

# Iron *N*-(2-hydroxy acetophenone) glycinate (FeNG), a non-toxic glutathione depletor circumvents doxorubicin resistance in Ehrlich ascites carcinoma cells in vivo

Avishek Ganguly · Paramita Chakraborty · Kaushik Banerjee ·  
Shilpak Chatterjee · Soumya Basu · Avijit Sarkar ·  
Mitali Chatterjee · Soumitra Kumar Choudhuri

Received: 12 May 2011 / Accepted: 1 September 2011 / Published online: 14 September 2011  
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**Abstract** Multidrug resistance-associated protein 1 (MRP1) reduces intracellular anticancer drug accumulation either by co transporting them with glutathione (GSH) or extruding drug-GSH conjugates outside of the cell. Thus, MRP1 confers multidrug resistance (MDR) and worsen successful chemotherapeutic treatment against cancer. Although the exact mechanism of MRP1 involved in MDR remains unknown, the elevated level of intracellular GSH is considered as a key factor responsible for MDR in cancer. Hence the quest for non-toxic molecules that are able to deplete intracellular GSH has profound importance to subdue MDR. The present preclinical study depicts the resistance reversal potentiality of an iron complex; viz. Ferrous *N*-(2-hydroxy acetophenone) glycinate (FeNG) developed by us in doxorubicin resistant Ehrlich ascites carcinoma (EAC/Dox) cells. FeNG potentiate cytotoxic effect of doxorubicin on EAC/Dox cells ex vivo and also increases the survivability EAC/Dox bearing Swiss albino mice in vivo as well. Moreover, in vivo administration of

FeNG significantly depletes intracellular GSH with ensuant increase in doxorubicin concentration in EAC/Dox cells without alternation of MRP1 expression. In addition, intra-peritoneal (i.p.) application of FeNG in normal or EAC/Dox bearing mice does not cause any systemic toxicity in preliminary trials in mouse Ehrlich ascites carcinoma model. Therefore, the present report provides evidence that FeNG may be a promising new resistance modifying agent against drug resistant cancers.

**Keywords** Multi drug resistance · Glutathione · Iron complex · Resistance modifying agent

## Abbreviations

MDR	Multi drug resistance
MRP1	Multidrug resistance-associated protein 1
GSH	Glutathione
FeNG	Iron <i>N</i> -(2-hydroxy acetophenone) glycinate
EAC/Dox	Doxorubicin resistant Ehrlich ascites carcinoma cells
MTT	(3-[4,5-Dimethylthiazol- 2-yl]-2, 5-diphenyltetrazolium bromide
BSO	DL-buthionine ( <i>S,R</i> ) sulfoximine
EA	Ethacrynic acid
DTNB	5,5'-Dithio bis(2-nitrobenzoic acid)
RMA	Resistance modifying agent
ROS	Reactive oxygen species
DCFDA	2',7'-Dihydrodichlorofluorescin diacetate

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A. Ganguly · P. Chakraborty · K. Banerjee ·  
S. Chatterjee · S. Basu · S. K. Choudhuri (✉)  
Department of In Vitro Carcinogenesis and Cellular  
Chemotherapy, Chittaranjan National Cancer Institute,  
37, S.P. Mukherjee Road, Calcutta 700 026, India  
e-mail: soumitra01@yahoo.com

A. Sarkar · M. Chatterjee  
Department of Pharmacology, Institute of Post Graduate  
Medical Education and Research, Kolkata, India

## Introduction

The successful treatment of cancer with chemotherapeutic drugs is frequently impaired as tumor cells exhibit multi drug resistance (MDR), a phenotype of cross resistance to many structurally and functionally unrelated anticancer drugs (Ye et al. 2007). Multi drug-resistance associated protein (MRP1), an 190 kDa ATP-binding cassette (ABC) membrane transporter is found to be over expressed in numerous drug-resistant cell line and extrudes a wide range of chemotherapeutic drugs out side drug resistant cells, thus confer at least in part, MDR in cancer (Borst et al. 2000). In physiology, MRPs are involved in phase II cellular detoxification process together with the glutathione *S*-transferase (GST) group of enzymes and reduced glutathione (GSH) (Majumder et al. 2006). GSTs catalyze the process of conjugation of many amphipathic drugs with GSH that renders them high affinity substrates for MRP. These monogluthionyl conjugates thus formed are then transported out of the cells in an ATP-dependant manner (Borst et al. 2000). All most all the members of MRP/ABCC family of transporters are known to transport GSH conjugates and/or GSH itself, and thus are likely involved in chemoresistance. However, over expression of MRP and GST combined with elevated level of intracellular GSH can increase the rate of conjugation and subsequent detoxification of chemotherapeutic agents and thereby reduce their effectiveness (Ballatori et al. 2009). Therefore, the disruption of the GSH/MRP cooperation, especially depletion of GSH may be an attractive strategy to circumvent MDR in cancer. Several GSH depleting agents namely, DL-buthionine (*S,R*) sulfoximine (BSO), ethacrynic acid (EA), sulfasalazine has been employed so far as resistance modifying agents (RMA) for sensitizing drug resistant cancer cells to conventional anti cancer drugs (Efferth and Volm 1993). Unfortunately most these RMAs developed by depleting GSH was unacceptable due to their dose-limiting toxicity in pre clinical or clinical settings (Chi et al. 2007; Estrela et al. 2006; Linares et al. 2011; Yamamotoa et al. 2002). These limitations have impelled us to search for new compounds or RMAs having low toxicity and high efficacy in modulating MDR.

We earlier synthesized and reported that an iron chelate viz. iron *N*-(2-hydroxy acetophenone)

glycinate (FeNG) overcomes drug resistance by involving ROS for induction of apoptosis in drug resistant cell line in vitro. Moreover, we also found that FeNG has the potential to deplete intracellular GSH in drug resistant T lymphoblastic leukaemia CEM/ADR 5000 cells (Ganguly et al. 2010). These observations inspired us to hypothesize that, FeNG might be effective to overcome MDR either by directly eradicating drug resistant cells or by sensitizing resistant cell to anticancer drugs through depletion of intracellular GSH in drug resistance cancer cells. Based on these rationales we tried to validate the possibility of use of FeNG by employing its GSH depleting ability to overcome MDR in cancer where resistance is due to elevated level of GSH. Therefore, in this communication we have evaluated the potential role of FeNG as resistance modifier in doxorubicin resistant Ehrlich ascite carcinoma cell (EAC/Dox) in vivo. Herein we report the underlying resistance reversing mechanism of FeNG in EAC/Dox cells. In addition, we conducted a study on the systemic toxicity of FeNG in normal and EAC/Dox bearing mice as a model system. Our preclinical data, suggest that FeNG may have clinical relevance to improve the efficacy of chemotherapeutic drugs in MDR in cancers.

## Material and method

### Reagents

*N*-(2-hydroxy) acetophenone, glycine, ferrous sulphate, MTT dye (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide, EA, BSO, verapamil, doxorubicin were purchased from Sigma Chemical Company, St. Louis, MO. 2',7'-dihydrodichlorofluorescein diacetate (H2-DCFDA, Molecular Probes), DTNB (SRL India) and FITC-labeled secondary antibody (Sigma) were procured for the present study.

### Synthesis of the iron complex

FeNG was synthesized from the ligand, potassium *N*-(2-hydroxy acetophenone) glycinate by its reaction with ferrous sulphate (Ganguly et al. 2010); in brief, 460 mg potassium (*N*-(2-hydroxyacetophenone) glycinate (NG) and 280 mg ferrous sulphate was dissolved in 5 ml double distilled water separately. Both

the solutions were cooled to 8–10°C. The solution of PHAG was added drop wise to ferrous sulphate solution kept in ice bath. The mixture was rotated in a magnetic stirrer for 25–30 min maintaining the temperature at 7–8°C. Deep brown colored precipitate deposited and was allowed to settle for 30 min in refrigerator. The precipitate was isolated by centrifugation and recrystallised in water-alcohol. Yields 40%, mp. >400°C.

### Treatment

A stock solution of FeNG ( $10^{-2}$  M or 50 mg/ml) was prepared just before the experiments by dissolving the lyophilized compounds in water. For in vitro work FeNG treatments were performed with a concentration range of  $10^{-3}$  M to  $10^{-5}$  at 37°C in RPMI medium supplemented with FBS; for in vivo experiments the treatments were given with different doses of FeNG, diluting with PBS. As for vehicles control, equal volumes of medium or PBS were added to untreated cells or injected i.p. in vitro or in vivo experiments respectively. As considerable level of GSH was depleted at 2–3 h after FeNG (25 mg kg<sup>-1</sup>) treatment in EAC/Dox cells, we hypothesise that administration of doxorubicin, 2 h later of FeNG treatment would cause better doxorubicin accumulation within the EAC/Dox cells. Therefore, this time gap (i.e. FeNG treatment 2 h prior to doxorubicin treatment) was maintained throughout the experiment.

### Animals

Swiss albino mice, originally obtained from National Institute of Nutrition, Hyderabad, India and reared in our institute animal facilities were used for all in vivo experiments with prior approval of the institutional animal ethics committee (IAEC). The experimental protocols described herein were approved by the IAEC (Registration No.: 175/99/CPCSEA, dated 28.01.2000) in accordance with the ethical guidelines laid down by the Committee for the purpose of Control and Supervision of Experiments on Animals (CPCSEA) by the Ministry of Social Justice and Empowerment, Government of India. Adult male Swiss albino mice weighing 18–20 g were kept for a quarantine period of 1 week at a temperature of  $25 \pm 2^\circ\text{C}$ , relative humidity of  $55 \pm 2\%$  and with photo cycle of

12 h light/dark. Water and food pellets were provided ad libitum.

### Cell line, tumor implantation and experimental protocol

EAC cell was maintained as an ascitic tumor in female Swiss albino mice. Doxorubicin resistant Ehrlich ascites carcinoma (EAC/Dox), which is also resistant against cisplatin, cyclophosphamide and vinblastin was developed and maintained according to the methods described previously (Choudhuri and Chatterjee 1998; Friche et al. 1992). Briefly, the treatment regime consisted of 2 mg/kg/week doxorubicin intraperitoneally (i.p.). The daily treatment dose was 0.4 mg/kg for 5 days (Majumder et al. 2006). Doxorubicin was started 24 h after inoculation of  $10^6$  EAC/Dox cells i.p. into mice. EAC/Dox cells were maintained in doxorubicin-free condition for at least one passage before the start of all the experiments. During experiments, ascitic fluid containing EAC/Dox cells was drawn out aseptically from peritoneal cavity of mice having 7–8 days of ascitic tumor growth. Tumor cells were washed in phosphate buffer saline (PBS pH 7.4) and counted in a haemocytometer and viability was checked by trypan blue exclusion method.

### Cytotoxicity assay (MTT assay)

Ascitic fluid was aseptically isolated from the mice within 7–10 days of inoculation of tumor cells. No treatment was given during the last passage before an in vitro experiment. Cells ( $4 \times 10^4$ ) were collected and washed thrice in PBS, and then plated in each well of flat bottomed 96-well plate with RPMI 1640 medium (Gibco BRL), containing HEPES (Sigma), penicillin–streptomycin and 10% FCS (Gibco BRL). Cells were incubated without drug for 24 h. EAC/Dox cells were treated with FeNG ( $10^{-5}$  M) and then after 2 h, various doses of doxorubicin ( $10^{-5}$  M to  $10^{-7}$  M) were added. The cells were then incubated for an additional 72 h with 5% CO<sub>2</sub> at 37°C and cell viability was determined using the MTT assay. After completion of incubation cells were further incubated with 5 mg per ml of MTT dye (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide; Sigma, France) for 4 h at 37°C. The monolayer was suspended in 0.1 ml of DMSO and the absorbance at 540 nm was read by ELISA reader (Tecan 200). The control value

corresponding to untreated cells was taken as 100% and the viability of treated samples was expressed as a percentage of the control. The data generated were from three separate experiments, each performed in triplicate.

#### In vivo survival study

Fifteen groups (each group containing 20 mice) of animals were taken for animal survival studies. Each mouse was inoculated (i.p.) with either  $10^6$  EAC/S cells (seven groups) or  $10^6$  EAC/Dox cells (eight groups); after 7 days single doses of FeNG (15, 20, 75 mg kg<sup>-1</sup>) were injected to EAC/S bearing mice. Similarly, either single doses (15, 25, 50, 75 mg kg<sup>-1</sup>) or triple doses (25 mg kg<sup>-1</sup> × 3 and 30 mg kg<sup>-1</sup> × 3, each dose at every 6th day) or vehicle (no FeNG treatment) were injected (i.p.) to EAC/Dox bearing mice in presence or absence of 2 mg kg<sup>-1</sup> (or 1 mg kg<sup>-1</sup> × 3 doses) doxorubicin (following 2 h of FeNG treatment) of appropriate groups. In addition, 7 days after inoculum of EAC/Dox cell to mice (i.p.), single dose of EA (25 mg kg<sup>-1</sup>) and BSO (25 mg kg<sup>-1</sup>) were injected to EAC/Dox bearing mice followed by 2 mg kg<sup>-1</sup> doxorubicin (2 h later of EA or BSO treatment) treatment. Animals were checked daily for assessment of ascitic growth and body weights were measured.

Two weeks after the implantation of EAC cells, total ascitic fluid (TAF) and packed cell volume (PCV) were measured; the average value of three independent experiments of each set was presented in Table 1. Time of death was recorded for calculation of mean survival time (MST). The MSTs were recorded following various drug treatment protocols (Choudhuri and Chatterjee 1998; Tsuoro et al. 1981). The statistical significance of the survival data, i.e., survival of the only doxorubicin treated groups versus drug treated groups was evaluated by ANOVA test with post hoc Tukey tests. Mouse survival times in different groups were also compared as treated/control (T/C) ratio (percent), i.e., the ratio of the survival time (in days) for treated mice to untreated control mice. As in standard National Cancer Institute protocols for screening new anticancer drugs, it is considered that the increase in survival time corresponding to T/C ratios around 120% to “marginal”, T/C ratios between 120 and 150% to be “clear” and T/C ratios equal or superior to 150% to be “marked”.

#### Determination of doxorubicin penetration/retention in EAC/Dox cells

To determine the doxorubicin retention of EAC/Dox bearing mice after FeNG treatment, EAC/Dox bearing mice were injected either with FeNG (25 mg kg<sup>-1</sup>) or PBS (vehicle treated) 2 h before doxorubicin (3 mg kg<sup>-1</sup>) administration or doxorubicin (3 mg kg<sup>-1</sup>) alone. After doxorubicin injection, at various time points (1–4 h)  $5 \times 10^6$  cells were collected and washed twice with ice cold PBS. Cell pellets were then suspended and lysed with extraction buffer (50% ethanol and 0.3 N HCl) and incubated for 15 min at 37°C in dark. The lysates were then centrifuged for 15 min at 3000 rpm and OD of doxorubicin in supernatant was measured at 496 nm using spectrophotometer (Deng et al. 2007).

#### Determination of intracellular glutathione (GSH) contents

Determination of intracellular GSH content was performed by a modification of the reported method (Utsugi et al. 2002). EAC/Dox bearing mice were injected with FeNG or PBS (vehicle treated). FeNG treatments were given at different time intervals (1–5 h) and the cells were collected and  $5 \times 10^5$  cells were washed three times with cold washing buffer [0.1 M sodium phosphate, 5 mM ethylenediaminetetraacetate (EDTA), pH 7.5] and immediately lysed in 100 µl lysis buffer (0.1% Triton-X, 0.1 M sodium phosphate, 5 mM EDTA, pH 7.5). Five minutes later, the lysates were acidified with 15 µl of 0.1 N HCl and protein was precipitated with 15 µl of 50% sulfosalicylic acid. After centrifugation at 12000 g, supernatants were collected for GSH measurement. The supernatants were assayed for GSH content by spectrophotometric determination of the reduction of DTNB to 5'-thio-2-nitrobenzoic acid at a wavelength of 412 nm. Protein was measured by Bradford Method (Bradford 1976).

#### Determination of MRP1 expression by flowcytometry

For the determination of MRP1 expression, treated (24, 48 and 72 h) or untreated EAC/Dox cells were harvested, fixed, permeabilized and incubated with anti-MRP1 polyclonal antibody for 30 min at 4°C

**Table 1** Effect of FeNG, EA and BSO as RMA on survivality of EAC/Dox and EAC/S bearing female Swiss albino mice

	RMA (mg kg <sup>-1</sup> )	Dox (mg kg <sup>-1</sup> )	EAC/S bearing mice			EAC/Dox bearing mice		
			MST (days)	No. of animals	T/C (%)	MST (Days)	No. of animals	T/C (%)
FeNG	0	0	15 ± 1.2	20	100	–	–	–
	0	2	29 ± 2.3	20	193	13 ± 0.96	20	100
	15	0	12 ± 0.9	20	80	–	–	–
		2	–	–	–	19 ± 1.6***	20	146.15
	25	0	18 ± 1.6	20	120	–	–	–
		2	–	–	–	22 ± 1.7***	20	169.23
	50	2	–	–	–	21.5 ± 1.5***	20	165.38
	75	0	19 ± 1.4	20	126	–	–	–
		2	–	–	–	23 ± 1.8***	20	176.92
	25 × 3	1 × 3	–	–	–	28 ± 2.1***	20	215.38
30 × 3	1 × 3	–	–	–	20 ± 1.3***	20	153.84	
EA	25	0	18.3 ± 2.9	20	122	–	–	–
		2	–	–	–	19.8 ± 4.3***	20	152.3
BSO	25	0	16.5 ± 3.8	20	110	–	–	–
		2	–	–	–	17.2 ± 2.1***	20	132.3

In EAC/Dox cell bearing mice, doxorubicin resistance was reversed with FeNG treatment. FeNG works better as resistance modifying agent (RMA) in EAC/Dox bearing mice than as an anticancer agent in EAC/S bearing mice. The combined effect of FeNG (at 25 mg kg<sup>-1</sup> × 3 doses) and 2 h later doxorubicin (at 1 mg kg<sup>-1</sup> × 3 doses) treatment increased the mean survival time (MST) of EAC/Dox cells bearing mice from 13 to 28 days. T/C value (expressed in percentage) was calculated by dividing the MST value for mice receiving treatment by MST value for the control group, which comprised of EAC/Dox cells bearing mice receiving no treatment. Values represent mean ± standard deviation (SD) for 20 mice in each group. Statistical significance of MST of different groups were analysed by ANOVA test ( $P_{ANOVA} < 0.0001$ ) followed by a post hoc Tukey test (\*\*\*) statistically significant difference from only doxorubicin treated control group at \*\*\* $P < 0.001$ )

(Tazzari et al. 2007). Cells were then washed and incubated with anti-rabbit IgG-FITC conjugated secondary antibody. Following washing MRP1 expression was analysed by flow cytometry using Cell Quest software (FACS calibur, Becton–Dickinson). Appropriate isotype control was taken in all cases. A total of 10,000 events were acquired and analysis of flow cytometric data was performed using WinMDI 2.9 software. A histogram of FITC-fluorescence ( $X$ -axis) versus counts ( $Y$ -axis) has been displayed.

#### Intra cellular ROS accumulation assay

Levels of ROS generation in cells were assessed fluorometrically using 2',7'-dihydrodichlorofluorescein diacetate (H2-DCFDA, Molecular Probes) with slight modification. EAC/Dox bearing mice were injected with FeNG or PBS (vehicle treated). After, different time interval (1–72 h) cells were collected and  $5 \times 10^5$  cells were then washed with PBS and further incubated with H2-DCFDA for 30 min at 37°C in

dark. After incubation, cells were washed twice in PBS at room temperature for 5 min each time. The fluorescence was measured at excitation and emission wavelengths of the oxidized form were 504 and 529 nm respectively (Ganguly et al. 2010).

#### In vivo subacute toxicity testing for FeNG

To determine the subacute toxicity of FeNG, seven (indicated in Tables 2, 3, 4) experimental groups (six mice per group) of mice were taken. During the study period, the mice were weighed twice a week. Clinical appearance of the mice was evaluated daily. After 2 weeks of continuous treatment, blood was collected from untreated as well as from treated mice. Blood was obtained via closed cardiac puncture with the help of a 22-gauge hypodermic needle by subxiphoid approach. Blood from each group was pooled into separate glass tubes and treated with anticoagulant (heparin) or left untreated for serum collection (Bürger et al. 2005; Nirwana et al. 2011). After that

**Table 2** Effect of FeNG singly and in combination with doxorubicin on haematological parameter of EAC/Dox bearing female Swiss albino mice or normal female Swiss albino mice

Parameters	Groups						
	Normal Untreated mice (i)	Normal + 3 mg/kg Dox treated mice (ii)	EAC/Dox bearing mice (iii)	EAC/ Dox + 3 mg/kg Dox treated mice (iv)	Normal + 75 mg/kg FeNG treated mice (v)	EAC/ Dox + 75 mg/kg FeNG treated mice (vi)	EAC/Dox + 75 mg/kg FeNG + 3 mg/kg Dox treated mice (vii)
WBC ( $\times 10^3/\mu\text{l}$ )	11.0 $\pm$ 1.1	11.2 $\pm$ 1.0	8.3 $\pm$ 0.7	9.2 $\pm$ 0.9	10.8 $\pm$ 0.9 ns	9.7 $\pm$ 0.8	10.1 $\pm$ 0.9 ns
RBC ( $\times 10^6/\mu\text{l}$ )	10.08 $\pm$ 0.8	9.24 $\pm$ 0.8	10.1 $\pm$ 0.9	8.11 $\pm$ 0.7	10.36 $\pm$ 0.7 ns	9.14 $\pm$ 0.9	8.96 $\pm$ 0.6 ns
HGB (g/dl)	14.9 $\pm$ 1.5	14.8 $\pm$ 1.2	15.3 $\pm$ 1.4	12.8 $\pm$ 1.1	14.9 $\pm$ 1.6 ns	14.0 $\pm$ 1.2	14.3 $\pm$ 1.4 ns
HCT (%)	52.2 $\pm$ 3.4	48.1 $\pm$ 2.7	52.7 $\pm$ 3.3	44.3 $\pm$ 2.8	52.9 $\pm$ 3.6 ns	48.0 $\pm$ 3.1	48.7 $\pm$ 3.0 ns
MCV (fL)	51.8 $\pm$ 2.0	52.1 $\pm$ 1.9	52.2 $\pm$ 2.1	52.2 $\pm$ 2.0	51.1 $\pm$ 1.7 ns	50.3 $\pm$ 1.4	51.6 $\pm$ 1.8 ns
MCH (pg)	14.8 $\pm$ 1.7	16.0 $\pm$ 2.0	15.1 $\pm$ 1.5	15.8 $\pm$ 1.8	14.4 $\pm$ 1.2 ns	15.3 $\pm$ 1.6	16.0 $\pm$ 1.9 ns
MCHC (g/dl)	28.5 $\pm$ 1.7	30.8 $\pm$ 1.9	29.0 $\pm$ 1.7	30.3 $\pm$ 1.8	28.2 $\pm$ 1.5 ns	30.4 $\pm$ 1.5	31.0 $\pm$ 1.6 ns
PLT ( $\times 10^3/\mu\text{l}$ )	992 $\pm$ 162	1498 $\pm$ 211	1085 $\pm$ 179	857 $\pm$ 124	1003 $\pm$ 157 ns	852 $\pm$ 129	947 $\pm$ 161 ns

The data are mean  $\pm$  SD of three independent experiments. FeNG (75 mg kg<sup>-1</sup>) alone or in combination with doxorubicin (3 mg kg<sup>-1</sup>) showed no hematological toxicity when compared with untreated and EAC/Dox plus doxorubicin (3 mg kg<sup>-1</sup>) control, respectively. Statistical significance of different groups were analysed by ANOVA test (WBC  $P_{\text{ANOVA}} = 0.0144$ , RBC  $P_{\text{ANOVA}} = 0.0359$ , HGB  $P_{\text{ANOVA}} = 0.3874$ , HCT  $P_{\text{ANOVA}} = 0.0386$ , MCV  $P_{\text{ANOVA}} = 0.8483$ , MCH  $P_{\text{ANOVA}} = 0.8631$ , MCHC  $P_{\text{ANOVA}} = 0.2792$ , PLT  $P_{\text{ANOVA}} = 0.004$ ) followed by a post hoc Tukey test [*ns* statistically not significant difference between group (i) vs. (v) and group (iv) vs. (vii)]

**Table 3** Effect of FeNG singly and in combination with doxorubicin on spleen and bone marrow of EAC/Dox bearing female Swiss albino mice or normal female Swiss albino mice

Group	Percentage of viable cells	
	Bone marrow cells	Spleen cells
Normal untreated mice (i)	96.2% $\pm$ 3.1	86.75% $\pm$ 2.8
Normal + 3 mg kg <sup>-1</sup> Dox treated mice (ii)	83.06% $\pm$ 2.4	90.74% $\pm$ 2.9
EAC/Dox bearing mice (iii)	80.95% $\pm$ 2.3	89.47% $\pm$ 2.8
EAC/Dox + 3 mg kg <sup>-1</sup> Dox treated mice (iv)	92.88% $\pm$ 2.8	90.74% $\pm$ 2.1
Normal + 75 mg kg <sup>-1</sup> FeNG treated mice (v)	90.36% $\pm$ 2.2 ns	95.06% $\pm$ 3.0*
EAC/Dox + 75 mg kg <sup>-1</sup> FeNG treated mice (vi)	75.65% $\pm$ 1.9	91.66% $\pm$ 2.3
EAC/Dox + 75 mg kg <sup>-1</sup> FeNG + 3 mg kg <sup>-1</sup> doxorubicin treated mice (vii)	89.3% $\pm$ 2.7 ns	94.11% $\pm$ 2.9 ns

The data are mean  $\pm$  SD of three independent experiments. FeNG (75 mg kg<sup>-1</sup>) alone or in combination with doxorubicin (3 mg kg<sup>-1</sup>) were non-toxic to spleen and bone marrow when compared with untreated and EAC/Dox plus doxorubicin (3 mg kg<sup>-1</sup>) control respectively. Statistical significance of different groups were analysed by ANOVA test (bone marrow cells  $P_{\text{ANOVA}} < 0.0001$  and spleen cells  $P_{\text{ANOVA}} = 0.0342$ ) followed by a post hoc Tukey test between group (i) vs. (v) and group (iv) vs. (vii). (*ns* statistically not significant difference, \*statistically significant difference at \* $P < 0.05$ )

haematological parameters were analyzed by automated haematology analyzer (Sysmex, KX-21) and haematologic biochemical analyses (blood urea,

creatinin, alkaline phosphatase, ALT, AST) was determined by automated clinical chemistry analyzer (Olympus, AU400).

**Table 4** Effect of FeNG singly and in combination with doxorubicin on hepatic and renal functions of EAC/Dox bearing female Swiss albino mice or normal female Swiss albino mice

Group	Alkaline phosphatase (U/L)	Aspartate Trans aminase (AST) (U/L)	Alanine Trans aminase (ALT) (U/L)	Urea (mg/dl)	Creatinin (mg/dl)
Normal Untreated mice (i)	129 ± 3.6	183 ± 2.8	65 ± 1.2	71 ± 2.9	0.5 ± 0.03
Normal + 3 mg/kg Dox treated mice (ii)	133 ± 3.8	222 ± 3.9	80 ± 1.4	47 ± 1.9	0.4 ± 0.02
EAC/Dox bearing mice (iii)	63 ± 1.8	783 ± 21	107 ± 1.9	64 ± 2.7	0.3 ± .01
EAC/Dox + 3 mg/kg Dox treated mice (iv)	106 ± 2.2	234 ± 4.4	62 ± 1.2	56 ± 2.2	0.4 ± 0.02
Normal + 75 mg/kg FeNG treated mice (v)	139 ± 4.1*	143 ± 2.3**	59 ± 1.1**	73 ± 3.1 ns	0.5 ± 0.02 ns
EAC/Dox + 75 mg/kg FeNG treated mice (vi)	87 ± 1.9	384 ± 5.7	68 ± 1.2	63 ± 2.6	0.4 ± 0.02
EAC/Dox + 75 mg/kg FeNG + 3 mg/kg Dox treated mice (vii)	141 ± 4.6***	287 ± 4.3***	57 ± 1.1**	64 ± 2.7*	0.4 ± 0.01 ns

The data are mean ± SD of three independent experiments. FeNG (75 mg kg<sup>-1</sup>) alone or in combination with doxorubicin (3 mg kg<sup>-1</sup>) showed no hepatic or renal toxicity when compared with untreated and EAC/Dox plus doxorubicin (3 mg kg<sup>-1</sup>) control, respectively. Statistical significance of different groups was analysed by ANOVA test (ALP, AST, ALT, Urea, Creatinin  $P_{ANOVA} < 0.0001$ ) followed by a post hoc Tukey test between group (i) vs. (v) and group (iv) vs. (vii). (ns statistically not significant difference, statistically significant difference at \* $P < 0.05$ , \*\* $P < 0.01$  and \*\*\* $P < 0.001$ )

#### Isolation of spleen cell

After completion of in vivo drug treatment, different groups of normal and EAC/Dox bearing female Swiss albino mice were anaesthetized and spleen was removed aseptically. After that spleen was rubbed against the fine wire mesh of tissue grinder. The cell suspension centrifuged at 1000 rpm for 5 min. The supernatant was discarded and the cells centrifuged in PBS twice at room temperature for washing. Cell viability was tested by trypan blue exclusion method and cells were counted in a phase contrast microscope. The experiment was repeated thrice. The average value of percentage of viable spleen cell was calculated from three independent experiments.

#### Isolation of bone marrow cell

Following in vivo drug treatment, different groups of normal and EAC/Dox bearing female Swiss albino mice were anaesthetized and the femur bone was cut with a vertebrate scissor. Bone marrow was flushed with 0.56% KCl solution and centrifuged at 3000 rpm for 15 min at 37°C. The supernatant was discarded and the cells were washed in PBS twice at

room temperature. Cell viability was tested by trypan blue exclusion method and counted in a phase contrast microscope. The experiment was repeated thrice. The average value of percentage of viable spleen cell was calculated from three independent experiments.

#### Measurement of tissue GSH

Single dose of FeNG (25 mg kg<sup>-1</sup>) was administered i.p. to EAC/Dox bearing mice. At various time points (2, 4 and 24 h) after FeNG injection, mice were sacrificed and tissue samples from lung, spleen, liver, kidneys and heart were collected. Tissue GSH was measured following the method of Sedlack and Lindsay (1968). Briefly, tissue homogenate in 0.1 ml PBS was mixed with 2.4 ml EDTA (0.02 M), 2 ml deionized water and 0.5 ml 50% TCA and centrifuged at 500 × g for 15 min at 4°C. 2 ml of supernatant was mixed with 2 ml 0.4 M Tris buffer (pH 8.9). 50 µl 5,5'-dithio bis (2-nitrobenzoic acid) [DTNB] (0.01 M) was added to the mixture. Within 2–3 min of addition of DTNB, optical density (OD) was measured at 412 nm. Protein was measured by the Bradford Method (1976).

## Measurement of glutathione peroxidase (GPx) activity

Single dose of FeNG (25 mg kg<sup>-1</sup>) was administered (i.p.) to EAC/Dox bearing mice. At various time points (2, 4 and 24 h) following FeNG injection, mice were sacrificed and tissue samples from lung, spleen, liver, kidneys and heart were collected. GPx activity was measured from the tissue homogenate, prepared according to reported method. In brief, the organs were dissected, dried and weighed. The homogenate was prepared with 0.15 M KCl solution and centrifuged at 10,000 rpm for 20 min at 4°C. The supernatant was analysed following the reported method (Hafemann et al. 1974; Sazuka et al. 1989).

## Statistical analysis

All data reported are the arithmetic mean from three independent experiments performed in triplicate  $\pm$  SD unless stated otherwise. Analysis of variance (ANOVA) tests prior to unpaired Student's *t*-test or ANOVA test with post hoc comparisons by Tukey tests were carried out to assess the statistical significant differences between groups, accepting  $P < 0.05$  as a level of significance. Data analyses were performed using the Prism software (GraphPad, San Diego, CA).

## Result

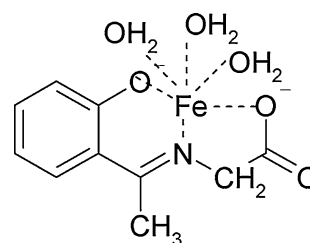
### Ex vivo cytotoxic effect of FeNG

In an initial approach, we performed MTT assay to determine the cytotoxic effect of FeNG (Fig. 1) on EAC/Dox cell line. Cells were treated with various doses of FeNG (10<sup>-5</sup>–10<sup>-3</sup> M) for different hours. FeNG at any concentration did not show significant impact on cellular viability of EAC/Dox cells as reflected in the IC<sub>10</sub> values of FeNG (10<sup>-4</sup> M) (Fig. 2a). On the other hand, ten fold lower concentration of IC<sub>10</sub> value of FeNG, i.e., 10<sup>-5</sup> M in combination with doxorubicin showed significant cytotoxic effect on EAC/Dox cells. Result presented in Fig. 2b clearly show that FeNG induces approximately 23, 42 and 23% reduction in cellular viability when used in different combinations of doxorubicin (10<sup>-5</sup>–10<sup>-7</sup> M) respectively as compared to only doxorubicin treated control. We also tested the effect of another established

RMA, verapamil (10<sup>-5</sup> M) in combination with 10<sup>-6</sup> and 10<sup>-7</sup> M of doxorubicin on EAC/Dox cells and it showed more or less comparable effect (43 and 11% reduction in cellular viability respectively) as FeNG. Thus, FeNG showed the ability to reverse resistance against doxorubicin of EAC/Dox cells.

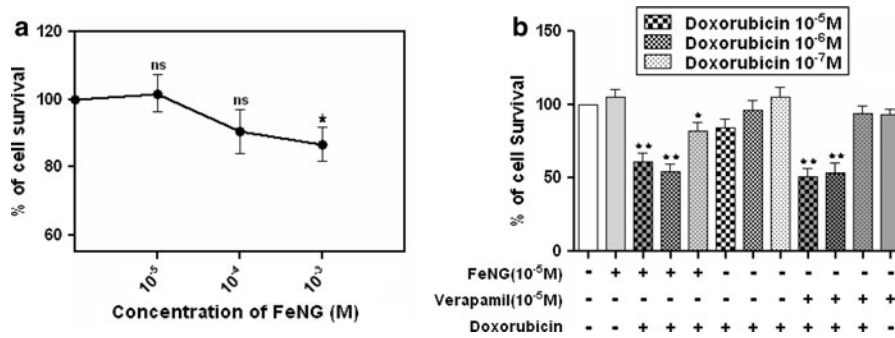
### Resistance modifying effect of FeNG in Swiss albino mice bearing doxorubicin resistant Ehrlich ascites carcinoma (EAC/Dox)

To extrapolate the in vitro data we evaluated the efficacy of FeNG as RMA in in vivo system. The ability of FeNG to enhance the antitumoural efficacy of doxorubicin was tested on doxorubicin resistant EAC/Dox cell bearing Swiss albino mice (Table 1). The female Swiss albino mice were inoculated (i.p.) with the sensitive and doxorubicin resistant subline of Ehrlich ascites carcinoma (EAC/S and EAC/Dox) cells and 7 days after development of tumor the animals were treated with or without doxorubicin in presence or absence of different single or triple doses of FeNG. Animals were monitored for any discomfort or pain. Survivability of EAC/Dox bearing mice were found to be significantly enhanced (T/C value 215.3%) by administration of FeNG (25 mg kg<sup>-1</sup>  $\times$  3 doses, each dose at every 4th day) 2 h before doxorubicin (1 mg kg<sup>-1</sup>) as compared with only doxorubicin treated group (Table 1). In contrast, the other treatment regimen (FeNG: 30 mg kg<sup>-1</sup>  $\times$  3 doses, each at every 4th day, doxorubicin: 1 mg kg<sup>-1</sup>) was less effective (T/C value 153.8%) (Table 1). Obviously, doxorubicin alone had an antitumoural effect on sensitive EAC/S bearing mice (Table 1). The T/C values for single 15, 25, 50, 75 mg kg<sup>-1</sup> FeNG followed by 2 mg kg<sup>-1</sup> doxorubicin and triple 25 mg kg<sup>-1</sup> FeNG followed by 1 mg kg<sup>-1</sup> doxorubicin treated groups were 146.1, 169.2, 165.4, 176.9 and 215.38%, respectively. Therefore, T/C values for FeNG treated



**Fig. 1** Structure of iron complex, iron (II) *N*-(2-hydroxyacetophenone) glycinate (FeNG)





**Fig. 2** In vitro cytotoxic effect of iron complex alone or in combination with doxorubicin in EAC/Dox bearing mice. **a** Dose response curves for iron complex (FeNG) using EAC/Dox cells as assessed by MTT assay. Cells were seeded into 96-well plates ( $4 \times 10^4$  cells/well) and allowed to overnight incubation at 37°C in 5%CO<sub>2</sub> incubator. Next day, cells were treated with increasing concentrations of FeNG for 72 h incubation. Results are expressed as percentage viability of vehicle-treated control cells. Value represents the mean  $\pm$  SD of three independent experiments with four replicates in each. Statistically significant difference from untreated control at \* $P < 0.05$ . **b** Cytotoxic effects of FeNG and verapamil in presence or absence of different concentration of doxorubicin on

EAC/Dox cells as assessed by MTT assay. Cells were seeded into 96-well plates ( $4 \times 10^4$  cells/well) and allowed to overnight incubation at 37°C in 5% CO<sub>2</sub> incubator. Next day, cells were pre treated with fixed concentration of either FeNG or verapamil followed by (2 h later) different concentration of doxorubicin for 72 h incubation. Results are expressed as percentage viability of vehicle-treated control cells. Value represents the mean  $\pm$  SD of three independent experiments with four replicates in each. Statistical significance was analysed by ANOVA test ( $P_{ANOVA} < 0.0001$ ) followed by unpaired Student's t test (statistically significant difference from only doxorubicin treated control at \* $P < 0.05$  and \*\* $P < 0.01$ , respectively)

groups were 1.5–2.1 times higher in comparison to only doxorubicin treated control (i.e. EAC/Dox cell bearing mice). The average packed cell volume (PCV) and ascitic fluid collected from FeNG treated group (FeNG: 25 mg kg<sup>-1</sup>  $\times$  3 doses, each at every 6th day, doxorubicin: 1 mg kg<sup>-1</sup>) were  $5.0 \pm 0.3$  and  $8.5 \pm 0.6$  ml, respectively. The corresponding values of average packed cell volume (PCV) and ascitic fluid for EAC/Dox bearing mice receiving only doxorubicin treatments were  $5.91 \pm 0.2$  and  $10 \pm 0.4$  ml, respectively. Hence, there was significant reduction in ascitic growth in FeNG treated group. We have also evaluated the effect of two different standard resistance modifiers BSO (25 mg kg<sup>-1</sup>) and EA (25 mg kg<sup>-1</sup>) on survivability of EAC/Dox bearing mice. Results represented in the table shows that the T/C values for BSO and EA treated group was 132.3 and 152.3%, respectively.

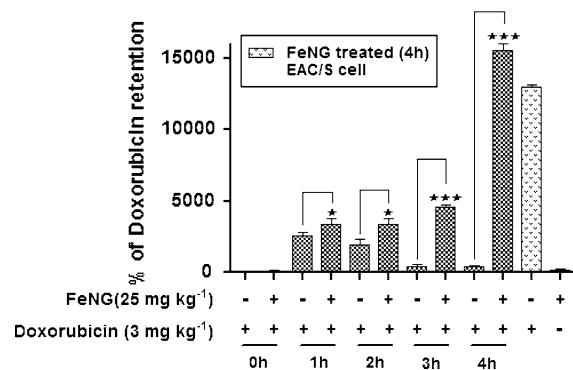
**Effect of FeNG on accumulation of doxorubicin in EAC/Dox cell in vivo**

Ex vivo and in vivo results demonstrated that FeNG reverses the doxorubicin resistance in EAC/Dox cell and therefore we enquired whether FeNG can increase intracellular accumulation of doxorubicin in EAC/Dox cells. To this end EAC/Dox bearing mice were injected with FeNG or PBS (vehicle treated) 2 h before

doxorubicin (3 mg kg<sup>-1</sup>) administration. Cells were collected and intracellular doxorubicin was measured from treated and untreated group at 0–4 h following doxorubicin administration (Fig. 3). We found that FeNG increased intracellular doxorubicin concentration in EAC/Dox cells in time dependent fashion as compared to only doxorubicin treated control cells, where we observed a decrease in doxorubicin concentration as time progressed (Fig. 3).

**GSH depletion but not MRP1 expression might be involved in FeNG induced doxorubicin accumulation in EAC/Dox cell**

Cancer cells circumvent the effect of chemotherapeutic agents by increasing cellular GSH and/or enhancing the conjugation of these drugs to GSH. Nearly all members of the MRP/ABCC family of transporters are known to transport GSH conjugates and/or GSH itself, and thus are likely involved in chemoresistance (Ballatori et al. 2009; Calvert et al. 1998). Hence, depletion of intracellular GSH and reduction of MRP1 expression may enhance intracellular accumulation of anticancer drugs by reducing their efflux. Therefore, to investigate the effect of FeNG on intracellular GSH level of EAC/Dox cells, EAC/Dox bearing mice were injected with FeNG (25 mg kg<sup>-1</sup>) or PBS (vehicle



**Fig. 3** Effect of FeNG on doxorubicin retention by EAC/DOX cells with EAC/S serving as control. EAC/DOX bearing mice (with 7 days of ascitic growth) were injected with the doxorubicin alone or in combination with FeNG i.p. (2 h prior to doxorubicin administration). After a period of different time interval (0 h to 4 h), cells were collected from the intraperitoneal cavity and doxorubicin uptake was measured using spectrophotometer. Values represent mean  $\pm$  SD of three independent experiments. Statistical significance was analysed by ANOVA test ( $P_{ANOVA} < 0.0001$ ) followed by unpaired Student's *t* test (Differences between without FeNG treated and FeNG treated cells are significant at  $*P < 0.05$  and  $***P < 0.001$ )

treated). Cells were collected at different time intervals and intracellular GSH was measured. We found that GSH was depleted gradually after FeNG treatment up to 3 h and then it reaches its normal levels as compared to untreated control (Fig. 4a).

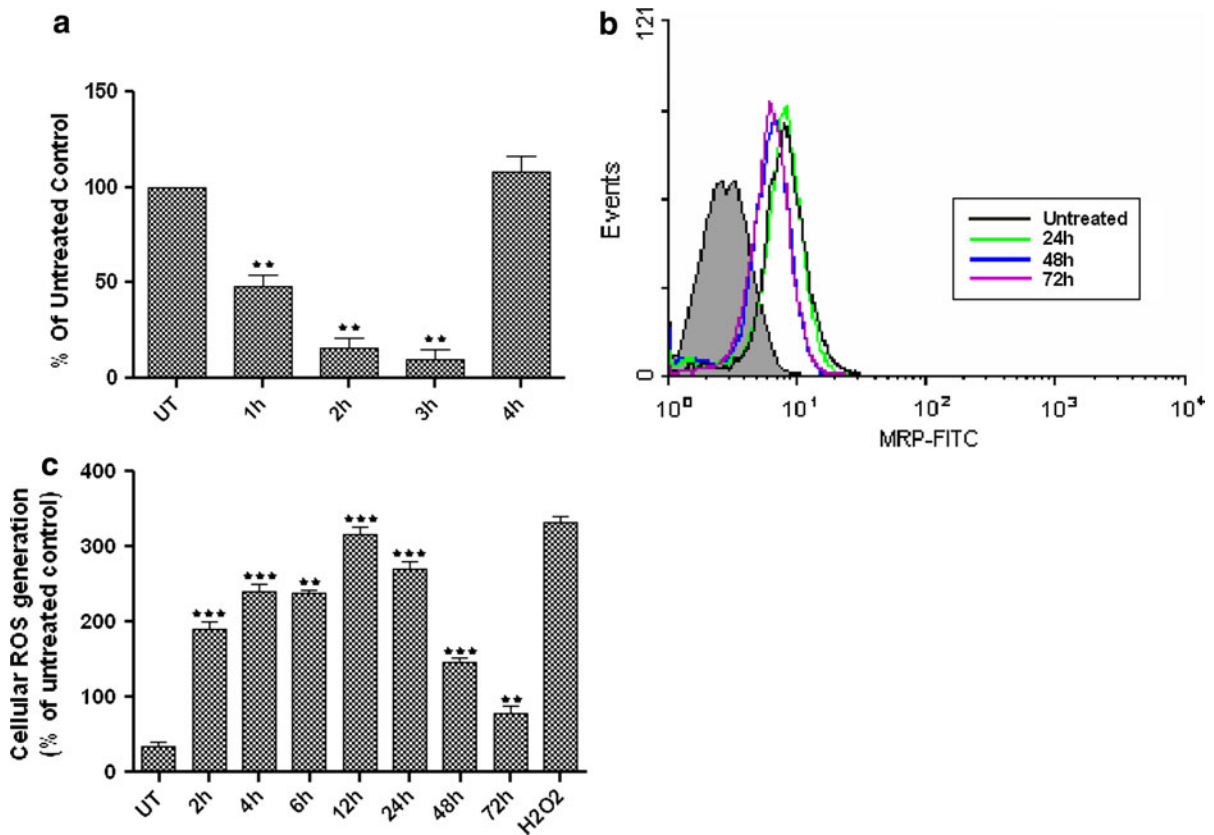
As reduction of MRP1 expression directly correlates with intracellular accumulation of anticancer drugs, we tried to explore whether FeNG could exert any effect upon the expression of this protein (MRP1). Temporal kinetics of MRP1 expression of EAC/DOX cells was ascertained by flowcytometric analysis following FeNG treatment in EAC/DOX bearing mice. Figure 4b showed that FeNG (25 mg kg<sup>-1</sup>) fails to exert any effect on MRP1 expression of EAC/DOX cells. Earlier we demonstrated that in vivo CuNG treatment induced ROS generation and thereby reduced MRP1 expression in EAC/DOX cells (Mookerjee et al. 2006). Therefore, we raised the question: does FeNG fail to induce ROS generation and subsequent alternation MRP1 expression? So we enquired whether FeNG (structurally similar to CuNG) induces ROS generation in EAC/DOX cell. To determine FeNG induced ROS generation, EAC/DOX bearing mice were injected with FeNG (25 mg kg<sup>-1</sup>), cells were isolated at different time intervals and intracellular H<sub>2</sub>O<sub>2</sub> was measured using oxidation sensitive

fluorescence dye DCFDA in EAC/DOX cells. We found, quite surprisingly that FeNG increases intracellular H<sub>2</sub>O<sub>2</sub> in EAC/DOX cells and maintained a sustained elevated level of H<sub>2</sub>O<sub>2</sub> as compared to untreated control (Fig. 4c). However, in vivo FeNG treatment although increases intracellular H<sub>2</sub>O<sub>2</sub>, it fails to alter MRP1 expression in EAC/DOX cells.

### In vivo toxicity of FeNG

We carried out subacute toxicity study (14 days chronic toxicity) in normal Swiss albino and EAC/DOX bearing mice with different combination of FeNG (75 mg kg<sup>-1</sup>, 3 doses each 25 mg kg<sup>-1</sup>) and doxorubicin (3 mg kg<sup>-1</sup>, 3 doses each 1 mg kg<sup>-1</sup>). This study was designed to investigate the toxicological effects of repeated (3 doses) administration (i.p.) of FeNG followed by doxorubicin (2 h later FeNG treatment) treatment on every 6th day for a period of 2 weeks. Following 2 days of total treatment schedule blood, bone marrow cell and spleen cells were isolated from treated and untreated mice. Our study did not reveal any significant pathological changes in treated groups as compared to the control group of animals (Tables 2, 3). No mortality or symptoms of toxicity were observed in test and control groups of animals. No significant differences were observed in the body weight gain/loss pattern, organ weight, haematological or biochemical parameters related to liver and kidney toxicity (see “Materials and methods”) of the entire test groups when compared to the control group (Tables 2, 4).

Several reports have been found that GSH depleting resistance modifier has potential role in organ toxicity by reducing GSH level (Lee et al. 2010; Nazmi et al. 2011; Turner et al. 2005). Therefore, we tried to explore whether FeNG has any prolonged effect on GSH level of vital organs of EAC/DOX bearing mice. We found that although initial GSH level decreased after FeNG treatment in vital organs at 2 h, the level GSH was restored to its normal level at 24 h, thus indicating that FeNG only temporarily depletes GSH in vital organ of EAC/DOX bearing mice (Fig. 5a). As FeNG initially depleted GSH in vital organs in EAC/DOX bearing mice, there was a possibility that vital organs confronted oxidative stress. Therefore, we examined the effect of FeNG on the activity of antioxidant enzyme, glutathione peroxidase (GPx) in vital organs of EAC/DOX bearing mice (Fig. 5b). We found that GPx activity was increased immediately



**Fig. 4** FeNG induces GSH depletion but not change in MRP1 expression in EAC/Dox cells. **a** FeNG depletes intra cellular glutathione (GSH) contents of EAC/Dox cells in vivo. EAC/Dox bearing mice were injected with FeNG i.p. After different time interval (1–4 h), cells were collected from intraperitoneal cavity and intra cellular GSH was measured as described under Materials and Methods. Results are presented as mean  $\pm$  SD of 3 independent experiments. Statistical significance was analysed by ANOVA test ( $P_{ANOVA} < 0.0001$ ) followed by unpaired Student's *t* test (differences between untreated control and FeNG treated cells are significant at  $**P < 0.01$ ). **b** Effect of FeNG on MRP1 expression of EAC/Dox cells. FeNG was administered in vivo in EAC/Dox bearing mice. After indicated time interval cells were collected and were labeled with anti

MRP1 antibody. Immunofluorescence analysis was performed by flow cytometry. Representative data of 3 independent experiments is presented. **c** Represents FeNG induces ROS generation in EAC/Dox cells. After in vivo administration FeNG in EAC/Dox bearing mice, EAC/Dox cells were isolated at indicated time points and intra cellular ROS generation was measured [in terms of peroxide using dichlorofluorescein diacetate (DCF-DA)] as described under Materials and Methods. Data are expressed as percent of control and are presented as mean  $\pm$  SD of 3 independent experiments. Statistical significance was analysed by ANOVA test ( $P_{ANOVA} < 0.0001$ ) followed by unpaired Student's *t* test (differences between control and FeNG treated cells are significant at  $**P < 0.01$  and  $***P < 0.001$ )

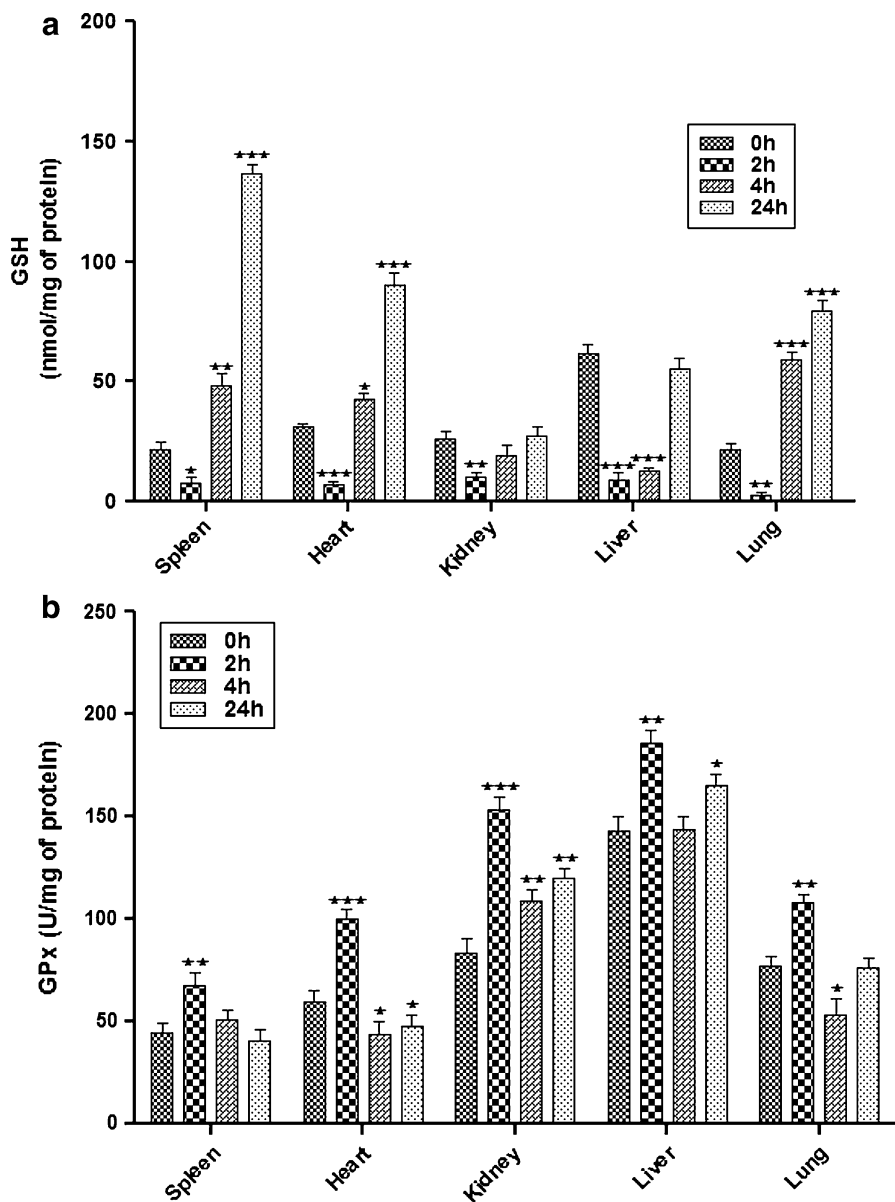
after FeNG treatment at 2 h and then gradually decreased to the level of untreated control.

## Discussion

Tumour resistance to chemotherapeutic agents remains a stumbling block to achieve long-term therapeutic advantage in cancer. Therefore, search of new molecules that can overcome tumour resistance may provide clinical benefits to patients with refractory cancer.

Besides several molecular events, cross resistance to multiple drugs in neoplastic cells has been linked to increased cellular levels of GSH (Ballatori et al. 2009). Several RMAs developed so far deplete GSH and sensitize drug resistant cancer cells to anticancer drugs but unfortunately their use has also been limited owing to their toxic side effects (Chi et al. 2007; Estrela et al. 2006; Linares et al. 2011; Yamamotoa et al. 2002).

In this context, we report in the present communication the resistance modifying property of FeNG. Earlier we reported that FeNG induces apoptosis



**Fig. 5** Effect of in vivo FeNG treatment on tissue GSH level and GPx activity in vital organ of EAC/Dox bearing mice. **a** Effect of in vivo FeNG treatment on GSH levels at 2, 4 and 24 h in spleen, heart, kidney, liver and lung of EAC/Dox bearing mice. Results are compared with corresponding controls. Results presented as mean  $\pm$  SD of 3 independent experiments. GSH levels decreased significantly in all organs at 2 h but at 24 h tissue GSH level significantly increases following FeNG treatment in vivo in EAC/Dox bearing animals compared to their 0 h treated counterparts. Statistical significance was analysed by ANOVA test (spleen, heart, liver, lung  $P_{ANOVA} < 0.0001$ ; kidney  $P_{ANOVA} = 0.0233$ ) followed by unpaired Student's t test (differences between 0 h treated control and 2, 4, 24 h treated

mice are significant at  $*P < 0.05$ ,  $**P < 0.01$  and  $***P < 0.001$ ). **b** Effect of in vivo FeNG treatment GPx activity in spleen, heart, kidney, liver and lung of normal and EAC/Dox bearing mice. Results are presented as mean  $\pm$  SD of 3 independent experiments. Compared to 0 h treated EAC/Dox bearing animals, In vivo FeNG treatment significantly increased GPx activity in all vital organs of EAC/Dox bearing mice at 2 h but after that at 24 h GPx activity reaches its normal level. Statistical significance was analysed by ANOVA test (spleen  $P_{ANOVA} = 0.0011$ ; heart, kidney, liver, lung  $P_{ANOVA} < 0.0001$ ) followed by unpaired Student's t test (differences between 0 h treated control and 2, 4, 24 h treated mice are significant at  $*P < 0.05$ ,  $**P < 0.01$  and  $***P < 0.001$ )

through generation of reactive oxygen species and FeNG also depletes intracellular GSH in doxorubicin resistance P-gp expressing T lymphoblastic leukemia CEM/ADR 5000 cells (Ganguly et al. 2010). This result inspired us to investigate the anti cancer effect of FeNG in MRP1 expressing doxorubicin resistant EAC/Dox cell in vivo. We tested the cytotoxic potential of FeNG and used ex vivo EAC/Dox cells; we found that FeNG has no toxic effects on EAC/Dox cells but in combination with doxorubicin FeNG kills EAC/Dox cells and the effect is almost equal to that of doxorubicin plus verapamil combination.

We also tried to extrapolate our in vitro data and carried out in vivo study in preclinical model of Ehrlich ascites carcinoma. The results of our study disclose that FeNG enhances anti tumour activity of doxorubicin and thereby increases T/C value in EAC/Dox bearing mice. We have used different combination of doses of FeNG and doxorubicin to evaluate RMA property of FeNG; 75 mg kg<sup>-1</sup> dose of FeNG splitted into three doses (25 mg kg<sup>-1</sup> × 3) followed by doxorubicin injection shows highest T/C value as compared to single 75 mg kg<sup>-1</sup> and other combination of doses of FeNG in EAC/Dox bearing mice. We have also evaluated the resistance reversing effect of two established resistance modifiers, viz., BSO and EA but both these compounds show lower potential than FeNG in terms of MST value of EAC/Dox bearing mice. This result suggests that FeNG reverses doxorubicin resistance in EAC/Dox cells ex vivo and in vivo. In this study, we further observed that even a single administration of 25 mg kg<sup>-1</sup> dose of FeNG accumulates doxorubicin in EAC/Dox cells to the level beyond of the parental doxorubicin-sensitive cell line. These results suggest that FeNG renders resistant EAC/Dox cell vulnerable to doxorubicin by increasing concentration as well as retention time of doxorubicin within the cell and thus reverse the doxorubicin resistance.

Chemotherapeutic failure of anticancer drug to treatment of cancer is often associated with cancer cell having higher amount of GSH. Elevated expression of glutathione S-transferases combined with high GSH levels increase the rate of conjugation and detoxification of chemotherapeutic agents. Besides this, increased expression levels of the MRP1 transporters can also contribute to chemoresistance by effluxing drug-GSH conjugates out side of the cell (Ballatori et al. 2009). Although several reports disclose the

efflux of doxorubicin by MRP1, controversy exists regarding the formation of monogluthionyl conjugate of doxorubicin. However, the active efflux process requires GSH for co-transport of doxorubicin by MRP1 (Borst et al. 2000; Loe et al. 1998; Versantvoort et al. 1995). Thus, decrease in intracellular GSH level and MRP1 expression causes accumulation of anticancer drugs in drug resistance cells. Therefore, targeting either GSH and/or MRP1 by small molecules might be a logical strategy to reverse drug resistance in cancer. Keeping this in mind we have evaluated the effect of FeNG on intracellular GSH and MRP1 expression in vivo in EAC/Dox cells. We observed that though FeNG significantly depletes GSH level but FeNG do not mount any significant impact on the expression level of MRP1 in EAC/Dox cells. Therefore, FeNG belongs to the categories of compounds, which deplete intracellular GSH and possess the potential to restrict the drug efflux traffic without affecting MRP1 expression. This may ultimately result in impaired efflux machinery causing doxorubicin treatment to be highly effective. Earlier we reported that CuNG reduces MRP1 expression in EAC/Dox cells through induction of ROS generation (Mookerjee et al. 2006). Hence, herein we try to evaluate the effect of FeNG on ROS generation of EAC/Dox cells and we find that although FeNG increases ROS generation it does not alter the MRP1 expression. This result indicates that, the amount of ROS generation produced by FeNG may not be sufficient to alter the MRP1 expression in EAC/Dox cells.

Moreover, we conducted a standardized study (14-day chronic toxicity) on the systemic toxicity of FeNG when applied in mice as a model system. Repeated doses of FeNG administration (i.p.) do not show any systemic toxicity at the total dose (75 mg kg<sup>-1</sup>) in our preliminary treatment trials of FeNG in a mice Ehrlich ascites carcinoma cell model. Hematological parameters show that FeNG alone (75 mg kg<sup>-1</sup>) and in combination with Dox (i.e., FeNG + Dox) has no toxic effect. No significant change in spleen and bone marrow cell count has been observed with the application of FeNG alone or in combination with doxorubicin (Tables 2, 3). Moreover, the body weight of treated mice remains unchanged when compared with untreated group (control) (data not shown). Drug-induced liver injury is a significant and still unresolved clinical problem.

Drugs that cause injury or dysfunction of liver are often manifested by abnormal increase in the level of liver enzymes, e.g., alanine transaminase (ALT) and alkaline phosphatase (ALP). Liver injury is defined as an alanine aminotransferase (ALT) level of more than three times the upper limit of the normal range, an alkaline phosphatase (ALP) level of more than twice the upper limit of normal, or a total bilirubin (TBL) level of more than twice the upper limit of normal if associated with any elevation of the alanine aminotransferase or alkaline phosphatase level (Navarro and Senior 2006). But, treatment of FeNG alone or in combination with doxorubicin does not show any alteration in serum enzyme level of AST, ALP and ALT as per defined criteria for liver dysfunctions. This observation clearly indicates that FeNG does not hamper the liver functions in normal or EAC/Dox bearing Swiss albino mice. Similarly treatment of FeNG alone or in combination with doxorubicin does not show any significant alteration in serum level of urea and creatinin as compared to untreated groups. These findings thus disclose that FeNG may not have any effect on normal renal functions in normal or EAC/Dox bearing Swiss albino mice.

Besides performing the role of a potent antioxidant, GSH also is a key player in phase II detoxification systems. Plenty of evidences disclose that several drugs deplete GSH in vital organs and exacerbate them to oxidative stress and organ toxicity (Mookerjee et al. 2006). As FeNG displayed the potential of GSH depletion in EAC/Dox cell, we then further enquire whether FeNG has any effect on GSH in vital organ of EAC/Dox bearing mice. We found that FeNG decrease GSH in vital organ at 2 h after that level of GSH was gradually increased as compared to untreated control at 24 h on the other hand highest GPx activity was found at 2 h and at 24 h it reaches its normal level. This result indicates that FeNG only temporarily decrease GSH in vital organs and probably oxidative stress generated due to GSH depletion at initial hour was well tolerated by increased activity of GPx in vital organs of EAC/Dox bearing mice. Thus, FeNG plays a tissue protective role by employing anti oxidant defence mechanisms of tissue.

In conclusion, our ex vivo results clearly indicate that FeNG alone has no cytotoxic potential against EAC/Dox cells, on the other hand when used in combination with doxorubicin, FeNG caused marked decrease in the survivability EAC/Dox cell and thus

reverses the doxorubicin resistance in EAC/Dox cells. Moreover, effect of FeNG in in vivo model reveals a typical mirror image of our ex vivo results. Furthermore, administration of FeNG in vivo significantly increases doxorubicin concentration in EAC/Dox cells by concomitant depletion of intracellular GSH whereas; FeNG does not have any effect on expression of MRP1 in EAC/Dox cells. In addition, FeNG does not show any systemic toxicity at concentrations that is used in preliminary trials in mice Ehrlich ascites carcinoma model. Therefore, our present preclinical study provides evidence that non-toxic FeNG a potent resistance modifier might have tremendous clinical relevance to improve the efficacy of chemotherapeutic drugs in MDR in cancers.

**Acknowledgments** This investigation received financial support from Indian Council of Medical Research (ICMR), New Delhi, no. 3/2/2/200/2009NCD-III and 5/13/53/2008NCD-III. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

**Conflict of interest** The authors have declared that no conflicts of interests exist.

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