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Selective inhibition of endogenous antioxidants with Auranofin causes mitochondrial oxidative stress which can be countered by selenium supplementation

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ABSTRACT

Auranofin is a thiol-reactive gold (I)-containing compound with potential as a chemotherapeutic. Auranofin has the capacity to selectively inhibit endogenous antioxidant enzymes thioredoxin reductase (TrxR) and glutathione peroxidase (GPx), resulting in oxidative stress and the initiation of a pro-apoptotic cascade. The effect of Auranofin exposure on TrxR and GPx, and the potential for cellular protection through selenium supplementation was examined in the non-cancerous human cell line Swan-71. Auranofin exposure resulted in a concentration dependent differential inhibition of selenoprotein antioxidants. Significant inhibition of TrxR was observed at 20 nM Auranofin with inhibition of GPx from 10 μM. Significant increases in reactive oxygen species (ROS) were associated with antioxidant inhibition at Auranofin concentrations of 100 nM (TrxR inhibition) and 10 μM (TrxR and GPx inhibition), respectively. Evaluation of mitochondrial respiration demonstrated significant reductions in routine and maximal respiration at both 100 nM and 10 μM Auranofin. Auranofin treatment at concentrations of 10 μM and higher concentrations resulted in a ~68% decrease in cellular viability and was associated with elevations in pro-apoptotic markers cytochrome c flux control factor (FCFc) at concentration of 100 nM and mitochondrial Bax at 10 μM. The supplementation of selenium (100 nM) prior to treatment had a generalized protective affect through the restoration of antioxidant activity with a significant increase in TrxR and GPx activity, a significant reduction in ROS and associated improvement in mitochondrial respiration and cellular viability (10 μM ~48% increase). Selenium supplementation reduced the FCFc at low doses of Auranofin (<10 μM) however no effect was noted on either FCFc or Bax at concentrations above 10 μM. The inhibition of antioxidant systems in non-cancerous cells by Auranofin is strongly dose dependent, and this inhibition can be altered by selenium exposure. Therefore, Auranofin dose and the selenium status of patients are important considerations in the therapeutic use of Auranofin as an agent of chemosensitization.

1. Introduction

Auranofin [2,3,4,6-tetra-o-acetyl-1-thio-β-D-glucopyranosato-S-(triethyl-phosphine)gold] is a thiol-reactive gold (I)-containing compound that has been utilized to treat rheumatoid arthritis inflammation, pain and swelling [1]. In this respect, Auranofin mediates basophilic histamine release, limiting the production of leukotriene C4, a critical factor in inflammatory processes [2,3]. Auranofin has also been shown to exert physiological effects as a potent inhibitor of endogenous antioxidants [4,5], capable of modulating oxidative stress *in vivo*. Therefore, Auranofin may be an effective regulator of intracellular signals resulting in cell death. The capacity of Auranofin to increase H_2O_2 concentration [6,7], and resultant increases in oxidative stress, triggers the intrinsic activation of the apoptosis cascade [8,9]. *In vitro* studies have confirmed this pro-apoptotic capacity in ovarian cancer cells resistant to standard chemotherapy [4,10]. As such, Auranofin may prove beneficial in the treatment apoptosis-resistant tumour cells, for patients undergoing chemotherapy [11–13].

The mechanism of action for Auranofin's pro-apoptotic activity is through the selective inhibition of antioxidant enzymes such as thioredoxin reductase (TrxR) and glutathione peroxidase (GPx) [4,5]. Theses endogenous antioxidants are responsible for the maintenance of redox homeostasis and play critical roles in the cytoplasm and mitochondria of all cells. TrxR and GPx are selenoproteins (proteins that incorporate the essential trace element

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selenium), and as such are dependent on an adequate supply of selenium. Selenium is incorporated in the active site of TrxR and GPx enzymes as selenocysteine, and is critical for their catalytic activity. The supply of selenium regulates the production of TrxR and GPx, and many studies in a variety of biological models and human systems have demonstrated the capacity of selenium supplementation to regulate the *in vivo* expression and activity of these selenoproteins [14–18].

Because TrxR and GPx activity is influenced by selenium concentration, the therapeutic potential of Auranofin may be significantly altered by the seleno-status of the patient. In this study we sought to investigate the interplay between the inhibition of TrxR and GPx by Auranofin and the capacity of selenium supplementation to mediate this effect.

2. Material and methods

2.1. Cell culture

The Swan71 cell line (kindly provided by Professor Gil Mor, Yale University) was chosen as a model of highly metabolically active non-cancerous cells. The Swan71 cell line was derived from primary trophoblasts isolated from a normal seven week placenta immortalized via telomerase reverse transcriptase. Swan71 cells were cultured at 37 °C with 5% CO_2 in Dulbecco's Modified Eagles/F12 media (DMEM/F12; Invitrogen, Australia) supplemented with 10% fetal bovine serum (FBS; Gibco, US) and 500 U/mL penicillin-streptomycin (P/S; Gibco, US). Cells were supplemented with 100 nM sodium selenite solubilized in water (Sigma-Aldrich, Australia) or Dulbecco's phosphate-buffered saline (PBS; Sigma-Aldrich, Australia) vehicle control for 24 h. After supplementation, cells were exposed to a range of concentrations of Auranofin (Sigma-Aldrich, Australia; 10 nM, 20 nM, 50 nM, 100 nM, 500 nM, 1 μM, 4 μM, 10 μM, 25 μM, 50 μM, 100 μM) or dimethyl sulfoxide (DMSO; Sigma-Aldrich, Australia) vehicle control for one hour.

2.2. Protein lysate extraction

Cells were maintained in 75 cc flasks and collected by standard trypsinization when 60–80% confluent. Cells were collected and washed in cold PBS then resuspended in 250 μL of cell lysis reagent (100 mM Tris – pH 7.4, 300 mM NaCl, 10% nonyl phenoxypolyethoxylethanol-40, 10% sodium deoxycholate, 1% Sodium dodecyl sulfate, 50 μM phenylmethylsulfonyl fluoride) (Sigma-Aldrich, Australia) and incubated on ice for 15 min. The lysed cell suspension was centrifuged at 17,000*g* for 10 min to pellet cell debris and the protein containing supernatant was collected and stored at −80 °C until required.

2.3. Mitochondrial fractionation isolation

The isolation of a mitochondrial fractions was performed described by Clayton and Shadel [19] with minor modifications. In brief, cells were collected by standard trypsinisation, washed with PBS, and resuspended in hypotonic buffer (Milli-Q filtered water). Cells were allowed 20 min to swell prior to transfer into Douser Homogenizer. Force was applied for three minutes continuously in order to break open cells. The degree of homogenization was visually checked using phase-contrast microscopy. A total of 8 mL of 2.5 X homogenization buffer (525 mM mannitol; 175 mM sucrose; 12.5 mM Tris-HCl (pH 7.5); 2.5 mM EDTA) (Sigma-Aldrich, Australia) was added to the homogenizer prior to covering the top with paraffin and inversion. The homogenate was transferred into a tube for centrifugation, with the homogenizer rinsed with 1 mL of 1 X homogenization buffer (210 mM mannitol; 70 mM sucrose; 5 mM Tris-HCl (pH 7.5); 1 mM EDTA) (Sigma-Aldrich, Australia) and this rinse added to the main homogenate. The resultant homogenate was brought up to a volume of 30 mL using 1 X homogenization buffer before centrifugation at 1300*g* for five minutes. The supernatant was transferred to a new tube and this step was repeated twice. The new supernatant was transferred to a new tube and centrifuged at 17,000*g* for 15 min to pellet mitochondria. The supernatant was transferred to another tube and this was considered the non-mitochondrial fraction. The pellet was resuspended in 1 X homogenization buffer and centrifuged at 17,000*g* for 15 min. The final mitochondrial pellet was resuspended in 80 μL lysis buffer and stored at −80 °C until required.

2.4. Protein quantification

Quantification of protein content in total lysates and subcellular fractions (mitochondrial and non-mitochondrial) was performed using a BCA Protein Assay kit (Pierce, Rockford, USA) following the manufacturer's protocol.

2.5. Biochemical analysis

Antioxidant enzyme activity was measured in protein lysates and standardized to protein content. Thioredoxin reductase activity was determined using a commercially available colorimetric assay (Cayman Chemical Company, Australia) following the manufacturer's instructions. Glutathione peroxidase activity was quantified following the protocol originally described by Flohe & Gunzler [9].

2.6. Cellular viability

Cellular viability was assessed using the MTT (3-(4,5-diemthyl thiazol-2-yl)-2,5-diphenylterazolium bromide) end point assay (Invitrogen, Australia). Briefly, cells were seeded into 96-well plates at 10,000 cells/well and treated as described above with sodium selenite or PBS vehicle control for 24 h, prior to treatment with Auranofin or DMSO vehicle control for one hour. After treatment, each well was washed twice with PBS then MTT solution (50 μL of 1 mg/mL suspended in DMEM/F12 without FBS or P/S) was added and incubated for two hours. A dose of 125 μL of DMSO (Sigma-Aldrich, Australia) was added to each well and incubated for 45 min. The degree of formazan product formation was determined by absorbance read at 560 nm using Multiskan Ascent plate reader with SkanIt software. Each reading was standardized to untreated control cells, and cell viability expressed as a percentage of survival in contrast to untreated control cells.

2.7. Mitochondrial respiration

Confluent cells (in 75 cc flasks) were treated with the optimal enzyme inhibitory concentrations of Auranofin as determined through enzyme activity testing: 100 nM (TrxR inhibition) and 10 μM (TrxR and GPx inhibition). Following incubation, cells were collected and resuspended at 1 million cells/mL in MiR05 mitochondrial respiration medium (110 mM sucrose, 0.5 mM EGTA, 3.0 mM $MgCl_2$ [Sharlab, Spain], 60 mM K-lactobionate, 10 mM KH_2PO_4, 20 mM Taurine, 20 mM HEPES, 1.0 g/l BSA, pH 7.1) (All reagents from Sigma-Aldrich, Australia aside from specified) or DMEM/F12. Mitochondrial respiration was assessed through O_2 consumption measured using the O2k oxygraph (Oroboros Instruments, Austria).

After the addition of cells into the oxygraph chamber, two separate protocols were used in order to assess mitochondrial function

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