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 pyrvuate 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 0512094721; fax: +39 0512094746.
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 (1600*g*, 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