Supplementary Information:

Apoptosis induction and inhibition of drug resistant tumor growth *in vivo* involving daunorubicin loaded graphene-gold composites

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Materials and methods

Materials

DNR was purchased from Sigma-Aldrich (St. Louise, MO). The RPMI 1640 cell culture medium was obtained from GIBCO (Invitrogen, Carlsbad, CA). GSH was purchased from Sigma. Fetal calf serum (FCS) was from Hyclone (Thermo Scientific, Waltham, MA). Penicillin, streptomycin, 3-(4,5-Dimethyl-2-thiazolyl)-2,5-diphenyl-2H-tetrazolium bromide (MTT) were all purchased from Sigma.

Preparation and characterization of GGN nanocomposites

Functionalized gold nanoparticles were synthesized by adding dropwise freshly prepared $NaBH_4$ (0.3 mL, wt 0.08%) and trisodium citrate (1 mL, wt 1.15%) to 0.32 mL $HAuCl_4$ (wt 1%) in 30 mL water while keeping magnetic stirring for 20 min at room temperature.[1] RGO was prepared from purified natural graphite according to our previous report.[2] RGO (1 mg) was first introduced in a 1 mL chitosan (0.5 mg/mL) aqueous solution and sonicated for several hours before being mixed with 0.5 mL (1 μg/mL) of freshly prepared gold nanoparticles solution. The chitosan was used in preparation of GGN because of its bioadhesive property so that chitosan can not only improve the relative solubility of grapheme but also act as a safe excepient in relevant drug formulations for drug delivery. The mixture was then stirred overnight before being dried under vacuum at room temperature. Transmission electron microscopy (JEM-2100) was used to characterize sizes and morphologies of the samples. RGO, GGN suspensions were also characterized by UV visible (Shimadzu UV3150) and Raman (Renishaw Invia Reflex system with Peltier-cooled charge-coupled device detectors) spectroscopies.

Antibody adsorption analysis

1mg/mL monoclonal P-gp antibody (abcam, USA) was absorbed on GGN by letting the solution antibody/GGN, v/v, 1:10 react overnight. The resulting nanocomposite was centrifuged at 4,000 rpm during 20 min. The antibody

remaining in supernatant was quantified using a Protein Assay kit (Thermo Scientific, USA), (see Fig. 1D in main text). The same antibody without adsorption on GGN was kept to be used as control.

Drug loaded P-gp-GGN

The desired concentration of DNR (100 μL, 2 mg/mL) was added dropwise while stirring to 1 mL P-gp-GGN. Stirring was continued overnight (16 h) at room temperature. The ensuing solution was then centrifuged at 10,000 rpm during 20 min. 20 μL of the supernatant was collected and analyzed with a high performance liquid chromatography (HPLC, Agilent, 1100 with a Kromasil, 100-5C18 analytical column) by comparison to a standard DNR concentration curve. The mobile phase consisted of 90% (v/v) methanol with triethylamine (95/5, v/v); the excitation wavelength was 488 nm. The drug loading (DL) and encapsulation efficiency (EE) were evaluated as: DL% = Ws/Wp×100%, EE% = Ws/Wt×100%, where Ws and Wp was the weight of loaded DNR and antibody-GGN nanocomposite, respectively, while Wt was that of total DNR.

Fluorescence and electrochemical study on drug control release by GSH

The DNR release studies were performed in a glass apparatus and measured by electrochemical and fluorescence assays. 0.5 mg P-gp-GGN were dispersed in (1 mL, 0.2 mg/mL) DNR aqueous solution. A known concentration (100 μM, 1 mM, 10mM) GSH was added and incubated at 37°C for 10 hours before fluorescence measurements (PerkinElmer LS-55), (Fig. 1E) and electrochemical analysis (CHI660b electrochemical workstation), (Fig. 1F).

Cell lines and culture

Adriamycin-resistant Leukemia cell lines KA were purchased from the Institute of Hematology of Tianjin, Chinese Academy of Medical Sciences. The drug resistant leukemia KA cells were cultured in cell culture medium containing 1 μg/mL adriamycin (Sigma, U.S.A.). Cell lines were maintained in RPMI-1640 medium containing 10% FCS, 100 U/ mL of penicillin, and 100 μg/mL of streptomycin at 37°C with 5% CO_2.

Scanning electron microscopic observations (SEM) analysis of morphological images of KA cells

KA cells were seeded on top of cover glass slips; GGN, DNR-loaded P-gp-GGN, gold nanoparticles were then administered to these KA cells. After 1 h incubation, the cells were fixed using 2.5% glutaraldehyde overnight. The cover slips were then washed with 30%, 50%, 70%, 80%, 90%, 95% and 100% ethanol before being carefully dried for SEM experiments.

TEM study of sub-cellular distribution of gold nanoparticles

KA cells were treated with drug loaded P-gp-GGN, washed with PBS, and then fixed in 2.5% glutaraldhyde. Ultrathin sections were cut and mounted on copper grids. The sections were viewed under a JEOL 1200-EX transmission electron microscope equipped with energy dispersive X-ray spectroscopy (EDS). Gold nanoparticles were identified by EDS analysis, and their sub-cellular distribution was investigated by TEM micrographs.

The study of KA membrane permeability

The permeability of cellular membranes following the exposures was determined by measuring the amount of released lactate dehydrogenase (LDH) enzyme from KA cells. The CytoTox 96® -kit (Promega, Fitchburg, WI, USA) was used according to the manufacturer instructions. LDH release of KA cells treated by different concentration drug loaded P-gp-GGN for 48 h was determined, and subsequently measuring the LDH from the culture medium. Absorbance values after the colorimetric reaction were measured at the wavelength of 490 nm.

Intracellular DNR accumulation

Initially, KA cells were treated with DNR alone (at the same concentration as that loaded on P-gp-GGN) or DNR-loaded P-gp-GGN respectively without or with treatment by 1 mM GSH (cells were treated with GSH within 30 min after treatment by DNR-loaded P-gp-GGN). Afterwards, cells were maintained for 2 h at 37 °C and then washed three times with PBS. The fluorescence intensity due to intracellular DNR concentration was measured by confocal fluorescence microscope (ZEISS, LSM 510) at 530 nm with a 488 nm excitation wavelength.

The intracellular DNR accumulation was confirmed by relevant HPLC analysis. Firstly, 1×10^7 KA cells treated during 24 hours with DNR (at the same concentration as that loaded on P-gp-GGN), DNR-loaded P-gp-GGN without or with treatment by GSH. The cells were then harvested by centrifugation at 2,000 g for 10 min and washed three times with PBS. After addition of chloroform/methanol (1/1, v/v), (10^7 cells/mL), the cellular extracts were sonicated and centrifuged at 10,000 g for 10 min, and the upper layer solution analyzed by HPLC (see HPLC conditions as described in Preparation and characterization of GGN nanocomposites). The intracellular DNR amount was then evaluated as DNR% = (amount of intracellular DNR)/(total amount of DNR or DNR in DNR-loaded P-gp-GGN) ×100%. The intracellular DNR amount by DNR-loaded IgG-GGN was evaluated as above.

MTT assay on cytotoxicity

The KA cells were initially cultured in cell culture medium containing 1 μg/mL adriamycin (Sigma, U.S.A.). KA cells were maintained in RPMI-1640 medium containing 10% FCS, 100 U/mL of penicillin, and 100 μg/mL of streptomycin at 37°C with 5% CO_2, KA Cells before being plated in 96-well plates (2×10^3 cells/well). After overnight incubation, various concentrations of DNR, P-gp-GGN, DNR-loaded P-gp-GGN, or DNR-loaded P-gp-GGN after treatment by GSH were added into specified wells. After 36 h, a 20 μL MTT solution (5 mg/mL) aliquot was added to each well. After four hours incubation, the supernatant was removed and 100 μL DMSO added to each well. Samples were then shaken for 15 min before the optical density (OD) was read at a wavelength of 540 nm. All experiments were performed in triplicates. Relative inhibition of cell growth was expressed as follows: Cell viability% = ([OD]test/[OD]control)×100%.

Acridine Orange/Ethidium Bromide (AO/EB) staining for detection of apoptotic KA cells

KA cells (5×10^5/well) were incubated during 72 hours in DNR-loaded P-gp-GGN or DNR-loaded IgG-GGN with treatment by GSH. Cells were then centrifuged for 5 min before specific staining of apoptotic cells through addition of l mL of AO/EB dye mixture (100 μg/mL AO and 100 μg/mL EB) and examination under fluorescent light microscope.

DNA fragmentation assay

KA cells (5×10^5/well) were incubated during 72 hours with DNR at the concentration used for loading onto P-gp-GGN, or DNR-loaded P-gp-GGN without or with treatment by GSH. DNA fragmentation was assessed under UV light.

Flow cytometry analysis

KA cells were seeded in 12 well plates (1×10^5 cells/well). After incubation during 72 h at 37 °C, 5% CO_2, KA cells were treated during 48 h with DNR, P-gp-GGN, or DNR-loaded P-gp-GGN without or with treatment by GSH. Annexin-V-FITC Apoptosis Detection Kit (KeyGEN, China) was used to detect apoptosis. Flow cytometric analysis was conducted using a BD FACSCanto flow cytometer (BD, USA).

Intravenous injection of reagents and tumor growth inhibition study in vivo

Nude mice were provided by the Animal Feeding Farm of National Institute for the control of Pharmaceutical and Biological Products (P.R. China). All mice were housed in the Southeast University animal facility and

experiments conducted following the guidelines of the Animal Research Ethics Board of Southeast University. KA cells (5×10^6) were suspended in culture medium and inoculated subcutaneously into the right flank of a 35 mice cohort. The nude mice inoculated with KA cells were divided into 5 groups (7 mice in each group) aimed to receive different treatments after the tumor size became ca. 50 mm^3 (i.e., after one week of inoculation): (1) None (control); (2) 8 mg/Kg DNR; (3) 16.96 mg/Kg P-gp-GGN; (4) 8+16.96 mg/Kg DNR-loaded P-gp-GGN; (5) 8+16.96 mg/Kg DNR-loaded P-gp-GGN with treatment by GSH (a volume of 10 μL of GSH was subcutaneously injected at the tumor location within 30 min after DNR-loaded P-gp-GGN treatment). Each group 2-5 received the above treatment specified through an intravenous injection administered by tail vein. The tumor dimensions of each nude mouse were measured on the 20th day after treatment and their volumes evaluated by: $Volume=\pi/6\times[(a+b)/2]^3$ (a being the largest diameter of a given tumor and b being the smallest one).

In situ detection of apoptosis by TUNEL staining

Apoptotic cell death amounts were detected in deparaffinized tumor tissue sections using terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) with a Klenow DNA fragmentation detection kit (Roche, USA). Sections were permeabilized with 20 μg mL^{-1} protease K, and endogenous peroxidase inactivated by 3% H_2O_2 in methanol. Apoptosis was detected by labelling the 3'-OH ends of the fragmented DNA with biotin-dNTP using Klenow at 37ºC for 1.5 hours. The tumor slides were then incubated with streptavidin horseradish peroxidase conjugate, followed by incubation with 3, 3'-diaminobenzidine and H_2O_2. Apoptotic cells were identified by their dark brown nuclei under light microscope.

Statistical analysis

All results are presented as Mean ± SD. A t-test was performed for each data group at each time point. A value of $P < 0.05$ was considered statistically significant.

(1) Ankanna S, Prasad TN, Elumalaiand EK, Savithramma N. *Dig J Nanomater Bios,* 2010, **5**, 369.

(2) Li PP, Chen Y, Zhu JH, Feng MA, Zhuang XD, Linand Y, et al. *Chem Eur J,* 2011,**17**,780.

Supporting information:

Methods

1. Antibody adsorption analysis (Fig. S4D)

1mg/mL monoclonal immunoglobin G antibody (IgG antibody, boster, China) was absorbed on GGN by letting the solution antibody/GGN, v/v, 1:10 react overnight. The resulting nanocomposite was centrifuged at 4,000 rpm for 20 min. The antibody remaining in supernatant was quantified using a Protein Assay kit (Thermo Scientific, USA). The same antibody without adsorption on GGN was kept to be used as control.

2. Drug loaded IgG-GGN

The desired concentration of DNR (100μL, 2 mg/mL) was added dropwise while stirring to IgG-GGN (0.5 mg/mL). Stirring was continued overnight (16 h) at room temperature. The ensuing solution was then centrifuged at 10,000 rpm for 20 min. 20 μL of the supernatant was collected and analyzed with a high performance liquid chromatography (HPLC, Agilent, 1100 with a Kromasil, 100-5C18 analytical column) by comparison to a standard DNR concentration curve. The mobile phase comprised of 90% (v/v) methanol with triethylamine (95/5, v/v); the excitation wavelength was 488 nm. The drug loading (DL) and encapsulation efficiency (EE) was evaluated as: DL% = Ws/Wp×100%, EE% = Ws/Wt×100%, where Ws was the weight of (DNR loaded in antibody-GGN nanocomposite) and Wp was the weight of DNR loaded in (DNR antibody-GGN nanocomposite), and Wt was that of total DNR.

3. Fluorescence and electrochemical study on drug control release by GSH (Fig. S1)

The release studies were performed in a glass apparatus and measured by electrochemical and fluorescence assays. 0.5 mg RGO were dispersed in (1 mL, 0.2 mg/mL) DNR. A known concentration (100 μM, 1 mM, 10mM) GSH was added and incubated at 37°C for 10 hours before fluorescence measurements (PerkinElmer LS-55).

4. Study on intracellular DNR accumulation (Fig. S3D, S4E)

Initially, K562, KA cells were treated with DNR and DNR loaded P-gp-GGN without or with treatment by 1 mM GSH (in each case, cells were treated with GSH within 30 min after treatment by DNR loaded P-gp-GGN). KA cells were treated with IgG-GGN, DNR loaded IgG-GGN, or DNR loaded IgG-GGN after treatment by 1 mM GSH (cells were treated with GSH within 30 min after treatment by DNR loaded IgG-GGN). Afterwards, cells were maintained for 2 h at 37 °C and then washed three times with PBS. The fluorescence intensity due to intracellular DNR concentration was measured by confocal fluorescence microscope (ZEISS, LSM 510) at an excitation wavelength at 488 nm and an emission wavelength at 530 nm.

5. MTT assay on cytotoxicity (Fig.S3A, S4A)

The drug resistant leukemia KA cells were initially cultured in cell culture medium containing 1 μg/mL adriamycin (Sigma, U.S.A.). K562, KA cells were maintained in RPMI-1640 medium containing 10% FCS, 100 U/mL of penicillin, and 100 μg/mL of streptomycin at 37°C with 5% CO_2, K562, KA Cells (2×10^3/well) before being plated in 96-well plates. After overnight incubation, various concentrations of DNR, IgG-GGN, DNR loaded IgG-GGN, or DNR loaded IgG-GGN after treatment by GSH were added into specified wells. KA cells were treated with various concentrations of (DNR+GSH) or (Gold nanoparticles+DNR) with or without GSH respectively. After 36 h, a 20 μL MTT solution (5 mg/mL) aliquot was added to each well. After four hours incubation, the supernatant was removed and 100 μL DMSO added to each well. Samples were then shaken for 15

min before the optical density (OD) was read at a wavelength of 540 nm. All experiments were performed in triplicates. Relative inhibition of cell growth was expressed as follows: Cell viability% = ([OD]test/[OD]control)×100%.

6. Acridine Orange/Ethidium Bromide (AO/EB) staining for detection of apoptotic KA cells (Fig. S3B, S4B)

KA, K562 cells (5×10^5/well) were incubated (72 hours) in DNR loaded P-gp-GGN, DNR loaded IgG-GGN with treatment by GSH. K562 cells (5×10^5/well) were incubated (72 hours) in DNR loaded P-gp-GGN with treatment by GSH. Cells were then centrifuged for 5 min before staining apoptotic cells by adding 1 mL of AO/EB dye mixture (100 μg/mL AO and 100 μg/mL EB) and examination under fluorescent light microscope.

7. DNA fragmentation assay (Fig. S3C, S4C)

K562, KA cells (5×10^5/well) were incubated during 72 hours with DNR at the concentration used for loading onto P-gp-GGN, or DNR loaded P-gp-GGN without or with treatment by GSH. DNA fragmentation was visualized under UV light.

8. Flow cytometry analysis (Fig. S3E, S4F)

KA cells were seeded in 12 well plates at 1×10^5 cells /well. After incubation during 72 h at 37 °C, 5% CO_2, K562, KA cells were treated with DNR, P-gp-GGN, or DNR loaded P-gp-GGN without or with treatment by GSH for 48 h, K562 cells were treated with DNR, IgG-GGN, or DNR loaded IgG-GGN without or with treatment by GSH for 48 h. Annexin-V-FITC Apoptosis Detection Kit (KeyGEN, China) was used to detect apoptosis. Flow cytometric analysis was conducted using a BD FACSCanto flow cytometer (BD, USA).

9. Western blotting analysis in vitro (Fig. S5)

After 72 hours treatment with DNR, or DNR-loaded P-gp-GGN without or with treatment by GSH, KA cells (1×10^7 cells/well) lysates were prepared by application of a lysis buffer. The lysates were then subjected to SDS-PAGE/ Western blot analysis. Proteins were detected by enhanced chemiluminescence (ECL, Thermo scientific).

10. Intravenous injection of reagents and tumor growth inhibition study in vivo (Fig. S6C)

Nude mice were provided by the Animal Feeding Farm of National Institute for the control of Pharmaceutical and Biological Products (P.R. China). All mice were housed in the animal facility and experiments on animals were conducted following the guidelines of the Animal Research Ethics Board of Southeast University. KA cells (5×10^6) were suspended in culture medium and inoculated subcutaneously into the right flank of 35 mice. The nude mice inoculated with KA cells were divided into other 5 groups (7 mice in each group) aimed to receive different treatments: (1) None (control); (2) 8 mg/Kg DNR; (3) 16.96 mg/Kg IgG-GGN; (4) 8+16.96 mg/Kg DNR loaded IgG-GGN; (5) 8+16.96 mg/Kg DNR loaded IgG-GGN with treatment by GSH (a volume of 10 μL of GSH was subcutaneously injected to tumor within 30 min after DNR loaded GGN treatment). When the tumor volume became ca. 50 mm^3 (i.e., after one week of inoculation), each group 2-5 received the above treatment specified through an intravenous injection administered by tail vein. The tumor dimensions of each nude mouse were measured (a is the largest and b being the smallest diameters of the tumor) on the 20th day after treatment. The tumor volume was evaluated by: $\text{Volume}=\pi/6\times[(a+b)/2]^3$.

11. In situ detection of apoptosis by TUNEL staining (Fig. S6A)

Apoptotic cell death amounts were detected in deparaffinized tumor tissue sections using terminal deoxynucleotidyl transferase-mediated dUTP nick end-labeling (TUNEL) with a Klenow DNA fragmentation detection kit (Roche, USA). Sections were permeabilized with 20 μg mL^{-1} protease K, and endogenous peroxidase inactivated by 3% H_2O_2 in methanol. Apoptosis was detected by labeling the 3'-OH ends of the fragmented DNA with biotin-dNTP using Klenow at 37ºC for 1.5 hours. The tumor slides were then incubated with streptavidin horseradish peroxidase conjugate, followed by incubation with 3, 3'-diaminobenzidine and H_2O_2. Apoptotic cells were identified by their dark brown nuclei when observed under light microscope.

12. Statistical analysis

All results are presented as Mean ± SD. A t-test was performed for each data group at each time point. A value of $P < 0.05$ was considered statistically significant.

Additional results

1. DNR release from RGO induced by GSH

The release process of DNR from RGO by GSH was investigated by fluorescence assay (Fig. S1). Cyan curve (e) shows the strong fluorescence spectra of (1mL, 0.2 mg/L) DNR alone. After the addition of 0.5 mg RGO to the above solution, the fluorescence intensity of the system decreased to 10% (red curve a). Then, GSH aliquots were added to the system. At GSH 100 μM, only 20% of the total fluorescence intensity was recovered. Upon increasing the GSH concentration to 1 mM, the fluorescence intensity reached to 27%. Further additions of GSH up to a final concentration of 10mM only resulted in a slight fluorescence intensity increases up to 28%.

2. MTT assay for evaluation of cytotoxicity

The cytotoxic effects of Gold nanoparticles+DNR or Gold nanoparticles+DNR in presence of GSH were explored on KA cell line. The cytotoxic effects were monitored after 36 h increased in a dose-dependent manner. The cytotoxic effect was significantly increased when KA cells were treated with Gold nanoparticles+DNR and GSH evidencing that Gold nanoparticles+DNR lead to significant enhancement of cytotoxic effect to KA cells, especially when associated to GSH, vs. DNR+GSH alone (Fig. S2).

3. Effect of the composite (and of its individual components) on pgp-negative cells (*in vitro*)

MTT assays were used to evaluate the cytotoxic effects of composite (and of its individual components) on pgp-negative cells (K562 cells) and compare them to the case of the targeted KA cells used elsewhere in this work. As shown in Fig. S3, K562 cells viability was significantly reduced by increasing concentrations of DNR, P-gp-GGN, DNR loaded P-gp-GGN, DNR loaded P-gp-GGN with GSH in a dose-dependent manner. The cell growth inhibition rate was higher when treated with DNR loaded P-gp-GGN with GSH than other treatments (Fig. S3A).

K562 cells were treated for 72 hours. Cells were then stained with AO/EB to detect cell apoptosis. The apoptotic nuclei of K562 were identified by their distinctively fragmented appearance (Fig. S3B). We further confirmed the apoptosis induced by DNR, P-gp-GGN, DNR loaded P-gp-GGN, DNR loaded P-gp-GGN with GSH using DNA fragmentation assay. DNA fragmentations of K562 cells were observed with DNR treatment (Fig. S3C, lane 2), but treatment with P-gp-GGN was not clear (lane 3). Stronger DNA fragmentation was detected in K562 cells treated with DNR loaded P-gp-GGN (lane 4). K562 cells treated with DNR loaded P-gp-GGN and GSH showed a much larger amount of fragmented chromosomal DNA (lane 5) than other ones.

Cellular extracts were collected for confirmation and quantification of the intracellular DNR accumulation by HPLC. The accumulation of DNR in K562 cells was about 21% after treatment with DNR loaded P-gp-GGN,

while the intracellular accumulation of DNR in cells was only 14% when cells were treated with DNR alone. In K562 cells treated with DNR loaded P-gp-GGN and GSH, the accumulation of DNR was about 35% (Fig. S3D).

Cellular extracts were collected for confirmation and quantification of the intracellular DNR accumulation by HPLC. In accumulation of DNR in KA cells was about 32% after treatment with DNR-loaded P-gp-GGN and 43% in KA cells treated with DNR-loaded P-gp-GGN and GSH; the accumulation of DNR in KA cells was about 19% after treatment with gold nanoparticles+DNR and 17% in KA cells treated with graphene oxide+DNR. Conversely, when cells were treated with DNR alone its intracellular accumulation was only 8%. So the DNR-loaded P-gp-GGN has significant effects compared to gold nanoparticles or RGO alone for drug delivery (Fig. S3E).

The apoptotic rates of K562 cells treated with DNR, P-gp-GGN, DNR loaded P-gp-GGN, DNR loaded P-gp-GGN with GSH were 29%, 16%, 46%, 58% respectively (Fig. S3F).

4. Effect of a modified composite, using a non-binding antibody, on K562/A02 cells *in vitro*

The results of the protein assay of IgG antibody self-association onto GGN are presented in Fig. S4D. This evidenced a loading of ca. 3.7%. The relevant drug loading efficiency was 31 %. MTT assays were used to demonstrate the cytotoxic effect of IgG-composite (and its parts) on KA (K562/A02) cells. As shown in Fig. S4, KA cells viability was significantly reduced with the increasing concentrations of DNR, IgG-GGN, DNR loaded IgG-GGN, DNR loaded IgG-GGN with GSH. Therefore, all treatments elicited cytotoxicity on KA cells in a dose-dependent manner. The cell growth inhibition rate was higher when treated with DNR loaded IgG-GGN with GSH than other treatments (Fig. S4A).

KA cells were treated for 72 hours. Cells were then stained with AO/EB to detect cell apoptosis. The apoptotic nuclei of KA were identified by their distinctively fragmented appearance (Fig. S4B). We further confirmed the apoptosis induced by DNR, IgG-GGN, DNR loaded IgG-GGN, DNR loaded IgG-GGN with GSH using DNA fragmentation assay. DNA fragmentations of KA cells were not clearly observed with DNR treatment or with treatment of IgG-GGN (Fig. S4C, lane 2, 3). Stronger DNA fragmentation was detected in KA cells treated with DNR loaded IgG-GGN (lane 4). KA cells treated with DNR loaded IgG-GGN with GSH showed a larger amount of fragmented chromosomal DNA (lane 5) than other ones.

Cellular extracts were collected for confirmation and quantification of the intracellular DNR accumulation by HPLC. The accumulation of DNR in KA cells was ca. 17% after treatment with the DNR loaded IgG-GGN, while the intracellular accumulation of DNR in cells was ca. 8% when cells were treated with DNR alone. In KA cells treated with DNR loaded IgG-GGN and GSH, the accumulation of DNR was ca. 33%. These results evidence that IgG-GGN readily facilitates the uptake of DNR into KA cells when the presence of GSH (Fig. S4E).

The apoptotic rates of KA cells treated with DNR, IgG-GGN, DNR loaded IgG-GGN, DNR loaded IgG-GGN with GSH were ca. 17%, 18%, 36%, 48% respectively (Fig. S4F).

5. Western blotting analyses

Western blotting analyses were used for investigating the apoptosis mechanisms *in vitro*. This evidenced that, compared with untreated control groups, KA cells treated with DNR-loaded P-gp-GGN experienced a drastic decrease in P-gp protein expression (Fig. S5A). The expression of apoptosis-related proteins was thus investigated (Fig. S5B). In agreement with their multidrug resistance, KA cells did not modify significantly their apoptosis-related proteins expression when treated separately with DNR or P-gp-GGN alone. Conversely, treatment by DNR-loaded P-gp-GGN and GSH caused a strong increase in expression of active caspases 8,3 in phase with activation of PARP (note that PARP is a downstream target of active caspase 3, itself being a downstream target of active caspase 8).

6. Effect of a modified composite, using a non-binding antibody, on K562/A02 cells *in vivo* in the xenograft model

To investigate the effects of modified composite on KA (K562/A02) cells *in vivo*, nude mice were inoculated with KA cells and the subsequent tumor growth was recorded after various treatments. KA mice without any treatment showed the largest tumor volume (3700 mm^3, Fig. S6C, a). DNR alone treatment did not significantly inhibit tumor growth (3500 mm^3, b), IgG-GGN or DNR loaded IgG-GGN treatments inhibited the tumor growth in KA mice (3400 mm^3 or 3000 mm^3, c or d). The group 5 mice were treated with DNR loaded IgG-GGN in the presence of GSH and the tumor growth was also moderately suppressed (2800 mm^3, e).

In the xenograft tumors excised from the KA nude mice, the apoptotic rate in the control group 1 was ca. 12% (Fig. S6Ba). The number of apoptotic cells increased slightly in group 2 and 3 xenograft tumors (26%, or 27%, b or c). In group 4 treated with DNR loaded IgG-GGN (34%, d) and group 5 treated with DNR loaded IgG-GGN in the presence of GSH (42%, e), did not efficiently induce cell apoptosis in tumor tissues. The result of apoptotic rate correlated well with that of tumor growth inhibition *in vivo*.

Figures

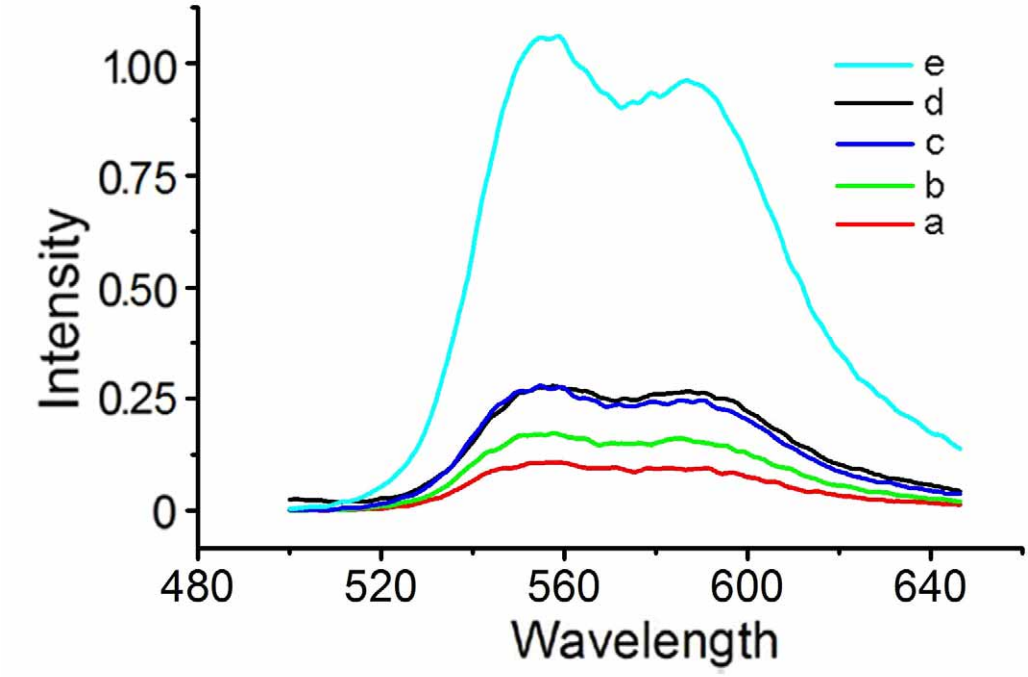

Fig. S1 Drug release assay: relative fluorescence intensity of DNR released from 1 mg/mL RGO after successive additions of GSH (b=100μM, c=1 mM, d=10mM). The maximum fluorescence intensity of 0.2 mg/mL DNR (e) was taken as 1.0.

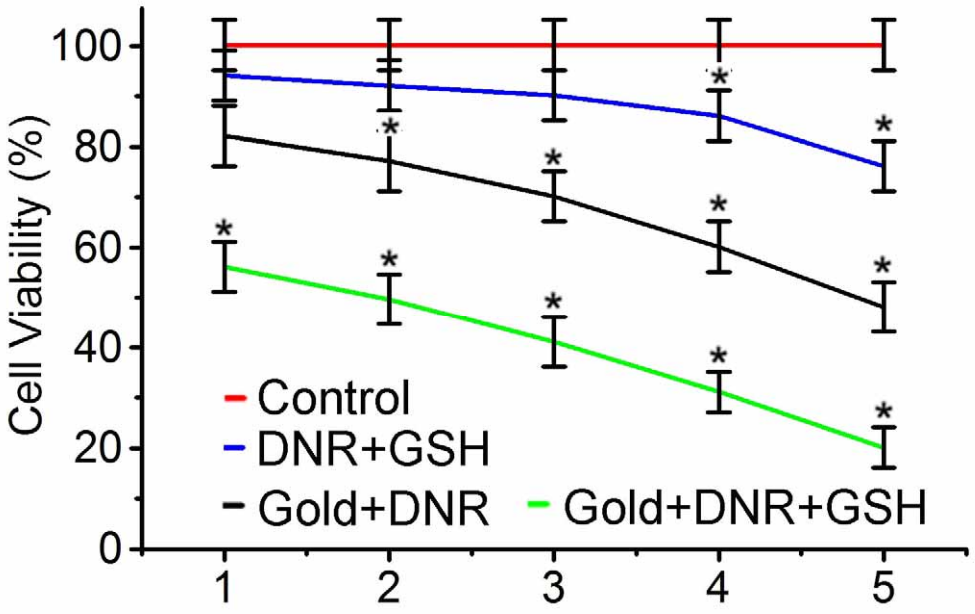

Fig. S2 Cytotoxic effect induced in KA cells after various treatments using the MTT assays. Blue curve: after treatment with 0.5 mg/L, 2 mg/L, 8 mg/L, 32 mg/L, and 128 mg/L of DNR with 1mM GSH. Black curve: after treatment with DNR+Gold nanoparticles (0.5 mg/L DNR+1.06 mg/L Gold nanoparticles, 2 mg/L DNR+4.24 mg/L Gold nanoparticles, 8 mg/L DNR+16.96 mg/L Gold nanoparticles, 32 mg/L DNR+68 mg/L Gold nanoparticles, 128 mg/L DNR+272 mg/L Gold nanoparticles). Green curve: after treatment with DNR+Gold nanoparticles (0.5 mg/L DNR+1.06 mg/L Gold nanoparticles, 2 mg/L DNR+4.24 mg/L Gold nanoparticles, 8 mg/L DNR+16.96 mg/L Gold nanoparticles, 32 mg/L DNR+68 mg/L Gold nanoparticles, 128 mg/L DNR+272 mg/L Gold nanoparticles) with 1mM GSH. * $P < 0.05$, compared to the control treatment.

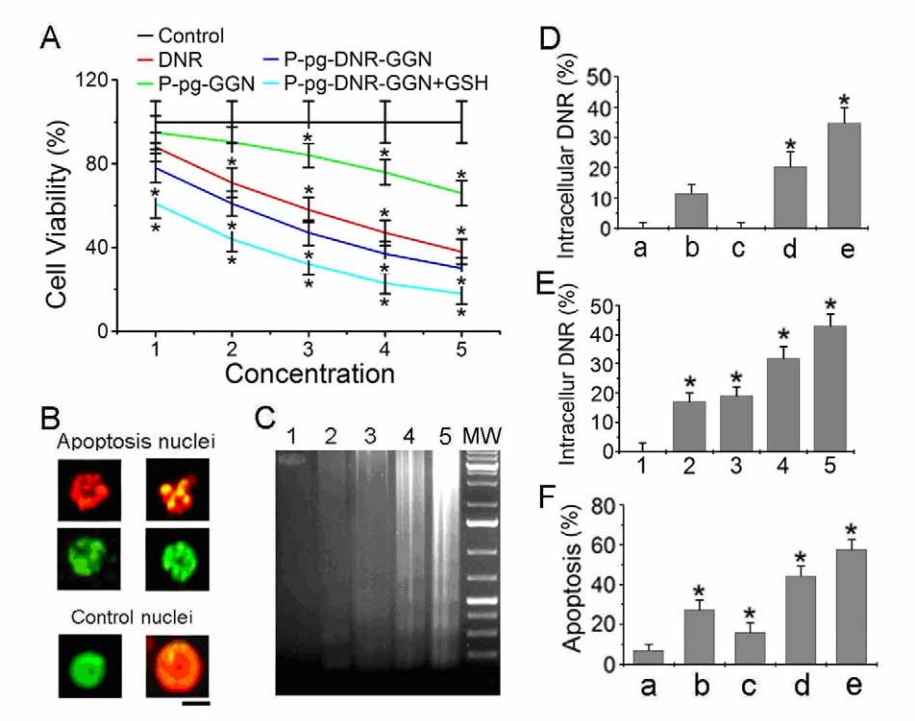

Fig. S3 (A) Cytotoxic effects induced in K562 cells after various treatments as evidenced by MTT assays. K562 cells were treated with (0.5, 2, 8, 32, 128 mg/L DNR), (1.06, 4.24, 16.96, 68, 272 mg/L P-gp-GGN), DNR loaded P-gp-GGN (0.5 mg/L DNR+1.06 mg/L P-gp-GGN, 2 mg/L DNR+4.24 mg/L P-gp-GGN, 8 mg/L DNR+16.96 mg/L P-gp-GGN, 32 mg/L DNR+68 mg/L P-gp-GGN, 128 mg/L DNR+272 mg/L P-gp-GGN), or DNR loaded P-gp-GGN (0.5 mg/L DNR+1.06 mg/L P-gp-GGN, 2 mg/L DNR+4.24 mg/L P-gp-GGN, 8 mg/L DNR+16.96 mg/L P-gp-GGN, 32 mg/L DNR+68 mg/L P-gp-GGN, 128 mg/L DNR+272 mg/L P-gp-GGN) and GSH respectively. (B) Detection of apoptotic and normal K562 cells by AO/EB staining. Control cell nuclei and apoptotic ones are shown. Bar: 10 μm. (C) DNA fragmentation in untreated K562 cells (1) or after different treatments: (2) 8 mg/L DNR, (3) 16.96 mg/L P-gp-GGN, (4) DNR loaded P-gp-GGN (8 mg/L DNR +16.96 mg/L P-gp-GGN); (5) DNR loaded P-gp-GGN (8 mg/L DNR +16.96 mg/L P-gp-GGN) and GSH, compared to a DNA Marker (MW). (D) Quantitative characterization by HPLC of the accumulation of DNR in untreated K562 cells (a; control) or after treatment with (b) 8 mg/L DNR, (c) 16.96 mg/L P-gp-GGN, (d) DNR loaded P-gp-GGN (8 mg/L DNR +16.96 mg/L P-gp-GGN), and (e) DNR loaded P-gp-GGN (8 mg/L DNR +16.96 mg/L P-gp-GGN) and GSH. (E) Quantitative characterization by HPLC of the accumulation of DNR in untreated KA cells (1; control) or after treatment with (b) (8 mg/L DNR +16.96 mg/L RGO), (c) (8 mg/L DNR +16.96 mg/L gold nanoparticles), (d) DNR loaded P-gp-GGN (8 mg/L DNR +16.96 mg/L P-gp-GGN), and (e) DNR loaded P-gp-GGN (8 mg/L DNR +16.96 mg/L P-gp-GGN) and GSH. (F) Quantitative analysis of apoptotic cells using Annexin-V-FITC method after various treatments (a) Controls or after treatment during 36 h with: (b) 8 mg/L DNR, (c) 16.96 mg/L P-gp-GGN, (d) DNR loaded P-gp-GGN (8 mg/L DNR +16.96 mg/L P-gp-GGN), (e) DNR loaded P-gp-GGN (8 mg/L DNR +16.96 mg/L P-gp-GGN) and GSH. * $P < 0.05$, compared to the control treatment.

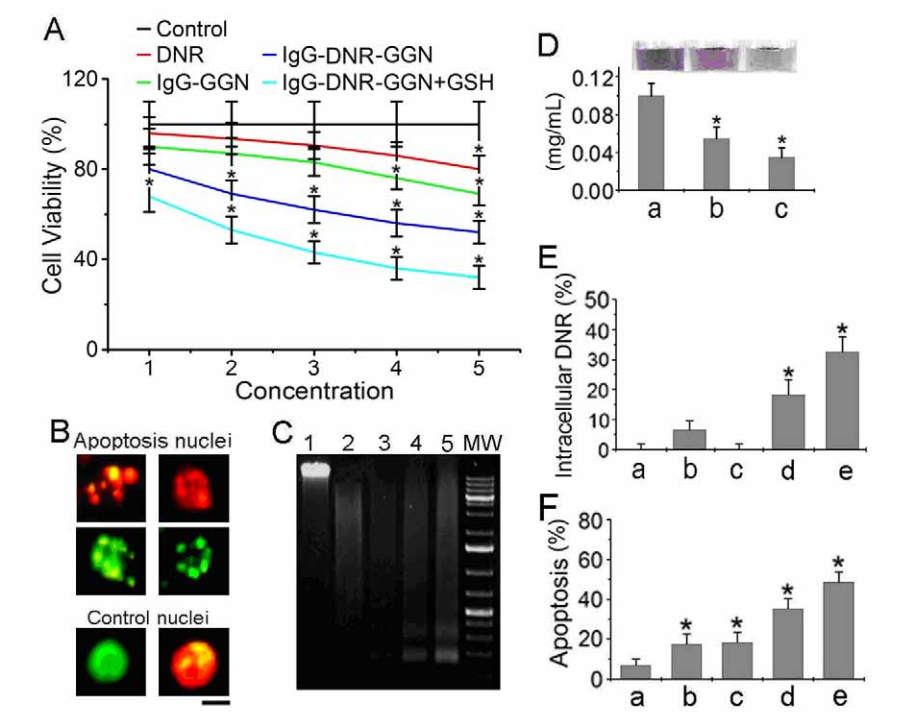

Fig. S4 (A) Cytotoxic effects induced in KA cells after various treatments as evidenced by MTT assays. KA cells were treated with (0.5, 2, 8, 32, 128 mg/L DNR), (1.06, 4.24, 16.96, 68, 272 mg/L IgG-GGN), DNR loaded IgG-GGN (0.5 mg/L DNR+1.06 mg/L IgG-GGN, 2 mg/L DNR+4.24 mg/L IgG-GGN, 8 mg/L DNR+16.96 mg/L IgG-GGN, 32 mg/L DNR+68 mg/L IgG-GGN, 128 mg/L DNR+272 mg/L IgG-GGN), or DNR loaded IgG-GGN (0.5 mg/L DNR+1.06 mg/L IgG-GGN, 2 mg/L DNR+4.24 mg/L IgG-GGN, 8 mg/L DNR+16.96 mg/L IgG-GGN, 32 mg/L DNR+68 mg/L IgG-GGN, 128 mg/L DNR+272 mg/L IgG-GGN) and GSH respectively. (B) Detection of apoptotic and normal KA cells by AO/EB staining. Control cell nuclei and apoptotic ones are shown. Bar: 10 μm. (C) DNA fragmentation in untreated KA cells (1) or after different treatments: (2) 8 mg/L DNR, (3) 16.96 mg/L IgG-GGN, (4) DNR loaded IgG-GGN (8 mg/L DNR +16.96 mg/L IgG-GGN); (5) DNR loaded IgG-GGN (8 mg/L DNR +16.96 mg/L IgG-GGN) and GSH, compared to a DNA Marker (MW). (D) Quantification of IgG antibody remaining in solution before (a) and after 1 h (b) or 24 h (c) exposure to GGN. (E) Quantitative characterization by HPLC of the accumulation of DNR in untreated KA cells (a; control) or after treatment with (b) 8 mg/L DNR, (c) 16.96 mg/L IgG-GGN, (d) DNR loaded IgG-GGN (8 mg/L DNR +16.96 mg/L IgG-GGN), and (e) DNR loaded IgG-GGN (8 mg/L DNR +16.96 mg/L IgG-GGN) and GSH. (F) Quantitative analysis of apoptotic cells using Annexin-V-FITC method after various treatments (a) Controls or after treatment during 36 h with: (b) 8 mg/L DNR, (c) 16.96 mg/L IgG-GGN, (d) DNR loaded IgG-GGN (8 mg/L DNR +16.96 mg/L IgG-GGN), (e) DNR loaded IgG-GGN (8 mg/L DNR +16.96 mg/L IgG-GGN) and GSH. * $P < 0.05$, compared to the control treatment.

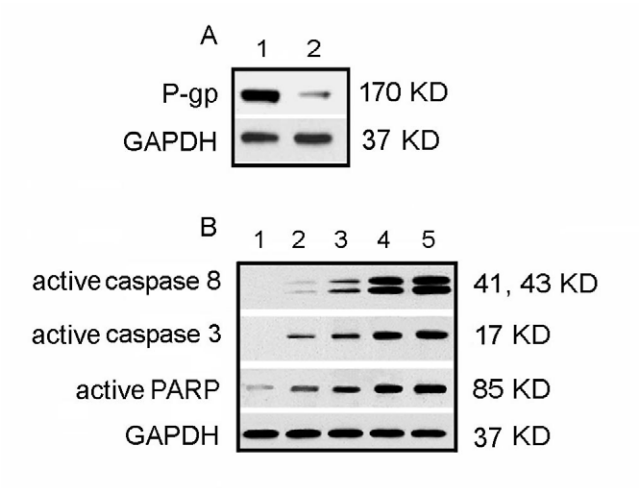

Fig. S5. Signal pathway analysis. (A) Western blotting analysis of P-gp and GAPDH in KA cells lysates obtained from untreated cells used as control (lane 1) or treated with DNR-loaded P-gp-GGN (8 mg/L DNR+16.96 mg/L P-gp-GGN) (lane 2). (B) Western blot analysis of active caspase 8, active caspase 3, active PARP and GAPDH in KA cells lysates: untreated cells (control, lane 1) or after treatment with 8 mg/L DNR (lane 2), 16.96 mg/L P-gp-GGN (lane 3), DNR-loaded P-gp-GGN (8 mg/L DNR+16.96 mg/L P-gp-GGN) (lane 4), or DNR-loaded P-gp-GGN (8 mg/L DNR+16.96 mg/L P-gp-GGN) and GSH (lane 5). GAPDH served as control.

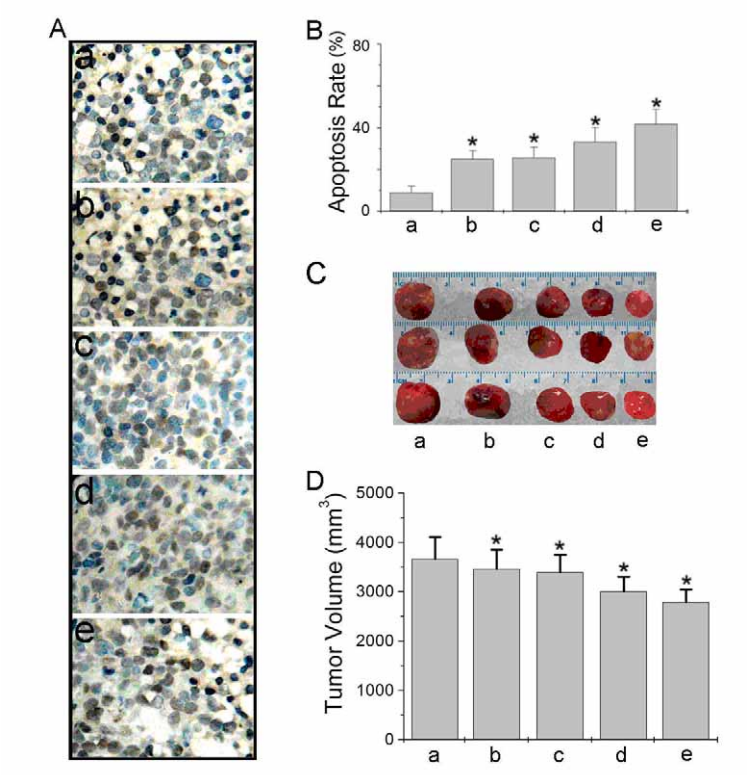

Fig. S6 (A) TUNEL staining of KA xenografted tumors (a, control group 1), or after treatment: b: group 2, DNR; c: group 3, IgG-GGN; d: group 4, DNR loaded IgG-GGN; e: group 5, DNR loaded IgG-GGN and GSH. (B) Quantitative analysis of apoptotic cells after the various treatments disclosed in A (same labels). (C) Inhibition of tumor growth in KA nude mice after different treatments (three tumors are shown in each case): a: group 1, (control group); b: group 2, DNR; c: group 3, IgG-GGN; d: group 4, DNR loaded IgG-GGN, e: group 5, DNR loaded IgG-GGN with GSH treatment. (D) Quantitative analysis of tumor volume after various treatments shown in a~e. Same labels as in A.