Anticancer activity of a Gold(I) phosphine thioredoxin reductase inhibitor in multiple myeloma

Jun Hui Szea,b,1, Prahlad V. Raningac,1, Kyohei Nakamura1, Mika Caseyd, Kum Kum Khannac, Susan J. Berners-Pricee, Giovanna Di Trapania2,*, Kathryn F. Tonissenb,h,∗∗

1 School of Environment and Science, Griffith University, Nathan, QLD, 4111, Australia
2 Griffith Institute for Drug Discovery, Griffith University, Nathan, QLD, 4111, Australia
3 Signal Transduction Laboratory, QIMR Berghofer Medical Research Institute, Herston, QLD, 4006, Australia
4 Immunology in Cancer and Infection Laboratory, QIMR Berghofer Medical Research Institute, Herston, QLD, 4006, Australia
5 Institute for Glycomics, Griffith University, Southport, QLD, 4222, Australia

ABSTRACT

Multiple myeloma (MM), the second most common haematological malignancy, is a clonal plasma B-cell neoplasm that forms within the bone marrow. Despite recent advancements in treatment, MM remains an incurable disease. Auranothine, a linear gold(I) phosphine compound, has previously been shown to exert a significant anti-myeloma activity by inhibiting thioredoxin reductase (TrxR) activity. A bis-chelated tetrahedral gold(I) phosphine complex [Au(d2pype)2]Cl (where d2pype is 1,2-bis(di-2-pyridylphosphino)ethane) was previously designed to improve the gold(I) compound selectivity towards selenol- and thiol-containing proteins, such as TrxR. In this study, we show that [Au(d2pype)2]Cl significantly inhibited TrxR activity in both bortezomib-sensitive and resistant myeloma cells, which led to a significant reduction in cell proliferation and induction of apoptosis, both of which were dependent on ROS. In clonogenic assays, treatment with [Au(d2pype)2]Cl completely abrogated the tumourigenic capacity of MM cells, whereas auranothine was less effective. We also show that [Au(d2pype)2]Cl exerted a significant anti-myeloma activity in vivo in human RPMI8226 xenograft model in immunocompromised NOD/SCID mice. The MYC oncogene, known to drive myeloma progression, was downregulated in both in vitro and in vivo models when treated with [Au(d2pype)2]Cl. This study highlights the “proof of concept” that improved gold(I)-based compounds could potentially be used to not only treat MM but as an alternative tool to understand the role of the Trx system in the pathogenesis of this blood disease.

1. Introduction

Multiple myeloma (MM) is the second most common haematological malignancy after non-Hodgkin’s lymphoma and is characterised by the presence of terminally differentiated, antibody-secreting plasma cells (PCs), which proliferate uncontrolled in the bone marrow. Despite recent advancements in MM treatment, including autologous stem cell transplantation, the proteasome inhibitors bortezomib and carfilzomib, and the immunomodulatory agents thalidomide and lenalidomide, MM remains incurable as a result of acquired drug resistance and the heterogeneity of myeloma cells [1,2]. Hence, there is a dire need to discover better therapeutic interventions to tackle this disease.

Tumour cells generally have higher metabolic demands due to their highly proliferative nature and as such, production of intracellular by-products including free reactive oxygen species (ROS) are also increased [3–5]. ROS can cause oxidative damage to biomolecules, and hence, cells evolved to express a network of antioxidant molecules to maintain a reduced cellular environment to prevent further cell damage [5,6]. Unfortunately, these “redox shields” have been exploited by the tumour cells in order to evade the cytotoxic effects of anticancer drugs that induce oxidative stress [7–10].

One of the major antioxidant systems that maintains the intracellular redox homeostasis is the thioredoxin (Trx) system [11]. It is comprised of thioredoxin (Trx), thioredoxin reductase (TrxR) enzyme, and nicotinamide adenine dinucleotide phosphate (NADPH), all of which as one maintains cellular redox balance by either directly
scavenging ROS [12] or by regulating enzymes and redox-sensitive transcription factors that promote cancer cell proliferation [13–16]. Various studies have shown that human cancers, including MM, have high Trx and TrxR expression, which correlates with increased cell proliferation, evasion of programmed cell death and chemoresistance [17–22].

Gold-based medicines have been widely studied and used as anti-inflammatory, anti-cancer, and anti-microbial agents to treat various pathologies [23–26,70]. Gold compounds have a high affinity for thiol and selenol groups, which makes the Trx system vulnerable to these compounds [27], since TrxR contains a selenocysteine residue in its C-terminal active site [28]. Auranofin, an FDA-approved gold-based drug for treating arthritis [25,29], is currently being tested in clinical trials for various cancers including chronic lymphocytic leukemia (CLL), small lymphocytic lymphoma (SLL) (NCT01419691) and ovarian cancer (NCT01747798). However, there are concerns that auranofin may react non-discriminately with protein thiols (including serum proteins), which may render this drug ineffective and potentially introduce negative side effects in patients [26,30,31]. Hence, a range of gold compounds have been synthesized to enhance their anti-cancer properties through better selectivity in targeting selenoproteins like TrxR [32–34,43]. Nevertheless, their effectiveness in in vitro and in animal models must first be established before testing can be justified in patients.

An improved gold(I) complex [Au(d2pype)2]Cl (Fig. 1), which has been shown to have high specificity towards TrxR when compared to auranofin [24,34], has not yet been tested against any blood cancers. Therefore, this compound may have potential as an alternative therapy to treat cancer cells that are highly dependent on the Trx system for their survival and drug resistance properties. As MM patients have been shown to have an upregulated Trx system compared to normal controls [35,36], we investigated the effectiveness of the gold compound, [Au(d2pype)2]Cl, in bortezomib-sensitive and resistant myeloma cells and showed that it significantly induced ROS-dependent apoptosis in both cell phenotypes. It also significantly inhibited the clonogenic activity of both bortezomib-sensitive and resistant myeloma cells. Finally, we showed that [Au(d2pype)2]Cl exerted a potent anti-tumour activity in the human RPMI8226 xenograft model of MM in vivo via MYC downregulation.

2. Materials and methods

2.1. Cells and reagents

Three human myeloma cell lines (JJN3, RPMI8226 and U266) were obtained from Dr. Slavica Vuckovic (QIMR Berghofer Medical Research Institute, Brisbane) and have been authenticated by the Gri. Alternatively, human peripheral blood mononuclear cells (PBMCs) were collected and isolated from the whole blood of healthy individuals under the Gri

Abbreviations

<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ac-DEVD-AMC</td>
<td>Acetyl-Asp-Glu-Val-Asp-AMC</td>
</tr>
<tr>
<td>H2DCFDA</td>
<td>2′,7′-Dichlorodihydrofluorescein diacetate</td>
</tr>
<tr>
<td>DTNB</td>
<td>dithionitrobenzoic acid</td>
</tr>
<tr>
<td>TrxR</td>
<td>thioredoxin reductase</td>
</tr>
<tr>
<td>TrxR1</td>
<td>cytosolic thiredoxin reductase</td>
</tr>
<tr>
<td>Auranofin</td>
<td>2,3,4,6-tetra-O-acetyl-1-thio-β-D-glucopyranosato-S-triethylphosphine gold(I)</td>
</tr>
<tr>
<td>[Au(d2pype)2]Cl</td>
<td>bis[1,2-bis(di-2-pyridylphosphino)ethane] gold(I) chloride</td>
</tr>
<tr>
<td>MM</td>
<td>multiple myeloma</td>
</tr>
<tr>
<td>MTS</td>
<td>3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium</td>
</tr>
<tr>
<td>PBMCs</td>
<td>peripheral blood mononuclear cells</td>
</tr>
</tbody>
</table>

Fig. 1. Chemical structures of gold(I) phosphate complexes [Au(d2pype)2]Cl and auranofin.
determine cellular ROS generation in myeloma cells with method as previously [36]. Briefly, 0.5 × 10^6 of myeloma cells were treated for 24 h before incubation with 10 μM H2DCFDA (Molecular probes, CA, USA) for 30 min. H2DCFDA oxidation was assessed using the FLUOstar Optima plate reader (BMG Labtech, Germany) using Ex480/Emission parameters to obtain fluorescence values that were normalized to protein content to obtain relative ROS levels.

2.4. Reverse transcriptase-quantitative PCR (RT-qPCR)

Total RNA was extracted from RPMI8226 and JJN3 myeloma cells (1 × 10^6 cells) using TRIzol™ Total RNA Lysis Solution (Bioline) as per manufacturer's instructions. Single stranded cDNA was synthesized from total RNA using the GoScript™ Reverse Transcript Reaction Mix (Promega). Resultant cDNA was analysed by RT-qPCR using SensiFAST™ SYBR® No-Rox Kit (Bioline). The RT-qPCR primers used were: Ribosomal Protein L32 (RPL32) [forward 5′-CAGGTGCTGATGGAGG-3′ and reverse 5′-CTTGAGGAAACATTGTGACGATC-3′], C-MYC [forward 5′-GCAGTCTGTTAGACGGTGGATT-3′ and reverse 5′-GTTCTTTCTCTGGTCAAGTAAATA-3′] and Cyclin D1 [forward 5′-GGCCCTGGTGTCCATCTCTGAA-3′ and reverse 5′-CTGCA GGGGCTTCTTTTCA-3′] (Integrated DNA Technologies, Singapore). Quantification was carried out on Bio-Rad CFX96 Real-Time PCR Detection System (Bio-Rad, USA) according to the manufacturer's guidelines. Reaction conditions were 95 °C for 2 min followed by 40 cycles of 95 °C for 5 s, 55 °C for 10 s, and 72 °C for 20 s. Gene expression was analysed based on the comparative cycle threshold (CT) method and normalized against Ribosomal Protein L32 (RPL32) expression.

2.5. Cell viability assay

Relative cell growth/proliferation following [Au(d2pype)2]Cl treatment was assayed either using the CellTitre-Blue® Cell Viability Assay (Promega) or by using MTS assays (Promega), as per manufacturer's instructions.

2.6. TrxR activity assay

TrxR activity was measured by DTNB reduction assays as described previously [35]. Briefly, cell lysates of treated and untreated cells were prepared using 0.5% (v/v) Nonidet P-40 cell lysis buffer (150 mM NaCl, 50 mM Tris-Cl, pH 8; 0.5% (v/v) Nonidet P-50, 0.5 mM EDTA, 2 mM PMFS, 1 μl/ml protease inhibitor cocktail VI and 1X PBS). To omit non-TrxR-specific DTNB reduction, cell lysates were treated with or without 8 μM auranoﬁn for 30 min at room temperature. The TrxR activity was measured in a buffer containing 125 mM potassium phosphate pH 7.5, 2.5 mM EDTA, 0.25 mM NADPH, and 3.125 mM DTNB. Units (U) of TrxR activity (μmoles of TNB produced/minute) were calculated using an extinction coefficient of 13.6 × 10^3 M\(^{-1}\) cm\(^{-1}\) of TNB at 412 nm. The specific TrxR activity (U/mg protein) was calculated by normalizing the units of TrxR activity with the quantity of protein in each sample (as determined using the BioRad DC protein assay reagent (BioRad) as per manufacturer's instructions).

2.7. Caspase-3 activity assay

The measurement of caspase activity in treated and untreated MM cells was measured by monitoring the cleavage of a caspase-3 substrate, Ac-DEVD-AMC (Cayman Chemical Company, USA) as described previously [36].

2.8. Methylcellulose clonogenic assay

The colony formation assay was carried out in methylcellulose (R&D Systems) as per manufacturer's instructions. Briefly, MM cells were treated with specified concentrations of the gold compounds for 24 h at 37 °C, 5% CO₂. These treated and untreated MM cells were washed with 1X PBS to remove residual compound before resuspension in RPMI-1640 media containing 1.4% methylcellulose, 25% (v/v) FBS, 100 U/ml penicillin and 100 μg/ml streptomycin, and 1% (v/v) BSA. The cells were then plated at the density of 1000 cells/ml in 24-wells plate and incubated at 37 °C, 5% CO₂ for 9–15 days. Colonies (> 50 cells per colony) formed were observed by staining with 0.5 mg/ml 3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) and left for 3 h before being counted using an inverted light microscope (Nikon Eclipse TS100).

2.9. Western blot analysis

MM cell extracts were prepared by washing the cells in cold 1X PBS followed by resuspension in 0.5% (v/v) Nonidet P-40 cell lysis buffer. Protein samples were then electrophoresed on a 10% SDS/polyacrylamide gel. Separated proteins were transferred onto a polyvinylidene difluoride (PVDF) membrane and probed with various specific antibodies including anti-PARP1 (Cell Signalling), anti-MYC (Cell Signalling), anti-H2AX (Millipore), and anti-HSP90 (clone 4F10, Santa Cruz) Appropriate horseradish peroxidase-conjugated secondary antibodies were used followed by detection with enhanced chemiluminescence (ECL) kit (GE Healthcare).

2.10. Animal studies

All experiments were conducted in accordance to the guidelines and ethics approval of the QIMR Berghofer Medical Research Institute Animal Ethics Committee. Luciferase-tagged human RPMI8226 cells (3 × 10^6 cells) were injected subcutaneously into 8-weeks old female NOD/SCID mice. Once tumour size reached ~30–50 mm^2, mice were treated with vehicle, auranoﬁn (5 mg/kg, Monday-Friday, IP), and [Au(d2pype)2]Cl (5 mg/kg, Monday-Friday, IP) for two weeks. Tumour growth was measured thrice weekly using a digital caliper. To calculate tumour area the following formula was used: tumour area = B*S, where B = largest tumour measurement and S = the smallest, based on two-dimensional caliper measurements. Tumour growth was measured by bioluminescence imaging during and at the end of two-weeks treatment (every alternate day) using a Xenogen IVIS Spectrum (Caliper Life Sciences).

2.11. Immunohistochemistry

Immunohistochemistry analysis was performed on RPMI8226 xenografts as described previously [38]. ApopTag staining was performed using the ApopTag peroxidase in situ apoptosis detection kit (S7100; MilliporeSigma, Billerica, MA, USA).

2.12. Statistical analysis

All values are presented as mean ± SEM. Data were analysed using GraphPad Prism 7 software (GraphPad Software, CA, USA). Statistical significance was determined by One-way ANOVA followed by Tukey’s post-test, paired/unpaired student t-test, and Two-way ANOVA followed by Sidak’s post-test. Where applicable, statistical significance is denoted by * for P ≤ 0.05, ** for P ≤ 0.01, *** for P ≤ 0.001, and **** for P ≤ 0.0001.

3. Results

3.1. (Au(d2pype)2)Cl inhibits myeloma cell proliferation and induces apoptosis in myeloma cells via TrxR inhibition

Auranoﬁn has been shown to inhibit cell proliferation, TrxR redox activity and to induce ROS-dependent apoptosis in myeloma cells treated with vehicle or auranoﬁn. TrxR activity was measured by DTNB reduction assays as described previously [36]. Briefly, cell lysates of treated and untreated cells were prepared using 0.5% (v/v) Nonidet P-40 cell lysis buffer (150 mM NaCl, 50 mM Tris-Cl, pH 8; 0.5% (v/v) Nonidet P-50, 0.5 mM EDTA, 2 mM PMFS, 1 μl/ml protease inhibitor cocktail VI and 1X PBS). To omit non-TrxR-specific DTNB reduction, cell lysates were treated with or without 8 μM auranoﬁn for 30 min at room temperature. The TrxR activity was measured in a buffer containing 125 mM potassium phosphate pH 7.5, 2.5 mM EDTA, 0.25 mM NADPH, and 3.125 mM DTNB. Units (U) of TrxR activity (μmoles of TNB produced/minute) were calculated using an extinction coefficient of 13.6 × 10^3 M\(^{-1}\) cm\(^{-1}\) of TNB at 412 nm. The specific TrxR activity (U/mg protein) was calculated by normalizing the units of TrxR activity with the quantity of protein in each sample (as determined using the BioRad DC protein assay reagent (BioRad) as per manufacturer's instructions).
We then investigated the effectiveness of \([\text{Au(d2pype)}_2]\text{Cl}\) on TrxR activity in myeloma cells. First, RPMI8226, U266 and JJN3 myeloma cells were treated with \([\text{Au(d2pype)}_2]\text{Cl}\) (0–4 \(\mu\text{M}\)) for 24 h. After 24 h treatment, TrxR redox activity was analysed using DTNB reduction assays. Results showed that \([\text{Au(d2pype)}_2]\text{Cl}\) significantly inhibited TrxR redox activity in all three myeloma cell lines tested (Fig. 2A). JJN3 cells were the most sensitive cell line, with almost 70\% inhibition of its TrxR activity when treated with 0.25 \(\mu\text{M}\) of \([\text{Au(d2pype)}_2]\text{Cl}\). Since TrxR is a selenoprotein and cell culture medium is often deficient in selenium, resulting in low basal TrxR activity levels [39,40], we confirmed that both auranoïn and \([\text{Au(d2pype)}_2]\text{Cl}\) also inhibited the higher TrxR activity level observed in myeloma cells supplemented with sodium selenite (Fig. S3).

We next tested if \([\text{Au(d2pype)}_2]\text{Cl}\) could inhibit myeloma cell viability and induce apoptosis. Fig. 2B, C and D showed that \([\text{Au(d2pype)}_2]\text{Cl}\) was capable of reducing cell viability in all of the myeloma cell lines tested. The IC_{50} values were observed to be 2.6 \(\mu\text{M}\), 2.3 \(\mu\text{M}\), and 0.6 \(\mu\text{M}\) in RPMI8226, U266, and JJN3 cells, respectively, while the \([\text{Au(d2pype)}_2]\text{Cl}\) compound had no significant cytotoxic effect on non-cancerous PBMCs (Fig. 2E). Next, we examined if \([\text{Au(d2pype)}_2]\text{Cl}\) can induce apoptosis in myeloma cells. RPMI8226, U266, and JJN3 cells were treated with \([\text{Au(d2pype)}_2]\text{Cl}\) (0–4 \(\mu\text{M}\)) for 24 h and caspase-3 activity was measured using a caspase-3 specific substrate Ac-DEVD-AMC. Our results show that \([\text{Au(d2pype)}_2]\text{Cl}\) significantly increased caspase-3 activity in all three myeloma cell lines, suggesting that myeloma cells are undergoing apoptosis following \([\text{Au(d2pype)}_2]\text{Cl}\) treatment (Fig. 2F). Furthermore, \([\text{Au(d2pype)}_2]\text{Cl}\) treatment resulted in cleavage of PARP1, a classical marker of apoptotic cell death, and increased \(\gamma\text{H2AX}\) accumulation, a marker of DNA fragmentation (Fig. 2G), suggesting that \([\text{Au(d2pype)}_2]\text{Cl}\) induced apoptosis in myeloma cells.

3.2. \([\text{Au(d2pype)}_2]\text{Cl}\) induces intracellular ROS and growth inhibitory effect is ROS dependent

The Trx system maintains intracellular redox homeostasis by...
scavenging ROS, and inhibition of TrxR using aurano
din has been shown to increase intracellular ROS, disrupt intracellular redox home-
istasis and lead to myeloma cell death [35,41]. We therefore examined whether [Au(d2pype)2]Cl induces ROS following TrxR inhibition. RPMI8226 and JNN3 cells were treated with [Au(d2pype)2]Cl (0–4 μM) for 24 h and intracellular ROS levels were measured using a ROS-sensitive H2DCFDA dye. Our results show that [Au(d2pype)2]Cl treatment significantly increased ROS in both RPMI8226 and JNN3 cells in a concentration-dependent manner (Fig. 3A and B), suggesting that [Au(d2pype)2]Cl disrupts intracellular redox homeostasis in myeloma cells. To confirm if [Au(d2pype)2]Cl induces myeloma cell apoptosis via ROS induction, we treated RPMI8226 and JNN3 cells with 2 μM and 1 μM [Au(d2pype)2]Cl, respectively, with or without 10 mM N-acetyl cysteine (NAC) for 24 h and subsequently measured caspase-3 activity and cell viability. Results showed that while [Au(d2pype)2]Cl significantly increased caspase-3 activity (Fig. 3C and D) and reduced cell viability (Fig. 3E and F) in RPMI8226 and JNN3 cells, addition of NAC was able to rescue RPMI8226 and JNN3 cells from undergoing apoptosis and cell death (Fig. 3E and F). To further confirm the involvement of ROS-dependent myeloma cell death in response to [Au(d2pype)2]Cl treatment, RPMI8226 and JNN3 cells were co-treated with [Au(d2pype)2]Cl in the presence or absence of the glutathione system inhibitor, buthionine.

Fig. 3. [Au(d2pype)2]Cl induces intracellular ROS and growth inhibitory effect is ROS dependent. (A, B) RPMI8226 and JNN3 cells were treated with 0–4 μM concentration of [Au(d2pype)2]Cl for 24 h followed by assessment of H2DCFDA oxidation. (C, D) Caspase-3 activity was measured by monitoring the cleavage of Ac-DEVD-AMC in myeloma cells pre-treated with 10 mM NAC prior to addition of [Au(d2pype)2]Cl for 24 h. (E, F) Cell viability was assessed by CellTiter-Blue® Cell Viability Assays in RPMI8226 and JNN3 cells following treatment with 2 μM and 1 μM of [Au(d2pype)2]Cl (respectively), with or without 10 mM NAC for 24 h. Values indicate mean ± SEM of three independent experiments. One-way ANOVA followed by Tukey’s post-test was employed. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.
sulphoximine (BSO). We found that [Au(d2pype)2]Cl and BSO co-treatment significantly reduced myeloma cell viability compared to a single agent treatment (Fig. S4), resulting in complete cell death.

3.3. [Au(d2pype)2]Cl induces apoptosis in bortezomib resistant myeloma cells

Upon prolonged treatment with standard-of-care therapy including bortezomib and immunomodulatory agents, myeloma patients acquire resistance to these therapies and can no longer be treated by the same therapy [42,44,45]. Hence, new therapies are required to overcome such drug resistance in MM patients. We therefore determined the cytotoxic activity of [Au(d2pype)2]Cl on bortezomib-resistant myeloma cells. Results showed that [Au(d2pype)2]Cl treatment significantly reduced RPMI8226-BR and U266-BR cell viability in a concentration- and time-dependent manner (Fig. 4A and B). We also measured TrxR redox activity and caspase-3 activity in RPMI8226-BR and U266-BR cells following 24 h treatment with [Au(d2pype)2]Cl (0–4 μM). Results demonstrated that TrxR activity was significantly inhibited (Fig. 4C and D) and caspase-3 activity was increased (Fig. 4E and F) upon [Au(d2pype)2]Cl treatment. Thus, our data suggest that [Au(d2pype)2]Cl induces apoptosis in bortezomib-resistant myeloma cells via TrxR inhibition.

3.4. Clonogenic potential of myeloma cells is diminished upon treatment with [Au(d2pype)2]Cl

Clonogenic assays are generally used to determine effectiveness of the anti-cancer drug by assessing the resistance and ability of cancer cells to form a colony after treatment with the drug [46,47]. Bortezomib sensitive parent RPMI8226 cells and bortezomib-resistant RPMI8226-BR cells were treated with 0–2 μM auranofin and [Au(d2pype)2]Cl for 24 h. It was observed that exposure of RPMI8226 and RPMI8226-BR cells to auranofin caused a significant reduction in the number of colonies formed in a concentration dependent manner (Fig. 5A, C and E). On the other hand, [Au(d2pype)2]Cl completely abrogated the tumourigenic capacity of myeloma cells after treatment with 2 and 4 μM of the gold compound for 24 h (Fig. 5B, D and F).

3.5. [Au(d2pype)2]Cl suppresses myeloma tumour growth in vivo

We evaluated the anti-myeloma activity of auranofin and [Au(d2pype)2]Cl in vivo using human RPMI8226 xenograft model in immunocompromised NOD/SCID mice. We found that both auranofin and [Au(d2pype)2]Cl exerted a significant growth suppression activity in the in vivo RPMI8226 xenograft model (Fig. 6A and B). These data suggested that both of these gold-based compounds can suppress myeloma growth. We also measured the percentage of apoptotic cells in vehicle-, auranofin, and [Au(d2pype)2]Cl-treated RPMI8226 tumours using ApopTag staining. Results showed that both auranofin and [Au(d2pype)2]Cl significantly increased the percentage of apoptotic cells (approximately 25%) in RPMI8226 tumours compared to 5% in vehicle-treated tumours (Fig. 6C and D). Since both auranofin and [Au(d2pype)2]Cl inhibited TrxR redox activity in vitro, we evaluated TrxR redox activity in vehicle-, auranofin-, and [Au(d2pype)2]Cl-treated RPMI8226 primary tumours. Results showed that auranofin and [Au(d2pype)2]Cl treatment inhibited TrxR redox activity by approximately 75% in RPMI8226 primary tumours (Fig. 6E). Hence, our data suggest that [Au(d2pype)2]Cl has the potential to exert an anti-myeloma activity that is comparable to auranofin.

3.6. [Au(d2pype)2]Cl suppresses myeloma tumour growth via MYC inhibition

Next, we aimed to delineate the underlying molecular mechanism for anti-myeloma activity of the [Au(d2pype)2]Cl compound. Several studies have reported that the MYC oncogene is amplified in more than half of myeloma patients [48,49]. Moreover, MYC has been shown to play a key role in MM disease progression as alterations in MYC promote the transition from MGUS to MM [50]. Hence, MYC represents an attractive therapeutic target in MM.

To investigate the effect of [Au(d2pype)2]Cl treatment on MYC expression in myeloma cells, RPMI8226 and JJN3 cells were treated with 0–2 μM [Au(d2pype)2]Cl for 24 h before MYC mRNA and protein levels were determined by RT-qPCR and western blot analysis, respectively. Results showed that [Au(d2pype)2]Cl treatment significantly reduced MYC mRNA levels (Fig. 7A) and markedly reduced MYC protein levels (Fig. 7B), suggesting that [Au(d2pype)2]Cl inhibits MYC at the transcriptional level. In addition to MYC mRNA, [Au(d2pype)2]Cl treatment also significantly reduced the mRNA levels of Cyclin D1 (Fig. 7A), a downstream transcriptional target of MYC, suggesting inhibition of the MYC signalling pathway. We also examined if auranofin can inhibit the MYC pathway in myeloma cells. RPMI8226 and JJN3 cells were treated with 0–2 μM auranofin for 24 h and MYC protein levels were determined. Interestingly, auranofin at the IC50 concentration (2 μM) had no effect on MYC protein levels (Fig. 7B) in either cell line, but at a cytotoxic concentration (4 μM) auranofin reduced MYC protein levels in RPMI8226 cells, but not in JJN3 cells (Fig. S5). Moreover, [Au(d2pype)2]Cl treatment markedly reduced MYC protein levels in RPMI8226 primary tumours (Fig. 7C). Hence, our data demonstrated that [Au(d2pype)2]Cl may exert anti-myeloma activity through not only TrxR inhibition but MYC inhibition as well.

We then aimed to examine if the cytotoxic activity of [Au(d2pype)2]Cl is MYC dependent in myeloma settings. JJN3 cells were transfected with either HA-tagged empty vector or HA-tagged wild-type MYC to exogenously increase MYC protein levels (Fig. 7D). Then 24 h post-transfection, cells were treated with or without 1 μM [Au(d2py-pe)2]Cl for 24 h and cell viability was analysed. Following transfection with wild-type MYC plasmid, MYC protein levels were markedly increased in JJN3. Interestingly, MYC overexpression had partially rescued JJN3 cells from undergoing cell death in response to [Au(d2py-pe)2]Cl treatment, suggesting that [Au(d2pype)2]Cl exerts its cytotoxic effect in part via MYC inhibition.

4. Discussion

Disease relapse due to acquisition of drug resistance during the course of the treatment is a major cause of death amongst MM patients [51–53]. Hence there is a desperate need to develop new anti-myeloma therapies that can also efficiently eradicate drug resistant myeloma cells.

It is known that cancer cells have increased metabolic demands due to their highly proliferative nature. This causes production of excessive free radicals or ROS that may cause oxidative damage to the cells if the levels of ROS exceed the antioxidant capacity in these cells [22]. Hence, cancer cells are heavily dependent on antioxidants including the Trx system, to maintain redox homeostasis in order to survive [18,19]. While cancer and normal cells depend on this system to maintain a reduced cellular state, the susceptibility of ROS damage-related cell death becomes more apparent in cancer cells as these cells have a narrower margin in reaching the maximum cytotoxicity threshold as compared to normal cells when treated with an anti-cancer drug [54]. Hence, targeting cellular antioxidants, such as the Trx system can be an effective approach to eradicate cancer cells without causing much collateral damage to surrounding normal cells [10].

Our lab has previously shown that myeloma cells have higher intrinsic oxidative stress accompanied with high basal expression of the Trx1 and TrxR1 proteins as compared to normal cells [36]. While the gold(I) drug auranofin (TrxR inhibitor) was able to disrupt the redox homeostasis and cause apoptosis in both sensitive and resistant myeloma cells [35], auranofin acts non-discriminately against many other thiol-containing compounds [30,31]. Therefore, our focus was to
investigate other available gold-based compounds that could potentially overcome the blood disease via specific inhibition on the Trx system in myeloma cells.

Various gold compounds have been designed that have improved reactivity and specificity towards selenol-containing proteins, including TrxR, in order to eradicate cancer cells with fewer side effects. In this study, we showed that [Au(d2pype)2]Cl was able to significantly inhibit the TrxR redox activity in RPMI8226, U266 and JIN3 myeloma cells. The effectiveness of the compound towards TrxR caused significant reduction in cell proliferation through induction of apoptotic cell death as demonstrated by increased caspase 3 activity, cleavage of PARP-1 as well as an increase in the DNA repair biomarker, γH2AX in myeloma cells. These results correlate with previous studies, which demonstrated survival dependency of myeloma cells on the Trx system in protecting against their high intrinsic oxidative stress for survival and growth. Additionally, there were no significant cytotoxic effects against normal PBMCs isolated from healthy individuals, which reaffirms that the Trx system can be

Fig. 4. [Au(d2pype)2]Cl induces apoptosis in bortezomib resistant myeloma cells. (A, B) Bortezomib-resistant myeloma cell lines RPMI8226-BR and U266-BR were treated with indicated concentrations of [Au(d2pype)2]Cl for 24, 48 and 72 h. Cell viability was measured using CellTiter-Blue® Cell Viability Assays. One-way ANOVA followed by Tukey’s post-test was employed. *, #, +, P < 0.0001 where treated samples are compared to corresponding untreated control. (C, D) TrxR activity was also analysed after RPMI8226-BR and U266-BR cells were treated with indicated concentrations of the [Au(d2pype)2]Cl for 24 h by measuring the NADPH-dependent reduction of DTNB by TrxR. (E, F) Caspase-3 activity was measured by monitoring the cleavage of Ac-DEVD-AMC. Values indicate mean ± SEM of three independent experiments performed in triplicate. One-way ANOVA followed by Tukey’s post-test was employed. *, P < 0.05; **, P < 0.01; ***, P < 0.001; ****, P < 0.0001.
selectively targeted in MM. We also showed that myeloma cell death via TrxR inhibition by \([\text{Au(d2pype)}_2]\)Cl is influenced by an increase in intracellular oxidative stress (Fig. 3). An un-controlled increase in ROS levels has been shown to be responsible for causing cellular damage and induce apoptotic cell death in cancer cells [58–60]. Our study showed that treatment of myeloma cells with the antioxidant N-acetyl cysteine (NAC) rescued the myeloma cells from undergoing apoptotic cell death. Furthermore, the addition of the glutathione synthesis inhibitor BSO significantly decreased the cell viability of the myeloma cells when co-treated with the TrxR-targeting gold compound \([\text{Au(d2pype)}_2]\)Cl. Together, these results showed that the ROS generated as a consequential event from TrxR inhibition plays an important role in stimulating apoptosis.

Through the years MM patients have been treated with a range of anti-myeloma drugs including bortezomib, dexamethasone and thalidomide [61]. Unfortunately, this has also resulted in an increase in the number of patients who relapse due to acquired resistance gained from prolonged exposure to these drugs [62,63]. Previously our lab showed that small molecule inhibitors of the Trx system sensitized bortezomib-resistant myeloma cells to bortezomib and overcame acquired hypoxia-induced bortezomib resistance in these cells [35,36]. In this study we showed that \([\text{Au(d2pype)}_2]\)Cl induced apoptosis in both bortezomib-resistant lines, RPMI8226-BR and U266-BR via TrxR inhibition (Fig. 4). Following this, we used methylcellulose assays to test for the clonogenic potential of bortezomib-sensitive and resistant myeloma cells (RPMI8226 and RPMI8226-BR) treated with auranofin or \([\text{Au(d2pype)}_2]\)Cl. Since clonogenic assays are a useful tool for testing and predicting the efficacy of drugs for a particular disease, the results could provide early projections of a parallel correlation between in vitro drug sensitivity and in vivo or clinical patient sample.

![Auranofin and [Au(d2pype)2]Cl](image)

Fig. 5. Clonogenic potential of myeloma cells is diminished upon treatment with \([\text{Au(d2pype)}_2]\)Cl. (A, B) Methylcellulose clonogenic assay for RPMI8226 cells treated with auranofin and \([\text{Au(d2pype)}_2]\)Cl at 0, 2 and 4 \(\mu\)M. (C − F) Number of RPMI8226 and RPMI8226-BR colonies formed after treatment with auranofin and \([\text{Au(d2pype)}_2]\)Cl with indicated concentrations. Values indicate mean ± SEM of three independent experiments performed in triplicate. One-way ANOVA followed by Tukey’s post-test was employed. **, \(P < 0.01\); ***, \(P < 0.001\); ****, \(P < 0.0001\).
We found overwhelming positive results, which showed complete abrogation of tumourigenic capacity in both parental and resistant myeloma cells when treated with [Au(d2pype)2]Cl, as compared to auranofin (Fig. 5). These results confirm that [Au(d2pype)2]Cl can potently eradicate bortezomib-resistant myeloma cells and therefore represents a viable therapy option for bortezomib-resistant or refractory MM patients.

In this study, we have tested for the first time an in vivo anti-cancer activity of auranofin and the gold compound using a human RPMI8226 xenograft model. Although anti-myeloma activity of auranofin has been tested previously using in vitro models, its in vivo anti-myeloma activity has not been tested to date. Our results convincingly show that both auranofin and [Au(d2pype)2]Cl significantly inhibited MM tumour growth in vivo and induced apoptosis as measured by caspase-3 cleavage. We showed that both auranofin and [Au(d2pype)2]Cl exerted anti-myeloma activity via TrxR inhibition in vivo, suggesting that both compounds are able to inhibit their primary target TrxR (Fig. 6). These results warrant further testing of the gold compound for the next phase involving pharmacokinetics and toxicology studies as there is currently little pharmacokinetic data on gold(I) compounds in animals that involved pharmacokinetics and toxicology studies as there is currently little pharmacokinetic data on gold(I) compounds in animals that.

Fig. 6. [Au(d2pype)2]Cl myeloma tumour growth in vivo. (A–E) The tumour growth analysis in human RPMI8226 multiple myeloma xenograft model treated with vehicle, auranofin (5 mg/kg, Monday-Friday, IP), or [Au(d2pype)2]Cl (5 mg/kg, Monday-Friday, IP) for two weeks. The mean tumour size (A) of each treatment group and bioluminescence images of RPMI8226 tumours (B) from each mouse is presented (n = 5 mice/group). (C) Representative images of ApopTag staining of primary RPMI8226 tumours treated with vehicle, auranofin, or [Au(d2pype)2]Cl for 2-weeks. (D) Quantification for percentage of apoptotic cells in primary RPMI8226 tumours treated with vehicle, auranofin, or [Au(d2pype)2]Cl for 2-weeks. (E) TrxR redox activity was measured in RPMI8226 tumours treated with vehicle, auranofin, or [Au(d2pype)2]Cl for 2-weeks. Two-way ANOVA followed by Sidak’s post-test was employed for tumour growth analysis. Paired student t-test was employed for apoptotic cell analysis and TrxR redox activity assays. Values indicate mean ± SEM (n = 3). **, P < 0.01, ****, P < 0.0001.
evaluate the bioavailability and effectiveness of the compound. This will not only encourage the development of other gold(I)-based compounds, but also to discover the potential of using TrxR targeting compounds as effective anti-myeloma therapies in the future.

To address the underlying molecular mechanism that may be involved upon treatment with [Au(d2pype)2]Cl, we assessed the expression of the MYC oncogene since several studies have reported its involvement in promoting the disease progression from non-symptomatic MGUS to active MM [48–50]. In addition, MYC amplification in MM patients is correlated with acquisition of drug resistance and disease relapse as approximately 55% of relapsed MM patients show MYC amplification [67]. Although MYC is amplified in many cancers, targeting MYC has been a huge challenge in cancer therapy due to its complicated structure as well as its predominant location in the nucleus [68,69] and therapies targeting MYC have not been successful in clinical trials, thus leaving MYC undruggable. Hence, there is a great interest in discovering drugs that can indirectly inhibit the expression and oncogenic activity of MYC. Our results (Fig. 7) showed that MYC mRNA and protein levels were significantly reduced in myeloma cells in vitro and in vivo upon treatment with [Au(d2pype)2]Cl, indicating that the gold compound may exert its anti-cancer activity via MYC inhibition. In contrast, the MYC expression levels were less affected by aurano in RPMI8226 and JNN3 myeloma cells, suggesting that aurano and [Au(d2pype)2]Cl may act via their own distinct mechanism. Further investigations are needed to understand the effects of TrxR inhibition by [Au(d2pype)2]Cl that contributed to the downregulation of MYC.

5. Conclusion

In conclusion, our findings show that [Au(d2pype)2]Cl significantly induced ROS-dependent apoptosis in bortezomib-sensitive and resistant myeloma cells via TrxR inhibition. [Au(d2pype)2]Cl also completely inhibited the clonogenic activity of bortezomib-sensitive and resistant myeloma cells suggesting that [Au(d2pype)2]Cl is likely to be effective in both newly diagnosed and relapsed/refractory MM patients. Moreover, our results showed that [Au(d2pype)2]Cl significantly inhibited myeloma tumour growth in vivo. In this study we also delineated a novel mechanism for the underlying anti-myeloma activity of [Au(d2pype)2]Cl by showing that [Au(d2pype)2]Cl inhibited MYC in myeloma cell lines in vitro as well as in primary tumours in vivo. Taken together, our data provides a strong rationale for using improved gold (I)-based compounds as not only potential drugs to be used as part of the current MM co-treatment regimen for more effective therapeutic approach, but also as a more specific tool to better understand the role.

![Fig. 7. [Au(d2pype)2]Cl suppresses myeloma tumour growth via MYC inhibition. (A, B) RPMI8226 and JNN3 cells were treated with indicated concentrations of [Au(d2pype)2]Cl for 24 h, and MYC and Cyclin D1 mRNA levels were analysed by RT-qPCR (A) while MYC protein levels were analysed by western blot analysis (B). (C) MYC protein levels were analysed in vehicle-, [Au(d2pype)2]Cl-, and aurano-treated RPMI8226 primary tumours. HSP90 was used as a loading control. (D) JNN3 cells were transfected with empty vector (EV) and HA-tagged wild-type MYC plasmid (Wt-MYC) for 24 h. Following 24 h transfection, transfected cells were treated with indicated concentrations of [Au(d2pype)2]Cl for 24 h and cell viability was analysed by CellTiter-Blue® Cell Viability Assays. Two-way ANOVA followed by Tukey’s post-tests was employed. Values indicate mean ± SEM (n = 3).](image-url)
of the TRX system in controlling the resistant nature of this disease.

Acknowledgements

J.H. Sze was supported by a Griffith University Postgraduate Research Scholarship and a Griffith University International Postgraduate Scholarship. P.R was supported by a grant from Cure Cancer Australia (1147230). K.N was supported by the Naito Foundations and NHMRC Project Grant (1159593). We also thank QIMR Berghofer Histology facility staff for their help with immunohistochemistry analysis and Griffith University for providing funding to support the student's research.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.redox.2019.101310.

References


