CHARACTERIZING THE CYTOTOXIC EFFECT OF THE GOLD COMPLEX AURANOFIN IN EPITHELIAL OVARIAN CANCER CELLS

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Abstract

Drug repurposing is a common strategy used to identify new targets for drugs approved in the clinical setting. This approach is cost-effective, less time-consuming than those involving the synthesis of new drugs and utilizes the biological overlap between diseases to place existing drugs for other purpose use. Drug repurposing could be a viable option to develop a second-line treatment for epithelial ovarian cancer (EOC). EOC is a fatal gynecological disease with a 5-year survival rate of 50%. EOC is comprised of distinct histotypes but the standard of care against EOC histotypes is similar: cytoreductive surgery followed by platinum-based chemotherapy; unfortunately, most patients initially respond to the standard of care, but approximately 80% of patients undergo relapse of the disease and become platinum-resistant, with only 15-20% of patients remaining responsive to a second round of the standard treatment. This calls for novel second lines of treatment that can help improve the overall and progression-free survival of patients with EOC.

In this thesis, we employed the gold complex auranofin originally developed to treat autoimmune diseases and rheumatoid arthritis and repurposed it against EOC. The rationale for the use of auranofin against this disease is based on results from clinical studies in which Kaplan Meir survival analyses demonstrated the existence of an association between the upregulation of the primary target of auranofin, the antioxidant thioredoxin reductase (TrxR), and poor disease prognosis in patients with EOC. Auranofin has been repurposed in other preclinical studies against lung, breast, and colon cancers. Using clinically platinum-sensitive and clinically platinumresistant EOC cells isolated from the same patient, we determined the mechanisms of cytotoxicity triggered by auranofin regardless of platinum sensitivity. We identified the role of auranofininduced cytotoxicity via TrxR inhibition in EOC cells. This was done to potentially use

auranofin as a therapeutic agent that can overcome the platinum resistance that is associated with the advanced stages of the disease.

Using multiple drug combinations with auranofin, such as auranofin and the glutathione inhibitor buthionine sulfoximine (L-BSO) and auranofin and cisplatin, a drug interaction analysis was performed to determine the ability of each drug combination to interact and potentiate their cytotoxicity against EOC cells. We demonstrated the successful potentiation in each of the drug combinations that were tested in the EOC cells. This potentiation was characterized by the disruption of multiple cellular pathways.

Overall, this thesis identifies the efficacy of auranofin alone or in combination with either L-BSO or the platinating agent cisplatin against EOC cells. It also provides a comprehensive understanding of the mechanism of action of the anti-rheumatic drug in EOC cells in the context of platinum resistance.

Resumé

Le repositionnement de médicaments est une stratégie courante utilisée pour identifier de nouvelles cibles pour des médicaments approuvés par la FDA dans un cadre clinique. Cette approche est rentable, moins chronophage et exploite le chevauchement biologique entre les maladies pour utiliser des médicaments existants à des fins multiples. Le repositionnement de médicaments pourrait être une option viable pour développer un traitement de seconde ligne contre le cancer épithélial de l'ovaire (CEO). Le CEO est une maladie gynécologique mortelle avec un taux de survie à 5 ans de 50 %. Il comprend cinq histotypes distincts, parmi lesquels le cancer séreux de haut grade de l'ovaire (CSHGO) est le plus invasif et le plus fréquent en clinique. Le traitement standard contre le CSHGO repose sur une chirurgie cytoréductrice suivie d'une chimiothérapie à base de platine, à laquelle la plupart des patientes répondent initialement. Cependant, environ 80 % des patientes rechutent, et seulement 15 à 20 % répondent au traitement standard après le développement de la résistance au platine. Cela souligne la nécessité d'un traitement de seconde ligne capable d'améliorer la survie globale et la survie sans progression des patientes atteintes de CSHGO.

Dans cette thèse, nous avons utilisé l'auranofine, un complexe à base d'or initialement développé pour traiter les maladies auto-immunes et la polyarthrite rhumatoïde, et l'avons repositionné contre le CSHGO. Une analyse de survie de Kaplan-Meier a révélé une association entre la surexpression de la cible principale de l'auranofine, la thioredoxine réductase (TrxR), un antioxydant, et un mauvais pronostic chez les patientes atteintes de cancer de l'ovaire. L'auranofine a été repositionnée dans des études précliniques contre les cancers du poumon, du sein, du côlon et les cancers de l'ovaire non-CSHGO. À l'aide de modèles *in vitro* de cellules humaines de

CSHGO cliniquement sensibles et résistantes au platine isolées d'une même patiente, nous avons déterminé les mécanismes de cytotoxicité de l'auranofine, indépendamment de la sensibilité au platine. Nous avons identifié le rôle de la cytotoxicité induite par l'auranofine via l'inhibition de TrxR dans le CSHGO, afin d'explorer son utilisation comme agent thérapeutique pour surmonter la résistance au platine associée aux stades avancés du CSHGO.

En combinant l'auranofine avec d'autres médicaments, tels que l'auranofine et le L-BSO ou l'auranofine et le cisplatine, nous avons effectué une analyse des interactions médicamenteuses pour évaluer la capacité de chaque combinaison à synergiser et à améliorer la cytotoxicité contre les cellules de CSHGO et de CEO. Nous avons démontré une potentialisation réussie pour chaque combinaison de médicaments contre le CSHGO ainsi que les carcinomes ovariens clairs résistants au platine et les carcinomes endométrioïdes de l'ovaire. Cette potentialisation a été caractérisée par la perturbation de multiples voies cellulaires.

Dans l'ensemble, cette thèse met en évidence l'efficacité de l'auranofine contre le CSHGO et les cellules de CEO résistantes au platine. Elle fournit également une compréhension approfondie du mécanisme d'action de ce médicament antirhumatismal dans les cellules de CEO dans le contexte de la résistance au platine.

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The past 4.5 years have been an unforgettable experience that instilled in me academic and personal growth. It allowed me to learn so much about myself while meeting great people and making lifelong friends who have contributed to the work described in this thesis.

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Contribution to Original Knowledge

This thesis contains three manuscripts, one of which is a literature review/perspective article, and two of which are original research articles. The literature review in Chapter 2 is the first to combine a brief history of auranofin with its role in cancer, with an updated revision on preclinical studies that use auranofin as a monotherapy and in combination with other drugs against various cancers.

The second manuscript in Chapter 3 is an original research article that is the first to use auranofin as a monotherapy against HGSOC cell lines that represent the early and advanced stages of the disease. Furthermore, we explored the combination of auranofin and L-BSO, a drug combination that has not been tested before in HGSOC.

The third manuscript in Chapter 4 is an original research article that is the first to combine auranofin with cisplatin in EOC cells. This manuscript characterized the efficacy of auranofin to sensitize invasive EOC cells to cisplatin, a combination that has not been previously tested *in vitro* in EOC.

Contribution of Authors

In Chapter 2, FHA prepared the original draft of the manuscript and created the table and the illustrations. CMT supervised the graduate student, provided conceptualization and writing, reviewed the first draft and provided edits.

In Chapter 3, FHA, SS, and CMT contributed to the conceptualization. FHA and ET collaborated on the methodology. EMJ did the MTT assays on auranofin in the PEO1 and PEO4 cells. BF completed the experiments on cisplatin in PEO1 and PEO4 cells. FHA, AAG, EZ and CMT completed data analysis. FHA wrote the original draft of the manuscript, and revision and editing were done by CMT. Critical revision was provided by EZ, and AAG contributed to project administration.

In Chapter 3, the investigation was done by FHA with the collaboration of BF. Data analysis and preparation of the first draft were completed by FHA. The experiments on the hypodiploid content analysis were done by BF. The methodology was optimized by FHA and AAG. Review and editing were completed by BF, EZ, AAG, and ASMN. CMT supervised the graduate student and provided conceptualization, resources, and project administration. The funding was provided by ASMN and CMT.

List of Abbreviations

ΔψM: Transmembrane potential ·OH: Hydroxyl radical

7-AAD: 7-Aminoactinomycin D

AF: Auranofin

AIF: Apoptosis inducing factor

AKT: Protein kinase B

ALL: Acute lymphoblastic leukemia

AML: Acute myeloid leukemia

APC: Antigen presenting cells

ARE: Antioxidant response element ASK: Apoptosis signaling kinase

ATM: Ataxia telangiectasia mutated

ATP: Adenosine triphosphate

BCA: Bicinchoninic acid

BCL-2: B-cell lymphoma 2

BCL-xL: B-cell lymphoma-extra large

BRCA1: Breast cancer gene 1 BRCA2: Breast cancer type 2 susceptibility protein

BSO: l-buthionine sulfoximide

BZ: Bortezomib
CDDP: Cisplatin

CI: Combination index

CLL: Chronic lymphocytic leukemia

CML: Chronic myeloid leukemia

CO2: Carbon dioxide

CRT: Calreticulin DAMPs: Danger- or damage-associated molecular patterns

DEVD: DNA-binding peptide; substrate for caspase-3

DHE: Dihydroethidium DMARS: Disease-modifying antirheumatic drugs

DNA: Deoxyribonucleic acid

DTNB: 5,5′-dithio-bis (2-nitrobenzoic acid)

DTT: Dithiothreitol

DUBs: Deubiquitinases EGFR: Epidermal growth factor receptor eIF2α: α-Subunit of eukaryotic initiation factor 2 (eIF2)

EOC: Epithelial ovarian cancer
ER: Endoplasmic reticulum

FBS: Fetal bovine serum

FDA: US Food and Drug Administration

FOXO3: Forkhead box O3

GLOBOCAN: Global Cancer Observatory

GSH: Glutathione

H2AX: Histone variant H2AX

H2O2: Hydrogen peroxide

HCl: Hydrochloric acid

HDACi: Histone deacetylase inhibitor

HEPES: 4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid

HGSOC: High-grade serous ovarian cancer

HIF1α: Hypoxia-inducible factor 1 alpha

HIV: Human immunodeficiency virus

HMGB1: High mobility group box 1

HRP: Horseradish peroxidase

HSP90: Heat-shock protein of 90 kDa IC50: Half-maximal inhibitory concentration

ICD: Immunogenic cell death

IgG: Immunoglobulin G

IKK: IκB kinase

IL-6: Interleukin-6

IL-8: Interleukin-8

JAK: Janus kinase

JNK: C-JUN N-terminal kinase

MAPK: Mitogen-activated protein kinase

MCL-1: Myeloid cell leukemia 1

mTOR: Mammalian target of rapamycin

MTT:3-(4,5-dimethylliazol-2-yl)-2,5-diphenyltetrazolium bromide

NAC: N-acetyl-L-cysteine

NADPH:Nicotinamide adenine dinucleotide phosphate

NF-kB: Nuclear factor kappa B

NRF2: Nuclear factor erythroid 2-related factor 2

NSAIDs: Nonsteroidal anti-inflammatory drugs

NSLC: Non-small cell lung cancer OD: Optical density

P3Cy5: Phycoerythrin-Cyanine®5

PARP: Poly-adenosine diphosphate (ADP) ribose polymerase

PBS: Phosphate-buffered saline

PD-L1: Programmed death-ligand 1

PD1: Programmed cell death protein 1

PDX: Patient-derived xenograft

PE: Phycoerythrin

PERK: Protein kinase R (PKR)-like endoplasmic reticulum kinase

PFA: Paraformaldehyde

PI3K: Phosphoinositide 3-kinase

PMSF: Phenylmethylsulfonyl fluoride

PS: Phosphatidylserine

PT: Platinum

PTEN: Phosphatase and tensin homolog

ROS: Reactive oxygen species

SDS: Sodium dodecyl sulfate

SCLC: Small-cell lung cancer

SP: Side population

TBS-T: Tris-buffered saline 0.1% Tween 20

TNB: 5′-thio-2-nitrobenzoic acid

TNF: Tumor necrosis factor

TP53: Tumor suppressor protein p53

Trx: Thioredoxin

TrxR: Thioredoxin reductase

TUSC2: Tumor suppressor candidate 2

uPA: Urokinase-type plasminogen activator

UPS: Ubiquitin proteasome system

VEGF: Vascular endothelial growth factor

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CHAPTER 1

General Introduction

1.1 Epithelial Ovarian Cancer

Ovarian cancer is the most fatal gynecological disease [1]. This is largely due to the diagnosis of the disease in the advanced stages and the various regions, apart from the ovaries, from which the cancer originates, for example, most being from the fallopian tube [2, 3]. Ovarian cancer is comprised of epithelial ovarian cancer (EOC), germ-cell ovarian cancer, and sex-cordstromal ovarian cancer [2]. EOC is the most common, making up 95% of ovarian cancers [4]. EOC encompasses five histotypes, including endometrioid, clear cell, mucinous, high-grade-serous, and low-grade-serous, which are characterized by unique mutational profiles and sites of origin [5].

High-grade serous ovarian cancer (HGSOC) is the most common and lethal ovarian cancer histotype in the clinical setting [6]. Contrary to its name, HGSOC originates from the secretory cells of the distal fallopian tube epithelium (FTSECs) [6]. The FTSECs contain a mutation for the tumour suppressor gene *TP53* that is present in 96% of HGSOC tumours [7] and highly expresses the double-strand DNA damage marker γH2AX [8]. These genetic mutations contribute to the development of invasive serous carcinoma from lesions known as serous tubular intra-epithelial carcinomas (STICs) [9]. This invasive subtype is characterized by high copy number variants and increased genomic instability [9]. Aside from *TP53*, other genetic mutations that are less prevalent in HGSOC include *BRCA1* and *BRCA2,* which are present in 12.5% and 11.5% of cases, respectively [7]. *BRCA1* and *BRCA2* are tumour suppressor genes that play a role in DNA damage repair and contribute to the regulation of genes involved in cell cycle and apoptosis [10]. Furthermore, HGSOC contains frequent focal amplifications of *CCNE1* involved in cell cycle progression, tumour suppressor *MYC,* and transcriptional regulator *MECOM* [7, 11-13].

Although this disease is characterized by genomic instability and a mutational profile, patients are unlikely to undergo genetic testing unless they have a hereditary risk for *BRCA1* or *BRCA2* mutations. The current methods used for the screening of this disease include the measurement of CA-125 serum levels, which follow up disease recurrence after initial chemotherapy but it does not impact ovarian cancer mortality rates [14]. The lack of sensitive screening tests for this disease contributes to the short overall and progression-free survival of patients. Aside from the genomic instability that characterizes this disease, the dissemination of HGSOC within the peritoneal cavity and the production of pleural effusions add to its invasive characteristics. Metastasis of HGSOC occurs in the adipose tissue of the omentum, where it forms solid tumours [15]. Cytoreductive or 'debulking' surgery is the first intervention used to eliminate tumour masses that are present within the peritoneal cavity and the removal of reproductive organs [9]. Due to most patients with HGSOC being diagnosed at advanced stages of the disease, they are required to undergo platinum-based chemotherapy following the invasive procedure.

1.2 Platinum-Based Chemotherapy

Platinum-based chemotherapy for EOC was introduced in the late 1970s with the approval of cisplatin [16]. Thereafter, with the discovery of paclitaxel in the 1990s, the current standard form of care became platinum-taxane combination therapy with the platinum agent being the essential component and paclitaxel being a potentiator [16]. HGSOC is initially more responsive to platinum-based chemotherapy in comparison to other subtypes of EOC [17]. However, approximately 75% of patients will undergo disease recurrence following the initial round of chemotherapy [17]. Patients who relapse 6 months following the first treatment with platinum-based chemotherapy are deemed resistant and have less than 10% response rate to a second round

of treatment [17]. Unfortunately, around 80-90% of patients diagnosed at the advanced stages of the disease will undergo platinum resistance [18]. Although there have been various clinical studies on the use of targeted therapies against ovarian cancer, there have yet to be treatments discovered that improve the overall survival rate of patients with this disease. This highlights the need for feasible drug options to be used for second-line treatment against HGSOC.

1.3 Auranofin

Auranofin is a gold complex that was approved by the FDA in 1985 as the first oral treatment against rheumatoid arthritis [19, 20]. Auranofin was given as a chronic treatment following drugs that were initially used to alleviate the pain caused by rheumatoid arthritis. The mechanism of action of auranofin was to reduce the inflammatory response of the autoimmune disease by inhibiting the recruitment of immune cells to the inflamed region [21]. Auranofin primarily acted as an anti-inflammatory drug through various mechanisms, including inhibiting lysosomal enzymes and pro-inflammatory cytokine secretion [19, 22]. Other drugs, for example, ones that specifically inhibit cytokine release, have replaced auranofin as the chronic treatment of rheumatoid arthritis [23]. Auranofin was also replaced in treating rheumatoid arthritis for commercial reasons, which was not as profitable in the market as other anti-rheumatic drugs [24]. However, as an inflammatory drug, auranofin is a promising therapeutic agent against cancer, which involves pro-inflammatory reactions that promote tumorigenesis [25]. This renders auranofin a potential option for drug repurposing against cancer.

Drug repurposing is a commonly used approach that employs drugs that are already approved in the clinic, which can target new pathways [26]. This is a cost-effective strategy which also saves time from employing new agents against complex diseases [26]. In addition, the concept

of developing a new molecule or drug for a single target does not consider the redundancy of molecular pathways among different diseases. More specifically, there is an overlap in the biological pathways that give rise to different diseases, which can be strategically targeted by single agents [26]. In addition to the anti-inflammatory functions of auranofin, extensive studies on the pharmacological functions of the drug revealed that it targets the antioxidant enzyme thioredoxin reductase (TrxR) [27]. TrxR helps to maintain the levels of oxidative stress within the cell to keep the levels of reactive oxygen species (ROS) below the threshold of what cells can tolerate for survival [28]. The upregulation of TrxR has been implicated in cancer, including ovarian cancer [29].

Normal cells undergo metabolic reactions that produce oxidative radicals as a by-product, disrupting the oxygen homeostasis within the cell [29]. This results in the production of ROS, which, at normal levels, are beneficial for the cell in signal transduction and regulation of transcription factors through activating NF-κB [30]. However, in events where the cell produces excess ROS, antioxidant response elements (ARE) are activated by the ROS-sensitive transcription factor Nrf2 [29]. This activates antioxidant systems within the cell to diminish the levels of toxic radicals and maintain only a beneficial amount of oxidative stress. However, cancer cells overproduce ROS because of increased metabolism that promotes cancer cell proliferation and tumorigenesis. As a result, cancer cells upregulate antioxidant systems to maintain ROS levels below the threshold to enable survival [29]. These findings suggest that auranofin is an attractive anti-cancer agent.

1.4 Hypothesis and Objectives

The objectives of this study are to characterize the role of auranofin in high-grade serous ovarian cancer (HGSOC) cells and to explore the interaction between auranofin and cisplatin in platinum-resistant EOCs to develop a second-line treatment for this disease. The final goal of this work is to provide preclinical evidence that can be used in clinical trials aiming to ultimately improve the survival rate of patients with HGSOC and platinum-resistant EOC. The first objective was to characterize the cytotoxic effects of auranofin in two HGSOC cell lines. The first cell line is clinically platinum-sensitive and represents the early stage of the disease, and the second cell line is clinically platinum-resistant and represents an advanced stage of the disease. The second aim of this study was to explore the interaction of auranofin and the pro-oxidative agent plus glutathione inhibitor, L-buthionine sulfoximine (L-BSO), in HGSOC cells. Finally, the third aim of this study was to explore the interaction between auranofin and cisplatin in spontaneously platinum-resistant endometrioid ovarian carcinoma cells and in clear-cell ovarian carcinoma cells which underwent prolonged *in vitro* exposure to cisplatin to achieve platinum resistance. It was first hypothesized that auranofin targets HGSOC cells regardless of platinum sensitivity through the inhibition of TrxR and synergizes with L-BSO in cancer cells. Secondly, it was hypothesized that auranofin sensitizes platinum-resistant EOC cells to cisplatin through a synergistic interaction by the drug combination.

CHAPTER 2

Literature Review

2.1 Preface

Many cancers develop resistance to their standard of care treatments, resulting in short overall survival and poor disease prognosis. A current common strategy to combat this issue is to repurpose drugs that are already approved by the US Food and Drug Administration (FDA) as a cost-effective and efficient way to develop long-term treatments against cancer. Drug repositioning allows for the identification of new targets for drugs that are already in the clinic. In the context of our research on EOC, we have selected the drug auranofin under the brand name Ridaura for the drug repositioning against this disease. First, we wanted to conduct an extensive literature review of auranofin and its present application in preclinical studies and clinical trials.

Auranofin was originally developed as an anti-inflammatory drug against rheumatoid arthritis, in which it inhibits the immune response. More specifically, auranofin acts as an immune suppressor by inhibiting immune cell infiltration and pro-inflammatory cytokine secretion. Aside from managing the immune response, the primary mechanism of action of auranofin is to inhibit the antioxidant enzyme thioredoxin reductase (TrxR), resulting in the overproduction of ROS within the cancer cell. Upregulation of TrxR expression is associated with poor disease prognosis in several cancers, including ovarian cancer. The combined targeting of TrxR and anti-inflammatory effects by auranofin render this drug a suitable agent against cancer. Cancer cells undergo increased metabolism that promotes the growth and increased proliferation of cancer cells, resulting in high levels of ROS. To tolerate the high levels of ROS, cancer cells upregulate antioxidant systems to endure oxidative stress and prevent cell death. The inhibition of TrxR would surpass ROS levels beyond the threshold of what cells can endure and survive. In addition to its role in promoting oxidative stress, auranofin elicits cytotoxic effects in various cancers through inhibition of glycolysis, induction of DNA damage, disruption of the ubiquitin-proteasome system, and more.

Auranofin has been reported to be successful in various cancers due to the range of ROS-mediated mechanisms of killing that the drug uses.

Due to the promising preclinical studies of the anti-rheumatic drug in cancer, we also mention the enrollment of auranofin in clinical trials. Auranofin has been enrolled in clinical trials as a monotherapy to treat different kinds of leukemia and ovarian cancer. However, the histotype of ovarian cancer patients who were enrolled in the trial was not specified. Furthermore, there are several clinical trials of auranofin in combination with sirolimus, an anti-fungal and immunosuppressant [31], against ovarian cancer and small-cell and non-small-cell lung cancer.

As this literature review also acts as a perspective article, we proposed a new approach to combine auranofin with platinum-based chemotherapy to treat cancer. The goal of this combination is to target cancer through two methods which aim to enhance cytotoxicity against cancer. First, the combination can enhance the killing of cancer cells via ROS-induced DNA damage since auranofin has been reported to induce DNA damage, and the platinating agents predominantly cause DNA damage. Second, we anticipate that auranofin will elicit immunogenic cell death. Although we start the review by discussing how auranofin regulates the immune response to reduce inflammation, there is an interesting study on auranofin inducing the release of markers that characterize immunogenic cell death in non-small lung carcinoma. Overall, this review helped us develop a better understanding of the role of auranofin in certain types of cancer and the gaps in the literature on studying auranofin in HGSOC and EOC. We used this as an opportunity to help us design our research questions in addressing the role of auranofin in EOC.

The Gold Complex Auranofin: New Perspectives for Cancer Therapy

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2.2 Abstract

Advanced stages of cancer are highly associated with short overall survival in patients due to the lack of long-term treatment options following the standard form of care. New options for cancer therapy are needed to improve the survival of cancer patients without disease recurrence. Auranofin is a clinically approved agent against rheumatoid arthritis that is currently enrolled in clinical trials for potential repurposing against cancer. Auranofin mainly targets the anti-oxidative system catalyzed by thioredoxin reductase (TrxR), which protects the cell from oxidative stress and death in the cytoplasm and the mitochondria. TrxR is over-expressed in many cancers as an adaptive mechanism for cancer cell proliferation, rendering it an attractive target for cancer therapy, and auranofin as a potential therapeutic agent for cancer. Inhibiting TrxR dysregulates the intracellular redox state causing increased intracellular reactive oxygen species levels and stimulates cellular demise. An alternate mechanism of action of auranofin is to mimic proteasomal inhibition by blocking the ubiquitin–proteasome system (UPS), which is critically important in cancer cells to prevent cell death when compared to non-cancer cells, because of its role on cell cycle regulation, protein degradation, gene expression, and DNA repair. This article provides new perspectives on the potential mechanisms used by auranofin alone, in combination with diverse other compounds, or in combination with platinating agents and/or immune checkpoint inhibitors to combat cancer cells, while assessing the feasibility for its repurposing in the clinical setting.

Keywords: Auranofin, cancer, Thioredoxin reductase, immunogenic cell death, cisplatin

2.3 Main Text

Auranofin: a brief history

Auranofin is a gold (I)-containing compound that was approved by the United States Food and Drug Administration in 1985 as a primary treatment against rheumatoid arthritis [32]. Gold compounds were early on used to treat tuberculosis and other diseases including syphilis and even psychiatric conditions [33]. Originally, gold compounds were selected as a form of treatment against rheumatoid arthritis because the disease was first thought to be caused by mycobacterium tuberculosis. The original gold compounds were highly toxic, until (2,3,4,6-tetra-O-acetyl-1-thiob-D-glucopyranosato-S) (triethylphosphine) gold (I) was synthesized: it was termed auranofin **(Fig.2.1)** [32, 34]. This drug is a metal gold (I) complex that acts as a prodrug, which is metabolized to their pharmacologically active derivatives after being given to the patient. The drug complex consists of two parts, a water-soluble aurothioglucose entity with a sulfur donor group, and a phosphine ligand that provides lipophilicity. The drug undergoes irreversible oxidation of the thioglucose tetraacetate accompanied by hydrolysis, leading to progressive deacetylation forming two main active forms: a triethylphosphinenegold (I) cation, and a gold (I) thioglucose species, with a variable number of acetyl groups [35]. Due to its chemical nature, auranofin has high selectivity for sulfur and selenium ligand proteins with exposed free cysteines, which are modified through coordination of the gold-triethylphosphine fragment [36, 37]. For instance, classic proteomic strategies and mass spectrometry-based redox proteomics demonstrated that auranofin affects proteins primarily involved in cell redox homeostasis and oxidizes over five hundred cysteine-containing peptides [38, 39]. The drug is administered orally for the treatment of active progressive rheumatoid arthritis and is commercialized under the brand name of Ridaura [40].

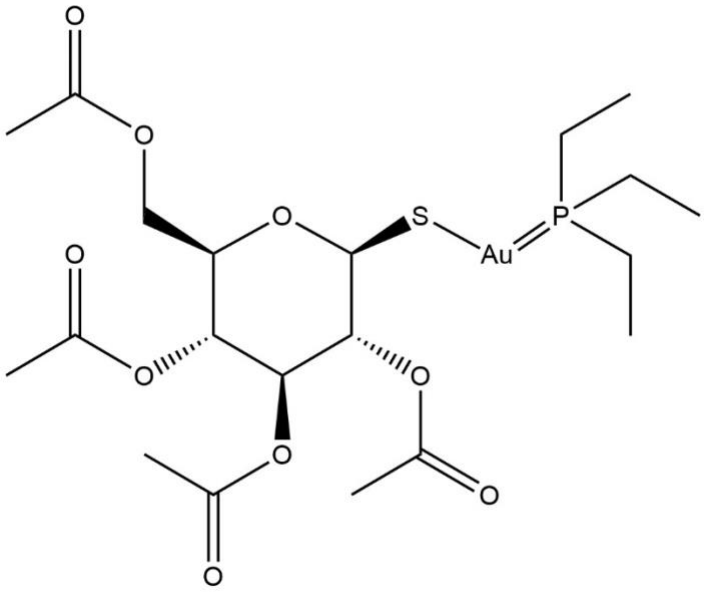

Figure 2.1. Structural formula of auranofin. The molecule consists of a monomeric linear complex with triethylphosphine and thiolate moieties bounded to an Au (I) center [41]. The molecule was adapted from the structure published in the CheBI database [42] using ChemDraw software.

Rheumatoid arthritis is a systemic, chronic, polyarticular inflammatory autoimmune disease that primarily targets the joints and causes increased swelling and inflammation in hands and feet. The disease has no cure and treatment goals are to reduce the pain and slow down further joint damage. Acute symptoms of the disease can be treated with nonsteroidal anti-inflammatory drugs (NSAIDs), such as acetylsalicylate, naproxen or ibuprofen, to alleviate pain, swelling and decrease inflammation. Corticosteroids are also used as more potent anti-inflammatory medications for acute symptoms yet with greater side effects. For the second line or chronic treatment, the gold complex auranofin was originally used to ameliorate the progression of the disease; however, the drug was displaced by a plethora of compounds jointly termed diseasemodifying antirheumatic drugs or DMARDs including methotrexate, hydroxychloroquine,

and sulfasalazine, all considered as synthetic immunosuppressive DMARDs. In addition, newer medications termed biological DMARDs are currently used, in combination with methotrexate, and consist mainly of inhibitors of cytokine targets such as TNF inhibitors, IL-6 and IL-1 receptor inhibitors, and JAK signaling inhibitors that prevent the recruitment of cells that cause a proinflammatory cascade associated with rheumatoid arthritis [43-47].

The major mechanism of action of auranofin against rheumatoid arthritis is to manage the autoimmune response via the inhibition of immune cell infiltration to the site of inflammation. This occurs via the suppression of T cell mitogenesis and macrophage cytotoxicity. Of interest, auranofin was shown to inhibit leukocytes and macrophages in a greater extent on cells drawn from patients with active rheumatoid arthritis [48]. Auranofin appears to be effective in decreasing lysosomal enzyme release; for instance, the gold in auranofin is concentrated within lysosomes of tissue macrophages. These lysosomal bodies were found in chondrocytes, synovial membrane cells, and the subsynovial macrophages of the joint. The lysosomal bodies containing soluble gold compounds have a particular morphological pattern, and have been early on termed ‘aurosomes’ [49]. Auranofin has been shown to be a potent inhibitor of lysosomal enzymes [50], including bglucuronidase, lysozyme, acid phosphatase, and cathepsin, which have been shown to be enriched in the synovial fluid of patients with rheumatoid arthritis [19]. Auranofin also indirectly inhibits the secretion of pro-inflammatory cytokines, such as IL-8 and IL-6 from macrophages and monocytes, by inhibiting the NF-kB signaling pathway [51-54]. Furthermore, auranofin suppresses the immune response via the inhibition of antibody-dependent complement lysis and chemotaxis, or migration of monocytes in the bloodstream to phagocytose the arthritic cells [50]. Auranofin was also reported to inhibit prostaglandin synthesis, suppress the stimulatory action of prostaglandins [19], inhibit platelet aggregation [55], and inactivate the complement [56]. These

functions and more recent investigations [57] demonstrate the role of auranofin against chronic inflammation, which poses it as a strong agent against various diseases, such as cancer, in which pro-inflammatory reactions readily occur and promote all stages of tumorigenesis within the tumor microenvironment [25].

Auranofin is a pro-oxidative agent targeting the thioredoxin reductase system

A primary mechanism of action discovered for auranofin is acting as a pro-oxidant agent by disrupting the reduction/oxidation (redox) system within the cell. This occurs via the inhibition of thioredoxin reductases (TrxRs) represented by two selenoenzyme isoforms, a cytoplasmic form or TrxR1, and a mitochondrial form or TrxR2. They act as antioxidants regulating the levels of reactive oxygen species (ROS), thus protecting the cells from the deadly consequences of damage due to oxidative stress [47, 58].

TrxRs have a redox active selenocysteine moiety [33, 59]; mass spectrometry studies suggest that TrxRs bind to approximately four triethylphosphinegold (I) cation (AuPet3+) fragments, while biochemical assays show that the gold compound greatly alters the active selenocysterine site of the enzymes [60-64]. It seems that the cytotoxicity of auranofin is related to the inhibition of both, cytoplasmic TrxR1 and mitochondrial TrxR2 [60]. TrxRs act in a nicotinamide adenine dinucleotide phosphate (NADPH)-dependent manner, by transferring electrons from NADPH to the active disulfide site on the oxidized Trx protein [65, 66]. The interaction between the active site dithiol in reduced Trx and oxidized cysteines of many proteins induces the process of thiol/disulfide exchange reaction to form an oxidized Trx; in this manner, Trx catalyzes the reduction of ROS from oxidized cysteines of proteins and in the process, Trx itself becomes oxidized [29, 67, 68].

The presence of cytoplasmic Trx1 in its reduced state is critical due to its multifunctional role in cellular processes such as inhibition of cell death via the binding of reduced Trx1 to apoptosis signalling kinase 1 (ASK1). The oxidation of Trx1 by ROS leads to the dissociation of Trx1 from the pro-apoptotic molecule ASK1 thus activating ASK1-induced cell death via the cJUN N-terminal kinase (JNK) and p38 MAP kinase pathways in the cytoplasm [58]. Trx1 is also involved in cell growth and proliferation by inhibiting phosphatase and tensin homolog (PTEN) and increasing AKT activity [69]. Finally, it was shown that Trx1 translocates from the cytoplasm to the nucleus and activates a number of transcription factors including NF-kB and tumor suppressor p53 [67]. Based on these cellular effects of Trx1, it is expected that the function of auranofin in inhibiting TrxR1 would lead to increased ROS, promotion of ASK-induced apoptosis, and blockage of cell growth, proliferation, and survival due to reduced AKT activity and NF-kB- and p53-mediated transcription.

Thioredoxin reductase 1 is over-expressed in cancer cells in association with decreased patient survival

The overexpression of TrxR1/Trx1 has been shown in breast, ovarian, colorectal, lung, pancreatic, and gastric cancers [68, 70-73]. Of interest, TrxR1 overexpression was detected in aggressive mammary tumors, in comparison with non-aggressive tumors [74]. Another interesting analysis was completed using an online public genomic database that indicated an association between the expression of the TrxR1 isoform and ovarian cancer prognosis. A Kaplan Meir survival curve was generated on a sample of over one-thousand ovarian cancer patients, which were grouped separately based on high and low TrxR1 expression; results showed that there was a significant difference in the overall survival of patients in the high TrxR1 expression group in

comparison to the low TrxR1 expression group; a hazard ratio of 1.5 indicated that patients with a high TrxR1 expression have 1.5 times chances of reaching a fifty percent overall survival in a shorter time than patients in the low TrxR1 expression group [68]. This signifies that TrxR1 overexpression is closely associated with tumorigenesis and poor prognosis of the disease. Consequently, this upregulation makes auranofin an interesting anti-cancer molecule as target of the TrxR1/Trx1 system [68]. The upregulation of TrxR1/Trx1 allows the maintenance of a normal redox balance in the context of high metabolism of the cancer cell, which because of its nature produces higher ROS levels and maintains a thither antioxidant control than non-cancer cells. The upregulation of TrxR1 occurs through the activation of Nrf2, a redox-sensitive transcription factor stimulated by oxidative stress, upon which it translocates to the nucleus and activates several antioxidant response elements (ARE)-regulated genes, including TrxR1 [29, 75, 76]. It is anticipated that auranofin causes the accumulation of overwhelming cellular levels of ROS that surpass the antioxidant buffering capacity of the cancer cells leading to their demise because of DNA damage [58, 75, 77-79]. Another important finding about auranofin that opens its use as an anticancer drug is the amplitude of cancers it may target considering that a large number of cancers carry a p53 gene mutation, while auranofin inhibits TrxR1 in a p53-independent manner [80].

Drugs that have been developed for other uses are also capable of inhibiting the TrxR1/Trx1 system. One is the case of cisplatin, a highly popular and first metal-based anti-cancer drug [81] that inhibits TrxR1 in an irreversible manner [82]. Cisplatin is a DNA-damaging agent known to cause cell death via direct toxicity towards the mitochondria, and by hybridizing the DNA forming DNA cross-links that are difficult to repair, leading to cell death [83, 84]. Another group of compounds that inhibit TrxR1 are histone deacetylase inhibitors (HDACi), which target cancer cells by their chromatin modifying effects [85]. One HDACi termed SAHA inactivates the function of Trx1 by binding to it, thereby leading to oxidative stress and induction of apoptosis [67].

Furthermore, in lung cancer cells, it was detailed that SAHA induced the down-regulation of Trx1, leading to the activation of ASK, which induces apoptotic cell death by triggering the ASK-JNK or ASK-p38 kinase pathways [86].

Preclinical evidence of auranofin monotherapy eliciting anti-cancer effects

Increasing evidence on the preclinical mechanistic effects of auranofin against cancer cells was mounted in the last ten to fifteen years, particularly based on the capacity of the compound to increment the oxidative stress environment within the cancer cells. For instance, a study reveals that head and neck squamous carcinoma rely heavily on Trx1 for survival. This was confirmed by treating the head and neck cancer cells with auranofin plus or minus the ROS scavenger N-acetyl cysteine (NAC), which ameliorates ROS-induced DNA damage [87, 88]. Pre-treatment with NAC counteracted the cancer cell killing effects of auranofin, indicating that regulation of ROS is a primary mechanism used by head and neck cancers to multiply and spread [89]. In another study, auranofin inhibited the proliferation of mesothelioma cells in a dose-dependent manner; the anticancer effect was associated with caspase-independent apoptosis and necrosis, and increased ROS levels. The toxicity of ROS in these cells was demonstrated by the fact that NAC also prevented auranofin-induced lethality [90]. Of interest, the DNA damage caused by auranofin-induced ROS seems to be favored by increased membrane fluidity as shown in ovarian cancer cells with higher membrane fluidity (IGROV-1) being more sensitive to auranofin than ovarian cancer cells with lower membrane fluidity (OVCAR-5) [91]. Altogether, these evidence emphasize that ROS regulation plays a primary role in cancer cell survival and proliferation, and further supports the possible repurposing of auranofin against various cancers due to the pro-oxidative mechanism it triggers.

One of the major pathways that auranofin targets is the PI3K/AKT/mTOR pathway. This pathway is involved in the regulation of cell proliferation, apoptosis, and angiogenesis, and it is commonly associated with disease progression and tumorigenesis. Evidence shows that auranofin induces cytotoxicity in human pancreatic adenocarcinoma and non-small cell lung cancer via the inhibition of the PI3K/AKT/mTOR pathway [92]. The anti-cancer effect of auranofin was also analyzed in a human pancreatic adenocarcinoma *in vivo* model, a type of cancer in which proangiogenic factors, hypoxia-inducible factor 1α (HIF-1α), and vascular endothelial growth factor (VEGF), are overexpressed [93]. This study revealed that auranofin inhibits pancreatic tumor growth at the primary tissue site and inhibits metastasis in distant organs as well. Additionally, auranofin inhibits the cancer cell response to hypoxia, demonstrated by a decrease in HIF-1α expression and VEGF secretion upon auranofin treatment under hypoxic conditions [93]. The authors conclude that this decreased expression of HIF-1α induced by auranofin is possibly due to the inhibition of AKT, which normally helps stabilize HIF-1α via VEGF production [93].

Another mechanism of action that auranofin uses to elicit cytotoxicity in cancer is by disrupting protein homeostasis. The action of auranofin in the inhibition of protein homeostasis was detected in HepG2 liver hepatocellular carcinoma cells and MCF-7 breast cancer cells [94]. In this study, auranofin induced apoptosis in HepG2 and MCF-7 cells by inhibiting the proteasome, a protein complex that degrades damaged or misfolded proteins [95]. In ovarian cancer cells, it was also shown via a proteomic analysis, that auranofin highly downregulated the expression of proteasome-related proteins [96]. The cytotoxic effect induced by auranofin has also been shown to be dependent on the inhibition of proteasome-associated deubiquitinases (DUBs), which are associated with cell growth and cancer progression [97]. These proteasome-associated DUBs are attractive therapeutic targets due to their role in regulating protein homeostasis within the cell. The

inhibition of DUBs by auranofin results in the inhibition of tumor growth in mice with HepG2 and MCF-7 tumors [94]. Additionally, the cytotoxicity of auranofin by inhibition of proteasomeassociated DUBs was also observed in chronic myeloid leukemia (CML) [98]. These findings are significant because auranofin can be considered one of the first DUB inhibitors already been in clinical use.

Aside from the evidence that auranofin targets different pathways within the cancer cell, the drug has been shown to target cancers of various genetic backgrounds. For instance, auranofin was shown to induce caspase-3-mediated apoptosis in human ovarian carcinoma SKOV-3 cells, which are deficient in the p53 tumor suppressor gene; this work also reported that auranofin toxicity was dependent on FOXO3 expression [99]. The efficacy of auranofin regardless of p53 functionality was also observed in p53 mutant refractory B-cell lymphoma, which happens to also carry a deletion in the tumor suppressor PTEN [100]. Another study using ovarian cancer cells demonstrated that the sensitivity of the cells to auranofin is enhanced by BRCA1 deficiency; BRCA1 is involved in DNA repair and in regulating the stability of antioxidant transcription factor Nrf2 via protein-protein interaction. BRCA1 deficient cells are usually more susceptible to oxidative stress. The BRCA1 deficient cells treated with auranofin had increased DNA doublestrand breaks, while the overall lethality of auranofin was prevented by NAC, indicating ROSmediated damage [101].

Another study highlights the efficacy of auranofin regardless of the sensitivity of the cancer cells to platinum drugs, one of the most widely used anti-cancer agents [84]. Auranofin induced cell death in a pair of sibling ovarian cancer cell lines that are either sensitive (OV2008) or resistant (OV2008/C13) to cisplatin [61]. The efficiency of auranofin against the cisplatin resistant cells was attributed to increased TrxR1 activity associated with the acquisition of resistance [61]. These findings signify that auranofin could be used to overcome platinum resistant diseases. For instance,

we recently demonstrated that auranofin is equally potent in inhibiting the function, growth, and viability of high-grade serous ovarian cancer cells termed PEO1, which were obtained from a patient when platinum sensitive, than of PEO4 cells, which were obtained from the ascites of the same patient when she became platinum resistant following treatment [102-104]. We showed that auranofin-induced cytotoxicity was also associated with apoptotic cell death and required the induction of oxidative stress, as the lethality was prevented by the antioxidant NAC [104].

It is important to mention that novel TrxR1 inhibitors have been discovered eliciting similar mechanisms than auranofin against cancer cells. For instance, a small molecule MJ25, which was developed to increase the level of p53-dependent transactivation in malignant melanoma cells, was also identified as an irreversible TrxR1 inhibitor. In these cells, MJ25 and auranofin were both lethal at low concentrations and their effectiveness were ameliorated by supplementation of selenium, demonstrating the selenoprotein dependency of the lethal effect of the compounds [105]. In this study the authors also demonstrated that if they further depleted the cells of their antioxidant systems, but abrogating intracellular glutathione (GSH) levels with L-buthionine sulfoximide (BSO), co-treatment with MJ25 or auranofin led to complete cellular eradication [105] demonstrating that simultaneous depletion of the glutathione and TrxR antioxidant systems led to enhanced cytotoxicity [75, 76, 90, 106]. Very recently, it was shown in acute lymphoblastic leukemia (ALL), the most relevant pediatric cancer, that auranofin had a selective anti-cancer activity upon a screen of a library of 3,707 approved drugs and pharmacologically active compounds; auranofin killed ALL cells via increasing ROS in the context of highly reduced levels of antioxidant glutathione [107].

Another compound that operates similarly to auranofin blocking the TrxR1/Trx1 system is piperlongumine, which is an alkaloid isolated from the fruit of the long pepper; results show that piperlongumine induces a lethal endoplasmic reticulum (ER) stress response by increasing the

levels of ROS thus causing apoptosis [108]. Other new TrxR1 inhibitors result from the synthesis of hybrid compounds among azelaic acid (AZA) and organic arsenicals. One of these derivatives, termed A-Z2 [for N-(4-(1,3,2-dithiarsinan-2-yl) phenyl)-azelamide], shows stronger activity against TrxR1 activity in acute myeloid leukemia (AML) cell lines than did AZA or arsenicals separately. The compound activates the intrinsic apoptotic pathway by selectively targeting TrxR1/Trx1 and indirectly inhibiting NF-kB; the compound also demonstrated efficacy *in vivo* against a patient-derived xenograft (PDX) AML model [109].

Anti-cancer effects of auranofin derivatives

Various gold (I) phosphine complex derivatives were developed since the anticancer properties of auranofin were revealed. These compounds incorporate a variety of ligands aiming to increasing the anticancer activity of the complex, but without removing the triethylphosphine moiety, which is critically important to maintain the cytotoxic potency of the molecule [33, 110]. Thus, a gold (I) complex with chelated diphosphines such as $[Au(dppe)_2]Cl$ was synthesized and showed high toxicity against various cancer types [111]; yet, this compound never reached the clinic due to unfavourable preclinical toxicity studies [33] Next, a related compound that included the propyl-bridged 2-pyridyl phosphine ligand (d2pypp) identified as $[Au(d2pypp)_2]^+$ was developed with the idea of enhancing the reactivity against TrxR while increasing its accumulation in the mitochondria [112]. This compound showed selectivity against breast cancer cells while sparing normal breast cells [113]. Other derivatives with anticancer effect are the lipophilic cationic gold (I) phosphine complex [Au(dppp)(PPh3)Cl] [114], the hydrophilic four-coordinate complex $[Au(P(CH_2OH_3)_4]Cl$ [115], and the highly lipophilic cationic Au (I) N-heterocyclic carbene complexes (NHC) of the form $[R_2Im)_2Au]^+$[116]. Two structurally related gold (I)-Nheterocyclic carbenes complexes tested in ovarian cancer cells show, similarly to auranofin, a potent inhibition

of TrxR, yet display differences in the magnitude of protein regulation as assessed by comparative proteomic analysis; the dicarbene derivative showed many more proteins regulated than its monocarbene counterpart, while displaying higher antiproliferative properties in three different ovarian cancer cell lines [117].

Potent and selective TrxR inhibition was also achieved by a series of linear gold (I) compounds resembling auranofin, all containing the $[Au(PEt_3)]^+$ synthon with different ligands—Cl^-, Br^-, cyanate, thiocyanate, ehtylxanthate, diethyldithiocarbamate or thiourea—and having antiproliferative effects towards multidrug-resistant and platinum-resistant cancer cells [118]. More recently, the derivatives of $[Au(PEt3)]^+$ with iodide or chloride ligands (also termed Et3PAuI and Et3PAuI) were shown to have high cytotoxicity against colorectal cancer cells *in vitro* and *in vivo* and with a potency similar to that of auranofin, while suggesting that the presence of the thiosugar moiety in auranofin is not mandatory for the pharmacological action [119]. Moreover, in an orthotopic mouse model of ovarian cancer, it was demonstrated that not only replacement of the thiosugar in auranofin is not required for its pharmacological function, but that its iodide analogue, the idodide (thiethylphosphine) gold I complex (or Et3PAuI), has much higher anticancer potency than that of auranofin while being well tolerated [120]. Such replacement of the thiosugar with iodine involves a higher reactivity of the molecule towards certain amino acids such as histidine, cysteine, methionine, and selenocysteine, which could explain the enhanced antitumoral activity of this iodide analogue of auranofin [121]. Furthermore, Et3PAuI demonstrated its efficacy against ovarian cancer cells resistant to platinum, yet it retains the crossresistance towards auranofin in auranofin-resistant ovarian cancer cells [122]. Other analogues of auranofin where synthesized with the general formula $Au(Pet_3)X$, where X is a pseudohalide group (cyanide, thiocyanate, or azide) that replaces the thiosugar in auranofin; the compounds have biological activity yet showed lesser cytotoxicity than auranofin towards colon cancer cells [123].

Auranofin anti-cancer activity in combination treatments

In human lung cancer, auranofin induces cytotoxicity via ROS production in stem cell-like cancer cells, a side-population (SP) that has high expression of ATP-binding cassette transporter 2 (ABCG2) [124]. SP cells are significant because they are able to develop drug resistance due to increased drug export via ABCG2. Interestingly, it was found that auranofin inhibits ABCG2 function by depleting cellular ATP via inhibition of glycolysis [124]. The disruption in cellular ATP levels by auranofin leads to the inhibition of ABCG2 function in drug export, preventing the SP cells from developing drug resistance. This suggests that auranofin can be used to improve the efficacy of other drugs by inhibiting the drug-resistant mechanism of SP cell populations. This is demonstrated in human lung cancer cells, in which auranofin synergizes with adriamycin to kill the cancer cells more potently than when the cells are treated with adriamycin alone [124]. Also in lung cancers auranofin was shown to be highly efficient when the glutathione antioxidant system, which usually complements the thioredoxin system, is compromised; this is a case of synthetic lethality in which auranofin is highly cytotoxic by inhibiting TrxR in a background of glutathione deficiency [125].

Of interest, cancer cells can develop resistance to auranofin by reduced drug accumulation caused by the dysregulation of influx and efflux drug transporters, as demonstrated in ovarian cancer cells that were made approximately 20-fold resistant to the gold I complex upon stepwise exposure of a parental cell line to increasing auranofin concentrations during an 8-month selection period [126]. The development of resistance to auranofin seems to be very specific as the resistant cells retained sensitivity to other investigational gold compounds, as well as to approved chemotherapeutics such as oxaliplatin, vinblastine, doxorubicin, etoposide, and paclitaxel [126].

In malignant B-cells, one study demonstrates that when exposed to high concentrations of L-ascorbate, the cells die due to autoxidation and generation of H_2O_2; this mechanism is

ameliorated by the counterbalancing activation of the Trx1 system. However, when cells were exposed to L-ascorbate in combination with auranofin, the H_2O_2 scavenging capacity of malignant B-cells was depleted. Of interest, in this combination therapy, the lethality was associated with the accumulation of radical hydroxyl ($^{\cdot}OH$) [127], generated by the catalysis of Fe^{2+} into Fe^{3+} via the Fenton reaction triggering iron-dependent cytotoxicity or ferroptosis [128]. This mechanism of toxicity induced by auranofin was also observed in human retinal pigment epithelial cells, in which auranofin-induced lethality was prevented by the presence of the ferroptosis inhibitor ferrostatin1 [129].

In lung cancer cells, auranofin was shown to interact with the natural inhibitor of TrxR1, selenocysteine, both *in vitro* and *in vivo*, by enhancing the accumulation of ROS [130]. Likewise, auranofin synergizes with another Trx1 inhibitor, piperlongumine, in killing gastric cancer cells in association with ROS-mediated ER stress response and mitochondrial dysfunction. The synergistic lethality of auranofin and piperlongumine was also observed *in vivo* in a xenograft tumor model and is consequence of the overwhelming ROS producing capacity of the cells when the drugs are combined [131]. Another case in which the toxicity of auranofin is associated with the overproduction of ROS is in both platinum sensitive OV2008 ovarian cancer cells and their resistant counterparts, OV2008/C13, when the gold complex is combined with either selenite or tellurite [132]. Such strategy of raising intracellular ROS to levels that cannot be managed by the antioxidant systems was demonstrated in MCF-7 breast cancer cells. In this case, a synergistic interaction was found between auranofin and mesupron blocking the growth of MCF-7 cancer cells. Mesupron is a small molecule that inhibits urokinase-type plasminogen activator (uPA) and causes mitochondrial dysfunction. When associated to the overproduction of ROS triggered by auranofin, the cells undergo synergistic apoptosis mediated by caspase-3 activation, and downregulation of antiapoptotic mitochondrial factors including BCL-2, BCL-xL, and MCL-1. All

these effects are associated with disruption of the mitochondrial potential [133] and mediated by the generation of ROS, as the toxicity was abrogated by NAC [134]. Along the same line of research, it was reported in colon cancer *in vitro* and *in vivo* that auranofin synergises with the antiinflammatory drug celecoxib via a mechanism that includes severe ROS production and oxidative stress and disturbance of mitochondrial redox homeostasis leading to a depletion of ATP [135]. Finally, a report shows that oxidative stress can be over-powered in A549 lung cancer cells by the combination of auranofin and KU55933, an inhibitor of the serine/threonine kinase ATM that is involved in sensing DNA damage upon DNA double strand breaks [136]. The toxicity of the combination therapy was rescued by ROS scavengers, demonstrating once again the critical role of redox homeostasis to maintain the wellbeing of cancer cells that operate with dangerously high background levels of ROS [77].

An interesting mechanism of toxicity was reported in brain tumor cells. In this case auranofin was used to kill glio- and neuroblastoma cells by inhibiting TrxR1 in combination with CyPPA [137], which is an opener of the small-conductance calcium-activated potassium channels [138]. The toxicity of the combination of auranofin and CyPPA results in massive mitochondrial damage, which was not only observed in two-dimensional cell cultures, but also was recapitulated in glioblastoma neurospheres. Another case in which mitochondrial stress was reported upon the action of auranofin is in breast cancer cells when auranofin was combined with the mitogenactivated protein kinase (MAPK) inhibitor trabetinib. In this case, MCF-7 cells treated with the combination auranofin/trabetinib underwent apoptosis associated with activation of executer caspases-3/7, activation of the p38 MAPK signaling pathway, and translocation of apoptosis inducing factor (AIF) from the mitochondria to the nucleus [139].

Using a high-throughput viability screen and reliable in silico data allowed the rational identification of two pathways that can lead to synergistic interaction towards cell death in ovarian

cancer. The study discovered that the TrxR inhibitor and ROS inducer auranofin synergized in the killing of ovarian cancer cells with the heat-shot protein 90 (HSP90) chaperone antagonist, AUY922 [140].

Finally, there is even a case in which auranofin synergizes with a combination treatment leading to a triplet of molecules targeting the same cancer cells. Auranofin was reported to interact in a synergistic manner with a combination of erlotinib [an inhibitor of activated epidermal growth factor receptor (EGFR)] killing non-small cell lung carcinoma cells which have been restored of tumor suppressor candidate 2 (TUSC2, also known as FUS1). In this case, the combination of TSUC2 restoration with erlotinib generates the vulnerability to allow auranofin-mediated toxicity [141]. A summary of preclinical studies done with auranofin and other compounds is presented in **Table 2.1**.

Table 2.1. Cytotoxicity of auranofin in combination treatments against different cancers

Drug combination	**Mechanism(s) of action**	**Cancer**	**Cells /animal models**	**References**
AF + Celecoxib	ROS mediated inhibition of hexokinase and glycolysis Disruption in mitochondrial oxidative phosphorylation	Colon	HCT116, HT-29, DLD-1, DLD-1 tumors in nude mice	[135]

AF + Adriamycin	ROS-mediated inhibition of glycolysis, ATP production, and ABCG2 transporter expression Decreased drug resistance	Lung	A549, NCI-H460, A549 tumors in athymic nude mice	[124]
AF + L-ascorbate	Iron-dependent inhibition of H_2O_2 scavenging capacity H_2O_2 -dependent cell death	Burkitt Lymphoma Chronic lymphocytic leukemia	Malignant B cells Raji and Mec-1, human CLL cells	[127]
	DNA damage			
AF + Piperlongumine	ROS-mediated ER stress and mitochondrial dysfunction Caspase-3/PARP1dependent apoptosis	Gastric	BGS-823, SGC-7901, KATO III	[131]

AF + Mesupron	ROS-mediated caspase-3-dependent apoptosis AIF nuclear translocation	Breast	MCF-7	[134]
AF + KU55933	ROS-mediated oxidation of antioxidant protein PRDX1/3 via TrxR and ATM inhibition	Lung	A549, MLF	[136]
AF + CyPPA	Cell death and mitochondrial damage via SK channel activation	Glioblastoma Neuroblastoma	SK-N-AS, U251	[137]
AF + Trametinib	Caspase-3/7-dependent apoptosis	Breast	MCF-7	[139]

	AIF nuclear translocation via p38/MAPK phosphorylation			

AF + Selenocysteine	ROS-mediated apoptosis via inhibition of PI3K/AKT and MEK/ERK pathways DNA damage	Lung	A549 and A549 tumor xenografts in mice	[130]
AF + selenite	ROS-mediated inhibition of TrxR, GPx, and GR Apoptosis	Ovarian	2008 (cisplatinsensitive) and C13* (cisplatin-resistant)	[132]
AF + AUY922	Cell death via ROS and HSP90 inhibition	Ovarian	A1847, OVCAR4, PEO4, SKOV3, OVCAR8	[140]
AF + erlotinib + TUSC2	NRF2-mediated oxidative stress ROS-mediated apoptosis and inhibition of colony formation	Lung	Wild-type EGFR NSCLC Calu-3, Calu-6, and H522, H157, H1299, human NSCLC H1299 tumors in mice	[141]

	DNA damage Increased animal survival			

AF Auranofin, *ROS* Reactive oxygen species, *ATP* Adenosine triphosphate, *ABCG2* ATP Binding Cassette Subfamily G Member 2, *ER* Endoplasmic Reticulum, *PARP* Poly(ADP-Ribose) Polymerase-1, *AIF* Apoptosis-inducing factor, *PRDX1/3* Peroxiredoxin 1, *TrxR* Thioredoxin Reductase, ATM kinase Ataxia-telangiectasia mutated, SK Small-conductance calcium-activated potassium channel (SK/K_{Ca}), *PI3K* Phosphatidylinositol 3-kinase, *GPx* Glutathione Peroxidase, *GR* Glutathione Reductase, *TUSC2* Tumor Suppressor Gene TUSC2, *EGFR* Epidermal Growth Factor Receptor, *NSCLC* Non-small cell lung cancer, *NRF2* Nuclear factor erythroid 2-like factor 2, p53 Tumor suppressor p53, *HSP90* Heat Shock Protein 90

Summary of mechanisms of action of auranofin as an anti-cancer agent

All mechanisms of action previously described for auranofin as an anti-cancer agent are summarized below in **Fig. 2.2.**

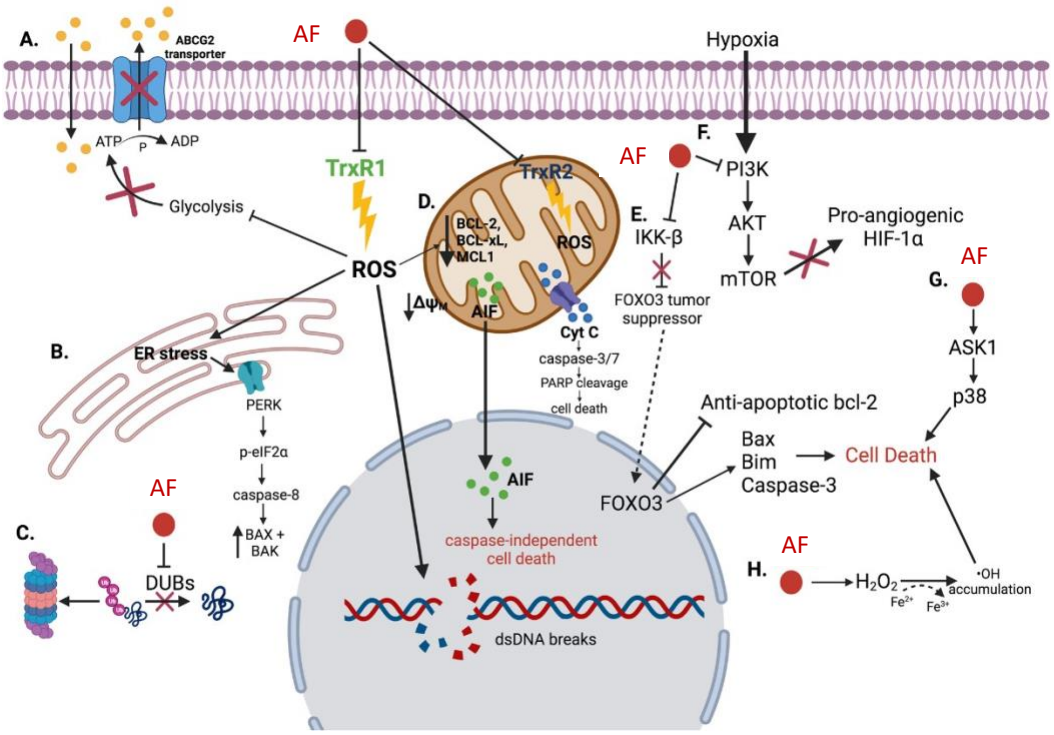

Figure 2.2. Auranofin (AF) inhibits the antioxidant enzymes thioredoxin reductase 1 (TrxR1) and 2 (TrxR2) resulting in an increase in the level of intracellular reactive oxygen species (ROS), mitochondrial permeability, and DNA damage. A, AF inhibits glycolysis resulting in reduced ATP levels and inhibition of the function of drug transporter, ABCG2, preventing development of drug resistance. B, AF-mediated ROS production causes ER stress and PERK activation, leading to cell death. C, AF inhibits the function of deubiquitinases enzymes (DUBs) in protein homeostasis and induction of tumor growth. D, Increased ROS induced by AF causes decreased membrane potential in the mitochondrial membrane, resulting in a decrease in anti-apoptotic proteins, caspase-dependent cell death, and translocation of apoptosis-inducing factor (AIF) into the nucleus to trigger caspase-independent cell death. E, AF inhibits the IKK-β signaling pathway, which normally induces FOXO3 tumor suppressor degradation. The inhibition of IKKβ by AF allows the nuclear translocation of FOXO3, activating proapoptotic proteins, resulting in cell death. F, AF

inhibits the PI3K/AKT/mTOR pathway, resulting in the inhibition of proangiogenic factors like HIF-1α, preventing tumorigenesis. G, AF activates ASK1 leading to p38mediated cell death. H, AF promotes the conversion of H2O2 to ·OH via the Fenton reaction, resulting in the induction of cell death. Cartoon created with Biorender.

Clinical translational advances on auranofin in cancer

Due to the promising evidence in the literature on the use of auranofin as an anti-cancer agent, the drug has been enrolled in phase I/II clinical trials to treat patients with the following diseases: chronic lymphocytic leukemia, non-small cell lung cancer or small cell lung cancer, and ovarian, peritoneal, and fallopian tube cancers (see United States National Library of Medicine; www.clinicaltrials.gov, trial numbers NCT01419691, NCT01737502, NCT01747798, and NCT03456700). The aim of these clinical trials is to measure the overall response rate to auranofin, to address any adverse effects of the drug, and to assess the survival rate of the patients following exposure to the treatment. Additionally, these clinical trials include patients with metastatic or recurrent disease, in which auranofin is being used as a possible consolidation therapeutic agent to improve overall survival rate. However exciting these clinical trials are, their outcomes have yet to be reported.

New viewpoint of research with auranofin against cancer: the link with platinum-based chemotherapy, immunogenic cell death, and immunotherapy

We propose that a potential synergism of platinum agents with auranofin would have two folds: 1) irreversible toxicity caused by accumulation of ROS and irreparable DNA damage; and

2) simultaneous induction of immunogenic cell death (ICD) with negative consequences for the survival of the tumor**.**

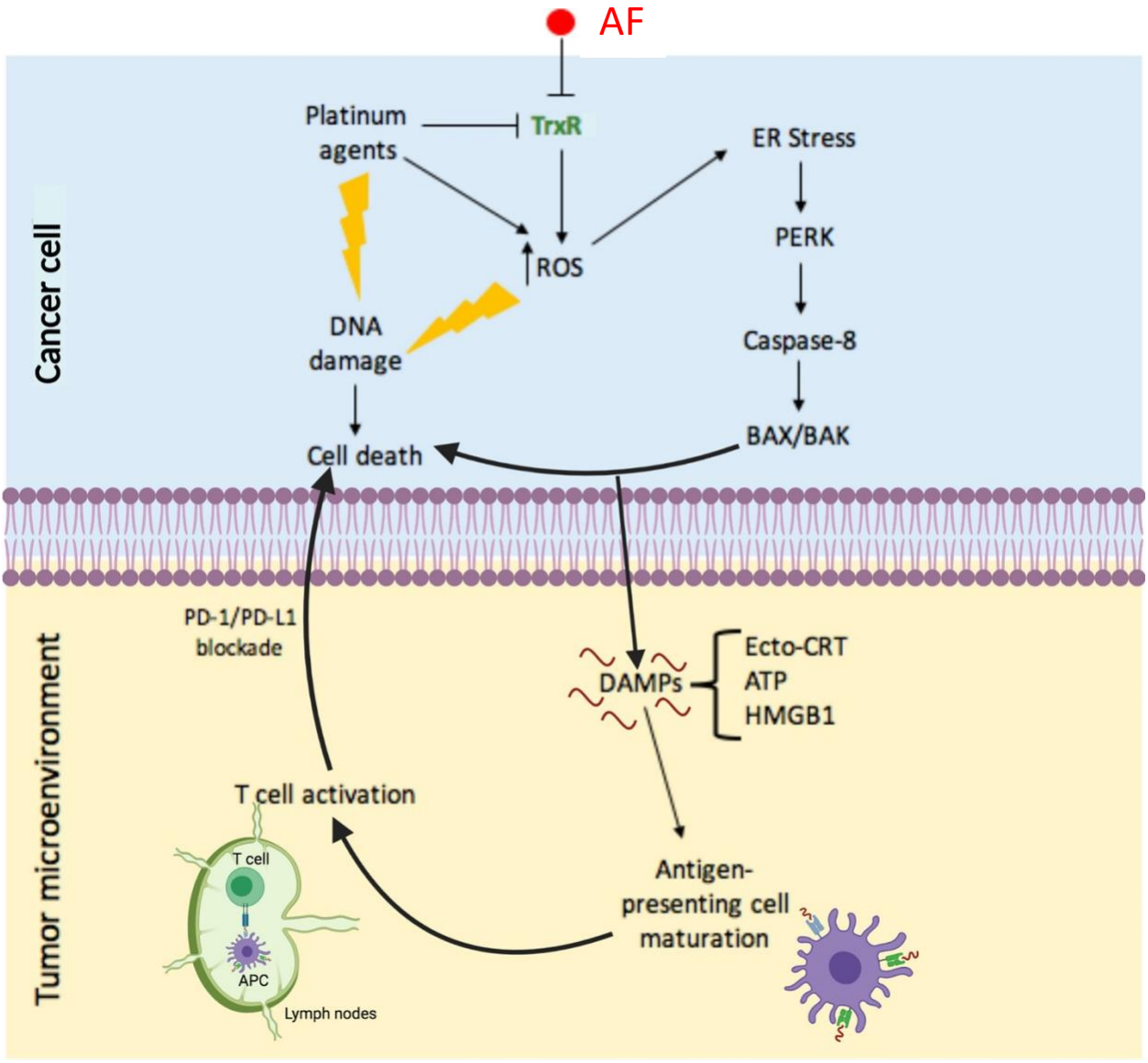

Figure 2.3. Targeting cancer cells with a combination of auranofin (AF) and a platinum agent in the context of the tumor microenvironment. AF and platinum agents inhibit the function of TrxR, inducing an overproduction of reactive oxygen species (ROS), mitochondrial permeability, and DNA damage, resulting in cancer cell death. Accumulation of intracellular ROS induces ER stress, activation of PERK and caspase-8, and the upregulation of pro-apoptotic proteins BAX and BAK, further potentiating cell death. This pathway triggers the release of danger or damage associated

molecular patterns (DAMPs) such as calreticulin (ecto-CRT), high mobility group box 1 (HMGB1) protein and ATP into the tumor microenvironment where they collectively activate antigen presenting cells (APCs). Mature APCs migrate into the lymph nodes and present tumor antigens to immature T cells, which develop into CD8+ T cells with anti-cancer cytotoxic activity. The inhibition of the interaction between PD-1 on T cells and PD-L1 on cancer cells prevents the neutralization of T cells by cancer cells, maintaining T cell cytotoxicity and favoring tumor cell death. This release of DAMPs into the tumor microenvironment via ER stress and the consequent activation of T cells is known as immunogenic cell death or ICD. Cartoon created with Biorender.

First, the toxicity of the widely used platinating agent cisplatin is proportional to the level of expression of TrxR1 [142], and cisplatin also inhibits TrxR1 with high specificity [82]. Furthermore, cancer cells that operate with increased oxidative stress are more vulnerable to damage by further ROS insults induced by exogenous agents [143] when compared to normal cells [77]. Based on this background, it is tempting to hypothesize that the oxidative stress caused by the combination auranofin/cisplatin will be high enough to disrupt the antioxidant capacity of cancer cells and to cause sufficient DNA damage that the cells will not be able to repair, resulting in cell death.

Second, gold compounds were suggested to be able to induce ICD [144] based on the observations that different gold derivatives caused ROS-mediated necroptosis in colon cancer cells [145], and triggered ER-stress mediated apoptosis and autophagy in non-small cell lung carcinoma [146]. However, it was not until very recently that auranofin was reported, in non-small cell lung cancer cells, to actually trigger the release of biomarkers that are characteristics of ICD [147]. This is a type of cell death involving an atypical apoptotic process, in which the cells, while dying, release a series of molecules to the tumor microenvironment. These ‘alarm’ molecules, also

known as danger signals, alarmins, or more precisely damage-associated molecular patterns (DAMPs), makes such type of apoptotic cell death 'immunogenic' because they promote an inflammatory response. DAMPs attract innate immune cells, especially antigen presenting cells (APCs) such as dendritic cells, leading to the phagocytosis of the dying cells followed by transportation to the lymphatics where tumor antigens are presented to T cells [40, 144, 148-151]. The most critical DAMPs released during ICD are calreticulin (CLR), ATP, and high-mobility group box 1 (HMGB1) protein [152]. The mechanism whereby CLR moves from its original site—the ER—to the plasma membrane, requires the ER stress associated phosphorylation of the eukaryotic translation initiation factor eIF2a, resulting in arrest of mRNA translation, activation of caspase-8, upregulation of pro-apoptotic BCL-2 family members BAX and BAK, the transport of CRT to the Golgi apparatus, and exocytosis of CRT-containing vesicles [153]. The presentation of CRT on the surface of the plasma membrane (a.k.a. ecto-CRT) occurs very early during the process of ICD, even before lipid molecules carrying phosphatidylserine residues are exposed to the outer leaflet of the plasma membrane of cells undergoing apoptosis [154]. The release of ATP and the non-chromatin DNA chaperone HMGB1 protein occurs later on in the cell death process and is mediated via an autophagy-associated mechanism [40, 155]. All three molecular components are essential for the promotion of maturation of APCs [149].

We anticipate that because auranofin causes ICD in non-small cell lung carcinoma [147], the overall lethality would be increased by the presence of one of the DNA damaging platinating agents that constitute the standard of care for this cancer [156, 157]. It is tempting to speculate that other platinum-responsive cancers, such as cervical, head and neck, testicular, bladder, and ovarian to mention some [83], may carry the basic molecular backgrounds to also be responsive to auranofin-mediated ICD. It is imperative that the scientific community investigates in detail

whether the lethal effect of auranofin towards platinum-sensitive and resistant cancers involve all or some of the components of the ICD pathway. This knowledge may create opportunities for combining auranofin with platinating derivatives known not to be per se effective inducers of ICD, such as cisplatin or carboplatin [158, 159]. Of the three platinum derivatives, cisplatin, carboplatin, and oxaliplatin, that are clinically approved worldwide against cancer, the latter is the only one demonstrated to be highly efficient in inducing ICD, yet it is only widely used to treat mostly colorectal carcinomas or platinum-sensitive ovarian cancer patients that had become allergic to carboplatin [160-163]. Thus, the capacity of auranofin to complement cisplatin and carboplatin in effectively inducing ICD may be efficient against many cancers. Auranofin and cisplatin, or carboplatin, may interact and act as a 'vaccine' in which cancer cells dying by ICD, via DAMPs, activate APCs; these, in turn, present the cancer antigens to T cells in the lymph nodes, leading to the generation of active tumor specific CD8+ T cells with the capacity to attacking and killing other cancer cells within the tumor microenvironment [40, 148, 151, 152].

In another exciting study, in this case in breast cancer, it was shown that auranofin was highly effective in causing cell death and impairing the growth of triple negative breast cancer cells grown as spheroids. In this work, also auranofin was effective *in vivo* in patient-derived tumor xenografts by inhibiting TrxR1 activity and increasing CD8+ T cell infiltration [164]. All these effects occurred in combination with the upregulation of PD-L1, a member of the PD-1/PD-L1 immune checkpoint [164]. As expected, combination of auranofin with an anti-PD-L1 antibody synergistically impaired the growth of syngeneic 4T1.2 primary tumors [164]. PD-L1 is expressed on the surface of cancer cells and usually engages with PD-1 expressed on the surface of T cells to neutralize their activity [165]. Additionally, PD-L1 is overexpressed in many cancers and is associated with tumor chemoresistance and poor clinical outcomes [166]. Furthermore, anti-PDL1 therapy was effective in synergizing with the ICD properties of oxaliplatin in hepatocellular

carcinoma *in vivo* [167]. Thus, adding anti-PD-1 or anti-PD-L1 antibodies on the top of auranofin/cisplatin combination therapy seems a rational combinational therapeutic approach. This idea is further supported by recent literature showing that breast cancer tissues were sensitive to mifepristone—an antiprogestin and antiglucocorticoid agent [168], which was able to induce ICD and thus synergize with PD-L1 blockade [169]. We previously reported that the anti-cancer effects of mifepristone [170, 171] were associated with the induction of proteotoxic ER stress [172], a pathway needed to be active for ICD to succeed.

Finally, another important avenue to pursue if auranofin and platinum agents synergize in killing cancer cells is to develop a therapeutic approach by synthesizing a hybrid compound between cisplatin and auranofin. Such hybrid compound would have higher lethality than the individual drugs against cancer cells resistant to standard platinum-based therapy at the time of recurrence. Hybrid compounds simultaneously targeting different points of signaling networks and various structures within cancer cells were explored extensively in recent years [173]. It has become clear that it is very difficult to achieve desirable chemotherapeutic effects in the treatment of advanced cancers using single drug therapy. Thus, a multi-target approach utilizing a hybrid molecule should provide greater therapeutic anticancer benefits and better safety while reducing the risk of developing drug resistance [173, 174]; we anticipate that a hybrid drug between cisplatin and auranofin will achieve such a goal.

Concluding remarks

Auranofin is considered safe for human use in treating rheumatoid arthritis; thus, this gold derivative can reach the clinic for other diseases relatively quickly and at a low cost, taking into account that the drug has a well-known toxicity profile [175]. Auranofin and other gold-related compounds emerge as highly promising agents to be repurposed for cancer therapy mainly in

combination with platinum derivatives thus encompassing a large number of tumor types. Synergistic interaction between auranofin and platinating agents may not only involve unrepairable ROS-induced DNA damage, but also induction of an immune response due to the capacity of auranofin to induce ICD. This places the combination auranofin-platinum combination therapy, working either as complementary drugs, or via a hybrid molecule, within the field of immune cancer therapies. As it is clear that combination treatments are most likely the reason whereby cancer will be hopefully under control in the near future, we suggest including as part of a multiplex therapy, in addition to auranofin and a platinum agent, immune checkpoint inhibitors to increase the possibility of transforming a lethal disease into a controllable chronic one.

Abbreviations

AF: Auranofin

AIF: Apoptosis inducing factor

ALL: Acute lymphoblastic leukemia

AML: Acute myeloid leukemia

AKT: Protein kinase B

APC: Antigen presenting cells

ARE: Antioxidant response element

ASK: Apoptosis signaling kinase

ATM: Ataxia telangiectasia mutated
BCL-2: B-cell lymphoma 2

BCL-xL: B-cell lymphoma-extra large

BRCA1: Breast cancer gene 1

BSO: l-buthionine sulfoximide

CML: Chronic myeloid leukemia

CRT: Calreticulin

DAMPs: Danger- or damage-associated molecular patterns

DMARS: Disease-modifying antirheumatic drugs

DUBs: Deubiquitinases

EGFR: Epidermal growth factor receptor

eIF2α: α-Subunit of eukaryotic initiation factor 2 (eIF2)

ER: Endoplasmic reticulum

FOXO3: Forkhead box O3

GSH: Glutathione

HDACi: Histone deacetylase inhibitor

HIF1α: Hypoxia-inducible factor 1 alpha

HMGB1: High mobility group box 1

H2O2: Hydrogen peroxide

HSP90: Heat-shock protein of 90 kDa

ICD: Immunogenic cell death

IKK: IκB kinase

IL-6: Interleukin-6
IL-8: Interleukin-8

JAK: Janus kinase

JNK: C-JUN N-terminal kinase

MAPK: Mitogen-activated protein kinase

MCL-1: Myeloid cell leukemia 1

mTOR: Mammalian target of rapamycin

NAC: N-Acetyl cysteine

NADPH: Nicotinamide adenine dinucleotide phosphate

NF-kB: Nuclear factor kappa B

NSAIDs: Nonsteroidal anti-inflammatory drugs

·**OH:** Hydroxyl radical

PI3K: Phosphoinositide 3-kinase

PD1: Programmed cell death protein 1

PD-L1: Programmed death-ligand 1

PDX: Patient-derived xenograft

PERK: Protein kinase R (PKR)-like endoplasmic reticulum kinase

PTEN: Phosphatase and tensin homolog

ROS: Reactive oxygen species

SP: Side population

TNF: Tumor necrosis factor

TP53: Tumor suppressor protein p53

Trx: Thioredoxin

TrxR: Thioredoxin reductase
TUSC2: Tumor suppressor candidate 2

uPA: Urokinase-type plasminogen activator

UPS: Ubiquitin proteasome system

VEGF: Vascular endothelial growth factor

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CHAPTER 3

Study of the Cytotoxicity Triggered by Auranofin Monotherapy in High-Grade Serous Ovarian Cancer Cells

3.1 Preface

The literature review focused on the cytotoxic effects of auranofin in various cancers, which overexpress the TrxR1 antioxidant enzyme. TrxR1 overexpression has been associated with poor prognosis in ovarian cancer. In addition, auranofin has already been enrolled in clinical trials for ovarian cancer patients. There have been limited studies on the use of auranofin against the most prevalent histotype of epithelial ovarian cancer, high-grade serous ovarian cancer (HGSOC) [9]. In this chapter, two different cell lines that represent HGSOC at different stages of the disease were used. The first cell line is clinically cisplatin-sensitive and is termed PEO1; the original cells were isolated from a patient following their first relapse 22 months post-treatment with platinumbased chemotherapy. The second cell line used in this chapter is their sister cell line, PEO4, which was isolated from the same patient following its second relapse in which the tumour was no longer responsive to platinum-based chemotherapy [9]. Since the PEO4 cells are clinically resistant to the standard of care, there is a need to develop a treatment that would combat the recurrent stage of the disease.

In parallel with the association between TrxR1 overexpression and poor diagnosis in ovarian cancer patients, we hypothesized that auranofin is a suitable therapeutic agent to treat the early and recurrent stages of HGSOC. We first confirmed the sensitivities of the PEO1 and PEO4 cells to cisplatin via a long-term clonogenic survival assay. A preliminary assessment of the effect of auranofin in both cell lines based on the cellular vitality or the metabolic state of the cell was completed [176]. To confirm the primary role of auranofin in targeting TrxR, we evaluated TrxR activity in response to auranofin and compared the basal TrxR activity among the cell lines. As TrxR inhibition is presumably associated with ROS overproduction, we measured ROS levels following exposure to auranofin in both cell types. This was complemented with cell-based assays

that evaluated the state of the nuclear DNA, mitochondria, and apoptosis following treatment with auranofin and whether the mechanisms of cytotoxicity used by the drug are dependent on ROS overproduction.

Some of the pathways explored in the following manuscript were based on molecular mechanisms of auranofin in other cancers, which we mentioned in our literature review. On the other hand, the manuscript initially highlights the efficacy of auranofin as a monotherapy against HGSOC. The manuscript reveals a compensatory mechanism used by the HGSOC cells in response to auranofin. As a result, we decided to carry out further experiments combining auranofin with L-buthionine sulfoximine (L-BSO), a pro-oxidative synthetic amino acid that depletes glutathione (GSH) levels. Studies on the auranofin and L-BSO combination in other cancers were not listed in the literature review. However, the drug combination was tested in human lung cancer, rhabdomyosarcoma, and more recently in glioblastoma. Most of the combination studies with auranofin that were listed in the review included drugs that are already FDA-approved or enrolled in clinical trials, whereas L-BSO has not been approved for use in the clinic. Nonetheless, our upcoming study is the first to explore the interaction of auranofin and L-
BSO in HGSOC.

Auranofin Induces Lethality Driven by Reactive Oxygen Species in High-Grade Serous Ovarian Cancer Cells

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3.2 Simple Summary

High-grade serous ovarian cancer (HGSOC) is the most prevalent type of ovarian cancer, accounting for 70% of ovarian cancer deaths. This is primarily due to the development of resistance against standard platinum-based chemotherapy. Several drugs are currently undergoing repurposing against ovarian cancer, including auranofin (AF), an anti-rheumatoid agent. The mechanism of action of AF has been studied in various cancers, however, there have been fewer studies on the effects of AF in HGSOC. In this study, we explore the mechanisms of action of AF in human HGSOC cells that are sensitive or resistant to platinum. We demonstrate the various cytotoxic effects of AF in HGSOC via the targeting of multiple pathways, suggesting the potential use of AF in a long-term consolidation therapy against this disease.

3.3 Abstract

High-grade serous ovarian cancer (HGSOC) accounts for 70% of ovarian cancer cases, and the survival rate remains remarkably low due to the lack of effective long-term consolidation therapies. Clinical remission can be temporarily induced by platinum-based chemotherapy, but death subsequently results from the extensive growth of a platinum-resistant component of the tumor. This work explores a novel treatment against HGSOC using the gold complex auranofin (AF). AF primarily functions as a pro-oxidant by inhibiting thioredoxin reductase (TrxR), an antioxidant enzyme overexpressed in ovarian cancer. We investigated the effect of AF on TrxR activity and the various mechanisms of cytotoxicity using HGSOC cells that are clinically sensitive or resistant to platinum. In addition, we studied the interaction between AF and another prooxidant, L-buthionine sulfoximine (L-BSO), an anti-glutathione (GSH) compound. We demonstrated that AF potently inhibited TrxR activity and reduced the vitality and viability of HGSOC cells

regardless of their sensitivities to platinum. We showed that AF induces the accumulation of reactive oxygen species (ROS), triggers the depolarization of the mitochondrial membrane, and kills HGSOC cells by inducing apoptosis. Notably, AF-induced cell death was abrogated by the ROS-scavenger N-acetyl cysteine (NAC). In addition, the lethality of AF was associated with the activation of caspases-3/7 and the generation of DNA damage, effects that were also prevented by the presence of NAC. Finally, when AF and L-BSO were combined, we observed synergistic lethality against HGSOC cells, which was mediated by a further increase in ROS and a decrease in the levels of the antioxidant GSH. In summary, our results support the concept that AF can be used alone or in combination with L-BSO to kill HGSOC cells regardless of their sensitivity to platinum, suggesting that the depletion of antioxidants is an efficient strategy to mitigate the course of this disease.

Keywords: auranofin; high-grade serous ovarian cancer; TrxR; apoptosis; DNA damage; ROS; Lbuthionine sulfoximine; cisplatin; N-acetyl cysteine; drug repurposing; GSH

3.4 Main Text

Introduction

Ovarian cancer is the eighth leading cause of cancer-related deaths among women worldwide [9]. According to GLOBOCAN, there were 313,959 new ovarian cancer cases and 207,252 deaths due to ovarian cancer in 2020 [177]. Over the past few decades, there has been a small reduction in the incidence of ovarian cancer, primarily due to preventative measures, such as the introduction of oral contraceptives [178], and the decline in the use of menopausal hormonal therapy [179]. In contrast, there has been minimal improvement in the overall survival of patients with this disease [9, 180]. Treatment with platinating agents is very efficient at the beginning of the illness following diagnosis and debulking surgery, leading to an initial response rate of 80% [181, 182]. However, over time the disease almost always recurs with a platinum resistant phenotype that is extremely challenging to treat, thus explaining its high mortality [183]. Acquired platinum resistance occurs via multipronged mechanisms with decreased intracellular accumulation, increased drug detoxification, and increased activity of the DNA repair machinery among the most often investigated [180].

To reduce the high mortality of ovarian cancer, platinum-based chemotherapy needs to be coupled with an additional treatment that can be given chronically to maintain the disease in a dormant stage. Drugs with cytotoxic activity that are currently approved for treating other conditions are ideal candidates for this role. Drug repurposing is a cost-effective approach in which drugs approved for one condition can be administered for a different disorder [184, 185]. With this goal in mind our laboratory has shown that the antiprogesterone/antiglucocorticoid agent mifepristone is efficient as upfront therapy or after cisplatin and/or paclitaxel therapy against ovarian cancer cells [170, 172, 180, 186, 187]. We have also shown the efficacy of the HIV

inhibitor nelfinavir against ovarian cancer cells sensitive or resistant to platinum [188]. Here, we added auranofin, a gold complex approved in 1985 to treat rheumatoid arthritis [20], to the list of anti-ovarian cancer drugs emerging from repurposing.

There have been studies exploring the cytotoxic effect of auranofin in ovarian cancer. However, such studies were conducted using cell lines that do not represent the high-grade serous histotype we report here. For instance, studies have used A2780 cells [120, 126] that have been identified as representing an endometrial ovarian carcinoma [189], OV2008 [61], which have been reported to be misidentified [190] and likely of cervical nature [191], SKOV-3 [99, 101] that have been genetically identified to represent a clear ovarian adenocarcinoma [189], and OVCAR-5 [101], which were incorrectly identified as of ovarian origin while they are actually of gastrointestinal nature [192]. Finally other studies have used the platinum resistant OV2008 (C13*) [61, 193], which are cells that were developed *in vitro* from OV2008 cells after doseescalating exposure to cisplatin. In this work we explore the cytotoxicity of auranofin towards a pair of cell lines that are respectively sensitive or resistant to platinum after being isolated from a patient when she was clinically sensitive or resistant to the drug [102]; these cells have been demonstrated to genomically represent the most prevalent histotype of the disease: high-grade serous ovarian cancer (HGSOC) [103, 183].

The primary mechanism of action of auranofin is to act as a pro-oxidative agent, increasing the production of reactive oxygen species (ROS) as a consequence of inhibiting the thioredoxin reductase (TrxR) anti-oxidant system [194]. TrxR is overexpressed in various cancers, including non-small cell lung cancer [195], breast cancer [196], and cisplatin-resistant ovarian cancer [61]. The TrxR system is involved in the overall promotion of tumor progression by preventing cell death triggered by oxidative stress [68]. Of interest, TrxR overexpression is associated with shorter overall survival in patients with ovarian cancer based on a Kaplan-Meier survival analysis [68].

These findings suggest that TrxR is an attractive therapeutic target against ovarian cancer, and auranofin is a potent TrxR inhibitor and pro-oxidative agent that can be used to combat this disease. Previous reports on various cancer cells have demonstrated that auranofin induces inhibition of cell proliferation by causing overproduction of ROS [197], caspase-independent apoptosis [198], and cell death triggered by DNA damage [199]. Additionally, auranofin has been shown to inhibit angiogenesis [93], protein homeostasis [96, 200], and deubiquitinases involved in proteasomemediated protein degradation [201]. These findings indicate that auranofin is a potent anti-cancer agent that negatively targets multiple metabolic pathways of cancer cells. In this study, we identified the mechanisms of cytotoxicity induced by auranofin in HGSOC cells that have different clinical sensitivities to platinum. We show that auranofin causes ROS-dependent inhibition of cell proliferation, caspase-associated apoptosis, mitochondrial membrane depolarization, DNA damage, cleavage of poly-ADP ribose polymerase (PARP), and polyubiquitination of proteins [201]. Additionally, we show a synergistic lethal interaction between auranofin and a second prooxidant agent, the glutathione (GSH) inhibitor, L-buthionine sulfoximine (L-BSO); this drug interaction involving two blockers of key antioxidant pathways that cancer cells rely upon is dependent on the presence of ROS.

Materials and Methods

Reagents and Cell Lines

PEO1 cells are epithelial ovarian cancer cells isolated from a patient after its first relapse 22 months following treatment with cisplatin, 5-fluorouracil, and chlorambucil, while the patient was still sensitive to platinum-based chemotherapy. PEO4 cells were subsequently isolated from

the same patient after the second relapse in which the patient was no longer sensitive to chemotherapy. These cell lines were histologically characterized in 1988 and sequenced in 2010 [102, 103], whereas we authenticated them in 2020 based on their autosomal short-tandem repeats [188]. The cells were cultured in RPMI 1640 medium (Mediatech, Manassas, VA, USA) supplemented with 5% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA, USA), 5% bovine serum (Life Technologies, Auckland, New Zealand), 0.01 mg/mL of human insulin (Roche, Indianapolis, IN, USA), 10 mM HEPES (Corning, Corning, NY, USA), 100 IU penicillin (Mediatech), 100 µg/mL streptomycin (Mediatech), 2 mM L-Alanyl-L-Glutamine (Glutagro™, Corning) and 1 mM sodium pyruvate (Corning). The cells were incubated at 37 °C in a humidified incubator with 5% CO_2. The drugs used in this study include: auranofin (Sigma Chemical Co., St. Louis, MO, USA), bortezomib (BZ) (Velcade®, Millennium Pharmaceuticals, Cambridge, MA, USA), L-buthionine sulfoximine (L-BSO) (Sigma), and N-acetyl cysteine (NAC; Sigma).

Cellular Vitality

To determine the wellbeing of the cells, we performed a cellular vitality assay [202] evaluating mitochondrial enzyme activities as surrogate markers of drug toxicity in control conditions versus drug toxicity. PEO1 and PEO4 cells growing at 70% confluency were harvested and seeded in triplicate in 96-well plates at a density of $2.5x10^3$ cells/well in HGSOC medium and allowed to adhere overnight at 37°C in 5% CO_2. The cells were then treated with varying concentrations of auranofin for 72 hours. Cell vitality was measured by adding 10 µl/well of 5 mg/ml MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] (Life Technologies, Burlington, ON, Canada) in PBS solution. The cells were incubated for 4 hours at 37°C in 5% CO_2 where the tetrazolium dye was reduced to insoluble formazan. One hundred microliters/well of

10% sodium dodecyl sulfate (SDS)/ 0.01 M HCl was added to stop the reaction. The absorbance was recorded at 570 nm following overnight incubation. Blank controls were subtracted and the percentage of cell vitality relative to the control was calculated.

Cellular Viability

Clinically platinum-sensitive (PEO1) and platinum-resistant (PEO4) HGSOC cells were treated with increasing concentrations of auranofin for 72 hours. The cells were collected, centrifuged, and the remaining cell pellet was resuspended in 1 mL of cell culture medium. An aliquot of cells was taken and stained with the Muse® count and viability reagent (Luminex, Austin, TX, USA) for 5 minutes; this reagent contains a DNA-binding dye that tags nucleated cells, and a second dye that differentiates live from dead cells by penetrating cells with compromised membrane integrity (i.e., non-viable cells). Stained cells were analyzed using the Muse® micro-capillary cytometer (Millipore, Hayward, ON, Canada), and the viable cell number and total cell number were determined.

Clonogenic Survival

To assess the residual toxicity of auranofin on HGSOC cells, 1000 viable cells were taken from the cell culture treated with auranofin for 72 hours and plated in media devoid of drug in a 6-well plate for 2 weeks. The experiment was terminated once the vehicle-treated group contained positive colonies. Positive colonies refer to colonies that contain 50 or more cells; this is used as a measure of assessing the proliferative capacity of the cells in a long-term period even though they survived the initial 72 hours of drug treatment; in other words, we tested how the exposure to auranofin affected their long-term proliferative capacity.

Cell Cycle Distribution

Following the 72-hour treatment with auranofin, PEO1 and PEO4 cells were fixed using 4% paraformaldehyde (PFA) and stored at 4°C overnight. Samples were centrifuged and the cell pellet was washed with 500 µL of 1 X phosphate-buffered saline (PBS) (Corning, Manassas, VA, USA). Two hundred thousand cells were collected and centrifuged at 2000 x *g* for 5 minutes. The supernatant was discarded, and the pellet was resuspended in 200 µL of cell cycle buffer containing 0.5 mg/mL propidium iodide, a cell permeable DNA intercalating agent that serves to analyze the status of DNA content. Cell cycle analysis was completed using the Muse® micro-capillary cytometer (Millipore). This method was previously described in detail [188].

Protein Lysate Preparation and Western Blot Analysis

PEO1 and PEO4 cells were treated with increasing concentrations of auranofin for 72 hours, and whole cellular extracts were collected at the end of the incubation. The cells were centrifuged at 1500 x *g* for 6 minutes, the supernatant was removed, and the cell pellet was resuspended in 1 mL of cold 1 X PBS. The samples were centrifuged at 2,000 x *g* for additional 5 minutes, the supernatant was removed, and the cell pellets were snap frozen in liquid nitrogen and stored at -80˚C until further processing. The proteins were isolated by first adding lysis buffer to the cell pellets. The lysis buffer was prepared as follows: 0.5% NP-40, 1 mM dithiothreitol (DTT), 1 mM phenylmethylsulphonyl fluoride (PMSF), 2 µg/mL aprotinin, 2 µg/mL pepstatin, 2 µg/mL leupeptin, 50 mM sodium fluoride, and 1 mM sodium orthovanadate. The cell pellets were resuspended in the lysis buffer by gentle vortexing, and were placed on ice on a shaker for 30 minutes at 4˚C. The samples were centrifuged at 12,000 x *g* for 15 minutes at 4˚C. The proteins in the supernatant were collected and transferred to separate tubes. Protein samples were then

quantified using the Pierce BCA Protein colorimetric assay purchased from Thermo Fisher Scientific (Rockford, IL, USA), and absorbance was measured at 562 nanometers using the BioTek Cytation 3 Multi-Mode Reader (Agilent, Santa Clara, CA, USA). The proteins were electrophoresed on 10% SDS-polyacrylamide gels. Following the transfer onto the PVDF membranes, the membranes were blocked in 5% non-fat dry milk at room temperature for 1 hour and incubated with the primary antibodies at 4˚C overnight. The membranes were washed 5 times in 1X TBS-T for 5 minutes each and incubated with the secondary antibody for 1 hour. The secondary antibody was removed, and the membranes were washed again 5 times in 1X TBS-T for 5 minutes each. The membranes were then imaged using the Bio-Rad ChemiDoc Touch Imaging System (Bio-Rad, Hercules, CA, USA). The primary antibodies used were monoclonal anti-b-actin produced in mouse as clone AC-15 (A5442, Sigma), polyclonal anti-PARP produced in rabbit (9541, Cell Signalling Technology, Danvers, MA, USA), and polyclonal anti-ubiquitin produced in rabbit (3933S, Cell Signalling). Secondary antibodies were goat anti-rabbit IgG (H+L) conjugate (1706515, BioRad) and goat anti-mouse IgG (H+L)-HRP conjugate (1706516, BioRad).

Detection of DNA Damage

To determine whether auranofin induces DNA damage in HGSOC, PEO1 and PEO4 cells were treated with 1, 2, or 4 μM auranofin for 72 hours. The cells were collected and centrifuged at 300 x *g* for 5 minutes, and the supernatant was removed. The cells were resuspended in 50 μL of 1 X assay buffer per 100,000 cells, and equal volume of fixation buffer was added to the cells. The samples were incubated on ice for 10 minutes, spun down at 300 x *g* for 5 minutes, and the supernatant was discarded. The cells were resuspended in 90 μL of 1X assay buffer for every 100,000 cells. Cells were then stained with 10 μL of antibody working solution that was prepared

by combining 5 µL of anti-phospho-ATM (Ser1981) labelled with phycoerythrin (PE), and 5 µL of anti-phospho-histone H2A.X (Ser139) labelled with PE-Cyanine®5 (PeCy5). The samples were incubated at room temperature for 30 minutes protected from light. One hundred microliters of 1X assay buffer were added, and samples were centrifuged at 300 x *g* for 5 minutes. The supernatant was discarded, and the cells were resuspended in 200 µL of 1X assay buffer. Cells were analyzed using the multicolor DNA damage protocol (Luminex) with the Guava Muse® cell analyzer (Millipore).

Detection of Annexin-V Binding

PEO1 and PEO4 cells were treated with 2 or 4 µM auranofin for 72 hours. The treated cells were collected and resuspended in different volumes of media to obtain 1 x 10^5 to 5 x 10^5 cells per mL. A 100 µL cell suspension containing approximately 1 x 10^6 cells was placed in a tube, and 100 µL of the annexin-V and dead cell reagent (Luminex) was added for 20 minutes at room temperature in the dark. Annexin-V is a calcium-dependent phospholipid binding protein that binds to phosphatidylserine (PS), which translocates to the extracellular surface of the plasma membrane during early apoptosis. The dead cell reagent differentiates live and dead cells, by integrating into the membrane of late apoptotic and dead cells owing to the loss of membrane structural integrity. The cells were analyzed using the annexin-V and dead cell protocol in the Guava Muse® cell analyzer (Millipore).

Measurement of Caspase-3/7 Activation

PEO1 and PEO4 cells treated with 2 or 4 µM auranofin for 48 hours were collected. The Muse caspase-3/7 kit (Luminex) was used. A 50 µL suspension containing approximately 5 x 10^5 cells was placed in a tube. Five microliters of the caspase-3/7 reagent working solution, prepared by diluting the caspase-3/7 stock 1:8 with 1 X PBS, were added to the cells and incubated in the

dark for 30 minutes in a 37˚C, 5% CO_2 incubator. This reagent binds to a DNA-binding DEVD peptide substrate that, upon activation of caspase- 3/7, is cleaved and then translocates to the nucleus to bind DNA and emit fluorescence. Cells were then stained for 5 minutes at room temperature in the dark with 150 µL of Muse caspase-7-AAD substrate working solution prepared at a 1:75 dilution using 1X assay buffer. 7-AAD is a cell permeable DNA-binding dye that integrates into cells that have lost their membrane structural integrity. The analysis was performed using the caspase- 3/7 protocol on the Guava Muse® cell analyzer (Millipore).

Treatment with a Caspase Inhibitor

PEO1 and PEO4 cells were pre-treated with 50 µM z-DEVD-fmk for 2 hours (Selleck Chemicals, Houston, TX, USA). This is a specific irreversible caspase-3 inhibitor that also potently inhibits caspase-6, caspase-7, caspase-8, and caspase-10. Two or 4 µM auranofin were added for 24 hours to PEO1 cells, and for 48 hours to PEO4 cells, and cell viability was assessed by cytometry and analyzed using the Guava Muse® cell analyzer (Millipore).

Detection of Mitochondrial Membrane Depolarization

PEO1 and PEO4 cells treated with 2 or 4 µM of auranofin for 24 hours were collected. The cells were resuspended in 500 µL of 1X assay buffer for a final concentration of 5 x 10^5 cells per mL. One hundred microliters of the cell suspension were placed in a 1.5 mL centrifuge tube and were incubated for 20 minutes at 37˚C 5% CO_2 with 95 µL of Mito-Potential working solution prepared by diluting the Muse Mito-Potential dye at 1:1000 in 1X assay buffer. Five microliters of the Muse Mito-Potential 7-AAD reagent (Luminex) were added to each tube and incubated for 5 minutes at room temperature. The analysis was performed using the Mito-Potential protocol on the Guava Muse® cell analyzer (Millipore).

Assessment of Intracellular ROS Levels

To assess whether auranofin stimulates the production of ROS in HGSOC, PEO1 and PEO4 cells were treated with 8 μM AF for 4 hours. Intracellular superoxide levels were measured using an oxidative stress assay (Luminex). This assay uses the cell permeable reagent dihydroethidium (DHE), which binds to DNA and produces red fluorescence upon interaction with superoxide ions. Following treatment, the cells were collected and prepared in 1X assay buffer at 1 x 10^6 to 1 x 10^7 cells per mL. An intermediate solution of the Muse oxidative stress reagent was prepared by diluting it 1:100 with 1X assay buffer. To prepare the Muse oxidative stress working solution, the intermediate solution was diluted 1:80. One hundred and ninety microliters of the Muse oxidative stress working solution were added to 10 μL of cells and mixed thoroughly by pipetting up and down. The samples were incubated for 30 minutes at 37˚C. The stained samples were analyzed using the oxidative stress protocol on the Muse® cell analyzer (Millipore).

Drug Interaction between Auranofin and L-BSO

Two hundred thousand PEO1 and PEO4 cells were plated per well in 6-well plates and allowed to attach overnight. The drug interaction was studied in triplicate experiments using two doses of auranofin with a fixed dose of L-BSO: 2 or 4 μM auranofin with or without 5 μM of LBSO for 72 hours. Upon treatment, the cells were collected, and viability and cell number were measured using the Muse® count and viability reagent (Luminex). The combination index (CI) was then calculated using the method of Chou and Talalay [203] utilizing the CompuSyn Software (ComboSyn Inc. Paramus, NJ, USA). For a specific drug combination, a CI>1 was considered antagonistic, CI=0 indicated no drug interaction, CI=1 indicated additivism, and CI<1 denoted synergism.

Measurement of TrxR Activity

We used a thioredoxin reductase (TrxR) assay kit purchased from Abcam (Cambridge, MA, USA). In this colorimetric assay, TrxR activity was measured by the reduction of 5,5'dithiobis (2-nitrobenzoic) acid (DTNB) using NADPH to 5-thio-2-nitrobenzoic acid (TNB^{2-}), and absorbance was measured at 412 nanometers. Two million PEO1 or PEO4 cells were treated with 1, 2, or 4 μM auranofin for 24 hours. The cells were placed on ice, collected by scraping, and washed twice with 1 X PBS; they were centrifuged at 1,500 x *g* for 6 minutes, and the supernatant was removed. The cells were resuspended again in 1 mL of 1X PBS and centrifuged at 2,000 x *g* for 5 minutes. The supernatant was decanted, and the cell pellet was snap frozen in liquid nitrogen and stored at -80°C until further processing. The cell pellet was homogenized on ice with 150 μL of cold assay buffer containing 1 X protease inhibitor cocktail (Abcam), and centrifuged at 12,000 x *g* for 15 min at 4°C. The protein concentration in the supernatant was quantified using the BCA protein assay (Pierce). Two sets of 50 μg of protein for each sample, and 10 μL of the TrxR positive control, were loaded into a 96 well plate, and the volume was adjusted to 50 μL with TrxR assay buffer. Ten microliters of TrxR inhibitor were added to one set to test the background enzyme activity, and 10 μL of assay buffer was added to the other set to measure total DTNB reduction. A standard curve was generated with 0, 10, 20, 30, 40, and 50 nmol/well, which was adjusted to a final volume of 100 μL with assay buffer. A reaction mix containing TrxR assay buffer, DTNB solution, and NADPH was prepared, and 40 μL of the reaction mix was added to the positive control and to each sample and mixed well. Optical density (OD) was measured at 412 nanometers (nm) to obtain A_{1AB} and A_{1INH}, and the samples were incubated for 20 minutes at 25°C and measured at 412 nm again to obtain A_{2AB} and $A_{2INH.}$ To determine the optical density of TNB^{2-} generated by TrxR, the following calculation was used: $\Delta A_{412nm} = (A_{2AB} - A_{2INH}) - (A_{1AB} - A_{1INH})$; where AB is assay buffer and INH is the inhibitor. TrxR activity was determined using the following formula: TrxR

activity = ΔB/[(T2-T1) x V] x sample dilution factor = nmol/min/mL = mU/mL; ΔB: nmol was calculated by applying ΔA_{412nm} to the TNB standard curve; T_1: time of the first reading (min); T_2: time of the second reading (min); and V: pretreated sample volume
(mL). One unit of TrxR is the amount of enzyme that generates 1.0 µmol of TNB per minute at 25°C.

In Vitro Analysis of Total GSH

The GSH assay kit was purchased from Abcam. This colorimetric assay measures the concentration of reduced GSH *in vitro.* The kit contains a chromophore, and the reduction of the chromophore by an enzyme can be determined kinetically by measuring the absorbance at 450 nanometers. The absorbance is directly proportional to the amount of GSH that is present in each sample. PEO1 and PEO4 cells were treated with 1 or 2 µM auranofin in the presence or absence of 5 µM L-BSO for 24 hours. Cells were placed on ice, collected by scraping, and washed twice with 1 X PBS. Cells were centrifuged at 1,500 x *g* for 6 minutes, and the supernatants were removed. Cells were resuspended again in 1 mL of 1X PBS, split into two tubes to obtain two sets of samples, and centrifuged at 2,000 x *g* for 5 minutes. The supernatants were decanted, and the cell pellets were snap frozen in liquid nitrogen and stored at -80°C until further processing. One set of samples was used to determine the protein concentration in mg/mL. The other set of samples was homogenized on ice using 100 µL of 5% sulfosalicylic acid, vortexed, and kept on ice for 10 minutes. The samples were then centrifuged at 12,000 x *g* at 4°C for 20 minutes, and the supernatant was collected and kept on ice. The samples were diluted 5-fold using the GSH assay buffer, and 10 µL of the diluted samples were added per well in a 96-well plate for the sample well and the sample background control well. The volume of each sample was adjusted to 20 µL/well with GSH assay buffer. A standard curve was produced with 0, 0.4, 0.8, 1.2, 1.6, and 2 nmol/well

of the GSH standard, which was adjusted to 20 µL/well with GSH assay buffer. A reaction mix containing substrate mix A, diluted enzyme mix A, enzyme mix B, enzyme mix C, and substrate mix B was prepared; 80 µL of the reaction mix was added to each sample wells and the GSH standard wells. A background control mix was prepared containing everything except the diluted enzyme mix A, and 80 µL of the background control mix was added to the wells of the sample background controls. The absorbance was then measured kinetically at 450 nanometers for 60 minutes at room temperature, and the absorbances at two time-points within the linear range were selected for each sample. The concentration of GSH was then determined using a formula recommended by the provider as follows: first, the rate of each standard reading and sample reading was calculated; rate = $[(OD_2 – OD_1)/t_2 – t_1)]$ where OD_2 is the optical density at the second time point, and OD_1 is the optical density for the first time point; t_1 is the initial time in min, and t_2 is the second time in min. The 0-standard rate was subtracted from all the standard rates, and the GSH standard curve was plotted to obtain the slope of the curve; thereafter, the rate of the background corrected samples was calculated by subtracting the sample background control rate from the sample rate; rate background corrected samples = $[\text{rate}_{\text{sample}} - \text{rate}_{\text{background control}}]$; then, the rate of the background corrected samples was applied to the GSH standard curve to calculate the amount of GSH in each sample: B = Rate background corrected samples /slope of the standard curve; and, finally, the GSH amount in sample was calculated as (B/ [VxP]) x D = nmol/mg, where B is the amount of GSH from the standard curve (nmol), V is the volume of sample added in each well (mL), P is the protein concentration in mg/mL, and D is the sample dilution factor.

Statistics

For tests involving Western blot analysis, the experiments were repeated at least twice with a similar outcome. All other data represent triplicate experiments and are expressed as the mean ±

SEM. Differences were considered statistically significant if $p<0.05$. GraphPad Prism 9 (GraphPad Software, La Jolla, CA, USA) was used for statistical analysis of data using *t*-test to compare two groups, or one-way ANOVA followed by Tukey's multiple comparison test to compare more than two groups within an experiment.

Results

Auranofin Reduces the Vitality of HGSOC Cells Regardless of Their Sensitivities to Cisplatin

To test whether blocking TrxR impairs the metabolic activity or wellbeing of ovarian cancer cells, we exposed sibling cell lines to auranofin and assessed their vitality by measuring the activity of mitochondrial enzymes. We used a pair of cell lines, termed PEO1 and PEO4, which have different sensitivities to platinum. For instance, we recently demonstrated that PEO4 cells are approximately ten times less sensitive to cisplatin than their PEO1 siblings obtained from the same patient earlier during disease evolution [188]. Despite the large difference in platinum sensitivity between the two cell lines (**Figures 3.1A and 3.1B**), both cell types responded to increased concentrations of auranofin with similar impairment in wellbeing as denoted by the similar decrease in their vitality demonstrated by the IC50s, which were similar in both cell lines (**Figures 3.1C and 3.1D**; see actual IC50s on the right corner of the panels). This signifies that both cell lines are equally sensitive to auranofin, at least in terms of the impairment of their mitochondrial metabolic activities.

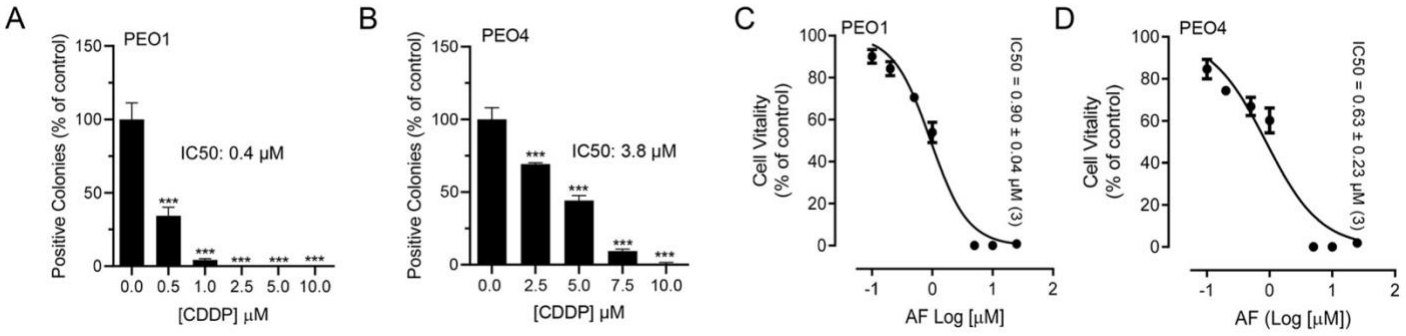

Figure 3.1. Effect of auranofin (AF) on the vitality of PEO1 or PEO4 cells. Cells were treated with DMSO (vehicle) or with various concentrations of cisplatin (CDDP) or auranofin (AF) for 72 hours. At the end of the treatment, the cells were subjected to a clonogenic survival assay (for CDDP) and vitality assay (for AF), as detailed in Materials and Methods. Panels A and B show contrasting clonogenic survival among the cell lines in response to CDDP whereas panels C and D show a similar decrease in vitality caused by AF in the two cell lines.

Auranofin Inhibits TrxR Activity

Since auranofin is known to inhibit TrxR [197], we tested whether indeed this occurred in PEO1 and PEO4 cells. PEO1 cells had relatively high basal TrxR activity, which was comparable to that of the rat liver homogenate used as a positive control (data not shown). Once treated with different concentrations of auranofin for 24 hours, there was a potent inhibition of TrxR activity (**Figure 3.2A**). In PEO4 cells, basal TrxR enzymatic activity was lower than that found in PEO1 cells; nevertheless, the activity of TrxR was significantly reduced further by auranofin in a concentration-dependent manner (**Figure 3.2B**). Our results demonstrate that in HGSOC cells auranofin hinders the activity of its primary target, TrxR.

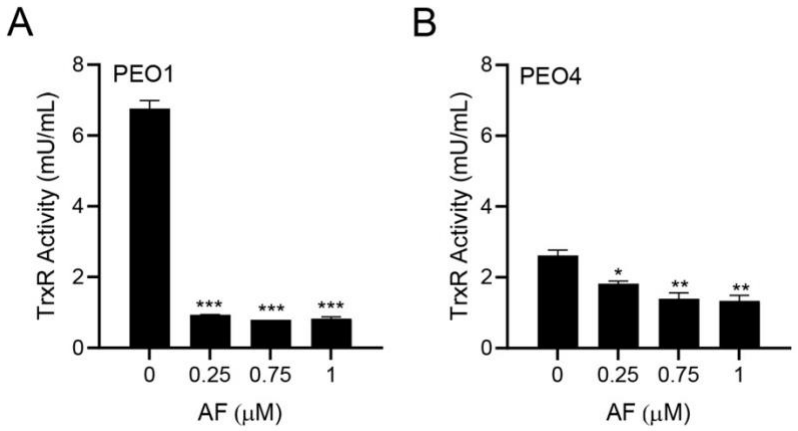

Figure 3.2. Effect of auranofin (AF) on the activity of the enzyme TrxR. In this colorimetric assay, TrxR activity was measured by the reduction of 5, 5'-dithiobis (2-nitrobenzoic) acid (DTNB) using NADPH, to 5-thio-2-nitrobenzoic acid (TNB2-) as detailed in Materials and Methods. *$p<0.05$, **$p<0.01$, and ***$p<0.001$ when compared to vehicle.

Auranofin Triggers the Accumulation of Reactive Oxygen Species

Although TrxR activity was assessed, a direct measure of the influence of TrxR inhibition is the causation of oxidative stress. Thus, to confirm the effect of auranofin on oxidative stress, we measured ROS production in HGSOC cells in the presence or absence of auranofin for 4 hours. **Figures 3.3A** and **3.3B** show the significant increase in the percentage of ROS positive cells in response to auranofin in both platinum sensitive PEO1 cells and platinum resistant PEO4 cells.

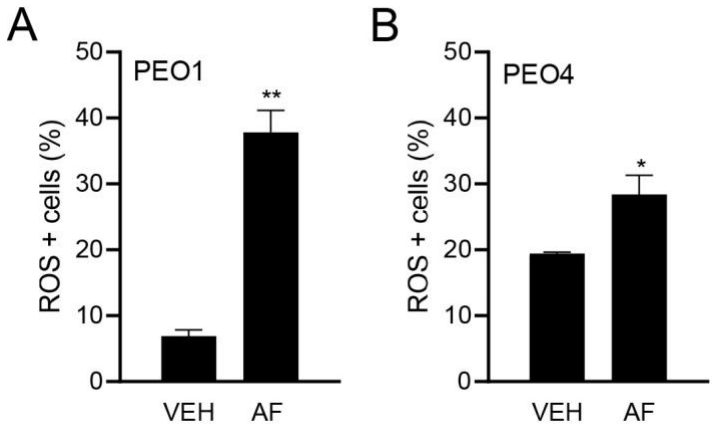

Figure 3.3. Effect of vehicle (VEH) or auranofin (AF) on the production of ROS. The oxidative stress method measures the levels of a cell permeable reagent named dihydroethidium (DHE), which upon interaction with superoxide binds to DNA and produces red fluorescence. Treatment was done with 8 μM AF for 4 hours. *$p<0.05$ and **$p<0.01$ compared to vehicle.

Auranofin Kills HGSOC Cells in Association with Induction of Apoptosis

The reduction in vitality of cells exposed to auranofin shown in **Figure 3.1** suggests that the drug may have cytotoxic effects. Indeed, auranofin reduced cell viability (**Figures 3.4A** and **3.4E**), which was associated with an increase in markers of apoptotic cell death, such as the double labelling of Annexin-V and 7-AAD (**Figures 3.4B** and **3.4F**). Confirmation of apoptosis induced by auranofin was elicited by the accumulation of cells with hypodiploid DNA content (**Figures 3.4C** and **3.4G**). Finally, if cells that were still viable after 72 hours of treatment (**Figures 3.4A** and **3.4E**) were placed in a clonogenic survival plate in the absence of auranofin, the long-term toxicity of the previous exposure to the drug was clearly depicted by the concentration-dependent reduction of viable colonies (**Figures 3.4D** and **3.4H**). In contrast to effects on vitality, in which both platinum-sensitive and resistant cells respond to auranofin in a similar fashion, measuring

lethality-related parameters clearly showed that auranofin is more potent against PEO1 cells than to PEO4 cells. Clonogenic survival depicts a five-fold difference, with an IC50 of 0.53 μM for PEO1 cells (**Figure 3.4D**) and an IC50 of 2.8 μM for PEO4 cells (**Figure 3.4H**).

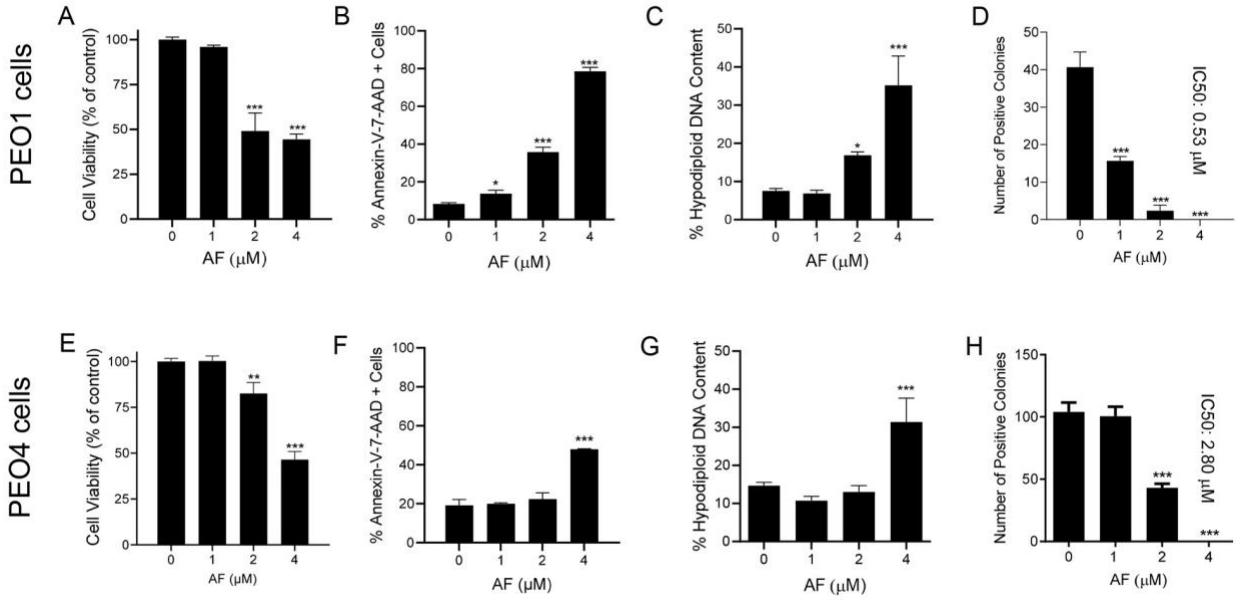

Figure 3.4. Viability of PEO1 and PEO4 cells after 72 hours of treatment with auranofin (AF) assessed using a cytometric viability assay (A and E). In a similar experiment, cells were stained with Annexin V and 7-AAD to determine apoptosis (B and F). Cells remaining from the viability experiment were also stained with propidium iodide and studied for cell cycle distribution; only the hypodiploid DNA content is shown (C and G). Finally, cells that were still alive after 72 hours of treatment with AF shown in (A) and (E), were subjected to a clonogenic survival assay to define their long-term reproductive capacity (D and H). * $P<0.05$, ** $P<0.01$ and *** $P<0.001$ compared with cells treated with vehicle.

Auranofin Induces Dissipation of the Mitochondrial Potential, a Phenomenon That Is Prevented by the Presence of the ROS Scavenger N-acetyl Cysteine

Cellular energy produced during mitochondrial respiration is stored as an electrochemical gradient across the mitochondrial membrane, and this accumulation of energy in healthy cells creates a mitochondrial trans-membrane potential ($\Delta\Psi_m$) that enables the cells to drive the synthesis of ATP. The collapse of this potential is believed to coincide with the opening of the mitochondrial permeability transition pores, leading to the release of cytochrome C into the cytosol, which then triggers the downstream events in the apoptotic cascade [204, 205]. Thus, depolarization of the inner mitochondrial membrane potential is a reliable indicator of mitochondrial dysfunction and cellular death by apoptosis [206]. Here, we show that treatment of PEO1 or PEO4 cells with auranofin caused the loss of $\Delta\Psi_m$ and further illustrated that the presence of the ROS scavenger N-acetyl cysteine (NAC) [207] prevented the depolarization of the mitochondrial membrane (**Figures 3.5A** and **3.5B**).

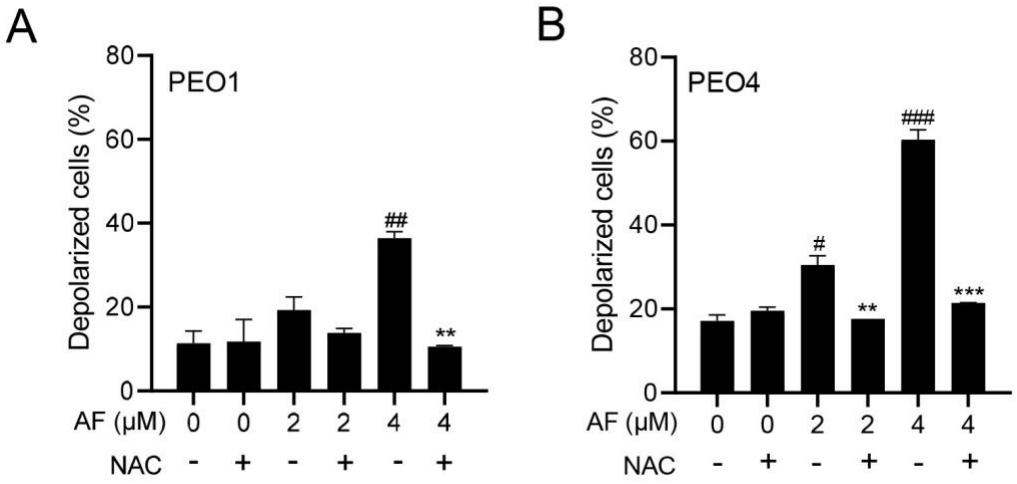

Figure 3.5. Auranofin (AF) increases the percentage of cells with depolarized mitochondria in a dose-dependent fashion. # P<0.05, ## P<0.01, and ### P <0.001 when compared to vehicle-treated controls; the addition of NAC blocked the effect; ** P<0.01 and *** P<0.001 when compared to

the corresponding AF-treated groups. Mitochondrial depolarization was assessed by a cytometric method that utilizes a Mito-Potential dye. A high membrane potential drives the dye into the inner membrane of intact mitochondria, resulting in high fluorescence. Cells with depolarized mitochondria showed decreased fluorescence. NAC, N-acetyl cysteine.

Auranofin-Induced Cell Death Is Prevented by N-acetyl Cysteine

To determine whether the mechanism of cytotoxicity by auranofin is dependent on the production of ROS, PEO1 and PEO4 cells were cultured in the presence of 2 or 4 μM auranofin with or without the addition of 5 mM NAC. The results presented in **Figure 3.6** show that auranofin reduced cell viability in a concentration-related manner and this effect was prevented by the antioxidant NAC (left panels in **Figure 3.6**). The right panels in **Figure 3.6** clearly show the morphological deterioration of the cell cultures in the presence of auranofin and how the deterioration was prevented by NAC.

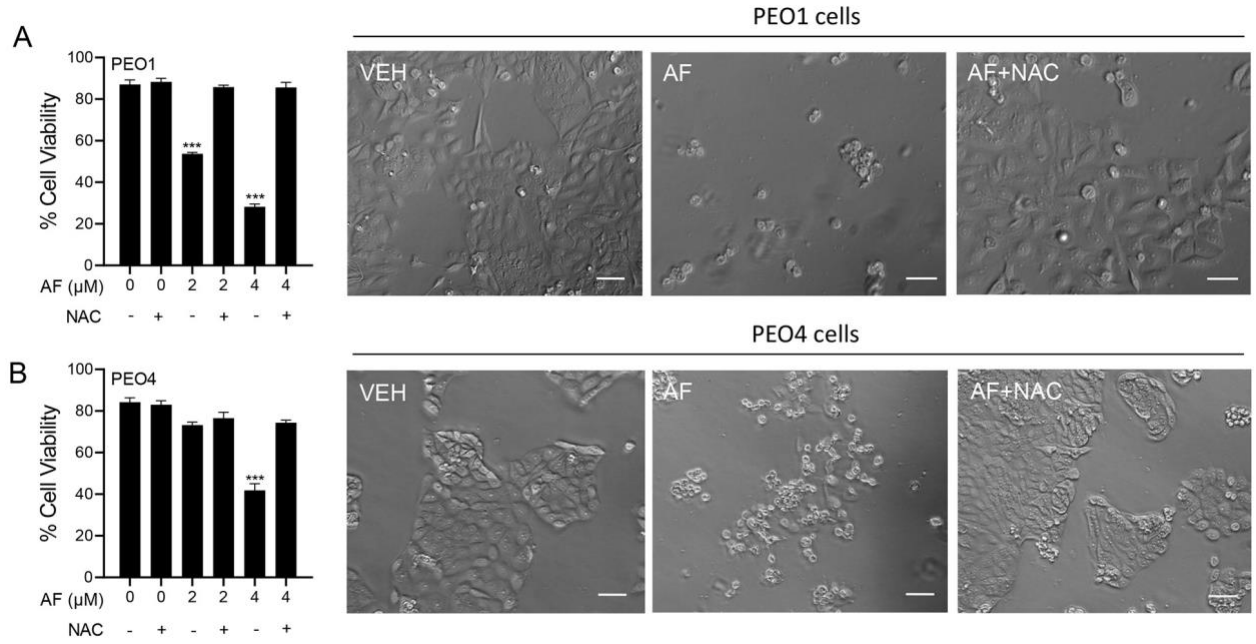

Figure 3.6. PEO1 (A) or PEO4 cells (B) were treated with auranofin (AF) in the absence or presence of 5 mM NAC. Cell viability was assessed after 72 hours using a microcytometer. *** P<0.001 vs. AF+NAC. In the right panels, phase-contrast images were obtained after 72 hours of incubation with the indicated drugs. VEH, vehicle; AF, auranofin; NAC, N-acetyl cysteine. Scale bars = 50 μm.

NAC Prevented Auranofin-Induced Caspase-3/7 Activation, Cleavage of PARP, and Induction of γH2AX

A caspase-3/7 cytometric assay was utilized to quantify the activation of executioner caspases in response to auranofin. The results indicate that auranofin was more effective in PEO1 than in PEO4 cells; nonetheless, such activation was prevented by the presence of NAC (**Figures 3.7A** and **3.7C**). Likewise, and in both cell lines, when assessing the induction of apoptosis by measuring the cleavage of PARP, we observed that auranofin was effective in inducing such a

cleavage, which was, at least in part, prevented by NAC (**Figures 3.7B** and **3.7D**). Finally, auranofin triggered the accumulation of the DNA damage marker γH2AX in both PEO1 (**Figures 3.7E**) and PEO4 (**Figure 3.7F**) cells and this was entirely abrogated by NAC. These results suggest that auranofin mediates both executer caspase activation and DNA damage by the generation of reactive oxygen species (ROS).

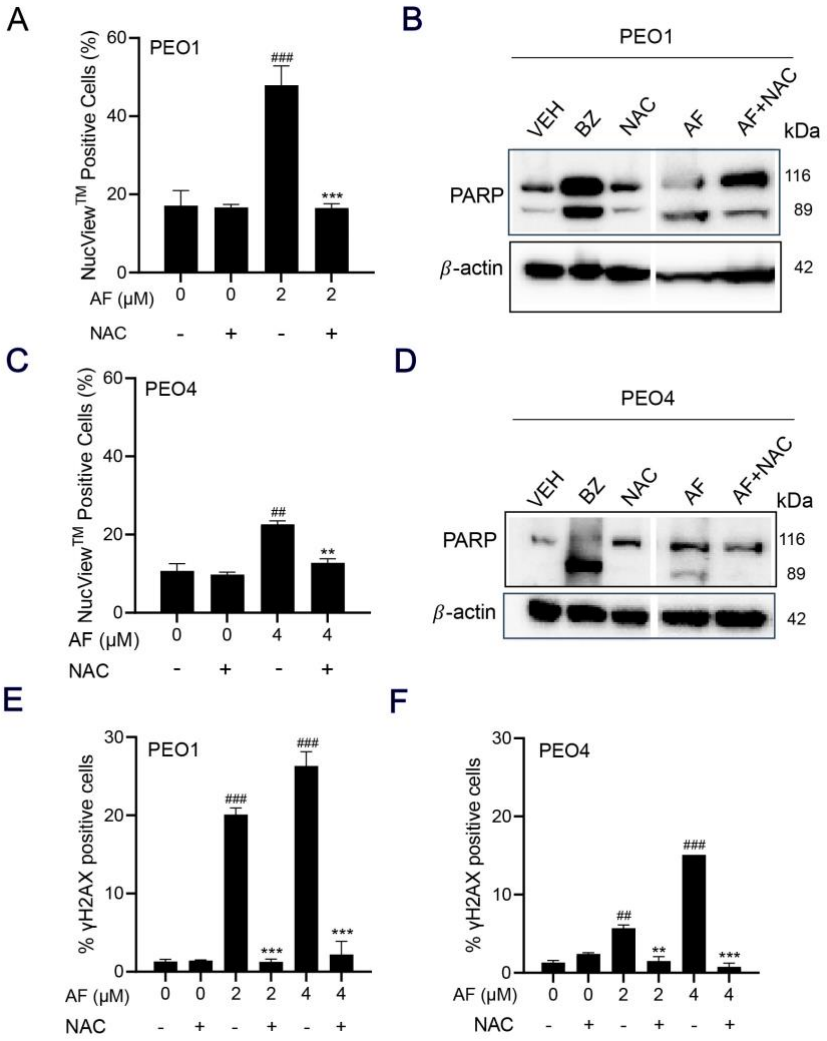

Figure 3.7. PEO1 (A) or PEO4 cells (C) were treated with auranofin (AF) in the absence or presence of 5 mM NAC for 24 hours (to measure PARP), 48 hours (to measure caspase-3/7 activity) or 72 hours (to measure accumulation of γH2AX). In A and C, cells were exposed to the

Muse® Caspase-3/7 reagent, which is cell membrane permeable, in combination with a dead cell dye (7-AAD). ## P<0.01 and ###P<0.001 compared to vehicle. **P<0.01 and ***P<0.001 compared to AF. (B) and (D) depict the effect of AF and NAC on the cleavage of PARP as detected by western blotting. In this experiment, cells treated with 20 nM bortezomib (BZ) were used as a positive control of PARP cleavage. E and F show the accumulation of γH2AX in response to AF with and without NAC. ## p<0.01 and ###p<0.01 compared to vehicle. ** p<0.01 and ***p<0.001 compared to the respective concentration of AF.

The Cytotoxic Effect of Auranofin and L-BSO against HGSOC Is Synergistic and Associates with Enhanced ROS Production and Reduced Levels of GSH

We hypothesized that the toxicity of auranofin could be augmented by blocking an additional, likely compensatory antioxidant system: GSH. Thus, we decided to simultaneously block TrxR with auranofin and GSH with L-BSO [208]. **Figures 3.2** and **3.4** show that auranofin blunted TrxR activity and caused cell death. **Figure 3.8** documents that the combination of auranofin and L-BSO leads to a further reduction in viability when compared to auranofin alone (**Figures 3.8A** and **3.8E**). The interaction between auranofin and L-BSO was synergistic based on the CI method of Chow and Talalay (**Figures 3.8B** and **3.8F**). Furthermore, the elevation of ROS induced by auranofin in both cell types was significantly increased in combination with L-BSO (**Figure 3.8C** and **3.8G**). Finally, we showed that blocking TrxR by auranofin alone leads to a compensatory increase in GSH, which is significantly reduced by the presence of L-BSO (**Figures 3.8D** and **3.8H**).

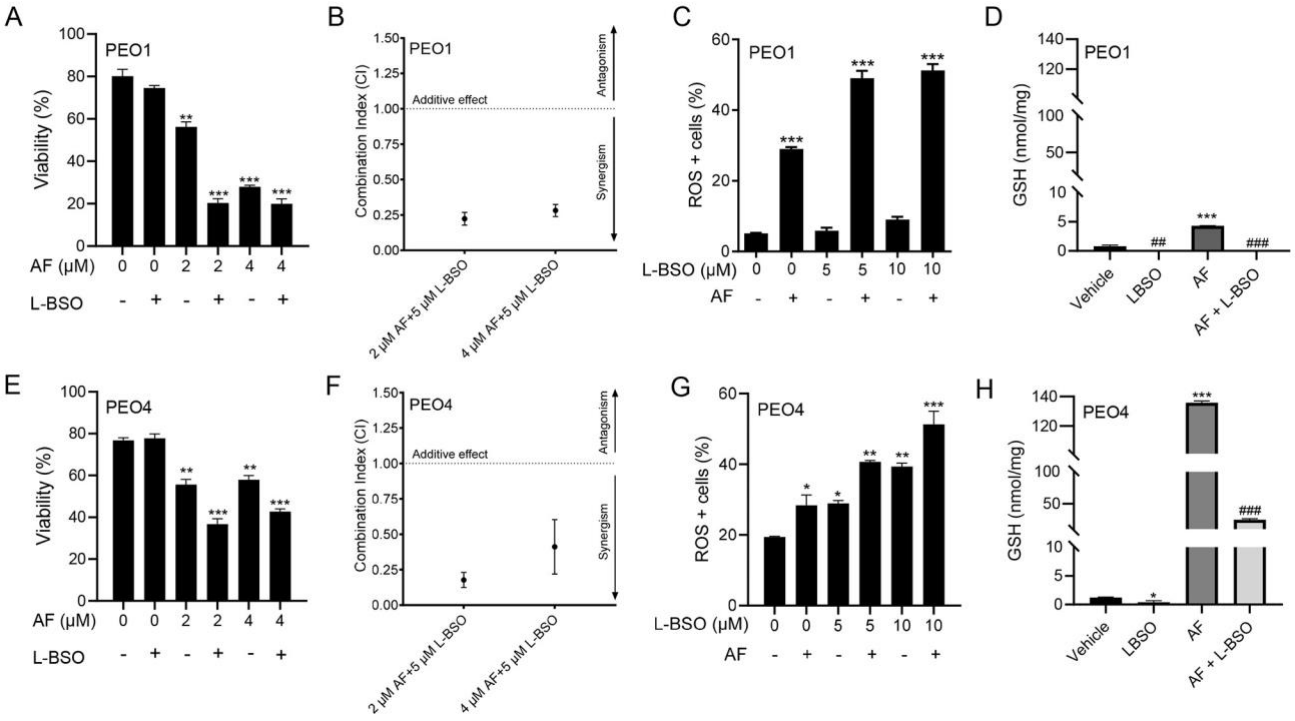

Figure 3.8. PEO1 (A) or PEO4 (E) cells were treated with 2 or 4 μM auranofin (AF) for 72 hours in the presence (+) or absence (-) of L-buthionine sulfoximine (L-BSO), and viability was recorded by cytometry. (B) and (F) The combination indexes of the drug interaction using different combinations of AF and L-BSO showed a CI <1 indicating synergism between the drugs. The CIs were calculated using the viability data of cells treated with the specified concentrations of AF and/or L-BSO in three independent experiments. (C) and (G) show ROS levels in response to AF with (+) or without (-) L-BSO. (D) and (H) display GSH levels respectively in PEO1 cells or in PEO4 cells treated with vehicle, 2 mM AF, or the combination of 2 mM AF and 5 mM L-BSO. In (A), (C), (E), and (G), **p<0.01 and ***p<0.001 vs. control. In (D) and (H), ***p<0.001 vs. vehicle, #p<0.05 and ##p<0.01 vs. vehicle, and ###p<0.001 vs. AF.

The Lethal Effect of Auranofin and L-BSO against HGSOC Is Prevented by the Presence of the ROS Scavenger NAC

We investigated whether the cytotoxicity of auranofin in combination with L-BSO was prevented by the ROS scavenger and antioxidant NAC. In **Figure 3.9A** for PEO1 cells and **Figure 3.9C** for PEO4 cells, we show that cell death caused by 2 μM auranofin was greatly enhanced by the presence of 5 mM L-BSO. Of interest, such reduction in cell viability was almost totally reversed by the presence of NAC. The phase-contrast panels in **Figures 3.9B** and **3.9D** show that the morphology and growth of the cells were negatively affected by auranofin alone and further impaired by auranofin in combination with L-BSO. Consistent with the viability data shown in panels A and C, the presence of NAC prevented the cytotoxic effects, and the cell cultures resemble that of vehicle-treated controls.

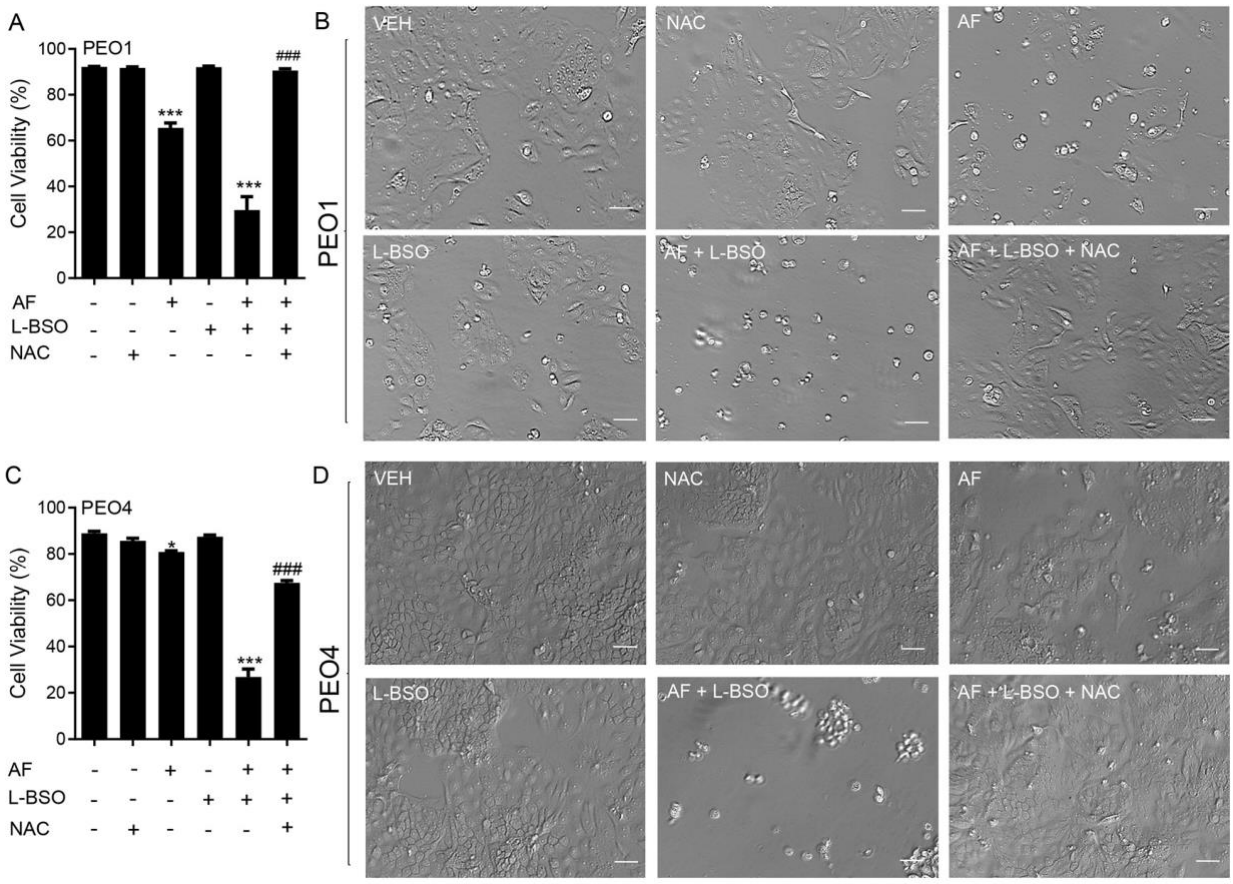

Figure 3.9. Cell viability (A and C) and phase-contrast images (B and D) of PEO1 and PEO4 cells receiving vehicle (VEH), N-acetyl cysteine (NAC; 5 mM), auranofin (AF; 2 mM), L-buthionine sulfoximine (L-BSO; 5 mM), or the combination of AF/L-BSO or AF/L-BSO/NAC. *P<0.05 and ***P<0.001 compared to vehicle; ###P<0.001 compared to the AF/L-BSO group. Scale bars = 50 μm.

Discussion

The cytotoxic properties of auranofin as a monotherapy or in combination with other drugs have been studied in various cancers, including lung [125, 136, 209, 210], breast [134, 139, 211], pancreatic adenocarcinoma [93], colorectal [119], gastric [131], mesothelioma [90], melanoma [105], malignant B-cells and acute lymphoblastic leukaemia (ALL) [100, 107, 127]. In ovarian cancer, auranofin has been shown to block the growth of A2780, SKOV-3, and IGROV-1 cells [61, 96, 101, 117, 126], but none of these cell lines represent the most common histotype which is HGSOC [189, 212]. In our study, we used two cell types, PEO1 (platinum sensitive) and PEO4 (platinum resistant), which were isolated from the same patient throughout the course of the disease [102] and genotypically identified as HGSOC [103]. These provide a valuable model of cellular changes during disease progression, in which PEO1 cells contain a *BRCA2* germline mutation, whereas PEO4 cells have a restored version of the gene [103, 213]. One previous study reported that PEO4 cells were resistant to the combination of auranofin with HSP90 inhibitors [140], but our results showed cytotoxicity by auranofin alone or in combination with the GSH inhibitor L-BSO against both the platinum sensitive PEO1 cells and their sibling platinum resistant PEO4 cells.

Auranofin would be particularly advantageous to improve the therapy for HGSOC because the gold complex has been already approved by the FDA against rheumatoid arthritis, and it is currently enrolled in several clinical trials as a monotherapy and in combination with other drugs (reviewed in [197]). This indicates the feasibility of repurposing auranofin against HGSOC as it has been shown to be clinically tolerable.

The effect of auranofin on cellular vitality or wellbeing [202] assessed through the surrogate activation of mitochondrial enzymes, revealed that both PEO1 and PEO4 cells were equally affected by the drug suggesting that there is no cross resistance between the platinum agent and the gold complex. However, when we studied viability (i.e., the capacity of auranofin to kill the cancer cells), we observed that the drug was more effective against PEO1 cells than PEO4 cells, suggesting that in terms of lethality, there is some degree of cross resistance between platinum and auranofin. This was supported by a series of additional studies on short-term cytotoxic responses to stress in which we showed that PEO4 cells were again less sensitive to the drug than the PEO1 cells.

The induction of apoptosis/death by auranofin represented by positive staining of Annexin-V and 7-AAD was greater in PEO1 cells than in PEO4 cells. Induction of apoptosis has been documented as one of the principal short-term cytotoxic effects of auranofin in other malignancies, including multiple myeloma, acute myeloid leukemia, murine triple-negative breast cancer, lung cancer, and mesothelioma [107, 198, 209, 214]. We further confirmed the induction of apoptosis with the accumulation of hypo-diploid DNA during exposure to auranofin, which concurs with similar effects reported in non-small cell lung carcinoma (NSCLC) cells [215]. The colony formation assay showed that even when plasma membrane permeability was not altered in the short term, auranofin caused long-term inhibition of cellular reproduction by inhibiting their clonogenic survival, which was again more pronounced (~five-fold) in PEO1 cells than in PEO4 cells.

Inhibition of positive colony formation by auranofin has also been demonstrated in a stemlike cancer cell side population and in SKOV-3 epithelial ovarian cancer cells in a p53-independent manner [124, 216].

When we explored the direct effect of auranofin against TrxR activity, the presumed target of the drug when acting against rheumatoid arthritis [194], we observed a marked inhibition of the activity as expected. However, this inhibition was achieved at much lower concentrations than those needed to achieve a cytotoxic effect, suggesting that the inhibition of TrxR is not sufficient to kill HGSOC cells. Similarly, TrxR activity was inhibited by auranofin at non-cytotoxic doses in Calu-6 lung cancer cells [209]. However, other studies have found that auranofin inhibition of TrxR in various cancers requires cytotoxic concentrations, including the endometroid ovarian carcinoma cell line A2780 [164, 189, 209, 214, 217, 218]. Notably, in our studies the inhibition of TrxR was lower in PEO4 cells than in PEO1 cells, and PEO4 cells also displayed less basal TrxR1 activity. This may explain the greater resistance of PEO4 cells to auranofin cytotoxicity, as they may rely less on the TrxR antioxidant system for survival when compared to PEO1 cells. Inhibition of TrxR is a well documented cause of oxidative stress [219]. When confirming the TrxR inhibition by auranofin in HGSOC we showed that the drug elevated ROS production in PEO1 and PEO4 cells and the increase was greater in PEO1 cells. We also noted that basal levels of ROS were higher in the platinum resistant PEO4 cells. This observation is in agreement with evidence that high basal expression of ROS associates with increased resistance to platinum [220]. These elevated levels of ROS are tolerated via the expression of antioxidant genes resulting from the interaction of mutated p53 with the ROS-sensitive transcription factor nuclear factor erythroid 2related factor 2 (NRF2) [221, 222]. This indicates that p53 mutations found in HGSOC can lead to increased ROS levels with a compensatory elevation of antioxidant activity for protection [220]. Once we confirmed that ROS production in HGSOC was induced by auranofin, we then explored whether ROS plays

a role in the cytotoxic effects elicited by the gold complex. We used N-acetyl cysteine (NAC), an agent that reduces ROS indirectly via the upregulation of NRF2 and increases synthesis of the antioxidant, glutathione (GSH) by providing cysteines [207]. NAC can also scavenge ROS molecules directly when it is metabolized into sulfane sulfur species thus exhibiting a cytoprotective role [223]. NAC reversed the lethality and the detrimental morphological effects induced by auranofin in PEO1 and PEO4 cells, demonstrating that these cytotoxic effects are primarily dependent on ROS-induced damage.

In most of the parameters of cytotoxicity that we assessed, PEO4 cells were less sensitive to auranofin than PEO1 cells. Interestingly, however, when analyzing the mitochondrial membrane potential in response to auranofin, PEO4 cells show a higher fraction of cells with dissipation of the mitochondrial potential compared to PEO1 cells. The mitochondrial membrane potential plays a critical role initiating death as part of the intrinsic apoptotic pathway [224]. Studies done in platinum-resistant NSCLC cells and OV2008 C13* cells showed increased mitochondrial mass in comparison to sensitive cell lines [225, 226]. In contrast, other studies found that cisplatinsensitive ovarian cancer cells contain increased mitochondrial content and mitochondrial ROS in comparison to those less sensitive to cisplatin [227, 228]. Thus, controversy exists concerning the role of mitochondrial content on platinum sensitivity. In our case, platinum resistant PEO4 cells may have higher mitochondrial function, reflected by their increased sensitivity to depolarization of the mitochondrial membrane by auranofin in comparison to platinum sensitive PEO1 cells. Notably, the depolarization of the mitochondrial membrane by auranofin in PEO1 and in PEO4 cells was dependent on the production of ROS, as shown by others [229].

In addition to the disruption of the mitochondrial membrane potential by auranofin, we also found a differential increase in the activation of the executor caspases-3/7 and cleavage of

PARP in PEO1 and PEO4 cells, with the effect being greater in platinum-sensitive PEO1 cells. Activation of caspase-3/7 by auranofin has also been reported in mutant p53 NSCLC cells [195], in p53-null SKOV3 ovarian cancer cells [216], in human gastric cancer cells [131], and in chronic lymphocytic leukemia (CLL) [230]. The cell death induced by auranofin in HGSOC was not dependent on the activation of executer caspases as the decrease in viability was not prevented by the presence of a pan-caspase inhibitor (**Figure 3.S1**). Additionally, increased caspase-3/7 activity and PARP cleavage induced by auranofin was dependent on ROS production as it was blocked by NAC. Similarly, cleavage of caspase-3 and PARP by auranofin is ROS-dependent in A549 human lung cancer cells [209], gastric cancer cells [131], and in CLL [230].

Apoptosis commonly occurs as a secondary response to sufficient DNA damage to prevent the survival of cells with genomic instability [231]. Since we found that auranofin elicited caspase3/7-associated apoptosis in HGSOC, we explored the occurrence of DNA damage and detected an increase, which was more significant in PEO1 cells than in PEO4 cells, following short-term exposure to auranofin. The higher sensitivity of PEO1 cells was likely due to their defective homologous recombination DNA repair machinery and germline mutation in *BRCA2* [103]. Accordingly, the decreased sensitivity of PEO4 cells to DNA damage may be primarily due to their functional DNA repair machinery resulting from the reversion of the *BRCA2* mutation [232], which confers platinum resistance by restoring genome stability allowing the cancer cells to proliferate [233]. The DNA damage induced by auranofin was ROS dependent, as it was prevented by the antioxidant NAC. A similar finding was observed in acute lymphoblastic leukemia (ALL), in which auranofin induced DNA damage by increasing ROS levels [107].

Aside from the cytotoxic effects already mentioned, auranofin has been proposed to be a proteasomal deubiquitinase inhibitor [194, 211, 234]. The proteasome degradation cycle is heavily used by cancer cells to regulate protein homeostasis, making this pathway an attractive therapeutic

target in cancer [235]. In this cycle, proteins that are meant for degradation are tagged with ubiquitin [236]. Interestingly, we detected an accumulation of polyubiquitinated proteins in PEO1 and PEO4 cells following treatment with auranofin and demonstrated that such ubiquitination is blocked by NAC, indicating that auranofin-induced polyubiquitination is dependent on ROS production (**Figure 3.S2**). It is unknown from our results, however, whether the polyubiquitination is a consequence of proteasomal inhibition by auranofin, or, as reported by others, because of inhibition of a deubiquitinase enzyme [201].

Since we demonstrated that PEO4 cells were less sensitive to auranofin than PEO1 cells, we conclude that auranofin as a monotherapy may not be sufficient for treating recurrent stages of HGSOC associated with platinum resistance. Auranofin has been shown to elicit anti-cancer effects in various combination treatments, which have been studied in human lung cancer, malignant B cells, breast cancer, gastric cancer, non-HGSOC ovarian cancer, brain tumor cells, and NSCLC (revisited in [197]). However, few studies have combined auranofin with another agent to target HGSOC cells. To develop a proficient consolidation therapy against HGSOC, we combined auranofin with another pro-oxidant: L-buthionine sulfoximine (L-BSO). L-BSO is an inhibitor of the rate-limiting enzyme, γ-glutamcysteine synthetase, which is involved in the synthesis of glutathione (GSH) [237]. At moderate levels, GSH plays protective roles within the cell, including the removal of ROS, regulation of the cell cycle, and regulation of apoptosis and necrosis [238]. Elevated levels of GSH have been detected in various cancers, including breast, ovarian, and lung [239] in association with tumor progression and drug resistance, including resistance to cisplatin [240]. Thus, GSH is an attractive target in platinum-resistant cancer cells, so we combined auranofin with L-BSO to block GSH and simultaneously target both antioxidant systems, TrxR and GSH, in HGSOC. This drug combination is also of interest in mesothelioma, lung cancer, rhabdomyosarcoma, and pancreatic cancer [198, 209, 218, 241]. In HGSOC we documented an

increase in GSH in both PEO1 and PEO4 cells after treatment with auranofin, suggesting that the cells may compensate for the oxidative environment caused by the block of TrxR. However, the addition of L-BSO reduced the elevation of GSH, an effect that was associated with further lethality compared to auranofin alone. The addition of L-BSO also led to higher levels of ROS than those caused by auranofin alone. Of particular interest, L-BSO alone was not able to increase ROS in PEO1 cells, but it did in PEO4 cells. This difference could be related to the higher basal expression of the antioxidant TrxR in PEO1 cells or because PEO4 cells handless higher basal levels of ROS. In summary, compared with monotherapy, the combination of auranofin and L-BSO increased the levels of ROS beyond those triggered by auranofin alone, regardless of the cellular sensitivity to platinum. This suggests that combining these drugs to inactivate both major antioxidant systems may be valuable as a chronic treatment to overcome platinum resistance in HGSOC.

Conclusions

We report that the gold complex auranofin is efficient in impairing the functionality of HGSOC cells that are clinically sensitive or resistant to cisplatin (**Figure 3.10**). We further show that the drug was more efficient in killing the platinum sensitive cells. The mechanism of cell death induced by auranofin involves inhibition of TrxR, depolarization of the mitochondrial membrane, production of ROS and caspase-associated apoptosis linked to DNA damage. This toxicity can be blocked by the antioxidant NAC indicating the relevance of ROS in the toxicity of auranofin. Furthermore, we provide evidence that in compensation for the pro-oxidant effect of auranofin, there is upregulation of GSH, which if blocked with L-BSO diminishes the antioxidant systems and potentiates the toxicity of auranofin in both platinum-sensitive and platinum-resistant HGSOC cells. We anticipate that auranofin can be repurposed to chronically treat HGSOC after initial

cytotoxic chemotherapy as a maintenance therapy alone or in combination with the antioxidant LBSO.

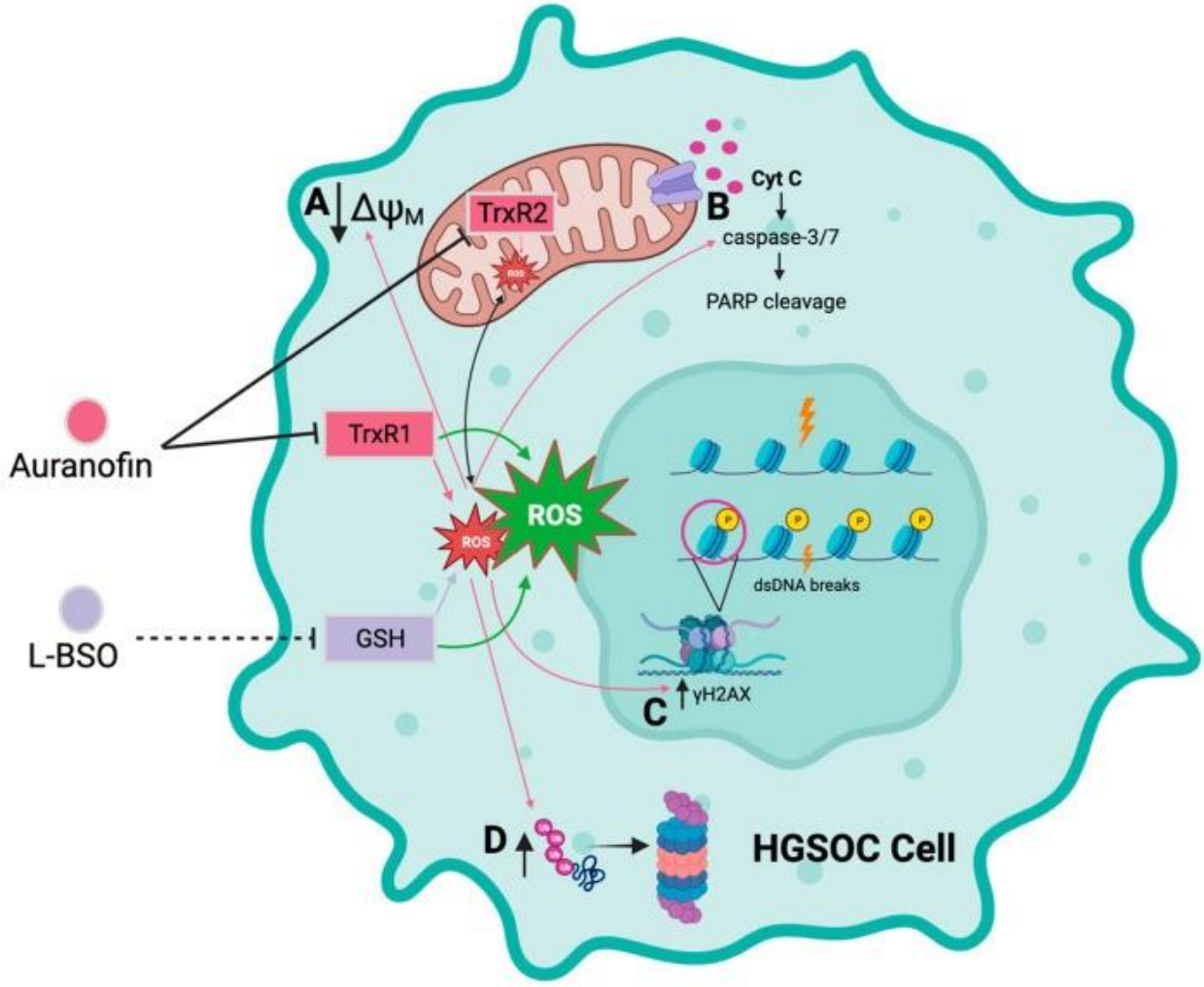

Figure 3.10. Diagrammatic model depicting the cytotoxicity of auranofin alone or in combination with L-BSO in HGSOC cells. Auranofin (AF) inhibits the activity of the antioxidant enzymes thioredoxin reductase 1 (TrxR1) and 2 (TrxR2), inducing an increase in reactive oxygen species (ROS). In turn, increased ROS induced by AF causes; A decreased membrane potential in the mitochondrial membrane, B increased activation of the executor caspase-3/7 and cleavage of polyADP ribose polymerase (PARP), C double-stranded DNA (dsDNA) breaks and phosphorylation of the serine 139 residue of the histone H2AX (γH2AX), and D accumulation of

polyubiquitinated proteins. L-buthionine sulfoximine (L-BSO) indirectly inhibits the production of the antioxidative protein, glutathione (GSH), resulting in an increase in ROS. The combination of AF and L-BSO results in a further increase in the production of ROS compared to the amount of ROS produced by each drug separately. Arrows in pink correspond to the effects caused by ROS as induced by

AF alone. Arrows in green indicate a greater induction of ROS by the combination of AF and LBSO. Created with BioRender.com.

Author Contributions

Conceptualization, F.H.A., S.S. and C.M.T.; methodology, F.H.A. and E.T.; experiments with cisplatin, B.N.F.; formal analysis, A.A.G., E.Z., F.H.A. and C.M.T.; MTT assays, E.M.-J.; original draft preparation, F.H.A.; writing—review and editing, C.M.T.; critical revision, E.Z.; project administration, A.A.G. All authors have read and agreed to the published version of the manuscript.

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Abbreviations

ΔψM	Transmembrane potential
7-AAD	7-Aminoactinomycin D
AF	Auranofin
ALL	Acute lymphoblastic leukemia
ATP	Adenosine triphosphate
BCA	Bicinchoninic acid
BRCA2	Breast cancer type 2 susceptibility protein
BZ	Bortezomib
CI	Combination index
CLL	Chronic lymphocytic leukemia
CO2	Carbon dioxide
DEVD	DNA-binding peptide; substrate for caspase-3
DHE	Dihydroethidium
DNA	Deoxyribonucleic acid
DTNB	5,5′-dithio-bis (2-nitrobenzoic acid)
DTT	Dithiothreitol
FBS	Fetal bovine serum
FDA	US Food and Drug Administration
GLOBOCAN	Global Cancer Observatory
GSH	Glutathione
H2AX	Histone variant H2AX
HCl	Hydrochloric acid
HEPES	4-(2-hydroxyethyl)-1-piperazine ethane sulfonic acid
HGSOC	High-grade serous ovarian cancer

HIV	Human immunodeficiency virus
HRP	Horseradish peroxidase
IC50	Half-maximal inhibitory concentration
IgG	Immunoglobulin G
L-BSO	L-buthionine sulfoximine
MTT	3-(4,5-dimethylliazol-2-yl)-2,5-diphenyltetrazolium bromide
NAC	N-acetyl-L-cysteine
NADPH	Nicotinamide adenine dinucleotide phosphate
NRF2	Nuclear factor erythroid 2-related factor 2
NSLC	Non-small cell lung cancer
OD	Optical density
PARP	Poly-adenosine diphosphate (ADP) ribose polymerase
PBS	Phosphate-buffered saline
PE	Phycoerythrin
P3Cy5	Phycoerythrin-Cyanine®5
PFA	Paraformaldehyde
PMSF	Phenylmethylsulfonyl fluoride
PS	Phosphatidylserine
ROS	Reactive oxygen species
SDS	Sodium dodecyl sulfate
TBS-T	Tris-buffered saline 0.1% Tween 20
TNB	5′-thio-2-nitrobenzoic acid
TrxR	Thioredoxin reductase
z-DEVD-fmk	Caspase-3 inhibitor

Supplementary Figures

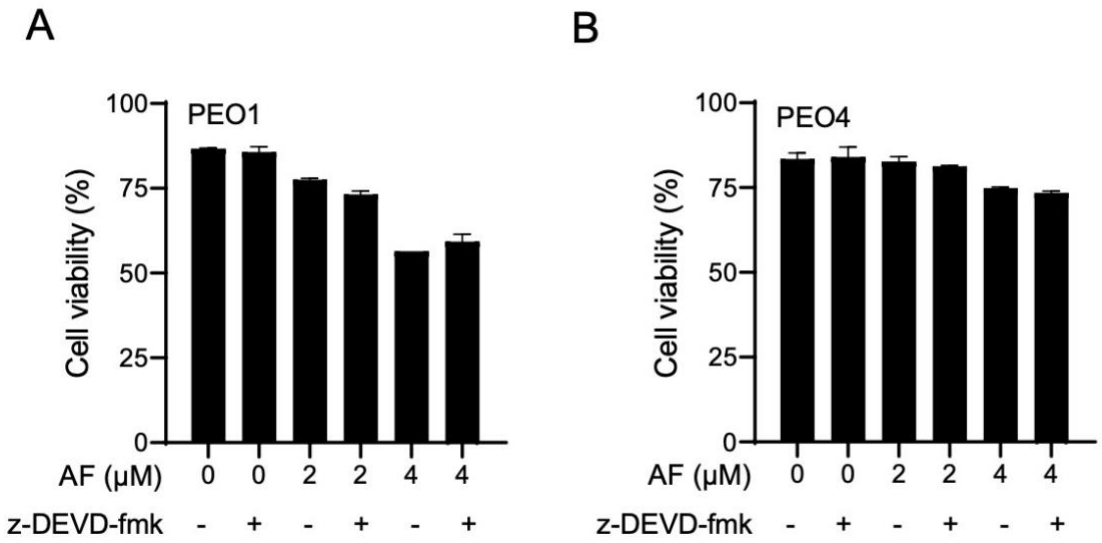


Figure S3.1. Induction of apoptosis by auranofin is not prevented by caspase inhibition

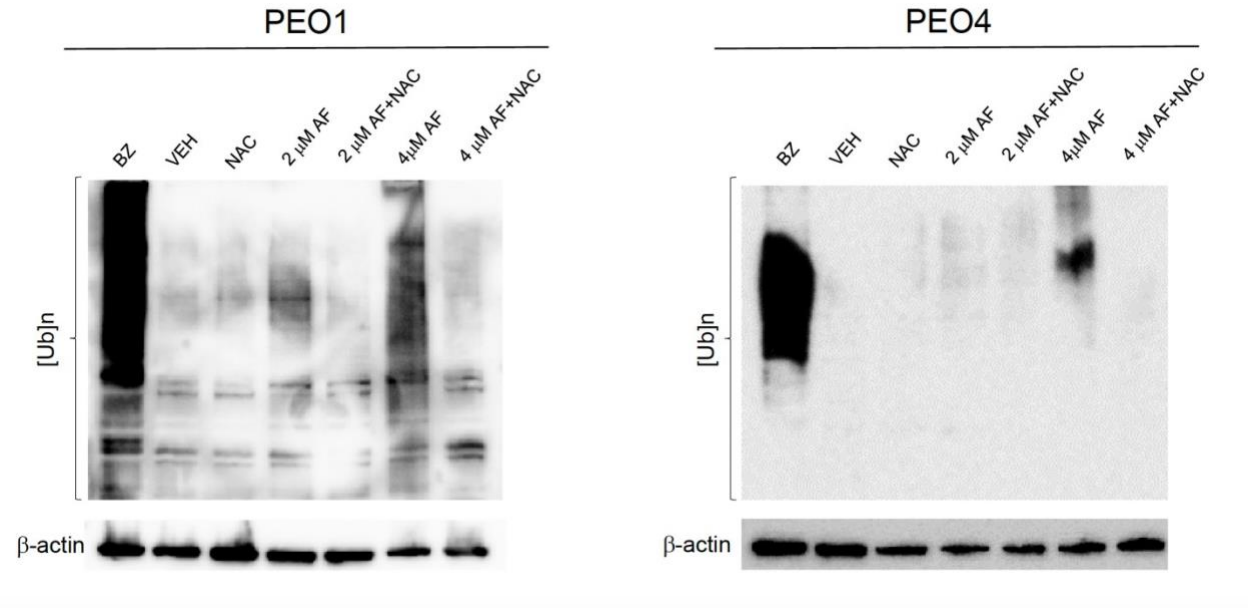


Figure S3.2. Auranofin induces accumulation of polyubiquitinated proteins, an effect prevented by NAC

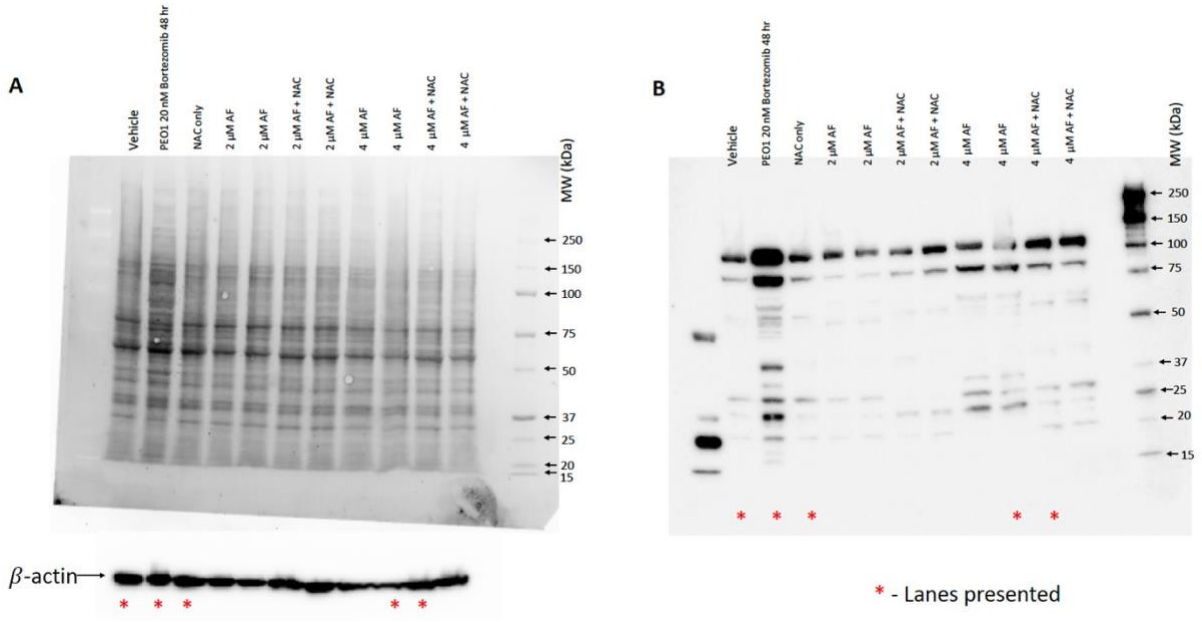
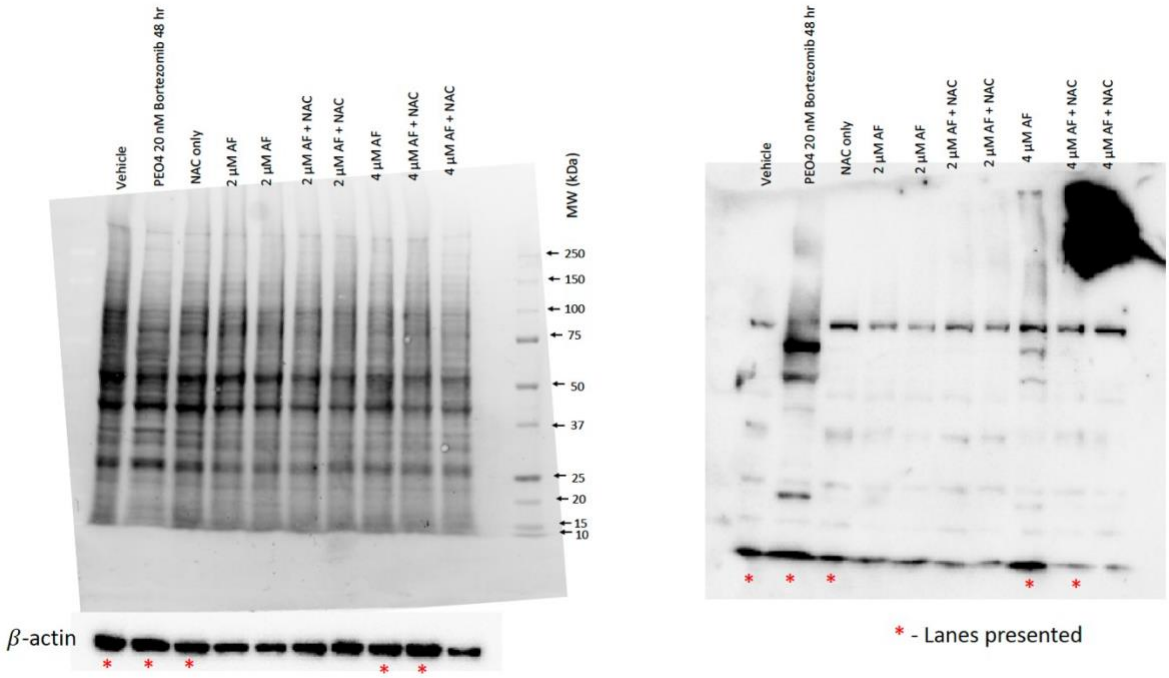
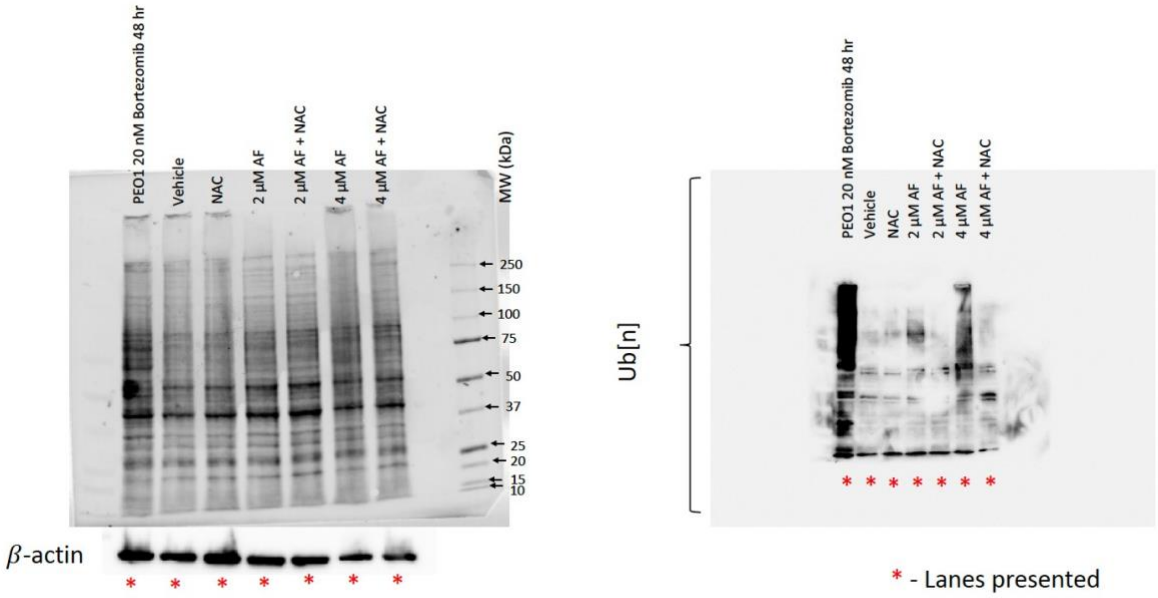


Figure S3.3. Original membranes containing detailed information of the uncropped immunoblot Images

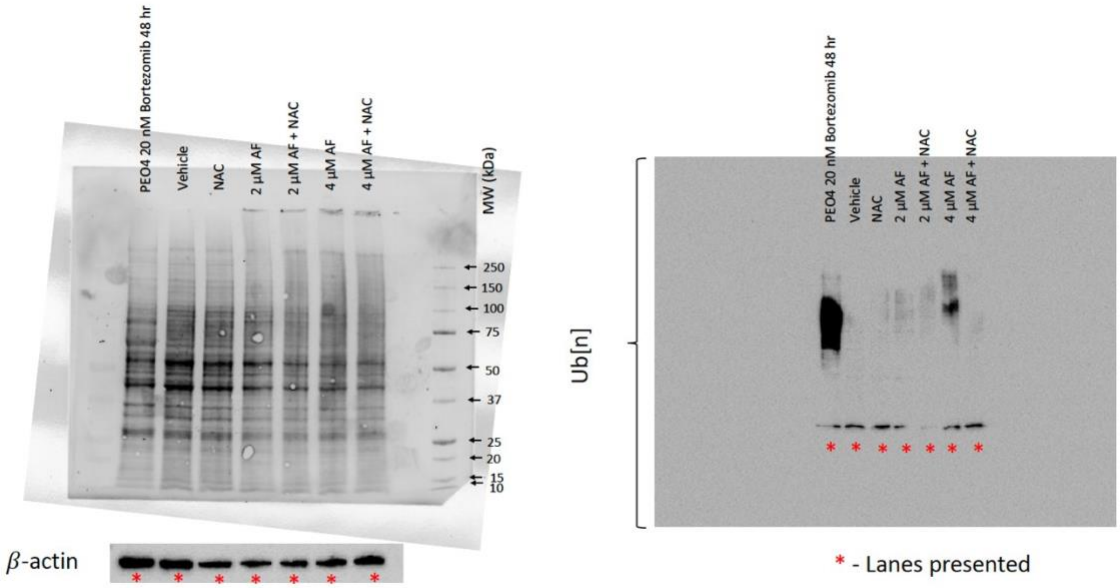
Blot S3.3.1. PEO1 cells were treated with the indicated concentrations of AF with or without the presence of 5 mM NAC for 24 hr. PEO1 cells treated with 20 nM bortezomib (Bz) for 48 hr were used as a positive control. Extracted proteins from the samples were run for 35 min at200V on TGX stain free fast cast acrylamide gel (10 %). After the run, the gel was activated by UV light, and the proteins on the gel were transferred for 7 min using TransBlot Turbo. After the transfer, total protein load was visualized in the unstained membrane using BioRad ChemiDoc imager (A). After 1 h of blocking with 5% non-fat dry milk, the blot was incubated with anti-PARP antibody overnight at 4℃. (B). The blot was then incubated for β-actin (A). Data from this blot was presented in Figure 7B.



Blot S3.3.2. PEO4 cells were treated with the indicated concentrations of AF with or without the presence of 5 mM NAC for 24 hr. PEO4 cells treated with 20 nM bortezomib (Bz) for 48 hr were used as a positive control. Extracted proteins from the samples were run for 35 min at 200V on TGX stain free fast cast acrylamide gel (10 %). After the run, the gel was activated by UV light, and the proteins on the gel were transferred for 7 min using TransBlot Turbo. After the transfer, total protein load was visualized in the unstained membrane using BioRad ChemiDoc imager (A). After 1 h of blocking with 5% non-fat dry milk, the blot was incubated with anti-PARP antibody overnight at 4℃. (B). The blot was then incubated for β-actin (A). Data from this blot was presented in Figure 7D.



Blot S3.3.3. PEO1 cells were treated with the indicated concentrations of AF with or without the presence of 5 mM NAC for 24 hr. PEO1 cells treated with 20 nM bortezomib (Bz) for 48 hr were used as a positive control. Extracted proteins from the samples were run for 35 min at 200V on TGX stain free fast cast acrylamide gel (10 %). After the run, the gel was activated by UV light, and the proteins on the gel were transferred for 7 min using TransBlot Turbo. After the transfer, total protein load was visualized in the unstained membrane using BioRad ChemiDoc imager (A). After 1 h of blocking with 5% non-fat dry milk, the blot was incubated with anti-ubiquitin antibody overnight at 4℃. (B). The blot was then incubated for β-actin (A). Data from this blot was presented in Supplementary Figure S2.



Blot S3.3.4. PEO4 cells were treated with the indicated concentrations of AF with or without the presence of 5 mM NAC for 24 hr. PEO4 cells treated with 20 nM bortezomib (Bz) for 48 hr were used as a positive control. Extracted proteins from the samples were run for 35 min at 200V on TGX stain free fast cast acrylamide gel (10%). After the run, the gel was activated by UV light, and the proteins on the gel were transferred for 7 min using TransBlot Turbo. After the transfer, total protein load was visualized in the unstained membrane using BioRad ChemiDoc imager (A). After 1 h of blocking with 5% non-fat dry milk, the blot was incubated with anti-ubiquitin antibody overnight at 4°C. (B). The blot was then incubated for β-actin (A). Data from this blot was presented in Supplementary Figure S2.

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CHAPTER 4

The Gold Complex Auranofin Sensitizes Platinum-Resistant Epithelial Ovarian Cancer Cells to Cisplatin

4.1 Preface

Chapter 3 focused on the cytotoxic effects of auranofin in HGSOC cells that are representative of different stages of the invasive disease. We demonstrated that auranofin reduced the functionality of the HGSOC cells regardless of their platinum sensitivities when the overall metabolic activity of the cells was tested. However, the anti-rheumatic drug had more potency in the platinum-sensitive cells under other parameters, such as viability and long-term colony formation. This cytotoxicity was elicited through ROS-mediated DNA damage, PARP cleavage, apoptosis, and accumulation of poly-ubiquitinated proteins. Interestingly, the clinically platinumresistant PEO4 cells were more susceptible to mitochondrial membrane depolarization by auranofin than the PEO1 cells. To overcome the cross-resistance between cisplatin and auranofin in the PEO4 cells, we combined auranofin and L-BSO against the HGSOC cells. This resulted in a favourable synergistic interaction between auranofin and L-BSO, which particularly enhanced the killing of the PEO4 cells.

As we proposed the combination of auranofin and cisplatin against cancer in the perspective article, Chapter 4 explores the interaction between the two drugs as a second-line treatment in platinum-resistant epithelial ovarian cancers. The combination of auranofin and cisplatin has not been previously studied in EOC cells. We use two models of EOC cells that represent different sensitivities to cisplatin, one of which, like the PEO4 cells, is spontaneously resistant to cisplatin (TOV112D) and was isolated from a patient with endometrioid ovarian carcinoma. The second model uses clear-cell ovarian carcinoma cells (IGROV-1), which had prolonged exposure to cisplatin to achieve cisplatin resistance (IGROV-1/CP). Approximately 70% of patients with ovarian cancer will relapse and develop resistance to platinum-based chemotherapy, signifying the critical need for a second line of treatment [242].

Cisplatin resistance occurs through various mechanisms, including increased drug efflux, enhanced DNA repair and upregulation of antioxidant systems like GSH [243]. In this chapter, we assessed whether the addition of auranofin to cisplatin helped to prevent those mechanisms of drug resistance from being acquired by the cells. In addition, as outlined in the literature review, the combination of auranofin and cisplatin could theoretically enhance the killing of cancer cells through multiple cellular targets. It was proposed in the literature review that this would occur via ROS-induced DNA damage, immunogenic cell death, and ER stress. However, in the upcoming manuscript, we only assessed the effects of the drug combination in eliciting ROS overproduction and DNA damage in EOC cells. In addition, we focused our study on whether cisplatin resistance was associated with the mitochondrial state. This is of particular interest since the PEO4 cells had prominent changes in the mitochondrial membrane potential in response to auranofin, in comparison to the PEO1 cells. Furthermore, as discussed in Chapter 3, there is a continued controversy regarding whether there is a distinct association between the mitochondria and cisplatin resistance, which the following chapter aimed to address.

Overall, the upcoming communication focused more on the ability of auranofin to sensitize the platinum-resistant EOC cells to cisplatin and explore a few mechanisms of cytotoxicity of which the drugs use to potentiate. This chapter provides further proof of the efficacy of auranofin in overcoming platinum resistance in EOC cells when combined with other therapeutic agents, this one being cisplatin.

The Gold Complex Auranofin Sensitizes Platinum Resistant Epithelial Ovarian Cancer Cells to Cisplatin

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4.2 Abstract

Although there are numerous studies on drug development for ovarian cancer (OC), survival rates for this disease remain low due to platinum (Pt) resistance. Following several rounds of Pt-based chemotherapy, OC cells develop resistance by increasing DNA repair and antioxidant systems. This study aimed to design a treatment approach to combat recurrent stages of OC by repurposing the anti-rheumatic gold complex auranofin (AF). Here we demonstrate that AF enhances the efficacy of cisplatin (CDDP) in Pt-resistant epithelial OC (EOC) cells. The drug combination induces mitochondrial-dependent apoptosis, PARP cleavage, DNA damage, and ROS overproduction. These results suggest the high potential for combining AF with CDDP as a second-line therapy for recurrent EOCs.

Keywords: Ovarian cancer, Auranofin, Cisplatin, Reactive oxygen species, Intrinsic apoptosis, DNA damage

4.3 Main Text

Introduction

The survival rate for ovarian cancer (OC) patients is only 30-40%, largely due to chemotherapy resistance [244]. The present first-line treatment for epithelial OC (EOC), cytoreductive surgery followed by platinum (Pt)-based chemotherapy, was developed in the late 1970s [18, 245]. The Pt derivative cisplatin (CDDP) was approved by the FDA for the treatment of advanced EOC and testicular cancer in 1978 (revisited in [180]). CDDP induces DNA damage via the formation of crosslinks [180, 246]. Other cellular targets include the mitochondria through the induction of reactive oxygen species (ROS), and the plasma membrane through changes in membrane fluidity [180]. Mechanisms of CDDP resistance acquired by cancer cells include increased DNA repair, clearance of misfolded proteins by the ubiquitin proteasome system, and reduced ROS levels via increased abundance of the antioxidant glutathione (GSH) [180]. Consequently, 80% of patients with EOC will experience disease recurrence, with a tumor that is resistant to CDDP [180, 247]. This highlights the need for a therapeutic agent to be coupled with CDDP as a second-line therapy to increase progression-free survival for this fatal disease.

Auranofin (AF), a gold complex approved in 1985 for rheumatoid arthritis, elicits cytotoxic effects in various cancers, including some subtypes of EOC [21, 248]. We previously demonstrated the potent cytotoxicity of AF against the most frequent histological subtype of EOC, high-grade serous ovarian cancer (HGSOC) [249], in which AF targets the thioredoxin reductase (TrxR) antioxidant system and induces depolarization of the mitochondrial membranes [250].

AF and CDDP target similar pathways through various mechanisms, including overproduction of ROS and inhibition of the protein degradation system [180, 194, 235, 251], suggesting that combining these drugs may enhance cytotoxicity in EOC cells.

Few studies have explored the combination of AF and CDDP against cancer, particularly in EOC. Studies on urothelial carcinoma [252] and small-cell lung cancer (SCLC) [253], have shown that AF increased the therapeutic effect of CDDP through cell cycle arrest and ROS accumulation [253]. Consequently, we hypothesized that combining AF with CDDP in EOC cells would enhance cytotoxicity compared to the individual drugs. We explored the cytotoxic efficacy of the AF/CDDP combination in two models of CDDP-resistant EOC cells.

We provide evidence of a synergistic cytotoxic interaction when combining AF and CDDP in CDDP-resistant EOC cells, involving intrinsic caspase-dependent apoptosis, DNA damage, mitochondrial membrane depolarization, and oxidative stress. Combining these two metal-derived drugs was better than either one of them alone in triggering cell death in EOC.

Materials and methods

Reagents and cell lines

TOV112D cells, isolated from a 42-year-old patient, are spontaneously resistant to clinically achievable concentrations of CDDP, and were classified as a dedifferentiated endometrioid ovarian carcinoma based on extensive transcriptional profiling [254, 255]. IGROV1/CP is a cell line derived from IGROV-1 cells which had acquired resistance to CDDP by prolonged exposure in culture to the Pt drug [256]; the original IGROV-1 cells were isolated from a grade 3 solid primary ovarian tumour in a 47-year-old patient [257] with a diagnosis of clearcell ovarian carcinoma [254, 258]. The cell lines were cultured in RPMI 1640 media (GibcoÔ, Hampton, New Hampshire, USA). The composition of the media was reported in detail recently [250]. The drugs used in this study include the following: auranofin (AF; A6733, Sigma Chemical

Co., St. Louis, MO, USA), *cis*-diammineplatinum (II) dichloride (cisplatin [CDDP], P4394, Millipore, Burlington, Massachusetts, USA), and N-acetyl-L-cysteine (NAC) (A7250, SigmaAldrich, St. Louis, Missouri, USA).

Measuring IC50s and combination indexes

The concentration of CDDP that kills 50% of cells (IC50) with or without AF was determined based on cellular viability. IGROV-1/CP or TOV112D were incubated with increasing concentrations of CDDP for 3 h followed by 72-h exposure or not to 2 μM AF. The viability was measured using the Muse® count and viability reagent (MCH600103, Luminex, Austin, TX, USA) [250]. The combination index (CI), which signifies the drug interaction between CDDP and AF, was calculated using the Chou and Talalay method [203], in which a CI>1 means antagonism, a CI of 0 means no interaction, a CI of 1 means addition, and a CI<1 means synergism.

Annexin-V staining

IGROV-1/CP and TOV112D were treated with 10 mM CDDP for 3 h followed by 72-h incubation in the presence or absence of 2 mM or 0.5 mM AF, respectively. Cells were stained with Annexin-V and 7-AAD (Luminex). Annexin-V is localized on the extracellular surface of the plasma membrane when the cell undergoes early apoptosis, whereas 7-AAD enters the cytoplasm due to plasma membrane rupture marking late apoptosis. Details of this technique are reported in our previous publication [250].

Cell cycle analysis

IGROV-1/CP cells and TOV112D cells were treated with 10 μM CDDP for 3 h followed by a 72-h treatment with 2 μM or 0.5 μM AF, respectively. Cell cycle distribution was assessed by microcytometry as previously described [188, 250].

Analysis of the mitochondrial membrane potential

IGROV-1/CP cells were treated with 15 μM CDDP for 3 h followed by a 72-h exposure to 2 μM AF, whereas the TOV112D cells were treated with 10 μM CDDP for 3 h followed by a 72h exposure to 0.5 μM AF. The cells were stained with a working solution consisting of the cationic lipophilic dye, Mito-Potential (Luminex), followed by 7-AAD. This protocol was outlined in detail previously [250].

Isolation of the cytosol for the detection of cytochrome c

IGROV-1/CP cells were treated for 3 h with 10 μM CDDP followed by a 48-h exposure to 2 μM AF. We followed the specific protocol for the isolation and measurement of cytochrome c as developed by others [259].

Protein expression measurement

IGROV-1/CP and TOV112D cells were treated with 10 μM CDDP alone for 3 h, and 2 μM or 0.5 μM AF for 72 h, respectively, or the combination of CDDP for 3 h and AF for 72 h. The method of preparation, extraction, and quantification of the protein lysates was previously outlined in detail [250]. The primary antibodies used were monoclonal anti-beta actin clone AC-15 (A5442, Sigma), polyclonal anti-PARP (9542S, Cell Signaling Technology, Danvers, MA, USA), monoclonal anti-phospho H2AX (Ser139) (9718, Cell Signaling), poly-clonal anti-caspase-3

(9662, Cell Signaling), monoclonal anti-caspase-9 (9508, Cell Signaling), monoclonal anticaspase-7 (D2Q3L1; 12827S, Cell Signaling), polyclonal anti-cytochrome c (4272, Cell Signaling), and monoclonal anti-ubiquitin (58395S, Cell Signaling). Secondary antibodies used were goat anti-rabbit IgG (H + L) conjugate (1706515, BioRad, City, State, Country) and goat anti-mouse IgG (H + L)-HRP conjugate (1706516, BioRad).

Treatment with the caspase-inhibitor z-DEVD-fmk

IGROV-1/CP and TOV112D cells were exposed for 3 h to 10 μM CDDP and for 48 h to 2 μM AF or 0.5 μM AF, respectively, in the presence or absence of 100 μM z-DEVD-fmk (S7312, Selleck Chemicals, Houston, USA). Z-DEVD-fmk is an irreversible inhibitor of caspase-3, which also potently inhibits caspase-6, -7, -8, and -10 (reviewed in [260]). The cell viability was assessed using the Guava Muse® cell analyzer (Millipore).

Measurement of intracellular levels of reactive oxygen species

IGROV-1/CP cells were treated for 3 h with 15 μM CDDP followed by 72-h exposure to 2 μM AF, whereas TOV112D cells were exposed to 10 μM CDDP for 3 h followed by 48-h exposure to 0.5 μM AF. Superoxide levels were detected using an oxidative stress assay previously described [250].

Treatment with antioxidant NAC

To determine whether the cytotoxicity induced by the combination of AF and CDDP was ROS-dependent, IGROV-1/CP cells and TOV112D cells were treated with 10 μM CDDP for 3 h and 2 μM AF or 0.5 μM AF, respectively for 48 h in the presence or absence of 5 mM N-acetyl

cysteine (NAC) (Sigma). Cell viability was determined using the Guava Muse® cell analyzer (Millipore).

Statistics

For tests involving western blot analysis, the experiments were repeated at least twice with a similar outcome. All other data represent triplicate experiments and are expressed as the mean ± SEM. Differences were considered statistically significant if $p<0.05$. GraphPad Prism 9 (GraphPad Software, La Jolla, CA, USA) was used for multiple comparison analysis of data using one-way ANOVA followed by Tukey's multiple comparison test. To compare the IC50s, a non-linear regression curve fit was used.

Results

AF re-sensitizes different subtypes of EOC cells to CDDP

To determine whether AF increases the sensitivity of Pt-resistant EOC cells to CDDP, we exposed IGROV-1/CP cells or TOV112D cells to a fixed concentration (2 μM) of AF and increasing concentrations of CDDP. In IGROV-1/CP cells, the concentration of CDDP that killed 50% of the cells (i.e., the IC50) was 200 ± 23.4 μM (n=3), which decreased to the clinically achievable concentration of 17 ± 3.12 μM (n=3) following the addition of AF. In TOV112D cells, the IC50 for CDDP was 80 ± 23.6 μM (n=3), which decreased to 25 ± 1.1 μM (n=3) in the presence of AF (**Figure 4.1A**). Furthermore, the interaction between the various concentrations of CDDP and the fixed concentration of AF in both cell lines was pharmacologically synergistic, with CI values below 1 **(Figure 4.1B**).

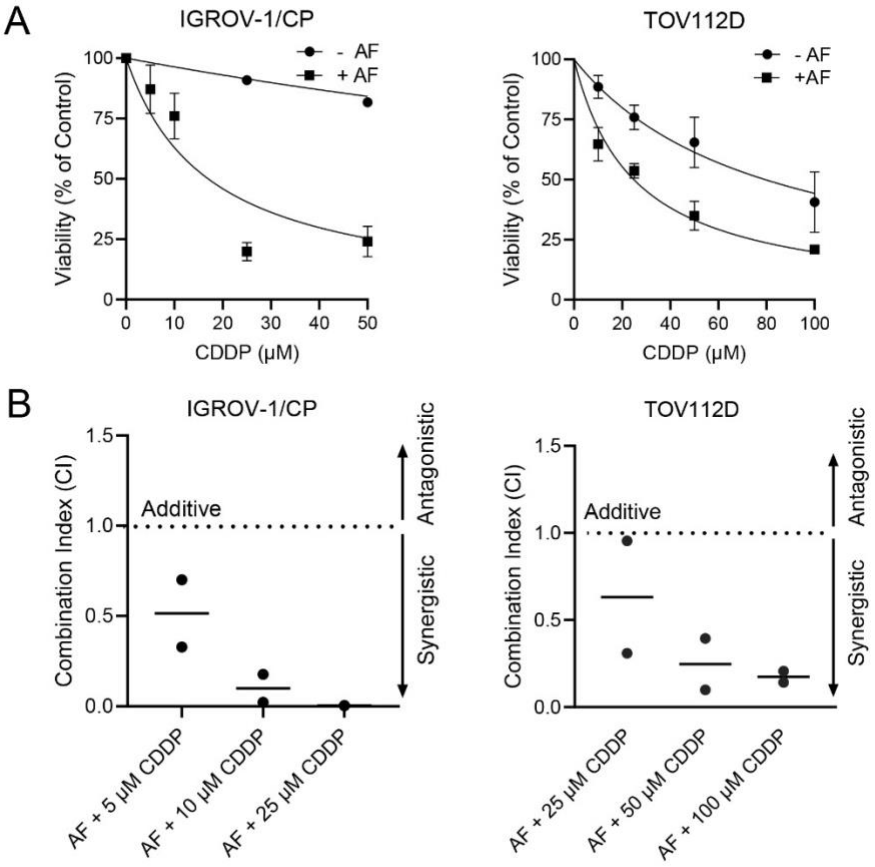

Figure 4.1. Effect of AF on the sensitization of IGROV-1/CP and TOV112D cells to CDDP. Cells were treated with increased concentrations of CDDP for 3 h followed or not by a fixed concentration (2 μM) of AF for 72 h. The viability was determined using microcytometry. Panel A shows a decrease in the IC50s of CDDP in both cell lines following the addition of AF. Panel B displays the synergistic interaction between AF and CDDP (CI<1) in the two cell lines.

The cytotoxicity of AF and CDDP in EOC occurs via apoptosis

The cytotoxic combination of AF and CDDP was associated with an increase in the marker of apoptotic cell death, Annexin-V **(Figure 4.2A**). CDDP alone was able to induce Annexin-V labeling in IGROV-1/CP cells, which was notably enhanced by AF. In TOV112D cells, there was

a slight increase in Annexin-V labeling by CDDP alone, while the addition of AF markedly enhanced it, suggesting apoptotic cell death. At the concentrations used, AF alone failed to induce apoptosis in either of the cell types. In addition to the increased Annexin-V labeling, the drug combination caused an accumulation of hypodiploid DNA content in both IGROV-1/CP cells and TOV112D cells (**Figure 4.2B**) further suggesting the occurrence of apoptotic cell death [261].

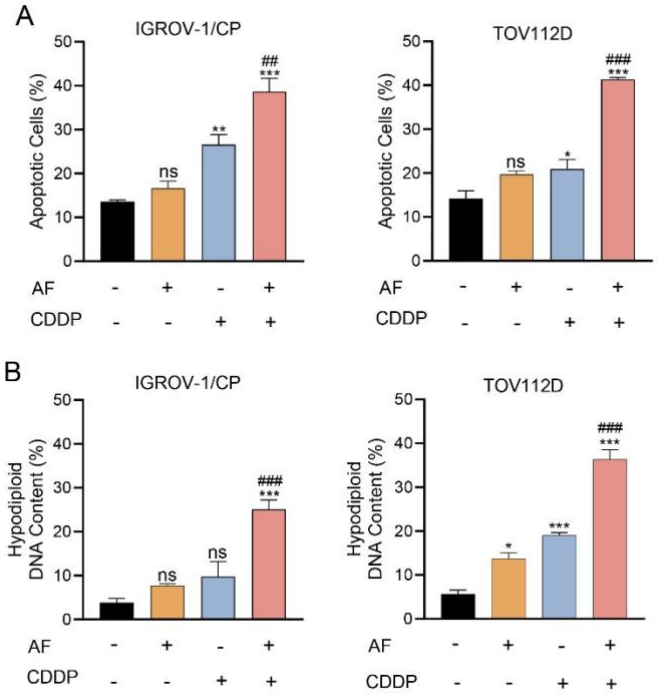

Figure 4.2. Effect of AF/CDDP combination on the induction of apoptosis in IGROV-1/CP and TOV112D cells. IGROV-1/CP and TOV112D cells were treated with 10 μM CDDP for 3 h followed by a 72-h exposure to 2 μM or 0.5 μM AF, respectively; at the end of the incubation cells were stained either with Annexin V and 7-AAD to determine apoptosis (Panel A) or propidium iodide to assess cell cycle analysis (Panel B). * $p < 0.05$, ** $p < 0.01$ and *** $p < 0.001$ when compared to vehicle. ## $p < 0.01$ and ### $p < 0.001$ when compared to AF- or CDDP-treated cells.

The cytotoxicity caused by the combination AF/CDDP is associated with intrinsic apoptosis, PARP cleavage, and DNA damage

To further confirm that apoptosis induced by the combination AF/CDDP in IGROV-1/CP and TOV112D cells is mediated by the intrinsic or mitochondrial pathway, we assessed the cleavage of the initiator caspase-9. Caspase-9 activation by the combination AF/CDDP was evident by the cleavage of the 49 kilodalton (kDa) pro-caspase form into 39 and 37 kDa fragments. Interestingly, the cleavage of pro-caspase-9 form was also induced by CDDP alone in both cell types (**Figure 4.3A**).

Caspases-3 and -7 are executioner caspases in the apoptotic pathway, which cleave a number of common substrates during programmed cell death [262]. In the IGROV-1/CP cells, CDDP induced a slight cleavage of caspases-3 and -7, which was enhanced by the addition of AF. To further document the induction of apoptosis by the drug combination, we measured the cleavage of poly ADP-ribose polymerase-1 (PARP), a nuclear enzyme that is heavily involved in DNA repair [263]. PARP can be cleaved into 89 kDa and 24 kDa fragments by caspases-3 and -7, which inactivates its role in the DNA repair pathway [263]. In IGROV-1/CP, PARP cleavage was highly evident only when cells were exposed to both AF and CDDP. In contrast, PARP cleavage was apparent in TOV112D cells following treatment with either AF or CDDP alone; but it was enhanced by the combination of both drugs (**Figure 4.3A**).

Early events in the DNA damage response, including the cleavage of PARP, also involve the phosphorylation of histone H2AX at serine-139, resulting in the formation of γH2AX. The expression of γH2AX is thought to increase DNA accessibility by DNA repair enzymes [264]. In IGROV-1/CP cells, both AF and CDDP increased the expression of gH2AX, and this was greatly enhanced by the drug combination. In the TOV112D cells, AF alone had little effect on gH2AX,

but there was a notable enhancement by exposure to CDDP and by both drugs in combination (**Figure 4.3A**).

Since we observed the induction of mitochondrial-dependent apoptosis via caspase-9 cleavage by the AF/CDDP combination, we decided to explore the state of the mitochondria in response to the treatment. We found clear changes in the mitochondrial membrane potential of IGROV-1/CP and TOV112D cells following exposure to AF/CDDP when compared to each drug alone (**Figure 4.3B**). The mitochondrial membrane was depolarized slightly by CDDP and more dramatically by the addition of AF to CDDP in IGROV-1/CP cells. In TOV112D cells, there was a slight depolarization of the mitochondrial membrane caused by AF, more so by CDDP, and even more enhanced by the combination AF/CDDP.

Finally, we observed that the cytotoxicity induced by AF and CDDP in IGROV-1/CP cells and TOV112D cells was partially, yet significantly reversed in the presence of z-DEVD-fmk, an irreversible inhibitor of the primary executioner caspase, caspase 3 [262] (**Figure 4.3C**).

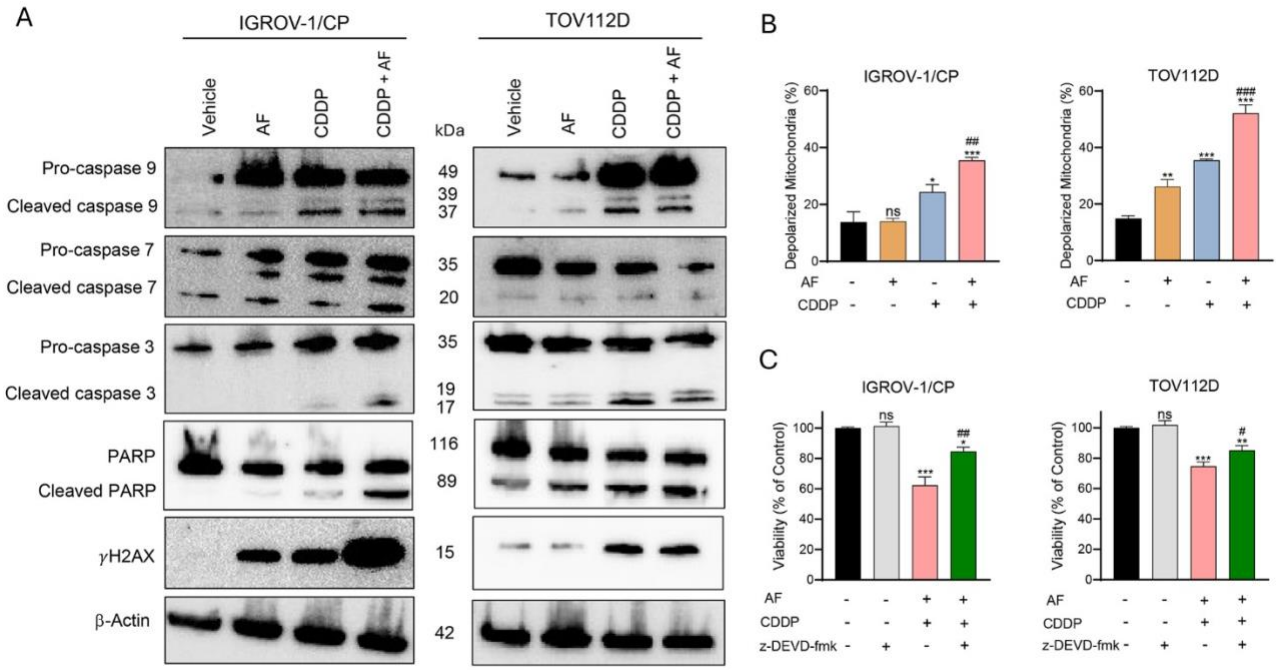

Figure 4.3. A) Effect of CDDP (3 h), AF (72 h), or their combination on the cleavage of caspases9, -7, and -3, and PARP, and the expression of γH2AX in IGROV-1/CP and TOV112D cells as detected by western blotting. β-actin was used as a protein loading control. B) AF/CDDP combination increases mitochondrial membrane depolarization in comparison to each drug alone. * $p < 0.05$, ** $p < 0.01$, and *** $p < 0.001$ compared to vehicle; ### $p < 0.001$ when compared to each drug separately. C) The caspase-3 inhibitor z-DEVD-fmk partially rescues the cells from AF/CDDP-induced cytotoxicity following 48 h treatment; * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ when compared to vehicle. # $p < 0.05$ and ## $p < 0.01$ when compared to AF/CDDP treatment.

AF and CDDP increase oxidative stress in EOC cells

We hypothesized that the disruption of the mitochondrial membrane potential elicited by the AF/CDDP combination in IGROV-1/CP and TOV112D cells was likely caused by ROS accumulation. To determine whether these two metals together act as pro-oxidants we measured the total intracellular levels of ROS in response to AF, CDDP, or the combination of both drugs. In IGROV-1/CP cells, there was a slight induction of ROS by AF, and a greater induction by CDDP, which did not increase further with the addition of AF; in TOV112D cells the drug combination produced a higher ROS accumulation than either drug alone (**Figure 4.4A**).

To determine whether the cytotoxic effect of the combination AF/CDDP is dependent on ROS production, we treated the EOC cells with the drugs in the presence of the antioxidant NAC. NAC rescued IGROV-1/CP and TOV112D cells from undergoing death elicited by the drug combination (**Figure 4.4B**). The healthy appearance of the IGROV-1/CP cells and TOV112D cells in untreated conditions changed to substantial toxicity in the presence of AF/CDDP; however, NAC was able to reverse in part the cell deterioration induced by AF/CDDP (**Figure 4.4C**).

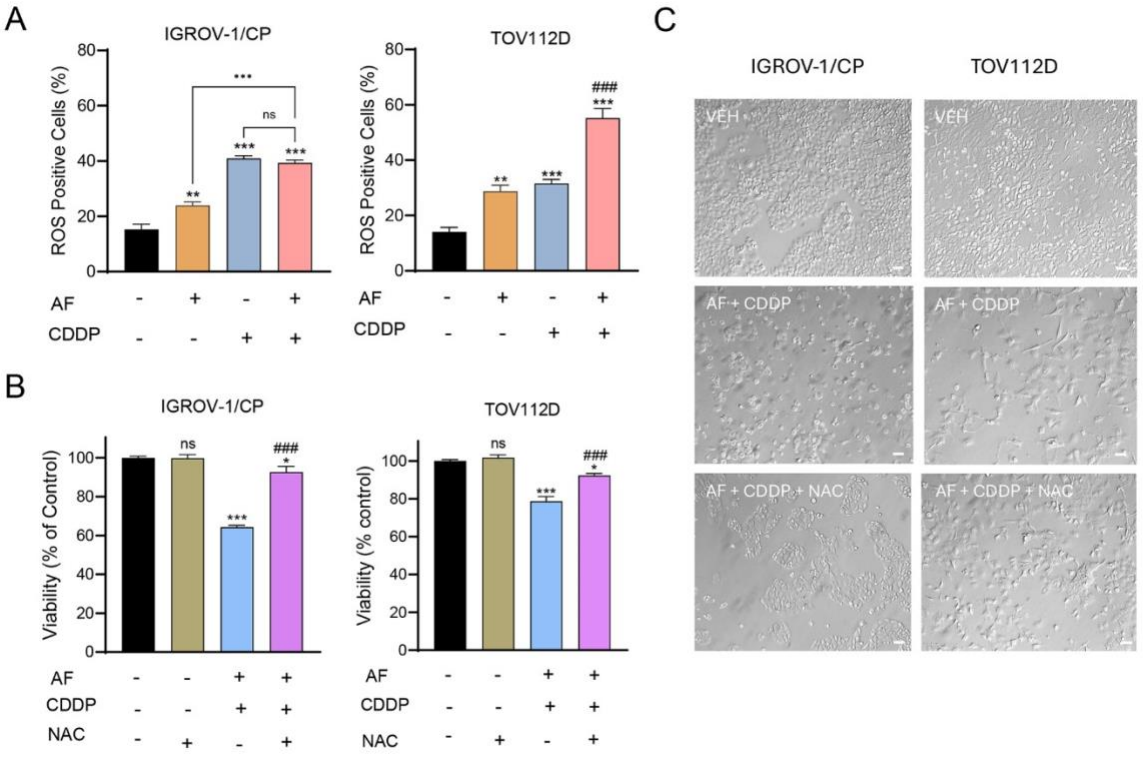

Figure 4.4. A) Effect of AF, CDDP, or their combination on the production of ROS. Treatment was performed with 15 μM CDDP for 3 h and 2 μM AF for 72 h in IGROV-1/CP cells, and 10 μM CDDP for 48 with 0.5 μM AF for 48 h in TOV112D cells. ** $p < 0.01$ and *** $p < 0.001$ when compared to vehicle. ### $p < 0.001$ when compared to AF or CDDP alone. B) Protection of AF/CDDP-induced cytotoxicity in the presence of 5 mM NAC for 48 h. * $p < 0.05$ and *** $p < 0.001$ compared to vehicle. ### $p < 0.001$ when compared to AF/CDDP. C) Phase-contrast images obtained after 48 h incubation with the indicated drugs. VEH, vehicle; AF, auranofin; CDDP, cisplatin; NAC, N-acetyl-L-cysteine. Scale bars = 50 μm.

Discussion

The present study focused on the interaction between the gold complex AF and the DNA damaging agent CDDP in Pt-resistant EOC cells. We demonstrated a synergistic increase in the killing of two EOC cell types when AF was combined with CDDP [254, 256]. Despite the large difference in sensitivity to CDDP between the two cell lines, the addition of AF significantly decreased the IC50s for CDDP in both types of EOC cells to the range of ~20 μM, which is within clinically reachable concentrations [265]. While a similar effect has been reported in small-cell lung carcinoma cells (SCLC) [253] and urothelial carcinoma cells [266], our study is the first to document that AF can restore Pt sensitivity in Pt-resistant EOCs establishing the basis for potential success of this combination in treating this fatal disease.

The combination of AF and CDDP strongly induced apoptosis in both tumor types tested. Apoptosis was triggered through the intrinsic pathway, as the addition of AF to CDDP enhanced the cleavage of caspase-9 and the executioner caspases-3 and -7 [262]. The cytotoxicity of the combination therapy in both cell types is partially dependent on caspase-3 activation, a finding that has not been reported in previous studies on this drug combination [252, 253].

PARP cleavage was also enhanced by combining AF with CDDP, particularly in the more Pt-resistant IGROV1/CP cells, and a similar effect has been reported in SCLC cells [253]. DNA damage was increased by CDDP in both EOC cell lines, indicated by the upregulation of gH2AX. In IGROV-1/CP cells but not TOV112D, the addition of AF to CDDP further increased gH2AX expression.

The AF/CDDP combination induced mitochondrial membrane depolarization in both types of tumor cells, with a more pronounced effect in the TOV112D cells which are somewhat more sensitive to Pt. This is consistent with a study showing less mitochondrial membrane depolarization

in CDDP-sensitive OVCAR-3 and OVCAR-4 cells compared to Pt-resistant OVCAR-8 cells [227]. This depolarization likely triggers the release of cytochrome c [267], which we demonstrated in IGROV-1/CP cells subjected to cell fractionation (**Supplementary Figure 4.1**).

Disruption of the mitochondrial membrane potential can be caused by mitochondrial ROS accumulation [180, 268, 269]. We previously reported that AF acts as a pro-oxidant by inhibiting the TrxR antioxidant system in HGSOC cells [250], an effect also observed in other cancers [20, 270, 271]. Similarly, CDDP generates ROS through mitochondrial damage and electron transport chain impairment [180] in lung and prostate cancer cells [272]. The AF/CDDP combination treatment enhances ROS production in TOV112D cells, but in IGROV-1/CP cells, ROS production seems to be primarily driven by CDDP. This is possibly due to upregulated antioxidant defences as a compensatory mechanism for survival [273]. Of interest, the cytotoxicity elicited by the AF/CDDP combination is ROS-dependent, as demonstrated by the rescue of viability observed when using the ROS scavenger, NAC (**Figure 4.4B**). NAC acts as an antioxidant via GSH replenishment or direct scavenging of oxidant species [274].

AF and CDDP have each been reported to disrupt protein homeostasis [21]. We observed an accumulation of poly-ubiquitinated proteins in IGROV-1/CP cells treated by the AF/CDDP combination, suggesting proteasome inhibition (**Supplementary Figure 4.2**).

In conclusion, we have documented a synergistic cytotoxic interaction between AF and CDDP in Pt-resistant EOC cells that is associated with intrinsic caspase-dependent apoptosis, DNA damage, and ROS accumulation (**Figure 4.5**). These results indicate that AF can overcome Pt resistance in EOC cells and suggests a promising strategy to improve the low survival in patients with recurrent EOC.

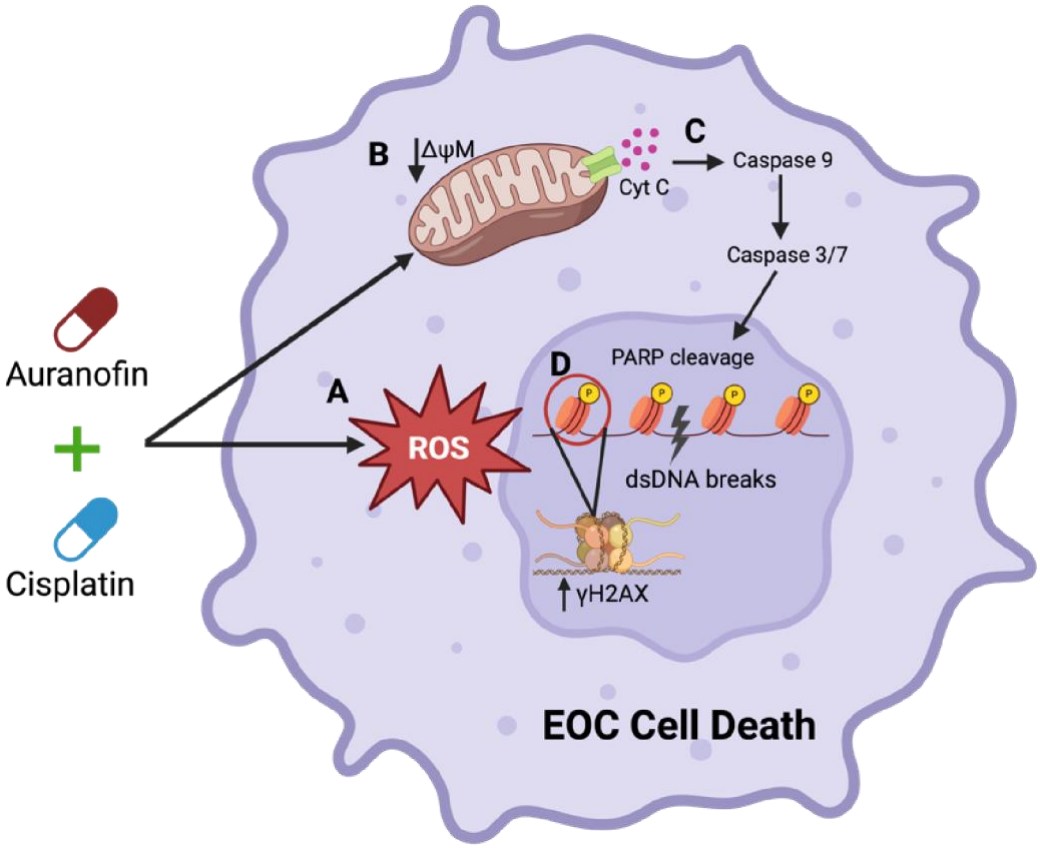

Figure 4.5. Schematic diagram showcasing the effect of the auranofin-cisplatin combination on epithelial ovarian cancer (EOC) cells. The combination elicits enhanced cytotoxicity against EOC via A) overproduction of reactive oxygen species (ROS), B) mitochondrial membrane depolarization C) activation of intrinsic apoptosis via cytochrome C release into the cytosol, caspase-9 activation, cleavage of executioner caspase-3/7, and cleavage of poly-ADP ribose polymerase (PARP) D) induction of DNA damage via phosphorylation of serine 139 residue on histone H2AX producing γH2AX. Created using BioRender.com.

Author Contributions: Farah H. Abdalbari: Investigation, Formal analysis, Methodology, Writing first draft. Benjamin N. Forgie: Investigation, Review and Editing. Edith Zorychta: Review and Editing. Alicia A Goyeneche: Supervision, Methodology, Review and Editing. Abu Shadat M Noman: Funding acquisition, Review and Editing. Carlos M Telleria: Conceptualization, Supervision, Resources, Project administration, Funding acquisition.

Data availability: Data will be made available upon reasonable request.

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Supplementary Figures:

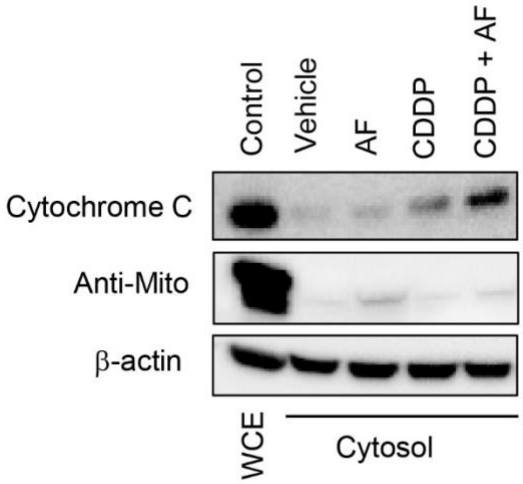


Figure S4.1. AF/CDDP in combination induces cytochrome c release into the cytosol, as detected by western blotting. We used whole cell extracts (WCE) to control for the presence of mitochondrial proteins in the WCE and the absence of mitochondrial proteins in the cytosol using

an anti-mitochondrial antibody (Anti-Mito). It is evident how the combination of AF/CDDP enhances the release of cytochrome c into the cytosolic fraction. IGROV-1/CP cells were treated for 3 h with 10 μM CDDP, for 72 h with 2 μM AF, or with the combination of both before isolation of cytosols.

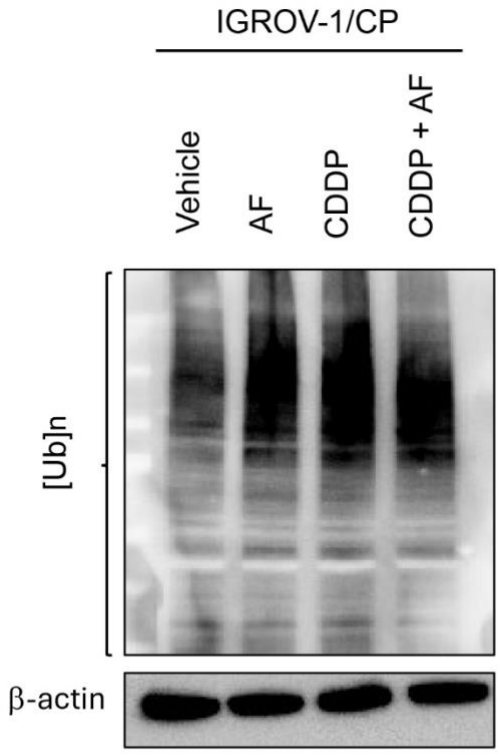
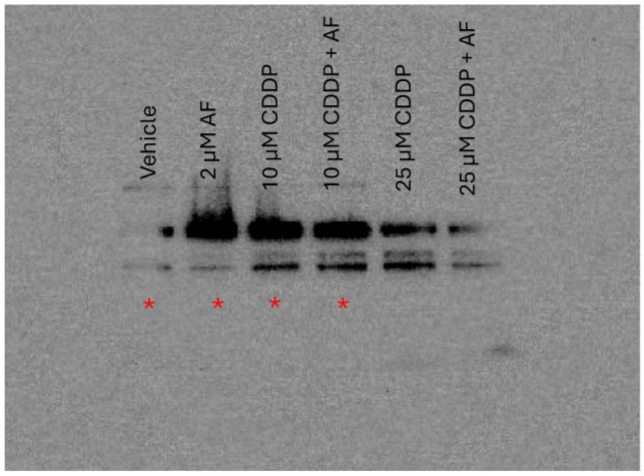


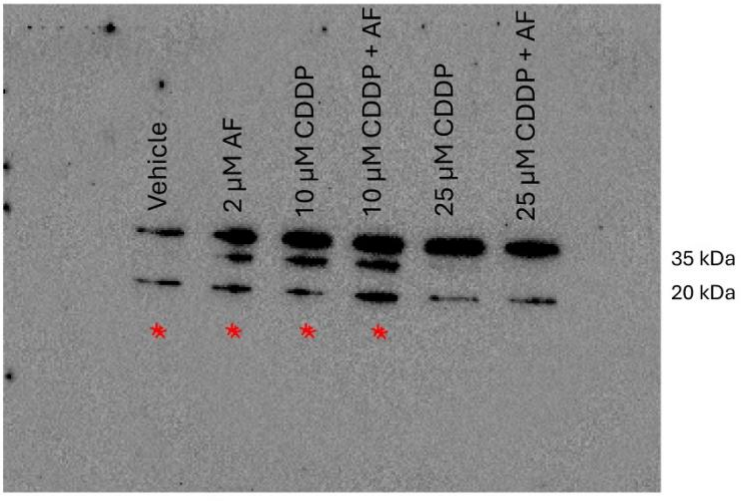
Figure S4.2. Accumulation of poly-ubiquitinated proteins in IGROV-1/CP cells following treatment with 10 μM CDDP for 3 h alone, with 2 μM AF for 72 h alone, or with the combination of both. [Ub]n denotes poly-ubiquitinated proteins as detected by western blotting.

Figure S4.3. Original membranes containing detailed information of the uncropped immunoblot images

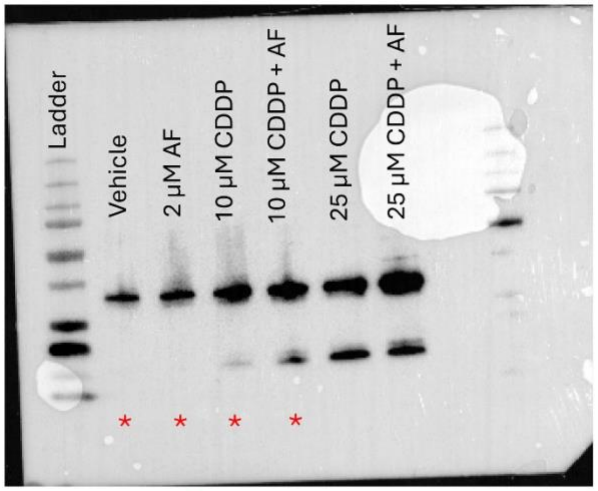
IGROV-1/CP Caspase-9 - Fig. 3A



IGROV-1/CP Caspase-7 - Fig. 3A

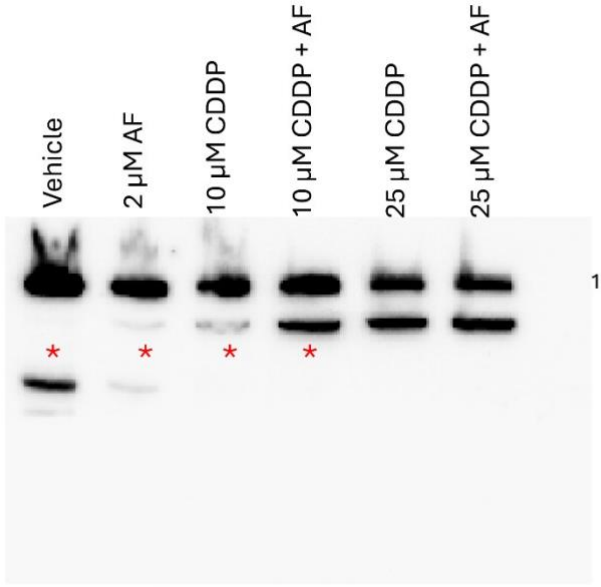


IGROV-1/CP caspase-3 - Fig. 3A



* Lanes presented

IGROV-1/CP PARP - Fig. 3A



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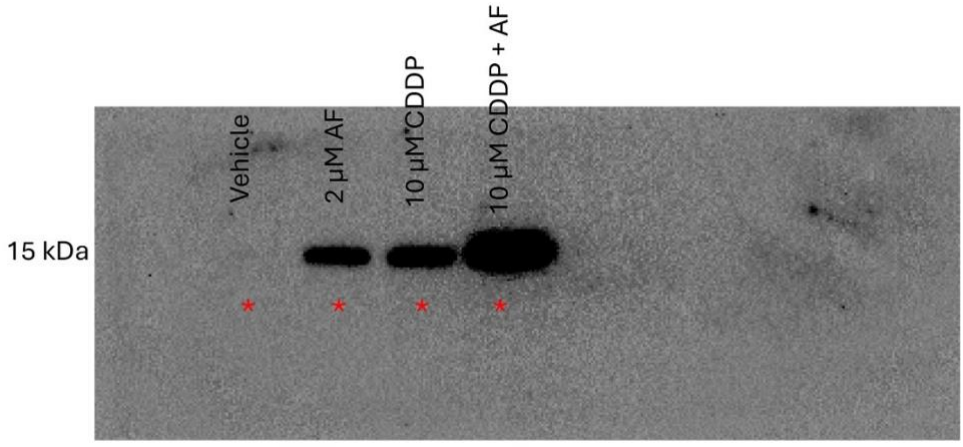
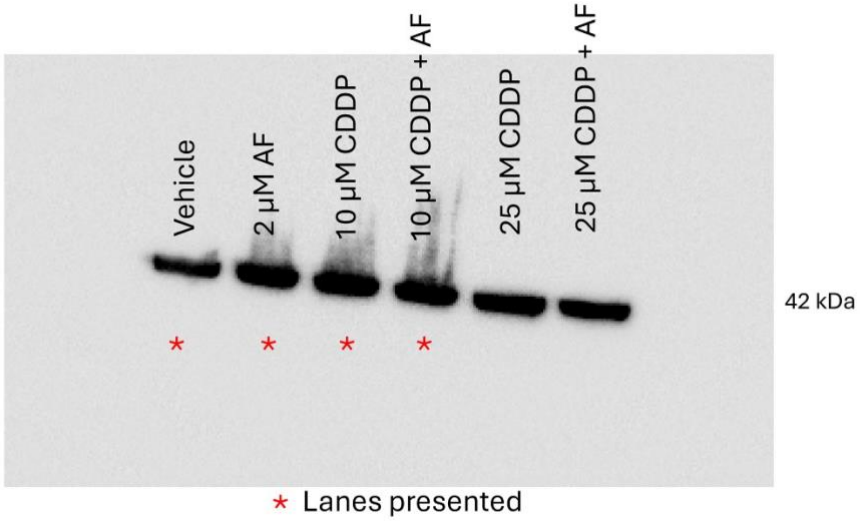
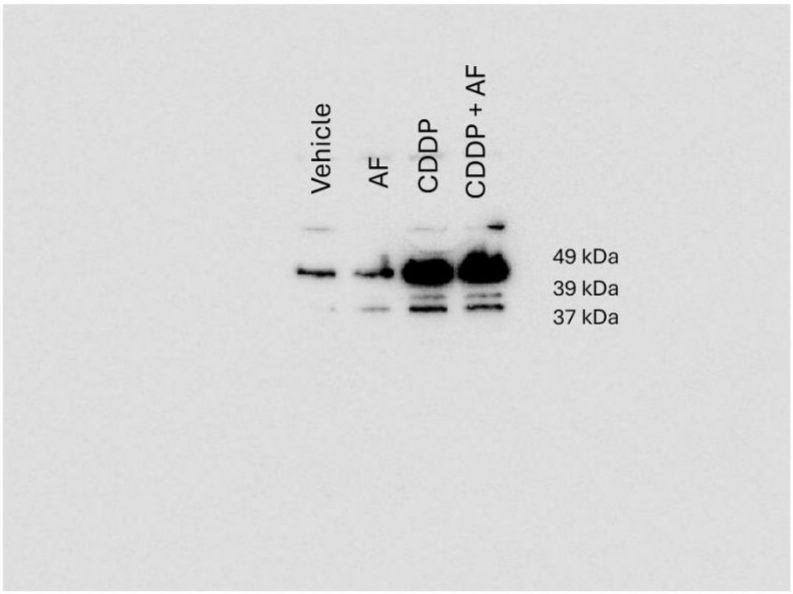
IGROV-1/CP γH2AX - Fig. 3A



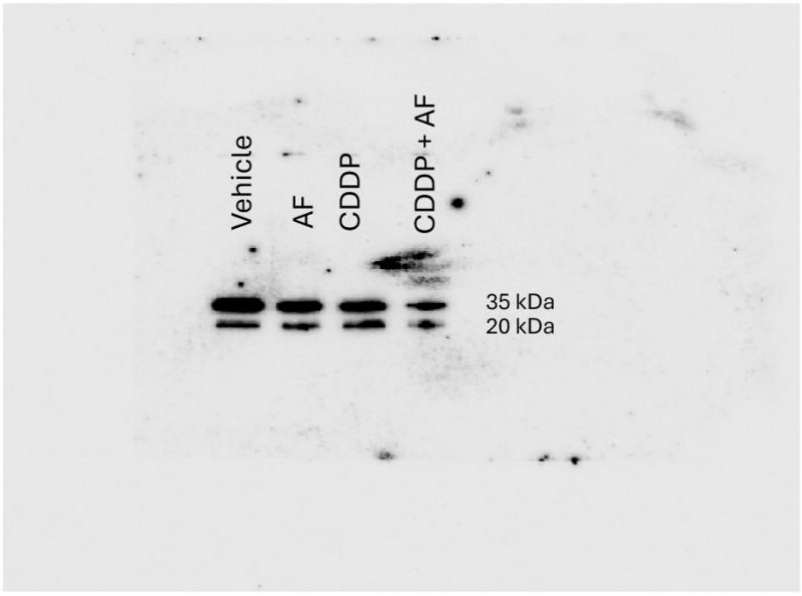
IGROV1CP β-actin - Fig. 3A



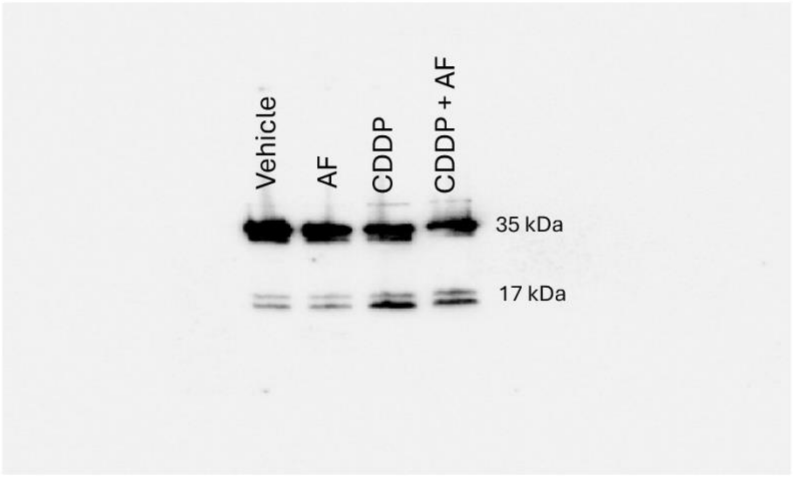
TOV112D caspase-9 - Fig. 3A



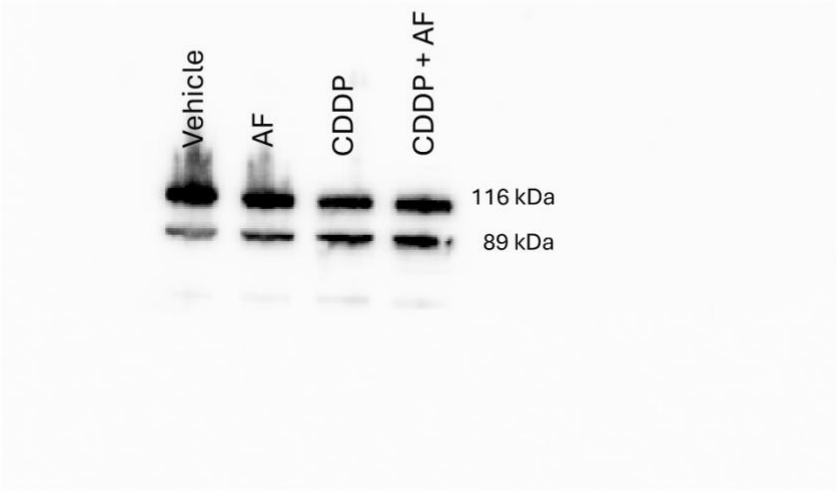
TOV112D caspase-7 - Fig. 3A



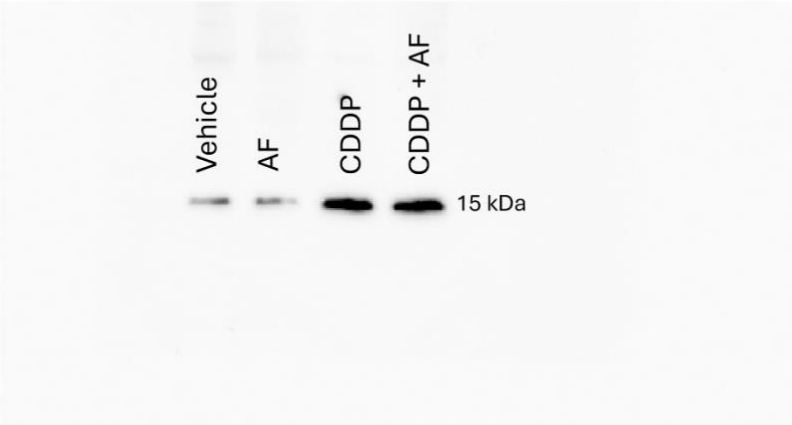
TOV112D caspase-3 - Fig. 3A



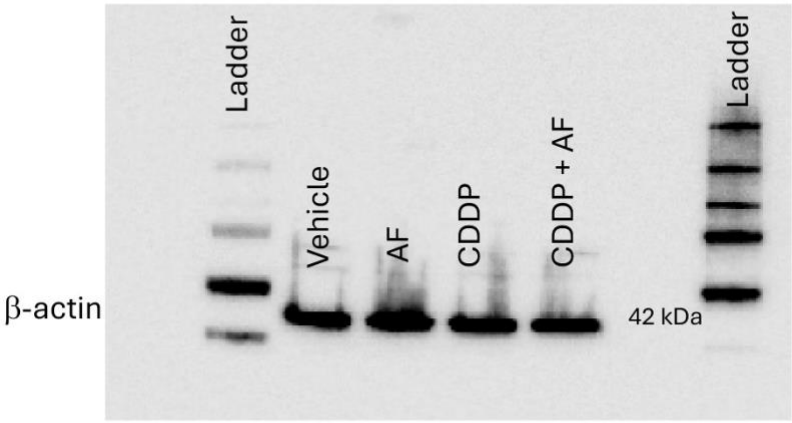
TOV112D PARP - Fig. 3A



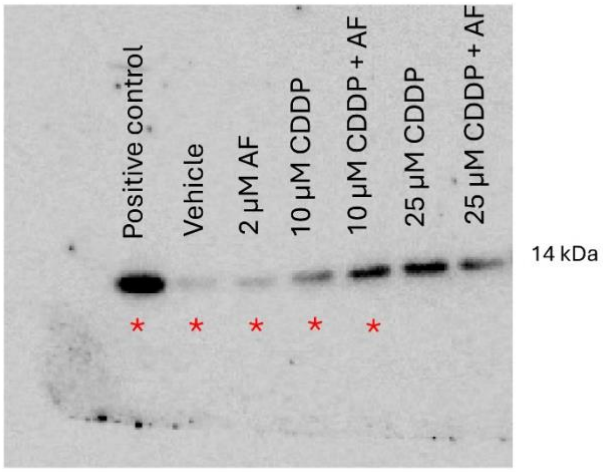
TOV112D γH2AX - Fig. 3A



TOV112D β-actin - Fig. 3A

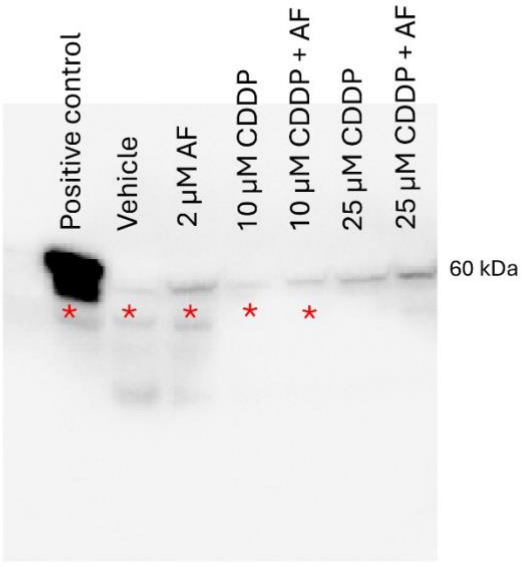


IGROV-1/CP Cytochrome C - Supp. Fig.1



* Lanes presented

IGROV-1/CP anti-mitochondrial - Supp. Fig.1



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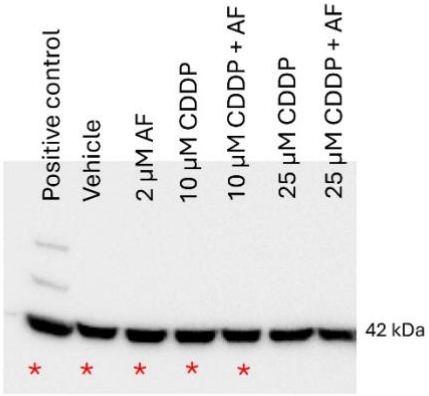
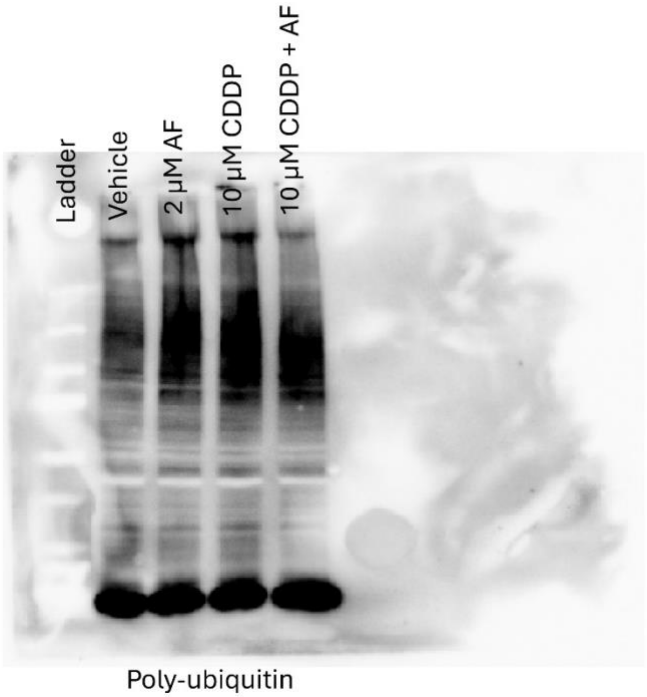
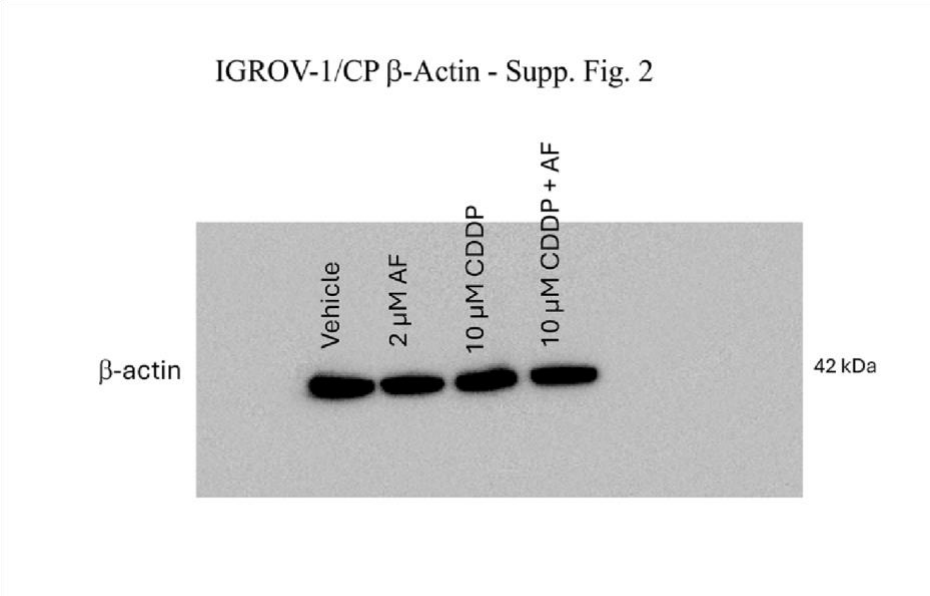
IGROV-1/CP β-actin Supp. Fig. 1



IGROV-1/CP Poly-ubiquitin - Supp. Fig. 2





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CHAPTER 5

General Discussion

The goal of this thesis was to study the repurposing of the anti-rheumatic gold complex auranofin against epithelial ovarian cancer. The perspective article in Chapter 2 provided a comprehensive literature review of the history of auranofin as an anti-rheumatic agent and the repurposing of auranofin against cancer, including an updated review of the studies using auranofin in cancer.

Chapters 3 and 4 contributed to the understanding of the therapeutic efficacy of auranofin in HGSOC and EOC, an invasive disease with a low overall survival rate due to the increased incidence of developing resistance to the first line of treatment. The extensive literary review provided an understanding of the pharmacological activity and effect of auranofin on the human body. It explained the chemical interaction of the drug with other molecules, the effect of its active state, as well as its overall side effects. We provided context on how auranofin was first developed as an agent against the autoimmune disease, rheumatoid arthritis, through its anti-inflammatory and immunosuppressive effects. This was integrated with a concise introduction to auranofin in cancer, a disease with increased inflammation. The association between auranofin and cancer originally started because the primary target of auranofin, TrxR, is upregulated within different cancers, one of which is ovarian cancer. The context of auranofin in cancer was combined with a thorough update on the preclinical and clinical studies employing auranofin in different cancers. Furthermore, we proposed the idea of using auranofin in combination with cisplatin to achieve enhanced cancer cell killing, an approach that we tested in Chapter 4. In the context of this thesis, the literature review which included past and current studies employing auranofin and cancer, highlighted a gap in the research on extensively exploring the cytotoxic effect of auranofin in the most invasive subtype of EOC, HGSOC [275].

As a result, Chapter 3 focused on exploring the cytotoxicity of auranofin in HGSOC cells that were isolated from the same patient at the early and advanced stages of the disease. The

HGSOC cells PEO1 and PEO4, are representative of clinically platinum-sensitive and clinically platinum-resistant states of the disease, respectively. More specifically, auranofin was employed in both states of platinum resistance *in vitro* to first evaluate its ability to overcome platinum resistance in HGSOC. Platinum resistance occurs in most patients with HGSOC, with only a 1520% response rate in subsequent exposures to platinum-based chemotherapy [276]. This chapter indicated the efficacy of auranofin in the platinum-sensitive PEO1 cells more than the platinumresistant PEO4 cells in most parameters that we tested. We were also surprised to find that despite TrxR being heavily reported as the primary target of auranofin, TrxR inhibition by auranofin in the PEO1 and PEO4 cells was not dependent on cytotoxicity induced by the drug. We uncovered that although PEO4 cells were already more resistant to cisplatin than PEO1 cells, they were dependent on other antioxidants as their basal expression of TrxR was a lot lower than that of PEO1, justifying their resistance to auranofin. Furthermore, the PEO4 cells managed to adapt other mechanisms of resistance in the presence of auranofin by upregulating the antioxidant protein, GSH. This highlights that the use of an inhibitor of a single antioxidant system, in this case, TrxR, is not sufficient to overcome platinum resistance in these HGSOC cells. We overcome this problem by pairing auranofin with the synthetic amino acid, L-BSO [277], which successfully achieved enhanced cytotoxicity and synergy in both cell types. This combination was explored in several cancers [209, 218, 230, 278, 279], however, it was not studied before in EOC, or more specifically HGSOC. The addition of L-BSO to auranofin to PEO1 cells was able to completely reduce GSH levels. In PEO4 cells, the GSH levels were also reduced following the addition of L-BSO, as the compensatory mechanism of the cells was successfully targeted.

The feasibility of implicating L-BSO with auranofin in the clinic is undetermined. A study on the clinical development of L-BSO involved frequent infusions of the GSH inhibitor to achieve sufficient maximal GSH depletion. This was achieved 12 h after the sixth dose of L-BSO [237], in

which L-BSO alone showed minimal toxicity. However, the clinical study revealed that L-BSO cross-reacted with the drug that it was combined with, melphalan [237]. The pharmacological interaction of auranofin and L-BSO has yet to be determined *in vivo*. A preclinical study on the auranofin-L-BSO combination is crucial in the context of HGSOC, as we have determined that GSH upregulation is a compensatory mechanism when platinum-resistant HGSOC cells are treated with auranofin. The administering of this combination is useful in the HGSOC clinically platinumsensitive cells, as well as clinically platinum-resistant cells, since the addition of L-BSO bypassed the compensatory mechanism used when exposed to auranofin alone. Since L-BSO has not been approved in the clinic, we did not do further studies with L-BSO and auranofin in Chapter 4.

There were additional research questions that we were aiming to address in Chapter 3, such as whether auranofin can elicit immunogenic cell death in EOC. We introduced ICD in the literature review, in which auranofin has been reported to successfully induce ICD in NSCLC, represented by the release of calreticulin, HMGB1, and ATP, as well as dendritic cell (DC) maturation [195]. We wanted to assess whether auranofin can elicit a similar immunogenic effect in HGSOC by measuring the release of ATP using a luminescent assay, HMGB1 by an ELISA, and ecto-calreticulin by immunofluorescence. The assays which were initially tested in PEO1 cells demonstrated that auranofin resulted in an increase in ATP release. This coincided with the anti-cancer effects of auranofin that were discussed in Chapter 2, in which auranofin reduced cellular ATP levels via the inhibition of glycolysis, consequently inhibiting drug transporter ABCG2 and preventing drug efflux. Interestingly, the addition of cisplatin to auranofin in the PEO1 cells resulted in the prevention of ATP release by auranofin alone. Furthermore, auranofin successfully induced HMGB1 release and ecto-calreticulin, suggesting that auranofin could potentially induce ICD in clinically platinum-sensitive HGSOC cells. However, we did not observe the release of the

three DAMPs by auranofin in the PEO4 cells. Further studies are needed in the clinically platinumresistant cells to make conclusive remarks on whether auranofin can elicit ICD, DC maturation and T-cell activation successfully in the recurrent stages of HGSOC.

In Chapter 3, we attempted to explore whether auranofin cytotoxicity was associated with *TP53* expression. We demonstrated that auranofin was proficient in the killing of the p53-mutant HGSOC cells. The *TP53* mutation contributes to platinum resistance as the mutated p53 interacts with the redox-sensitive transcription factor, Nrf2, to activate the antioxidant response elements (ARE). A similar mechanism could be contributing to the mechanism of resistance by the PEO4 cells to auranofin to reduce ROS levels. To further determine whether auranofin cytotoxicity is linked to p53 expression, p53-null clear cell ovarian carcinoma (SKOV-3) cells, p53-mutant clear cell ovarian carcinoma (IGROV1), and p53 wildtype endometrioid ovarian carcinoma cells (A2780) [254] were treated with increasing concentrations of auranofin for 72 h. The p53 wildtype (A2780) cells were the most sensitive to auranofin, followed by the p53-mutant (IGROV1) cells and p53-null (SKOV-3) respectively. This suggests that the efficacy of auranofin is partially dependent on p53 expression. However, an interesting study revealed that extremely high doses of auranofin elicit cytotoxicity in p53-null SKOV-3 cells by activating the FOXO3 tumour suppressor, independent of p53 [216].

Chapter 4 continues to explore the ability of auranofin to overcome platinum resistance in combination with cisplatin, and we expand on the models of platinum resistance in EOC. The first model of platinum resistance in the TOV112D cells has a spontaneous resistance to platinum, whereas the platinum resistance in IGROV-1/CP cells was induced *in vitro* by prolonged exposure to cisplatin. The second model provides a better understanding of the efficacy of auranofin when platinum resistance in the advanced stages is higher than normal, further highlighting the effectiveness of the drug. However, testing the combination of auranofin and cisplatin in multiple

cell lines, which represent each model of platinum resistance, is crucial to make conclusive remarks. The results published in Chapter 4 submitted for publication are different from the study done in Chapter 3, which was more focused on using auranofin to overcome platinum resistance in HGSOC.

Chapter 4 demonstrated that the addition of auranofin to cisplatin significantly helped to reduce the IC50 of cisplatin within the IGROV-1/CP and TOV112D cells, regardless of the difference in cisplatin sensitivities among the cell lines. An interesting finding that was not presented in the manuscript demonstrated that the IGROV-1/CP cells were more resistant to auranofin as a monotherapy in comparison to the TOV112D cells. This finding may be related to the subtype of the cells in which the endometrioid ovarian carcinoma cells A2780 and TOV112D were more sensitive to auranofin than the clear cell ovarian carcinoma cells SKOV-3 and IGROV-1 or IGROV-1/CP. Regardless of the difference in sensitivity to auranofin or cisplatin among the ovarian cancer cell histotypes, the combination of auranofin and cisplatin resulted in a synergistic interaction in both the IGROV-1/CP and TOV112D cells.

As suspected in the perspective article in Chapter 2, the mechanism of potentiation between the anti-rheumatic drug and the platinating agent cisplatin occurs via ROS overproduction and DNA damage. However, we did not explicitly test whether the potentiation in DNA damage by the combination is mediated by ROS overproduction. The cytotoxicity elicited by the combination was associated with PARP cleavage and caspase-3-dependent apoptosis. In addition, the combination was successful in disrupting the mitochondrial membrane potential in both the IGROV-1/CP and TOV112D cells. Interestingly, treatment with auranofin alone was sufficient to depolarize the mitochondrial membrane in TOV112D cells but not in IGROV-1CP, although it was not comparable to the mitochondrial membrane depolarization elicited by auranofin in the PEO4 cells. However, the addition of cisplatin to auranofin helped to achieve the same level of mitochondrial

membrane depolarization in TOV112D as auranofin alone in the PEO4 cells. On the other hand, the level of mitochondrial membrane depolarization by the drug combination was not as sufficient as that induced by auranofin alone in the TOV112D cells and PEO4 cells, respectively. Based on other reported findings, cisplatin induces a less prominent effect in disrupting the mitochondrial membrane potential in more cisplatin-resistant OVCAR-8 cells compared to the more cisplatin-sensitive OVCAR-3 and OVCAR-4 [227]. This validates our findings in which the less prominent effect of auranofin alone and the drug combination on mitochondrial membrane depolarization in the IGROV-1/CP cells is associated with their increased cisplatin resistance in comparison to the TOV112D and PEO4 cells. Nonetheless, auranofin alone and in combination with cisplatin plays a role in disrupting the mitochondrial state and eliciting mitochondrial stress, as characterized in IGROV-1/CP by cytochrome c release from the mitochondria into the cytosol, an early event that initiates intrinsic apoptosis.

The disruption of the mitochondrial membrane potential in all three EOC cells that we studied was caused by increased accumulation of ROS. This can occur through increased cytoplasmic and/or mitochondrial ROS [226]. In our studies, we focused on measuring total intracellular ROS through superoxide levels. Although superoxide is a by-product of the electronic transport chain (ETC) reaction by the mitochondria, several enzymes, like NADPH oxidase which is present within the cytosol, produce superoxide [280]. Auranofin alone induced a slight increase in ROS levels in IGROV-1/CP cells, more so in the TOV112D cells, and more enhanced in the PEO4 cells. However, the addition of cisplatin to auranofin in the TOV112D cells resulted in enhanced overproduction of ROS in comparison to what was induced by auranofin alone in PEO4 cells. It would be interesting to determine how the addition of cisplatin to auranofin would contribute to ROS accumulation within the PEO4 cells. Interestingly, in the IGROV-1/CP cells, we observed that the addition of cisplatin to auranofin did not enhance ROS increase more than what

was elicited by cisplatin alone, suggesting that ROS production in these cells is primarily driven by cisplatin. One of the mechanisms of acquired resistance to cisplatin involves GSH upregulation, which helps to detoxify the effects of the platinating drug [281]. The lack of enhanced ROS overproduction by the combination of auranofin with cisplatin is possibly due to the IGROV-1/CP being more resistant to cisplatin via GSH upregulation. It would have been interesting to measure levels of reduced GSH protein within the EOC cells in the presence of auranofin or cisplatin separately and in combination. Furthermore, it would have been interesting to explore the effect of the combination of auranofin and L-BSO combination in IGROV-1/CP cells, particularly since GSH is likely the mechanism of resistance used by the IGROV-1/CP cells to maintain ROS levels and consequently prevent the enhanced disruption of the mitochondrial membrane potential.

The combination of auranofin and L-BSO in PEO4 cells showed similar levels of ROS production to that induced by auranofin and cisplatin combination in the TOV112D cells. Likely, the combination of auranofin and L-BSO may successfully enhance ROS production in the IGROV-1/CP cells, like that encountered in PEO4 cells. One of the research questions to be addressed in the future is whether the combination of auranofin, L-BSO, and cisplatin would enhance the killing of platinum-resistant EOC cells *in vivo*. This would be a valuable study to have a better mechanistic understanding of the behavior of EOC cells and to eliminate any possible strategies for compensation that arise when either of the drugs is employed separately. However, the preclinical feasibility, cross-reactivity, and clinical safety of combining the three compounds in EOC cells is undetermined.

In the literature review, we touched upon the effect of auranofin in disrupting the protein degradation pathway via inhibition of the deubiquitinase enzyme in the cisplatin-resistant endometrioid ovarian cancer cells, A2780. Although we did not assess the direct effect of auranofin on the deubiquitinase enzyme in the EOC cells, we indirectly measured the impact of auranofin on

the proteasome-ubiquitin pathway. It was revealed that auranofin treatment in PEO1, PEO4 and IGROV-1/CP resulted in the accumulation of misfolded proteins, suggesting a disruption in the clearing of misfolded proteins within the cell. In HGSOC cells, the accumulation of poly-ubiquitinated proteins was mediated by ROS, as represented by the lack of ubiquitin expression following the addition of NAC to auranofin. The rescuing by NAC could be attributed to the molecule encompassing a dual role as a ROS scavenger and an antagonizer for proteasome inhibitors [282]. This strongly suggests that auranofin acts as a proteasome inhibitor in HGSOC. In the IGROV-1/CP cells, the level of ubiquitin expression by auranofin alone, cisplatin alone, or the combination is equivalent, indicating that proteasome inhibition in these cells can be mediated by either drug or both. It is plausible for auranofin and cisplatin to be potentiating as proteasome inhibitors in the platinum-resistant clear-cell ovarian carcinoma cells since the two drugs inhibit different components of the proteasome-ubiquitin system [234, 251].

Future directions could explore the impact of the combination of auranofin, L-BSO, and cisplatin in platinum-resistant EOC cells while identifying the direct mechanism of action of auranofin in inhibiting the proteasome-ubiquitin system in EOC cells. In addition, it would be useful to explore the efficacy of auranofin in inducing ICD in platinum-resistant HGSOC as well as other histotypes of EOC. The immunogenic capacity of this anti-rheumatic drug would help to transform EOC tumours from “cold” to “hot” by eliciting DC maturation and increasing T-cell infiltration [283]. Furthermore, a more comprehensive study on the auranofin and cisplatin combination could be carried out in the IGROV-1/CP cells and their cisplatin-sensitive sister cell line, IGROV-1, to further characterize the association of auranofin cytotoxicity and cisplatin resistance in the clear cell ovarian carcinoma histotype.

Conclusion

The primary objectives of this thesis were to identify the cytotoxic effects of auranofin in HGSOC cells with different platinum sensitivities and the ability of auranofin to sensitize platinum-resistant EOC cells to cisplatin. In the first aim, we found that PEO1 and PEO4 cells were equally sensitive to auranofin when the overall metabolic activity of the cells was tested. However, PEO1 cells were more sensitive to auranofin via strongly reduced TrxR activity, increased apoptosis, long-term inhibition of their proliferative capacity, ROS-dependent DNA damage, PARP cleavage, caspase-3/7 activation, and protein polyubiquitination. The PEO4 cells were more sensitive than PEO1 cells in terms of auranofin-induced mitochondrial membrane depolarization. The first aim also explored the interaction of auranofin and L-BSO against HGSOC, which revealed a synergistic interaction that enhanced the killing of PEO1 and PEO4 cells. The enhanced cytotoxicity by auranofin and L-BSO combination in the HGSOC cells was mediated via an enhanced increase in ROS production due to GSH inhibition.

In the second aim, the auranofin and cisplatin combination resulted in a synergistic interaction within the platinum-resistant EOC cells IGROV-1/CP and TOV112D. This was mediated through ROS accumulation, DNA damage, PARP cleavage, intrinsic apoptosis, and mitochondrial membrane depolarization.

This thesis highlighted the effectiveness of auranofin in mediating cytotoxicity against different EOC subtypes, and the ability of the anti-rheumatic drug to potentiate with other compounds to enhance cytotoxicity against platinum-resistant EOC cells.

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