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Original Article

# Resveratrol and capsaicin used together as food complements reduce tumor growth and rescue full efficiency of low dose gemcitabine in a pancreatic cancer model

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ABSTRACT

Pancreatic adenocarcinoma, highly resistant to all current anti-cancer treatments, necessitates new approaches promoting cell death. We hypothesized that combined actions of several Bioactive Food Components (BFCs) might provide specific lethal effect towards tumor cells, sparing healthy cells. Human tumor pancreatic cell lines were tested *in vitro* for sensitivity to resveratrol, capsaicin, piceatannol, and sulforaphane cytotoxic effects. Combination of two or three components showed striking synergetic effect with gemcitabine *in vitro*. Each BFC used alone did not affect pancreatic tumor growth in a pre-clinical *in vivo* model, whereas couples of BFCs had anti-tumor activity. In addition, tumor toxicity was similar using gemcitabine alone or a combination of BFCs and two thirds of gemcitabine dose. Moreover, BFCs enhanced fibrotic response as compared to gemcitabine treatment alone. Reactive oxygen species (ROS) and apoptosis increases were observed, while cell cycle was very mildly affected. This study raises the possibility to use BFCs as beneficial food complements in the therapy of pancreatic adenocarcinoma, especially for patients unable to receive full doses of chemotherapy.



## Introduction

Pancreatic ductal adenocarcinoma (PDAC) is the 4th cause of cancer mortality in occidental countries [1]. At the time of diagnosis 5-year survival is very limited (1–4%). Surgical resection offers the best chances of survival but is possible for only less than 20% of the patients, since pancreatic cancers are often diagnosed at advanced stages. However, even resectable PDACs display a high rate of recurrence [2]. Systemic chemotherapy includes the nucleoside analog gemcitabine, and more recently the association of oxaliplatin, irinotecan and 5 Fluorouracile (FOLFIRINOX) or nab-paclitaxel but with limited efficiency [3,4]. Extensive efforts have been made to identify adjuvant or neoadjuvant therapies capable of improving the poor prognosis of PDAC, based on the molecular targets involved in cancer progression, such as *KRAS* or *TP53*. Unfortunately, phase III studies of targeted therapies in combination with gemcitabine have shown limited or even no improvement in patient survival [5]. Other non-chemotherapy approaches such as radiotherapy, targeted therapies and non-conventional therapies have also failed to produce valuable clinical benefits [6]. Even the recent hope residing in metformin failed to improve the outcome of patients with locally advanced or metastatic pancreatic cancer [7]. Thus, there is still an urgent need to find new therapeutic options to help manage this deadly cancer.

Bioactive food components (BFCs) are physiologically active constituents in foods or dietary supplements, including those needed to meet basic human nutrition needs, that have been demonstrated to have a role in health and to be safe for human consumption in intended food and dietary supplement uses (as to

*Abbreviations:* PDAC, Pancreatic ductal adenocarcinoma; BFCs, Bioactive Food Components or Compounds; BITC, Benzyl Isothiocyanate; ROS, Reactive Oxygen Species; CSC, Cancer Stem Cell; PDX, Patient Derived Xenograft; FOLFIRINOX, Association of Oxaliplatin, Irinotecan and 5 Fluorouracile; $IC_{50}$, Half maximal inhibitory concentration; CI, Combination Index; C, Capsaicin; R, Resveratrol; S, Sulforaphane; P, Piceatannol; G, Gemcitabine; MAP kinase, Mitogen Activated Protein kinase; TRPV, Transient Receptor Potential cation channel Vanilloid.

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the American Dietetic Association). Some can have direct antitumor effects or be valuable as cancer preventing agents. They seem to be efficient because they can control both the apoptosis response and the metabolic pathways of the cancer cells [8]. Moreover they affect cancer stem cell (CSC) maintenance by inhibiting several CSC-promoting pathways, limiting self-renewal and inducing differentiation and apoptosis [9]. Resveratrol, capsaicin and isothiocyanates BFCs are of particular interest in the field since: (i) they were already tested as single BFC combined with conventional treatment, (ii) they induce strong oxidative stress, (iii) they were already evaluated in clinical trials and (iv) they were tested on pancreatic cancer models.

Resveratrol is a polyphenol, belonging to stilbenes, involved in the so-called "French paradox" and has been extensively analyzed as a protective or therapeutic agent [10]. In pancreatic cancer models, resveratrol was shown to exert an antitumor effect synergistic with gemcitabine [11]. Many other *in vitro* and clinical studies have been conducted involving anticancer activity of resveratrol through many cellular pathways [1,12], including increased reactive oxygen species (ROS) production [2,13]. In the $KRAS^{G12D}$ mice, a model of spontaneous pancreatic adenocarcinoma, resveratrol treatment over a 10-month period reduced pancreatic lesions compared with mice that did not receive resveratrol treatment, indicating that resveratrol reduced spontaneous pancreatic tumors [3,4,14]. Several clinical trials have shown that resveratrol can be safe and reasonably well-tolerated at doses of up to 5 g/day [5,15]. Capsaicin, found in hot peppers, is an alkaloid that has been used to treat pain and inflammation. It is used in human, mostly as a pain reliever [6,16]. Like resveratrol, capsaicin suppresses the growth of cancer cells by NF-κB inactivation, ROS generation [7,17–19]. Moreover, young LSL-$Kras^{G12D}$/Pdx1-Cre mice treated with a single dose of caerulin develop pancreatic cancer lesions. This is significantly reduced when they are fed daily for eight weeks with capsaicin [8,20]. The isothiocyanates found in cruciferous vegetables such as broccolis, cabbages or cauliflower have well-documented anticancer activities. For example, benzyl isothiocyanate (BITC) reduces pancreatic cancer tumor growth by ways common to resveratrol and capsaicin such as NF-kB inhibition [9,21] or ROS induction [10,22], but also through antiangiogenic effect dependant on STAT3 [11,23]. The isothiocyanate sulforaphane sensitizes pancreatic CSCs to chemotherapy by inhibiting their self-renewal potential, apoptosis resistance and NF-kB activity [24,25]. A prospective clinical trial with sulforaphane-enriched broccoli sprout extracts is ongoing in Germany to examine its action on pancreatic cancer treatment (ClinicalTrials.gov Identifier NCT01879878, Trials 2014, 15:204).

Most of the studies reporting antitumor action of BFCs evaluated them as a unique component used with the conventional drug for the cancer model. In this work, we attempted to evaluate whether combinations of BFCs could exert enhanced anticancer activity with or without conventional treatment *in vitro* and in a preclinical xenograft model.

## Materials and methods

### *Animals, pancreatic cell lines and antibodies, tissue specimens*

The 8–12 week-old NOD/Shi-SCID IL2Rγnull mice (NSG) were produced and housed at the University of Bordeaux animal facility A2, according to the rules and regulations of the Institutional Animal Care and Use Committee (agreement number A33063916). Pancreatic cell lines origins have been described earlier [26]. Genomic *KRAS* PCR amplification and sequencing were carried out to control the identity of the cells [27]. Human fibroblasts were obtained from mammoplasties after the patients have given consent. MCF7 cells were obtained from the ATCC (Teddington, United Kingdom). The BXPC-3 and CAPAN-2 were maintained in RPMI (Invitrogen, Saint Aubin, France) with 10% Fetal Calf Serum (FCS, Invitrogen) with Penicillin/Streptomycin 1/100 (Invitrogen). Human fibroblasts, MiaPaCa-2 and PANC-1 cells were maintained in DMEM with 10% FCS and 1/100 Penicillin/Streptomycin (Invitrogen). The HPNE cells were maintained in 75% DMEM 1 g/L glucose, 25% M3 Base Medium (Fisher Scientific, Illkirch, France) 10 ng/mL of Epidermal Growth Factor (Sigma Aldrich, Lyon, France), 10% FCS and 2 mM of L-glutamine (Invitrogen). Resveratrol (R), sulforaphane (S), capsaicin (C) and BITC were from Sigma Aldrich. Piceatannol (P) was purchased from Enzo life sciences (Villeurbanne, France) and Gemcitabine (Gemzar©, Lilly, Neuilly-sur-Seine, France) at the pharmacy of Bordeaux University hospital.

### *In vitro cell survival assay*

Cells were plated at $3 \times 10^3$ cells per well in 96-well plates. The day after, increasing doses of BFCs or combinations of BFCs at their $IC_{50}$ with or without gemcitabine (G) were applied and cells were kept in culture for 48 h. Each point was done at least in quadruplet. The experiments were carried out more than 3 times. Results are expressed as cell viability: $(OD_{treated}/OD_{untreated}) \times 100$. Cells were grown in 96-well plates, then stained and fixed with 0.5% crystal violet solution in methanol for 15 min at room temperature. Cells were washed twice in PBS, air-dried and observed with an inverted microscope.

### *Isolation of RNA, cDNA synthesis, PCR analysis for KRAS mutational status and protein extraction and western-blotting*

Total RNA was isolated using Trizol® and treated by DNAse according to the manufacturer's instructions (Invitrogen and Ambion, Saint Aubin, France). cDNAs were synthesized using Reverse transcriptase cDNA synthesis kit (Roche Applied Science, Meylan, France). PCRs for *KRAS* were carried out with the Promega PCR master mix (Promega) [28]. Protein extracts and western blotting were performed as already described [26]. At least 3 independent blots were performed for each protein.

### *Cell cycle analysis, apoptosis detection and ROS detection*

Cell cycle analyses were performed as already described [26]. Apoptosis was revealed by FITC-Annexin V staining of the cells (Apoptosis Kit, Beckman Coulter) followed by detection by flow cytometry. For ROS detection, cells were seeded in 12-well plate ($2 \times 10^5$ cells per well), then treated with single BFCs or combinations at their $IC_{50}$ for 3 h. To determine cell viability, MTS (3-(4,5-dimethylthiazol-2-yl)-5-(3-carboxymethoxyphenyl)-2-(4-sulfophenyl)-2H-tetrazolium) solution (Promega, Charbonnières, France) was applied for 1.5 h after cells were washed with PBS. Optical densities (OD) were read at 490 nm.

### *Xenografts of the CAPAN-2 cells*

Mice were injected subcutaneously with $8 \times 10^5$ cells in 100 µl serum-free medium in the right and the left flanks. When tumors were visible, measures with a caliper were done 3 times a week. When the tumors reached about 100 $mm^3$, mice were treated by BFCs and/or gemcitabine 3 times a week during 3 weeks. Gemcitabine was diluted in PBS and injected intraperitoneally and BFCs diluted in ethanol were given by gavage, with doses as indicated in the legend of Fig. 6. Control mice were administrated with ethanol (2% v/v in PBS) or PBS. Treatment tolerance was evaluated everyday by observing the mice and using the Lloyds' distress animal scoring system [29]. Tumors were resected and weighed between 19 and 21 days after starting the treatments.

### *Statistical analysis*

Statistical tests were performed using the Graph-Pad Prism software (6.04, GraphPad). *In vitro* results are expressed as mean ± SD, analyzed by unpaired, bilateral Student's *t* tests. Results *in vivo* are expressed as mean ± SEM, analyzed by 2-tailed Mann–Whitney test.

## Results

### *Combinations of BFCs are very potent to kill pancreatic tumor cell lines in vitro*

Half maximal inhibitory concentration ($IC_{50}$) of BFCs were determined on several pancreatic tumor cell lines (BXPC-3, CAPAN-2, MiaPaCa-2 and PANC-1) and on one mammary tumor cell line (MCF7) and compared to toxicity on normal human fibroblasts and Human Pancreatic Normal Epithelial Cells (HPNE). All tumor cell lines were sensitive to the tested BFCs in a dose-dependent manner (Fig. 1A–D, Table 1). Of note, low doses of all the tested compounds boosted cell survival of fibroblasts, but not tumor cells. Importantly, BFCs used at pancreatic cell line $IC_{50}$ were not toxic on human fibroblast.

Next, combination treatments were performed in cell culture using the mean $IC_{50}$ for each BFC, on two of the pancreatic cell