Alma Mater Studiorum - Università di Bologna

DOTTORATO DI RICERCA IN

Scienze Biomediche: progetto n°3 " Fisiologia Applicata e Fisiopatologia"

Ciclo XXVI

Settore consorsuale di afferenza: 06/A2
Settore scientifico disciplinare: MED/04

THE INHIBITION OF AEROBIC GLYCOLYSIS AS A THERAPEUTIC APPROACH TO IMPROVE CANCER CHEMOTHERAPY

Presentata da: Dott.ssa Marina Eleonora Vettraino

Coordinatore Dottorato:
Prof. Lucio Cocco

Relatore:
Prof.ssa Giuseppina Di Stefano

Esame finale anno 2014

INDEX

Chapter I

Introduction

Cellular energy metabolism is one of the main process that is affected during the transition from normal to cancer cells. This was recognized several years ago by Warburg (1930) who observed that neoplastic cells produce high amounts of lactic acid even in the presence of oxygen (Warburg O, 1930). This metabolic behaviour, named aerobic glycolysis, was attributed by Warburg to an impairment of cell respiration with a consequent enhancement of glycolysis to cope with the high energetic needs of neoplastic cells.

Warburg also proposed that the impaired cell respiration was the prime cause of cancer. However, this theory was considered too simplistic, since it did not directly link the molecular mechanism of uncontrolled cell growth to the impaired respiration. Interest in the metabolic property of cancer continued up to the 1960s and began to decline concomitantly with the widespread application of newer molecular techniques (Gatenby RA and Gillies RJ, 2004). So, the view of cancer as a metabolic disease was gradually displaced with the view of cancer as a genetic disease. However, in recent years, interest has been renewed as it has become clear that many of the signalling pathways that are affected by genetic mutations have a profound effect on core metabolism, making this topic once again one of the most intense areas of research in cancer biology.

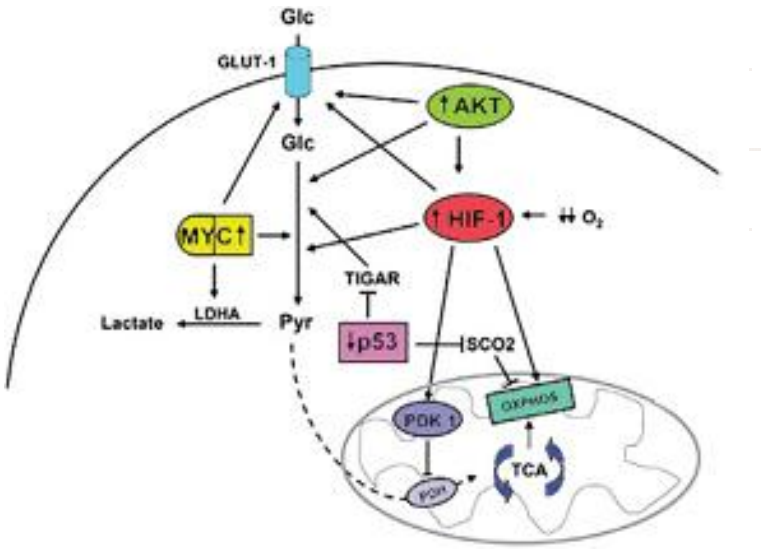
To date it is well documented that the metabolic reprogramming of cancer cells is the direct result of alterations in oncogenes and oncosuppressors (Levine AJ et al, 2010; Buchakjian MR et al, 2010). For instance, *c-myc* is known to transactivate most of genes of glycolytic enzymes, including lactate dehydrogenase A (LDH-A) and the glucose transporter 1 (GLUT1), and to enhance both glucose uptake and

lactate production (Osthus RC et al, 2000; Shim H et al, 1997). It has been proposed that the hypoxic environment, where tumors develop, promotes the stabilization of hypoxia inducible factor 1 alpha (HIF1α) which in turn induces the expression of glycolytic enzymes (aldolase, phosphoglycerate kinase, phosphofructokinase, pyruvate kinase and LDH-A) and GLUT1 (Semenza GL et al, 1994; Elbert BL et al, 1995). As a further example, hyperactivation of Akt, which is one of the most frequently observed mutations in human cancers, was reported to contribute both to the increased proliferation of cancer cells and to their highly glycolytic phenotype (Robey RB and Hay N, 2009).

The tumor suppressor protein p53 has also been involved in the glycolytic shift of cancer cells. On the one hand, overexpression of a dominant negative mutant p53 can be found in some tumors and leads to the induction of hexokinase II (HK-II) and to an increased glucose uptake (Mathupala SP et al, 1997; Smith TA et al, 2006). On the other hand, loss-of-function of the tumor suppressor p53 promotes an enhanced glycolytic phenotype by the repression of TIGAR (TP53-induced glycolysis and apoptotic regulator) expression, which normally down-regulates fructose-2,6-bisphosphatase, thus decreasing the glycolytic rate. Likewise, loss-of-function of p53 diminishes the expression of SCO-2, a gene required for the appropriate assembly of cytochrome c oxidase, and thus limits the activity of mitochondria in the cancer cells (Bensaad K et al, 2006).

The glycolysis-based metabolism of cancer cells is further promoted by the expression of a spliced variant form of pyruvate kinase (PKM2), which is less

active and was found to cause reduced oxygen consumption and increase lactate production (Vander Heiden MG et al, 2010).

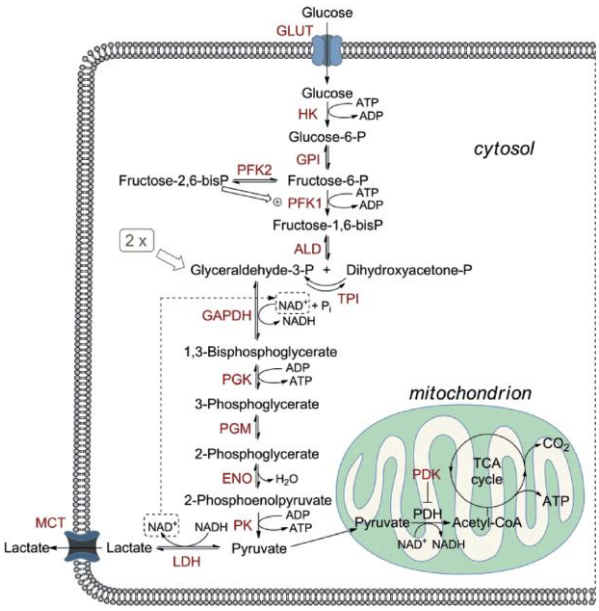


Modified from Ortega AD et al, 2009

Figure 1. Genetic alterations underlying the glycolytic phenotype of cancer cells.

Glycolysis and "aerobic glycolysis"

The first biochemical reactions of glucose metabolism, which generate two residues of reduced NAD and of pyruvic acid from one molecule of sugar, are the same in normal and in neoplastic cells. In normal cells, NADH is then re-oxidized by the highly active respiratory chain and pyruvic acid, after decarboxylation to acetyl-CoA, enters the TCA cycle. On the contrary, in neoplastic cells reduced NAD is re-oxidized by pyruvic acid, a reaction catalyzed by the enzyme lactate dehydrogenase (LDH) which converts pyruvic into lactic acid. The regeneration of NAD by LDH activity allows a sustained glucose consumption with a build up of lactate (aerobic glycolysis). In aerobic glycolysis each molecule of glucose is metabolized to two residues of lactic acid, with a net gain of two ATP residues.



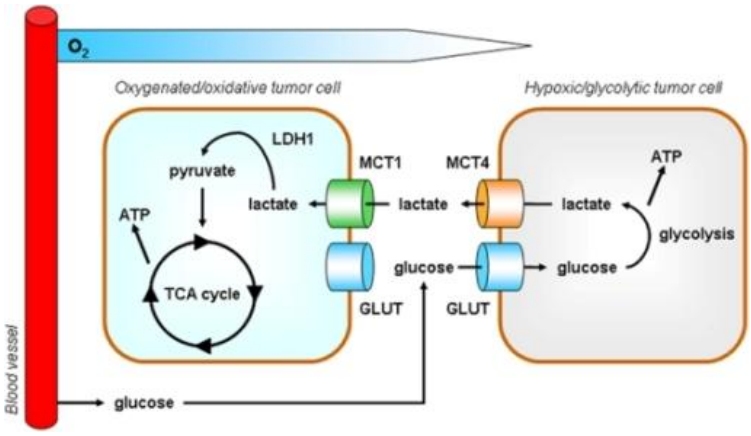
Granchi C et al, 2012

Figure 2. Glucose metabolism through the glycolytic flux.

Contrary to normal, non proliferating cells, which typically adopt a catabolic metabolism focused on maximizing energy yield through the complete demolition of glucose to CO2, cancer cells are characterized by an anabolic-type metabolism, aimed at biomass construction. As a result of increased glucose uptake and glycolysis, high levels of glycolytic intermediates are generated, which can be used to obtain the building blocks needed for macromolecular biosynthesis. Anabolic pathways branching from glycolysis are in fact responsible for producing some amino acids, as well as both lipid and nucleotide precursors (Christofk HR et al, 2008).

It is worth noting that this anabolic-type metabolism is not exclusive of cancer cells, but can also be observed in normal tissues when a rapid cell division is required, such as during embryonic development, in wound healing or during the activation of immune responses. Contrary to what supposed in past years, proliferating normal cells and a large part of cancer cells use glycolysis mainly to generate new macromolecules, and still derive a significant fraction of their ATP from oxidative phosphorylation (Bellance N et al, 2009). Glycolytic ATP becomes a more important energetic resource when the hypoxic microenvironment hinders mitochondrial function, such as in the healing wound bed or in hypo-vascularized tumor regions. The increased lactate production operated by LDH also contributes to the development of extracellular acidosis, a factor which finally facilitates tumor invasion and metastasis (Neri D and Supuran CT, 2011), although it should be reiterated that lactic acid is not just a discharge product; instead it actively participates in angiogenesis, migration, immune and

radio-resistance (Sonveaux P et al, 2008). Moreover it is involved in energy production thanks to a particular cell-cell shuttle system, also known as the "lactate shuttle" (Draoui N and Feron O, 2011). Tumor cells usually can be classified into two categories: "normoxive/glycolytic", which are closer to blood vessels, or "hypoxic/glycolytic", which are further away from the vascular network; these two cell types establish a symbiotic cell-cell shuttling of lactate. In general, glucose is actively taken up through the transporter GLUT into less oxygenated cells, which use it in the glycolytic process to produce ATP. The final conversion of pyruvate to lactate is catalyzed by lactate dehydrogenase 5 (LDH-5). The monocarboxylate transporter 4 (MCT4) then extrudes lactic acid from hypoxic cells into the extracellular milieu. Lactate then functions as a metabolic fuel in oxidative tumor cells, where it is taken up through the monocarboxylate transporter 1 (MCT1) and oxidized to pyruvate by LDH-1, thus entering the TCA cycle in mitochondria with production of energy and CO2.



Porporato PE et al, 2011

Figure 3. The lactate-shuttle system.

In some tumors, a symbiosis between lactate producing cells and lactate consuming cells was also observed. By studying the metabolic profile of neoplastic cells and associated fibroblasts obtained from breast cancer specimens, Pavlides et al. (Pavlides S et al, 2009) observed that cells from tumor stroma display metabolic features similar to those of wound-healing fibroblasts and secrete high levels of lactate and pyruvate originating from aerobic glycolysis. The Authors hypothesized that these energetic metabolites can be taken by epithelial cancer cells and used to fuel the TCA cycle, resulting in efficient energy production and in a consequent proliferative advantage for cancer cells. This proposed metabolic relationship between cancer stromal and parenchymal cells has been termed "reverse Warburg effect".

Inhibition of aerobic glycolysis as a possible strategy to hinder cancer cell proliferation

The observation that cancer cells exhibit increased glycolysis and are more dependent on this pathway to ATP generation has led to the evaluation of glycolytic inhibitors as potential anticancer agents. One of the first studied inhibitors was 3-bromopyruvate, a compound which inhibits hexokinase and has been shown to abolish ATP production and cause severe depletion of cellular ATP. 3-Bromopyruvate was shown to exhibit potent cytotoxic activity against cancer cells with mitochondrial respiratory defects and cells in hypoxic conditions (Pelicano H et al, 2006).

Another glycolytic inhibitor is 2-deoxyglucose (2-DG) which is a glucose analogue and has long been known to act as a competitive inhibitor of glucose metabolism. 2-DG is phosphorylated by hexokinase to 2-DG-phosphate, but unlike glucose-6-phosphate, cannot be further metabolized by phosphohexokinase isomerase which converts glucose-6-phosphate to fructose-6-phosphate. In this manner, 2-DG causes ATP depletion, leading to blockage of cell cycle progression and cell death in vitro (Pelicano H et al, 2006).

Several other glycolysis inhibitors have been shown to have promising anticancer activity in vitro and in vivo, and some of them have entered clinical trials; however, it should be recognized that there are potential obstacles in using glycolytic inhibitors for cancer treatment: in fact, glycolytic enzymes are also necessary to glucose metabolism of normal cells and their inhibition can cause

severe disorders produced by the impaired glucose utilization. It is known that certain normal tissues including brain, retina and testis use glucose as the main energy source. In this way, inhibition of glycolysis may be potentially toxic for these tissues.

In the laboratory where I worked during my PhD project, studies were focused on lactate dehydrogenase (LDH), which converts pyruvate to lactate. LDH is the only enzyme whose inhibition should allow a blocking of aerobic glycolysis of tumor cells without damaging normal cells which in conditions of normal functional activity and sufficient oxygen supply do not need this enzyme. The LDH inhibition as an approach to anticancer was proposed several years ago (Fiume L, 1960; Papacostantinou J and Colowick SP, 1961a; Papacostantinou J and Colowick SP, 1961b) and is currently under active investigation (Manerba M et al, 2012; Granchi C et al, 2010; Ward RA et al, 2012). Interest in LDH as an anticancer therapeutic target also comes from the observation that this enzyme (namely its –A isoform) becomes constantly up regulated during neoplastic change (Levine AJ et al, 2010; DeBerardinis RJ et al, 2008; Buchakjian MR et al, 2010; Dang CV and Gao P, 2009), offering the possibility of a therapeutic intervention aimed at correcting this altered activity. LDH-A is trascriptionally up-regulated by HIF-1, and it was found to be correlated with aggressive phenotypes and poor prognosis in several tumor types (Koukourakis MI et al, 2003; Kolev Y et al , 2008). Early literature data indicated that up-regulation of LDH-A under *c-myc* control ensures efficient aerobic glycolysis in tumor cells, conferring growth advantage (Shim H et al, 1997), whereas this enzyme does not

seem to be as necessary for healthy cells under normal conditions, as they generally use the aerobic oxidation pathway. A definitive confirmation of the key role of LDH-A in tumor maintenance was obtained by inhibition of LDH-A expression by shRNA, which decreased the ability of tumors to proliferate under hypoxic conditions and stimulated mitochondrial respiration (Fantin VR et al, 2006; Wang ZY et al, 2012). Moreover, increased expression of LDH-A plays an important role in resistance of human breast cancer cells against paclitaxel and trastuzumab: this observation was proven by genetically down-regulating LDH-A, which led to a significantly increased sensitivity of resistant cells to both anticancer agents (Zhou M et al 2010; Zhao Y et al, 2011). Furthermore, LDH-A knockdown by shRNA, in fumarate hydratase deficient cells which rely on glycolytic metabolism, results in a significant decrease in tumor growth in a xenograft mouse model of renal cancer (Xie H et al, 2009).

Interest in LDH as an anticancer therapeutic target also comes from the observation that, in normal cells, inhibition of LDH enzymatic activity should not cause harmful effects, as can be deduced by studying individuals with LDH genetic deficiencies; in fact it is known that patients with a hereditary deficiency of the A (Kanno T et al, 1988) or B (Motoshi K et al,1971; Miwa S et al, 1971; Okumura N et al, 1999) LDH isoform are free of symptoms, except for muscle rigidity and myoglobinuria, which can appear in individuals with LDH-A (Kanno T et al, 1988) deficiency after strenuous exercise. Overall, these observations encourage to study the validity of an anticancer therapeutic approach based on

LDH inhibition and justify the present efforts aimed at identifying small molecule inhibitors of this enzyme.

Lactate dehydrogenase (LDH)

LDH enzyme inter-converts pyruvate and lactate using nicotinamide adenine dinucleotide (NAD) as a cofactor.

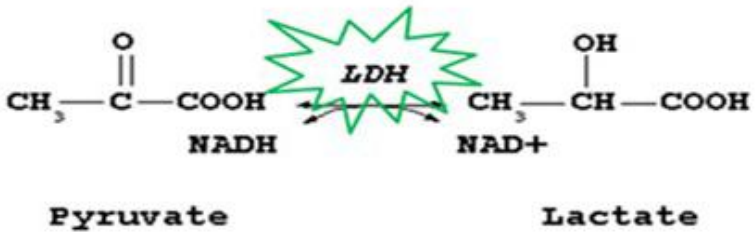


Figure 4. LDH enzymatic reaction.

This (or similar) enzymatic activity has been characterized in organisms of the three domains of life (Archaea, Bacteria and Eukarya) (Madern D, 2002) and was evolutionarily conserved since it can be an important source of ATP and oxidized NAD for living organisms during periods of transient anaerobiosis.

As well as the enzyme of other vertebrates, human LDH is a 140 kDa tetrameric molecule which exists in different isozymes, generally composed by the association of two different 35 KDa subunits (Markert CL et al, 1975). In vertebrates (including humans) three LDH-encoding loci have been identified in DNA: *ldh-a*, *ldh-b* and *ldh-c*, which produce the M (muscle) subunit, also named

LDH-A, H (heart) subunit, also name LDH-B and X subunit, respectively. The three genetic loci show 71-75% sequence identity, indicating extensive conservation in amino acid composition of the LDH subunits (Holmes RS et al, 2009). In humans, the X subunit is almost exclusively found in spermatozoa; the isozyme composed by X subunits is usually called LDH-C and has been supposed to play a role in male fertility (Yu Y et al, 2001). The association of M and H subunits can give rise to five isozymes: the tetramers H4, H3M1, H2M2, H1M3 and M4.

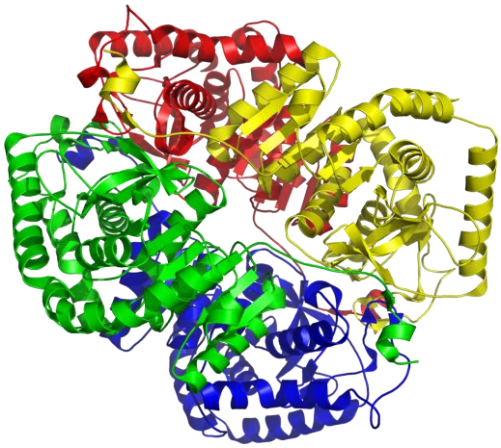


Figure 5. Lactate dehydrogenase M4.

These five isozymes have different electrophoretic mobility, which is dependent on their H subunit content. H4 is the fastest moving form and for this reason it is also reported as LDH-1; on the contrary, the slowest form, M4, is also named LDH-5. The LDH forms enriched in M subunit predominate in skeletal muscle (fast twitch glycolytic fibers) and liver, while those enriched in H subunits are primarily found in heart and brain. LDH-A and –B also display different kinetic

properties (Read JA et al, 2001). LDH-A requires higher pyruvate concentrations to reach the maximum activity: the Km for pyruvate is 158 μM for LDH-A and 58 μM for LDH-B (Eszes CM et al, 1996; Hewitt CO et al, 1999). Moreover, at substrate concentrations needed for optimal LDH-A activity, the –B isozyme is inhibited: the Ki for pyruvate is 770 μM for LDHB and 3900 μM for LDH-A (Eszes CM et al, 1996; Hewitt CO et al, 1999).

On the basis of these differences, Kaplan (1975) hypothesized a different physiological function for the LDH isozymes (Everse J et al, 1975). He proposed the so-called "aerobic-anaerobic theory" which postulates that the B type of LDH is suited to aerobic metabolism, while the A type is more suited to tissues exposed to oxygen limitation, such as the overworking skeletal muscle. However, later data put into question this theory, since it was observed that human erythrocytes, which do not have mitochondria, predominantly synthesize LDH-B and that individual organs of different animal species display large differences in the LDH isozyme pattern.

In normal conditions, the different LDH isozyme pattern expressed in tissues does not seem to play a different physiological role and in cytosol, in spite of their different kinetic parameters, both LDH-A and –B preferentially catalyze the reduction of pyruvate to lactate (Quistorff B et al, 2011).

Since in most tissues the total enzymatic activity is high compared to the metabolic flux observed in steady state conditions, the LDH catalyzed reaction is always close to equilibrium and, as a consequence, both LDH-A and –B operate in the same way, as it is the same chemical reaction being catalyzed. LDH

reaction could significantly deviate from its equilibrium only during extensive changes in glycolytic flux and energy metabolism. Therefore, it is only in these situations that a different physiological role of the isozyme pattern can be perceived.

LDH engaged in glycolysis is located in the cytosol; however, the enzyme is also present in peroxisomes, mitochondria and nuclei, where it exerts peculiar functions which so far may have not received due consideration in the studies aimed at inhibiting its activity.

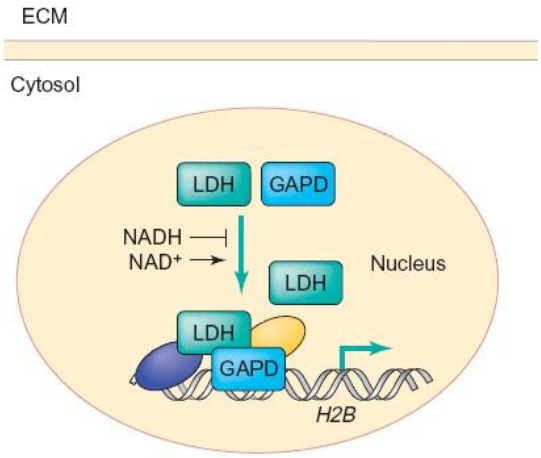
Peroxisomal LDH - In peroxisomes LDH is present in the matrix, where the A isoform predominates (Baumgart E et al, 1996; McClelland GB et al, 2003). One of the main function of peroxisomes is to shorten the longchain fatty acids (C > 22), which cannot be handled by mitochondria. The NADH generated by β-oxidation of the longchain fatty acid cannot be re-oxidized by the respiratory chain. A solid evidence was obtained that it can be reconverted to NAD by LDH in the presence of pyruvate (Baumgart E et al, 1996; McClelland GB et al, 2003). Lactate/pyruvate exchange is made possible by the monocarboxylate transporter present in the peroxisome membrane (McClelland GB et al, 2003).

Mitochondrial LDH – Glycolysis derived lactate can be re-oxidized and used as an energy source or as a precursor of gluconeogenesis (Brooks GA, 2009); re-oxidation takes place in mitochondria and can occur both in the cells of origin and in adjacent or distant cells, after lactate release in extracellular medium. The facilitated transport of lactate across cell membranes is accomplished by a family of monocarboxylate transport proteins differentially expressed in tissues (Brooks

GA, 2009). Through these transporters, lactate can also gain access to mitochondria, where it is oxidized to pyruvic acid by the mitochondrial form of LDH. In mitochondria, pyruvate dehydrogenase rapidly metabolizes pyruvic acid, reducing its concentrations and favoring the oxidation of lactate by LDH.

Nuclear LDH - Besides its widely known role in cell energy metabolism, the M subunit of LDH also displays an additional function in nuclei. Some years ago several authors observed that the LDH M subunit binds to single-stranded DNA (ssDNA) (Patel GL et al, 1980; Kaiserman HB et al, 1989). In analogy with what observed for other ssDNA-binding proteins (Shamoo Y, 2002) an involvement of the enzyme in DNA transcription and/or replication was suggested. This possibility was supported by the finding that nuclear LDH was localized on transcriptionally active regions of chromosomes (Patel GL et al, 1980; Williams KR et al, 1985). Moreover, the injection of the enzyme in cells of Chironomus salivary glands stimulated RNA synthesis in polytenic chromosomes, which on the contrary was inhibited by antibodies binding to LDH M subunit (Egyhazi E et al, 1989). More recently, a decisive proof of a role played by LDH in DNA transcription was obtained by Zhang, Luo and collaborators, who demonstrated that the enzyme is an essential component of the transcriptional complex of the histone 2B gene (H2B) (Dai RP et al, 2008). In this transcriptional complex, LDH M subunit is in association with glyceraldehyde 3-phosphate dehydrogenase (GAPDH) and the coordinated activity of the two enzymes maintain the proper NAD/NADH ratio necessary for optimal H2B expression (Dai RP et al 2008; He H et al, 2013). In contrast with cytoplasmic LDH, for which the minimal

functional unit was shown to be a dimmer (Wang XC et al, 1987), the nuclear enzyme is monomeric and is presumably maintained catalytically active by the supramolecular structure formed with GAPDH and with the other components of the transcriptional complex. GAPDH and LDH monomers were also found to form catalytically active complexes in vitro (Levashov AV et al, 1997). Since LDH M subunit is widely distributed within cell nuclei (Cattaneo A et al, 1985), its involvement in regulating the transcription of other genes cannot be excluded; these observations suggest that LDH could also participate in regulating survival pathways and that its inhibition in anticancer treatment might have effects not simply limited to the energy metabolism of transformed cells.



Modified from Kim JW and Dang CV, 2005

Figure 6. LDH in the transcriptional complex of the histone 2B gene.

The search for LDH inhibitors

The success of a therapeutic approach based on LDH inhibition chiefly depends on the availability of inhibitors with good target specificity. Unfortunately, in spite of extensive research, LDH proved to be a very difficult target for the development of new inhibitors, since all high throughput screening experiments have been usually characterized by a very low success rate (Ward et al, 2012).

One of the approaches adopted in the search of small molecules which could inhibit LDH enzyme is represented by the modification of already known inhibitors, like oxamic acid and gossypol, two longtime known inhibitors, displaying no selectivity between the –A and –B LDH isoforms. Oxamic acid is an analogue of pyruvic acid, the enzyme substrate. Although displaying a good selectivity for LDH (Papacostantinou and Colowick, 1961a) and a weak toxicity in healthy animals (Papacostantinou and Colowick, 1961b), oxamic acid has the drawback of a poor cellular penetration; as a consequence, it was found to inhibit the proliferation of tumor cells cultured in vitro only at millimolar concentrations, which cannot be expected to be reached in vivo. Starting from oxamic acid as a lead compound, some libraries of derivatives have been created, with the aim of improving the therapeutic potential of the molecule. Several of these molecules were synthesized and tested on LDH of *Plasmodium falciparum*, a parasite which relies on aerobic glycolysis for its energetic needs in order to improve the chemotherapy of malaria (Choi S et al, 2007; Deck LM et al, 1998).

Encouraging results were also obtained on the α-hydroxyacid dehydrogenase (HADH) of Trypanosoma cruzi, which shows similarities with the human LDH-C. Simple derivatives of oxamic acid such as N-isopropyl and N-propyl oxamate were found more active in inhibiting HADH in vitro than the parent compound and their ethyl-ester pro-drugs orally administered to Trypanosoma cruzi infected mice also demonstrated therapeutic efficacy in vivo (Aguirre-Alvarado C et al, 2007). To our knowledge, no oxamic acid derivative with anticancer potential has been identified with this approach.

Gossypol is a natural polyphenolic aldehyde derivative found in cotton seeds and it is a well-known nonselective LDH inhibitor competitive with NADH. However, it also inhibits other NAD-dependent enzymes such as glyceraldehydes-3-phosphate dehydrogenase (GADPH) (Gomez MS et al, 1997). Moreover, because of its structural characteristics gossypol can interact with different cellular components affecting several biological functions, which causes unspecific toxicity (Dodou K et al 2005; Lee CY et al, 1982). Contrary to oxamic acid, it displays in vitro activity at the micromolar level; for this reason, many gossypol analogues have been designed with the aim of reducing the molecule toxicity. The most promising one is a derivative called FX-11, selected as a potential anticancer drug for its ability to preferentially inhibit the A isoform of LDH (Le A et al, 2010). Furthermore, it was demonstrated that FX-11-mediated inhibition of LDH-A negatively affected cellular energy supply by depleting ATP levels, diminished cellular production of lactate, induced oxidative stress, and caused cell death. FX-11 also decreased growth in tumor xenograft models, such as human lymphoma

and pancreatic cancer (Le A et al, 2010). However, further studies have challenged the validity of FX-11, giving rise to the suspicion that some of the observed effects could be not specifically ascribed to LDH inhibition, but to the reactive nature of the catechol group of the molecule (Kohlmann A et al, 2013; Lee CY et al, 1982).

Another commonly procedure adopted for the search of pharmacologically active molecules that potentially could inhibit LDH is the screening of already established compound collections by using high throughput technologies. It was recently employed by researchers from Genentech Inc., who tested for LDH-A inhibition the compounds available in their own Company Repository and in the Roche collections. This screening identified 2-thio-6-oxo-1,6-dihydropyrimidine as a moderately potent LDH-A and LDH-B inhibitor, with only weak effects on other structurally related dehydrogenases, suggesting some specificity for LDH. In following experiments, a series of chemical modifications were introduced in the molecule structure with the aim of improving its LDH-A inhibition properties. Unfortunately, none of the obtained inhibitors, tested on cultured cell up to 50 μM, was found to reduce lactate production (Dragovich PS et al, 2013); additional studies are then needed to improve the biological properties of these inhibitors.

Recently researchers from GlaxoSmithKline announced the discovery of quinoline 3-sulfonamides as potent (nanomolar) LDH inhibitor, with good selectivity towards the A isoform (Billiard J et al, 2013). The details of the high throughput screening procedure leading to this discovery are at present un-published.

Moreover another strategy to find new potential LDH inhibitors is that adopted by a research team from the University of Pisa, which used an ex-novo design method to obtain new chemical structures by inserting –OH and –COOH groups into a N-hydroxyindole (NHI) scaffold (Granchi C et al, 2011). These compounds were designed on the basis of the "OH-COOH" pharmacophore motif often present in previously reported LDH inhibitors, as the active site normally hosts an α-hydroxy group (lactate) or an α-keto acid (pyruvate).

Several molecules obtained with this procedure were found to inhibit the lactate production in cancer cells, but no further experiments were performed with this class of compounds; a thorough examination of their biological characteristics would be useful to better evaluate their potential.

Fragment-based lead generation also proved to be a successful approach with a number targets (Rees DC et al, 2004). It requires a preliminary screening aimed at identifying small chemical fragments which, although not inhibiting the activity of target protein, show the property of binding to critical sites needed for its function, as, in the case of LDH, the NADH and the pyruvate binding regions. This preliminary selection is usually performed with the aid of NMR spectroscopy and/or surface plasmon resonance. The identified fragments are then linked together by the use of flexible spacers, yielding compounds with greatly improved potency and selectivity. This approach has been applied the first time to the search of LDH inhibitors by Moorhouse et al (Moorhouse AD et al, 2011). More recently, it has been simultaneously used by two different teams of researchers, in Astra-Zeneca and ARIAD Laboratories. By using this approach, Astra-Zeneca

researchers succeeded in obtaining some of the most potent LDH inhibitors now available (Ward RA et al, 2012); in particular the most active molecule showed in the enzyme inhibition assay a IC50 as low as 0.27 μM, but, unfortunately, was reported to lack activity in cell based assays, probably because of scarce cellular penetration. However, its dimethyl ester derivative, although displaying reduced enzyme inhibitory potency compared to the parental compound, was found to inhibit lactate production in cultured cells (IC50 = 4.8 μM). Researchers at ARIAD Pharmaceuticals obtained similarly potent molecules (Kohlmann A et al, 2013); one of the most promising was able to heavily reduce lactate production in cultured cells, although at rather high doses (200 μM).

Both the Astra-Zeneca and the ARIAD study are quite recent and to our knowledge further published reports are still lacking. New advances leading to the generation of more bioavailable molecules are expected, to make possible the start of anticancer experiments in vivo.

Finally, the structure-based virtual screening approach is a further method to search new LDH inhibitors. My research team in collaboration with the Department of Pharmaceutical Sciences of the University of Bologna, by adopting a virtual screening protocol based on the crystal structure of the human M subunit of LDH and applied to the molecules of the National Cancer Institute diversity set, identified a molecule, named galloflavin which is able to inhibit both the –A and –B LDH isoforms. The description of this new inhibitor and all its effects, showed in several cellular models, are presented in this thesis.

REFERENCES TO INTRODUCTION

- Aguirre-Alvarado C, Zaragoza-Martinez F, Rodriguez-Paez L et al. In vitro and in vivo trypanocydal activity of ethyl esters of N-allyl and N-propyl oxamates using different Trypanosoma cruzi strains. J. Enzyme Inhib. Med. Chem. 22, 227-233 (2007).

- Baumgart E, Fahimi HD, Stich A et al. L-Lactate dehydrogenase A4- and A3B isoforms are bone fide peroxisomal enzymes in rat liver. Evidence for involvement in intra-peroxisomal NADH oxidation. J. Biol. Chem. 271, 3846-3855 (1996).

- Bellance N, Lestienne P, Rossignol R. Mitochondria: from bioenergetics to the metabolic regulation of carcinogenesis. Front Biosci. 14, 4015-4034 (2009).

- Bensaad K, Tsuruta A, Selak MA et al. TIGAR, a p53-inducible regulator of glycolysis and apoptosis, Cell 126 (1) 107–120 (2006).

- Billiard J, Dellison JB, Brain J et al. Quinoline 3-sulfonamides inhibit lactate dehydrogenase A and reverse aerobic glycolysis in cancer cells. Cancer and Metab. 1, 19 (2013).

- Brooks GA. Cell-cell and intracellular lactate shuttles. J. Physiol. 587 23, 5591-5600 (2009).

- Buchakjian MR, Kornbluth S. The engine driving the ship: metabolic steering of cell proliferation and death. Nat. Rev. Mol. Cell Biol. 11, 715-727 (2010).

- Cattaneo A, Biocca S, Corvaja N et al. Nuclear localization of a lactic dehydrogenase with single-stranded DNA-binding properties. Exp. Cell Res. 161, 130-140 (1985).

- Choi S, Beeler AB, Pradhan A, Watkins EB, Rimoldi JM, Tekwani B, et al. Generation of oxamic acid libraries: antimalarials and inhibitors of Plasmodium falciparum lactate dehydrogenase. J Comb Chem 9, 292-300 (2007).

- Christofk HR, Vander Heiden MG, Wu N et al. Pyruvate kinase M2 is a phosphotyrosine-binding protein. Nature 452, 181-186 (2008).

- Dai RP, Yu FX, Goh SR et al. Histone 2B (H2B) expression is confined to a proper NAD+/NADH redox status. J. Biol. Chem. 283, 26894-26901 (2008).

- Dang CV, Le A and Gao P. MYC-induced cancer cell energy metabolism and therapeutic opportunities. Clin. Cancer Res. 15, 6479-6483 (2009).

- DeBerardinis RJ, Lum JJ, Hatzivassiliou G et al. The biology of cancer: metabolic reprogramming fuels cell growth and proliferation. Cell Metab. 7, 11-20 (2008).

- Deck LM, Royer RE, Chamblee BB, Hernandez VM, Malone RR, Torres JE, et al. Selective inhibitors of human lactate dehydrogenase and lactate dehydrogenase from malarial parasite Plasmodium falciparum. J Med Chem 41, 3879-87 (1998).

- Dodou K, Anderson RJ, Small DA et al. Investigations on gossypol: past and present developments. Expert Opin. Drugs 14, 1419-1434 (2005).

- Dragovich PS, Fauber BP, Corson LB et al. Identification of substituted 2-thio-6-oxo- 1,6 dihydropyrimidines as inhibitors of human lactate dehydrogenase. Bioorg. Med. Chem. Lett. 23, 3186–3194 (2013).

- Draoui N, Feron O. Lactate shuttles at a glance: from physiological paradigms to anti-cancer treatment Dis. Model. Mech. 4, 727–732 (2011).

- Ebert BL, Firth JD, Ratcliffe PJ. Hypoxia and mitochondrial inhibitors regulate expression of glucose transporter-1 via distinct Cis-acting sequences. J. Biol. Chem. 270 (49) 29083–29089 (1995).

- Egyhazi E, Holst M, Pigon A et al. Effects of a DNA helix-destabilizing protein on transcription in living cells. Eur. J. Cell Biol. 48, 88-94 (1989).

- Eszes CM, Sessions RB, Clarke AR et al. Removal of substrate inhibition in a lactate dehydrogenase from human muscle by a single residue change. FEBS Lett. 399, 193-197 (1996).

- Everse J, Kaplan NO. Mechanisms of action and biological functions of various dehydrogenase isozymes. In: Isoenzymes II – Physiological function. Markert CL (Ed.), Academic Press, New York, USA, 29-43 (1975).

- Fantin VR, St-Pierre J, Leder P. Attenuation of LDH-A expression uncovers a link between glycolysis, mitochondrial physiology, and tumor maintenance. Cancer Cell 9, 425 –434 (2006).

- Fiume L. Inhibition of aerobic glycolysis in Yoshida ascites hepatoma by tartronic acid. Nature 187, 792-793 (1960).

- Gatenby RA, Gillies RJ. Why do cancer have high aerobic glycolysis? Nature Rev Cancer 4, 891-899 (2004).

- Gillies RJ, Robey I, Gatenby RA. Causes and consequences of increased glucose metabolism of cancers. J. Nucl. Med. 49, 24S-42S (2008).

- Granchi C, Bertini S, Macchia M et al. Inhibitors of lactate dehydrogenase isoforms and their therapeutic potentials. Curr. Med. Chem. 17, 672-697 (2010).

- Granchi C, Roy S, Giacomelli C et al. Discovery of N-hydroxyindole-based inhibitors of human lactate dehydrogenase isoform A (LDH-A) as starvation agents against cancer cells. J. Med. Chem. 54, 1599-1612 (2011).

- Gomez MS, Piper RC, Hunsaker LA, Royer RE, Deck LM, Makler MT, Vander Jagt DL, Mol. Substrate and cofactor specificity and selective inhibition of lactate dehydrogenase from the malarial parasiteBiochem. Parasitol. 90, 235 –246 (1997).

- He H, Lee MC, Zheng LL et al. Integration of the metabolic / redox state, histone gene switching, DNA replication and S-phase progression by moonlighting metabolic enzymes. Biosci. Rep. 33, 187-197 (2013).

- Hewitt CO, Eszes CM, Sessions RB, et al. A general method for relieving substrate inhibition in lactate dehydrogenases. Protein Eng. 12, 491-496 (1999).

- Holmes RS, Goldberg E. Computational analyses of mammalian lactate dehydrogenases: human, mouse, opossum and platypus LDHs. Comput. Biol. Chem. 33, 379-385 (2009).

- Kaiserman HB, Odenwald WF, Stowers DJ et al. A major single-stranded DNA binding protein from ovaries of the frog, Xenopus laevis, is lactate dehydrogenase. Biochim. Biophys. Acta 1008, 23-30 (1989).

- Kanno T, Sudo K, Maekawa M, Nishimura Y, Ukita M, Fukutake K. Lactate dehydrogenase M-subunit deficiency: a new type of hereditary exertional myopathy.Clin. Chim. Acta 173, 89– 98 (1988).

- Kohlmann A, Zech SG, Li F et al. Fragment growing and linking lead to novel nanomolar lactate dehydrogenase inhibitors. J. Med. Chem. 56, 1023-1040 (2013).

- Kolev Y, Uetake H, Takagi Y, Sugihara K. Lactate dehydrogenase-5 (LDH-5) expression in human gastric cancer: association with hypoxia-

inducible factor (HIF-1alpha) pathway, angiogenic factors production and poor prognosis.Ann. Surg. Oncol 15, 2336 –2344 (2008).

- Koukourakis MI, Giatromanolaki A, Sivridis E, Bougioukas G, Didilis V, Gatter KC, Harris AL, Tumour and Angiogenesis Research Group, author. Lactate dehydrogenase-5 (LDH-5) overexpression in non-small-cell lung cancer tissues is linked to tumour hypoxia, angiogenic factor production and poor prognosis Br. J. Cancer 89, 877 –885 (2003).

- Le A, Cooper CR, Gouw AM et al. Inhibition of lactate dehydrogenase A induces oxidative stress and inhibits tumor progression. Proc. Natl. Acad. Sci. USA 107, 2037-2042 (2010).

- Lee CY, Moon YS, Yuan JH et al. Enzyme inactivation and inhibition by gossypol. Mol. Cell. Biochem. 47, 65-70 (1982).

- Levashov AV, Ugolnikova AV, Ivanov MV et al. Formation of homo- and heterooligomeric supramolecular structures by D-glyceraldehyde-3-phosphate dehydrogenase and lactate dehydrogenase in reversed micelles of aerosol OT in octane. Biochem. Mol. Biol. Int. 42, 527-534 (1997).

- Levine AJ, Puzio-Kuter AM. The control of the metabolic switch in cancers by oncogenes and tumor suppressor genes. Science 330, 1340-1344 (2010).

- Madern D. Molecular evolution within the L-malate and L-lactate dehydrogenase super-family. J. Mol. Evol. 54, 825-840 (2002).

- Manerba M, Vettraino M, Fiume L et al. Galloflavin (CAS 568-80-9): a novel inhibitor of lactate dehydrogenase. ChemMedChem 7, 311-317 (2012).

- Markert CL, Shaklee JB, Whitt GS. Evolution of a gene. Multiple genes for LDH isozymes provide a model of the evolution of gene structure, function, and regulation. Science 189, 102-114 (1975).

- Mathupala SP, Heese C, Pedersen PL. Glucose catabolism in cancer cells. The type II hexokinase promoter contains functionally active response elements for the tumor suppressor p53, J. Biol. Chem. 272 (36) 22776–22780 (1997).

- McClelland GB, Khanna S, Gonzalez GF et al. Peroxisomal membrane monocarboxylate transporters: evidence for a redox shuttle system? Biochem. Biophys. Res. Commun. 304, 130-135 (2003).

- Miwa S, Nishina T, Kakehashi Y, Kitamura M, et al. Studies of erythrocytes metabolism in a case with hereditary deficiency of H-subunit of lactate dehydrogenase. Acta Haem. Jap. 34, 228– 232 (1971).

- Moorhouse AD, Spiteri C, Sharma P et al. Targeting glycolysis: a fragment based approach towards bifunctional inhibitors of hLDH-5. Chem. Commun. 47, 230-232 (2011).

- Motoshi K, Naoko I, Fumiko H, Atsushi H. Hereditary deficiency of subunit H of lactate dehydrogenase Clin. Chim. Acta 34, 419– 423(1971).

- Neri D, Supuran CT. Interfering with pH regulation in tumours as a therapeutic strategy. Nat. Rev. Drug Discovery 10, 767–777 (2011).

- Okumura N, Terasawa F, Ueno I, Oki K, Tamauchi K, Hidaka H, et al. Genetic analyses in homozygous and heterozygous variants of lactate dehydrogenase-B (H) subunit--LD-B Matsumoto I and II (LD-B W323R) Clin. Chim. Acta 287, 163 –171 (1999).

- Osthus RC, Shim H, Kim S, et al. Deregulation of glucose transporter 1 and glycolytic gene expression by c-Myc. J. Biol. Chem 275 (29) 21797–21800 (2000).

- Papacostantinou J, Colowick SP. The role of glycolysis in the growth of tumor cells. I. Effects of oxamic acid on the metabolism of Ehrlich tumor cells in vitro. J. Biol. Chem 236, 278-284 (1961a).

- Papacostantinou, J, Colowick SP. The role of glycolysis in the growth of tumor cells. II. The effect of oxamic acid on the growth of HeLa cells in tissue culture. J. Biol. Chem. 236, 285–288 (1961b).

- Patel GL, Thompson PE. Immunoreactive helix-destabilizing protein localized in transcriptionally active regions of Drosophila polytene chromosomes. Proc. Natl. Acad. Sci. USA 77, 6749-6753 (1980).

- Pavlides S, Whitaker-Menezes D, Castello-Cros R et al. The reverse Warburg effect. Aerobic glycolysis in cancer associated fibroblasts and the tumor stroma. Cell Cycle 8, 3984-4001 (2009).

- Pelicano H, Martin DS, Xu RH et al. Glycolysis inhibition for anticancer treatment. Oncogene 25, 4633-4646 (2006).

- Quistorff B, Grunnet N. The isoenzyme pattern of LDH does not play a physiological role; except perhaps during fast transitions in energy metabolism. Aging 3, 457-460 (2011).

- Read JA, Winter VJ, Eszes CM et al. Structural basis for altered activity of M- and H isozyme forms of human lactate dehydrogenase. Proteins 43, 175-185 (2001).

- Rees DC, Congreve M, Murray CW et al. Fragment based lead discovery. Nat. Rev. Drug Discovery 3, 660−672 (2004).

- Robey RB, Hay N. Is Akt the "Warburg kinase"? Akt-energy metabolism interactions and oncogenesis. Semin Cancer Biol 19, 25-31 (2009).

- Semenza GL, Roth PH, Fang HM, Wang GL. Transcriptional regulation of genes encoding glycolytic enzymes by hypoxiainducible factor 1. J. Biol. Chem. 269 (38) 23757–23763 (1994).

- Shamoo Y. Single-stranded DNA-binding proteins. In: Enciclopedia of Life Sciences Macmillan Publisher Ltd (Ed.), Nature Publishing Group, 1-7 (2002).

- Shim H, Dolde C, Lewis BC, et al. c-Myc transactivation of LDH-A: implications for tumor metabolism and growth, Proc. Natl. Acad. Sci. USA 94 (13) 6658–6663 (1997).

- Smith TA, Sharma RI, Thompson AM, Paulin FE. Tumor 18F-FDG incorporation is enhanced by attenuation of P53 function in breast cancer cells in vitro, J. Nucl. Med. 47 (9) 1525–1530 (2006).

- Sonveaux P, Végran F, Schroeder T, Wergin MC, Verrax J et al. Targeting lactate-fueled respiration selectively kills hypoxic tumor cells in mice. J. Clin. Invest. 118, 3930–3942 (2008).

- Vander Heiden MG, Locasale JW, Swanson KD et al. Evidence for an alternative glycolytic pathway in rapidly proliferating cells. Science 329, 1492-1499 (2010).

- Warburg O. Uber den Stoffwechsel der Tumoren. Verlag Springer, Berlin (1926). Translated into English by Dickens F under the title The metabolism of tumors Constable, London (1930).

- Ward RA, Brassington C, Breeze AL et al. Design and Synthesis of Novel Lactate Dehydrogenase A Inhibitors by Fragment-Based Lead Generation. J. Med. Chem. 55, 3285-3306 (2012).

- Wang XC, Jiang L, Zhou HM. Minimal Functional Unit of Lactate Dehydrogenase. J. Prot. Chem. 16, 227-231 (1987).

- Wang ZY, Loo TY, Shen JG, Wang N, Wang DM, Yang DP, Mo SL, Guan XY, Chen JP. LDH-A silencing suppresses breast cancer tumorigenicity through induction of oxidative stress mediated mitochondrial pathway apoptosis. Breast Cancer Res. Treat. 131, 791 – 800 (2012).

- Ward RA, Brassington C, Breeze AL et al. Design and Synthesis of Novel Lactate Dehydrogenase A Inhibitors by Fragment-Based Lead Generation. J. Med. Chem. 55, 3285-3306 (2012).

- Williams KR, Reddigari S, Patel GL. Identification of a Nucleic Acid Helix- Destabilizing Protein from Rat Liver as Lactate Dehydrogenase-5. Proc. Natl. Acad. Sci. USA 82, 5260-5264 (1985).

- Xie H, Valera VA, Merino MJ, Amato AM, Signorotti S, Linehan WM, et al. LDH-A inhibition, a therapeutic strategy for treatment of hereditary leiomyomatosis and renal cell cancer. Mol. Cancer Ther. 8, 626– 635 (2009).

- Yu Y, Deck JA, Hunsaker LA et al. Selective active site inhibitors of human lactate dehydrogenases A4, B4, and C4. Biochem. Pharmacol. 62, 81-89 (2001).

- Zhao Y, Liu H, Liu Z, Ding Y, Ledoux SP, Wilson GL et al. Overcoming trastuzumab resistance in breast cancer by targeting dysregulated glucose metabolism. Cancer Res. 71, 4585-4597 (2011).

- Zhou M, Zhao Y, Ding Y, Liu H, Liu Z, Fodstad O, Riker AI, Kamarajugadda S, Lu J, Owen LB, Ledoux SP, Tan M. Warburg effect in chemosensitivity: targeting lactate dehydrogenase-A re-sensitizes taxol-resistant cancer cells to taxol. Mol. Cancer 9-33 (2010).

Chapter II

Scientific production

Impairment of Aerobic Glycolysis by Inhibitors of Lactic Dehydrogenase Hinders the Growth of Human Hepatocellular Carcinoma Cell Lines

Short Communication

Pharmacology

Pharmacology 317519
DOI: 10.1159/000317519

Received: May 5, 2010
Accepted after revision: June 21, 2010
Published online: ■■■

Impairment of Aerobic Glycolysis by Inhibitors of Lactic Dehydrogenase Hinders the Growth of Human Hepatocellular Carcinoma Cell Lines

Luigi Fiume Marcella Manerba Marina Vettraino Giuseppina Di Stefano

Department of Experimental Pathology, University of Bologna, Bologna, Italy

Key Words
Cancer bioenergetics · Lactic dehydrogenase, inhibition · Aerobic glycolysis · Hepatocellular carcinoma

Abstract

Background/Aims: By reducing the number of ATP molecules produced via aerobic glycolysis, the inhibition of lactic dehydrogenase (LDH) should hinder the growth of neoplastic cells without damaging the normal cells which do not rely on this metabolic pathway for their energetic needs. Here, we studied the effect of oxamic and tartronic acids, 2 inhibitors of LDH, on aerobic glycolysis and cell replication of HepG2 and PLC/PRF/5 cells, 2 lines from human hepatocellular carcinomas. ***Methods:*** Aerobic glycolysis was measured by calculating the amounts of lactic acid formed. The effect on replication was assessed by culturing the cells in both standard conditions and glucose-deprived medium, which was used to shut down aerobic glycolysis. ***Results:*** The oxamic and tartronic acids inhibited aerobic glycolysis, impaired the growth of both cell lines and also induced an increased expression of p53-upregulated modulator of apoptosis, a signal of cell death. A strong impairment of cell replication by oxamic acid was only found when the cells were cultured in the presence of glucose, indicating that it was for the most part owing to inhibition of aerobic glycolysis. ***Conclusions:*** Inhibition of aerobic glycolysis achieved by blocking LDH could be useful in the treatment of human hepatocellular carcinomas. Without interfering with glucose metabolism in normal cells, it could hinder cell growth by itself and could also enhance the chemotherapeutic index of associated anticancer agents by decreasing the levels of ATP selectively in neoplastic cells.

Introduction

According to the finding of Warburg [1], reevaluated in recent years [2], neoplastic cells derive much of their energy for survival and growth from aerobic glycolysis. This property prompted many efforts to deprive neoplastic cells of ATP by inhibiting the enzymes of glycolysis [3]. However, glycolytic enzymes are also necessary for the glucose metabolism of normal cells, and their inhibition can cause the severe disorders induced by impaired glucose utilization [4]. Lactic dehydrogenase (LDH) is the only glycolytic enzyme whose inhibition should allow a blocking of the aerobic glycolysis of tumor cells without damaging the normal cells which, in conditions of normal functional activity and sufficient oxygen supply, do not need this enzyme [5, 6]. In normal cells, pyruvate, the end product of the glycolysis pathway, is not converted into lactate, but is decarboxylated to acetyl-CoA, which enters the Krebs cycle. Support for the possibility that LDH activity is not necessary for normal cells also comes

KARGER

Fax +41 61 306 12 34
E-Mail karger@karger.ch
www.karger.com

0031–7012/10/0000–0000$26.00/0

Accessible online at:
www.karger.com/pha

Prof. G. Di Stefano
Department of Experimental Pathology, University of Bologna
Via San Giacomo 14
IT–40126 Bologna (Italy)
Tel. +39 0512 094 721, Fax +39 0512 094 746, E-Mail giuseppina.distefano@unibo.it

from the findings that humans with a hereditary deficiency in the activity of LDH-A (the isoform of the muscle and liver) do not display any symptoms under normal circumstances [7], and also that a complete hereditary deficiency in LDH-B (the isoform of the heart) is usually asymptomatic [8].

In the present experiments, we studied whether the inhibition of aerobic glycolysis achieved by LDH blocking can be exploited to impair the growth of human hepatocellular carcinoma (HCC) cells, the treatment of which with systemic chemotherapy has met with little success. We examined two HCC lines with different biological properties: HepG2 cells, a p53-native, well-differentiated hepatoma [9], and PLC/PRF/5 cells, a p53-mutant, poorly differentiated hepatocarcinoma [10].

In these cell lines, the activity of LDH was blocked by using oxamic and tartronic acids, two competitive inhibitors of the enzyme [11, 12]; the former inhibitor was found to reduce HCC cell viability by selectively preventing aerobic glycolysis.

Materials and Methods

Cell Culture and Treatments

HepG2 and PLC/PRF/5 cells were cultured in DMEM with 25 mmol/l glucose, supplemented with 10% fetal bovine serum, nonessential amino acids, 100 IU/ml penicillin, 100 μg/ml streptomycin and 2 mmol/l glutamine, and deprived of sodium pyruvate. All products were obtained from BioWhittaker. Cells were incubated in a humidified atmosphere of 5% CO_2 and 95% air at 37°C. Prior to the experiments, 1.6×10^4 cells suspended in 500 μl culture medium were seeded into each well of a 12-well Nunclon Multidish plate. After 24 h, the culture medium was discarded and the experiment was started by addition of 500 μl of fresh medium with or without the sodium salts of oxamic acid or tartronic acid (obtained from Sigma). Each concentration of the drug was tested in quadruplicate. The medium was replaced every 24 h. 72 h after the start of the experiment, cell growth was evaluated using the neutral red assay, which gives a measure of the number of viable cells that actively endocytose this compound [13]. In the present experiments, this assay was preferred to that based on the reduction of MTT since this last reagent is also reduced by cytosolic NADH [14], whose pool can be modified by inhibitors of LDH. The IC_{50} (dose of compound producing a 50% inhibition of cell growth) was calculated from the second-order polynomial regression of the experimental data. In the different determinations, r^2 values ranged from 0.95 to 0.98.

A second set of experiments was performed by growing the cells in glucose-free medium to divert their metabolism from aerobic glycolysis [15–17]. In these experiments, DMEM was prepared by substituting 25 mmol/l glucose with 10 mmol/l galactose and supplemented with dialyzed fetal bovine serum. The culture and treatment conditions were as described above.

Evaluation of Aerobic Glycolysis

Aerobic glycolysis was evaluated by determining the amounts of lactic acid formed. 5×10^5 cells in 2 ml of culture medium (see above) were seeded in each well (35 mm in diameter) of a 6-well Nunclon plate. The cells were incubated for 24 h. The medium was then aspirated off and replaced with 1 ml of Krebs-bicarbonate buffer containing 25 mmol/l glucose (with or without the inhibitor of LDH), prewarmed at 37°C. Each dose of LDH inhibitor was tested in triplicate. Lactic acid was measured in 3 wells at the start of the experiment ('zero time') and 3 h after incubation at 37°C in wells with or without the compound. To assess the concentrations of lactic acid in the wells (intracellularly plus released in medium), 100 μl of 100% trichloroacetic acid solution was added to each well; the cell lysate was collected and the well washed with 1 ml of 10% trichloroacetic acid. After centrifugation, the concentration of lactic acid in the supernatant was measured according to the method of Barker and Summerson [18]. It had previously been verified that neither oxamic nor tartronic acid interferes with the lactic acid measure. The amount of lactic acid formed during the 3-hour incubation with or without the LDH inhibitor was calculated by subtracting the value measured at 'zero time'. Each experiment was repeated twice.

Expression of p53-Upregulated Modulator of Apoptosis

To verify whether the inhibition of aerobic glycolysis can trigger death signals in cells, we evaluated the expression of the p53-upregulated modulator of apoptosis (PUMA; a protein of the BH3-only family, which is considered a general sensor of cell death [19]) in cultures of HepG2 or PLC/PRF/5 cells (1×10^6 cells, seeded in 25-cm^2 flasks) maintained for 3 days in the presence of 80 mmol/l oxamic acid. At the end of treatment, the oxamic acid-exposed cells and cells from a control culture were lysed in 100 μl of 100 mmol/l potassium phosphate buffer, pH 7.5, containing 1% NP-40 and a protease inhibitor cocktail (Sigma). The cell homogenates were left 30 min on ice and then centrifuged 15 min at 10,000 *g*. 15 μg of proteins of the supernatants (measured according to Lowry) was loaded into 12% polyacrylamide gel for electrophoresis. The separated proteins were blotted on a low fluorescent polyvinylidene difluoride membrane (GE Life Sciences) using a standard apparatus for wet transfer with an electrical field of 300 mA for 2 h. The blotted membrane was blocked with 5% BSA in TBS-Tween. PUMA was revealed using a rabbit polyclonal antibody (Abcam), visualized by a Cy5-labeled secondary antibody (GE Life Sciences). β-Actin was used as a loading control. It was revealed by using a rabbit polyclonal antibody (Sigma), visualized by the Cy5-labeled secondary antibody. All incubation steps were performed according to the manufacturer's instructions. Fluorescence of the blots was assayed by the Pharos FX scanner (BioRad) at a resolution of 100 μm, using the Quantity One software (BioRad).

Results

Aerobic Glycolysis

As shown in figure 1, oxamic and tartronic acids inhibited the aerobic glycolysis of both HCC cell lines. The inhibition by oxamic acid was stronger than that by tartronic acid, probably because of a more efficient block of

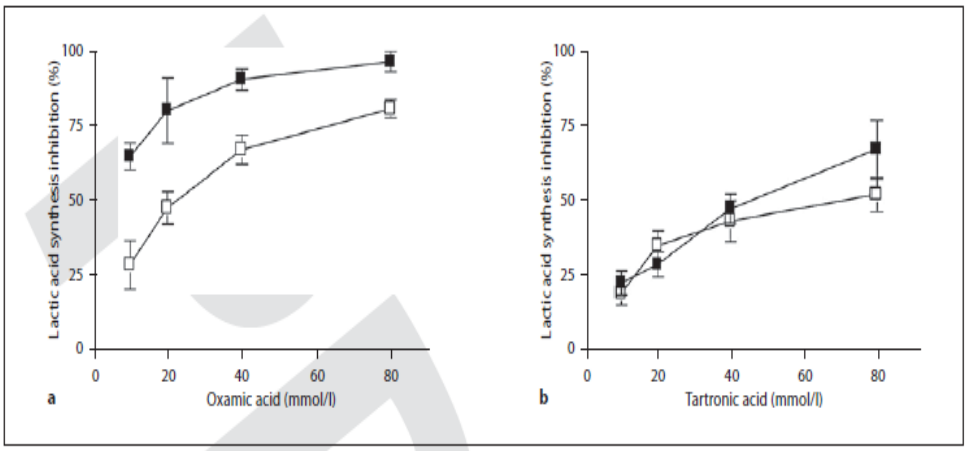


Fig. 1. Effect of oxamic acid (**a**) and tartronic acid (**b**) on the amounts of lactic acid produced in 3 h by HepG2 (■) and by PLC/PRF/5 (□) cells. Each entry is the mean value of 6 determinations performed in 2 different experiments. Results were statistically evaluated using the t test. p was <0.001 for all the data, except for the lower concentration of tartronic acid (10 mmol/l; $p < 0.01$). In the absence of the inhibitors, the amounts of lactate formed in 3 h by HepG2 and by PLC/PRF/5 cells were 2.57–2.85 and 3.83–3.91 μmol/10^6 cells, respectively.

LDH. The effective concentrations of the 2 inhibitors were very similar to those acting in inhibiting the aerobic glycolysis in Ehrlich ascites tumor cells and in HeLa cells [5, 20].

Cell Growth

The effect of oxamic and tartronic acids on the growth of HCC cell lines was tested using the same concentrations which inhibited aerobic glycolysis. As shown in figure 2a, b, oxamic acid displayed a similar activity on both HepG2 and PLC/PRF/5 cell lines. The IC_{50} were 62.3 and 50.8 mmol/l for HepG2 and PLC/PRF/5, respectively. Although tartronic acid hindered aerobic glycolysis to a lower extent than oxamic acid (fig. 1), the percentages of cell growth inhibition caused by the former compound were similar (PLC/PRF/5 cells: IC_{50} = 59.5 mmol/l) or even higher (HepG2 cells: IC_{50} = 19.6 mmol/l) than those produced by equal concentrations of the latter compound. This result was likely due to the hindering effect of tartronic acid on glutamine oxidation (see below). When neoplastic cells are cultured in the presence of glucose, they derive their energy from aerobic glycolysis and from the oxidation of glutamine, which, after conversion first into glutamate and then into α-ketoglutarate, is metabolized via the Krebs cycle [17]. To verify whether oxamic and tartronic acids impair cell growth only by inhibiting aerobic glycolysis or also by interfering with the Krebs cycle, we studied their effect on the growth of the HCC cells cultured in the absence of glucose and in the presence of galactose (see Materials and Methods). Under these conditions, the cell lines practically did not display aerobic glycolysis [15–17, 21] and, for survival and growth, relied almost completely on glutamine oxidation [17]. Figure 2c shows that oxamic acid tested at the concentrations used in the present experiments impaired the growth of cells cultured on galactose only by 0–16% (HepG2 cells) and by 5–24% (PLC/PRF/5 cells), indicating that this compound interferes with the metabolic pathway of the Krebs cycle only to a small extent, and that the inhibition of cell replication measured when the cells were cultured on glucose in the presence of oxamic acid (fig. 2a) could for the most part be attributed to the impairment of aerobic glycolysis. Tartronic acid hindered the growth of HepG2 and PLC/PRF/5 cells by 4–49% and 10–77%, respectively, indicating that in these cells, this compound also interfered with glutamine oxidation (fig. 2d).

Expression of PUMA

PUMA is a protein of the BH3-only family with a molecular mass of about 20 kDa, normally expressed at very low levels, but rapidly induced in response to a wide range of harmful stimuli [19]. It transduces apoptosis signals to the mitochondria, but under ATP-depleted conditions, it primarily causes necrosis [22]. PUMA can therefore be considered a general sensor of cell death

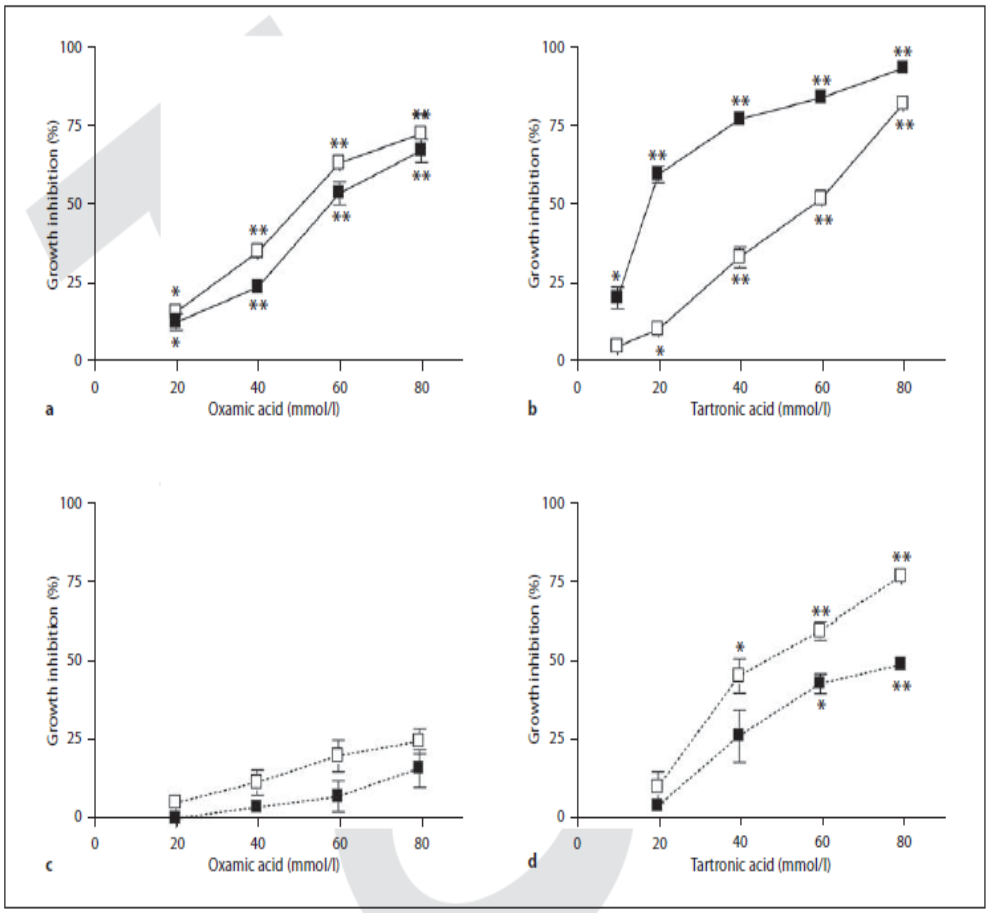


Fig. 2. a, b Effect of oxamic (**a**) and tartronic acid (**b**) on the growth of HepG2 (■) and PLC/PRF/5 (□) cells. **c, d** Effect of oxamic (**c**) and tartronic acid (**d**) on the growth of HepG2 (■) and PLC/PRF/5 (□) cells cultured in the absence of glucose. Each concentration of the compound was tested in triplicate. Data are mean values. Standard errors ranged from 0.1 to 10.6% of the mean values. Results were statistically evaluated using the t test. * $p < 0.01$; ** $p < 0.001$.

[19]. The Western blot analysis reported in figure 3 shows that treatment with oxamic acid increased PUMA expression in both HepG2 and PLC/PRF/5 cells. As shown in figure 3c, d, the intensity of the PUMA fluorescent signal, normalized on that of β-actin (loading control), is 2.2 (HepG2) and 3.6 (PLC/PRF/5) times higher in treated cells than in control cultures. This result indicates that the reduced cell growth observed in cultures treated with oxamic acid (fig. 2a) derives, at least in part, from the killing of cells. A similar experiment was not performed with tartronic acid since we found that this compound is a nonspecific inhibitor of aerobic glycolysis (see above).

Discussion

The principal result of the present experiments was the finding that oxamic acid, an inhibitor of LDH, hindered the replication of HCC cells by selectively blocking aerobic glycolysis. The increased expression of PUMA indicated that the block of aerobic glycolysis resulted in the death of the cells. In contrast, tartronic acid was shown also to interfere with glutamine oxidation. This interference might be due to the inhibition of mitochondrial malic dehydrogenase [23]. The present data are in agreement with those of Papaconstantinou and Colowick [20], who studied the effect of oxamic acid on HeLa cell replication,

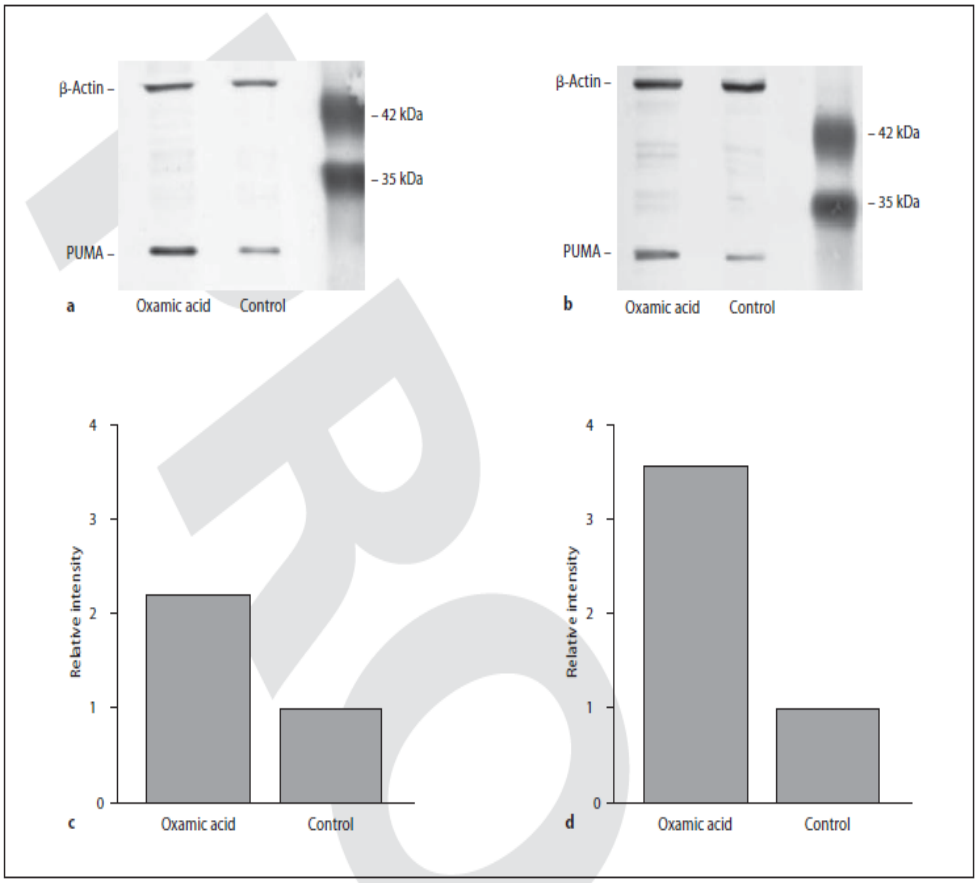


Fig. 3. a, b Western blot analysis of PUMA expression in HepG2 (**a**) and PLC/PRF/5 cells (**b**) treated with oxamic acid (see Materials and Methods). **c, d** Intensity of the PUMA fluorescent signal in treated cells compared to that of control cultures. The measure is given in arbitrary units and is normalized on the intensity of the loading control signal (β-actin). **c** HepG2 cells. **d** PLC/PRF/5 cells.

and fit in with the finding that mammary tumor cell lines in which LDH activity was reduced by means of short hairpin RNA showed a retarded rate of cell proliferation [24]. Oxamic and tartronic acids are competitive inhibitors of LDH and hinder aerobic glycolysis in vitro at high concentrations that can hardly be reached in vivo [20]. However, the present results suggest that research using drug design technologies to find a strong and selective LDH inhibitor hindering aerobic glycolysis also in vivo can be a worthwhile attempt to improve the systemic chemotherapy of HCC. Different from the inhibitors of glycolytic enzymes other than LDH, the inhibitors of this enzyme should impair HCC growth without interfering with the oxidation of glucose to CO_2 in normal cells. Moreover, by decreasing the ATP levels selectively in the neoplastic cells, they could also increase the chemotherapeutic index of associated anticancer agents, for instance by hindering the mechanisms of cell repair which require an energy supply [25], or by impeding the activity of the ATP-dependent efflux pumps, a major cause of the acquired multidrug resistance [26].

Acknowledgments

This study was supported by the Fondazione del Monte, the Roberto and Cornelia Pallotti Legacies for Cancer Research and the University of Bologna.

References

1 Warburg O: Über den Stoffwechsel der Tumoren. Berlin, Springer, 1926. [English translation Dickens F (transl): The Metabolism of Tumours. London, Constable, 1930.]
2 Garber K: Energy deregulation: licensing tumors to grow. Science 2006;312:1158–1159.
3 Pelicano H, Martin DS, Xu R-H, Huang P: Glycolysis inhibition for anticancer treatment. Oncogene 2006;25:4633–4646.
4 Guyton AC, Hall JE: Textbook of Medical Physiology. Philadelphia, WB Saunders, 2000.
5 Fiume L: Inhibition of aerobic glycolysis in Yoshida ascites hepatoma by tartronic acid. Nature 1960;187:792–793.
6 Papacostantinou J, Colowick SP: The role of glycolysis in the growth of tumor cells. 1. Effects of oxamic acid on the metabolism of Ehrlich tumor cells in vitro. J Biol Chem 1961;236:278–284.
7 Kanno T, Sudo K, Maekawa M, Nishimura Y, Ukita M, Fukutake K: Lactate dehydrogenase M-subunit deficiency: a new type of hereditary exertional myopathy. Clin Chim Acta 1988;173:89–98.
8 Okumura N, Terasawa F, Ueno I, Oki K, Tamauchi K, Hidaka H, Tozuka M, Okura M, Katsuyama T: Genetic analyses in homozygous and heterozygous variants of lactate dehydrogenase-B (H) subunit-LD-B Matsumoto I and II (LDH-B W323R). Clin Chim Acta 1999;287:163–171.
9 Aden DP, Fogel A, Plotkin S, Damjanov I, Knowles BB: Controlled synthesis of HBsAg in a differentiated human liver carcinoma-derived cell line. Nature 1979;282:615–616.
10 Alexander JJ, Bey EM, Geddes EW, Lecatsas G: Establishment of a continuously growing cell line from primary carcinoma of the liver. S Afr Med J 1976;50:2124–2128.
11 Novoa WB, Winer AD, Glaid AJ, Schwert GW: Lactic dehydrogenase. 5. Inhibition by oxamate and by oxalate. J Biol Chem 1959; 234:1143–1148.
12 Quastel JH, Wooldridge WR: Some properties of the dehydrogenating enzymes of bacteria. Biochem J 1928;22:689–702.
13 Fotakis G, Timbrell JA: In vitro cytotoxicity assays: comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. Toxicol Lett 2006;160:171–177.
14 Berridge MV, Herst PM, Tan AS: Tetrazolium dyes as tools in cell biology: new insights into their cellular reduction. Biotechnol Ann Rev 2005;11:127–152.
15 Eagle H, Barban S, Levy M, Schulze HO: The utilization of carbohydrates by human cell cultures. J Biol Chem 1958;233:551–558.
16 Goldberg EB, Nitowski HM, Colowick SP: The role of glycolysis in the growth of tumor cells. 4. The basis of glucose toxicity in oxamate-treated, cultured cells. J Biol Chem 1965; 240:2791–2796.
17 Reitzer LJ, Wice BM, Kennell D: Evidence that glutamine, not sugar, is the major energy source of cultured HeLa cells. J Biol Chem 1979;254:2669–2676.
18 Barker SB, Summerson WH: The colorimetric determination of lactic acid in biological material. J Biol Chem 1941;138:535–554.
19 Yu J, Zhang L: PUMA, a potent killer with or without p53. Oncogene 2009;27:S71–S83.
20 Papaconstantinou J, Colowick SP: The role of glycolysis in the growth of tumor cells. 2. The effect of oxamic acid on the growth of HeLa cells in tissue culture. J Biol Chem 1961;236: 285–288.
21 Rossignol R, Gilkerson R, Aggeler R, Yamagata K, Remington SJ, Capaldi RA: Energy substrate modulates mitochondrial structure and oxidative capacity in cancer cells. Cancer Res 2004;64:985–993.
22 Toth A, Jeffers JR, Nickson P, Min JY, Morgan JP, Zambetti GP, Erhardt P: Targeted deletion of PUMA attenuates cardiomyocyte death and improves cardiac function during ischemia-reperfusion. Am J Physiol Heart Circ Physiol 2006;291:H52–H60.
23 Harada K, Wolfe RG: Malic dehydrogenase. 6. Kinetic study of hydroxymalonate inhibition. J Biol Chem 1968;243:4123–4130.
24 Fantin VR, St-Pierre J, Leder P: Attenuation of LDH-A expression uncovers a link between glycolysis, mitochondrial physiology and tumor maintenance. Cancer Cell 2006; 9:425–434.
25 Kinoshita E, van der Linden E, Sanchez H, Wyman C: RAD50, an SMC family member with multiple roles in DNA break repair: how does ATP affect function? Chromosome Res 2009;17:277–288.
26 Cole SPC, Tannock IF: Drug resistance; in Tannock IF, Hill RP, Bristow RG, Harrington L (eds): The Basic Science of Oncology. New York, McGraw-Hill, 2005, pp 376–399.

Inhibition of lactic dehydrogenase as a way to increase the anti-proliferative effect of multitargeted kinase inhibitors

Pharmacological Research 63 (2011) 328–334

Contents lists available at ScienceDirect

Pharmacological Research

journal homepage: www.elsevier.com/locate/yphrs

Inhibition of lactic dehydrogenase as a way to increase the anti-proliferative effect of multi-targeted kinase inhibitors

Luigi Fiume, Marina Vettraino, Marcella Manerba, Giuseppina Di Stefano*

Department of Experimental Pathology, University of Bologna, via San Giacomo, 14 - 40126 Bologna, Italy

ARTICLE INFO

Article history:
Received 4 November 2010
Received in revised form 9 December 2010
Accepted 10 December 2010

Keywords:
Aerobic glycolysis
Kinase inhibitors
Hepatocellular carcinoma
Lactic dehydrogenase

ABSTRACT

Protein kinase inhibitors are a relatively new class of promising anticancer drugs, most of which exert their action by binding to the ATP site on the targeted kinases. We hypothesized that a decrease in ATP levels in neoplastic cells could reduce the competition for the same enzymatic site, thus increasing the efficacy of kinase inhibitors. Using oxamic acid, an inhibitor of lactic dehydrogenase (LDH) which hinders aerobic glycolysis, we decreased ATP levels in PLC/PRF/5 cells (a line from a hepatocellular carcinoma). We found that in these cells oxamic acid potentiated the antiproliferative activity of sorafenib, imatinib and sunitinib, three kinase inhibitors. When aerobic glycolysis was shut down by culturing the cells in the absence of glucose, oxamic acid did not reduce the ATP levels, suggesting that in normal tissues, which do not rely on aerobic glycolysis for their ATP synthesis, the block of LDH should not impair cellular metabolism.

In conclusion, the inhibition of LDH could enhance anticancer activity of sorafenib, imatinib and sunitinib without increasing their side effects on normal cells, which in conditions of normal functional activity and sufficient oxygen supply do not need the activity of this enzyme.

1. Introduction

In recent years a growing number of studies were addressed at interfering with the metabolic pathways which generate energy in neoplastic cells [for a review, see 1]. It has long been known that, to meet their energy demand, cancer cells largely rely on aerobic glycolysis [2]. This process can be hindered by blocking lactic dehydrogenase (LDH), which causes a failing in the regeneration of NAD, reduced during the conversion of glyceraldehyde 3-phosphate to 1,3-diphosphoglycerate, and necessary to maintain the glycolytic flow. Apart from other glycolytic enzymes, LDH is probably unessential for normal tissues in conditions of normal functional activity and sufficient oxygen supply. The inhibition of LDH could therefore result in a selective depletion of ATP in tumour cells [3,4]. In previous experiments, oxamic acid, a competitive inhibitor of LDH [5], was shown to hinder the growth of HeLa [6], HepG2 and PLC/PRF/5 cells [7] cultured in vitro. Moreover, by causing a selective decrease of ATP levels in neoplastic cells, oxamic acid could also improve the chemotherapeutic index of associated anticancer drugs. An emerging class of antineoplastic agents are multi-targeted kinase inhibitors. These are relatively recent developed compounds whose use will likely increase in the next future because of broad-spectrum antitumor activity, ease of administration and good tolerability. Most of them are small molecules which bind to the ATP site on the protein kinases target of their activity [8]. We considered the possibility that a decrease in ATP levels in neoplastic cells could reduce the competition for the same enzymatic site, thus increasing the efficacy of the kinase inhibitors. To explore this possibility, in PLC/PRF/5 cells we studied the effect of three commonly used kinase inhibitors (sorafenib, sunitinib and imatinib) given alone or in combination with oxamic acid. PLC/PRF/5 cells are derived from a human hepatocellular carcinoma (HCC) [9] and were considered an appropriate model to perform this study since this tumour is often refractory to chemotherapeutic interventions and even the benefit observed with the introduction of kinase inhibitors (sorafenib) was modest, so that the search for new and more effective therapeutic alternatives for treating HCC is still ongoing.

2. Materials and methods

2.1. Compounds

Sodium oxamate was purchased from Sigma; it was dissolved in DMEM without sodium pyruvate.

Sorafenib *p*-toluenesulfonate, sunitinib malate and imatinib methanesulfonate were purchased from LC Laboratories (USA). For the experiments, sorafenib and sunitinib were dissolved in 100%

* Corresponding author. Tel.: +39 0512094721; fax: +39 0512094746.
E-mail address: giuseppina.distefano@unibo.it (G. Di Stefano).

1043-6618/$ – see front matter © 2010 Elsevier Ltd. All rights reserved.
doi:10.1016/j.phrs.2010.12.005

DMSO (Sigma) and diluted with DMEM to the desired concentrations. For all the tested doses of the drug final DMSO concentration was kept to 0.05%, which was also added to the cultures of untreated cells (controls). Imatinib was dissolved in PBS and diluted to the desired concentrations with DMEM.

2.2. Cell cultures

PLC/PRF/5 cells were cultured in DMEM with 25 mM glucose, supplemented with 10% foetal bovine serum, non-essential amino acids, 100 IU/ml penicillin, 100 mg/ml streptomycin, 2 mM glutamine and devoid of sodium pyruvate. All products were obtained from Bio-Wyttacker. Cells were incubated in a humidified atmosphere of 5% CO_2, 95% air at 37 °C.

2.3. Inhibition of LDH by oxamic acid

Oxamic acid is a known inhibitor of LDH, which compete with pyruvate for its binding site on the enzyme molecule. As a first step, we evaluated the inhibition constant (Ki) of oxamic acid for the enzyme from PLC/PRF/5 cells. Cells from two semi-confluent 75 cm^2 flasks were harvested, pelleted and suspended in 3 vol. of PBS. The cell suspension was then lysed by sonication and centrifuged (1600 × g, 30 min at 4 °C) to discard the cellular debris. The supernatant was used to measure LDH activity, as follows: it was diluted 1:1000 in 100 mM phosphate buffer pH 7.5 containing 0.12 mM NADH and pyruvate. The enzymatic activity was measured by recording for 5 min the decrease in absorbance at $\lambda_{340\ nm}$, produced by NADH oxidation in the presence of scalar concentrations of pyruvate (0.1–1 mM). The effect of oxamic acid was evaluated in a competitive assay by adding 0–0.5 mM compound to the reaction mix in the presence of the scalar concentrations of pyruvate. The Ki was calculated using the software GraphPad Prism 5. A similar assay was also performed on purified bovine LDH (type M) obtained from Sigma.

2.4. Inhibition of lactate production by oxamic acid

To verify whether LDH blocking by oxamic acid can hinder aerobic glycolysis, we measured the amounts of lactate formed in the presence of the inhibitor. 5×10^5 cells in 2 ml of culture medium were seeded in each well of a 6-well Nunclon plate and let to adhere overnight. The medium was then replaced with 1 ml of Krebs-bicarbonate buffer containing 25 mM glucose (with or without scalar concentrations of oxamic acid), pre-warmed at 37 °C. Each dose of oxamic acid was tested in triplicate. Lactate was measured in 3 untreated wells at the start of experiment (baseline value) and 3 h after incubation at 37 °C. In each well, we simultaneously measured released in medium and intracellular lactate: at the end of incubation 100 μl of 100% trichloroacetic acid (TCA) solution was added; the cell lysate was collected and the well was washed with 1 ml 10% TCA. After centrifugation, lactate in the supernatant was measured according to the method of Barker and Summerson [10]. The amount of metabolite formed during the 3 h incubation with or without oxamic acid was calculated by subtracting the baseline value. The experiment was repeated twice.

2.5. Measure of ATP levels

ATP levels were measured using the CellTiter-Glo Luminescent Cell Viability Assay from Promega. For this experiment, 2×10^4 cells in 200 μl of culture medium were seeded into each well of a 96-multiwell plate and allowed to adhere overnight. Oxamic acid was added and ATP levels were measured after 3, 6 and 24 h incubation at 37 °C. Each dose of compound was tested in triplicate. After incubation, the plate was allowed to equilibrate at room temperature for 30 min and the CellTiter-Glo reactive was directly added to each well, as indicated by the manufacturer. The plate was kept on a shaker for 10 min to induce cell lysis and its luminescence was measured using a Fluoroskan Ascent FL reader (Labsystems). A second set of experiments was performed after seeding the cells in glucose-free medium, to divert their metabolism from aerobic glycolysis [11–13]. In these experiments DMEM was prepared by substituting 25 mM glucose with 10 mM galactose and was supplemented with dialyzed foetal bovine serum. In these conditions cells derive their ATP from the TCA cycle using glutamine as the main substrate. In fact galactose does not enter the glycolytic pathway, but is needed as a source of precursors for DNA synthesis.

2.6. Cell viability assay

Cell growth was evaluated using the neutral red assay, which gives a measure of the numbers of viable cells that actively endocytose this compound [14]. In the present experiments this assay was preferred to that based on the reduction of MTT, since this last reagent is also reduced by cytosolic NADH [15], whose pool can be modified by LDH inhibitors.

Cells (10^5/well) were seeded in 24-multiwell plates, allowed to adhere overnight and treated with the multi-kinase inhibitors (sorafenib, imatinib, sunitinib) at 37 °C for 24 h, in the presence or absence of oxamic acid. Each dose of compound was tested in quadruplicate and each experiment was replicated three times. After the treatments, cells were incubated 3 h at 37 °C with the neutral red dye dissolved in DMEM at the final concentration of 30 μg/ml. After this time, the medium was removed and the cells were solubilised with 1 ml of 1% acetic acid in 50% ethanol. The solution from each well (200 μl) was then transferred to a 96-multiwell plate and its absorbance at λ_{540} was measured using a microplate reader (Multiskan Ascent FL, Labsystems).

2.7. Morphologic observation of treated cells

Cells were grown on coverslips to 70–80% confluence. They were treated for 6 h at 37 °C with 16 μM sorafenib and 80 mM oxamic acid given alone or in combination. After treatment, they were fixed with 2% paraformaldehyde in PBS for 5 min at 4 °C, washed twice in PBS and in distilled water and incubated with Giemsa solution (Sigma) for 3 min at room temperature. Coverslips were then washed thoroughly in distilled water, dehydrated with alcohol, xylene-cleared and mounted with Canada balsam.

2.8. Incorporation of [^{14}C]thymidine

PLC/PRF/5 cells were seeded in 24-multiwell plates (2×10^4 cells/well) and let to adhere overnight. Oxamic acid (80 mM), sorafenib (16 μM) and their combination were added together with 1 μCi [^{14}C]thymidine (Perkin Elmer). For each compound, six wells were prepared; moreover, an additional six wells were treated only with [^{14}C]thymidine, to serve as control. Plates were incubated at 37 °C in a humidified atmosphere and after 1 h and 6 h the acid-insoluble radioactivity incorporated by the cells was counted. For each time interval, three wells per treatment were used. Briefly, cells from each well were lysed with 200 μl 0.5 N NaOH; lysates were recovered with 500 μl H_2O and macromolecules were denatured with TCA (final concentration of TCA = 10%). The acid precipitate was washed with 2 × 5 ml 5% TCA over a glass microfiber filter. The radioactivity of the acid insoluble material adsorbed onto the filters was then measured using a β-counter.

2.9. Immunoblot analysts of ERK 1/2 phosphorylation

Cultures of PLC/PRF/5 (1.5×10^6 cells, seeded in 25 cm^2 flasks) were exposed to 80 mM oxamic acid, 16 μM sorafenib or their combination. After 1 h incubation at 37 °C, the treated cells and cells from a control culture were lysed in 150 μl M-PER buffer (Pierce) containing protease inhibitors (Roche) and a phosphatase inhibitor cocktail (Pierce). The cell homogenates were left 30 min on ice and then centrifuged 15 min at 10,000 × g. 20 μg proteins of the supernatants (measured according to Lowry) was loaded into 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 300 mA for 2 h. The blotted membrane was blocked with 5% BSA in TBS-Tween and probed with a rabbit anti-ERK 1/2 antibody (Cell Signaling Technology) or with a rabbit anti-phosphoERK 1/2 monoclonal antibody (Thr202/Tyr204, Cell Signaling Technology). Binding was revealed by a Cy5-labelled secondary antibody (GE Lifescience). 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).

2.10. Statistical analysts

Statistical significance of the results was calculated using the softwares GraphPad InStat version 3.10 and GraphPad Prism version 5. ANOVA followed by Bonferroni's post test was used when the software indicated a Gaussian distribution of the experimental data. Otherwise, analysis was performed using Kruskal-Wallis and Dunn's tests.

3. Results

3.1. Inhibition of LDH and aerobic glycolysts

Inhibition by oxamic acid of LDH from PLC/PRF/5 cells measured in cell free extracts is reported in Fig. 1. The Ki value with respect to pyruvate was 81.4 μM, similar to that determined using crystalline LDH from bovine muscles (99 μM).

Fig. 2 shows the reduction of lactate production measured in PLC/PRF/5 cells after 3 h incubation in the presence of oxamic acid; these data indicate that LDH inhibition by oxamic acid can interrupt aerobic glycolysis. Fig. 2 shows that the effective concentrations of inhibitor were about 1000 times higher than those active on LDH from cell free extracts. This discrepancy might be mainly due to a poor capacity of oxamic acid to cross the cell membrane, considering that its pKa is as low as 2.1 [16] and polar and anionic molecules, cannot easily penetrate the cell membrane unless they use a membrane transporter.

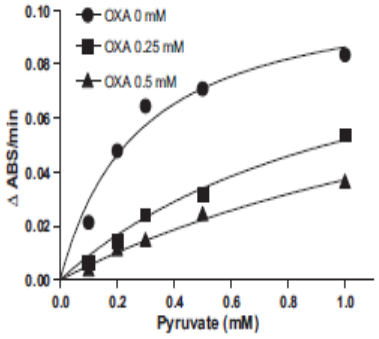


Fig. 1. Effect of oxamic acid (OXA) on PLC/PRF/5 cells LDH. The enzymatic assay was performed as described in Section 2. The Ki (measured using the GraphPad Prism 5 software) was 81.4 μM.

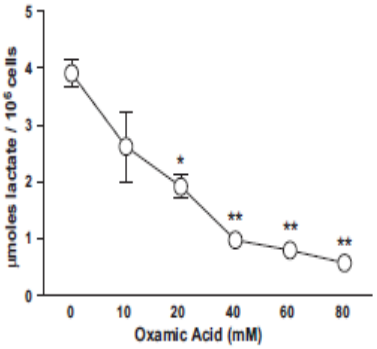


Fig. 2. Production of lactic acid by PLC/PRF/5 cells after 3 h incubation in the presence of scalar concentration of oxamic acid. Each entry is the mean value ± SE of results from two experiments, performed as described in Section 2. (*, **) A statistically significant reduction compared to controls with $p < 0.05$ and $p < 0.01$, respectively.

3.2. Effect of oxamic acid on ATP levels

In PLC/PRF/5 cells exposed for 3–24 h to oxamic acid at the concentrations which inhibited aerobic glycolysis (40–80 mM), the levels of ATP were reduced by 25–50% (Fig. 3A and B). The results obtained by culturing the cells in the presence (Fig. 3A) or in the absence (Fig. 3B) of glutamine were practically superimposable, demonstrating that, when maintained in conventional DMEM medium, PLC/PRF/5 cells mainly derive their ATP from glucose metabolism. When the aerobic glycolysis was abolished by culturing the cells in the absence of glucose [11–13], oxamic acid did not decrease the levels of ATP (Fig. 3C), indicating that in glycolysing cells the reduced ATP concentrations were only caused by the inhibition of aerobic glycolysis.

3.3. Effect of kinase inhibitors and of their combination with oxamic acid on PLC/PRF/5 cells viability

Fig. 4 shows the effect of sorafenib, sunitinib and imatinib on PLC/PRF/5 cells viability, as measured by the neutral red assay after 24 h-exposure to the compounds. To test their effect when given in combination with oxamic acid, for each drug we chose doses causing a statistically significant inhibition on PLC/PRF/5 cell proliferation, not higher than 30%, in order to facilitate the evidence of a possible potentiating effect in the combination with oxamic acid. In Fig. 4 the chosen doses have been highlighted by a shaded area. When given in combination with the kinase inhibitors, oxamic acid was used at the concentrations of 60 and 80 mM, since these doses were found to almost completely inhibit aerobic glycolysis (Fig. 2) and to produce a 24 h-sustained 50% reduction in cellular ATP (Fig. 3A and B). Given to PLC/PRF/5 cells for 24 h, these doses of oxamic acid produced only a small and not statistically significant effect on cell proliferation (see below). Fig. 5 shows the effect on cell growth of sorafenib (8 and 16 μM), sunitinib (8 μM) and imatinib (32 μM), given to PLC/PRF/5 cells in combination with 60 and 80 mM oxamic acid, as evaluated by the neutral red assay. The graphs also report the effects of oxamic acid and of the kinase inhibitors given alone, which were measured in the same experiment together with the drug combination. In the different tests, the growth inhibitory effect caused by oxamic acid ranged from 9% to 20% and resulted not statistically significant. Given alone, the three kinase inhibitors confirmed the effect already measured in the

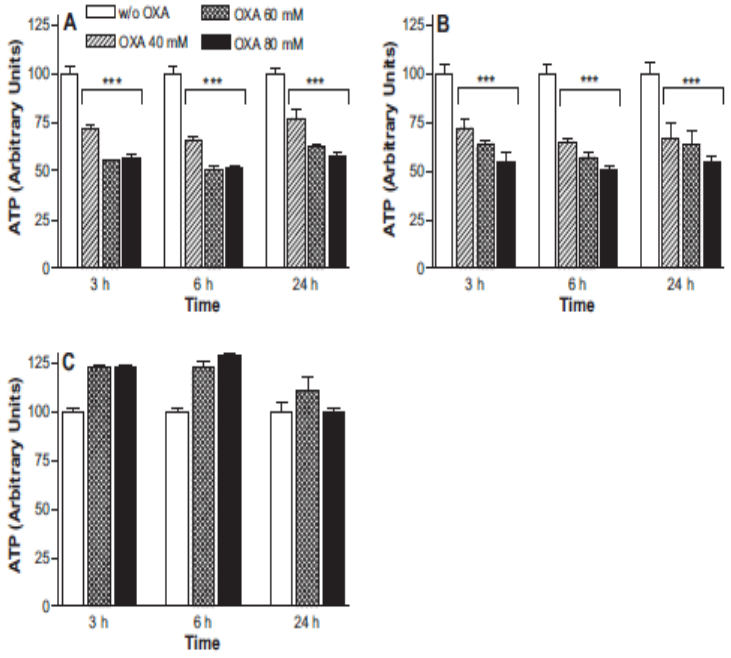


Fig. 3. Effects caused by oxamic acid (OXA) on ATP levels of PLC/PRF/5 cells cultured in DMEM with (A) or without (B) glutamine or in glucose deprived medium (C). (***) Significant reduction compared to controls with $p < 0.001$.

growth inhibition curve reported in Fig. 4. When these compounds were given in combination with oxamic acid, an enhancement of their anti-proliferative activity was observed, that, for the 80 mM dose, always resulted statistically significant when compared to the effect of the kinase inhibitor given alone. In the case of 16 μM sorafenib, the combination with 80 mM oxamic acid had a so heavy effect to bring about the almost complete inhibition of cell growth (>97%) over the 24 h.

3.4. Effects caused by sorafenib and by its combination with oxamic acid on the morphology of PLC/PRF/5 cells

Morphological examination of treated cells confirmed the potentiating effect exerted by the drugs combination. It was performed only on cells treated with sorafenib since, compared to the other kinase inhibitors, the gain of efficacy observed with the oxamic acid combination of this compound was the most remarkable (see Fig. 5). PLC/PRF/5 cells were fixed and stained after a 6 h-exposure to sorafenib, oxamic acid or their combination. Fig. 6B shows that the only effect caused by oxamic acid was a moderate vacuolization of cytoplasm. The morphology of nuclei of the cells treated with this compound appeared to be similar to that of control cells (Fig. 6A). On the contrary, after sorafenib treatment (Fig. 6C) most cell nuclei appeared reduced in size, with a more intensely stained chromatin or even picnotic. The combination of the two compounds (Fig. 6D) definitively worsened the changes produced by sorafenib: almost all cells exhibited shrunken nuclei in which nucleoli were no longer detectable and severely vacuolated cytoplasms. Many picnotic cells were visible.

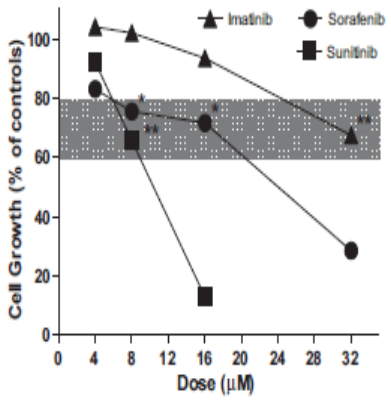


Fig. 4. Effect of kinase inhibitors on PLC/PRF/5 cells growth, measured by the neutral red assay after 24 h incubation with the compounds. The shaded area indicates the selected doses for studying the effect of oxamic acid combination. Asterisks indicate a statistically significant difference with $p < 0.05$ (*) or $p < 0.01$ (**).

3.5. [^{14}C]thymidine incorporation by cells treated with sorafenib given alone or in combination with oxamic acid

For the same reason stated above (Section 3.4), this experiment was performed only in PLC/PRF/5 cells treated with sorafenib. After 1 h incubation with [^{14}C]thymidine in the presence of 16 μM sorafenib, 80 mM oxamic acid, their combination or saline, not statistically significant differences were observed in radioactivity incorporation among the PLC/PRF/5 cultures, as evaluated by ANOVA (data not shown). The treatment was then continued for an additional 5 h in order to measure the increase in radioactivity incorporation in the treated cultures, which is an indicator of active DNA synthesis. Results are reported in Fig. 7. ANOVA test showed that, compared to control cultures treated with saline, only cells treated with the combination oxamic acid/sorafenib displayed a statistically significant delay of DNA synthesis at this time. The result of this experiment together with the morphologic observation of cells, which was performed only after a 6 h treatment, suggested a very rapid onset for the anti-proliferative action caused by the combination of sorafenib with oxamic acid.

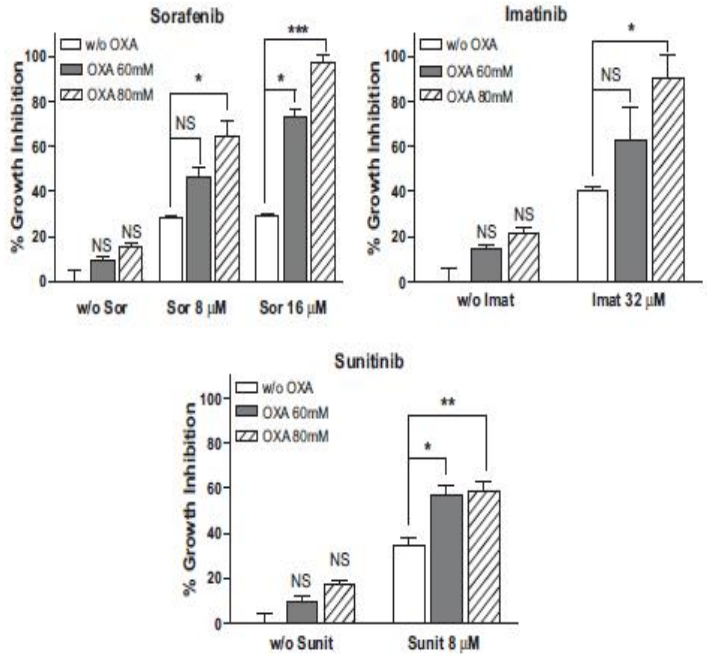


Fig. 5. Effect of the oxamic acid (OXA) combination with the kinase inhibitors on PLC/PRF/5 growth, measured by the neural red assay after 24 h incubation with the compounds. Asterisks indicate a statistically significant difference with $p < 0.05$ (*), $p < 0.01$ (**) or $p < 0.001$ (***). NS, not significant.

3.6. *Immunoblot analysis of ERK 1/2 phosphorylation in cells treated with sorafenib given alone or in combination with oxamic acid*

The immunoblot experiment was performed only in PLC/PRF/5 cells treated with sorafenib.

One of the primary targets of sorafenib is the RAF kinase, the first signaling element of the MAPK pathway, which has an essential role in HCC cell proliferation. The blocking of RAF causes a downstream reduction of MEK and ERK phosphorylation, ultimately resulting in the inhibition of cell proliferation. Fig. 8 shows that, in agreement with published data [17], 1 h treatment with 16 μM sorafenib

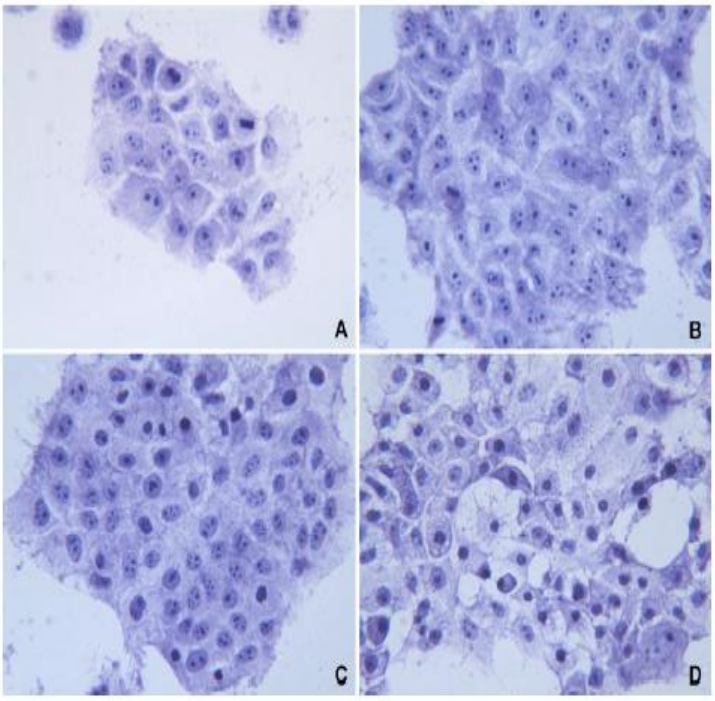


Fig. 6. Morphology of PLC/PRF/5 cells exposed for 6 h to oxamic acid (B), sorafenib (C) or their combination (D). A control culture, treated with saline, is shown in (A).

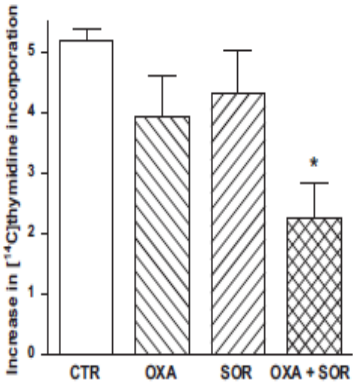


Fig. 7. Increase of [^{14}C]thymidine incorporation measured in PLC/PRF/5 cells from 1 to 6 h incubation in the presence of 80 mM oxamic acid, 16 μM sorafenib or their combination. Data were analyzed by ANOVA followed by the Bonferroni post test. (*) A statistically significant difference compared to controls, with $p < 0.05$.

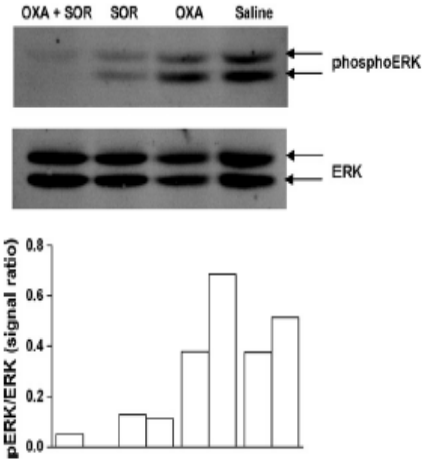


Fig. 8. Immunoblot analysis of ERK 1/2 phosphorylation in PLC/PRF/5 cells exposed to sorafenib (SOR), oxamic acid (OXA) or their combination. For both protein bands, the ratio of the fluorescent signals between phosphoERK and ERK was calculated and has been reported in the bar graph.

reduced the phosphorylation of ERK1/2. The bar graph reporting the ratios between the fluorescence signals of phosphoERK and ERK indicates that 80 mM oxamic acid given for 1 h to PLC/PRF/5 cells did not affect ERK phosphorylation, which on the contrary was heavily reduced in cultures treated with the 16 μM sorafenib/80 mM oxamic acid combination, where the low molecular weight band of phosphoERK was not detected. The further reduction of phosphoERK observed in these cells, compared to those treated with sorafenib alone, is a confirmation of the enhancing effect caused by oxamic acid on the signaling inhibition produced by sorafenib.

4. Discussion

The present experiments showed that oxamic acid decreased the ATP levels in glycolysing PLC/PRF/5 cells and potentiated the anti-proliferative activity of kinase inhibitors on these cells. Our results are in agreement with those of Zhou et al. [18], who found that the inhibition of LDH by oxamic acid significantly increased the sensitivity to taxol of mammary cancer cells resistant to this drug. In performing our study we deliberately chose conditions (a 24 h only exposure to drugs) in which oxamic acid produced a not significant effect on cell growth; as a consequence, the observation that the effect of its combination with the kinase inhibitor was significantly higher than that of the inhibitor given alone suggests that the combination of the two molecules could result not only in an additive, but also in a synergistic effect [19]. This seems reasonable mainly for the combination sorafenib/oxamic acid, for which we observed a striking increase of the anti-proliferative effect compared to sorafenib given alone, with a rapid impairment in DNA synthesis, as indicated by the reduced rate of [^{14}C]thymidine incorporation (Fig. 7).

According to the rationale which suggested these experiments, the enhanced anti-proliferative effect of oxamic acid combination with kinase inhibitors can at least in part be due to the reduced number of ATP molecules competing with the drugs for the binding to the common enzymatic site. This is also supported by the stronger signaling inhibition produced by sorafenib when given in combination with oxamic acid (Fig. 8). When aerobic glycolysis was shut down by culturing PLC/PRF/5 cells on galactose [11–13], oxamic acid did not cause a depletion of ATP, indicating that this compound does not interfere with the oxidative phosphorylation, still operative in this culture condition. As a consequence, in glycolysing cells the reduced ATP concentrations caused by oxamic acid can only be ascribed to the inhibition of aerobic glycolysis. This suggests that in normal cells, which do not produce ATP through aerobic glycolysis, the inhibition of LDH should not exacerbate the adverse reactions of antineoplastic drugs. Support to the possibility that the LDH activity is not necessary to normal cells in conditions of normal functional activity and sufficient oxygen supply also comes from the finding that humans with a hereditary deficiency of LDH isoforms do not show any symptoms under normal circumstances [20,21].

Oxamic acid is a non-toxic substance in laboratory animals up to 3 g/kg [6]. On the other hand, it inhibits the aerobic glycolysis in cells cultured in vitro only at high concentrations which are not expected to be reached in vivo. However, changes of the molecule can be studied using oxamic acid as a lead compound to synthesize a selective and more powerful inhibitor of LDH. It should display a lower Ki than that of oxamic acid and/or a higher capacity to cross the cell membrane (see Section 3.1). A similar attempt was performed to improve the chemotherapy of malaria [22,23]: several derivatives of the oxamic acid molecule were synthesized and tested on LDH of Plasmodium falciparum, a parasite which relies on aerobic glycolysis for its energetic needs. Encouraging results are also those of experiments addressed to the inhibition of the isoform II of α-hydroxyacid dehydrogenase (HADH II) of Trypanosoma cruzi, which shows a substrate specificity similar to that of LDH-C4 of spermatozoa. Simple derivatives of oxamic acid such as N-isopropyl and N-propyl oxamate were found more active in inhibiting HADH II in vitro than the parent compound [24,25] and their ethyl-ester pro-drugs orally administered to Trypanosoma cruzi infected mice at a dose of 10 mg/kg/day for 60 days completely eliminated amastigote nests in the muscles and myocardium, demonstrating their efficacy in vivo [26]. Moreover, by applying the virtual screening procedure [27] to the crystal structure of human LDH, also molecules chemically unrelated to oxamic acid but able to inhibit LDH can be searched.

5. Conclusions

The results of our experiments encourage the search for a LDH inhibitor more powerful than oxamic acid and also active in vivo. Its combination with kinase inhibitors could enhance the anti-proliferative efficacy of these drugs in the treatment of responsive tumours without exacerbating their effects on normal cells, whose ATP levels should not be affected by the block of the enzyme.

Acknowledgements

This work was supported by Fondazione del Monte di Bologna e Ravenna, Roberto and Cornelia Pallotti Legacies for Cancer Research and University of Bologna.

References

[1] Pelicano H, Martin DS, Xu R-H, Huang P. Glycolysis inhibition for anticancer treatment. Oncogene 2006;25:4633–46.
[2] Warburg O. Uber den Stoffwechsel der Tumoren. Berlin: Verlag Springer; 1926. Translated into English by Dickens F under the title The metabolism of tumours. Constable, London, 1930.
[3] Fiume L. Inhibition of aerobic glycolysis in Yoshida ascites hepatoma by tartronic acid. Nature 1960;187:792–3.
[4] Papacostantinou J, Colowick SP. The role of glycolysis in the growth of tumor cells. I. Effects of oxamic acid on the metabolism of Ehrlich tumor cells in vitro. J Biol Chem 1961;236:278–84.
[5] Novoa WB, Winer AD, Glaid AJ, Schwert GW. Lactic dehydrogenase. V. Inhibition by oxamate and by oxalate. J Biol Chem 1959;234:1143–8.
[6] Papacostantinou J, Colowick SP. The role of glycolysis in the growth of tumor cells. II. The effect of oxamic acid on the growth of HeLa cells in tissue culture. J Biol Chem 1961;236:285–8.
[7] Fiume L, Manerba M, Vettraino M, Di Stefano G. Impairment of aerobic glycolysis by inhibitors of lactic dehydrogenase hinders the growth of human hepatocellular carcinoma cell lines. Pharmacology 2010;86:157–62.
[8] Sebolt-Leopold JS, English JM. Mechanisms of drug inhibition of signalling molecules. Nature 2006;441:457–62.
[9] Alexander JJ, Bey EM, Geddes EW, Lecatsas G. Establishment of a continuously growing cell line from primary carcinoma of the liver. S Afr Med J 1976;50:2124–8.
[10] Barker SB, Summerson WH. The colorimetric determination of lactic acid in biological material. J Biol Chem 1941;138:535–54.
[11] Reitzer LJ, Wice BM, Kennell D. Evidence that glutamine, not sugar, is the major energy source of cultured HeLa cells. J Biol Chem 1979;254:2669–76.
[12] Rossignol R, Gilkerson R, Aggeler R, Yamagata K, Remington SJ, Capaldi RA. Energy substrate modulates mitochondrial structure and oxidative capacity in cancer cells. Cancer Res 2004;64:985–93.
[13] 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;97:539–47.
[14] Fotakis G, Timbrell JA. In vitro cytotoxicity assays: comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. Toxicol Lett 2006;160:171–7.
[15] Berridge MV, Herst PM, Tan AS. Tetrazolium dyes as tools in cell biology: new insights into their cellular reduction. Biotechnol Annu Rev 2005;11:127–52.
[16] Xin Z, Liu G, Abad-Zapatero C, Pei Z, Szczepankiewicz BG, Li X, et al. Identification of a monoacid-based, cell permeable, selective inhibitor of protein tyrosine phosphatase 1B. Bioorg Med Chem Lett 2003;13:3947–50.
[17] Liu L, Cao Y, Chen C, Zhang X, McNabola A, Wilkie D, et al. Sorafenib blocks the RAF/MEK/ERK pathway, inhibits tumor angiogenesis, and induces tumor cell apoptosis in hepatocellular carcinoma model PLC/PRF/5. Cancer Res 2006;66:11851–8.
[18] Zhou M, Zhao Y, Ding J, Liu H, Liu Z, Fodstad O, et al. Warburg effect in chemosensitivity: targeting lactate dehydrogenase-A re-sensitizes taxol-resistant cancer cells to taxol. Mol Cancer 2010;9:33–45.
[19] Tallarida R. The interaction index: a measure of drug synergism. Pain 2002;98:163–8.
[20] Kanno T, Sudo K, Maekawa M, Nishimura Y, Ukita M, Fukutake K. Lactate dehydrogenase M-subunit deficiency: a new type of hereditary exertional myopathy. Clin Chim Acta 1988;173:89–98.
[21] Okumura N, Terasawa F, Ueno I, Oki K, Yamauchi K, Hidaka H, et al. Genetic analyses in homozigous and heterozigous variants of lactate dehydrogenase-B (H) subunit -LD-B Matsumoto I and II (LD-B W323R)-. Clin Chim Acta 1999;287:163–71.
[22] Choi S, Beeler AB, Pradhan A, Watkins EB, Rimoldi JM, Tekwani B, et al. Generation of oxamic acid libraries: antimalarials and inhibitors of Plasmodium falciparum lactate dehydrogenase. J Comb Chem 2007;9:292–300.
[23] Deck LM, Royer RE, Chamblee BB, Hernandez VM, Malone RR, Torres JE, et al. Selective inhibitors of human lactate dehydrogenase and lactate dehydrogenase from malarial parasite Plasmodium falciparum. J Med Chem 1998;41:3879–87.
[24] Elizondo S, Chena MA, Rodriguez-Paez L, Nogueda B, Baeza I, Wong C. Inhibition of Trypanosoma cruzi α-hydroxyacid dehydrogenase-isozyme II by N-isopropyl oxamate and its effect on intact epimastigotes. J Enzyme Inhib Med Chem 2003;18:265–71.
[25] Aguirre-Alvarado C, Zaragoza-Martinez F, Rodriguez-Paez L, Nogueda B, Baeza I, Wong C. In vitro and in vivo trypanocydal activity of ethyl esters of N-allyl and N-propyl oxamates using different Trypanosoma cruzi strains. J Enzyme Inhib Med Chem 2007;22:227–33.
[26] Aguirre-Alvarado C, Zaragoza-Martinez F, Rodriguez-Paez L, Tellez-Rendon JL, Nogueda B, Baeza I, et al. Trypanocydal activity of the ethyl ester of N-propyl and N-isopropyl oxamates on intracellular amastigotes of Trypanosoma cruzi acute infected mice. J Enzyme Inhib Med Chem 2010;25:111–5.
[27] Rester U. From virtuality to reality, virtual screening in lead discovery and lead optimization: a medicinal chemistry perspective. Curr Opin Drug Discov Devel 2008;11:559–68.

Effect of sorafenib on the energy metabolism of hepatocellular carcinoma cells

European Journal of Pharmacology 670 (2011) 39–43

Contents lists available at SciVerse ScienceDirect

European Journal of Pharmacology

journal homepage: www.elsevier.com/locate/ejphar

Molecular and Cellular Pharmacology

Effect of sorafenib on the energy metabolism of hepatocellular carcinoma cells

Luigi Fiume, Marcella Manerba, Marina Vettraino, Giuseppina Di Stefano *

Department of Experimental Pathology, University of Bologna, Italy

ARTICLE INFO

Article history:
Received 22 February 2011
Received in revised form 12 August 2011
Accepted 27 August 2011
Available online 10 September 2011

Keywords:
Sorafenib
Hepatocellular carcinoma chemotherapy
Aerobic glycolysis
Tumor cell metabolism

ABSTRACT

Recent data demonstrated that sorafenib impaired the oxidative phosphorylation of a rat myogenic cell line and suggested that this biochemical lesion can contribute to the cardiac toxicity caused by the drug. With the experiments reported here, we verified whether sorafenib inhibits oxidative phosphorylation also in cells from human hepatocellular carcinomas (HCCs), which are treated with this drug. By using the HCC cell lines PLC/PRF/5 and SNU-449 we studied the effects of the drug on ATP cellular levels, oxygen consumption and aerobic glycolysis, a metabolic pathway generally used by neoplastic cells to meet their energy demand. The effect of sorafenib on ATP cellular levels was also studied in cells grown in a glucose-free medium, which only derive their energy from oxidative phosphorylation. We found that at clinically relevant concentrations sorafenib hindered oxidative phosphorylation, whereas at the same time stimulated aerobic glycolysis in glucose-grown cells, thus attenuating the cellular ATP depletion. These results support the impairment of oxidative phosphorylation as a mechanism contributing to the antineoplastic activity of sorafenib in the treatment of HCCs.

1. Introduction

Sorafenib is a multi-kinase inhibitor approved for the treatment of non-resectable hepatocellular carcinomas (HCCs) (Llovet et al., 2008). Its clinical success has been attributed to the ability of inhibiting the target kinases (Liu et al, 2006). However, in experiments aimed at elucidating the mechanism(s) underlying the cardiac side effects of sorafenib, Will et al. (2008) observed that the drug, at clinically relevant concentrations, inhibited the oxidative phosphorylation (OXPHOS) of a myoblast cell line (H9c2) and directly impaired the functions of rat heart isolated mitochondria. In the present experiments, we studied whether this mechanism of cell damage can also take part in the anti-proliferative effect of sorafenib on HCC cells. To address this issue, we used PLC/PRF/5 and SNU-449 cell cultures (two lines derived from human HCCs (Alexander et al., 1976; Park et al., 1995)), which, for some experiments, were adapted to grow in glucose-deprived medium. PLC/PRF/5 and SNU-449 cells, similar to the majority of neoplastic cells, largely rely on aerobic glycolysis for their ATP generation (Pelicano et al., 2006). When maintained in glucose-deprived medium, these cells can obtain ATP solely through OXPHOS, using glutamine as the main substrate for the TCA cycle (Marroquin et al., 2007; Reitzer et al., 1979; Rossignol et al., 2004). We exposed cell cultures to sorafenib added at the concentrations raised in plasma of patients treated with the drug (4–16 μM) (Strumberg et al., 2005, 2007) and studied its effects on OXPHOS and aerobic glycolysis. It was observed that treatment with sorafenib caused an early reduction of ATP cellular levels and that this reduction was more marked in cultures which only rely on OXPHOS for their energetic needs. Evidence was obtained that the decrease in ATP levels in glycolysing cells, although moderate, can contribute to the antiproliferative effect of sorafenib.

2. Materials and methods

2.1. Materials

Sorafenib (p-toluensulfonate salt) was obtained from LC Labs (USA). The stock solutions used for the experiments were prepared in DMSO and were added to the cell cultures at the indicated concentrations (see below), taking care that the final DMSO content of cell cultures never exceeded 0.05% vol/vol. In all experiments, DMSO was also added to control cultures, treated with saline. All other chemicals were from Sigma-Aldrich.

Cell culture media and supplements were obtained from Bio-Wyttacker. Plastics for cell cultures were from Falcon. The CellTiter-Glo Luminescent cell viability reagent was purchased from Promega. The phosphorescent oxygen-sensitive probe (MitoXpress probe) was from Luxcel Biosciences.

2.2. Cell cultures

PLC/PRF/5 and SNU-449 are cell lines derived from human HCCs with HBV etiology, taken from patients prior to cytotoxic therapy (Alexander et al., 1976; Park et al., 1995). They were cultured in DMEM with 25 mM glucose, supplemented with 10% fetal bovine serum, non-

* Corresponding author at: Department of Experimental Pathology, University of Bologna, via San Giacomo, 14-40126 Bologna, Italy. Tel.: +39 0512094721; fax: +39 0512094746.
E-mail address: giuseppina.distefano@unibo.it (G. Di Stefano).

0014-2999/$ – see front matter © 2011 Elsevier B.V. All rights reserved.
doi:10.1016/j.ejphar.2011.08.038

essential aminoacids, 100 IU/ml penicillin, 100 mg/ml streptomycin, 2 mM glutamine. Cells were incubated in a humidified atmosphere of 5% CO_2, 95% air at 37 °C.

A sub-culture of the two cell lines was maintained in glucose-free medium containing 10 mM galactose (needed as a source of precursors for DNA synthesis (Marroquin et al., 2007; Reitzer et al., 1979; Rossignol et al., 2004)) and supplemented with 10% dialyzed fetal bovine serum, non-essential aminoacids, 100 IU/ml penicillin, 100 mg/ml streptomycin, 6 mM glutamine. Adaptation of cell metabolism to these conditions required a minimum of 4 weeks culture.

2.3. Assay of ATP levels

ATP levels were measured using the CellTiter-Glo Luminescent Cell Viability Assay. For these experiments, 2×10^4 cells in 200 µl of culture medium were seeded into each well of a 96-multiwell opaque body plate and allowed to adhere overnight. Sorafenib (4–16 µM) was added and ATP levels were measured after 3 and 6 h incubation at 37 °C. Each dose of drug was tested in quadruplicate. After incubation, the plate was allowed to equilibrate at room temperature for 30 min and the CellTiter-Glo reactive was directly added to each well, as indicated by the manufacturer. The plate was kept on a shaker for 10 min to induce cell lysis and luminescence was measured by using a Fluoroskan Ascent FL reader (Labsystems). This experiment was performed both on glycolytically poised and on galactose grown cells.The effect of oxamic acid (an inhibitor of lactate dehydrogenase (Novoa et al., 1959)) (40–80 mM) was also tested using this assay.

2.4. Measure of lactate levels

To study the effect of the drug on aerobic glycolysis, we measured the amount of lactic acid produced by PLC/PRF/5 and SNU-449 cells after 1 and 3 h incubation in the presence of 4–16 µM sorafenib. 5×10^5 cells in 2 ml of culture medium were seeded in each well of a 6-well Nunclon plate and let to adhere overnight. The medium was then replaced with 1 ml of Krebs-bicarbonate buffer containing 25 mM glucose and scalar concentrations of sorafenib, pre-warmed at 37 °C. Each dose of the drug was tested in triplicate. Lactate (intracellular + released in medium) was measured according to the method of Barker and Summerson (1941), following the procedure described by Fiume et al. (2010).

2.5. Cell respiration assay

PLC/PRF/5 and SNU-449 cells were plated at 5×10^4 cells/well in 96 multi-well clear bottom, black body plates and allowed to adhere overnight. After the addition of the MitoXpress phosphorescent oxygen-sensitive probe (10 pmoles/well), plates were placed in a Victor™ fluorescence reader (Perkin Elmer) at 30 °C and were monitored for about 20 min to reach temperature and gas equilibrium and to obtain basal signals. Then, sorafenib (4 and 8 µM) was quickly added, wells were sealed with mineral oil and monitoring of the signal was resumed for the next 60 min. During this interval, the increase of fluorescence signal, which indicates oxygen consumption, was measured every 60 s with 340/642 nm excitation/emission filters, a delay time of 30 µsec and a measurement window of 100 µsec. All procedures of the experiment were performed at 30 °C with pre-warmed solutions. Each dose of sorafenib was tested in quadruplicate. Evaluation of cell respiration was performed by applying the linear regression analysis to the time profiles of fluorescence signals obtained from each well, in order to determine the slopes of each profile.

2.6. Evaluation of cell growth

Cell growth was evaluated using the neutral red assay, which gives a measure of the number of viable cells that actively endocytose this compound (Fotakis and Timbrell, 2006).

PLC/PRF/5 and SNU-449 cells (3×10^4/well) were seeded in 24-multiwell plates, allowed to adhere overnight and treated with scalar concentrations of sorafenib or oxamate in order to calculate the IC_{50} of the two drugs. Each dose was tested in triplicate. Treated cultures were incubated at 37 °C for 24 h (treatment with sorafenib) or 72 h (treatment with oxamate). At the end of incubation periods, untreated cultures (controls) and drug-treated cells were maintained for an additional 3 h at 37 °C with the neutral red dye dissolved in DMEM at the final concentration of 30 µg/ml. After this time, the medium was removed and the cells were solubilised with 1 ml of 1% acetic acid in 50% ethanol. The solution from each well (200 µl) was then transferred to a 96-multiwell plate and its absorbance at λ_{540} was measured using a microplate reader (Multiskan Ascent FL, Labsystems).

Experiments were also performed to compare the effect caused on cell growth by 8 µM sorafenib and 40 mM oxamate (PLC/PRF/5 cells) and 8 µM sorafenib and 80 mM oxamate (SNU-449 cells), which produced a similar ATP depletion in the cell cultures (see below). The results were evaluated after 24, 48 and 72 h exposure times.

2.7. Statistical analysis

All experimental data were statistically evaluated by ANOVA followed by the Dunnet post test using the GraphPad softwares Prism 5 and InStat 3.

3. Results

3.1. Effect of sorafenib on OXPHOS

To evaluate the effect of sorafenib on OXPHOS we measured ATP cellular levels of PLC/PRF/5 and SNU-449 cultures maintained both in conventional and in glucose-deprived medium, containing galactose (see Materials and Methods). ATP was measured after 3 and 6 h exposure. At these times the number of viable cells is not yet affected by the drug. Fig. 1 shows that when cell cultures were treated with oxamic acid, ATP levels were reduced in glycolysing cells only. Oxamic acid is an inhibitor of lactate dehydrogenase (Novoa et al., 1959), which selectively blocks aerobic glycolysis (Fiume et al., 2010; Papacostantinou and Colowick, 1961). This observation confirmed that in cells grown in galactose this metabolic pathway was not operative and that these cells had to resort only to OXPHOS to obtain ATP. Fig. 1 (upper graphs) shows that sorafenib had a marked effect on ATP levels of these cultures, causing an almost complete deprivation of energy supply at the dose of 16 µM, which is very close to the drug IC_{50}s (Fig. 2). In glycolysing cells (Fig. 1, bottom graphs), the effect of sorafenib was milder and the ATP content appeared to be decreased by only 12–32%. Unpredictably, in glycolysing cells sorafenib caused a decrease of ATP level which was significantly higher at the dose of 8 µM than at 16 µM. This contradictory result can be explained by the higher implementation effect exerted by the 16 µM dose of sorafenib on aerobic glycolysis (see below).

In a further experiment the effect of imatinib and sunitinib (two other multi-kinase inhibitors (Sebolt-Leopold and English, 2006)) was also studied. These drugs, tested on cell cultures at ten-fold higher concentrations than those raised in patients (Will et al., 2008), did not cause any decrease in ATP levels both in glycolysing and in galactose-maintained PLC/PRF/5 cells (data not shown).

3.2. Effect of sorafenib on aerobic glycolysis

In PLC/PRF/5 and SNU-449 cells sorafenib stimulated aerobic glycolysis, as indicated by the higher amount of lactic acid formed in the presence of the drug (Fig. 3). The enhancement of aerobic glycolysis accounts for the small depletion of ATP produced by 16 µM sorafenib in cultures maintained in standard medium containing glucose,

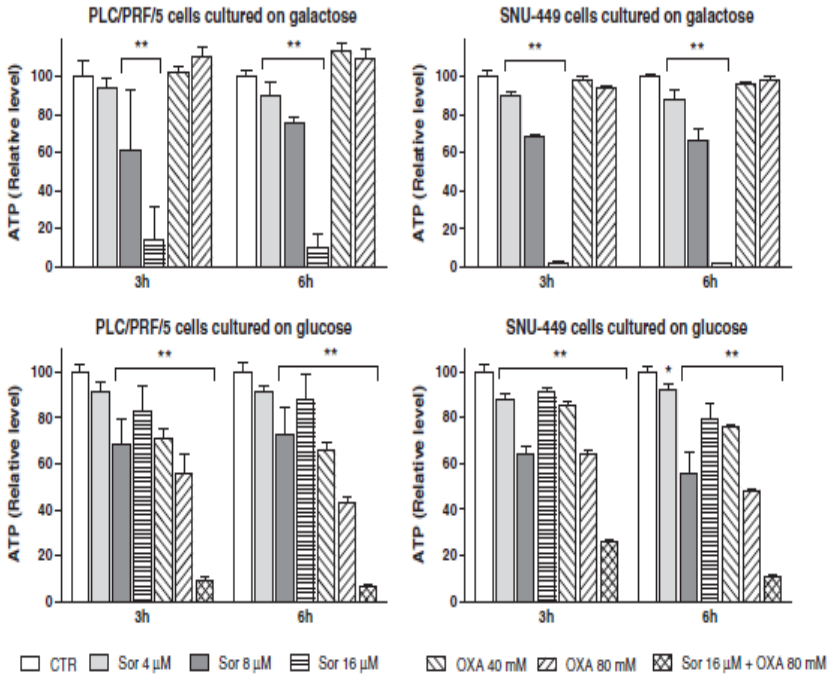


Fig. 1. Effect of sorafenib on ATP levels of PLC/PRF/5 and SNU-449 cells cultured on galactose (upper graphs) or in standard DMEM containing glucose (bottom graphs). The effect of oxamate (an inhibitor of LDH) was also tested. Each entry is the mean value ± SD. Data were analyzed by ANOVA, followed by the Dunnet post test. Asterisks indicate a statistically significant increase compared to controls, with $P<0.05$ (*) and $P<0.01$ (**).

compared to that caused in cells grown on galactose. As a further confirmation, when glycolysing cells were exposed to 16 μM sorafenib in association with 80 mM oxamic acid (which blocks aerobic glycolysis), their ATP content dropped to very low levels (<10% of control cells after a 6 h incubation) (Fig. 1, bottom graphs).

3.3. Effect of sorafenib on cell respiration

To further confirm that sorafenib impairs mitochondrial functions also in cells grown in standard medium containing glucose, we studied the effect of the drug on the respiration of these cells, which maintain their physiological status. Respiration was evaluated by measuring cell oxygen consumption for a period of 60 min after the addition of the drug. Fig. 4 shows that even at the lower concentration (4 μM), sorafenib significantly decreased oxygen consumption in both cell lines. A more marked effect was observed in PLC/PRF/5 cells, in which the 8 μM concentration of the drug completely abolished respiration. In SNU-449 cells the effects caused by the two tested concentrations were superimposable. This finding definitively demonstrates that sorafenib can markedly hinder the mitochondrial function also in glycolysing HCC cells.

3.4. Effect of ATP reduction on cell viability

Fig. 5 shows the effects of oxamic acid and sorafenib on the viability of cells cultured in glucose medium and exposed for three days to concentrations of drugs (40 mM oxamic acid and 8 μM sorafenib in PLC/PRF/5 cells; 80 mM oxamic acid and 8 μM sorafenib in SNU-449 cells) causing a similar depletion of ATP (about 30%) (Fig. 1, bottom graphs). Oxamic acid, which inhibits lactate dehydrogenase, causes cell death by only reducing ATP cellular levels (Goldberg et al., 1965; Papacostantinou and Colowick, 1961). It caused a decrease in the number of viable cells which, although lower than that produced by sorafenib, was statistically significant. This suggests that a depletion of ATP to an extent similar to that produced by 8 μM sorafenib in glycolysing cells can contribute to the antiproliferative effect of the drug on these cells.

4. Discussion

Liu et al. (2006) found that in PLC/PRF/5 cells sorafenib at clinically relevant concentrations inhibited RAF/MEK/ERK signaling pathway, thus impairing cell viability. In the experiments reported here, by using the same cell line and similar drug concentrations we showed that sorafenib also markedly impaired the OXPHOS. The same effect was also observed in SNU-449 cells, another cell line derived from a human HCC. Because of the link between the two energetic pathways (Krebs, 1972), the depletion of mitochondrial ATP in glycolysing cells caused an increase of glycolysis so that the ATP content in these cells was only 12–32% lower than that of control cells. This decrease, although moderate, is statistically significant and can impair cell growth, as supported by the finding that in both cell lines a dose of oxamic acid which reduces ATP levels to the same extent as sorafenib after a 3 days exposure significantly reduced the number of viable cells by about 30% (Fig. 4). An inhibitory effect on cell growth caused by a moderate decrease of ATP levels (~30%) was also reported by Lieberthal et al. (1998), who reduced cellular ATP by using 2-deoxyglucose and observed a 20–30% inhibition of the replication of cultured mouse proximal tubular cells. Moreover, in the case of sorafenib, which blocks the target kinases by binding to their ATP sites (Sebolt-Leopold and English, 2006), the depletion of ATP could also enhance the anti-kinase activity of the drug by reducing the competition for the common enzymatic site. Therefore, the present results point out that the loss of ATP caused by the drug interference with OXPHOS probably contributes to the anticancer activity of sorafenib on human HCC cells. Contrary to sorafenib, imatinib and sunitinib did not impair the OXPHOS of PLC/PRF/5 cells.

Before the introduction of sorafenib, systemic chemotherapy of HCCs was practically unused. This drug, however, produced only modest results on patient survival (Llovet et al., 2008) and, consequently, the

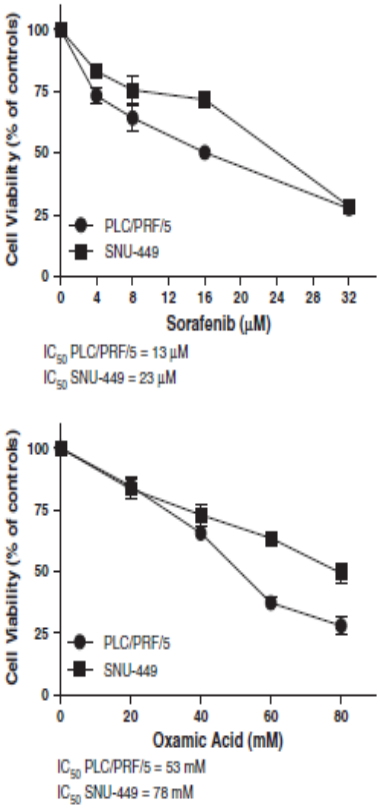


Fig. 2. Effect of sorafenib and oxamate on PLC/PRF/5 and SNU-449 cell growth. Cells were treated for 24 h (sorafenib) and 72 h (oxamate). Experiments were performed as described in Materials and methods.

search of new therapeutic strategies to fight this tumor form is still in progress. The observation of an increased ratio between glycolytic and OXPHOS-derived ATP in HCC cells treated with this drug suggests that a possible way to enhance the therapeutic power of sorafenib could be its combination with glycolytic inhibitors, such as oxamate, which blocks lactate dehydrogenase. As shown by our results, the association of sorafenib/oxamate caused the almost complete ATP depletion in treated cells. Unfortunately, oxamate has the drawback of a scarce

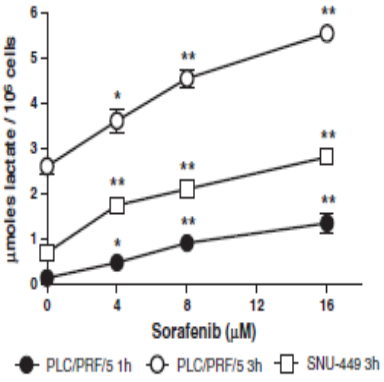


Fig. 3. Amounts of lactate (intracellular + released in medium) produced by glycolysing PLC/PRF/5 and SNU-449 cells exposed to scalar concentrations of sorafenib. The reported data are mean values ± S.D. They were analyzed by ANOVA, followed by the Dunnet post test. Asterisks indicate a statistically significant increase compared to controls, with $P<0.05$ (*) and $P<0.01$ (**).

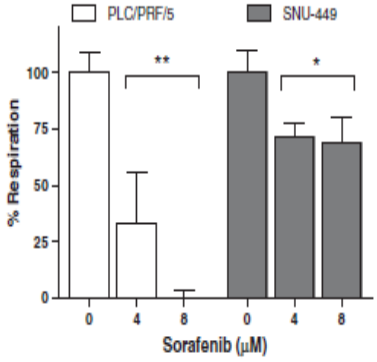


Fig. 4. Effect of sorafenib on cell respiration. Each dose of compound was tested in quadruplicate. Oxygen consumption of each sample was monitored for 60 min by registering the fluorescence signals of the oxygen-sensitive probe. Time profiles of fluorescence signals were analyzed by applying the linear regression in order to calculate the slopes. The slope of control cultures, treated with saline, has been fixed to 100. Asterisks indicates a significantly different slope of the line compared to controls, with $P<0.05$ (*) and $P<0.001$ (**).

cellular penetration and, consequently, it inhibits aerobic glycolysis at concentrations which cannot be reached in vivo. However, novel inhibitors of aerobic glycolysis are under study (Granchi et al., 2010) and will probably be available for clinical trials in the near future. Their administration in combination with sorafenib could be a worthwhile attempt to enhance the results of HCC chemotherapy.

In this prospect, an important issue to be addressed is to ascertain whether sorafenib also hinders OXPHOS in normal cells, a possibility supported by the finding that the drug damages mitochondria isolated from non-neoplastic tissues (Will et al., 2008). Normal cells have an OXPHOS capacity much higher than that of human HCC cells, whose

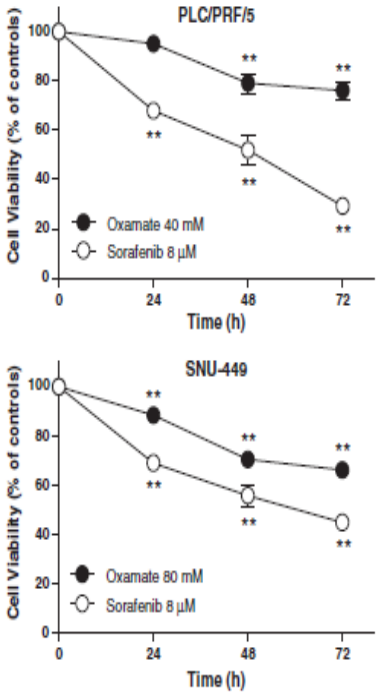


Fig. 5. Comparison of growth inhibition produced in PLC/PRF/5 cells by 40 mM oxamate and 8 μM sorafenib and in SNU-449 cells by 80 mM oxamate and 8 μM sorafenib. These doses of the compounds caused a comparable reduction of ATP cellular levels (see Fig. 1). Data (mean values ± S.D.) were analyzed by ANOVA, followed by the Dunnet post test. ** indicate a statistically significant difference compared to controls, with $P<0.01$.

mitochondrial content is very low (Cuezva et al., 2002). Therefore, in contrast to HCC cells, normal cells have an energetic reserve in the mitochondrial compartment to which they can resort when the physiological work increases and the consequent reduction of ATP/ADP ratio stimulates the OXPHOS (Krebs, 1972). It can be speculated that even when sorafenib impairs OXPHOS in normal cells, it does not decrease the amount of ATP synthesized in mitochondria in basal conditions, but only reduces their energetic reserve with a consequently decreased capacity of coping with a higher energy demand.

Acknowledgments

This work was supported by Fondazione del Monte di Bologna e Ravenna, Roberto and Cornelia Pallotti Legacies for Cancer Research and University of Bologna.

References

Alexander, J.J., Bey, E.M., Geddes, E.W., Lecatsas, G., 1976. Establishment of a continuously growing cell line from primary carcinoma of the liver. S. Afr. Med. J. 50, 2124–2128.

Barker, S.B., Summerson, W.H., 1941. The colorimetric determination of lactic acid in biological material. J. Biol. Chem. 138, 535–554.

Cuezva, J.M., Krajewska, M., de Heredia, M.L., Krajewski, S., Santamaría, G., Kim, H., Zapata, J.M., Marusawa, H., Chamorro, M., Reed, J.C., 2002. The bioenergetic signature of cancer: a marker of tumor progression. Cancer Res. 62, 6674–6681.

Fiume, L., Manerba, M., Vettraino, M., Di Stefano, G., 2010. Impairment of aerobic glycolysis by inhibitors of lactic dehydrogenase hinders the growth of human hepatocellular carcinomas cell lines. Pharmacology 86, 157–162.

Fotakis, G., Timbrell, J.A., 2006. In vitro cytotoxicity assays: comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride. Toxicol. Lett. 160, 171–177.

Goldberg, E.B., Nitowsky, H.M., Colowick, S.P., 1965. The role of glycolysis in the growth of tumor cells. IV. The basis of glucose toxicity in oxamate-treated cultured cells. J. Biol. Chem. 240, 2791–2796.

Granchi, C., Bertini, S., Macchia, M., Minutolo, F., 2010. Inhibitors of lactate dehydrogenase isoforms and their therapeutic potential. Curr. Med. Chem. 17, 672–697.

Krebs, H.A., 1972. The Pasteur effect and the relations between respiration and fermentation. Essays Biochem. 8, 1–34.

Lieberthal, W., Menza, S.A., Levine, J.S., 1998. Graded ATP depletion can cause necrosis or apoptosis of cultured mouse proximal tubular cells. Am. J. Physiol. Renal Physiol. 274, F315–F327.

Liu, L., Cao, Y., Chen, C., Zhang, X., McNabola, A., Wilkie, D., Wilhelm, S., Lynch, M., Carter, C., 2006. Sorafenib blocks the RAF/MEK/ERK pathway, inhibits tumor angiogenesis, and induces tumor cell apoptosis in hepatocellular carcinoma model PLC/PRF/5. Cancer Res. 66, 11851–11858.

Llovet, J.M., Ricci, S., Mazzaferro, V., Hilgard, P., Gane, E., Blanc, J.F., de Oliveira, A.C., Santoro, A., Raoul, J.L., Forner, A., Schwartz, M., Porta, C., Zeuzem, S., Bolondi, L., Greten, T.F., Galle, P.R., Seitz, J.F., Borbath, I., Häussinger, D., Giannaris, T., Shan, M., Moscovici, M., Voliotis, D., Bruix, J., SHARP Investigators Study Group I, 2008. Sorafenib in advanced hepatocellular carcinoma. N. Engl. J. Med. 359, 378–390.

Marroquin, L.D., Hynes, J., Dykens, J.A., Jamieson, J.D., Will, Y., 2007. Circumventing the Crabtree effect: replacing media glucose with galactose increases susceptibility of HepG2 cells to mitochondrial toxicants. Toxicol. Sci. 97, 539–547.

Novoa, W.B., Winer, A.D., Glaid, A.J., Schwert, G.W., 1959. Lactic dehydrogenase. V. Inhibition by oxamate and by oxalate. J. Biol. Chem. 234, 1143–1148.

Papacostantinou, J., Colowick, S.P., 1961. The role of glycolysis in the growth of tumor cells. II. The effect of oxamic acid on the growth of HeLa cells in tissue culture. J. Biol. Chem. 236, 285–288.

Park, J.G., Lee, J.H., Kang, M.S., Park, K.J., Jeon, Y.M., Lee, H.J., Kwon, H.S., Park, H.S., Yeo, K.S., Lee, K.U., 1995. Characterization of cell lines established from human hepatocellular carcinoma. Int. J. Cancer 62, 276–282.

Pelicano, H., Martin, D.S., Xu, R.H., Huang, P., 2006. Glycolysis inhibition for anticancer treatment. Oncogene 25, 4633–4646.

Reitzer, L.J., Wice, B.M., Kennell, D., 1979. Evidence that glutamine, not sugar, is the major energy source of cultured HeLa cells. J. Biol. Chem. 254, 2669–2676.

Rossignol, R., Gilkerson, R., Aggeler, R., Yamagata, K., Remington, S.J., Capaldi, R.A., 2004. Energy substrate modulates mitochondrial structure and oxidative capacity in cancer cells. Cancer Res. 64, 985–993.

Sebolt-Leopold, J.S., English, J.M., 2006. Mechanisms of drug inhibition of signalling molecules. Nature 441, 457–462.

Strumberg, D., Richly, H., Hilger, R.A., Schleucher, N., Korfee, S., Tewes, M., Faghih, M., Brendel, E., Voliotis, D., Haase, C.G., Schwartz, B., Awada, A., Voigtmann, R., Scheulen, M.E., Seeber, S., 2005. Phase I clinical and pharmacokinetic study of the novel Raf kinase and vascular endothelial growth factor receptor inhibitor BAY 43–9006 in patients with advanced refractory solid tumors. J. Clin. Oncol. 23, 965–972.

Strumberg, D., Clark, J.W., Awada, A., Moore, M.J., Richly, H., Hendlisz, A., Hirte, H.W., Eder, J.P., Lenz, H.J., Schwartz, B., 2007. Safety, pharmacokinetics, and preliminary antitumor activity of sorafenib: a review of four phase I trials in patients with advanced refractory solid tumors. Oncologist 12, 426–437.

Will, Y., Dykens, J.A., Nadanaciva, S., Hirakawa, B., Jamieson, J., Marroquin, L.D., Hynes, J., Patyna, S., Jessen, B.A., 2008. Effect of the multitargeted tyrosine kinase inhibitors imatinib, desatinib, sunitinib and sorafenib on mitochondrial function in isolated rat heart mitochondria and H9c2 cells. Toxicol. Sci. 106, 153–161.

Galloflavin (CAS 568-80-9): A Novel Inhibitor of Lactate Dehydrogenase

FULL PAPERS

DOI: 10.1002/cmdc.201100471

Galloflavin (CAS 568-80-9): A Novel Inhibitor of Lactate Dehydrogenase

Marcella Manerba,[a] Marina Vettraino,[a] Luigi Fiume,[a] Giuseppina Di Stefano,*[a] Andrea Sartini,[b] Elisa Giacomini,[b] Rosa Buonfiglio,[b] Marinella Roberti,[b] and Maurizio Recanatini[b]

One of the most prominent alterations in cancer cells is their strict dependence on the glycolytic pathway for ATP generation. This observation led to the evaluation of glycolysis inhibitors as potential anticancer agents. The inhibition of lactate dehydrogenase (LDH) is a promising way to inhibit tumor cell glucose metabolism without affecting the energetic balance of normal tissues. However, the success of this approach depends chiefly on the availability of inhibitors that display good selectivity. We identified a compound (galloflavin, CAS 568-80-9) which, in contrast to other inhibitors of human LDH, hinders both the A and B isoforms of the enzyme. To determine the mechanism of action, we collected LDH-A and -B inhibition data in competition reactions with pyruvate or NADH and evaluated the results using software for enzyme kinetics analysis. We found that galloflavin inhibits both human LDH isoforms by preferentially binding the free enzyme, without competing with the substrate or cofactor. The calculated K_i values for pyruvate were 5.46 μM (LDH-A) and 15.06 μM (LDH-B). In cultured tumor cells, galloflavin blocked aerobic glycolysis at micromolar concentrations, did not interfere with cell respiration, and induced cell death by triggering apoptosis. To our knowledge, the inhibition of LDH is, to date, the only biochemical effect described for galloflavin. Because galloflavin is not commercially available, we also describe herein a procedure for its synthesis and report its first full chemical characterization.

Introduction

Although highly heterogeneous in their genetic features, nearly all tumor cells, regardless of their tissue origin, display a common metabolic phenotype characterized by elevated glucose uptake with lactic acid production in the presence of oxygen (aerobic glycolysis).[1] This observation, which originally dates back to Warburg,[2] suggests that a rational approach to cancer management can be found in therapies that specifically target this metabolic profile. Lactic acid dehydrogenase (LDH, EC 1.1.1.27) is involved in the final step of aerobic glycolysis, which converts pyruvate into lactate. Inhibition of LDH as an approach to anti-neoplastic chemotherapy was proposed several years ago[3–5] and has been recently re-evaluated[6] following two observations: a) neoplastic cells with decreased LDH levels induced by shRNA[7] or by siRNA[8] show decreased tumorigenicity; b) humans with a hereditary deficiency of the A[9] or B[10–15] LDH isoform are free of symptoms, except for muscle rigidity and myoglobinuria, which can appear in individuals with LDH-A[9] deficiency after strenuous exercise.

LDH is a very well-characterized enzyme. The mammalian enzyme is a tetrameric complex composed of two different subunit types: LDH-A (or LDH-M, muscle) and LDH-B (or LDH-H, heart). The combination of these two isoforms can give rise to homotetrameric structures (LDH-A or M4 and LDH-B or H4), or to heterogeneous enzyme complexes (LDH-AB3, LDH-A2B2, LDH-A3B).[16] In humans, an additional isoform has been described, producing the complex LDH-X (C4) which is expressed only in testis.[6] All LDH isoenzymes share a very similar structure but differ in their tissue distribution, kinetic and regulatory properties.[16] The A subunit is prevalent in skeletal muscles, liver, and neoplastic tissues; the B subunit is expressed more in the heart, kidney, spleen, and brain.[6]

Until recently, the only well-characterized and specific inhibitor of LDH was oxamic acid, a small molecule that inhibits both the A and B isoforms of LDH by competing with pyruvic acid, the enzyme's natural substrate.[5,17] Although it displays good selectivity for LDH[5,18] and weak toxicity in healthy animals,[19] oxamic acid has the drawback of poor cellular penetration; as a consequence, it was found to inhibit aerobic glycolysis and the proliferation of tumor cells cultured in vitro only at high concentrations,[5,18] which cannot be expected to be reached in vivo.

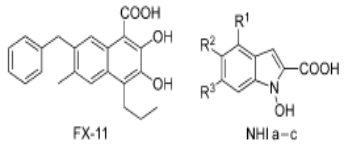
Over the past years, LDH inhibitor research was aimed at the discovery of improved antimalarial therapy, as LDH activity is also essential to the metabolism of *Plasmodium falciparum*.[20–26] These studies led to the identification of 3-dihydroxy-6-methyl-7-(phenylmethyl)-4-propylnaphthalene-1-carboxylic

[a] Dr. M. Manerba, Dr. M. Vettraino, Prof. L. Fiume, Prof. G. Di Stefano
Department of Experimental Pathology, University of Bologna
Via San Giacomo 14, 40126 Bologna (Italy)
E-mail: giuseppina.distefano@unibo.it

[b] Dr. A. Sartini, Dr. E. Giacomini, Dr. R. Buonfiglio, Prof. M. Roberti, Prof. M. Recanatini
Department of Pharmaceutical Sciences, University of Bologna
Via Belmeloro 6, 40126 Bologna (Italy)

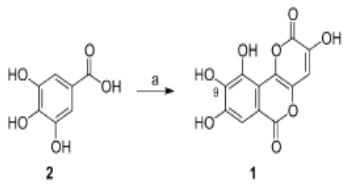
Supporting information for this article is available on the WWW under http://dx.doi.org/10.1002/cmdc.201100471.

acid (FX-11), which also strongly inhibits the A isoform of mammalian LDH by competing with NADH, the cofactor of the enzyme.[21,22] FX-11 was also found to be active in experimental



animals, in which it hindered the growth of tumor xenografts.[8] However, the possibility that off-target effects of this inhibitor also contributed to its biological action cannot be ruled out. More recently, some *N*-hydroxyindole-based compounds (NHIa–c) were also found to inhibit the LDH-A isoform.[27]

Herein we describe the identification of a novel LDH inhibitor, galloflavin (**1**; Scheme 1 and Table 2 below), which was selected by virtual screening (VS) of a molecular dataset and carefully biologically characterized. Because galloflavin (**1**) is not available from commercial sources, we also set up a procedure for its synthesis,[28] and report herein its full chemical characterization for the first time.



Scheme 1. Synthesis of galloflavin (**1**) from gallic acid (**2**). *Reagents and conditions*: a) KOH (5 M), H_2O/EtOH (1:1), air, RT, 12 h.

Virtual screening

To identify novel LDH inhibitors, a structure-based VS protocol was developed. The RCSB Protein Data Bank (PDB)[29] was searched, and 75 LDH crystal structures were found. Among them, we chose the 2.3 Å crystal structure of human muscle form (LDH-A, PDB ID: 1I10[30]), which was the only A isoform LDH structure available at the time. The NCI Diversity Set (1880 compounds when it was downloaded) was explored as a first step toward a more comprehensive investigation of the chemical space. The GOLD software package, implementing a genetic algorithm, was used for the VS procedure.

Chemistry

Galloflavin (**1**) was obtained by oxidation of a basic solution of gallic acid (**2**)[28] in air (Scheme 1). Following the previously reported procedure,[28] we sought to identify reproducible conditions for the synthesis suitable to provide adequate quantities of substance for full chemical characterization and biological studies.

Biology

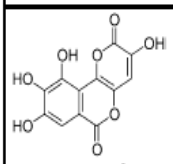
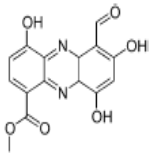
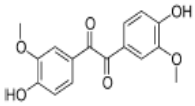
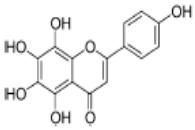
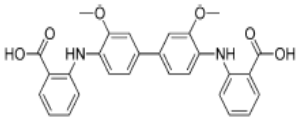
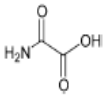
Our VS search for the identification of LDH inhibitors was initially focused on the structure of the LDH-A, as this isoform is up-regulated in the majority of human cancer cells.[31] Twenty compounds selected from the VS procedure were preliminarily probed against purified human LDH-A and -B, in the presence of non-limiting concentrations of both substrate (pyruvate) and cofactor (NADH). Five compounds were found to be active at the micromolar level, and, by using the PLC/PRF/5 human cell line, were additionally characterized for their activity on some cellular metabolic processes (lactate production, respiration, ATP synthesis). The PLC/PRF/5 cell line is derived from an undifferentiated hepatocellular carcinoma;[32] it was chosen for studying the effects of LDH inhibitors both because of its human origin, and because this tumor form usually displays a highly glycolytic phenotype due to a marked decrease in cellular mitochondria content.[33] Taken together, the results of the early evaluations focused our interest on galloflavin, which was further studied to investigate the inhibition pattern on human LDH-A and -B. We also verified that the inhibition of aerobic glycolysis effected by galloflavin in PLC/PRF/5 cells triggered cell death via apoptosis.

Results and Discussion

After ranking all molecules of the NCI Diversity Set for their affinity to LDH-A, we selected 20 (Supporting Information, table A) from the top 10% of compounds on the bases of careful inspection of the putative binding mode as well as availability. In particular, in the absence of objective criteria for prioritizing the top-scoring compounds (see Experimental Section), we checked for several properties: reasonable inhibitor binding mode, realistic binding conformation, and synthetic accessibility of the molecular skeleton. Samples of the selected compounds were obtained from the NCI and were tested on purified human LDH-A and -B, as described in the Experimental Section. Only five of them (Table 1) were found to cause enzyme inhibition at the micromolar level. A PubChem search (http://pubchem.ncbi.nlm.nih.gov) of the structures of these five compounds allowed retrieval of their common denomination and published bioassay data. The compounds are: galloflavin (NCS107022), lomofungin (NCS156939), redoxal (NCS73735), vanillil (NCS16722), and nortangeretin (NCS76988). According to the information in the PubChem database, neither LDH inhibitory activity nor other effects on glycolytic enzymes have been reported yet for these compounds. For each molecule, we obtained a concentration–response plot for LDH inhibition, in which experimental data were fitted by using the second-order polynomial regression in order to calculate the minimum dose required for complete inhibition of enzyme activity (IC_{100}; Table 1). Although less commonly used than IC_{50}, this parameter was chosen with the aim of identifying a fully active inhibitor dose for testing on cells cultured in vitro for preliminary evaluation of their biological activity.

The IC_{100} values calculated for LDH-A were tested with PLC/PRF/5 cultures to evaluate their effect on lactate production,

Table 1. Summary of the biological effects of test compounds found to inhibit purified LDH.

Compd		IC_{100}[a] [μM]		Inhibition[b] [%]			
		hLDH-A	hLDH-B	Lactate Production	ATP Levels	Cell Growth	Respiration
	Galloflavin (NCS107022)	201	200	44.0±4.1	34.2±5.2	61.8±5.0	0.89±6.7
	Lomofungin (NCS156939)	202	182	24.5±9.9	28.2±0.6	>95	>95
	Vanillil (NCS16722)	205	178	20.0±5.2	2.53±0.2	0.0±14.4	ND
	Nortangeretin (NCS76988)	270	212	53.3±0.6	17.3±0.1	79.5±25.5	68.3±7.0
	Redoxal (NCS73735)	177	158	74.6±20.0	1.63±0.0	>95	>95
	Oxamic acid[c]	690	1175	67.0±2.9	28.7±2.4	34.5±0.5	5.71±2.5

[a] The IC_{100} value represents the minimal concentration required to effect complete LDH inhibition. [b] Inhibitory effects were determined in PLC/PRF/5 cells using a compound concentration equal to the LDH-A IC_{100} value. [c] Inhibition data given for oxamic acid are the effects observed at 4×10^4 μM.

cell respiration, ATP levels, and cell proliferation; the results obtained are listed in Table 1. For comparison, the IC_{100} value of oxamic acid—the only well-characterized selective LDH inhibitor[5,18]—is also included, having been evaluated in the same way as for the newly identified inhibitors. As apparent in Table 1, oxamic acid elicits biological effects on cells at concentrations as high as ~4×10^4 μM (no effects at IC_{100}), because it has limited cell penetration. Galloflavin appears to have a good capacity of cell penetration, as it shows a marked effect on lactate production in cultured PLC/PRF/5 cells at the same concentration that it blocks purified LDH-A (201 μM). Good correspondence was observed between the decreased lactate levels and the decrease in cellular ATP, as observed in PLC/PRF/5 cells after the selective block of aerobic glycolysis caused by treatment with oxamic acid. Moreover, galloflavin inhibited cell growth and did not affect oxygen consumption (Table 1).

Among the other compounds, vanillil was not deemed worthy of further study, as it caused only a small decrease in lactate levels and did not affect cellular ATP and PLC/PRF/5 growth. The respiration assay was not performed with this compound, because we observed interference of vanillil with the signal of the oxygen-sensitive probe.

The remaining three compounds (redoxal, lomofungin, and nortangeretin), added to the assay cell cultures at their respective LDH IC_{100} values, hindered oxygen consumption, denoting lack a of specificity toward this enzyme. No bioassay data for nortangeretin are available in the PubChem database. In contrast, lomofungin and redoxal are previously studied compounds for which other biological effects have been described. Lomofungin is an antibiotic that inhibits nucleic acid synthesis in yeast;[34] redoxal is known to induce pyrimidine depletion[35] by inhibiting dihydroorotate dehydrogenase.[36]

From our biological evaluations, galloflavin proved to be the only potentially useful LDH inhibitor. Therefore, a more detailed characterization of its biological properties was performed. Figure 1 shows the galloflavin docking pose with human LDH-A, obtained by the VS procedure. As detailed in Figure 2, this molecule apparently binds mainly to the enzyme by hydrogen bonds. In particular, the carbonyl oxygen atoms of galloflavin act as hydrogen bond acceptors with Thr247 and Gln99, and the compound's hydroxy groups establish hydrogen bond interactions with Asn137 and Ala95. Finally, the 9-hydroxy group could make contact with either Ala97 or

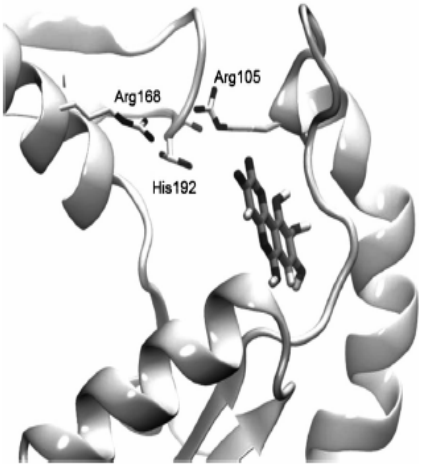


Figure 1. Calculated binding pose of galloflavin with human muscle LDH-A (PDB ID: 1I10) as the outcome of the VS protocol. Catalytically relevant His192 and Arg105 residues, as well as Arg168 (numbering from the human muscle enzyme) are shown as sticks, with the main chain of the protein displayed in ribbon form; some residues have been omitted for clarity.

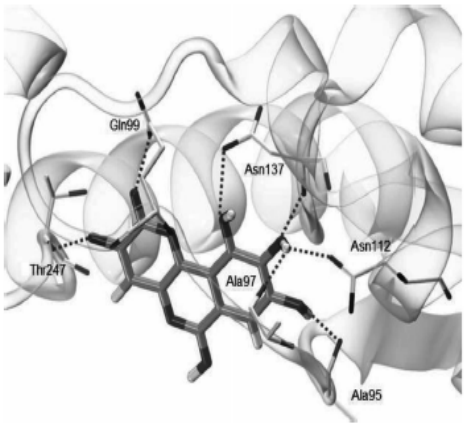


Figure 2. Putative binding mode of galloflavin at the human LDH-A binding site (displayed in ribbon form). Atoms likely involved in hydrogen bonds are linked by dashed lines, and selected relevant residues are depicted as sticks; some residues have been omitted for clarity.

Asn112 and with the backbone nitrogen atom of Asn137 as well.

Galloflavin was also docked into the human LDH-B isoform, and a pose quite similar to that observed with human LDH-A was found (figure 1, Supporting Information). This was not unexpected, considering the high sequence similarity (~75%)[6] of the binding site domains.

As an initial step in the biological characterization of galloflavin, we tried to better define the pattern of enzyme inhibition by using purified human LDH-A and LDH-B. For this, we performed enzymatic assays in the presence of scalar concentrations of the substrate (pyruvate) or cofactor (NADH). For comparison and to verify the accuracy of our evaluation, a similar test was also performed with oxamic acid, which, as stated above, is a competitor of pyruvate[17] in the LDH reaction. LDH inhibition data obtained with galloflavin and oxamic acid were evaluated by GraphPad Prism 5.0, using the software's built-in enzyme kinetics analyses. Briefly, several datasets of LDH activity were collected in the presence of various inhibitor concentrations, starting at zero. These datasets were plotted as substrate concentration versus enzyme activity, fitted by second-order polynomial regression, and analyzed by applying the mixed-model inhibition fit. This procedure was preferred over analysis by Lineweaver–Burk plots, as it avoids the approximation errors linked to the transformation of experimental data.[37] The mixed-model fit is defined by Equations (1–3), which include competitive, uncompetitive, and noncompetitive inhibition terms: V_{max} is the maximum enzyme velocity without inhibitor, K_M is the Michaelis–Menten constant, K_i is the inhibition constant, α determines the mechanism of action, X is the substrate concentration, Y is the enzyme activity, and I is the inhibitor concentration.

$$^{App}V_{max} = V_{max}/[1 + I/(\alpha \cdot K_i)] \quad (1)$$

$$^{App}K_M = K_M \cdot (1 + I/K_i)/[1 + I/(\alpha \cdot K_i)] \quad (2)$$

$$Y = {}^{App}V_{max} \cdot X/({}^{App}K_M + X) \quad (3)$$

This calculation model includes the parameter α, which can be indicative of the mechanism of inhibition. The α value is an index of the degree to which the binding of inhibitor changes the affinity of the enzyme for substrate. An α value equal to one indicates that the inhibitor has equal affinity for both the free enzyme and the enzyme–substrate complex. If $\alpha > 1$, the inhibitor preferentially binds to the free enzyme, and if $\alpha < 1$, the inhibitor binds with greater affinity to the enzyme–substrate complex. A very large α value denotes that inhibitor binding prevents binding of the substrate, and is indicative of competitive inhibition.[38] Results are reported in Table 2.

According to the global fitting of data, galloflavin seems to preferably bind the free enzyme, without being competitive with either pyruvate or NADH, as indicated by the obtained α values. The discovery of galloflavin's inhibitory action against both human LDH isoforms (see also Table 1) suggests broader and more interesting properties than those of FX-11 and the *N*-hydroxyindole-based inhibitors, which were found to act specifically on LDH-A.[22,27] In fact, data were recently reported that indicate a pivotal role for LDH-B in tumorigenesis mediated by hyperactive mTOR (mammalian target of rapamycin) signaling.[39]

To further characterize the biological effects of galloflavin, PLC/PRF/5 cells were treated with various doses of this compound. As shown in Figure 3a, cell respiration was largely unaffected by galloflavin at all concentrations, suggesting a lack of toxicity toward mitochondrial functions. For the other studied parameters, the effects caused by galloflavin were found to be concentration dependent, without evidence of a threshold dose. The inhibition of lactate production (Figure 3b), which is indicative of impairment of aerobic glycolysis, and the decrease in ATP synthesis (Figure 3c) can explain the decreased cell viability (Figure 3d).[40] By applying the second-order poly-

Table 2. LDH inhibition data obtained with galloflavin in competition experiments with pyruvate and NADH.

	hLDH-A		hLDH-B	
	Pyruvate	NADH	Pyruvate	NADH
Galloflavin				
K_i [μM]:	5.46	56.0	15.1	23.2
α:[a]	9.94	0.59	3.36	4.44
r^2:[b]	0.989	0.983	0.963	0.961
Oxamic acid[c]				
K_i [μM]:	61.4	–	5.76	–
α:[a]	2.44×10^{16}	–	2.66×10^{3}	–
r^2:[b]	0.987	–	0.978	–

[a] The α value is indicative of the mechanism of inhibition; see text for a detailed explanation. [b] Goodness of fit. [c] For comparison, the inhibitory data obtained using oxamic acid, a LDH inhibitor that competes with pyruvate, is also shown.

nomial regression to the data in Figure 3, we calculated that the concentration of galloflavin causing a 50% decrease in lactate production is 140 μM. This dose fits well with the concentration required to decrease cell viability by 50% (184 μM), suggesting that in PLC/PRF/5 cells exposed to galloflavin, the decrease in viability could be ascribed mainly to inhibition of aerobic glycolysis caused by the compound. Figure 3c also shows that 140–184 μM galloflavin can be expected to reduce ATP levels by only 25–30%. This result can be easily explained by considering that even in cells where glycolysis is active, mitochondrial function is not completely abolished and that mitochondrial respiration is more productive in ATP synthesis than glycolysis. This limited effect on ATP levels appeared to have a positive consequence, as by studying the cell death mechanism triggered by galloflavin (Figure 4) we observed the induction of apoptosis, with no evidence of necrosis. Cell death by necrosis is the inevitable consequence of deep ATP depletion; this sets off a damaging inflammatory reaction, often linked to increased risks of tumor progression.

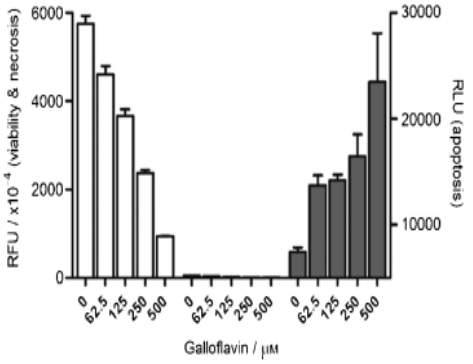


Figure 4. Study of the cell death mechanism induced by galloflavin in PLC/PRF/5 cells after 24 h exposure: viability (□), apoptosis (▒), and necrosis (■). The results show apoptosis as the only cell death pathway that occurs as a result of treatment. A similar experiment, repeated after 48 h exposure, produced superimposable results (data not shown).

To our knowledge, the inhibition of LDH isoenzymes is at present the only biochemical effect described for galloflavin. Preliminary toxicity data for this molecule are available from the NCI (http://dtp.nci.nih.gov/), and remarkably they suggest good tolerability: in mice the maximum tested dose (400 mg kg^{-1} injected i.p.) did not produce lethal effects. In conclusion, galloflavin might be a promising lead candidate in the field of tumor metabolism inhibitors, and warrants a more exhaustive evaluation as a potential anticancer agent.

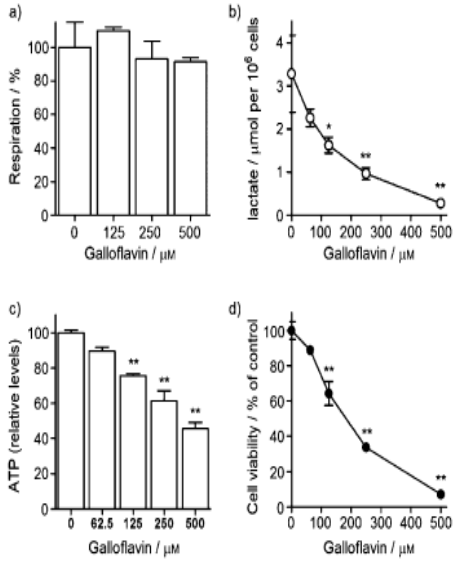


Figure 3. Synopsis of the biological characterization of galloflavin in PLC/PRF/5 cultures: a) cell respiration, b) inhibition of lactate synthesis, c) decrease in ATP levels, and d) reduction of cell viability. For all experiments (performed as described in the Experimental Section), galloflavin was dissolved in DMSO. Cultures exposed to galloflavin contained 0.6% DMSO, and the same DMSO amount was also added to control cells; we verified that 0.6% DMSO has no effect on PLC/PRF/5 cultures. The results were evaluated by ANOVA followed by Dunnet's post-test, using GraphPad Prism 5 software. Asterisks indicate statistically significant difference from controls: *$p<0.05$, **$p<0.01$.

Experimental Section

Structure-based VS protocol on human LDH-A

The crystal structure of human muscle L-lactate dehydrogenase M chain complexed with oxamic acid and NADH (PDB ID: 1I10)[30] was downloaded from the PDB (http://www.rcsb.org). Among the different polypeptide chains present in the .pdb file, the A chain was taken as the starting structure for performing the VS on the basis of the overall resolution and the *B*-factor. Water molecules, oxamic acid, NADH, and acetate ion were removed, and hydrogen atoms were added by using the BIOPOLYMERS module of Sybyl 7.3 (Tripos Inc., St. Louis, MO, USA). The protonation states of ionizable groups were set up, and the tautomeric forms of histidine were protonated according to their putative hydrogen bond patterns in the crystal structure. The VS of the NCI Diversity Set II (http://dtp.cancer.gov/branches/dscb/div2_explanation.html) was performed by using GOLD v. 4.0.1 docking software (CCDC, Cambridge, UK). The 3D models of ligands were downloaded from the ZINC database (http://zinc.docking.org) and the active site was defined as a sphere of 15 Å radius centered at atom 1056 to comprise

the cavity formerly occupied by oxamic acid and NADH. The genetic search algorithm was set up at an accuracy of 100%, with default settings; in particular, the population size was 100, the selection pressure was 1.1, the number of operations was 10^5, the number of islands was 5, the niche size was 2, migrate was 10, mutate was 95, and crossover was 95. The GoldScore scoring function was used, and 100 runs were performed for each molecule of the subset. The top 10% of virtual hits were selected for visual inspection, which is a recommended step in VS protocols, although it is time consuming, and lacking in objectivity.[41] In fact, criteria such as a good predicted binding affinity (calculated by a combination of hydrogen bonding, van der Waals interactions, hydrophobic contacts) or the evaluation of key interactions characteristic of each biological system are difficult to implement into a computer algorithm. Finally, 20 compounds were selected for biological evaluation, and among them, galloflavin (**1**) showed the best profile.

Chemistry

General: Reagents, starting materials, and solvents were purchased from commercial suppliers (Aldrich). Reaction progress was monitored by TLC on pre-coated silica gel plates (Kieselgel 60 F_{254}, Merck) and visualized by UV light ($\lambda = 254$ nm). ^{1}H and ^{13}C NMR spectra were recorded on Varian Gemini instruments operating at 400 and 100 MHz, respectively, with DMSO as solvent. Chemical shifts (δ) are reported relative to $(CH_3)_4Si$ as internal standard. FTIR spectra were collected on a Nicolet Avatar 320 ESP instrument; ν_{max} is expressed in cm^{-1}. MS data were recorded on a VG 7070E spectrometer or on a Waters ZQ 4000 apparatus operating in electrospray (ES) mode. The purity of galloflavin was determined to be $\geq$99.5% by HPLC [Varian 5000 apparatus equipped with a diode array Hewlett–Packard 104 AA detection system set at $\lambda = 220$ nm, UV range: 210–450 nm; column: C_{18} Phenomenex 5 μm (150 × 4.6 mm)].

Synthesis of galloflavin (1): Gallic acid **2** (1.00 g, 5.87 mmol) was dissolved in H_2O/EtOH 1:1 (8.00 mL) in a large beaker. KOH (5.00 M, 4.70 mL, 23.5 mmol) was added dropwise, and the reaction mixture was stirred vigorously in air at room temperature for 12 h. The precipitated green potassium salt was filtered and re-dissolved in H_2O (5.00 mL) at 50 °C, then acidified to pH 4 with 2.00 N HCl. The precipitated galloflavin was filtered, washed with Et_2O and CH_2Cl_2, and evaporated to dryness in vacuo to give a green solid (165 mg, 10%); ^{1}H NMR (400 MHz, [D_6]DMSO): $\delta = 6.92$ (s, 1H), 7.19 ppm (s, 1H); ^{13}C NMR (100 MHz, [D_6]DMSO) $\delta = 107.4$, 110.7, 111.2, 111.8, 132.8, 133.9, 141.0, 141.1, 141.8, 147.2, 157.5, 159.4 ppm; IR (Nujol): $\nu = 3352$, 1682, 1613, 1584, 1462, 1413, 1376, 1345, 1299, 1176, 1091 cm^{-1}; UV/Vis (CH_3CN/H_3PO_4 aqueous solution 8.67 mM, 15:85 *v/v*): $\lambda_{max} = 224$, 258, 378 nm; MS (ES): *m/z* 279 $[M+H]^+$, 301 $[M+Na]^+$, 317 $[M+K]^+$; HPLC $t_R = 6.1$ min ($\geq$99.5%).

Biological evaluation

Assay on purified human LDH: As a first step, the 20 compounds identified in the VS were tested for their ability to inhibit the purified human LDH isoforms. Human LDH-A (from human liver) and LDH-B (from human heart) were obtained from Lee Biosolutions (St. Louis, MO, USA). Stock solutions (500 mM) of the compounds were prepared in DMSO. They were added in scalar amounts (0–500 μM final concentrations) to a reaction mix containing 100 mM phosphate buffer (pH 7.5), LDH (0.015 U mL^{-1}), 1 mM pyruvate, and 150 μM NADH. For all the determinations (including those without compounds), final DMSO concentration was always kept at 0.6%. The enzymatic activity was measured by monitoring NADH oxidation for a period of 3 min. Because the majority of compounds were found to interfere with the UV reading of NADH oxidation, we adopted the procedure of Moran and Schnellmann,[42] by which LDH activity is measured by recording the decrease in NADH fluorescence. At the wavelengths used for NADH determinations ($\lambda_{ex} =$ 340 nm, $\lambda_{em} = 460$ nm) the tested compounds showed no appreciable fluorescence. The assay was performed in 96-well white-body plates using a Fluoroskan Ascent FL reader (Labsystems). The minimal concentration of compounds leading to 100% inhibition of LDH activity (IC_{100}) was calculated from the second-order polynomial regression of the experimental data, using GraphPad Prism 5 software. Following the results of the biological evaluation performed on the selected LDH inhibitors (see below), a more detailed study using purified LDH was repeated on galloflavin to ascertain its mechanism of inhibition. Galloflavin (0–500 μM) was probed in the presence of scalar concentrations of pyruvate (0–2 mM) or NADH (20–150 μM). In the first case, NADH in the reaction mix was maintained at 150 μM; in the second case pyruvate was kept at 2 mM. The two sets of experimental data were analyzed by applying the mixed-model fit, using GraphPad Prism 5 software.

Inhibition of lactic acid production: This test was performed on five NCI compounds, selected on the basis of their LDH inhibitory activity, using PLC/PRF/5 cells cultured in vitro. Cell culture conditions were as described.[18] Cells (5×10^5) were seeded in each well of a six-well Nunclon plate and let to adhere overnight. The IC_{100} dose of inhibitors (tested in triplicate) were then added to the cultures. Lactate was measured in three untreated wells at the start of experiment (baseline value) and 3 h after incubation at 37 °C. In each well, lactate levels—both free in the medium and intracellular—were simultaneously measured: at the end of incubation 100% trichloroacetic acid (TCA, 100 μL) was added; the cell lysate was collected, and the well was washed with 1 mL 10% TCA. After centrifugation (4 °C, 5 min, 2500 *g*), lactate in the supernatant was measured according to the method of Barker and Summerson.[43] The amount of metabolite formed during the 3 h incubation with or without inhibitor was calculated by subtracting the baseline value.

Effects on cellular respiration: This assay was performed with a phosphorescent oxygen-sensitive probe (MitoXpress) from Luxcel Biosciences. PLC/PRF/5 cells were plated at 5×10^4 cells per well in 96-well clear-bottom black-body plates and allowed to adhere overnight. After the addition of the MitoXpress phosphorescent oxygen-sensitive probe (10 pmol $well^{-1}$), plates were placed in a Victor fluorescence reader (PerkinElmer) at 30 °C and were monitored for ~20 min to reach temperature and gas equilibrium and to obtain basal signals. The inhibitor to be tested was quickly added, wells were sealed with mineral oil, and monitoring of the signal was resumed for the next 60 min. During this interval, the increase in fluorescence signal, which indicates oxygen consumption, was measured every 60 s with 340/642 nm excitation/emission filters, a delay time of 30 μs, and a measurement window of 100 μs. All dispensing steps of the experiment were performed at 30 °C with pre-warmed solutions. In this experiment the same compound doses used in the lactate inhibition assay were studied; they were tested in quadruplicate. Evaluation of cell respiration was performed by applying linear regression analysis to the time profiles of fluorescence signals obtained from each well, in order to determine the slope of each profile; GraphPad Prism 5 software was used.

Inhibition of ATP synthesis: ATP levels were measured using the CellTiter-Glo Luminescent Cell Viability Assay from Promega. For

this experiment, 2×10^4 PLC/PRF/5 cells in 200 µL culture medium were seeded into each well of a 96-well white-body plate and allowed to adhere overnight. Inhibitors (same doses of previous tests) were added, and ATP levels were measured after 3 h incubation at 37 °C. Each dose of compound was tested in triplicate. After incubation, the plate was allowed to equilibrate at room temperature for 30 min, and the CellTiter-Glo reagent was directly added to each well, as per the manufacturer's instructions. The plate was kept on a shaker for 10 min to induce cell lysis, and luminescence was measured with a Fluoroskan Ascent FL reader (Labsystems).

Effects on cell proliferation: The effects caused by the LDH inhibitors on cell proliferation were studied by using PLC/PRF/5 cells and evaluated with the neutral red assay.[44] Cells (1.6×10^4 per well) were seeded in 24-well plates, allowed to adhere overnight, and treated for 72 h at 37 °C with the IC_{100} dose of LDH inhibitors. Each compound was tested in triplicate. After the treatments, cells were incubated for 3 h at 37 °C with the neutral red dye dissolved in DMEM at the final concentration of 30 µg mL^{-1}. After this time, the medium was removed, and the cells were solubilized with 1 mL 1% acetic acid in 50% EtOH. The solution from each well (200 µL) was then transferred to a 96-well plate and its absorbance at $\lambda =$ 540 nm was measured using a microplate reader (Multiskan Ascent FL, Labsystems).

Mechanism of cell death: The cell death mechanism caused by galloflavin was determined by using the ApoTox-Glo™ Triplex Assay (Promega), which measures viability, cytotoxicity, and caspase-3/7 activation in the same sample. The test was performed on PLC/PRF/5 cells seeded in 96-well white-body plates (2×10^4 cells per well) after 24 h exposure to various galloflavin concentrations, following the instructions of the manufacturer.

Acknowledgements

The authors thank the US National Cancer Institute for supplying the compounds studied. This work was supported by Roberto and Cornelia Pallotti's Legacy for Cancer Research and the University of Bologna (Italy).

Keywords: cancer · drug discovery · enzymes · kinetics · metabolism

[1] R. A. Gatenby, R. J. Gillies, *Nat. Rev. Cancer* **2004**, *4*, 891–899.
[2] O. Warburg in *Über den Stoffwechsel der Tumoren*, Verlag Springer, Berlin, **1926**, Translated into English by F. Dickens, *The Metabolism of Tumours*, Constable, London, **1930**.
[3] H. Busch, P. V. Nair, *J. Biol. Chem.* **1957**, *229*, 377–387.
[4] L. Fiume, *Nature* **1960**, *187*, 792–793.
[5] J. Papacostantinou, S. P. Colowick, *J. Biol. Chem.* **1961**, *236*, 278–284.
[6] C. Granchi, S. Bertini, M. Macchia, F. Minutolo, *Curr. Med. Chem.* **2010**, *17*, 672–697.
[7] V. R. Fantin, J. St-Pierre, P. Leder, *Cancer Cell* **2006**, *9*, 425–434.
[8] A. Le, C. R. Cooper, A. M. Gouw, R. Dinavahi, A. Maitra, L. M. Deck, R. E. Royer, D. L. Vander Jagt, G. L. Semenza, C. V. Dang, *Proc. Natl. Acad. Sci. USA* **2010**, *107*, 2037–2042.
[9] T. Kanno, K. Sudo, M. Maekawa, Y. Nishimura, M. Ukita, K. Fukutake, *Clin. Chim. Acta* **1988**, *173*, 89–98.
[10] K. Motoshi, I. Naoko, H. Fumiko, H. Atsushi, *Clin. Chim. Acta* **1971**, *34*, 419–423.
[11] S. Miwa, T. Nishina, Y. Kakehashi, M. Kitamura, A. Hiratsuka, K. Shizume, *Acta Haem. Jap.* **1971**, *34*, 228–232.
[12] R. Joukyuu, S. Mizuno, T. Amakawa, T. Tsukada, T. Nishina, M. Kitamura, *Clin. Chem.* **1989**, *35*, 687–690.
[13] H. Wakabayashi, M. Tsuchiya, K. Yoshino, K. Kaku, H. Shigei, *Int. Med.* **1996**, *35*, 550–554.
[14] K. Hidaka, N. Ueda, I. Hirata, Y. Watanabe, Y. Minatogawa, I. Iuchi, *J. Hum. Genet.* **1999**, *44*, 69–72.
[15] N. Okumura, F. Terasawa, I. Ueno, K. Oki, K. Tamauchi K, H. Hidaka, M. Tozuka, M. Okura, T. Katsuyama, *Clin. Chim. Acta* **1999**, *287*, 163–171.
[16] J. Everse, N. O. Kaplan, *Adv. Enzymol.* **1973**, *37*, 61–133.
[17] W. B. Novoa, A. D. Winer, A. J. Glaid, G. W. Schwert, *J. Biol. Chem.* **1959**, *234*, 1143–1148.
[18] L. Fiume, M. Manerba, M. Vettraino, G. Di Stefano, *Pharmacology* **2010**, *86*, 157–162.
[19] J. Papacostantinou, S. P. Colowick, *J. Biol. Chem.* **1961**, *236*, 285–288.
[20] M. S. Gomez, R. C. Piper, L. A. Hunsaker, R. E. Royer, L. M. Deck, M. Makler, D. L. Vander Jagt, *Mol. Biochem. Parasitol.* **1997**, *90*, 235–246.
[21] L. M. Deck, R. E. Royer, B. B. Chamblee, V. M. Hernandez, R. R. Malone, J. E. Torres, L. A. Hunsaker, R. C. Piper, M. T. Makler, D. L. Vander Jagt, *J. Med. Chem.* **1998**, *41*, 3879–3887.
[22] Y. Yu, J. A. Deck, L. A. Hunsaker, L. M. Deck, R. E. Royer, E. Goldberg, D. L. Vander Jagt, *Biochem. Pharmacol.* **2001**, *62*, 81–89.
[23] S. Choi, A. Pradhan, N. L. Hammond, A. G. Chittiboyina, B. L. Tekwani, M. A. Avery, *J. Med. Chem.* **2007**, *50*, 3841–3850.
[24] S. Choi, A. B. Beeler, A. Pradhan, E. B. Watkins, J. M. Rimoldi, B. Tekwani, M. A. Avery, *J. Comb. Chem.* **2007**, *9*, 292–300.
[25] A. Cameron, J. Read, R. Tranter, V. J. Winter, R. B. Sessions, R. L. Brady, L. Vivas, A. Easton, H. Kendrick, S. L. Croft, D. Barros, J. L. Lavandera, J. J. Martin, F. Risco, S. Garcia-Ochoa, F. J. Gamo, L. Sanz, L. Leon, J. R. Ruiz, R. Gabarro, A. Mallo, F. Gomez de Las Heras, *J. Biol. Chem.* **2004**, *279*, 31429–31439.
[26] R. Conners, F. Schambach, J. Read, A. Cameron, R. B. Sessions, L. Vivas, A. Easton, S. L. Croft, R. L. Brady, *Mol. Biochem. Parasitol.* **2005**, *142*, 137–148.
[27] C. Granchi, S. Roy, C. Giacomelli, M. Macchia, T. Tuccinardi, A. Martinelli, M. Lanza, L. Betti, G. Giannaccini, A. Lucacchini, N. Funel, L. G. Leon, E. Giovannetti, G. J. Peters, R, Palchaudhuri, E. C. Calvaresi, P. J. Hergenrother, F. Minutolo, *J. Med. Chem.* **2011**, *54*, 1599–1612.
[28] R. D. Haworth, J. M. McLachlan, *J. Chem. Soc.* **1952**, 1583–1589.
[29] J. Westbrook, Z. Feng, L. Chen, H. Yang, H. M. Berman, *Nucleic Acids Res.* **2003**, *31*, 489–491.
[30] J. A. Read, V. J. Winter, C. M. Eszes, R. B. Sessions, R. L. Brady, *Proteins* **2001**, *43*, 175–185.
[31] R. D. Goldman, N. O. Kaplan, T. C. Hall, *Cancer Res.* **1964**, *24*, 389–399.
[32] J. J. Alexander, E. M. Bey, E. W. Geddes, G. Lecatsas, *S. Afr. Med. J.* **1976**, *50*, 2124–2128.
[33] J. M. Cuezva, M. Krajewska, M. Lopez de Heredia, S. Krajewski, G. Santamaria, H. Kim, J. M. Zapata, H. Marusawa, M. Chamorro, J. C. Reed, *Cancer Res.* **2002**, *62*, 6674–6681.
[34] M. Cannon, A. Jimenez, *Biochem. J.* **1974**, *142*, 457–463.
[35] E. S. Cleaveland, A. Monks, A. Vigro-Wolff, D. W. Zaharevitz, K. Paull, K. Ardalan, D. A. Cooney, H. J. Ford, *Biochem. Pharmacol.* **1995**, *49*, 947–954.
[36] W. Knecht, M. Löffler, *FEBS Lett.* **2000**, *467*, 27–30.
[37] R. A. Copeland, "Reversible Inhibitors" in: *Enzymes: a Practical Introduction to Structure, Mechanism, and Data Analysis, 2nd ed.*, Wiley-VCH, Weinheim, **2000**.
[38] R. A. Copeland, "Reversible Modes of Inhibitor Interactions with Enzymes" in: *Evaluation of Enzyme Inhibitors in Drug Discovery*, Wiley-VCH, Weinheim, **2005**.
[39] X. Zha, F. Wang, Y. Wang, S. He, Y. Jing, X. Wu, H. Zhang, *Cancer Res.* **2011**, *71*, 13–18.
[40] W. Lieberthal, S. A. Menza, J. S. Levine, *Am. J. Physiol. Renal Physiol.* **1998**, *274*, F315–F327.
[41] D. M. Krüger, A. Evers, *ChemMedChem* **2010**, *5*, 148–158.
[42] J. H. Moran, R. G. Schnellmann, *J. Pharmacol. Toxicol. Methods* **1996**, *36*, 41–44.
[43] S. B. Barker, W. H. Summerson, *J. Biol. Chem.* **1941**, *138*, 535–554.
[44] G. Fotakis, J. A. Timbrell, *Toxicol. Lett.* **2006**, *160*, 171–177.

Received: August 6, 2011
Revised: October 10, 2011
Published online on November 4, 2011

Galloflavin, a new lactate dehydrogenase inhibitor, induce the death of human breast cancer cells with different glycolytic attitude by affecting distinct signalling pathways

European Journal of Pharmaceutical Sciences 47 (2012) 729–738

Contents lists available at SciVerse ScienceDirect

European Journal of Pharmaceutical Sciences

journal homepage: www.elsevier.com/locate/ejps

Galloflavin, a new lactate dehydrogenase inhibitor, induces the death of human breast cancer cells with different glycolytic attitude by affecting distinct signaling pathways

F. Farabegoli[a,1], M. Vettraino[a,1], M. Manerba[a], L. Fiume[a], M. Roberti[b], G. Di Stefano[a,*]

[a] Department of Experimental Pathology, University of Bologna, via San Giacomo 14, 40126 Bologna, Italy
[b] Department of Pharmaceutical Sciences, University of Bologna, via Belmeloro 6, 40126 Bologna, Italy

ARTICLE INFO

Article history:
Received 16 January 2012
Received in revised form 31 July 2012
Accepted 16 August 2012
Available online 30 August 2012

Keywords:
Aerobic glycolysis
Apoptosis
Galloflavin
Lactate dehydrogenase

ABSTRACT

Galloflavin (GF), a recently identified lactate dehydrogenase inhibitor, hinders the proliferation of cancer cells by blocking glycolysis and ATP production. The aim of the present experiments was to study the effect of this compound on breast cancer cell lines reproducing different pathological subtypes of this tumor: MCF-7 (the well differentiated form), MDA-MB-231 (the aggressive triple negative tumor) and MCF-Tam (a sub-line of MCF-7 with acquired tamoxifen resistance).

We observed marked differences in the energetic metabolism of these cell lines. Compared to MCF-7 cells, both MDA-MB-231 and MCF-Tam cells exhibited higher LDH levels and glucose uptake and showed lower capacity of oxygen consumption. In spite of these differences, GF exerted similar growth inhibitory effects. This result was explained by the finding of a constitutively activated stress response in MDA-MB-231 and MCF-Tam cells, which reproduce the poor prognosis tumor forms. As a further proof, different signaling pathways were found to be involved in the antiproliferative action of GF. In MCF-7 cells we observed a down regulation of the ERα-mediated signaling needed for cell survival. On the contrary, in MCF-Tam and MDA-MB-231 cells growth inhibition appeared to be contributed by an oxidative stress condition. The prevalent mechanism of cell death was found to be apoptosis induction.

Because of the clinical relevance of breast cancer forms having the triple negative and/or chemoresistant phenotype, our results showing comparable effects of GF even on aggressively growing cells encourage further studies to verify the potential of this compound in improving the chemotherapy of breast cancer.

1. Introduction

The recent years have witnessed a rediscovered interest in the bioenergetic properties of cancer cells, now regarded as novel molecular targets to develop therapeutic strategies (Pelicano et al., 2006). Unlike normal cells, cancer cells obtain a high percentage of their ATP through the degradation of glucose to lactate, which can occur even in condition of sufficient oxygen supply (Garber, 2006). This metabolic feature is basically the consequence of a reprogramming of the cell energetic machinery caused by altered oncogenes and oncosuppressors (Levine and Puzio-Kuter, 2010), but could also be promoted by the hypoxic microenvironment often found in solid tumor lesions, which prevents the proceeding of oxidative phosphorylation (Gatenby and Gillies, 2004).

While the mechanisms underlying the metabolic remodeling of cancer cells remain elusive, increasing evidences suggest that the inhibition of glycolysis could be a rational approach to cancer management (Pelicano et al., 2006). Among the key enzymes of the glycolytic process, lactate dehydrogenase (LDH) is emerging as the most interesting target for the development of inhibitors (Granchi et al., 2010). LDH catalyses the conversion of pyruvate to lactate, utilizing NADH as a co-factor. This is the last step of glycolysis and is not active in normal cells, in conditions of normal functional activity and sufficient oxygen supply. The active LDH enzyme is a tetramer composed of two types of subunits (A and B), with different kinetic and regulatory properties. The combination of these two isoforms can give rise to both homotetrameric structures and heterogeneous enzyme complexes (Everse and Kaplan, 1973). The A subunit predominates in liver and skeletal muscle and was found to be up-regulated in various cancer cells (Granchi et al., 2010), as a result of hypoxic microenvironment and gene mutation (Pelicano et al., 2006; Levine and Puzio-Kuter, 2010; Gatenby and Gillies, 2004). In these cells increased LDH-A activity

* Corresponding author. Tel.: +39 051 2094721; fax: +39 051 2094746.
E-mail address: giuseppina.distefano@unibo.it (G. Di Stefano).
[1] These authors contributed equally to this work.

0928-0987/$ - see front matter © 2012 Elsevier B.V. All rights reserved.
http://dx.doi.org/10.1016/j.ejps.2012.08.012

accelerates ATP production by regenerating oxidized NAD for glycolysis continuation. LDH B subunit is inhibited by high pyruvate concentrations and predominates in heart (Everse and Kaplan, 1973). LDH-B was recently found to be involved in mTOR-mediated tumorigenesis (Zha et al., 2011) and was also found to be up-regulated in stromal cells of human breast cancer samples with unfavorable prognosis (Bonuccelli et al., 2010). This last observation suggested that LDH-B could also have a role in supporting the growth of epithelial neoplastic cells by supplying them with energetic metabolites in a paracrine fashion. Inhibition of LDH as an approach to antineoplastic chemotherapy was proposed several years ago (Fiume, 1960; Papacostantinou and Colowick, 1961a) and has been recently re-evaluated following two observations: (a) neoplastic cells with a reduction of LDH levels induced by shRNA or by siRNA showed a decreased tumorigenicity (Fantin et al., 2006; Le et al., 2010); (b) humans with a hereditary deficiency of the A or B isoform of LDH did not display any symptom (Hidaka et al., 1999; Joukyuu et al., 1989; Kanno et al., 1988; Kitamura et al., 1971; Miwa et al., 1971; Okumura et al., 1999; Wakabayashi et al., 1996) except for muscle rigidity and myoglobinuria, complained after strenuous exercise by individuals with LDH-A deficiency (Miwa et al., 1971).

We recently identified a molecule (galloflavin, GF) which inhibits both the A and B isoforms of LDH (Manerba et al., 2012). GF was selected by using a structure-based virtual screening procedure, applied to the compounds in the Open Chemical Collection of the NCI. This molecule is a gallic acid derivative for which no other biochemical effect has been described in literature to date. In our first study we found that GF inhibited lactate production and ATP synthesis of human hepatocellular carcinoma cells cultured in vitro without affecting their capacity of oxygen consumption. Moreover, at doses hindering the cell metabolism, GF induced cell death in the form of apoptosis (Manerba et al., 2012).

The aim of the present paper was to investigate the effects of GF on cells from tumors of different pathological subtypes, suggestive of a different metabolic state of the transformed cells. As a model for our study we chose human breast cancer, a neoplasm which is known to exhibit remarkable heterogeneity in phenotype, usually classified by reporting the expression profile of ER, PR and HER-2. The MCF-7 cell line was used as a model of the well differentiated human breast cancer (ER and PR positive) and the MDA-MB-231 as representative of the more aggressive triple-negative form of the tumor. The experiments were also performed on a sub-line of MCF-7, in which ER function has been lost. This line (MCF-Tam) was obtained by maintaining the parental cells in the presence of clinically relevant levels of 4-hydroxy-tamoxifen (10^{-7} M) (Farabegoli et al., 2007) and shows an activated EGFR signaling pathway (Farabegoli et al., 2010).

2. Materials and methods

2.1. Cell cultures

MCF-7 and MDA-MB-231 cell lines were obtained from ATCC. They were maintained in DMEM, supplemented with 10% FBS, 100 U/ml penicillin/streptomycin, 2 mM glutamine and 1 mM sodium pyruvate. The sub-line MCF-Tam was selected by maintaining the parental line for 1 year in the presence of 10^{-7} M 4-hydroxy-tamoxifen (Farabegoli et al., 2007). It was grown in α-MEM without phenol red, supplemented with 10% charcoal-stripped FBS, 100 U/ml penicillin/streptomycin, 2 mM glutamine, 1 mM sodium pyruvate and 10^{-7} M 4-hydroxy-tamoxifen. All media and supplements were from Bio-Whyttaker. During the course of experiments, cells were routinely screened for Mycoplasma contamination and found to be free. In all experiments, GF was added to the culture media in the presence of 0.6% DMSO. The same amount of DMSO was always added to the control, untreated cultures. In some experiments, cultures of normal human lymphocytes and of lymphoblasts were also used. Normal lymphocytes were purified from peripheral blood following the procedure described by Hornung et al. (2002). Lymphoblasts (clone GM130C) were from the Coriell Cell Repositories (USA); they are normal, non-transformed lymphocytes induced to proliferate by EBV infection. Both lymphocytes and lymphoblasts were maintained in RPMI medium, supplemented as described above.

2.2. Compounds and reagents

Galloflavin (GF) was synthesized according to the procedure described by Manerba et al. (2012). Briefly, 1 g gallic acid was dissolved in 8 ml water/ethanol (1:1). 4.7 ml of 5 M potassium hydroxide was added drop wise and the reaction mixture was vigorously stirred in air at room temperature for 12 h. The precipitated green potassium salt was filtered and re-dissolved in 5 ml water at 50 °C, then acidified to pH 4 with 2 N HCl. The precipitated GF was filtered, washed with diethyl ether and dichloromethane and evaporated to dryness in vacuo. The final product was chemically characterized as described by Manerba et al. (2012).

All other compounds and all reagents used for the experiments were from Sigma–Aldrich.

2.3. LDH activity and composition

Cells from semi-confluent 25 cm^2 flasks were harvested, pelleted and suspended in 3 vol. of PBS. The cell suspension was then lysed by sonication and centrifuged (1600g, 30 min at 4 °C) to discard the cell debris. Protein content of the supernatant was measured according to the method of Bradford. This cell extract was used to measure LDH activity, as follows. An amount of 50–100 µl was diluted in 3 ml 100 mM phosphate buffer pH 7.5 containing 0.12 mM NADH and 2 mM pyruvate. The enzymatic activity was measured by recording for 5 min the decrease in absorbance produced by NADH oxidation at λ_{340} nm. The global LDH activity was expressed as mU/mg of cell proteins or as mU/10^6 cells.

To determine the LDH isoform composition of the cellular extracts from the three cell lines we utilized the coenzyme analogue ratio method, originally set up by Goldman et al. (1964), which is based on the comparison of the enzyme activity obtained with a reaction mixture containing reduced nicotinamide hypoxanthine dinucleotide (NHXDH) and low pyruvate concentration to that measured in the presence of NADH and high pyruvate concentration.

Two reaction mixtures were prepared: the first containing 0.22 mM NHXDH and 0.33 mM pyruvate and the second containing 0.13 mM NADH and 10 mM pyruvate, both in a final volume of 3 ml of 100 mM phosphate buffer (pH 7.5). The reactions were started by adding the cellular extract to each mixture and the optical density change at λ_{340} nm between 30 and 120 s was determined. The detailed procedure for calculating the relative amounts of A and B isoforms was described by Goldman et al. (1964).

2.4. Assay of cell respiration

This assay was performed by using a phosphorescent oxygen-sensitive probe (MitoXpress probe) from Luxcel Biosciences. Cells from each line (5×10^4/well, in phenol red free medium) were seeded in four wells of a 96-multiwell clear bottom, black body plate and allowed to adhere overnight. After the addition of the MitoXpress phosphorescent oxygen-sensitive probe (10 pmoles/well), plate was placed in a Victor™ fluorescence reader (Perkin

Elmer) at 30 °C and was monitored for about 20 min to reach temperature and gas equilibrium and to obtain basal signals. The wells were then sealed with mineral oil and monitoring of the signal was resumed for the next 60 min. During this interval, the increase of fluorescence signal, which indicates oxygen consumption, was measured every 60 s with 340/642 nm excitation/emission filters, a delay time of 30 μs and a measurement window of 100 μs. All dispensing steps of the experiment were performed at 30 °C with pre-warmed solutions. For each cell line, evaluation of respiration was performed by applying the linear regression analysis to the time profiles of fluorescence signals obtained from the four wells, in order to determine the slope of the profile. The Prism 5 GraphPad software was used. The steepest slope, indicative of the highest oxygen consumption, was obtained from MCF-7 cells and was arbitrarily set as 100.

2.5. 2-Deoxy-[^{14}C]glucose uptake assay

Cells (5×10^5/well) were seeded in six-well plates and allowed to adhere overnight. The next day, cells were washed thrice with Krebs Ringer buffer pH 7, 4 and incubated for 15 min at 37 °C in 1 ml Krebs Ringer buffer containing 0.5 μCi 2-deoxy-D-[1-^{14}C]glucose (55.5 mCi/mmol) (Moravek, USA). Cells were then washed twice with ice-cold Krebs Ringer buffer and lysed with 500 μl NaOH 0.5% for 1 min to generate extract samples for liquid scintillation counting. The remaining lysate was assayed for protein concentration using the Bradford reagent. 2-Deoxy-D-[1-^{14}C]glucose uptake rates were calculated as nmol/min per milligram protein.

2.6. Effects of GF on lactate production

Cells (5×10^5) in 1 ml of culture medium were seeded in each well of a 6-well Nunclon plate and let to adhere overnight. Scalar amounts of GF (0–250 μM, tested in duplicate) were then added to the cultures. Lactate was measured in 3 untreated wells at the start of experiment (baseline value) and 3 h after incubation at 37 °C. In each well, we simultaneously measured released in medium and intracellular lactate: at the end of incubation 100 μl of 100% trichloroacetic acid (TCA) solution was added; the cell lysate was collected and the well was washed with 1 ml 10% TCA. After centrifugation, lactate in the supernatant was measured according to the method of Barker and Summerson (1941). The amount of metabolite formed during the 3 h incubation with or without the inhibitor was calculated by subtracting the baseline value. By using the same procedure, in some experiments lactate levels were also measured in GM130C lymhoblasts.

2.7. Effect of GF on ATP cellular levels

ATP levels were measured using the CellTiter-Glo Luminescent Cell Viability Assay from Promega. For this experiment, 2×10^4 cells in 200 μl of culture medium were seeded into each well of a 96-multiwell white body plate and allowed to adhere overnight. GF (0–250 μM, tested in duplicate) was added and ATP levels were measured after 3 h incubation at 37 °C. After incubation, the plate was allowed to equilibrate at room temperature for 30 min and the CellTiter-Glo reactive was directly added to each well. The plate was kept on a shaker for 10 min to induce cell lysis and its luminescence was measured by using a Fluoroskan Ascent FL reader (Labsystems). By using the same procedure, in some experiments ATP levels were also measured in GM130C lymhoblasts.

2.8. Effect of GF on cell growth

The effects caused by GF on the growth of breast cancer cells were evaluated with the neutral red assay (NR). This dye is actively endocytosed by living cells, allowing a precise estimate of their number. Before each experiment, a plot reporting the NR absorbance values obtained from scalar amounts of cells was obtained from each cell line. These data were fitted by using the linear regression analysis with the GraphPad Prism 5 software; the resulting mathematical equation was used to calculate the number of cells at the beginning and at the end of experiments. Cells (1.0×10^4/well) were seeded in 96-multiwell plates, allowed to adhere overnight and treated for 24 and 48 h at 37 °C with scalar doses of GF (0–250 μM, tested in duplicate). After incubation, cells were maintained 3 h at 37 °C with the NR dye dissolved in medium at the final concentration of 30 μg/ml. Medium was then removed and the cells were solubilized with 200 μl of 1% acetic acid in 50% ethanol. Absorbance of the solutions was measured at λ_{540} using a microplate reader (Multiskan Ascent FL, Labsystems). The cell growth during the period of treatment (with or without GF) was calculated by subtracting the cell number at the start of experiment from that found after the 24 or 48 h incubation. For comparison, this experiment was also performed on normal human lymphocytes and on GM130C lymphoblasts, which were cultured (1×10^5 cells/ml) in 24 well plates and exposed to GF (0–250 μM) for 24 h. After incubation, cells were counted under a light microscope and their viability was determined by Trypan blue exclusion. The effect caused by GF on lymphoblasts was evaluated by measuring the inhibition of cell growth; the effect on lymphocytes (which do not divide) was evaluated according to the following equation:

$$100 - [(\text{final cell number} - \text{initial cell number})/\text{initial cell number}] \times 100$$

2.9. Characterization of cell death

Information on the mechanism of cell death caused by GF was obtained by using the ApoTox-Glo™ Triplex Assay (Promega), which measures viability, cytotoxicity and caspase 3/7 activation in the same sample. The test was performed on cells seeded in 96-well white body plates (2×10^4 cells/well) after a 6 h exposure to different GF concentrations, following the instructions of the manufacturer.

2.10. Reactive oxygen species visualization and quantification

This assay was performed by using 2′,7′-dichlorofluorescin diacetate (DCF-DA). When applied to intact cells, DCF-DA crosses cell membranes and is hydrolyzed enzymatically by intracellular esterases to non-fluorescent DCF. In the presence of reactive oxygen species (ROS), DCF is oxidized to highly fluorescent dichlorofluorescein (DCF). Cells were grown on cover slips (2×10^4/well) in 24 well plates and they were treated for 4 h with 125 μM GF. After washing with PBS, the cells were incubated at room temperature in the dark for 20 min with 10 μM DCF-DA dissolved in PBS. After incubation, the cells were rinsed three times with PBS and mounted with a solution of Hoechst (10 μg/ml) and DABCO. The samples were observed at a Nikon epifluorescence microscope equipped with filters for Hoechst and FITC. Cells showing a bright and intense fluorescence were counted as positive, whereas cells having no or low fluorescence were counted as negative. At least three fields for sample were analyzed and 100–200 cells for sample were counted. To obtain a quantitative index of ROS generation, we adopted the method described by Wang and Joseph (1999), with modifications. Briefly, cells (6×10^5/well) were grown in six well plates and treated for 4 h with 125 μM GF. After further 1 h incubation with 100 μM DCF-DA, they were washed with PBS and lysed with 250 μl of 1% Triton dissolved in water. Lysates were placed in

a 96 well microplate and their fluorescence (λ_{exc} 485; λ_{em} 530) was measured by using the Fluoroskan Ascent FL reader (Labsystems) in kinetic mode for 30 min at 37 °C. The increase in fluorescence was calculated by the following formula: $[(Ft_{30} - Ft_0)/Ft_0]$, where Ft_{30} is the fluorescence measured at 30 min and Ft_0 is the fluorescence at time 0. Experiment was performed in triplicate.

2.11. ER and EGFR expression

ERα and EGFR expression was evaluated by measuring mRNA levels after a 24 h exposure to scalar doses of GF. RNA was extracted by guanidinium–phenol–isoamylic alcohol method according to Chomczynski and Sacchi (1987), with modifications. RT-PCR reaction was performed using the cMaster RT plus PCR system kit according to the instruction of the supplier (Eppendorf, Hamburg, Germany).

The following primers were used: ERα, F: 5′-CCG-CTCATGATCA AACGCTCTAAG-3′ R: 5′-GCCCTCTACACATTTTCCCTGGTT-3′, which originate a 375 bp DNA fragment; EGFR F: 5′-CTCACGCAGTTGGG-CACTTT-3′, R: 5′-TCATGGGCAGCTCCTTCAGT-3′ (301); β-actin F: 5′-GGCATCGTGATGGACTCCG-3′, R: 5′-GCTGGAAGGTGGACAGCGA-3′ (613). Annealing temperature was 58 °C. β-actin primers were used together with ERα (MCF-7 cells) or EGFR primers (MDA-MB-231 and MCF-7 Tam) into the same tube.

2.12. Western blots

Cell cultures (1.5×10^6 cells, seeded in 25 cm^2 flasks) were exposed to 125 μM GF for 16 h. After incubation they were lysed in 150 μl M-PER buffer (Pierce) containing protease inhibitors (Roche) and a phosphatase inhibitor cocktail (Pierce). The cell homogenates were left 30 min on ice and then centrifuged 15 min at 10000g. 20–40 μg proteins of the supernatants (measured according to Bradford) was loaded into 12% 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 300 mA for 2 h. The blotted membrane was blocked with 5% BSA in TBS-Tween and probed with the primary antibody. In the different experiments, the used antibodies were: rabbit anti-ERK 1/2 and rabbit monoclonal anti-ERK 1/2-(phosphoThr202/Tyr204) (Cell Signaling Technology), rabbit anti-p38, anti-p38-(phospho Tyr182/Thr180), anti-JNK, anti-JNK-(phospho Thr183/Tyr185) anti-PUMA and anti-Bax (Abcam), rabbit anti-actin (Sigma–Aldrich). Binding was revealed by a Cy5-labelled secondary antibody (GE Lifescience). 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).

3. Results

3.1. Characterization of cell metabolism and metabolic effects of GF

The metabolic characteristics of the three cell lines was estimated by quantifying their glucose uptake, lactate production and relative oxygen consumption. Glucose uptake was evaluated by exposing the cells to 2-deoxy-[^{14}C]glucose. Inside the cells this analogue enters the glycolytic pathway but, after phosphorylation by hexokinase, it cannot be further processed (Nelson et al., 1996). For this reason, it can be usefully employed to track glucose uptake by cells. Moreover, by performing LDH enzymatic assays on cell extracts in the presence of NADH or NHXDH as cofactors, we quantified LDH activity and typified the enzyme isoform composition of the three lines. The results of these determinations are reported in Table 1.

They showed marked differences in the metabolic profile of the studied cells, suggesting MCF-Tam and MDA-MB-231 as the most glycolysis-dependent lines. These cells exhibited highly increased LDH activity and 2-deoxy-[^{14}C]glucose uptake, which inversely correlated with their capacity of oxygen consumption. Their lactate production appeared, however, only modestly changed (+18–30%) when compared to that of MCF-7 cells. This result can be explained at least in part by considering that in both MCF-Tam and MDA-MB-231 about 50% of LDH activity is due to the B isoform, which is less efficient in lactate production in the presence of high substrate concentrations (Everse and Kaplan, 1973).

The effects of GF on cell metabolism were evaluated by exposing the three cell lines to the compound for 3 h, followed by the determination of lactate and ATP cellular levels. For comparison, a similar experiment was also performed with oxamic acid (OXA). OXA, a pyruvate analogue, is to date the only well characterized and specific inhibitor of LDH (Papacostantinou and Colowick, 1961a; Novoa et al., 1959); it inhibits both the A and B isoforms of the enzyme by competing with its substrate (Manerba et al., 2012).

The obtained results are shown in Fig. 1. GF appeared to reduce both lactate and ATP production in all the three cell lines; these effects resulted to be dose-dependent, with no evidence of a threshold dose. The inhibition data obtained with GF were analyzed using the second order polynomial regression, in order to calculate the IC_{50}s (doses causing 50% inhibition). In the case of MDA-MB-231 and MCF-Tam the calculated IC_{50} for lactate production was in good agreement with that evaluated for ATP synthesis (244 μM vs 230 μM for MDA-MB-231; 130 μM vs 160 μM for MCF-Tam), suggesting again that these cell lines are highly reliant on glycolysis for ATP generation. On the contrary, in MCF-7 cells the IC_{50} for ATP synthesis (340 μM) was about 3-fold higher than that calculated for lactate production (104 μM), an indication that in these cells oxidative phosphorylation remains operative and can be a source of ATP supply.

The highest tested dose of OXA (80 mM) (Fig. 1, panel B) never caused inhibitions higher than 50%. The limited solubility of this compound in cell culture medium did not allow to study the effects of doses >80 mM.

3.2. Effects of GF on cell growth

Fig. 2 shows the effects of GF on the growth of breast cancer cells. GF non only inhibited cell growth, but in treated cultures at the highest dose (250 μM) also caused a reduction in cell number, which at the end of treatment appeared to be lower than that seeded at the beginning of experiment. This observation suggests that GF could have not only anti-proliferative effects, but also the capacity of inducing cell death. As a confirm, evidences of cell death were also observed when the cultures were exposed to the GF dose causing 50% inhibition of cell growth (IC_{50}), which after 16 h incubation produced increased expression of proteins involved in apoptosis signals (see below and Fig. 5 and 6).

The data reported in Fig. 2 also show that, in spite of the differences in the metabolic profile observed in the three lines (Table 1), the effect caused by GF on cell growth was quite similar. The IC_{50} doses reported in the bar graph (Fig. 2B) were calculated by applying the second order polynomial regression to the data of the growth inhibition plots. The statistical analysis, performed by two-way ANOVA, did not show statistical significant differences among the three cell lines. This result conflicts with the much higher glycolysis dependence shown by MDA-MB-231 and MCF-Tam cells (Table 1 and Fig. 1A) and can be justified by other characteristics of these cells (see below and Fig. 5). As shown in Fig. 2B, the calculated IC_{50} for the breast cancer cell lines was 90–150 μM GF.

Table 1
Overview of the metabolic characteristics of the studied cell lines.

		MCF-7	MCF-Tam	MDA-MB-231
Global LDH activity	mU/10^6 cells	185 ± 15	320 ± 27	357 ± 22
	mU/mg cell proteins	358 ± 29	864 ± 73	990 ± 61
LDH composition	% LDH-A	71.5 ± 5.5	51.5 ± 7.0	47.5 ± 6.1
	% LDH-B	28.5 ± 5.5	48.5 ± 7.0	52.5 ± 6.1
2-deoxy-[^{14}C]Gluc. uptake	pmoles/min/mg cell proteins	69.8 ± 1.1	225 ± 17	189 ± 2.0
Lactic acid produced in 3 h	μmoles/10^6 cells	3.78 ± 0.05	4.94 ± 0.06	4.47 ± 0.11
Cell respiration	Relative O_2 consumption (%)	100 ± 2.2	38.4 ± 14.8	30.9 ± 10.3

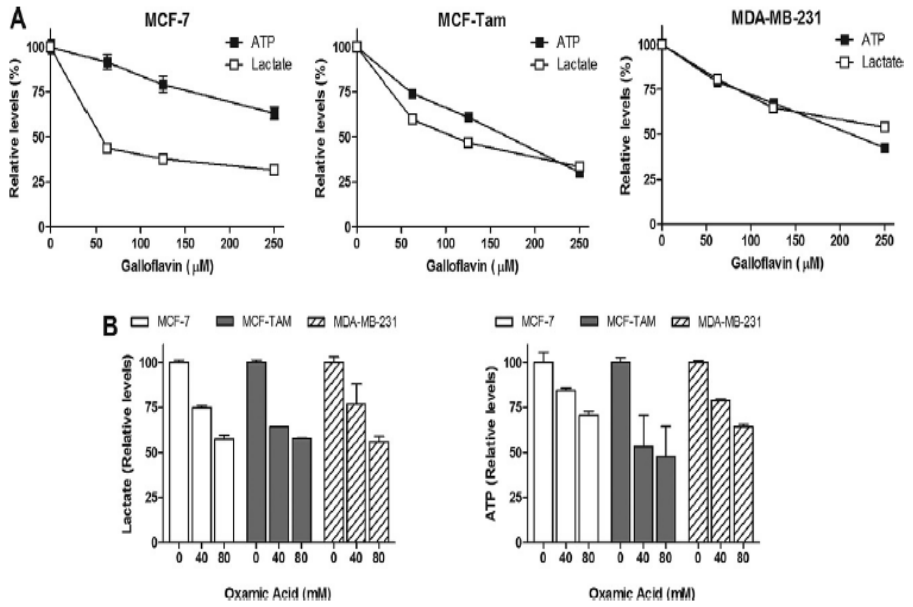


Fig. 1. Panel A: effects of galloflavin on lactic acid and ATP production in the studied cell lines. Experimental data were fitted using the second order polynomial regression in order to calculate the IC_{50} doses with the GraphPad Prism 5 software. Results are reported in the text. Panel B: Effects caused by oxamic acid, a known specific LDH inhibitor.

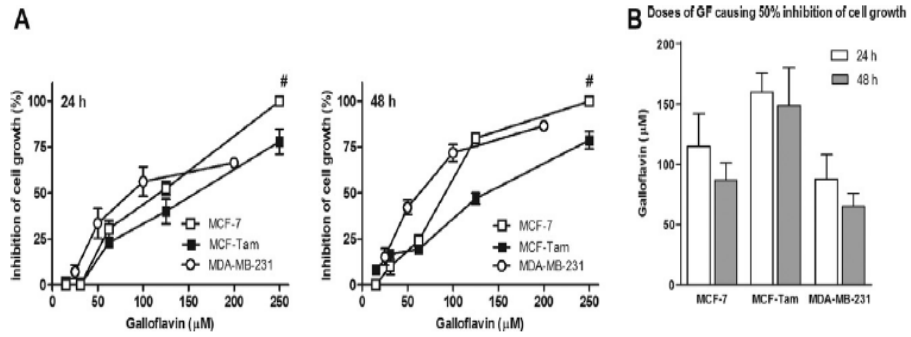


Fig. 2. Inhibition of cell growth caused by galloflavin on breast cancer cells, evaluated as described in Section 2. The symbol #indicates a final cell number lower than that seeded at the beginning of experiments. Data were fitted using the second order polynomial regression in order to calculate the IC_{50} doses with the GraphPad Prism 5 software. Results (reported in panel B as mean values ± SEM) were then analyzed by using the two-way ANOVA. No statistical significant difference was found ($p > 0.05$, $n = 3$–5) among the three breast cancer cell lines.

As a first attempt to explore the possible mechanisms involved in the cell death caused by GF, we evaluated: (a) whether, by causing ATP depletion, this compound could induce the down-regulation of the signaling pathways needed for cell survival; (b) the presence of an oxidative stress condition in the treated cultures. In previous studies LDH inhibition was in fact found to induce reactive oxygen species (ROS) generation, as a consequence of a compensatory increase in cell respiration set out by cells attempting

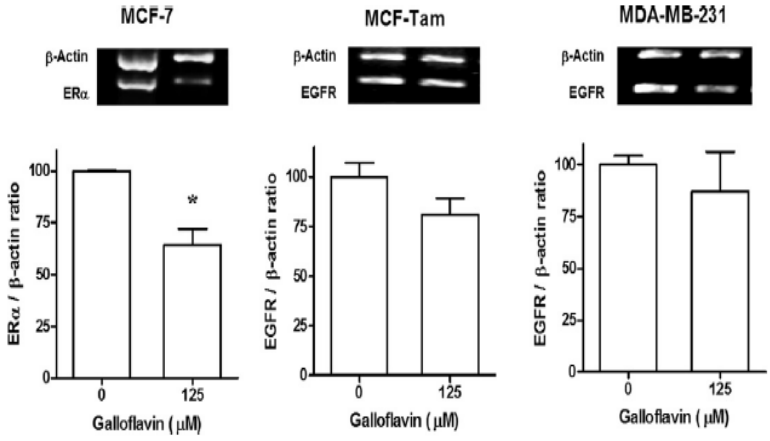


Fig. 3. Effects of galloflavin on ERα mRNA in MCF-7 cells and EGFR mRNA in MCF-Tam and MDA-MB-231 cells experiments were performed as described in Section 2. Results (mRNA band intensities from 2 experiments, normalized on β-actin expression) were analyzed by the Student's *t*-test using the GraphPad Prism 5 software. *Indicates a statistically significant difference, with $p < 0.05$.

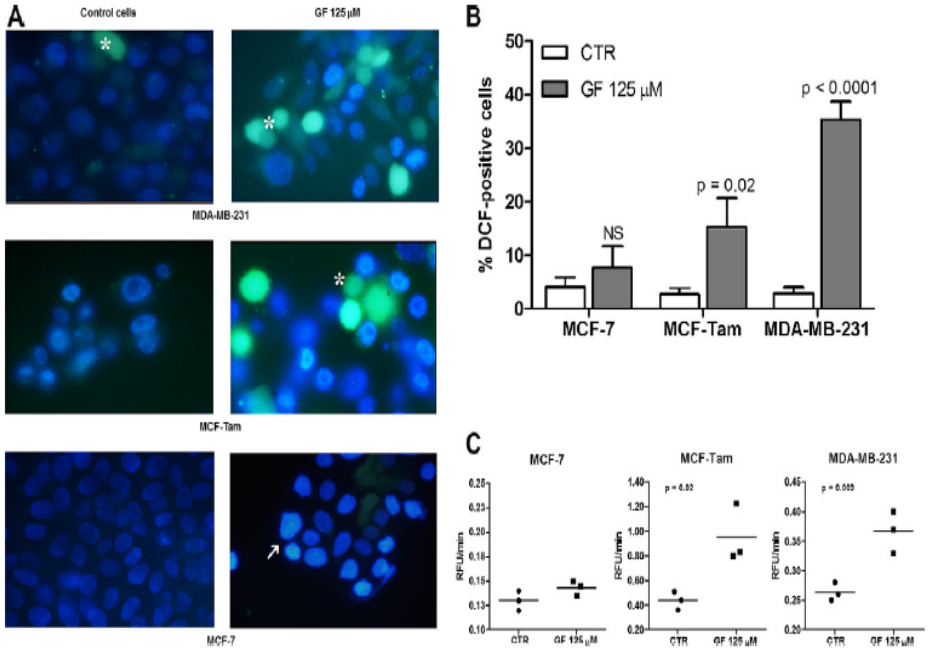


Fig. 4. Panel A: microscopic view of GF treated cells, exposed to DCF-DA. White asterisks indicate DCF-labeled cells, with bright, diffuse cytoplasmic fluorescence. Arrow indicates apoptotic cells with condensed nuclei. Panel B: number of DCF-labeled cells counted at the microscopic examination. Panel C: intensity of the generated fluorescence signal, evaluated by DFC-DA assay as described in Section 2. Each symbol in graphs indicates a different cell culture. Results reported in B and C were statistically evaluated by the Student's *t*-test, using the GraphPad Prism 5 software. Statistically significant differences are indicated on the graphs.

to restore their ATP supply (Le et al., 2010). These effects were studied by incubating breast cancer cell cultures to the mean IC_{50} dose of GF (125 μM).

We found that in MCF-7 cells GF caused a statistically significant reduction of ERα mRNA expression (Fig. 3), thus leaving these cells deprived of their most important survival signal (Levenson and Jordan, 1997). On the contrary, in MDA-MB-231 and MCF-Tam cultures the EGFR-mediated signaling needed for inducing cell growth (Farabegoli et al., 2007; Yamasaki et al., 2007) did not seem significantly affected by GF, as shown by the level of EGFR mRNA measured in the cells treated with the compound (Fig. 3). Conversely, in both these cell lines, we observed that a 4 h exposure to GF was responsible of an evident generation of reactive oxygen species, which did not appear significantly increased during GF treatment of MCF-7 cells. These results are reported in Fig. 4, which shows the microscopic view of the breast cancer cells treated with GF and exposed to DCF-DA (panel A), together with a quantitative assessment of the number of DCF-labeled cells counted at the microscopic examination (panel B). The same figure also shows an evaluation of the intensity of the fluorescence signal generated by DCF in treated cells (panel C). The results reported in Fig. 4 suggest that, contrary to MCF-7 cells, which according to our data display a good capacity of oxygen utilization (Table 1), the highly glycolysing MDA-MB-231 and MCF-Tam cells are not able to cope

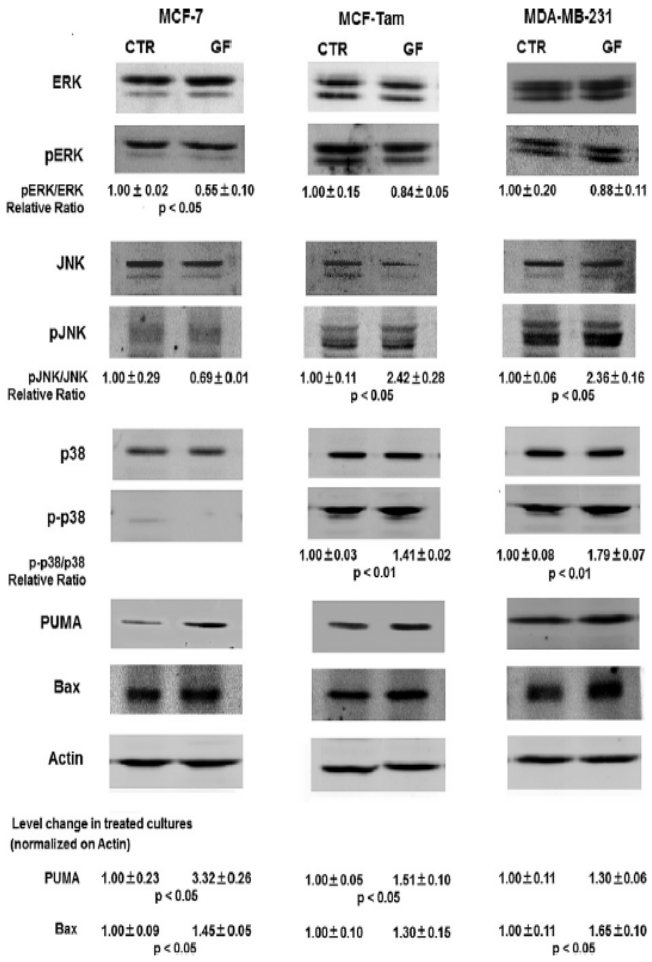


Fig. 5. Expression of sample proteins involved in cell replication, stress response or death, evaluated by western blot in breast cancer cells exposed to 125 μM GF. Experiment was performed as described in Section 2. To obtain a semi-quantitative evaluation of the change in protein level after GF treatment, band intensities were measured by reading the fluorescence of the Cy5-labelled secondary antibody, using the Pharos FX scanner (BioRad).

with the ATP depletion caused by LDH inhibition by simply increasing the mitochondrial function. They also indicate oxidative stress as a possible cause contributing to the growth inhibition caused by GF in MDA-MB-231 and MCF-Tam cells.

As a second step, we studied the expression of sample proteins involved in cell replication, stress response and death in breast cancer cells treated with 125 μM GF (Fig. 5) and tried to ascertain the prevalent death mechanism triggered by the compound in these cells (Fig. 6).

As shown in Fig. 5, in MCF-7 cells GF caused a statistically significant reduction of ERK phosphorylation, together with an increased expression of PUMA (P-53 Upregulated Modulator of Apoptosis) and Bax. These data, together with the already observed reduction of ERα mRNA confirmed that in these cells GF caused the shutdown of the survival (and/or growth) signals and the induction of apoptosis. This modality of cell death was also confirmed by the results reported in Fig. 6. In MCF-Tam and MDA-MB-231 cells, ERK phosphorylation was found to remain unchanged. In spite of the modest increase observed in Bax and PUMA expression, which can be justified by the lack in these cells of a functionally active p53 protein (Antony et al., 2012), apoptosis appeared to contribute to the cell death (Fig. 6). Fig. 5 also shows that in MCF-Tam and MDA-MB-231 cells (but not in MCF-7) GF induced the activation of the stress–response pathway, as witnessed by the statistically significant increase of JNK and p38 phosphorylation. Together with the sustained ERK phosphorylation, which was already observed in ROS exposed cells (Yu and Kim, 2001; Akool et al., 2012) and was found to mediate death events (Cagnol and Chambard, 2010), the increased JNK and p38 phosphorylation confirmed the oxidative stress induced by GF as the main cause of cell death in MCF-Tam and MDA-MB-231 cells and justified the appearance of necrosis, clearly detected in both these cells (Fig. 6).

The study of phospho-JNK and phospho-p38 expression also allowed to explain why, in spite of their much higher glycolysis dependence, MDA-MB-231 and MCF-Tam did not exhibit higher sensitivity to the antiproliferative effects of GF (Fig. 2). As shown in Fig. 5, in these cells, which are representative of aggressive forms of breast cancer, the stress response pathway is constitutively activated, since both JNK and p38 appeared phosphorylated also in the untreated cultures. This constitutive activation can render the cells less susceptible to harmful stimuli (Davidson et al., 2006). Constitutive activation of p38 was already observed both in tumor samples from patients with recurrent breast cancer and in drug resistant breast cancer cell lines (Gutierrez et al., 2005;

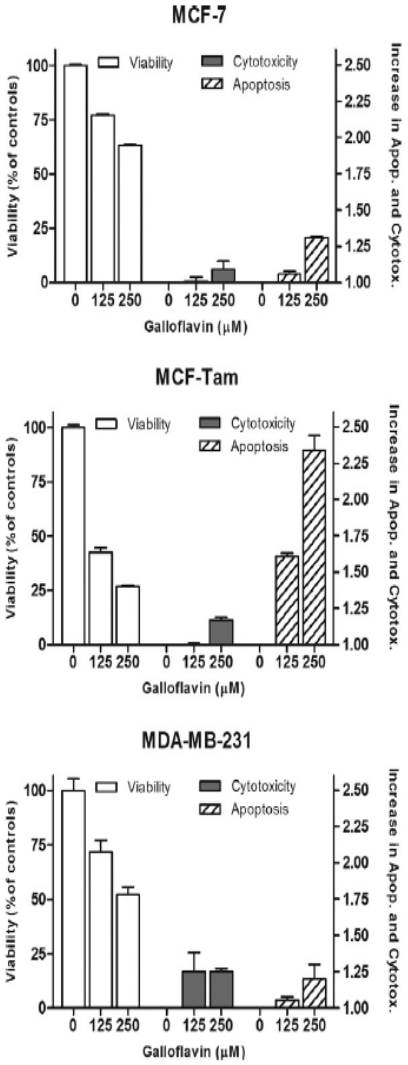


Fig. 6. Antiproliferative mechanisms triggered after a 6 h exposure to galloflavin. Experiments were performed by using the ApoTox-Glo™ Triplex Assay (Promega), as described in Section 2. This test is based on a fluorogenic peptide not able to cross the cell membrane, giving a fluorescent signal when degraded by proteases released by necrotic cells and by a luminogenic substrate degraded by caspases 3 and 7.

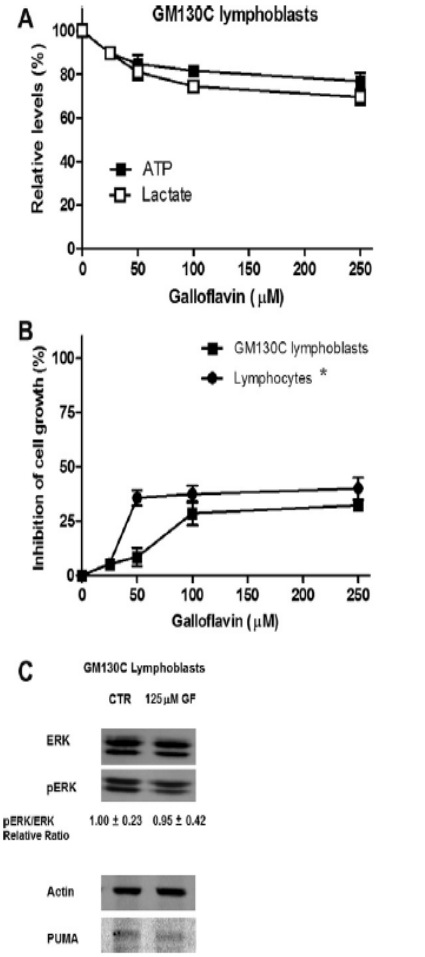


Fig. 7. Experiments performed on normal lymphocytes and lymphoblasts. Panel A: decrease of ATP and lactate production in GM130C lymphoblasts after 6 h exposure to GF. Panel B: effect of GF on lymphocytes survival and lymphoblasts growth, evaluated as described in Section 2. Panel C: immunoblot evaluation of replication and death signals in GM130C lymphoblasts exposed to 125 μM GF.

Davidson et al., 2006). This feature has been proposed as an adaptive mechanism to bypass the cytotoxic effects of chemoterapeutics and as a marker of tumor progression (Davidson et al., 2006). The observation that these signals are not activated in the parental MCF-7 cells even after GF treatment can be a further, final confirm that the growth inhibition effects caused by the compound on the studied breast cancer cell lines occurs through the activation of different pathways.

3.3. Effects of GF on normal lymphocytes and lymphoblasts

Compared to commonly used chemotherapeutics, GF inhibits the growth of breast cancer cells at higher dose (125 μM) (see above and Fig. 2). For this reason, we tried to acquire some indications about the effect of this compound on non neoplastic cells. As a model, we used normal human lymphocytes purified from peripheral blood and GM130C human lymphoblasts (see Section 2). They were preferred to the commonly used MCF-10 breast cells since, although not tumorigenic in mice, the MCF-10 cultures exhibit several signs of a proceeding neoplastic transformation, such as lack of senescence and growth in semisolid medium (Marella et al., 2009; see also the cell line data on www.atcc.org). A further motivation for this choice is that proliferating lymphocytes are one of the cell populations more susceptible to the toxic effects of conventional chemotherapeutics, which commonly cause serious bone marrow toxicity. The performed experiments are reported in Fig. 7. Because of the low yield in purified cells from blood samples and of their reduced lifespan, normal lymphocytes were only used to test the effect of GF on cell survival (panel B). Fig. 7A shows that, compared to the effect caused on breast cancer cells (Fig. 1A), GF affected lactate and ATP production of lymphoblasts to a lower extent, causing a <30% reduction at the highest tested dose. A similarly milder effect was observed on lymphocytes survival and lymphoblasts growth (Fig. 7B), with a low evidence of a dose/response result. Finally, Fig. 7C shows that 125 μM GF (the dose causing 50%

growth inhibition and induction of death in breast cancer cells) (see Figs. 2B and 5) did not increase the expression of PUMA, suggesting that at this dose the growth inhibition caused by the compound on lymphoblasts (panel B) could be due to a cytostatic effect and not to cell death. These results suggest a better tolerability of GF for normal lymphocytes/lymphoblasts when compared to breast cancer cells.

4. Conclusions

Taken together, our experimental data show that the inhibition of LDH caused by GF can exert comparable growth inhibitory effects on breast cancer cells, even when these cells are characterized by different metabolic states. Different mechanisms can contribute to the antiproliferative effect, as the down-regulation of survival signaling pathways (ERα reduction in MCF-7), or the induction of an oxidative stress condition (MCF-Tam and MDA-MB-231).

The inhibition of bioenergetic metabolism of cancer cells obtained by LDH blockade is a promising therapeutic strategy and is actually a field of active investigation (Pelicano et al., 2006). Several previously published data (Nagai et al., 1988; Zaho et al., 2009) indicated that LDH also plays a critical role on growth properties and tumor maintenance of breast cancer cells. Importantly, inhibition of LDH-A was found to be a way to overcome the acquired resistance of breast cancer cells to taxol (Zhou et al., 2010) and trastuzumab (Zhao et al., 2011), two chemotherapeutics widely used in the treatment of this tumor form. However, the success of a therapeutic approach based on LDH inhibition chiefly depends on the availability of inhibitors with good target specificity. In previous studies LDH inhibition was obtained by using oxamic acid, an analogue of pyruvic acid, the enzyme substrate. Although displaying a good selectivity for LDH (Papacostantinou and Colowick, 1961a) and a weak toxicity in healthy animals (Papacostantinou and Colowick, 1961b), oxamic acid has the drawback of a poor cellular penetration; as a consequence, it was found to inhibit the proliferation of tumor cells cultured in vitro only at millimolar concentrations, which cannot be expected to be reached in vivo. Unfortunately, in spite of extensive research, LDH proved to be a very difficult target for the development of new inhibitors, since all high throughput screening experiments have been usually characterized by a very low success rate (Ward et al., 2012). GF, a compound recently identified by our research group (Manerba et al., 2012) in the Open Chemical repository of NCI, was found to be active in vitro at micromolar concentrations. As shown by our results (Fig. 2) the mean IC_{50} of GF for all the breast cancer cell lines appeared to be around 125 μM, which is undoubtedly higher than the active dose of commonly used chemotherapeutics. However, the actual absence of other compounds active on both the enzyme subunits offers a good motivation to further studies aimed at better defining the therapeutic potential of GF. Some data on GF toxicity are available from NCI web site (http://dtp.nci.nih.gov/) and suggest a good tolerability: in mice the maximum tested dose (400 mg/kg) injected i.p. did not produce lethal effects. In this context, quite promising results are also those described in Fig. 7, showing reduced toxicity of GF on normal human lymphocytes and lymphoblasts.

The breast cancer cell lines selected for this study are representative of different pathological subtypes of the tumor. In spite of the unquestionable progresses achieved in breast cancer therapy, the treatment of some forms of this tumor, such as the triple negative or the drug resistant lesions, still poses a challenge to the clinical oncologists. In this context, our observation of GF activity even on the breast cancer cells which mimic these tumor forms with unfavorable prognosis, for its clinical relevance adds further interest in the properties of this compound.

Acknowledgements

This work was supported by Cornelia and Roberto Pallotti Legacies for Cancer Research and by University of Bologna, RFO funds.

References

Akool, E.S., Gauerb, S., Osman, B., Doller, A., Schulz, S., Geiger, H., Pfeilschifter, J., Eberhardt, W., 2012. Cyclosporin A and tacrolimus induce renal Erk1/2 pathway via ROS-induced and metalloproteinase-dependent EGF-receptor signaling. Biochem. Pharmacol. 83, 286–295.
Antony, M.L., Kim, S.H., Singh, S.V., 2012. Critical role of p53 upregulated modulator of apoptosis in benzyl isothiocyanate-induced apoptotic cell death. PLoS ONE 7, e32267.
Barker, S.B., Summerson, W.H., 1941. The colorimetric determination of lactic acid in biological material. J. Biol. Chem. 138, 535–554.
Bonuccelli, G., Whitaker-Menezes, D., Castello-Cros, R., Pavildes, S., Pestell, R.G., Fatatis, A., Witkiewicz, A.K., Vander Heiden, M.G., Migneco, G., Chiavarina, B., Frank, P.G., Capozza, F., Flomenberg, N., Martinez-Outschoorn, U.E., Sotgia, F., Lisanti, M.P., 2010. The reverse Warburg effect. Glycolysis inhibitors prevent the tumor promoting effects of caveolin-1 deficient cancer associated fibroblasts. Cell Cycle 9, 1960–1971.
Cagnol, S., Chambard, J.C., 2010. ERK and cell death: mechanisms of ERK-induced cell death – apoptosis, autophagy and senescence. FEBS J. 277, 2–21.
Chomczynski, P., Sacchi, N., 1987. Single-step method of RNA isolation by acid guanidinium thiocyanate–phenol–chloroform extraction. Anal. Biochem. 162, 156–159.
Davidson, B., Konstantinovsky, S., Kleinberg, L., Nguyen, M.T.P., Bassarova, A., Kvalheim, G., Nesland, J.M., Reich, R., 2006. The mitogen-activated protein kinases (MAPK) p38 and JNK are markers of tumor progression in breast carcinoma. Gynecol. Oncol. 102, 453–446.
Everse, J., Kaplan, N.O., 1973. Lactated dehydrogenases: structure and function. Adv. Enzymol. 37, 61–133.
Fantin, V.R., St-Pierre, J., Leder, P., 2006. Attenuation of LDH-A expression uncovers a link between glycolysis, mitochondrial physiology and tumor maintenance. Cancer Cell 9, 425–434.
Farabegoli, F., Barbi, C., Lambertini, E., Piva, R., 2007. (−)-Epigallocatechin-3-gallate down-regulates estrogen receptor alpha function in MCF-7 breast carcinoma cells. Cancer Detect. Prev. 31, 499–504.
Farabegoli, F., Papi, A., Orlandi, M., 2010. (−)-Epigallocatechin-3-gallate down-regulates EGFR, MMP-2, MMP-9 and EMMPRIN and inhibits the invasion of MCF-7 tamoxifen-resistant cells. Biosci. Rep. 30, 99–108.
Fiume, L., 1960. Inhibition of aerobic glycolysis in Yoshida ascites hepatoma by tartronic acid. Nature 187, 792–793.
Garber, K., 2006. Energy deregulation: licensing tumors to grow. Science 312, 1158–1159.
Gatenby, R.A., Gillies, R.J., 2004. Why do cancer have high aerobic glycolysis? Nat. Rev. Cancer 4, 891–899.
Goldman, R.D., Kaplan, N.O., Hall, T.C., 1964. Lactic dehydrogenase in human neoplastic tissues. Cancer Res. 24, 389–399.
Granchi, C., Bertini, S., Macchia, M., Minutolo, F., 2010. Inhibitors of lactate dehydrogenase isoforms and their therapeutic potentials. Curr. Med. Chem. 17, 672–697.
Gutierrez, M.C., Detre, S., Johnston, S., Mohsin, S.K., Shou, J., Allred, D.C., Schiff, R., Osborne, C.K., Dowsett, M., 2005. Molecular changes in tamoxifen-resistant breast cancer: relationship between estrogen receptor, HER-2, and p38 mitogen-activated protein kinase. J. Clin. Oncol. 23, 2470–2476.
Hidaka, K., Ueda, N., Hirata, I., Watanabe, Y., Minatogawa, Y., Iuchi, I., 1999. First case of missense mutation (LDH-H:R171P) in exon 4 of the lactate dehydrogenase gene detected in a Japanese patient. J. Hum. Genet. 44, 69–72.
Hornung, V., Rothenfusser, S., Britsch, S., Krug, A., Jahrsdorfer, B., Giese, T., Endres, S., Hatmann, G., 2002. Quantitative expression of toll-like receptor 1–10 mRNA in cellular subsets of human peripheral blood mononuclear cells and sensitivity to CpG oligodeoxynucleotides. J. Immunol. 168, 4531–4537.
Joukyuu, R., Mizuno, S., Amakawa, T., Tsukada, T., Nishina, T., Kitamura, M., 1989. Hereditary complete deficiency of lactate dehydrogenase H-subunit. Clin. Chem. 35, 687–690.
Kanno, T., Sudo, K., Maekawa, M., Nishimura, Y., Ukita, M., Fukutake, K., 1988. Lactate dehydrogenase M-subunit deficiency: a new type of hereditary exertional myopathy. Clin. Chim. Acta 173, 89–98.
Kitamura, M., Iijima, N., Hashimoto, F., Hiratsuka, A., 1971. Hereditary deficiency of subunit II of lactate dehydrogenase. Clin. Chim. Acta 34, 419–423.
Le, A., Cooper, C.R., Gouw, A.M., Dinavahi, R., Maitra, A., Deck, L.M., Royer, R.E., Vander Jagt, D.L., Semenza, G.L., Dang, C.V., 2010. Inhibition of lactate dehydrogenase A induces oxidative stress and inhibits tumor progression. PNAS 107, 2037–2042.
Levenson, A.S., Jordan, V.C., 1997. MCF-7: the first hormone-responsive breast cancer cell line. Cancer Res. 57, 3071–3078.
Levine, A.J., Puzio-Kuter, A.M., 2010. The control of the metabolic switch in cancers by oncogenes and tumor suppressor genes. Science 330, 1340–1344.
Manerba, M., Vettraino, M., Fiume, L., Di Stefano, G., Sartini, A., Giacomini, E., Roberti, M., Recanatini, M., 2012. Galloflavin (CAS 568–80-9): a novel inhibitor of lactate dehydrogenase. ChemMedChem. 7, 311–317.

Marella, N.V., Malyavantham, K.S., Wang, J., Matsui, S., Liang, P., Berezney, R., 2009. Cytogenetic and cDNA microarray expression analysis of MCF10 human breast cancer progression cell lines. Cancer Res. 69, 5946–5953.

Miwa, S., Nishina, T., Kakehashi, Y., Kitamura, M., Hiratsuka, A., Shizume, K., 1971. Studies on erythrocyte metabolism in a case with hereditary deficiency of H-subunit of lactate dehydrogenase. Acta Haematol. Jpn. 34, 228–232.

Nagai, M.A., Sonohara, S., Brentani, M.M., 1988. Estrogen control of lactate dehydrogenase isoenzyme-5 in human breast cancer. Int. J. Cancer 41, 10–16.

Nelson, C.A., Wang, J.Q., Leav, I., Crane, P.D., 1996. The interaction among glucose transport, hexokinase, and glucose-6-phosphatase with respect to 3H-2-deoxyglucose retention in murine tumor models. Nucl. Med. Biol. 23, 533–541.

Novoa, W.B., Winer, A.D., Glaid, A.J., Schwert, G.W., 1959. Lactic dehydrogenase. V. Inhibition by oxamate and by oxalate. J. Biol. Chem. 234, 1143–1148.

Okumura, N., Terasawa, F., Ueno, I., Oki, K., Tamauchi, K., Hidaka, H., Tozuka, M., Okura, M., Katsuyama, T., 1999. Genetic analyses in homozygous and heterozygous variants of lactate dehydrogenase-B (H) subunit – LD-B Matsumoto I and II (LD-B W323R). Clin. Chim. Acta 287, 163–171.

Papacostantinou, J., Colowick, S.P., 1961a. The role of glycolysis in the growth of tumor cells. I. Effects of oxamic acid on the metabolism of Ehrlich tumor cells in vitro. J. Biol. Chem. 236, 278–284.

Papacostantinou, J., Colowick, S.P., 1961b. The role of glycolysis in the growth of tumor cells. II. The effect of oxamic acid on the growth of HeLa cells in tissue culture. J. Biol. Chem. 236, 285–288.

Pelicano, H., Martin, D.S., Xu, R.H., Huang, P., 2006. Glycolysis inhibition for anticancer treatment. Oncogene 25, 4633–4646.

Wakabayashi, H., Tsuchiya, M., Yoshino, K., Kaku, K., Shigei, H., 1996. Hereditary deficiency of lactate dehydrogenase H-subunit. Int. Med. 35, 550–554.

Wang, H., Joseph, J.A., 1999. Quantifying cellular oxidative stress by dichlorofluorescein assay using microplate reader. Free Rad. Biol. Med. 27, 612–616.

Ward, R.A., Brassington, C., Breeze, A.L., Caputo, A., Critchlow, S., Davies, G., Goodwin, L., Hassall, G., Greenwood, R., Holdgate, G.A., Mrosek, M., Norman, R.A., Pearson, S., Tart, Jonathan, Tucker, Julie A., Vogtherr, Martin, Whittaker, David, Wingfield, Jonathan, Winter, Jon, Hudson, Kevin, 2012. Design and synthesis of novel lactate dehydrogenase A inhibitors by fragment-based lead generation. J. Med. Chem. 55, 3285–3306.

Yamasaki, S., Zhang, D., Bartholomeusz, C., Sudo, T., Hortobagyi, G.N., Kurisu, K., Ueno, N.T., 2007. Sensitivity of breast cancer cells to erlotinib depends on cyclin-dependent kinase 2 activity. Mol. Cancer Ther. 8, 2168–2177.

Yu, J.S., Kim, A.K., 2001. Wogonin induces apoptosis by activation of ERK and p38 MAPKs signaling pathways and generation of reactive oxygen species in human breast cancer cells. . Cells 31, 327–335.

Zaho, Y.H., Zhou, M., Liu, H., Ding, Y., Khong, H.T., Yu, D., Fodstad, O., Tan, M., 2009. Upregulation of lactate dehydrogenase A by ErbB2 through heat shock factor 1 promotes breast cancer cell glycolysis and growth. Oncogene 28, 3689–3701.

Zha, X., Wang, F., Wang, Y., He, S., Jing, Y., Wu, X., Zhang, H., 2011. Lactate dehydrogenase B is critical for hyperactive mTOR-mediated tumorigenesis. Cancer Res. 71, 13–18.

Zhao, Y., Liu, H., Liu, Z., Ding, Y., LeDoux, S.P., Wilson, G.L., Voellmy, R., Lin, Y., Lin, W., Nahta, R., Liu, B., Fodstad, O., Chen, J., Wu, Y., Price, J.E., Tan, M., 2011. Overcoming trastuzumab resistance in breast cancer by targeting dysregulated glucose metabolism. Cancer Res. 71, 4585–4597.

Zhou, M., Zhao, Y., Ding, J., Liu, H., Liu, Z., Fodstad, O., Riker, A.I., Kamarajugadda, S., Lu, J., Owen, L.B., Ledoux, S.P., Tan, M., 2010. Warburg effect in chemosensitivity: targeting lactate dehydrogenase-A re-sensitizes taxol-resistant cancer cells to taxol. Mol. Cancer 9, 33–45.

Galloflavin suppresses lactate dehydrogenase activity and causes MYC downregulation in Burkitt lymphoma cells through NAD/NADH dependent inhibition of sirtuin-1

Galloflavin suppresses lactate dehydrogenase activity and causes MYC downregulation in Burkitt lymphoma cells through NAD/NADH-dependent inhibition of sirtuin-1

Marina Vettraino, Marcella Manerba, Marzia Govoni and Giuseppina Di Stefano

Activation of the *myc* oncogene in cancer cells upregulates lactate dehydrogenase A (LDH-A) expression, leading to a sustained glycolytic flux that is needed to produce ATP under hypoxic conditions. We studied the effects of galloflavin (GF), a recently identified LDH inhibitor, on *myc* overexpressing Burkitt lymphoma (BL) cells. Epstein–Barr virus-infected lymphoblasts were used as a non-neoplastic control. Our results showed that *myc* overactivation induced a two- to seven-fold increase in LDH-A expression in BL cells compared with non-neoplastic lymphoblasts; this result is consistent with previously reported data. Moreover, GF treatment suppressed LDH activity and inhibited BL cell replication but did not affect lymphoblast viability. Surprisingly, we found that increased levels of the MYC and LDH-A proteins did not lead to a metabolic shift in BL cells toward glycolytic ATP generation. BL cells were treated with GF at doses that achieved 50% inhibition of cell growth and lactate production, and ATP levels were scarcely affected after GF treatment. The same results were also obtained by suppressing LDH activity with oxamate, an LDH specific inhibitor. Our data suggest that LDH activity is important for maintaining a correct NAD/NADH balance in BL cells. LDH inhibition led to decreased NAD cellular levels, which resulted in sirtuin-1 inhibition. Confirming previous studies, sirtuin-1 inhibition caused a reduction in MYC protein levels, depriving BL cells of their most important survival signal. This study further describes the biological functions of the LDH enzyme and suggests that LDH inhibition could be useful for the treatment of cancer. *Anti-Cancer Drugs* 24:862–870 © 2013 Wolters Kluwer Health | Lippincott Williams & Wilkins.

Anti-Cancer Drugs 2013, **24**:862–870

Keywords: Burkitt lymphoma, cancer cell metabolism, lactate dehydrogenase, MYC protein

Department of Experimental Diagnostic and Specialty Medicine, University of Bologna, Bologna, Italy

Correspondence to Giuseppina Di Stefano, Department of Experimental Diagnostic and Specialty Medicine, University of Bologna, via San Giacomo, 14-40126 Bologna, Italy
Tel: +39 051 209 4700; fax: +39 051 209 4746;
e-mail: giuseppina.distefano@unibo.it

Received 20 March 2013 **Revised form accepted** 29 May 2013

Introduction

The metabolic properties of cancer cells can be exploited for the development of therapeutic strategies [1]. Unlike normal cells, cancer cells can generate ATP through the degradation of glucose to lactate even under adequate oxygen conditions. This metabolic feature, which has been termed aerobic glycolysis, is the consequence of oncogenes and oncosuppressors reprogramming the cellular energetic machinery [2]. In addition, aerobic glycolysis is promoted by the hypoxic microenvironment found in solid tumor lesions, and this environment prevents oxidative phosphorylation [3]. Activation of the *myc* oncogene is a common DNA alteration that induces the glycolytic phenotype of cancer cells [4], and this alteration is frequently observed in human tumors. MYC is a helix–loop–helix leucine zipper transcription factor that dimerizes with its partner protein MAX. The dimer then binds to specific DNA sequences and transactivates genes involved in cell proliferation, differentiation, and apoptosis. Further, MYC plays an important role in the regulation of glucose metabolism [5]. It directly transactivates the A subunit of lactate dehydrogenase (LDH-A), which is directly involved in *myc*-mediated cell transformation [4,6]. LDH catalyzes the conversion of pyruvate to lactate, using NADH as a cofactor. This conversion is the last step in glycolysis, and this process is not active in normal cells under physiological conditions. The LDH enzyme is a tetramer composed of two types of subunits (A and B); the subunits have different kinetic and regulatory properties [7]. The A subunits are predominately found in liver and skeletal muscles and are upregulated in various cancer cells [1–3,8]. In these cells, increased LDH-A activity not only accelerates ATP production but also regenerates oxidized NAD for glycolysis. B subunits of LDH are inhibited by high pyruvate concentrations and are found at high levels in aerobic tissues, such as the heart. LDH-B is involved in mTOR-mediated tumorigenesis [9], and its increased expression in squamous carcinoma significantly affects the survival rate among patients [10].

Several published studies [11–14] suggest that LDH is an interesting target for the development of antineoplastic agents. We recently identified a molecule [galloflavin (GF)] that inhibits both the A and B isoforms of this enzyme [15]. GF was selected through a structure-based virtual screening procedure applied to the compounds in the Open Chemical Collection of the National Cancer Institute. To our knowledge, no other biochemical effects

0959-4973 © 2013 Wolters Kluwer Health | Lippincott Williams & Wilkins DOI: 10.1097/CAD.0b013e328363ae50

of GF have been described in the literature. Preliminary data on the antineoplastic effects of GF have been reported previously [15,16].

The *myc*-dependent linkage between altered cellular metabolism and tumorigenesis [4,5] prompted us to investigate the effects of GF-mediated LDH inhibition in *myc* hyperexpressing cancer cells. This study allowed us to verify whether this alteration could predict a better response to LDH inhibitors. Thus, we studied the effects of GF on Burkitt lymphoma (BL) cells. Deregulated *myc* expression is considered as the molecular hallmark of BL and is observed in greater than 90% of BL cases [17]. BL cells are characterized by *myc* overexpression, in addition to a chromosomal translocation. The most common translocation is t(8:14), which places the oncogene under the control of the immunoglobulin heavy chain promoter. For comparison, the effects of GF were also tested in non-neoplastic lymphoblastoid cells, which were immortalized by Epstein–Barr virus (EBV) infection. The two cell lines are based on the same cellular type (B lymphocyte) and were modified by infection with the same virus. Essentially, these cells differ only in their level of *myc* expression; the t(8:14) translocation causes elevated MYC levels and leads to neoplastic transformation.

Materials and methods

Cell lines and culture conditions

The Raji and Daudi cell lines are derived from BLs and are positive for EBV [18]; the Loucke cell line is derived from a sporadic and EBV-negative BL [19]. GM00130C and AG3138A are B lymphocyte cell lines immortalized by EBV infection; they were obtained from Coriell Cell Repositories (Coriell Institute for Medical Research, Camden, New Jersey, USA). The cells were grown as suspension cultures in RPMI 1640 containing 10% (Raji, Daudi, and Loucke) or 20% (GM00130C and AG3138A) fetal bovine serum, 100 U/ml penicillin/streptomycin, and 4 mmol/l glutamine. Cells were maintained at a concentration of $1–2 \times 10^5$ viable cells/ml. All media and supplements were purchased from Bio-Whittaker (Verviers, Belgium). GF was added to the culture media in the presence of 0.6% dimethyl sulfoxide for all experiments. Equivalent amounts of dimethyl sulfoxide were added to the control (untreated) cultures.

Compounds

GF can be obtained from the National Cancer Institute. A simple synthesis method for this compound was also developed and has been described previously [15]. Some of the experiments described below were also performed with oxamate (OXA), a known inhibitor of LDH [12,20]. OXA and all of the other compounds and reagents used were purchased from Sigma-Aldrich (St Louis, Missouri, USA).

LDH activity and composition

Cells ($3–8 \times 10^6$) were harvested, pelleted, and suspended in 1 ml of PBS. The suspension was lysed by sonication and centrifuged (1600*g* for 30 min at 4°C) to remove cell debris. The protein content of the supernatant was measured using the Bradford method. This cell extract (30–180 µl amounts) was used to measure LDH activity and the isoform composition (% of A and B isoforms) using a method previously described by Goldman *et al.* [21]. This method is based on comparing the enzymatic activity measured in (i) a reaction mixture containing 220 µmol/l reduced nicotinamide hypoxanthine dinucleotide (an NADH analog) and limited pyruvate (330 µmol/l) with that in (ii) a reaction mixture containing 130 µmol/l NADH and excess pyruvate (10 mmol/l). The detailed procedure has been reported previously [16,21]. LDH activity was expressed as mU/mg of protein or as mU/10^6 cells.

Cell respiration assay

This assay was performed using a phosphorescent oxygen-sensitive probe (MitoXpress probe; Luxcel Biosciences, Cork, Ireland). Raji, Daudi, and Loucke cells (3×10^5 cells/well, in phenol-red-free medium) were seeded in a 96-well clear bottom and black body plate. Four wells were used for each cell line. After the addition of the MitoXpress phosphorescent oxygen-sensitive probe (10 pmoles/well), the plate was placed in a Victor fluorescence reader (Perkin Elmer, Waltham, Massachusetts, USA) at 30°C and monitored for ~20 min, which allowed the plate to reach temperature and gas equilibrium and allowed a basal signal to be obtained. The fluorescence signal was measured every 60 s with 340/642 nm excitation/emission filters, a delay time of 30 µs, and a window of 100 µs. The wells were then sealed with mineral oil, and signal monitoring was resumed for the next 50 min. The increase in the fluorescence signal during this interval indicates oxygen consumption by the cells.

Effects of GF on lactate and ATP production

Both lactate and ATP production were measured after 6 h of incubation in the presence of varying amounts of GF (0–200 µmol/l, tested in duplicate). Lactate measurements were performed on samples of 5×10^5 cells according to the method described by Barker and Summerson [22]. ATP levels were measured using the CellTiter-Glo Luminescent Cell Viability Assay (Promega, Madison, Wisconsin, USA); 1×10^4 cells were used for these assays. The detailed methods have been reported previously [15,16]. A second-order polynomial regression was applied to experimental data to calculate the dose of GF causing a 50% reduction in ATP or lactate levels (IC_{50}). Prism 5 GraphPad software (GraphPad, La Jolla, California, USA) was used for statistical analyses.

Effects of GF on cell growth

To study the effect of GF on cell proliferation, 1×10^5 cells were seeded in 24-well plates and treated with varying doses of GF (0–200 µmol/l, tested in duplicate) or OXA (0–60 mmol/l, tested in duplicate) for 24 h at 37°C. After incubation, cells were counted under a light microscope using a Neubauer chamber, and cell viability was determined by Trypan blue exclusion. The difference between the number of viable cells at 24 h and at the beginning of the experiment (cell growth) was reported graphically. The data were analyzed using Prism 5 GraphPad software to determine the slope of the growth inhibition curve. The GF dose causing a 50% inhibition of cell growth (GI_{50}) was calculated only when a statistically significant growth inhibition was observed (when the curve slope was significantly different from 0). A second-order polynomial regression was applied to the experimental data to calculate the GI_{50} value. GF doses causing a reduction in cell number to values lower than 1×10^5 cells/well (the number of cells initially seeded in the plate) were excluded from this calculation.

Quantification of NAD and NADH levels

Cellular levels of NAD and NADH were assessed using the protocol described by Umemura and Kumar [23], with some modifications. This method uses a mild alkaline buffer, which extracts both NAD and NADH; the two dinucleotide forms can subsequently be distinguished by their respective heat stabilities. A detailed explanation of the method has been reported previously [23]. The assay was performed on samples of 6×10^6 cells. After 16 h of incubation with the tested compound (GF or OXA), cells were counted, pelleted at 4°C, and lysed with ice-cold extraction buffer (1 ml/6×10^6 cells; 20 mmol/l sodium bicarbonate, 100 mmol/l sodium carbonate, 10 mmol/l nicotinamide, and 0.1% Triton-X100). Cell lysates were centrifuged at 16 000*g* for 5 min at 4°C to remove insoluble material. A 50 µl aliquot of the sample was incubated at 60°C for 30 min to selectively denature NAD. A separate 50 µl aliquot was mixed with 840 µl of a buffer containing 100 mmol/l Tris-HCl (pH 8), 5 mmol/l EDTA, 0.5 mmol/l MTT, and yeast alcohol dehydrogenase (0.2 mg/ml). After addition of 200 mmol/l phenazine ethosulfate (10 µl), the solution was incubated for 5 min at 25°C. Thereafter, 100 µl of 6 mol/l ethanol was added; the mixture was centrifuged at 16 000*g* for 30 s at 25°C. The absorbance of the supernatant was measured at 570 nm for 120 s at 10-s intervals using a UV/visible spectrophotometer set to the 'kinetics' mode. This sample was used to measure the total combined NAD and NADH content. The same reaction was then repeated on the sample incubated at 60°C, and this sample was used to measure the NADH content. The measured absorbance change per second is proportional to the amount of the dinucleotide present in the sample. The NAD and NADH concentrations in the experimental samples were calculated using a calibration curve previously generated using known amounts of NAD(H) standards. Differences in NAD and NADH levels after GF treatment were statistically evaluated by analysis of variance and using the Bonferroni post-hoc test. All of the analyses were carried out using Prism 5 GraphPad software.

Western blots

Cell cultures (3×10^6 cells, seeded in 25 cm^2 flasks) were incubated for 16 h in the presence of GF or OXA. After incubation, cells were lysed in M-PER buffer (80 µl, Pierce, Rockford, Illinois, USA) containing protease inhibitors (Roche, Indianapolis, Indiana, USA). The cell homogenates were incubated on ice for 30 min and then centrifuged at 10 000*g* for 15 min. Supernatants (20 µg of total protein measured using the Bradford assay) were loaded onto a 10% polyacrylamide gel for electrophoresis. The separated proteins were transferred onto a low fluorescence polyvinylidene difluoride membrane (GE Life Sciences, Pittsburgh, Pennsylvania, USA) for 2 h at 300 mA using standard wet transfer methods. The blotted membrane was blocked with 3% BSA and 3% casein in tris buffered saline–Tween and probed with the primary antibody. The following antibodies were used: rabbit anti-c-myc [Y69] monoclonal antibody (Abcam), rabbit anti-p53 (acetyl K382) [EPR358(2)] monoclonal antibody (Abcam, Cambridge, UK), and rabbit anti-actin (Sigma-Aldrich). A Cy5-labeled secondary antibody (GE Life Sciences) was used to detect the presence of bound primary antibodies. All of the incubation steps were conducted according to the manufacturers' instructions.

Table 1 LDH activity and composition measured in BL and lymphoblastoid cells

	Burkitt lymphoma cells			Lymphoblastoid cells	
	Raji	Daudi	Louckes	GM130C	AG3138A
Global LDH activity					
Cells (mU/10^6)	159	173	256	113	159
Cell proteins (mU/mg)	2430	1790	3308	319	458
LDH composition (%)					
LDH-A	33	33	27	15	4.5
LDH-B	67	67	73	85	95.5
Lactic acid produced in 6 h (µmol/10^6)					
Cells	5.79±0.14	4.60±0.10	3.27±0.05	1.25±0.06	2.48±0.11

BL, Burkitt lymphoma; LDH-A, lactate dehydrogenase A subunit; LDH-B, lactate dehydrogenase B subunit.

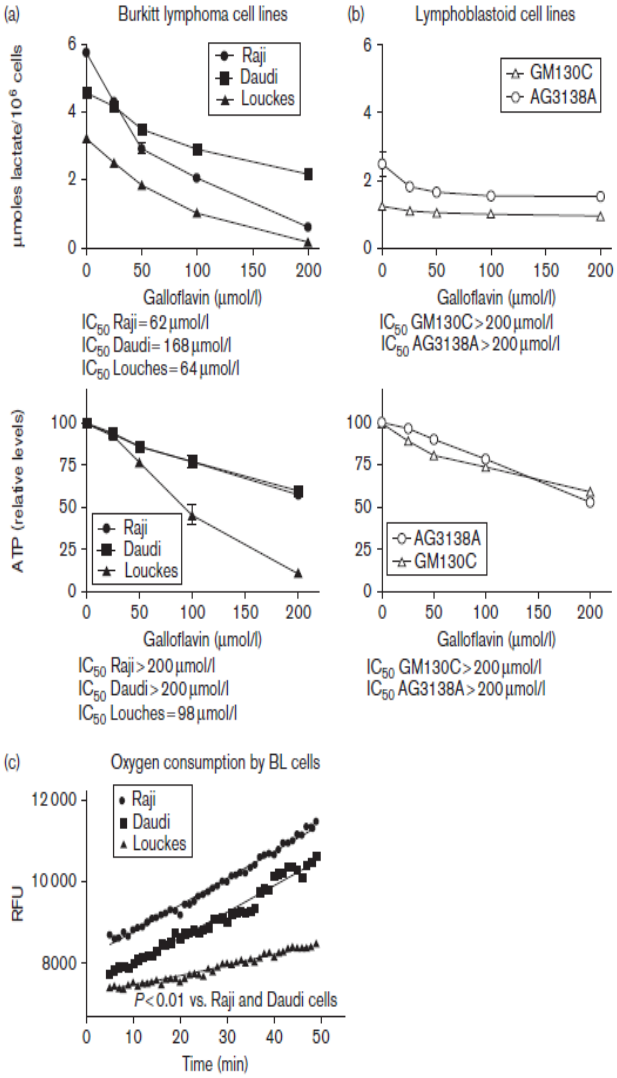
A Pharos FX scanner (BioRad, Hercules, California, USA) was used to detect fluorescence at a resolution of 100 µm, and the Quantity One software (BioRad) was used to analyze images. This experiment was repeated twice. MYC and acetyl-p53 band intensities of the different samples were normalized using actin expression as a reference. Differences in MYC and acetyl-p53 levels caused by GF treatment were statistically evaluated on normalized band intensities by analysis of variance followed by the Bonferroni post-hoc test. Prism 5 GraphPad software was used for all statistical analyses.

Results

Characterization of LDH activity and effects of GF on lactate and ATP production

Table 1 indicates that *myc* hyperexpression in BL cells enhances the activity of the LDH-A subunit, which is in accordance with previously reported data [4,6]. However, LDH-A activity did not exceed one-third of the total enzymatic activity in any of the tested cell lines. In addition to enhanced LDH-A activity, a corresponding increase in lactate production was observed in these cells. Table 1 also shows that the LDH activity (measured

Fig. 1



Lactate and ATP levels measured in (a) BL and (b) lymphoblastoid cells treated with varying doses of GF for 6 h. The GF concentrations causing a 50% reduction in metabolite levels (IC_{50}) were calculated by applying the second-order polynomial regression to the experimental data using the Prism5 GraphPad software. (c) Respiration assay performed in BL cells. The slope of the curves (calculated from the linear regression of experimental data) is proportional to cellular oxygen consumption. Slope differences were statistically evaluated by ANOVA followed by the Bonferroni post-hoc test. ANOVA, analysis of variance; BL, Burkitt lymphoma; GF, galloflavin; IC, inhibitory concentration.

as mU/mg of cell protein) was three- to 10-fold higher in BL cells than in lymphoblastoid cell lines, indicating that neoplastic changes also increased the activity of the LDH-B subunit. The results shown in Fig. 1b demonstrate that GF did not greatly affect either lactate production or ATP synthesis in lymphoblastoid cells, suggesting that LDH activity is not essential for the metabolism of these cells. Fig. 1a shows that increased expression of LDH-A does not drive cell metabolism towards glycolytic ATP generation, which is an unexpected result and is discordant with previously published data. Despite notable effects on lactate production, GF only modestly hindered ATP synthesis in two of the three tested BL cell lines (Raji and Daudi). These results can be explained by assuming that some BL cell lines maintain adequate mitochondrial function even in the presence of overexpressed *myc*, and the mitochondria can support these cells by providing ATP. Oxygen consumption was measured in the three BL cell lines, and data from this experiment (Fig. 1c) support the hypothesis that these cells have functional mitochondria. Raji and Daudi cells display a significantly higher capacity for mitochondrial respiration compared with Louckes cells. This result justifies the effects of GF on Louckes cell ATP levels.

LDH inhibition by GF affects the growth of *myc* overexpressing cells independent of their ATP reserve

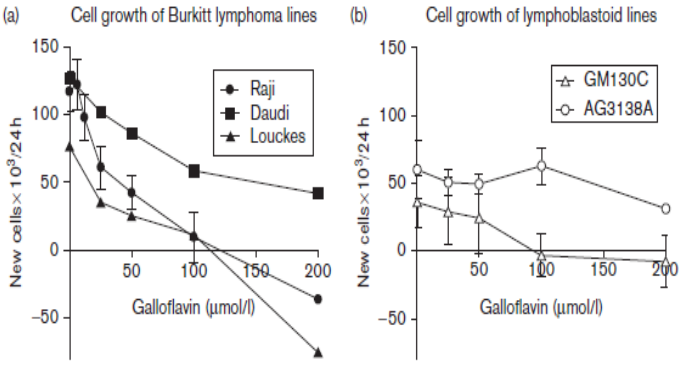
Figure 2 illustrates the effect of GF on the growth of lymphoblastoid (Fig. 2b) and BL cells (Fig. 2a). Compared with previously studied human cancer cell lines [15,16], GF showed greater inhibition of BL cell growth, and calculated GI_{50} doses ranged from 27 to 88 µmol/l. The effect of GF on lymphoblastoid cells was not statistically significant; this observation, together with the results reported in Fig. 1, suggests that LDH activity is dispensable in lymphoblastoid cells and that normal tissues should tolerate GF treatment.

As shown in Figs 1a and 2a, GF doses causing a 50% reduction in BL cell lactate production correlate with doses causing a 50% inhibition in cell growth. The GF dosage correlation suggests that the GF-mediated reduction in cell viability is related to its inhibition of LDH activity. These results also indicate that inhibition of cell growth by LDH cannot be fully attributed to ATP depletion because GF GI_{50} doses reduced ATP levels by only 7.8, 13.6, and 20% in Louckes, Raji, and Daudi cells, respectively. To identify a potential mechanism for the induction of cell death, we focused our subsequent studies on Raji cell cultures, as these cells are sensitive to GF, but their ATP levels are essentially unaffected by GF treatment. The same experiments were also conducted on the GM130C lymphoblastoid cell line as a control.

Effects of GF on the NAD/NADH balance and *myc* downregulation

In aerobic glycolysis, the LDH enzyme functions to regenerate oxidized NAD, which is necessary for sustained glucose degradation. Figure 3a shows that 50 µmol/l GF

Fig. 2



Is the slope of the growth inhibition curve significantly different from 0?

Raji	$P<0.0001$	$GI_{50}=33.4$ µmol/l	GM130C	$P=0.0753$	GI_{50} not calculated
Daudi	$P=0.0169$	$GI_{50}=88.7$ µmol/l	AG3138A	$P=0.1679$	GI_{50} not calculated
Louckes	$P=0.0044$	$GI_{50}=27.2$ µmol/l			

Growth inhibition caused by exposure to GF for 24 h in (a) BL and (b) lymphoblastoid cells. The initial number of seeded cells was subtracted from the number of cells present after the 24 h of incubation and this value was plotted. A negative value indicates a final cell number that was lower than the number of seeded cells at the beginning of the experiment. Growth inhibition was evaluated by determining whether the slope of the growth inhibition curve was statistically different from 0. The same data (excluding negative values) were used to calculate the GF dose that caused a 50% reduction in cell growth (GI_{50}). Prism5 GraphPad software was used. BL, Burkitt lymphoma; GF, galloflavin.

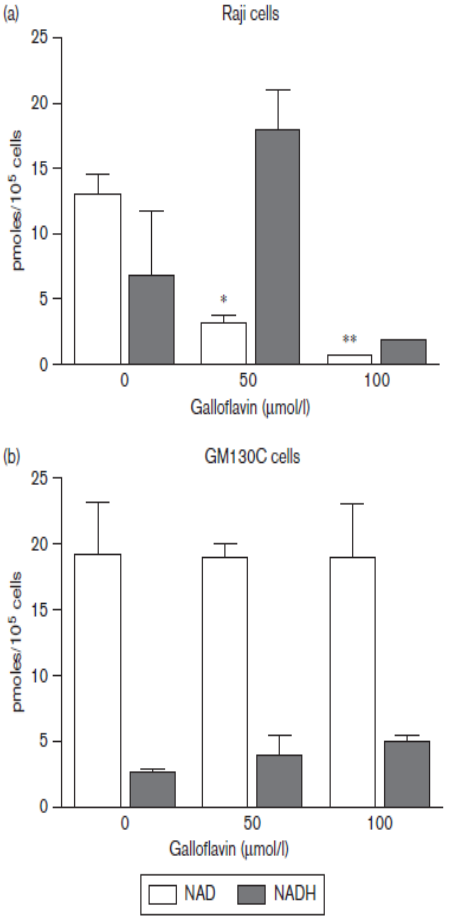
markedly affected the NAD/NADH balance in Raji cells, causing a four-fold reduction in NAD levels and an increase in NADH levels. These effects were not observed after GF treatment of GM130C lymphoblastoid cells (Fig. 3b). Further, 100 µmol/l GF had no effect on NAD concentration, and this dosage caused a slight, but not statistically significant, increase in NADH levels in GM130C cells. In Raji cells, the higher GF dose (100 µmol/l, three times the GI_{50} value) strongly affected cell viability, inducing the depletion of both dinucleotide forms. NAD participates in multiple metabolic reactions as a cofactor, and it can also mediate biologic processes by acting as a substrate for a wide range of proteins [24,25]. Among the NAD-consuming enzymes, sirtuins, particularly SIRT1, have high K_m values for NAD [24]. Therefore, these enzymes are highly susceptible to changes in the cellular levels of this dinucleotide. Moreover, previous studies have demonstrated that the MYC protein is a substrate of SIRT1, which deacetylates MYC and promotes its transcription factor functions [26]. On the basis of this knowledge, we investigated the effect of GF on MYC protein levels and SIRT1 activity. In the absence of a commercially available, specific, acetyl-MYC antibody, SIRT1 activity was evaluated by measuring the acetylation levels of the p53 protein, a known SIRT1 target [27]. Figure 4 shows that 50 µmol/l GF led to a notable decrease in MYC protein levels and an increase in p53 acetylation, which is indicative of SIRT1 inhibition. Because *myc* activity is related to its protein level [28], these data suggest that GF can suppress MYC-driven signals in Raji cells. These signals are required for cell growth, and these GF-mediated effects can be regulated by SIRT1 inhibition. Interestingly, MYC protein levels and p53 acetylation were unaffected in GM130C cells exposed to 100 µmol/l GF.

Myc downregulation through SIRT inhibition is a consequence of LDH inhibition

GF is a recently identified molecule with inhibitory activity against LDH. Presently, the properties of this molecule have not been fully characterized and effects other than LDH inhibition cannot be excluded. To verify whether the effects of GF observed in Raji cells are a result of LDH inhibition, we treated Raji cultures with OXA. OXA is a known inhibitor specific to LDH that affects both the A and B subunits [7,20]. The limitation of OXA is its inefficient cell penetration, which renders this molecule active only at millimolar concentrations.

Figure 5 shows that treatment with OXA inhibited the growth of Raji cells, and the GI_{50} was calculated as 52.5 mmol/l (Fig. 5a). Like GF, OXA did not significantly affect cellular ATP levels (Fig. 5b). However, Raji cells treated with OXA at its GI_{50} dose had highly reduced NAD levels (Fig. 5c), decreased MYC protein levels, and suppressed SIRT1 activity (Fig. 5d). Taken together, these observations strongly suggest that the effects of GF observed in Raji cells could be attributed to LDH inhibition.

Fig. 3

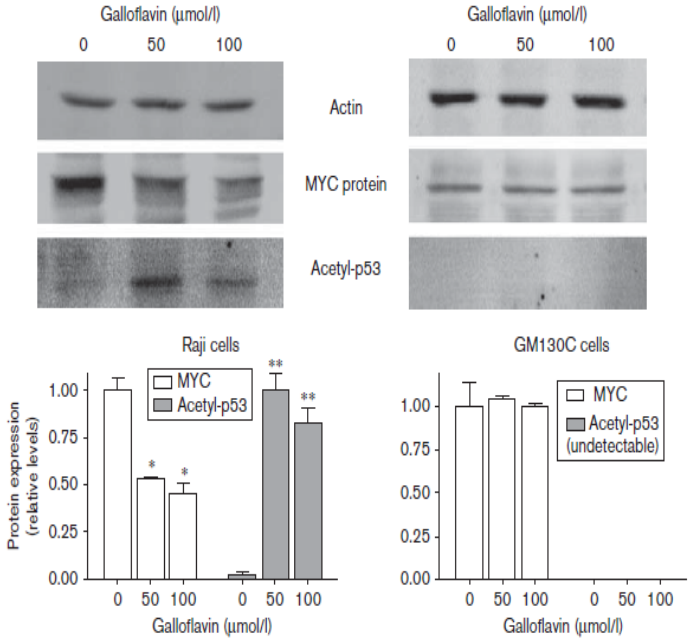


NAD and NADH levels in (a) Raji and (b) GM130C cells after exposure to 50 and 100 µmol/l GF for 16 h. See text for further details. Differences in NAD and NADH levels were statistically evaluated by ANOVA and using the Bonferroni post-hoc test. *$P<0.05$ compared with control cells; **$P<0.01$ compared with control cells.
A concentration of 100 µmol/l GF drastically depletes NAD and NADH levels in Raji cells as it strongly affects cell viability (see text and Fig. 2a). ANOVA, analysis of variance; GF, galloflavin.

Discussion

Increased LDH-A levels in various cancer cells suggested that this enzyme is a promising candidate for the development of inhibitors [1–3,8,11–14]. Conventionally, the upregulation of LDH-A in cancer cells was thought to be important for meeting the high energy needs of these cells and for promoting cell proliferation [6]. LDH-A can accomplish this task by regenerating NAD, which drives a continuous glycolytic flux and promotes ATP production even when mitochondrial function is suppressed or

Fig. 4

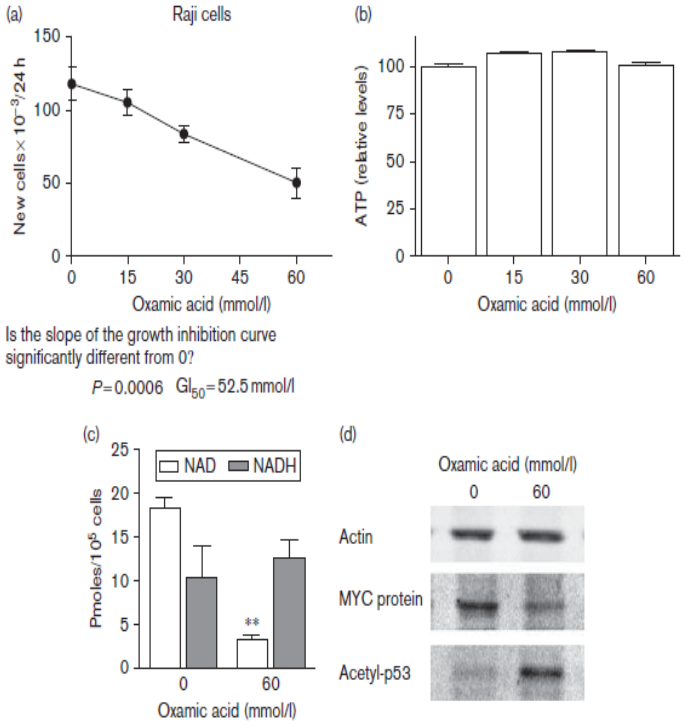


Downregulation of MYC and inhibition of SIRT1 enzymatic activity in Raji cells treated with GF. Inhibition of SIRT1 activity was measured by assessing the acetylation level of p53. These effects were not observed in GM130C cells treated with GF. Protein expression levels were measured as described in the Materials and Methods section. Differences were statistically evaluated by ANOVA and using the Bonferroni post-hoc test. *$P<0.05$ compared with control cells; **$P<0.01$ compared with control cells. ANOVA, analysis of variance; GF, galloflavin.

oxygen levels are low. However, recent reviews have compared the metabolic properties of cancer cells and normal tissues and they have concluded that although mitochondrial DNA mutations and reduced mitochondrial biogenesis can be observed in some cancer cells, several tumors derive most of their ATP from oxidative phosphorylation and not from glucose degradation [29]. Our data indicate that *myc* overexpression in BL cells increases the expression of the LDH-A subunit, which is normally expressed at low levels in this cell type. However, higher LDH-A levels do not drive cell metabolism toward glycolytic ATP generation, which was an unexpected result. GF and OXA treatment, at doses that cause a 50% reduction in cell growth and lactate production, did not affect ATP levels in any of the three BL cell lines. Interestingly, our data suggest that LDH functions to sustain cellular levels of NAD^+ in addition to its role in regulating energy supplies within the cell.

NAD^+ acts not only as a coenzyme for oxidoreductases but also as a substrate for several enzymatic reactions, in which it contributes as an ADP–ribose donator [24,25]. NAD^+-cleaving enzymes (sirtuins, poly ADP–ribose polymerases, and c-ADP synthases) are involved in the control of energy metabolism, cell survival, and aging; these proteins have also been proposed to function as sensors that trigger adaptive responses to unbalanced redox conditions. To accomplish this task, these enzymes must adjust their activity on the basis of physiological changes in NAD^+ levels. Among the NAD^+-consuming enzymes, SIRT1 is the best characterized, although the mechanisms involved in its activation have not yet been elucidated [30]. SIRT1 has a relatively high K_m for NAD^+ (150–200 μmol/l) [24], making it highly responsive to physiological changes in NAD^+ levels. Further, SIRT1 activation is commonly observed under conditions of energy stress (i.e. fasting or low glucose supply), leading to increased NAD^+ levels [24,31]. SIRT1 is a histone deacetylase that promotes cell survival under stressful conditions by deacetylating key cell cycle molecules and apoptosis regulatory proteins, including p53 [27,30]. Several reports have revealed that SIRT1 is upregulated in various human tumors [30], including B-cell lymphoma, for which enhanced expression of this protein was detected in greater than 70% of patients [32]. In B-cell lymphoma, SIRT1 expression was also associated with decreased patient survival. These data suggested that SIRT1 may be involved in tumor progression and may also serve as a prognostic marker and/or a therapeutic target. Recently, SIRT1 was found to directly affect MYC function, promoting MYC transcriptional activity and cell

Fig. 5



Effects of OXA on (a) Raji cell growth, (b) ATP levels, (c) NAD/NADH balance, and (d) MYC expression and SIRT1 activity. $^{**}P < 0.01$ compared with control cells. Because OXA is a specific inhibitor of LDH, these data strongly suggest that the effects of GF on Raji cells can be attributed to its ability to suppress LDH activity. GF, galloflavin; LDH, lactate dehydrogenase; OXA, oxamate.

proliferation [26,33]. Aberrant *myc* expression is frequently observed in tumors as a consequence of induction by upstream oncogenic signals or as a result of mutations within *myc* itself. For this reason, MYC is also considered to be an attractive target for therapeutic purposes [34]. Interestingly, experimental data have suggested that partial or temporary inhibition of *myc*-mediated signaling can induce tumor differentiation and regression [35]. Maintenance of the tumor phenotype is dependent on a precise threshold level of MYC protein expression [28]; in the absence of MYC expression at or below the threshold, cells enter a phase of proliferative arrest and apoptosis. Moreover, several reports have shown that SIRT1 and MYC inhibition induces different effects in normal and neoplastic cells. In noncancerous cultures, SIRT1 silencing was responsible for arresting growth but had no apparent effects on cell viability [36,37]. In mice, systemic and reversible *myc* inhibition through transgene expression [35] was well tolerated in normal tissues. Our data further support these results. By affecting cellular levels of NAD^+, LDH inhibition on GF treatment led to the downregulation of SIRT1 activity and MYC expression and deprived BL cells of their most important survival signaling. These effects were not observed on LDH inhibition in nontumor lymphoblastoid cells.

The results from our study further characterize the biological functions of the LDH enzyme and suggest that LDH inhibition may be useful as a treatment for cancer.

Acknowledgements

This work was supported by Fondazione del Monte di Bologna e Ravenna and by the Roberto and Cornelia Pallotti Legacies for Cancer Research.

Conflicts of interest

There are no conflicts of interest.

References

1 Pelicano H, Martin DS, Xu RH, Huang P. Glycolysis inhibition for anticancer treatment. *Oncogene* 2006; **25**:4633–4646.
2 Levine AJ, Puzio-Kuter AM. The control of the metabolic switch in cancers by oncogenes and tumor suppressor genes. *Science* 2010; **330**: 1340–1344.
3 Gatenby RA, Gillies RJ. Why do cancer have high aerobic glycolysis? *Nat Rev Cancer* 2004; **4**:891–899.
4 Dang CV, Le A, Gao P. MYC-induced cancer cell energy metabolism and therapeutic opportunities. *Clin Cancer Res* 2009; **15**:6479–6483.

5 Kim JW, Dang CV. Cancer's molecular sweet tooth and the Warburg effect. *Cancer Res* 2006; **66**:8927–8930.
6 Shim H, Dolde C, Lewis BC, Wu CS, Dang G, Jungmann RA, *et al.* c-Myc transactivation of LDH-A: implications for tumor metabolism and growth. *Proc Natl Acad Sci USA* 1997; **94**:6658–6663.
7 Everse J, Kaplan NO. Lactated dehydrogenases: structure and function. *Adv Enzymol* 1973; **37**:61–133.
8 Granchi C, Bertini S, Macchia M, Minutolo F. Inhibitors of lactate dehydrogenase isoforms and their therapeutic potentials. *Curr Med Chem* 2010; **17**:672–697.
9 Zha X, Wang F, Wang Y, He S, Jing Y, Wu X, *et al.* Lactate dehydrogenase B is critical for hyperactive mTOR-mediated tumorigenesis. *Cancer Res* 2011; **71**:13–18.
10 Kinoshita T, Nohata N, Yoshino H, Hanazawa T, Kikkawa N, Fujimura L, *et al.* Tumor suppressive microRNA-375 regulates lactate dehydrogenase B in maxillary sinus squamous cell carcinoma. *Int J Oncol* 2012; **40**:185–193.
11 Fiume L. Inhibition of aerobic glycolysis in Yoshida ascites hepatoma by tartronic acid. *Nature* 1960; **187**:792–793.
12 Papacostantinou J, Colowick SP. The role of glycolysis in the growth of tumor cells. I. Effects of oxamic acid on the metabolism of Ehrlich tumor cells in vitro. *J Biol Chem* 1961; **236**:278–284.
13 Fantin VR, St-Pierre J, Leder P. Attenuation of LDH-A expression uncovers a link between glycolysis, mitochondrial physiology and tumor maintenance. *Cancer Cell* 2006; **9**:425–434.
14 Le A, Cooper CR, Gouw AM, Dinavahi R, Maitra A, Deck LM, *et al.* Inhibition of lactate dehydrogenase A induces oxidative stress and inhibits tumor progression. *Proc Natl Acad Sci USA* 2010; **107**:2037–2042.
15 Manerba M, Vettraino M, Fiume L, Di Stefano G, Sartini A, Giacomini E, *et al.* Galloflavin (CAS 568-80-9): a novel inhibitor of lactate dehydrogenase. *ChemMedChem* 2012; **7**:311–317.
16 Farabegoli F, Vettraino M, Manerba M, Fiume L, Di Stefano G. Galloflavin, a new lactate dehydrogenase inhibitor, induces the death of human breast cancer cells with different glycolytic attitude by affecting distinct signaling pathways. *Eur J Pharm Sci* 2012; **47**:729–738.
17 Slack GW, Gascoyne RD. MYC and aggressive B-cell lymphomas. *Adv Anat Pathol* 2011; **18**:219–228.
18 Just T, Burgwald H, Broe MK. Flow cytometric detection of EBV (EBER snRNA) using peptide nucleic acid probes. *J Virol Methods* 1998; **73**: 163–174.
19 Marchini A, Longnecker R, Kieff E. Epstein–Barr virus (EBV)-negative B-lymphoma cell lines for clonal isolation and replication of EBV recombinants. *J Virol* 1992; **66**:4972–4981.
20 Novoa WB, Winer AD, Glaid AJ, Schwert GW. Lactic dehydrogenase. V. Inhibition by oxamate and by oxalate. *J Biol Chem* 1959; **234**:1143–1148.
21 Goldman RD, Kaplan NO, Hall TC. Lactic dehydrogenase in human neoplastic tissues. *Cancer Res* 1964; **24**:389–399.
22 Barker SB, Summerson WH. The colorimetric determination of lactic acid in biological material. *J Biol Chem* 1941; **138**:535–554.
23 Umemura K, Kimura H. Determination of oxidized and reduced nicotinamide adenine dinucleotide in cell monolayer using a single extraction procedure and a spectrophotometric assay. *Anal Biochem* 2005; **338**:131–135.
24 Houtkooper RH, Cantò C, Wanders RJ, Auwerx J. The secret life of NAD+: an old metabolite controlling new metabolic signaling pathways. *Endocr Rev* 2010; **31**:194–223.
25 Lin SJ, Guarente L. Nicotinamide adenine dinucleotide, a metabolic regulator of transcription, longevity and disease. *Curr Opin Cell Biol* 2003; **15**:241–246.
26 Mao B, Zhao G, Lv X, Chen HZ, Xue Z, Yang B, *et al.* Sirt1 deacetylates c-Myc and promotes c-Myc/Max association. *Int J Biochem Cell Biol* 2011; **43**:1573–1581.
27 Li K, Luo J. The role of SIRT1 in tumorigenesis. *N Am J Med Sci* 2011; **4**:104–106.
28 Sachaf GM, Gentles AJ, Elchuri S, Sahoo D, Soen Y, Sharpe O, *et al.* Genomic and proteomic analysis reveals a threshold level of MYC required for tumor maintenance. *Cancer Res* 2008; **68**:5132–5142.
29 Bellance N, Lestienne P, Rossignol R. Mitochondria: from bioenergetics to the metabolic regulation of carcinogenesis. *Front Biosci* 2009; **14**: 4015–4034.
30 Milner J. Cellular regulation of SIRT1. *Curr Pharm Des* 2009; **15**:39–44.
31 Rodgers JT, Lerin C, Haas W, Gygi SP, Spiegelman BM, Puigserver P. Nutrient control of glucose homeostasis through a complex of PGC-1α and SIRT1. *Nature* 2005; **434**:113–118.
32 Jang KY, Hwang SH, Kwon KS, Kim KR, Choi HN, Lee NR, *et al.* SIRT1 expression is associated with poor prognosis of diffuse large B-cell lymphoma. *Am J Surg Pathol* 2008; **32**:1523–1531.
33 Menssen A, Hydbring P, Kapelle K, Vervoorts J, Diebold J, Lüscher B, *et al.* The c-MYC oncoprotein, the NAMPT enzyme, the SIRT1-inhibitor DBC1, and the SIRT1 deacetylase form a positive feedback loop. *Proc Natl Acad Sci USA* 2012; **109**:E187–E196.
34 Hermeking H. The MYC oncogene as a cancer drug target. *Curr Cancer Drug Targets* 2003; **3**:163–175.
35 Soucek L, Whitfield J, Martins CP, Finch AJ, Murphy DJ, Sodir NM, *et al.* Modeling Myc inhibition as a cancer therapy. *Nature* 2008; **455**:679–683.
36 Knight JRP, Milner J. SIRT1, metabolism and cancer. *Curr Opin Oncol* 2012; **24**:68–75.
37 Ford J, Jiang M, Milner J. Cancer-specific functions of SIRT1 enable human epithelial cancer cell growth and survival. *Cancer Res* 2005; **65**:10457–10463.

Galloflavin prevents the binding of lactate dehydrogenase A to single stranded DNA and inhibits RNA synthesis in cultured cells

Biochemical and Biophysical Research Communications 430 (2013) 466–469

Contents lists available at SciVerse ScienceDirect

Biochemical and Biophysical Research Communications

journal homepage: www.elsevier.com/locate/ybbrc

Galloflavin prevents the binding of lactate dehydrogenase A to single stranded DNA and inhibits RNA synthesis in cultured cells

Luigi Fiume, Marina Vettraino, Domenica Carnicelli, Valentina Arfilli, Giuseppina Di Stefano *, Maurizio Brigotti

Department of Experimental, Diagnostic and Specialty Medicine (DIMES), University of Bologna, Italy

ARTICLE INFO

Article history:
Received 28 November 2012
Available online 10 December 2012

Keywords:
Galloflavin
ssDNA binding proteins
LDH-A binding to ssDNA
LDH-A inhibitors

ABSTRACT

Lactate dehydrogenase A (LDH-A) binds single stranded DNA (ssDNA) and stimulates cell transcription. Binding is prevented by NADH, suggesting that the coenzyme site is involved in the interaction LDH-A/ssDNA. We recently identified an inhibitor of LDH-A enzymatic activity (Galloflavin, GF) which occupies the NADH site. In the experiments reported here we studied whether GF can also hinder the binding of LDH-A to ssDNA and investigated its effects on RNA synthesis in cultured cells. Using a filter binding assay we observed that 4 μM GF inhibited the binding of human LDH-A to a single stranded [^{3}H]DNA sample by 50%. After only 0.5–1 h, 50–100 μM GF inhibited RNA synthesis in SW620 cells maintained in a medium in which galactose substituted glucose. In these culture conditions, SW620 cells did not produce lactic acid and effects caused by the inhibition of the enzymatic activity of LDH-A could be excluded.

Novel LDH-A inhibitors which hinder aerobic glycolysis of cancer cells are at present actively searched. Our results suggest that: (i) inhibitors which bind the NADH site can exert their antiproliferative activity not only by blocking aerobic glycolysis but also by causing an inhibition of RNA synthesis independent from the effect on glycolysis; (ii) GF can be a useful tool to study the biological role of LDH-A binding to ssDNA.

1. Introduction

In DNA replication and transcription the double-stranded DNA (dsDNA) has to be opened in order to provide a single-stranded template (ssDNA). ssDNA binding proteins (ssBP) exert a helix destabilising activity and their interaction to ssDNA prevents premature annealing and protects the strand from attack by endonucleases. By the same mechanism, ssBPs play a fundamental role in DNA recombination and repair [1]. In eukaryotic cells, including those from humans, the best studied ssBP is the replication protein A (RPA) [2]. However, also some glycolytic enzymes were found to bind ssDNA [3,4]. Particularly investigated for this property was lactate dehydrogenase (LDH) (EC. 1.1.1.27). The A (M) isoform of this enzyme binds ssDNA [5–8] and facilitates the activity of DNA polymerase α-primase complex in vitro [6,9]. Evidence was obtained that interaction of LDH-A with ssDNA also occurs in vivo: antibodies reacting with LDH-A were concentrated in the transcriptionally active puffs of polytenic chromosomes of Drosophila salivary gland cells [10,11] and in the chromatin of mammalian cells from which they were released after DNAase treatment [12]. Moreover injection of LDH-A in nuclei of Chironomus tentans salivary gland cells by means of a micromanipulation technique stimulated the transcription of polytenic chromosomes which on the contrary was inhibited by antibodies binding to LDH-A [13]. More recently, LDH-A was found to be a component of the transcriptional complex involved in S-phase-dependent histone H2B transcription [14].

LDH-A binding to ssDNA is prevented in vitro by NADH [5,10], suggesting that the coenzyme site is involved in the interaction LDH-A/ssDNA. In the present experiments we investigated whether galloflavin (GF) (Fig. 1), a recently identified small molecule which inhibits the enzymatic activity of LDH-A by occupying the NADH site [15], also hinders the interaction of the enzyme with ssDNA. Targeting ssBPs to prevent their interaction with ssDNA is a new strategy for drug development towards better cancer treatment [16,17]. Small molecules which inhibit ssDNA binding activity of RPA prevent cell cycle progression, induce cytotoxicity and increase the efficacy of chemotherapeutic DNA damaging agents [18].

In the experiments reported here we found that GF inhibited LDH-A/ssDNA interaction and given the data which indicate an enhancing effect of LDH-A binding to ssDNA on the transcription in vivo [13], we studied the drug capacity to hinder the synthesis of RNA, by using a human colorectal cell line (SW620) [19] adapted to replicate in a medium in which galactose substituted glucose.

* Corresponding author. Address: Department of Experimental, Diagnostic and Specialty Medicine, University of Bologna, Via San Giacomo 14, I-40126 Bologna, Italy. Fax: +39 0512094746.
E-mail address: giuseppina.distefano@unibo.it (G. Di Stefano).

0006-291X/$ - see front matter © 2012 Elsevier Inc. All rights reserved.
http://dx.doi.org/10.1016/j.bbrc.2012.12.013

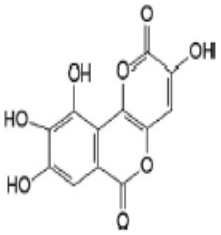


Fig. 1. Galloflavin (GF).

Since lactate is not produced in cells growing in the absence of glucose [20], the biological effects caused by GF in these cells could not be ascribed to an interference with the enzymatic activity of LDH-A.

2. Materials and methods

2.1. Reagents

NADH was obtained from Sigma–Aldrich. GF was synthesised and characterised as described by Manerba et al. [15]. [^{3}H]-uridine (20 Ci/mmol) and [^{3}H]-thymidine (17 Ci/mmol) were purchased from Perkin Elmer.

2.2. Filter DNA-binding assay

[^{3}H]DNA (2251 bp) was obtained by PCR amplification of the 731–2981 region of the pBR322 plasmid in the presence of [8-^{3}H]dATP [21]. Separation of the two strands of [^{3}H]DNA was achieved by heat denaturation (50 ng of [^{3}H]DNA in 10 μl of 20 mM Tris/HCl, pH 7.5 in eppendorf tubes, 3 min 100 °C and rapid cooling on ice) and checked on 1% agarose gel on larger DNA amounts (450 ng). The filter DNA-binding assay was performed in 200 μl of buffer A (20 mM Tris/HCl, pH 7.5, 50 mM NaCl, 1 mM EDTA, 2 mM mercaptoethanol, 5% glycerol, 0.05 mg/ml BSA) [6] containing 50 ng of heat denaturated [^{3}H]DNA (10^4 dpm) and LDH-A (0.25–1 units). After 10 min at 30 °C the formation of the [^{3}H]DNA/LDH-A complex was measured by spotting the reaction mixtures on nitrocellulose filters (Millipore), pre-soaked (20 min) in buffer A without BSA. After four washes with 5 ml of buffer A the radioactivity collected on the filters was quantified in a liquid-scintillation β-counter. Where indicated, LDH-A (10 units in 1 ml buffer A) was dialysed (3 h at 4 °C) against 500 ml buffer A without BSA before the filter-binding assay in order to remove the ammonium sulphate present in the commercial preparation. When GF was assayed as inhibitor of the [^{3}H]DNA/LDH-A interaction, the reaction mixtures (including the controls) contained 1% DMSO required for GF solubility. The same amount of DMSO was added when the inhibitory effect of NADH was tested.

2.3. Cell culture

SW620 cells, derived from a human colorectal cancer [19], were cultured in Leibovitz L-15 medium (Sigma–Aldrich), which does not contain glucose. The medium was supplemented with 10% dialysed FCS (Sigma–Aldrich). In all experiments GF was added to the culture medium in the presence of 0.6% DMSO. The same amount of DMSO was always added to control, untreated cultures.

2.4. LDH activity and lactic acid determination

To measure the LDH activity in SW620 cells, 8×10^6 cells were harvested, pelleted and suspended in 1 ml PBS. The suspension was lysed by sonication and centrifuged to discard the cell debris. Protein content of the supernatant was measured according to Bradford. This cell extract (100 μl amount) was used to measure LDH activity and composition (% of A and B isoforms), following the procedure originally set up by Goldman et al. [22].

For determination of lactic acid, cells (5×10^5 in 2 ml medium) were seeded in wells (35 mm diameter) of a Nunclon plate, let to adhere overnight and then cultured for 24 h. Afterwards, the cells were counted and lactic acid of cells and medium was measured according to Barker and Summerson [23].

2.5. Effect of GF on cell replication

Cells (2×10^4/well) were seeded in 96-multiwell plates, let to adhere overnight and then treated with GF (0–100 μM); four wells were used for each condition. The medium was replaced after 24 h and cell replication was evaluated after a total 48 h exposure to GF by using the neutral red assay, which gives a measure of the number of viable cells [24]. Cell growth was assessed by subtracting the number of seeded cells from that measured at the end of experiment. The detailed procedure is described in Farabegoli et al. [25].

2.6. Effects of GF on DNA and RNA synthesis

DNA and RNA synthesis were evaluated by measuring the incorporation in the cell acid-insoluble material of [^{3}H]-thymidine (17 Ci/mmol) or [^{3}H]-uridine (20 Ci/mmol), respectively. Cells were seeded in 24-multiwell plates (2×10^5/well) and let to adhere overnight. They were then treated with GF (0–100 μM) for 0.5 or 1 h and labelled with the radioactive nucleosides (2 μCi) in the last 30 min of treatment. When treatment with GF lasted only 0.5 h nucleosides were added at the same time with GF. Incorporation of [^{3}H]-thymidine was also measured after 4 h of GF treatment. After the end of treatment cells were lysed with 200 μl 0.5 N NaOH and the lysates were recovered with 500 μl H_2O. Macromolecules were then denatured in 10% TCA. The acid precipitate was collected over a glass microfiber filter and washed with 5×5 ml of 5% TCA; the radioactivity adsorbed onto the filters was measured using a β-counter.

3. Results

3.1. Effect of GF on LDH-A binding to ssDNA

To measure the extent of binding of LDH-A to ssDNA, we employed a filter binding assay with heat-denaturated 2251 bp [^{3}H]DNA (2×10^5 dpm/μg) obtained by PCR amplification of the 731–2981 region of the pBR322 plasmid in the presence of [8-^{3}H]dATP [21]. LDH-A from human liver (150 U/mg protein; 1140 U/ml) was obtained from Lee Biosolutions (St. Louis, MO, USA). The enzyme was the A4 isoform, i.e. all the four subunits where encoded by the LDH-A gene. The detailed procedure of the binding assay is described in Section 2. Fig. 2A shows the electrophoresis analysis of the nucleic acid before (lane 1) and after heat denaturation (lane 2), followed by incubation (5–15 min at 30 °C) under the same ionic conditions of the filter-binding assay (lanes 3–5). Densitometric analysis performed on ethidium bromide stained-gels showed that after heat treatment 10% of [^{3}H]DNA was double stranded (lane 2) and that, during the 10 min-incubation time chosen for the filter-binding assay (lane 4), negligible DNA re-annealing occurred (4%). We can conclude that during the filter binding assay 86% of [^{3}H]DNA was in single stranded form. The formation of the [^{3}H]DNA/LDH-A complex is shown in Fig. 2B. The addition of increasing amounts of LDH-A allowed a progressive rising in complex formation until a plateau was reached when ~90% of [^{3}H]DNA was captured. It should be noted

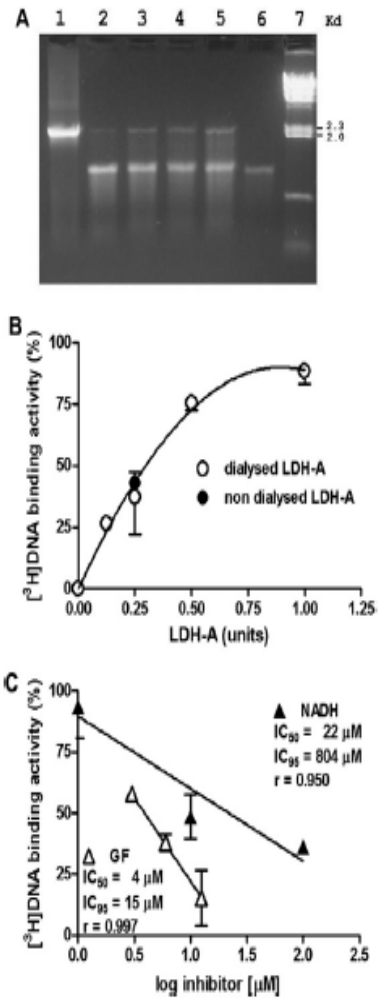


Fig. 2. Inhibition of the binding of LDH-A to ssDNA caused by GF. (A) Gel electrophoresis of the 2251 bp DNA employed in the filter-binding assay. Gel electrophoresis analysis was performed on ethidium bromide containing-1% agarose gel. Lane 1, 2251 bp DNA (450 ng); lane 2, heat denaturated 2251 bp DNA (450 ng); lane 3, heat denaturated 2251 bp DNA (450 ng) incubated 5 min at 30 °C; lane 4, heat denaturated 2251 bp DNA (450 ng) incubated 10 min at 30 °C; lane 5, heat denaturated 2251 bp DNA (450 ng) incubated 15 min at 30 °C; lane 6, heat denaturated 2251 bp DNA (225 ng); lane 7, size markers (λ phage *HindIII* digested). (B) Dose–response curve of [^{3}H]DNA binding activity displayed by LDH-A. The filter DNA-binding assay was performed as described in Section 2. LDH-A was either dialysed before the assay (empty circles) or non dialysed (filled circle). Values are means ± SEM. Data were fitted using the second order polynomial regression with the GraphPad Prism 5 software ($r = 0.99$). (C) Effect of GF and NADH on the formation of [^{3}H]DNA/LDH-A complex. IC_{50} and IC_{95} were calculated by the linear regression between the percent activity and the log of inhibitor concentrations. The regression parameters were analysed by GraphPad Prism 5 software and the difference in slope of two lines was statistically significant ($p < 0.05$).

that this value corresponds to the percentage of ssDNA present in the assay, in keeping with the notion that LDH-A scarcely binds to dsDNA [5]. The dose–response experiments described above were performed after dialysis of the enzyme, aimed at removing the ammonium sulphate (3.1 M) present in the commercial LDH-A preparation. To gain information on the effect of GF on the DNA-binding activity of LDH-A we used a low fixed amount of enzyme (0.25 units) omitting the dialysis step. Under these conditions, about 40% of the [^{3}H]DNA was found to bind to LDH-A using either dialysed (Fig. 2B, empty circle) or non dialysed (Fig. 2B, filled circles) enzyme. GF showed a marked inhibitory effect compared to the reference inhibitor NADH (Fig. 2C). The IC_{50} of GF was ~5-fold lower with respect to that obtained with NADH. Moreover, since the slopes of the two curves were significantly different, the extrapolated IC_{95} indicated a ~50-fold difference between the two inhibitors (Fig. 2C). It is worth noting that in these experiments LDH-A was preincubated (10 min at 30 °C in 190 µl buffer A) with the inhibitors (GF or NADH) before the addition of [^{3}H]DNA, in order to facilitate their docking into the active site. Interestingly, the IC_{50} of GF obtained without (6 µM) or with preincubation (4 µM) were quite similar, confirming the considerable power of GF as inhibitor of LDH-A/ssDNA interaction in vitro.

3.2. Effects of GF on SW620 cells

LDH activity in SW620 cells, measured as described in [22] was 196 mU/10^6 cells with 55% and 45% of the total enzymatic activity due to the A and B isoforms, respectively. These values are similar to those measured in neoplastic cell lines cultured in the presence of glucose and displaying aerobic glycolysis [25].

In agreement with previous data obtained on cells growing in a medium in which galactose substituted glucose [20], lactic acid, measured as described in [23], was not detected in SW620 cells. The limit of sensitivity of the assay was 50 nmoles/10^6 cells.

Fig. 3A shows that GF inhibited the cell replication: after 48 h of treatment the IC_{50} calculated as described in the legend was 34.7 µM.

After only 0.5–1 h GF produced significant inhibitions of RNA synthesis at the concentrations of 50 and 100 µM (Fig. 3B). The effect of GF was also tested on DNA synthesis, which was not inhibited even in cells treated for 4 h with the drug given at 100 µM (data not shown).

4. Discussion

The results reported here showed that GF, which occupies the NADH site of LDH-A, strongly hindered the interaction of the enzyme with ssDNA in vitro. After a short time of exposure, GF inhibited RNA synthesis in SW620 cells cultured in the absence of glucose, in which effects of GF due to an impairment of LDH-A enzymatic activity could be excluded. Therefore, the anticancer activity exerted by GF on normally glycolysing neoplastic cells [15,25] was likely caused not only by the block of aerobic glycolysis, but also by an inhibition of RNA synthesis, independent from the effect on glycolysis. Since LDH-A is bound to chromosomes and enhances their transcription in vivo [11,13] the impairment of ssDNA/LDH-A interaction caused by GF can account for this inhibition of RNA synthesis. The observation that GF inhibited RNA synthesis in SW620 cells without affecting the synthesis of DNA fits with the finding of Patel et al. [10]. The authors examined *Shara coprophila* salivary gland chromosomes by immunofluorescence staining procedure in the presence of antibodies binding LDH-A. At certain developmental stages *Shara* chromosomes exhibit distinct DNA-synthesising puffs; these replicative puffs showed little LDH-A immunofluorescence, while transcriptional puffs in the same chromosomes exhibited intense staining.

Small molecules interfering with the enzymatic activity of LDH-A are now searched to selectively block aerobic glycolysis for an approach to antineoplastic chemotherapy proposed several years ago [26,27] and at present actively pursued [28,29]. Our data indicate that inhibitors which occupy the NADH site of the enzyme besides blocking aerobic glycolysis might also prevent LDH-A/ssDNA interaction and hamper transcription.

Finally, the present results suggest that GF by hindering the binding of LDH-A to ssDNA can be a tool to study the biological role of this interaction in the cells.

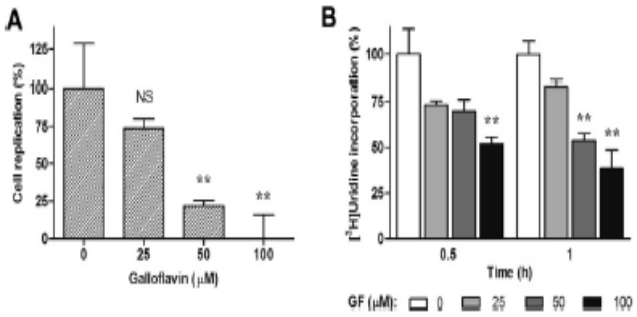


Fig. 3. Effect of GF on replication of SW620 cells and on [^{3}H]-uridine incorporation. (A) Inhibition of SW620 cell replication after 48 h exposure to GF. Each value was obtained from four determinations. Data were fitted using the second order polynomial regression in order to calculate the IC_{50} with the GraphPad Prism 5 software. Results (mean values ± SEM) were statistically evaluated using the *t*-test. $^{**}p < 0.01$. (B) Effect of GF on [^{3}H]-uridine incorporation in SW620 cells after 0.5–1 h exposure to GF. Experiments were run in triplicate and repeated twice. Data were analysed by ANOVA, followed by the Dunnett post test. $^{**}p < 0.01$.

Acknowledgments

This work was supported by Cornelia and Roberto Pallotti Legacies for Cancer Research and by University of Bologna, RFO funds.

References

[1] Y. Shamoo, Single-stranded DNA-binding proteins, in: Macmillan Publisher Ltd. (Ed.), Encyclopedia of Life Sciences, Nature Publishing Group, 2002, pp. 1–7.
[2] C. Iftode, Y. Daniely, J.A. Borowiec, Replication protein A (RPA): the eukaryotic SSB, Crit. Rev. Biochem. Mol. Biol. 34 (3) (1999) 141–180.
[3] Z. Ronai, Glycolytic enzymes as DNA binding proteins, Int. J. Biochem. 25 (7) (1993) 1073–1076.
[4] J.W. Kim, C.V. Dang, Multifaceted roles of glycolytic enzymes, Trends Biochem. Sci. 30 (3) (2005) 142–150.
[5] P. Calissano, C. Volonté, S. Biocca, A. Cattaneo, Synthesis and content of a DNA-binding protein with lactic dehydrogenase activity are reduced by nerve growth factor in the neoplastic cell line PC12, Exp. Cell Res. 161 (1) (1985) 117–129.
[6] F. Grosse, H.P. Nasheuer, S. Scholtissek, U. Schomburg, Lactate dehydrogenase and glyceraldehyde-phosphate dehydrogenase are single-stranded DNA-binding proteins that affect the DNA-polymerase-α-primase complex, Eur. J. Biochem. 160 (3) (1986) 459–467.
[7] F.S. Sharief, S.H. Wilson, S.S. Li, Identification of the mouse low-salt-eluting single-stranded DNA-binding protein as a mammalian lactate dehydrogenase-A isoenzyme, Biochem. J. 233 (3) (1986) 913–916.
[8] H.B. Kaiserman, W.F. Odenwald, D.J. Stowers, E.H.A. Poll, R.M. Benbow, A major single-stranded DNA binding protein from ovaries of the frog, Xenopus laevis, is lactate dehydrogenase, Biochim. Biophys. Acta 1008 (1) (1989) 23–30.
[9] O. Popanda, G. Fox, H.W. Thielmann, Modulation of DNA polymerases a, d and e by lactate dehydrogenase and 3-phosphoglycerate kinase, Biochim. Biophys. Acta 1397 (1) (1998) 102–117.
[10] G.L. Patel, S. Reddigari, K.R. Williams, E. Baptist, T.E. Thompson, S. Sisodia, A rat liver helix-destabilizing protein: properties and homology to LDH-5, in: P. Skean, S.J. Friedman (Eds.), Growth, Cancer, and the Cell Cycle, Humana Press Inc., Clifton, New Jersey, 1984, pp. 41–58.
[11] K.R. Williams, S. Reddigari, G.L. Patel, Identification of a Nucleic Acid Helix-Destabilizing Protein from Rat Liver as Lactate Dehydrogenase-5, Proc. Natl. Acad. Sci. USA 82 (16) (1985) 5260–5264.
[12] A. Cattaneo, S. Biocca, N. Corvaja, P. Calissano, Nuclear localization of a lactic dehydrogenase with single-stranded DNA-binding properties, Exp. Cell Res. 161 (1) (1985) 130–140.
[13] E. Egyhazi, M. Holst, A. Pigon, J. Stigare, G.L. Patel, Effects of a DNA helix-destabilizing protein on transcription in living cells, Eur. J. Cell Biol. 48 (1) (1989) 88–94.
[14] L. Zheng, R.G. Roeder, Y. Luo, S phase activation of the histone H2B promoter by OCA-S, a coactivator complex that contains GAPDH as a key component, Cell 114 (2) (2003) 255–266.
[15] M. Manerba, M. Vettraino, L. Fiume, G. Di Stefano, A. Sartini, E. Giacomini, R. Buonfiglio, M. Roberti, M. Recanatini, Galloflavin (CAS 568-80-9): A novel inhibitor of lactate dehydrogenase, ChemMedChem 7 (2) (2012) 311–317.
[16] Y. Zou, Y. Liu, X. Wu, S.M. Shell, Functions of human replication protein A (RPA): from DNA replication to DNA damage and stress responses, J. Cell Physiol. 208 (2) (2006) 267–273.
[17] V.J. Anciano Granadillo, J.N. Earley, S.C. Shuck, M.M. Georgiadis, R.W. Fitch, J.J. Turchi, Targeting the OB-folds of replication protein A with small molecules, J. Nucl. Acids (2010), http://dx.doi.org/10.4061/2010/304035.
[18] S.C. Shuck, J.J. Turchi, Targeted inhibition of replication protein A reveals cytotoxic activity, synergy with chemotherapeutic DNA-damaging agents, and insight into cellular function, Cancer Res. 70 (8) (2010) 3189–3198.
[19] A. Leibovitz, J.C. Stinson, W.B. McCombs, C.E. McCoy, K.C. Mazur, N.D. Mabry, Classification of human colorectal adenocarcinoma cell lines, Cancer Res. 36 (12) (1976) 4562–4569.
[20] H. Eagle, S. Barban, M. Levy, H.O. Schulze HO, The utilization of carbohydrates by human cell cultures, J. Biol. Chem. 233 (3) (1958) 551–558.
[21] M. Brigotti, L. Barbieri, P. Valbonesi, F. Stirpe, L. Montanaro, S. Sperti, A rapid and sensitive method to measure the enzymatic activity of ribosome-inactivating proteins, Nucleic Acids Res. 26 (18) (1998) 4306–4307.
[22] R.D. Goldman, N.O. Kaplan, T.C. Hall, lactic dehydrogenase in human neoplastic tissues, Cancer Res. 24 (1964) 389–399.
[23] S.B. Barker, W.H. Summerson, The colorimetric determination of lactic acid in biological material, J. Biol. Chem. 138 (1941) 535–554.
[24] G. Fotakis, J.A. Timbrell, In vitro cytotoxicity assays: comparison of LDH, neutral red, MTT and protein assay in hepatoma cell lines following exposure to cadmium chloride, Toxicol. Lett. 160 (2) (2006) 171–177.
[25] F. Farabegoli, M. Vettraino, M. Manerba, L. Fiume, M. Roberti, G. Di Stefano, Galloflavin, a new lactate dehydrogenase inhibitor, induces the death of human breast cancer cells with different glycolytic attitude by affecting distinct signalling pathways, Eur. J. Pharm. Sci. 47 (4) (2012) 729–738.
[26] L. Fiume, Inhibition of aerobic glycolysis in Yoshida ascites hepatoma by tartronic acid, Nature 187 (1960) 792–793.
[27] J. Papaconstantinou, S.P. Colowick, The role of glycolysis in the growth of tumor cells. I. Effects of oxamic acid on the metabolism of Ehrlich ascites tumor cells in vitro, J. Biol. Chem. 236 (1961) 278–284.
[28] C. Granchi, S. Bertini, M. Macchia, F. Minutolo, Inhibitors of lactate dehydrogenase isoforms and their therapeutic potentials, Curr. Med. Chem. 17 (7) (2010) 672–697.
[29] R.A. Ward, C. Brassington, A.L. Breeze, A. Caputo, S. Critchlow, G. Davies, L. Goodwin, G. Hassall, R. Greenwood, G.A. Holdgate, M. Mrosek, R.A. Norman, S. Pearson, J. Tart, J.A. Tucker, M. Vogtherr, D. Whittaker, J. Wingfield, J. Winter, K. Hudson, Design and synthesis of novel lactate dehydrogenase A inhibitors by fragment-based lead generation, J. Med. Chem. 55 (7) (2012) 3285–3306.

General discussion

Impairment of Aerobic Glycolysis by Inhibitors of Lactic Dehydrogenase Hinders the Growth of Human Hepatocellular Carcinoma Cell Lines

According to the finding of Warburg (Warburg O, 1930), reevaluated in recent years (Garber K, 2006) neoplastic cells derive much of their energy for survival and growth from aerobic glycolysis.

Lactate dehydrogenase (LDH) is the only glycolytic enzyme whose inhibition should allow a blocking of the aerobic glycolysis of tumor cells without damaging the normal cells which, in conditions of normal functional activity and sufficient oxygen supply, do not need this enzyme (Fiume L, 1969; Papacostantinou J and Colowick SP, 1961a).

In this preliminary work my research team and I demonstrated that oxamic acid and tartronic acid, two LDH competitive inhibitors, impaired aerobic glycolysis and replication of HepG2 and PLC/PRF 5 cells, two lines derived from a well differentiated and a poorly differentiated human hepatocellular carcinoma (HCC), respectively (Aden DP et al, 1979; Alexander JJ et al, 1976).

We have also shown that oxamic acid impairs cell growth by only inhibiting aerobic glycolysis, without interfering with Krebs cycle. Cultured cells derive their energy from glucose metabolism and glutamine oxidation. When cells are posed in a glucose free medium, they can not glycolyse and almost completely rely for their survival and growth on glutamine oxidation.

We found that under these conditions oxamic acid practically do not affect HepG2 and PLC growth, indicating that this compound does not interfere with the Krebs cycle and that the growth inhibition caused by oxamic acid when cells are cultured on glucose could be attributed to the impairment of aerobic glycolysis; on the contrary tartronic acid was shown to interfere with glutamine oxidation and this interference might be due to the inhibition of mitochondrial malic dehydrogenase (Harada K et al, 1968).

Moreover the increased expression of the p53-Upregulated Modulator of Apoptosis (PUMA) indicated that the block of aerobic glycolysis resulted in the death of the cells.

The present data are in agreement with those of Papaconstantinou and Colowick (Papaconstantinou J and Colowick SP, 1961b), who studied the effect of oxamic acid on HeLa cell replication and fit in with the finding that mammary tumor cell lines in which LDH activity was reduced by means of short hairpin RNA showed a retarded rate of cell proliferation (Fantin VR et al, 2006).

In conclusion, inhibition of aerobic glycolysis achieved by blocking LDH could be useful in the treatment of human hepatocellular carcinomas. Without interfering with glucose metabolism in normal cells, it could hinder cell growth by itself and could also enhance the chemotherapeutic index of associated anticancer agents by decreasing the levels of ATP selectively in neoplastic cells.

Inhibition of lactic dehydrogenase as a way to increase the anti-proliferative effect of multitargeted kinase inhibitors

Protein kinases play a critical role in the modulation of growth factor signalling. Activated forms of these enzymes can cause increase of tumor cell proliferation, induce apoptotic effects and promote angiogenesis and metastasis. Because these effects are initiated and proceed through by a sequential activation of proteins of the kinase family, these enzymes are a good target for anticancer treatment (Arora A et al, 2005). Multi-targeted inhibitors of protein kinases are an emerging class of antineoplastic agents. They are quite recently developed compounds, whose use will likely increase in the next future because of broad spectrum antitumor activity, ease of administration and good tolerability. Most of them are small molecules which bind to the ATP site on the protein kinases target of their activity (Sebolt-Leopold JS and English JM, 2006).

We considered the possibility that a decrease of ATP levels in neoplastic cells could reduce the competition for the same enzymatic site, thus increasing the efficacy of the kinase inhibitors. To explore this possibility, in this work it was studied on PLC/PRF/5 cells the effect of three commonly used kinase inhibitors (sorafenib, sunitinib and imatinib) given alone or in combination with oxamic acid, an LDH inhibitor. PLC/PRF/5 cells derived from HCC (Alexander JJ et al, 1976) and were an appropriate model to perform this study since this tumor is often refractory to chemotherapeutic interventions and even the benefit observed with the introduction of kinase inhibitors (sorafenib) was modest, so that the

search for new and more effective therapeutic alternatives for treating HCC is still ongoing.

As a first step to verify our experimental hypothesis, cell viability assays were performed to evaluate the antiproliferative effect on PLC/PRF/5 cells of the kinase inhibitors given alone. To test their effect when given in combination with oxamic acid, for each kinase inhibitor we selected doses causing a statistically significant inhibition on PLC/PRF/5 cell proliferation, not higher than 30%, in order to facilitate the evidence of a possible potentiating effect in the association with oxamic acid. In subsequent experiments the selected doses of kinase inhibitors (sorafenib 8-16 μM, sunitinib 8 μM; imatinib 32 μM) were combined with oxamic acid. Oxamic acid was used at concentrations of 60 and 80 mM which block aerobic glycolysis and reduce cellular ATP levels, but causing minimal effects on cell growth, not higher than 20% (Fiume L et al, 2010).

When the kinase inhibitors were given in combination with oxamic acid, an enhancement of their anti-proliferative effect was observed, that, for the 80 mM dose, always resulted statistically significant when compared to the effect of the kinase inhibitor given alone. In the case of 16 μM sorafenib, the combination with 80 mM oxamic acid had a so heavy effect to bring about the almost complete inhibition of cell growth (> 97%) over the 24h.

Morphological examination of treated cells confirmed the potentiating effect exerted by the drugs association. It was performed only on cells treated with sorafenib since, compared to the other kinase inhibitors, the gain of efficacy

observed with the oxamic acid association of this compound was the most remarkable.

Moreover to confirm the enhanced effect produced by the oxamic acid / sorafenib, we also evaluated the phosphorylation level of ERK protein, involved in the MAPK kinase signalling pathway. One of the primary targets of sorafenib is RAF kinase, the first signalling element of MAPK pathway, which has an essential role in HCC cell proliferation (Liu L et al, 2006). Blocking of RAF causes a downstream reduction of MEK and ERK phosphorylation, ultimately resulting in the inhibition of cell proliferation.

By immunoblot analysis we observed that 1h treatment with 16 μM sorafenib reduced the phosphorylation of ERK1/2 and 80 mM oxamic acid given for 1h to PLC/PRF/5 cells did not affect ERK phosphorylation, which on the contrary was heavily reduced in cultures treated with the 16 μM sorafenib / 80mM oxamic acid association, where the low molecular weight band of phosphoERK was not detected. The further reduction of phosphoERK observed in these cells, compared to those treated with sorafenib alone, is a confirm of the enhancing effect caused by oxamic acid on the signalling inhibition produced by sorafenib.

According to the rationale which suggested these experiments, the enhanced antiproliferative effect of oxamic acid association with kinase inhibitors can at least in part be due to the reduced number of ATP molecules competing with the drugs for the binding to the common enzymatic site.

We showed that when aerobic glycolysis was shut down by culturing PLC/PRF/5 cells on galactose (Reitzer LJ et al, 1979; Rossignol R et al, 2004), oxamic acid

did not cause a depletion of ATP, indicating that this compound does not interfere with the oxidative phosphorylation, still operative in this culture condition. As a consequence, in glycolysing cells the reduced ATP concentrations caused by oxamic acid can only be ascribed to the inhibition of aerobic glycolysis. This suggests that in normal cells, which do not produce ATP through aerobic glycolysis, the inhibition of LDH should not exacerbate the adverse reactions of antineoplastic drugs.

Oxamic acid is a non toxic substance in laboratory animals, up to 3g/kg (Papaconstantinou J and Colowick SP, 1961b). On the other hand, it inhibits the aerobic glycolysis of cells cultured in vitro only at high concentrations, which are about 1000 times higher than those active on LDH from cell free extracts and can not expected to be reached in vivo.

However, changes of the molecule can be studied using oxamic acid as a lead compound to synthesize a selective and more powerful inhibitor of LDH. A similar attempt was performed to improve the chemotherapy of malaria, by testing several derivatives of the oxamic acid molecule on LDH of Plasmodium falciparum, a parasite which relies on aerobic glycolysis for its energetic needs (Choi S et al, 2007; Deck LM et al, 1998). Encouraging results are also those of experiments addressed to the inhibition of the isoform II of α-hydroxyacid dehydrogenase (HADH II) of Trypanosoma cruzi, which shows a substrate specificity similar to that of LDH-C4 of spermatozoa. Moreover, by applying the virtual screening procedure (Rester U, 2008) to the crystal structure of human LDH, also molecules chemically unrelated to oxamic acid but able to inhibit LDH

can be searched; therefore the results of our experiments encourage the search for a LDH inhibitor more powerful than oxamic acid and also active in vivo. Its association with kinase inhibitors could enhance the anti-proliferative efficacy of these drugs in the treatment of responsive tumours without increasing their side effects on normal cells, which in conditions of normal functional activity and sufficient oxygen supply do not need the activity of this enzyme.

Effect of sorafenib on the energy metabolism of hepatocellular carcinoma cells

In our previous work (Fiume L et al, 2011) we demonstrated that oxamic acid, an inhibitor of lactate dehydrogenase, potentiated the activity of multitargeted kinase inhibitors (sorafenib, sunitinib, imatinib), by depleting ATP thus reducing the competition for the common enzymatic site; in particular we observed the most marked effect with the association oxamic acid /sorafenib. The enhancing effect caused by oxamic acid on the signalling inhibition produced by sorafenib could represent an interesting result because to date it is the only drug used for hepatocellular carcinomas (HCCs) chemotherapy, even though it produced only modest results on patient survival (Llovet JM et al, 2008).

Recent data demonstrated that sorafenib impaired the oxidative phosphorylation (OXPHOS) of a rat myogenic cell line and suggested that this biochemical lesion can contribute to the cardiac toxicity caused by the drug (Will Y et al, 2008). In this paper, we studied whether this mechanism of cell damage can also take part in the anti-proliferative effect of sorafenib.

We elucidated that sorafenib at the concentrations raised in plasma of patients treated with the drug (4-8 uM) decreased the oxygen comsuption in PLC/PRF 5 and SNU-449 cell cultures, two lines derived from human HCCs (Alexander JJ et al, 1976; Park JG et al, 1995).

We also showed that in glycolysing cells the mitochondrial ATP depletion, caused by sorafenib, stimulated aerobic glycolysis, as indicated by the higher amount of

lactic acid formed in the presence of the drug. Moreover when the cell coltures were treated with the association oxamic acid/sorafenib, both the metabolic pathways were inhibited, and only after 3 hours of exposure, it could be observed a drastic decrease of ATP levels. Because of the link between the two energetic pathways (Krebs HA, 1972), as stated above, the depletion of mitochondrial ATP in glycolysing cells caused an increase of glycolysis so that the ATP content in these cells was only 12–32% lower than that of control cells. This decrease, although moderate, is statistically significant and can impair cell growth, as supported by the finding that in both cell lines a dose of oxamic acid which reduces ATP levels to the same extent as sorafenib after a 3 days exposure significantly reduced the number of viable cells by about 30%. Moreover, in the case of sorafenib, which blocks the target kinases by binding to their ATP sites (Sebolt-Leopold JS and English JM, 2006), the depletion of ATP could also enhance the anti-kinase activity of the drug by reducing the competition for the common enzymatic site. Therefore, the present results point out that the loss of ATP caused by the drug interference with OXPHOS probably contributes to the anticancer activity of sorafenib on human HCC cells.

The observation of an increased ratio between glycolytic and OXPHOS-derived ATP in HCC cells treated with this drug suggests that a possible way to enhance the therapeutic power of sorafenib could be its combination with glycolytic inhibitors, such as oxamic acid, which blocks LDH. Unfortunately, oxamic acid has the drawback of a scarce cellular penetration and, consequently, it inhibits aerobic glycolysis at concentrations which cannot be reached in vivo. However,

our results encourage the search of novel LDH inhibitors more potent and active in vivo than the oxamic acid and they will probably be available for clinical trials in the near future. Their administration in combination with sorafenib could be a worthwhile attempt to enhance the results of HCC chemotherapy.

Galloflavin (CAS 568-80-9): A Novel Inhibitor of Lactate Dehydrogenase

The inhibition of lactate dehydrogenase (LDH) is a promising way to inhibit tumor cell glucose metabolism without affecting the energetic balance of normal tissues. However, the success of this approach depends chiefly on the availability of inhibitors that display good selectivity.

The only well-characterized and specific inhibitor of LDH is oxamic acid which displays a good selectivity for LDH and weak toxicity in healthy animals, although has the drawback of poor cellular penetration (Papaconstantinou J and Colowick SP, 1961b) . In our previous works (Fiume L et al 2010; Fiume L et al, 2011), we studied its effect in neoplastic cells and we confirmed its ability in hindering aerobic glycolysis. Our results suggest that, research using drug design technologies to find a strong and selective LDH inhibitor hindering aerobic glycolysis also in vivo, can be a worthwhile attempt to improve the common antineoplastic chemotherapy.

In this work we described the identification of a novel LDH inhibitor, galloflavin, a gallic acid derivative, which was selected by virtual screening of a molecular dataset and carefully biologically characterized. This approach, adopted by our colleagues of the Department of Pharmaceutical Sciences, was based on the crystal structure of the A isoform of LDH, obtained from the Protein Data Bank and it was applied to the molecules of the National Cancer Institute diversity set and led to the identification of 20 putative inhibitors, which were subsequently

tested on the purified human enzyme. Only five of them were found to cause enzyme inhibition at micromolar level. The compounds are: galloflavin (NCS107022), lomofungin (NCS156939), redoxal (NCS73735), vanillil (NCS16722), and nortangeretin (NCS76988). According to the information in the PubChem database, neither LDH inhibitory activity nor other effects on glycolytic enzymes have been reported yet for these compounds. For each compound we calculated the minimum dose required for complete inhibition of enzyme activity (IC100). Although less commonly used than IC50, this parameter was chosen with the aim of identifying a fully active inhibitor dose for testing on cells cultured in vitro for preliminary evaluation of their biological activity. The IC100 values calculated for LDH-A were tested with PLC/ PRF/5 cultures to evaluate their effect on lactate production, cell respiration, ATP levels, and cell proliferation; galloflavin was found to reduce the lactate production, proving good cell permeability. Moreover, it was shown to be not harmful for mitochondrial respiration, suggesting tolerability for normal cell metabolism.

Among the other compounds, vanillil was not deemed worthy of further study, as it caused only a small decrease in lactate levels and did not affect cellular ATP and PLC/PRF/5 growth. The remaining three compounds (redoxal, lomofungin, and nortangeretin), added to the assay cell cultures at their respective LDH IC100 values, hindered oxygen consumption, denoting lack a of specificity toward this enzyme. From our biological evaluations, galloflavin proved to be the only potentially useful LDH inhibitor. Therefore, a more detailed characterization of its biological properties was performed.

The docking made with human LDH-A isoform showed that galloflavin apparently binds mainly to the enzyme by hydrogen bonds. In particular, the carbonyl oxygen atoms of galloflavin act as hydrogen bond acceptors with Thr247 and Gln 99, and the compound's hydroxy groups establish hydrogen bond interactions with Asn137 and Ala 95. Finally, the 9- hydroxy group could make contact with either Ala 97 or Asn112 and with the backbone nitrogen atom of Asn 137 as well. Galloflavin was also docked into the human LDH-B isoform, and a pose quite similar to that observed with human LDH-A was found. This was not unexpected, considering the high sequence similarity (~75%) (Granchi C et al, 2010) of the binding site domains.

Galloflavin was found to inhibit both the isoform A and B of LDH and to determine the mechanism of action, we collected LDH-A and -B inhibition data in competition reactions with the substrate (pyruvate) or the cofactor (NADH) and evaluated the results using software for enzyme kinetics analysis. We found that galloflavin inhibited both human LDH isoforms by preferentially binding the free enzyme, without competing with the substrate or cofactor. The calculated Ki values for pyruvate were 5.46 mm (LDH-A) and 15.06 mm (LDH-B).

The discovery of galloflavin's inhibitory action against both human LDH isoforms suggests broader and more interesting properties than those of some inhibitors, which were found to act specifically on LDH-A (Yu Y et al, 2001; Granchi C et al, 2011). In fact, data were recently reported that indicate a pivotal role for LDH-B in tumorigenesis mediated by hyperactive mTOR (mammalian target of rapamycin) signaling (Zha X et al, 2011).

We calculated that the concentration of galloflavin causing a 50% decrease in lactate production is 140 μM. This dose fits well with the concentration required to decrease cell viability by 50% (184 μM), suggesting that in PLC/PRF/5 cells exposed to galloflavin, the decrease in viability could be ascribed mainly to inhibition of aerobic glycolysis caused by the compound. However these doses (140–184 μM) reduced ATP levels by only 25-30%. This result can be easily explained by considering that even in cells where glycolysis is active, mitochondrial function is not completely abolished and that mitochondrial respiration is more productive in ATP synthesis than glycolysis. This limited effect on ATP levels appeared to have a positive consequence, as by studying the cell death mechanism triggered by galloflavin, we observed the induction of apoptosis, with no evidence of necrosis caused by a deep ATP depletion.

To our knowledge, the inhibition of LDH isoenzymes is the only biochemical effect described for galloflavin. Preliminary toxicity data for this molecule are available from the NCI (http://dtp.nci.nih.gov/), and remarkably they suggest good tolerability: in mice the maximum tested dose (400 $mgkg^{-1}$ injected i.p.) did not produce lethal effects.

In conclusion, galloflavin was found to be a promising lead candidate in the field of tumor metabolism inhibitors and led us to continue with further studies aimed at better define its therapeutic potential.

Galloflavin, a new lactate dehydrogenase inhibitor, induce the death of human breast cancer cells with different glycolytic attitude by affecting distinct signalling pathways

After the identification of galloflavin, a new lactate dehydrogenase (LDH) inhibitor (Manerba M et al, 2012), the aim of our next work was to investigate the effects of this compound on cells from tumors of different pathological subtypes, suggestive of a different metabolic state of the transformed cells. As a model for our study we chose human breast cancer, a neoplasm which is known to exhibit remarkable heterogeneity in phenotype, usually classified by reporting the expression profile of ER, PR and HER-2. The MCF-7 cell line was used as a model of the well differentiated human breast cancer (ER and PR positive) and the MDAMB-231 as representative of the more aggressive triple-negative form of the tumor. The experiments were also performed on a sub-line of MCF-7, in which ER function has been lost. This line (MCF-Tam) was obtained by maintaining the parental cells in the presence of clinically relevant levels of 4-hydroxy-tamoxifen (10^{-7} M) (Farabegoli F et al, 2007) and showed an activated EGFR signalling pathway (Farabegoli F et al, 2010).

As first we characterized these three cell lines by quantifying their LDH enzymatic activity, glucose uptake, lactate production and relative oxygen consumption and we observed that they showed marked differences in the metabolic profile; in particular, MDAMB-231 and MCF-Tam exhibited a

phenotype more dependent on glycolysis, contrary to MCF-7 in which oxidative phosphorylation remained still operative.

The data reported in this paper showed that, in spite of the differences in the metabolic profile observed in the three lines, the effect of galloflavin on the cell growth was quite similar and this result could be in conflict with the much higher glycolysis dependence shown by MDA MB-231 and MCF-Tam; only later we showed that it could be justified by other characteristics of these cells. In this way, we continued investigating about the different antiproliferative mechanisms which contribute to cell death in the three cell lines; in particular, we wanted to evaluate if galloflavin, by depleting ATP levels, could down-regulate the signalling pathway involved in cell survival, and if this compound could be responsible for the induction of stress oxidative in the treated cultures.

In previous studies LDH inhibition was in fact found to induce reactive oxygen species (ROS) generation, as a consequence of a compensatory increase in cell respiration set out by cells attempting to restore their ATP supply (Le A et al., 2010). These effects were studied by incubating breast cancer cell cultures to the mean IC50 dose of galloflavin (125 μM).

While in MCF-7 galloflavin 125 μM, by depleting ATP levels, caused a down-regulation of the expression of mRNA of ERα, thus depriving cells of the most important survival signal (Levenson AS and Jordan VC, 1997), in MDA-MB-231 and MCF-Tam galloflavin was found to activate a stress response as a result of increased ROS generation. In this case, the highly glycolysing MDA-MB-231 and MCF-Tam cells were not able to cope with the ATP depletion caused by LDH

inhibition by simply increasing the mitochondrial function and the induction of the oxidative stress is a possible cause contributing to the growth inhibition. The activation of the stress-response pathway in these cells was witnessed by the statistically significant increase of JNK and p38 phosphorylation. The study of phospho-JNK and phospho-p38 expression also allowed to explain why, in spite of their much higher glycolysis dependence, MDA-MB-231 and MCF-Tam did not exhibit higher sensitivity to the antiproliferative effects of galloflavin. In these cells, which are representative of aggressive forms of breast cancer, the stress response pathway is constitutively activated, since both JNK and p38 appeared phosphorylated also in the untreated cultures. This constitutive activation can render the cells less susceptible to harmful stimuli (Davidson B et al., 2006). Finally, these different antiproliferative mechanisms led to the apoptosis induction in all the three cell lines.

As shown by our results, galloflavin inhibits the growth of breast cancer cells at higher dose (125 μM) compared to commonly used chemotherapeutics. However we demonstrated that non neoplastic cells present a better tolerability to galloflavin as compared with breast cancer cells, by studying its effect on lymphocytes from peripheral blood and GM130C human lymphoblasts. These data, together with those ones on galloflavin toxicity available from NCI web site (http://dtp.nci.nih.gov/), suggesting a good tolerability in mice, offers a good motivation to test this new inhibitor on other cellular models in order to continue defining its therapeutic potential. Our results on breast cancer cells confirm that the inhibition of bioenergetic metabolism obtained by LDH blockage is a

promising therapeutic strategy. Moreover they agree with other several previously published data indicating that LDH play a critical role on growth properties and tumor maintenance of breast cancer cells (Nagai MA et al, 1988; Zaho YH et al, 2009) and with other two works in which inhibition of LDH-A was found to be a way to overcome the acquired resistance of breast cancer cells to taxol (Zhou M et al, 2010) and trastuzumab (Zhao X et al, 2011).

In spite of the unquestionable progresses achieved in breast cancer therapy, the treatment of some forms of this tumor, such as the triple negative or the drug resistant lesions, still poses a challenge to the clinical oncologists. In this context, our observation of galloflavin activity even on the breast cancer cells which mimic these tumor forms with unfavorable prognosis, for its clinical relevance adds further interest in the properties of this compound.

Galloflavin suppresses lactate dehydrogenase activity and causes MYC downregulation in Burkitt lymphoma cells through NAD/NADH dependent inhibition of sirtuin-1

In this paper we continued investigating about the effects caused by galloflavin on the observation that aerobic glycolysis, is the consequence of oncogenes and oncosuppressors reprogramming the cellular energetic machinery (Levine AJ and Puzio-Kuter AM, 2010). One of the most characterized DNA alteration that induces glycolytic phenotype in cancer cells is the activation of the *myc* oncogene (Dang CV et al, 2009).

MYC is a helix–loop–helix leucine zipper transcription factor that dimerizes with its partner protein MAX. The dimer then binds to specific DNA sequences and transactivates genes involved in cell proliferation, differentiation, and apoptosis. Further, MYC plays an important role in the regulation of glucose metabolism (Kim JW and Dang CV, 2006). It directly transactivates the A subunit of lactate dehydrogenase (LDH-A), which is directly involved in *myc*-mediated cell transformation (Dang CV et al, 2009; Shim H et al, 1997).

The *myc*-dependent linkage between altered cellular metabolism and tumorigenesis (Dang CV et al, 2009; Kim JW and Dang CV, 2006) prompted us to investigate the effects of galloflavin-mediated LDH inhibition in *myc*-hyperexpressing cancer cells. Thus, we studied the effects of galloflavin on Burkitt's lymphoma (BL) cells (Raji, Daudi, Loukes), representing a tumor characterized for the 90% of cases by *myc* overexpression, in addition to a

chromosomal translocation (the most common translocation is t(8:14)) (Slack GW and Gascoyne RD, 2011).

Our data indicated that *myc* overexpression in BL cells increased the expression of the LDH-A subunit, which is normally expressed at low levels in this cell type. Compared to other tumor models, BL cells were found to be very susceptible to galloflavin effects (IC 50~30 uM), while on two non neoplastic lymphoblastoid cell lines used as control (GM130C and AG3138A) the effect of galloflavin was not statistically significant. However, we found that higher LDH-A levels do not drive cell metabolism toward glycolytic ATP generation, and this observation was an unexpected result. In fact, galloflavin at doses that cause a 50% reduction in cell growth and lactate production, did not affect ATP levels in any of the three BL cell lines, suggesting that in these cells oxidative phosphorylation remains operative and can be a source of ATP supply.

Since in these cells the growth inhibition is not to be ascribed to the ATP depletion, we tried to investigate to find another possible antiproliferative mechanism. For this purpose, we considered only Raji cell cultures, as these cells were more sensitive to galloflavin, but their ATP levels were essentially unaffected by galloflavin treatment.

In aerobic glycolysis, the LDH enzyme functions to regenerate oxidized NAD, is necessary for sustained glucose degradation. NAD participates in multiple metabolic reactions as a cofactor, and it can also mediate biologic processes by acting as a substrate for a wide range of proteins (Houtkooper RH et al, 2010; Lin SJ et al, 2003). Among the NAD-consuming enzymes, sirtuins, particularly,

SIRT1, have high Km values for NAD (Houtkooper RH et al, 2010). Therefore, these enzymes are highly susceptible to changes in the cellular levels of this dinucleotide.

SIRT1 is a histone deacetylase that promotes cell survival under stressful conditions by deacetylating key cell cycle molecules and apoptosis regulatory proteins, including p53 (Li K and Luo J, 2011; Milner J, 2009). Several reports have revealed that SIRT1 is upregulated in various human tumors (Milner J, 2009), including B-cell lymphoma, for which enhanced expression of this protein was detected in greater than 70% of patients (Jang KY et al, 2008). Moreover, previous studies have demonstrated that the MYC protein is a substrate of SIRT1, which deacetylates MYC and promotes its transcription factor functions (Mao B et al, 2011). On the basis of this knowledge, we investigated the effect of galloflavin on MYC protein levels and SIRT1 activity. In the absence of a commercially available, specific, acetyl-MYC antibody, SIRT1 activity was evaluated by measuring the acetylation levels of the p53 protein, a known SIRT1 target (Li K and Luo J, 2011).

We observed that galloflavin at 50 μM markedly affected the NAD/NADH balance in Raji cells causing a four-fold reduction in NAD levels and an increase in NADH levels. Furthermore galloflavin led to a notable decrease of MYC protein levels and an increase in p53 acetylation, which is indicative of SIRT-1 inhibition. Since *myc* activity is related to its protein levels (Sachaf GM et al, 2008), these data suggest that galloflavin can suppress MYC-driven signals in Raji cells.

The same experiments were also conducted on the GM130C lymphoblastoid cell line as a control and interestingly, MYC protein levels and p53 acetylation were unaffected in GM130C cells exposed to 100 μM galloflavin.

Since galloflavin is a recently identified LDH inhibitor, and other effects than LDH inhibition cannot be excluded, to verify whether its effects observed on Raji cells were a result of LDH inhibition, we treated Raji cultures with oxamic acid (OXA), a known specific inhibitor of this enzyme. OXA inhibited the growth of Raji cells but like galloflavin did not significantly affect cellular ATP levels. However at its IC50 dose highly reduced NAD levels, decreased MYC protein levels, and suppressed SIRT1 activity. Therefore, these observations strongly suggested that the effects of galloflavin observed in Raji cells could be attributed to LDH inhibition.

There is a lot of literature suggesting that SIRT1 and MYC could be considered attractive targets for therapeutic purposes (Milner J, 2009; Jang KY et al, 2008; Menssen A et al, 2012; Hermeking H, 2003). Therefore the results from our study not only further characterize the biological functions of the LDH enzyme, but also suggest that LDH inhibition may be useful as a treatment for cancer.

Galloflavin prevents the binding of lactate dehydrogenase A to single stranded DNA and inhibits RNA synthesis in cultured cells

Besides its widely known role in cell energy metabolism, lactate dehydrogenase A (LDH-A) also displays an additional function in nuclei. Some years ago several authors observed that the LDH-A binds to single-stranded DNA (ssDNA) (Patel GL and Thompson PE, 1980; Williams KR et al, 1985; Calissano P et al, 1985; Grosse F et al, 1986; Sharief FS et al, 1986; Kaiserman HB et al, 1989). In analogy with what observed for other ssDNA-binding proteins (ssBPs) (Shamoo Y, 2002) an involvement of the enzyme in DNA transcription and/or replication was suggested; thus targeting ssBPs to prevent their interaction with ssDNA is a new strategy for drug development towards better cancer treatment (Zou Y et al, 2006; Anciano Granadillo VJ et al, 2010).

LDH-A binding to ssDNA is prevented in vitro by NADH (Calissano P et al 1985, Patel GL et al, 1984), suggesting that the coenzyme site is involved in the interaction LDH-A/ssDNA. In this paper we investigated whether galloflavin, a recently identified small molecule which inhibits the enzymatic activity of LDH-A by occupying the NADH site (Manerba M et al, 2012), also hinders the interaction of the enzyme with ssDNA.

Using a filter binding assay we observed that 4 μM of galloflavin inhibited the binding of human LDH-A to a single stranded [3H]DNA sample by 50%. After only 0.5–1 h, 50–100 μM galloflavin inhibited RNA synthesis in SW620 colorectal cancer cells maintained in a medium in which galactose substituted

glucose. In these culture conditions, SW620 cells did not produce lactic acid and effects caused by the inhibition of the enzymatic activity of LDH-A could be excluded. Therefore, the anticancer activity exerted by galloflavin on normally glycolysing neoplastic cells (Farabegoli F et al, 2012; Manerba M et al, 2012) was likely caused not only by the block of aerobic glycolysis, but also by an inhibition of RNA synthesis, independent from the effect on glycolysis.

Small molecules interfering with the enzymatic activity of LDH-A are now searched to selectively block aerobic glycolysis for an approach to antineoplastic chemotherapy proposed several years ago (Fiume L, 1960; Papacostantinou J and Colowick SP, 1961a) and at present actively pursued (Granchi C et al, 2010; Ward RA et al, 2012). Our data indicate that inhibitors which occupy the NADH site of the enzyme besides blocking aerobic glycolysis might also prevent LDH-A/ssDNA interaction and hamper transcription. Finally, the present results suggest that galloflavin, by hindering the binding of LDH-A to ssDNA, can be a tool to study the biological role of this interaction into the cells.

Conclusion

In recent years a growing number of studies were addressed at interfering with the metabolic pathways which generate energy in neoplastic cells. It has long been known that, to meet their energy demand, cancer cells largely rely on aerobic glycolysis (Warburg O, 1930). This process can be hindered by blocking lactate dehydrogenase (LDH), which causes a failing in the regeneration of NAD, reduced during the conversion of glyceraldehyde 3-phosphate to 1,3-diphosphoglycerate, and necessary to maintain the glycolytic flow. As stated before, at difference from other glycolytic enzymes, LDH is probably unessential for normal tissues in conditions of normal functional activity and sufficient oxygen supply. Moreover, support for the possibility that LDH activity is not necessary for normal cells also comes from the findings that humans with a hereditary deficiency in the activity of LDH-A (the isoform of the muscle and liver) do not display any symptoms under normal circumstances (Kanno T et al, 1988), and also that a complete hereditary deficiency in LDH-B (the isoform of the heart) is usually asymptomatic (Okumura N et al, 1999). Therefore, in the field of the metabolic inhibitors, LDH is considered one of the most promising therapeutic targets. An important result obtained from our preliminar experiments was the finding that oxamic acid, an LDH inhibitor, hindered the replication of cells from human hepatocellular carcinomas (HCCs) by selectively blocking aerobic glycolysis, thus without interfering with glucose metabolism in normal cells which do not rely on aerobic glycolysis. These data led us to suppose that the selective depletion of ATP levels in neoplastic cells could be a good strategy to increase the chemotherapeutic index of associated anticancer agents, like the

multitargeted kinase inhibitor molecules, which exert their action by binding to the ATP site on the targeted kinases. An interesting result, in fact, was the striking increase of the antiproliferative effect of sorafenib, a multitargeted kinase inhibitor used for the HCCs chemotherapy, in association with a LDH inhibitor like oxamic acid. Our results showed that the combination of the two drugs caused a strong depletion of ATP levels in cultured cells, which caused a rapid induction of cell death. This so marked decrease of ATP levels was also due to the sorafenib interference with oxidative phosphorylation, which made cells more dependent on aerobic glycolysis. As just mentioned before, oxamic acid inhibits the aerobic glycolysis in cells cultured in vitro only at high concentrations which are not expected to be reached in vivo. Nevertheless our preliminary works encouraged us to search for LDH inhibitors more powerful than oxamic acid and also active in vivo; thus we identified a new molecule, galloflavin, able to inhibits both A and B isoforms of LDH enzyme. We described its effects on PLC/PRF/5 cells from HCC in which it hindered cellular metabolism by depleting lactic acid and ATP production, thus leading cells to death. Galloflavin was also tested on breast cancer cell lines with different metabolic phenotypes, causing in these cells different antiproliferative mechanisms responsible for apoptosis induction. The most promising results were obtained in Burkitt's lymphoma cells characterized by *myc* overexpression which in turn up-regulates LDH-A levels: in fact, among all the studied cellular models, these cells were found to be the most susceptible to galloflavin effect (IC50 ~ 30 μM). However, an unexpected result was to point out. In spite of the higher LDH-A levels, these cells did not present a much higher

glycolysis dependence, indeed they maintained an adequate mitochondrial function which provided for ATP supply. LDH inhibition in these cells did not produce a significant decrease of ATP levels, but it caused an alteration in the balance NAD/NADH leading to the inhibition of Sirt-1 activity, which was found to down-regulate Myc expression, thus depriving the cells of their most important survival factor; interestingly, our data suggest that LDH functions to sustain cellular levels of NAD in addition to its role in regulating energy supplies within the cell.

Finally, we also showed that galloflavin hindered RNA synthesis by preventing LDH-A/single stranded DNA (ssDNA) interaction, thus exerting its anticancer activity not only by the block of aerobic glycolysis, but also by inhibition of RNA synthesis, independent from the effect on glycolysis. As discussed before, LDH-A seems to be implicated in the transcription of histone 2B gene (Dai RP et al, 2008) and its involvement in regulating the transcription of other genes cannot be excluded; in this way galloflavin could be a useful tool to study the biological role of LDH-A binding to ssDNA.

At present, the only small molecule inhibitor tested on cultured cancer cells representative of human tumors with different metabolic states and/or pathological subtypes is galloflavin (Farabegoli F et al, 2012; Vettraino M et al, 2013). However, this compound is active at quite high doses, when compared to conventionally used chemotherapeutics; moreover, it shows solubility problems hindering its administration in vivo.

To make possible a more exhaustive evaluation of the real therapeutic potential of LDH inhibition, critical issues are still to be solved. A first crucial point is the generation of more bioavailable inhibitors. LDH proved to be a very difficult target for medicinal chemistry researches; the identification of new inhibitors is made difficult by the characteristics of the enzyme active site, which is poorly accessible and highly polar. Furthermore, permeability through cell membranes is frequently hampered by excessive polarity or explicit negative changes present in the inhibitors, which then compromise their activity in cell-based assays and in vivo. Moreover, the LDH cofactor is shared by a large number of cellular enzymes, which are expected to display high structural homology in their NADH binding site. As a consequence, potential inhibitors hindering NADH binding are at high risk of reduced specificity.

The availability of molecules showing good inhibitory power on cultured cells will also allow studies aimed at identifying the molecular alterations predictive of a good response of neoplastic cells to LDH inhibition. Consistently with the observation of Myc-induced LDH-A expression, experiments already performed with galloflavin have shown that neoplastic cells originating as a consequence of Myc over-activation are highly responsive to LDH inhibition (Vettraino M et al, 2013). This result suggests that these tumor forms could be appropriate clinical conditions to test the validity of the LDH-targeted therapeutic approach.

However, the metabolic features of cancer cells are under the control of several oncogenic signals, among which a major role is played by p53, Akt, PI3K and

Ras. Further study is needed to understand the effects of LDH inhibition on tumors critically depending for their growth on these specific alterations.

REFERENCES TO DISCUSSION

- Aden DP, Fogel A, Plotkin S, Damjanov I, Knowles BB. Controlled synthesis of HBsAg in a differentiated human liver carcinoma-derived cell line. Nature 282, 615-616 (1979).

- Alexander JJ, Bey EM, Geddes EW, Lacatsas G. Establishment of a continuously growing cell line from primary carcinoma of the liver. S Afr Med J 50, 2124-2128 (1976).

- Anciano Granadillo VJ, Earley JN, Shuck SC, Georgiadis MM, Fitch RW, Turchi JJ. Targeting the OB-folds of replication protein A with small molecules. J. Nucl. (2010).

- Arora A, Scholar EM. Role of tyrosine kinase inhibitors in cancer therapy. J Pharmacol Exp Ther 315, 971-979 (2005).

- Calissano P, Volontè C, Biocca S et al. Synthesis and content of a DNA-binding protein with lactic dehydrogenase activity are reduced by nerve growth factor in the neoplastic cell line PC12. *Exp. Cell Res.* 161, 117-129 (1985).

- Choi S, Beeler AB, Pradhan A, Watkins EB, Rimoldi JM, Tekwani B, et al. Generation of oxamic acid libraries: antimalarials and inhibitors of Plasmodium falciparum lactate dehydrogenase. J Comb Chem 9, 292-300 (2007).

- Dai RP, Yu FX, Goh SR et al. Histone 2B (H2B) expression is confined to a proper NAD+/NADH redox status. J. Biol. Chem. 283, 26894-26901 (2008).

- Dang CV, Le A, Gao P. MYC-induced cancer cell energy metabolism and therapeutic opportunities. Clin Cancer Res 15, 6479–6483 (2009).

- Davidson B, Konstantinovsky S, Kleinberg L, Nguyen NTP, Bassarova A, Kvalheim G, Nesland JM, Reich R. The mitogen-activated protein kinases (MAPK) p38 and JNK are markers of tumor progression in breast carcinoma. Gynecol. Oncol. 102, 453-446 (2006).

- Deck LM, Royer RE, Chamblee BB, Hernandez VM, Malone RR, Torres JE, et al. Selective inhibitors of human lactate dehydrogenase and lactate dehydrogenase from malarial parasite Plasmodium falciparum. J Med Chem 41, (20) 3879-87 (1998).

- Fantin VR, St-Pierre J, Leder P. Attenuation of LDH-A expression uncovers a link between glycolysis, mitochondrial physiology and tumor maintenance. Cancer Cell 9, 425–434 (2006).

- Farabegoli F, Barbi C, Lambertini E, Piva R. (-)-Epigallocatechin-3-gallate down-regulates estrogen receptor alpha function in MCF-7 breast carcinoma cells. Cancer Detect. Prev. 31, 499–504 (2007).

- Farabegoli F, Papi A, Orlandi M. (-)-Epigallocatechin-3-gallate downregulates EGFR, MMP-2, MMP-9 and EMMPRIN and inhibits the invasion of MCF-7 tamoxifen-resistant cells. Biosci. Rep. 30, 99–108 (2010).

- Farabegoli F, Vettraino M, Manerba M et al. Galloflavin, a new lactate dehydrogenase inhibitor, induces the death of human breast cancer cells with different glycolytic attitude by affecting distinct signalling pathways. Eur. J. Pharm. Sci. 47, 729-738 (2012).

- Fiume L. Inhibition of aerobic glycolysis in Yoshida ascites hepatoma by tartronic acid. Nature 187, 792–793 (1960).

- Fiume L, Manerba M, Vettraino M, Di Stefano G. Impairment of aerobic glycolysis by inhibitors of lactic dehydrogenase hinders the growth of

human hepatocellular carcinoma cell lines. Pharmacology 86, 157-62 (2010).

- Fiume L, Vettraino M, Manerba M, Di Stefano G. Inhibition of lactic dehydrogenase as a way to increase the anti-proliferative effect of multi-targeted kinase inhibitors. Pharmacol Res. 63 (4), 328-34 (2011).

- Garber K. Energy deregulation: licensing tumors to grow. Science; 312: 1158–1159 (2006).

- Granchi C, Bertini S, Macchia M, Minutolo F. Inhibitors of lactate dehydrogenase isoforms and their therapeutic potentials. Curr. Med. Chem. 17 (7) 672–697 (2010).

- Granchi C, Roy S, Giacomelli C, Macchia M, Tuccinardi T, Martinelli A. Discovery of N-hydroxyindole-based inhibitors of human lactate dehydrogenase isoform A (LDH-A) as starvation agents against cancer cells. J. Med. Chem 54, 1599 –1612 (2011).

- Grosse F, Nasheuer HP, Scholtissek S et al. Lactate dehydrogenase and glyceraldehyde-phosphate dehydrogenase are single-stranded DNA-binding proteins that affect the DNA-polymerase-α–primase complex. Eur. J. Biochem. 160, 459-467 (1986).

- Harada K, Wolfe RG. Malic dehydrogenase 6. Kinetic study of hydroxymalonate inhibition. J Biol Chem 243, 4123–4130 (1968).

- Hermeking H. The MYC oncogene as a cancer drug target. Curr Cancer Drug Targets 3, 163–175 (2003).

- Houtkooper RH, Canto` C, Wanders RJ, Auwerx J. The secret life of NAD: an old metabolite controlling new metabolic signalling pathways. Endocr Rev 31, 194–223 (2010).

- Jang KY, Hwang SH, Kwon KS, Kim KR, Choi HN, Lee NR, et al. SIRT1 expression is associated with poor prognosis of diffuse large B-cell lymphoma. Am J Surg Pathol. 32, 1523–1531 (2008).

- Kanno T, Sudo K, Maekawa M, Nishimura Y, Ukita M, Fukutake K. Lactate dehydrogenase M-subunit deficiency: a new type of hereditary exertional myopathy. Clin Chim Acta 173, 89–98 (1988).

- Kaiserman HB, Odenwald WF, Stowers DJ et al. A major single-stranded DNA binding protein from ovaries of the frog, Xenopus laevis, is lactate dehydrogenase. Biochim. Biophys. Acta 1008, 23-30 (1989).

- Kim JW and Dang CV. Cancer's molecular sweet tooth and the Warburg effect. Cancer Res 66, 8927–8930 (2006).

- Krebs HA. The Pasteur effect and the relations between respiration and fermentation. Essays Biochem. 8, 1–34 (1972).

- Le A, Cooper CR, Gouw AM et al. Inhibition of lactate dehydrogenase A induces oxidative stress and inhibits tumor progression. Proc. Natl. Acad. Sci. USA 107, 2037-2042 (2010).

- Levenson AS and Jordan VC. MCF-7: the first hormone-responsive breast cancer cell line. Cancer Res. 57, 3071–3078 (1997).

- Levine AJ and Puzio-Kuter AM. The control of the metabolic switch in cancers by oncogenes and tumor suppressor genes. Science 330, 1340–1344 (2010).

- Li K and Luo J. The role of SIRT1 in tumorigenesis. N Am J Med Sci 4, 104–106 (2011).

- Lin SJ and Guarente L. Nicotinamide adenine dinucleotide, a metabolic regulator of transcription, longevity and disease. Curr Opin Cell Biol 15, 241–246 (2003).

- Liu L, Cao Y, Zhang X, McNabola A, Wilkie D, Wilhelm S, Lynch M, Carter C Sorafenib blocks the RAF/MEK/ERK pathway, inhibits tumor angiogenesis, and induces tumor cell apoptosis in hepatocellular carcinoma model PLC/PRF/ 5. Cancer Res 66, 11851-11858 (2006).

- Llovet, JM, Ricci, S, Mazzaferro V, Hilgard P, Gane E, Blanc JF et al. SHARP Investigators Study Group l. Sorafenib in advanced hepatocellular carcinoma. N. Engl. J. Med. 359, 378–390 (2008).

- Manerba M, Vettraino M, Fiume L et al. Galloflavin (CAS 568-80-9): a novel inhibitor of lactate dehydrogenase. ChemMedChem 7, 311-317 (2012).

- Mao B, Zaho G, Lv X, Chen HZ, Xue Z, Yang B, et al. Sirt1 deacetylates c-Myc and promotes c-Myc/Max association. Int J Biochem Cell Biol 43, 1573–1581 (2011).

- Menssen A, Hydbring P, Kapelle K, Vervoorts J, Diebold J, Luscher B, et al. The c-MYC oncoprotein, the NAMPT enzyme, the SIRT1-inhibitor DBC1, and the SIRT1 deacetylase form a positive feedback loop. Proc Natl Acad Sci USA 109, E187–E196 (2012).

- Milner J. Cellular regulation of SIRT1. Curr Pharm Des 15, 39–44 (2009).

- Nagai MA, Sonohara S, Brentani MM. Estrogen control of lactate dehydrogenase isoenzyme-5 in human breast cancer. Int. J. Cancer 41, 10–16 (1988).

- Okumura N, Terasawa F, Ueno I, Oki K, Tamauchi K, Hidaka H, Tozuka M, Okura M, Katsuyama T. Genetic analyses in homozygous and heterozygous variants of lactate dehydrogenase-B (H) subunit-LD-B Matsumoto I and II (LDH-B W323R). Clin Chim Acta 287, 163–171 (1999).

- Papacostantinou J, Colowick SP. The role of glycolysis in the growth of tumor cells. I. Effects of oxamic acid on the metabolism of Ehrlich ascites tumor cells in vitro. J. Biol. Chem. 236, 278-284 (1961a).

- Papaconstantinou J, Colowick SP. The role of glycolysis in the growth of tumor cells. 2. The effect of oxamic acid on the growth of HeLa cells in tissue culture. J Biol Chem 236, 285–288 (1961b).

- Park JG, Lee JH, Kang MS, Park KJ, Jeon YM, Lee HJ, Kwon HS, Park HS, Yeo KS, Lee KU. Characterization of cell lines established from human hepatocellular carcinoma. Int. J. Cancer 62, 276–282 (1995).

- Patel GL and Thompson PE. Immunoreactive helix-destabilizing protein localized in transcriptionally active regions of Drosophila polytene chromosomes. Proc. Natl. Acad. Sci. USA 77, 6749-6753 (1980).

- Patel GL, Reddigari S, Williams KR, Baptist E, Thompson TE, Sisodia S. A rat liver helix destabilizing protein: properties and homology to LDH-5, in: P. Skean, S.J. Friedman (Eds.), Growth, Cancer, and the Cell Cycle, Humana Press Inc., Clifton, New Jersey, 41–58 (1984).

- Reitzer LJ, Wice BM, Kennell D. Evidence that glutamine, not sugar, is the major energy source of cultured HeLa cells. J Biol Chem 254, 2669-76 (1979).

- Rester U. From virtuality to reality. Virtual screening in lead discovery and lead optimization: a medicinal chemistry perspective. Curr Opin Drug Discov Devel 11, 559-68 (2008).

- Rossignol R, Gilkerson R, Aggeler R, Yamagata K, Remington SJ, Capaldi RA. Energy substrate modulates mitochondrial structure and oxidative capacity in cancer cells. Cancer Res 64, 985-93 (2004).

- Sachaf GM, Gentles AJ, Elchuri S, Sahoo D, Soen Y, Sharpe O, et al. Genomic and proteomic analysis reveals a threshold level of MYC required for tumor maintenance. Cancer Res 68, 5132–5142 (2008).

- Sebolt-Leopold JS, English JM. Mechanism of drug inhibition of signalling molecules. Nature 441, 457-462 (2006).

- Shamoo Y. Single-stranded DNA-binding proteins. In: Enciclopedia of Life Sciences Macmillan Publisher Ltd (Ed.), Nature Publishing Group, 1-7 (2002).

- Sharief FS, Wilson SH, Li SS. Identification of the mouse low-salt-eluting singlestranded DNA-binding protein as a mammalian lactate dehydrogenase-A isoenzyme. Biochem. J. 233, 913-916 (1986).

- Shim H, Dolde C, Lewis BC, Wu CS, Dang G, Jungmann RA, et al. c-Myc transactivation of LDH-A: implications for tumor metabolism and growth. Proc Natl Acad Sci USA 94, 6658–6663 (1997).

- Slack GW and Gascoyne RD. MYC and aggressive B-cell lymphomas. Adv Anat Pathol 18, 219–228 (2011).

- Vettraino M, Manerba M, Govoni M, et al. Galloflavin suppresses lactate dehydrogenase activity and causes MYC downregulation in Burkitt lymphoma cells via NAD/NADH-dependent inhibition of sirtuin-1. *Anti-Cancer Drug* 24, 862-870 (2013).

- Warburg O: Über den Stoffwechsel der Tumoren. Berlin, Springer, 1926. [English translation Dickens F (transl): The Metabolism of Tumours. London, Constable, 1930].

- Ward RA, Brassington C, Breeze AL et al. Design and Synthesis of Novel Lactate Dehydrogenase A Inhibitors by Fragment-Based Lead Generation. J. Med. Chem. 55, 3285-3306 (2012).

- Will Y, Dykens JA, Nadanaciva S, Hirakawa B., Jamieson J, Marroquin LD, Hynes J, Patyna S, Jessen BA. Effect of the multitargeted tyrosine kinase inhibitors imatinib, desatinib, sunitinib and sorafenib on mitochondrial function in isolated rat heart mitochondria and H9c2 cells. Toxicol. Sci. 106, 153–161 (2008).

- Williams KR, Reddigari S, Patel GL. Identification of a Nucleic Acid Helix- Destabilizing Protein from Rat Liver as Lactate Dehydrogenase-5. Proc. Natl. Acad. Sci. USA 82, 5260-5264 (1985).

- Yu Y, Deck JA, Hunsaker LA, Deck LM, Royer RE, Goldberg E, Vander Jagt DL. Selective active site inhibitors of human lactate dehydrogenases A4, B4, and C4.Biochem. Pharmacol. 62, 81–89 (2001).

- Zaho YH, Zhou M, Liu H., Ding Y, Khong HT, Yu D, Fodstad O, Tan M. Upregulation of lactate dehydrogenase A by ErbB2 through heat shock factor 1 promotes breast cancer cell glycolysis and growth. Oncogene 28, 3689–3701 (2009).

- Zha X, Wang F, Wang Y et al. Lactate dehydrogenase B is critical for hyperactive mTOR-mediated tumorigenesis. Cancer Res. 71, 13-18 (2011).

- Zhao Y, Liu H, Liu Z, Ding Y, Ledoux SP, Wilson GL, Voellmy R, Lin Y, Lin W, Nahta R, Liu B, Fodstad O, Chen J, Wu Y, Price JE, Tan M. Overcoming trastuzumab resistance in breast cancer by targeting dysregulated glucose metabolism. Cancer Res. 71, 4585 –4597 (2011).

- Zhou M, Zhao Y, Ding Y, Liu H, Liu Z, Fodstad O, Riker AI, Kamarajugadda S, Lu J, Owen LB, Ledoux SP, Tan M. Warburg effect in chemosensitivity: targeting lactate dehydrogenase-A re-sensitizes taxol-resistant cancer cells to taxol. Mol. Cancer 9-33 (2010).

- Zou Y, Liu Y, Wu X, Shell SM. Functions of human replication protein A (RPA): from DNA replication to DNA damage and stress responses. J. Cell Physiol. 208 (2) 267-273 (2006).

ACKNOWLEDGEMENTS

Thanks to Professor Di Stefano for her helpfulness and for having allowed me to realize this project.

A special thanks goes to a big scientist, Professor Fiume, for his precious teachings.

Finally thanks to my family and my friends who always supported me and to my "young colleagues" with whom I spent amusing moments despite of problems and difficulties related to our job.