

# Leukemia growth is inhibited by benzoxime without causing any harmful effect in rats bearing RBL-1 xenotransplants

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**Abstract.** The present study aimed to investigate the effect of benzoxime on leukemia RBL-1 cell proliferation and a leukemic Sprague-Dawley rat model. Proliferation of RBL-1 cells was determined using an MTT assay. Sprague-Dawley rats were assigned randomly into three groups of 10 animals each, where the positive control group was administered an intravenous injection of normal saline, the negative control group was administered  $1 \times 10^6$  RBL-1 cells and the treatment group was administered with  $1 \times 10^6$  RBL-1 cells and then benzoxime (50 mg/kg/day) for 1 week. Increased dosage of benzoxime reduced RBL-1 cell viability from 92 at  $2 \mu\text{M}$  to 21% at  $12 \mu\text{M}$  after 24 h. Benzoxime treatment prevented the loss of body weight in the rats with leukemia. Compared with the negative control rats, the body weight was determined to be significantly reduced ( $P < 0.05$ ) in the positive control rats. The weight of the spleen and liver was determined to be significantly increased ( $P < 0.02$ ) in the positive control rats and the benzoxime-treated rats compared with that in the negative control group on day 35 of RBL-1 cell implantation. Analysis of leukocytes in rats on day 35 demonstrated a significant reduction ( $P < 0.05$ ) in the cluster of differentiation (CD)11b and CD45 level in the positive control group compared with that in the negative control group. The level of CD11b and CD45 was determined to be similar in the rats in the benzoxime treatment and negative control groups. Analysis of the level of serum glutamic pyruvic transaminase, serum glutamic oxaloacetic transaminase and blood urea nitrogen indicated that all three components exhibited no significant changes in the rats following treatment with benzoxime compared with the component levels in the negative control group. The levels of these three components were in the normal range in rats treated with benzoxime on day 35 of cell implantation. These data demonstrated that the liver and kidneys are not influenced

by benzoxime in rats with leukemia. In summary, the present study demonstrated that benzoxime efficiently prevents leukemia growth without inducing any harmful effects in rat models through targeting CD11b and CD45 level; thus, benzoxime should be evaluated further regarding its use in the treatment of leukemia.

## Introduction

Leukemia is one of the malignant clonal disease types involved with hematopoietic stem cells. In 2015 the incidence rate of leukemia was reported to be 5.68 cases/100,000 individuals in China (1). Leukemia is the leading cause of cancer-associated mortalities globally in children and adults <35 years old, and various studies have been conducted in order to understand its mechanisms (1-4). Leukemia is characterized by the upregulation of cell proliferation and its failure to undergo differentiation into hematopoietic cells (5-7). The treatment strategy for leukemia consists of transplantation of bone marrow, and chemo- and radiotherapy (8-10). Despite these available treatment strategies, leukemia continues to be the leading cause of mortality globally; therefore, clinicians and researchers require novel drug candidates in order to treat leukemia efficiently. Natural compounds isolated from diverse sources act as therapeutic candidates for the treatment and prevention of various disorders including cancer and arthritis (11-14). Natural products have been determined to act as neuroprotective, antioxidant (15), anti-inflammatory (16) and anti-apoptotic agents (17), as well as reducing autophagy (18). Sanguinarine is located in the plant *Sanguinaria canadensis*. Sanguinarine is a member of the alkaloid family and has been determined to act as a potential agent against inflammation, tumor growth and hypertension (19,20).

The present study aimed to investigate the effect of benzoxime (Fig. 1) on RBL-1 leukemia cell proliferation and on leukemia Sprague-Dawley rat models. The results demonstrated that benzoxime treatment reduced RBL-1 leukemia cell proliferation *in vitro* and prevented damage to the spleen and liver, and changes in the biochemical profile of blood *in vivo*.

## Materials and methods

**Cell culture.** The leukemia RBL-1 cell line was supplied by the Chinese Academy of Sciences (Shanghai, China). Cell culture was performed in 75-cm<sup>2</sup> tissue culture flasks,

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which contained RPMI-1640 medium (Gibco; Thermo Fisher Scientific, Inc., Waltham, MA, USA). L-glutamine (2 mM) and 10% fetal bovine serum were added to the medium (Gibco; Thermo Fisher Scientific, Inc.). The medium also contained 1% penicillin-streptomycin (100 U/ml penicillin and 100  $\mu$ g/ml streptomycin). The cells were incubated at 37°C in a humidified atmosphere of 5% CO<sub>2</sub>.

**Analysis of cell viability.** The effect of benzoxime on leukemia RBL-1 cell viability was analyzed with an MTT assay. RBL-1 cells were seeded onto 96-well cell culture plates at a density of  $2 \times 10^4$  cells/well and cultured for 24 h. Benzoxime dissolved in dimethyl sulfoxide (DMSO) at 2–14  $\mu$ M doses was added to the RPMI-1640 medium and incubation was conducted for 24 h. The conditions for incubation used were a temperature of 37°C in an atmosphere containing 5% CO<sub>2</sub>. Following incubation, the cells were washed twice with PBS and subsequently exposed to 0.5 mg/ml MTT. Incubation of the cells was continued for 4 h at 37°C and then the culture medium was removed. DMSO was added to the plates for solubilization of the formazan crystals. Measurement of the absorbance values for each plate was performed in triplicate independently at 485 nm. The microplate autoreader (BioTek Instruments Inc., Winooski, VT, USA) was used for recording absorbance.

**Handling of animals.** The male Sprague-Dawley rats (8-week old; weight, ~200 g; n=30) were purchased from the Guangzhou University's Laboratory Animal Center for Traditional Chinese Medicine [license no. scxk-129(Yue)2014-0129; Guangzhou, China]. The animals were accommodated under 12-h light and dark cycles in an animal house under conditions of controlled humidity and a temperature of 20°C. The rats had free access to the fresh drinking water and standard laboratory diet *ad libitum*. The working protocols involving animals were approved by the Committee for Care and Use of Animal of Guangzhou University of Traditional Chinese Medicine (approval no. 2014A123).

**Leukemia rat model development.** The 30 Sprague-Dawley rats were randomly assigned into three groups of 10 animals each. To induce malignancy,  $1 \times 10^6$  RBL-1 cells in 200  $\mu$ l sterile RPMI-1640 medium were inoculated subcutaneously into the postauricular region of the animals (18). The treatment group was inoculated with  $1 \times 10^6$  RBL-1 cells subcutaneously and then treated with benzoxime (50 mg/kg/day) for 1 week through an intravenous tail injection. The positive control group was administered with an intravenous injection of normal saline alone (100  $\mu$ l). The animals in negative control group were given  $1 \times 10^6$  RBL-1 cells subcutaneously followed by administration of 100  $\mu$ l normal saline alone. During the study, the rat body weight was recorded every week. The animals were sacrificed on day 35 of the study using established CO<sub>2</sub> euthanasia method where the flow rate of CO<sub>2</sub> displaced >30% of the chamber volume/minute, in order to extract the liver and spleen, and collect the blood samples. The liver and spleen of each animal was weighed as previously reported (19,20). Tumor diameter was measured using calipers, and the tumor volume was calculated. The tumors were measured in 2 dimensions and tumor volume was calculated according to the formula  $V = (D \times d^2)/2$ , in which D and d are the major and minor perpendicular tumor diameters, respectively.

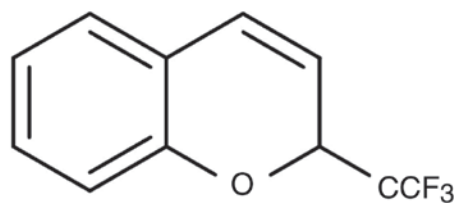


Figure 1. Chemical structure of benzoxime.

**Immunofluorescence staining.** The blood samples (~600  $\mu$ l) from the rats were collected and then treated with lysing buffer (Pharm Lyse; BD Biosciences, San Jose, CA, USA). Following lysis of the blood cells, the samples were subjected to centrifugation for 10 min at 4°C at 1,500 rpm to isolate the leukocytes. The leukocytes were cultured on glass coverslips and fixed in 4% paraformaldehyde for 15 min at room temperature. Slips were washed in PBS three times for 30 min at room temperature and incubated with 0.1% Triton X-100 for 30 min at room temperature. Following washing, the slips were blocked in goat serum (10%; Thermo Fisher Scientific, Inc.) for 20 min at room temperature. The cells were then incubated with anti-CD3 (cat. no. SAB4700040; dilution 1:200), anti-CD19 (cat. no. SAB5500047; dilution 1:200) and anti-CD11b (cat. no. SAB4700386; dilution 1:200; all from Sigma-Aldrich; Merck KGaA, Darsmttdt, Germany) antibodies at 4°C overnight. Subsequently, the cells were washed for 15 min twice with PBS at room temperature and incubated with polyclonal peroxidase-conjugated goat anti-rabbit antibody (cat. no. ZDR-5306; dilution 1:200, ZSGB-BIO) at room temperature for 1 h. The cells were observed under a fluorescence microscope (BX53; Olympus) at x250 magnification. Flow cytometry was used for the analysis of surface markers using the previously reported procedures (21–23).

**Determination of biochemical profiles.** The level of various components, including serum glutamate pyruvate transaminase (sGPT), serum glutamate oxaloacetic transaminase (sGOT) and blood urea nitrogen (BUN), in the rat blood serum samples was determined using the previously described procedures (24,25).

**Statistical analysis.** The presented data are the mean  $\pm$  standard error of the mean of three experiments performed independently. The data were analyzed using one-way analysis of variance followed by Student-Newman-Keuls test for multiple comparisons. All statistical analyses were performed using SPSS 17.0 software package (SPSS, Inc., Chicago, IL, USA).  $P < 0.05$  was considered to indicate a statistically significant difference.

## Results

**Benzoxime has an inhibitory effect on RBL-1 cell viability.** RBL-1 cells were exposed for 24 h to a range of benzoxime doses from 2–14  $\mu$ M and the effect on viability of the cells was examined using an MTT assay. It was observed that an increase in the dosage of benzoxime from 2 to 12  $\mu$ M reduced RBL-1 cell viability from 92 to 21%. Further increase in benzoxime concentration did not significantly decrease the viability inhibition, compared with 12  $\mu$ M benzoxime. The

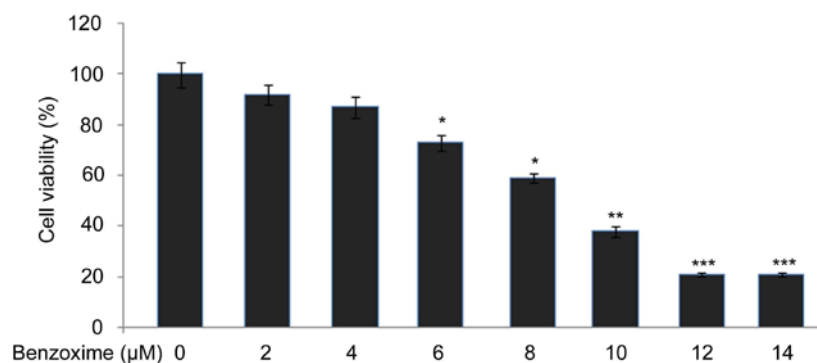


Figure 2. Benzoxime reduces RBL-1 cell viability. Treatment of RBL-1 cells for 24 h with various concentrations (2, 4, 6, 8, 10, 12 and 14  $\mu$ M) of benzoxime was followed by an MTT assay. The data are presented as the mean  $\pm$  standard deviation of three independent experiments. \* $P<0.05$ , \*\* $P<0.02$ , \*\*\* $P<0.01$  vs. 0  $\mu$ M benzoxime.

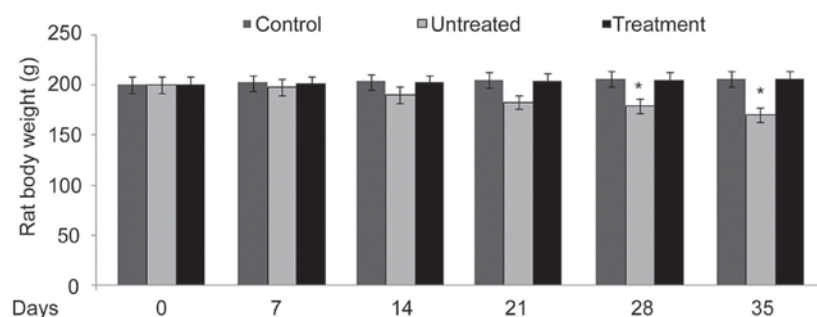


Figure 3. Benzoxime treatment of RBL-1 cell rat allograft model prevents loss of body weight. The body weight presented for each group is the mean  $\pm$  standard deviation of all the rats in the group. The weights presented are between the day of implantation and day 35 after implantation of the tumor cells. \* $P<0.05$  vs. negative control and benzoxime treatment group.

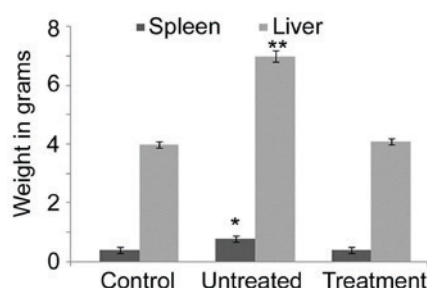


Figure 4. Benzoxime treatment of RBL-1 cell rat allograft model prevents the increase in spleen and liver weight. The expressed data are presented as the mean  $\pm$  standard error of the mean of the weight of spleens and livers of all the rats in the group on day 35 following implantation of the tumor cells. \* $P<0.05$  and \*\* $P<0.02$  vs. negative control group.

viability of the RBL-1 cells following treatment with 14  $\mu$ M benzoxime was determined to be 24% after 24 h (Fig. 2).

*Development of leukemia in Sprague-Dawley rats is inhibited following treatment with benzoxime.* In benzoxime-treated rats, body weight was determined to be similar to that of the rats in the negative control group. Compared with the negative control group rats, the positive control group presented with significantly ( $P<0.05$ ) reduced body weight (Fig. 3). The weights of the spleen and liver were determined to be significantly increased in the positive control rats compared with those in the negative control and benzoxime-treated groups, after 35 days (Fig. 4). The liver and spleen were also determined

to be enlarged in the positive control rats compared with those in the negative control and benzoxime-treated groups, after 35 days (Fig. 5). The average tumor size in the liver of the positive control group was 540 mm<sup>3</sup>, while no tumor growth was observed in the rats of the negative control and treatment groups. In the spleen of the positive control group, the tumor size was determined to be 435 mm<sup>3</sup>, but no tumor was present in the rats of the negative control and treatment groups (Fig. 5).

*Blood cell surface markers in rats with leukemia are affected by benzoxime.* Analysis of leukocytes from positive control rats after 35 days demonstrated a significant increase ( $P<0.05$ ) in CD11b and CD45 levels compared with those in negative control rats. The level of leukocyte surface markers CD11b and CD45 was determined to be similar in the rats of the benzoxime treatment and negative control groups (Fig. 6).

*Benzoxime treatment prevents alteration in hematological, renal and hepatic parameters in rats with leukemia.* Determination of general body weight, and weight of spleen and liver demonstrated no significant changes between rats of the benzoxime treatment and negative control groups. Additionally, analysis of the level of sGPT, sGOT and BUN indicated that all the three components had no significant changes between the rats of the benzoxime treatment and negative control groups. At 35 days, the levels of these three components in rats treated with benzoxime were close to those in the control animals (Fig. 7). These data demonstrated that

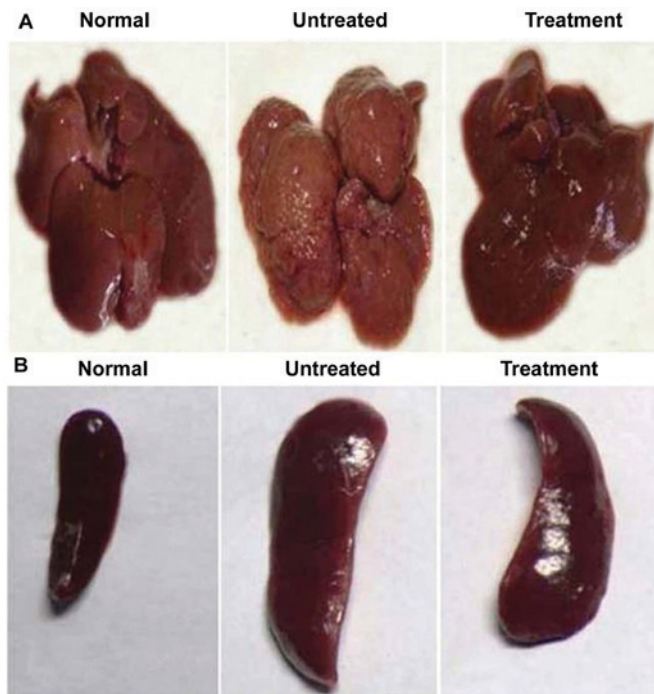


Figure 5. Benzoxime treatment maintains the size of (A) liver and (B) spleen in RBL-1 cell rat allograft models. The livers and spleens of all the rats were extracted on day 35 after implantation of the tumor cells. Images were captured at a magnification of x200.

the liver and kidneys are not influenced by benzoxime in rats with leukemia.

## Discussion

The present study aimed to investigate the effect of benzoxime on leukemia RBL-1 cell viability *in vitro* and in RBL-1 cell rat leukemia allograft models *in vivo*. Upregulation of proliferation and failure to undergo differentiation into hematopoietic cells comprise the characteristic features of leukemia (5-7); therefore, suppression of leukemia cell proliferation is considered to be of notable importance for its treatment. The present study demonstrated that the synthetic compound benzoxime has the potential to inhibit the proliferation of leukemia RBL-1 cells. Benzoxime inhibited the proliferation of RBL-1 cells in a dose-dependent manner without inducing any harmful effects *in vivo*. These data indicated that benzoxime should be evaluated for its potential as an anti-leukemia agent; thus, an *in vivo* leukemia rat model was established by transplantation of leukemia RBL-1 cells into Sprague-Dawley rats using the previously reported procedures (22,23). Anti-leukemic studies for the evaluation of various molecules are generally performed using murine allograft models, due to the quick and easy developmental procedures (26,27).

Numerous studies have evaluated the anti-leukemic potential of a number of chemotherapeutic agents such as 2-benzyloxybenzaldehyde, chloroquine and chrysin; however, leukemia continues to be a challenge for clinicians and researchers (22,27,28). The present study demonstrated that benzoxime has an anti-leukemia effect in leukemia RBL-1 cell rat models *in vivo*. Benzoxime treatment of the leukemic rat model resulted in the prevention of loss of body weight

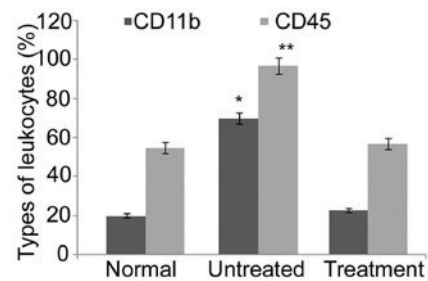


Figure 6. Benzoxime treatment of leukemia Sprague-Dawley rats reduces the expression of cell markers in leukocytes. On day 35 after tumor cell implantation, the blood samples of rats were collected and subjected to flow cytometry for analysis of CD11b and CD45 levels. Expressed data are presented as the mean  $\pm$  standard error of the mean of all the rats at day 35 after implantation of the tumor cells. \* $P < 0.05$  and \*\* $P < 0.02$  vs. negative control group. CD, cluster of differentiation.

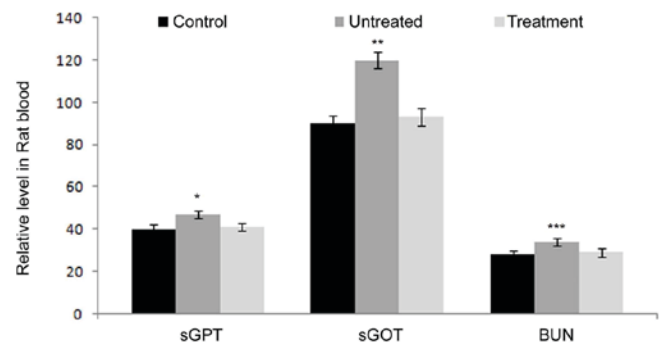


Figure 7. Effect of benzoxime on blood biochemical profile in RBL-1 cell rat leukemia allograft models. The blood samples were collected on day 35 after tumor cell implantation for examination of the biochemical profile. \* $P < 0.05$  and \*\* $P < 0.02$  vs. negative control and treatment group. sGPT (U/l), serum glutamic pyruvic transaminase; sGOT (U/l), serum glutamic oxaloacetic transaminase; BUN (mg/dl), blood urea nitrogen.

compared with the positive control group. The body weight in the positive control rats was significantly reduced compared with the benzoxime treatment and negative control rats. The weight of the liver and spleen was significantly increased in the positive control rats compared with that in the negative control group. It was determined that the level of monocyte surface marker CD11b and CD45 in the positive control rats was significantly increased compared with that in the negative control group; however, a significant increase in the level of CD11b was prevented by the treatment of leukemia rats with benzoxime.

In conclusion, the present study demonstrated that benzoxime reduces leukemia RBL-1 cell proliferation *in vitro* without causing any harmful effects *in vivo*. It also prevented damage to the spleen and liver, and changes in sGPT, sGOT and BUN. Thus, the present study demonstrated that benzoxime acts as a potential candidate for the treatment of leukemia. However, further experiments need to be performed to confirm these results.

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## Availability of data and materials

The datasets used and/or analyzed during the current study are available from the corresponding author on reasonable request.

## Authors' contributions

YL designed the study and wrote the paper. HW, RZ and GZ conducted the experiments. YY and ZL performed the literature study and compiled the data. All the authors wrote and approved the article for publication.

## Ethics approval and consent to participate

The working protocols involving animals were approved by the Committee for Care and Use of Animal of Guangzhou University of Traditional Chinese Medicine (approval no. 2014A123).

## Patient consent for publication

Not applicable.

## Competing interests

The authors declare that they have no conflict of interest.

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