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## Original article

# Protective Effects of Tannins in *Sanguisorbae Radix* on Myelosuppression Mice

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ABSTRACT

**Objective** To explore the protective effects of tannins in *Sanguisorba Radix* (TSR) on myelosuppression mice induced by cyclophosphamide (CTX). **Methods** TSR was ig given at the dose of 20 mg/kg for 10 d after ip administration of CTX (200 mg/kg). **Results** TSR could significantly increase the numbers of white blood cells, red blood cells, and platelets of myelosuppression in mice. And it could accelerate bone marrow haemopoietic stem/progenitor cells (HSPCs) in myelosuppression mice and enhance cell proliferation by promoting cell cycles from $G_0/G_1$ phase to access into S and $G_2$/M phases, then the reduced number of HSPCs induced by CTX was reversed. Moreover, TSR could increase the mRNA and protein expression levels of O(6)-methylguanine-DNA methyltransferase (MGMT) in HSPCs of myelosuppression mice. **Conclsion** TSR has a protective function against CTX-induced myelosuppression. The mechanism might be related to protecting hematopoietic stem cells of bone marrow, stimulating hematopoiesis recovery, as well as preventing the apoptosis of hematopoietic stem cells induced by CTX.

*Key words*

bone marrow cells; cyclophosphamide; haemopoietic stem/progenitor cells; myelosuppression; tannins; *Sanguisorbae Radix*



## 1. Introduction

During the past several years, a number of novelly targeted anticancer drugs have been integrated into clinical practice. Despite the efficacy and variety of new agents, traditional myelosuppressive chemotherapy remains the backbone of cancer treatment. In addition, the new biologicals often increase myelotoxicity when added to standard chemotherapy regimens. Therefore, myelosuppression remains the most common toxicity encountered in the oncology clinic today and this complication is likely to remain a serious problem indefinitely (Davey and Jeffery, 2006; Khuri, 2007).

So far, no effective method is used to treat myelosuppression once it occurs. Transfusions could be effective in replenishing red blood cells (RBC) and platelets (PLT), and another alternative way is growth factor injections-included erythropoietin, granulocyte colony-stimulating factor (G-CSF or filgrastim), granulocyte macrophage

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colony-stimulating factor (GM-CSF), and interleukin 11, which can boost bone marrow performance (Crawford et al, 1991; Nemunaitis et al, 1991), but both of them can not radically protect bone marrow and improve the white blood cell (WBC) level. In the past several years, lots of experimental studies on the effects of Chinese herbal medicines to prevent and treat myelosuppression induced by chemotherapy or radiotherapy have been carried out in China (Raposo et al, 2006).

*Sanguisorbae Radix* originates from the dried roots of plants in *Sanguisorba* L. of Rosaceae, such as *S. officinalis* L. or *S. officinalis* var. *longifolia* (Bert.) Yu et Li, with the functions of cooling blood and hemostasis, detoxification, and sore healing. *S. officinalis* L. var. *longifolia* contains a large amount of tannins (about 20%), and further contains compounds such as saponins and flavones. Currently, most researches focus on saponins in *Sanguisorbae Radix* (SSR). It is generally believed that SSR are active ingredients in the plants of *Sanguisorba* L. to increase WBC and protect bone marrow. Domestic fundamental research on effect of *Sanguisorbae Radix* on increasing white cells indicated that SSR were the main active parts to enhance the *in vitro* proliferation of bone marrow cells of mice; Moreover, SSR also could increase the numbers of WBC, RBC, and platelets in myelosuppression of mice (Zou et al, 2012). However, tannins in *Sanguisorbae Radix* (TSR) and flavones in *Sanguisorbae Radix* can not promote the proliferation of bone marrow cells, instead of causing bone marrow inhibition in high concentration (Gao et al, 2006).

Currently, researches on the efficacy of TSR are relatively rare. It was reported in the foreign literatures that TSR had anticancer functions (Bastow et al, 1993). There were some reports about the TSR extract, for example, TSR extract containing 43.86% tannins was obtained through extraction with 70% hydrous acetone (Gao et al, 2006). The TSR extract was obtained using acetone by the above process, with not only tannins but also saponins, which comprised 15% saponins by detection (Wang et al, 1993).

So far, researches on efficacy of TSR are relatively rare. As yet, no report related to bone marrow protection and leucopenia therapy of TSR has been known. The aim of this work is to investigate the effects of TSR on myelosuppression mice induced by cyclophosphamide (CTX) and their possible mechanism of protection on bone marrow cells.

## 2. Materials and methods

### *2.1 Preparation of TSR*

TSR was extracted according to the patent (PCT/ N2011/079633) as follows: *Sanguisorbae Radix* (200 g) was pulverized into coarse powders, and then decocted with water twice, each for 1.5 h. The water amount was 10 times of the powders in the first decoction, and eight times in the second decoction. Then, the filtrates were merged and concentrated under reduced pressure to a proper amount. The precipitation was removed. The solution was separated by macroporous resin, wherein it was firstly eluted by water to colorless, then by 10% ethanol with an amount of two times of column volume, finally by 60% ethanol with an amount of three times of column volume. The 60% ethanol eluent was collected and concentrated under reduced pressure to a proper amount. After the concentrated solution was spray dried, it was dissolved in water, then the obtained solution was stood for 1 h, and the precipitation is filtered. The filtrate was placed in an evaporating dish, into which gelatin solution was sprayed slowly. After standing for 1 h, the precipitation was collected, and taken out after lyophilization for 12 h. The precipitation was then ground into fine powders, and resolved with 90% acetone for 2 h. The obtained solution was fully distilled at 45 °C under reduced pressure to recover acetone, and the concentrate was poured out and freeze dried for 12 h. Thus highly purified TSR with the tannins content of 98.5% could be obtained.

### *2.2 Reagents*

Hemolysin used in this experiment was purchased from Sysmex Shanghai Ltd. and propidium iodide (PI) from Sigma-ldrich, Inc. (China). Cyclophosphamide (batch No. 1210221) from Jiangsu Hengrui Medicine Company (China). *Sanguisorbae Radix* was from Sichuan Neautus Traditional Chinese Medicine Co., Ltd. (batch No. 130801, China)

Antimouse CD34 monoclonal antibodies were purchased from Biolengend Inc. (batch No. R-1209S, USA) and Trizol from Invitrogen Co. (batch No. D0026, China). cDNA reverse transcriptase kit was from Bestbio Co. (batch No. K1622, China) and quantitative real-time PCR kit from Genecopoeia Inc. (batch No. T03S12, USA).

### *2.3 Animal grouping and treatment*

Forty Balb/c mice (specific pathogen free, ♀/♂, 20–25 g) were purchased from Laboratory Animal Center of Chengdu University of Traditional Chinese Medicine, certification No. SCXK(chuan)2004-11. The mice were used for the experiments after an acclimatization period of 7 d. Mice were given food and water throughout the experiments. The experimental protocol was approved by the Local Ethics Review Committee for Animal Experimentation of Chengdu University of Traditional Chinese Medicine.

After 3 d of acclimatization, mice were randomly divided into four groups such as normal (NR), model (MD), G-CSF, and TSR groups, 10 in each group.

All the mice except NR group were ip injected with 200 mg/kg CTX by human and mice body surface area dose conversion on days 8, 9, and 10 during the experiment. The mice in NR and MD groups were given 20 mL/kg distilled water, while the mice in G-CSF group were sc injected with 30 μg/kg G-CSF from day 7 to the end of the experiment, and the mice in TSR group were ig administered with 20 mg/kg of TSR aqoeous solution during the experiment.

On day 11, the blood from each animal was collected, placed into a lavender top collection tube containing EDTA,

and kept at ambient temperature. Then all the animals were sacrificed by anesthesia and their thigh bones were taken out under sterile conditions.

### *2.4 Hemocyte count*

The blood samples were sent to LiLai Diagnostic Center (Chengdu, China) and WBC, RBC, and PLT were measured by a Bayer Advia 120 Hematology Analyzer at different time within 24 h after blood collection.

### *2.5 Colors of bone marrow*

Pictures of thigh bones were taken, and colors of bone marrow were compared among groups.

### *2.6 Hematopoietic stem/progenitor cells count*

Thigh bone marrow cavity was washed with saline to collect marrow cells with 200 wells filter. After the cell suspension was centrifuged at 2000 *g* for 10 min, and 4 mL of 70% ethanol was added into deposit for fixation after supernatant was removed, and then oscillated to make hematopoietic stem/progenitor cell (HSPC) suspension for next steps.

Mouse IgG FITC (20 μL) and CD45 PE (20 μL) were added into control tubes, while CD34 PE (20 μL) and CD45 FITC (20 μL) were added into other group tubes. And then 50 μL HSPC suspension was added into each tube, incubated for 15 min, and later mixed together and protected from light. After that, 2 mL hemolysin was added into each tube and incubated for another 10 min after mixed together. Tubes were centrifuged at 3000 *g* for 5 min, the supernatant was removed, and 1 mL of 1% PFA was added into tubes for HSPC count by FCM.

### *2.7 HSPC cycle analysis*

HSPC suspension (2 mL) was washed with PBS twice, and then PI dye was added for cell cycles detection. The percentage of cell phases was tested by FCM.

### *2.8 Quantitative RT-PCR analysis*

Total RNA of HSPC was isolated using Trizol, and the quantity and integrity were examined using NanoDrop ND−1000 Spectrophotometer and Agilent BioAnalyzer 2100, respectively. Total RNA was reversely transcribed using cDNA by random hexamer primers and the High Capacity cDNA Reverse Transcription Kit according to the protocol of manufacturers. Primers were designed using Primer 3.0 software with the maximum self-complementarity score set at 5 and the maximum 3 self-complementarity score set to 0 to minimize the primer dimer formation (Table 1). Quantitative real time QRT-PCR was done by using SYBR Green. Samples were run in triplicate using standard conditions on an ABI 7500 Sequence Detector and the data were analyzed by using β-actin as an endogenous control. Dissociation curves were done after each experiment to confirm the specificity of product amplification.

**Table 1 Primer sequences and products length**

| Genes | Primer sequences (5'-3') | Product lengths /bp |
|---|---|---|
| MGMT | F:TTTCGACGTTCGTAGGTTTAG<br>R:GAATCCACGAGCAGAGCAACG | 127 |
| β-actin | F:TGGACACTACCGTCTCTCGTG<br>R:CGTGCAGAGTTGAGTCAGATG | 96 |

### *2.9 Western blotting analysis*

Preparation of HSPC protein and Western blotting were performed as described previously. Protein concentration was determined by Bradford protein assay (Bio-Rad, USA) with BSA used as the standard. The lysates (20 μg) were solubilized in Laemmli's sample buffer by boiling and then subjected to 10% SDS-PAGE followed by electro-transfer onto a nitrocellulose filter. Anti-MGMT monoclonal antibody was purchased from NeoMarkers (USA). Peroxidase conjugated anti-mouse IgG was used in the secondary reaction. The immune complex was visualized with an ECL Western blotting detection system (Amersham Pharmacia Biotech). The quality and amount of protein samples applied on the gel were confirmed by detection with anti-α-tubulin antibody. The bands were quantified by autoradiographic signal intensities of the bands on Western blotting by densitometric scanning, normalized by internal control (α-tubulin).

### *2.10 Statistical analysis*

All data were analyzed using Solutions Statistical Package for the Social Sciences 13.0. Measurement data were compared with One-way analysis of variance, multiple groups means were compared with single factor analysis of variance, and the comparison among groups was performed with LSD method.

## 3. Results

### *3.1 Effects of TSR on hemocyte count*

As shown in Table 2, compared with NR group, the MD group had a significant decline in WBC ($P < 0.05$); Compared with MD group, the TSR group had a significant rise in WBC ($P < 0.05$). Table 2 also indicates the number of RBC in

**Table 2 Effect of TSR on hemocyte count in myelosuppression mice ($\bar{x} \pm s$, $n = 10$)**

| Groups | WBC count / ($\times 10^9 \cdot L^{-1}$) | RBC count / ($\times 10^9 \cdot L^{-1}$) | PLT count / ($\times 10^9 \cdot L^{-1}$) |
|---|---|---|---|
| NR | $6.72 \pm 0.55^{*}$ | $32.57 \pm 4.60^{*}$ | $523.09 \pm 67.86^{*}$ |
| MD | $1.13 \pm 0.15^{\triangle}$ | $12.28 \pm 2.09^{\triangle}$ | $203.22 \pm 39.88^{\triangle}$ |
| G-CSF | $7.04 \pm 0.53^{*}$ | $30.71 \pm 2.99^{*}$ | $457.18 \pm 45.34^{*}$ |
| TSR | $5.97 \pm 0.36^{*}$ | $29.10 \pm 3.25^{*}$ | $412.54 \pm 39.80^{*}$ |

$^{\triangle}P < 0.05$ $^{\triangle\triangle}P < 0.01$ *vs* NR group; $^{*}P < 0.05$ $^{**}P < 0.01$ *vs* MD group, same as below

the MD group decreased significantly ($P < 0.05$ *vs* NR group), while it was significantly raised after TSR treatment ($P < 0.05$). The decline of PLT induced by CTX also was significantly reversed by TSR ($P < 0.05$ *vs* MD group).

### 3.2 Effects of TSR on color of bone marrow

Bone marrow of normal mouse is red in color, while it changed to be white after the nistration of CTX. After TSR treatment, the color of bone marrow clearly recovered to be red (Figure 1).

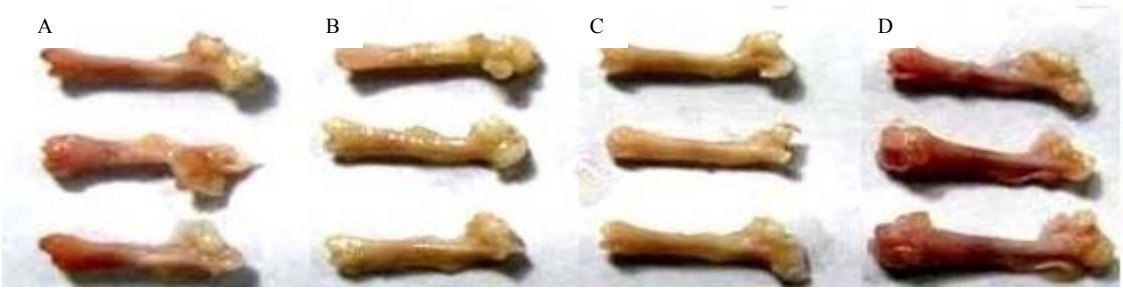


**Figure 1 Effects of TSR on thigh bone color of myelosuppression mice**

### 3.3 Effects of TSR on HSPC count

As shown in Table 3, compared with NR group, the bone marrow HSPC in MD group was significantly decreased ($P < 0.01$); Compared with MD group, and bone marrow HSPCs were significantly increased in TSR group ($P < 0.01$). HSPC count detection pictures are shown in Figure 2.

**Table 3 Effects of TSR on count of HSPC in myelosuppression mice ($\bar{x} \pm s$, $n = 10$)**

| Groups | HSC count /× ($10^6$·mL$^{-1}$) |
|---|---|
| NR | $4.73 \pm 0.75^{**}$ |
| MD | $1.08 \pm 0.13^{\triangle\triangle}$ |
| G-CSF | $3.15 \pm 0.34^{*}$ |
| TSR | $3.67 \pm 0.28^{**}$ |

### 3.4 Effects of TSR on HSPC cycle

As shown in Table 4, compared with NR group, the bone marrow HSPC in $G_1$ phase was significantly increased ($P < 0.05$), and those in S and $G_2$ phases were significantly decreased ($P < 0.05$). Compared with MD group, FCM analysis showed that the cells in $G_1$ phase were significantly decreased in the TSR groups ($P < 0.01$), however, increased in

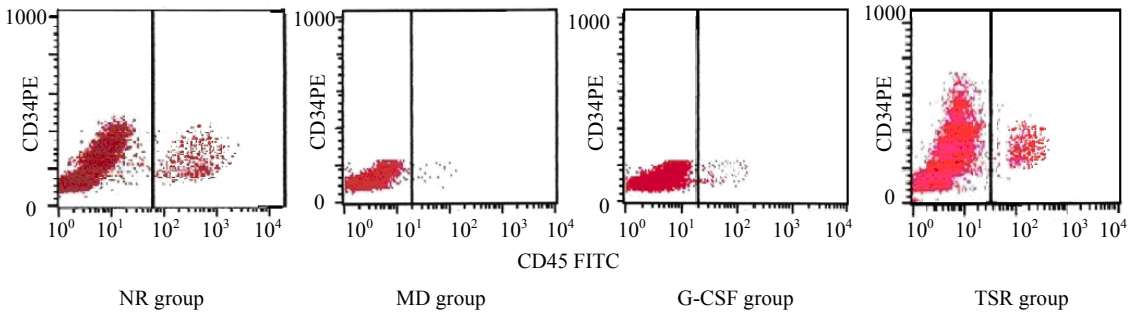


**Figure 2 HSPC count in each group by flow cytometry detection**

**Table 4 Effects of TSR on cycle of HSPC in myelosuppression mice ($\bar{x} \pm s$, $n = 10$)**

| Groups | $G_1$ / % | S / % | $G_2$ / % |
|---|---|---|---|
| NR | $54.58 \pm 4.23^{*}$ | $29.25 \pm 3.23^{*}$ | $9.20 \pm 1.28^{**}$ |
| MD | $66.78 \pm 6.11^{\triangle}$ | $20.36 \pm 2.18^{\triangle}$ | $4.23 \pm 0.81^{\triangle\triangle}$ |
| G-CSF | $55.23 \pm 4.39^{*}$ | $27.67 \pm 2.76^{*}$ | $9.38 \pm 0.96^{**}$ |
| TSR | $51.44 \pm 3.87^{**}$ | $30.82 \pm 3.51^{**}$ | $8.47 \pm 0.75^{**}$ |

S and $G_2$ phases with statistically significant difference ($P < 0.01$). HSPC cycle detection pictures are shown in Figure 3.

### 3.5 Effects of TSR on mRNA expression of MGMT

To investigate the influence of TSR on MGMT, we observed the effect on reducing mRNA expression of MGMT ($P < 0.01$ *vs* NR group) after CTX administration by QRT-PCR, while the significant increase in MGMT mRNA levels ($P < 0.01$ *vs* MD group) could be detected in the HSCs of bone marrow undergoing TSR treatment (Figure 4).

### 3.6 Effects of TSR on protein expression of MGMT

To further confirm the result of QRT-PCR detection, the protein levels of MGMT in the hematopoietic stem cells were also measured by Western blotting. As depicted in Figure 5, TSR treatment markedly antagonized the reduction in MGMT protein levels ($P < 0.01$ *vs* MD group) induced by CTX administration.

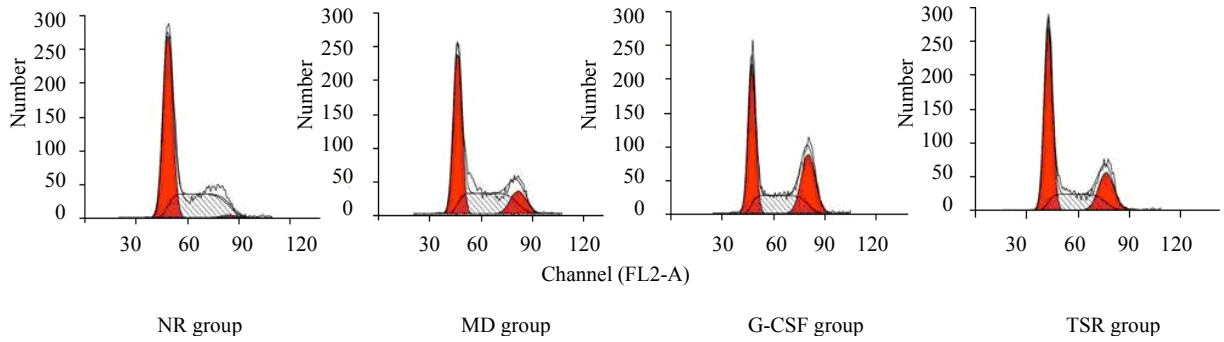


**Figure 3 HSPC cycle analysis in each group by flow cytometry detection**

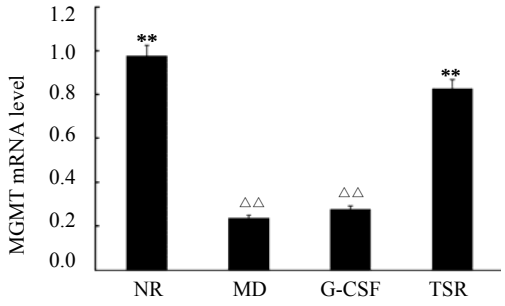


**Figure 4 MGMT mRNA levels in each group**

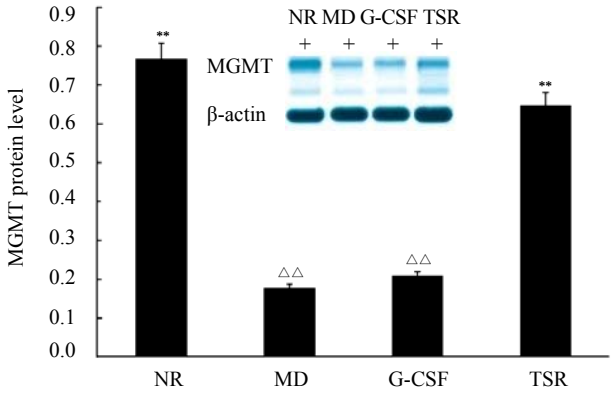


**Figure 5 MGMT protein level in each group**

## 4. Discussion

Most antineoplastic drugs have diverse side-effects on the hematopoietic system. Certain drugs are directly cytotoxic to myeloid stem cells or their progenitors whereas others affect hematopoiesis indirectly (Kaina et al. 2007). In most instances, drugs with a direct effect cause the reductions in circulating blood cells and in bone marrow cellularity, and at very high doses they may irreversibly decrease the proliferative potential of hematopoietic stem cells (Kaufmann et al, 1996). Experimental evidence indicates that such permanent stem cell damage may lead to the failure of the hemopoietic bone marrow to produce the sufficient numbers of blood cells. Myelosuppression is the most common and formidable side-effects of cytotoxic chemotherapy.

In the present study, the CTX was used to induce myelosuppression (Khuri, 2007). Clinical hematopoietic parameters such as WBC, RBC, and PLT were routinely used to accurately monitor the chemotherapeutic adverse effects (Milsom and Williams, 2007). In our study, TSR administration induced a marked increase in WBC, RBC, and PLT, which might facilitate the recovery of pancytopenia.

At the same time, our study discovered that the color of bone marrow turned to be white after CTX administration, which indicated a sharp injury in bone marrow or its microenvironment. After the treatment of TSR, the color of bone marrow clearly recovered to be red.

To further verify the above phenomenon, HSPC count assay was performed to evaluate the proliferation of hemopoietic progenitor, which is regarded as the premise of peripheral blood cell recovery after bone marrow suppression. CD34 is a molecular marker; Phosphorylation of CD34 glycoprotein mainly expresses in the early primary HSPCs, vascular endothelial cells, and embryonic fibroblast cells. In hematopoietic tissues, CD34 expression is highest in early hematopoietic progenitor cells, with cell differentiation and maturation. Therefore, the detection of bone marrow CD34 cells may reflect the number of bone marrow hematopoietic stem cells. The pattern of CD34 expression suggests that it plays a significant role in early hematopoiesis. So it is chosen as a surface marker for very early hematopoietic stem cells in this paper (Nemunaitis et al, 1991; Raposo et al, 2006).

Our studies indicated that the number of HSPC in the bone marrow could be significantly increased by TSR.

The overwhelming majority cells in the bone marrow after receiving chemotherapy may stay in $G_1$ phase and can not pass $G_1$/S check point (Sabharwal and Middleton, 2006). Therefore, it is a hot area for the antitumor treatment by driving cells from $G_1$ phase into S phase and then entering $G_2$/M phase.

Our studies found that TSR could promote normal and myelosuppression cells into proliferation cycles. And also they could increase the rate of S + M/$G_2$ cells, while decrease $G_0$/$G_1$ phase cells and promote the cell proliferation. From the results we concluded that TSR might take the effect by promoting cells in $G_2$/M phase into $G_1$ phase, and it could promote the production of DNA photolyase and repair the damaged DNA after chemotherapy damage.

MGMT is a suicide enzyme that repairs the pre-mutagenic, pre-carcinogenic and pre-toxic DNA damage O(6)-methylguanine (Sun and Peng, 2008). It is therefore a key node in the defense against commonly found carcinogens, and a marker of resistance of normal and cancer cells exposed to alkylating therapeutics. It also likely protects against therapy-related tumor formation caused by these highly mutagenic drugs (Steidl et al, 2002). Since the amount of MGMT determines the repair level of toxic DNA alkylation adducts, MGMT may be an effective agent to reduce the harmful effects of chemotherapy.

Previous studies report that chemoselection of MGMT variants resistant to pseudosubstrates of MGMT administered in association with alkylating agents may decrease myelotoxicity (Sun and Peng, 2008; Ng et al, 2004). Consistent with previous reports, our studies showed that TSR could significantly promote the gene and protein expression of MGMT in myelosuppression mice.

In conclusion, our study demonstrated for the first time that TSR can effectively protect and treat CTX-induced myelosuppression. The findings may be of great importance for the development of TSR as a therapeutic agent for the prevention and treatment of human myelosuppression.

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