Effect of mitochondrial metabolism-interfering agents on cancer cell mitochondrial function and radio/chemosensitivity

Achilleas G. Mitrakas, Dimitra Kalamida and Michael I. Koukourakis

Abnormal mitochondrial function is common in cancer cells and activates metabolic pathways suppressed in normal tissues. Experimental and clinical studies suggest that mitochondria might serve as targets for novel anticancer therapies. We investigated whether mitochondrial metabolism-interfering agents (MMIAs) available currently in clinical practice affect cancer cell mitochondrial metabolism and synergize with chemotherapy and radiotherapy. Two cancer cell lines A549 (lung cancer) and DU145 (prostate cancer) were treated with a variety of MMIAs (metformin, nimodipine, memantine, oxytetracycline, amiodarone, and sodium azide) and their response was assessed using a resazurin reduction method and confocal microscopy. Focusing on amiodarone and metformin, we investigated their potential sensitizing effect on cancer cells when treated with ionizing radiation, cisplatin, and docetaxel. Resazurin reduction was increased by metformin and decreased by amiodarone at nontoxic concentrations. Amiodarone induced mitochondrial swelling, whereas metformin exerted no apparent effect on their morphology. Amiodarone and metformin exerted a weak radiosensitization effect on A549, whereas a synergetic activity with cisplatin and docetaxel was evident in both cell lines. It can be concluded that amiodarone and metformin, being well-established drugs in clinical practice, constitute two potential drugs for further experimental and clinical evaluation as cancer cell sensitizers to chemotherapy and radiotherapy. ***Anti-Cancer Drugs*** **25:1182–1191 © 2014 Wolters Kluwer Health | Lippincott Williams & Wilkins.**

Anti-Cancer Drugs 2014, **25**:1182–1191

Keywords: amiodarone, cancer, cisplatin, docetaxel, metformin, mitochondria, radiotherapy

Department of Radiotherapy/Oncology, Radiobiology and Radiopathology Unit, Democritus University of Thrace, Alexandroupolis, Greece

Correspondence to Michael I. Koukourakis, MD, Department of Radiotherapy/Oncology, Radiobiology and Radiopathology Unit, Democritus University of Thrace, Alexandroupolis 68100, Greece
Tel: +30 25510 74622; fax: +30 25510 30349; e-mail: targ@her.forthnet.gr

Received 20 March 2014 **Revised form accepted** 24 June 2014

Introduction

Mitochondria are vital organelles with their own genome, playing a key role in cell metabolism through oxidative phosphorylation. Within mitochondria, ATP, the source of energy for a variety of metabolic reactions, is produced by oxidation of various carbon fuels in the citric acid cycle. Through oxidative phosphorylation, reactive oxygen species are also produced and their accumulation can damage the cell and lead to a variety of diseases. Furthermore, mitochondria are considered to be sensors of O_2, calcium, carbohydrates, and lipid acids. Apart from cell metabolism, mitochondrial function is directly linked to cell proliferation and apoptosis [1]. Mitochondria are, therefore, critical organelles for cell survival and recent studies consider these worthy targets for the development of new anticancer agents either as targets of cytotoxic drugs or as sensitizers to chemotherapy and radiotherapy [2].

Several drugs used in the clinical practice for the treatment of endocrine diseases, cardiological, or neurological disorders also target discrete mitochondrial functions. Among these, dimethylbiguanide (metformin), an antihyperglycemic drug, effective against type II diabetes [3], inhibits complex I of mitochondria. Interestingly, in a recent meta-analysis, this drug improved survival of cancer patients with type II diabetes treated with chemotherapy [4]. Memantine, a blocker of *N*-methyl-D-aspartate receptor used in Alzheimer's disease, increases complex I and decreases complex IV mitochondrial activity [5]. Oxytetracycline (terramycin), a widely used topical antibiotic, inhibits mitochondrial protein synthesis [6] and accelerates mitochondrial lipid peroxidation [7,8]. Nimodipine, a di-hydrophilic calcium channel blocker, used against subarachnoid brain hemorrhage, appears to also stimulate calcium-ATPase activity in mitochondria [9] and improve mitochondrial respiratory rates [10]. Amiodarone, a class III antiarrhythmic agent, inhibits mitochondrial beta-oxidation of fatty acids and suppresses the respiratory chain at complex I and II, decreasing ATP production [11]. Finally, the inorganic compound sodium azide, used in research for its bacteriostatic activity and also as a pesticide, inhibits cytochrome c oxidase and mitochondrial respiration [12].

In this study, we have investigated the effect of the above mitochondrial metabolism-interfering agents (MMIAs) to assess their effect on cancer cell mitochondrial function using the AlamarBlue resazurin reduction assay and confocal microscopy focusing on mitochondria using MitoTracker. The effect of these agents on cell viability and on cancer cells' sensitization to chemotherapy and radiotherapy has also been studied.

Materials and methods

Cell culture

DU145 prostate cancer and A549 lung cancer cell lines were used for experiments. Both cell lines were cultured in Dulbecco's modified Eagle medium (Gibco DMEM;

0959-4973 © 2014 Wolters Kluwer Health | Lippincott Williams & Wilkins DOI: 10.1097/CAD.0000000000000152

Gibco, Life Technologies, Grand Island, New York, USA) with 10% fetal bovine serum and 37°C, 21% oxygen/5% CO_2.

MMIAs

To assess the effect of MMIAs on cell viability, titrations with various concentrations at different time points have been used on the basis of a bibliographic study: (a) metformin (Merck Co., Hunterdon, New Jersey, USA) at a range of 25–2000 μmol/l, (b) nimodipine (Pharmathen Hellas S.A., Marousi, Athens, Greece) at a range of 25–200 μmol, (c) memantine (Lundbeck S.A., Lundebeck S.A., Valby, Denmark) at a range of 10–80 μmol/l, (d) oxytetracycline (Pfizer Hellas S.A., Athens, Greece) at 10–80 μmol/l, (e) amiodarone (Sanofi Aventis, Athens, Greece) at a range of 50– 400 μmol/l, and (e) sodium azide (Sigma-Aldrich, Saint Louis, Missouri, USA) at a range of 7.5–60 mmol/l for incubation times of 30.5 h [13,14], 54.5 h [15], 54.5 h [15], 8.5 h [16,17], and 2 h [18], respectively. Fluorescence measurements have been performed for AlamarBlue (ThermoScientific, Marietta, Ohio, USA) and the CyQuant (Invitrogen Ltd, Paisley, UK) according to the company's instructions.

For radiosensitization and chemosensitization experiments, a range of concentrations have been used for metformin and amiodarone with various concentrations of cisplatin or docetaxel.

AlamarBlue Assay

The AlamarBlue assay is a reliable method for cell viability [19]. This assay, using the metabolic activity of cells to reduce resazurin (oxidized form: 7-hydroxy-3H-phenoxazin-3-1-10-oxide) to resorufin, counts the number of cells with active mitochondria, given that resazurin reduction is performed by mitochondrial enzymes [20]. The resultant fluorescence of the reduced and the oxidized form is measured at 590 nm emission wavelength with an excitation at 530–560 nm. This assay is considered to be a simple reliable, nontoxic, and safe method of cell viability that is measured without requiring fixation of cells so that these can continue their growth for further experimentation. Given that the cell viability is measured on the basis of the mitochondrial function, this method can also be used to monitor this very function with nontoxic agents, provided that these interfere with the enzymic activity involved in resazurin metabolism. However, if cytotoxic agents interfere with resazurin reduction, the method may become unreliable as the effect on cell viability and the effect on mitochondrial resazurin reduction become components of the same measurement. In the latter case, additional methods measuring cell viability by mitochondrial independent assays should be used.

Cancer cells in the appropriate culture medium were plated in a 96-well plate at a concentration of 500 cells/well. 10% v/v of AlamarBlue was added in each well. As a negative control (background measurement), culture medium without cells was used. In addition, vitamin C was used as a positive control for full reduction of resazurin (5 μl/well). Then, the relative fluorescence (in RFU units: fluorescence in each well minus background fluorescence) was measured every 30 min for 6.5 h in a FLUOstar Omega microplate reader (BMG LABTECH GmbH, Ortenberg, Germany).

CyQuant Assay

Given that the AlamarBlue assay monitors cell viability, on the basis of mitochondrial function and use in the current study of mitochondrial metabolism-targeting drugs that may confound the method's accuracy, cell viability was further and comparatively assessed using an additional method. The CyQUANT Cell Proliferation Assay Kit (C7026; Invitrogen) is based on the fluorescence of a dye bound to cellular nucleic acids. This assay provides a direct index of the DNA content of the viable, attached to the bottom of the plate, cells.

Briefly, 500 cells per well in culture medium were plated in 96-well plates and titrated with various concentrations of the examined agents. The culture medium was removed and 50 μl of the CyQUANT reagent was added at the attached cancer cells. Fluorescence measurements were obtained following a 45 min incubation time using a microplate reader (FLUOstar Omega; BMG LABTECH GmbH) at 480 nm (excitation) and 520 nm (emission).

Confocal microscopy

The integrity and functionality of mitochondria were also assessed by confocal microscopy using specific staining compounds that accumulate in active mitochondria, MitoTracker (Invitrogen) and AlamarBlue. Mitochondrial pyruvate dehydrogenase (PDH) enzyme immunostaining was also used.

MitoTracker is cell-permeant and passively enters into the cell [21]. Thereafter, this enters mitochondria only within live cells utilizing the negative mitochondrial membrane potential. As the MitoTracker is chemically reactive, it links to thiol groups in the mitochondria, becoming permanently bound to the mitochondria, so that the dye persists after the cell is fixed or when cells die [22]. Altered mitochondrial potential may affect MitoTracker staining. The fluorescence of MitoTracker Deep Red FM can be monitored at 644 nm excitation wavelength and 665 nm emission wavelength. Changes in mitochondrial potential can cause loss of the mitotracker signal. Thus, we have selected Mitotracker to stain active mitochondria and monitor mitochondria potential and functionality changes by mitochondrial agents. AlamarBlue was also used to assess mitochondria activity in confocal microscopy by detecting the intensity of fluorescence of resorufin in stained cells excited by a red laser line at 562 nm.

For immunofluorescence staining, A549 and DU145 cells grown on no. 1 glass coverslips were treated with five drugs at representative concentrations following screening with various incubations times, aiming to visualize their potential activity on mitochondria: metformin (400 μmol/l for 24 h), oxytetracycline (80 μmol/l for 2 h), memantine (80 μmol/l for 48 h), nimodipine (200 μmol/l for 24 h), and amiodarone (400 μmol/l for 2 h). For the control experiments, untreated cells were used. The cells were stained using either AlamarBlue 10% v/v for 4 h or Mitotracker (250 nmol/l) for 30 min. DNA was counterstained with Hoechst 33342 (1 μg/ml; Sigma-Aldrich) for 10 min at 37°C in a CO_2 incubator (5% CO_2).

Commonly, cells were fixed in 3.7% formaldehyde/PBS pH 7.4 for 20 min at 37°C, omitting the permeabilization step to sustain the AlamarBlue dye inside the cells. For PDH staining, following the PFA fixation process, cells were permeabilized in PBS/0.1% v/v Triton X-100 pH 7.4 for 5 min at room temperature. Cells were blocked in PBS/5% w/v BSA pH 7.4 and stained with anti-PDH E2/E3bp rabbit polyclonal antibody (1 : 1000, ab110333; Abcam, Cambridge, UK) for 1 h at room temperature. Cells were washed in PBS pH 7.4, incubated with the appropriate CF 488 secondary antibody (1 : 500; Biotium Inc., Hayward, California, USA) at room temperature for 30 min. After final washes, coverslips were mounted in homemade Mowiol mounting medium. Imaging of fixed samples was performed on a customized Andor Revolution Spinning Disk Confocal System built around a stand (IX81; Olympus Corporation, Tokyo, Japan) with a ×60 lens and a digital camera (Andor Ixon + 885; Andor Technology Ltd., Belfast, UK) (CIBIT Facility; MBG-DUTH, Alexandroupolis, Greece). Image acquisition was performed in Andor IQ 2 software (Andor Technology). Optical sections were recorded every 0.3 μm.

Cell chemosensitivity experiments

In chemosensitization experiments, cells were incubated with clinically established drugs, 8 μmol/l of cisplatin [23] and 0.62 and 1.8 nmol/l of docetaxel [24], for 24 h h in PBS. The viability of cells was assessed at 24 h. Cell proliferation and survival experiments were conducted using the Cyquant. The RFU were recorded for each well at specific time points (8 and 12 days). For every irradiated well, the RFU ratio was calculated as follows:

$$\text{RFU ratio} = \frac{\text{RFU irradiated} - \text{RFU negative controls}}{\text{RFU nonirradiated} - \text{RFU negative controls}}.$$

Cell radiosensitivity clonogenic assay

Cells were irradiated using 2, 4, 6, and 9 Gy after 24 h incubation with 400 μmol/l of metformin or 2 h incubation with 400 μmol/l of amiodarone. Then, they were harvested using trypsinization and 100 cells were seeded in six-well plates in duplicate. Plates were placed in the incubator and colonies were observed every 2 days while the medium was changed every third day. Twelve days after treatment, medium was removed and cells were washed with 2 ml of PBS. Then, cells were fixed by adding 2 ml of methanol/acetic acid 3 : 1 for 10 min in − 20°C and stained with 0.5% crystal violet. Colonies with at least 50 cells were counted on an inverted microscope. The percentage of colonies counted at various dose points as compared with nonrradiated cells was used to plot dose–response curves for clonogenic survival.

Results

AlamarBlue reduction by cancer cell lines after incubation with MMIAs

Before studying the effect of the examined agents on cancer cell mitochondrial function, we tested whether these drugs had a reductive effect on resazurin *per se*. The RFU of various concentrations of the drugs on cell-deprived culture medium with AlamarBlue remained unaffected for 7 h (data not shown).

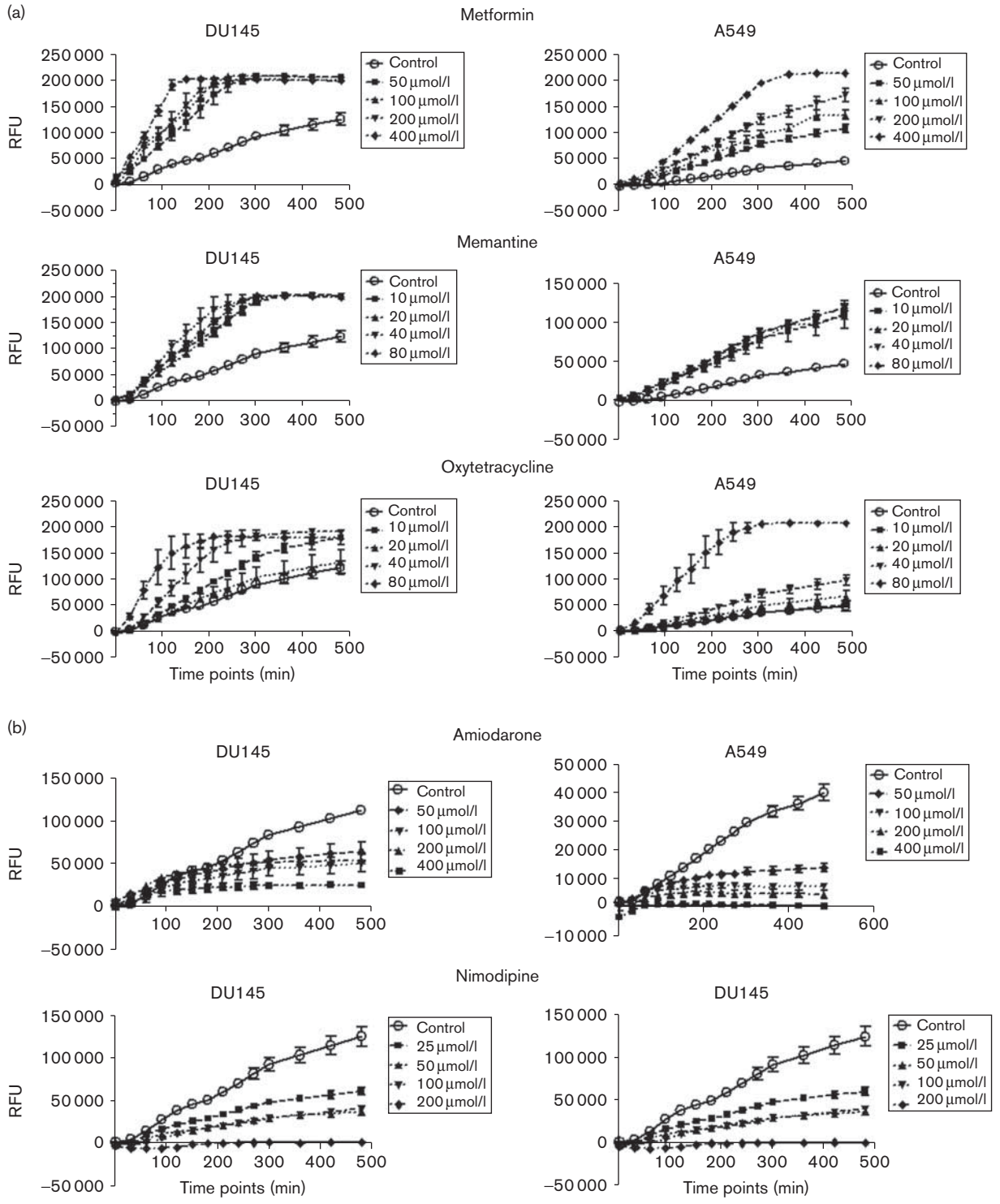
Figure 1a shows the effect of preincubation of cells with different concentrations of the agents on AlamarBlue reduction according to the protocol described in methods. An intense, dose-dependent, stimulatory effect on mitochondrial resazurin reduction, as indicated by increased RFUs, was observed for all tested concentrations of metformin and memantine in both DU145 and A549 cell lines. A similar effect was also observed in the case of oxytetracycline for concentrations above 40 μmol/l.

In contrast, amiodarone suppressed the mitochondrial resazurin reduction in a dose-dependent manner (Fig. 1b) in both cell lines, although the effect was more profound for A549. In particular, 400 μmol/l of amiodarone almost completely blocked mitochondrial function with RFU similar to the background. Similar patterns have been noted for nimodipine, with a stronger effect in DU145. Nimodipine level at 200 μmol/l completely blocked resazurin reduction in both cell lines. Finally, sodium azide did not induce a clear inhibitory effect on mitochondrial reductive activity on resazurin (data not shown).

Confocal microscopy after incubation with MMIAs in A549 and DU145 cell lines

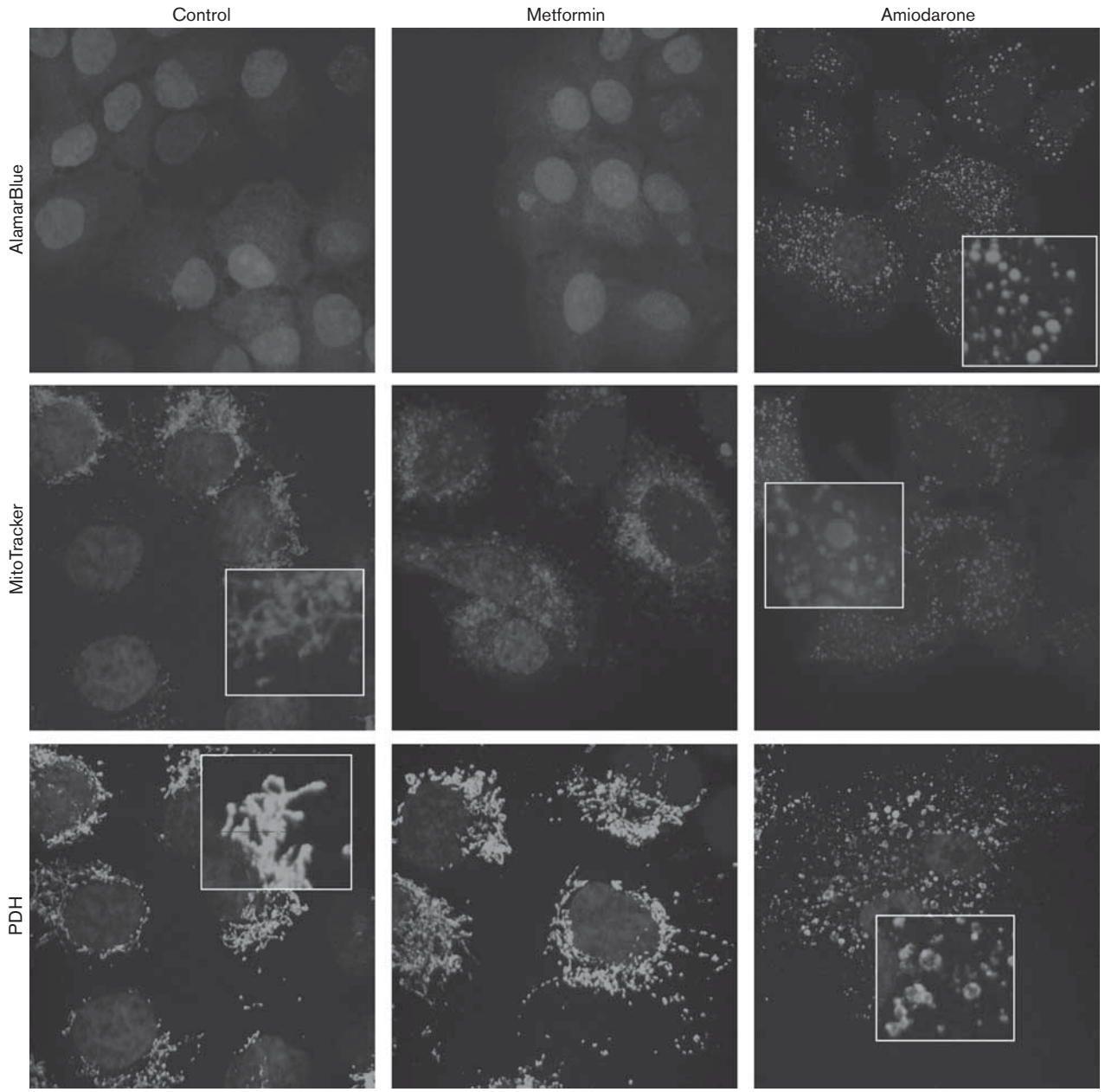
In control cells, MitoTracker showed the presence of elongated structures in the cytoplasm corresponding to mitochondria. AlamarBlue showed a diffuse staining, apparently representing the reduced resazurin flowing out of mitochondria. Following exposure to the MMIAs, there was no apparent effect in the morphology of the cytoplasmic staining, with the exception the treatment with amiodarone. In the latter case, both staining methods showed a dilation of mitochondria that assumed a round shape (Fig. 2). This direct effect on mitochondria swelling was evident for amiodarone concentrations above 100 μmol/l as shown in titration experiments (data not shown).

Fig. 1



(a) Relative fluorescence units (RFUs) obtained after incubation of DU145 and A549 cell lines with four different concentrations of metformin (24 h incubation time), memantine (48 h incubation time), and oxytetracycline (2 h incubation time). (b) RFU following incubation of cells with four different concentrations of amiodarone (2 h incubation time) and nimodipine (24 h incubation time). RFUs are recorded every 30 min for 6.5 h.

Fig. 2



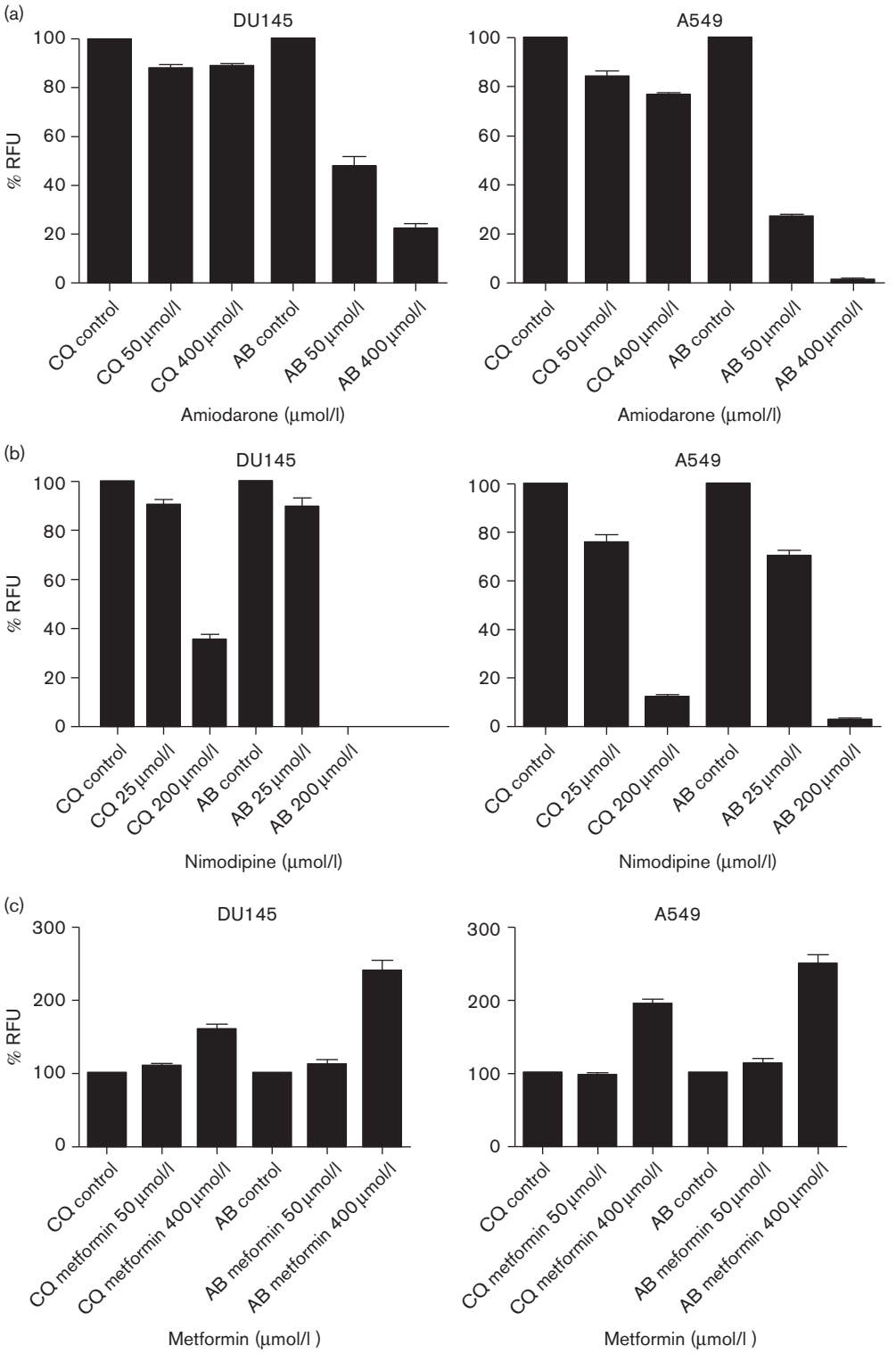
Confocal immunofluorescence images of the mitochondria staining in DU145 cells with Mitotracker, AlamarBlue and immunostaining with anti-PDH antibody following incubation with metformin (400 μmol/l for 24 h) and of amiodarone (400 μmol/l for 2 h). Note the swelling of mitochondria, following exposure to amiodarone, compared with the elongated shape of mitochondria evident in control images stained with MitoTracker and with anti-PDH (magnified in white-bordered boxes). PDH, pyruvate dehydrogenase.

Cell viability versus resazurin reduction

To confirm that the changes in the rates of resazurin reduction were without a doubt an effect on mitochondrial metabolism and not an indication of cell death or increased proliferation, DU145 and A549 cell lines were incubated with the lowest and highest concentrations of nimodipine and amiodarone (showing blockage of resazurin reduction) and of metformin (showing induction of the effect); (nimodipine: 25 and 200 μmol/l, amiodarone: 50 and 400 μmol/l and metformin: 25 and 400 μmol/l). Cell viability was measured using both AlamarBlue and CyQuant assays as described previously in the methods section (Fig. 3).

CyQuant assays showed that amiodarone exerted a weak cytotoxic effect, showing cell viability at around 80% of

Fig. 3



Percentage of relative fluorescence unit (RFU) changes using the CyQuant and the AlamarBlue assays in DU145 and A549 cell lines following incubation with different concentrations of amiodarone (a), nimodipine (b), and metformin (c).

the control cell population, even following exposure to the highest concentration, whereas AlamarBlue assays showed a sharp dose-dependent reduction in the RFUs. Therefore, we can safely conclude that amiodarone exerts a strong inhibitory effect on mitochondrial reductive activity on resazurin.

In contrast, nimodipine showed a cytotoxic effect on cells, especially at the highest concentration, as further confirmed by CyQuant assays. Thus, an eventual effect of the drug on resazurin reduction by mitochondrial enzymes cannot be measured reliably in the context of nimodipine-induced cytotoxicity.

In addition, metformin clearly increased the cell proliferation as indicated by CyQuant, showing that the RFU values of the cells incubated with 400 μmol/l metformin were two-fold increased compared with the control values for the A549 and half-fold increased for DU145. Moreover, it seems that metformin also affected the mitochondrial function by enhancing the activity of the complex responsible for resazurin metabolism as RFUs were 2.5-fold increased compared with the control values for both cell lines.

Radiosensitization with metformin and amiodarone

Twenty-four hour incubation with nontoxic concentrations of metformin (400 μmol/l) and amiodarone (400 mmol/l) was used to assess an eventual effect on cancer cell postradiation clonogenic survival (Fig. 4). The dose allowing 50% clonogenic survival of the DU145 cells (compared with nonirradiated cells) was 3.2 Gy and was reduced to 1.9 and 1.5 Gy when cells were incubated with metformin and amiodarone, respectively. The dose allowing 50% clonogenic survival of the A549 cells was 2.85 Gy and was reduced to 1.7 and 2.1 Gy when cells were incubated with metformin and amiodarone, respectively.

Chemosensitization with metformin and amiodarone

Metformin and amiodarone exerted the strongest effect on the cell mitochondria function. Amiodarone inhibited and metformin enhanced the metabolism of AlamarBlue. Thus, we have investigated whether the coincubation of metformin or amiodarone with cisplatin or docetaxel affects cell sensitivity to chemotherapy. Cells were incubated with different concentrations of either metformin or amiodarone and cisplatin or docetaxel. We used a common incubation time of 24 h for all drugs and afterwards the number of cells was measured using CyQuant assay.

Representative results for the experiments are presented in Fig. 5. Figure 5a shows that incubation with 100 μmol/l amiodarone exerted a minor cytotoxic effect in DU145 and A549 cells. Moreover, treatment with 8 μmol/l of cisplatin induced a 20% cell population reduction in the two cell lines. Simultaneous incubation with two different concentrations of amiodarone confirmed a synergistic effect between the two drugs. The mild effect on the cell viability of docetaxel at 0.6 and 1.8 nmol/l on DU145 and A549 cell lines is shown in Fig. 5b. Combined incubation with amiodarone resulted in significant sensitization in both cell lines.

Discussion

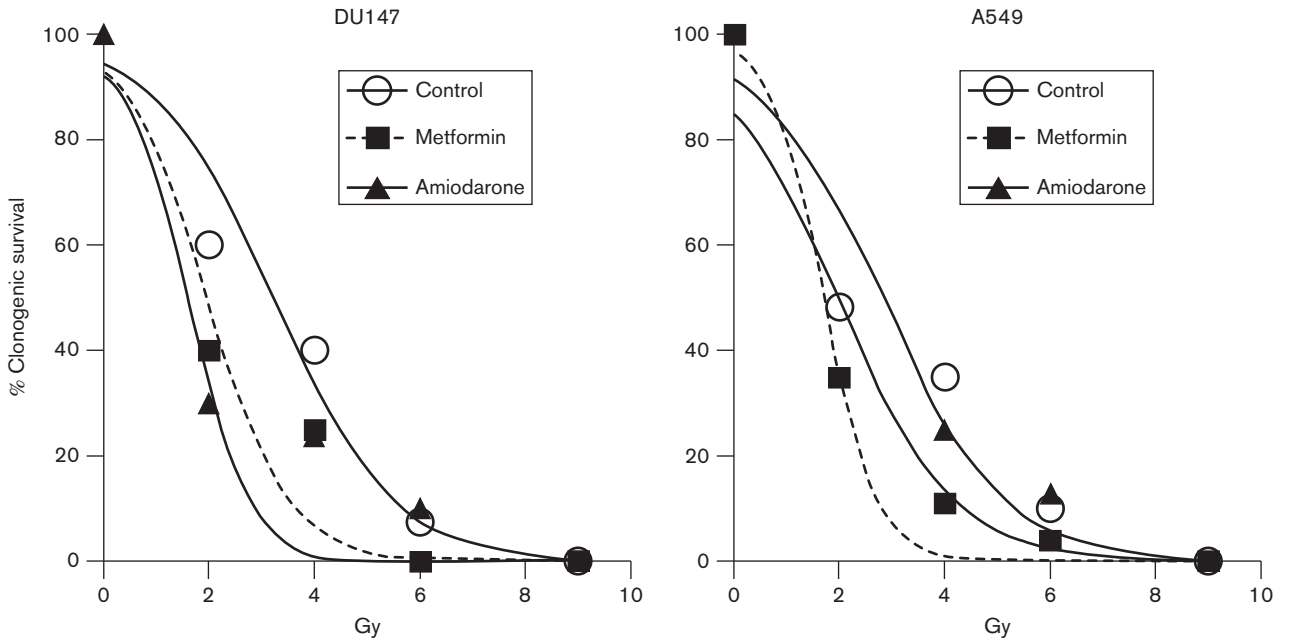
In this in-vitro study, taking into consideration the highly important role of mitochondria in a variety of cell function and molecular pathways, we investigated an ultimate effect of different MMIAs on cell chemosensitization and radiosensitization [2]. A variety of different drugs that target the mitochondrial function on different mitochondrial complexes were used to facilitate our investigation.

Using the AlamarBlue assay, we measured the effect of the drugs on resazurin metabolism. The interesting finding of this part of our study is that although MMIAs are inhibitors of different complexes of mitochondria, the response of cancer cells, as measured by the reduction intensity of resazurin, differed among drugs. In particular, memantine, metformin, and oxytetracycline increased, whereas amiodarone reduced resazurin reduction. This was a direct interference on mitochondrial as the CyQuant assay excluded dependence on cell viability. It is, therefore, evident that MMIAs exert a complex effect on mitochondrial metabolism, as despite being blockers of some complex, they seem to directly or indirectly accentuate side mitochondrial metabolic activities. Various combinations of such drugs may more efficiently interfere with mitochondrial enzyme function, resulting in potent blockage of mitochondrial metabolism, a hypothesis that requires further investigation.

Surprisingly, in contrast to published studies showing an antiproliferative effect of metformin [25,26], also, here, confirmed for high drug concentrations, low concentrations of metformin exerted a stimulatory effect on cancer cell proliferation. This compound inhibits complex I of mitochondria. In leukemic cells, metformin decreases oxygen consumption and mitochondrial ATP synthesis, while stimulating glycolysis for ATP and lactate production [27]. A similar effect has also been confirmed in mouse liver [28]. Moreover, a direct effect on increased glucose transporter expression on cell membranes has also been reported [29]. Interestingly, high ambient glucose levels have been shown to block the apoptotic and antiproliferative activity of metformin [30]. This stimulatory effect on anaerobic glycolysis, in the presence of high glucose, may at least partially explain the proliferation advantage of the cells that have been exposed to low metformin concentrations.

Amiodarone, in contrast, inhibits mitochondrial β-oxidation of fatty acids and suppresses the respiratory chain at complex I and II, decreasing the production of ATP [11].

Fig. 4



Clonogenic radiation dose–response cell survival curves of DU145 and A549 cell lines, respectively, in control cells and cells incubated with amiodarone (400 μmol/l for 2 h) or metformin (400 μmol/l for 24 h).

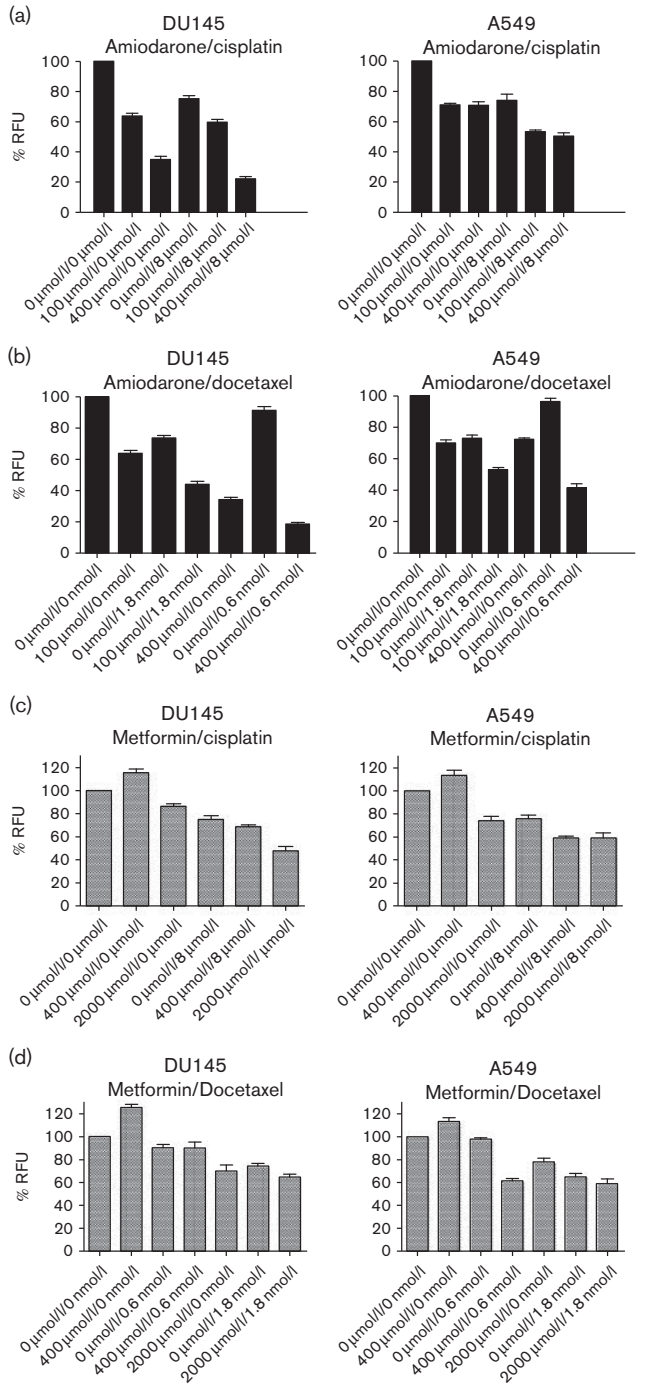
At concentrations above 100 μmol/l, we noted a clear effect on mitochondria shape, implying a direct effect on organelle membrane permeability and eventually mitochondria damage, in addition to its activity on mitochondrial enzymes. At this concentration, 24 h incubation has a mild cytotoxic activity. Interestingly, in contrast to the metformin effect, amidarone blocked resazurin reduction, further suggesting different effects of these two drugs' mitochondria metabolism. The dose-dependent effect of amiodarone on mitochondrial permeability transition and respiratory chain function has been reported previously [31].

Despite their different mechanistic actions on mitochondria, both drugs seem to block oxidative phosphorylation, induce anaerobic metabolism, and, depending on the concentration, can by themselves become toxic for cancer cells. As they are well-tested and widely used drugs in the clinical practice, an eventual potentiation of antitumor activity of established anticancer agents such as radiotherapy and chemotherapy may have an immediate clinical application. Experiments with escalating doses of radiotherapy at therapeutic levels showed, however, a mild radiosensitization effect only in the A549 cell line. The blockage of oxygen consumption and the increase in anaerobic metabolism noted in other tumor models [32] may in fact lead to resistance to radiotherapy, minimizing an eventual additive effect of drug cytotoxicity on radiation efficacy. This, however, triggers a further hypothesis that MMIAs, by blocking oxidative phosphorylation, would strongly sensitize cells to anaerobic metabolism inhibitors such as HIF or PDH-kinase inhibitors. Such double metabolic combinations with radiotherapy may be very important and should be investigated thoroughly.

In addition, we have provided clear evidence that amiodarone has a synergistic effect with cisplatin and docetaxel, even at mildly cytotoxic concentrations of the latter agents. It is noteworthy that although 0.62 nmol/l of docetaxel did not exert any cytotoxic effect on A549, coincubation with 400 μmol/l of amiodarone increased the percentage of reduction by 30%. The same pattern was observed when A549 were incubated with 400 μmol/l of metformin and 0.62 nmol/l of docetaxel. Although hypoxia contributes overall to chemoresistance, cisplatin and docetaxel, in contrast to radiation, do not require the formation of oxygen free radicals for their cytotoxic activity. Cisplatin, being an intercalating agent [33], damages DNA independent of oxygen and docetaxel by acting on mitochondria and the nuclear envelop [34,35], and is well expected to synergize with MMIAs to induce mitochondria-mediated apoptosis. Interestingly, mitochondrial interference produced by both drugs showed sensitization of both cell lines to radiotherapy, an effect that should be examined thoroughly in further *in vitro* and animal experiments.

Taking all the aforementioned data into consideration, it is clear that several drugs that are well established in clinical practice for the treatment of noncancer diseases

Fig. 5



Chemosensitization experiments with metformin and amiodarone in DU145 and A549 cell lines using the CyQuant assay. (a) Percent reduction of cells incubated with cisplatin and amiodarone. (b) Percent reduction of cells incubated with docetaxel and amiodarone. (c) Percent reduction of cells incubated with cisplatin and metformin. (d) Percent reduction of cells incubated with docetaxel and metformin.

target cancer cell mitochondrial function and may have an important antineoplastic effect in combination with radiotherapy and commonly used chemotherapy drugs such as cisplatin and docetaxel. Given the fact that cancer cells mostly rely on anaerobic glycolysis, MMIAs may be valuable in blocking an eventual switch of cancer energetics to mitochondria respiration when tumors are treated with anaerobic pathway targeting molecules such as HIF inhibitors or 2-deoxy-D-glucose.

Acknowledgements

Conflicts of interest

There are no conflicts of interest.

References

1 Lord CE, Dauphinee AN, Watts RL, Gunawardena AH. Unveiling interactions among mitochondria, caspase-like proteases, and the actin cytoskeleton during plant programmed cell death (PCD). *PLoS One* 2013; **8**:e57110.

2 Ralph SJ, Low P, Dong L, Lawen A, Neuzil J. Mitocans: mitochondrial targeted anti-cancer drugs as improved therapies and related patent documents. *Recent Pat Anticancer Drug Discov* 2006; **1**:327–346.

3 El-Mir MY, Nogueira V, Fontaine E, Avéret N, Rigoulet M, Leverve X. Dimethylbiguanide inhibits cell respiration via an indirect effect targeted on the respiratory chain complex I. *J Biol Chem* 2000; **275**:223–228.

4 Yin M, Zhou J, Gorak EJ, Quddus F. Metformin is associated with survival benefit in cancer patients with concurrent type 2 diabetes: a systematic review and meta-analysis. *Oncologist* 2013; **18**:1248–1255.

5 McAllister J, Ghosh S, Berry D, Park M, Sadeghi S, Wang KX, *et al*. Effects of memantine on mitochondrial function. *Biochem Pharmacol* 2007; **75**:956–964.

6 Van den Bogert C, Holtrop M, Melis TE, Roefsema PR, Kroon AM. Different effects of oxytetracycline and doxycycline on mitochondrial protein synthesis in rat liver after long-term treatment. *Biochem Pharmacol* 1987; **36**:1555–1559.

7 Gnanasoundari M, Pari L. Impact of naringenin on oxytetracycline-mediated oxidative damage in kidney of rats. *Ren Fail* 2006; **28**:599–605.

8 Fréneaux E, Labbe G, Letteron P, Le Dinh T, Degott C, Genève J, *et al*. Inhibition of the mitochondrial oxidation of fatty acids by tetracycline in mice and in man: possible role in microvesicular steatosis induced by this antibiotic. *Hepatology* 1988; **8**:1056–1062.

9 Chen L, Yang Y, Liu L, Cao M. Changes of neuronal calcium channel following brain damage induced by injection of pertussis bacilli in rats. *Chin J Traumatol* 2002; **5**:224–227.

10 Rodriguez y Baena R, Gaetani P, Silvani V, Spanu G, Marzatico F. Effect of nimodipine on mitochondrial respiration in different rat brain areas after subarachnoid haemorrhage. *Acta Neurochir Suppl (Wien)* 1988; **43**:177–181.

11 Fromenty B, Fisch C, Labbe G, Degott C, Deschamps D, Berson A, *et al*. Amiodarone inhibits the mitochondrial beta-oxidation of fatty acids and produces microvesicular steatosis of the liver in mice. *J Pharmacol Exp Ther* 1988; **255**:1371–1376.

12 Bowler MW, Montgomery MG, Leslie AG, Walker JE. How azide inhibits ATP hydrolysis by the F-ATPases. *Proc Natl Acad Sci USA* 2006; **103**:8646–8649.

13 Owen MR, Doran E, Halestrap AP. Evidence that metformin exerts its anti-diabetic effects through inhibition of complex 1 of the mitochondrial respiratory chain. *Biochem J* 2000; **348**:607–614.

14 Marroquin LD, Hynes J, Dykens JA, Jamieson JD, Will Y. Circumventing the Crabtree effect: replacing media glucose with galactose increases susceptibility of HepG2 cells to mitochondrial toxicants. *Toxicol Sci* 2007; **97**:539–547.

15 Wu HM, Tzeng NS, Qian L, Wei SJ, Hu X, Chen SH, *et al*. Novel neuroprotective mechanisms of memantine: increase in neurotrophic factor release from astroglia and anti-inflammation by preventing microglial activation. *Neuropsychopharmacology* 2009; **34**:2344–2357.

16 Lokeshwar BL, Selzer MG, Zhu BQ, Block NL, Golub LM. Inhibition of cell proliferation, invasion, tumor growth and metastasis by an oral non-antimicrobial tetracycline analog (COL-3) in a metastatic prostate cancer model. *Int J Cancer* 2002; **98**:297–309.

17 Abu-Amero KK, Bosley TM. Detection of mitochondrial respiratory dysfunction in circulating lymphocytes using resazurin. *Arch Pathol Lab Med* 2005; **129**:1295–1298.

18 Bogucka K, Wojtczak L, Erecińska M. Effect of amyl azide on respiration and oxidative phosphorylation in mitochondria. *Acta Biochim Pol* 1970; **17**:239–246.

19 Zachari MA, Chondrou PS, Pouliliou SE, Mitrakas AG, Abatzoglou I, Zois CE, Koukourakis MI. Evaluation of the AlamaBlue assay for adherent cells irradiation experiments. *Dose Response* 2013; **12**:246–258.
20 Page B, Page M, Noel C. A new fluorometric assay for cytotoxicity measurements in-vitro. *Int J Oncol* 1993; **3**:473–476.
21 Cottet-Rousselle C, Ronot X, Leverve X, Mayol JF. Cytometric assessment of mitochondria using fluorescent probes. *Cytometry A* 2011; **79**:405–425.
22 Chazotte B. Labeling mitochondria with MitoTracker dyes. *Cold Spring Harb Protoc* 2011; **2011**:990–992.
23 Lee K, Tanaka M, Kanamaru H, Hashimura T, Yamamoto I, Konishi J, Kuze F. In vitro antagonism between cisplatin and vinca alkaloids. *Br J Cancer* 1989; **59**:36–41.
24 Ichite N, Chougule MB, Jackson T, Fulzele SV, Safe S, Singh M. Enhancement of docetaxel anticancer activity by a novel diindolylmethane compound in human non-small cell lung cancer. *Clin Cancer Res* 2009; **15**:543–552.
25 Ben Sahra I, Regazzetti C, Robert G, Laurent K, Le Marchand-Brustel Y, Auberger P, *et al*. Metformin, independent of AMPK, induces mTOR inhibition and cell-cycle arrest through REDD1. *Cancer Res* 2011; **71**:4366–4372.
26 Storozhuk Y, Hopmans SN, Sanli T, Barron C, Tsiani E, Cutz JC, *et al*. Metformin inhibits growth and enhances radiation response of non-small cell lung cancer (NSCLC) through ATM and AMPK. *Br J Cancer* 2013; **108**:2021–2032.
27 Scotland S, Saland E, Skuli N, de Toni F, Boutzen H, Micklow E, *et al*. Mitochondrial energetic and AKT status mediate metabolic effects and apoptosis of metformin in human leukemic cells. *Leukemia* 2013; **27**:2129–2138.
28 Silva FM, da Silva MH, Bracht A, Eller GJ, Constantin RP, Yamamoto NS. Effects of metformin on glucose metabolism of perfused rat livers. *Mol Cell Biochem* 2010; **340**:283–289.
29 Grisouard J, Timper K, Radimerski TM, Frey DM, Peterli R, Kola B, *et al*. Mechanisms of metformin action on glucose transport and metabolism in human adipocytes. *Biochem Pharmacol* 2010; **80**:1736–1745.
30 Wahdan-Alaswad R, Fan Z, Edgerton SM, Liu B, Deng XS, Arnadottir SS, *et al*. Glucose promotes breast cancer aggression and reduces metformin efficacy. *Cell Cycle* 2013; **12**:3759–3769.
31 Varbiro G, Toth A, Tapodi A, Veres B, Sumegi B, Gallyas F Jr. Concentration dependent mitochondrial effect of amiodarone. *Biochem Pharmacol* 2003; **65**:1115–1128.
32 Batchuluun B, Inoguchi T, Sonoda N, Sasaki S, Inoue T, Fujimura Y, *et al*. Metformin and liraglutide ameliorate high glucose-induced oxidative stress via inhibition of PKC-NAD(P)H oxidase pathway in human aortic endothelial cells. *Atherosclerosis* 2014; **232**:156–164.
33 Baruah H, Barry CG, Bierbach U. Platinum-intercalator conjugates: from DNA-targeted cisplatin derivatives to adenine binding complexes as potential modulators of gene regulation. *Curr Top Med Chem* 2004; **4**:1537–1549.
34 André N, Carré M, Brasseur G, Pourroy B, Kovacic H, Briand C, Braguer D. Paclitaxel targets mitochondria upstream of caspase activation in intact human neuroblastoma cells. *FEBS Lett* 2002; **532**:256–260.
35 Haldar S, Jena N, Croce CM. Inactivation of Bcl-2 by phosphorylation. *Proc Natl Acad Sci USA* 1995; **92**:4507–4511.