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Antiproliferative effect of gold(I) compound auranofin through inhibition of STAT3 and telomerase activity in MDA-MB 231 human breast cancer cells

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Signal transducer and activator of transcription 3 (STAT3) and telomerase are considered attractive targets for anticancer therapy. The *in vitro* anticancer activity of the gold(I) compound auranofin was investigated using MDA-MB 231 human breast cancer cells, in which STAT3 is constitutively active. In cell culture, auranofin inhibited growth in a dose-dependent manner, and *N*-acetyl-L-cysteine (NAC), a scavenger of reactive oxygen species (ROS), markedly blocked the effect of auranofin. Incorporation of 5-bromo-2′-deoxyuridine into DNA and anchorage-independent cell growth on soft agar were decreased by auranofin treatment. STAT3 phosphorylation and telomerase activity were also attenuated in cells exposed to auranofin, but NAC pretreatment restored STAT3 phosphorylation and telomerase activity in these cells. These findings indicate that auranofin exerts *in vitro* antitumor effects in MDA-MB 231 cells and its activity involves inhibition of STAT3 and telomerase. Thus, auranofin shows potential as a novel anticancer drug that targets STAT3 and telomerase. [BMB Reports 2013; 46(1): 59-64]

INTRODUCTION

Metal-containing compounds possess a wide spectrum of biological functions and have attracted interest because of their applications in medicinal chemistry (1, 2). For example, the platinum compound cisplatin is a well-known anticancer drug, and gold compounds including auranofin have long been used to treat rheumatoid arthritis (1, 3, 4).

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Auranofin [2, 3, 4, 6-tetra-*O*-acetyl-1-thio-β-$_D$-glucopyranosato-S-(triethylphosphine)gold(I)] is a gold(I)-based drug. The compound reacts with thiol and selenol groups; therefore, proteins that contain reactive cysteine and selenocysteine in their active sites are targets of auranofin (5, 6). The selenocysteine-containing thioredoxin reductase (TrxR) is specific target of this compound (7). TrxR catalyzes the NADPH-dependent reduction of oxidized thioredoxin (Trx), which contributes to intracellular redox regulation, antioxidant defense, apoptosis inhibition, and cell proliferation (8, 9). Elevated levels of Trx and TrxR have been detected in several cancer cells, and high Trx/TrxR plays a role in cell proliferation and resistance to chemotherapy. Therefore, the Trx/TrxR system has been targeted in cancer therapy (10, 11). Auranofin is a potent TrxR inhibitor that induces mitochondrial permeability transition, cytochrome c release, reactive oxygen species (ROS) generation, and apoptotic cell death (12, 13). Based on its ability to inhibit TrxR and stimulate ROS production, auranofin has shown promise as an anticancer agent in cancer cell lines including human leukemia, ovarian cancer, cervical cancer, lung cancer, and breast cancer (13-18). However, the precise mechanisms underlying its effects on cell growth and targeted proteins are still largely unknown.

Signal transducers and activator of transcription3 (STAT3) is a transcription factor that mediates cell growth and differentiation, and contributes to tumorigenesis by promoting cell proliferation and angiogenesis (19, 20). Constitutively active STAT3 is frequently detected in human cancer cell lines and metastatic malignant tumor including breast cancer (21, 22). Recent study demonstrated that STAT3 also regulates the expression of telomerase reverse transcriptase (23), which maintains telomere length during DNA replication and is needed for tumor cell survival. Accordingly, STAT3 is an attractive target for anticancer therapy in cancers with elevated STAT3 activity (24).

In a previous study, we reported that the anticancer effects of auranofin in acute promyelocytic leukemia cell lines were mediated by ROS production and p38 mitogen-activated protein kinase activation (14, 15). The ability of auranofin to inhibit IL-6-induced activation of STAT3 was also demonstrated in HepG2 human hepatoma cells (25). Nakaya *et al*. reported that auranofin induced apoptosis in human multiple myeloma cells by down-regulating STAT3 and inhibiting nuclear factor-kappa B activity

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(26). In addition, intracellular ROS can regulate STAT3 and telomerase activity (27). These findings raise the possibility of a potent anticancer function of auranofin via ROS-dependent STAT3 and telomerase inhibition in cancer cells that require high STAT3 and telomerase activity for cell survival and antiapoptosis.

It is not clear whether auranofin inhibits cell growth and the activity of STAT3 and telomerase in breast cancer cells in a ROS-dependent manner. Therefore, in the present study, we investigated the effects of auranofin on STAT3 and telomerase and its *in vitro* tumorigenic potential using MDA-MB 231 human breast cancer cells, in which constitutively active STAT3 is implicated in cell proliferation and survival.

RESULTS AND DISCUSSION

Antiproliferative effects of auranofin in MDA-MB 231 cells

To investigate the effect of auranofin on the growth of MDA-MB 231 cells, 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyl tetrazolium bromide (MTT) assay was performed to evaluate cell growth after 12-72 h treatment with various concentrations of auranofin. At doses up to 2 μM, auranofin dose-dependently decreased growth. This effect was inhibited by a 30 min preincubation with 10 mM *N*-acetyl-L-cysteine (NAC), which is a well known scavenger of ROS (Fig. 1A, B). It suggests ROS participation in the auranofin-mediated growth inhibition. Although our previous study showed that 2 μM auranofin blocked IL-6-induced STAT3 activation in HepG2 hepatoma cells, 2 μM auranofin was cytotoxic to MDA-MB 231 cells (Fig. 1A). Therefore, in the following experiments, 0.5 or 1 μM auranofin was used.

To examine the effect of auranofin on cell proliferation, the 5-bromo-2'-deoxyuridine (BrdU) incorporation assay was performed. As shown in Fig. 2, lower levels of incorporated BrdU were detected in the nuclei of auranofin-treated cells than in untreated control cells. These results indicate that auranofin inhibits cell proliferation and reduces *in vitro* growth of MDA-MB 231 cells.

Inhibition of anchorage-independent growth by auranofin

Tumorigenicity is correlated with anchorage-independent cell growth. To investigate the effect of auranofin on the tumorigenic potential of MDA-MB 231 cells, the soft agar colony formation assay was performed. As shown in Fig. 3, auranofin markedly reduced the number of colonies in soft agar. After 24 h, the number of colonies was 38 ± 2.5% (0.5 μM auranofin) and 23 ± 3.2% (1 μM auranofin) that of the untreated control. After 48 h, the number of colonies was 35 ± 18% (0.5 μM auranofin) and 11 ± 3.2% (1 μM auranofin) that of the untreated control. These findings indicate that auranofin reduces the *in vitro* tumorigenicity of MDA-MB 231 cells.

Downregulation of STAT3 phosphorylation and telomerase activity by auranofin

To explore whether the antiproliferative action of auranofin is associated with STAT3 regulation, we investigated the effects of auranofin on STAT3 phosphorylation and telomerase activity. Treatment with 1 μM auranofin for 3-12 h markedly blocked STAT3 phosphorylation (Fig. 4A, B), and STAT3 phosphorylation was restored by preincubation with 10 mM NAC for 30 min before auranofin treatment (Fig. 4C). Our data suggest that auranofin inactivates STAT3 through ROS-dependent mechanism. However, it could not be excluded that auranofin may interact

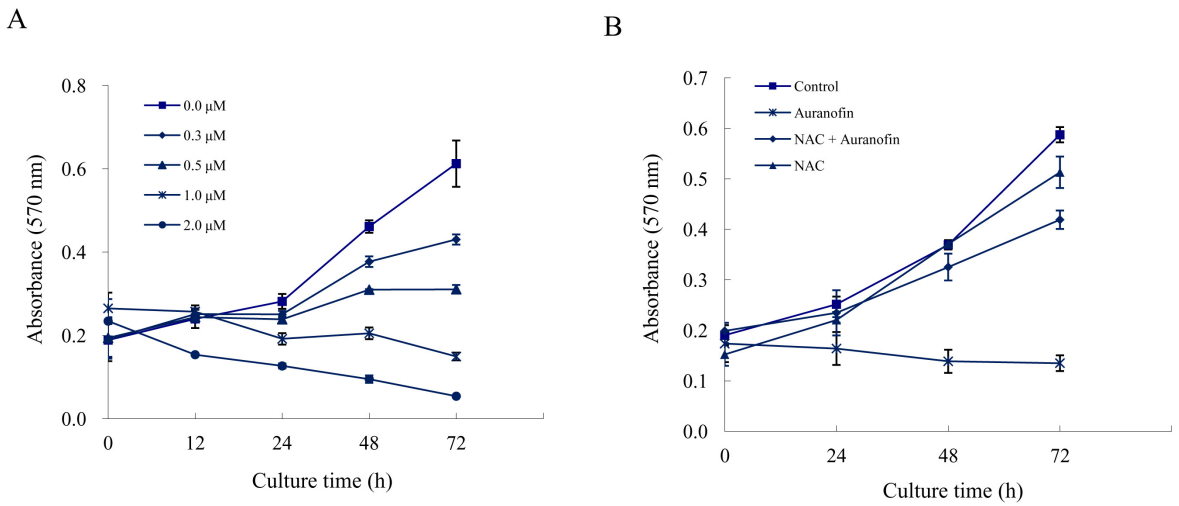

Fig. 1. Growth inhibition of MDA-MB 231 cells by auranofin. MDA-MB 231 cells were seeded in 96-well plates (1×10^4/well) and incubated overnight. (A) Cells were treated with auranofin (0.3-2 μM) and cultured for 12-72 h. At the indicated times, viable cells were determined by the MTT assay. (B) After preincubation with 10 mM NAC for 30 min, the cells were cultured in the presence of 1 μM auranofin for the indicated times, and then MTT assay was performed. Results are expressed as mean ± standard deviation (SD) of triplicate wells. Experiments were performed three times with similar results.

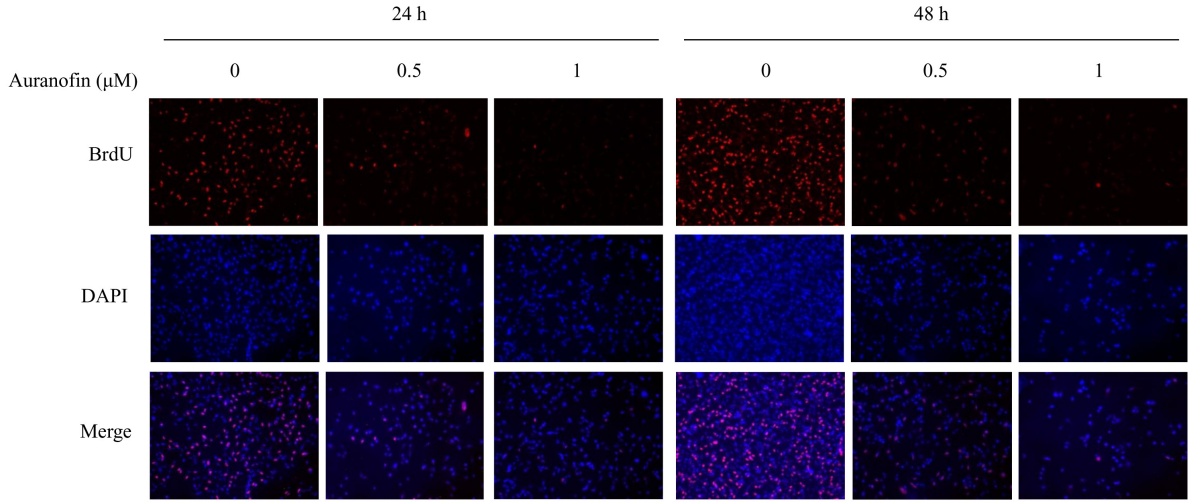

Fig. 2. The inhibitory effect of auranofin on MDA-MB 231 cell proliferation. Cells were treated with auranofin (0.5 or 1 μM) and incubated for 24 or 48 h. BrdU (10 μM) was added to each culture 30 min before the end of incubation. To detect BrdU incorporation into DNA, immunocytochemistry was carried out using anti-BrdU antibodies and Alexa Fluor 488-conjugated secondary antibodies as described in Materials and Methods. DAPI staining was performed simultaneously. The stained cells were observed using a fluorescence microscope and photographed (×200). Experiments were performed twice with similar results, and representative results are shown.

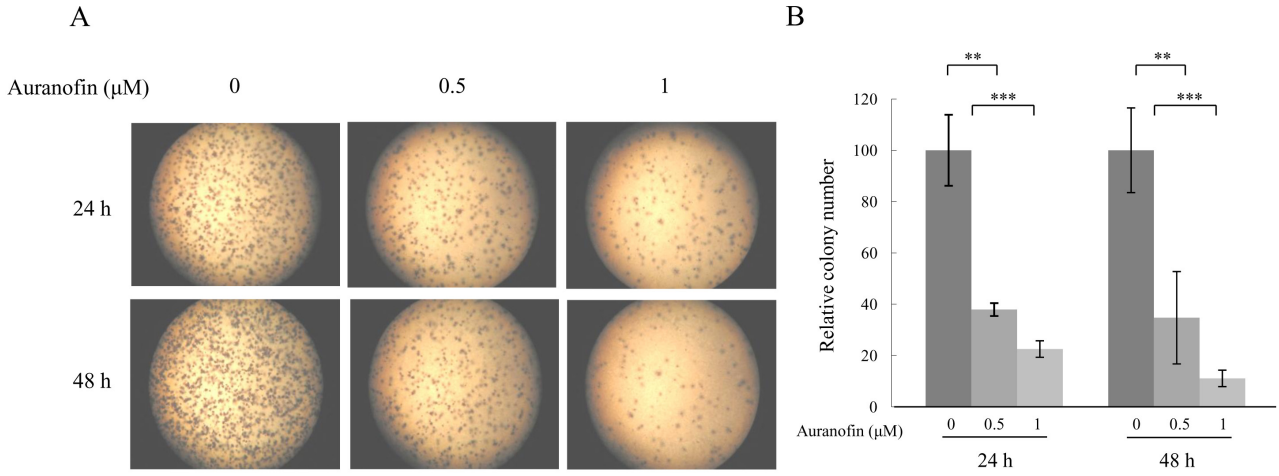

Fig. 3. Inhibition of anchorage-independent growth of MDA-MB 231 cells by auranofin. Cells were treated with auranofin (0.5 or 1 μM) for 24 or 48 h. After harvesting cells and suspending them in culture medium containing 0.3% soft agar, the cells were plated over a 0.6% bottom agar layer. The cells were cultured for 14 days, and the 1 ml culture medium covering the top agar layer was changed every 3 days. (A) Colonies that formed in the soft agar were stained with 1 mg/ml MTT. (B) Colonies in three random fields of each well were counted and expressed as a ratio to the untreated control cells. Results are expressed as mean ± SD of three separate experiments. $^{**}P < 0.005$, $^{***}P < 0.0005$ versus untreated control.

directly with reactive cysteine-bearing kinases or phosphatases which participate in STAT3 regulation. Then, thiol-containing NAC might interrupt the interaction and prevent the action of auranofin on STAT3 inhibition.

Cellular telomerase activity was determined using the TRAPeze telomerase detection kit, which is based on polymerase chain reaction (PCR) amplification of telomeric repeats. Untreated actively growing cells produced obvious telomerase products, showing the same pattern as the positive control (telomerase-positive cell extract), whereas the negative control using heat-inactivated telomerase did not show these products. Cells exposed to 1 μM auranofin exhibited weaker telomerase

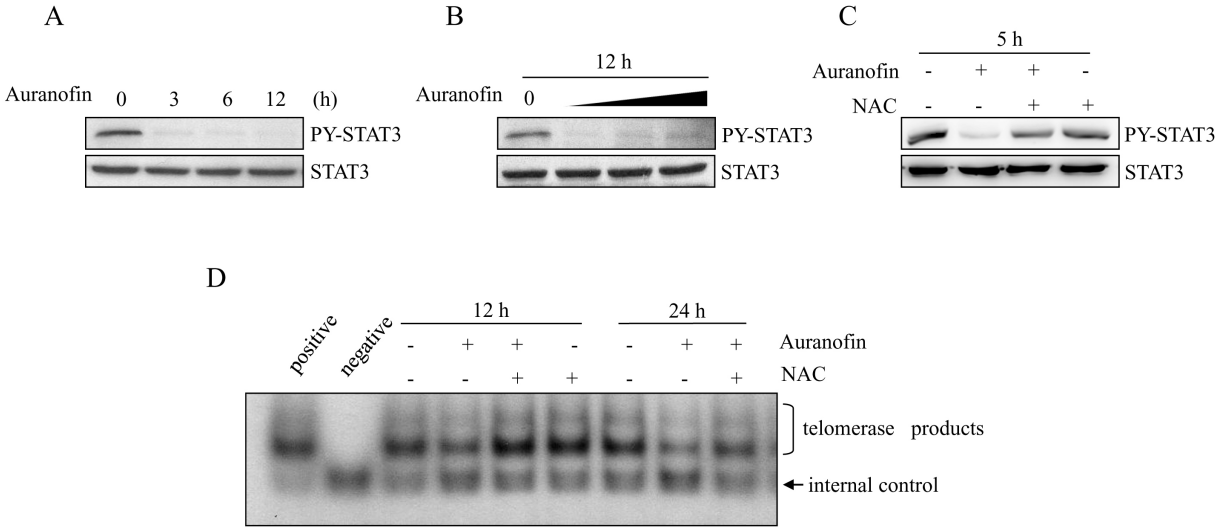

Fig. 4. Inhibition of STAT3 and telomerase activity in auranofin-treated MDA-MB 231 cells. Cells were treated with 1 μM auranofin for the indicated times (A) or treated for 12 h with increasing auranofin concentrations (0.3, 0.5, or 1 μM) (B). (C) Cells were preincubated with 10 mM NAC for 30 min before auranofin treatment (1 μM) for 5 h. Using cell lysates, STAT3 and tyrosine-phosphorylated STAT3 (PY-STAT3) were detected by Western blot analysis. (D) Cells were preincubated for 30 min in the absence or presence of 10 mM NAC and then exposed to 1 μM auranofin for 12 or 24 h. Telomerase activity was determined using the TRAPeze telomerase detection kit as described in Materials and Methods. Positive and negative controls were the cell extract from telomerase-positive cells supplied in the kit and heat-inactivated (85°C for 10 min) positive cell extract, respectively. All experiments were carried out three times with similar results

activity; however, telomerase activity was restored by NAC pretreatment (Fig. 4D). Although, auranofin only partially inhibited telomerase activity, the compound completely inhibited STAT3 phosphorylation (Fig. 4A, D). These results suggest that MDA-MB 231 cells may also possess a STAT3-independent pathway that regulates telomerase reverse transcriptase.

The telomerase assay includes an additional primer/template to amplify an internal control in each reaction sample. Because amplification of the telomerase products and the internal control are semi-competitive, samples with high telomerase activity (e.g., positive control and untreated samples) produce less of the internal control. PCR-amplified telomerase products generally appear as a ladder with 6-bp increments, but in our experiments the telomerase products appeared as a broad diffuse smear (Fig. 4D). This result may have been caused by poor electrophoretic separation of the PCR products.

Our results indicate that auranofin inhibited cell growth, *in vitro* tumorigenicity, and the activity of STAT3 and telomerase. Because ROS scavenger NAC blocked the inhibitory ability of this compound, it is thought that auranofin-elevated oxidative stress inactivates STAT3 and attenuates telomerase activity, thereby inhibiting cell proliferation. Recently, Watson *et al*. showed that TrxR knockdown by siRNA transfection in HeLa cells did not induce ROS generation, even though TrxR activity was completely inhibited (28). Therefore, it is not clear whether ROS-related STAT3 inhibition is linked to the auranofin-reduced TrxR activity. Further study is required to investigate the relationship between auranofin-regulated STAT3 inhibition and decreased TrxR activity and ROS production in MDA-MB 231 cells.

In summary, auranofin exerts an antitumorigenic effect on MDA-MB 231 human breast cancer cells, and this effect is related to the inhibition of STAT3 and telomerase. Our findings suggest the potential of auranofin as a novel anticancer drug that targets STAT3 and telomerase, which are required for proliferation and tumorigenesis in several cancers, including breast cancer. We used MDA-MB 231 cells in this study because they express constitutively active STAT3 and are sensitive to auranofin. However, to confirm our findings, further study is needed using other breast cancer cells that express high levels of active STAT3 for survival and tumor development.

MATERIALS AND METHODS

Cell culture and treatment

MDA-MB 231 breast cancer cells, purchased from the American Type Culture Collection (Rockville, MD), were maintained in RPMI 1640 medium (Gibco Life Technology, Gaithersburg, MD) supplemented with 10% heat-inactivated fetal bovine serum (Gibco) at 37°C in 5% CO_2. To investigate the effect of auranofin, cells were treated with 0.3-2 μM auranofin (Alexis, Lausen, Switzerland) for the indicated times.

Cell viability was determined by MTT assay. After culturing cells in the absence or presence of auranofin, 10 μM MTT (Sigma, Louis, MO) was added, and the cells were incubated for an additional 3 h. The cells were lysed by adding 100 μl isopropanol containing 0.04 M HCl solution. After 10 min, 100 μl

distilled water was added and the dissolved blue formazan was detected by measuring absorbance at 570 nm.

BrdU incorporation assay

Cell proliferation was evaluated by detecting BrdU incorporation into cellular DNA. MDA-MB 231 cells were seeded on glass slides and grown overnight. The next day, 0.5-1 μM auranofin was added to the cells, which were then cultured for an additional 24-48 h. For BrdU incorporation, 10 μM BrdU (Sigma, St Louis, MO) was added to the cell culture 30 min before the end of incubation. The cells were fixed with 4% paraformaldehyde for 20 min and then treated with 1 N HCl, followed by 0.1 M boric acid. After blocking with 10% normal donkey serum (Sigma) for 1 h at 37°C, the cells were incubated with anti-BrdU antibody (Sigma) at 4°C overnight, washed with phosphate-buffered saline (PBS) three times, and then stained with Alexa Fluor 488-conjugated secondary antibody (Abcam, Cambridge, MA) for 2 h. The stained cells were observed and photographed using a fluorescence microscope (Axiovert 200, Zeiss, Germany).

Colony formation assay

A 0.6% soft agar solution was poured into a 12-well plate and solidified at room temperature. Onto the bottom layer, 0.3% top agar containing MDA-MB 231 cells (1×10^5 cells) was poured. An extra 1 ml cell culture medium was added to cover the top agar layer and changed every 3 days for 2 weeks. Colonies were stained with 1 mg/ml MTT, and the colonies in three random fields of each well were counted under the microscope.

Western blot analysis

Cells were washed with ice-cold PBS and lysed in lysis buffer [25 mM Tris-HCl (pH 7.2), 0.1% sodium dodecyl sulfate (SDS), 0.1% Triton X-100, 1% sodium deoxycholate, 150 mM NaCl, 1 mM ethylenediaminetetraacetic acid, 1 mM sodium orthovanadate, 10 μg/ml aprotinin, 5 μg/ml leupeptin, and 1 mM phenylmethylsulfonyl fluoride] for 30 min on ice. Equal amounts of cell lysates were separated on 12% SDS-polyacrylamide gel, transferred to nitrocellulose membrane, and analyzed with antibodies against human STAT3 and tyrosine-phosphorylated STAT3 (Cell Signalling Technology, Beverly, MA). Target proteins were detected using an enhanced chemiluminescence detection kit (Amersham-Pharmacia Biotech, Piscataway, NJ).

Telomerase activity assay

Telomerase activity was determined using the TRAPeze telomerase detection kit (Chemicon, Temecula, CA), according to the manufacturer's instructions. Briefly, 10^6 cells were suspended in 200 μl CHAPS lysis buffer and incubated on ice for 30 min. After centrifuging the cells for 20 min at 4°C, the supernatant was transferred into a fresh tube. The protein concentration of the cell extract was determined, and 20 ng protein was used in each 50-μl reaction mixture containing ^{32}P-TS primer end-labeled with [γ-^{32}P] ATP, TRAP primer mix, dNTP mix, 1× TRAP reaction buffer, and Taq polymerase (Promega Corporation, Madison, WI). To add telomeric repeats to the 3'end of substrate oligonucleotides, the reaction mixture was incubated at 30°C for 30 min in a thermocycler. The telomerase products were amplified by PCR (27 cycles of 94°C for 30 sec and 59°C for 30 sec) and separated by electrophoresis in a 12% non-denaturing polyacrylamide gel. The gel was dried and autoradiographed. For positive and negative controls, we used protein extracts from the telomerase-positive cells supplied in the kit and heat-inactivated (85°C for 10 min) positive cell extract, respectively.

Statistical analysis

Student's *t*-test for one-way analysis of variance was used to assess differences between untreated control and auranofin-treated groups. $P < 0.05$ was considered significant.

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