Journal of Zhejiang University SCIENCE B
ISSN 1673-1581 (Print); ISSN 1862-1783 (Online)
www.zju.edu.cn/jzus; www.springerlink.com
E-mail: jzus@zju.edu.cn

A novel thioredoxin reductase inhibitor inhibits cell growth and induces apoptosis in HL-60 and K562 cells*

Zuo-fu PENG[1], Lin-xiang LAN[1], Fang ZHAO[1], Jing LI[1], Qiang TAN[2], Han-wei YIN[3], Hui-hui ZENG[†‡1]

([1]*Department of Chem-Biology, School of Pharmaceutical Sciences, Peking University, Beijing 100083, China*)

([2]*Department of Geriatric Medicine, Peking University First Hospital, Beijing 100034, China*)

([3]*College of Life Sciences, Peking University, Beijing 100075, China*)

[†]E-mail: zenghh@bjmu.edu.cn

Received Nov. 30, 2007; revision accepted Dec. 5, 2007

Abstract: Human thioredoxin reductase (TrxR) system is associated with cancer cell growth and anti-apoptosis process. Effects of 1,2-[bis(1,2-benzisoselenazolone-3(2H)-ketone)]ethane (BBSKE), a novel TrxR inhibitor, were investigated on human leukemia cell lines HL-60 and K562. BBSKE treatment induced cell growth inhibition and apoptosis in both cell lines. Apoptosis induced by BBSKE is through Bcl-2/Bax and caspase-3 pathways. Ehrlich's ascites carcinoma-bearing mice were used to investigate the anti-tumor effect of BBSKE in vivo. Tumor-bearing mice treated with BBSKE showed an increase of life span with a comparable effect to cyclophosphamide (CTX). These results suggest a potential usage of BBSKE as a therapeutic agent against non-solid tumors.

Key words: Thioredoxin reductase (TrxR), Novel TrxR inhibitor, 1,2-[bis(1,2-benzisoselenazolone-3(2H)-ketone)]ethane (BBSKE), Apoptosis

doi:10.1631/jzus.B071605 **Document code:** A **CLC number:** R73

INTRODUCTION

Human thioredoxin (Trx), which was initially termed as adult T-cell leukemia-derived factor (ADF) (Teshigawara *et al*., 1985), is over-expressed in many human primary tumors compared to the levels in the equivalent normal tissues, such as childhood T-cell acute lymphoblastic leukemia (Shao *et al*., 2001), non-small cell lung carcinoma (Soini *et al*., 2001), and melanoma cell lines (Barral *et al*., 2000). Moreover, expression level of Trx correlates well with the resistance of several T-cell leukemia cell lines to adriamycin (Wang *et al*., 1997) and is involved in the drug sensitivity of bladder and prostate cancer cells to cisplatin, mitomycin C, doxorubicin, and etoposide (Yokomizo *et al*., 1995).

Pathological and biological functions of Trx are accomplished mainly by its reduced form, with thioredoxin reductase (TrxR) serving as the primary catalyst for the NADPH-dependent reduction of the oxidized Trx (Holmgren, 1985). It has been reported that expression and activity of TrxR are also elevated in many tumors (Kahlos *et al*., 2001; Sasada *et al*., 1999; Bjorkhem-Bergman *et al*., 2002), and our previous data showed that there exist some relationships between TrxR inactivation and growth/proliferation inhibition, cell cycle arrest or apoptosis in the investigated tumor cell lines (Zhao *et al*., 2006).

In this report, we used a novel TrxR inhibitor, 1,2-[bis(1,2-benzisoselenazolone-3(2H)-ketone)]ethane (BBSKE), to further investigate the role of TrxR activity in non-solid tumor growth. BBSKE could inhibit cell growth and induce apoptosis in two human leukemia cell lines, HL-60 and K562 cells, through Bcl-2/Bax and caspase-3 pathways. Furthermore, BBSKE could significantly increase the life span of mice with Ehrlich's ascites carcinoma.

‡ Corresponding author

* Project (No. 30472036) supported by the National Natural Science Foundation of China

MATERIALS AND METHODS

Agents

BBSKE was synthesized and purified in our laboratory, stored as a 20 mmol/L solution in absolute DMSO at −20 °C, and diluted with the medium prior to use. The final concentration of DMSO in medium is maximum 0.25%.

Cell lines and culture conditions

Human leukemia cell lines HL-60 and K562 were purchased from Cell Center of Peking University Health Science Center, China and cultured at 37 °C in a humidified atmosphere of 5% CO_2 in RPMI-1640 medium (Gibco, Grand Island, NY, USA) supplemented with 10% heat-inactivated fetal bovine serum, 100 U/ml penicillin and 100 U/ml streptomycin.

Trypan blue exclusion test of cell viability

The effects of BBSKE on cell growth of HL-60 and K562 cells were estimated using the trypan blue exclusion test as described by Coligan *et al*.(1997). Briefly, cells were seeded in 6-well flat-bottom plates at the concentration of 1×10^5 cells/ml. After 24, 48 or 72 h of BBSKE treatment, trypan blue (400 μg/ml in HBSS or PBS, pH 7.2~7.4) was added to cells after washing twice with PBS. After 5 min, cells were rinsed twice with PBS and cell numbers were counted. Nuclei of non-viable cells should be stained as blue.

Flow cytometry assay

The percentage of apoptotic cells was tested by propidium iodide staining method. After BBSKE treatment about 10^6 cells were fixed in 75% pre-cooled ethanol overnight and stained with propidium iodide (50 μg/ml) in the presence of RNase (20 μg/ml) for 30 min at 37 °C. DNA content was analyzed using a Coulter EPICS-XL cytometer.

Reverse-transcriptase-polymerase chain reaction (RT-PCR)

Total RNA was extracted from cells using Trizol according to the supplier's instructions and reverse transcribed cDNA was analyzed for the expression of Bcl-2 and Bax. Sequences of the primers were as follows: Bcl-2, forward GAA CTG GGG GAG GAT TGT GG and reverse CCG GTT CAG GTA CTC AGT CA; Bax, forward CTG GAC AGT AAC ATG GAG CTG and reverse GGC GTC CCA AAG TAG GAG A.

Western blot

After drug treatment, total cellular proteins were extracted with lysis buffer containing 25 mmol/L Tris (pH 7.4), 137 mmol/L sodium chloride, 1% Triton X-100, 1% sodium deoxycholate, 0.1% SDS, 1 mmol/L PMSF, and 10 μg/ml leupeptin, aprotinin, and pepstatin. The protein concentration was determined by Bradford assay. Cellular protein (50 μg) was resolved by SDS-PAGE and electrophoretically transferred onto polyvinylidene difluoride membranes (Millipore, Bedford, MA, USA). The membranes were blocked with 5% skim milk in Tris-buffered saline (50 mmol/L Tris-HCl, pH 7.5), and 150 mmol/L NaCl containing 0.05% Tween-20, and then incubated with specific primary polyclonal/monoclonal antibodies including Bcl-2, Bcl-xL, cytochrome C, caspase-3 (Santa Cruz Biotechnology, USA). The primary antibody complex was then stained with horseradish peroxidase-conjugated secondary antibodies (Jackson ImmunoResearch Laboratories, USA). Protein bands were visually detected by enhanced chemiluminescence (ECL Western blotting detection system, Amersham Pharmacia Biotech., USA).

Ehrlich's ascites carcinoma (EAC)

Adult male ICR mice (10 to 12 weeks old) were purchased from the Central Animal House, Peking University Health Science Center, China. Ehrlich's ascites carcinoma (EAC) was propagated into wild type mouse by intra-peritoneal injection of 0.2 ml freshly drawn ascites fluid (diluted 1:5 in sterile saline) from a mice-bearing ascites tumor. Mouse mean body weight before EAC propagation is (20±2) g. Transplantation was carried out using a sterile disposable syringe under aseptic conditions. Forty mice were randomly divided into 5 groups, and on the next day after transplantation, mice were treated p.o. daily with or without BBSKE: (1) control group: 8 mice receiving only sterile saline; (2) cyclophosphamide (CTX) group: 8 mice receiving 60 mg/kg of CTX; (3) BBSKE-1 group: 8 mice receiving 72 mg/kg of BBSKE; (4) BBSKE-2 group: 8 mice receiving 36 mg/kg of BBSKE; and (5) BBSKE-3 group: 8 mice receiving 18 mg/kg of BBSKE.

RESULTS

BBSKE, a TrxR inhibitor, inhibiting growth of human leukemia cell lines HL-60 and K562 in vitro

Our previous data showed that BBSKE can inhibit growth of several human carcinoma cell lines. To investigate the effect of BBSKE on non-solid tumors, human leukemia cell lines HL-60 and K562 were utilized. By using trypan blue exclusion test, the live cell numbers were counted after treated with BBSKE for designed period of time. Fig.1 indicates that BBSKE had cytotoxic effect on both cell lines in a dose-dependent manner. The IC_{50} values at 24 h of BBSKE on HL-60 and K562 cells were 3.74 μmol/L and 4.01 μmol/L, respectively. These results demonstrate that BBSKE can inhibit the growth of HL-60 and K562 cells dose- and time-dependently.

BBSKE inducing apoptosis in HL-60 and K562 cells

To determine whether the growth inhibition effect of BBSKE on HL-60 and K562 cells was due to apoptosis, propidium iodide staining was performed on cells after BBSKE treatment and assayed for apoptosis by flow cytometer. After 24 h, apoptotic cells could be observed in BBSKE-treated cells (Fig.2). In each cell line the percentage of apoptosis increased dose-dependently. Thus BBSKE can induce apoptosis in HL-60 and K562 cells, which leads to the growth inhibition of both cell lines.

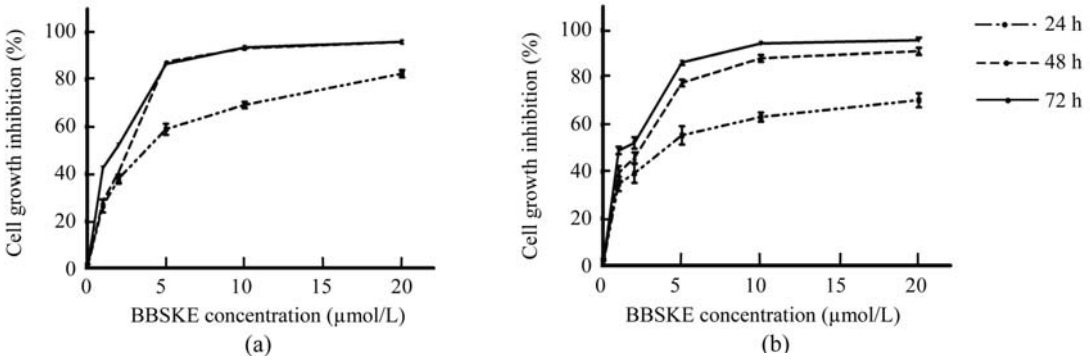

Fig.1 Cells cultured with different concentrations of BBSKE for 24, 48 or 72 h were analyzed by trypan blue exclusion test. (a) HL-60; (b) K562

Data were expressed as percentage of decreased cell numbers from untreated cells. Means±*SD* of at least three independent experiments were shown

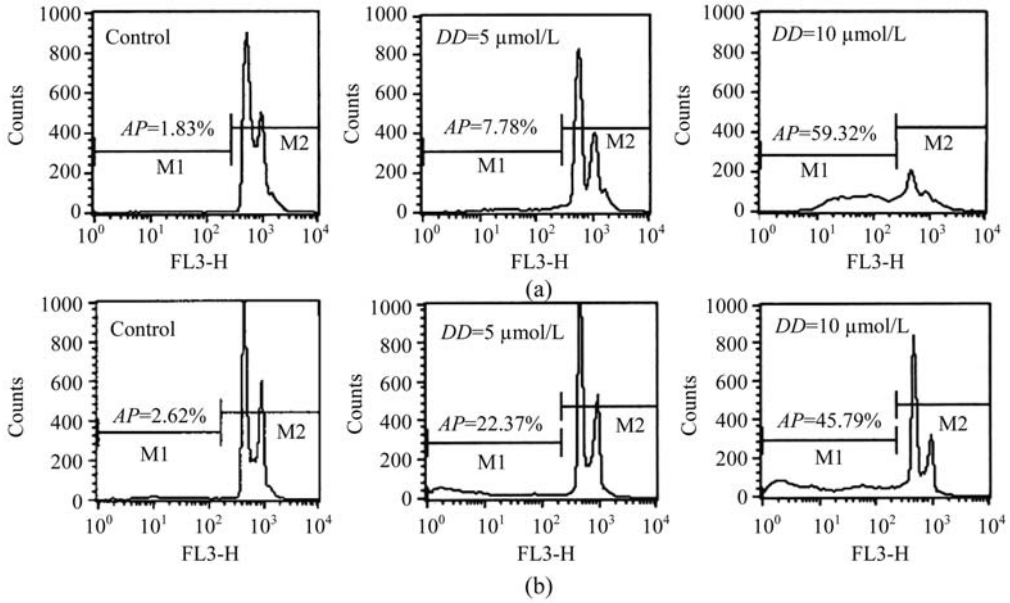

Fig.2 Effect of BBSKE on apoptosis. HL-60 (a) and K562 (b) cells treated with or without various dosages of BBSKE for 24 h were assayed by propidium iodide staining method

Drug dosages (DD) and apoptotic proportions (AP) were labeled in each figure

Bcl-2/Bax pathway playing a role in the apoptosis induced by BBSKE

To elucidate the possible mechanism of apoptosis induced by BBSKE, expressions of two Bcl-2 family members were tested. As shown in Figs.3 and 4, Bcl-2 expression was downregulated by BBSKE, with upregulation of Bax expression at both RNA and protein levels in each cell line.

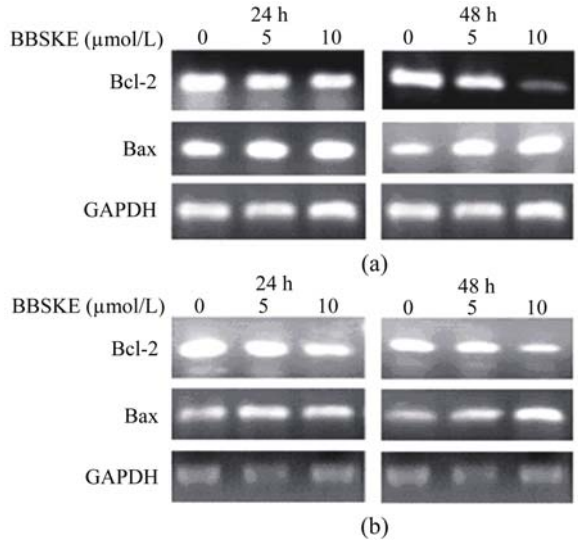

Fig.3 The mRNA levels of Bcl-2 and Bax in BBSKE-treated and -untreated HL-60 (a) and K562 (b) cells were analysed by RT-PCR. GAPDH expression was monitored for control

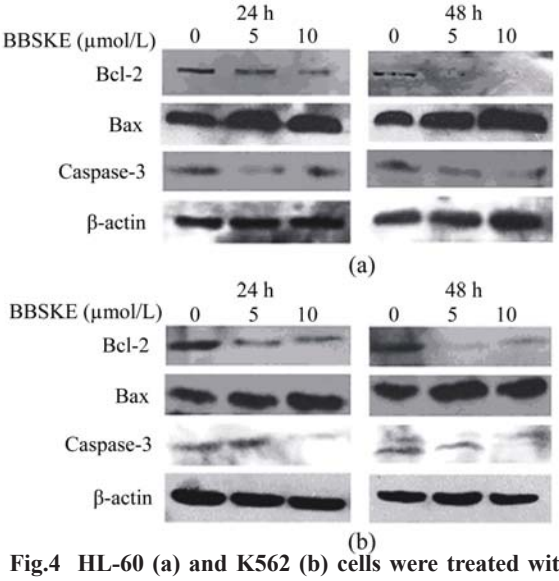

Fig.4 HL-60 (a) and K562 (b) cells were treated with the indicated concentrations of BBSKE for 24 or 48 h. Bcl-2, Bax and caspase-3 were assayed by Western blot. Equal amounts of total cellular protein (50 μg) were resolved on 15% SDS-PAGE and β-actin was used as a lane loading control

BBSKE increases the life span of mice with Ehrlich's ascites carcinoma

To test if BBSKE administration could inhibit non-solid tumor growth in vivo, EAC-bearing ICR mice received high or low dose of BBSKE after 24 h of intra-peritoneal tumor inoculation. The life spans of mice with or without treatment were listed in Table 1. High dose (72 mg/kg) of BBSKE treatment increased survival of EAC-bearing mice to 16.0 d. CTX was used as positive control. As shown in Table 1, the effect of high dose BBSKE is comparable to CTX.

Table 1 BBSKE expanded the lifespan of mice with Ehrlich's ascites carcinoma

Group	Dose (mg/kg)	Survival (d)	Increase of life span (%)
Control	0	12.1	0
CTX	60	16.5	36.4
BBSKE-1	72	16.0	32.2
BBSKE-2	36	14.6	21.0
BBSKE-3	18	14.2	17.4

Forty ICR mice were randomly divided into 5 groups (as indicated in the table). On the next day after transplantation, mice were treated p.o. daily with or without BBSKE. Control mice received only sterile saline and mice receiving cyclophosphamide (CTX) were used as positive control

DISCUSSION

As a cofactor, binding partner, protein reductant and cytokine-like factor, Trx performs many biological functions, such as supplying reducing equivalents to ribonucleotide reductases and peroxiredoxins (Chae *et al*., 1994; Laurent *et al*., 1964), the regulation of the activity of several transcription factors (Hirota *et al*., 1997; Makino *et al*., 1999), and the control of apoptosis signal-regulating kinase 1 (ASK-1) activity (Saitoh *et al*., 1998). Trx levels, balanced by TrxR modulation, are positive with cell proliferation and negative with apoptosis (Powis *et al*., 2000). Because of the multiple roles of Trx in tumorogenesis, Trx is regarded as a marker for prognosis (Raffel *et al*., 2003; Kakolyris *et al*., 2001) and several Trx inhibitors have been identified (Powis *et al*., 1998; Pallis *et al*., 2003). Furthermore, the function of Trx as a disulfide reductase in mammalian cells is generally dependent upon the activity of TrxR, so we have chosen TrxR as a target for chemotherapeutic drug design.

BBSKE is a novel TrxR inhibitor designed and synthesized by our group. Previous studies have shown that BBSKE can significantly inhibit TrxR activities in several human carcinoma cell lines and TrxR inactivation by BBSKE correlates with cell death/apoptosis in the investigated cell lines. In this work, we showed that BBSKE could also inhibit the growth of two human leukemia cell lines, HL-60 and K562, in dose- and time-depend manners (Fig.1).

Compared to the other human carcinoma cell lines that we have investigated (Zhao *et al*., 2006), HL-60 and K562 are the most sensitive cell lines to BBSKE treatment with IC_{50} values at 24 h of 3.74 μmol/L and 4.01 μmol/L, respectively. Another human histiocytic/monocytic leukemia cell line U-937 has been shown to have much higher TrxR expression compared to peripheral blood monocytes and lymphocytes (Söderberg *et al*., 2000). Whether the superior sensitivities of HL-60 and K562 cells to BBSKE relate to the constitutive cellular TrxR activity level is still under investigation.

Moreover, the mechanism of apoptosis induced by BBSKE in some human carcinoma cell lines has been investigated. BBKSE induces apoptosis with alterations in Bcl-2, Bax, and caspase-3 expressions in human cancer cell lines A549 (lung cancer), Bel-7402 (epithelial hepatoma), BGC823 (stomach adenocacinoma), HeLa (cervical cancer), KB (nasopharyngeal epidermal carcinoma), PC-3 (human prostatic cancer) and DU145 (prostate cancer) (Zhao *et al*., 2006; Shi *et al*., 2003). In A549 cells, BBSKE inhibits the activity of TrxR, leading to the accumulation of oxidated Trx; the change of the redox state of Trx results in the decrease of NF-κB DNA-binding activity which consequently down-regulates the expressions of anti-apoptosis genes, such as Bcl-2, Bcl-xL, cIAP-2 and XIAP; finally, the mitochondrion-dependent apoptosis occurs in BBSKE-treated A549 cells (Lan *et al*., 2007). Here, we observed the similar mechanism in HL-60 and K562 cells that BBSKE could induce apoptosis in HL-60 and K562 cells through Bcl-2/Bax pathway and cleavage of pro-caspase-3 (Figs.3 and 4). The present results are in agreement with the reported findings on solid tumors. Further study needs to be done for a better understanding of the apoptosis mechanisms in BBSKE-treated HL-60 and K562 cells.

To further test the effect of BBSKE on non-solid tumor in vivo, mice with EAC were treated with different doses of BBSKE (18, 36 or 72 mg/kg) until death. Table 1 indicates that BBSKE could increase the life span of tumor-bearing mice dose-dependently. CTX is used in this experiment as positive control, which is clinically used nowadays to treat several types of cancer such as lymphomas, cancers of the ovary, breast and bladder, and chronic lymphocytic leukemia. Moreover, Andreani *et al*.(1983) have suggested that an increase in the life span of ascites bearing animals by 25% can be considered as an indicative of significant drug activity. To our surprise, high dose (72 mg/kg) BBSKE treatment (32.2% of life span increase) is comparable to the effect of CTX (36.4% of life span increase). Whether the therapeutic effect of BBSKE in EAC mouse model is due to the induction of tumor cell apoptosis needs to be further analyzed.

In conclusion, here we for the first time report the effects of BBSKE on some non-solid tumors. BBSKE could inhibit cell growth and induce apoptosis in two human leukemia cell lines, HL-60 and K562 cells, which is partially through Bcl-2/Bax pathway. In vivo experiment using EAC mouse model indicates that BBSKE could increase the life span of tumor-bearing mice with a comparable effect to CTX, suggesting a potential usage of BBSKE as a therapeutic agent against non-solid tumors.

References

Andreani, A., Scapini, G., Galatulas, I., Bossa, R., 1983. Potential antitumor agents IX: synthesis and antitumor activity of two analogues of ketocaine. *J. Pharm. Sci.*, **72**(7):814-815. [doi:10.1002/jps.2600720724]

Barral, A.M., Källström, R., Sander, B., Rosén, A., 2000. Thioredoxin, thioredoxin reductase and tumour necrosis factor-alpha expression in melanoma cells: correlation to resistance against cytotoxic attack. *Melanoma Res.*, **10**(4): 331-343. [doi:10.1097/00008390-200008000-00004]

Bjorkhem-Bergman, L., Jonsson, K., Eriksson, L.C., Olsson, J.M., Lehmann, S., Paul, C., Bjornstedt, M., 2002. Drug-resistant human lung cancer cells are more sensitive to selenium cytotoxicity. Effects on thioredoxin reductase and glutathione reductase. *Biochem. Pharmacol.*, **63**(10): 1875-1884. [doi:10.1016/S0006-2952(02)00981-4]

Chae, H.Z., Chung, S.J., Rhee, S.G., 1994. Thioredoxin-dependent peroxide reductase from yeast. *J. Biol. Chem.*, **269**(44):27670-27678.

Coligan, J.E., Kruisbeek, A.M., Margulies, D.H., Shevach, E.M., Strober, W., 1997. Current Protocols in Immunology. John Wiley & Sons, New York.

Hirota, K., Matsui, M., Iwata, S., Nishiyama, A., Mori, K., Yodoi, J., 1997. AP-1 transcriptional activity is regulated by a direct association between thioredoxin and Ref-1. *Proc. Natl. Acad. Sci. USA*, **94**(8):3633-3638. [doi:10.1073/pnas.94.8.3633]

Holmgren, A., 1985. Thioredoxin. *Annu. Rev. Biochem.*, **54**(1):237-271. [doi:10.1146/annurev.bi.54.070185.001321]

Kahlos, K., Soini, Y., Saily, M., Koistinen, P., Kakko, S., Paakko, P., Holmgren, A., Kinnula, V.L., 2001. Up-regulation of thioredoxin and thioredoxin reductase in human malignant pleural mesothelioma. *Int. J. Cancer*, **95**(3):198-204. [doi:10.1002/1097-0215(20010520)95:3<198::AID-IJC1034>3.0.CO;2-F]

Kakolyris, S., Giatromanolaki, A., Koukourakis, M., Powis, G., Souglakos, J., Sivridis, E., Georgoulias, V., Gatter, K.C., Harris, A.L., 2001. Thioredoxin expression is associated with lymph node status and prognosis in early operable non-small cell lung cancer. *Clin. Cancer Res.*, **7**(10):3087-3091.

Lan, L., Zhao, F., Wang, Y., Zeng, H., 2007. The mechanism of apoptosis induced by a novel thioredoxin reductase inhibitor in A549 cells: possible involvement of nuclear factor-kappaB-dependent pathway. *Eur. J. Pharmacol.*, **555**(2-3):83-92. [doi:10.1016/j.ejphar.2006.10.037]

Laurent, T.C., Moore, E.C., Reichard, P., 1964. Enzymatic synthesis of deoxyribonucleotides VI. Isolation and characterization of thioredoxin, the hydrogen donor from *Escherichia coli* B. *J. Biol. Chem.*, **239**(12):3436-3444.

Makino, Y., Yoshikawa, N., Okamoto, K., Hirota, K., Yodoi, J., Makino, I., Tanaka, H., 1999. Direction association with thioredoxin allows redox regulation of glucocorticoid receptor function. *J. Biol. Chem.*, **274**(5):3182-3188. [doi:10.1074/jbc.274.5.3182]

Pallis, M., Bradshaw, T.D., Westwell, A.D., Grundy, M., Stevens, M.F., Russell, N., 2003. Induction of apoptosis without redox catastrophe by thioredoxin-inhibitory compounds. *Biochem. Pharmacol.*, **66**(9):1695-1705. [doi:10.1016/S0006-2952(03)00471-4]

Powis, G., Kirkpatrick, D.L., Angulo, M., Baker, A., 1998. Thioredoxin redox control of cell growth and death and the effects of inhibitors. *Chem. Biol. Interact.*, **111-112**(1):23-34. [doi:10.1016/S0009-2797(97)00148-8]

Powis, G., Mustacich, D., Coon, A., 2000. The role of the redox protein thioredoxin in cell growth and cancer. *Free Radic. Biol. Med.*, **29**(3-4):312-322. [doi:10.1016/S0891-5849(00)00313-0]

Raffel, J., Bhattacharyya, A.K., Gallegos, A., Cui, H., Einspahr, J.G., Alberts, D.S., Powis, G., 2003. Increased expression of thioredoxin-1 in human colorectal cancer is associated with decreased patient survival. *J. Lab. Clin. Med.*, **142**(1):46-51. [doi:10.1016/S0022-2143(03)00068-4]

Saitoh, M., Nishitoh, H., Fujii, M., Takeda, K., Tobiume, K., Sawada, Y., Kawabata, M., Miyazono, K., Ichijo, H., 1998. Mammalian thioredoxin is a direct inhibitor of apoptosis signal-regulating kinase (ASK) 1. *EMBO J.*, **17**(9):2596-2606. [doi:10.1093/emboj/17.9.2596]

Sasada, T., Nakamura, H., Ueda, S., Sato, N., Kitaoka, Y., Gon, Y., Takabayashi, A., Apyrou, G., Holmgren, A., Yodoi, J., 1999. Possible involvement of thioredoxin reductase as well as thioredoxin in cellular sensitivity to cis-diamminedichloroplatinum(II). *Free Radic. Biol. Med.*, **27**(5-6):504-514. [doi:10.1016/S0891-5849(99)00101-X]

Shao, L., Diccianni, M.B., Tanaka, T., Gribi, R., Yu, A.L., Pullen, J.D., Camitta, B.M., Yu, J., 2001. Thioredoxin expression in primary T-cell acute lymphoblastic leukemia and its therapeutic implication. *Cancer Res.*, **61**(10):7333-7338.

Shi, C., Yu, L., Yang, F., Yan, J., Zeng, H., 2003. A novel organoselenium compound induces cell cycle arrest and apoptosis in prostate cancer cell lines. *Biochem. Biophys. Res. Commun.*, **309**(3):578-583. [doi:10.1016/j.bbrc.2003.08.032]

Söderberg, A., Sahaf, B., Rosn, A., 2000. Thioredoxin reductase, a redox-active selenoprotein, is secreted by normal and neoplastic cells: presence in human plasma. *Cancer Res.*, **60**(4):2281-2289.

Soini, Y., Kahlos, K., Näpänkangas, U., Kaarteenaho-Wiik, R., Säily, M., Koistinen, P., Pääakkö, P., Holmgren, A., Kinnula, V.L., 2001. Widespread expression of thioredoxin and thioredoxin reductase in non-small cell lung carcinoma. *Clin. Cancer Res.*, **7**(6):1750-1757.

Teshigawara, K., Maeda, M., Nishino, K., Nikaido, T., Uchiyama, T., Tsudo, M., Wano, Y., Yodoi, J., 1985. Adult T leukemia cells produce a lymphokine that augments interleukin 2 receptor expression. *J. Mol. Cell. Immunol.*, **2**(1):17-26.

Wang, J., Kobayashi, M., Sakurada, K., Imamura, M., Moriuchi, T., Hosokawa, M., 1997. Possible roles of an adult T-cell leukemia (ATL)-derived factor/thioredoxin in the drug resistance of ATL to Adriamycin. *Blood*, **89**(7):2480-2487.

Yokomizo, A., Ono, M., Nanri, H., Makino, Y., Ohga, T., Wada, M., Okamoto, Y., Yodoi, J., Kuwano, M., Kohno, K., 1995. Cellular levels of thioredoxin associated with drug sensitivity to cisplatin, mitomycin C, doxorubicin, and etoposide. *Cancer Res.*, **55**(19):4293-4296.

Zhao, F., Yan, J., Deng, S., Lan, L., He, F., Kuang, B., Zeng, H., 2006. A thioredoxin reductase inhibitor induces growth inhibition and apoptosis in five cultured human carcinoma cell lines. *Cancer Lett.*, **236**(1):46-53. [doi:10.1016/j.canlet.2005.05.010]