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Thioredoxin reductase inactivation as a pivotal mechanism of ifosfamide in cancer therapy

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Abstract

Thioredoxin reductase reduces thioredoxin, thereby contributing to multiple cellular events related to carcinogenesis including cell proliferation, apoptosis, and cell signaling. This selenium-containing oxidoreductase is over-expressed in many malignant cells and has been proposed as a target for cancer therapy. Ifosfamide is an oxazaphosphorine alkylating agent with a broad spectrum of antineoplastic activity. The purpose of this study is to test the hypothesis that anticancer efficacy of ifosfamide may rely on its ability to inhibit thioredoxin reductase in tumor. To inspect the consequence of thioredoxin reductase inhibition by ifosfamide on tumor cell proliferation, mice bearing hepatoma 22 (H22) cells in ascites were injected with 350 mg/kg ifosfamide. Thioredoxin reductase activity was maximally inhibited by half at 6 h, and a subsequent pronounced cellular proliferation inhibition due to cell cycle arrest in G_1 phase was found. Moreover, at 6 h, except thioredoxin reductase inhibition, ifosfamide did not affect cell cycle or other measured antioxidant enzymes activity in the tumor cells. Intriguingly, when these cells were injected into healthy mice, they totally lost the capacity of causing either ascitic or solid tumors. Thioredoxin reductase inhibition could also be found in solid H22 tumor by 62%, bladder by 74% and kidney by 37% at 6 h. Overall, these observations provide direct evidence that inhibition of thioredoxin reductase activity in malignant cells by ifosfamide is highly associated with its anticancer effect and the mechanism of ifosfamide systemic toxicity may be related to multi-organ inhibition of thioredoxin reductase activity.

Keywords: Ifosfamide; Thioredoxin reductase; Thioredoxin; Cancer

1. Introduction

Ifosfamide, an oxazaphosphorine-type anticancer alkylating agent, is known to have considerable activity against a wide range of tumors in both adults and children (Furlanut and Franceschi, 2003).

Ifosfamide is a prodrug that has first to be transformed by hepatic cytochrome *P-450* enzymes (Dechant et al., 1991; Wagner, 1994). Via 4-hydroxylation ifosfamide yields isofosforamide mustard and acrolein, and via *N*-dechloroethylation ifosfamide liberates chloroacetaldehyde (Furlanut and Franceschi, 2003). Among various metabolites, isofosforamide mustard probably represents the most important cytotoxic compound which could react with DNA molecules to form intra- and interstrand crosslinks, leading to DNA strand-breaks, inability to synthesize DNA and ultimately cell apoptosis and/or necrosis, thereby being considered as a major anticancer mechanism of ifosfamide (Zhang et al., 2005). Recently, chloroacetaldehyde has been proposed to play a role in antitumor efficacy of ifosfamide through influencing the oxidative phosphorylation in mitochondria and causing strand-breaks and strong inhibition of DNA-synthesis in tumor cells (Bruggemann et al., 2006). In addition, acrolein, which inactivates the DNA repair protein *O*6-methylguanine-DNA methyltransferase (Lee et al., 1992), also partially contributes to the cellular toxicity of ifosfamide.

Thioredoxin reductase is a homodimeric selenoprotein that catalyzes NADPH-dependent reduction of thioredoxin. Thioredoxin is a cofactor in protein disulfide reduction and DNA synthesis, but independently, it inhibits apoptosis, stimulates

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cell proliferation and angiogenesis, and increases transcription factor activity (Biaglow and Miller, 2005). Thioredoxin reductase is a potential molecular target of anticancer agents since thioredoxin reductase is over-expressed in many tumor cells, exhibits pro-survival signaling attributes and incites a pro-survival effect, enhances tumor proliferation and resistance to therapeutic modalities (Nguyena et al., 2006). It has been shown that decreased expression of thioredoxin reductase by a small interference RNA construct reverses the tumor morphology and the tumorigenic properties of lung carcinoma cancer cells (Yoo et al., 2006). As a selenoprotein, thioredoxin reductase contains a selenocysteine on its flexible C-terminal arm which is very reactive and well-accessible during catalysis (Mustacich and Powis, 2000), and, many electrophilic compounds selectively and irreversibly modify this active site (Nordberg and Arnér, 2001). A recent study showed that prototype compounds representative for the major classes of clinically used anticancer alkylating agents could effectively inhibit thioredoxin reductase *in vitro* (Witte et al., 2005). More recently, it was reported that targeting thioredoxin reductase is a basis for cancer therapy by arsenic trioxide (Lu et al., 2007).

We have found that cyclophosphamide potently inhibited the activity of tumor thioredoxin reductase *in vivo* (Wang et al., 2007). As a structural analog of cyclophosphamide, ifosfamide has not yet been studied for possible interaction with mammalian thioredoxin reductase. Therefore we investigated the effect of ifosfamide on thioredoxin reductase activity in mice.

2. Materials and methods

2.1. Chemicals and drugs

NADPH, HEPES, insulin, 5,5′-dithiobis (2-nitrobenzotic acid) (DTNB), thioredoxin (*E. coli*), guanidine hydrochloride, reduced glutathione (GSH), bovine serum albumin, 1-chloro-2,4-dinitrobenzene (CDNB), RNase A, propidium iodide (PI), and ifosfamide were all purchased from Sigma (St. Louis, MO, USA). Other chemicals were of the highest grade available.

2.2. Animals

Healthy male Kunming mice (body weight of 20–22 g) and their diet were all purchased from Shanghai SLAC Laboratory Animal Co. Ltd., PR China. The mice were housed in plastic cages (5–8/each) in a room with controlled temperature (22±1 °C) and humidity (50±10%) and 12 h light/dark circle. The mice were allowed ad libitum to obtain food and water. All experiments were performed strictly adhering to the ethical guidelines issued by the University of Science and Technology of China.

2.3. Tumor cell inoculation

For ascites growth, murine hepatoma 22 (H22) cells were injected into the peritoneal cavity of mice (20×10^6 viable cells/each). For solid tumor growth, H22 cells were inoculated subcutaneously at right axilla of mice (5×10^6 viable cells/each).

2.4. Animals treatment

2.4.1. Treatment in mice bearing ascites

To inspect the effects of ifosfamide on thioredoxin reductase, cell proliferation, etc., mice bearing H22 ascites were randomly divided into two groups: (i) control set (which were treated with saline, $n=5$), (ii) ifosfamide-treated set ($n=5$). Ifosfamide (350 mg/kg) was injected intraperitoneally after cell inoculation for 3 days. Animals were sacrificed by cervical dislocation at a designed time as shown in the results.

After sacrifice of ascites-bearing mice, ascitic fluid was carefully collected, and the peritoneal cavity was washed three times with ice-cold saline to fully obtain ascitic cells. The ascitic fluid along with the washed saline was merged and further adjusted to a fixed volume. The viable cells were counted in a hemocytometer using the Trypan blue dye exclusion method.

The ascitic fluid sampled from each mouse was washed and resuspended twice by ice-cold saline, and divided in two vials. One vial was stored at −30 °C for the determination of thioredoxin reductase, GSH level, catalase, superoxide dismutase, glutathione peroxidase, and glutathione-*S*-transferase activities at designed time as shown in results.

The other vial was immediately used to check proliferative capacity of H22 cells *in vivo*. For this purpose: (i) Viable cells (20×10^6) obtained from 6 h saline and ifosfamide-treated mice, were injected into the abdominal cavity of mice, and the respective cell number was counted after 6 days; (ii) Viable cells (5×10^6) obtained from 6 h saline and ifosfamide-treated mice, were injected subcutaneously into the right front axilla of mice. The day of cell implantation was designated as day 0. After cell inoculation, tumor size was measured every five days for 25 days using vernier calipers. The long and short dimensions of the tumors were measured and the tumor volumes were calculated in the following formula: tumor volume = 0.5 × long diameter × short diameter × short diameter.

2.4.2. Treatment in mice bearing solid tumor

In the first set of experiment, to observe the effect of ifosfamide on thioredoxin reductase in different tissues, 24 mice that were implanted with H22 cells were randomly divided into three groups with eight mice per group. The day of cell implantation was designated as day 0. Treatment was initiated on day 10 when the tumor grew to approx. 700 mm^3 in size. Group I was treated with saline as control, groups II and III were intraperitoneally injected with a single dose of ifosfamide (350 mg/kg). Animals in groups II and III were sacrificed by cervical dislocation at 6 and 54 h respectively after ifosfamide administration, and group I was sacrificed by cervical dislocation at 6 h after saline administration.

In the second set of experiment, to investigate whether the high dose of ifosfamide has an impact on brain thioredoxin reductase, 16 mice were randomly divided into two groups with eight mice in each group. Group I was treated with saline as control, and group II was intraperitoneally injected with 1000 mg/kg ifosfamide. Animals were sacrificed by cervical dislocation at 6 h after saline or ifosfamide administration.

After sacrifice, tumors, brains, bladders and kidneys in animals of the first experiment and only brains in animals of the

second experiment were immediately excised and rinsed in ice-cold saline and then stored at −30 °C.

2.5. Cell cycle analysis

The cancer cells harvested from 6 and 96 h saline and ifosfamide-treated mice were prepared to measure cell cycle distribution. Samples of the cells (2×10^6) were washed twice with PBS and fixed in 70% ethanol at 4 °C for 24 h. Immediately before analysis the fixed cells were washed with PBS again to remove ethanol. The pellet was resuspended in PBS containing 1 mg/ml of RNase A and incubated at 37 °C for 30 min. Then, PI was added to a concentration of 40 μg/ml and the resulting mixture was further incubated for another 30 min in the dark at 4 °C. Cell cycle phase distribution of nuclear DNA was determined on FACS Calibur; fluorescence detector was equipped with 488 nm argon laser light source and 623 nm band pass filter (linear scale) using Cell Quest software (Becton Dickinson). A total of 10,000 events were acquired and analysis of flow cytometric data was performed using ModFit software. For each sample, data were obtained through two gates, FSC versus SSC and FL2W versus FL2A, to reduce debris and other contamination. A histogram of DNA content (*x*-axis, PI-fluorescence) versus counts (*y*-axis) was displayed.

2.6. Biomakers

Tumor tissues and cells were homogenized with ice-cold 150 mM NaCl and centrifuged at 15,000 g at 4 °C for 15 min. The resulting supernatants were used for determination. Protein levels were determined by the Bradford dye-binding assay with bovine serum albumin as standard.

2.6.1. Thioredoxin reductase activity assay

Thioredoxin reductase activity was measured based on the method of Holmgren and Bjŏrnstedt (1995) with some modifications. A stock mixture was composed of HEPES buffer (0.25 M), NADPH (2.5 mM), EDTA (10 mM), and insulin (1 mM), with the final pH 7.6. In a 96-well plate, 7 μl stock mixture, 3 μl thioredoxin (0.17 mM), 40 μl HEPES (50 mM, pH 7.6), and 20 μl sample (with 20–30 μg protein except brain with 100–120 μg protein) were added into each well. The enzymatic reaction was maintained at 37 °C for 20 min, and then was ceased by adding 240 μl terminative solution (0.5 mM DTNB/6 M guanidine hydrochloride in 0.2 M Tris–HCl, pH 8.0). Each sample contained a non-enzymatic reaction as the control. The non-enzymatic reaction included all components except thioredoxin which was substituted by the same volume of water. The 96-well plates were read at 412 nm. A_{412} change was calculated according to the following process. The absorbance of control was subtracted from the absorbance of the sample. A background control, which was the subtraction of absorbance with and without thioredoxin in the absence of tissue homogenate, was further subtracted from the absorbance of all samples. Thioredoxin reductase activity unit was defined as A_{412} change × 1000/min and thioredoxin reductase activity was expressed as U/mg protein (Ganther and Ip, 2001).

2.6.2. GSH and antioxidant enzymes assays

2.6.2.1. GSH level. Immediately after homogenate, a certain volume of homogenate was taken out to mix with trichloroacetic acid (20% w/v), at the ratio of 10:1 in volume. This procedure has been confirmed to be able to precipitate all proteins in the homogenate and make GSH in the homogenate stable at 4 °C for at least 2 h. The trichloroacetic acid treated homogenate was centrifuged at 10,000 g and 4 °C for 5 min. Within 2 h after the centrifugation, the resulting supernatant was mixed with DTNB (20 mg/ml in 0.2 M phosphate buffer, pH 8.0) and was read at 412 nm (Ellman, 1959). The amount of GSH was expressed as nmol/mg protein.

2.6.2.2. Catalase activity. The breakdown of hydrogen peroxide on addition of catalase leads to a decrease in light absorption of hydrogen peroxide solution in the ultraviolet region (Claiborne, 1985). In brief, 150 μl sample (containing approx. 0.9 mg protein) was mixed with 3 ml phosphate buffered solution (50 mM, pH 7.0; containing 16 mM hydrogen peroxide). The mixture was incubated at 25 °C, and the reduction of light absorption at 240 nm was read for 15 min. Catalase activity was calculated as nmol of H_2O_2 consumed/min/mg of tissue protein and expressed as U/mg of tissue protein.

2.6.2.3. Superoxide dismutase activity. Xanthine–xanthine oxidase can generate a superoxide flux which reduces nitroblue tetrazolium. Superoxide dismutase reduces the light absorption through scavenging superoxide anion (Sun et al., 1988). A superoxide dismutase kit based on this principle was obtained (Trevigen, Gaithersburg, MD, USA), and the provided instruction was followed. One unit of SOD activity was defined as the amount of protein that inhibits the rate of nitroblue tetrazolium reduction by 50%. Data are expressed as U/mg of tissue protein.

2.6.2.4. Glutathione peroxidase activity. Glutathione peroxidase catalyzes the reaction of GSH and hydrogen peroxide (Rotruck et al., 1973). Therefore, GSH consumption in the presence of hydrogen peroxide and the enzyme can be used for the assessment of glutathione peroxidase activity. At first, 100 μl phosphate buffer (0.1 M, pH 7.0, containing 1 mM GSH) and 50 μl sample (containing approx. 300 μg protein) were mixed. Then, the reaction was started by adding 50 μl hydrogen peroxide (100 mM) and was terminated by the addition of 1 ml saturated $(NH_4)_2SO_4$ solution after 5 min incubation at 37 °C. Following the centrifugation to separate protein precipitated by $(NH_4)_2SO_4$, a portion of the supernatant was taken out for the assessment of GSH according to the description in GSH assay. A non-enzymatic reaction is necessary for the calculation of GSH decrease caused by the enzymatic reaction. For this purpose, the procedure was similar to the sample assay only replacing the sample with the phosphate buffer. The activity of glutathione peroxidase is calculated by the decrease of GSH and expressed as U/mg protein; a unit was defined as 1 μmol of GSH oxidized/min.

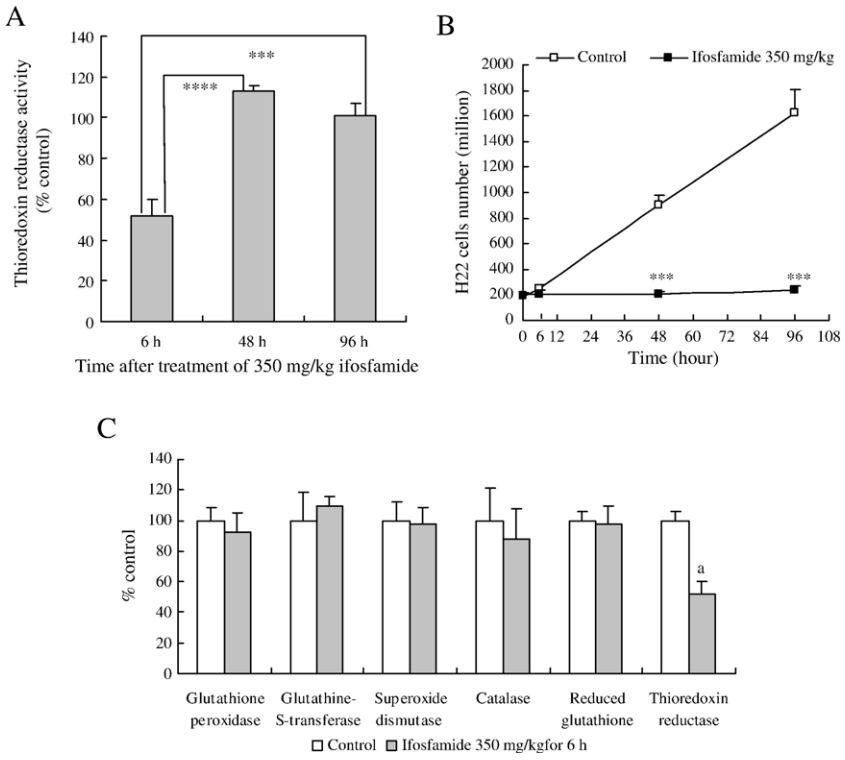

Fig. 1. Effects of 350 mg/kg ifosfamide on ascitic H22 cells. (A) Inhibition and subsequent recovery of thioredoxin reductase activity. Changes of thioredoxin reductase activity are expressed as the percentage of the control value, ****, $P<0.0001$ compared with 6 h. (B) H22 cell proliferation.***, $P<0.001$ compared with control. (C) Effect of ifosfamide on redox parameters (thioredoxin reductase, glutathione peroxidase, glutathione-*S*-transferase, superoxide dismutase and catalase activities as well as GSH level). Changes are expressed as the percentage of the control value. Open bars indicate control; Solid bars represent the treatment of 350 mg/kg ifosfamide for 6 h. a: $P<0.0001$ compared with control. The control activity of glutathione peroxidase, glutathione-*S*-transferase, superoxide dismutase, catalase, and thioredoxin reductase was 70.6±5.8, 65.8±7.2, 2.7±0.3, 173.1±25.1, and 131.1±8.2 U/mg protein, respectively, and the control GSH level was 19.4±1.1 nmol/mg protein. Data represented means±S.D. ($n=5$).

2.6.2.5. Glutathione S-transferase activity. Glutathione *S*-transferase activity assay was carried out by the method of Habig et al. (1974) using CDNB as substrate. Reaction was started by the addition of 25 µl sample (containing approx. 150 µg protein) to 2 ml reaction mixture (pH 6.5, 100 mM sodium phosphate buffer with 1 mM CDNB and 1 mM GSH). The changes in absorbance were recorded for 10 min at 340 nm. One unit of glutathione-*S*-transferase activity was calculated in terms of nmol CDNB conjugate formed/min/mg of tissue protein and expressed as U/mg of tissue protein.

2.7. Statistical analysis

Data are presented as the means±S.D. The differences between the groups were examined using Student's *t*-test. A *P* value of less than 0.05 was considered statistically significant.

3. Results

3.1. The time course of thioredoxin reductase activity in ascitic H22 cells and cell proliferation after ifosfamide treatment

When 350 mg/kg ifosfamide was administrated intraperitoneally, thioredoxin reductase activity in extracts of the ascitic cells changed over time, substantially reduced to 52% of the control at 6 h ($P<0.0001$), and recovered to normal level at 48 and 96 h (Fig. 1A). Cell number significantly reduced at 48 and 96 h after injection (P all <0.001; Fig. 1B), but did not change at 6 h as compared with the control.

3.2. Changes of redox parameters in ascitic H22 cells

In parallel with thioredoxin reductase, after 350 mg/kg ifosfamide treatment for 6 h, the activities of antioxidant enzymes (glutathione peroxidase, glutathione-*S*-transferase, superoxide dismutase and catalase), as well as GSH level in H22 cells were also determined. Only thioredoxin reductase activity considerably decreased ($P<0.0001$), and other redox parameters had no significant variation (Fig. 1C).

3.3. Cell cycle analysis

We performed DNA cell cycle analysis on ascitic H22 cells after being treated with 350 mg/kg ifosfamide for 6 and 96 h. At 6 h, there was no significant change in cell cycle distribution between control and treated cells (Fig. 2A and B). However, at 96 h, ifosfamide treatment resulted in an accumulation of cells in G_1 phase compared with vehicle treatment (Fig. 2C and D).

3.4. The tumorigenicity of 6 h ifosfamide-treated ascitic H22 cells

At 6 h after 350 mg/kg ifosfamide was administrated, thioredoxin reductase activity in ascitic cells was maximally inhibited

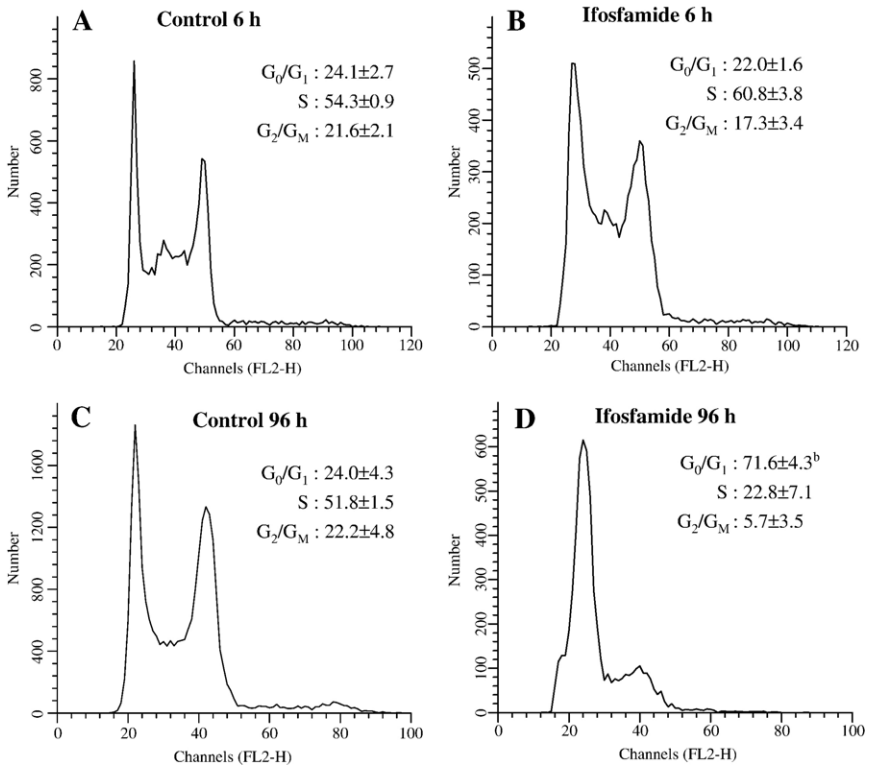

Fig. 2. Effects of ifosfamide on cell cycle distribution in ascitic H22 cells. Mice bearing ascitic cells were treated with saline (A and C) or 350 mg/kg ifosfamide (B and D) for 6 and 96 h. Cell cycle analysis was performed by flow cytometry as detailed under "Materials and methods". Data (% of cells in the indicated phases) represented the means ± S.D. of five independent measurements. $^{b}P<0.001$ compared with control.

(Fig. 1A), whereas cell cycle and other antioxidant parameters had no significant variation (Figs. 2A, B and 1C). In order to assess the tumorigenicity of these cells, they were injected into the abdominal cavity and the right front axilla of healthy mice. Cells in ascites were analyzed after 6 days. There was 99% inhibition of cell number in comparison with control group ($P<0.01$; Fig. 3A). The volume of solid tumor in control mice approached 3000 mm^3 on the 25th day, while no tumor was found in mice injected with ifosfamide-treated cells (Fig. 3B). The dramatic changes in tumorigenicity of thioredoxin reductase-inactivated cells imply thioredoxin reductase is essential for tumor growth.

3.5. Inactivation and subsequent induction of thioredoxin reductase activity by ifosfamide treatment in various tissues

The major limitation of cancer chemotherapy of ifosfamide is the injury of normal tissues, leading to multiple organ toxicity, such as damage to the bladder (hemorrhagic cystitis), nephrotoxicity, and neurotoxicity (Furlanut and Franceschi, 2003; Lerch et al., 2006). Therefore, we determined the activity of thioredoxin reductase in H22 solid tumor, bladder, kidney, and brain of mice treated with ifosfamide. Six hours after 350 mg/kg ifosfamide was administrated intraperitoneally, thioredoxin reductase activities maximally decreased by 62%, 74% and 37% in solid H22 tumor, bladder and kidney respectively, and recovered to 62% of normal level in tumor and normal level in bladder and kidney respectively at 54 h (Fig. 4A, B and C); while the activity of cerebral thioredoxin reductase did not significantly decrease at designed time (Fig. 4D).

In order to determine whether ifosfamide inactivates thioredoxin reductase activity in brain, 16 animals were injected intraperitoneally with 1000 mg/kg ifosfamide. This dose is

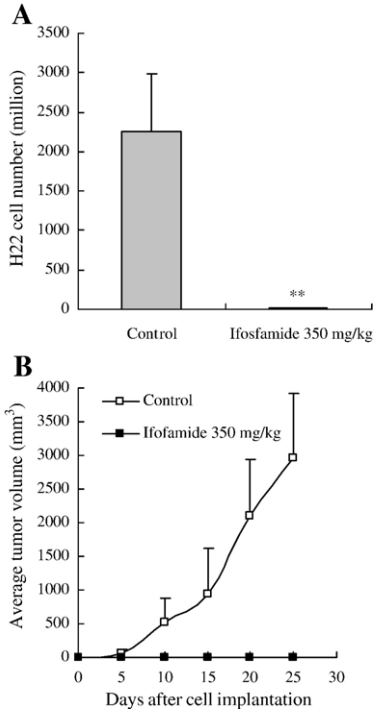

Fig. 3. Tumorigenicity of 6 h ifosfamide-treated ascitic H22 cells. (A) Growth of ascitic cells and (B) Growth of tumors. All data represented means ± S.D. ($n=5$).

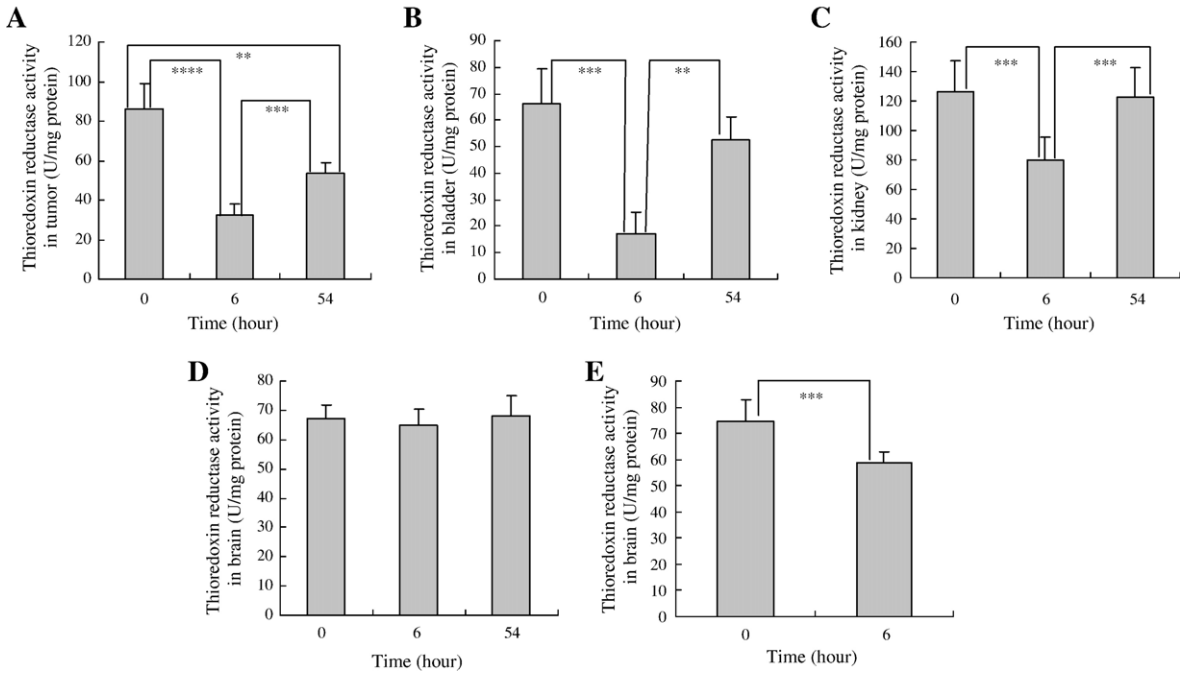

Fig. 4. Effect of ifosfamide on thioredoxin reductase activity in tissues. A–D, 350 mg/kg ifosfamide was intraperitoneally administrated and thioredoxin reductase activity was tested at designed time in solid H22 tumor, bladder, kidney and brain, respectively. (E) Ifosfamide of 1000 mg/kg was intraperitoneally administrated and thioredoxin reductase activity was tested at 6 h. All bars represented means ± S.D. ($n=8$). Statistically relevant decreases or increase in thioredoxin reductase activity are indicated in the figure with asterisks. ****, $P<0.0001$; ***, $P<0.001$; **, $P<0.01$.

chosen based on a previous report studying the mechanism of ifosfamide-associated encephalopathy (Lerch et al., 2006). We found 1000 mg/kg ifosfamide caused lethargy, severe hind limb paralysis and impending death 6 h later, so the mice were killed by cervical dislocation at 6 h after administration of ifosfamide and cerebral thioredoxin reductase activity was tested. As shown in Fig. 4E, the activity of cerebral thioredoxin reductase showed a significant reduction to 78% of the control ($P<0.001$).

4. Discussion

In this study, we demonstrated ifosfamide inhibited thioredoxin reductase activity *in vivo*, and a subsequently dramatic deceleration in tumor progression was found. The inhibition of thioredoxin reductase activity was relatively specific since other antioxidant parameters were not significantly affected. Interestingly, the ifosfamide-treated cancer cells with decreased thioredoxin reductase activity lost total tumorigenicity after being inoculated into mice.

Mammalian thioredoxin reductases contain a reactive and solvent accessible selenocysteine residue which is located on a flexible C-terminal arm of the protein. This selenocysteine is essentially involved in the catalytic cycle of thioredoxin reductase and thus represents an attractive binding site for many electrophilic compounds (Nordberg and Arnér, 2001). The mechanism by which thioredoxin reductase is inactivated by ifosfamide is not clearly defined, but there are several possibilities considered to be relevant. Firstly, the inhibition of thioredoxin reductase is likely to be pertinent to the metabolism of this drug. As active metabolites of ifosfamide, isofosforamide mustard and chloroacetaldehyde generate electrophilic species (Furlanut and Franceschi, 2003), which can readily react with highly reactive C-terminal selenocysteine residues of thioredoxin reductase. That acrolein inactivates thioredoxin reductase *in vitro* and in cells has been reported (Yang et al., 2004; Park et al., 2005). Secondly, isofosforamide mustard produces nitric oxide through induction of nitric oxide synthase, leading to peroxynitrite formation in cells (Korkmaz et al., 2006). Thioredoxin reductase is irreversibly inactivated by peroxynitrite *in vivo* via a reaction with the selenocysteine residue (Park et al., 2002). Thirdly, as with cyclophosphamide, ifosfamide acts through the loss of a chlorine atom with formation of a positively charged reactive intermediate that irreversibly binds to a nucleophilic site (Moore, 1991). Inhibition of thioredoxin reductase by ifosfamide may occur by direct targeting of the active-site selenolate by the intermediate produced from ifosfamide (Witte et al., 2005).

It is notable that thioredoxin reductase activity in tumors was rapidly inhibited by ifosfamide with a quick restoration; however, a dramatic deceleration in tumor progression was found (Fig. 1A and B). Reduced thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase ASK-1 (Saitoh et al., 1998). Inactivation of thioredoxin reductase by ifosfamide leads to oxidation of thioredoxin (Lu et al., 2007), and thus induce apoptosis via ASK-1 activation. Furthermore, inhibition of the disulfide-reducing activity of thioredoxin reductase by the alkylating agent DNCB, the anticancer drug cisplatin, or the chemopreventive agent curcumin forms selenium-compromised thioredoxin reductase with NADPH oxidase activity, which

produces ROS and causes oxidative stress (Fang et al., 2005; Nordberg and Arnér, 2001; Sasada et al., 1999). On the other hand, even selenium-compromised thioredoxin reductase without NADPH oxidase activity can induce rapid cell death (Anestal and Arnér, 2003; Cenas et al., 2006). Selenium-compromised thioredoxin reductase-derived apoptosis proteins (SecTRAPs) is a potent killer of cancer cells (Arnér, 2006). Overall, although thioredoxin reductase activity decrease was transient, underlying this, SecTRAPs may last for longer duration, thereby leading to substantial suppression of cancer cell proliferation as seen herein (Fig. 1A and B).

With a uniquely accessible selenocysteine residue, thioredoxin reductase may therefore be more susceptible to inactivation by ifosfamide than most thiol-containing cellular scavengers like GSH. Ifosfamide can decrease intracellular GSH levels in malignant cell lines *in vitro* and in peripheral blood lymphocytes *in vivo* (Lind et al., 1989; Meier et al., 1994). Moreover, pretreatment with buthionine sulfoximine, an inhibitor of GSH synthesis with less cytotoxic effect per se, could enhance the cellular toxicity of ifosfamide *in vitro* (Zaki et al., 2003; Springate et al., 1999). Thus the sulfhydryl status of cells, particularly the intracellular concentration of GSH, plays a role in the response of cells to ifosfamide. In the present study, 350 mg/kg ifosfamide, which was a relatively high pharmacological dose in animal experiment, considerably reduced thioredoxin reductase activity ($P<0.0001$), whereas did not significantly change GSH level of cancer cells (Fig. 1C). Albeit 1100 mg/kg ifosfamide could significantly deplete GSH level in H22 ascitic cells, it also caused complete host death (data not shown). Therefore, as compared with GSH, ifosfamide's impact on thioredoxin reductase in cancer cells may be more relevant to its pharmacological action.

Therapeutic application of high-dose ifosfamide is limited by several side-effects, among which, hemorrhagic cystitis, neurotoxicity and nephrotoxicity are given the greatest concern (Furlanut and Franceschi, 2003). The presence of these side-effects is likely to be connected with the metabolism of this drug. Acrolein may induce hemorrhagic cystitis, whereas chloroacetaldehyde may be responsible for both nephro- and neurotoxicity (Furlanut and Franceschi, 2003; Lerch et al., 2006). We determined the activity of thioredoxin reductase in bladder, kidney and brain of mice treated with ifosfamide, and found that bladder and kidney showed the same phenomena of inhibition and subsequent induction as tumor. Thioredoxin reductase activity significantly decreased by 74% in bladder and 37% in kidney at dose of 350 mg/kg (Fig. 4B and C), and 22% in brain at dose of 1000 mg/kg (Fig. 4E). Thus, the inhibition of thioredoxin reductase activity by ifosfamide is a nonspecific effect for cancer cells. Our results also suggest that the mechanism of ifosfamide systemic toxicity may be related to multi-organ inhibition of thioredoxin reductase activity.

The other major limitation to the use of ifosfamide is tumor resistance, which is due to multiple mechanisms that include increased DNA repair, increased cellular thiol levels, glutathione *S*-transferase and aldehyde dehydrogenase activities, and altered cell-death response to DNA damage (Zhang et al., 2005). Thioredoxin reductase 1 is upregulated under oxidative stress due to the release of NF-E2-related factor 2 from keap1 and the binding of NF-E2-related factor 2 to antioxidant response element in thioredoxin reductase1 gene promoter (Sakurai et al., 2005). In present study, we observed thioredoxin reductase recovery in both ascitic and solid H22 *in vivo* within 54 h. Thioredoxin reductase recovery to normal level in ifosfamide-treated tumors may be related to the resistance of tumor cells. If the cells survive the significant oxidative stress, increased expression of newly synthesized thioredoxin reductase that is not being inhibited, could possibly restore the physiological redox balance in the cell (Nordberg and Arnér, 2001). Our results suggest modulating thioredoxin reductase recovery after ifosfamide treatment may be a rational therapeutic strategy to overcome ifosfamide-resistance.

To sum up, we found that ifosfamide preferentially inhibited the activity of thioredoxin reductase *in vivo* over other antioxidant parameters. Furthermore the quick recovery of thioredoxin reductase activity might be closely associated with the development of cellular resistance to ifosfamide. The thioredoxin reductase activity inhibition could be considered as a pivotal mechanism contributing to anticancer effect of ifosfamide.

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