

Linking genotoxicity and cytotoxicity with membrane fluidity: a comparative study in ovarian cancer cell lines following exposure to auranofin

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Abstract

Auranofin, an organogold compound classified as an anti-rheumatic agent is under phase 2 clinical trials for re-purposing to treat recurrent epithelial ovarian cancer. We have reported earlier that Breast cancer 1, early onset (BRCA1) mutant ovarian cancer cells exhibit increased sensitivity to auranofin. BRCA1 is a DNA repair protein whose functional status is critical in the prognosis of ovarian cancer. Apart from DNA repair capability of cancer cells, membrane fluidity is also implicated in modulating resistance to chemotherapeutics. We report here that membrane fluidity influences the sensitivity of ovarian cancer cell lines (OVCAR5 and IGROV1) to auranofin. Electron spin resonance (ESR) analysis revealed a more fluidized membrane in IGROV1 compared to OVCAR5. Interestingly, IGROV1 cells were more sensitive to auranofin induced cytotoxicity than OVCAR5. In comparison to OVCAR5, IGROV1 cells also exhibited an increased number of DNA double strand breaks (DSBs) upon auranofin treatment as assessed by 53BP1 immunostaining. Furthermore, correlation analysis demonstrated a strong positive correlation ($r = 0.856$) between membrane fluidity and auranofin sensitivity in these cell lines. Auranofin-treated IGROV1 cells were also exhibited increased cellular oxidation and apoptosis. Anti-oxidant, N-acetyl cysteine (NAC) inhibited the cellular oxidation and apoptosis in auranofin-treated ovarian cancer cells suggesting reactive oxygen species (ROS) mediates the anti-cancer properties of auranofin. Overall, our study suggests that auranofin mediates its cytotoxicity via ROS production in ovarian cancer cells which correlates positively with membrane fluidity.

Key words

Membrane fluidity, auranofin, drug sensitivity, ovarian cancer, DNA damage, Cell survival

Introduction

The dynamic nature of plasma membrane inferred as membrane fluidity is a critical physiochemical property that modulates cellular functions. Over the years, a significant number of studies have been undertaken to understand how membrane fluidity influences drug sensitivity in cancer cells (1). Analysis of membrane dynamics of chemo-resistant cancer cells reveals rigidification of cell membranes (2). The observed membrane rigidity of resistant cancer cells has been found to be due to the presence of relatively high amounts of cholesterol and sphingomyelin (3,4). Breast cancer cells were reported to have reduced expression of sphingomyelinase that catalyses the hydrolysis of sphingomyelin (5). Recovering sphingomyelinase expression fluidizes the membrane and enhances drug transport which eventually leads to reversal of resistance (6). Interestingly, the threshold concentration of internalised doxorubicin is the same for both resistant and sensitive cells suggesting drug uptake could be a major factor deciding the sensitivity of cancer cells (2). Modulation of membrane fluidity by pharmacological agents has been demonstrated to increase the drug uptake and thereby sensitivity of cancer cells to chemotherapeutic agents (7). The membrane lipid analysis of cisplatin and doxorubicin resistant breast cancer cells showed a high content of cholesterol and sphingomyelins which results in membrane rigidity (4). Conversely, resistant cancer cells possess fewer amounts of diacyl and triacyl glycerols that are known to constitute a more fluid membrane (4). A similar analysis of membrane phospholipids demonstrated qualitative and quantitative differences between malignant, benign and normal breast tissues (3). Significantly distinguishable was lysophosphatidylcholine where its presence was remarkably decreased in malignant and benign tissues compared to normal breast tissues (3). However, a comparative study of membrane fluidity and drug sensitivity of cancer cells of the same histological origin has not yet been performed.

Auranofin, a thioredoxin reductase inhibitor has been widely used for the treatment of rheumatoid arthritis under the proprietary name Ridaura. Auranofin has also been demonstrated to possess anti-cancer properties in both in vitro and in vivo conditions (8,9). Moreover, auranofin is currently undergoing phase 2 clinical trials for re-purposing to treat recurrent epithelial ovarian cancer (<https://clinicaltrials.gov/ct2/show/NCT01747798>). We have recently reported that genetic depletion of BRCA1 sensitizes ovarian cancer cells to auranofin (10). Auranofin induces lethal DNA double strand breaks (DSBs) and apoptosis in BRCA1 deficient ovarian cancer cells (10). Anti-oxidant mediated protection of BRCA1 deficient cells suggest that reactive oxygen species (ROS) plays a critical role in auranofin induced DNA damage and apoptosis (10).

Membrane fluidity is often studied in association with intracellular drug uptake (2,7). However, early increase in membrane fluidity is critical in modulating cellular response to chemotherapeutic drugs such as cisplatin (11). Interestingly, blocking the early increase in membrane fluidity by pharmacological means inhibit apoptosis despite the intracellular uptake and formation of cisplatin DNA adducts (11). This suggests that changes in membrane fluidity induced by chemotherapeutic drugs may regulate cellular fate which is a critical and independent process from intracellular drug uptake and its targets. Furthermore, this also points that membrane fluidity is a critical physiochemical parameter that could potentially modulate multiple processes from drug entry to induced cellular outcome. However, a correlative study investigating the role of inherent membrane fluidity in regulating the genotoxic and cytotoxic effects of drugs has yet to be established. Adopting an integrated approach, in this study we therefore aimed to elucidate how plasma membrane fluidity could modulate cytotoxic and genotoxic responses of ovarian cancer cell lines (IGROV1 & OVCAR5) of adenocarcinoma origin to auranofin.

Materials and methods

Chemicals

All chemicals were purchased from Sigma–Aldrich, UK unless indicated otherwise. Anti-53BP1 antibody (Rabbit polyclonal) was purchased from Novus Biologicals, UK. Rabbit polyclonal antibodies against PARP, Nrf2 and Actin were purchased from Cell Signalling Technology, Danvers, MA. Alexa Fluor 488-labelled anti-rabbit antibody was purchased from Molecular Probes, UK.

Cell culture and treatments

Ovarian cancer cell lines were a kind gift from Prof Hani Gabra, Hammersmith Hospital, Imperial College London, UK. Cells were maintained in RPMI-1640 (Gibco, Paisley, UK) and the medium was supplemented with 10 % fetal bovine serum (Gibco) 50 U/ml penicillin (Gibco) and 50 mg/ml streptomycin (Gibco) and maintained at 37 °C in a humidified 5 % CO₂ atmosphere. Auranofin was purchased from Sigma-Adrich, UK. Auranofin is insoluble in water and soluble in highly polar organic solvents such as DMSO. A detailed chemical and physical properties is given in pubchem

(<https://pubchem.ncbi.nlm.nih.gov/compound/16667669#section=Experimental-Properties>)

For treatment with auranofin, a stock solution of 14.73 mM was made in DMSO. Final concentration (v/v) of DMSO in the experimental cell culture medium was 0.013 % for achieving 2 μM of auranofin. For concentration viability study, the concentration of DMSO (v/v) was 0.0678 % to achieve 10 μM of auranofin. Respective higher concentration (v/v) of DMSO was added to the control cells. For pre-treating cells with N-acetyl cysteine (NAC), NAC (2.5 mM) was added to the cells for 2 h before treating the cells with auranofin.

Determination of membrane fluidity

Membrane fluidity was measured by Electron spin resonance (ESR) spectroscopy, using the spin label 5-doxyl stearic acid (5-DS). 2 μl of a 10^{-3} M solution of 5-DS in ethanol was introduced to an Eppendorf tube and the ethanol was evaporated by a stream of nitrogen to leave a film of the spin label. Approximately 10^6 cells in 1 ml phosphate buffered saline (PBS) were added to the tube and vortexed for 30 s to allow the label to be taken up by the cells. The cell pellet was washed with fresh PBS and re-suspended in 10-20 μl of PBS, before being sucked into a 5 cm length of gas permeable Teflon tubing (Zeus, Donegal, Ireland) which was folded in half and inserted into an open ended 4 mm i.d. quartz tube and positioned in the microwave cavity, where the temperature was controlled by a flow of air. Measurements were made using a Bruker EMXmicro ESR spectrometer (Coventry, UK). Measurement conditions were approximately 9.4 GHz microwave frequency, 10 mW microwave power, 3355 gauss magnetic field and sweep width 100 gauss. Spectra were collected with a time constant of 82 ms. and sweep time of 110 s. The separation of the outer extrema ($2T_{\parallel}$) corresponds approximately to $2T_{zz}$ and that of the inner extrema ($2T_{\perp}$) to approximately $2T_{yy}$ or $2T_{xx}$ (Supplementary figure 1). As motional freedom increases, $2T_{\parallel}$ decreases and $2T_{\perp}$ increases. In this intermediate to slow motion range, where the correlation time is between 2×10^{-7} and 3×10^{-9} s, the rigidity of the label in a specific environment can be defined in terms of an order parameter, S, such that S=1 for a completely rigid system and S=0 for a completely fluid system. The order of a given environment is given by

$$S = [2T_{\parallel} - (2T_{\perp} + 1.6)] / [2T_{zz} - (T_{xx} + T_{yy})]$$

where T_{xx} , T_{yy} and T_{zz} are the hyperfine tensors for the nitroxide in a crystal and are assigned the values 6.3, 5.8 and 33.6G. In calculation of the order parameter S, a correction of 0.8 G is applied to the measured value of T_{\perp} (12,13).

Cell survival assay

Percentage of cell survival was determined by 3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyl tetrazolium bromide (MTT) assay. MTT was purchased from Sigma–Aldrich, UK. Briefly, cells were treated with the indicated concentration of auranofin for 48 h. Following 1 mg/ml concentration of MTT was added to the wells and incubated at 37 °C for 3 h. The reaction was stopped with DMSO. The colour intensity was measured at 550 nm by spectrophotometry and relative cell viability (%) was expressed as a percentage relative to the DMSO treated control samples set to 100 %.

DNA double strand breaks (DNA DSBs) analysis by immunofluorescence microscopy

DNA damage was determined by assessing double strand breaks as reported earlier by us and other workers (14,15). Auranofin treated or untreated cells were permeabilised in 0.5% solution of Triton X-100 in phosphate buffered saline (PBS) and then blocked with a solution of 0.1% Triton X-100, 10 % FBS in PBS. After blocking, cells were incubated with anti-53BP1 rabbit polyclonal antibodies for 1 h at room temperature. Following primary antibody incubation, cells were washed with a 0.1% Triton X-100 in PBS washing buffer and incubated with Alexa Fluor 488-labeled anti-rabbit IgG secondary antibody for 1 h at room temperature. Cells were washed in PBS and counterstained with 4,6-diamidino-2-phenylindole (DAPI) 1 µg/10 ml in PBS. Washed once with PBS and mounted with Fluorosave reagent (Calbiochem, Merck-Millipore, UK) and viewed using Zeiss Axiovert 200 M microscope (Carl Zeiss Micro Imaging, LLC, USA).

Western blotting

Following experimental treatments, cells (0.3×10^6) were removed from the flasks by scraping. The whole cell lysate preparation and Western blotting were carried out as previously reported (14).

Statistical analysis

Results are reported as the mean \pm SEM. Two tailed independent Student's t test without equal variance assumption was used to determine p-values (*, $P < 0.05$; **, $P < 0.005$, ***, $P < 0.0005$),). Significance of differences in 53BP1 foci numbers between auranofin treated OVCAR5 and IGROV1 cells were assessed using unpaired Student's two-tailed t-test and significance of differences in 53BP1 foci numbers between auranofin treated and respective control cells were assessed using paired Student's two-tailed t-test. Regression analysis was performed to determine the linear relationship between membrane fluidity and % cell survival. Analysis was performed using GraphPad Prism version 6.00 for Windows, GraphPad Software, La Jolla California USA, www.graphpad.com.

Results

Positive correlation between membrane fluidity and auranofin sensitivity: IGROV1 is more fluid and sensitive than OVCAR5

The studies on membrane fluidity and chemo resistance have mainly been restricted to a single breast cancer cell line MCF-7 and its drug resistant variant (2). Our aims were to do a comparative analysis of membrane fluidity in two ovarian cancer cell lines (OVCAR5 & IGROV1) of adenocarcinoma origin (16,17) and to further investigate how membrane fluidity influences sensitivity of these cell lines to auranofin. Electron spin resonance (ESR) using the spin label 5-DS allowed the determination of membrane order parameter S, which is inversely proportional to membrane fluidity. ESR analysis revealed relatively rigid membranes in

OVCAR5 as inferred by an increased S value (Figure 1A). In contrast, IGROV1 appeared to have a relatively fluidised membrane as demonstrated by a decreased S value (Figure 1A).

Auranofin is known to exhibit cytotoxic activity against various cancer cells (8,9). Assessment of viability at a wide range of concentrations showed IGROV1 cells were more sensitive to auranofin induced cytotoxicity than OVCAR5 cells (Figure 1 B). Treatment of auranofin at 1 μ M resulted in more than 55 % cell death in IGROV1 compared to less than 10 % in OVCAR5 (Figure 1 C). Interestingly, IGROV1 possess a more fluid membrane compare to OVCAR5 (Figure 1 A). This prompted us to perform a correlation analysis between S values and % cell survival upon auranofin (1 μ M) treatment. The scatter plot revealed a strong positive correlation ($r = 0.0.856$) between membrane fluidity and cell survival upon exposure to auranofin (1 μ M). We fitted a linear regression model ($R^2 = 0.7319$, $p = 0.02982$) to the data as demonstrated in figure 1 D. IGROV1, which possess a more fluid membrane, was more sensitive to auranofin than OVCAR5 with a rigid membrane (Figure 1 A, B & C). Furthermore, OVCAR4, another ovarian cancer cell line which also possess a relatively rigid membrane was comparatively resistant than IGROV1 (supplementary figure 2A & B).

Auranofin induces increased DNA double strand breaks (DSBs) in IGROV1 cells

We have previously reported that BRCA1-depleted ovarian cancer cells exhibit increased sensitivity to auranofin due to the accumulation of unrepaired lethal DNA DSBs (10). In order to understand whether decreased survival exhibited by IGROV1 cells correlate with DNA damage, generation of DSBs were analysed by immunofluorescence using anti-bodies against 53BP1. Upon double strand breaks, 53BP1 along with other DNA repair proteins relocates to the sites of DSBs to form discrete foci. Hence, analysing 53BP1 leads to indirect quantitative assessment of DSBs as reported earlier (18,19). Immunostaining of 53BP1 showed discrete foci marking DSBs in auranofin treated cells (Figure 2 A). As shown in the figure 2 A and B,

the number of foci is significantly higher in IGROV1 compared to OVCAR5 upon auranofin treatment. Intriguingly, there was more DSBs in untreated OVCAR5 cells and the number of DSBs does not significantly change after auranofin treatment (Figure 2B). However, the number of cells exhibiting more than 10 foci has increased significantly after 6 h and 18 h following auranofin treatment of IGROV1 cells (Figure 2B). Furthermore, auranofin induced DSBs were significantly higher in IGROV1 cells than OVCAR4, another ovarian cancer cell line which possess a comparatively rigid membrane (Supplementary figure 3). Taken together, the increased number of DSBs in IGROV1 is corroborated by the decreased survival which in turn correlated with the increased membrane fluidity.

Increased cellular oxidation and apoptosis in auranofin-treated IGROV1 cells

We have reported earlier that auranofin induces oxidative stress and apoptosis in BRCA1-depleted OVCAR5 cells. Cellular oxidative stress was determined by the expression of the biological marker, Nrf2 (20). Cellular oxidative stress activates Nrf2 which in turn drives the expression of anti-oxidant genes. As shown in figure 3 A, auranofin (1 μ M) treated IGROV1 cells exhibited strong induction of Nrf2 after 6 h compared to OVCAR5 where induction of Nrf2 is minimal. This suggests that auranofin causes increased cellular oxidation in IGROV1 cells compared to OVCAR5. Western blot analysis of PARP cleavage was performed to assess apoptosis in auranofin-treated cancer cells. As presented in figure 3 B, there was increased cleavage of PARP in auranofin (2 μ M) treated IGROV1 cells. On the other hand, auranofin (2 μ M) treated OVCAR5 cells exhibited less cleavage of PARP1. Overall, the data suggest that IGROV1 cells are comparatively more sensitive to auranofin induced cellular oxidation and apoptosis.

Anti-oxidant N-Acetyl Cysteine (NAC) abrogates the cellular oxidation and apoptosis induced by auranofin in IGROV1 cells

Previous studies have established that ROS mediate the cytotoxic effects of auranofin (8). We also reported NAC protects BRCA1-depleted ovarian cancer cells from auranofin induced apoptosis (10). In order to determine whether NAC ameliorates cellular oxidation and apoptosis induced by auranofin, cells were pre-treated with NAC. As presented in figure 4 A, IGROV1 cells that were pre-treated with NAC expressed less Nrf2 upon treatment with auranofin (1 μ M). However, there was no considerable decrease in Nrf2 expression when IGROV1 cells were treated at higher concentration (2 μ M). As mentioned in the previous section, Nrf2 expression was comparatively less in auranofin treated OVCAR5 cells. Unlike in IGROV1 cells, OVCAR5 cells exhibited a dose dependent increase in Nrf2 expression as there was increased expression at higher concentration of auranofin (2 μ M) compared to lower concentration (1 μ M) (Figure 4 A). Pre-treatment with NAC blocked the expression of Nrf2 in OVCAR5 cells upon auranofin treatment (Figure 4 A). Interestingly, there was decreased cleavage of PARP in auranofin (2 μ M)- treated IGROV1 cells that were pre-treated with NAC (Figure 4 B). This stated that NAC protects cells from apoptosis induced by auranofin (2 μ M). On the other hand, auranofin induced less cleavage of PARP in OVCAR5 cells which was slightly altered by pre-treatment with NAC.

Discussion

Our study demonstrated that there is a positive correlation between membrane fluidity and auranofin sensitivity. Even though the lipid profile of IGROV1 is yet to be determined, the fluidic nature suggests that the membrane might consists of an increased proportion of unsaturated acyl side chains of phospholipids along with ceramide as reported for drug sensitive cancer cells (1). Conversely, the presence of large amounts of cholesterol and sphingomyelin rigidifies membrane and is found to be involved in developing resistance to various anti-cancer drugs (1). How membrane fluidity modulates drug resistance is still unknown and probably involves several different pathways. Drugs cross the plasma membrane

barrier by diffusion and diffusion rate of drugs is lower through a higher ordered rigid membrane compared to a lesser ordered fluid membrane (1). Pharmaceutical intervention to fluidise the membrane had shown that the drug could effectively diffuse into the membrane and could reverse the resistance (7). A rapid increase in plasma membrane fluidity has been reported in cisplatin treated tumour cells (21). Interestingly, in our study, auranofin does not induce any significant change in the membrane fluidity of ovarian cancer cell lines (unpublished data).

Studies have demonstrated that membrane fluidity of cancer cells is generally greater than that of the normal cells and increased membrane fluidity correlates with malignant and metastatic potential (22). This suggests that IGROV1 might potentially be more invasive than OVCAR5 which possess a comparatively rigid membrane. Further studies on metastatic potential of these cell lines provide valuable information on the relation between membrane fluidity and the invasive nature of ovarian cancer cells. Interestingly, our studies with the ovarian cancer cell lines of the same histological origin showed that sensitivity to auranofin increases with membrane fluidity. Future studies investigating whether cholesterol imparts resistance to auranofin in IGROV1 cells strengthen our observations since cholesterol has been implicated in rigidifying the membrane and thereby reduces the metastatic potential of cancer cells (23).

We have reported that the un-repaired DSBs results from auranofin treatment cause the increased sensitivity of BRCA1-depleted ovarian cancer cells (10). The increased number of DSBs in auranofin treated IGROV1 cells compared to OVCARs (OVCAR5 & OVCAR4) suggests that drug sensitivity correlates with DNA damage which in turn correlates with membrane fluidity. Regardless of the relatively high background DNA damage in OVCAR5 cells, the number of DSBs remained unchanged upon auranofin treatment. However, IGROV1 exhibited a significant rise in DSBs upon auranofin treatment. Moreover, the potential of auranofin to cause DNA damage increases its potential as an anti-cancer drug since cancer cells

in general are more susceptible to DNA damaging agents due to the deregulated and faulty DNA repair machinery (24).

It has been previously reported that ROS mediate the cytotoxic effects of auranofin (8). We also reported that anti-oxidant NAC protects BRCA1-depleted ovarian cancer cells from oxidative DNA damage and apoptosis induced by auranofin (10). NAC mediated inhibition of Nrf2 expression in auranofin (1 μ M) treated IGROV1 cells conclusively proved that auranofin induced cellular oxidation is the mechanism behind the cytotoxic effects of auranofin. This was further confirmed by the inhibition of apoptosis by NAC in auranofin (2 μ M) treated IGROV1 cells. However, the induction of Nrf2 at higher concentration of auranofin (2 μ M) was not significantly changed in NAC pre-treated cells. Interestingly, apoptosis induced by auranofin at this concentration (2 μ M) was blocked by NAC. This suggests that the concentration of NAC used in this experiment could neutralize the ROS from reaching a threshold to activate apoptosis. This however could not prevent the amount of ROS signalling the expression of Nrf2.

In conclusion, our study suggests that there is a positive correlation between membrane fluidity and auranofin sensitivity in the ovarian cancer cell lines. As there is only limited information in the literature, more studies have to be performed with agents known to reduce membrane fluidity (e.g. cholesterol) to elucidate the role of membrane fluidity in modulating the sensitivity of malignant cells to auranofin and other drugs of interest.

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Figure legends

Figure 1. Auranofin sensitivity positively correlates with membrane fluidity: IGROV1 is more fluid and sensitive to auranofin than OVCAR5. (A) Membrane fluidity was determined by ESR using the spin label 5-doxyl stearic acid. Membrane order parameter S which is inversely proportional to the membrane fluidity was calculated from the spectra as mentioned in the methods. Values are the mean of three independent experiments \pm SEM at mean temperature. S values at mean temperature were plotted. Two-tailed unpaired t test was performed to determine the significance difference in S values between OVCAR5 and IGROV1 cells. * $P < 0.05$. (B) % Cell survival was determined by MTT assay. Relative cell viability (%) was expressed as a percentage relative to the DMSO treated control samples set to 100 %. Values are the mean of three independent experiments \pm SEM. ** $P < 0.005$. (C) % Cell survival was determined by MTT assay. *** $P < 0.0005$ (D) Regression analysis showing a positive correlation between % cell survival at 1 μ M of auranofin and membrane order parameter S .

Figure 2. Auranofin induces more DNA DSBs in IGROV1 cells. (A) Immunofluorescence staining of 53BP1. (B) Number of cells with more than 10 53BP1 foci per 200 cells were counted manually per slide for each sample and the results were plotted. Data point shows the mean of two independent experiments \pm SEM. * $p < 0.05$, ** $p < 0.005$.

Figure 3. Auranofin induces increased cellular oxidation and apoptosis in IGROV1 cells. (A) Expression of Nrf2 and cleavage of PARP (B) were determined by Western blotting using antibodies against Nrf2 and PARP respectively. Cells were treated with auranofin (1 μ M or 2 μ M) for indicated time points and samples were processed for Western blotting. Actin was determined as loading control.

Figure 4. Anti-oxidant N-acetyl cysteine ameliorates the cellular oxidation and apoptosis in auranofin-treated IGROV1 cells. Western blot analysis was performed by using antibodies against Nrf2 (A) PARP (B). Cells were pre-treated with NAC before treating with auranofin (2 μ M) for indicated time points and samples were processed for Western blotting. Actin was determined as loading control.

Supplementary Figure 1. A representative ESR spectrum of 5-doxyl stearic acid spin label from ovarian cancer cells showing the separation of the outer extrema $2T_{parallel}$ and inner extrema $2T_{perp}$.

Supplementary Figure 2. (A) IGROV1 cells possess a more fluid membrane and exhibit increased sensitivity to auranofin. Membrane fluidity was determined by ESR using the spin label 5-doxyl stearic acid. Membrane order parameter S which is inversely proportional to the membrane fluidity was calculated from the spectra as mentioned in the methods. Values are the mean of three independent experiments \pm SEM at mean temperature. S values at mean temperature was plotted. (B) % Cell survival was determined by MTT assay. Values are the mean of three independent experiments \pm SEM. Two-tailed unpaired t test was performed to determine the significance difference in % survival between auranofin treated OVCAR4 and IGROV1 cells. * $P < 0.05$.

Supplementary Figure 3. IGROV1 exhibits more sensitivity to auranofin induced DSBs than OVCAR4. Number of cells with more than 10 53BP1 foci per 200 cells were counted manually per slide for each sample and plotted. Significance of differences in 53BP1 foci numbers between auranofin treated cells were assessed using unpaired Student's two-tailed t -test. Data point shows the mean of two independent experiments \pm SEM. * $p < 0.05$. ** $p < 0.005$.