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The thioredoxin reductase inhibitor auranofin triggers apoptosis through a Bax/Bak-dependent process that involves peroxiredoxin 3 oxidation

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ABSTRACT

Thioredoxin reductase (TrxR) is a key selenoprotein antioxidant enzyme and a potential target for anti-cancer drugs. One potent inhibitor of TrxR is the gold (I) compound auranofin, which can trigger mitochondrial-dependent apoptosis pathways. The exact mechanism of apoptosis induction by auranofin is not yet clear, but there are indications that mitochondrial oxidative stress is a central event. We assessed the redox state of the peroxiredoxins (Prxs) in Jurkat T-lymphoma cells treated with auranofin, and found that mitochondrial Prx3 was considerably more sensitive to oxidation than the cytosolic Prx1 and 2, indicating selective mitochondrial stress. Prx3 oxidation was detected at apoptotic doses of auranofin in several cell types, and occurred before other mitochondrial events including cytochrome c release and mitochondrial depolarisation. Auranofin was also able to sensitise U937 cells to TNF-α-mediated apoptosis. Auranofin-induced apoptosis was effectively blocked by the overexpression of Bcl-2, and Bax/Bak deficient mouse embryonic fibroblasts were also resistant to apoptosis, indicating a central role for the pro-apoptotic proteins of this family in auranofin-triggered apoptosis. Auranofin exposure inhibited the proliferation of apoptosis-resistant cells, and at higher doses of auranofin could cause cell death through necrosis. We conclude that auranofin induces apoptosis in cells through a Bax/Bak-dependent mechanism associated with selective disruption of mitochondrial redox homeostasis in conjunction with oxidation of Prx3.

1. Introduction

Thioredoxin reductase (TrxR) is a selenoprotein that plays a critical role in maintaining redox homeostasis in cells through the NADPH-dependent reduction of thioredoxin (Trx) [1,2]. One of the major antioxidant roles of Trx is to reduce a ubiquitous family of thiol peroxidases known as peroxiredoxins (Prxs). These enzymes decompose peroxides using a highly reactive cysteine thiolate in their active site [3]. In the presence of peroxides the Prx active site cysteine forms a disulfide bond with a neighbouring cysteine residue, which Trx reduces to complete the catalytic cycle. Mammals have six Prxs, with Prx3 localised to the mitochondrion. Prx3 is kept reduced through the action of mitochondrial Trx2 and TrxR2, and this coupled system is seen as important in protecting mitochondria from H_2O_2 generated by respiratory complexes during metabolism [4].

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There is growing evidence that these coupled mitochondrial antioxidant enzyme systems may also play a role in the regulation of apoptosis. Overexpression of Prx3 provides protection against induction of apoptosis by serum deprivation, hypoxia and cytotoxic drugs [5–9]. The proposed mechanism is scavenging of H_2O_2 that may otherwise promote the release of pro-apoptotic factors from mitochondria. Consistent with this hypothesis we observed Prx3 oxidation during the initiation of receptor-mediated apoptosis [10]. We have also found that pro-apoptotic isothiocyanates are able to inhibit TrxR and cause selective Prx3 oxidation at concentrations that trigger apoptosis [11]. The mechanism of Prx3 oxidation in both models is currently unclear, but may be due to an increase in mitochondrial H_2O_2 generation or impairment of the TrxR/Trx system. Previous studies have shown that lowering of Prx3 levels with siRNA resulted in increased cellular levels of H_2O_2, and this sensitized cells to the induction of apoptosis by staurosporine or TNF [8]. Similar deficiencies in Trx2 [12,13] or TrxR2 [14] also promote apoptosis in some cell types or organs. Prx3 has previously been identified as a target gene of c-Myc essential for the transformation associated with this oncogenic transcription factor [5], and overexpression of Prx3 has been reported in breast and lung carcinomas, mesothelioma and hepatocellular carcinomas [15–18]. A recent study has also revealed a link between Fanconi anemia and deregulation of Prx3 function [19].

Several anti-cancer agents have been identified as TrxR inhibitors [20–25]. Auranofin, an organic gold compound widely used for the treatment of rheumatoid arthritis [26], has also been tested as an anti-cancer agent along with other gold (I) compounds [27–30]. The pharmacological effect of auranofin is thought to be due to its high reactivity with cellular nucleophiles such as selenocysteine and cysteine, making auranofin a potent inhibitor of TrxR [31,32]. The predominantly cytosolic isoform TrxR1 has also been detected in the intermembrane space of mitochondria, where its inhibition by auranofin may inversely correlate to apoptosis through interactions with Cu,Zn-superoxide dismutase [33], illustrating the complexity of compartmentalization and interactions in subcellular redox system networks. Rigobello et al. have undertaken a series of studies on the ability of auranofin to trigger apoptosis in cultured cells, and propose a general model in which TrxR inhibition causes oxidative stress in the mitochondria that leads to apoptosis [34–37]. Here we have examined the effect of auranofin treatment on cytoplasmic and mitochondrial Prxs, and show selective oxidation of mitochondrial Prx3 at doses that induce apoptosis. We also used mouse embryonic fibroblasts deficient in Bax and Bak to delineate a specific role for this mitochondrial pathway in auranofin-mediated apoptosis.

2. Materials and methods

2.1. Reagents

Cell culture materials RPMI 1640, fetal bovine serum (FBS), penicillin, streptomycin, and geneticin were from Gibco BRL (Auckland, New Zealand). Auranofin was from ICN Biomedicals Inc (Costa Mesa, CA, USA). Human TNF was from R&D Systems (Minneapolis, MN, USA). Monoclonal antibody to cytochrome *c* (clone 7H8.2C12) was from BD Biosciences (San Jose, CA, USA). Rabbit polyclonal antibodies to Prx1, 2, 3 and Prx-SO_2H were from Ab Frontier (Seoul, Korea). Hybond-PVDF membrane and enhanced chemiluminescence (ECL™) Western blotting system were from Amersham Biosciences (Buckinghamshire, England). 5-Iodoacetamidofluorescein and MitoSox were from Molecular Probes (San Diego, CA, USA). Complete™ protease inhibitors were from Roche Diagnostics (Manheim, Germany). The artificial caspase substrate Asp-Glu-Val-Asp-7-amino-4-methylcoumarin (DEVD-AMC) was from the Peptide Institute Inc (Osaka, Japan). All other chemicals and reagents were from Sigma Chemical Co (St. Louis, MO, USA) and BDH Laboratory Supplies (Poole, England). All water was deionized and ultrafiltrated using a Milli-Q filtration system.

2.2. Cell culture

The human Jurkat T-lymphoma and U937 monocytic cell lines were acquired from the ATCC (Rockville, MD, USA) and grown in RPMI 1640 supplemented with 10% fetal bovine serum (FBS), 100 U/ml penicillin, and 100 μg/ml streptomycin. Jurkat transfectants overexpressing Bcl-2 and neo controls, generated as previously described [38], were grown in RPMI 1640 supplemented with 10% FBS and 315 μg/ml geneticin. SV40 immortalised MEFs derived from wild-type and Bax/Bak DKO mice were generously provided by Dr David Huang of the Walter and Eliza Hall Institute, Melbourne. MEFs were maintained in DMEM supplemented with 10% FBS, 50 μM β-mercaptoethanol and 100 μM asparagine. Cells were maintained in a humidified incubator at 37 °C and 5% CO_2/air. Cell lysates were made by harvesting 1×10^6 Jurkat cells or 0.2×10^6 MEFs in 100 μl of lysis buffer (40 mM HEPES, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, protease inhibitors, 1% CHAPS pH 7.4).

2.3. TrxR assay

The activity of TrxR was measured using a modified DTNB reduction assay [39]. In short, sample cell lysates (50 μl, 50 μg total protein) were transferred to a microplate and mixed with 50 μl of 10 mM DTNB and the change in absorbance at 412 nm was monitored for 2 min to give a baseline DTNB reduction. After this, 10 μl of 2 mM NADPH was added to the reaction mix in order to determine the NADPH-dependent DTNB reduction. The relative activity of TrxR was determined as the difference between $\Delta A_{412\ nm}$ before and after the addition of NADPH.

2.4. Determination of caspase activity

Caspase activity within treated cells was determined fluorometrically by following the cleavage of DEVD-AMC. Treated cells (5×10^5) were pelleted and frozen at −80 °C. Frozen pellets were resuspended in 10 μl PBS and transferred to a 96 well plate. Ninety μl of caspase buffer (5 mM dithiothreitol, 100 mM HEPES, 10% sucrose, 0.1% NP-40 and 0.1% CHAPS at pH 7.25) containing 50 μM DEVD-AMC was added to the sample and the rate of AMC production (excitation 370 nm, emission 445 nm) was followed at 37 °C with a POLARstar Galaxy

fluorescent platereader (BMG Labtechnologies Pty. Ltd. Mt Eliza, Australia).

2.5. Detection of mitochondrial reactive oxygen species

The mitochondrial-targeted dihydroethidium dye MitoSox was used to determine the level of mitochondrial oxidants, according to the method of Mukhopadhyay et al. [40]. Following treatment cells were harvested and resuspended in Hanks buffered saline solution containing 5 μM MitoSox. Samples were incubated with MitoSox for 10 min before fluorescence was analysed by flow cytometry with excitation 488 nm and emission 585 nm (FL2 channel).

2.6. Flow cytometry analysis of apoptotic markers

Phosphatidylserine (PS) exposure and propidium iodide (PI) uptake were assessed by resuspending cells in binding buffer containing 1 μg Annexin-V-FITC and 5 μg PI according to manufacturer's instructions (Biosource Annexin V detection kit, Invitrogen). The cell suspension was incubated in the dark for 10 min and then 10,000 cells were analysed using a Cytomics FC500 MPL flow cytometer (Beckman Coulter, Fullerton, CA) to determine the percentage of PS- and PI-positive cells. Mitochondrial permeability transition was assessed by using the potentiometric dye tetramethylrhodamine ethyl ester (TMRE) as previously described [41]. The method involved staining treated cells with 50 nM TMRE for 15 min before being analysed by flow cytometry and monitoring FL2 fluorescence. For the quantification of DNA fragmentation (%Sub G_0/G_1), PI staining of cells was carried out in PBS containing 50 μg/ml PI, 0.1% Triton X-100, and 0.1% sodium citrate [42].

2.7. Immunoblot detection of the Prxs

Treated cells were washed and resuspended in NEM containing buffer (100 mM NEM, 40 mM HEPES, 50 mM NaCl, 1 mM EDTA, 1 mM EGTA, protease inhibitors, pH 7.4) supplemented with 10 μg/ml catalase. Cells were incubated at room temperature for 15 min and CHAPS was added to a final concentration of 1% (Jurkat cells) or 2% (U937 cells). Protein extracts (20 μg) were combined in sample loading buffer (62.5 mM Tris–HCl pH 6.8, 10% glycerol, 2% SDS, 0.025% bromophenol blue) and resolved by SDS-PAGE. Proteins were transferred to PVDF membrane by Western blotting and probed with the appropriate primary antibody in 2% skim milk $TBST_{20}$ overnight at 4 °C. Immunoreactivity was visualized by using a peroxidase system with enhanced chemiluminescence (ECL) (Amersham Biosciences). Densitometry of scanned images was undertaken using Quantity One® software.

2.8. Cytochrome c release assay

Auranofin-treated Jurkat cells (2×10^6 cells) were harvested and resuspended in 30 ml isotonic buffer (10 mM HEPES, 0.3 M mannitol, Complete™ protease inhibitors) supplemented with 1 μg digitonin. After 1 min incubation on ice samples were centrifuged at 13,000 × *g* for 10 min. The cytosolic supernatant was removed immediately for immunoblot analysis. Protein content of the cytosolic fractions was determined by using the BioRad DC assay. Supernatant aliquots were subjected to SDS-PAGE followed by Western blotting against cytochrome *c*. Immunoreactivity was visualized by using a peroxidase system with enhanced chemiluminescence (ECL) (Amersham Biosciences).

2.9. Cell proliferation assay

To quantify cell proliferation, experiments were performed using initial cell concentrations of 1×10^6cells/ml for Jurkat and B9 cultures and 2×10^5 cells/200 mm^2 well for MEFs. Cells were harvested after 24 h in the presence or absence of auranofin and the total number of viable cells remaining was determined by staining cells with trypan blue under a hemocytometer.

2.10. Statistics

Values are shown as the mean and standard error of three or more independent experiments, and all blots are representative of at least three independent experiments. Statistical analyses were performed with the software package SigmaStat (Systat, San Rafael, CA, USA).

3. Results

3.1. Inhibition of TrxR by auranofin correlates closely with induction of apoptosis at doses that cause oxidation of Prx3

Jurkat T-lymphoma cells were treated with auranofin at a range of concentrations, whereupon TrxR inhibition, Prx oxidation and viability were assessed. Auranofin had an IC_{50} of 0.2 μM for total cellular TrxR activity after 30 min, with virtually complete loss of activity at doses in excess of 1 μM auranofin (Fig. 1A). Separation of the cells into cytoplasmic and mitochondrial fractions indicated that auranofin had slightly greater efficacy against cytosolic (predominantly TrxR1) than mitochondrial (predominantly TrxR2) activity (Fig. 1B). Assessment of cell viability 24 h after auranofin exposure showed an LD_{50} of 1.4 μM (Fig. 1C). Cell death was associated with an increase in caspase-3 activity and PS exposure, both of which peaked at 2–3 μM auranofin (Fig. 1D). At higher doses of auranofin there was a decline in both apoptotic markers, consistent with increased necrotic cell death. Prx oxidation was also measured by visualizing conversion of the reduced monomer to oxidized dimer by non-reducing SDS-PAGE and Western blotting. Oxidation of all the Prxs was observed, but Prx3 was clearly the most sensitive (Fig. 2A). Prx3 oxidation was detectable with 0.5 and 1 μM auranofin, and complete oxidation occurred at approximately 2 μM auranofin. This oxidation was complete within 10 min of treatment (Fig. 2B).

To determine if the sensitivity of Prx3 to oxidation is common to TrxR inhibitors we investigated the effect of a second known TrxR inhibitor dinitrochlorobenzene (DNCB) [43]. Jurkat cells exposed to DNCB displayed a dose-dependent

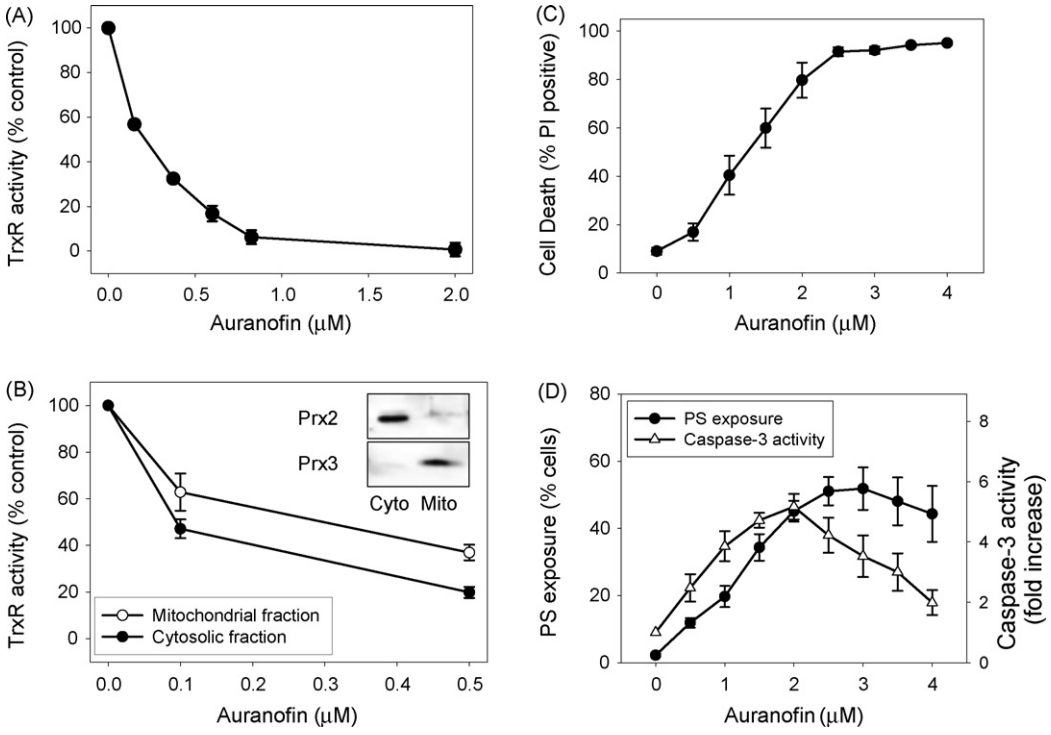

Fig. 1 – Auranofin induces apoptosis in Jurkat cells. (A) Auranofin inhibits TrxR activity in Jurkat cells. Jurkat cells were treated for 30 min with the indicated concentration of auranofin before cells were harvested. Cell lysates were assessed for TrxR activity by measuring NADPH-dependent reduction of DTNB. (B) TrxR activity of the cytosolic (●) and mitochondrial (○) fractions prepared from Jurkat cells exposed for 30 min to the indicated concentrations of auranofin. Inset, western blots of Prx2 (cytosolic) and Prx3 (mitochondrial) confirm the localisation of the enzymes to the expected fractions. (C) Auranofin is cytotoxic to Jurkat cells. Jurkat cells were exposed to the indicated concentrations of auranofin for 24 h before being stained with propidium iodide and analysed by flow cytometry. (D) Auranofin exposure induces apoptosis in Jurkat cells. Caspase-3 activity was examined in Jurkat cells exposed to auranofin after 6 h by monitoring the cleavage of DEVD-AMC. PS exposure was monitored by flow cytometry 8 h after auranofin exposure. Values represent the mean ± S.E. of four independent experiments.

inhibition of TrxR and a concomitant increase in cell death (Fig. 3A). As with auranofin, Prx3 was considerably more sensitive to oxidation than the cytoplasmic Prxs, and of these, Prx2 was more sensitive to oxidation than Prx1 (Fig. 3B).

3.2. *Auranofin sensitises U937 cells to TNF-α-mediated apoptosis*

We have previously shown that Prx3 oxidation occurs during receptor-mediated apoptosis, in particular, activation of the Fas pathway in Jurkat cells and the TNF-α pathway in U937 cells [10]. The proportion of U937 cells in a population that undergo apoptosis following treatment with TNF-α alone is typically restricted to 30%, which corresponds to the extent of Prx3 oxidation. Therefore, we wanted to test whether auranofin could sensitise cells to TNF-α-mediated apoptosis. The synergistic potential of auranofin was tested by pre-treating U937 cells with 1 μM auranofin for 30 min prior to TNF-α stimulation. TNF-α or auranofin alone had only a limited effect on cell viability under the conditions utilized here; however, upon combination of the two compounds there was a dramatic increase in cell death (Fig. 4A). Similarly, auranofin significantly enhanced both PS exposure and caspase-3 activity following TNF-α treatment after 6 h, confirming that auranofin was sensitising U937 cells to apoptosis (Fig. 4B and C).

3.3. *Auranofin activates the mitochondrial pathway of apoptosis, and this is blocked by Bcl-2 overexpression*

The release of cytochrome c and loss of mitochondrial membrane potential are common events leading to the induction of caspase activity in many models of apoptosis. A substantial loss in mitochondrial membrane potential and cytochrome c release did not occur until after 2 h auranofin treatment (Fig. 5A and B), and this timing was closely associated with caspase activation (Fig. 5C). Overexpression of the anti-apoptotic protein Bcl-2 completely blocked all of the apoptotic changes triggered by auranofin (Fig. 5A–C). These results were confirmed by the absence of PS exposure at 6 h (Fig. 6A). Bcl-2 overexpression inhibited auranofin-induced cytotoxicity until doses that triggered necrosis were used (Fig. 6B).

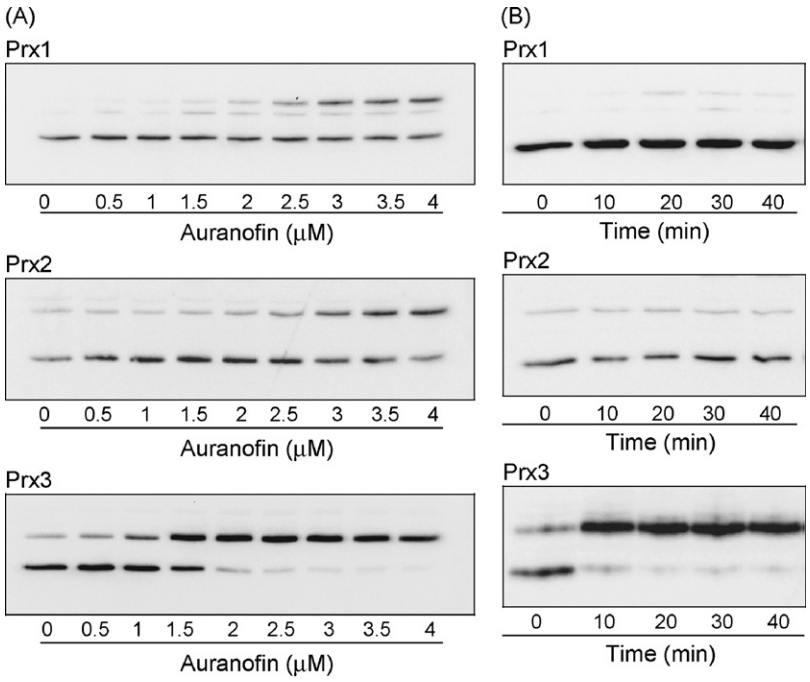

Fig. 2 – Auranofin exposure leads to the preferential oxidation of mitochondrial Prx3. (A) Dose-dependent oxidation of Prx1, 2 and 3 after 40 min auranofin exposure. (B) Time course of Prx oxidation following exposure to 2 μM auranofin. Prx oxidation to the dimer was monitored by non-reducing Western blotting. Gels are representative of three independent experiments.

To determine if Prx3 oxidation occurred before or after commitment to apoptosis we assessed oxidation in Bcl-2 overexpressing cells. The extent of Prx3 oxidation was similar regardless of Bcl-2 expression (Fig. 7A), indicating that oxidation was not a consequence of apoptosis induction. One potential consequence (or cause) of Prx3 oxidation is an increase in mitochondrial oxidant levels. To assess mitochondrial oxidation status, we used the lipophilic cationic dihydroethidium probe (MitoSox), which localises exclusively to the mitochondria. Apoptotic doses of auranofin (2 μM) caused a time dependent increase in mitochondrial oxidant production with a doubling of fluorescence over 2 h (Fig. 7B). Bcl-2 overexpression did not block mitochondrial oxidant production (Fig. 7B). Antimycin A, which is known to increase electron leakage from complex III in the mitochondrial respiratory chain [40,44], increased MitoSox fluorescence to about the same degree in both Jurkat and B9 cells (Fig. 7B).

3.4. *Bax and Bak are required for auranofin-induced apoptosis*

To elucidate the role of other Bcl-2 family members in the regulation of auranofin-induced apoptosis we compared the response of wild-type mouse embryonic fibroblasts (WT MEFs) to MEFs deficient in the pro-apoptotic Bcl-2 proteins Bax and Bak (DKO MEFs). Viability studies demonstrated that Bax/Bak were important for auranofin-induced cytotoxicity (Fig. 8A). The WT MEFs had an LD_{50} of approximately 2.3 μM, while cell death was not seen in the Bax/Bak DKO MEFs until higher doses of auranofin were used. Caspase-3 activation and DNA fragmentation were dramatically inhibited in the Bax/Bak DKO MEFs (Fig. 8B and C), confirming that Bax and Bak are essential for auranofin-induced apoptosis. Prx3 was oxidised by auranofin in both WT and DKO MEFs (Fig. 8D).

3.5. *Auranofin inhibits proliferation of cells resistant to apoptosis*

Previous studies have shown that impairment of TrxR activity by antisense technology or chemical inhibition decreases the proliferative capacity of cells [45–47]. To probe such effects in our system, Jurkat and B9 cells were cultured for 24 h in the presence or absence of 2 μM auranofin. After this time the total number of viable cells had doubled in untreated Jurkat and B9 cultures, while Jurkat cells exposed to auranofin showed a dramatic reduction in cell number, consistent with the induction of apoptosis (Fig. 9A). In contrast, auranofin exposure to apoptosis-resistant B9 cells prevented any increase in the total number of viable cells, thus remaining at the starting concentration of 1×10^6 cells/ml after 24 h. In a similar manner, Bax/Bak DKO MEFs exposed to 3 μM auranofin failed to proliferate over 24 h when compared to untreated controls (Fig. 9B). Cell cycle analyses of growth-arrested B9s and Bax/Bak DKO MEFs did not show any clear signs of G_2/M arrest but were rather suggestive of a delayed progression through the cell cycle (data not shown). Together these results demonstrate that auranofin can efficiently inhibit cell proliferation in cells that are resistant to apoptosis.

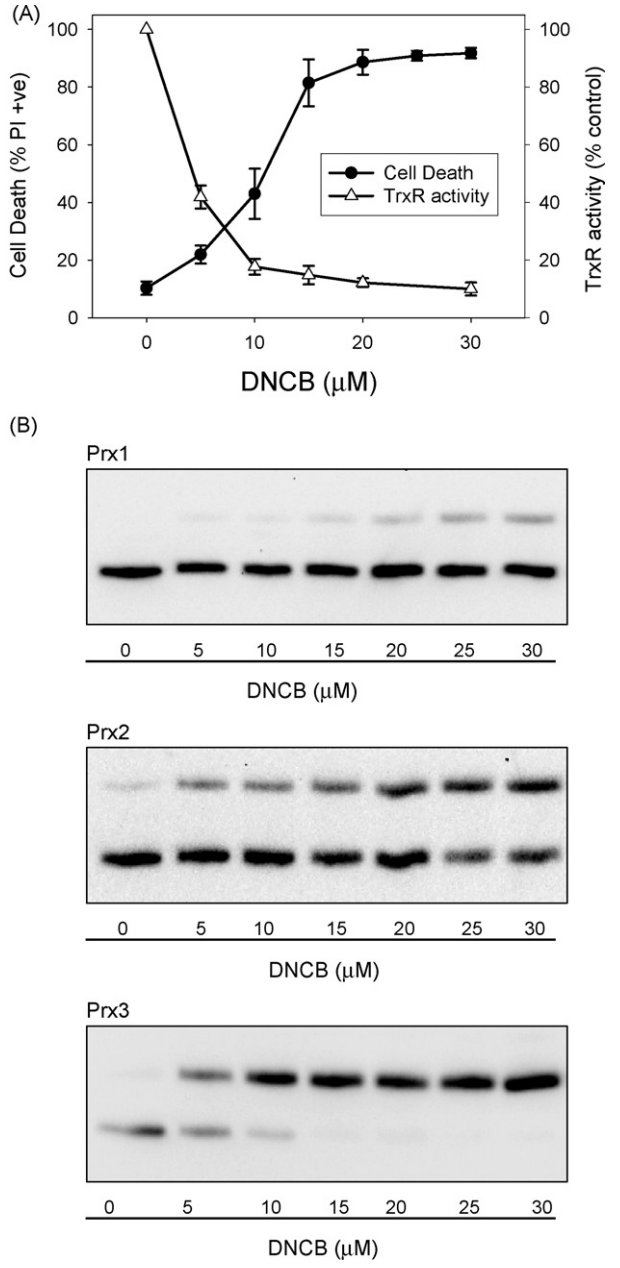

Fig. 3 – An alternative TrxR inhibitor DNCB also promotes Prx3 oxidation. (A) DNCB inhibits TrxR and causes cell death in Jurkat cells. DNCB treated cells were harvested after 30 min and cell lysates were assessed for TrxR activity by measuring NADPH-dependent reduction of DTNB. Cell viability was assessed 24 h after DNCB exposure by staining cells with propidium iodide and analysing them by flow cytometry. (B) DNCB exposure leads to the oxidation of Prxs in a dose (after 40 min exposure) dependent manner. Prx oxidation to the dimer was monitored by non-reducing Western blotting. Values represent the mean ± S.E. of four independent experiments. Gels are representative of three independent experiments.

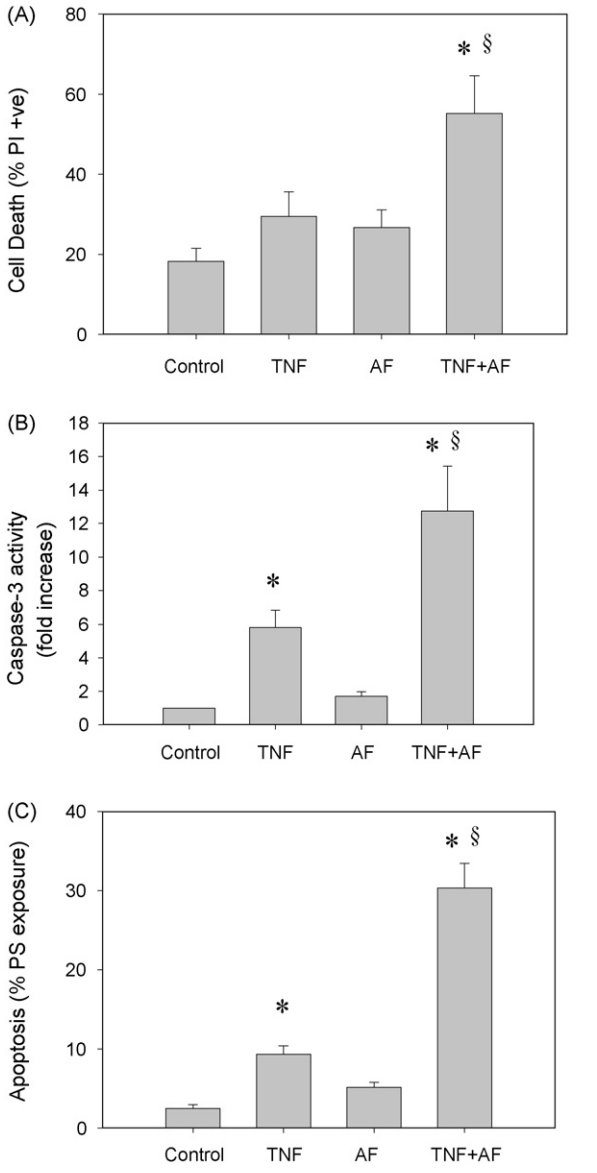

Fig. 4 – Auranofin sensitises U937 cells to TNF-α induced apoptosis. (A) Cell death in TNF-α stimulated cells pre-treated with auranofin. U937 cells treated with TNF-α (20 ng/ml) in the presence or absence of auranofin (1 μM) were incubated for 24 h before being stained with propidium iodide and analysed by flow cytometry. (B) Caspase-3 activity in TNF-α stimulated cells pre-treated with auranofin. The caspase-3 activity of cell lysates was determined in monitoring cleavage of Asp-Glu-Val-Asp-AMC. (C) Phosphatidylserine (PS) exposure in TNF-α stimulated cells pre-treated with auranofin. PS exposure was monitored in U937 cells by flow cytometry following a 6 h treatment. Values represent the mean ± S.E. of four independent experiments. '*' Indicates a significant difference ($p < 0.05$) compared to control cells. '§' Indicates a significant difference ($p < 0.05$) compared to TNF alone. Significant differences were determined by a two-way repeated measures analysis of variance with Bonferroni multiple comparison (Systat).

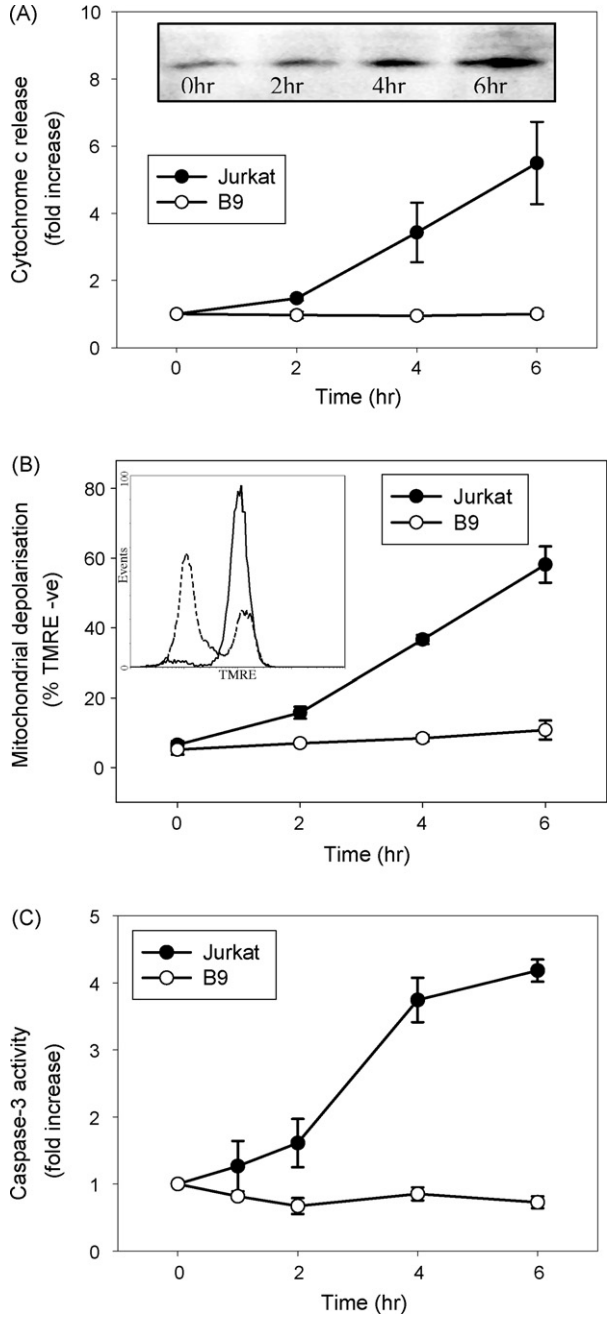

Fig. 5 – Auranofin-induced apoptosis operates via the mitochondrial pathway and is inhibited by the overexpression of Bcl-2. (A) Cytochrome c release in Jurkat cells following exposure to 2 μM auranofin. Cells were harvested at the indicated times and digitonin permeabilised cytosolic fractions were prepared. The amount of cytochrome c in each fraction was assessed by Western blotting. (B) Mitochondrial membrane depolarisation in Jurkat cells exposed to 2 μM auranofin. Auranofin exposed Jurkat cells were stained with 50 nM TMRE for 10 min and analysed by flow cytometry. Inset: FACS histogram showing TMRE staining of Jurkat cells incubated in the presence (hashed line) or absence (solid line) of auranofin for 6 h. (C) Caspase-3 activation in Jurkat cells exposed to 2 μM auranofin. The caspase-3 activity of cell lysates was determined by monitoring cleavage of DEVD-AMC. Values represent the mean ± S.E. of four independent experiments. Gels are representative of four independent experiments. FACS plots are representative of four independent experiments.

4. Discussion

In this study we have shown that auranofin caused selective oxidation of mitochondrial Prx3 at concentrations that were able to trigger apoptosis. Prx3 oxidation was detectable well before major apoptotic events could be measured, and it still occurred when apoptosis was blocked by overexpression of Bcl-2 or by the removal of the pro-apoptotic mediators Bax and Bak. This indicates that Prx3 oxidation was a direct effect of auranofin exposure rather than a consequence of downstream apoptotic events in the mitochondria.

These findings support earlier studies proposing that mitochondrial TrxR2 is a primary auranofin target leading to mitochondrial oxidative stress and apoptosis [32,36,37]. It is not clear why Prx3 is considerably more sensitive to oxidation than cytoplasmic Prx1 and Prx2 since auranofin showed similar efficacies against mitochondrial and cytoplasmic TrxR activity. One possibility is that the mitochondrial environment is more oxidising as a consequence of increased hydrogen peroxide derived from respiratory complexes, and that disruption of mitochondrial TrxR activity therefore has more severe consequences. This hypothesis is supported by selective Prx3 oxidation in response to DNCB treatment (this study), and with pro-apoptotic isothiocyanates that also have TrxR-inhibitory activity [11]. These results also parallel a series of studies by Jones and co-workers, demonstrating that mitochondrial Trx2 is considerably more sensitive to oxidation than cytosolic Trx1 following oxidative stress [48–50]. A recent study demonstrated that apoptosis-inducing heavy metals, several of which are known thioredoxin reductase inhibitors [23,51–53], caused selective Trx2 oxidation and activation of the apoptosis signalling kinase (ASK1) [54].

Prx3 oxidation appears to be a sensitive marker of mitochondrial oxidative stress. It is also tempting to speculate that Prx3 oxidation is closely associated with the initiation of apoptosis. One mechanism for this could be an increase in mitochondrial H_2O_2 due to impairment of Prx3 antioxidant activity. Prx3 is vital to H_2O_2 detoxification as it is more abundant than glutathione peroxidase in mitochondria [8,55]. It has been proposed that mitochondrial H_2O_2 plays a prominent role in apoptotic processes, including triggering the release of cytochrome c from the intermembrane space [56], however, direct evidence is currently lacking. The consumption of endogenous peroxides by Prx3 in the presence of a TrxR inhibitor would also drive the oxidation of Trx2 since Trx2 is used for regeneration of Prx3. Indeed, Prx3 oxidation occurred at auranofin concentrations that inhibited TrxR activity by >90%, and since Prx3 is present at higher concentrations than Trx2 [55], oxidized Trx2 will accumulate rapidly. One consequence of Trx oxidation will be activation of ASK1 forms located in cytoplasmic or mitochondrial membranes, which are inhibited by the reduced forms of Trx1 and Trx2, respectively [57–59].

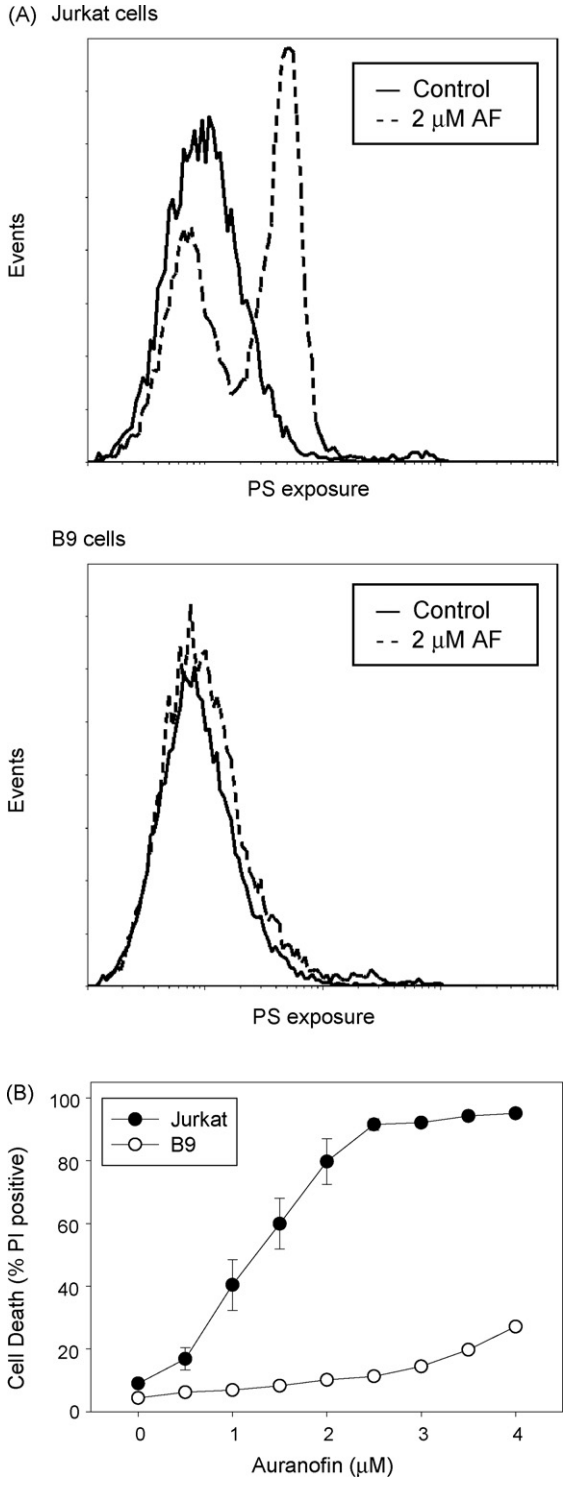

Fig. 6 – Bcl-2 overexpression blocks auranofin-induced cytotoxicity. (A) FACS histogram showing PS exposure in Jurkat and B9 cells incubated in the presence (hashed line) or absence (solid line) of 2 μM auranofin for 8 h. (B) Cell viability in Jurkat (○) and B9 (●) cells treated with auranofin. Cell viability was assessed 24 h after auranofin exposure by staining cells with propidium iodide and analysing them by flow cytometry. Values represent the mean ± S.E. of four independent experiments. FACS plots are representative of four independent experiments.

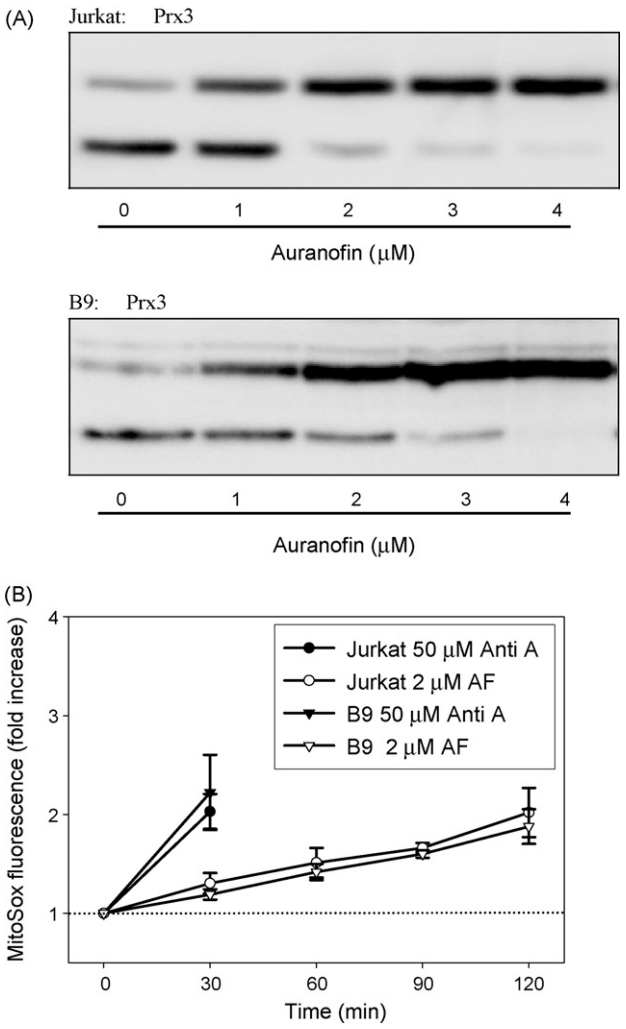

Fig. 7 – Auranofin causes a rapid increase in the production of mitochondrial oxidants in both Jurkat cells and transfectants overexpressing Bcl-2. (A) Auranofin exposure for 30 min leads to the selective oxidation of Prx-3 in Jurkat cells and Bcl-2 transfectants (B9 clones). Prx oxidation to the dimer was monitored by non-reducing Western blotting. (B) Mitochondrial oxidant production in Jurkat and B9 cells exposed to auranofin. Cells were exposed to 2 μM AF or 50 μM antimycin A for the indicated times before being harvested and resuspended in HBSS with 5 μM MitoSox for 10 min. Cells were analysed by flow cytometry monitoring fluorescent changes in the FL2 channel. Values represent the mean ± S.E. of four independent experiments. Gels are representative of three independent experiments.

We have previously shown that mitochondrial Prx3 is oxidised during the initiation of death receptor and isothiocyanate-mediated apoptosis [10,11], and it has been reported that mitochondrial Trx2 is preferentially oxidised during TNF-mediated apoptosis [60]. Furthermore, disruption of mitochondrial redox homeostasis by auranofin was able to sensitise U937 cells to TNF. A recent study has demonstrated that arsenic trioxide can sensitise cells to TNF-α-mediated

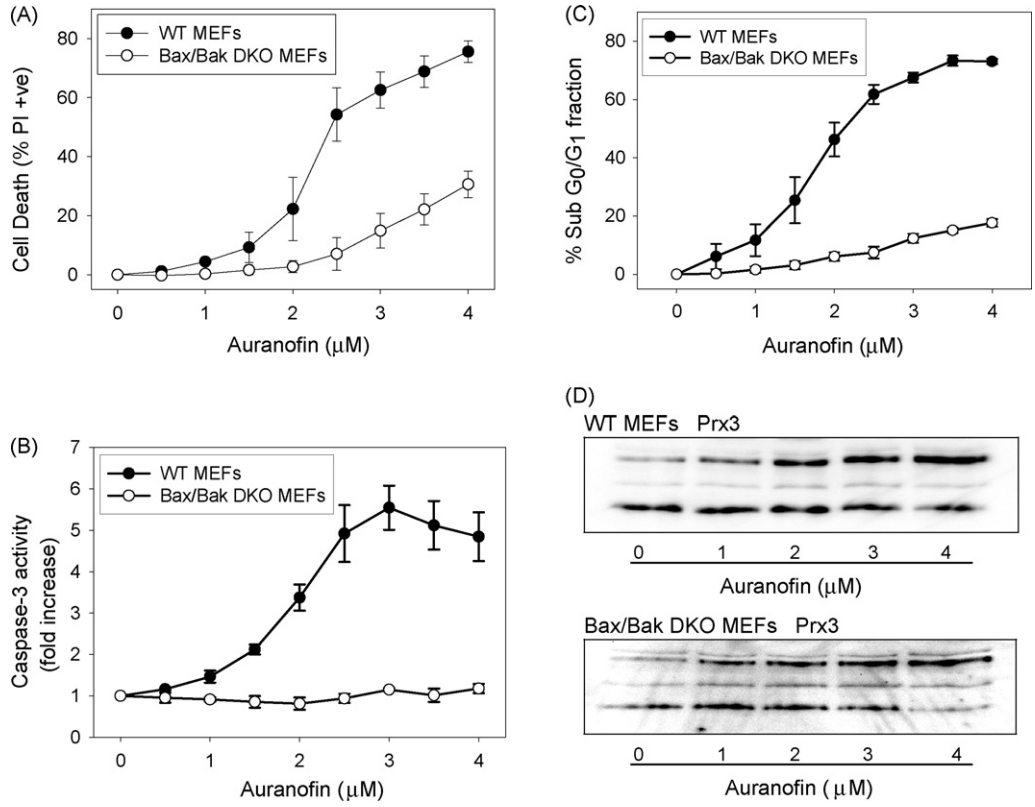

Fig. 8 – Bax and Bak are required for auranofin-induced apoptosis. (A) Loss of cell viability in MEFs exposed to auranofin. WT and Bax/Bak DKO MEFs were exposed to the indicated concentrations of auranofin for 24 h before being stained with propidium iodide and analysed by flow cytometry. (B) Caspase-3 activity in MEFs exposed to auranofin. Auranofin exposed MEFs were harvested after 8 h and caspase-3 activity was assessed by monitoring the cleavage DEVD-AMC. (C) DNA fragmentation (%Sub G_0/G_1 fraction) in MEFs exposed to auranofin after 24 h. For figures (A–C), ○ = WT MEFs and ● = $Bax^{-/-}$, $Bak^{-/-}$ DKO MEFs. (D) Auranofin exposure leads to the oxidation of Prx3 in both WT and DKO MEFs. Prx oxidation to the dimer was monitored by non-reducing Western blotting. Values represent the mean ± S.E. of four independent experiments. Gels are representative of three independent experiments.

apoptosis via p38 MAPK activation of the mitochondrial pathway [61]. Given that arsenic trioxide is an efficient irreversible inhibitor of TrxR [23], it seems possible that TrxR inhibition is the common mechanism by which both auranofin and arsenic trioxide sensitise cells to receptor-mediated apoptosis.

Rigobello et al. have shown that in isolated mitochondria auranofin induces the mitochondrial membrane permeability transition (MPT), which leads to the depolarisation of mitochondria [36] and the release of cytochrome *c* [37]. Recently, they demonstrated that the MPT inhibitor cyclosporin A fails to prevent cytochrome *c* release in cells exposed to auranofin [35]. Our finding that auranofin-induced apoptosis is completely blocked in cells either overexpressing Bcl-2 or being deficient in Bax and Bak is of interest in this context. It suggests that auranofin-triggered apoptosis is regulated by the Bcl-2 family rather than the mitochondrial permeability transition (MPT) pore. Interestingly, recent studies have revealed that the MPT pore plays an essential role in mitochondrial membrane disruption during necrosis [62–64]. It is therefore possible that the MPT pore may regulate auranofin-induced cell death at necrotic doses. It will be of interest in future studies to characterise which BH3-only proteins, if any, are involved in Bax/Bak activation following cellular exposure to auranofin.

While this study has focused on apoptosis in auranofin-treated cells, the oxidative stress following inhibition of TrxR can promote a combination of apoptotic and necrotic cell death, depending on concentration and cell type. These deleterious effects may be due to inhibition of various Trx and TrxR-dependent pathways, or due to the formation of SecTRAPs that are derivatives of TrxR killing cells by a pro-oxidant gain of function [65–67]. It is known that certain compounds targeting TrxR may lead to Trx oxidation, whereas knockdown of the enzyme or inhibition to the same extent with other compounds apparently does not necessarily give Trx oxidation [68]. More recently, reduced Trx has been proposed to facilitate the denitrosylation of caspases [69,70], and that inhibition of TrxR by auranofin inhibits apoptosis by promoting the accumulation of nitrosylated caspases [70]. It is not clear how this mechanism fits with the pro-apoptotic properties of auranofin, and the observed oxidation of Prx3 and Trx2 during apoptosis [10,49,60].

Despite our ignorance of the details surrounding redox changes during apoptosis, it is becoming increasingly clear that inhibition of TrxR may be an important molecular

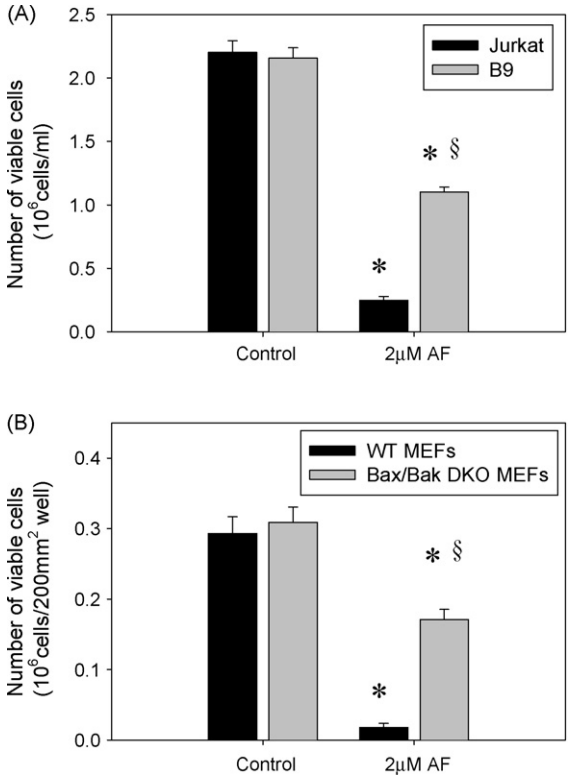

Fig. 9 – Auranofin inhibits proliferation of apoptosis-resistant cells. (A) Monitoring growth of Jurkat (black bar) and B9 (grey bar) cells incubated in the presence or absence of 2 μM auranofin for 24 h. Cultures were treated at an initial cell concentration of 1×10^6 cells/ml. (B) Monitoring growth of WT (black bar) and Bax/Bak DKO (grey bar) MEFs incubated in the presence or absence of 3 μM auranofin. MEFs were treated at an initial cell concentration of 0.2×10^6 cells/200 mm^2 well. The total number of viable cells in each culture was determined by counting cells on a hemocytometer in the presence of trypan blue. Values represent the mean ± S.E. of four independent experiments. '*' Indicates a significant difference ($p < 0.05$) between control and auranofin-treated cells. '§' Indicates a significant difference ($p < 0.05$) between cell types treated with auranofin. Significant differences were determined by a two-way repeated measures analysis of variance with Bonferroni multiple comparison (Systat).

mechanism leading to cell death upon use of electrophilic compounds in anti-cancer therapy [20]. There are a number of organic gold compounds that are currently being investigated as anti-cancer drugs, several of which induce apoptosis by targeting the mitochondria or inhibiting TrxR [28,32,71–75]. For instance, Rackham et al. recently showed that a gold (I) phosphine complex selectively induces apoptosis in transformed cells by inhibiting Trx and TrxR [73]. They also demonstrated that the delocalised lipophilic cation accumulated in the mitochondria. Another recent study used two dimensional proteomic expression profiling to investigate the action of a gold (III) complex that was selectively toxic to cancer cells [75]. Interestingly, the authors found that one of the few proteins with altered expression was Prx3, which exhibited a 3-fold decrease in expression [75]. Auranofin has been shown to induce apoptosis in cisplatin-resistant cancer cells [76], suggesting that anti-cancer drugs targeting TrxR may overcome some forms of drug resistance. It is proposed that TrxR inhibitors are selectively toxic to transformed cells because such cells rely on elevated TrxR activity to maintain DNA synthesis and redox homeostasis [20,23,77,78]. In line with this notion, studies using organic gold complexes similar to auranofin have found that transformed cells exhibit a greater sensitivity to the drug compared to normal cells [71,73,75]. We found that overexpression of the oncogenic protein Bcl-2 effectively blocked auranofin-triggered apoptosis. Considering that many cancers overexpress anti-apoptotic Bcl-2 family members [79], it is possible that drugs targeting TrxR may encounter similar resistance problems as traditional chemotherapy. As such, it would also be of interest to determine whether small molecule inhibitors of the Bcl-2 family, such as ABT-737 [80], can act synergistically with TrxR inhibitors to promote cancer cell death. Despite this potential limitation, auranofin was still able to inhibit the proliferation of cells resistant to apoptosis. This is consistent with recent studies demonstrating that knockdown of TrxR causes a dramatic reduction in tumour progression in vivo [45]. These findings reinforce the idea that TrxR is a key drug target, as its inhibition can have multiple effects including triggering death in cells with intact apoptotic machinery and inhibiting proliferation of apoptosis-resistant cells.

Until recently auranofin was the primary agent used to treat rheumatoid arthritis [81]. Overall auranofin is well tolerated at doses of 6 mg/day. However, a minority of patients on auranofin can exhibit adverse side effects such as diarrhoea, gastro-intestinal upset and skin rash. It remains to be seen whether the potent cytotoxicity of auranofin is responsible for such side effects.

In summary, we have shown that auranofin disrupts mitochondrial redox homeostasis and induces apoptosis via mitochondrial outer membrane permeabilization and apoptotic signalling events regulated by the Bcl-2 family (Fig. 10). Cell death triggered by auranofin occurred in conjunction with TrxR inhibition and Prx3 oxidation; two early events not dependent upon downstream apoptotic signalling. These

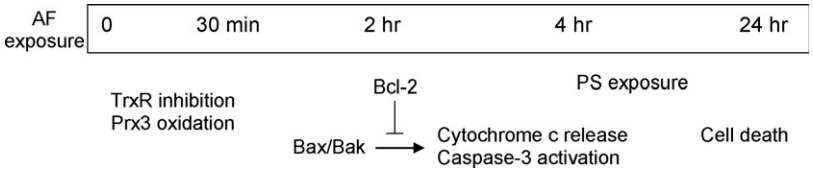

Fig. 10 – Summary of the events associated with auranofin exposure.

results support a model in which auranofin inhibits TrxR, resulting in the rapid accumulation of oxidised Prx3. Additional work is required to determine if oxidation of Prx3, in addition to being an important antioxidant enzyme and a sensitive marker of mitochondrial stress, also plays an active role in the initiation of apoptosis.

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