Department of Biochemistry and Molecular Biology[1], School of Basic Medical Sciences, Lanzhou University; Institute of Modern Physics[2], Chinese Academy of Sciences; Laboratory Center for Medical Science[3], Lanzhou University, Key Laboratory of Preclinical Study for New Drugs of Gansu Province, Lanzhou, China

Inhibition of thioredoxin reductase by auranofin induces apoptosis in adriamycin-resistant human K562 chronic myeloid leukemia cells

Jing-jing Liu[1], Qing Liu[2], Hu-lai Wei[1,3], Juan Yi[3], Huai-shun Zhao[3], Li-ping Gao[1,3]

Received November 1, 2010, accepted December 3, 2010

Li-ping Gao, Department of Biochemistry and Molecular Biology, School of Basic Medical Sciences, Lanzhou University, Lanzhou, People's Republic of China
gaolp@lzu.edu.cn

Pharmazie 66: 440–444 (2011) *doi: 10.1691/ph.2011.0835*

Mammalian thioredoxin reductase (TrxR) catalyzes the NADPH-dependent reduction of oxidized thioredoxin (Trx) and plays a central role in regulating cellular redox homeostasis, cell growth and apoptosis. Increasing evidence shows that TrxR is over-expressed or constitutively active in many tumor cells. Moreover, TrxR appears to contribute to increased tumor cell growth and a resistance to chemotherapy. In this study, we evaluated the activity of TrxR in adriamycin-resistant leukemic cells (K562/ADM) and adriamycin-sensitive parental lines (K562), and found that TrxR activity was higher in the drug resistant cell sublines K562/ADM than in K562 drug sensitive parental cells. Auranofin, a gold(I) compound clinically used as an antirheumatic agent, reduced TrxR activity and was more effective than adriamycin in decreasing cell viability in K562/ADM cells. In addition, auranofin induced apoptosis in dose-dependent manners, accompanied by caspase-3 activation in K562/ADM cells. Our results demonstrate that inhibition of TrxR and induction of apoptosis by auranofin provides its ability in overcoming adriamycin resistance in K562/ADM cells.

1. Introduction

The thioredoxin system consists of thioredoxin (Trx), thioredoxin reductase (TrxR) and nicotinamide adenine dinucleotide phosphate (NADPH). This system is one of the main thiol-dependent electron donor systems in the cell and participates in several cell processes, including regulation of cellular redox homeostasis and other cellular processes, such as cell proliferation, angiogenesis, apoptosis and signaling (Arnér and Holmgren 2000). Mammalian TrxR contains a conserved N-terminal redox active disulfide motif -Cys-Val-Asn-Val-Gly-Cys-, and a C-terminal active site sequence -Gly-Cys-Sec-Gly-, the latter of which is essential for catalysis and access by different substrates and inhibitors (Sandalova et al. 2001; Liu et al. 2009). So it reduces not only the disulfide in oxidized Trx, but also some other protein disulfides or a wide spectrum of oxidized low molecular weight compounds (Nordberg and Arnér 2001; Becker et al. 2001; Wipf et al. 2004). Most of these substrates are involved in cellular redox regulation. At early stages, TrxR may be beneficial for preventing cancer due to its capability to counteract the oxidative stress caused by many carcinogens (Tonissen and Di Trapani 2009). Once a cell has initiated a cancer phenotype, then high levels of TrxR may assist cancer development due to its growth promoting, through the disulfide reduction of ribonucleotide reductase needed for deoxyribonucleotide synthesis (Nordlund and Reichard 2006) and anti-apoptotic functions (Lincoln et al. 2003; Nguyen et al. 2006). A growing body of evidence indicates TrxR is over-expressed or constitutively active in many tumor cells (Biaglow and Miller 2005; Engman et al. 2003). Thus, TrxR is a potential molecular target for cancer therapy. Inhibition of TrxR may contribute to the mechanism of some anticancer drugs (Witte et al. 2005). Targeting TrxR is a basis for cancer therapy by arsenic trioxide (Lu et al. 2007). In addition, the activity of TrxR is higher in some drug resistant cell sublines compared with the drug sensitive parental lines (Björkhem-Bergman et al. 2002). TrxR is regarded as a contributing factor leading to resistance to chemotherapeutic drugs (Nguyen et al. 2006).

Gold(I) compounds have been used for the treatment of rheumatoid arthritis for a long time (Champion et al. 1990). Moreover, gold compounds were shown to act as anticancer agents (McKeage et al. 2002), to inhibit mitochondrial functions (Rigobello et al. 2002), to stimulate the release of Cytochrome c (Rigobello et al. 2004), and to induce apoptosis (Liu et al. 2000; Park and Kim 2005; Venardos et al. 2004). Auranofin (Fig. 1) is a gold (I) compound well known as an antirheumatoid agent. It reacts with selenolcontaining residues, and inhibits the reduced form of TrxR (isolated from human placenta) at nanomolar levels (Ki = 4 nM)(Gromer et al. 1998). Auranofin was shown to possess *in vivo* antitumor activity against P388 murine leukemia (Simon et al. 1981). More recently, it was reported that auranofin is able to overcome cisplatin resistance in human ovarian cancer cells (Marzano et al. 2007), highlighting the potential of this drug in the treatment of drug resistant tumors.

In the present paper, we compared the activity of TrxR in K562/ADM multidrug-resistant leukemic cells and the drug sensitive parental lines (K562). We demonstrate that the activity of TrxR is higher in the drug resistant cell sublines K562/ADM compared to the drug sensitive parental lines (K562). Auranofin, a gold(I) compound clinically used as an antirheumatic

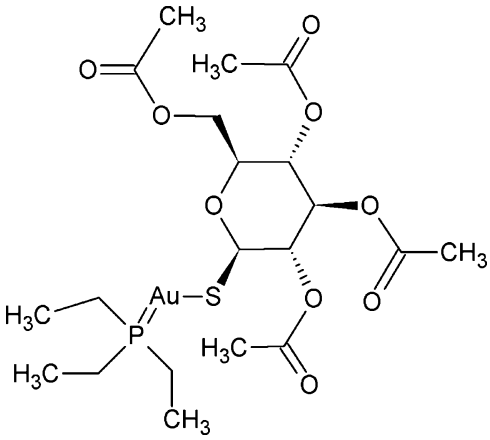

Fig. 1: Chemical structure of auranofin

agent, inhibits TrxR activity, decreases cell viability and induces apoptosis in K562/ADM.

2. Investigations, results and discussion

The emergence of adriamycin (ADM)-resistance constitutes a frequent barrier to successful treatment of a number of tumors. Many mechanisms are involved in the development of drug resistance. Of note, TrxR 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). However, whether the activity of TrxR is increased in the drug resistant cell sublines K562/ADM compared with the drug sensitive parental lines (K562) has not been investigated. Therefore, the TrxR activity was assessed in K562/ADM multidrug-resistant leukemic cells and the drug sensitive parental lines (K562). Our result show that the basal level of active TrxR in the resistant K562/ADM cells was 3.6-fold higher than in drug sensitive parental lines (Fig. 2). This result indicates that high TrxR activity may contribute to the acquired adriamycin resistance in K562/ADM cells.

In clinical practice, gold(I) drugs have been extensively used to treat rheumatoid arthritis (Kean and Kean 2008), but they have also been studied for their potential antitumor properties (von Essen 1978). Among gold-containing drugs, auranofin, a phosphine gold(I) compound, has been found to selectively inhibit TrxR in near-stoichiometric amounts (Gromer et al. 1998). Whether auranofin shows inhibition of TrxR activity and cytotoxity in K562/ADM cells was further studied. Treatment for 24 h with various concentrations of auranofin drastically decreases TrxR activity in the K562/ADM cells (Fig. 3). To evaluate the effect of auranofin on cell growth, cell viability was performed by MTT assay. As shown in Fig. 4, auranofin reduced the viability of K562/ADM cells in a dose and time-dependent manner. It was noted that auranofin reduces cell viability at short incubation time (6 h, Fig. 4A). At concentrations of 0.25, 0.5, 1, 2 and 3 μM of auranofin for 24 h, the proliferation of K562/ADM cells was decreased by 45, 75, 81, 96 and 100%, respectively (Fig. 4B), when compared with control cells. Moreover, proliferation of the cells which exposed to only 0.5 μM auranofin for 6 h, 24 h and 48 h (Fig. 4A, 4B and 4C) was decreased by 34, 75 and 86%, respectively, while the application of the same doses of adriamycin decreased the proliferation of K562/ADM cells by 1.1, 12 and 33% respectively. These results clearly demonstrate that auranofin reduces cell viability at very low concentrations and short incubation time and is able to overcome the acquired adriamycin resistance.

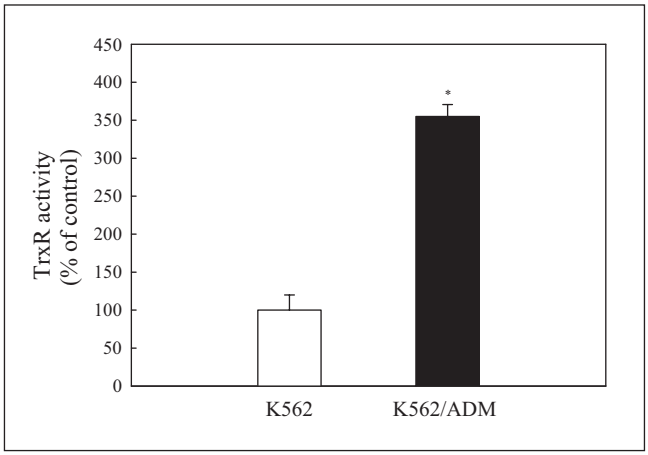

Fig. 2: Estimation of TrxR activity in K562/ADM multidrug-resistant leukemic cells and the drug sensitive parental lines (K562). TrxR activity was evaluated by means of the Thioredoxin Reductase Assay Kit as described in the Materials and Methods. The data are represented as means ± SD of triplicate experiments *$p < 0.05$, K562/ADM vs K562 cells

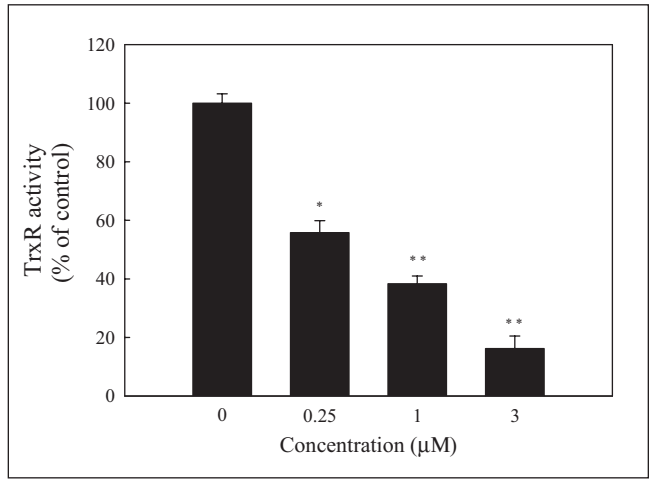

Fig. 3: Inhibition of TrxR activity by auranofin in K562/ADM cells. K562/ADM cells were treated with the indicated concentrations of auranofin for 24 h. TrxR activity was evaluated by means of the Thioredoxin Reductase Assay Kit as described in the Materials and Methods. The data are represented as means ± SD of triplicate experiments. *$p < 0.05$, **$p < 0.01$ as compared with the control group

Apoptosis is one of major forms of cell death. The cytotoxicity of chemotherapeutic agents is attributed to apoptosis (Nakanishi and Toi 2005). To determine whether the cytotoxic effect of auranofin is due to apoptosis, we examined the characteristic events of apoptosis. The quantitative assessment of sub-G1 cells by flow cytometry was used to estimate the number of apoptotic cells. The percentage of apoptotic cells with the treatment of auranofin for 24 h was increased concentration-dependently from 2.14% to 24.6%, which was significantly higher than that in the adriamycin treated cells (from 2.14% to 4.8%)(Fig. 5). This result suggests that auranofin is able to induce cell apoptosis in K562/ADM cells.

Caspases have been shown to be activated during apoptosis in many cells and play critical roles in both the initiation and execution of apoptosis. Among the identified caspases, activation of caspase-3 is crucial in numerous types of cells leading to the execution of apoptosis (Porter and Jänicke 1999; Yang et al. 2007). Therefore, we examined whether caspase-3 was involved in apoptosis induced by auranofin in K562/ADM cells. As shown in Fig. 6, the caspase-3 activity was increased by 53% in k562/ADM cells after 12 h of treatment with 1 μM auranofin. Compared to auranofin, the caspase-3 activity of K562/ADM cells after adriamycin treatment was not significantly increased

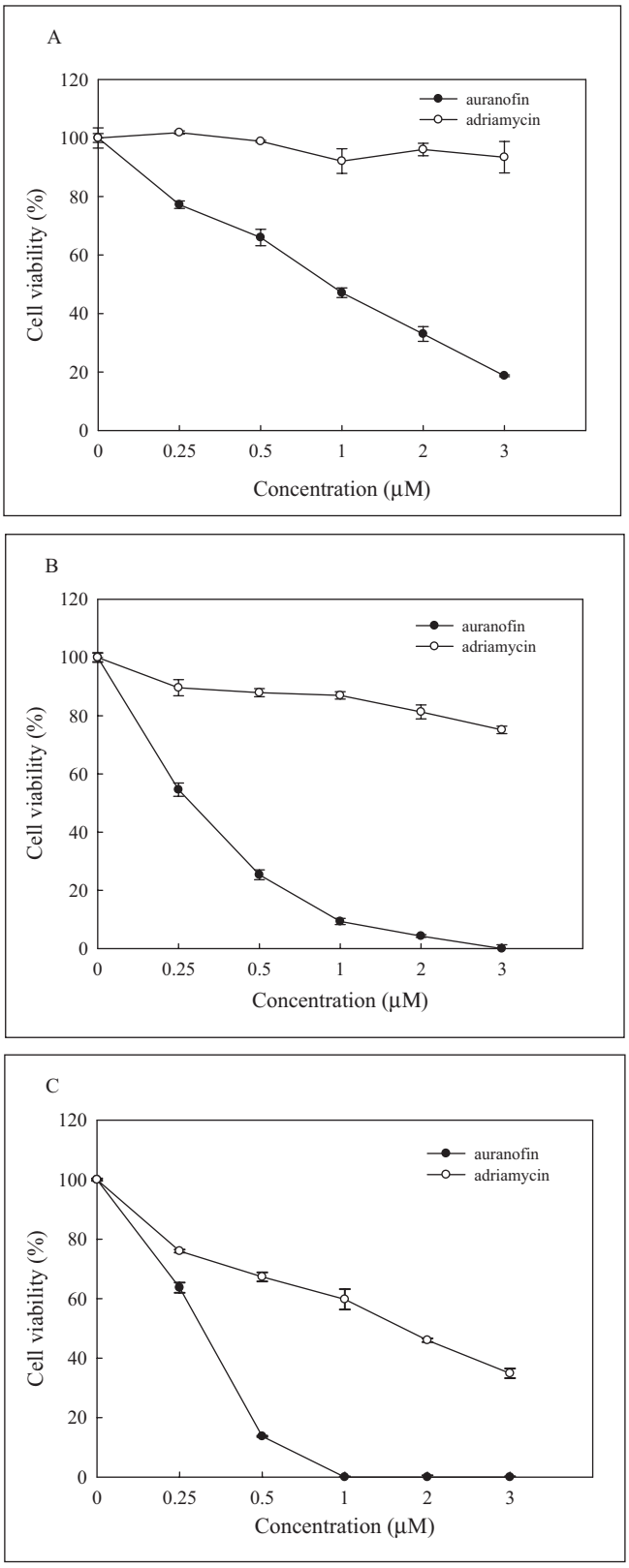

Fig. 4: Inhibition of K562/ADM proliferation by auranofin. K562/ADM cells were treated with the indicated concentrations of auranofin and adriamycin for 6 (A), 24 (B), 48 (C) h. Cell viability was determined by MTT assay and expressed as the percentage of the untreated control. The data are represented as means ± SD of triplicate experiments

(4.5% after 24 h).The result shows that auranofin activates caspase-3 in K562/ADM cells.

In conclusion, our results showed that the activity of TrxR was higher in adriamycin-resistance cell sublines (K562/ADM cells) compared to adriamycin -sensitive parental lines (K562 cells).

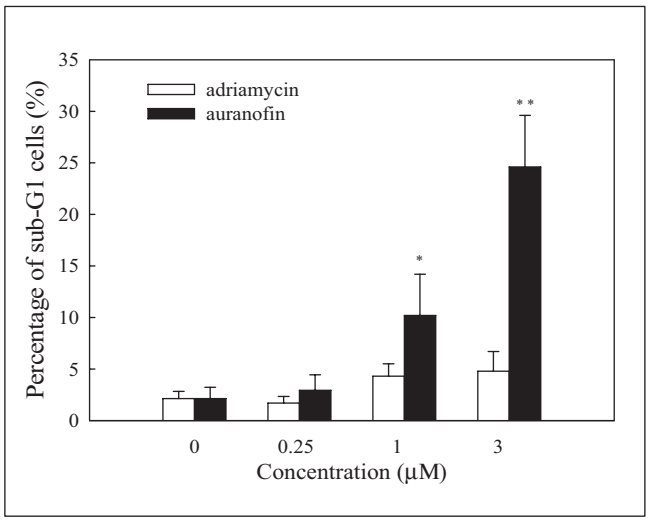

Fig. 5: Auranofin induces apoptosis in K562/ADM cells. K562/ADM cells were incubated with the indicated concentration of auranofin and adriamycin for 24 h, and The level of apoptosis was analyzed by flow cytometry as described in the Materials and Methods. Data are represented as means ± S.D. of three experiments. *$p < 0.05$, **$p < 0.01$ as compared with the control group

Auranofin exhibited cytotoxic effect on adriamycin-resistant human leukemia K562/ADM cell lines via cell apoptosis with a decrease in cellular TrxR activity. These results clearly demonstrate that auranofin is able to overcome the acquired adriamycin resistance in K562/ADM cells and it might be a candidate drug to overcome the drug resistance of human chronic myelogenous leukemia.

3. Experimental

3.1. Materials

Auranofin, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), propidium iodide (PI) and Thioredoxin Reductase Assay Kit were purchased from Sigma (St. Louis, MO, USA). RPMI-1640 medium, fetal bovine serum and RNase A were products of Gibco BRL (USA).

3.2. Cell line and culture conditions

Adriamycin-sensitive human chronic myeloid leukemia cells (K562) and adriamycin -resistent variant, K/ADM cells, which was generated by monthly selection with 5 μg/ml adriamycin, were kindly provided by

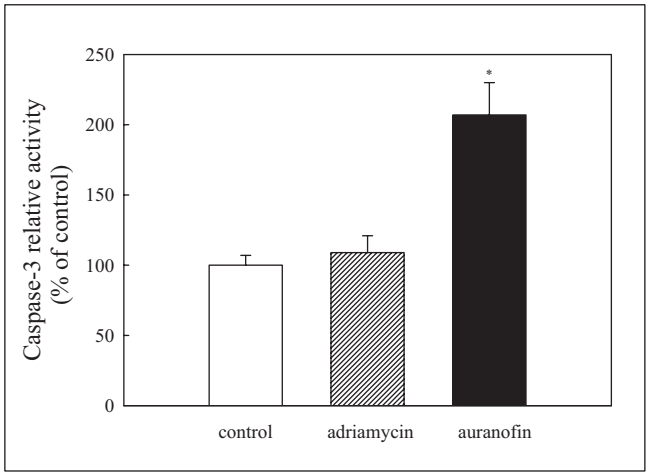

Fig. 6: Auranofin enhances caspase-3 activation in K562/ADM cells. K562/ADM cells were incubated for 12 h in the absence or in the presence of 1 μM of auranofin and adriamycin, respectively. Quantitation of the percentage of caspase-3 positive cells based on flow cytometry analysis, and data show the mean ± SD of duplicate realized experiments. *$p < 0.05$ as compared with the control group

Professor Hulai Wei (Department of Biochemistry and Molecular Biology, University of Lanzhou, China). The cells were cultured at 37 °C in a humidified atmosphere of 5% CO_2 in RPMI-1640 medium supplemented with 10% heat-inactivated fetal bovine serum, 100 units/ml penicillin and 100 μg/ml streptomycin.

3.3. MTT assay

Auranofin was dissolved in DMSO to achieve a stock solution, which was diluted to different concentrations prior to use. Cell viability was determined by MTT colorimetric assay (Mosmann 1983). Briefly, K562/ADM cells (5×10^4 per well) were seeded in 96-well plates and cultured in the presence of 0.25–3 μM auranofin for 4–48 h. At the end of treatment, cells were incubated with 0.5 mg/ml of MTT at 37 °C for 4 h. Then 10% SDS was added to each well to dissolve the formazan formed. The absorbance was measured at a wavelength of 570 nm using a microplate reader (Bio-Rad).

3.4. Estimation of TrxR activity

TrxR activity was determined by the Thioredoxin Reductase Assay Kit (Sigma-Aldrich) according to the manufacturer's instruction. Briefly, after treatment with different concentrations of auranofin for 24 h, the cells were harvested and lysed with a buffer containing 150 mM NaCl, 50 mM Tris–HCl, 1% Triton X-100, 1% SDS, 1% deoxycholate, 1 mM NaF, and 1 mM EDTA, and protease inhibitors. After centrifugation at $14{,}000 \times g$ for 10 min at 4 °C, protein concentrations of supernatants were determined using the Bio-Rad Protein Assay kit. 20 μg portion of protein was used to initiate the activity assay in reactions in a 96-well plate. Reduction of 5,5′-dithiobis(2-nitrobenzoic) acid was followed at 412 nm using a spectrophotometer. The percentage of TrxR activity in comparison with the control was determined (Cattaruzza et al. 2011).

3.5. Flow cytometric analysis

After treatment with different concentrations of auranofin and adriamycin for 24 h, the cells were harvested and washed with PBS. Cell pellets were fixed in cold 70% ethanol. After centrifugation at 1200 rpm for 5 min, cells were resuspended in 0.5 ml DNA staining reagent containing 50 μg/ml propidium iodide, 50 μg/ml RNase A, 0.1% Triton X-100 and 0.1 mM EDTA (pH 7.4). The samples were read on a Becton Dickson FACStar Plus cytometer, and the results were analysed with CellQuest software (Ho et al. 2006).

3.6. Measurement of caspase-3 enzyme activity

Caspase-3 activation was determined using the Active Caspase-3 Apoptosis kit (BD Pharmingen, San Diego, CA, USA) according to the manufacturer's instruction. Cells (1×10^6) stained with FITC conjugated anti-active caspase-3 antibody, were analyzed by flow cytometry (Augustin et al. 2010).

3.7. Statistical analysis

The data are expressed as means ± SD. Statistical comparisons were made by using Student's t-test. $P < 0.05$ was considered statistically significant.

Acknowledgements: This work was supported by Lanzhou University (grant number LZUYX200803).

References

Arnér ES, Holmgren A (2000) Physiological functions of thioredoxin and thioredoxin reductase. Eur J Biochem 267: 6102–6109.

Augustin E, Moś-Rompa A, Nowak-Ziatyk D, Konopa J (2010) Antitumor 1-nitroacridine derivative C-1748, induces apoptosis, necrosis or senescence in human colon carcinoma HCT8 and HT29 cells. Biochem Pharmacol 79: 1231–1241.

Becker K, Herold-Mende C, Park JJ, Lowe G, Schirmer RH (2001) Human thioredoxin reductase is efficiently inhibited by (2,2′:6′,2′ ′-terpyridine) platinum(II) complexes. Possible implications for a novel antitumor strategy. J Med Chem 44: 2784–2792.

Biaglow JE, Miller RA (2005) The thioredoxin reductase/thioredoxin system: novel redox targets for cancer therapy. Cancer Biol Ther 4: 6–13.

Björkhem-Bergman L, Jönsson K, Eriksson LC, Olsson JM, Lehmann S, Paul C, Björnstedt M (2002) Drug-resistant human lung cancer cells are more sensitive to selenium cytotoxicity. Effects on thioredoxin reductase and glutathione reductase. Biochem Pharmacol 63: 1875–1884.

Cattaruzza L, Fregona D, Mongiat M, Ronconi L, Fassina A, Colombatti A, Aldinucci D (2011) Antitumor activity of gold(III)-dithiocarbamato derivatives on prostate cancer cells and xenografts. Int J Cancer. 128: 206–215.

Champion GD, Graham GG, Ziegler JB (1990) The gold complexes. Baillieres Clin Rheumatol 4: 491–534.

Engman L, Al-Maharik N, McNaughton M, Birmingham A, Powis G (2003) Thioredoxin reductase and cancer cell growth inhibition by organotellurium antioxidants. Anticancer Drugs 14: 153–161.

Gromer S, Arscott LD, Williams CH Jr, Schirmer RH, Becker K (1998) Human placenta thioredoxin reductase. Isolation of the selenoenzyme, steady state kinetics, and inhibition by therapeutic gold compounds. J Biol Chem 273: 20096–20101.

Ho HY, Wei TT, Cheng ML, Chiu DT (2006) Green tea polyphenol epigallocatechin-3-gallate protects cells against peroxynitrite-induced cytotoxicity: modulatory effect of cellular G6PD status. J Agric Food Chem. 54:1638–45.

Kean WF, Kean IR (2008) Clinical pharmacology of gold. Inflammopharmacology 16: 112–125.

Lincoln DT, Ali Emadi EM, Tonissen KF, Clarke FM (2003) The thioredoxin-thioredoxin reductase system: over-expression in human cancer. Anticancer Res 23: 2425–2433.

Liu J, Akahoshi T, Namai R, Matsui T, Kondo H (2000) Effect of auranofin, an antirheumatic drug, on neutrophil apoptosis. Inflamm Res 49: 445–451.

Liu Z, Huang SL, Li MM, Huang ZS, Lee KS, Gu LQ (2009) Inhibition of thioredoxin reductase by mansonone F analogues: Implications for anticancer activity. Chem Biol Interact 177: 48–57.

Lu J, Chew EH, Holmgren A (2007) Targeting thioredoxin reductase is a basis for cancer therapy by arsenic trioxide. Proc. Natl. Acad. Sci. U.S.A. 104:12288–12293.

Marzano C, Gandin V, Folda A, Scutari G, Bindoli A, Rigobello MP (2007) Inhibition of thioredoxin reductase by auranofin induces apoptosis in cisplatin-resistant human ovarian cancer cells. Free Radic Biol Med 42: 872–881.

McKeage MJ, Maharaj L, Berners-Price SJ (2002) Mechanisms of cytotoxicity and antitumor activity of gold(I) phosphine complexes: the possible role of mitochondria. Coord Chem Rev 232:127–135.

Mosmann T (1983) Rapid colorimetric assay for cellular growth and survival: application to proliferation and cytotoxicity assays. J Immunol Methods 65: 55–63.

Nakanishi C, Toi M (2005) Nuclear factor-kappaB inhibitors as sensitizers to anticancer drugs. Nat Rev Cancer 5: 297–309.

Nguyen P, Awwad RT, Smart DD, Spitz DR, Gius D (2006) Thioredoxin reductase as a novel molecular target for cancer therapy. Cancer Lett 236: 164–174.

Nordberg J, Arnér ES (2001) Reactive oxygen species, antioxidants, and the mammalian thioredoxin system. Free Radic Biol Med 31: 1287–1312.

Nordlund P, Reichard P (2006) Ribonucleotide reductases. Annu Rev Biochem 75: 681–706.

Park SJ, Kim IS (2005) The role of p38 MAPK activation in auranofin-induced apoptosis of human promyelocytic leukaemia HL-60 cells. Br J Pharmacol 146: 506–513.

Porter AG, Jänicke RU (1999) Emerging roles of caspase-3 in apoptosis. Cell Death Differ 6: 99–104.

Rigobello MP, Scutari G, Boscolo R, Bindoli A (2002) Induction of mitochondrial permeability transition by auranofin, a gold(I)-phosphine derivative. Br J Pharmacol 136: 1162–1168.

Rigobello MP, Scutari G, Folda A, Bindoli A (2004) Mitochondrial thioredoxin reductase inhibition by gold(I) compounds and concurrent stimulation of permeability transition and release of cytochrome c. Biochem Pharmacol 67: 689–696.

Sandalova T, Zhong L, Lindqvist Y, Holmgren A, Schneider G (2001) Three-dimensional structure of a mammalian thioredoxin reductase: implications for mechanism and evolution of a selenocysteine-dependent enzyme. Proc Natl Acad Sci U.S.A 98: 9533–9538.

Simon T M, Kunishima DH, Vibert GJ, Lorber A (1981) Screening trial with the coordinated gold compound auranofin using mouse lymphocyte leukemia P388. Cancer Res 41: 94–97.

Tonissen KF, Di Trapani G (2009) Thioredoxin system inhibitors as mediators of apoptosis for cancer therapy. Mol Nutr Food Res 53: 87–103.

Venardos K, Harrison G, Headrick J, Perkins A (2004) Auranofin increases apoptosis and ischaemia-reperfusion injury in the rat isolated heart. Clin Exp Pharmacol Physiol 31: 289–294.

von Essen A (1978) [The aim: a multiform cancer campaign]. MMW Munch Med Wochenschr 120: 1173.

Wipf P, Lynch SM, Birmingham A, Tamayo G, Jimenez A, Campos N, Powis G. (2004) Natural product based inhibitors of the thioredoxin-thioredoxin reductase system. Org Biomol Chem 2: 1651–1658.

Witte AB, Anestål K, Jerremalm E, Ehrsson H, Arnér ESJ (2005) Inhibition of thioredoxin reductase but not of glutathione reductase by the major classes of alkylating and platinum-containing anticancer compounds. Free Radic Biol Med 39: 696–703.

Yang S, Zhou Q, Yang X (2007) Caspase-3 status is a determinant of the differential responses to genistein between MDA-MB-231 and MCF-7 breast cancer cells. Biochim Biophys Acta 1773: 903–911.