The effect of CELLFOOD™ on radiotherapy or combined chemoradiotherapy: preclinical evidence

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Abstract

Background: Based on previous observations that the nutraceutical CELLFOOD™ (CF), the 'physiological modulator' that aimed to make oxygen available 'on demand', inhibits the growth of cancer cells, this study was designed to investigate the role of CF in the regulation of hypoxia-inducible factor 1 alpha (HIF1α) and its correlated proteins, phosphoglycerate kinase 1 and vascular endothelial growth factor. Our idea was that CF, acting on HIF1α, in combination with current anticancer therapies could improve their effectiveness.

Methods: To evaluate the effect of CF in association with radiotherapy and chemotherapy, different human cancer cell lines and mice with mesothelioma were analysed by tumour growth, clonogenic assay, western blot and immunohistochemical analysis.

Results: CF in combination with radiation with or without cisplatin increases the death rate of cancer cells. In vivo, 70% of mice treated with CF before the mesothelioma graft did not show any tumour growth, indicating a possible preventive effect of CF. Moreover, in mouse mesothelioma xenografts, CF improves the effect of radiotherapy also in combination with chemotherapy treatment. Immunohistochemical analysis of tumour explants showed that HIF1α expression was reduced by the combination of CF and radiotherapy treatment and even more by the combination of CF and radiotherapy and chemotherapy treatment. Mechanistically, CF increases the fraction of oxygenated cells, making the radiotherapy more effective with a greater production of reactive oxygen species (ROS) that in turn, reduce the HIF1α expression. This effect is amplified by further increase in ROS from chemotherapy.

Conclusions: Collectively, results from preclinical trials suggest that CF could be a useful intervention to improve the efficacy of radiotherapy or combined treatment strategies and could be a promising treatment modality to counteract cancer.

Keywords: combined treatment strategies, CELLFOOD™, HIF1α, mesothelioma, radiosensitivity

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Introduction

Cancer is one of the leading causes of death worldwide. In 2012, there were 8.2 million cancer-related deaths and 14 million new cases worldwide. The numbers are predicted to increase, with 26 million new cancer cases and 17 million cancer deaths expected in 2030.1,2

Cancer prognosis depends on various factors, including the location, type, the grade, stage and response to treatments. Some cancer types are curable; others that are diagnosed early are treatable by surgical removal, while certain types, especially those that miss early diagnosis, have a devastating outcome. Some cancer types are difficult to diagnose at an early stage due to the long latency period and nonspecific symptoms and after diagnosis, are resistant to conventional and multimodal treatments.3-6 The complex molecular biology of cancer makes treatment difficult;
Therefore, efforts to develop more effective and less toxic treatments or to improve existing therapies such as chemotherapy and radiotherapy (RT) are critical to improve outcomes for cancer patients.

Most solid tumours develop regions of hypoxia or oxygen deficiency due to a mismatch between tumour growth and angiogenesis. Severe or prolonged hypoxia results in the death of normal as well as cancer cells. Cancer cells can adapt to the hostile environment and this adaptability benefits predicting the malignancy and aggression of a tumour. Cancer cells adapt to hypoxia and trigger a number of hypoxia-related molecules and pathways; the main one among them is the hypoxia-inducible factor 1 (HIF1). HIF1 is a heterodimeric protein with the O2-regulated HIF1α subunit and the constitutively expressed HIF1β subunit. Under normoxic conditions, the hydroxylation and the acetylation of two prolines and a lysine residue, at its oxygen-dependent degradation domain, promote the interaction of HIF1α with the von Hippel-Lindau (pVHL) ubiquitin E3 ligase complex and thus marks HIF1α for degradation by the ubiquitin–proteasome system. However, under hypoxic conditions, the low availability of oxygen results in the inhibition of prolyl hydroxylase activity and, consequently, in increasing HIF1α stability. HIF1 is overexpressed in common cancers and regulates the transcription of several genes involved in biological processes, such as angiogenesis, cell proliferation and survival, pH regulation and apoptosis. HIF1α is a central molecule involved in mediating cellular processes.

The induction of angiogenesis represents one of the major hallmarks of carcinogenesis since the growth of new vessels is crucial in providing malignant cells with an adequate supply of oxygen and nutrients. It is generally accepted that in physiological and pathological processes the vascular endothelial growth factor (VEGF) is a major inducer of the angiogenesis. Under oxygen-limited conditions, solid tumour cells utilize glycolytic enzymes such as phosphoglycerate kinase 1 (PGK1) to produce adenosine triphosphate (ATP). Several malignancies, including prostate cancer, breast cancer, pancreatic ductal adenocarcinoma, multidrug-resistant ovarian cancer and metastatic gastric cancer, have all been shown to exhibit an increased expression of PGK1. PGK1 is regulated by HIF1α. Therefore, inhibition of HIF1α synthesis or activity can contribute to blocking tumour angiogenesis, glycolysis and tumour-cell growth. Hypoxia in solid tumours is associated with resistance to radiation therapy and chemotherapy but is also related to a poorer outcome regardless of treatment modality. This evidence has prompted researchers to develop therapeutic strategies that target hypoxic cells by modifying the hypoxic microenvironment or targeting components of the HIF1 signalling pathway.

In this context, CELLFOOD™ (CF) provided by Eurodream srl (La Spezia, Italy), the ‘physiological modulator’ aimed at making oxygen available ‘on demand’, could increase the tumour partial pressure of oxygen. CF (also known as Everett Storey Formula Deutrosulfazyme™), is a nutraceutical supplement whose constituents, including purified water, Lithothamnium calcar-eum alga extract, thallus, trace elements, enzymes, amino acids and sulphates of hydrothermal origin, acidifier: (D2SO4) E513 0.009%, sodium selenite, are all naturally occurring substances which are essential to the body’s biochemical functions. In cells, CF mixture was always tested for the increased bioavailability of oxygen. Deuterium sulphate has been described as an original formula able to increase intracellular oxygen availability but synergies with the other components cannot be excluded. CF is a preparation capable of modulating the bioavailability of oxygen in cells by increasing ‘on demand’ the levels in case of hypoxia or lowering its concentration in case of hyperoxia. In fact, the increased level of molecular oxygen obtained by adding CF to distilled water shows that the formulation is able to produce oxygen from scratch, starting from the same water molecules. The synergistic action of deuterium sulphate and other components of CF, in particular, the enzymes with oxide-reductase action, create optimal conditions for the generation of molecular oxygen. CF induces death in several human tumour cells without damaging healthy cells.

In endothelial cells that are refined O2 sensors, the ability of CF to modulate O2 availability and mitochondrial respiratory metabolism without affecting their viability, and to inhibit HIF1α activation by hypoxia, was highlighted. Other studies indicate that CF treatment in leukaemia cell lines induces cell death due to apoptotic mechanisms and altering cell metabolism through HIF1α and glucose transporter 1 regulation. Based on this, and knowing the oxygenating
action of CF, we assumed that it can sensitize cancer cells to standard therapy through HIF1α modulation.

Pertinent to this, there are no studies investigating the effect of CF in combination with RT with or without chemotherapy. In our study, we show that CF reduces the expression of HIF1α and PGK1 and VEGF in colon carcinoma, tongue squamous carcinoma and mesothelioma cell lines. In addition, we assayed the effect of CF alone and in association with radiation on viability of these cell lines as well as on lung adenocarcinoma and breast adenocarcinoma.

Cisplatin (CISP) is a well-known chemotherapeutic drug currently in use for treatment of numerous solid tumours and it is one of the most commonly used agents for radiosensitization. Hypoxia increased resistance to CISP and hypoxia-induced chemoresistance is reversible after reoxygenation. In addition, mesothelioma and tongue squamous carcinoma cell lines, representing two cancer cell lines for receiving CISP as therapy, when tested for the association CF and CISP showed an additive effect on cellular death. Finally, the effect of CF alone or in association with irradiation with or without standard therapy was assayed in xenograft mesothelioma mouse model.

**Methods**

**Cell lines and materials**

Human colon carcinoma (HCT-116), squamous carcinoma of tongue (Cal27), lung adenocarcinoma (Calu3), breast adenocarcinoma (MDA-361) and mesothelioma (MSTO-211H, briefly MSTO) from the American Type Culture Collection (ATCC) were cultured according to ATCC protocols and gradually conditioned in Dulbecco’s Modified Eagle Medium/F12 + Glutamax (Invitrogen Life Technologies, Paisley, UK) supplemented with 10% foetal bovine serum and antibiotics and maintained at 37°C and 5% CO₂.

CF (liquid) was kindly provided by Eurodream srl (La Spezia, Italy) and stored at room temperature. CF was diluted in phosphate buffered saline (PBS) and sterilized using a 0.20 μm syringe filter before use. CISP and pemetrexed were kindly provided by Manipulating Cytotoxic Chemotherapics Unit of our Institute.

**Cell treatment**

**Hypoxic treatment.** Cobalt dichloride, CoCl₂, a hydroxylase inhibitor, promotes a response similar to hypoxia. For the CoCl₂ experiments, 1 × 10⁶ cell lines were seeded in 10 cm diameter dishes 24 h before being treated with 0 and 100 µmol/l CoCl₂ for 4 h and 6 h. For the other experiments, cells were treated with or without 100 µmol/l CoCl₂ and CF (5 µl per ml of medium corresponding to a 1:200 dilution) for 24 h.

**Irradiation treatment (RT).** HCT-116, MSTO, Cal27, Calu3, MDA-361 cells were cultured in a T25 flask and after 24 h, CF (5 µl per ml of medium corresponding to a 1:200 dilution) was added. The day after the flask were irradiated with 0, 4 and 6 Gray (Gy). Briefly, irradiation was conducted using a linear accelerator (Varian 2100CD, Varian Medical Systems, Palo Alto, California, USA) with 6MeV energy. The cell viability after 24 h from irradiation was quantified with trypan blue by manual cell counting.

**CISP treatment.** Viability of MSTO and Cal27 upon CF + CISP treatment was measured. MSTO and Cal27 cells were plated and after 24 h treated with CF (5 µl per ml of medium corresponding to a 1:200 dilution). The day after, the flask were treated with CISP at concentrations ranging from 0 to 12.5 µmol/l. Cell viability was evaluated after 24 h after treatment.

**CISP and RT treatment.** MSTO and Cal27 cells were plated and after 24 h treated with CF (5 µl per ml of medium corresponding to a 1:200 dilution). The day after the flask were treated with 0 and 0.780 µmol/l CISP (CISP) and after 4 h were irradiated with 4 Gy. The cell viability was evaluated after 24 h from irradiation. The cytotoxic effect obtained with the CF and RT or CF and CISP combinations was analysed according to the Chou and Talalay method. Combination index values above 1.1 indicate antagonistic, 0.9–1.1 additive, 0.7–0.9 moderately synergistic, 0.3–0.7 synergistic and <0.3 strongly synergistic. All experiments were repeated in triplicate and media values with standard deviation were calculated.

**Clonogenic assay**

Five hundred viable cells per well [untreated, with vehicle (CNTR), and treated with CF, 4 Gy, CF + 4 Gy] were plated in a 35 mm dish and allowed to grow in normal medium for 10–14 days and then stained for 30 min at room temperature.
with a 6% glutaraldehyde and 0.5% crystal violet solution. Pictures were captured digitally. Experiments were repeated in triplicate.

**Measurement of reactive oxygen species (ROS) generation**

Measurement of ROS generation was examined by using dichlorodihydrofluorescein diacetate (DCFH-DA) in cells according to previously published procedures. Oxidation of DCFH-DA by ROS converts the molecule to 2’,7’-dichlorodihydrofluorescein diacetate (DCF), which is highly fluorescent. Briefly, MSTO cells were plated for 24 h at 37°C and 5% CO₂. The cells were washed with PBS and incubated for 30 min with DCFH-DA. Cells were then washed again with PBS to remove the exceeding probe prior to treatment with 50, 100, 200 μmol/l H₂O₂ or CNTR with and without 5 mmol/l N-acetyl-L-cysteine (NAC). The intensity of DCF fluorescence was determined using a fluorescence microplate reader (Fluoroskan Ascent FLTM, Labsystems, Milford, MA, USA) using an excitation and an emission wavelength of 485 nm and 530 nm, respectively. ROS levels were calculated as a percent increase of fluorescence intensity when compared with the control sample according to the following formula: $\text{ROS} = \left( \frac{\text{fluorescence intensity of treated sample}}{\text{protein concentration of treated sample}} \right) \times 100 \times \left( \frac{\text{protein concentration of vehicle-treated control}}{\text{protein concentration of treated control}} \right)$. Student t test was used to determine significant differences.

**In vivo animal models**

Male CD1 nude mice (6–8 weeks old; weight 18–25 g) were obtained from Charles River. Mice were housed in the animal facility of the Istituto di Ricovero e Cura a Carattere Scientifico Regina Elena National Cancer Institute for 2 weeks before each experiment; animals had ad libitum water and food. The Ethics Committee of the Cancer Institute approved (CE/534/12 and CE/823/16) all the experimental protocols that were carried out in accordance with the Italian regulations (Legislative Decree 4 March 2014, no. 26) and with the Guide for the Care and Use of Laboratory Animals. A mouse xenograft model of mesothelioma was created as previously described. MSTO cell suspensions (2.5 × 10⁶) in 0.2 ml complete medium were injected subcutaneously into the flank of CD1 nude mice (n = 10/treatment group) and growth was measured twice weekly with callipers and calculated by the formula: $\frac{4}{3} \pi \times (\text{large diameter}) \times (\text{small diameter})^2$. In order to test the influence of doses on combined treatment, two different experimental designs were planned, as schematically summarized in Figure 1. In particular, two control groups were conceived, one receiving no CF at all (CNTR), the other (CFCNTR) receiving CF only before xenograft [Figure 1(a)]. Regarding CF administration, two groups of mice, CFCNTR and CFCF, were treated with CF, 12 drops intraperitoneally (i.p.) 7 days before the inoculation of MSTO cells (pre-CF administration), with the CFCF group continuing daily CF administration. All mice were xenografted; after palpable lesions were established (average diameter > 5 mm), mice (n = 10/treatment group) were assigned to treatment groups: CFCNTR, CFCF, CF, increasing doses of CF (CFID), RT treatment with 8 Gy [RT(8)], CF + RT(8) and CNTR, untreated. The unpre-CF administered groups were treated with CF 12 drops (i.p.) 5 days a week (CF group); CFID group, treated i.p. the first day with three drops of CF and then increasing CF gradually until 24 drops on the 15th day; this same dose was used until the end. Regarding irradiation, animals underwent RT(8) to the mass on the 1st and 21st day. For RT treatment, animals were anesthetized and placed on a polymethyl methacrylate (PMMA) box, tumours were treated with RT while the rest of body was shielded using jaws, multileaf collimators and 2 cm lead shielding. In addition, the tumour and body dosage were verified using gafchromic films as described previously. Combined treatment groups receiving CF and RT(8) treatment were identified as CFID + RT(8) group [Figure 1(a)].

In a second experiment, the mice (n = 10/treatment group) were inoculated with MSTO as described above and allocated in accordance to mass appearance, in eight groups [Figure 1(b)]: CNTR, untreated group; CF group, treated with 12 CF drops i.p. 5 days a week; RT(4) group irradiated with 4 Gy on the 1st and 42nd day; standard therapy (ST) group undergoing standard chemotherapy with CISP 3 mg/kg i.p. and pemetrexed 150 mg/kg i.p. once every 21 days; ST + CF group undergoing standard chemotherapy and 12 CF drops i.p. 5 days a week; ST + RT(4), CF + RT(4), CF + ST + RT(4) groups were treated as already described, but together with 4 Gy RT on the 1st and 42nd day. Mice were followed for tumour growth size up until 60 days
from starting treatment. The mice were sacrificed and the tumour masses were removed. Xenograft tumour tissues were divided into two pieces immediately after removal from the mouse and replaced in 10% neutral buffered formalin or homogenates. Tissue lysates were typically prepared by tissue homogenization that was first minced and thoroughly rinsed in PBS to remove any residual blood.

**Western blot of protein from xenograft mesothelioma homogenate tissues**

Wet tissues were homogenates in ice-cold lysis buffer [20 mmol/l Tris (pH 8.0), 1% NP40, 10% glycerol, 137 mmol/l NaCl, 10 mmol/l ethylenediamine tetracetic acid (EDTA) and protease inhibitor]. The homogenates were treated for 1 min with a homogenizer ultra-Turrax T8 (IKA, Staufen, Germany) followed by 1 min on ice. This treatment was repeated five times. The lysate was incubated on ice for 20 min and cleared by centrifugation at 18,000 \( \times g \) for 20 min at 4°C to pellet the cell debris, and the supernatant was immediately stored at \(-80°C\). Protein concentration was measured using Bradford Protein Assay (Bio-Rad Laboratories; Hercules, California, US). Lysates were denatured and separated on 10% sodium dodecyl sulphate-polyacrylamide gel electrophoresis.
Goat antiamouse immunoglobulin G horseradish peroxidase-conjugated secondary antibodies (Bio-Rad Laboratories) were used. Subsequently, the blots reacted with ECL western blotting detection reagents (Amersham Pharmacia, Uppsala, Sweden) according to the manufacturer’s instructions. Scion Image software (Scion Corporation, Frederick, MD) was used for relative quantification of band proteins obtained in western blot.

**Histology and immunohistochemical analysis of xenograft mesothelioma tissues**

The formalin-fixed, paraffin-embedded samples were sectioned at 2 μm and stained with haematoxylin and eosin. The histological diagnosis was examined by a pathologist. In addition, the most representative blocks were selected and cut into new 2 μm-thick sections for immunohistochemical studies. The immunohistochemical assessment of CD45, HIF1α, PGK1 and VEGF was performed in formalin-fixed paraffin-embedded tissues, obtained from the biological specimens collected through biotic procedures in untreated/treated mice, using the following antibodies: CD45 (2B11/PD7/26) Leica, mouse monoclonal antibody, at a dilution of 1:100, after antigen retrieval in citrate buffer; α (H-206), rabbit polyclonal antibody, at the dilution 1:50, after antigen retrieval in citrate buffer; PGK1 (Y-12; Santa Cruz Biotechnology), goat polyclonal antibody at the dilution 1:50, after antigen retrieval in citrate buffer; VEGF(A-20; Santa Cruz Biotechnology), rabbit polyclonal antibody at a dilution of 1:50, after antigen retrieval in citrate buffer; immunohistochemical staining was performed in an automated autostainer (BOND-III, Leica, Milan, Italy) by a biotin-free polymeric horseradish peroxidase (HRP)-linker antibody conjugate system (Bond polymer refine, Leica) and counterstain in haematoxylin. For each tumour, four different 2 μm paraffin sections were analysed and examined by light microscopy for histological evaluations based on staining intensity of the above-mentioned protein (0, negative; 1, weak; 2, moderate; and 3, strong level of detection).

**Statistics**

Cell-culture-based assays were repeated at least three times; mean ± standard deviation (SD) were calculated. Cell lines were examined separately. Differences in xenograft tumour size in vivo were assessed using a 2-tailed student’s t test. Significance was set at $p < 0.05$.

**Results**

**CF reduces HIF1α expression in cancer cells**

The expression of HIF1α protein was analysed in Cal27, MSTO and HCT-116 after treatment with 100 μmol/l CoCl2 for 4, 6 and 24 h. As shown in [Figure 2(a)], HIF1α expression was not detectable in control cells and CoCl2 treatment induced an increase in HIF1α expression in both cell lines at 4 h that declined at 24 h. PGK1 and VEGF expressions were induced also by CoCl2 at 4 h, 6 h and 24 h [Figure 2(a)]. Consequently, the effect of CF in hypoxia was evaluated at 4 h for HIF1α and at 24 h for PGK1 and VEGF. CF treatment induced a reduction of HIF1α [Figure 2(b)] which was confirmed at 24 h with a reduction in the expression of PGK and VEGF [Figure 2(c)]. Results indicate that in cancer cells CF under hypoxia induces a reduction of the expression of HIF1α and its correlated proteins, PGK1 and VEGF.

**CF in combination with radiation increases cellular death in comparison with radiation alone**

In addition to Cal27, MSTO and HCT-116 cells, other two lines from human cancer, lung (Calu3) and breast (MDA-361), were treated with CF (5 μl per ml of medium corresponding to a 1:200 dilution) alone or in association with irradiation using 4 or 6 Gy (Figure 3). At 24 h post-treatment to compare the treatments in different cell lines, the percentage of vital cells versus CNTR [RT(0) + 0CF, 100% of live cells] were evaluated as described above. CF induced significant cellular death in HCT-116, MSTO, Cal27, Calu3 and MDA-361. Dose–response effect of 4 or 6 Gy on cellular death was evident in HCT-116, MSTO, MDA-361 and Cal27 cells. No significant differences were evident in Calu3 after 6 Gy compared with 4 Gy treatment. Significant ($p < 0.05$) cumulative effect of CF and irradiation versus CF or irradiation alone was appreciable in all other cell lines [Figure 3(a)]. Treatment with 6 or 4 Gy did not show great variation in the different cell lines.
Results indicate that CF induces cellular death in cancer cells and the combined treatment with radiation increases the death rate.

**CF in combination with cisplatin increases cellular death compared with cisplatin alone**

We chose MSTO and Cal27 cell lines to assay the association between CF-CISP and CF-CISP-RT (Figure 4). The choice was justified by the fact that CISP and RT are the standard therapy for mesothelioma and tongue cancer in clinical practice. Cell lines were treated with different concentrations of CISP (ranging from 0 µmol/l to 12.5 µmol/l) and a single concentration of CF (5 µl per ml of medium corresponding to a dilution of 1:200). After 24 h, to compare the treatments, the percent of vital cells versus the CNTR (100% of vital cells) was measured. MSTO [Figure 4(a)] and Cal27 [Figure 4(b)] showed a reduction in viability with an increasing concentration of CISP. The combination with CF (5 µl per ml of medium corresponding to a dilution of 1:200) further decreased the MSTO [Figure 4(a)] and Cal27 [Figure 4(b)] cell viability. Afterwards, we tested the association CF-CISP and irradiation on MSTO [Figure 4(c)] and Cal27 [Figure 4(d)] cell lines. To appreciate the effectiveness of this association, we used 0.780 µmol/l CISP and RT(4), while CISP, taken individually, induces a low level of death in both cell lines. The cell lines were untreated (CNTR) and treated with 1:200 CF (CF) or 0.780 µmol/l CISP (CISP) or RT(4) or 1:200 CF + 0.780 µmol/l CISP (CF + CISP) or 1:200 CF + 4Gy [CF + RT(4)] or 0.780 µmol/l CISP + 4Gy [CISP + RT(4)] or 1:200 CF + 0.780 µmol/l CISP + 4Gy [CF + CISP + RT(4)]. The associations CF + CISP, CF + RT(4) and CISP + RT(4) were more effective at reducing vital cells than single treatment, and a synergistic effect of the treatment was highlighted (Table 1).

When radiation treatment was added to CF + CISP, further reduced cell vitality versus the association of CISP + RT(4) was observed [Figure 4(c), (d)]. The results indicate that by adding CF to nonlethal CISP dose, it induces significant cancer cell death. This effect is amplified by RT.

**ROS are involved in HIF1α, VEGF and PGK1 modulation**

It is known that ROS are generated by RT, CISP and pemetrexed treatment. Therefore, we...
investigated MSTO cells, the line used to develop the mesothelioma model on nude mice, to test the effect of ROS on the modulation of HIF1α, VEGF and PGK1. To generate ROS, we used H2O2. First, we performed a dose curve, as the different cell lines have various sensitivity thresholds.
to \( \text{H}_2\text{O}_2 \), and we chose for the following experiments the dose range of 50–200\( \mu \text{mol/l} \) of \( \text{H}_2\text{O}_2 \) because it induces stress without killing MSTO cells dramatically. The addition of 50–200\( \mu \text{mol/l} \) \( \text{H}_2\text{O}_2 \) to MSTO cells resulted in enhanced ROS generation, while the cotreatment with NAC attenuates the ROS levels [Figure 5(a)].

Using western blotting, the ROS-modulated proteins have been further investigated. We detected HIF1\( \alpha \) at 100\( \mu \text{mol/l} \) and 200\( \mu \text{mol/l} \) of \( \text{H}_2\text{O}_2 \) but not at 0\( \mu \text{mol/l} \) and 50\( \mu \text{mol/l} \), while VEGF and PGK1 were detectable from 0\( \mu \text{mol/l} \) to 200\( \mu \text{mol/l} \) of \( \text{H}_2\text{O}_2 \) [Figure 5(b)]. We found an increasing expression of HIF1\( \alpha \) at 100\( \mu \text{mol/l} \) \( \text{H}_2\text{O}_2 \), while at 200\( \mu \text{mol/l} \) \( \text{H}_2\text{O}_2 \) it decreased [Figure 5(b), (c)]. Vice versa PGK1 and VEGF showed greater expression after the addition of 200\( \mu \text{mol/l} \) \( \text{H}_2\text{O}_2 \) [Figure 5(b), (c)].

The cotreatment with 5\( \text{mmol/l} \) NAC abrogated the effect of ROS on the PGK1 and VEGF expression [Figure 5(b), (d)]. Conversely, the addition of 200\( \mu \text{mol/l} \) \( \text{H}_2\text{O}_2 \) and NAC treatment increased the HIF1\( \alpha \) expression compared with the treatment with 200\( \mu \text{mol/l} \) \( \text{H}_2\text{O}_2 \) alone [Figure 5(b), (c), (d)].

On the other hand, the levels of ROS generated after 200\( \mu \text{mol/l} \) \( \text{H}_2\text{O}_2 \) and NAC treatment, were comparable with those obtained with 100\( \mu \text{mol/l} \) \( \text{H}_2\text{O}_2 \) alone [Figure 5(a)]. The results indicate that high levels of ROS reduce the expression of HIF1\( \alpha \) and increase PGK and VEGF expression.

**Figure 4.** Combined CF, CISP and irradiation treatment increases cancer cell death.

Histograms of MSTO (a) and Cal27 (b) cells survival percentage after the treatment with cisplatin doses ranging between 0.78 and 12.5\( \mu \text{mol/l} \) and CF at dose fixed alone and in association compared to the untreated (CNTR, cell survival 100%).

Data are expressed as mean ± SD (standard deviation) of at least three independent experiments. *\( p < 0.05 \) versus CNTR and and *\( p < 0.05 \) versus CISP. Histograms of MSTO (c) and Cal27 (d) cells survival after the treatment with 0.78\( \mu \text{mol/l} \) CISP, CF and RT(4) alone or in association. Data are expressed as mean ± SD of at least three independent experiments. *\( p < 0.05 \) versus CF. *\( p < 0.05 \) versus CISP. Histograms of MSTO (c) and Cal27 (d) cells survival after the treatment with 0.78\( \mu \text{mol/l} \) CISP, CF and RT(4) alone or in association. Data are expressed as mean ± SD of at least three independent experiments. *\( p < 0.05 \) versus CNTR.

**CF has preventive effects**

We performed a pilot study to test the effect of different treatments with CF [as described in Figure 1(a)] on tumour growth of xenograft...
MSTO mice [Figure 6(a)]. The CNTR group showed a gradual increase in the size of the tumour mass in each mouse; the CF group did not show a decrease in size of the tumour mass compared with CNTR. After CF pre-administration (CFCNTR group), 60% of mice did not show any growth of tumour masses and the remaining 40% manifested a tumour growth in metastasis cells inoculum; of these, 20% were growing gradually and homogeneously while the remaining 20% showed an irregular growth pattern. When, after the pre-administration, CF receiving was not interrupted (CFCF group), 70% of the mice did not develop a tumour mass. CFCNTR and CFCF groups showed a significant ($p=0.0005$ and $p=0.00014$, respectively) reduction of the tumour mass versus CNTR. SD is not represented in the CFCNTR and CFCF groups because it is too high due to the elevated number of total tumour mass regression. To understand the irregularity on tumour growth of CFCNTR compared with the CNTR group, the masses were removed and characterized by histological and immunohistochemistry (IHC) analyses at the end of the experiment. Microscopic examination by haematoxylin–eosin staining confirmed mesothelioma tissues present in CNTR [Figure 6(b, 1)] while, surprisingly in CFCNTR group, masses with irregular growth were not mesothelioma but tissues with lymphocytes [Figure 6(b, 2)]. The immunohistochemical staining of lesions with CD45 protein that presents on the surface of almost all haematolymphoid cells, was negative in CNTR [Figure 6(b, 3)] and positive in CFCNTR [Figure 6(b, 4)], suggesting further investigations on potentially

| Cell line | Schedule       | Fraction affect | Combination index | Effect          |
|-----------|----------------|-----------------|-------------------|----------------|
| MSTO      | 0.78 µmol/l CISP + CF | 0.61            | 0.10              | Strongly synergistic |
|           | 1.56 µmol/l CISP + CF | 0.97            | 0.06              | Strongly synergistic |
|           | 3.13 µmol/l CISP + CF | 0.82            | 0.20              | Strongly synergistic |
|           | 6.25 µmol/l CISP + CF | 1.00            | 0.21              | Strongly synergistic |
|           | 12.5 µmol/l CISP + CF | 1.00            | 0.43              | Synergistic      |
|           | 4Gy + CF        | 0.53            | 0.06              | Strongly synergistic |
|           | 6Gy + CF        | 0.57            | 0.09              | Strongly synergistic |
|           | 0.78 µmol/l CISP + 4Gy | 0.56          | 0.41              | Synergistic      |
| Cal27     | 0.78 µmol/l CISP + CF | 0.58            | 0.05              | Strongly synergistic |
|           | 1.56 µmol/l CISP + CF | 0.64            | 0.05              | Strongly synergistic |
|           | 3.13 µmol/l CISP + CF | 0.82            | 0.02              | Strongly synergistic |
|           | 6.25 µmol/l CISP + CF | 1.00            | 0.01              | Strongly synergistic |
|           | 12.5 µmol/l CISP + CF | 1.00            | 0.43              | Synergistic      |
|           | 4Gy + CF        | 0.78            | 0.05              | Strongly synergistic |
|           | 6Gy + CF        | 0.85            | 0.07              | Strongly synergistic |
|           | 0.78 µmol/l CISP + 4Gy | 0.72          | 0.63              | Synergistic      |

Fraction affect is the level of inhibition induced by treatments. Combination index values above 1.1 indicate antagonistic, 0.9–1.1 additive, 0.7–0.9 moderately synergistic, 0.3–0.7 synergistic and <0.3 strongly synergistic.

MSTO-H211 mesotelioma cell line; Cal27, carcinoma of the tongue cell line; CF, CELLFOOD™; CISP, cisplatin. 4Gy, irradiation treatment (RT) with 4Gy; 6Gy, RT with 6Gy.
induced immunological response elicited by pre-administration with CF.

To this end, we reported that CF pre-administration prevents engraftment of the tumour; however, on the other hand, in the CFCF group, 70% of the mice did not show masses supporting the preventive effect. No therapeutic effect of CF at the dosage used was evident after the appearance of the tumour mass. The results indicate that, although CF has no therapeutic effect, it could be used for tumour prevention.

**CF increases the radiation efficacy alone and in association with ST in MSTO xenograft mice**

Since CF (12 drops) treatment does not induce significant tumour shrinkage versus control, we conducted a pilot study *in vivo* using the experimental mesothelioma mice model to identify the conditions for treatment with CFID and RT alone and in combination [Figure 1(a)]. Literature reported treatment of 20 and 15 Gy for MSTO xenografts and thus, we decided to deliver 8 Gy, also considering *in vitro* data. We compared the effect of CFID and RT(8) alone and the association of CFID + RT(8) versus CNTR [Figure 6(c)]. RT(8), CFID and CFID + RT(8) were significantly effective* in reducing tumour growth versus CNTR. No consideration on the combined action of CFID + RT(8) versus CFID or RT(8) alone was possible because despite adding RT(8), treatment with CFID significantly reduced the tumour mass compared with only CFID treatment; CFID + RT(8) did not significantly improve response when compared with only RT(8). During the experiment, benefits in the status of mice receiving CF compared with those treated with RT was noted (8); thus, we decided to record the survival rate. Figure 6(d) shows the average survival rate for each group of mice according to treatment. The RT(8) group (47 ± 12 days) showed a significant incidence (*p* = 0.000452) of early death in comparison with the CNTR group (65 ± 4 days), indicating that the RT(8) administered was too high. By adding CF to RT(8) treatment [CFID + RT(8) group] the days of survival increased to 55 ± 11 days, thus suggesting that CFID benefits radiation treatment. CFID (64 ± 3 days) and CNTR (65 ± 4 days) showed

![Figure 5. Effect of ROS on HIF1α, PGK1 and VEGF expression.](image)

(a) The histogram shows the results of three independent experiments: the percentage of levels of ROS with respect to untreated MSTO cells (CNTR) after the addition of 50–200 μmol/l H₂O₂ (−NAC) and after cotreatment with 5 mmol/l NAC (+ NAC). Data are expressed as mean ± SD of at least three independent experiments. ROS effect on the expression of the HIF1α protein, PGK1 and VEGF was detected by western blot in all experiments, (b) represents a single experiment. The relative bands intensities of the proteins of interest after 50–200 μmol/l H₂O₂ (c) and 50–200 μmol/l H₂O₂ with NAC (d) were quantified by the Scion Image software in all experiment.

* *p* < 0.05 versus CNTR.
CNTR, with vehicle; HIF1α, hypoxia-inducible factor 1 alpha; NAC, N-acetyl-L-cysteine; PGK1, phosphoglycerate kinase 1; ROS, reactive oxygen species; SD, standard deviation; VEGF, vascular endothelial growth factor.
the same days of survival, indicating that CFID alone had no effect on survival compared with CNTR mice. All these results led us to believe that the dose of RT(8) was too strong and that in order to appreciate the possible combined therapeutic effect, it was necessary to reduce the RT(8) and CF dosage. Remarkably, CF improved the well-being of the mice.

For subsequent in vivo experiments, in light of the results obtained, for subsequent in vivo experiments, we decided to reduce the dose to 4 Gy, give two doses of RT (day 1 and 42), reduce the dosage of CF to 12 drops as described above and start all treatments when the tumour mass was palpable. RT(4) and ST were administered at day 1 and day 42 because the first RT(4) treatment induced a significant reduction in tumour mass from the 30th to the 40th day. Figure 7 summarizes the tumour growth of mesothelioma xenograft mice upon CF or CF + RT(4) [Figure 7(a)] or CF + ST [Figure 7(b)] or CF + RT(4) + ST [Figure 7(c)] treatment versus untreated (CNTR). CF alone was not effective in inhibiting the tumour growth; the effect of irradiation was evident at 30 days after irradiation treatment, and an effect combined of RT(4) and CF was evident in the CF + RT(4) group in the same amount of time. After the second dose of RT(4), an increased and reduced tumour mass was present in the RT(4) and CF + RT(4) groups, respectively, further confirming a combined effect of CF and RT(4) [Figure 7(a)].

The action of CF with ST was evaluated [Figure 7(b)]. ST significantly reduced the tumour mass, compared with CNTR, 19 days after treatment. Adding CF to ST does not change the effect of ST alone. However, it did differ when mice were treated with ST + RT(4) or CF + ST + RT(4) compared with RT(4) alone [Figure 7(c)]. Tumour masses of ST + RT(4) mice group were significantly reduced.
HIF1α expression in vivo was reduced by CF in combination with RT or ST + RT

All xenograft tumour tissues of each single mouse undergoing different treatments as well as untreated mice were divided into two pieces to analyse the HIF1α, PGK1 and VEGF expressions by western blot and IHC. Figure 7(d) shows a representative western blot of lysates from mice tumour masses. HIF1α was detected, as well as VEGF and PGK1 in untreated mice (CNTR). CF alone treatment did not induce a variation in the expression of all investigated proteins than untreated CNTR, while RT(4) treatment increased HIF1α expression. In the other treatment groups, HIF1α was undetectable and VEGF and PGK were detectable at different levels of expression [Figure 7(d), (e)]. IHC analysis of tissues of CNTR group exhibited the same staining of CF group for HIF1α, PGK1 and VEGF (data not shown). Higher staining intensity for HIF1α, as well as PGK1 and VEGF were revealed in CF and RT(4) treatment [Figure 7(f)]. After CF + RT(4) association, a reduction of HIF1α and PGK1 intensity staining was revealed, adding ST to CF + RT(4) [CF + ST + RT(4)] further lowers HIF1α staining but not PGK1 and VEGF. VEGF staining intensity was reduced by RT(4) treatment compared with CF treatment. Negative staining of VEGF was revealed after CF and RT(4) combination treatment [CF + RT(4)], while adding ST to CF + RT(4) [CF + ST + RT(4)] increased the intensity of VEGF staining compared with CF + RT(4) treatment. The protein expressions obtained in IHC were comparable with those of western blot, with the exclusion of the HIF1α in the group of CF + RT(4) but we speculated that HIF1α was degraded during the preparation of lysate from tissues. With these results, we showed that with IHC HIF1α expression in vivo was reduced by CF in combination with RT(4) [CF + RT(4)] and especially for CF, when delivered in combination with ST + RT(4) [CF + ST + RT(4)].

PGK1 and VEGF expression, proteins with HIF1α correlation, were positively regulated by HIF1α in CF + RT(4) treatment but not in CF + ST + RT(4) treatment, suggesting a strengthening effect of RT(4) by CF.

Discussion

Hypoxia is a feature of most tumours that contributes to different processes, including chemoresistance, radioresistance, angiogenesis and altered metabolism. Most solid tumours have hypoxic areas due to the presence of a network of highly irregular and messy blood vessels that fail to supply oxygen and nutrients to the rapidly growing tumour cells. To survive this hostile environment, cancer cells have developed a set of responses that enable them to adapt to hypoxic conditions. A critical mediator of the hypoxic response is HIF1α. Under hypoxic conditions, the HIF1α complex binds hypoxic-response elements in the promoter region of different target genes including enzymes involved in glycolysis and pH regulation, such as PGK1 and angiogenesis, such as VEGF. Overexpression of HIF1α is associated with advanced disease stages, poor prognosis and treatment resistance among cancer patients, while inhibition of HIF1α retards tumour growth in animal models. Therefore, research efforts towards discovering novel HIF1α inhibitors for the treatment of cancer are currently underway. The aim of these efforts is to discover new agents that target only tumour cells without causing general cytotoxicity-related side effects. In this context, we hypothesized that CF could be a promising agent for the inhibition of HIF1α.

CF modulates the bioavailability of oxygen either increasing or decreasing it ‘on demand’, when hypoxic or hyperoxic conditions occur, respectively. Therefore, it is plausible that CF reaches the hypoxic tumour mass and oxygenates it, thus, to induce HIF1α degradation. Previous studies report the role of CF in HIF1α modulation only in endothelial and leukaemic cells. Here, we have investigated the CF effect on HIF1α modulation in cells from solid cancers. We have shown that in hypoxia, CF oxygenation reduces the expression of HIF1α and related proteins, such as PGK1 and VEGF, in cancer cell lines.

Hypoxia is associated with increased tumour resistance to radiation treatment. Tumour cells become about two to three times more radioresistant than those in normoxic conditions due to
Figure 7. Effect of CF in association with therapeutic treatments on tumour growth and HIF1α, PGK1 and VEGF expression in mesothelioma mice xenograft.

(a) Action of the CF alone and with RT(4) on the tumour growth. * significant versus CNTR; ¤ significant versus CNTR + RT(4);
(b) action of the ST alone and with CF on the tumour growth; * significant versus CNTR; (c) action of the RT(4) alone, with ST and with ST + CF on the tumour growth; * significant versus CNTR + RT(4); * significant versus ST + RT(4); (d) a representative western blot of HIF1α, PGK1 and VEGF of masses removed by mice untreated and treated with ST, RT(4) and CF alone and in combination, and quantification of protein band intensities by Scion Image software and tubulin-normalization [e]; (f) immunohistochemistry of tumour explants; the scale bar is 30 µmol/l.

Data are expressed as mean ± SD (standard deviation) of all determinations of explants for each group.

* p < 0.05 versus RT(4).
* ¤ p < 0.05 versus CF + RT(4).
CF, CELLFOOD™; CNTR, with vehicle; RT(4), irradiation treatment [RT] with 4 Gy; RT(8), RT with 8 Gy; ST, standard therapy; HIF1α, hypoxia-inducible factor 1 alpha; PGK1, phosphoglycerate kinase 1; VEGF, vascular endothelial growth factor; H/E, haematoxylin and eosin staining.
the effect that oxygen has on the generation of free radicals. In normoxia, radiation induces irreparable damage to deoxyribonucleic acid (DNA), while under hypoxic conditions, this effect is altered. Here, we showed that the addition of a physiologic modulator of oxygen, CF, to RT treatment on cancer cells improved efficacy of treatment compared with RT treatment alone.

Hypoxia occurs frequently in solid tumours and although its role in chemotherapy resistance has been recognized, various explanations have been provided. Among these include, the selection of hypoxia-resistant cells, the adaptation of cancer cells to hypoxia and the inhibition of cell proliferation by hypoxia. Notably, the lethal action of DNA-damaging drugs, like CISP, is strongly dependent on the growth rate, and the inhibition of cell proliferation by hypoxia could be an important mechanism leading to resistance to CISP. The resistance to CISP is reversed after reoxygenation of cancer cells. Here, we have shown that the low degree of cell death, obtained with low doses of CISP, increased after adding CF. In short, CF improved radio- and chemotherapeutic treatment of cancer cell lines.

To validate these findings in vivo, we performed a mesothelioma mouse xenograft. Malignant pleural mesothelioma is a disease for which there is no standardized therapeutic protocol, as all types of treatment are burdened by the high rate of recurrence and poor survival. The standard therapy for all subtypes is chemotherapy such as CISP, pemetrexed, carboplatin, gemcitabine or doxorubicin. The multimodal approach includes radical cytoreductive surgery followed by RT, chemotherapy or targeted therapy. Mesotheliomas are particularly hypoxic solid tumour masses. We speculated whether the standard therapy for pleural mesothelioma, such as CISP and pemetrexed or CISP and pemetrexed and RT, are more effective if they are administered in combination with CF, the ‘physiological modulator’ aimed at making oxygen available ‘on demand’ which is different to the hypoxic microenvironment. In addition, in line with this rationale, we supposed that CF could be an agent that inhibits the tumour engraftment. Here, in mice mesothelioma xenografts, we show that, although CF does not have any effects on mesothelioma therapy when treatment begins after tumour mass formation, CF prevents the development of mesothelioma when it is provided before the onset of tumours (CFCF, Figure 6(a)). In addition, when CF was given before the inoculum of mesothelioma cells, we observed the absence of tumour onset or small masses without mesothelioma in mice but like the tissues with lymphocytes (Figure 6(b)). Therefore, we highlight the preventive effect of CF for mesothelioma, even though further investigations are required.

In addition, we recognized the protective effect of CF. When mice were treated with RT(8), an early death rate was observed compared with CNTR. The combination of increasing CF (CFID) doses and RT(8) prolonged the number of days of the mice’s life without tumour mass changing significantly in comparison with RT(8) alone (Figure 6(d)).

The effect of the CF and RT(4) combination was evident on tumour growth and survival with delivering lower doses of CF and RT(4), and by adding ST with CF and RT(4), was found to be more efficient than ST and RT(4), further confirming the advantage in the use of CF in improving mesothelioma treatment (Figure 7).

At the molecular level, CF does not change the expression of HIF1α, demonstrated by IHC in comparison with CNTR. In in vivo mice studies, HIF1α expression increases from 18h to 24h after RT. This upregulation lasts up to 1 week. In an in vivo tumour model, it was shown that some HIF1α target genes are also regulated after irradiation, including VEGF. In our study, we detected an increased expression of HIF1α, VEGF and PGK1 in the RT(4) treatment group after 18 days post-RT treatment. Treatment of mesothelioma-bearing mice with CF + RT(4) reduces the HIF1α, PGK1 and VEGF expression versus RT(4) suggesting that CF, as shown on MSTO cells (Figure 2(b), (c)), acts on degradation of HIF1α. Adding ST to CF + RT(4) treatment restores the PGK1 and VEGF expression of RT(4) treatment, and HIF1α is downregulated, suggesting that alternative pathways can be induced when a single factor, such as HIF1α is blocked. For instance, SP1 is a key transcription factor in PGK1 and VEGF expression under hypoxia. ROS are capable of activating SP1, thereby modulating the regulation of gene transcription. ROS work by two opposing mechanisms on the HIF1α. In literature, both high and low expression of HIF1α was associated with high ROS levels. Interestingly, CISP and pemetrexed in mesothelioma cells act through ROS. In our hypoxic experimental model of mesothelioma cells, MSTO, we observed that the
highest levels of ROS downregulate HIF1α but not VEGF and PGK expression. Although further studies in this direction are underway, we believe that the addition of ST to RT(4) + CF increase the levels of ROS which contribute to induction of SP1 and therefore, VEGF and PGK1. In addition, it is plausible that ROS, by activating a proline hydroxylase and by increasing ubiquitin–proteasome activity degrades HIF1α at the post-transcriptional level; this could explain the absence of HIF1α in the group CF + ST + RT(4).58,59 On this basis, we reported a model to explain the action of CF with RT and chemotherapy (Figure 8).

Before irradiation, the tumour mass consists of well-oxygenated and nonoxygenated cells. RT is expected to kill a higher number of well-oxygenated than hypoxic cells with ROS production and increased HIF1α expression. In the case of CF + RT treatment, CF is expected to increase the fraction of oxygenated cells reducing the hypoxic fraction, making RT more effective (b). This implies a greater production of ROS and reduction of HIF1α (b) compared with RT alone (a). After the CF administration, when ST is added to RT (c), we expected a further increase in ROS, attributable to the action of CISP and an overall reduction in HIF1α expression.

ROS, reactive oxygen species; HIF1α, hypoxia-inducible factor 1 alpha; RT, irradiation therapy; CF, CELLFOOD™; ST, standard therapy.

Figure 8. Schematic representation of a model explaining the association between CF and radio- and chemotherapy. (a) Before irradiation (baseline), the tumour mass consists of well-oxygenated (green) and nonoxygenated (red) cells. RT kills a higher number of well-oxygenated than hypoxic cells with ROS production and increased HIF1α expression. In the case of CF + RT treatment, CF is expected to increase the fraction of oxygenated cells reducing the hypoxic fraction, making RT more effective (b). This implies a greater production of ROS and reduction of HIF1α (b) compared with RT alone (a). After the CF administration, when ST is added to RT (c), we expected a further increase in ROS, attributable to the action of CISP and an overall reduction in HIF1α expression.
attributable to CISP, and a reduction in HIF1α expression due to the capability of the whole treatment better overcoming tumour resistance.

Glycolytic metabolism in malignancies correlates with radioresistance.\(^{21,60}\) PGK1 is one of the glycolytic enzymes that is upregulated by HIF1α and its activity in cancer cells is greater than in normal cells.\(^{15–19}\) Overexpressed PGK1 is one of the extrinsic factors of tumour cell radiosensitivity.\(^{61}\) Recently, the suppression of PGK1 was reported to enhance the radiosensitivity of U251 xenografts and that PGK1 with Cofilin1 could be used to evaluate glioma radiosensitivity and prognosis.\(^{62}\) In our mice with mesothelioma, the reduction of PGK1 expression after CF+RT(4) and ST+CF+RT(4) versus RT(4) treatment was observed, showing greater radiosensitivity in irradiated mice in association with CF treatment.

A potential limitation of our approach is the determination of amount of CF to be administered in a cancer patient cohort, as well as the local availability of CF within large hypoxic tumours. In fact, the limited vascularization could represent an obstacle to the local distribution. These aspects need further investigation, although our in vivo results are very encouraging.

A further issue is the possibility of optimizing the fractionation scheme when combining multiple approaches. Of note, the application of hypofractionated regimes of RT could be further investigated thanks to its capability of improving efficacy on hypoxic and normoxic cells using photon\(^{63}\) or ion-beam RT.\(^{64}\)

In summary, these findings showed that the oxygen modulator CF, enhancing cellular oxygenation, decreases HIF1α stabilization. CF’s action in mesothelioma mice xenograft leads to an increase in the tumour growth mass radiosensitivity, consequently improving the result of therapies. These effects were mediated through triggering ROS production. Our study elucidated a possible mechanism of action for the CF+RT and CF+ST+RT combination in effecting cell death in malignant mesothelioma cells. Given that CF is a nutritional supplement used daily by healthy and sportspeople to improve performance, it could be evaluated in prospective clinical trials to improve the response of combined therapies.

Author contribution
BN and SB performed experiments and analysed data; BA and GC performed the mice treatments; BAn and MC performed the IHC and analysed results; AS and LS performed the irradiation treatment and analysed results; RG designed, conceived and supervised the study and wrote the paper.

All authors have contributed to and approved the final manuscript.

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