the final products were obtained after dialyzation and lyophilization. BSA nanoparticles of 100 nm diameter were prepared by adding 1% BSA in distilled water with a pH value of 9. The solution was then stirred at 500 rpm for 5 h, and the final products were obtained after dialyzation and lyophilization.

#### Optimization of drug loading capacity and encapsulation efficiency

On the basis of a single factor, orthogonal design was used to evaluate the effects of different factors on the loading efficiency and encapsulation capacity. The factors investigated were emulsifier, polyvinyl alcohol (PVA) concentration, poly lactic acid (PLA) concentration, drug carrier ratio, dispersion phase (oil phase), and volume ratio of the continuous phase (aqueous phase), respectively. Parallel experiments were conducted at three levels for each factor, and the encapsulation efficiency EE% and drug loading DL% were evaluated by HPLC. The HPLC conditions to quantify DNR and GA

in the complex are as follows: the complex suspensions were dissolved in 3 mL 0.1% mixed solution (TFA/MeOH = 3:1). Then, DNR and GA were quantified with the Agilent 1100 system and reversed phase C18 column. For DNR, the mobile phase was 0.1% mixed solution of TFA/MeOH = 3:1, and acetonitrile. The DNR concentration was determined at 480 nm under 1 mL/min flow rate. For GA, gradient elution was used containing 0.05% trifluoroacetic acid with 0.8 mL/min flow rate. GA concentration was measured at 254 nm with 15 min retention time.

#### Cell culture

The multidrug-resistant leukemia cell K562/A02 was purchased from the Shanghai Institute of Cell Biology, Chinese Academy of Sciences (Shanghai, China). K562/A02 cells were cultured in RPMI 1640 (Gibco, USA) supplemented with 10% FBS, 100 mg/mL streptomycin, and 100 IU/ml penicillin in 5%  $\text{CO}_2$  atmosphere at 37 °C.

#### Fluorescence microscopy

K562/A02 cells were divided into two groups and incubated with the dual drug-loaded PVA/PLA nanoparticles with and without P-glycoprotein, respectively. Drug concentrations were 1 and 10  $\mu\text{M}$  for DNR and GA, respectively. The cells were subjected to fluorescent microscopy and observed for 10 h to evaluate the cellular uptake of the nanoparticles.

#### Cell proliferation assay

Cell proliferation was analyzed using the Cell Proliferation Reagent Kit 1(MTT) (Sigma, USA). In total, K562/A02 cells were seeded in 96-well plates at  $5 \times 10^3$  per well and cultured for 24 h. MTT solution was added into each well for 4 h. After removing the medium, the absorbance was measured using a Microplate Reader (Bio-Rad, Hercules, CA, USA) at 490 nm. Each experiment was conducted in triplicate.

#### Cell apoptosis analysis

Cell apoptosis was performed using the Annexin V Apoptosis Detection kit I (BD Biosciences, USA). After incubation for 24 h, cells were digested with trypsin and washed in cold PBS twice. Subsequently, 500  $\mu\text{L}$  buffer,

5  $\mu$ L FITC annexin V, and 5  $\mu$ L propidium iodide (PI) were added. Finally, apoptosis was assessed by flow cytometry (FACScan, BD Biosciences).

#### qRT-PCR analysis

Total RNA was extracted from tissue samples and cultured cells using the Trizol reagent (Invitrogen) according to the manufacturer's instructions. Total RNA was reversed transcribed into cDNA using the TaqMan microRNA Reverse Transcription Kit (Applied Biosystems, USA). The qRT-PCR reactions were performed using the Fast Start Universal SYBR Green Master (Rox) (Roche Applied Science) on a 7500 Real-time system (Applied Biosystems, USA) according to the manufacturer's protocols. The levels of miR-10a and Tiam1 were normalized by U6 and  $\beta$ -actin, respectively. Primer pairs used in this study were the following:

Homo-Bcl-2: 5'-GGTGGGGTCATGTGTGTGG-3' (forward)

Homo-Bcl-2: 5'-CGGTTCAGGTAAGTCAAGTCATCC-3' (reverse)

Homo-Bax: 5'-CCCGAGAGGTCTTTTTCCGAG-3' (forward)

Homo-Bax: 5'-CCAGCCCATGATGGTTCTGAT-3' (reverse)

The qRT-PCR reactions were performed in triplicate and included no-template controls.

#### Western blot

Total proteins were extracted from cells using the RIPA lysis buffer (50 mM Tris-HCl, pH 7.4, 150 mM NaCl, 0.2% SDS, 1% Triton X-100, 2 mM EDTA) for 30 min on ice. The supernatant was collected by centrifuging at 12,000 rpm, 10 min. After the loading buffer was added, the mixtures were treated at 100 °C for 5 min to denature the protein. Protein concentration was determined by the BCA Quantification Kit (Beyotime, Beijing, China). The samples (30 mg) were separated on 10% SDS-PAGE and blotted onto a PVDF membrane. The membranes were blocked with 5% no-fat milk (*w/v*) at room temperature for 1 h and incubated with primary antibodies overnight at 4 °C: rabbit anti-human GAPDH antibody (1: 2000, Cell Signaling Technology, Beverly, MA, USA), rabbit anti-human Bax antibody (1: 2000,

Cell Signaling Technology, Beverly, MA, USA), and rabbit anti-human Bcl-2 antibody (1: 2000, Cell Signaling Technology, Beverly, MA, USA). After incubation, the membranes were washed with PBST three times again, and then incubated with a horseradish peroxidase (HRP)-conjugated secondary antibody (Boster Biotechnology, Wuhan, China) at room temperature for 2 h. The immuno-reactivity was visualized using the ECL Western blotting kit (Beyotime, Beijing, China) according to the manufacturer's protocols. GAPDH was employed as a loading control.

#### In vivo toxicity assay

Balb/c nude mice of 6 weeks old were purchased from Qing Long Shan company, Nanjing, China. Six mice were randomly divided into a control group and an experiment group with three mice in each group. Mice in the experiment group were injected with nanoparticles (1 mg/kg DNR and 1 mg/kg GA) every 2 days for 15 days. The free-drugs group was treated with 1 mg/kg DNR and 1 mg/kg GA without nano-encapsulation. The control group was injected with saline at the same time points. Hematoxylin-eosin (HE) staining was performed for the major organs after treatments. Animal experiments were carried out in accordance with Guidelines for the Care and Use of Laboratory Animals in Jiangsu Cancer Hospital.

#### Statistical analysis

The experiments in this study were performed for three times and data were presented as mean  $\pm$  SD. One-way ANOVA with Tukey–Kramer multiple comparison post hoc tests were used for statistical analysis. \* $P < 0.05$  and \*\* $P \leq 0.01$  were used to indicate that the difference is statistically significant.

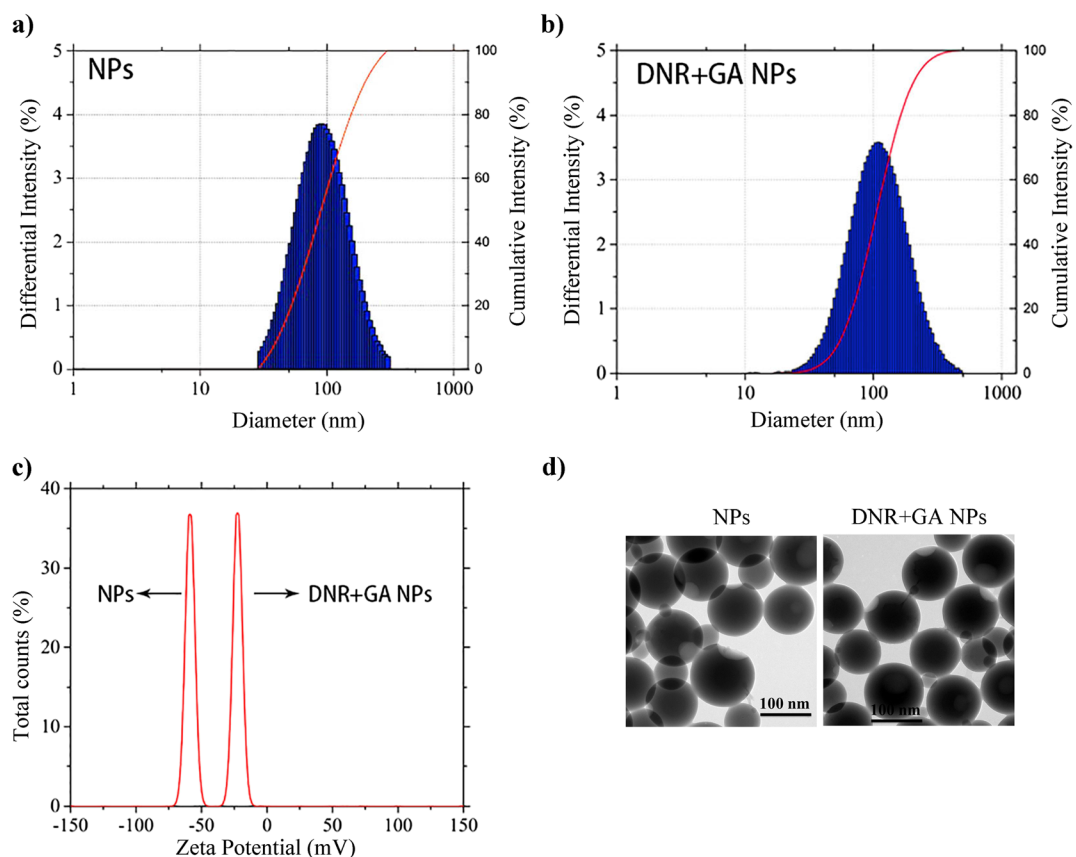
## Results and discussion

### Preparation and characterization of DNR/GA-loaded PVA/PLA nanoparticles

PVA/PLA nanoparticles were successfully prepared using the solvent evaporation method. DLS data showed that the mean diameter of the nanoparticles was about 90 nm, which increased to approximately 110 nm after P-p-glycoprotein antibody modification (Fig. 1a, b).

Also, Zeta potential shifted from  $-57$  to  $-20$  mV after antibody attachment (Fig. 1c). The narrow size distribution evidenced the overall homogenous property of our products. TEM images of the original (left) and antibody cross-linked (right) particles further confirmed the uniform distribution with spherical morphology, well consistent with the DLS data (Fig. 1d). To achieve an optimal drug loading efficiency and encapsulation capacity, the ingredients of the nanocarrier were carefully adjusted. The proportion of the main components, including the oil phase (PLA, poly lactic acid), water phase (DNR/GA), and surfactant phase (PVA, polyvinyl alcohol), was investigated. The results of the average EE% (encapsulation efficiency) and DL% (drug loadings) were used to provide a comprehensive evaluation of these factors (Fig. 2a). The optimal formulation was selected with 50% water, 40% PLA, and 10% PVA, achieving EE% of 68.1 and DL% of 1.29, respectively,

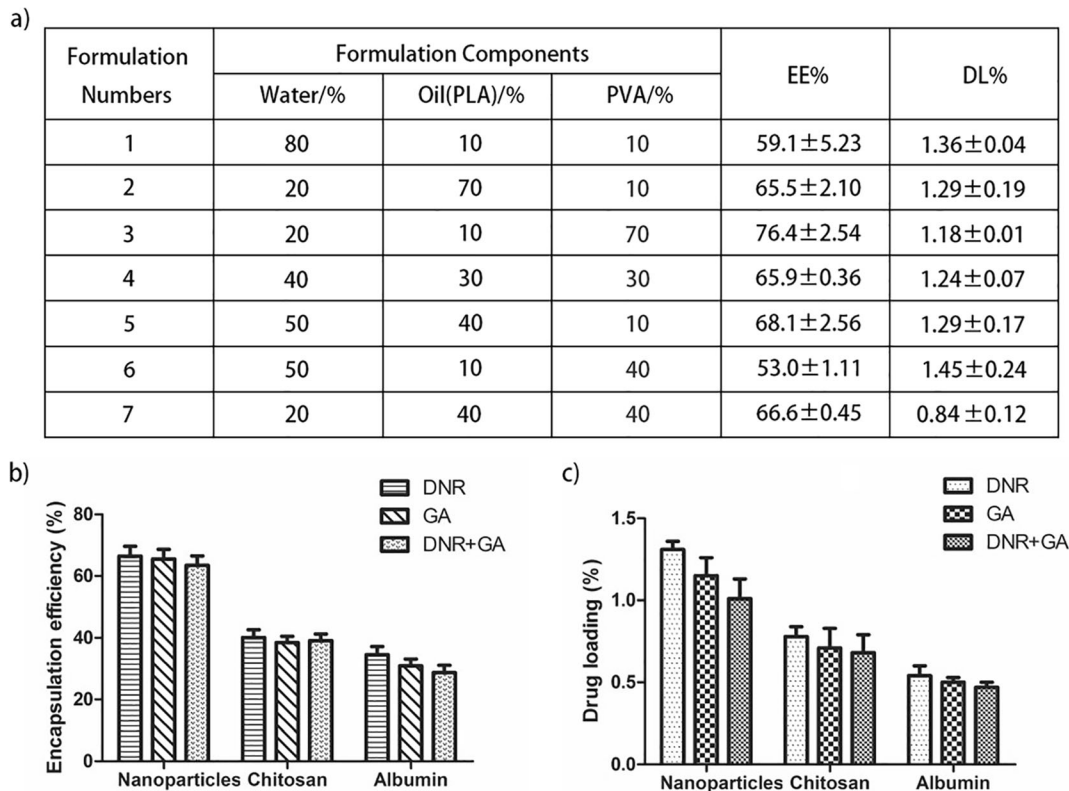
for DNR. Similarly, this formulation also resulted in favorable EE% (3) and DL% (1.13) for GA. Moreover, the EE and DL of our prepared nanoparticles were compared with other commonly used carriers including chitosan and albumin (Fig. 2b, c). The PLA nanoparticles demonstrated higher EE and DL for DNR and GA, probably because PLA nanoparticles were more appropriate to load hydrophilic drugs compared with chitosan and albumin. In vitro release of the loaded DNR and GA were tested at  $37$  °C in PBS solutions at a pH value of 7.4, which represented the normal physiological environments. The HPLC peaks and corresponding standard curves used for the calculation were shown in Fig. 3a. GA and DNR showed similar drug release profiles during the 96-h examination, which showed more than 90% release at 48 h and remained nearly stable afterwards (Fig. 3b).



**Fig. 1** Characterization of the prepared dual drug-loaded PVA/PLA nanoparticles. **a, b** Dynamic light scattering (DLS) analysis of the diameter of the nanoparticles before and after p-glycoprotein antibody modification. **c** Changes of ZETA potential before and

after antibody modification. **d** TEM images of the dual drug-loaded PVA/PLA nanoparticles before and after antibody modification





**Fig. 2** Evaluation and optimization of drug loading capacity and encapsulation efficiency. **a** Changes of drug loading capacity and encapsulation efficiency with different formulation components. **b** Comparison of encapsulation efficiency of PVA/PLA

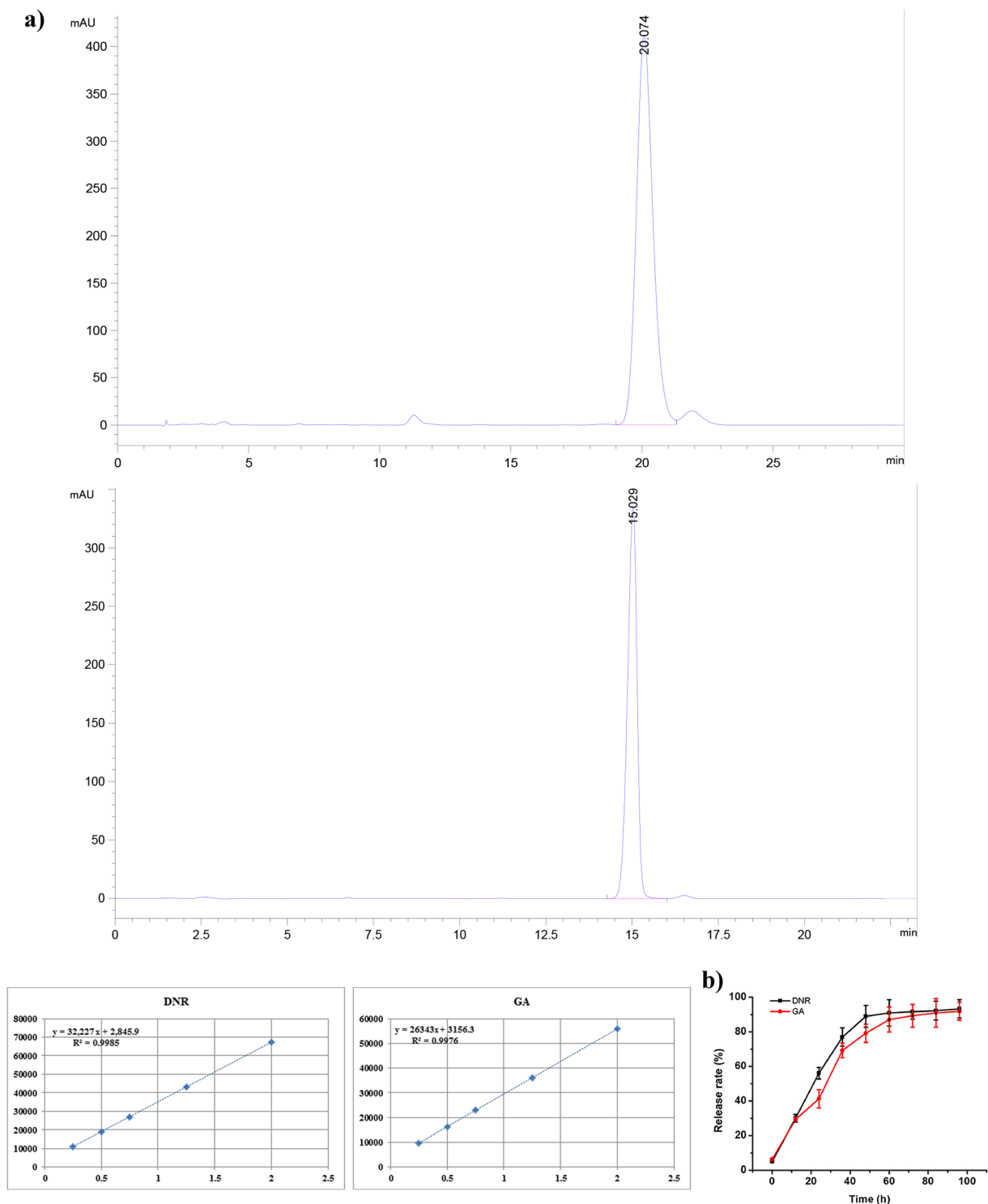
nanoparticles with conventional carriers of chitosan and albumin. **c** Comparison of drug loading capacity of PVA/PLA nanoparticles with conventional carriers of chitosan and albumin

#### Cellular uptake of DNR/GA-loaded PVA/PLA nanoparticles

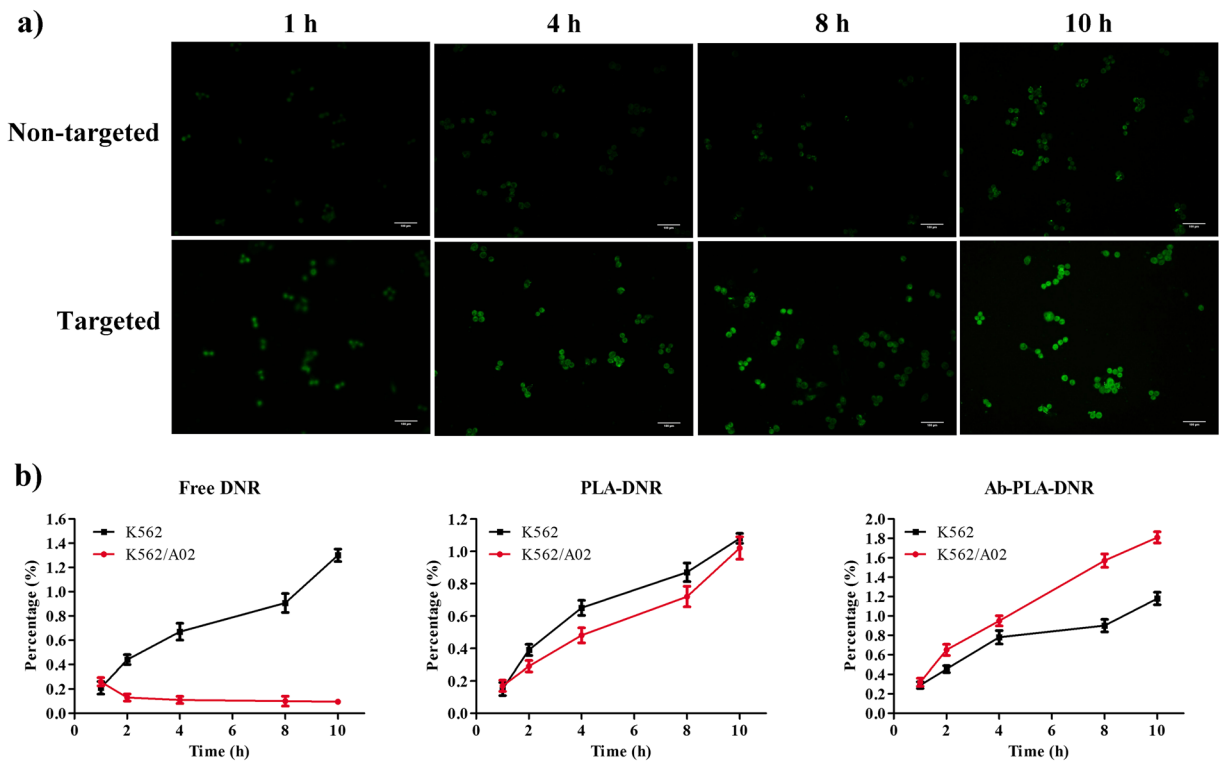
The overexpression of P-glycoprotein largely contributes to multidrug resistance (MDR) of cancer cells, which can also be used to differentiate malignant cells from normal ones. Thus, targeting P-glycoprotein has been extensively explored for specific drug delivery to MDR cells (Li et al. 2010). To improve the precision of the nanoparticles to kill leukemia cells, the P-glycoprotein antibody was modified on the particle surface. In this experiment, K562/A02 cells were incubated with the nanostructure or free drug for different time periods and then subjected to fluorescent microscopy. As a multidrug-resistant leukemia cell line, K562/A02 is derived from the parent K562 cells that are sensitive to chemotherapy. Fluorescent microscopy found that DNR encapsulated in PLA nanoparticles gradually accumulated in the drug-resistant cells during the 10-h monitoring, as illustrated by the increased fluorescence. Therefore, PLA-mediated drug delivery could overcome the

expulsion of DNR from drug-resistant cancer cells. For antibody conjugated nanoparticles, obvious DNR fluorescence was soon observed 1 h after incubation, which increased sustainably and peaked at 10 h, which was significantly stronger than that without antibody modification (Fig. 4). It was then believed that the P-glycoprotein antibody could further improve intracellular drug distribution by active targeting.

Further, we quantified the intracellular drug amount at different time points for various treatments in both K562 and K562/A02 cells. The percentages of cellular uptake were calculated by comparing the originally added drug and the drug remained in the supernatants using HPLC. As shown in Fig. 4b, the amount of free DNR gradually increased during the 10-h incubation time in parental K562 cells, to about 1.3% of the total incubated drug. For the resistant K562/A02 cells, intracellular DNR decreased from 0.25% at 1 h to about 0.1% at 10 h. For the PLA-encapsulated DNR, the uptake percentage in both K562 and K562/A02 cells increased to about 1% in 10 h, indicating that



**Fig. 3** a HPLC peaks of DNR and GA from instrument and corresponding standard curves. b Release profiles of DNR and GA from the PVA/PLA nanoparticles in PBS solution within 96 h

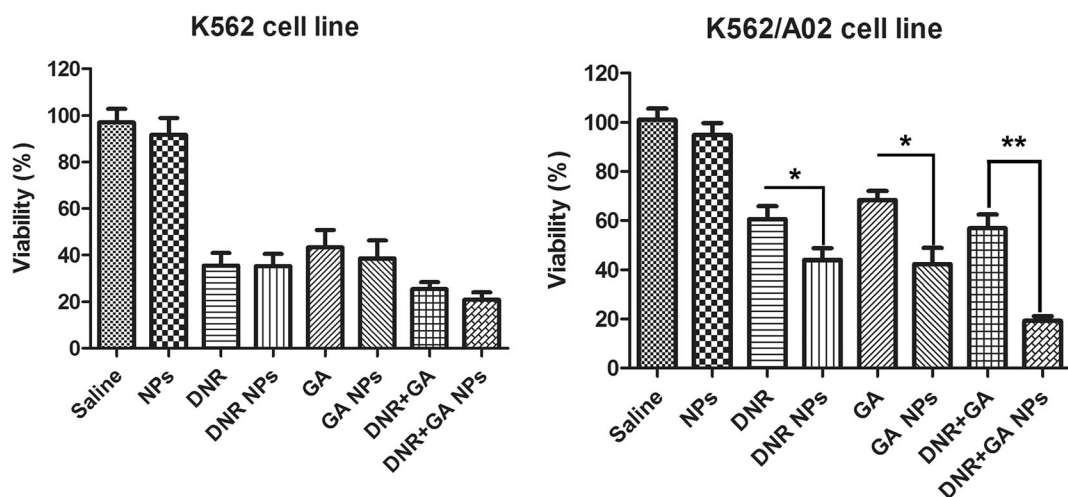


**Fig. 4** a Fluorescent imaging of cellular uptake of the drug-loaded nanocarriers with and without P-glycoprotein antibody modification at 1, 4, 8, and 10 h after incubation. b Quantification of the

intracellular drug amount at different time points for various treatments in K562 and K562/A02 cells. Scale bar = 100 μm

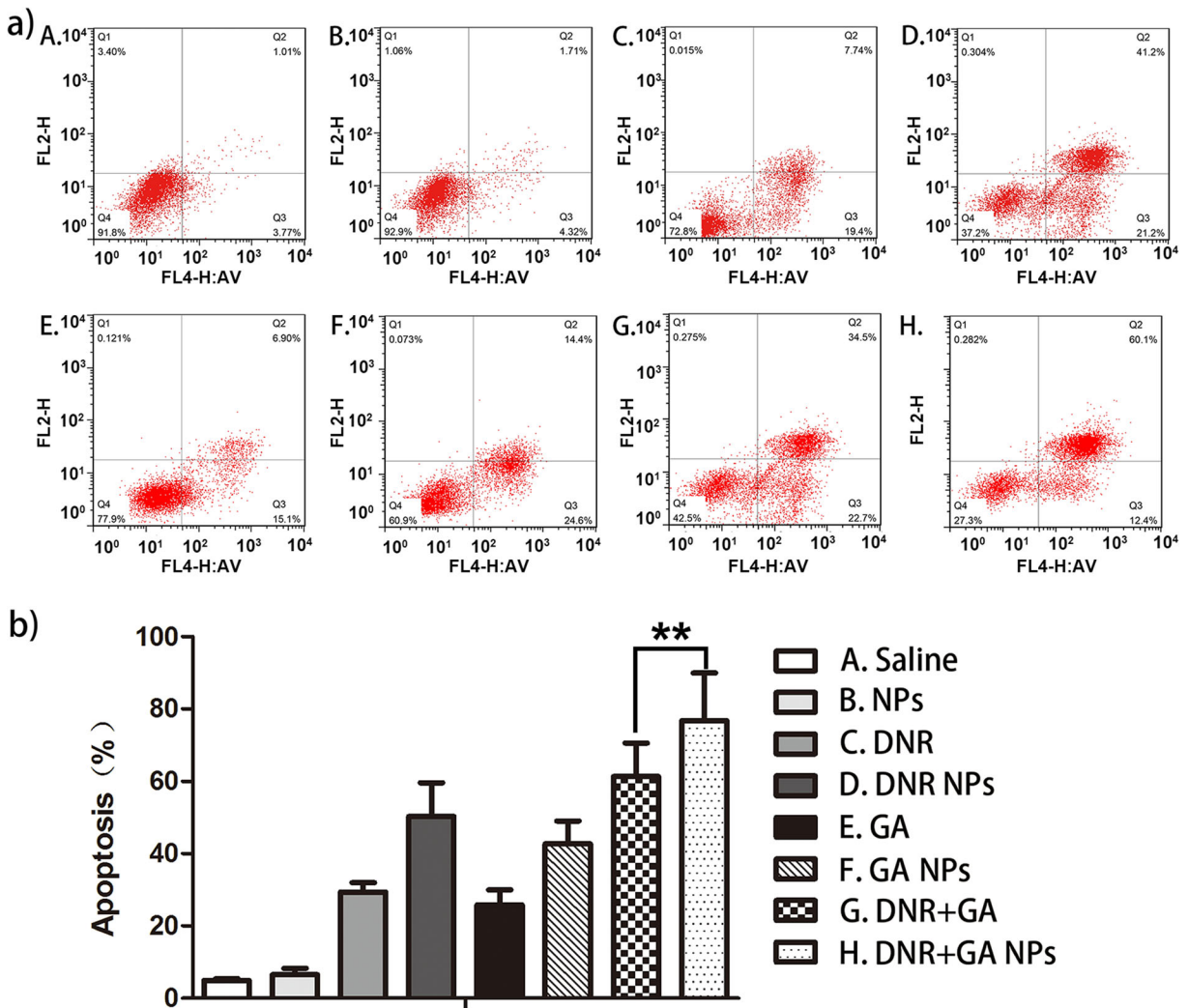
the uptake of nanoparticles was not compromised by drug resistance. Finally, the antibody conjugated nanoparticles demonstrated the most abundant drug

accumulation in the drug-resistant K562/A02 cell line, reaching 1.8% of the originally added drug. These data strongly supported the ability of the



**Fig. 5** MTT results of the efficacy of different treatments on K562 and K562/A02 cells. Data were presented as mean ± SD (n = 3). \*\*P < 0.01, \* P < 0.05





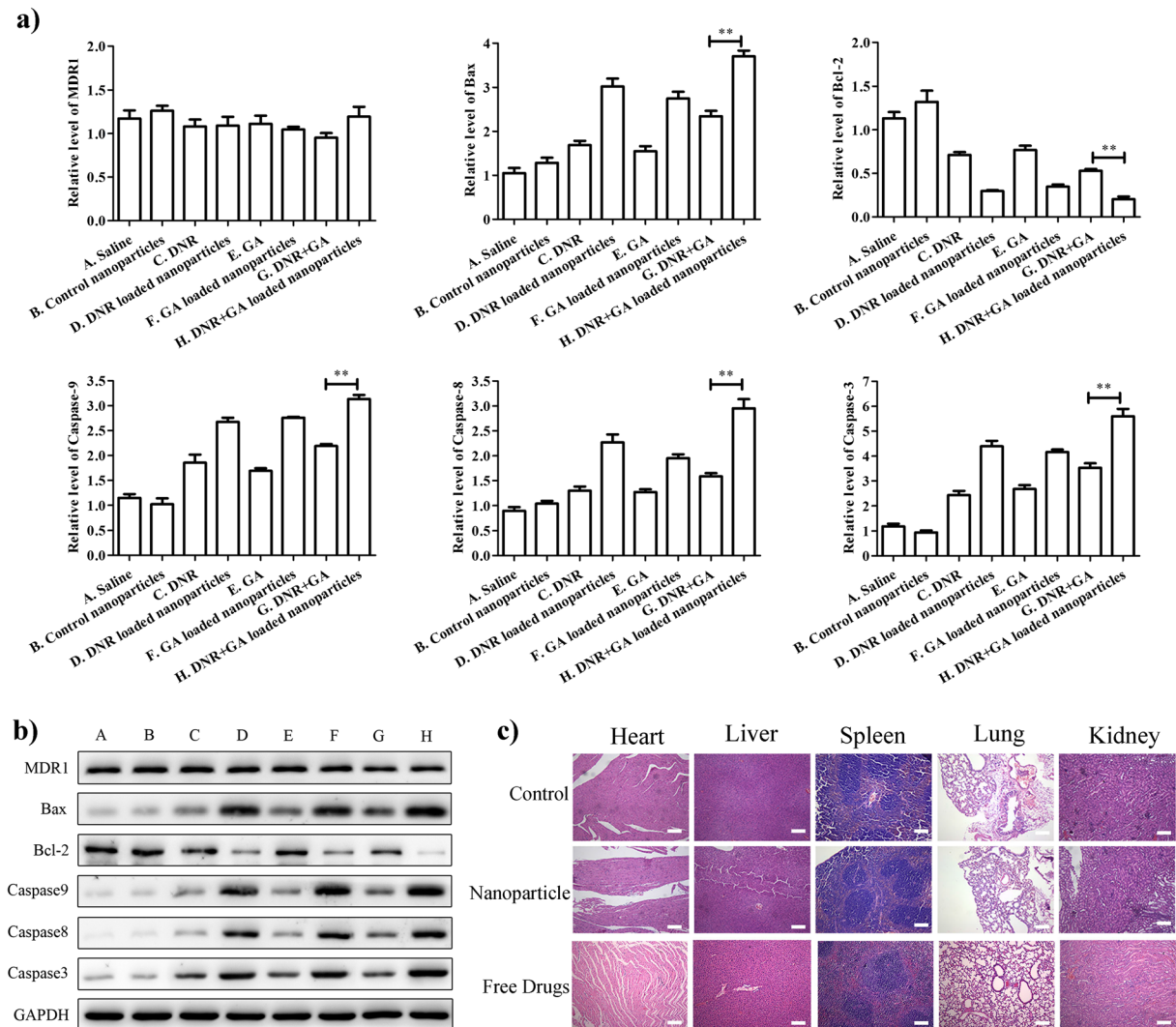
**Fig. 6** Flow cytometry analysis of the apoptosis of the K562/A02 cells with different treatments. Data were presented as mean  $\pm$  SD ( $n = 3$ ).  $**P < 0.01$

drug-loaded PLA nanoparticles to circumvent drug resistance and the effectiveness of P-glycoprotein antibody modification on improving drug internalization.

### Therapeutic efficacy of DNR/GA-loaded PVA/PLA nanoparticles

Given the nanosystem was readily internalized by K562/A02 cells, it was speculated that the drug resistance could be circumvented. We also used the parental K562 cell line without drug resistance as control. In this experiment, the cells were divided into 8 groups to comprehensively evaluate the therapeutic efficacy of

different treatments after 24 h incubation. The concentrations of the drugs were 1 and 10  $\mu$ M for DNR and GA, respectively. As shown in Fig. 5, the nanoparticles without drug loading showed favorable biocompatibility in both K562 and K562/A02 cells, demonstrating comparable cell viability to the saline control. For the non-resistant K562 cells, free DNR treatment effectively inhibited cell proliferation, eradicating more than 60% of cells after 24 h incubation. Free GA also potently suppressed cell growth. The combined use of DNR and GA induced a more pronounced cell killing effect. It was observed that K562 cells were sensitive to both drugs, and the nanoparticle delivery did not significantly



change the therapeutic efficacy, neither in single treatment nor combined therapy.

In comparison, K562/A02 cells were strongly resistant to the treatment of free DNR or GA, as more than 60% of cancer cells survived the therapy after 24 h. Dual drug therapy also failed to significantly reduce cell viability compared with that of a single drug. The poor antitumor effect is largely attributed to multidrug resistance of the cell. However, after being encapsulated into the nanosystem, the potency of each drug was significantly improved. The encapsulated DNR and GA

the treatments of cells in groups from A to H are the same as those from **a**. **c** HE staining of major organs from mice treated with saline, free drugs, and the nanoconstructs. Scale bar = 100  $\mu$ m. Data were presented as mean  $\pm$  SD ( $n = 3$ ). \*\* $P < 0.01$

achieved much enhanced cell inhibition compared to the non-encapsulated drugs, with approximately 50% of cancer cells eradicated. The most potent loss of cell viability was found in the group incubated with the dual drug-loaded nanoparticles with an inhibition ratio higher than 80%, probably due to the synergistic effect of DNR and GA with different acting mechanisms. Therefore, nano-encapsulation could increase drug toxicity against the MDR leukemia cells to a very large extent, which was mainly attributed to the circumvention of drug resistance by nanoparticles. Of note, the effectiveness

of the drug combination was only achieved after encapsulation, as evidenced by significantly improved cell toxicity compared to the simple blending ( $P < 0.01$ ). Flow cytometry results were consistent with the MTT observation, and the number of apoptosis cells was the largest in the group treated with DNR/GA-loaded nanoparticles (Fig. 6). Thus, in the discovery of drug combinations in the treatment of drug-resistant leukemia, using nanocarriers can provide a more objective evaluation of the therapeutic efficacy that is overshadowed by MDR.

#### Mechanism of the apoptosis induced by DNR/GA-loaded PVA/PLA nanoparticles

A major mechanism responsible for multiple drug resistance is the overexpression of the MDR1 gene, which encodes P-glycoprotein, an ATPase effluxing drug molecule out of the cell membrane. To explore how the nanosystem bypassed the resistance mechanism in K562/A02 cells, we evaluated the effects of different treatments on MDR1 and P-glycoprotein expressions. As shown in Fig. 7, the expressions of both MDR1 and P-glycoprotein were almost equal among the groups subjected to different treatments, and showed no significant difference compared with the saline control. Therefore, the enhanced therapeutic efficacy of the drug-loaded nanoparticles was not correlated with the regulation of MDR1 genes. Bcl-2 and Bax are responsible for the regulation of mitochondrial apoptosis induced by extracellular stresses and serve as anti-apoptotic and pro-apoptotic proteins, respectively. We then tested the expression of apoptosis-related genes and proteins by polymerase chain reaction and Western blot. K562/A02 cells treated with DNR + GA-loaded nanoparticles showed a significantly reduced expression of anti-apoptotic protein (Bcl-2) compared with other groups ( $P < 0.01$ ). On the other hand, the levels of activated Caspases 9, 8, and 3, the indicators of mitochondria-mediated apoptosis, were significantly increased in cells treated with the drugs encapsulated in nanoparticles compared to the free counterparts ( $P < 0.01$ ). These observations pointed to the superior efficacy of our nano-preparations to regulate the apoptosis of the drug-resistant leukemia cells. As the MDR-related proteins were not influenced by our treatments, the greatly enhanced pro-

apoptosis effect was most probably resulted from the targeted delivery of anticancer agents into cells, which increased intracellular drug concentration and bypassed the transmembrane effluxing mechanism. As in vivo toxicity is an important concern for further application of nanoparticles (Zhang et al. 2012), the toxicity of the nanoconstructs was tested in healthy mice. HE staining showed that free-drugs treatment caused obvious cardiotoxicity compared to the saline control, while the injection of the nanoparticles induced a negligible damage to normal organs including the heart, liver, spleen, lung, and kidney, which paved the way for future in vivo leukemia therapy.

#### Conclusion

Conclusively, we have developed a simple and versatile drug delivery strategy using PVA/PLA nanoparticles to circumvent the multiple drug resistance of leukemia cells. This system carries DNR and GA, a conventional broad-spectrum anticancer agent and a relatively new nature product recently proved effective in leukemia, respectively. With distinctly different mechanisms of action, the encapsulation of the two drugs in the nanoparticles has exhibited a remarkably enhanced therapeutic efficacy compared to the simple blending and single-drug treatments. Such observations suggest that the therapeutic potential of the drug combination can be fully unleashed by nano-encapsulation. The nanosystem did not alter the expression of the genes associated with drug resistance, but enhanced cell apoptosis by targeted drug delivery. Therefore, this strategy not only offers a potential strategy to bypass multidrug resistance by nano-encapsulation, but also inspires future design of new drug combinations using the biocompatible nanocarriers.

**Authors' contributions** HZ designed the experiments. LZ, YG, and XW performed the experiments and handed the data. PW wrote and edited the paper. PW and HZ contributed equally to this work.

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### Compliance with ethical standards

**Conflict interest** The authors declare that they have no conflict of interest.

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### References

- Bishop JF (1997) The treatment of adult acute myeloid leukemia. *Semin Oncol* 24:57–69
- Cai Y, Xu Y, Chan HF, Fang X, He C, Chen M (2016) Glycyrrhetic acid mediated drug delivery carriers for hepatocellular carcinoma therapy. *Mol Pharm* 13:699–709
- Chen FY, Zhang Y, Chen XY, Li JQ, Xiao XP, Yu LL, Tang Q (2017) Development of a hybrid paclitaxel-loaded arsenite nanoparticle (HPAN) delivery system for synergistic combined therapy of paclitaxel-resistant cancer. *J Nanopart Res* 19:155
- Csuk R (2014) Recent developments in the synthesis of antitumor-active glycyrrhetic acid derivatives. *Mini-Rev Org Chem* 11:253–261
- Cui F, Li Y, Zhou S, Jia M, Yang X, Yu F, Ye S, Hou Z, Xie L (2013) A comparative in vitro evaluation of self-assembled PTX-PLA and PTX-MPEG-PLA nanoparticles. *Nanoscale Res Lett* 8:301
- Danafar H, Rostamizadeh K, Davaran S, Hamidi M (2014) PLA-PEG-PLA copolymer-based polymersomes as nanocarriers for delivery of hydrophilic and hydrophobic drugs: preparation and evaluation with atorvastatin and lisinopril. *Drug Dev Ind Pharm* 40:1411–1420
- Döhner H, Weisdorf DJ, Bloomfield CD (2015) Acute myeloid leukemia. *N Engl J Med* 373:1136–1152
- Fox LC, Cummins KD, Costello B, Yeung D, Cleary R, Forsyth C, Tatarczuch M, Burbury K, Motorna O, Shortt J, Fleming S, McQuillan A, Schwarzer A, Harrup R, Holmes A, Ratnasingham S, Chan KL, Hsu WH, Ashraf A, Putt F, Grigg A (2017) The incidence and natural history of dasatinib complications in the treatment of chronic myeloid leukemia. *Blood Adv* 1:802–811
- Halley PD, Lucas CR, McWilliams EM, Webber MJ, Patton RA, Kural C, Lucas DM, Byrd JC, Castro CE (2016) Daunorubicin-loaded DNA origami nanostructures circumvent drug-resistance mechanisms in a leukemia model. *Small* 12:308–320
- He SQ, Gao M, Fu YF et al (2015) Glycyrrhizic acid inhibits leukemia cell growth and migration via blocking AKT/mTOR/STAT3 signaling. *Int J Clin Exp Med* 8:5175
- Hibasami H, Iwase H, Yoshioka K, Takahashi H (2005) Glycyrrhizin induces apoptosis in human stomach cancer KATO III and human promyelocytic leukemia HL-60 cells. *Int J Mol Med* 16:233–236
- Holohan C, Van Schaeybroeck S, Longley DB et al (2013) Cancer drug resistance: an evolving paradigm. *Nat Rev Cancer* 13:714–726
- Kihara R, Nagata Y, Kiyoi H, Kato T, Yamamoto E, Suzuki K, Chen F, Asou N, Ohtake S, Miyawaki S, Miyazaki Y, Sakura T, Ozawa Y, Usui N, Kanamori H, Kiguchi T, Imai K, Uike N, Kimura F, Kitamura K, Nakaseko C, Onizuka M, Takeshita A, Ishida F, Suzushima H, Kato Y, Miwa H, Shiraishi Y, Chiba K, Tanaka H, Miyano S, Ogawa S, Naoe T (2014) Comprehensive analysis of genetic alterations and their prognostic impacts in adult acute myeloid leukemia patients. *Leukemia* 28:1586–1595
- Kirtane AR, Kalscheuer SM, Panyam J (2013) Exploiting nanotechnology to overcome tumor drug resistance: challenges and opportunities. *Adv Drug Deliv Rev* 65:1731–1747
- Li R, Wu R, Zhao L et al (2010) P-glycoprotein antibody functionalized carbon nanotube overcomes the multidrug resistance of human leukemia cells. *ACS Nano* 4:1399–1408
- Li L, Osdal T, Ho Y, Chun S, McDonald T, Agarwal P, Lin A, Chu S, Qi J, Li L, Hsieh YT, Dos Santos C, Yuan H, Ha TQ, Popa M, Hovland R, Bruserud Ø, Gjertsen BT, Kuo YH, Chen W, Lain S, McCormack E, Bhatia R (2014) SIRT1 activation by a c-MYC oncogenic network promotes the maintenance and drug resistance of human FLT3-ITD acute myeloid leukemia stem cells. *Cell Stem Cell* 15:431–446
- Loos C, Syrovets T, Musyanovych A, Mailänder V, Landfester K, Simmet T (2014) Amino-functionalized nanoparticles as inhibitors of mTOR and inducers of cell cycle arrest in leukemia cells. *Biomaterials* 35:1944–1953
- Meng H, Mai WX, Zhang H, Xue M, Xia T, Lin S, Wang X, Zhao Y, Ji Z, Zink JI, Nel AE (2013) Codelivery of an optimal drug/siRNA combination using mesoporous silica nanoparticles to overcome drug resistance in breast cancer in vitro and in vivo. *ACS Nano* 7:994–1005
- Mura S, Nicolas J, Couvreur P (2013) Stimuli-responsive nanocarriers for drug delivery. *Nat Mater* 12:991–1003
- Nishida Y, Maeda A, Chachad D, Ishizawa J, Qiu YH, Komblau SM, Kimura S, Andreeff M, Kojima K (2015) Preclinical activity of the novel B-cell-specific Moloney murine leukemia virus integration site 1 inhibitor PTC-209 in acute myeloid leukemia: implications for leukemia therapy. *Cancer Sci* 106:1705–1713
- Picaud S, Fedorov O, Thanasopoulou A, Leonards K, Jones K, Meier J, Olzschka H, Monteiro O, Martin S, Philpott M, Tumber A, Filippakopoulos P, Yapp C, Wells C, Che KH, Bannister A, Robson S, Kumar U, Parr N, Lee K, Lugo D, Jeffrey P, Taylor S, Vecellio ML, Bountra C, Brennan PE, O'Mahony A, Velichko S, Muller S, Hay D, Daniels DL, Uhr M, la Thangue NB, Kouzarides T, Prinjha R, Schwaller J, Knapp S (2015) Generation of a selective small molecule inhibitor of the CBP/p300 bromodomain for leukemia therapy. *Cancer Res* 75:5106–5119



- Rochani AK, Girija AR, Borah A et al (2016) Heat-shock protein 90-targeted nano anticancer therapy. *J Pharm Sci* 105:1454–1466
- Sheng X, Tucci J, Mittelman SD (2015) Reduction of oxidative stress promotes daunorubicin resistance in acute lymphoblastic leukemia cells. *Cancer Res* 75(15 Supplement):1247
- Singh MR, Bart G, Zinke-Allmang M (2010) Polaron hopping in nano-scale poly (dA)–poly (dT) DNA. *Nanoscale Res Lett* 5: 501–504
- Stauss HJ, Morris EC (2013) Immunotherapy with gene-modified T cells: limiting side effects provides new challenges. *Gene Ther* 20:1029–1032
- Thirugnanam S, Xu L, Ramaswamy K, Gnanasekar M (2008) Glycyrrhizin induces apoptosis in prostate cancer cell lines DU-145 and LNCaP. *Oncol Rep* 20:1387–1392
- Wang C, Xu H, Liang C, Liu Y, Li Z, Yang G, Cheng L, Li Y, Liu Z (2013) Iron oxide@ polypyrrole nanoparticles as a multi-functional drug carrier for remotely controlled cancer therapy with synergistic antitumor effect. *ACS Nano* 7:6782–6795
- Whitlock JA, Greer JP, Lukens JN (1991) Epipodophyllotoxin-related leukemia. *Cancer* 68:1–604
- Zhang XY, Hu WB, Li J et al (2012) A comparative study of cellular uptake and cytotoxicity of multi-walled carbon nanotubes, graphene oxide, and nanodiamond. *Toxicol Res* 1:62–68
- Zhang W, Gao C, Konopleva M, Chen Y, Jacamo RO, Borthakur G, Cortes JE, Ravandi F, Ramachandran A, Andreeff M (2014) Reversal of acquired drug resistance in FLT3-mutated acute myeloid leukemia cells via distinct drug combination strategies. *Clin Cancer Res* 20:2363–2374
- Zhang Y, Chen H, Zhou S, Wang SX, Zheng K, Xu DD, Liu YT, Wang XY, Wang X, Yan HZ, Zhang L, Liu QY, Chen WQ, Wang YF (2015a) Sp1 and c-Myc modulate drug resistance of leukemia stem cells by regulating survivin expression through the ERK-MSK MAPK signaling pathway. *Mol Cancer* 14:56
- Zhang XY, Wang K, Liu MY et al (2015b) Polymeric AIE-based nanoprobe for biomedical applications: recent advances and perspectives. *Nanoscale* 7:11486–11508
- Zhao MX, Zhu BJ (2016) The research and applications of quantum dots as nano-carriers for targeted drug delivery and cancer therapy. *Nanoscale Res Lett* 11:207
- Zhu H, Chen H, Zeng X, Wang Z, Zhang X, Wu Y, Gao Y, Zhang J, Liu K, Liu R, Cai L, Mei L, Feng SS (2014) Co-delivery of chemotherapeutic drugs with vitamin E TPGS by porous PLGA nanoparticles for enhanced chemotherapy against multi-drug resistance. *Biomaterials* 35:2391–2400

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