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Abbreviations: BL, Burkitt's lymphoma; GF, galloflavin; LDH, lactate dehydrogenase; OXA, oxamate; PARP, poly-ADP-ribose polymerase; ROS, reactive oxygen species

Abstract

Up-regulation of glycolysis, a well recognized hallmark of cancer cells, was also found to be predictive of poor chemotherapy response. This observation suggested the attempt of sensitizing cancer cells to conventional chemotherapeutic agents by inhibiting glucose metabolism. Lactate dehydrogenase (LDH) inhibition can be a way to hinder glycolysis of cancer cells without affecting the metabolism of normal tissues, which usually does not require this enzymatic activity. In this paper, we showed that two LDH inhibitors (oxamate and galloflavin) can increase the efficacy of cisplatin in cultured Burkitt's lymphoma (BL) cells and that this potentiating effect is not exerted in proliferating normal lymphocytes. This result was explained by the finding that in BL cells LDH inhibition induced reactive oxygen species (ROS) generation, which was not evidenced in proliferating normal lymphocytes. In BL cells treated with the association of cisplatin and LDH inhibitors, these ROS can be a further cause of DNA damage, to be added to that produced by cisplatin, leading to the failure of the response repair.

At present LDH inhibitors suitable for clinical use are actively searched; our results can allow a better understanding of the potentiality of LDH as a possible target to develop innovative anticancer treatments.

Keywords: Cancer cell metabolism; Cisplatin; Lactate dehydrogenase; Lymphoma cells.

1. Introduction

In spite of the advances achieved with the introduction of the new generation, “biological” therapeutics, chemotherapy based on DNA damaging agents still maintains a central role in non-surgical cancer treatment. Initially, these agents usually show effectiveness at reducing tumor burden, but relapse at therapy discontinuation and/or resistance during sustained treatments are often observed and represent a significant clinical challenge. As a consequence, therapeutic strategies aimed at overcoming these drawbacks are actively searched.

Since one of the mechanisms governing intrinsic and acquired resistance to DNA damaging agents is activation of the cellular DNA repair machinery (Longley and Johnston, 2005), interference with DNA repair has been proposed as an adjuvant approach to improve the efficacy of chemotherapeutic drugs (Ding et al., 2006). However, this approach involves the risk that the enhanced therapeutic power of the genotoxic drugs can be accompanied by increased toxicity for normal cells, which use the same DNA repair mechanisms of neoplastic cells (Li, 2012; Fiume, 2014). Another risk to be considered is the occurrence of secondary malignancies because of potential mutagenesis and carcinogenesis following the inhibition of DNA repair in normal tissues (Li, 2012; Fiume, 2014).

Studies aimed at understanding the molecular features associated with drug resistance have shown that up-regulation of glycolysis can be predictive of poor chemotherapy response (Dorward and Singh, 1996; Song et al., 2014) and that inhibition of glucose metabolism can sensitize cancer cells to several commonly used chemotherapeutic agents (Ihrlund et al., 2008; Hernlund et al., 2008; Zhang and Aft, 2009; Loar et al., 2010; Xie et al., 2011; Zhao et al., 2014; Leung et al., 2014; Sullivan et al., 2014). Increased glucose uptake and metabolism are one of the hallmarks of neoplastic

change (Gillies et al., 2008); they provide cancer cells with ATP and precursors needed for the biosynthesis of macromolecules and are supposed to confer growth advantage to these cells. A possible way to hinder glycolysis of cancer cells without affecting glucose metabolism of normal tissues is the inhibition of lactate dehydrogenase (LDH) activity (Granchi et al., 2010; Fiume et al., 2014). By reducing pyruvate to lactate, LDH allows the rapid reoxidation of NADH needed for sustaining the glycolytitc flux and assuring ATP synthesis and biomass production. The A isoform of this enzyme is constantly up-regulated in cancer cells and is not necessary for normal cell survival (Granchi et al., 2010; Fiume et al., 2014).

By using two small molecule LDH inhibitors (oxamate (OXA) (Papacostantinou and Colowick, 1961) and galloflavin (GF) (Manerba et al., 2012)), we verified whether hindering LDH activity can selectively increase the efficacy of cisplatin in neoplastic cells. Although introduced in the clinics more than forty years ago, this drug is currently administered to treat a multitude of cancers. Cisplatin exerts anticancer activity by interacting with nucleophilic N7 sites of purine bases forming DNA intra- and inter-strand crosslinking that interfere with DNA synthesis (Jamieson and Lippard, 1999). However, its use is often limited by acquired or intrinsic resistance of the cancer cells (Kartalou and Essigmann, 2001). For our experiments, we used a cell line (Loukes) derived from a Burkitt's lymphoma, which is a tumor form highly responsive to LDH inhibition (Vettraino et al., 2013), and non-neoplastic lymphoblastoid cells, immortalized by Epstein Barr virus infection (GM00130C). We found that both LDH inhibitors increased cisplatin activity only in neoplastic cells, and explored the mechanisms underlying their sensitizing effect.

2. Materials and Methods

2.1 Cell lines and culture conditions

Loukes cells are derived from a sporadic, EBV negative Burkitt's lymphoma (Marchini et al., 1992). GM00130C is a B lymphocyte cell line immortalized by EBV infection (Vettraino et al., 2013). The cells were grown as a suspension culture in RPMI 1640 containing 10% FBS (20% for GM00130C), 100 U/ml penicillin/streptomycin, 4 mM glutamine and were maintained at a concentration of 1-2 x 10^5 viable cells/ml. All media and supplements were from Sigma-Aldrich.

2.2 Compounds

GF was synthesized according to the procedure described by Manerba et al. (2012). It was added to the culture media in the presence of 0.6% DMSO for all the experiments. Equivalent amounts of DMSO were also added to the control (untreated) cultures.

OXA, cisplatin (cis-diamminedichloroplatinum(II)) and all other compounds and reagents were purchased from Sigma-Aldrich.

2.3 LDH inhibition

LDH inhibition was evaluated by dosing the produced lactate. 5 x 10^5 cells in 1 ml of Krebs Ringer buffer were seeded in each well of a 6-well plate. Different amounts of inhibitors (tested in duplicate) were then added to the cultures. Lactate (both intracellular and released in medium) was measured 6h after incubation at 37°C. At the end of incubation, cells were lysed by adding 100 μl of 100% trichloroacetic acid (TCA) in the Krebs Ringer medium (TCA final concentration = 10%). After centrifugation to remove the cell debris and acid insoluble material, lactate was measured in the supernatant using the procedure described by Farabegoli et al. (2012). The dose of compound causing 50%

inhibition of lactate production (LDH IC_{50}) was calculated from the second order polynomial regression of experimental data, using the Prism 5 GraphPad software. For each inhibitor, the LDH IC_{50} dose was subsequently assayed on ATP cellular production, NAD/NADH ratio and viability. Inhibition of ATP production was measured after 6h incubation by using the CellTiter-Glo Luminescent Cell Viability Assay from Promega, as described by Vettraino et al. (2013). The effect on cell viability was measured at 24h as described on paragraph 2.4. NAD/NADH ratio was measured after 6h incubation as described on paragraph 2.5.

2.4 Combination experiment of cisplatin with OXA(GF)

Loukes and GM00130C cells (1 x 10^5 in 24-multiwell plates) were incubated for 24h in the presence of cisplatin (3 and 6 μM) and LDH inhibitors (LDH IC_{50} dose), given alone or in combination. At the end of incubation, the percentage of living cells was evaluated by Trypan blue exclusion. The interaction between LDH inhibitors and cisplatin was assessed by calculating the combination index according to the procedure described by Dos Santos Ferreira et al. (2012), which applies the following formula:

$$\frac{\text{Surviving cells treated with the association}}{\text{(Surviving cells treated with cisplatin) x (Surviving cells treated with OXA(GF))}}$$

According to Dos Santos Ferreira et al. (2012), a result ranging from 0.8 to 1.2 denotes an additive effect. Synergism is indicated by a result < 0.8; antagonism by a result > 1.2.

2.5 NAD/NADH assay

Cellular levels of NAD and NADH were assessed using the protocol described by Vettraino et al. (2013), using samples of 1.5 x 10^6 Loukes cells. After incubation (6h or 16h) with OXA(GF) (LDH IC_{50} dose, given alone or in combination with 6 μM cisplatin) cells were counted, pelleted at 4°C and lysed with ice-cooled extraction buffer (500 μl / 1.5 x 10^6 cells) containing 20 mM sodium bicarbonate, 100 mM sodium carbonate, 10 mM nicotinamide and 0.1% Triton-X100. The cell lysate was centrifuged at 16000g, 4°C for 5 min to remove the insoluble material. A 50 μl aliquot of the sample was kept at 60°C for 30 min to selectively decompose NAD. A further 50 μl was mixed with 840 μl of a buffer containing 100 mM Tris-HCl pH 8, 0.5 mM EDTA, 0.5 mM MTT, 0.2 mg/ml of yeast alcohol dehydrogenase. After addition of 10 μl of 200 mM phenazine ethosulfate, the solution was incubated for 5 min at 25°C. Then 100 μl of 6 M ethanol was added, the mixture was centrifuged at 16000g, 25°C for 30 sec, and the absorbance at 570 nm of the supernatant was measured for 120 sec with 10 sec intervals, using an UV/visible spectrophotometer, in the “kinetics” mode. This sample allowed measuring total NAD + NADH content. The same reaction was then repeated on the sample incubated at 60°C, to measure NADH. The measured absorbance change / second is proportional to the amount of the dinucleotide. NAD and NADH concentrations in the experimental samples were calculated using a calibration curve previously obtained with known amounts of NAD(H) standards.

2.6 Western blots analysis

Loukes cell cultures (1.5 × 10^6 cells) were exposed to cisplatin (6 μM) and OXA(GF) (LDH IC_{50} dose) by using the schedules reported in the Figure legends. After incubation they were lysed in 100 μl RIPA buffer containing protease and phosphatase inhibitors (Sigma Aldrich). The cell homogenates were left 30 min on ice and then centrifuged 15

min at 10000g. 50 µg proteins of the supernatants (measured according to Bradford) were loaded into 12% or 8% polyacrylamide gel for electrophoresis. The separated proteins were blotted on a low fluorescent PVDF membrane (GE Lifescience) using a standard apparatus for wet transfer with an electrical field of 80 mA for 4h. The blotted membrane was blocked with 5% BSA (or casein) in TBS-Tween and probed with the primary antibody. The used antibodies were: rabbit anti-gammaH2AX [phospho S139] (Abcam), mouse anti-PADPR [H10] (Abcam), rabbit anti-Rad51 (Thermo Fisher), rabbit anti-MYC [Y69] (Abcam), rabbit anti-Actin (Sigma Aldrich). Binding was revealed by a Cy5-labelled secondary antibody (anti rabbit-IgG, GE Lifescience; anti mouse-IgG, Jackson Immuno-Research). All incubation steps were performed according to the manufacturer's instructions. Fluorescence of the blots was assayed with the Pharos FX scanner (BioRad) at a resolution of 100 µm, using the Quantity One software (BioRad).

2.7 Flow cytometry assay of ROS production

Cells (1 x 10^6) were collected after 6h treatment with LDH inhibitors (LDH IC_{50} dose), cisplatin (6 µM) given alone or in association. After centrifugation and washing with PBS, the cells were maintained for 30 min in the dark in a solution containing 10 µM 2-7-dichlorofluorescein diacetate (DCF). ROS production was analyzed by flow cytometry using 10.000 cells in the FACSCalibur system (Becton-Dickinson, San Jose, CA, USA).

2.8 Colony formation assay

Loukes cells were treated with cisplatin (3 µM, 1h) and LDH inhibitors (15 mM OXA or 30 µM GF, 6h) given alone or in combination. In the cultures treated with the drug combination, cisplatin was added during the last hour of OXA/GF treatment. Each dose was tested in triplicate. These short exposure times were chosen since the heavy toxicity of longer treatments were previously found to completely abolish cell clonogenicity,

impeding the quantification of effects. After the treatment, cells were centrifuged to remove the drug containing medium and were seeded in 24 well plates (1000 cells/well) in 1 ml complete RPMI 1640 containing 1% methylcellulose and incubated at 37 °C in humidified atmosphere for 15 days. Colonies of more than 50 cells were scored visually under an inverted microscope by two independent observers.

2.9 Statistical analyses

All analyses were performed using the software GraphPad Prism 5. Each experiment was repeated twice with at least triplicate samples per treatment group. Results are expressed as mean ± SE of replicate values. Analysis was performed by ANOVA corrected by Dunnet's test or Student's t test. All tests of statistical significance were two-sided and p values < 0.05 were considered statistically significant.

3. Results

3.1 LDH inhibition

In order to calculate the doses causing 50% inhibition of LDH activity (LDH IC_{50}) in the selected cellular model, cultures of Loukes cells were incubated for 6h in the presence of scalar amounts of either compound. Inhibition of LDH was evaluated through the reduction of produced lactate; the calculated LDH IC_{50} doses were 15 mM for OXA and 30 μM for GF. Table 1 shows the effects caused by these doses of inhibitors on ATP production and NAD/NADH ratio (which were measured after 6h incubation) and on cell viability (assayed at 24h). LDH IC_{50} doses were used for all the reported experiments with cisplatin association. Both higher and lower doses of inhibitors were not included in the

study because the former would have heavily affected cell viability and the latter were considered to insufficiently hinder enzymatic activity.

3.2 LDH inhibitors cause additive effects on cisplatin toxicity in Loukes cells

Fig. 1 shows that both OXA and GF, given at their LDH IC_{50} dose, significantly increased the effects of cisplatin (3 and 6 μM) on Loukes cells. The statistical evaluations reported on the graph were performed by ANOVA and compared the results obtained with cisplatin with those caused by its association with either LDH inhibitor. A similar evaluation (for the sake of brevity not shown on the graph) was performed by comparing the effect of either LDH inhibitor with its combination with the two cisplatin doses and also gave indications of statistically significant differences ($p < 0.05$).

One of the most commonly followed methods for analyzing the effects of the interaction between pharmacologically active compounds requires the development of the so-called isobologram, which reports the proper compounds' combinations that produce an iso-effective curve (Breitinger, 2012). The need to maintain reliable levels of LDH inhibition in cells exposed to cisplatin did not allow the application of the isobole method. For this reason, to analyze the effect of the interaction between cisplatin and LDH inhibitors we followed the method described by Dos Santos Ferreira et al. (2012), which is based on the calculation of an Interaction Index (R), obtained through the equation reported in Materials and Methods (see above). This equation was applied to the results of three independent experiments. The results are reported in the Table shown on Fig. 1 and clearly indicate for both OXA and GF the capacity of causing additive effects on cisplatin toxicity.

3.3 Exploring mechanisms underlying the increased cisplatin toxicity

The major function of LDH is the regeneration of oxidized NAD, required to sustain the glycolytic flux (Fiume et al., 2014). Besides working as electron acceptor for cellular

dehydrogenases, NAD is also the substrate of poly-ADP-ribose polymerases (PARPs) (Di Stefano et al., 2013). PARPs are crucial enzymes in DNA repair and their catalytic activity is strongly induced by binding to single strand DNA breaks, one of the evidenced lesions after cisplatin treatment. Since DNA repair by PARPs requires high NAD amounts, needed to build up the poly-ADP-ribose moieties (Di Stefano et al., 2013), we hypothesized that, by hindering NAD regeneration, LDH inhibitors could affect the repair of cisplatin induced lesions.

Fig. 2A shows that after 16h exposure to the IC_{50} dose of OXA(GF), NAD content of the Loukes cells was reduced. A similar, but less prominent effect was caused by cisplatin, which is known to activate PARP mediated response repair (Olaussen, et al., 2013). NAD levels appeared more severely affected in cultures treated with the association of the LDH inhibitor and cisplatin. When poly-ADP-ribosylation of the cellular proteins was measured by Western Blot (Fig. 2B), we found markedly increased signals of poly-ADP-ribosylation in cells treated with the association of cisplatin and OXA(GF). This result suggested that the increased antineoplastic effect caused by the compounds' association might not be due to a compromised repair, but to an increased DNA damage.

To verify this hypothesis, we measured in treated cells the levels of phosphorylated histone H2AX and of Rad51. Serine 139 phosphorylation of H2AX is one of the earliest markers of DNA damage (single and double strand breaks) (Sharma et al., 2012). It was found to increase linearly with the severity of the damage and to mediate the formation of clusters of proteins involved in DNA repair. Rad 51 (a recombinase) is one the recruited proteins and its increased expression indicate the induction of the DNA damage response (Klein, 2008). To obtain better evidence of the effects caused by LDH inhibition on the cisplatin induced damage, this experiment was performed in two steps: A) Loukes cells were exposed to cisplatin (1h); B) after a 24h delay, treated and control (untreated) cultures received a further 16h treatment with OXA(GF). Results are summarized in Fig. 3

and showed that 1h treatment with 6 μM cisplatin was sufficient to induce in Loukes cells a DNA damage response, as evidenced by increased phospho-H2AX and Rad51 levels (Fig. 3A). At the end of step B (Fig. 3B), in cisplatin treated cells phospho-H2AX signal is slightly reduced, while signs of DNA damage are evidenced in cultures treated with the LDH inhibitor alone. Cells receiving the association cisplatin/OXA(GF) showed a markedly enhanced phospho-H2AX level which, however, was not joined with a concurrent increase in Rad51 protein. On the contrary, these cultures showed evidences of Rad51 signal reduction, suggesting that the severity of DNA damage might have caused the failing of the response repair. This is in agreement with the increased antineoplastic effect produced by the compounds' association (Fig. 1). A further confirmation came from the heavily reduced levels of MYC protein observed in these cells (Fig. 3B), which is expected to deprive Loukes cultures of survival-needed signaling (Brady et al., 2008; Zhao et al., 2013). The above described effects were observed by using both LDH inhibitors, although they seemed best evidenced in OXA treated cells.

The slightly increased H2AX phosphorylation observed in cells only treated with OXA(GF) indicated that signs of DNA damage can also be observed after LDH inhibition. In cells consuming high glucose amounts, such as cancer cells, high amounts of pyruvate can be produced, which can lead to saturation of the mitochondrial electron transport chain and ROS generation (Lu et al., 2015), especially in cells with a less efficient respiratory function. ROS generation after LDH inhibition was already evidenced in some cultured cancer cells (Le et al., 2010; Farabegoli et al., 2012). Fig. 4 shows that Loukes cells exposed for 6h to the LDH IC_{50} dose of each inhibitor produced ROS. In OXA treated cultures, cells showing ROS generation were increased by 40%, compared to control, untreated cultures; GF was found to cause a less marked increase: 30%, compared to control cells. The lower level of ROS in GF treated cells can be explained considering that the polyphenolic structure of GF molecule can contribute to a partial scavenging of ROS

(Di Meo, et al., 2013); this could also justify the less marked effects caused by the combination cisplatin/GF on the DNA damage markers shown in Fig. 3B. Fig. 4 also shows that, in agreement with published data (Marullo, et al., 2013), ROS generation is not evidenced within the first 6h of cisplatin exposure, and that the ROS levels measured at this time in Loukes cells treated with the association of cisplatin and OXA/GF can be ascribed to LDH inhibition.

DNA damage is considered an inevitable consequence of ROS derived metabolism (Cooke, et al., 2003). The occurrence of oxidative DNA lesions, which are added to those caused by cisplatin, can give an explanation of the increased antineoplastic effect observed in the cells treated with the association cisplatin/OXA(GF).

3.4 Clonogenic survival

In MYC over-expressing BL cells, LDH silencing was found to reduce cloning efficiency (Shim et al., 1997) and suppression of MYC activity was related with reduced cell survival (Zhao et al., 2013). On the basis of these findings, we evaluated the clonogenic growth of Loukes cells, treated with cisplatin given alone or in combination with OXA/GF. The results of this assay (Fig. 5) were in agreement with the effects observed on cell viability. A 6h exposure to LDH inhibitors (see Materials and Methods) did not significantly affect the clonogenic survival of Loukes cells. However, when the treatment with LDH inhibitors was combined with 1h exposure to cisplatin, cell clonogenicity was severely impaired.

3.5 Experiments on non-neoplastic cells

As non-neoplastic, control cells for our experiments, we chose the GM00130C lymphoblastoid line. GM00130C cells have been obtained by immortalizing B lymphocytes by Epstein Barr virus infection. They are actively dividing cells, but do not proliferate

indefinitely. Therefore, Loukes and GM00130C derive from the same cell type and their substantial difference lies in the neoplastic nature of the Loukes cells. Compared to Loukes cells, GM00130C have reduced levels of LDH and produce lower amounts of lactate (Vettraino et al., 2013). GM00130C cells were treated with the same doses of cisplatin and LDH inhibitors already used on Loukes cells, replicating the experiments reported on Fig. 1. The results obtained on GM00130C cells are reported on Fig. 6A. In agreement with previous results, GM00130C were found less responsive to LDH inhibitors; when cisplatin was given in combination with either OXA or GF, no statistically significant difference was observed in comparison with cells treated with cisplatin alone.

Fig. 6B shows that in GM00130C cells treated with either LDH inhibitor, no significant production of ROS was detected. According to this result, the postulated mechanism underlying the increase of cisplatin toxicity observed in Loukes cells did not seem to take place in normal lymphoblasts, thus explaining the unchanged cisplatin toxicity.

4. Discussion

Increased glucose uptake and metabolism, a widely accepted hallmark of neoplastic change (Gillies et al., 2008), offer a therapeutic window to develop innovative anticancer strategies. To date, the metabolic features of cancer cells have been successfully exploited for diagnostic purposes (Gallamini et al., 2014), while no anticancer agent active on energetic metabolism gained established clinical application. LDH-A is the most promising target to develop glycolysis inhibitors with selective activity on cancer cells. In fact, this enzyme is not necessary for normal tissue survival, as witnessed by the observation that humans with a hereditary deficiency of this LDH isoform do not show any symptom under normal circumstances (Kanno et al., 1988; Maekawa et al., 1990; Miyajima et al. 1993; Tsujno et al., 1994). Unfortunately, in spite of extensive studies also

involving multinational drug companies (Ward et al., 2012; Billiard et al., 2013; Fauber et al., 2014; Xie et al., 2014), the development of LDH inhibitors suitable for clinical use is proving a very difficult task. Nevertheless, the availability of in vitro active compounds can allow at present a better understanding of the potentiality of LDH inhibition in anticancer treatments. This was also the aim of the present experiments.

Although active only at the millimolar range, OXA is the most used LDH inhibitor. It specifically hinders enzymatic activity by competing with pyruvate (Papacostantinou and Colowick, 1961). GF is a polyphenol recently identified by our research group through a Virtual Screening campaign (Manerba et al., 2012); contrary to OXA, GF is active at the micromolar range, both on purified human LDH and on lactate production in cultured cells. Cisplatin is one of the most potent anticancer agents and has been used in chemotherapy for more than 30 years. Despite its success, its effectiveness in the treatment of some tumor forms is limited because of acquired or intrinsic resistance (Kartalou and Essigmann, 2001). The molecular mechanisms that underlie cisplatin resistance are poorly understood; however, the finding of an up-regulated glucose metabolism in resistant cells suggested the attempt of increasing cisplatin efficacy by glycolysis inhibition (Dorward and Singh, 1996; Song et al., 2014). In the present experiments we verified this hypothesis in a Burkitt's lymphoma cell line treated with the association of cisplatin with OXA or GF, and compared the results obtained in these neoplastic cells with those produced by the same treatment in proliferating normal lymphocytes. A sensitizing effect on cisplatin activity was only found in lymphoma cells. Since proliferating lymphocytes are one of the most susceptible cell populations to the toxic effects of cisplatin-like chemotherapeutics, this result is a further confirm of the tolerability of LDH inhibition for normal cells. LDH inhibition could be expected to increase the efficacy of genotoxic agents by different mechanisms. By reducing ATP supply and NAD regeneration, it could primarily be responsible of an inadequate response to DNA damage. In fact, ATP is required for the chromatin

remodeling necessary to the recruitment of proteins involved in DNA repair (Swygert and Peterson, 2009), and NAD is the substrate of PARP enzymes (Di Stefano et al., 2013), which restore DNA structure. On the contrary, in cell cultures treated with the association of cisplatin with LDH inhibitors we obtained evidence of increased DNA damage, which was presumably ROS mediated. ROS generation was already found to lower the apoptotic threshold for cytotoxicity and, through this mechanism, to increase the efficacy of cytostatic treatments (Trachootham et al., 2009). In cells under cisplatin treatment, which also activates the PARP mediated repair response (Olaussen, et al., 2013), ROS produced by LDH inhibition could be expected to cause relevant effects, since poly-ADP-ribosylation of histones opens chromatin structure, rendering it more accessible to other potentially harmful factors.

Our data show that LDH inhibition led to ROS generation only in neoplastic cells, which differ from their normal counterpart in both high glucose uptake and less efficient mitochondrial function (Gillies et al., 2008). They are also in agreement with data obtained by using dichloroacetate, a non toxic compound hindering pyruvate dehydrogenase kinase (Xie et al., 2011). Dichloroacetate causes a metabolic shift from aerobic glycolysis to glucose oxidation and was similarly found to cause ROS generation in neoplastic cells and to increase cisplatin efficacy.

As stated above, in spite of important research investments, the development of LDH inhibitors suitable for clinical use does not seem within reach. The results obtained by combining OXA/GF with cisplatin, which is administered intravenously at weeks intervals, could also have the advantage of facilitating the access to clinical trials of new molecules effective on LDH inhibition but showing low oral availability, which is a major reason for drug candidates failing to reach the market.

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Legends to Figures

1. Combination experiments of LDH inhibitors (OXA and GF) with cisplatin. OXA and GF were given at their LDH IC_{50} dose (15 mM OXA and 30 μM GF). After 24h incubation the viability of Loukes cells was determined by Trypan blue exclusion. The Table shows the Interaction Index (R) of the two LDH inhibitors with cisplatin, calculated according to Dos Santos Ferreira et al. (2012) and using the formula reported in Materials and Methods. "R" values are indicative of additive effects.
2. Frame A: Reduction of NAD levels measured in Loukes cells 16h after incubation with 15 mM OXA and 30 μM GF (LDH IC_{50} dose), given alone or in association with 6 μM cisplatin. Frame B: poly-ADP-ribosylation level of cellular proteins measured in Loukes cells 16h after incubation with 15 mM OXA or 30 μM GF, given alone or in association with 6 μM cisplatin.
3. Association of LDH inhibitors (OXA and GF) with cisplatin increases DNA damage. Frame A: Loukes cells were exposed for 1h to 6 μM cisplatin. After 24h increased levels of phospho-H2AX and Rad51 indicated the induction of a DNA damage response. Frame B: Loukes cells were subsequently exposed for 16h to OXA (15 mM) or GF (30 μM). A further increase of phospho-H2AX level was evidenced. The simultaneous heavy reduction of MYC concentration suggests a compromised cell survival.
4. ROS levels measured by DCF in Loukes cells after 6h exposure to 15 mM OXA, 30 μM GF and 6 μM cisplatin, given alone or in association. The bar graphs indicate the percentage of gated cells showing a fluorescence signal higher than the median value measured in untreated cells.
5. Colony formation assay performed on Loukes cells. Cispl., cisplatin. * Statistically significant difference ($p < 0.05$) from cultures treated with OXA and with cisplatin. **

Statistically significant difference ($p < 0.05$) from cultures treated with GF and with cisplatin.

6. Results obtained in normal immortalized lymphocytes. The table shows the effects caused by LDH inhibitors (6h incubation) on lactate and ATP production. The bar graph in frame A shows the effects caused on cell viability by cisplatin given alone or in combination with OXA (15 mM) or GF (30 μM). Frame B: ROS detection after 6h exposure to 15 mM OXA and 30 μM GF.

Table 1

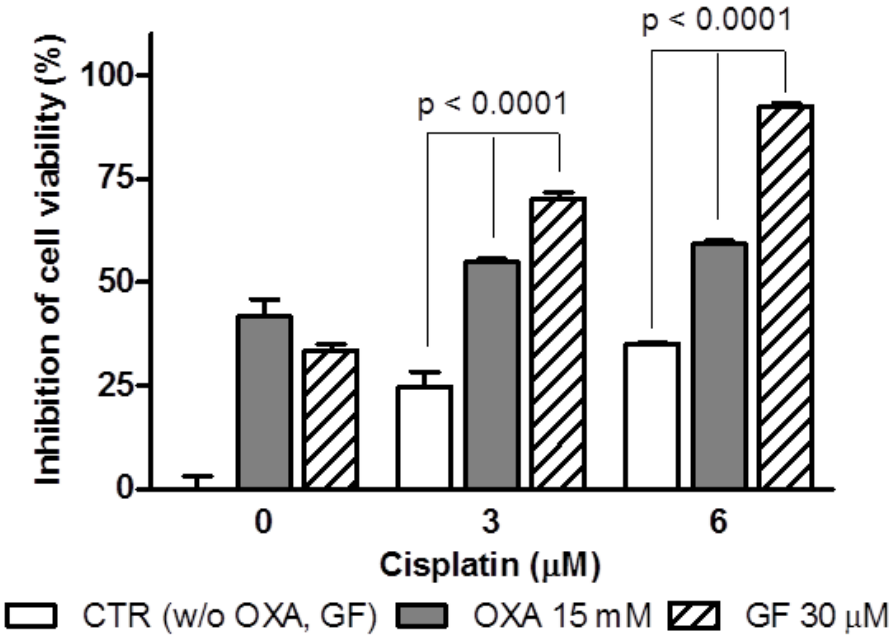
Effects of the selected doses of inhibitors on Loukes cells

% Reduction caused on:	OXA 15 mM	GF 30 μM
Lactate levels	50*	50*
NAD/NADH ratio	43 ± 3	46 ± 2
ATP	21 ± 4	25 ± 3
Cell Viability	42 ± 7	35 ± 5

Effects on lactate and ATP levels and on NAD/NADH ratio were assayed at 6h; cell viability was measured at 24h.

* Calculated from the second order linear regression of experimental data obtained by exposing Loukes cells to scalar doses of inhibitors (see Materials and Methods)

Figure 1



Interaction Index

LDH Inhibitor	Cisplatin (μM)	R
OXA 15 mM	3	1.04 ± 0.04
	6	1.08 ± 0.02
GF 30μM	3	0.96 ± 0.02
	6	0.90 ± 0.03

Figure 2

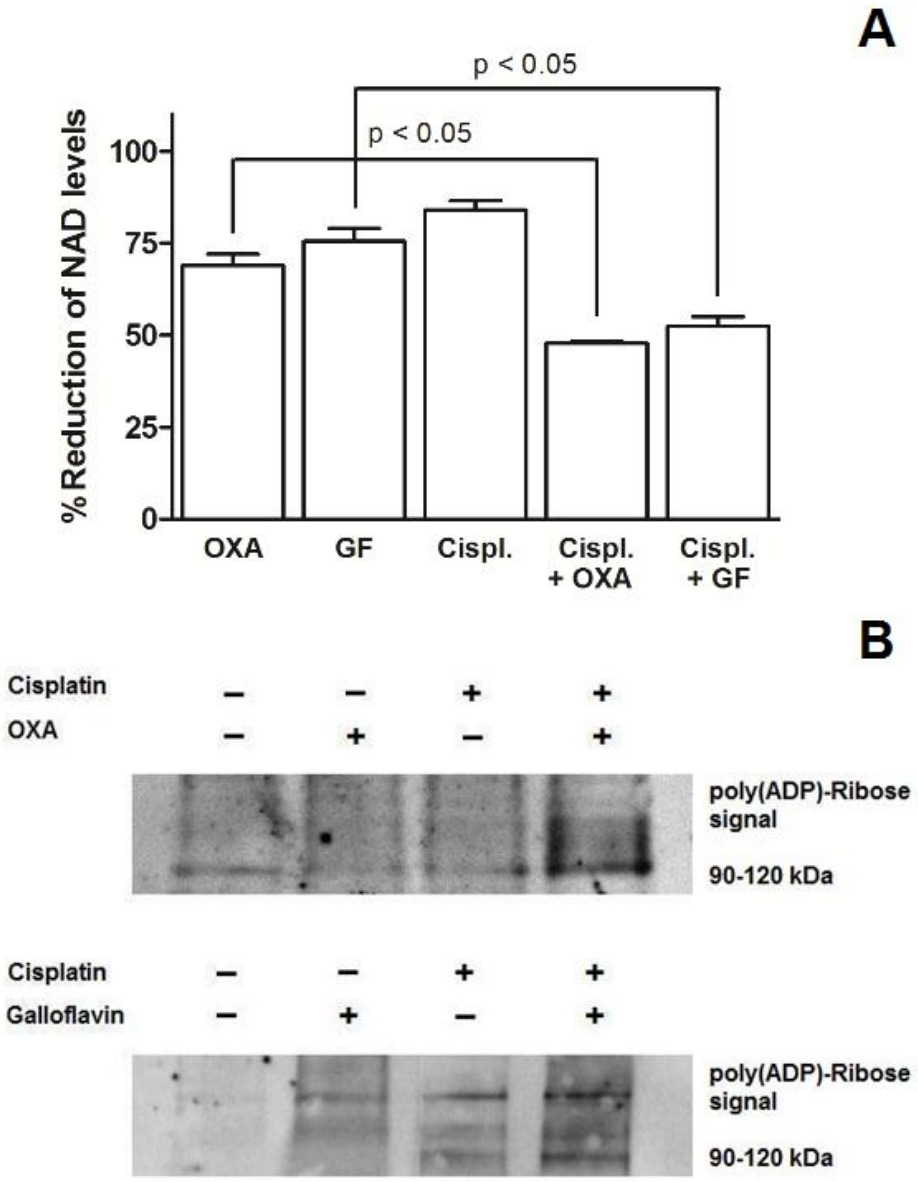

A
p < 0.05
p < 0.05
% Reduction of NAD levels
100
75
50
25
0
OXA
GF
Cispl.
Cispl. + OXA
Cispl. + GF
B
Cisplatin
OXA
poly(ADP)-Ribose signal
90-120 kDa
Cisplatin
Galloflavin
poly(ADP)-Ribose signal
90-120 kDa

Figure 3

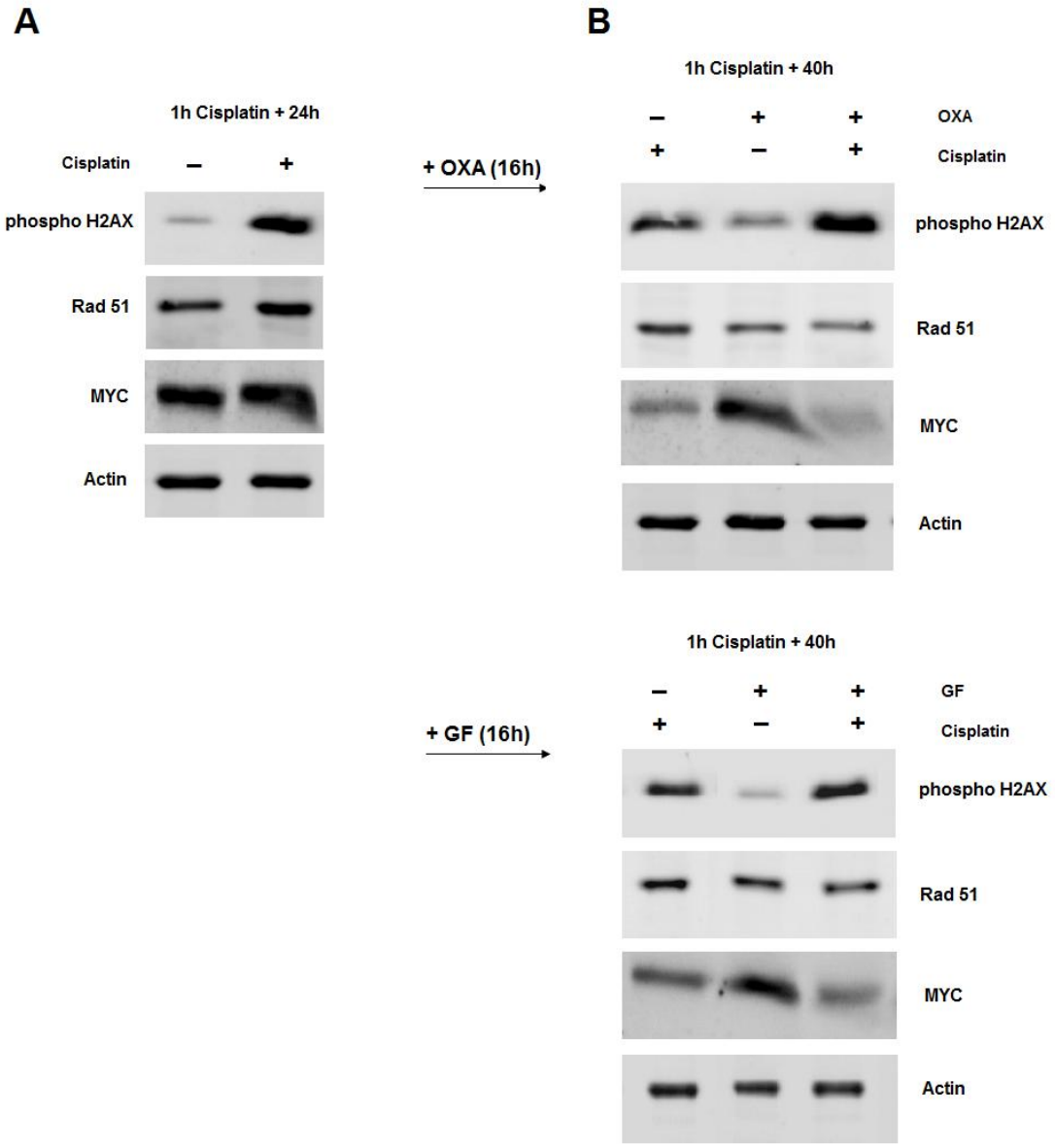

A
1h Cisplatin + 24h
Cisplatin – +
phospho H2AX
Rad 51
MYC
Actin
+ OXA (16h)
B
1h Cisplatin + 40h
– + + OXA
+ – + Cisplatin
phospho H2AX
Rad 51
MYC
Actin
+ GF (16h)
1h Cisplatin + 40h
– + + GF
+ – + Cisplatin
phospho H2AX
Rad 51
MYC
Actin

Figure 4

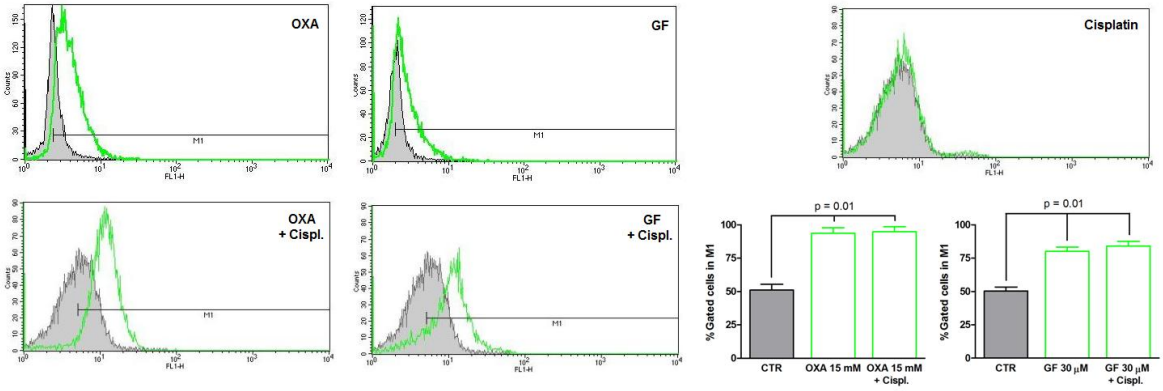

OXA
GF
Cisplatin
OXA
+ Cispl.
GF
+ Cispl.
M1
FL1-H
Counts
p = 0.01
% Gated cells in M1
CTR
OXA 15 mM
OXA 15 mM
+ Cispl.
p = 0.01
% Gated cells in M1
CTR
GF 30 μM
GF 30 μM
+ Cispl.

Figure 5

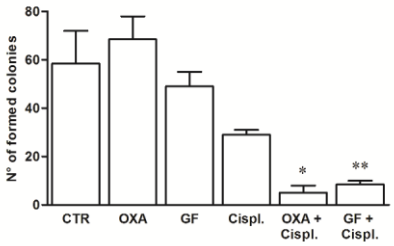
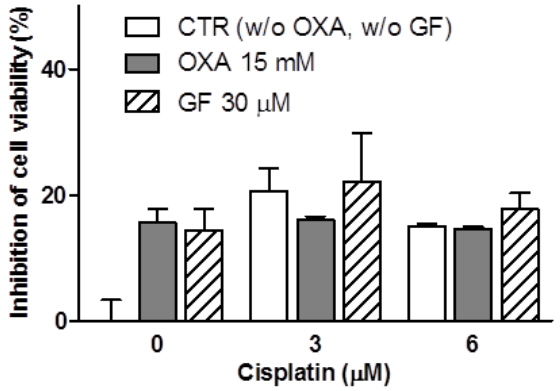

N° of formed colonies
80
60
40
20
0
*
**
CTR
OXA
GF
Cispl.
OXA + Cispl.
GF + Cispl.

Figure 6

Effect of LDH inhibitors on GM00130C cells

% Inhibition caused on:	OXA 15 mM	GF 30 μM
Lactate levels	11.6 ± 0.3	10.4 ± 1.6
ATP	13.3 ± 2.1	10.5 ± 0.8
Cell Viability	15.6 ± 2.2	14.3 ± 3.6

A



B