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Lactate dehydrogenase inhibitors sensitize lymphoma cells to cisplatin without enhancing the drug effects on immortalized normal lymphocytes

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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.

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

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

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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.

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

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 $1–2 \times 10^5$ 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. 5×10^5 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 6 h 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 6 h 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 24 h as described on paragraph 2.4. NAD/NADH ratio was measured after 6 h incubation as described in Section 2.5.

2.4. Combination experiment of cisplatin with OXA(GF)

Loukes and GM00130C cells (1×10^5 in 24-multiwell plates) were incubated for 24 h 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:

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 1.5×10^6 Loukes cells. After incubation (6 h or 16 h) 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/1.5×10^6 cells) containing 20 mM sodium bicarbonate, 100 mM sodium carbonate, 10 mM nicotinamide and 0.1% Triton-X100. The cell lysate was centrifuged at 16,000*g*, 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 16,000*g*, 25 °C for 30 s, and the absorbance at 570 nm of the supernatant was measured for 120 s with 10 s 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