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

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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.

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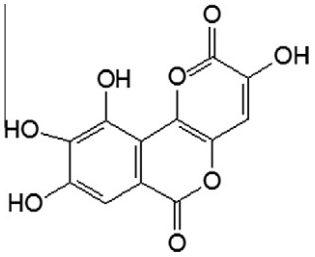

Fig. 1. Galloflavin (GF).

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

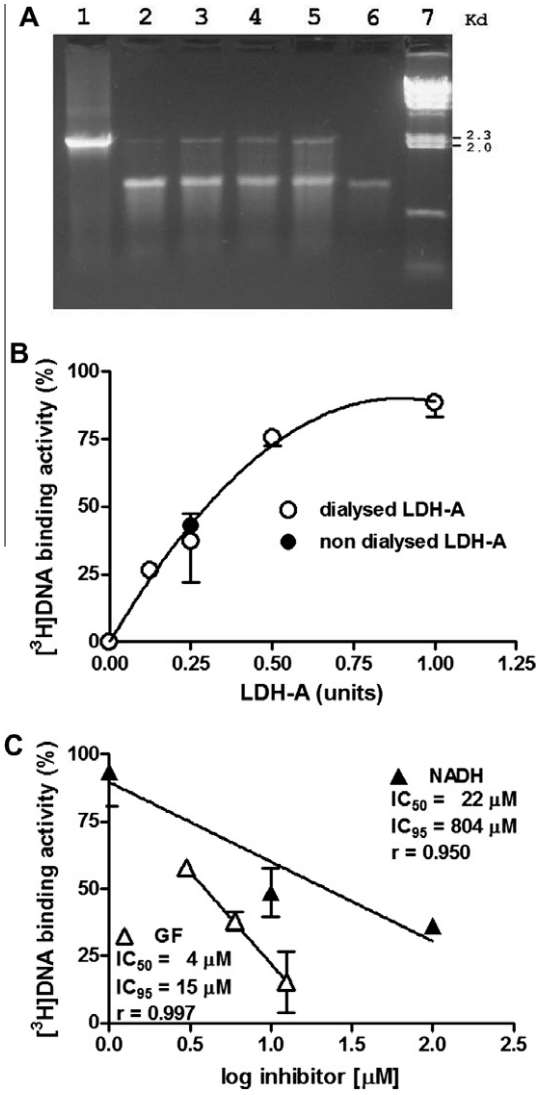

Fig. 2. Inhibition of the binding of LDH-A to ssDNA caused by GF. (A) Gel electrophoresis of the 2251 bp DNA employed in the filter-binding assay. Gel electrophoresis analysis was performed on ethidium bromide containing-1% agarose gel. Lane 1, 2251 bp DNA (450 ng); lane 2, heat denaturated 2251 bp DNA (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 *Hind*III 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 pre-incubation (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.

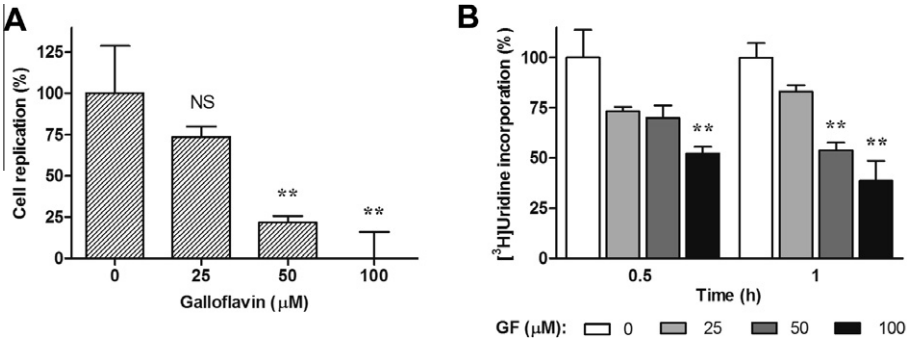

Fig. 3. Effect of GF on replication of SW620 cells and on [^{3}H]-uridine incorporation. (A) Inhibition of SW620 cell replication after 48 h exposure to GF. Each value was obtained from four determinations. Data were fitted using the second order polynomial regression in order to calculate the IC_{50} 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. $^{**}p < 0.01$.

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