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A mixture consisting of OPL-ROS and Galloflavin as an effective inhibitor of energy pathways in cancer cells.

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Summary

Otto Warburg[1] in the early years of the last century, observed that most of cancer cells showed reduced mitochondrial activity and high consumption of glucose with high production of lactic acid at the same time. This metabolic phenotype expressed by cells under anaerobic conditions, manifested in conditions of normal partial pressures of oxygen was named Aerobic Glycolysis and is known today in biochemistry as "Warburg Effect". Aerobic Glycolysis is not just being only observed in cancer cells but also in normal cells growing rapidly because of their metabolic sources required for the production of daughter cells. For some years, the suppression of aerobic glycolysis was considered a possible treatment strategy in cancer control. However, recent studies have shown that genetic ablation of aerobic glycolysis alone in some particular lines of cancer cells do not cause the hoped-for suppression of cell multiplication in response to the sharp reduction of lactic acid[2] production. This study analyzed the response of a cell line with an undifferentiated hepatocellular carcinoma characterized by an aerobic and glycolytic phenotype cultivated in presence of a saturated aqueous mixture of ROS, indicated by the acronym OPL (Poliatomic Liquid Oxygen) containing a non-competitive inhibitor of lactate dehydrogenase, Galloflavin. The inhibitor blocks the action of lactate dehydrogenase and ROS in OPL blocks mitochondrial respiration. The combined effect leads the cells to the inhibition of energetic ways of neoplastic leading them to apoptosis.

Hypothesis

The term cancer defines a cellular condition characterized and sustained by genetic alterations that leads to neoplastic transformation[3].
It is a genomic instability that involves the efficiency of cellular respiration[4] and metabolic reprogramming known as aerobic glycolysis, an energy pathway characterized by a much higher velocity than glycolysis expressed by cells endowed with a normal mitochondrial respiratory activity[5]. It is always the same aerobic glycolysis being expressed by neoplastic cells representing an additional factor in the exaltation of genomic instability[6], causing the appearance of mutations of genes that encode not only enzymes involved in DNA repair[7], but also particular proteins implicated in membrane transport of glucose as in the cell activation proliferation. Well known are the mutations involving the metabolic pathway PI3 / AKT[8] caused a cytoplasmic protein whose activation induces not only cell growth but also the appearance of aerobic glycolysis. Lots of studies have demonstrated the existence of a direct relationship between oxidative genetic mutations, p53 protein activity[9] and apoptosis. This is an important cellular signaling pathway that could be explored for the development of new therapeutic devices in the fight against tumors[10]. The Reactive Oxygen Species (ROS) and their products represent specific indices of cellular metabolic damage responsible for the neoplastic transformation. It is though unexplainable how any

therapeutic ROS contrast approach like massive doses of antioxidants has not yet led to expected results. History of anti-cancer therapy using antioxidants is still full of contradictory results. Failure of the antioxidant strategy in cancer could be attributed to the dual role of ROS, leading on one hand to cell proliferation and on the other to the opposite direction towards the activation of apoptosis and therefore to the reach the point where it could be possible for a ROS anti-cancer antioxidants[11] therapy strategy to work, bringing tumor cells towards apoptosis rather than to their proliferation[12]. This work (investigation) aims to demonstrate that cells cultures of undifferentiated hepatocellular carcinoma when incubated in the presence of ROS and Galloflavin, a non-competitive inhibitor of lactic dehydrogenase (LDH), inhibiting aerobic glycolysis and interfering with mitochondrial respiration directing them towards apoptosis[16], suggesting a new possible therapy strategy against primary liver tumors.

Introduction

In cells with efficient mitochondrial respiration, more than 90% of the molecular oxygen is transformed into water while the remaining is reduced to free superoxide anion which will activate the generational cascade of Oxygen Reactive Species[13] [14]:superoxide ion, hydrogen peroxide (H2O2), hydroxyl radical (OH ·) and the nitric oxide (NO ·). The endogenous production and their different intracellular distribution, makes ROS a possible "gas controllers" of important signaling pathways which leads to proliferation but also to cells apoptosis[16]. In neoplastic cells most mitochondria are unable to make the best use of the oxygen molecule; the electrons of the oxidative phosphorylation are diverted towards an increased production of superoxide ions, amplifying the oxidative respiratory stress[17], inducing mutations that will lead the p53 protein to direct the neoplastic cells towards apoptosis. Regarding this, many blood and urine studies of cancer pacients have shown a high levels of purine and oxidized pyrimidine (evidencing a serious index of oxidative damage in nucleic acids[18]). However it is still unexplainable why the approaches made by any contrast ROS therapy administrating massive doses of antioxidants, have not yet led to the expected therapeutic successes. It can be said with certainty that the history of anti-cancer therapy is more studded with contradictory than coherent data[19]. Information suggests that the failure of the antioxidant strategy in cancer can be attributed to the dual role of ROS, on one side leading to cell proliferation and on the other in the opposite direction and therefore to the activation of apoptosis offering a possible path for the antioxidant anti-cacer therapy strategy with ROS[20] [21] [22] possible. The genomic instability underlying the neoplastic transformation due to oxidative damage[23] is mainly caused by hydroxyl ions which in addition affects the chromatin proteins involving mainly the purines and especially the guanine; oxidative damage, especially due to the release in the blood and urine of 8-oxoguanine (8-oxoG) in patients with cancer[24]. It has also been observed that one of the causes of genomic instability and leading to neoplastic transformation is the presence of mutations affecting the TP53 gene. The TP53 gene encodes the tp53 phosphoprotein: a transcription factor able to regulate the cell cycle in the presence of ROS[25][26]. In the early phases of neoplastic transformation, the tp53 protein is able to prevent DNA mutations by ROS, reducing instability by suppressing tumors in their early phases. In the late phases of neoplastic transformation when DNA mutations are serious to the point that it is no longer possible to repair them, the protein is able to direct the neoplastic cell towards apoptosis[27]. Another important function of tumor suppression by ROS is the inhibition of angiogenesis through the action phosphoprotein tp53[28] .

Some observations indicate a possible relationship between the aerobic glycolysis of the neoplastic cells and the tissue oxidative state[29]. Neoplastic cells generally express an anaerobic metabolism despite the presence of normal partial oxygen pressures. This is the reason for it to be called "aerobic glycolysis" [30]. Although the homolactic fermentation is characterized by a low energy yield, in cancer cells is highly enhanced by a high level of

glycolytic enzymes[31]. Cancer cell glycolysis is one hundred times higher than normal. This gives the cancer cells a higher resistance even in conditions of poor or incomplete oxygenation as often happens in solid tumors[32]. In addition to this, aerobic glycolysis is particularly advantageous for cells in active replication including cancer cells as its intermediate metabolites are the precursors necessary for the duplication of nucleic acids and for the structures that will constitute the new daughter cells through the way metabolism of pentose phosphate[33]. The presence of aerobic glycolysis in tumor cells causes an abundant production of lactic acid which induces an acidification of the microenvironment, representing an important factor facilitating tumor invasion as for the acid degradation of the extracellular matrix and for the strong induction that the extracellular acidosis plays on the phenomenon of angiogenesis. Cells that compose a tumor mass can be found under two conditions of metabolism: one called "aerobic condition" characterized by an environment with normal partial oxygen pressures in which the pyruvate is completely oxidized to CO_2 and H_2O through the cycle of citric acid and the electron transport chain; the other one and more frequent called "aerobic glycolysis", consists of an energetic metabolic reprogramming performed by cells that in environmental conditions characterized by adequate partial oxygen pressures expresses the homolactic fermentation[34]. Lactic dehydrogenase (LDH) is the most important enzyme in aerobic glycolysis. This enzyme converts the pyruvate produced by glycolysis into lactic acid, reoxidizing the NADH obtained from Glyceraldehyde-3-phosphate dehydrogenase (GAPDH) thus supporting continuous flow of glycolysis which requires a constant addition of oxidized NAD as an electron acceptor[35]. The result of the enzymatic activity of the Lactic Dehydrogenase is a high production and accumulation of lactic acid associated with a high acidosis. Lactic dehydrogenase (LDH) is an enzyme that is ubiquitously present including humans. It is present in 5 different isoforms all tetrameric and formed by the combinations of two different types of subunits, M and H, encoded by two different ldh-a and ldh-b genes. It is the different combination of these monomers to form two homotetrameric isoforms called LDH-A (M4) and LDH-B (H4) and three heterotetrameric isoforms LDH-2 (M1H3), LDH-3 (M2H2), LDH-4 (M3H1) [36]. We also recognize in humans a different LDH-X isoform (C4) whose monomer is encoded by the ldhc gene and is phenotypically expressed mainly in the testes and spermatozoa. The five isoforms of LDH besides presenting a different tissue distribution[37], also have a different intracellular compartimentation and differ both in enzymatic kinetics and in their regulation[38][39]. The tissues characterized by an intense aerobic metabolism such as those of brain and heart, express isoforms constituted for the most part by H subunits such as LDH-B (or LDH-1) [40] and LDH-2 while isoforms composed mainly of M subunits (LDH-A or LDH-5 and LDH-4) are expressed especially in tissues that often undergo anaerobiosis conditions such as tumor tissues or skeletal liver and muscle cells[41]. The LDH-3 isoform is expressed in malignant tumor tissues and lymphatic tissues, whereas the LDH-A isoform is overexpressed in high proliferative neoplastic cells; this is associated with a high aerobic glycolytic velocity and the consequent high production of lactic acid.

This study aims to evaluate "in vitro" a possible antitumor strategy through the use of a saturated aqueous mixture of superoxide ion and other trace ROS and of galloflavin, which inhibits the lactic dehydrogenase[42][43] without damaging the healthy cells which with sufficient oxygen supply will not need this enzyme[44]. In support of this hypothesis, there is evidence that tumor cells with reduced LDH activity obtained by shRNA or si-RNA show a reduction in their carcinogenicity

Materials and methods

Polyatomic Liquid Oxygen (OPL).

OPL, an acronym for Oxygen Polyatomic Liquid is used to indicate a bidistilled, sterile and apyrogenic aqueous mixture in which ROS has been dissolved at a gram / liter obtained through a sputtering OPL-ROS generator Medical Device (DMe) n ° 000CO2017809000053 produced by Barcoline srl licensed by the Istituto Internazionale Barco S.p.A.The OPL-ROS production technology is patented. The OPL technology is based on the diffusion of a gaseous mixture rich in ROS produced in sputtering, sifted through a polysulfone semipermeable polymeric filter (PES) in countercurrent with respect to a flow of sterile water for injections. The membrane (PES) is a nonionic exchange material which allows to block the positive ions by means of the sulphonated groups while the ROS anions pass selectively into the bidistilled water solution, forming a saturated aqueous solution of ROS at 1g / L molality which can be used during the production of the mixture as a source of reactive oxygen species in the aqueous phase. The membranes for the production of OPL-ROS used in this study have been produced in such a way as to be characterized by a marked stability against oxidizing agents, a range of resistance to pH between 3 and 12 and the ability to inhibit any proliferation bacterial. Using the analytical methods and the probes subsequently listed, it has been shown that OPL is a saturated solution of ROS containing: superoxide anion ($O_2\text{-} \bullet$), OH radicals, NO radicals and singlet oxygen. With the same analytical methods the decay of the oxidative capacity of the OPL-ROS mixture was evaluated which corresponds to a half-life of about 6 hours, half-life that depends on the temperature, the pH and the purity of the bidistilled water. Singlet oxygen was detected by a specific probe (S36002-Life Technologies). This probe shows no appreciable response to the hydroxyl radical or superoxide. This indicator initially shows a weak blue fluorescence, but in the presence of singlet oxygen, it emits a green fluorescence (λexc = 488 nm; λem = 525 nm) similar to that of fluorescein. The superoxide anion ($O_2\text{-} \bullet$) was detected using ferrocytochrome c (from horse core C7752, Sigma-Aldrich Milan Italy) as a probe. The superoxide anion reacts by reducing f ferrycytochrome to ferrocytochrome and this reaction is followed by absorption spectroscopy at 550 nm. The superoxide anion was also revealed in UV at 258 nm (ε = 1010 M-1 cm-1 at 240 nm). Nitric oxide was determined using the DAF-2 probe (4,5-diaminofluorescein-2, D224 Sigma Aldrich Milan, Italy). In the presence of NO the DAF-2 becomes DAF-2T and increases the emission at 515 nm (λex = 485 nm).
Terephthalic acid (THA 185361 Aldrich) has been used as a specific probe for hydroxyl radicals. OH radicals convert terephthalic acid into 2-hydroxyterephthalic acid (HTA) [12, 13]. HTA was detected by fluorescence measurement (λexc = 310nm, λem = 425nm).

Galloflavin synthesis (CAS 586-80-9)

Galloflavin is a molecule that inhibits aerobic glycolysis through a non-competitive block for the pyruvate of LDH-A and LDH-B45. In addition, galloflavin has also the ability to reduce ATP production without interfering with cellular respiration. Galloflavin has the ability to penetrate undifferentiated hepatocellular carcinoma cells obtained from a primary neoplastic lesion from a male patient's lobe VIII 47 47. Since it was not available on the market, galloflavin was synthesized according to the acid synthesis procedure. gallic through oxidation in an alkaline environment (KOH) (pH> 10) in hydroalcoholic solution45, while the purity of galloflavin was determined to 99.5% by HPLC-DAD-TOF system (ESI + and ESI-)

Measurement method of lactic acid variations in cell cultures

The phosphoric acid for HPLC analysis was prepared from sodium phosphate monobasic monohydrate (BDH Laboratory Supplies, Poole, England) and phosphoric acid (V800287 VETEC ≥85%). Methanol for reverse phase chromatography (RP-HPLC) was purchased by Carlo Erba (Rodano, MI, Italy). The preparation / dilution of samples and solutions were

performed gravimetrically with ultrapure water (MilliQ, 18.2 MΩ cm-1 at 25 ° C, Millipore, Bedford, MA, USA).

Lactate analysis by RP-HPLC with UV detection
An HPLC system (1260 Infinity Agilent Technologies) with an autosampler, mechanical degaser and UV / vis diode array detector at 210 nm (volume injected 10 µL) were used for lactate analysis. The separations were carried out using a RP HPLC column Hydra RP C18 (Phenomenex) 250 x 4.6 mm (silica particle size 4 µm) in isocratic elution in 0.1% phosphoric acid and flow 0.8 ml min-1 . The chromatographic run is complete in 15 minutes.

Measurement of oxygen variations in cell cultures
In this study, mitochondrial respiration was evaluated through the consumption of oxygen in 24 hours in cell cultures of undifferentiated hepatocarcinoma being incubated in the absence of galloflavin and OPL-ROS (graph 1, control), in presence of 50 µM of OPL-ROS (graph 2), 200 µM of galloflavin (graph 3), 50 µM of OPL-ROS and 200 µM of galloflavin (graph 4). The oxygen variations were measured with PeriFlux Sistem 5000 (Perimed), Tc electrode sensor E5280-tcpO2 / tcpCO2 (REF-945-377) RADIOMETER. Fixing ring of the TC550 Perimed electrode. PSW ExM software.

Measurement of ATP in cell cultures
For the measurement of ATP production a MAK190-Adenosine triphosfate assay kit (Sigma-Aldrich) was used consisting of: ATP Assay Buffer 25 ml, ATP Probe, DMSO 0.2 mL, ATP Converter 1 vl, Developer Mix 1 vl, standard ATP 1.0 mmol 1 vl.

Cell culture of undifferentiated hepatocarcinoma cells
The undifferentiated hepatocarcinoma cells were obtained from a primary lesion placed in the hepatic lobe VIII and withdrawn through a 15 G needle. The cells were subsequently separated by trypsinization and suspended by centrifugation and washing in PBS. They were cultured with DMEM and / or 25 mM glucose. All cell growth media were supplemented with 10% FBS, non-essential amino acids, 100 U / mL of penicillin G and 100 µg / mL of streptomycin and 2mM L-glutamine. The cells were cultured at 37 ° C in a 5% humidified CO2 incubator. The cells were seeded in 75 cm2 piaste and used during their exponential growth phase. All experiments were performed on cells after 24 hours sowing.

Measurement of the intracellular content of OPL-ROS
Intracellular ROS-OPLs were measured through oxidation-sensitive fluorescent probes DHE and DCFH-DA. After undifferentiated hepatocarcinoma cells were seeded in 6-well multi-well plates at a concentration of 2 x 106 per well. The DHE probe detects the presence of superoxide anion. The DHE probe was added in the dark at different times until reaching the final concentration of 10 µM and then after 30 minutes adding 50 µM OPL-ROS.
During the dark probe, DCFH-DA probe was added to the final concentration of 10 µM, 30 minutes before the end of incubation with 50 µM of OPL-ROS. The cells were washed with PBS and suspended in 500 µL of PBS and then subjected to measurement of ROS-OPL contents by fluorimetric analysis with a fluorescence spectrophotometer. For the DHE probe the excitation wavelength of 392 nm was used, while the emission one of 538 nm was used. The excitation and emission window was set as for DHE probe and DCFH-DA to 10 nm. The use of these two probes showed a prolonged increase in fluorescence intensity only in the cells treated with the OPL-ROS mixture.

Determination of the PUMA expression by Western Blotting.
The PUMA48 test makes it possible to distinguish whether the growth inhibition of a cell culture of undifferentiated hepatocellular carcinoma is due to a cytostatic effect or to cell

death of the galloflavin-OPL-ROS mixture. For this purpose, 75 cm2 flasks were seeded with 2 x 106 cells of undifferentiated hepatocarcinoma cultivated with DMEM and / or 25 mM of glucose. All cell growth media were supplemented with 10% FBS, non-essential amino acids, 100 U / mL of penicillin, 100 g / mL of streptomycin, 2 mM glutamine. The cells were cultured at 37 ° C in a 5% humidified CO2 incubator.

For the PUMA test, the following were set up:

- 2 x 106 cells of undifferentiated hepatocellular carcinoma cell cultures seeded in 75 cm2 flasks and treated with 50 mM ROS of the OPL mixture (RosOPL)
- 2 x 106 cells of undifferentiated hepatocellular carcinoma cell cultures seeded in 75 cm2 flasks and treated with 200 µM galloflavin.
- 2 x 106 cells of undifferentiated hepatocellular carcinoma cell cultures are seeded in 75 cm2 flasks and treated with 200 µM galloflavin and 50 mM ROS of the OPL mixture (Galloflavin-RosOPL)
- The control is performed with 2 x 106 cells of undifferentiated hepatocellular carcinoma cell cultures seeded in 75 cm2 and 10 µM flasks of the OPL mixture without ROS and without galloflavin.

Daily for four days the treated cells and control cells are lysed with 100 µL of potassium phosphate buffer (100 mmol / L) at pH 7.5, containing 1% of NP40 and protease inhibitors. Once the homogenate of cells are obtained, it are centrifuged for 15 minutes after allowing it to stand for 30 minutes in the ice. With the Lowry method, 20 µg of protein centrifuge are measured and charged to the 12% polyacrylamide gel and subjected to electrophoresis. After the run of the proteins through an electric field of 300 mA for 2 hours, they are transferred to a low fluorescence PVDF membrane. Once the whole is immobilized with 5% BSA in TBS Tween, the membrane is incubated with an anti-PUMA polyclonal rabbit antibody, the protein-antibody binding will be evidenced by a secondary antibody labeled with the Cy5 fluorophore (A. Biosciences).

Determination of apoptosis

The apoptosis of undifferentiated hepatocellular carcinoma cells was assessed by the cytofluorimetric study of nuclei with hypodiploid DNA content.

Undifferentiated Hepatocarcinoma cells are incubated in 96 multi-well plates at the concentration of 1 X 104 cells per well and subsequently, are incubated for 24 hours with 200 µM galloflavin, respectively, with the OPL-ROS mixture at a dose of 50 µM, with the 200 µM mixture of galloflavin combined with 50 µM of OPL-ROS and for cell-only control without treatment. The cells are then detached by trypsinization and suspended by centrifugation and washing in PBS. The pellet obtained is resuspended in a hypotonic lysis solution containing 50 µg / mL of propidium iodide. After incubation at 4 ° C for 30 'cells are subjected to cytofluorimetric analysis to evaluate the presence of nuclei containing hypodiploid DNA.

Statistic analysis

The significance of the data was analyzed through the Student t test and one-way Anova followed by the Bofferoni post-tests.

RESULTS

In order to evaluate the inhibitory action of galloflavin on the enzymatic activity of lactic dehydrogenase, in this study the variations in lactate production were analyzed when cells of undifferentiated hepatocellular carcinoma44 equipped with an aerobic phenotype are incubated with this inhibitor. Lactic dehydrogenase (LDH) is a key enzyme of aerobic glycolysis as it guarantees the necessary NAD + for its maintenance, re-oxidizing NADH through the reduction of pyruvate to lactic acid. In this study, hepatocellular carcinoma cells,

incubated for 24 hours with a concentration of 200 µM of galloflavin expressed a clear reduction in the production of lactic acid, in line with the data available in the literature. That confirms the strong inhibitory function on the lactic dehydrogenase and in consequence the blocking of the aerobic glycolysis of neoplastic cells.

In the same study the inhibitory action on the lactic dehydrogenase of the aqueous mixture OPL-ROS saturated with ROS at the molality of 1000 mg / liter was also verified. Cell cultures of undifferentiated hepatocellular carcinoma were incubated for 24 hours with 50 µM of OPL-ROS verifying the synthetic activity of the lactic dehydrogenase through the quantification of lactic acid synthesized in culture. The lactic acid synthesis expressed by the undifferentiated hepatocellular carcinoma cells has not been influenced by the presence of OPL-ROS up to the point where it can be sustained that the lactic dehydrogenase is not inhibited by ROS. (Figure 1)
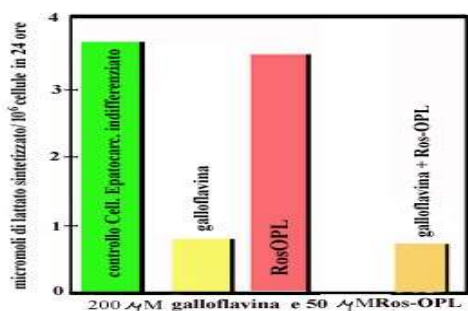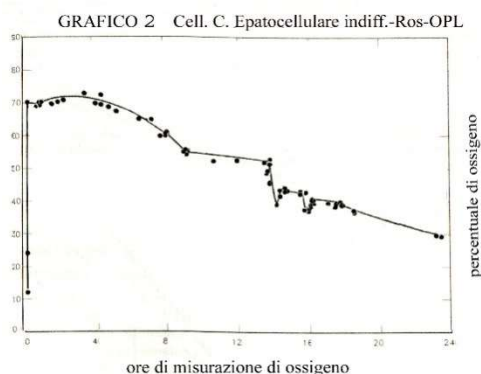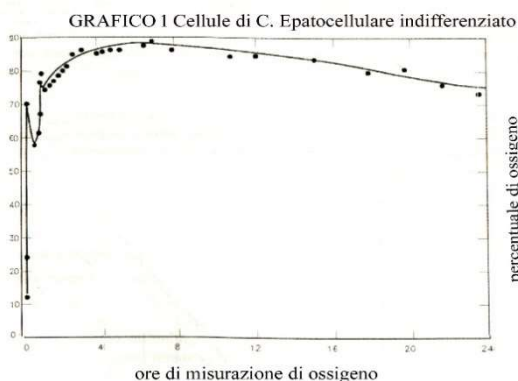

Figure 1. In this study the results obtained demonstrate a clear inhibitory action of galloflavin on lactic dehydrogenase, when the undifferentiated hepatocellular carcinoma cells capable of expressing the aerobic glycolytic phenotype is incubated for 24 hours with 200 µM of galloflavin. Always the same cell cultures when incubated with 50 µM of a saturated aqueous solution of OPL-ROS, are not inhibited in the synthesis of the lactic dehydrogenase. Also in the same study it was highlighted that there is no synergistic action towards the lactic dehydrogenase, when the neoplastic cells are incubated with the same amount of OPL-ROS and galloflavin.

To verify if the galloflavin in addition to performing a blocking action on aerobic glycolysis is also able to interfere on mitochondrial respiration, both oxygen consumption and ATP production in undifferentiated hepatocellular carcinoma cell cultures were measured when incubated with 200 µM of galloflavin. The measurement of oxygen consumption was performed on the four cell cultures, including the control culture consisting solely of undifferentiated hepatocarcinoma cells during 24 hours incubation. Through a probe it was possible to continuously measure the oxygen consumption of cells in order to evaluate the possible interferences that galloflavin or the mixture of OPL-ROS or the galloflavin-OPL-ROS mixture have on mitochondrial respiration[45] (Figure 2).
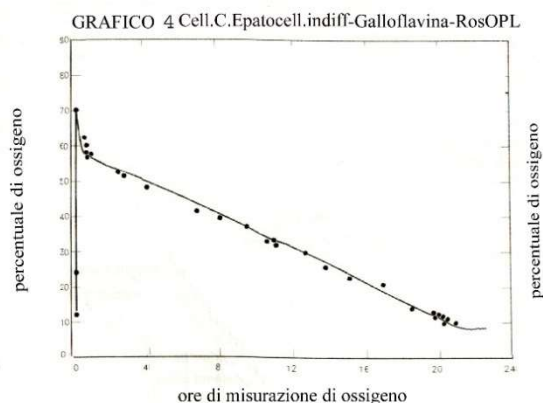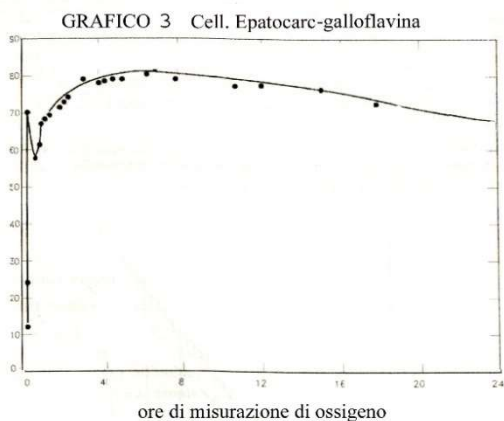
Figure 2. Oxygen consumption in 24 hours. Graph 1: Control of undifferentiated hepatocellular carcinoma cells shows normal oxygen consumption. Graph 2: incubating for 24 hours, cultures of undifferentiated hepatocellular carcinoma cells, with 50 µM of ROS of the OPL-ROS mixture, a regular reduction of oxygen consumption is observed over time. Graph 3: incubating for 24 hours, cultures of hepatocellular carcinoma cells, with only 200 µM of galloflavin, no effect on oxygen consumption is observed. Graph 4: incubating for 24 hours, cultures of hepatocellular carcinoma cells with 200 µM of galloflavin and 50 µM of ROS of the OPL-ROS mixture, a gradual reduction in oxygen consumption is observed.

In this case, it was also considered useful measure the production of ATP in culture since most of the production of cellular ATP even in neoplastic cells is due to the oxidative activity of mitochondrial respiration. Regarding this, the stoichiometric data indicated that for each glucose molecule oxidized in glycolysis alone, there is a net production of only two ATP molecules, while in glycolysis it is associated with mitochondrial respiration leading to a net gain for each glucose molecule at 36 molecules of which 34 of ATP derive from mitochondrial respiration activity. In this study, in cultures of undifferentiated hepatocellular carcinoma cells incubated with galloflavin only, just a slight flexion of ATP production was measured since it indicates that the neoplastic cells are still capable of expressing a mitochondrial oxidative energy metabolism capable of keeping them alive; therefore it is shown that galloflavin, although without toxic effects on normal cells is however ineffective for the treatment of tumors with an aerobic phenotype (figure 3).
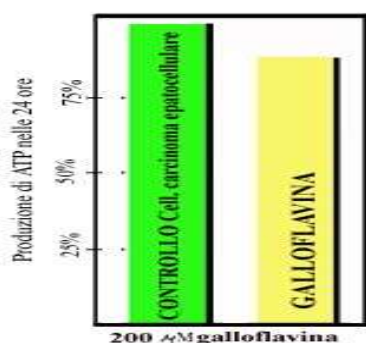

Figure 3. By incubating for 24 hours cultures of hepatocellular carcinoma cells with 200 µM of galloflavin, only a slight decrease in ATP production is observed.

In this study the inhibition of the energy yield of mitochondrial respiration was studied through the reduction of ATP synthesis when the cultures of hepatocellular carcinoma cells were incubated for 24 hours with the only mixture of OPL-ROS at a dose of 50 µM . The decline in the production of ATP synthesis suggests that the ROS contained in the OPL-

ROS mixture were able to interfere with the ATP synthesis of mitochondrial respiration, an action not expressed, as mentioned, by galloflavin (Figure 4).
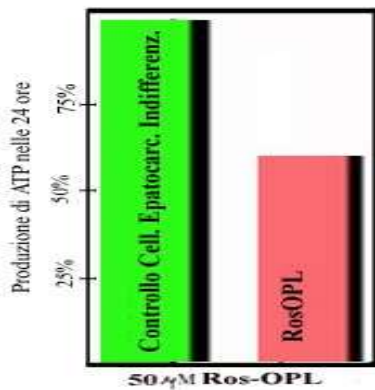

Figure 4. By incubating for 24 hours hepatocellular carcinoma cell cultures with 50 µM of ROS contained in the OPL-ROS mixture, an effective depression of ATP synthesis is observed.

Finally, to verify a possible synergy between galloflavin and ROS, a mixture consisting of 200 µM galloflavin and 50 µM of OPL-ROS was used in order to inhibit the energetic synthesis of cells of undifferentiated hepatocellular carcinoma, directing them towards cell death (apoptosis and / or necrosis). The obtained data, incubating the cells of undifferentiated hepatocellular carcinoma with the galloflavin-OPL-ROS mixture and measuring the reduction of ATP synthesis in 24 hours, confirmed a possible synergy of OPL-ROS-galloflavin in inhibiting the energetic syntheses of neoplastic cells which express aerobic glycolysis (Figure 5).
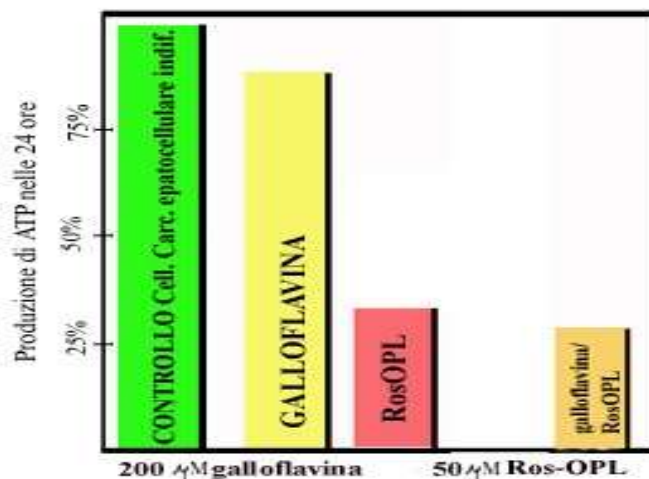

Figure 5. Falling of ATP synthesis, observable when the cells of undifferentiated hepatocellular carcinoma are incubated for 24 hours with 50 µM of OPL-ROS and 200 µM of Galloflavin, indicating a possible cellular energy block, which can be used to counteract the proliferation of neoplastic cells.

The unexpected synergistic inhibitory action of the mixture OPL-ROS and galloflavin in cells cultures of undifferentiated hepatocellular carcinoma on the energetic synthesis of ATP and on the inhibition of cell growth, allowed to continue the studies with the PUMA-48 test. A laboratory study that makes possible to distinguish whether the inhibition on the growth of a cell culture is due to a cytostatic effect or to cell death. The results obtained showed a 3.6-

fold increase in PUMA expression for the undifferentiated hepatocellular carcinoma cells treated with the galloflavin-OPL-ROS solution (Figure 6).
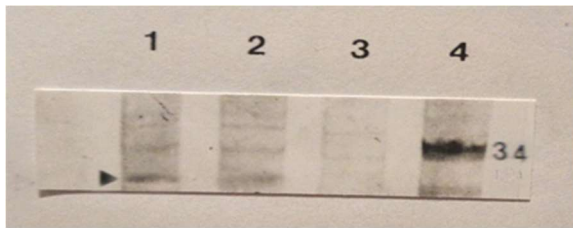

Figure 6. Results obtained with Western Blotting techniques showed an increase of 3.6-fold PUMA expression for undifferentiated hepatocellular carcinoma cells treated with galloflavin-OPL-ROS (1) solution, of 2.8 for cells treated with the only OPL-ROS solution (2) and 1.8 for cells treated with galloflavin alone (3). In this case the PUMA survey showed that the inhibition of the cell growth treated with the 200 µM galloflavin solution and 50 µM of ROS contained in the OPL-ROS mixture is caused by cell death and not by a cytostatic effect of the solution.

Cytotoxic effect of galloflavin

Morphological alterations induced by incubation for 24 hours with 200 µM galloflavin of undifferentiated hepatocellular carcinoma cells are compared with a control culture made up of the same cells but not treated with galloflavin. The cells from both cultures are incubated with propidium iodide and analyzed by cytofluorimetry.

Cytofluorimetric analysis of undifferentiated hepatocellular carcinoma cells incubated for 24 hours with 200 µM galloflavin compared to control culture cells showed a 43% increase in the number of apoptotic nuclei.

Cytotoxic effect of the OPL-ROS solution

The morphological alterations induced on the undifferentiated hepatocellular carcinoma cells by incubation for 24 hours with 50 µM of OPL-ROS are compared with a control culture made up of the same cells but not treated with OPL-ROS. The cells from both cultures are incubated with propidium iodide and analyzed by cytofluorimetry.

Cytofluorimetric analysis of undifferentiated hepatocellular carcinoma cells incubated for 24 hours with 50 µM of OPL-ROS compared to cells in the control culture showed a 47% increase in the number of apoptotic nuclei.

Cytotoxic effect of the Galloflavin-OPL-ROS solution

The morphological changes induced on the undifferentiated hepatocellular carcinoma cells by incubation for 24 hours with a mixture of 50 µM of OPL-ROS and 200 µM galloflavin, are compared with a control culture consisting of the same cells but not treated with OPL-ROS. The cells from both cultures are incubated with propidium iodide and analyzed by cytofluorimetry.

Cytofluorimetric analysis of undifferentiated hepatocellular carcinoma cells incubated for 24 hours with a mixture of 50 µM of OPL-ROS and 200 µM galloflavin, compared to the cells of the control culture showed a 90% increase in the number of apoptotic nuclei

Discussion

The data obtained in this study confirm that the cells of undifferentiated hepatocellular carcinoma are still equipped with an efficient mitochondrial respiration, one of the main causes for which the inhibitors of the lactic dehydrogenase, such as galloflavin, are not sufficient in the fight against tumors. Confirming this was the found of galloflavin in

hepatocellular carcinoma cells while causing a blockage of aerobic glycolysis. As confirmed by the documented reduction in lactate production, it does not cause a significant reduction in ATP synthesis and neither an evident decline in oxygen consumption, indicators of a mitochondrial respiratory activity still able to support neoplastic cells in their vital energy requirements. In this study, we wanted to overcome this important limitation of aerobic glycolysis inhibitors, associating a saturated aqueous solution of ROS to galloflavin, indicated as Liquid Poliatomic Oxygen (OPL) which interfering with the mitochondrial respiration of cancer cells is able to drastically reduce the intracellular levels of ATP by targeting neoplastic cells to death by apoptosis.

Conclusions

This study shows that an effective treatment of neoplasms must not necessarily proceed only through the blocking of mitochondrial respiration from which almost all cellular energy derives, but must also operate the inhibition of aerobic glycolysis which through the pentose cycle supports the synthesis of ribose necessary for the production of nucleotides which are essential to support rapid cell proliferation. For this purpose the association of an aerobic glycolysis inhibitor such as galloflavin with an aqueous mixture of OPL-ROS, could represent an effective antineoplastic treatment, even if it is necessary for further investigate modalities of ROS inhibition on oxidative phosphorylation since they can damage mitochondria isolated from normal cells.

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