

## Resveratrol Potentiates the Cytotoxic Oxidative Stress Induced by Chemotherapy in Human Colon Cancer Cells

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### Key Words

Reactive oxygen species • Antitumor drugs • Resveratrol • Uncoupling proteins • Colon cancer

inhibition of AKT and STAT3 proteins, which are known to have oncogenic potential in colorectal carcinomas.

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### Abstract

The treatment of advanced colorectal cancer with 5-fluorouracil has two major problems: development of tumor resistance and toxicity toward normal tissues. The aim of this study was to investigate the possible advantages of combining 5-fluorouracil (5-FU) with resveratrol (*trans*-3, 4', 5-trihydroxystilbene) for treating HT-29 and SW-620 colorectal carcinoma cell lines. Since combined treatment using 5-FU with resveratrol resulted in a significant decrease in long-term cell survival, we investigated the possible basis of this synergistic interaction at a molecular level, focusing on oxidative stress as a possible mediator of cell death. Resveratrol established interactions with the mitochondria of cancer cells and induced an imbalance in cellular antioxidant activities, leading to a significant increase in the levels of both intracellular reactive oxygen species (ROS) and lipid peroxides. Combined treatment with resveratrol sensitized colon cancer cells to 5-fluorouracil, inducing a further increase in oxidative stress, which was linked to the

### Introduction

Colorectal cancer is one of the leading causes of cancer mortality worldwide [1, 2]. Although ~70-80% of patients are eligible for curative surgical resection at the time of diagnosis, ~50% of all newly diagnosed patients ultimately develop metastatic disease [3]. These patients eventually receive systemic chemotherapy, but despite significant advances in the development of chemotherapeutic agents, no such drugs for the treatment of colorectal cancer have a non-relapsing cure rate. Successful tumor therapy using 5-fluorouracil is usually impeded by the development of tumor resistance mechanisms. Even though several molecules involved in the generation of tumor resistance have been identified, most of the essential aspects of the resistance mechanisms still remain elusive.

Oxygen free radicals are released as a consequence of chemotherapy and mediate, at least in part, its pro-

apoptotic effects in cancer cells [4]. High levels of anti-oxidant systems [5] and overexpression of uncoupling protein-2, a mitochondrial suppressor of ROS, have been shown to induce chemoresistance in cancer cells [6]. Therefore, modulation of oxidative stress in tumor cells has been suggested as an important strategy to sensitize tumors to cytotoxic drugs and even produce clinical responses in patients who were previously refractory [7, 8].

The search for novel and effective cancer chemopreventive agents has led to the identification of several naturally occurring compounds one of which is resveratrol (*trans*-3, 4', 5-trihydroxystilbene), a phytoalexin derived from the skin of grapes and other fruits; its potential chemopreventive and chemotherapeutic activities have been proved in all three stages of carcinogenesis (initiation, promotion and progression) [9]. Resveratrol exhibits anticancer properties in a wide variety of tumor cells, including colon carcinoma [10]. This agent not only provokes apoptosis in cancer cells, it has also been suggested that it sensitizes them to certain chemotherapeutic drugs [11]. However, little is known about the possible pro-oxidative effects of cytotoxic concentrations of resveratrol in tumor cells nor the possible importance of its redox interactions to potentiate cell death induced by antitumor drugs.

Taking the aforementioned into account, the aim of this study was to investigate whether pharmacological concentrations of resveratrol could modify the redox state of tumor cells, potentiating chemotherapy-induced cytotoxicity in human cancer cells. For this purpose, we analyzed the effects of resveratrol alone and in combination with 5-fluorouracil on proliferation rate, long-term cell survival, oxidative stress, levels of uncoupling proteins and the levels of inactive and active forms of AKT and STAT3 in HT-29 and SW-620 human colorectal carcinoma cell lines.

## Materials and Methods

### Chemicals

Routine chemicals used were obtained from Sigma-Aldrich (St. Louis, USA), Panreac (Barcelona, Spain) and Molecular Probes (Paisley, UK). Cell culture reagents were purchased from Invitrogen (Carlsbad, CA, USA) and Ractiva (Barcelona, Spain). The antibodies against uncoupling protein-5/BMCP1 (UCP5; cat. UCP51-A) and uncoupling protein-2 (UCP2; cat. UCP21-A) were purchased from Alpha Diagnostic International (San Antonio, USA). The antibodies against STAT3 (cat. 9139), phospho-STAT3 (Tyr 705; cat. 9145), AKT/PKB/Rac (cat. 9272)

and phospho-AKT (Ser 473; 9271) were purchased from Cell Signaling Technology, Inc. (Danvers, MA, USA).

### Cell culture and treatments

The human colon carcinoma cell lines HT-29 and SW-620 were a kind gift from Dr. S. Fernández de Mattos (University of the Balearic Islands). These cell lines were grown in Dulbecco's modified Eagle's medium (DMEM containing 25 mM glucose) supplemented with 10% (v/v) fetal bovine serum, 90 units/mL penicillin and 90 µg/mL streptomycin, in a 5% CO<sub>2</sub> humidified atmosphere at 37°C. Cisplatin (*cis*-diamminedichloroplatinum II or CDDP), 5-fluorouracil (5-FU), carmustine (BCNU) and etoposide were dissolved in DMSO, and added to cell culture dishes never exceeding a DMSO concentration of 0.01%. Stock solutions of antitumor drugs, *tert*-butyl hydroperoxide (*tert*-BuOOH), and sodium *L*-ascorbate (Vit. C) were freshly prepared before experiments. An equivalent volume of vehicle was added to controls. The protein content in each sample was determined by Bradford's method [12].

### Determination of cell survival using colony formation assay

For clonogenic assays, colon cancer cells were plated in six-well plates and treated the day after with 100 µM resveratrol alone or in combination with 10 µM 5-fluorouracil for a period of 6 h. After removal of the medium containing antitumor drugs, cells were trypsinized and plated at low density (2,000 cells per 60-mm plate). Cells were then cultivated for 14 days. Colonies were stained with crystal violet and clones were counted for each condition.

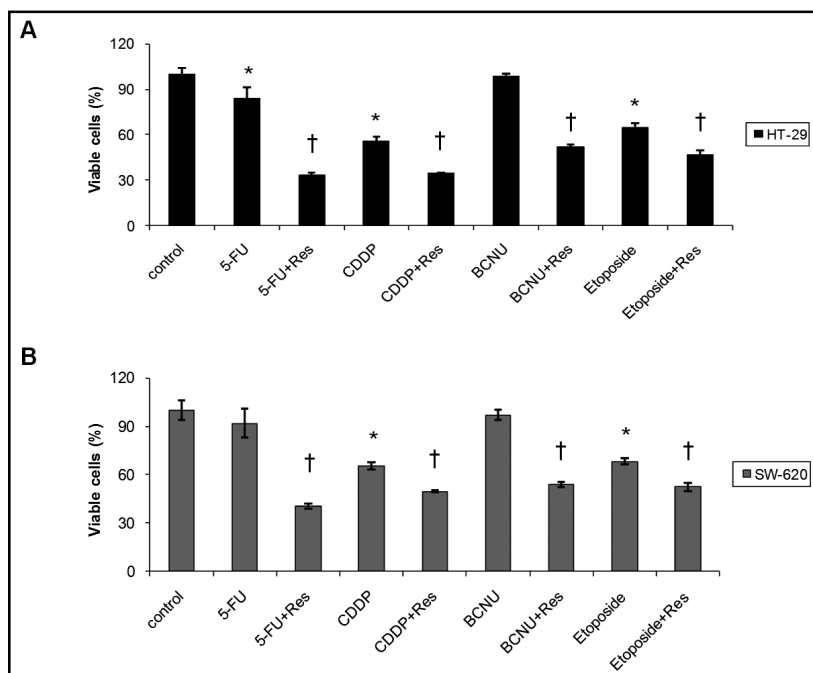
### Cell proliferation/viability assays

Cell proliferation was assessed by colorimetric analysis of 3-(4,5-dimethylthiazolyl-2)-2,5-diphenyltetrazolium bromide (MTT) reduction. One day after seeding ( $1 \times 10^4$  cells), HT-29 and SW-620 cell lines were treated with 100 µM resveratrol, 25 µM BCNU, 25 µM etoposide, 10 µg/mL cisplatin, 1 mM Vit. C and 25 µM *tert*-BuOOH or exposed to the vehicle for the times indicated. MTT solution in phosphate-buffered saline (5 mg/mL) was added 1/10 to the culture medium in each well. The plates were incubated for 1 hour at 37°C, after which the medium was replaced with dimethyl sulfoxide (DMSO) to dissolve any formazan crystals formed by the cells. The absorbance was recorded at 570 nm with background subtraction at 610 nm using a microplate reader.

### Mitochondrial isolation

Cells ( $6 \times 10^6$ ) were harvested by trypsinization, centrifuged at 1600 rpm for 5 min, washed with PBS and resuspended in 1 mL of cold isolation buffer (0.25 M sucrose, 10 mM Tris-HCl, and 0.5 mM EDTA-K<sup>+</sup>, pH 7.4). Samples were then immediately homogenized at 4°C; nuclei and cell debris were sedimented by differential centrifugation at 600 x g for 10 min and the pellets were discarded. Supernatant was subjected to a further centrifugation at 7000 x g for 10 min and the crude mitochondrial pellet obtained was resuspended with 0.1 mL of isolation buffer.

**Fig. 1.** Resveratrol potentiates the growth inhibition induced by mechanistically dissimilar antitumor drugs. A, effect of antitumor drugs on cell viability of HT-29 cells. B, effect of antitumor drugs on cell viability of SW-620 cells. Cell viability was measured after treating cells with a conventional antitumor drug alone or in combination with 100  $\mu$ M resveratrol (Res) for 48 hours and then results were expressed as % viable cells against control. The final concentration of each antitumor drug was the following: 5-fluorouracil (10  $\mu$ M), cisplatin (CDDP; 10  $\mu$ g/mL), carmustine (BCNU; 25  $\mu$ M) and etoposide (25  $\mu$ M). Values are expressed as mean  $\pm$  SEM from at least three independent experiments performed in duplicate. When they do not appear, error bars are smaller than the symbol size. Statistical significance ( $P < 0.05$ ): \*, antitumor drug-treated cells versus control cells; †, cells treated with an antitumor drug alone versus combined treatment with resveratrol.



#### Analysis of enzymatic activities

All spectrophotometric determinations were assayed in microtiter plate and performed in the same way as previously described protocols with some modifications. Ferrocyclochrome-c: oxygen oxidoreductase (COX or complex IV; EC 1.9.3.1) activity was measured in cell lysates and mitochondrial fractions in order to calculate the mitochondrial recovery [13]. Superoxide dismutase (SOD; EC 1.15.1.1) [14], glutathione peroxidase (GPx; EC 1.11.1.9) [15] and catalase (EC 1.11.1.6.) [16, 17] antioxidant activities were determined in the supernatant of samples previously disrupted by sonication (10,000 x g for 10 min).

#### Determination of intracellular ROS levels

Intracellular ROS levels were detected using 2',7'-dichlorofluorescein diacetate (DCFH-DA) as previously described protocols [18]. Cells were incubated with 10  $\mu$ M DCFH-DA for 30 min at 37°C, washed twice with PBS, and resuspended in PBS containing 10 mM glucose; fluorescence values were then immediately measured.

#### Measurement of mitochondrial hydrogen peroxide production

The rate of hydrogen peroxide generation was determined using the fluorescent probe Amplex Red, which is a specific reagent to measure hydrogen peroxide [19]. Mitochondria (0.12 mg protein/mL) were added to respiration buffer supplemented with 0.1 U/mL horseradish peroxidase and 50  $\mu$ M Amplex red reagent. The assays were performed in respiratory State 4 (absence of ADP) and in the presence of succinate (5 mM) as a substrate.

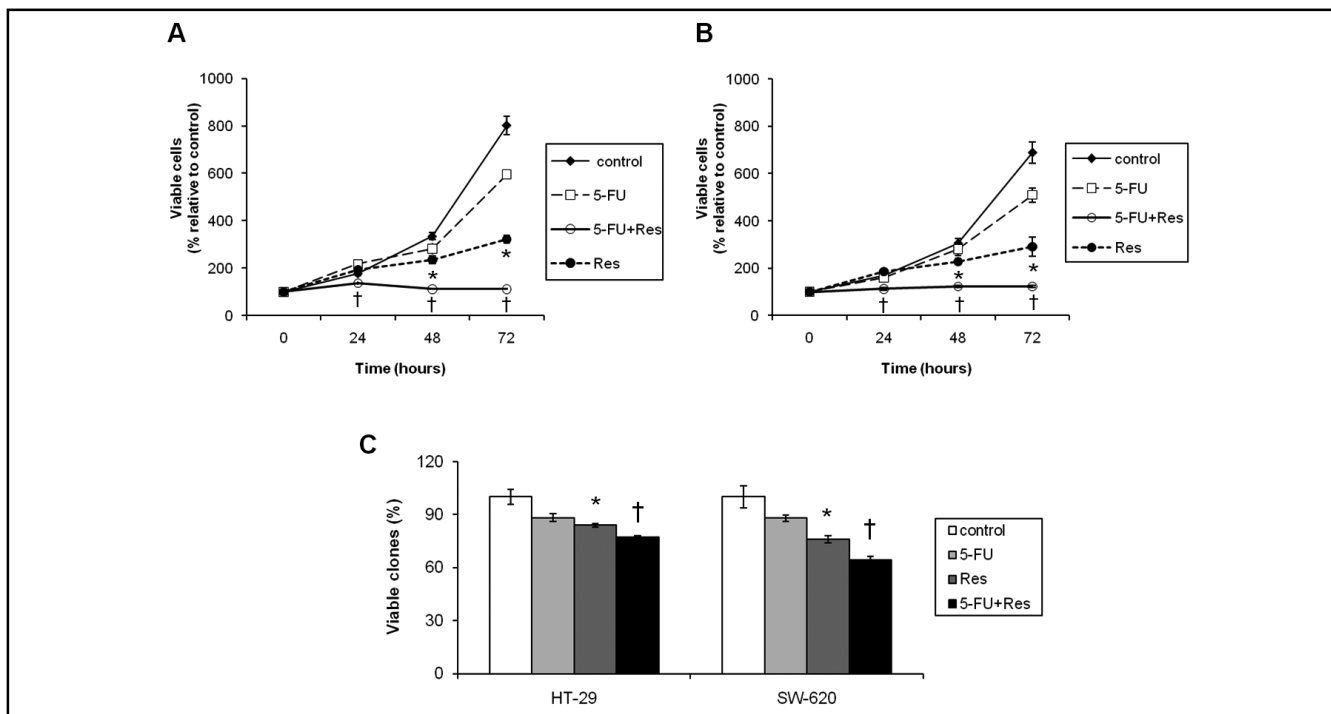
#### Determination of oxidative damage

Protein carbonyl groups were quantified in mitochondrial fractions using the Oxyblot™ protein oxidation detection kit (Chemicon, Chancellors Ford, UK) by means of a slot-blot system

(Bio-Rad, CA, USA) following the manufacturer's instructions. Lipid peroxidation was determined in mitochondrial fractions as 4-hydroxy-2-nonenal (4-HNE) adducts using a slot-blot immunoassay [20]; the antibody against 4-HNE (cat. num. HNE11-S) was obtained from Alpha Diagnostic International, San Antonio, USA. Total lipid peroxidation was determined in cell lysates as thiobarbituric acid reactive substances (TBARS) according to previously described methods [21]; butylated hydroxytoluene (BHT) was added to homogenizing buffer at a final concentration of 4 mM to stop autooxidation during the heating step; the assay was performed spectrophotometrically at 532 nm using a molar extinction coefficient of  $1.56 \times 10^5 \text{ M}^{-1} \cdot \text{cm}^{-1}$ .

#### Western blot analysis

Cells were rinsed with ice-cold PBS and then homogenized in ice-cold lysis buffer (20 mM Tris-HCl, 1.5 mM  $\text{MgCl}_2$ , 140 mmol/L NaCl, 0.5% Nonidet P-40, 10% glycerol, 1 mmol/L EGTA, 1 mmol/L  $\text{NaVO}_3$ , 1 mmol/L phenylmethylsulfonyl fluoride, 10  $\mu$ g/mL leupeptin, and 10  $\mu$ g/mL pepstatin; pH 7.4). Protein samples from total cell lysates (30  $\mu$ g) were subjected to electrophoresis on 8-10% SDS-polyacrylamide gels, electroblotted onto nitrocellulose membranes (Bio-Rad, CA, USA) and immunoblotted using the corresponding primary antibodies. Immunoreactive bands were visualized using Immuno-Star Western C Kit reagent® (Bio-Rad, CA, USA), according to the manufacturer's instructions. An anti- $\alpha$ -tubulin antibody (cat. num. sc-5286; Santa Cruz Biotechnology, Santa Cruz, USA) was used to normalize the amount of the samples analyzed. Densitometric analysis of the signals was performed using Quantity One software (Bio-Rad, CA, USA). The apparent molecular weights of phospho-AKT (60 kDa), AKT (60 kDa), phospho-STAT3 (86 kDa), STAT3 (86 kDa), UCP2 (33 kDa), UCP5 (36 kDa) and  $\alpha$ -tubulin (55 kDa) were estimated using protein molecular-mass standards (Bio-Rad, CA, USA).



**Fig. 2.** Cytotoxic concentrations of resveratrol sensitize colon cancer cells to 5-fluorouracil. A, growth curves of HT-29 cells treated with 5-fluorouracil (10  $\mu$ M) alone or combined with 100  $\mu$ M resveratrol (Res). B, growth curves of SW-620 cells treated with 5-fluorouracil (10  $\mu$ M) alone or combined with 100  $\mu$ M resveratrol. C, clonogenic analyses of HT-29 and SW-620 cell lines were performed after treating cells with a medium containing 5-fluorouracil (10  $\mu$ M) alone or in combination with 100  $\mu$ M resveratrol for 6 h; cells were then trypsinized, and plated at low density (2000 per 60-mm plate); after 2 weeks, formed colonies were stained with crystal violet and clones were counted for each condition. Values are expressed as mean  $\pm$  SEM from at least three independent experiments performed in duplicate. When they do not appear, error bars are smaller than the symbol size. Statistical significance ( $P < 0.05$ ): \*, resveratrol-treated cells versus control cells; †, cells treated with 5-fluorouracil versus combined treatment with resveratrol.

### Statistical analysis

All data were expressed as means  $\pm$  SEM from at least three independent experiments performed in duplicate. Statistical analysis was carried out using the Statistical Program PASW Statistics 18.0. Statistical differences between experimental groups were analyzed with unpaired Student *t* test. A level of  $P < 0.05$  was accepted as significant.

## Results

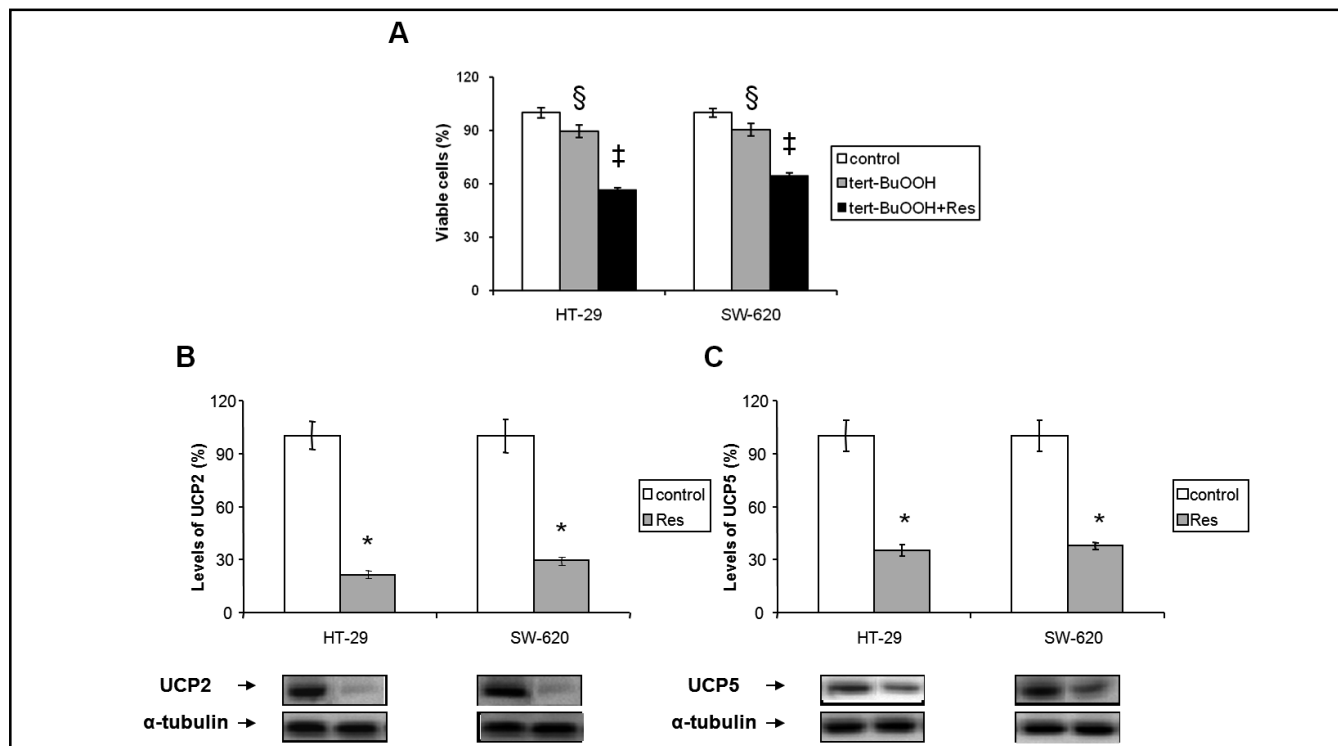
### *Resveratrol potentiates the growth inhibition induced by mechanistically dissimilar antitumor drugs*

Figs. 1A and B show the responsiveness of colon adenocarcinoma cells to conventional antitumor drugs. After 48-h treatment with 10  $\mu$ M 5-fluorouracil, > 80% of HT-29 cells and > 90% of SW-620 cells remained viable, reflecting that both cell lines show a marked resistance to the antiproliferative effects of 5-fluorouracil (5-FU). Similarly, HT-29 and SW-620 cells were resistant

to carmustine (BCNU) antiproliferative activity. On the other hand, cisplatin and etoposide were more effective at inhibiting colon cancer cell proliferation. Notably, combined treatment with resveratrol, used at pharmacological doses, sensitized cancer cells to all conventional anticancer agents tested ( $P < 0.001$ ). It is worth noting that resveratrol was more effective at reducing cell proliferation in combination with the drugs for which cancer cells showed greater resistance. Specifically, combined treatment of resveratrol with 5-FU for 48 hours significantly reduced the number of viable cells (60% for HT-29 cells and 56% for SW-620 cells) compared to 5-FU alone.

### *Cytotoxic concentrations of resveratrol sensitize colon cancer cells to 5-fluorouracil*

Fig. 2 shows growth curves for HT-29 and SW-620 colon cancer cells treated with resveratrol (100  $\mu$ M) alone or in combination with 10  $\mu$ M 5-FU. Combined treatment with resveratrol brought about a time-dependent inhibi-



**Fig. 3.** Resveratrol induces mitochondrial oxidative stress in colon cancer cells. A, cell viability was measured after treating cells with *tert*-butyl hydroperoxide (*tert*-BuOOH; 25  $\mu$ M) alone or in combination with 100  $\mu$ M resveratrol for 48 hours and then results were expressed as % viable cells against control. B, Western blot of mitochondrial uncoupling protein-2. C, Western blot of mitochondrial uncoupling protein-5. Colon cancer cells were treated with 5-fluorouracil (10  $\mu$ M) alone or combined with 100  $\mu$ M resveratrol (Res) for 48 hours; equal loading was confirmed using primary antibodies against  $\alpha$ -tubulin; representative bands are shown. Values are expressed as mean  $\pm$  SEM from at least three independent experiments performed in duplicate. Statistical significance ( $P < 0.05$ ): §, hydroperoxide-treated cells versus control cells; ‡, cells treated with hydroperoxide versus combined treatment with resveratrol; \*, resveratrol-treated cells versus control cells.

tion of cell proliferation in HT-29 cells (Fig. 2A) and SW-620 cells (Fig. 2B), thus counteracting the resistance to fluorouracil-induced cell death. To further investigate the long-term effects of this association, clonogenic assays were performed; HT-29 and SW-620 cells were treated for 6 hours and then viable colonies were counted after two weeks. The combined treatment of 5-FU with resveratrol caused a significant decrease in cancer cell survival compared to 5-FU alone (Fig. 2C;  $P < 0.05$  for HT-29 cells and  $P < 0.001$  for SW-620 cells).

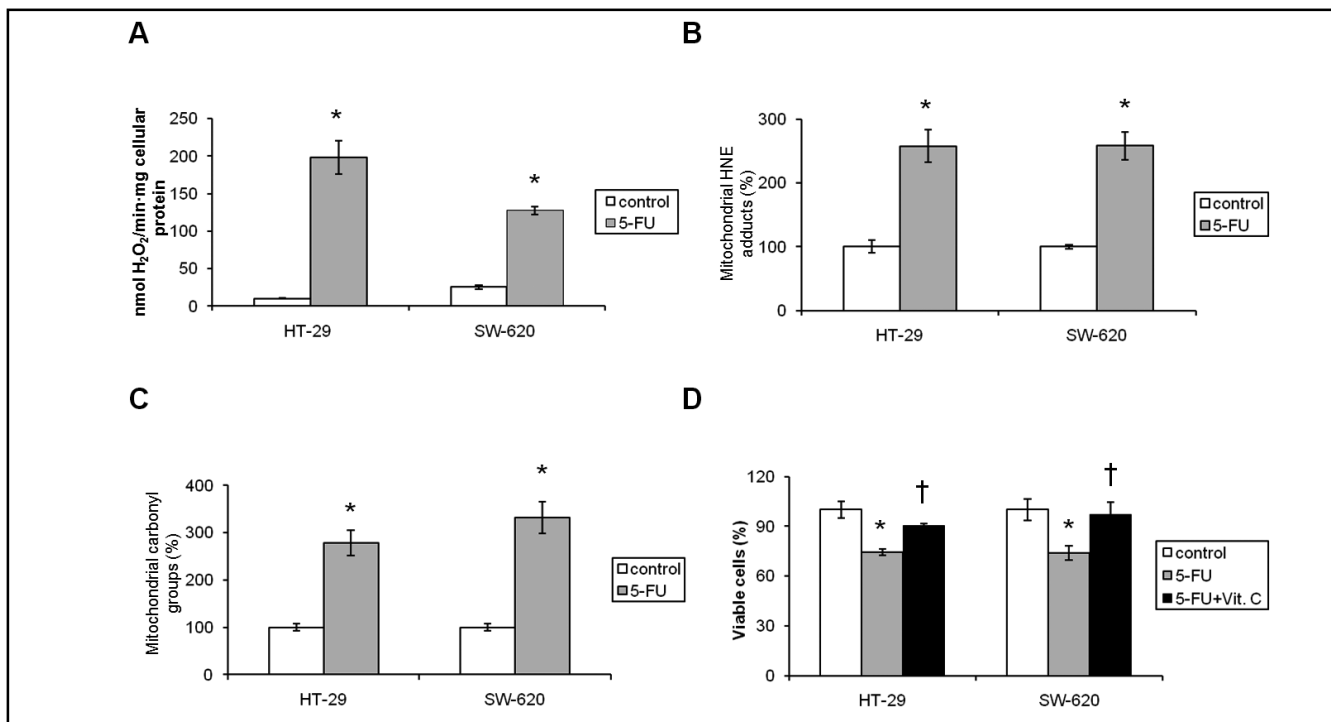
#### *Resveratrol induces mitochondrial oxidative stress in colon cancer cells*

Pharmacological concentrations of resveratrol (100  $\mu$ M) did not exert an antioxidant effect on tumor cells since resveratrol was not able to prevent oxidative cytotoxicity induced by exposing cells to a medium containing 25  $\mu$ M *tert*-butyl hydroperoxide for 48 hours (Fig. 3A). As shown in Figs. 3B and C, 48-hour treatment with 100  $\mu$ M resveratrol downregulated the levels of mitochon-

drial uncoupling protein-2 (to 22% and 29% of the control levels of HT-29 and SW-620 cells, respectively) and uncoupling protein-5 (to 35% and 38% of the control levels of HT-29 and SW-620 cells, respectively).

#### *Fluorouracil induces mitochondrial oxidative stress in colon cancer cells*

Treatment with 5-FU (10  $\mu$ M) for 48 hours had marked pro-oxidative effects on mitochondria. Isolated mitochondria from colon cancer cells treated with 5-FU for 48 hours showed greater ROS generation compared to those from controls (Fig. 4A;  $P < 0.001$ ). Additionally, treatment with 5-FU caused a 2.5-fold increase in mitochondrial lipid peroxidation (measured as HNE adducts; Fig. 4B;  $P < 0.005$ ) and also brought about a marked increase in mitochondrial protein carbonylation (2.8-fold in HT-29 cells and 3.3-fold in SW-620 cells), evidenced by the accumulation of carbonylated proteins (Fig. 4C;  $P < 0.005$ ) in mitochondrial fractions from HT-29 and SW-620 cells.



**Fig. 4.** Fluorouracil induces mitochondrial oxidative stress in colon cancer cells. A, H<sub>2</sub>O<sub>2</sub> production of isolated mitochondria from fluorouracil-treated cells was measured fluorometrically using the Amplex red reagent; succinate (5 mM) was used as substrate. B, levels of HNE adducts were measured by immunoblotting in mitochondrial fractions from colon cancer cells previously treated with 5-fluorouracil (10  $\mu$ M) for 48 h. C, protein carbonyl levels were measured by immunoblotting in mitochondrial fractions from colon cancer cells previously treated with 5-fluorouracil (10  $\mu$ M) for 48 h. D, cell viability was measured after treating cells with 5-fluorouracil (5-FU; 10  $\mu$ M) alone or combined with the antioxidant sodium ascorbate (Vit.C; 1 mM) for 72 hours and then results were expressed as % viable cells against control. Values are expressed as mean  $\pm$  SEM from at least three independent experiments performed in duplicate. Statistical significance ( $P < 0.05$ ): \*, fluorouracil-treated cells versus control cells; †, cells treated with 5-fluorouracil versus combined treatment with sodium ascorbate.

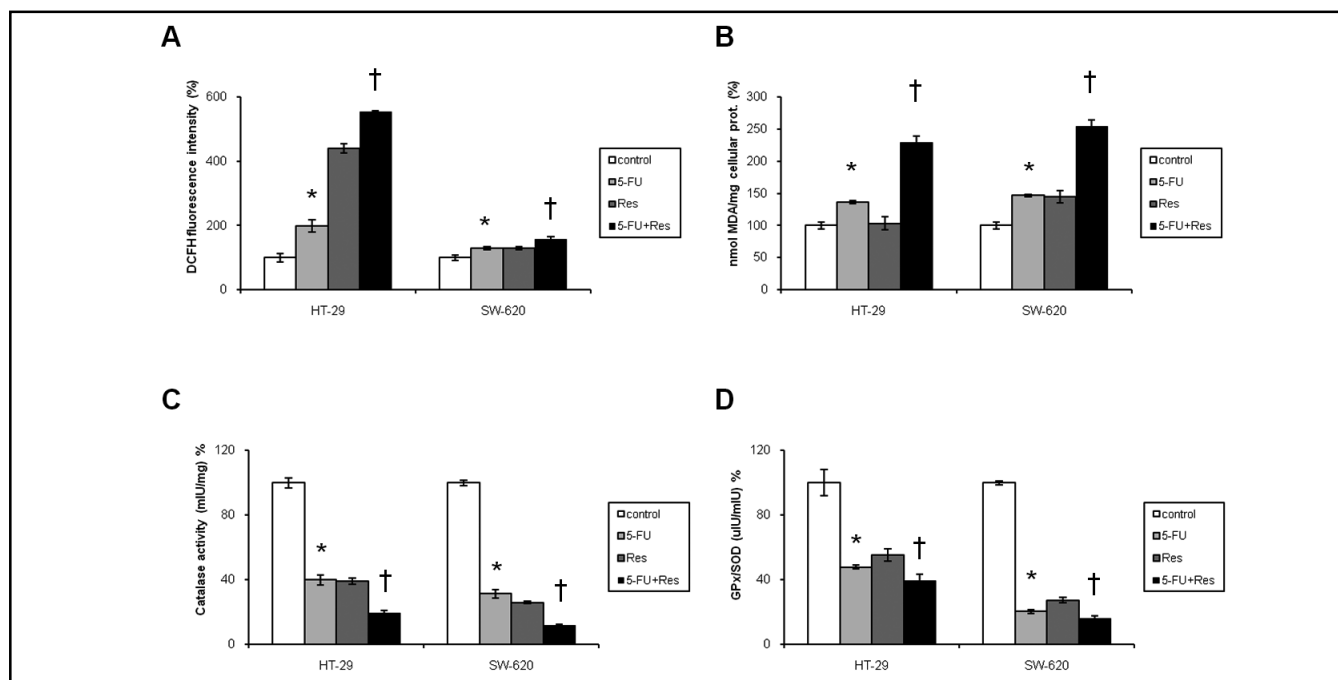
Interestingly, our results proved that ROS are required for the mechanism of action of 5-fluorouracil in colon cancer cells, since the antioxidant sodium ascorbate prevented cell death in response to 5-FU (Fig. 4D).

Our experiments also revealed that 5-FU was able to establish the same pattern of interactions at mitochondrial level than resveratrol. In this sense, treatment with 5-FU (10  $\mu$ M) for 48 hours caused a statistically significant downregulation of the levels of mitochondrial UCP2 (to 29.4% and 56.7% of the control levels of HT-29 and SW-620 cells, respectively) and UCP5 (to 58.9% and 73.7% of the control levels of HT-29 and SW-620 cells, respectively).

#### *Oxidative imbalance is associated to resveratrol-induced sensitization to chemotherapy*

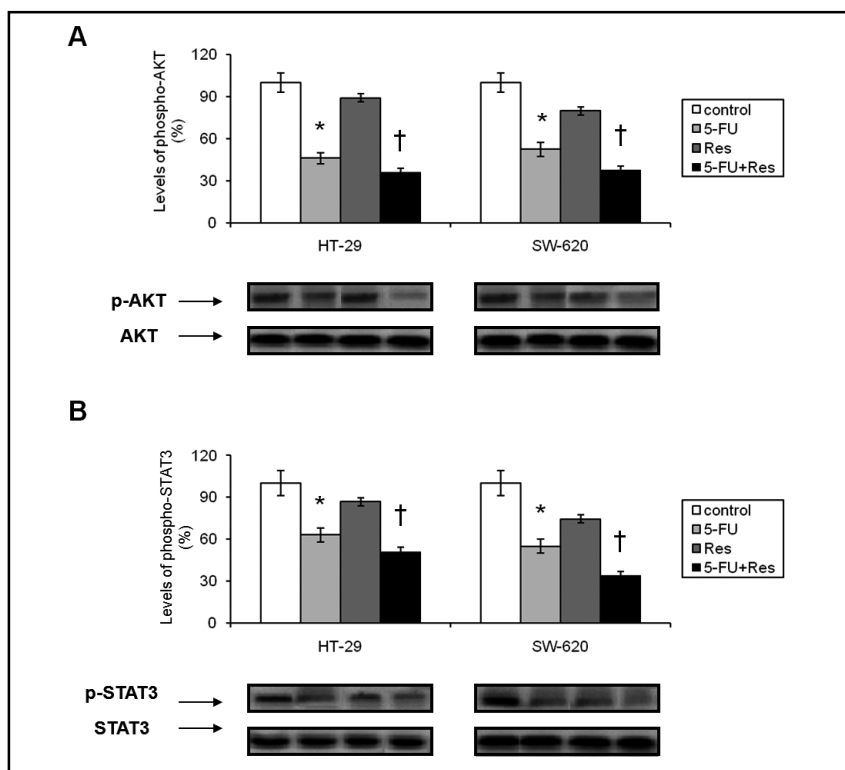
To prove that oxidative stress is relevant to increase the cytotoxicity induced by 5-FU in colon cancer cells,

we studied the effects of the treatment of 5-FU alone and in combination with resveratrol on antioxidant capacity, intracellular ROS levels, and cellular lipid peroxidation (Fig. 5). Intracellular ROS levels (Fig. 5A;  $P < 0.01$ ) and accumulation of lipid oxidative products, measured as TBARS (Fig. 5B;  $P < 0.01$ ), increased in response to 5-FU. Additionally, fluorouracil significantly inhibited the activity of enzymes that detoxify H<sub>2</sub>O<sub>2</sub> such as catalase (Fig. 5C;  $P < 0.001$ ) and GPx causing a concomitant increase in SOD activity (Fig. 5D;  $P < 0.001$ ), which reflects that 5-FU impairs hydroperoxide scavenging capacity in cancer cells. The combined treatment of resveratrol with 5-FU significantly potentiated the effects of this chemotherapeutic agent, promoting intracellular accumulation of ROS and causing a further increase in the levels of lipid peroxides (68.1% in HT-29 cells and 73.3% in SW-620 cells). Our results show that chemotherapy-induced cell death is a cell type-dependent re-



**Fig. 5.** Oxidative imbalance is associated to resveratrol-induced sensitization to chemotherapy. A, effects on intracellular ROS levels. B, effects on cellular lipid peroxidation. C, effects on catalase activity. D, effects on glutathione peroxidase-superoxide dismutase activity ratio. Colon cancer cells were treated with 5-fluorouracil (10  $\mu$ M) alone or combined with 100  $\mu$ M resveratrol (Res) for 48 hours. Values are expressed as mean  $\pm$  SEM from at least three independent experiments performed in duplicate. When they do not appear, error bars are smaller than the symbol size. Statistical significance ( $P < 0.05$ ): \*, fluorouracil-treated cells versus control cells; †, cells treated with 5-fluorouracil versus combined treatment with resveratrol.

**Fig. 6.** Combined therapy using resveratrol and 5-fluorouracil promotes the inhibition of redox-sensitive pro-survival pathways. A, Western blot of phospho-AKT. B, Western blot of phospho-STAT3. Colon cancer cells were treated with 5-fluorouracil (10  $\mu$ M) alone or combined with 100  $\mu$ M resveratrol (Res) for 48 hours; equal loading was confirmed using primary antibodies against  $\alpha$ -tubulin; representative bands are shown. Values are expressed as mean  $\pm$  SEM from at least three independent experiments performed in duplicate. Statistical significance ( $P < 0.05$ ): \*, fluorouracil-treated cells versus control cells; †, cells treated with 5-fluorouracil versus combined treatment with resveratrol.



sponse, which is not linear with ROS concentration, suggesting that other factors such as alterations in endog-

enous antioxidant capacity are important to promote cellular damage and death.

*Combined therapy using resveratrol and 5-fluorouracil promotes the inhibition of redox-sensitive pro-survival pathways*

ROS are very unstable biochemical compounds, therefore, the signal of cell death initiated by certain chemotherapeutic agents is likely to be communicated to the nucleus through modifications in signaling pathways sensitive to redox state. To explore this possibility, we determined the levels and variations in the phosphorylation state of proteins that promote cancer cell survival such as the protein kinase AKT and the transcription factor STAT3. As shown in Fig. 6A, we observed that treatment with 5-FU for 48 hours decreased phospho-AKT to 46% and 52% of the control levels of HT-29 and SW-620 cells, respectively. Similarly, the levels of the active form of STAT3 decreased in response to 5-FU to 63% and 55% to the control levels of HT-29 and SW-620 cells, respectively (Fig. 6B). Since the levels of total AKT and STAT3 were not significantly modified by the treatment, these results indicate that 5-FU partially inactivates both signaling molecules. Combined therapy using resveratrol and 5-FU enhanced the inhibition of AKT and STAT3 induced by the chemotherapeutic agent.

## Discussion

Plant polyphenols have been the focus of numerous studies investigating their biological attributes, which mainly include chemoprevention, growth-inhibiting activity, and anti-inflammatory and antioxidant properties. However, a further evaluation of the literature indicates that the anti-cancer mechanisms of plant polyphenols are complex and controversial. It does appear that the anti-carcinogenic activity of resveratrol may be related to, but not entirely due to, its above-mentioned properties. Additionally, a pro-oxidant action may be important in anti-cancer and apoptosis-inducing properties of this compound [22].

In the present study, we explored the redox behaviour of resveratrol when it acts as a cytotoxic compound in colon cancer cells, studying its potential to enhance the cytotoxic oxidative stress induced by 5-fluorouracil. Our results show that combination therapy of resveratrol and 5-fluorouracil, at concentrations achievable in patients receiving chemotherapy [23], induced cell death in human colon cancer cells through cytotoxic oxidative stress. Resveratrol, at pharmacological doses, was found to render tumor cells more susceptible to death induction

after chemotherapy and to significantly overcoming resistance to 5-FU.

The apoptotic response of a cell damaged by chemotherapy partly depends on the balance between levels of proteins that predispose cells to apoptosis and antagonize apoptosis. Therefore, we explored the mechanisms of growth inhibition of chemo-surviving colon cancer cells by resveratrol, studying the possible effects on the levels of mitochondrial uncoupling proteins and pro-survival signaling molecules. Interestingly, uncoupling proteins have been suggested as molecular sensors for oxidative stress [24, 25], since ROS can activate UCPs, and increased proton backflow through them could release mitochondrial membrane potential and thus prevent further production of ROS [26].

Our experiments reveal a marked mitochondrial oxidative stress after 48-hour treatment with 5-FU, which is concomitant with the downregulation of the mitochondrial levels of UCP2 and UCP5. To further explore the mechanisms involved in the induction of mitochondrial oxidative stress by 5-FU, we exposed isolated mitochondria from HT-29 and SW-620 cells to this chemotherapeutic agent for 45 minutes; under these conditions, fluorouracil was unable to stimulate a direct and immediate mitochondrial  $H_2O_2$  generation (data not shown). These observations are in accordance with the indirect stimulation of  $H_2O_2$  production through the decrease in uncoupling capacity of cancer cell mitochondria as one of the mechanisms involved in the induction of mitochondrial oxidative stress after treatment with 5-FU and resveratrol; interestingly, both agents establish a similar pattern of interactions with mitochondria of cancer cells, but resveratrol exhibits a greater capacity to deplete the mitochondrial content of UCP2 and UCP5. These findings could explain a mechanism for the enhanced ROS generation at mitochondrial level, which would disrupt the cytoprotective feed-back loop, thus facilitating the commitment to cell death following chemotherapy [6]. Previous experiments in our laboratory evidenced that mitochondrial uncoupling is overactivated in colon cancer cells, and this mechanism might help them to avoid cytotoxic concentrations of ROS [19]. Supporting this idea, other studies have reported a cytoprotective role for UCP2 and UCP5 against oxidative stress [6, 27].

Among the molecular mechanisms that were found to be associated with the sensitization of cancer cells to 5-FU by resveratrol is the inhibition of the endogenous antioxidant capacity of cancer cells. Combined treatment of 5-FU with resveratrol enhanced



the susceptibility of cancer cells to oxidative damage by inhibition of antioxidant enzymatic activities. Interestingly, the combined treatment promoted an imbalance between SOD and GPx activities. High SOD activity in conjunction with low GPx activity probably contributes to the observed intracellular ROS accumulation. In accordance with these results, the imbalance of SOD and GPx activities has been proposed to play a role in initiating and propagating oxidative damage, and thus predisposing to cytotoxic oxidative stress [28]. Oxidative stress observed after treatment with 5-FU was manifested by increased lipid peroxidation, which was strongly potentiated by the combined treatment with resveratrol.

The transcription factor STAT3 and the protein kinase AKT are important signaling molecules that have been found to be overexpressed or activated in most types of human tumors, therefore, it is now generally accepted that both proteins represent valid targets for novel anticancer drug design [29, 30]. Notably, both proteins are subjected to redox modifications that regulate their activity [31-33]. We analyzed the effects of the treatment with 5-FU alone or combined with resveratrol on these pathways to explore the possibility that their inhibition could be associated to cytotoxic oxidative stress in colorectal cancer cells. STAT3 is known to be activated by phosphorylation at Tyr705, which induces dimerization, nuclear translocation and DNA binding [34, 35]. Colon cancer cell lines were found to exhibit constitutive activation of survival promoting proteins such as AKT and STAT3. HT-29 and SW-620 cell lines showed a partial depletion in the levels of their activated forms after treatment with 5-FU. Importantly, resveratrol caused a complete inactivation of both the transcription factor STAT3 and

the kinase AKT in fluorouracil-based combination therapy.

In conclusion, our results show that resveratrol, an antioxidant compound in healthy tissues, behaves as a pro-oxidative agent in human colon cancer cells. Specifically, the synergistic interaction between resveratrol and 5-fluorouracil was found to rely strongly on the promotion of cytotoxic oxidative stress which was concomitant with the inactivation/down-regulation of redox-sensitive proteins that participate in crucial pathways that promote cancer cell survival.

## Abbreviations

AKT (V-akt murine thymoma viral oncogene homologue); BCNU (Carmustine); *tert*-BuOOH (*Tert*-butyl hydroperoxide); CDDP (Cisplatin); 5-FU (5-fluorouracil); GPx (Glutathione peroxidase); HNE (4-hydroxy-2-nonenal); Res (Resveratrol); ROS (Reactive oxygen species); SOD (Superoxide dismutase); STAT3 (Signal transducer and activator of transcription 3); UCP (Uncoupling protein); Vit. C (Sodium *L*-ascorbate).

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