



Evidence that the antiproliferative effects of auranofin in *Saccharomyces cerevisiae* arise from inhibition of mitochondrial respiration



Tania Gamberi^a, Tania Fiaschi^a, Alessandra Modesti^a, Lara Massai^b, Luigi Messori^b, Manuela Balzi^a, Francesca Magherini^{a,*}

^a Department of Experimental and Clinical Biomedical Sciences, University of Florence, V.le Morgagni, 50-50134 Firenze, Italy

^b Department of Chemistry "Ugo Schiff", University of Florence, Via della Lastruccia 3, 50019 Sesto Fiorentino (FI), Italy

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ABSTRACT

Auranofin is a gold based drug in clinical use since 1985 for the treatment of rheumatoid arthritis. Beyond its antiinflammatory properties, auranofin exhibits other attractive biological and pharmacological actions such as a potent *in vitro* cytotoxicity and relevant antimicrobial and antiparasitic effects that make it amenable for new therapeutic indications. For instance, auranofin is currently tested as an anticancer agent in four independent clinical trials; yet, its mode of action is highly controversial. With the present study, we explore the effects of auranofin in *Saccharomyces cerevisiae* and its likely mechanism. Notably, auranofin is reported to induce remarkable yeast growth inhibition. Solid evidence is provided that growth inhibition is the consequence of a direct cytotoxic insult occurring at the mitochondrial level; a profound depression of cell respiration is indeed clearly documented as the main cause of cell death while induction of ROS plays only a secondary role. More in detail, the mitochondrial NADH kinase Pos5 is identified as a primary target for auranofin. The implications of these results are discussed in the frame of current mechanistic knowledge on the cellular effects of auranofin and of its role as a prospective anticancer drug.

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1. Introduction

The metal compound 2,3,4,6-tetra-*o*-acetyl- β -D-glucopyranosato-S-(triethylphosphine) gold(I), manufactured as auranofin (AF)-RIDAURA[®]- was first developed in the 1980s and then extensively used for the clinical treatment of severe rheumatoid arthritis. AF consists of a linear, two-coordinated gold(I) complex with triethylphosphine and thiosugar ligands, thus affording a P-Au-S chromophore (Fig. 1). Upon activation, AF loses the sugar thiolate and binds biomolecules through direct coordination of the gold(I) center to suitable donor atoms. Beyond its antiarthritic and antiinflammatory properties, relevant cytotoxic effects were disclosed for AF *in vitro* against several human cancer cell lines. During the last few years, new potential therapeutic applications were proposed for this interesting metallodrug. In fact, inhibition of a few inflammatory pathways and of various thiol redox enzymes makes AF, an optimal candidate for cancer

therapy and for treatment of various parasitic and microbial infections in the frame of a more general "drug repositioning strategy". For instance, relevant therapeutic actions were reported for AF toward *Schistosoma japonicum* (Song et al., 2012), *Giardia lamblia* (Tejman-Yarden et al., 2013) and *Entamoeba histolytica* (Debnath et al., 2012). In addition, four clinical trials were started against various cancer types and are still ongoing. A recent review by Madeira et al. (Madeira et al., 2012) summarizes the "state of art" on the medical applications of AF.

Several lines of evidence suggest that AF mainly acts by modifying the overall cell redox state and by promoting a pronounced intracellular oxidative stress; accordingly, an increased level of ROS was highlighted in a few cases, ultimately leading to cell death. Also, AF was found to react directly with several thiol-redox enzymes such as thioredoxin reductase (Cox et al., 2008; Gandin et al., 2010; Rigobello et al., 2004) and glutathione-S-transferase (De Luca et al., 2013), that are most likely involved in the mediation of its anti-parasitic and antimicrobial effects (Angelucci et al., 2009; Sannella et al., 2008). Furthermore, in some bacterial strains, AF was found to inhibit selenoprotein synthesis (Jackson-Rosario et al., 2009; Jackson-Rosario and Self, 2009). A number of studies,

* Corresponding author. Tel.: +39 0 552751237.

E-mail address: francesca.magherini@unifi.it (F. Magherini).

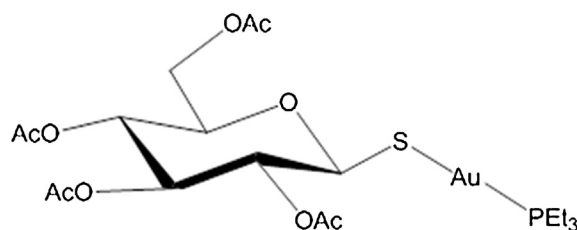


Fig. 1. AF structure.

now suggest that AF and various gold(I) carbene complexes produce significant cytotoxic effects in cancer cells, mainly through a direct mitochondrial damage, probably consequent to strong inhibition of the seleno-enzyme thioredoxin reductase (Rigobello et al., 2004; Marzano et al., 2007). Mammals express two isoforms of Thioredoxin reductase: TrxR1 and TrxR2, respectively localized in the cytosol and in the mitochondria. Both are selenoproteins encoded by distinct genes that are all essential, as their respective deletions are embryonically lethal in mice (Jakupoglu et al., 2005; Nonn et al., 2003). The anti-neoplastic activity of AF is most likely linked to the fact that many tumors overexpress TrxR with a consequent increased resistance to ROS (Grogan et al., 2000). Thus, inhibition of TrxRs, in particular of the mitochondrial isoform, can alter the mitochondrial membrane permeability and induce the release of segregated proapoptotic factors, ultimately triggering cancer cell apoptosis (Gandin et al., 2010; Marzano et al., 2007). Yet, contrasting reports have appeared on this issue and no conclusive consensus has been reached on the “true” mode of action of AF (Rigobello et al., 2008; Omata et al., 2006; Lothrop et al., 2009). In fact, increased ROS production is not a common feature for AF treated cells; indeed, Rigobello et al. (Rigobello et al., 2008) showed that, in Jurkat T cells, AF causes apoptosis with very limited oxidative stress and Omata et al. (Omata et al., 2006) proposed a ROS-independent inhibition of the mitochondrial activity as principally responsible for the pro-apoptotic effects of AF. Furthermore, the presence of a rare Sec residue in mammalian Thioredoxins, frequently cited as a main reason for the broad substrate enzyme specificity and as a target of gold(I) compounds, was recently discussed by Lothrop et al. (Lothrop et al., 2009). Interestingly, these authors found that the truncated variant, TrxR2Δ missing the Sec residue, still reduced DTNB almost as efficiently as full-length TrxR2 and that a few gold(I) compounds, such as AF and aurothioglucose, effectively inhibited both the full-length enzymes and the TrxR2Δ enzyme. Authors showed that the N-terminal redox center of this enzyme was also inhibited and concluded that AF and aurothioglucose do not selectively inhibit the Sec-containing active-site motif of human TrxRs.

To verify the above mechanistic hypotheses and elucidate further the cellular pharmacology of AF, we have analyzed its effects in yeast. Yeast is, indeed, an excellent biological model to monitor and identify the biochemical mechanisms of cytotoxic compounds (Matuo et al., 2012). A number of previous studies described the effects of various metal based anticancer drugs such as cisplatin and ruthenium compounds in yeast providing new valuable mechanistic information (Cunha et al., 2013; Singh et al., 2014; Stevens et al., 2013; Wang et al., 2012).

Yeast, as plants, does not contain selenoproteins and so represents a very interesting model to discover new potential targets of AF. In this study, we demonstrated that yeast growth inhibition is the consequence of a direct cytotoxic insult occurring at the mitochondrial level. Indeed, a profound reduction of O_2 consumption was clearly documented as an early event of AF treatment, although mitochondrial morphology and mitochondrial membrane potential were not affected. The screening of selected deletion strains of genes, involved in the mitochondrial function, allowed us to

identify Pos5 NADH kinase as a likely target for AF. Conversely, inhibition of mitochondrial thioredoxin reductase and induction of ROS appear not to be directly involved in the antiproliferative mechanism of AF. To the best of our knowledge no studies have been reported so far on the effects of AF in yeast.

2. Materials and methods

2.1. Strains and growth conditions

The haploid *Saccharomyces cerevisiae* BY4741 (MATa; his3Δ1; leu2Δ0; met15Δ0; ura3Δ0) and the deletion strains were obtained from EUROSCARF. The plasmid pYX142 + mGFP (Westermann and Neupert, 2000) was used to transform BY4741 in order to obtain a strain that constitutively expresses a mitochondria targeted GFP. Transformation was performed using lithium acetate method (Gietz and Woods, 2002). Yeast cells were grown overnight in YP (1% yeast extract, 2% peptone) supplemented with carbon sources: glucose 2% w/v (YPD), galactose 2% w/v (YPGal), ethanol 2% v/v (YPEt) or glycerol 3% (YPGly) v/v. Overnight cultures in early stationary phase were diluted to 0.2 OD₆₀₀ and AF (from 10 mM stock solution in DMSO) was added. Control cells were treated with equal amount of DMSO. Growth was monitored by measuring the turbidity of the culture at 600 nm on a spectrophotometer. For growth on solid medium, cells were grown in YPD, YPGal, YPEt or YPGly liquid media, then serially diluted to 0.1, 0.01, 0.001 and 0.0001 OD₆₀₀/ml. Five microliters of each dilution were spotted on the corresponding complete solid medium containing AF at different concentrations (5, 25 and 100 μM) or DMSO as control and incubated at 30 °C for 48 h.

Spotting assay of mutant deletion strains was performed with cells grown in YPGal (or YPGly) and then serially diluted to 0.1, 0.01 and 0.001 OD₆₀₀/ml. Five microliters of each dilution were spotted on YPGal (or YPGly) complete solid medium containing AF 5 μM or DMSO as control and incubated at 30 °C for 48 h. SD (yeast nitrogen base) minimal medium supplemented with histidine, leucine, methionine and uracil was used to verify the auxotrophy for arginine of AF treated wild-type strain.

2.2. Minimum inhibitory concentration (MIC) determination

For MIC determination we treated 10⁴ yeast cells with serial dilutions of drug in YPD medium. After growth for 24 h, OD₆₀₀ readings were taken. MIC₅₀ is defined as the concentration of drug for which the OD₆₀₀ after 24 h, is half of the OD₆₀₀ of untreated cells (Fleming et al., 2002).

2.3. Cell viability assays

To determine the replicative viability of the cells, the cell density was normalized to 1 × 10⁷ cells/ml and five-fold serial dilution was made. A 100 μl of the last two dilutions were plated in triplicate on YPD solid medium. The percentage of colony-forming units (C.F.U.) was obtained by relating the C.F.U. counts of AF treated cells to those of the control, which was considered to be 100%. Viability was defined as the ability of a single cell to form a colony within two days. To distinguish between metabolically active and dead yeast cells, fluorochrome FUN1 (Molecular Probes) was utilized. Inside the cell, this dye is converted from a diffusely distributed pool of green fluorescent intracellular stain to a compact form consisting of orange-red cylindrical intravacuolar structures. For the conversion of FUN1, both plasma membrane integrity and metabolic capability are required. Dead cells and metabolic inactive cells exhibit extremely bright, diffuse, green-yellow fluorescence and diffuse green fluorescence respectively.

2.4. Oxygen consumption measurements

The oxygen consumption was measured using a Clark-type O₂ electrode from Hansatech at 30 °C. A 10⁷ cells from culture at the indicated time points, were transferred to an airtight chamber maintained at 30 °C, containing respiration buffer (0.1 M K-Phthalate, pH5.0). Oxygen content was monitored for at least 10 min. The rate of decrease in oxygen content, related to the amount of cells, was taken as index of the respiratory ability (Magherini et al., 2009).

2.5. Fluorescence microscopy

For mitochondrial membrane potential estimation, cells grown in YPD and YPGly, were harvested at the indicated time. A 10⁷ cells were washed twice in 10 mM HEPES buffer, then suspended in the same buffer and incubated at 30 °C in the dark with rhodamine B hexylester (rhodamine B) (Molecular Probes) at a concentration of 50 nM for 10–15 min. The morphology of mitochondria was visualized using the plasmid pYX142-mtGFP that allows a constitutive expression of mitochondria targeted GFP (Westermann and Neupert, 2000). In all cases the cells were washed twice in 10 mM Hepes buffer, immobilized with 0.5% low melting point agarose and visualized on Leica TCF SP5 confocal microscope.

2.6. Flow cytometry

Samples (10⁶ cells) grown on YPD and YPGly were harvested at indicated time, pelleted in a microcentrifuge and washed once in PBS. For Rhodamine 123 cells were resuspended in 1 ml of 50 µM citrate and incubated with the probe (500 nM) at 30 °C for 10 min. For Dihydrorhodamine 123 and for MitoSox red cells were incubated in 10 mM Hepes for 2 h and 10 min, respectively. Flow cytometry was carried out on a Becton-Dickinson FACS Calibur model flow cytometer. All the probes were from Molecular Probes and the excitation and the emission lengths were selected according to the manufacturing protocols.

2.7. Ethanol and ATP determination

For ethanol determination, 1 ml of the growth from control and 25 µM treated cells was harvested at indicated time and then centrifuged. The cleared supernatant was collected to estimate the ethanol production. Ethanol was determined using alcohol-dehydrogenase/aldehyde-dehydrogenase method (Enzy-Plus, Diffchamb).

ATP amount was determined with ATP Determination Kit (Molecular Probes) according to manufacturing instruction.

2.8. Mitochondria purification

S. cerevisiae strain was grown in 500 ml YPGal medium until 1–2 O.D./ml of optical density was reached.

Cells were pelleted by centrifugation at 3000 × g for 10 min, washed twice with distilled water, and mitochondrial enriched fraction was prepared as previously described (Gamberi et al., 2009). The supernatant obtained after mitochondria recovery (SN) was conserved. The solution containing mitochondria was diluted in 100 mM Tris-HCl pH8 and mitochondria were disrupted by vortexing. After centrifugation the supernatant was recovered and used as mitochondrial fraction (MF).

2.9. Assay of NAD kinase activity

NAD kinase activity was assayed by a stop method as previously described, with slight modification (Kawai et al., 2000). Briefly

The mitochondrial extract was incubated with 1 ml of the reaction mixture (5.0 mM NAD⁺, 5.0 mM ATP, 5.0 mM MgCl₂ and 100 mM Tris-HCl pH 8.0) for 20 minutes at 30 °C in order to allow the NADP⁺ formation. The reaction was terminated by immersing the test tubes in boiling water for 5 min; then 5 mM glucose-6-phosphate was added to the mixture, and the amount of NADP formed was determined enzymatically with 0.5 U-6-phosphate dehydrogenase. The reaction was performed at 30 °C for 30 min and the formation of NADPH was measured at 340 nm.

Protein concentrations of mitochondrial extract was determined in accordance with the method of Bradford by using BSA as the standard (Bradford, 1976). One unit (U) of enzyme activity was defined as 1.0 µmol NADP produced in 1 min at 30 °C in 1 ml of mixture. Specific activity was expressed in U/mg of mitochondrial extract proteins. AF inhibition was performed incubating 50 µg of protein extract with AF 5 µM or DMSO as control, for 10 min at 30 °C.

2.10. Reproducibility of the results

At least three biological experiments were performed. Data are average ± SD of at least three independent experiments or are representative results of similar repetition. In fact, in some cases, such as in fluorescence measurements, absolute data were not comparable in the experiments performed on different days, although the observed trends were fully consistent among the independent experiments; in this cases a typical example is shown and the SD and statistical test are referred to triplicate measurement. Non-paired, two tailed *t*-test was used to control the statistical significance.

3. Results

3.1. Respiring cells show an increased sensitivity to AF treatment

We first evaluated the sensitivity of *S. cerevisiae* BY4741 strain to scalar AF concentrations in YPD liquid medium. Notably, treatment with AF resulted in a marked inhibition of yeast growth, with a MIC₅₀ falling in the 45–55 µM range (Fig. 1S). Then, to investigate whether AF biological activity might be influenced by yeast metabolism, the effects of AF on yeast growth were evaluated both on solid and liquid complete media, containing different fermentable (2% glucose (YPD), 2% galactose (YPGal)) and non-fermentable (3% glycerol (YPGly), 2% ethanol (YPE)) carbon sources. Fig. 2A shows the effects of three different drug concentrations (two below MIC: 5 and 25 µM; one above MIC: 100 µM) on yeast growth in solid media. AF treatment induced, in all media, a dose-dependent growth inhibition, but this inhibition was far more pronounced on glycerol, ethanol and galactose compared to glucose. To assess how different carbon sources affect yeast growth inhibition by AF in liquid media, cells were grown overnight and inoculated into fresh media, at the density of 0.2 O.D._{600nm}/ml. A concentration of 25 µM AF was used, since the experiment in solid media indicated that respiring cells are more sensitive than fermenting ones. Growth was monitored over 24 h. Again, we observed a dose dependent growth reduction, but the effect was far greater in non-fermentable carbon sources (reduction, referred to the maximal OD reached, of about 64 ± 3%, 50 ± 5% on YPGly, YPEt) and in YPGal (60 ± 7%) compared to YPD (36 ± 5%) (Fig. 2B). It is well known that when cells grow on glucose, their metabolism is exclusively fermentative since the glucose repression mechanism is fully operative. Conversely, on glycerol or ethanol, when respiration is activated, AF toxicity increases. With galactose (a fermentable substrate but unable to induce glucose repression), cells are more reliant on respiration (Fendt et al., 2010). On this medium we observed a response comparable to

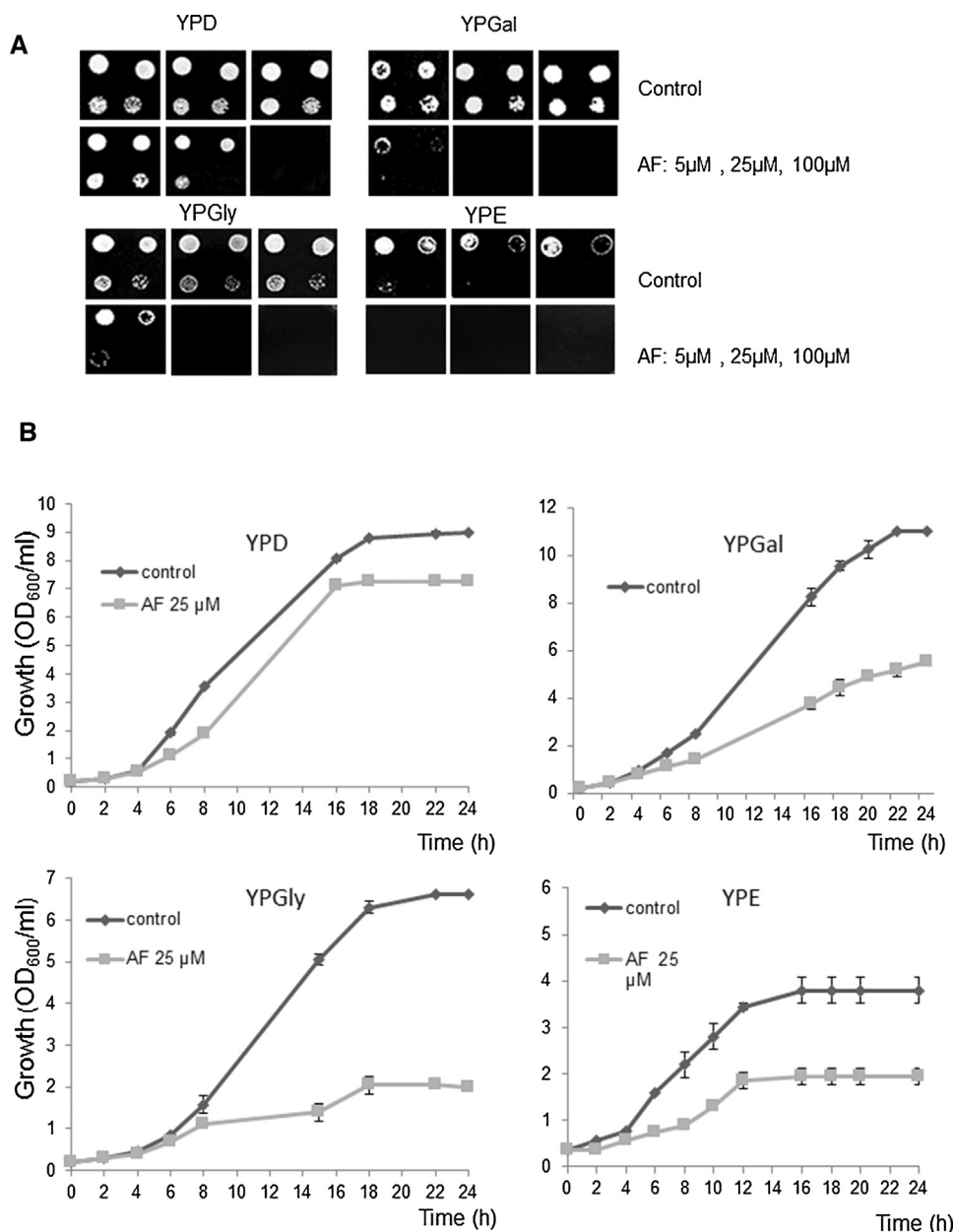


Fig. 2. Respiring cells show an increased sensitivity to AF treatment. (A) Growth on solid medium: cells were grown in YP liquid media supplemented with the indicated carbon source and then serially diluted to 0.1, 0.01, 0.001 and 0.0001 OD₆₀₀/ml. Five microliters of each dilution were spotted on complete solid medium containing AF at the indicated concentrations or DMSO as control. (B) Growth in liquid medium: cells were grown in complete medium with different carbon sources supplemented with DMSO (control) or AF 25 μ M.

the one observed in non-fermentable carbon sources. These data demonstrate that activation of mitochondrial metabolism greatly enhances yeast sensitivity to AF. In particular, yeast growth rate in YPGly, YPE and YPGal with AF decreases after 6–8 h indicating that, at this time, the effect induced by the drug is just consolidated. Thus, treatment with the complex for 6 h was chosen to investigate in depth the AF mechanism of action.

For comparison purposes, cisplatin, a well-known standard drug for many cancer types, was tested under the same experimental conditions. Cisplatin is known to induce cell death through DNA damage, both in mammalian and yeast cells. More precisely, the anticancer action of this drug arises from its capacity to form DNA-platinum covalent adducts, which ultimately lead to apoptosis (Cunha et al., 2013; Roos and Kaina, 2013). This mechanism should not be affected by a change in carbon source.

Indeed, cells treated with 100 μ M cisplatin show the same growth both on a fermentable and non-fermentable carbon sources (Fig. 2S).

3.2. The effects of AF on cell viability

Yeast growth inhibition induced by AF might arise either from cell death or from cell cycle arrest, i.e. from a cytotoxic or from a cytostatic action. Thus, to clarify this point, we performed two viability tests. At first, replicative cell viability was assessed through a plating test. Cells were grown on fermentable and non-fermentable liquid media (we selected YPD and YPGly as representative of these two conditions) supplemented with DMSO (control) or 25 μ M AF and plated on complete medium with 2% glucose. After 2 days, the colony-forming units (C.F.U.) were evaluated. After 6 h of AF

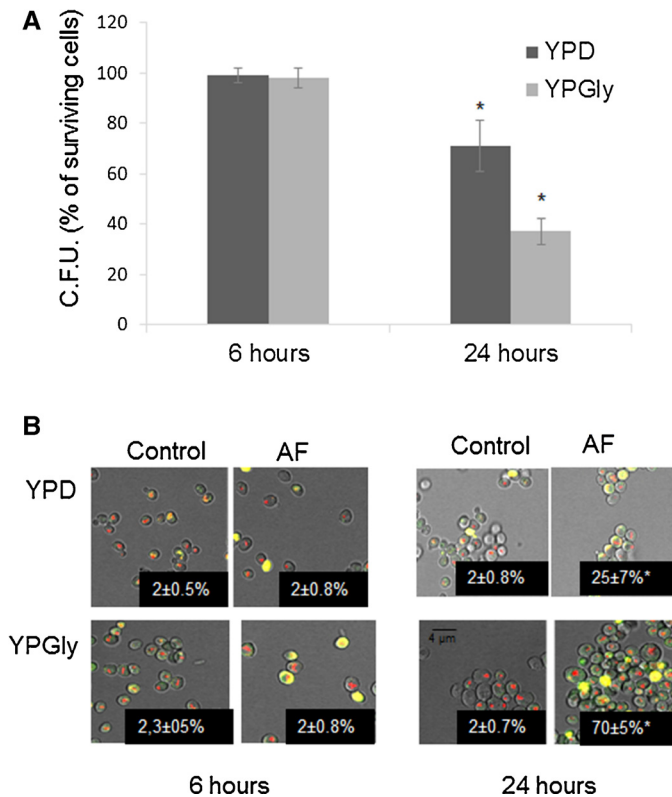


Fig. 3. Six hours of AF treatment does not affect yeast viability. (A) Replicative viability was performed by counting the C.F.U. after 6 or 24 h of treatment with AF 25 μ M in the indicated carbon source. The percentage of colony-forming units of yeast cells was obtained by relating the C.F.U. counts of treated cells to those of control cells which were considered to be 100%. The two-tailed, non-paired Student's *t*-test was performed; (*) indicates a *p*-value < 0.05. (B) FUN assay. Representative images of control and 25 μ M AF treated cells observed with confocal microscopy. Numbers in each figure indicate the percentage of yellow cells (\pm SD). Pictures shown are representative of three different experiments. The two-tailed, non-paired Student's *t*-test was performed. (*) indicates a *p*-value < 0.05.

treatment, yeast cells were viable on all tested media. To verify the long term effects of AF on viability, cells were also plated after 24 h of AF treatment. In this latter condition, AF markedly reduced yeast viability both in YPD and YPGly (viabilities of 71% and 37% have been measured respect to controls); again the effect is more pronounced with a non-fermentable carbon source (Fig. 3A). Metabolic viability was then assessed by using FUN-1: with this fluorescent stain metabolic inactive cells are bright yellow/green fluorescent, while metabolic active cells form cylindrical intravacuolar structures. Fig. 3B shows representative field of *S. cerevisiae* cells treated with AF for 6 and 24 h. The percentage of non-metabolic active cells, reported in each image, is the same in control and treated cells at 6 h, while is far higher in AF treated cells at 24 h. These results indicate that after 6 h of growth in the presence of AF, yeast cells are viable and have a functional metabolism; only after a more prolonged treatment, drug's effects on cell viability were detected.

3.3. The biological effects of AF in yeast are not related to mitochondrial thioredoxin reductase and glutathione reductase activities

Previous studies pointed out that thioredoxin reductase might constitute a primary target for AF in cancer cells (Cox et al., 2008; Rigobello et al., 2004; Marzano et al., 2007). Remarkably, in several parasitic organisms the thioredoxin and the glutathione reductase activities are replaced by a unique enzyme: the thioredoxin

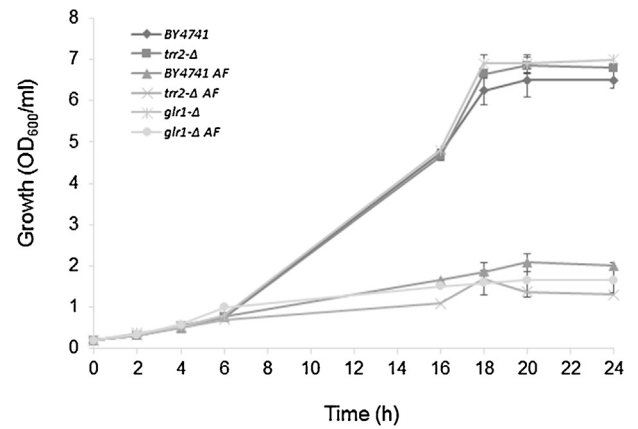


Fig. 4. In yeast the effect of AF is not mediated by thioredoxin reductase and glutathione reductase enzymes. BY4741, *trr2*- Δ and *glr1*- Δ strains were grown in YPGly supplemented with AF 25 μ M. Growth is expressed as OD₆₀₀/ml (mean \pm SD). The graphic is representative of three independent experiments.

glutathione reductase, that resulted inhibited by AF (Martínez-González et al., 2010). In *S. cerevisiae* these two enzymatic activities are separated and are represented by thioredoxin reductase (*trr*) a glutathione reductase (*glr1*) proteins. In order to verify if these enzymes could be a target, the AF effect on growth of the null mutant for *TRR2* (homologous to mammalian *TRX2*) and *GLR1* has been analyzed. As shown in Fig. 4, the *trr2*- Δ and *glr1*- Δ exhibit the same behavior of the wild-type strain indicating that, in *S. cerevisiae*, these enzymes are not implicated in AF's mechanism neither as a primary drug targets (no increase of resistance was detected), nor as an enzymes involved in drug sensitivity (no decrease in growth was detected). Furthermore, the same experiment was performed on null mutants of the three genes (*GTO1*, *GTO2*, *GTO3*) coding for glutathione transferase enzymes, since GST P1-P, one of the cytosolic mammalian GSTs, resulted to be inhibited by AF in vitro (De Luca et al., 2013). Also in this case no difference was detected between the wild-type and the null strains (Fig. 3S). Overall, these data demonstrate that the main enzymes involved in redox homeostasis control are not implicated in AF mechanism of action. Since the null mutant of *trr1* enzyme is not viable, it was not included in this screening and, as consequence, we cannot exclude that this enzyme could be involved in AF toxicity.

3.4. AF inhibits oxygen consumption

Since the cytotoxic effects of AF turned out to be far greater in respirable media, we further monitored the mitochondrial function by measuring oxygen consumption of whole cells in respirable carbon sources (YPGly and YPE) and in YPGal after 6 h of 25 μ M AF treatment. As shown in Fig. 5, AF treatment caused a net reduction of O₂ consumption in all tested media. This reduction is far more evident when glycerol and ethanol are used since these are only respirable carbon sources. This latter result further supports the concept that the cytotoxic effects of AF in yeast are the consequence of a strong inhibition of yeast respiration.

3.5. AF cytotoxicity does not involve ROS production

To establish whether the observed perturbations in the mitochondrial function might be assigned to a ROS-induced damage, ROS formation was explored. A time course of ROS production was performed by flow cytometry during AF treatment. Cells were incubated with dihydrorhodamine 123 (Fig. 6A) or MitoSOX™ Red (Fig. 6B) to detect both generic ROS and mitochondrial superoxide. No significant increase of ROS production was observed during the

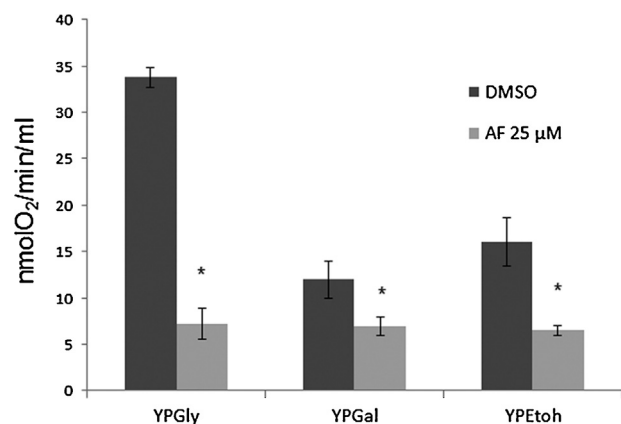


Fig. 5. O₂ consumption is reduced in AF treated yeast cells. O₂ consumption of whole yeast cells was evaluated after 6 h of growth in complete medium supplemented with the indicated carbon source. Data are expressed as nmolO₂/min/ml of 10.D. of yeast cells. Three independent biological replicates were performed. Two tailed, non-paired Student's *t*-test was performed; (*) indicates a *p*-value < 0.05.

treatment and also when a higher concentration of AF (100 μM) was used (data not shown). Lack of a ROS mediated damage is further proved by the fact that treatment with 5 mM ascorbic acid is unable to restore a normal growth (Fig. 4S). These results indicate

that, the inhibition of oxygen consumption, clearly detected after 6 h of treatment, cannot be related to an increase of ROS production. Although ROS production is undetectable, we cannot exclude that ROS could be produced locally and/or at a very low level and that, some antioxidant proteins or enzymes, could cooperate to AF cell resistance. To go deeper into this hypothesis we tested the drug effect on single gene deletion strains of non-essential genes involved in redox homeostasis. The deletion strains included proteins of non-enzymatic and enzymatic defense. The null mutants of *YAP1*, *SKN7*, *MSN2*, and *MSN4* were also included since they are the most important transcription factors that activate the transcription of anti-oxidant genes in response to oxidative stress (Ikner and Shiozaki, 2005; Moye-Rowley, 2002). The deleted strains were grown for 48 h in YPGal solid medium containing 5 μM AF and DMSO as control (Fig. 6C). A strong growth reduction was observed for the genes *SOD2* and *GSH1*, while *GSH2* and *SOD1*, appear less affected. All the others gene deletions have no effect on growth. Thus, *SOD2* and *GSH1* could be involved in drug resistance mechanism.

3.6. AF does not affect mitochondrial membrane potential and morphology

Afterward, we investigated whether AF treatment might affect the morphology and/or the membrane potential of mitochondria.

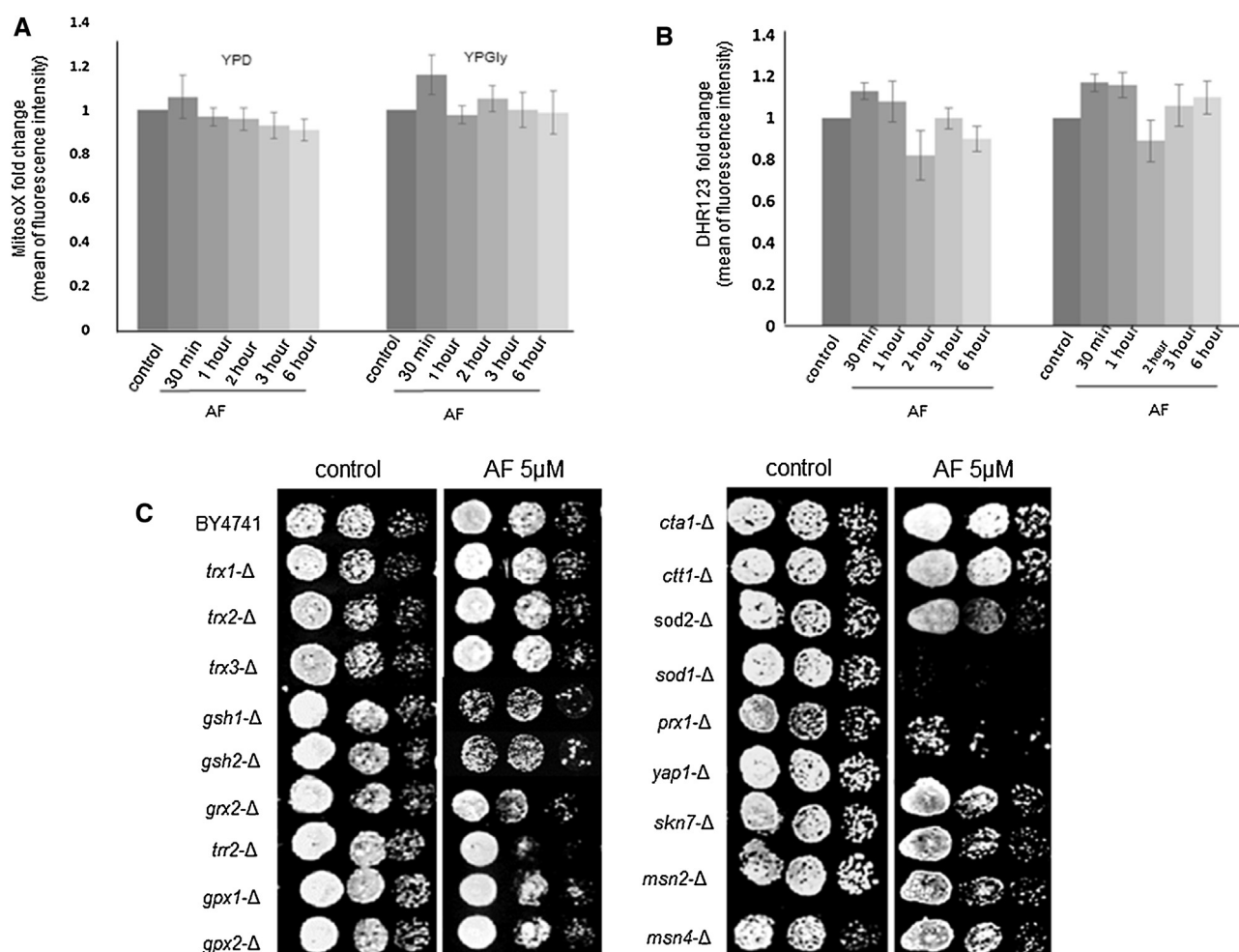


Fig. 6. AF does not induce early ROS production. Cells were grown in YPD and YPGly supplemented with 25 μM AF. Cells were incubated with DHR123 (A) or MitoSOX™ Red (B) in order to detect both generic ROS and mitochondrial superoxide. Data were analyzed by flow cytometer and the reported data are representative of three independent experiments with the same trend. (C) Deletion strains were grown in YPGal and then serially diluted to 0.1, 0.01 and 0.001 OD₆₀₀/ml. Five microliters of each dilution were spotted on YPGal complete solid medium containing AF 5 μM or DMSO as control.

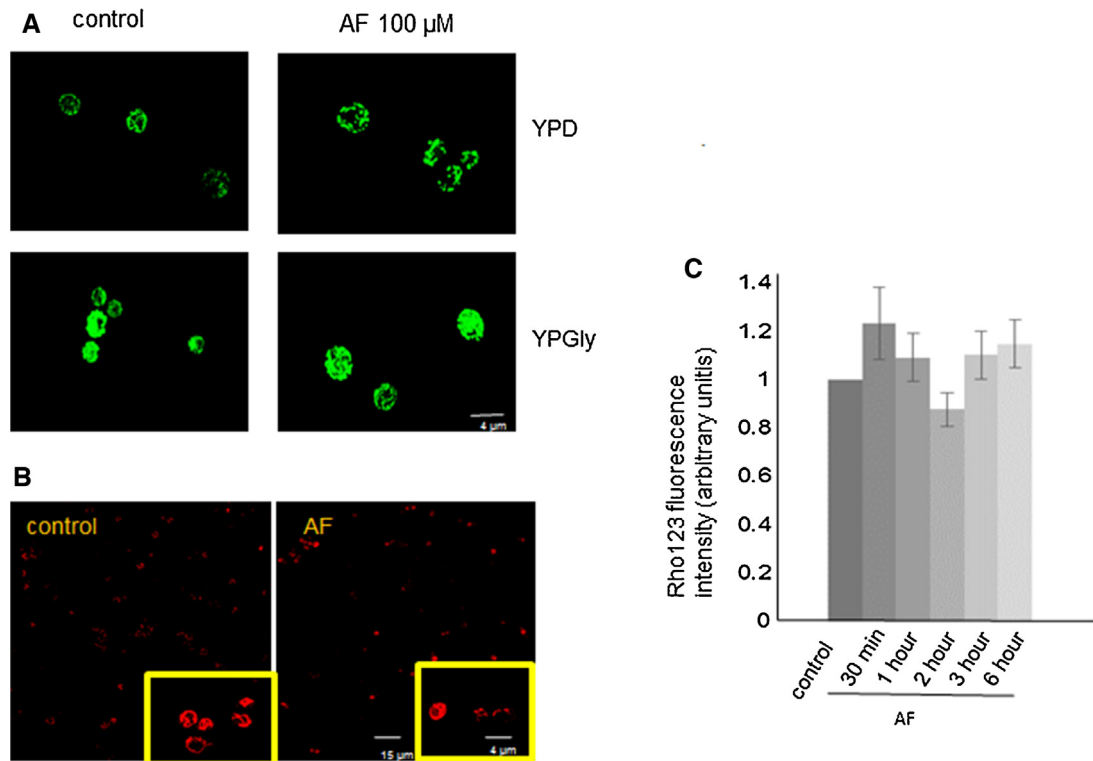


Fig. 7. Mitochondrial morphology and mitochondrial membrane potential are not affected by AF treatment. (A) Mitochondrial morphology were visualized on confocal microscopy using the plasmid pYX142-mtGFP that allows a constitutive expression of mitochondria targeted GFP. (B) Rhodamine B, hexyl ester was used to evaluate mitochondrial membrane potential on confocal microscopy. The observations (A and B) were performed after 6 h of 100 μ M AF treatment. (C) Mitochondrial membrane potential evaluated by flow cytometer analysis using Rhodamine 123 during 6 h of AF 25 μ M treatment.

Again, experiments were performed on yeast cells grown on YPD and YPGly supplemented with 25 (data not shown) and 100 μ M AF for 6 h. The mitochondrial morphology in control and AF treated cells was investigated using the plasmid pYX142-mtGFP that allows GFP to be specifically located in these organelles (Fig. 7A). As expected, mitochondria were more developed in respiring media where they form long filaments, creating a branched tubular network, both in control and in treated cells, with no differences detectable even when 100 μ M AF was applied. The membrane potential was evaluated using Rhodamine B hexyl ester probe (Rhodamine B) by confocal microscopy (Fig. 7B) (Reungpatthanaphong et al., 2003). To obtain a more quantitative data, a time course of Rhodamine B fluorescence was also carried out through flow cytometry analysis with 25 and 100 μ M (data not shown). None of these two methods, revealed a difference in the membrane potential, thus indicating that abrogation of O_2 consumption by AF is not due to an uncoupling process (Fig. 7C).

3.7. Active metabolism of yeast cells treated with AF is supported by fermentation

Moreover, we investigated whether and how cells treated with AF for 6 h and displaying a reduced mitochondrial respiration, maintain and/or adapt their metabolism, as suggested by the above FUN experiment. In line with expectations, AF treated cells shift their metabolism from respiration to fermentation. Fig. 8 illustrates ethanol (A) and ATP (B) production in AF treated cells; interestingly, treated cells show an increased ATP production in comparison to control cells and this increase is sustained by an increase in fermentation.

3.8. AF toxicity involves the respiratory function

Based on the above results, we can deduce that the potent growth inhibition effects of AF in yeast are primarily determined by a direct anti-mitochondrial mechanism. To investigate in more detail how AF impairs mitochondria function, we tested the effect of the drug on single gene deletion strains in which non-essential, nuclear and mitochondrial-encoded subunits of the respiratory chain complexes, were deleted. In this panel of genes, we also included proteins required for mitochondrial function, such as a key enzyme of Krebs cycle (Cit1), the ATP/ADP translocator proteins and the mitochondrial NADH kinase Pos5. The deletion strains were grown for 24 h in YGal liquid medium, in order to permit the growth also of the mutants unviable in strictly respiring medium, in the presence of DMSO or 25 μ M AF. For each strain, the growth of cells (OD_{600} after 24 h) incubated with DMSO was set at 100% and the percentage growth of AF-treated cells was calculated (Fig. 9A). The strains that showed an increased resistance ($p \leq 0.05$) to AF treatment were furthermore subjected to growth on glycerol plus 0.1% glucose (Fig. 9B). This glucose concentration does not induced glucose repression but at the same time allows null mutant strains to grow and thus, to bring out some possible resistances (Rolland et al., 2002). In order to not lose information concerning possible interesting genes, that could not arise when galactose was used, we also included in this selection the strains that showed an increased resistance with a low level of significance ($p \leq 0.1$). Several deleted strains showed a significant resistance increase in both conditions. These include deletion strains of respiratory chain complex (Ndi1, Sdh4, Qcr6, Rip1, and Atp2), one enzyme of Krebs metabolism (Cit1), and the NADH kinase (Pos5). Table 1S shows the growth data from which histogram was derived. These data were also confirmed by spotting the null strains in

YPGly solid medium containing 5 μ M AF and DMSO as control (Fig. 9C).

3.9. *Pos5* NADH kinase directly contributes to AF-induced mitochondrial dysfunction

Among these proteins, we focused our attention on *Pos5*. This enzyme is the only NADH kinase of the mitochondria and it is involved in resistance to a broad range of oxidative stress, in mitochondria iron homeostasis and in the stability of mitochondrial DNA (Outten and Culotta, 2003; Shi et al., 2011; Strand et al., 2003). Furthermore, *pos5*- Δ is auxotrophic for arginine (Outten and Culotta, 2003); this phenotype allows to check the *Pos5* enzyme activity or inhibition through a simple test of growth in minimal medium lacking arginine. Thus, we checked if the wild-type strain treated with AF was still able to grow on minimal medium without arginine. Fig. 10 clearly shows that yeast cells treated with AF display a reduced ability to grow without arginine also when a low dose of drug (5 μ M) was used. In order to rule out that this effect was due to a general inhibition exerted by AF on the pathway of arginine synthesis, we examined whether other null mutants of this pathway were less sensitive to the drug. All the mutant tested show the same behavior of the wild-type strain, indicating a specific action of AF on *Pos5* (Fig. 5S). In order to strengthen the *Pos5* involvement in AF toxicity we checked if the NAD kinase activity of the mitochondrial extract was inhibited by AF. NAD kinase activity was assayed both in the mitochondrial fraction (MF) and in the supernatant obtained after mitochondria recovery (SN) in BY4741 and in *POS5* null mutant. This mutant was included as negative control. The pretreatment of MF and SN with AF causes a reduction of NAD kinase activity only in the MF of BY4741 indicating that AF inhibits only the mitochondrial enzyme. The residual NAD kinase activity detected in *POS5* null mutant could be due to a contamination of cytosolic extract or to the activity of *Ipd1* (NADP⁺ specific isocitrate dehydrogenase) that partially contributing to mitochondrial NADPH generation (Shi et al., 2011). This activity is not affected by AF strengthening the finding that *Pos5* enzyme is inhibited by AF.

4. Discussion

The results of the several experiments and assays that we have carried out so far on yeast, upon AF treatment, provide new

valuable insight into the mode of action of this cytotoxic gold(I) drug.

At first, we have demonstrated that AF causes a remarkable inhibition of yeast growth that is strictly dependent on the functional state of mitochondria; indeed, a proper functioning of mitochondria greatly enhances the antiproliferative actions of AF thus, providing solid and unambiguous evidence that AF inhibits yeast growth through a mitochondrial damage.

Afterward, careful O₂ consumption measurements revealed that the antiproliferative effects of AF are the consequence of a strong depression of yeast respiration leading to cell death. This effect was already evident after 6 h of AF treatment but at this time treated cells were still viable and metabolically active as demonstrated by the FUN experiment and by the ATP assay. Cell death occurred later being massive at 24 h. These observations imply that inhibition of respiration comes first and that it is the “true” reason for the cytotoxic action of AF in yeast. This concept is further reinforced by the observation that yeast growth inhibition is not induced by an increase in ROS production. In fact, we did not detect any increase of ROS level after 6 h of AF treatment; furthermore, co-treatment with 5 mM ascorbic acid was unable to restore a normal cell growth (Fig. 3S).

Experiments performed on yeast null mutant strains of genes involved in redox homeostasis revealed a strong growth reduction for the mutant *sod2*- Δ and *gsh1*- Δ treated with AF. *Sod2* is important for the defense against O₂⁻ generated at the mitochondrial respiratory chain, suggesting that AF might induce ROS production at this level. *Gsh1* catalyzes the first, and rate-limiting, step in the glutathione (GSH) biosynthetic pathway. Since GSH plays a central role in the cell redox state regulation, it is possible that a deficiency of this molecule makes the cells unable to cope also with a low level of ROS. This finding also suggests that antioxidant enzymes are not direct targets of AF, but some of them (such as *Sod2* and *Gsh1*) contribute to the mechanisms of drug resistance. As a further proof of the mitochondrial nature of the cell damage induced by AF we showed a net metabolic shift from respiration to fermentation upon AF treatment. In fact, we demonstrated an increased ethanol and ATP production in AF treated cells. This behavior is similar to that shown by respiratory-deficient yeast strains. The main feature of these strains is the activation of alternative pathways that compensate for respiratory-deficient state (Liu and Butow, 1999). In particular, it is known that mitochondrial

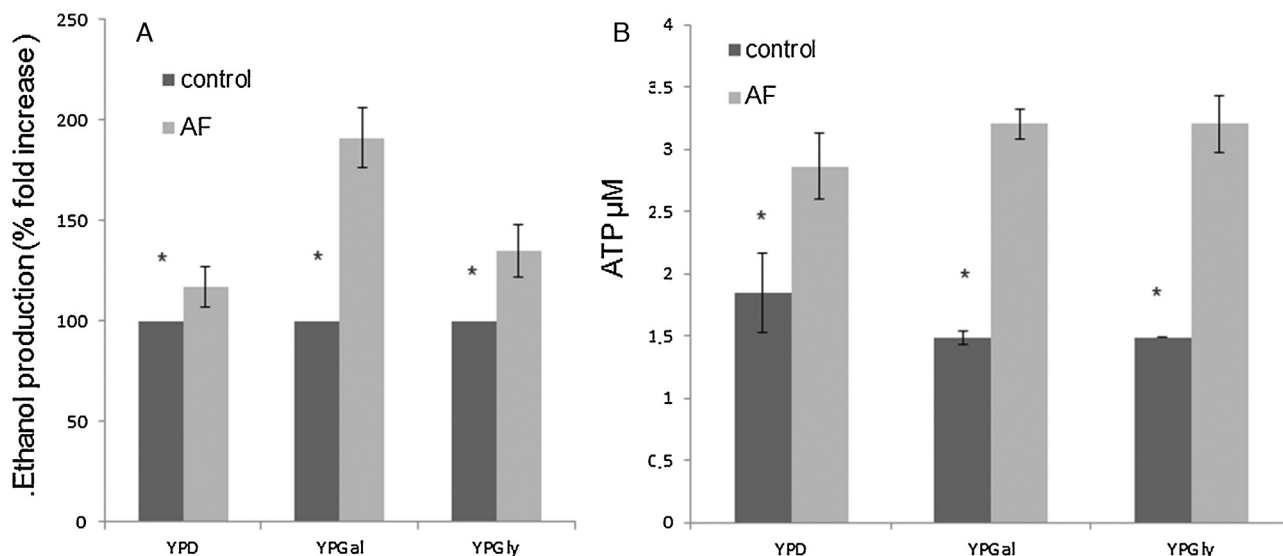


Fig. 8. AF treated cells metabolism is sustained by an increased fermentation. (A) % Fold increase of ethanol production was evaluated after 6 h of 25 μ M AF treatment in YP supplemented with different carbon sources. (B) ATP production determined using 1 OD of yeast cells. The image indicates the results from three biological replicates performed in duplicate. Two tailed, non-paired Student's *t*-test was performed; (*) indicates a *p*-value < 0.05.

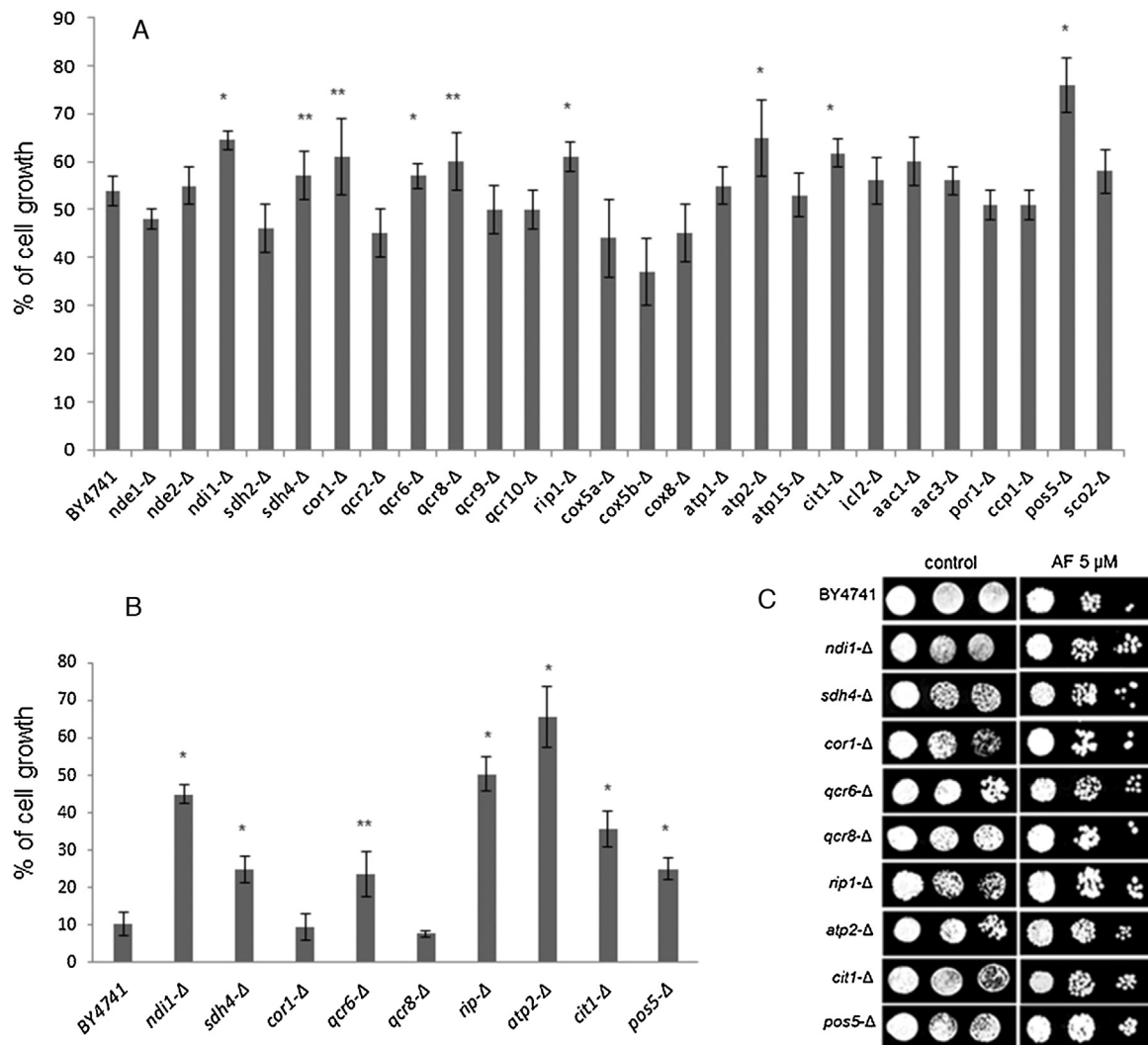


Fig. 9. AF toxicity is dependent on several mitochondrial proteins. (A) Null mutant and wild-type strains were treated for 24 h with AF 25 µM in YPGal. (B) The strains that showed an increased growth in galactose were also tested YPGly. Data are expressed as percentage of cell growth (OD_{600}) compared with that of the same strain incubated without AF (100%). Data represent means and SD of three independent experiments performed in duplicate. Two tailed, non-paired Student's *t*-test was performed; (*) and (**) indicates a *p*-value < 0.05 and 0.1 respectively. (C) Null mutant strains were grown in YPGly and then serially diluted to 0.1, 0.01 and 0.001 OD_{600} /ml. Five microliters of each dilution were spotted on YPGly complete solid medium containing AF 5 µM or DMSO as control.

damages enable the achievement of a fermentation rate higher than in wild-type strains (Hutter and OliverS.G., 1998). However, it is also reported that functional mitochondria are essential to maintain the tolerance to ethanol and, hence, a high growth rate (Hutter and OliverS.G., 1998). Also in our case, despite the increased ATP production, AF treated cells slowdown the growth rate after 6 h. All these data strengthen the idea of mitochondria as AF target. Notably, the screening of selected deletion strains of genes, involved in mitochondrial function, allowed us to identify, several strains more resistant to AF treatment in comparison to the wild-type strain. Among these, we focused our attention on *POS5*. This gene encodes for one of the three NAD kinases expressed by *S. cerevisiae* genome. The other two enzymes, *Utr1* and *Yef1* are localized in the cytosol. Since NADPH cannot cross the mitochondrial membrane, cytosolic and mitochondrial pools of NADPH are synthesized separately. Indeed, in the mitochondria, NADPH is mainly produced by *Pos5* (Outten and Culotta, 2003). Recently, Shi et al. (Shi et al., 2011) demonstrated that *pos5-Δ* mutant presents a reduction of the activity of complex II (succinate dehydrogenase), III (ubiquinol cytochrome c oxidoreductase) and IV (cytochrome oxidase). In particular, the activity of the cytochrome oxidase complex is reduced

by 75–95% in the mutant. In our study the involvement of *Pos5* in the mechanism of action of AF is supported by the fact that the wild-type strain treated with AF is unable to grow in minimal medium lacking arginine. In fact, mitochondrial NADPH is required for the third step in the conversion of glutamate to arginine that occurs in mitochondria. The essential NADHP production by *Pos5* in this process, cannot be rescued by the presence or overexpression of cytosolic NAD(H) kinases (*Utr1p* or *Yef1p*) (Bieganowski et al., 2006), and is only poorly compensated by *Idp1* (NAD⁺ specific isocitrate dehydrogenase) partially contributing to mitochondrial NADPH generation (Shi et al., 2011). This observation strongly confirms the selective action of AF on the above NADH kinase. The respiratory deficiency induced by AF could arise from NADPH deficiency. Indeed NADPH is a cofactor of several antioxidant enzymes and its deficiency might result in ROS accumulation, damaging in turn, some component of the respiratory chain. In our study we do not detect any increase of ROS level, although we cannot exclude that a very local and potentially damaging ROS production may occur. Another intriguing hypothesis is related to the involvement of *Pos5* in the correct biogenesis of iron–sulfur (Fe–S) clusters. The correct assembly of the Fe–S cluster is essential for the correct

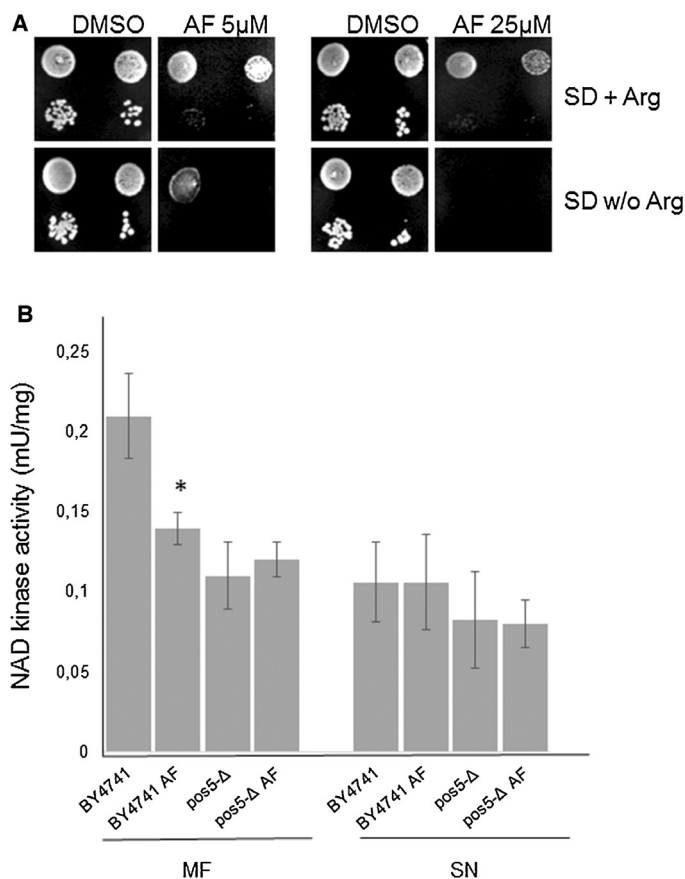


Fig. 10. AF treated cells are unable to grow in medium lacking arginine. (A) BY4741 was grown in YPD and then serially diluted to 0.1, 0.01, 0.001 and 0.0001 OD₆₀₀/ml. Five microliters of each dilution were spotted on solid SD minimal medium with and without arginine containing AF 5 μM, 25 μM or DMSO as control. (B) NAD kinase activities in mitochondrial (MF) and in cytosolic fractions (SN) was affected by AF. NAD kinase activity was assayed in presence of DMSO or AF 5 μM in MT or SN (50 μg) of BY4741 and pos-Δ null mutant.

function of respiratory chain complex such as complex II and III. POS5 deletion was in fact shown to cause mitochondrial iron accumulation and deficiency in Fe–S containing proteins (Outten and Culotta, 2003). Furthermore, Pain et al. demonstrated that Pos5 plays a direct role in F–S clusters synthesis and that the role of mitochondrial NADPH in Fe–S cluster biogenesis appears to be distinct from its function in anti-oxidant defense (Pain et al., 2010). Although Pos5 shows a low primary structure homology with the human enzyme, the tertiary structure and the key aminoacids of the NADH binding site appear conserved (Ando et al., 2011). Recently, the human mitochondrial NADH kinase (NADK2) has been identified (Ohashi et al., 2012) and mitochondrial NADP(H) deficiency due to a mutation in NADK2 was also proposed as cause of mitochondrial disorders (Houten et al., 2014). Further studies will be necessary to investigate if the increased resistance of other identified deleted strains is a consequence of Pos5 inactivation or due to a direct activity of AF.

Overall, a rather complete and exhaustive picture has been achieved to describe the cytotoxic mechanism of AF in yeast. The present results strongly suggest that AF behaves as an antimitochondrial cytotoxic agent, capable of inhibiting profoundly and selectively the respiratory chain. Owing to its cellular effects, AF should be qualified as a “mitocan” of class 5 according to the recent categorisation proposed by Neuzil et al. (Neuzil et al., 2013). The elucidation of the mode of action of AF in yeast, described here, will now serve as a reference model and a working hypothesis to decipher and interpret the cytotoxic mechanisms of AF and of related

gold compounds in animal cells for which controversial results and contrasting views have been reported so far.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.biocel.2015.05.016>

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